Université de Montréal

Streptococcus suis capsular type 2 interactions with phagocytic cells

par

Mariela Segura

Département de microbiologie et immunologie
Faculté de médecine

Thèse présentée à la Faculté des études supérieures
en vue de l'obtention du grade de
Philosophiæ Doctor (Ph.D.)
en microbiologie et immunologie

Avril 2002

©Mariela Segura, 2002

W
4
U58
2002
v. 083

Université de Montréal
Faculté des études supérieures

Cette thèse intitulée

Streptococcus suis capsular type 2 interactions with phagocytic cells

presentée par
Mariela Segura

a été évaluée par un jury composé des personnes suivantes:

Président-rapporteur

Dr Mario Jacques
Professeur titulaire,
Directeur du Groupe de Recherche sur les Maladies Infectieuses du Porc (GREMIP),
Directeur du "Canadian Research Network on Bacterial Pathogens of Swine",
Faculté de médecine vétérinaire,
Université de Montréal.

Directeur de recherche

Dr Marcelo Gottschalk
Professeur titulaire, GREMIP,
Faculté de médecine vétérinaire,
Université de Montréal.

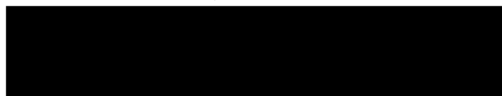
Membre du jury

Dr John M. Fairbrother
Professeur titulaire, GREMIP,
Faculté de médecine vétérinaire,
Université de Montréal.

Examinateur externe

Dr Daniel Grenier
Professeur titulaire,
Directeur du Groupe de Recherche en Ecologie Buccale (GREB),
Faculté de médecine dentaire,
Université de Laval.

Représentant du doyen de la FES



Thèse acceptée le ....

SOMMAIRE

Streptococcus suis est une bactérie impliquée dans des cas de méningite chez le porc, en plus d'être considérée un important agent de zoonose. Chez l'homme, cette bactérie peut causer, entre autres, des méningites et septicémies. La pathogenèse de l'infection causée par *S. suis* n'est pas bien connue. Cette bactérie est transmise par voie respiratoire et, à partir des amygdales, elle peut devenir septicémique et envahir les méninges et autres tissus, probablement en association avec des monocytes. Une première hypothèse suggérerait que les monocytes phagocyteraient les bactéries présentes dans le sang et les porteraient jusqu'au système nerveux central (SNC) (théorie du "Cheval de Troie"). Par contre, un faible pourcentage des monocytes contiennent des bactéries pendant une bactériémie. De plus, *S. suis* possède une capsule polysaccharidique (CPS) qui aurait des propriétés antiphagocytaires. Etant donné la controverse sur la phagocytose de *S. suis*, nous avons décidé d'entreprendre des études quantitatives de phagocytose en utilisant une technique de compte viable. Nous avons utilisé une souche capsulée et son mutant non capsulé, ce dernier obtenu préalablement dans notre laboratoire. Les résultats ont montré que la souche capsulée n'est pratiquement pas phagocytée par des macrophages murins. Par contre, le mutant non capsulé est phagocyté et éliminé rapidement de l'intérieur des cellules. Ces résultats indiquent que *S. suis* n'utiliserait pas la voie intracellulaire comme mécanisme de dissémination dans le sang. En fait, des résultats obtenus au cours de ces études ont démontré que *S. suis* est capable d'adhérer en grand nombre aux macrophages murins. Des épreuves d'inhibition ont suggéré que la fraction d'acide sialique présente dans la CPS de *S. suis* serait, en partie, responsable de cette adhésion. De plus, le complément présent dans le sérum augmenterait l'adhérence de *S. suis* aux phagocytes. Par contre, des concentrations élevées de certaines souches se sont avérées toxiques pour ces cellules. Nous avons démontré que l'hémolysine (suilysine) était responsable de cette activité.

Les conséquences inflammatoires de ces interactions avec les phagocytes ont ensuite été étudiées. Nous avons évalué la capacité de *S.*

suis à induire la production des cytokines proinflammatoires "tumor necrosis factor alpha" (TNF) et interleukine-6 (IL-6) par des macrophages murins. Les deux cytokines ont été induites par les cellules stimulées avec *S. suis* et ce, en l'absence de phagocytose, tel que révélé par des études avec la cytochalasine. Des études avec la paroi cellulaire purifiée ont démontré que celle-ci est responsable, en grand partie, de l'effet proinflammatoire observé. Par contre, la CPS et l'hémolysine purifiées ne joueraient pas un rôle important dans l'induction de ces deux cytokines.

Etant donné que *S. suis* est un important agent de zoonose, nous avons ensuite étudié la capacité de ce pathogène à induire la production de TNF, IL-1, IL-6, IL-8 et "monocyte chemotactic protein one" (MCP-1) par la lignée de monocytes humains THP-1. Des bactéries vivantes ou inactivées à la chaleur ont induit la production de quantités comparables de ces cytokines; l'IL-8 a été celle qui a été produite de façon prédominante. Nous avons aussi démontré que le récepteur CD14 est partiellement impliqué dans la production de TNF, IL-1, IL-6 et MCP-1 par les monocytes stimulés avec *S. suis*. Des récepteurs autres que le CD14 sembleraient être impliqués dans l'induction d'IL-8. Finalement, des études de blocage effectuées avec des anticorps anti-TNF et anti-IL-1 ont montré que ces deux cytokines ont un rôle d'amplification dans la cascade de cytokines proinflammatoires activées par *S. suis*.

Le fait que *S. suis* n'est pas phagocyté mais demeure adhéré aux phagocytes pourrait permettre le maintien du haut niveau de bactériémie couramment observé pendant les premières étapes de l'infection. Une des conséquences de cette adhérence serait une importante réaction inflammatoire qui provoquerait, entre autres, une infiltration de leucocytes et même une augmentation de la concentration de la bactérie au SNC. De plus, l'effet toxique de l'hémolysine produite par cette haute concentration de *S. suis* augmenterait les effets néfastes de la réponse inflammatoire pendant la méningite.

Mots clés: *S. suis, cellules phagocytaires, phagocytose, adhésion, cytotoxicité, induction de cytokines, capsule polysaccharidique, suilysine, paroi cellulaire, méningite*

SUMMARY

Streptococcus suis capsular type 2 is an important etiological agent of swine meningitis and it has been highlighted as a cause of occupational disease leading to meningitis and fulminant sepsis in humans. The pathogenesis of *S. suis* infections is still unclear. *S. suis* is transmitted via the respiratory route and remains localized in the palatine tonsils. From that site, bacteria may become septicemic and invade the meninges and other tissues, possibly in close association with monocytes/macrophages. Indeed, an early theory, called the "Trojan horse theory", suggested uptake of bacteria by monocytes, intracellular survival and invasion of the central nervous system (CNS). However, only a low number of monocytes actually contained intracellular bacteria. Furthermore, *S. suis* is a well-encapsulated bacterium and recent studies using isogenic mutants defective in capsule production suggested the antiphagocytic properties of the capsular polysaccharide (CPS). To further elucidate the role of CPS in *S. suis* interactions with phagocytes and in bacterial mechanisms of dissemination, we evaluated quantitatively the uptake and intracellular survival of *S. suis* type 2 in murine macrophages. The role of the capsule was also evaluated using a previously obtained unencapsulated isogenic mutant. The encapsulated *S. suis* wild type strain was practically not phagocytosed, whereas the unencapsulated mutant was easily ingested and killed by macrophages. These results further suggest that *S. suis* is able to resist uptake by phagocytes, and thus may use other mechanisms for bloodstream dissemination.

One possible mechanism is that bacteria interact with phagocytes and remain extracellularly bound to the cell surface. Indeed, by using an enzyme-linked immunosorbent assay technique, we demonstrated high levels of *S. suis* adhesion to murine macrophages. Inhibition studies showed that the sialic acid moiety of the *S. suis* capsule was, at least in part, responsible for bacterial recognition by macrophages. Serum pre-opsonization of bacteria increased adhesion levels, and complement would be partially involved in the serum-enhanced binding of *S. suis* to cells. High bacterial concentrations of some isolates were cytotoxic for cells, and these cytotoxic effects correlated with production of suilysin, the only cytolysin described to date for *S. suis*.

To further investigate the consequences of *S. suis* interactions with phagocytes in the inflammatory reaction, we evaluated the capacity of *S. suis* to induce the up-regulation pro-inflammatory cytokines tumor necrosis factor alpha (TNF) and interleukin-6 (IL-6) by murine macrophages. Results showed that *S. suis* stimulated both cytokines in a cytochalasin-insensitive fashion confirming previous results. Experiments with the unencapsulated mutant, purified CPS, or *S. suis* cell wall demonstrated that the latter would be the main bacterial component responsible for cytokine stimulation. Unexpectedly, suilysin showed no cytokine stimulating activity *in vitro*.

Since *S. suis* is an important agent of meningitis and toxic shock syndrome in humans, we were interested to evaluate the ability of *S. suis* to induce the up-regulation of TNF, IL-1, IL-6, IL-8 and monocyte chemotactic protein one (MCP-1), the two latter being related to leukocyte chemotaxis, by the human monocytic THP-1 cell line. Levels of these five cytokines, when induced by either heat-killed or live bacteria, were similar, and IL-8 levels were markedly higher compared to those obtained with the other cytokines. We also demonstrated that the CD14 receptor is partially involved in TNF, IL-1, IL-6 and MCP-1 production, whereas CD14-independent pathways seem to be responsible for IL-8 production following *S. suis* stimulation. In addition, blocking studies with anti-TNF and anti-IL-1 antibodies revealed that these cytokines are involved in amplification of the *S. suis*-induced cytokine cascade.

The adherence of *S. suis* to phagocytes in the absence of bacterial ingestion would allow a persistent high-grade bacteremia during the first steps of the infection. Following adherence, a substantial inflammatory reaction, with an up-regulation of pro-inflammatory cytokines, would be triggered by *S. suis*. This would lead to an infiltration of leukocytes and bacteria into the CNS. Furthermore, at high bacterial concentrations, the hemolysin-related cytotoxic effects at sites of inflammation could have serious consequences during meningeal infection.

Key words: *S. suis, phagocytic cells, phagocytosis, adhesion, cytotoxicity, cytokine induction, polysaccharide capsule, suilysin, cell wall, meningitis.*

TABLE OF CONTENTS

JURY IDENTIFICATION ii
SOMMAIRE iii
SUMMARY v
TABLE OF CONTENTS vii
LIST OF TABLES ix
LIST OF FIGURES x
LIST OF ABBREVIATIONS xii
DEDICATION xiii
ACKNOWLEDGEMENTS xiv

I. INTRODUCTION 1

II. LITERATURE REVIEW 5

1. *Streptococcus suis* 6
  1.1. General aspects of *S. suis* 6
  1.2. The capsular types (serotyping) 7
  1.3. *S. suis* infection and transmission 10
    1.3.1. In pigs 10
    1.3.2. In humans 12
    1.3.3. In other animal species 14
    1.3.4. Treatment and control of the infection 14
  1.4. Proposed virulence factors of *S. suis* capsular type 2 17
    1.4.1. The polysaccharide capsule 18
    1.4.2. Proteins 20
      1.4.2.1. MRP and EF 20
      1.4.2.2. Hemolysin (suilysin) 22
      1.4.2.3. IgG binding protein (HSP60) 23
      1.4.2.4. Hemagglutinins and adhesins 24
      1.4.2.5. Others 26
  1.5. The pathogenesis of *S. suis* infection 27
    1.5.1. *S. suis* meningitis: a first hypothesis 27
    1.5.2. The controversy about *S. suis* phagocytosis 31
2. The host inflammatory response in disease 33
  2.1. Central role of phagocytes 33
    2.1.1. Bacterial interactions with phagocytes: consequences in disease 34
      2.1.1.1. Bacterial uptake and intracellular survival . 39
      2.1.1.2. Bacterial adhesion and resistance to phagocytosis 40
      2.1.1.3. Cell activation 43
  2.2. Inflammatory mediators: the cytokine network 44
    2.2.1. The pro-inflammatory cytokines 45
      2.2.1.1. Tumor necrosis factor alpha 46
      2.2.1.2. Interleukin-1 48
      2.2.1.3. Interleukin-6 50
    2.2.2. The chemokines 54
      2.2.2.1. Interleukin-8 57
      2.2.2.2. Monocyte chemotactic protein-1 58

2.3. Bacterial modulins 59
2.3.1. Gram-negative bacteria: the example of LPS 60
2.3.2. Gram-positive bacteria: the example of the cell wall. 64
3. Pathogenesis of meningitis caused by other bacteria: the example of GBS 67
4. References 72

III. MATERIALS, METHODS AND RESULTS 91

Article I: *Streptococcus suis* and group B *Streptococcus* differ in their interactions with murine macrophages. **1998**. FEMS Immunol. Med. Microbiol. **21:** 189-195 92

Article II: *Streptococcus suis* interactions with the murine macrophage cell line J774: adhesion and cytotoxicity. **2002**. Infect. Immun. **70:** 4312-4323 108

Article III: Heat-killed *Streptococcus suis* capsular type 2 strains stimulate tumor necrosis factor alpha and interleukin-6 production by murine macrophages. **1999**. Infect. Immun. **67:** 4646-4654 138

Article IV: CD14-dependent and -independent cytokine and chemokine production by human THP-1 monocytes stimulated by *Streptococcus suis* capsular type 2. **2002**. Clin. Exp. Immunol. **127:** 243-254 163

IV. DISCUSSION 193

1. The controversy about *Streptococcus suis* phagocytosis 194
2. Consequences of *S. suis* interaction with phagocytes 205
2.1. Cytotoxic effects 205
2.2. The activation of the pro-inflammatory cytokine cascade 207
3. *Streptococcus suis* pathogenesis: a new hypothesis 219
4. References 223

V. GENERAL CONCLUSIONS 231

VI. ANNEXE xv

Article V: Pro-inflammatory cytokine and chemokine release by human brain microvascular endothelial cells stimulated by *Streptococcus suis* serotype 2. **2002**. FEMS Immunol. Med. Microbiol. *Submitted* xvi

Article VI: Up-regulation of ICAM-1, CD11a/CD18 and CD11c/CD18 on human THP-1 monocytes stimulated by *Streptococcus suis* serotype 2. **2002**. Infect. Immun. *Submitted* xxxvii

Article VII: The pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions. **2000**. Vet. Microbiol. **76:** 259-272 ... lxi

CURRICULUM VITAE lxxx

LIST OF TABLES

MATERIALS, METHODS AND RESULTS

Article II:

Table 1. Comparative studies with different *S. suis* capsular type 2 isolates 121
Table 2. Effect of bacterial pre-treatments on adhesion 122
Table 3. Role of bacterial products in cytotoxicity and inhibition of cytotoxic activity 126

Article IV:

Table 1. *S. suis* capsular type 2 strains of porcine and human origins used in this study 169
Table 2. Effect of anti-cytokine Mabs on *S. suis*-induced cytokine release by IFN-γ pre-activated THP-1 cells 177

ANNEXE

Article V:

Table 1. *S. suis* capsular type 2 strains of porcine and human origins used in this study xx

Article VI:

Table 1. Strains of *S. suis* serotype 2 used in this study xli

LIST OF FIGURES

LITERATURE REVIEW

Figure 1. First hypothesis of the pathogenesis of *S. suis* meningitis 31
Figure 2. Diagrammatic representation of cytokine mechanisms of action . 44
Figure 3. Systemic effects of pro-inflammatory cytokines 51
Figure 4. Sequential steps of the pathophysiology of bacterial meningitis .. 53
Figure 5. Sequential steps of leukocyte transmigration............................ 56
Figure 6. Biological functions of IL-8 57
Figure 7. Major biological functions of MCP-1 59
Figure 8. Comparison of the cell wall structures of Gram-positive and Gram-negative bacteria 61
Figure 9. Schematic representation of LPS binding to monocytes............ 63

MATERIALS, METHODS AND RESULTS

Article I:

Figure 1. Phagocytosis of *S. suis* and GBS strains 100
Figure 2. Time course of intracellular survival of *S. suis* and GBS strains . 102

Article II:

Figure 1. Kinetics of *S. suis* adhesion to J774 macrophages 119
Figure 2. Electron micrographs showing *S. suis* adhesion to J774 cells 120
Figure 3. Effect of bacterial pre-opsonization on adhesion to J774 cells 124
Figure 4. Effect of incubation time in presence of *S. suis* on J774 injury 125
Figure 5. TEM demonstrating J774 cell-injury after incubation with *S. suis* . 127

Article III:

Figure 1. Time-course production of TNF-α and IL-6 by *S. suis* 147
Figure 2. Effect of PMA-costimulation in cytokine induction by *S. suis* 149
Figure 3. Effect of bacterial concentration on TNF-α and IL-6 production ... 150
Figure 4. Effect of phagocytosis inhibition on cytokine production 151
Figure 5. Relative role of bacterial components in cytokine production 153

Article IV:

Figure 1. Kinetics of cytokine release by non-activated THP-1 174
Figure 2. Kinetics of cytokine release by IFN-γ pre-activated THP-1 174
Figure 3. Effect of bacterial concentration on cytokine production 175
Figure 4. Effect of CD14 blockade on cytokine production 178
Figure 5. Comparative study of cytokine production by different *S. suis* capsular type 2 strains 180

DISCUSSION

Figure 10. TEM showing *S. suis* lysis inside the phagosome 198
Figure 11. *S. suis* cytokine-inducing activity is not related to endotoxin contamination 208
Figure 12. Hypothetic model of *S. suis* interactions with macrophages 211
Figure 13. Effect of CD14 blockade on chemokine production 216
Figure 14. Proposed model for *S. suis*-induced cytokine cascade with THP-1 monocytes 218
Figure 15. Summary of the knowledge and proposed hypothesis for *S. suis* pathogenesis 222

ANNEXE

Article V:

Figure 1. Time course of IL-6, IL-8, and MCP-1 production by BMEC xxv
Figure 2. Time course of IL-6, IL-8, and MCP-1 production by HUVEC....... xxvi
Figure 3. Comparative study of cytokine production by different *S. suis* capsular type 2 strains xxix
Figure 4. Cytokine production by purified proposed virulence factors........ xxxi

Article VI:

Figure 1. Kinetics of up-regulated expression of ICAM-1, VCAM-1 and E-selectin on HUVEC xlv
Figure 2. Kinetics of the expression of ICAM-1 and CD11a,b,c/CD18 on THP-1 monocytes xlvi
Figure 3. Effect of the bacterial dose on adhesion molecules expression .. xlvii
Figure 4. Adhesion molecule up-regulation by different *S. suis* strains xlix
Figure 5. Adhesion molecule up-regulation by purified virulence factors..... l
Figure 6. Adhesion of *S. suis*- stimulated monocytes to endothelial cells .. li

LIST OF ABBREVIATIONS

BBB:	blood-brain-barrier
BMEC:	brain microvascular endothelial cells
C'MS:	complement from mouse serum
CNS:	central nervous system
CPS:	capsular polysaccharide
CSF:	cerebrospinal fluid
EF:	extracellular factor
FBS:	normal fetal bovine serum
FHA:	filamentous hemagglutinin
FN:	fibronectin
GAS:	group A *Streptococcus*
GBS:	group B *Streptococcus*
HUVEC:	human umbilical endothelial cells
IFN:	interferon
IgG:	immunoglobulin G
IL:	interleukin
kDa:	kilodaltons
LBP:	lipopolysaccharide-binding protein
LPS:	lipopolysaccharide
LTA:	lipoteichoic acid
Mab:	monoclonal antibody
MBP:	mannan-binding protein
MPS:	mononuclear phagocyte system
MRP:	muramidase-released protein
OMPs:	outer membrane proteins
Pab:	polyclonal antibody
PEM:	peritoneal exudate macrophages
PFGE:	pulsed-field gel electrophoresis
PG:	peptidoglycan
PMN:	polymorphonuclear cells or neutrophils
RAPD:	randomly amplified polymorphic DNA
RES:	reticulo-endothelial system
RGD:	Arg-Gly-Asp binding domain
SAS:	subarachnoidal space
SEM:	scanning electron microscopy
TEM:	transmission electron microscopy
TLR:	Toll-like receptor
TNF-α:	tumor necrosis factor alpha
VP:	Voges-Proskauer

A mis padres

ACKNOWLEDGEMENTS

Je tiens à remercier sincèrement tous ceux et celles qui ont contribué de près ou de loin à la réalisation de ce projet, et plus particulièrement :

Le docteur Marcelo Gottschalk, professeur titulaire au Département de pathologie et de microbiologie de la Faculté de médecine vétérinaire de l'Université de Montréal, pour m'avoir encouragé à poursuivre des études de troisième cycle, pour sa confiance et ses conseils judicieux, pour son soutien financier ainsi que sa grande disponibilité tout au long de ce projet;

Le docteur Jana Stankova, professeur titulaire au Département d'immunologie, Faculté de médecine de l'Université de Sherbrooke, pour sa codirection pendant mes études à la maîtrise ainsi que pour ses conseils scientifiques;

Le docteur Mario Jacques, directeur du Groupe de recherche sur les maladies infectieuses du porc (GREMIP) et du Réseau canadien de recherche sur les bactéries pathogènes du porc, pour sa disponibilité et ses conseils avisés lors de la révision de mes manuscrits scientifiques;

Madame Sonia Lacouture, assistante de recherche au laboratoire du Dr Gottschalk, pour sa collaboration technique, les discussions scientifiques, ainsi que pour ses encouragements et ses témoignages d'amitiés;

Mes compagnons de laboratoire et tous les membres du GREMIP pour leur soutien et leur amitié;

Le docteur Martin Olivier, chercheur au Centre de Recherche en Infectiologie, CHUL, Université de Laval, pour m'avoir facilité les dernières étapes nécessaires au dépôt de ma thèse et ce, pendant mon stage postdoctoral dans son laboratoire. Des remerciements s'adressent aussi à tous les membres de son laboratoire pour leurs encouragements;

Tous mes amis, d'ici et d'ailleurs, qui m'ont soutenue au cours de mes années d'études;

Ma famille, pour tous leurs encouragements, leur soutien moral et leur confiance.

Cette étude a été rendue possible grâce au support financier du Conseil de recherches en sciences naturelles et en génie du Canada (CRNSG), du Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR) et du Réseau Canadien de recherche sur les bactéries pathogènes du porc.

J'ai reçu une bourse d'étude de l'Agence Canadienne de Développement Internationale (ACDI) et une bourse d'excellence de l'Université de Montréal.

I. INTRODUCTION

Streptococcus suis is an important pathogen which has been associated with a wide variety of infections in swine such as meningitis, septicaemia, arthritis and endocarditis. To date, 35 different capsular types of *S. suis* have been described. *S. suis* capsular type 2 is considered to be the most virulent as well as the most prevalent capsular type in diseased pigs. *S. suis* has also been isolated from human cases of meningitis, endocarditis, septicaemia and toxic-shock syndrome. *S. suis* infection in humans is considered an occupational disease of increasing importance, and is one of the major causes of adult meningitis in some parts of the world.

The clinical presentation of *S. suis* infection may vary from asymptomatic bacteremia to fulminant systemic disease, resembling the clinical syndrome of Gram-negative sepsis. Meningitis is the most striking feature and the most common histopathological characteristics are the presence of fibrin, oedema and cellular infiltrates of the meninges and choroid plexus.

Knowledge on virulence factors and the pathogenesis of *S. suis* infection is still limited. *S. suis* is transmitted via the respiratory route and remains localized in the palatine tonsils. Some animals will remain healthy carriers and will never develop disease, whereas others will, sooner or later, develop bacteremia, sometimes septicaemia and finally, meningitis. Hence, in the latter cases, bacteria would travel and persist throughout the bloodstream and reach the central nervous system (CNS). An early theory, called the "Trojan horse theory", suggested uptake of bacteria by monocytes, intracellular survival and invasion of the CNS. This bacterial uptake by macrophages or by monocytes could take place directly in the tonsils, or once the bacteria are in the bloodstream. However, some studies carried out during the last decade suggest that bacteria may also use (an)other mechanism(s) to disseminate. In fact, *S. suis* is a well encapsulated bacterium and, as shown for other bacterial pathogens, the capsular polysaccharide (CPS) may confer antiphagocytic properties. Despite the fact that the CPS seems to be an important virulence factor, most avirulent strains are encapsulated, indicating that other important

virulence factors are essential. Indeed, among several proteins suggested as putative virulence factors, a hemolysin (suilysin), a thiol-activated toxin, which may have, by unknown mechanisms, a role in virulence, is produced by *S. suis*. Thus, bacterial attributes responsible for *S. suis* bloodstream survival and dissemination, leading to meningeal invasion, are still not clear.

Inflammation is a hallmark of *S. suis* infection, and in this regard, it has been suggested that once bacteria enter into the CNS, the induction of an acute inflammatory exudate increases the volume of the cerebrospinal fluid, leading to an increased intracranial pressure, which is responsible for the clinical signs of meningitis.

It is now recognized that several inflammatory and infectious diseases are associated with the overproduction of cytokines, which are important host mediators of inflammation. Cytokines are believed to mediate reactions associated with clinical deterioration, multiorgan system failure, and death during septic shock. They have also been implicated in meningeal inflammation caused by other bacterial species, such as Group B *Streptococcus* and *Streptococcus pneumoniae*, by alteration of the cerebrospinal fluid dynamics, brain metabolism, and the control of cerebral blood flow. In this regard, the induction of cytokines by monocytes/macrophages during *S. suis* infection has been proposed as playing a role in the pathogenesis of meningitis. However, this hypothesis has never been addressed.

Thus, despite the fact that mononuclear phagocytes have been implicated as playing a central role in the pathogenesis of the meningitis, the interactions of *S. suis* type 2 with phagocytic cells are still controversial. Furthermore, limited studies on the role of the inflammatory response in the pathogenesis of *S. suis* infections have been reported.

From this knowledge, our work **hypothesis** is that interactions of *S. suis* type 2 with phagocytic cells represent a key step in the pathogenesis of the infection caused by this pathogen. The result of this interaction is bacterial dissemination and induction of an acute inflammatory response which is responsible, at least in part, for the pathophysiology of *S. suis* meningitis.

General Aim: To study *S. suis* type 2 interactions with phagocytic cells.

Specific Objectives:

I. To study quantitatively the uptake and intracellular survival of *S. suis* type 2 in murine macrophages *in vitro*.

II. To evaluate and characterize the surface adhesion of *S. suis* type 2 to murine macrophages *in vitro*.

III. To study the induction of some important pro-inflammatory cytokines *in vitro* by murine macrophages and by human monocytes after stimulation with *S. suis* type 2.

II. LITERATURE REVIEW

1. *Streptococcus suis*

In 1951 Jansen and van Dorssen (158) described outbreaks of meningo-encephalitis in 1-6 months old pigs, occurring in several provinces of The Netherlands. Hemolytic streptococci were isolated from the brains and internal organs of these animals. Outbreaks of streptococcal meningitis and arthritis in piglets in East Anglia followed those described by Jansen and van Dorssen (98). In 1963 De Moor (74) originally proposed that those septicemic infections in pigs were caused by hemolytic streptococci of new Lancefield groups designated as R, S, and T. In 1987, chemotaxonomic and deoxyribonucleic studies carried out by Kilpper-Bälz and Schleifer indicated that all these strains belong, in fact, to one species, *Streptococcus suis*, within the Lancefield group D (166). Since 1951, and particularly during the last 10 years, *S. suis* infections have been considered as a major and worldwide problem in the swine industry.

1.1. General aspects of *S. suis*

S. suis is a Gram-positive coccus, possessing cell wall antigenic determinants related to Lancefield group D, although it is genetically unrelated to other members of this group (DNA relatedness values between strains of *S. suis* and *Enterococcus faecalis* are less than 10%). *S. suis* is a small nonmotile ovoid coccus, less than 2 μm in diameter, that occurs singly, in pairs, or rarely in short chains. All strains are α-hemolytic on sheep blood agar, and many strains produce β-hemolysis on horse blood agar. *S. suis* is chemo-organotroph with fermentative and facultatively anaerobic metabolism (166).

Many biochemical tests were initially proposed for the identification of *S. suis* and several biochemical variations have been noted among different strains (139, 207, 254). Despite this fact, a minimal number of tests carried out in combination with capsular typing (see section 1.2) may allow for the definitive

identification of most *S. suis* isolates recovered from pigs. Higgins and Gottschalk (139) proposed four tests for a presumptive identification of *S. suis*: no growth in 6.5% NaCl agar, a negative Voges-Proskauer (VP) test, and production of acid in trehalose and salicin broths. The NaCl (6.5%) test clearly differentiates *S. suis* and *Streptococcus bovis* from the genus *Enterococcus*. The VP test is critical and appears to be the most reliable for differentiating *S. suis* from *S. bovis*, two species which are commonly misidentified. Although some isolates are trehalose or salicin negative, very few are negative for both tests. Tarradas et al. (254) also proposed the hydrolysis of esculin and the absence of β-hemolysis on sheep blood agar as important biochemical parameters for *S. suis* identification. Some authors have used commercial multitest systems for biochemical identification of *S. suis*. However, results from these systems are, in some cases, not adequate and their use may be questioned (114, 139, 149).

1.2. The capsular types (serotyping)

S. suis can be classified into capsular types or serotypes according to the capsular polysaccharide antigens. The original classification of *S. suis* into Lancefield groups R, S, RS and T, which actually correspond to capsular types 2, 1, 1/2 and 15, respectively (115, 139, 209), is obsolete and should be avoided, since it was realized that the polysaccharides involved in serotyping originated from the capsular material rather than from the cell wall (139). During the last 10 years, several capsular types, reaching a total of 35 serotypes in 1995, have been described (140). Some of the reference strains originated from diseased pigs, whereas others were from the nasal cavities of clinically healthy pigs. Reference strains of capsular types 20, 31 and 33 were isolated from other animal species and capsular type 14 reference strain was recovered from a human case of meningitis (115, 139, 140). Some capsular types cross-react, indicating the possession of common capsular antigenic determinants. This is the case of capsular type 1/2 cross-reaction with types 1 and 2 antisera (209).

Two-way cross-reactions between types 6 and 16, and one-way cross-reaction between types 2 and 22, have also been demonstrated (115).

At this time, it is not possible to know whether any of the capsular types of *S. suis* belong to the normal flora of the nasal cavity or whether they represent real pathogens. So far, all known capsular types have been found in North America (113). Among them, capsular type 2 has always been considered the most virulent and prevalent type isolated from diseased pigs in most countries worldwide where the swine industry is important. However, the situation may be different depending on the geographical location and also, with time. For example, the frequency of isolation of *S. suis* strains of type 2 from diseased animals in Canada decreased from 32% to 16%, in the last 10 years (137). This situation is very different from that observed in some European countries, such as France, where almost 70% of *S. suis* isolates recovered from diseased animals belong to capsular type 2 (30). Under specific circumstances, some strains belonging to other capsular types appear to be highly prevalent, as it is the case for serotype 14 in United Kingdom (131, 132) and serotypes 1/2 and 5 in Canada (172). In Scandinavia, capsular type 7 predominated for several years, but within the last years capsular type 2 has come to prominence (138, 139). Nevertheless, the majority of isolates associated with pathogenic processes belong to the first nine capsular types. Some capsular types such as 18, 19, and 21 are generally recovered from clinically healthy animals (114, 115). Finally, the number of untypeable isolates is in general relatively low and, these isolates are recovered from cases of sporadic disease. Thus, it seems that there is no justification at the present time for the characterization of new capsular types (137).

Serotyping is an important step in the routine diagnostic procedure. Different techniques have been described, but most laboratories have adopted the coagglutination technique (112, 139). Since the majority of typable isolates belong to capsular types 1-8 and 1/2, it is advisable for diagnostic laboratories to only use antiserum corresponding to these serotypes and to send untypable isolates to a reference laboratory (138).

In the recent years, two independent research groups have studied the phylogenetic diversity of *S. suis* serotypes by comparison of 16 rRNA gene sequence (54, 220). Results showed that 32 of 35 reference strains have a nucleotide sequence similarity which ranged between 93 and 100%, and fell into a major group comprising three clusters. Comparison with nucleotide sequence from other streptococci indicated that, with the exception of serotypes 32, 33 and 34, *S. suis* reference strains did not cluster with any other *Streptococcus* species in the genus. However, there is no indication suggesting that members of these three serotypes (32-34) should be transferred to another species.

For epidemiological studies as well as for eventual eradication purposes, the detection of specific serotypes or strains of *S. suis* in live animals could be attempted by the use of PCR procedures. *S. suis*-specific 16S ribosomal sequences that might be used for specific detection of *S. suis* strains were identified (220, 242). More recently, a species-specific probe (serotypes 1 to 31) targeting 16S rRNA was designed and used for fluorescent in situ hybridization (35). In addition, the use of chaperonin 60 (HSP60) gene sequences for development of a rapid *S. suis* identification microarray system has recently been indicated. These sequences provided a higher level of discrimination between serotypes than the use of 16S rRNA sequences (37). On the other hand, PCR assays based on capsular genes have been developed for serotypes 1, 2, 7, and 9 (243, 246). Serotype-specific isolation from contaminated tissues, such as tonsils, may also be carried out using a recently reported immunocapture method (117).

Genetic diversity among members of the *S. suis* species is important, and this should be taken into account in diagnosis, surveillance, and control of the disease (53, 127, 128, 242). It has been suggested that isolates from clinically healthy animals were very heterogenous, in contrast to most isolates from cases of disease (7, 23, 251). On the other side, Hampson et al. (127) did not find any tendency of genetic variation between isolates recovered from healthy and those from diseased animals, or between isolates from animals with different disease syndromes. They concluded that virulence, as well as tissue tropism within the

species, does not appear to be confined to narrow genetic groupings of the bacteria. At present, there are no known common virulence markers for all *S. suis* serotypes (280).

1.3. *S. suis* infection and transmission

1.3.1. In pigs

S. suis is a worldwide cause of a variety of porcine infections. It is one of the most important swine meningitis agents. In addition, it is a cause of meningo-encephalitis, septicemia, arthritis, endocarditis, pericarditis, polyserositis, rhinitis, abortion and, it has also been associated with bronchopneumonia (138). Although *S. suis* is commonly isolated from the respiratory tract of pigs with respiratory disease, its relationship to pneumonia is unclear because *S. suis* is usually isolated in combination with other recognized respiratory pathogens, such as *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, *Bordetella brochiseptica* and others (103, 224). Thus, in these cases, *S. suis* may act as opportunistic or secondary pulmonary pathogen. This hypothesis is supported by the difficulty to reproduce respiratory clinical signs in experimentally infected animals. Indeed, in experimental trials, pre-infection with *B. brochiseptica* is needed for the induction of *S. suis* pneumonia (281). In contrast, in pigs with meningitis or meningo-encephalitis, *S. suis* is the sole bacterium isolated from brain, and thus is considered a primary pathogen (224).

S. suis type 2 affects growing pigs from soon after birth up to slaughter weight. However, the disease is most common following weaning and mixing, and the majority of cases occur between three and 12 weeks of age. The incidence of the disease varies from herd to herd and also varies within a herd over a period of time. No seasonal incidence has been discerned (62, 63). Different management practices and/or presence of other pathogens have been suggested as predisposing factors (75). Indeed, *S. suis* infection is associated

with times of stress in the pig's life, for example, weaning, mixing and/or moving animals, and with overcrowding and poor ventilation (75). Currently, there is an ongoing debate about the predisposition of pigs to *S. suis* infection due to concomitant viral infection such as Aujeszky's disease virus and Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) (86, 152).

S. suis reservoirs appear to be the swine themselves, as clinically healthy carriers; the species is readily harbored, primarily in the tonsils and nasal cavities and is also commonly recovered from the lungs, vagina, and prepuce of subjects exhibiting no clinical manifestations (64, 254). Thus, pigs carrying pathogenic *S. suis* serotypes and/or strains are known to be the source of infection for naive herds. Piglets born from sows with uterine and/or vaginal infections are either infected or become infected at, or soon after, birth while suckling. They also diversely and heterogeneously acquire the bacterium due to close contact with the sow, her faeces, and the surrounding structures. Infection of newborn piglets may also take place through the respiratory route from sow to piglets and among piglets (28, 138). Thus, both vertical and horizontal transmission are involved in spread of the disease (10, 67, 226). The carrier rate can range from 0% to 100%, and there are no significant differences between male and female pigs or between different age groups (66). Robertson and Blackmore (226) proposed that once a pig was infected with *S. suis* type 2, it remained a carrier for life.

Presumptive diagnosis of *S. suis* infections is based on clinical signs and macroscopic lesions. Diagnosis is confirmed by the isolation of the infectious agent and the recognition of microscopic lesions in tissues. Isolation of *S. suis* from lungs has to be interpreted with caution since, as mentioned above, this organism is almost constantly present in the upper respiratory tract. Pigs may harbor a variety of *S. suis* strains or serotypes in their nasal cavities and tonsils with no relationship with a specific pathological condition (188, 260). It is also possible to isolate multiple *S. suis* serotypes or strains from diseased animals within the same herd (138, 224). On the other hand, by the means of molecular tools, a prolonged persistence of an epidemic *S. suis* strain in a closed pig

population has been suggested to be responsible for the mortality in the farm under study (187, 260). The most consistent clinical signs reported are neurological signs, including opisthotonus, lateral recumbency, paddling, convulsions, and ataxia. Sudden death, without premonitory signs are also reported (223). Although *S. suis* is not isolated from every case of meningitis, compatible meningo-encephalitic lesions are found during the histopathological analysis, and *S. suis* may be isolated in those cases from other organs but not from the brain or meninges. In septicemia cases, *S. suis* is isolated from different organs, such as the spleen, liver, heart, lung, and brain. Inflammatory lesions are found in these organs on histopathological examination, but no typical pattern of lesions is observed (103). Suppurative or fibrinopurulent inflammation in the brain, heart, lungs, and serosae are the most common histopathological observations. Infected pigs generally have clinical signs and gross lesions referable to either the respiratory system or to the central nervous system, but not both (223).

1.3.2. In humans

During the period 1960 to 1966 Perch et al. (208) diagnosed for the first time, three cases of severe human meningitis and sepsis due to *S. suis* capsular type 2 in Denmark. Since 1966, the number of human cases of *S. suis* type 2 infections has been steadily increasing in different countries worldwide, such as the Netherlands, the United Kingdom, France, Belgium and Germany (55, 62, 68, 180, 185, 309). Furthermore, *S. suis* has been identified as the most common cause of meningitis in adults in Hong Kong (56). Despite the importance of the swine industry in Canada, human cases of meningitis were not recorded until 1982 (235), and the first case in Quebec was diagnosed in 1996 (186). In fact, the cumulative case reports are less than actual numbers since *S. suis* infection is under-diagnosed in humans. Unfortunately, because many laboratories are unaware of this organism, it can easily be misidentified as pneumococci, viridans streptococci, or enterococci (56, 173).

The clinical features of *S. suis* type 2 infection in man are similar to those in the pig. The most important manifestations are meningitis and septicemia. Fulminant fatal sepsis and toxic-shock syndrome have also been reported (39, 102, 173). In addition, other clinical manifestations, such as arthritis, endocarditis, pharyngitis, endophthalmitis, and spondylodiscitis are also observed in human cases of *S. suis* infection (13, 14, 206). Meningitis was observed mainly in persons previously in good health; however splenectomy, alcoholism, diabetes, and defects in humoral immunity have been suggested as predisposing factors (14, 58, 102, 173, 285, 306).

All infected patients have been adults and, with few exceptions, were men who handled pigs or pork by-products directly or indirectly. To date, *S. suis* infection in man has been considered an occupational disease (62, 88). Capsular type 2 is the serotype most frequently associated with human *S. suis* infections; however, capsular types 4 and 14 have also been isolated from human cases and thus all three types could be considered as epidemiologically implicated in zoonosis (14, 115, 131). Skin injuries or minor abrasions might represent the route of entry of the infection in most cases (88, 285). However, infection via other routes is not ruled out, because in some cases a definitive occupational exposure to pigs or pork could not be established (56, 58, 68, 173, 185). A ten-case report of fatal *S. suis* infection related to raw pork or uncooked pig's blood consumption was recently reported in Thailand (101). Since human carriage of the organism in the nose and throat has been postulated (14, 62, 228), and antibodies to *S. suis* type 2 have been found in pig farmers and meat inspectors, a sub-clinical infection in man has been suggested (225). The epidemiological importance of pigs to human infection was further established by molecular fingerprinting analysis, which showed common RAPD patterns between isolates of human and pig origin (53). These observations were recently confirmed by a study of genetic diversity of *S. suis* strains isolated from pigs and humans by pulsed-field gel electrophoresis (PFGE). Human strains were very homogenous and were statistically clustered in the same group of strains isolated from diseased pigs (29).

In general, *S. suis* disease in man has a positive prognosis if the diagnosis is established early and suitable treatment is started without delay. However, a striking feature is partial or total permanent loss of hearing as the most frequent sequela. The incidence of deafness in patients with meningitis due to *S. suis* infection is very high when compared to any other meningitides. Deafness also occurs in pigs with *S. suis* type 2 meningitis (182). As yet, no explanation can be given for this unusually high incidence of disturbance of the eighth cranial nerve in *S. suis* meningitis (14, 55). However, in an animal study, it was suggested that cochlear sepsis rather than eighth cranial nerve involvement was primarily responsible for hearing loss (163).

1.3.3. In other animal species

When De Moor in 1963 described septicemic infections in pigs caused by hemolytic streptococci, occurrence of these streptococci was confined entirely to swine. At this time, they had not been found in man, nor in other animal species tested (74). Since the isolation of the capsular types 20 and 31 reference strains from diseased calves, and capsular type 33 reference strain from a diseased lamb (115, 140), *S. suis* isolates of several different serotypes have been recovered from ruminants, horses, wild boar, cats, dogs, and birds. This suggests that *S. suis* may be pathogenic for more than one animal species (71, 79, 80, 140, 141, 150, 234). As described for pigs, tonsils seem to act as a carrier site for *S. suis* in these animal species (71, 78, 234). A possible role of these animal species as healthy carriers and as secondary reservoirs of *S. suis* should be further investigated.

1.3.4. Treatment and control of the infection

Streptococci are extremely sensitive to the beta-lactamins and this group of antibiotics has been used prophylactically in herds where *S. suis* type 2 is a problem, especially at periods of highest risk, e.g., weaning (138). Treatment of

individual cases has to be undertaken early in the course of the disease to be successful (62). Studies have pointed out that the inflammatory reaction against infection may be detrimental in some cases and have recommended treatment with anti-inflammatory medications (264, 273). In this regard, adjunctive therapy with an anti-inflammatory agent is recommended for treatment of *S. suis* meningitis in pigs (9). Indeed, excellent results were obtained when post-weaning meningitis was treated with both penicillin and dexamethasone in the very early stages of the disease (59). Therapeutic use of penicillin has also been reported as the first choice in human patients infected with *S. suis* capsular type 2, although some human cases have been difficult to treat using usual dosages of this antibiotic (14, 301). In human medicine, the use of anti-inflammatory agents in cases of bacterial meningitis is nonetheless controversial (138).

Despite the fact that *S. suis* type 2 has been shown to be extremely sensitive to penicillin, recent epidemiological studies have suggested that the overall susceptibility to penicillin of clinical isolates of *S. suis* has significantly changed. Furthermore, several cases have been reported to be difficult to treat with penicillin, despite the use of large doses of the antibiotic over a prolonged time lapse. In addition, several moderately susceptible and some resistant strains of *S. suis* have recently been isolated (122, 301). It has been suggested that modifications in the penicillin-binding proteins of certain field strains, rather than beta-lactamase production, are involved in the mechanism of resistance to penicillin (41, 122). Thus, penicillin sensitivity can no longer be assumed for all *S. suis* strains, and the routine treatment of *S. suis* infections with this antibiotic will have to be reevaluated if resistant strains become more prevalent (75, 122). In addition to penicillin, an increase in resistance to erythromycin, clindamycin and tetracycline has been reported (222, 252). It was suggested that conjugation of antibiotic resistance in clinical strains is possibly mediated by a transposon similar to Tn*916*, rather than being related to plasmid DNA (42, 252).

S. suis is an example of an emerging infection associated with the intensification of the swine industry. As mentioned above, multiple factors are involved, including the health status of the herd, the quality of the environment

and management. Thus, control of environmental and stress factors, such as mixing and moving, using "all in - all out" systems of housing with adequate cleaning and drying of rooms between batches, decreasing the population density, and improving the ventilation constitute the first line of defense against the disease. These management measures coupled with strategic medication of clinically ill animals should be used for control and prevention of mortality caused by streptococcosis (11, 62, 63, 75).

Production technologies such as medicated early weaning and segregated early weaning have been used to improve the health status of pigs and to eliminate some infectious organisms. It is now accepted that, although early weaning can succeed in controlling diseases such as pleuropneumonia and swine dysentery, its capacity to reduce or eliminate early colonizers, such as *S. suis*, is questionable (138). Indeed, early weaning procedures were shown to be not effective for the elimination of the carrier state of *S. suis*, because pigs are exposed during birth and while suckling (75, 226, 259). In addition, the organism persists in tonsils in the presence of circulating antibodies and in pigs receiving feed medication with penicillin. This may explain how the organism can persist indefinitely in a herd and why most attempts of control or eradication by blanket medication have failed (64). It has been suggested that *S. suis* could be eradicated only by use of extreme management procedures or by delivering all pigs by cesarean section (75). Hence, control measures have now centered on vaccination.

Currently, immunization efforts have focused on the use of autogenous bacterins. Vaccine failure in experimental trials has been reported in addition to poor disease control in the field with the use of bacterins. The exact reasons for vaccine failure are still unknown, but possible explanations are degradation of protective antigens or loss of antigenicity of the bacteria caused by heat or formalin processing (147), weak immunogenicity of the capsulated bacteria (76), production of antibodies to antigens not associated with virulence factors (148), or lack of cross-reactivity. In herds infected with multiple strains or serotypes of *S. suis*, multivalent vaccines or vaccines that provide a strong degree of cross-

immunity are needed to provide adequate control of infection (138, 224). Different types of vaccines have been or presently are under investigation, such as whole-cell bacterins, live attenuated strains, live avirulent strains, cell wall proteins, purified putative virulence factors, and unencapsulated isogenic mutants. For the future, an effective vaccine would provide a useful method of control in severely-affected herds (138).

1.4. Proposed virulence factors of *S. suis* capsular type 2

A virulence factor denotes a bacterial product or strategy that contributes to virulence or pathogenicity. Most studies on *S. suis* virulence factors have been carried out with capsular type 2 strains. Although there is confusion in the description of virulence, researchers agree at least on one point: the existence of virulent and avirulent strains of *S. suis* serotype 2. To date, several proposed virulence factors have been described for *S. suis* serotype 2 strains. However, the mere presence of these virulence factors does not necessarily define the strain as virulent or not. Some strains possessing a specific virulence factor are virulent, while other strains devoid of the same virulence factor are still virulent. Indeed, the concept of "virulence" may differ between different groups, since experimental infection models for *S. suis* can be misleading. For example, different studies have designated field strains as virulent or avirulent based on: (1) the clinical condition of the animal from which the strain was isolated (clinically healthy or diseased animals); (2) on the presence of virulence-related proteins; or (3) different experimental infection models, using (a) different strains of mice, or (b) colostrum-deprived piglets (pre-infected or otherwise with other micro-organisms), or (c) piglets of different ages from either conventional or specific-pathogen-free herds (24, 27, 104, 278). In fact, results from experimental infections of pigs with *S. suis* may depend, among other considerations, on the immunological status of the animals, the route of infection, the size of the inoculum and the presence of the organism as normal inhabitant of the upper respiratory tract of animals prior to the experimental

infection. Caution is therefore recommended before classifying a strain as virulent or avirulent. In fact, important discrepancies exist in the literature regarding even the virulence of the same strain of *S. suis* (116, 250).

Despite the fact that knowledge on virulence factors is limited, the most important candidates in *S. suis* are the capsular polysaccharide (CPS), the virulence-related proteins such as the muramidase-released protein (MRP) and the extracellular protein factor (EF), the hemolysin (suilysin), and the adhesins.

1.4.1. The polysaccharide capsule

Encapsulated bacteria are responsible for causing some of the most serious invasive infections, including septicemia, pneumonia, and meningitis (190). The role of capsules in bacterial virulence is to protect bacteria from the host's inflammatory response, i.e. complement activation and phagocyte-mediated killing (161). Indeed, the most common meningeal pathogens, *Neisseria meningitidis*, *Haemophilus influenzae* type b, *Streptococcus pneumonia*, and group B *Streptococcus* (GBS) are all encapsulated (264).

S. suis also possesses an integral, cell associated capsule (157). The CPS of *S. suis* type 2 has a molecular weight of 310-kDa and is composed of five different sugars: rhamnose, galactose, glucose, *N*-acetyl glucosamine, and *N*-acetyl neuraminic acid (sialic acid), in a molar ratio of 1.0:3.7:0.9:0.8:1.3 (93, 162). The conformational structure of the capsule is largely unknown, although the sialic acid moiety has been suggested as being at a possible terminal sequence and probably responsible of antigenicity (49, 50, 162). Even though antibodies against the type 2 CPS have been shown to have opsonizing activity, the CPS itself is a poor immunogenic material (92). In addition, antibodies against the capsular material only partially protect against infection (49) and convalescent (protected) animals produce low levels of these antibodies (76).

The CPS of *S. suis* serotype 2 is so far the only proven virulence factor, based on studies on nonencapsulated isogenic mutants obtained by insertional

mutagenesis. The absence of CPS correlated with increased hydrophobicity and phagocytosis by both murine and porcine phagocytes. In addition, unencapsulated mutants were shown to be avirulent in murine and pig models of infection (48).

Isolates of *S. suis* type 2 from diseased animals were shown to possess a thicker capsule than those from clinically healthy animals (120). An increase of capsular material thickness following *in vivo* growth was noted for virulent strains but not for avirulent ones (214). On the other hand, a study by Clifton-Hadley et al. (65) did not demonstrate any correlation between the thickness of capsular material and virulence. Capsular type 2 is considered to be one of the most virulent, but cells of the type reference strain were not covered by a thick layer of CPS, compared to other serotypes. It was suggested that the invasive ability of strains of this serotype may be due to the composition of the capsular material which contains sialic acid (157). This latter component has already been related to virulence for other bacterial agents of meningitis (287). However, Charland et al. (51) further demonstrated that virulent and avirulent strains possess a capsule of similar size with similar concentrations of sialic acid. In fact, resistance to clearance from the bloodstream does not rely only on the presence of the CPS, since a well encapsulated avirulent strain is eliminated from circulation within 48 h, whereas a virulent strain can stay in relatively high numbers in the blood for more than five days (unpublished observations). In conclusion, despite the fact that the CPS seems, based on the above mentioned mutational studies, to be a major virulence factor, most avirulent strains are encapsulated, indicating that other important virulence factors are essential.

Clearly, different strains within one capsular type vary in virulence and tropism, both within countries and between countries. There are also differences in pathogenicity between capsular types. For example, *S. suis* type 1 was generally associated with meningitis in baby piglets, and causes meningitis, arthritis, and septicemia, whereas *S. suis* type 2 occurred at any age and causes a wide variety of lesions. Several capsular types are found mainly in healthy pigs, notably type 21 and to a lesser extent, types 17, 18, and 19 (114,

115). It has been shown that *S. suis* type 9 produced a different distribution of lesions from that reported for *S. suis* type 2 (275). Correlation between capsular antigens and virulence is the basis for the suggestion that these antigens play a role in the pathogenesis of the disease (227). However, this suggested correlation is not widely accepted, and Reams et al. (223) indicated that neither clinical signs nor gross lesions were associated with specific capsular types. Indeed, some strains belonging to less common capsular types have been associated with severe cases of infection.

1.4.2. Proteins

1.4.2.1. MRP and EF

In addition to the CPS, cell-wall and extracellular proteins have been associated with virulence of *S. suis*. Two proteins, a muramidase-released protein (MRP) and an extracellular protein factor (EF), originally associated with virulent strains, have been reported in type 2 strains (279). MRP is a 136-kDa cell wall-associated protein, also released during bacterial growth, whereas EF is a 110-kDa protein only detected in culture supernatants (279). Type 2 strains with the phenotype MRP+EF+ induced severe specific clinical signs of disease, whereas strains with the phenotype MRP-EF- did not (277, 281). Molecular weight variants of these two proteins were later described. Enlarged or reduced forms of MRP, respectively called MRP* (> 136-kDa) and MRP^s (< 136-kDa), and higher molecular weight EF proteins, called EF* (> 150-kDa) can be found in phenotypes as MRP*EF-, MRP^sEF-, MRP^sEF+, MRP-EF*, and MRP+EF* of uncertain or variable virulence (119, 241, 297, 298). Strains of the last phenotype were isolated at high frequency from human patients, but caused almost no clinical signs of disease in experimentally infected pigs (241).

Although the genes encoding the EF and MRP proteins were cloned and characterized, the amino acid sequences did not provide information with respect to the possible functions of these proteins (241, 244). A particular region

within the MRP amino acid sequence, however, showed some similarity with the fibronectin-binding protein of *Staphylococcus aureus*, although binding of MRP to human fibronectin could not be confirmed (244). To date, the function of these two proteins in the pathogenesis of the *S. suis* infection remains unclear.

Isogenic mutants lacking both these proteins appeared to be as virulent as the wild type strain after experimental infection of newborn germfree pigs. Similar results were obtained with isogenic MRP-EF- mutants of *S. suis* type 1 (245). The authors suggested that the virulence of *S. suis* is a multifactorial process in which particular functions can be fulfilled by alternative factors. They also suggested that the synthesis of these proteins may only be coincidentally associated with virulence rather than being virulence factors *per se*. However, this association of MRP and EF with virulence is observed with strains of certain countries but not with others. For example, most North American strains isolated from acute cases of septicemia and/or meningitis (of either pig or human origin) were MRP and/or EF negative (53, 119). Interestingly, as demonstrated by randomly amplified polymorphic DNA analysis, the few MRP+EF+ North American stains were clustered in the same group as European strains which shared the same phenotype (53). A certain association of these proteins (specially the EF protein (298)) with virulence seems to exist, and most isolates harbouring these factors are virulent. However, the absence of one or more of these proteins cannot necessarily be associated with a lack of virulence. Again, since the term "virulence" is poorly defined for *S. suis* it is also possible that, under standardised conditions, MRP+ EF+ strains are potentially more virulent than MRP- EF- strains.

Both MRP and EF proteins are major antigens recognized by convalescent sera of infected pigs (279). A subunit vaccine containing MRP and EF protected pigs against challenge with a homologous or a heterologous *S. suis* type 2 strain MRP+ EF+ (299). The possible cross-protection of this type of vaccine would be of interest, since in addition to capsular type 2, strains of different types (from 1 to 22) have been reported to produce MRP and/or EF in several European countries (31, 298). On the other hand, this kind of vaccine

would not be useful in North America since, as mentioned above, most virulent strains lack MRP and EF (105).

1.4.2.2. Hemolysin (suilysin)

Hemolysin activity is associated with the virulence and pathogenicity of several bacterial species. A 54-kDa hemolysin, also known as suilysin, has been identified in *S. suis* type 2 by Jacobs et al. (154). Gottschalk et al. (123) also described a 65-kDa hemolysin in *S. suis* type 2. These two proteins were shown to be the same toxin and the molecular mass variation was in fact related to purification methods (123). Suilysin belongs to the family of toxins known as thiol-activated toxins, or more recently named as antigenically related cholesterol-binding cytolytic toxins. The most widely known members of this family are streptolysin O, listeriolysin, perfringolysin, and pneumolysin (204). Suilysin possesses several characteristics in common with these toxins, such as loss of activity upon oxidation, reactivation upon reduction, inhibition by small amounts of cholesterol, formation of transmembrane pores and a "multi-hit" mechanism of action (123). Membrane cholesterol is thought to be the toxin-binding site at the surface of eukaryotic cells (8, 204). In addition, the N-terminal amino acid sequence of suilysin shows many similarities with the respective deduced sequences of the above mentioned toxins (154). Furthermore, the gene encoding for suilysin (*sly*) has been recently sequenced, revealing a relative high similarity with the pneumolysin (237).

While several of these cytolysins have been shown to be determinants of bacterial pathogenicity, their biological roles may vary, as do the lifestyles of the bacteria secreting them (204). A role of suilysin in virulence has been suggested since it has been shown to be cytotoxic to endothelial cells (52), and also to epithelial cells (170, 198). On the other hand, hemolytic *S. suis* type 2 supernatant injected intraperitoneally failed to cause death in mice (97).

Despite the fact that antibodies against the suilysin could not be detected in pigs experimentally infected with a suilysin-positive strain of *S. suis* type 2

(123), a vaccine containing purified suilysin was highly immunogenic and induced an increase in hemolytic-neutralising antibodies. This vaccine protected mice against a lethal *S. suis* type 2 challenge and induced protection against clinical signs in pigs (154, 156). Since several strains of different serotypes (from 1 to 22) and field isolates from diseased pigs were shown to have hemolytic activity (97, 154, 155), suilysin was suggested as a promising cross-protection factor for use in vaccines (155). However, as observed for MRP and EF, most European strains are suilysin-positive, whereas variable production of this protein has been observed with North American strains (119, 202, 250). Indeed, the presence of the *sly* gene was demonstrated only in 7% of North America strains (237). In a recent study in France, authors reported that most of the type 2 isolates recovered form diseased pigs carried MRP+EF-suilysin- phenotype. Thus, despite the fact that suilysin, EF and MRP are highly associated with pathogenicity (7, 250, 251), they should not be universally used to determine virulence of *S. suis* (31, 198).

S. suis apparently has other virulence factors to compensate for the absence of suilysin. The available clinical data on field isolates are not sufficient to link the presence of suilysin to specific clinical symptoms (237). Nevertheless, since a certain role in the pathogenesis of the infection could be attributed to the suilysin, this toxin may be associated with high virulence in hemolytic *S. suis* strains. To the best of our knowledge, no avirulent, suilysin-positive *S. suis* type 2 strain has been reported to date (121).

1.4.2.3. IgG binding protein (heat shock protein 60)

A 60-kDa IgG-binding protein (previously identified as a 52-kDa protein) was purified and characterized from *S. suis* type 2 strains (26, 238). It was further observed that the *S. suis* IgG-binding protein is a member of the 60-kDa heat-shock protein family (25). This protein represents a common antigen found in all *S. suis* serotypes tested including both virulent and avirulent strains of *S. suis* serotype 2. Hence, its role in virulence is unknown (239). The IgG-binding

protein is associated with the cell surface, and is also released in a soluble form during bacterial growth. It reacts with a large variety of mammalian IgG and also with other plasma proteins, such as human fibronectin (238). It was suggested that these types of proteins would help bacteria to elude host defenses. Non-immune binding of IgG to the bacterial cell surface may mask antigens on the cell wall thereby limiting effective opsonization and antibody-complement-dependent bactericidal mechanisms.

1.4.2.4. Hemagglutinins and adhesins

Adhesins are important virulence factors which specifically mediate the attachment of pathogenic bacteria to host cells (200). *S. suis* was found to possess hemagglutinating properties (118). Further studies showed that the hemagglutinating activity could be inhibited with neutral monosaccharides, but not with trypsin or pronase treatment, suggesting that the host receptors are glycolipids rather than glycoproteins. More specifically, *S. suis* recognized the disaccharide sequence Galα1-4Gal that is present in the trihexosylceramide, GbO_3, a neutral glycolipid belonging to the P blood group antigens (125). Hence, the adhesin was named P adhesin, and classified into 2 subtypes, P_N and P_O, based on the differences in their binding specificity. Type P_O was inhibited by galactose only, whereas type P_N was inhibited by both galactose and N-acetylgalactosamine (126). The adhesin was later purified and shown to be a 18-kDa protein undergoing phase variation, and widely expressed among strains of different serotypes (256, 257). The purified adhesin was shown to be highly immunogenic, and induced bactericidal activity in mice (256). However, both virulent and non-virulent strains of *S. suis* posseses this adhesin (256), and no correlation could be made between hemagglutination activity and virulence for mice (118). *S. suis* was shown to bind to frozen sections of pig pharyngeal tissue in a Galα1-4Gal inhibitable manner (125), but there are at present no further clues to the biological role of this adhesion activity, nor to the presence of the P_N and P_O variants (124).

Fimbriae are important in bacterial adhesion to host surfaces; however, very few Gram-positive species have been shown to carry fimbriae, compared with the large number of Gram-negative species known to possess them (22). Interestingly, ultrastructural studies of surface components of *S. suis* revealed the presence of peritrichous, thin, and flexible fimbriae (157). Morphologically similar fimbriae were observed on hemagglutinating as well as on non-hemagglutinating strains of *S. suis* (118). Thus the possible role of fimbriae in hemagglutination and/or cell adhesion remains unclear.

Adherence of *S. suis* type 2 to porcine lung sections has been shown to correlate with the thickness of the capsular material, but it is probably not the only mechanism involved since an unencapsulated mutant had the same adherence capacity as the wild type strain. Thus, cell wall components, in addition to CPS, were suggested to be involved in adhesion (120). Indeed, Lalonde et al. (170) showed that the cell wall would be the main component responsible for *S. suis* attachment to porcine epithelial cells. Whereas the capsule partially masks adhesion to epithelial cells (170), it does not seen to affect adhesion to brain microvascular endothelial cells (52). A modulation of capsule expression was suggested during the different steps of bacterial colonization and dissemination in the host (52, 256). Thus, taking into account these observations, the CPS and the cell wall components may be included in the list of potential *S. suis* adhesins.

In addition, a binding activity of *S. suis* to albumin was reported for virulent as well as for non-virulent type 2 isolates. A 39-kDa protein was identified as being responsible, at least in part, for this binding activity. Furthermore, the addition of albumin to the culture broth resulted in an increase in virulence of *S. suis* strains in mice (213). Bacterial proteins which bind host proteins have been proposed to be involved in the pathogenesis of many Gram-positive infections; however, the role of *S. suis* albumin-binding protein is unknown to date (159). Nevertheless, a recent study reported that *S. suis* isogenic mutants defective in expression of the 39-kDa protein show a decrease of adhesion to bovine tracheal cells and porcine tracheal rings (36). Since non-

virulent strains also possess this binding activity, it would not be sufficient by itself to make the strain more virulent.

1.4.2.5. Others

In addition to the above mentioned putative virulence factors, superoxide dismutase activity was reported in *S. suis* type 2 strains. Superoxide is a major antibacterial substance in macrophage phagolysosomes, and thus the capacity to secrete this type of bacterial enzyme is important in virulence. However, no correlation was observed between specific superoxide dismutase activity and virulence. It is unlikely, therefore, that the production of this enzyme by *S. suis* would be related to phagocytic killing resistance of virulent isolates (171). Another report indicated that *S. suis* requires manganese, but not iron, for *in vitro* growth, and that manganese availability during growth affects the activity of the superoxide dismutase enzyme (194). On the other hand, iron-restriction-induced genes have been reported in *S. suis* (240), and therefore, the role of iron requirement in *S. suis* pathogenicity remains to be clarified.

In summary, more studies are needed to characterise virulence factors of *S. suis* type 2. The presence of MRP, EF and the suilysin in European isolates represents virulence potential. Conversely, the absence of one or more of these proteins in isolates from affected animals cannot be automatically associated with a lack of virulence. It is possible that MRP-, EF-, suilysin negative virulent strains from North America are comparatively less virulent than MRP+, EF+, suilysin positive European strains. Experimental infections with a well-standardised model using several strains representative of both groups are needed to confirm this hypothesis. Finally, virulent strains can also be isolated from healthy animals and clinical disease may sometimes be the consequence of the disturbance of the immune balance due to different causes, such as other infectious diseases, management, and stress. So far, the enigma of virulence factors and/or markers for virulent North American type 2 strains is still

unsolved. The lack of knowledge of virulence factors for other serotypes is even more marked.

1.5. The pathogenesis of *S. suis* infection

The pathogenesis of *S. suis* infections is poorly understood. Moreover, studies on this subject are almost limited to serotype 2 and only concern the development of meningitis. Reasons that may explain why *S. suis* will successfully colonise some piglets and not others are poorly known. Colonized animals will harbor the bacteria in their tonsils. Some animals will only be healthy carriers and will never develop disease, whereas others will, sooner or later, develop bacteremia, sometimes septicemia and finally, meningitis. Hence, in these cases, bacteria would travel throughout the bloodstream and reach the central nervous system (CNS). The pathogenesis of the infection is thus influenced by the immune status of the host, environmental factors, and virulence attributes of the infectious agent. In view of the variation in virulence and suggested tissue tropism (discussed in sections 1.3 and 1.4), it is not known whether the findings from pathogenesis studies can be extrapolated across all pathogenic *S. suis* isolates. Nevertheless, the acute inflammatory response that results from the invasion and multiplication of pathogenic strains in porcine tissues is a hallmark of *S. suis* infection and needs further investigation. The possible virulence factors that cause this response are still unknown.

1.5.1. *S. suis* meningitis: a first hypothesis

Early works showed that meningitis, arthritis, and septicemia could be reproduced experimentally in young early-weaned piglets by intravenous and sub-dural inoculation and, more importantly in relation to pathogenesis, by spraying liquid cultures of *S. suis* type 1 in the nose and the throat (91, 98). Later, typical syndromes were also reproduced experimentally with *S. suis* type

2 (63, 296). Capsular types 1 and 2 were thus shown to be primary pathogens capable of producing disease unaided. This has since been confirmed with other capsular types (e.g. type 7) (34).

In the 1970s and 80s it was thought that the organism probably must gain entry to the bloodstream and cause a sustained bacteremia. Early studies on the pathogenesis of *S. suis* provided evidence that bacterial antigens became intracellular in the epithelial cells of the tonsils and in the lymph nodes draining the tonsil. Macrophages may also engulf *S. suis* and carry it in. Presumably, this is a means of antigen sampling and natural immunization. It is likely to represent a delicate balance, which if upset, might result in septicemia (6, 289, 293). In addition to the tonsillar route, septicemia has been shown to result from experimentally infecting skin abrasions with *S. suis*. Young pigs frequently have skin abrasions and these may be another route of entry (6). A third possible route was postulated via the alveolae following inhalation of aerosol droplets, but attempts to demonstrate this met with negative results (6). Indeed, some pulmonary affections, such as interstitial pneumonia, may result from septicemia-induced vascular damage (275). In addition, as mentioned above, infected pigs generally have clinical signs and gross lesions referable to either the respiratory system or to the CNS, but not both (223).

Although many studies have demonstrated that a sustained bacteremia is important in the pathogenesis of generalized bacterial meningitis, the site(s) and mechanisms of bacterial entry into the CNS have not been established conclusively (265). Three routes of access to the CNS were thus suggested. One route proposed was that *S. suis* might enter the cerebrospinal fluid (CSF) via the back of the nose and olfactory nerves; however, experimental infection of the olfactory mucosa with large doses of a virulent strain of *S. suis* type 2 failed to reproduce meningitis (63). A second route was proposed on the basis that both humans and pigs often suffered hearing loss during *S. suis* type 2 meningitis, due to destructive multiplication of the organism in the inner ear. It was proposed that the organism may somehow gain entry to the meninges via the eustachian tubes and ear. However, experimental infections indicated that

the opposite was true. The inner ear infection was an extension of the CSF infection (6, 163). The third route postulated was that bacteria could be carried into the CSF in association with monocytes migrating into the CSF compartment to maintain populations of resident macrophages; that is, circulating organisms enter in a cell-associated form rather than as free organisms. This mechanism of entry would be analogous to that by which some viruses (e.g. human immunodeficiency virus) invade the CNS (290). It was shown that *S. suis* is engulfed by monocytes and macrophages and that, in the absence of anti-*S. suis* specific antibodies and complement, virulent strains survive and multiply, whereas non-virulent strains (including encapsulated non-pathogenic strains) are destroyed (289, 291, 292). Mononuclear phagocytes were thus implicated as playing a central role in the pathogenesis of bacterial meningitis by disseminating organisms to the site(s) where lesions subsequently develop. This hypothesis was called the "Trojan horse theory" (290).

Several reports have identified the choroid plexus as the most probable site of meningeal pathogens entry to the CNS (265). In keeping with the evidence for entry of the bacteria within monocytes via the choroid plexus, the very earliest histological lesions in meningitis were shown to be at this site. Pathological studies of experimentally infected pigs showed disruption of the plexus brush border, a decrease in the number of Kolmer cells, and exudation of fibrin and inflammatory cells into the ventricles. Intracellular bacteria were demonstrated in the parenchyma of the choroid plexus, in the ventricular exudate, and also within peripheral blood monocytes. Separate studies found that circulating monocytes containing phagocytosed bacteria-sized particles migrated into the CSF compartment with the choroid plexus acting as a major site of cellular ingress. These observations support the concept that in the pathogenesis of meningitis, bacteria may gain access to the CSF compartment in association with monocytes migrating along normal pathways (290, 291).

As mentioned above, the level and duration of bacteremia are recognized as major determinants in the development of generalized meningitis caused by a number of bacteria, such as *S. pneumoniae*, GBS, *H. influenzae* type b,

Escherichia coli K1 and *N. meningitidis* (264, 265). Williams and Blakemore (291) proposed that, as the level of *S. suis* bacteremia increases, more circulating organisms will be available to be ingested by monocytes, and thus the level of cell-associated bacteremia will increase and tend to persist since pathogenic *S. suis* strains are able to survive in these cells.

The sum of available evidence therefore pointed to the following theory (6) (Fig. 1). In the pathogenesis of meningitis the organism is taken in through the nose or the mouth and colonizes the upper respiratory tract mucosa (36). It is then swept to the tonsils by the ciliary clearance mechanisms. The organism resides subclinically in the crypts for a variable length of time and is occasionally sampled by the epithelial cells. Macrophages may also engulf and transport the bacteria. Under normal circumstances this may stimulate an immune response without resulting in disease, but under stress or possibly excessive intake of organisms, this mechanism goes wrong. The bacteria are engulfed by monocytes, the monocytes migrate through the choroid plexus, and meningtis follows rapidly. Alternatively, the organism may gain access to the blood through skin abrasions, cause a bacteremia, and then be taken up by monocytes. Once bacteria enter into the CNS within migrating monocytes, they multiply and stimulate the ingress of more inflammatory cells into the CSF. Bacteria and inflammatory cells are then washed by the CSF to sites of CSF efflux (e.g. the arachnoid villi) where they accumulate causing a blockage of draining and an increase in intra-cranial pressure. The combination of this pressure and the release of cytokines from phagocytic cells are probably factors in the manifestation of the typical acute clinical signs (47). The bacteria multiply inside macrophages and destroy them. Free extracellular bacteria can be observed in smears but whether they multiply in the CSF *in vivo* is not known. The spread of bacteria throughout the CSF space leads rapidly to generalized inflammation. The inflammatory response thus starts locally in the choroid plexus and the subsequent rapid distribution of lesions results from the flow of CSF laden with bacteria (290-292).

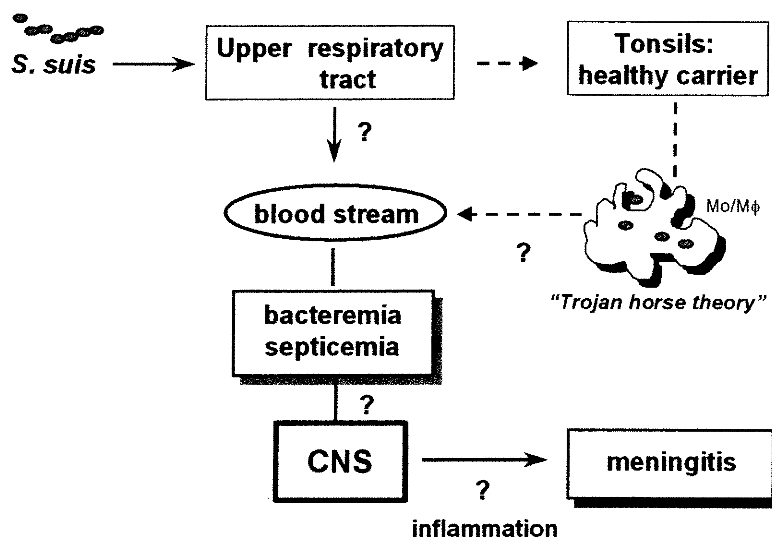


Fig. 1: First hypothesis of the pathogenesis of *S. suis* meningitis. Mo: monocytes. Mϕ: macrophages. CNS: central nervous system.

The concurrent development of meningitis, arthritis, and serositis is a feature of several bacterial meningitides. The CSF compartment, joint spaces, and serosal cavities are anatomically similar, being all open fluid-filled sacs that have large populations of resident macrophages. On the basis of these observations, Williams and Blakemore (291) extrapolated the Trojan horse theory to the pathogenesis of arthritis and serositis caused by *S. suis* entering these cavities within monocytes.

1.5.2. The controversy about *S. suis* phagocytosis

Despite the fact that the Trojan horse theory is largely addressed in the literature, Williams' findings that monocytes can phagocytose encapsulated *S. suis* in the absence of specific antiserum appears to be different from earlier *in vitro* observations (291). Indeed, earlier studies demonstrated that pathogenic

strains were able to resist phagocytosis in porcine or human blood in the absence of anti-*S. suis* specific antiserum. They multiplied freely in such blood. Antibodies against the type-specific CPS resulted in opsonization and destruction of these pathogenic strains and thus inhibited their growth. A strain of type 2 which had lost most of its capsule could not resist phagocytosis even in the absence of specific antibodies. It was thought, therefore, that it was the capsule of the pathogenic strains that was responsible for the resistance to phagocytosis and that enabled pathogenic strains to become septicemic in pigs early in the infection, before an antibody response had been launched (1, 63). These observations were recently confirmed by the obtention of isogenic acapsular mutants from a virulent *S. suis* type 2 strain (48). As mentioned above (section 1.4.1) unencapsulated isogenic mutants were more greatly phagocytosed than the encapsulated parental strain, by both murine macrophages and porcine monocytes. These mutants were shown to be avirulent for both mice and piglets, being cleared from circulation rapidly (48).

However, in contrast to the above suggested anti-phagocytic properties of *S. suis* CPS, and in agreement to the Trojan horse theory, a study carried out using flow cytometry also indicated a high level of *S. suis* uptake by swine and human phagocytes in a whole blood model of phagocytosis (40). To date, studies of the interaction between *S. suis* type 2 and phagocytic cells are contradictory and further investigations are needed to clarify the role of the CPS in the pathogenesis of *S. suis* infection.

2. The host inflammatory response in disease

Most mammals are continuously challenged by microorganisms, as a result of which defense mechanisms must be maintained throughout their life span. The first line of defense is provided by the skin or the mucosa of the respiratory and gastrointestinal tracts. However, when these barriers become damaged, an easy path of entry is provided for pathogenic microorganisms. Furthermore, some microorganisms are capable of penetrating these barriers and can thereby gain access to the underlying tissues. There, they are encountered by immunological defense mechanisms and may elicit an inflammatory reaction. Inflammation is the process by which the host attempts to counteract the invading pathogen. Although it is essential, inflammation can be harmful to the host and therefore it is also implicated in the pathology of infectious diseases. We usually focus on offensive mechanisms employed by bacterial pathogens, but it is often the defensive response of the host that cause the primary manifestations of disease (43, 135). Dinarello (84) stated that “inflammation is the price the host pays for an efficient and effective defense system. In the extreme, death is the price paid for an effective host response”.

2.1. Central role of phagocytes

The mononuclear phagocyte system (MPS) was first postulated more than 30 years ago. Today the MPS has been widely accepted as a replacement for the reticulo-endothelial system (RES) (274). Mononuclear phagocytes are a diverse, widely distributed group of cells, characterized primarily by their powerful phagocytic capacity, whose chief function is thought to be in host defense and the immune response. All macrophages are derived from common precursors in the adult bone marrow, circulate as mature monocytes for 1 to 3 days, and then leave the bloodstream to enter peripheral tissues as differentiated macrophages, where they remain for 1-3 months and then die.

According to their localization in tissues and body cavities, macrophages vary in their morphological and functional characteristics and therefore have different names, e.g., Kupffer cells in the liver, pulmonary and alveolar macrophages in the lung, and microglial cells in the CNS (310).

Macrophages play a central role in the initiation and carrying out of both innate and adaptive immune responses. Monocytes respond to chemotactic signals from infectious agents by leaving the blood vessels and migrating toward the site of inflammation, where they differentiate into exudate macrophages. Influx of monocytes into a site of acute inflammation generally occurs after the influx of neutrophils (PMN), and mononuclear cells (macrophages and lymphocytes) predominate at sites of chronic inflammation. The delayed and continued recruitment of monocytes is mediated by chemotactic factors derived from inflammatory cells, as well as from persistent infectious or inflammatory agents. Egress of leukocytes from blood vessels depends on margination and adhesion to the endothelial cells lining the vessels, events that are closely regulated (193).

Mononuclear phagocytes participate in immunological protection through classical pathways of phagocytosis and through novel mechanisms involving oxygen-free radicals, reactive nitrogen intermediates, and other mediators. Mononuclear phagocytes are now recognized as a source of many important secreted substances that are involved in different aspects of the immune and inflammatory systems. For example, macrophage-derived proteins act during mobilization of systemic inflammatory responses (such as the induction of the hepatic acute phase reaction), during homeostasis, hematopoiesis, wound healing, and so on (165, 310).

2.1.1. Bacterial interactions with phagocytes: Consequences in disease

Mononuclear phagocytes can phagocytose non-opsonized and opsonized microorganisms. Macrophages are able to restrict the growth of or to kill

ingested microorganisms. This antimicrobial activity is mediated by processes that can be grouped into oxidative and non-oxidative mechanisms. Oxidative mechanisms are mediated by the production of reactive oxygen intermediates and, in rodents, also by reactive nitrogen intermediates. Non-oxidative mechanisms include phagosomal acidification, nutrient deprivation, and the action of antimicrobial polypeptides (e.g., lysozyme, defensins, cathepsin, etc.). These mechanisms can occur alone or in concert to create an environment that is unfavorable for the multiplication of, or lethal to, ingested microorganisms (310).

Several receptors that are involved in microbial recognition are present on phagocytic cells. The criteria for a receptor are that it binds ligand in a specific and saturable manner and then transmits a biological signal to the cell. This is in contrast to a "binding site", which binds ligand but elicits no biological signal. Many binding sites have been described that have the potential to serve as microbial recognition structures by cooperating with phagocytic receptors on the cell. One example of these binding structures is the cell surface proteoglycans (57, 87, 231).

Phagocytic receptors may be grouped according to a broad interpretation of the primary ligands they recognize:

I. Receptors for particles opsonized with immunoglobulin G (IgG). They recognize the Fc protion of the IgG, and they are collectively termed the Fcγ receptors, and individually designated FcγRI, FcγRII, and FcγRIII. The Fcγ receptors are members of a large family that mediate a series of diverse functions critical to cellular immune responses, including phagocytosis, antibody-dependent cellular cytotoxicity, secretion of inflammatory mediators, generation of respiratory burst, and clearance of immune complexes (268). The contribution of Fcγ receptors in host defense is best inferred from *in vivo* observations. In the case of *S. pneumoniae* and GBS, there is an inverse relationship between type-specific antibody titers and susceptibility to disease (20, 96). In addition, several studies have demonstrated that type-specific

antibodies promote efficient bacterial clearance of pneumococcus, GBS, *H. influenzae* type b (19, 38, 253). There is clearly an accessory role for complement in many of these processes, which will be discussed subsequently.

II. Receptors for particles opsonized with complement. They are classified in three distinct receptors, named the CR1, CR3, and CR4, and recognize different components of the complement cascade. The CR1 is the receptor for C3b and C4b. The CR3, also termed Mac-1 or CD18/CD11b, preferentially recognizes the proteolytically inactivated fragment of fixed C3b, called iC3b, but also binds multiple diverse ligands, such as fibrinogen. It has been proposed that Mac-1 has two distinct binding sites, one for iC3b and a second lectin-like site, which may bind bacterial cell wall components, such as the lipopolysaccharide (LPS) of Gram-negative bacteria. The CR3 β chain (CD18) is common to two other related receptors, LFA-1 (CD18/CD11a) and CR4 or p150/95 (CD18/CD11c). These three receptors are commonly called the β2 integrins (5). CR4 binds iC3b and C3dg, and has also been implicated in bacterial binding to macrophages in the absence of opsonins (308). Complement-mediated mechanisms are especially evident both in the early clearance of bacteria by nonimmune hosts, and in the subsequent antibody-directed phagocytosis. The synergy between these receptors has long been appreciated. For example, *S. pneumoniae* fixes complement by the alternative pathway, but requires specific anti-capsular antibody to increase phagocytosis and killing (111). In the *H. influenzae* system, Noel et al. (197) demonstrated that even in the presence of opsonic antibody, optimal bacterial uptake depends on complement receptors.

III. Receptors for LPS (endotoxin). It is well established that exposure of macrophages to LPS causes a dramatic alteration in their physiology, such as the release of large amounts of inflammatory mediators implicated in the Gram-negative septic shock (that will be discussed in section 2.3.1). There are different proposed receptors for LPS (e.g., scavenger receptor, β2 integrins and CD14). However, not all of them transmit the macrophage-activating signals typically associated with endotoxemia. The CD14, a 55-kDa inositolphosphate-

linked glycoprotein, is the most important receptor for LPS implicated in endotoxemia (77, 303).

IV. Receptors for simple and complex saccharides. One of the most important is the mannose-receptor, capable of binding terminal mannose, fucose and to a lesser extent other hexoses. A galactose-specific and sialic acid-specific receptors have also been described (192). The mannose-receptor have been implicated in nonspecific microbial recognition and destruction. For example, mannose-containing capsular types of *Klebsiella pneumoniae* are ingested and killed while other capsular types are resistant (16). Internalization via this receptor is usually accompanied by a brisk respiratory burst (189). On the counter side, bacteria have several proteins that are also able to recognize mannose and other sugars on macrophage glycoproteins, such as the mannose-specific type 1 fimbriae of *E. coli*. This recognition mechanism that involves the interaction of a carbohydrate-binding protein (lectin) on the surface of one type of cell that combines with complementary sugars on the surface of another is called "lectinophagocytosis". Lectinophagocytosis is important in the host defense against bacteria in the absence of antibodies and/or complement (201).

V. The β1 and β3 integrin receptors. In addition to the well-described β2 integrins, mononuclear phagocytes express both β1 and β3 integrins that are implicated in both cell-cell as well as cell-matrix interactions (5, 189). The β1 integrin family, referred to as VLA antigens, is the largest family which includes receptors for fibronectin, collagen, and laminin (5). The fibronectin receptor (α5β1 or VLA-5) recognizes the Arg-Gly-Asp (RGD) binding domain of fibronectin. In addition, this receptor binds microorganisms that have been opsonized with fibronectin or that have fibronectin-like proteins on their surface (159). The presence of RGD sequences in proteins expressed on the surface of *Bordetella pertussis* suggests the possibility of an integrin-RGD interaction in the initial microbial recognition event (130). The addition of fibronectin to

macrophages also causes an increase in complement receptor-mediated phagocytosis (305).

There are two general concepts to be stressed concerning the recognition of microbes by phagocytic cells. The first is the concept of receptor redundancy and receptor cooperativity. Multiple receptors on the cell surface often participate in a given microbial recognition event. For example, macrophages use receptors for both IgG and complement to engulf encapsulated bacteria (146, 189). The second concept concerns the transduction of specific cellular signals following receptor ligation. Often, the receptor to which a microbe binds orchestrates many of the subsequent intracellular events during phagocytosis by transducing specific cellular signals. Some receptors, for example the mannose and Fcγ-receptors, are particularly well suited to direct particles to phagolysosomes and trigger a respiratory burst, whereas other receptors, for example the CR1, may not (165). Indeed, from this perspective, the immune response is designed to target microbes preferentially to those receptors on phagocytic cells capable of making the appropriate cellular responses.

Despite the existence of all this machinery for recognition and killing of pathogens, the latter have developed mechanisms for exploitation of mammalian host cell functions. As will be discussed in the following sections, intracellular bacteria can direct their own uptake by ensuring their intracellular survival at the same time, whereas extracellular bacteria may attach to cells by impairing or blocking the phagocytic machinery of the host cell. In addition, under different conditions the outcome of the interaction could be either cell activation or immunosuppression. Thus, the consequence of an interaction between a pathogen and a mammalian cell is determined firstly by which receptor is bound, and secondly by the bacterial adhesin engaged in the interaction. In addition to the ability to recognize an eukaryotic target, adhesins have inherent abilities to incite, subvert, or co-opt host defense systems (146). Virtually every pathogen has more than one adhesin, thereby conferring the capability to interact with more than one receptor and their attendant signal transduction systems (129, 146).

2.1.1.1. Bacterial uptake and intracellular survival

A surprising number of pathogens have co-opted the existing integrin-based system of targets and ligands. Knowing the communication between integrins and the internal cytoskeleton, intracellular pathogens engineer their own uptake, often beginning with recognition of integrins. Three strategies appear to be used to gain entry into the system. The first can be termed masking, whereby adsorption of the natural ligand for the integrin onto the bacterial surface (for example, C3b or iC3b) smuggles the pathogen along natural pathways. It has been demonstrated that uptake into phagocyte via Mac-1 may enable the microorganism to bypass a critical killing pathway; this is true for some intracellular bacteria such as *Rhodococcus equi*, *Legionella pneumophila*, and *Mycobacterium tuberculosis* (151, 205, 236). The second is most fittingly called ancillary ligand recognition, whereby the pathogen binds to carbohydrates on glycosylated integrins rather than to the RGD recognition site. It has been demonstrated that in the absence of opsonins, encapsulated GBS are able to enter and persist efficiently in macrophages by evading intracellular antibactericidal activities. Further studies indicated that GBS can interact with Mac-1 via the lectin-site in a C3-independent fashion, and that the GBS capsular polysaccharide mediates this interaction (3, 12, 269). The third process, true mimicry, is the rarest but perhaps the most sophisticated strategy, in which the pathogen expresses, for example, proteins containing the RGD binding motif (as shown for filamentous hemagglutinin or FHA of *B. pertussis*). In this system, the binding of the pertussis toxin to macrophage carbohydrates first up-regulates the integrin CR3. The activated CR3 then in turn binds the adhesin FHA, leading to bacterial uptake into the macrophage. It has been suggested that this binding is beneficial for *B. pertussis* since it may lead to ingestion of the bacteria without an accompanying respiratory burst (130). Therefore, under certain circumstances, complement receptors may represent a potential mechanism for entry into macrophages that is used by intracellular microorganisms to

circumvent the microbicidal responses associated with endocytic events mediated by other receptors, such as the Fcγ receptors.

The exploitation of host functions continues when bacterial pathogens become intracellular parasites. Nearly all invasive bacteria enter a membrane-bound vacuole as part of their invasion process, but their subsequent fates vary. Certain bacteria thrive within vacuoles that fuse with lysosomes, although little is known about their survival mechanisms. Others have developed mechanisms to prevent fusion of the pathogen-containing vacuole with lysosomes, thereby maintaining a protected niche inside the host cell. *Salmonella* is known to express several gene products that enhance intracellular survival by neutralizing lysosomal killing mechanisms. Pathogens such as *L. pneumophila*, and *M. tuberculosis*, which utilize an integrin receptor to enter macrophages, reside in host vacuoles that do not fuse with lysosomes. Other pathogens have developed more sophisticated mechanisms that allow them to escape from the vacuole and replicate in the cytosol. This is the case of *Shigella*, *Listeria* and *Rickettsia*. Interestingly, all three species have evolved a similar mechanism to propel themselves through the cytosol, by an actin-based motility. This direct cell-to-cell spread allows dissemination within tissues while the bacteria remain sheltered from bactericidal cells or host components such as circulating antibodies or complement (94, 99). In the case of *Salmonella*, which clearly persist in macrophages but with a limited extent of replication, macrophages could act as a "taxi" to deliver *Salmonella* to distal sites, where replication may occur in other cells, such as epithelial cells (94). This mechanism could be an analogue to the proposed "Trojan horse theory" for *S. suis* dissemination and pathogenesis (as discussed in section 1.5).

2.1.1.2. Bacterial adhesion and resistance to phagocytosis

Not all bacterial-host cell interactions result in cellular entry. Several bacteria that are pathogenic for the mammalian host adhere to the surface of

cells and remain extracellularly localized. Bacteria express specialized virulence factors to achieve attachment to host cells. Surface adherence may result from either direct attachment of the microorganism adhesins to receptors located on the cellular surface or from interaction with host polysaccharides and/or proteins (such as serum or extracellular matrix components), which in turn bind to host receptors (159, 283). For instance, many staphylococci and streptococci have been shown to bind fibronectin (159). In general microorganisms coated with fibronectin (which recognize multiple integrin receptors) are not efficiently phagocytosed (217, 262), although the adhesion of phagocytes to surface coated by fibronectin enhances the ability of other receptors to internalize microorganisms (142, 305). In addition, many pathogenic bacteria have learned to exploit cell surface glycoconjugates (such as glycoproteins, glycolipids, and proteoglycans) as receptors for attachment (57, 87, 160, 231).

An extracellular life-style is generally associated with highly encapsulated bacterial species. For example, the group B capsule of *N. meningitidis* is a major virulence determinant that decreases the affinity of the macrophage for the meningococcus. Only encapsulated organisms are recovered from the blood and CSF of patients with meningitis (221). Capsular polysaccharides are important surface determinants present on several pathogenic Gram-positive and Gram-negative bacteria. These structures morphologically represent the outermost layer of microorganisms and are polymers of repeating units. The CPS have antiphagocytic effects, in part due to their net electrostatic charge. It has been repeatedly shown that encapsulated organisms are more virulent than their unencapsulated variants. It is believed that the mechanism for enhanced virulence is related to the ability of encapsulated organisms to evade normal host-defense mechanisms (161). CPS are known to inactivate the alternative pathway of complement by at least two distinct mechanisms. One mechanism has been described for organisms that contain sialic acid in their CPS, such as group B *N. meningitidis* and GBS (221, 287). Sialic acid has a high affinity for inactivating proteins of the alternative pathway, particularly factor H, and renders the organism resistant to opsonophagocytosis in the absence of antibody.

Another mechanism is the decrease in binding affinity for factor B, implicated in the amplification loop of the alternative pathway of complement, as shown for type 7 and 12 pneumococcal capsules. Thus, virulent encapsulated bacteria have taken advantage of self-protective mechanisms against autoimmune disease by mimicking host oligosaccharides (161). The importance of the CPS in modulating the bacterial-cell interaction has also been largely demonstrated for *H. influenzae* type b. This microorganism binds to macrophages in the presence of nonimmune serum; however, binding is not evident in complement-depleted serum (197). This interaction leads largely to binding without ingestion. Thus, uptake inhibition by the CPS may involve not only the quantity and distribution of serum opsonins deposited on the bacterial surface but also their effect on direct bacteria-phagocyte interactions (195).

The bacterial CPS is able to modulate not only complement opsonization but also the deposition of other opsonins, such as the mannan-binding protein (MBP), an acute phase serum protein with potential anti-microbial activity. It has been shown that the majority of encapsulated pathogens causing meningitis (including *S. suis*) have a relatively low MBP binding capacity (272). In addition to the CPS, several pathogens have other surface components with the capacity to reduce bacterial opsonization. For example, Group A *Streptococcus* (GAS) express a virulence factor called M-protein, that inhibits complement fixation and prevents complement-mediated phagocytosis (168).

The ability of some bacterial pathogens to actively inhibit their uptake by professional phagocytes is another fascinating feature of exploitation of host functions. *Haemophilus ducreyi* associates with phagocytes and remains extracellular throughout infection and survives by resisting phagocytic killing *in vivo* (21). Similarly, *Helicobacter pylori* is able to inhibit its own uptake by an active process inhibiting the global function of professional phagocytes (219). Furthermore, extracellularly adherent *H. pylori* and *H.ducreyi* are able to induce and survive the extracellular release of toxic oxygen metabolites. Bacteria would be able to counteract the induced respiratory burst by producing enzymes such as the superoxide dismutase and catalase, which may protect the organism from

this lethal effect (21, 171, 218). Together, these bacterial properties would be likely to increase the tissue injury induced during infection. Other pathogens also inhibit their uptake or the uptake of other nonrelated prey by professional phagocytes. Enteropathogenic *E. coli* and *Yersinia* species use type III secretion mechanisms to insert effector proteins into the host cells, leading to an impairment of the general phagocytosis pathway (95, 110).

Finally, another mechanism to escape from phagocytic uptake and killing is by cell damage either by secreted protein toxins or by activation of the cellular program of apoptosis or programmed cell death (32, 99).

2.1.1.3. Cell activation

Cell activation is another consequence of bacterial interaction with phagocytic cells. In addition to the ability of directing leukocyte mobility and phagocytosis, several adhesins are now recognized to induce inflammatory mediators, such as cytokines, in host tissues (146). Binding of bacteria to phagocytes has been shown to be sufficient to stimulate the release of cytokines (4, 304). Whether or not the cytokines released as a result of this interaction actually play a role in adhesion remains to be determined. However, their overproduction may have pathological consequences for the host. Bacterial invasion was also shown to be accompanied by cytokine activation (15, 295). Furthermore, cytokines and/or their receptors are in some cases involved in the process of cell invasion (133).

Bacteria possess an intrinsic capacity to induce the production of a wide panel of cytokines. In the case of streptococci, whole bacteria and numerous surface compounds efficiently trigger the synthesis of cytokines by phagocytic cells. In addition, released bacterial proteins can also stimulate phagocyte-derived cytokines (45, 133). Thus, bacteria seem to be able to modulate the cytokine response during inflammation, by perturbation of cytokine networks (a concept that will be discussed in the following sections).

2.2. Inflammatory mediators: the cytokine network

The various strategies utilized by the immunoinflammatory system to deal with exogenous microorganisms rely heavily on the family of intercellular signalling proteins known as cytokines. These molecules are essential local and systemic hormones and major homeostatic mediators which form part of the system of innate immunity (Fig. 2).

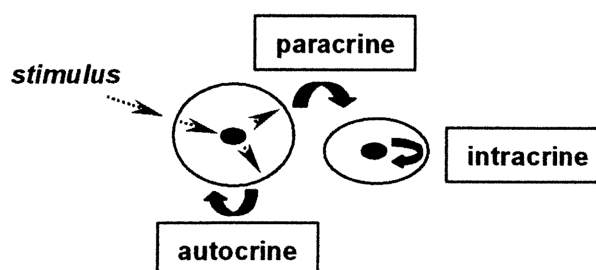


Fig. 2: Diagrammatic representation of cytokine mechanisms of action. Adapted from Henderson et al. (133).

To date, upwards of 200 cytokines have been identified and many have been cloned and their receptors defined. It is now clear that some of these cytokines are involved in the induction of inflammation, while other cytokines play a role in down-regulating inflammatory processes. It is envisaged that it is the balance between these cytokines, along with other factors (e.g. other mediators such as eicosanoids, nitric oxide, soluble cytokine receptors, etc.) that controls the induction, perpetuation and cessation of inflammation. Indeed, it is now realized that cytokines rarely, if ever, act in isolation but rather act to induce, or inhibit, other cytokines, creating a network of cytokines to which cells respond. Cytokine network will be thus defined as "the complex interactions of cytokines by which they induce or suppress their own synthesis or that of other

cytokines or their receptors, and antagonize and synergize with each of other in many different ways" (134, 295).

The immunoinflammatory system, however, contains a paradox. Cytokines, which are central to the protective role of this system, are also the major mediators of the pathology which accompanies infections. The most striking example of this is Gram-negative septic shock. Experimental septic shock can be prevented by neutralizing pro-inflammatory cytokines or by administering a immunomodulatory or anti-inflammatory cytokine. This illustrates the importance of the balance of the cytokine network in the control of tissue pathology and the pivotal nature of this balance to host survival (46, 134, 288).

2.2.1. The pro-inflammatory cytokines

Historically, cytokines have been subdivided into families such as the interleukins, chemokines, interferons, etc. However, this classification is not particularly useful and, in the case of the interleukins, is misleading. Indeed, one of the characteristic feature of cytokines is their pleiotropy, although this should not be interpreted as redundancy, as it so often is. Wilson et al. (295) proposed that in the immunoinflammatory system, the most appropriate subdivisions of cytokines are (I) those that modulate leukocytes to produce pro-inflammatory responses and (II) those that have the capacity to down-regulate inflammatory cells (which will not be discussed in this review).

Within hours of infection, phagocytic cells, and also antigen-presenting cells, produce interleukin-12 (IL-12), which is one of the most important links between natural and adaptive immunity (229). IL-12 orchestrates the high expression of interferon-gamma (IFN-γ), as well as other cytokines, which then act on phagocytic cells by potentiating their activity. Thus, IL-12 and IFN-γ comprise a powerful amplifying mechanism of macrophage-induced inflammatory cascade. If uncontrolled, this physiological response may result in pathology due to cytokine dysregulation. The most important pro-inflammatory

cytokines in this inflammatory cascade are tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6). Monocytes in the inflammatory exudate are the major origin of these pro-inflammatory cytokines (165, 288).

2.2.1.1. Tumor necrosis factor alpha

The tumor necrosis factor alpha (TNF-α) plays a pivotal role in the pathogenesis of inflammation, cachexia, toxic shock, and tissue injury (248). Cells derived from the monocyte/macrophage lineage are the principal source of TNF, but it is also made by glial cells in brain, Kupffer cells in the liver, keratinocytes in skin, mast cells, NK cells, T cells and B cells. Several factors produced by bacteria, viruses, and parasites are capable of inducing cells to produce TNF. The list of TNF-stimulating factors includes endotoxin (LPS), enterotoxins, toxic shock syndrome toxins, bacterial cell wall, and products of complement activation. LPS is an extremely potent stimulus to TNF biosynthesis (261).

TNF initially exits as a 26-kDa cell-surface associated molecule anchored by an N-terminal hydrophobic domain. Membrane-bound TNF possesses bioactivity and may function as nondiffusible mediator of cell-cell interaction in costimulatory signalling for B-cell activation by T-cells. TNF is subsequently processed by proteolytic cleavage to 17-kDa peptides, which form a biologically active homotrimeric complex. The serum half-life after intravenous administration of recombinant TNF is short (20 to 40 min). TNF disappears from circulation by binding to TNF receptors or following clearance of the protein by the kidneys and liver. TNF interacts with at least two distinct membrane-associated receptors termed TNF-R1 (55-kDa) and TNF-R2 (75-kDa), which exhibit similar receptor binding affinities for TNF (72). Both receptors are present virtually in all types of cell, excluding red blood cells and also exist in soluble forms in the serum. These soluble TNF receptors are also termed TNF-binding proteins, since they bind the cytokine and compete for TNF with cell associated receptors. Thus, interpretation of serum TNF concentrations during infection

may be confounded by its stereotypical pattern of transient appearance (so that the TNF peak may be missed), and by the release of soluble TNF receptors which remove bioactivity but may not block detection in immunoassays (261).

As mentioned above, TNF plays a major role in the septic shock syndrome (248). Most of the injurious sequelae of septic shock are attributable to the effects of TNF and TNF-triggered cytokine cascade. Once triggered, this process may proceed unimpeded for hours or days, is self-propagating and mediates potentially lethal toxicity by synergistic interactions. This sequence of events drives the septic shock syndrome, and explains the apparent dissociation of the clinical syndrome from the underlying infection. Indeed, Koch's postulates may be applied to an analysis of the role of TNF in this process. Firstly, TNF is produced in septic shock syndrome, both in patients and in experimental models of the disease. Secondly, passive transfer of TNF to normal animals or humans induces the septic shock syndrome. Thirdly, removing TNF activity from these animals prevents the development of lethal shock and tissue injury (261).

The hemodynamic, metabolic and pathologic sequelae of TNF are virtually indistinguishable from the syndrome caused by lethal bacteremia or endotoxemia. These manifestations included hypotension, increased fluid requirements, acute renal tubular necrosis, pulmonary edema, disseminated intravascular coagulopathy, initial increases followed by later decreases of serum glucose, and a cascade of cytokine and stress hormone release. The administration of other putative mediators of shock, such as IL-1, may trigger individual components of this syndrome, but TNF is the only endogenous mediator identified so far that is sufficient to trigger the entire spectrum of metabolic, hemodynamic, tissue and cytokine cascade responses (46, 248).

Whereas an acute overproduction of TNF triggers septic shock syndrome, chronic overproduction of TNF can cause a syndrome of anorexia, catabolism, weight loss and anemia. Termed cachexia, this state of protein and energy depletion contributes to the morbidity and mortality rates of chronic diseases. The metabolic defect in cachexia is related to the inability of the host to

conserve protein, and since mammals do not possess a pool of stored protein, net loss of protein correlates with net loss of function (46, 261).

The mentioned toxic effects occur not only by direct action of TNF on host cells, but also by the interaction with a cascade of other endogenous mediators of inflammation. Indeed, it appears that there is some kind of hierarchy with TNF inducing IL-1, and IL-6 being induced by either IL-1 or TNF (46).

2.2.1.2. Interleukin-1

The IL-1 family consists of three structurally related polypeptides. The first two are interleukin-1α and interleukin-1β, each of which has a broad spectrum of both beneficial and harmful biologic actions, and the third is IL-1 receptor antagonist, which inhibits the activities of IL-1 (82).

The two forms of IL-1, α and β, are the products of separate genes. They have different amino acid sequences, but are structurally related at the three-dimensional level, act through the same cell-surface receptors, and share biological activities. Both IL-1α and IL-1β are rapidly synthesized by mononuclear cells, primarily monocytic phagocytes, that have been stimulated by microbial or inflammation products. Most IL-1α remains in the cytosol of cells in its precursor form, where it may function as an autocrine messenger. There is also evidence that the precursor is transported to the cell surface and associated with the membrane. This membrane-bound precursor is biologically active, perhaps serving as a paracrine messenger to adjacent cells. On the other hand, a considerable amount of IL-1β, is released by the cell into the extracellular space and the circulation. The mechanisms of release include exocytosis from vesicles, active transport, or release after cell death. Unlike the IL-1α precursor, the IL-1β precursor must be cleaved for optimal biologic activity. Several common enzymes cleave the precursor into smaller, more active forms, but one protease appears to be highly specific for cleaving the IL-1β form from 31-kDa to 17.5-kDa, its most active form. This enzyme is known as the IL-1β-

converting enzyme. It is an intracellular protease, a member of the cysteine protease family. Although IL-1-β-converting enzyme does not cleave the IL-1α precursor, nonspecific proteases that do so have been identified (82, 83).

In some instances, the production of IL-1 is an important rapid and direct determinant of disease, whereas in other instances its production is an intermediate step. In septic shock, for example, IL-1 acts directly on the blood vessels to induce vasodilatation through the rapid production of small mediator molecules such as platelet-activating factor, prostaglandins, and nitric oxide. These substances are potent vasodilators and induce shock in laboratory animals. Other systemic effects of IL-1 are the ability to induce fever, sleep, anorexia, and hypotension. IL-1 also stimulates the release of pituitary hormones, and increases the synthesis of collagenases, resulting in the destruction of cartilage (84, 85).

Plasma levels of IL-1β are usually below the limit of detection of the available assays in normal subjects, but are detectable in patients with sepsis, or other pathologies. Plasma IL-1α is rarely detected in such patients, even though the assays for IL-1α are more sensitive than those for IL-1β. The lack of circulating IL-1α is consistent with the observation that cultured cells do not release the α form under conditions that result in the release of the β form (85).

A particularly important local property of IL-1 is thought to be the stimulation of endothelial adhesion molecules and of leukocyte emigration (84). The induction of PMN and monocyte chemotactic cytokines, which are important players in local inflammation, probably mediate some of the effects of IL-1 (discussed in section 2.2.2). On the other hand, IL-1 also plays a role in the production of PMN from the bone marrow. Indeed, IL-1 stimulates the early stem cell in the bone marrow, induces the production of colony-stimulating factor, with consequent proliferation of PMN and monocyte precursors (84).

Administration of IL-1, to humans or experimental animals, induces pathophysiologic changes which mimic the host response to infection. In addition, the biological properties of IL-1 share remarkable similarities to those

of TNF. When the two cytokines are used together in experimental studies, the net effect often exceeds the additive effect of each cytokine (84).

2.2.1.3. Interleukin-6

Interleukin-6 is a multi-functional cytokine that plays a central role in host defense mechanisms, regulation of the immune responses, B-cell differentiation, immunoglobulin production, and hematopoiesis. In addition, IL-6 is probably the major physiological inducer of acute phase protein synthesis in the liver, with IL-1 and TNF playing only subsidiary roles. IL-6 may also have an important role in corticosteroid release during inflammation (288).

IL-6 is produced by a variety of cells, but stimulated monocytes, fibroblasts and endothelial cells are probably the main sites of production *in vivo*. IL-6 is secreted by monocytes in at least five different molecular forms, that result of post-translational changes such as glycation and phosphorylation, with molecular masses between 21- and 28-kDa. The IL-6 receptor shows similarities to that of IL-1 in possessing an immunoglobulin-like domain (288). IL-6 receptor was found to belong to the cytokine receptor family. The receptor consists of two chains, gp80 implicated in ligand binding and gp130 which is the signalling transducer. A soluble IL-6 receptor was also described which actually enhances IL-6 activity by transporting IL-6 to the gp130 signal transducer unit of the receptor (72, 143). The distribution of specific high affinity receptors for IL-6 on different cells, including activated B cells, resting T cells, and several hematopoietic cell lines, reflects the broad distribution of responsive target cell types (2, 288). Indeed, IL-6 acts on a wide range of tissues, exerting growth-inducing, growth-inhibitory and differentiation-inducing effects, depending on the nature of the target cells (144).

IL-6 is produced early in inflammation (shortly after IL-1 and TNF) and displays several proinflammatory properties such as maturation and activation of PMN and macrophages, and differentiation and maintenance of cytotoxic T cells

and natural killer cells. In addition, *in vivo* and *in vitro* evidence supports the concept that the IL-6 system plays an unexpected positive role in local inflammatory reaction by amplifying leukocyte recruitment, at least in part by augmenting the local production of chemokines (2, 230, 300).

Like IL-1 and TNF, IL-6 is an endogenous pyrogen and an inducer of acute-phase responses. Since IL-1 and TNF induce IL-6 production, levels of IL-6 often correlate with the amount of fever and severity of disease in patients with infections. In fact, the best correlations of the severity of an infectious disease with any cytokine is clearly with the levels of IL-6, not IL-1 or TNF (45, 84).

As already mentioned TNF, IL-1 and IL-6 share similar biological properties and it is likely that the pathophysiologic events of infectious or inflammatory diseases is due to the synergism between these highly pleiotropic molecules. Fig. 3 summarizes the systemic effects of these three proinflammatory cytokines.

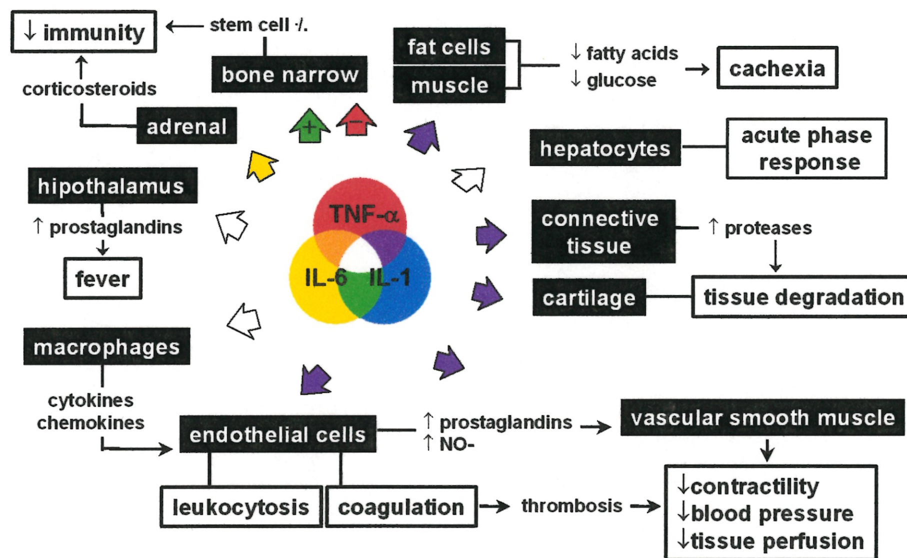


Fig. 3: Systemic effects of pro-inflammatory cytokines: TNF-α, IL-1β, and IL-6.

Cytokines not only play an important role in septic shock, but also in local inflammation, as is the case of CNS infection leading to meningitis. Bacterial infection of the CNS, after blood dissemination, is achieved by increased blood-brain-barrier (BBB) permeability and induction of local inflammation, which in turns facilitates further bacterial entry into the CNS (Fig. 4). It was suggested that common host mediators are responsible, at least in part, for these events. Intracisternal inoculation of IL-1 into rats led to increased BBB permeability, whereas TNF inoculation elicits subarachnoidal space (SAS) inflammation. Inoculation of both cytokines potentiates these effects (212, 264). Lymphocytes bind to CNS endothelial cells with lower affinity than to endothelium derived from other organs. After stimulation with proinflammatory cytokines, such as IL-6, TNF, and IL-1, lymphocyte adhesion to the cerebrovascular endothelium increases and becomes similar to that observed in other organs. This finding may explain why the BBB endothelium becomes permeable to hematogenous inflammatory cells during CNS inflammatory conditions (108, 249, 264). Indeed, elevated levels of TNF, IL-1, and IL-6 are present in the CSF during bacterial meningitis (45, 273) (Fig. 4). These proinflammatory cytokines will promote chemokine expression, that, in turn, will increase leukocyte transmigration and further BBB leakage, as described subsequently (108). Supporting evidence that the release of endogenous cytokines into the CSF causes injury comes from studies demonstrating that the CSF inflammatory response is reduced by the simultaneous inoculation of antibodies to TNF and IL-1 together with the meningeal agent (212). The early appearance of cytokines, such as TNF, IL-1, IL-6, in CSF prior to the increase of leukocytes shows that these cytokines are released from cells normally present in the CSF, such as endothelial cells, microglial cells, and astrocytes, which indicates that they play a role in the initial phase of the local inflammation reaction. Subsequently, the monocytes in the inflammatory exudate are the major origin of the cytokines (273).

Thus, the ability of meningeal pathogens to induce a marked SAS inflammatory response contributes to many of the pathophysiologic consequences of bacterial meningitis.

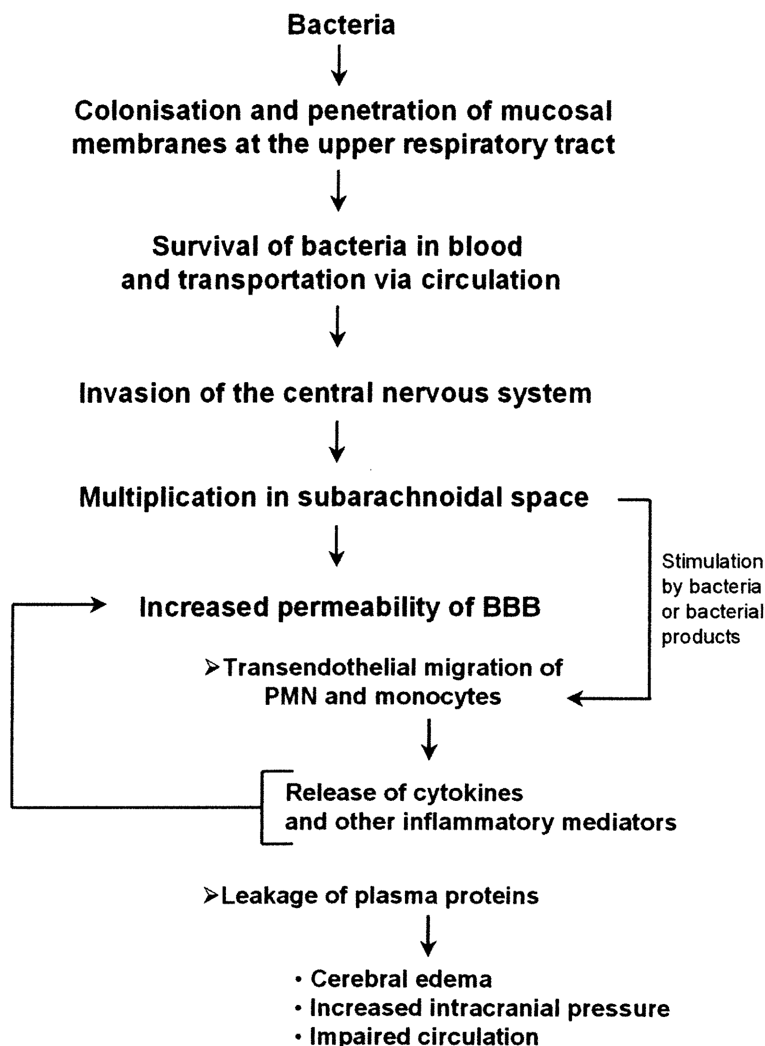


Fig. 4: Sequential steps of the pathophysiology of bacterial meningitis. First, bacteria that colonize epithelial cells of the upper respiratory tract cross the mucosal barrier and enter the bloodstream, where they survive mainly by virtue of their polysaccharide capsule. Next, bacteria invade the central nervous system and multiply in the subarachnoidal space. At that site, bacteria and bacterial products stimulate the production and release of cytokines by various types of cells, which in turn increase the permeability of the blood-brain-barrier (BBB). This results in transendothelial migration of granulocytes (PMN) and monocytes and leakage of plasma proteins into the subarachnoidal space, which play a role in the elimination of bacteria but can also have deleterious effects on the central nervous system. Adapted from van Furth et al. (273).

2.2.2. The chemokines

Leukocyte infiltration is a hallmark of inflammation. The accumulation of leukocytes at sites of inflammation is induced by the local production and secretion of chemotactic ligands by a wide variety of stimulated cell types. Recently, several host-derived cytokines, named chemokines, have been identified that stimulate chemotaxis *in vitro* and elicit the accumulation of various types of inflammatory cells *in vivo*. Monocytes/macrophages are a rich source of chemokines (18, 203).

Chemokines constitute a large family of small inflammatory cytokines involved in the recruitment and activation of a wide variety of cell types. The nomenclature of chemokines is based on the position of conserved cysteines within the amino acid sequence (18, 169, 203). Chemokines can be divided into four groups depending on whether the first pair of cysteines is separated (C-X-C) or not (C-C) by an intervening amino acid, whether the second cysteine is missing (C), or whether the first pair of cysteines is separated by three amino acids (C-X_3-C) (18, 72). The C-X-C or α subfamily of chemokines has strong PMN chemotactic and activating properties, with IL-8 being the prototype member. Members of the C-C or β subfamily have relative specificity for the elicitation of mononuclear cells (macrophages and T cells), with monocyte chemotactic protein one (MCP-1) being the best characterized member (18, 72). Finally, lymphotactin, the only C chemokine known so far, is mainly chemotactic for T cells, whereas the C-X_3-C chemokine fractalkine or neurotactin has been reported to act as a chemoattractant for T cells, monocytes, and PMN (72).

As mentioned above, meningitis is accompanied by a differential immigration of leukocytes into the SAS; however, the mechanisms regulating leukocyte invasion are still incompletely understood (249). Although the brain is considered a "privileged" organ, lacking a routine immunological surveillance and containing only 1-5 leukocytes/μl CSF under normal conditions, more than 10^5 leukocytes may accumulate during the acute phase of a bacterial meningitis.

Characteristically, PMN have been found to be the first leukocytes at the onset of the disease, but the cellular picture gradually changes to a mononuclear pattern consisting mainly of monocytes and lymphocytes (249). It is still unclear how leukocytes leave the circulation and migrate through the tight endothelial cell barrier of the brain vessels, the BBB (108). In particular the exact mechanism regulating the directed migration of distinct leukocyte populations to an inflammatory site within the CNS is poorly understood. Based on studies in other tissues, this process is thought to comprise the following steps: rolling of leukocytes along the vessel walls, activation of integrins on the surface of the leukocytes, activation dependent arrest of the cells and finally migration through the vessel wall (169) (Fig. 5). Chemokines are thought to play a role at two steps. Firstly, chemokines link the surfaces of endothelial cell and leukocytes, which results in the activation of integrins at the cellular membrane of leukocytes. Secondly, leukocytes are thought to migrate through the endothelial cell layer along a gradient of chemokines and other chemoattractant factors (169). The immobilization of chemokines by binding to surface proteoglycans or to components of the extracellular matrix is thought to be important for the maintenance of the chemokine gradient needed for leukocyte activation and transmigration into tissue spaces (18).

Chemokines mediate their activities by binding to target cell surface seven-transmembrane helix receptors. According to their ligands, the receptors are divided into families, the C-X-C receptor family and the C-C receptor family, which are mainly expressed on the surface of cells of the hematopoietic lineage. However, expression of several different chemokine receptors has been detected in the CNS, the functional significance being not yet clear (107, 169).

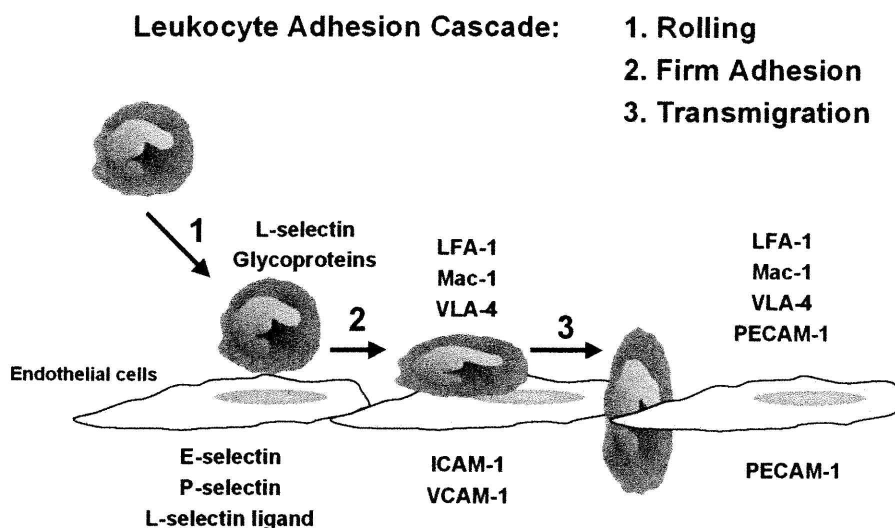


Fig. 5: Sequential steps of leukocyte migration through the endothelial layer. Leukocyte recruitment to sites of inflammation is mediated by several families of adhesion molecules present on the surface of leukocytes and endothelial cells. These include selectins (E-, P-, and L-selectin) which mediate rolling of leukocytes on endothelial cells by binding to glycoproteins and other glycoconjugates (184). Firm adhesion and transmigration is mediated mainly by the β_2 integrins (LFA-1 and Mac-1), the β_1 integrin VLA-4, and members of the immunoglobulin superfamily, such as intracellular adhesion molecule one (ICAM-1), vascular cell adhesion molecule one (VCAM-1), and platelet/endothelial cell adhesion molecule one (PECAM-1) (5).

High levels of chemokines are detected in the CSF of patients with bacterial meningitis (249), and neutralizing antibodies against different chemokines significantly decrease the recruitment of leukocytes into the CNS in experimental animal models (81). In addition, all CNS cell types crucial for proper BBB functioning are able to express chemokines *in vitro* (108). Thus, the interplay between chemokines, pathogenic microorganisms, and host inflammatory cells is likely to be major determinant of the clinical presentation and outcome of meningitis.

2.2.2.1. Interleukin-8

Interleukin-8 was first purified as a chemotactic factor for PMN. However, subsequent studies demonstrated that IL-8 exhibits multiple effects on PMN, including induction of shape change, release of lysosomal enzymes, induction of respiratory burst, generation of superoxide, hydrogen peroxide, and bioactive lipids. IL-8 also increases the expression of adhesion molecules on PMN, as well as on the endothelium, with consequent increased transendothelial migration (191) (Fig. 6). Most cell types produce little, if any, IL-8 constitutively. However, certain cell types, including endothelial cells, monocytes-macrophages, and PMN produce large amounts of IL-8 when stimulated by other cytokines, such as TNF and IL-1, or bacterial products, such as LPS. In the CNS, in addition to endothelial cells and macrophages, astrocytes can also synthesize IL-8 (18, 107, 179).

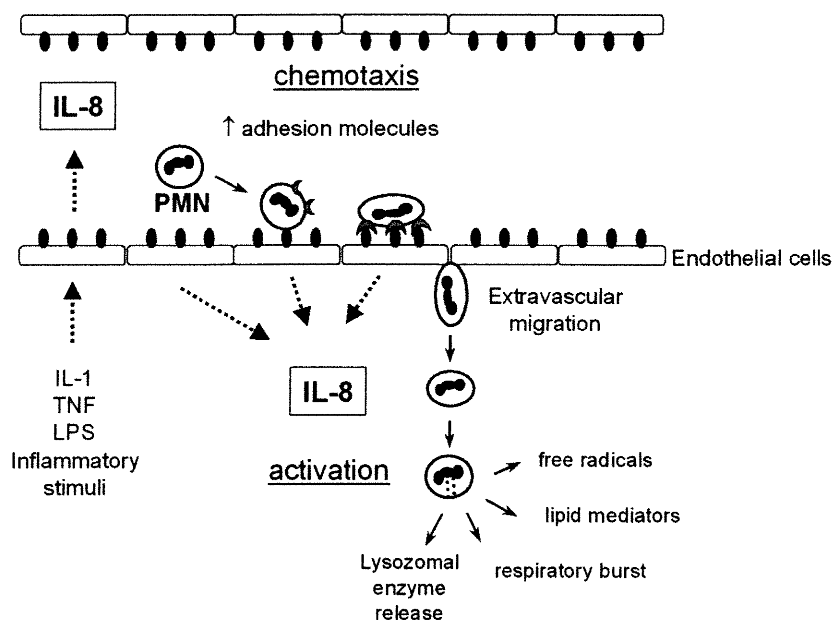


Fig. 6: Biological functions of IL-8 on chemotaxis, extravascular migration, and activation of neutrophils (PMN). Adapted from Mukaida et al. (191).

IL-8 plays a key role in host defense mechanism through its effects on PMN activation, but a continued presence of IL-8 in the circulation in response to inflammatory conditions may lead to a variable degree of tissue damage. In this regard, numbers of PMN in the CSF of patients with bacterial meningitis correlated with IL-8 levels. The consequence of activated PMN is pain, tissue damage, inflammation, and cell death (249).

2.2.2.2. Monocyte chemotactic protein-1

Monocyte chemotactic protein-1 was purified as a monocyte chemotactic factor from culture supernatants of various type of cells. Subsequent studies revealed that MCP-1 has multiple functions against monocytes/macrophages, such as induction of respiratory burst, expression of β2-integrins, release of lysosomal enzymes and induction of pro-inflammatory cytokine production, such as IL-1 and IL-6 (18, 191) (Fig. 7). MCP-1 is also a potent attractant for T lymphocytes and natural killer cells (17). As observed for IL-8, IL-1 and TNF are major stimuli of MCP-1 expression in a wide variety of cells, including endothelial and epithelial cells. MCP-1 is also produced by several CNS cells (107). However, monocytes are the major source of MCP-1 (18).

Elevated levels of MCP-1 were found in the CSF of patients with pyogenic meningitis. These patients suffered from pneumococcal, meningococcal and *H. influenzae* bacterial meningitis (108). Low levels of MCP-1 are also found in the CSF of normal individuals, thus the presence of some monocytes and T cells in the CSF of healthy people may be due to the low constitutive expression of MCP-1. Taken together, MCP-1 may contribute to the recruitment of monocytes in both physiological conditions and in acute bacterial meningitis. Furthermore, administration of anti-MCP-1 antibodies significantly decreased macrophage infiltration in the CNS during experimental bacterial meningitis (81, 108, 169).

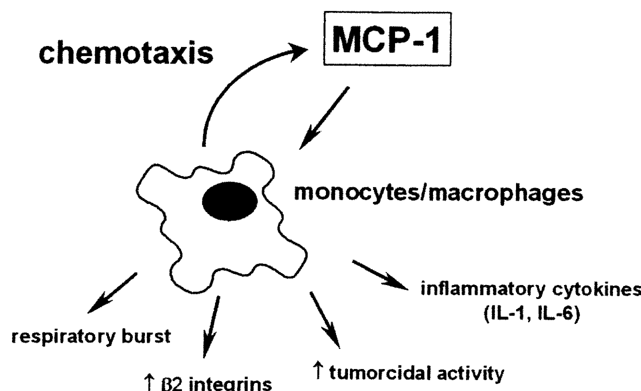


Fig. 7: Major biological functions of MCP-1. Adapted from Mukaida et al. (191).

2.3. Bacterial modulins

Currently, it is accepted that the interaction between microorganisms and host cells is a linear causal chain of events in which the exogenous organism induces the host to produce cytokines which warn and protect the host or induce tissue pathology, or both. There is growing evidence that this is a simplistic picture of the true system and that microorganisms exert a more fundamental control of the cytokine network than had previously been thought possible. The clearest example of this is the recent finding that viruses produce a range of cytokine homologues, including soluble forms of cytokine receptors and anti-inflammatory cytokines such as IL-10, which act to inhibit host protective cytokine networks. Possibly, the most significant finding in this regard is that knockout mice for certain transgenic cytokine genes (IL-2, IL-10) die as a result of their response to their own normal bacteria flora. Finally, recent studies have revealed that bacteria produce a wide range of molecules able to induce both pro- and anti-inflammatory cytokines and thus influence host cytokine networks (134). Henderson et al. (133) suggested the term "modulin" to describe these

bacterial cytokine-inducing molecules which modulate cell activity. On the other hand, Wilson et al. (295) proposed that "the ability of bacterial components (or bacterial activities) to induce cytokine release from host cells can be regarded as an aspect of bacterial virulence, only when this response results in pathology due to its intensity and/or chronicity".

2.3.1. Gram-negative bacteria: the example of LPS

The surface components of Gram-negative bacteria form an extremely complex structure consisting of an inner membrane, a periplasmic space containing peptidoglycan (PG) and an outer membrane containing a range of so-called outer membrane proteins and a unique polymorphic molecule, the LPS (Fig. 8). LPS is one of the most potent inflammatory bacterial mediators and has been strongly implicated in the inflammatory response associated with Gram-negative sepsis. It is thus important to define the terms "LPS" and "endotoxin". The term LPS should be reserved for purified bacterial extracts which are reasonably free of detectable contaminants, particularly protein. In contrast, the term endotoxin should be used to refer to products of extraction procedures which result in macromolecular complexes of LPS, protein and phospholipid (145). Thus, it is important to realize that the terms endotoxin and LPS are not interchangeable and that the proteins in endotoxin have a range of potent biological activities in their own right (134).

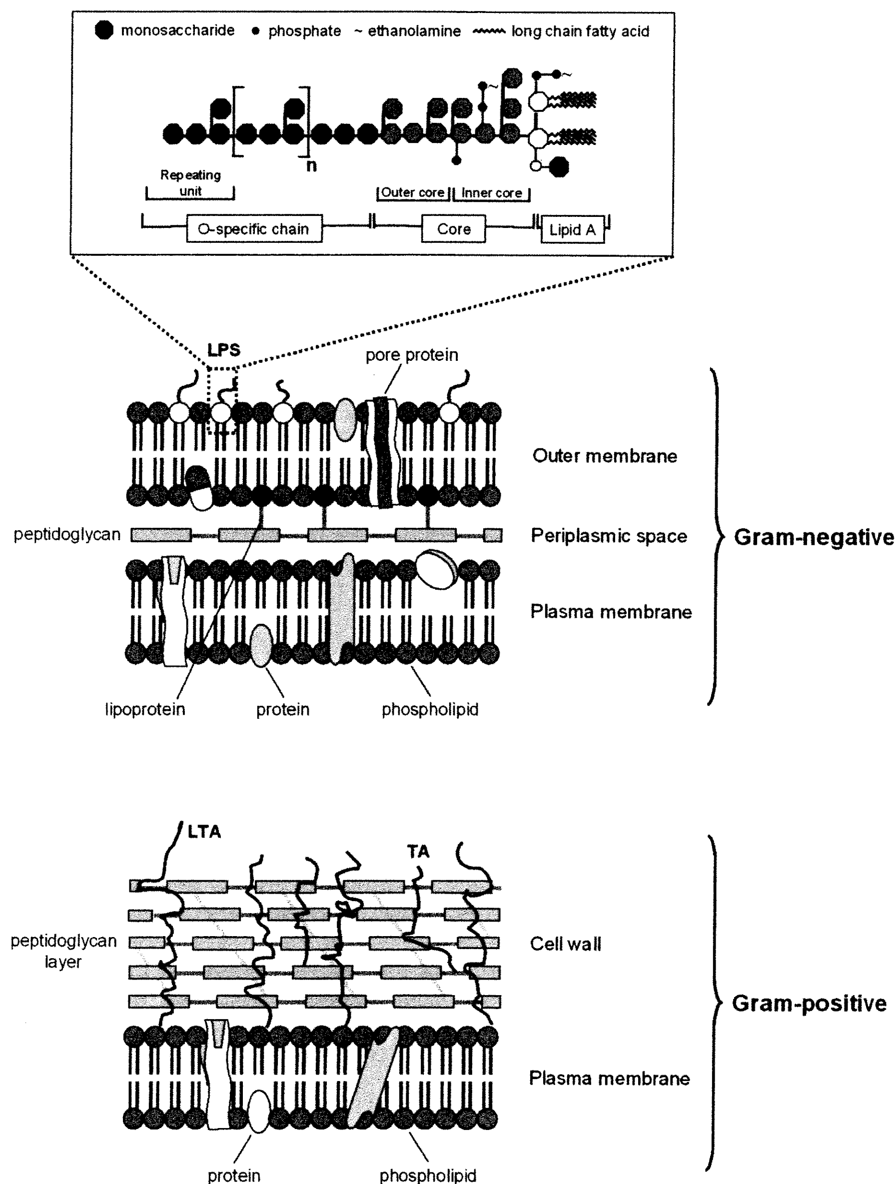


Fig. 8: Comparison of the cell wall structures of Gram-positive and Gram-negative bacteria, with an insert showing the structure of lipopolysaccharide (LPS). Teichoic acids (TA) and lipoteichoic acids (LTA) are major components of Gram-positive cell wall. TA consist of chains of glycerol, ribitol, mannitol, or sugars linked by phosphodiester bonds and are attached to muramic acid residues in the peptidoglican. LTA consist of chains of glycerol phosphate, with D-alanine and sugar substituents, attached to a glycolipid in the plasma membrane. Adapted from Glauser (109).

The LPS molecule can be divided into 3 regions. That part of the LPS molecule furthest from the outer membrane contains repeating carbohydrate units that represent the O-antigens (and is termed the O-antigenic side chain). Moving inwards, the next region of the LPS is termed the core region and consists of an outer and inner core. The outer core exhibits a range of structural variation, depending on the bacterium, whereas the inner core is more uniform and is characterized by the presence of the unusual sugars heptose and 2-keto-3-deoxyoctonoic acid (KDO). The final component of the LPS molecule is lipid A (Fig. 8). LPS is very tightly associated with the outer membrane proteins via the lipid A structures. It has been recognized for many years that the "endotoxic" activities of LPS are due to this lipid A region, with the fatty acid residues being important determinants of activity (133, 134).

One of the most important discoveries, in terms of understanding how the cytokine network is controlled in Gram-negative infections, is that endotoxin is, by itself, relatively inactive. It is only the complex of the active component of endotoxin with host factors which confers on endotoxin its remarkable biological potency. Indeed, LPS exhibits binding activity to a variety of host proteins, the most important being CD14 and the LPS-binding protein (LBP) (69, 133, 134, 303). CD14, which is a 55-kDa protein, exists both as a glycosylphosphatidylinositol (GPI)-anchored membrane protein on circulating monocytes and PMN (mCD14), and as a relatively abundant plasma protein, known as soluble CD14 (sCD14). The LBP is a 60-kDa glycoprotein, synthesized by liver hepatocytes and found in the acute-phase serum. It binds with high affinity to the lipid A region, and the resultant LBP-LPS complex promotes and accelerates the binding of LPS to CD14, increasing LPS activity. LBP also functions as an opsonin for Gram-negative bacteria (303). The sCD14 in complex with LPS-LBP confers LPS-responsiveness to cells that to do not express mCD14, such as endothelial cells (211).

One of the major enigmas of LPS activation of cells was the fact that CD14 is unable to induce intracellular signalling, in despite of several reports indicating that LPS does stimulate many pathways of intracellular activation, and

that blockade of CD14 considerably inhibits cellular responses to LPS, both *in vitro* and *in vivo* (174, 302). It has been thus suggested that the function of CD14 is to bind and amplify responses to LPS, as a part of a multi-component receptor (267). At high LPS concentrations, other mechanisms of cell activation have also been proposed (70, 109, 153, 181) (Fig. 9).

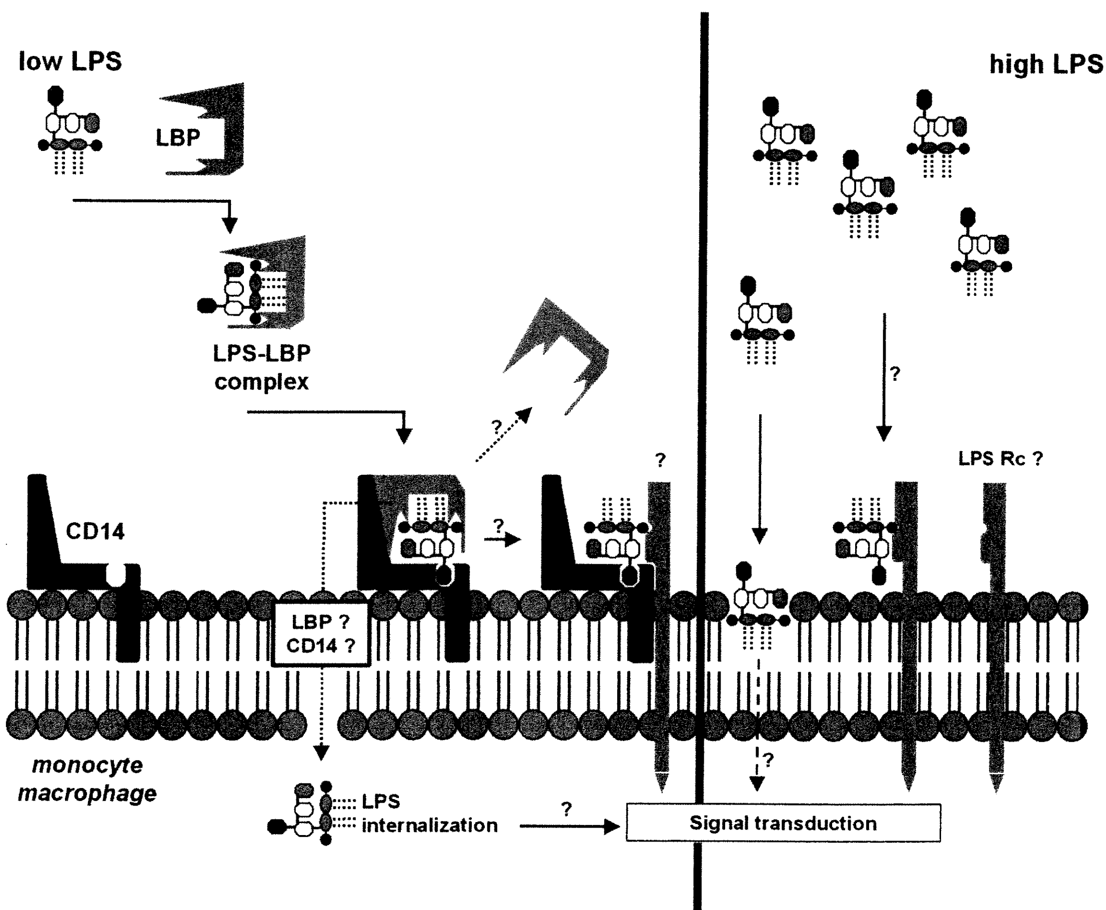


Fig. 9: Schematic representation of lipopolysacharide (LPS) binding to monocytes and macrophages at low and high concentrations. LPS is bound by a plasma protein, the LPS-binding protein (LBP). The resulting LPS-LBP complex is a ligand for the CD14 receptor on the cell surface. In the final step of the activation process, the CD14 complex attaches to an unidentified receptor (Rc), and signal transduction, followed by cell stimulation, occurs. Stimulation by this mechanism occurs at very low levels of LPS. Co-internalization of LBP and CD14 with LPS has been proposed (267). At higher LPS concentrations, direct signalling via the outer membrane or the unidentified Rc can occur. Adapted from Glauser (109).

Recently, the discovery of the Toll family of receptors (first described in fruit fly *Drosophila*) provides us with the long sought transmembrane molecules linking the extracellular compartment, where contact with and recognition of microbial pathogens occur, and the intracellular compartment, where signalling cascades leading to cellular responses are initiated (276). The 10 human Toll-related proteins characterized to date are referred to as Toll-like receptors (TLRs). Both, TLR2 and TLR4 have been implicated in cellular responses to LPS, although TLR4 is the major mammalian LPS signal transducer (215), whereas TLR2 may be implicated in signalling at high doses of LPS (178). Several lines of evidence suggest that LPS triggers monocyte cytokine production following interaction with mCD14 and activation of TLRs. CD14 may potentiate signalling activity by TLRs (216, 307).

2.3.2. Gram-positive bacteria: the example of the cell wall

Although it has been known for many years that Gram-positive bacteria also induce cytokine synthesis, the nature of the cytokine-inducing bacterial constituents has not been delineated. It is only in very recent years that the range of bacterial components and products which are capable of inducing cytokine synthesis has begun to be appreciated (134).

Although the cell membrane is very similar in both Gram-positive and Gram-negative bacteria, the cell wall structure differs greatly between the two types of organisms (as shown in Fig. 8). The Gram-positive cell wall consists of a large PG layer, teichoic or lipoteichoic acids (LTA) and various proteins, any of which could be involved in the activation of host cell systems leading to inflammation (109, 282).

Unlike the pathophysiology of shock caused by Gram-negative bacteria, little is known about the sequence of events that leads to septic shock caused by Gram-positive bacteria. Undoubtedly, cytokines play a central role in these events. For Gram-negative sepsis, these cytokines are mainly released on

stimulation of macrophages and other cells by endotoxin or LPS. In patients with serious, life-threatening, Gram-positive bacterial infection with shock, these events may be triggered by cell-wall components and exotoxins (282). Indeed, there have been several reports indicating that the cell-wall products of Gram-positive bacteria can also induce the release of cytokines. For example, PG has long been recognized to have potent immunomodulatory actions, and was shown to activate monocytes to produce cytokines (136, 258). In addition to PG, LTA of various Gram-positive bacterial species also stimulates these cells to release TNF, IL-1, IL-6, IL-8 and several other cytokines (33, 45, 61, 164). No obvious correlation was found between biological activity and chain length, the type of glycosyl substituent or glycolipid structure, or the fatty acid composition of LTAs (164). However, the acetyl chains are essential for binding of LTA to the surface of macrophages (164), and also to induce cell activation (164, 263). On the other hand, in comparative studies of the LTAs from a range of Gram-positive bacterial species, Bhakdi et al. (33) reported that there were major differences in their capacities to stimulate cytokine release by monocytes.

LTA can be regarded as the Gram-positive equivalent of LPS, although the latter has a much greater potency and range of biological activities (133). However, whole cell wall preparations are more potent stimuli for cytokine release than individual components and, in fact, it has been shown that PG and LTA act in synergy to cause shock and multiple organ failure associated with Gram-positive organisms (73).

In this regard, pneumococcal cell wall components have been shown to reproduce many features of pneumonia, as well as of meningitis and otitis media (266). Indeed, the intracisternal inoculation of purified cell walls can induce inflammation and impair the BBB as observed during meningeal infection (212). Although covered by capsular polysaccharide, the cell wall is accessible to interact with the host, as evidenced by its ability to fix some components of the acute phase response, such as complement. The cell wall is a dynamic structure and its components are continuously inserted into and released from the bacterium. In addition, cell wall components released by enzymatic degradation

are more potent chemotactic factors than are intact cell walls, a finding relevant to the consequences of bacterial lysis induced by antibiotics (266).

Although various bacterial species differ in the composition of their cell walls, the host reaction to invasion of these different species is remarkably similar. Thus, common pathways of cell activation have been proposed for Gram-negative and Gram-positive bacteria. In addition to being a high affinity receptor for LPS, CD14 has been implicated in the responses to several bacteria or bacterial products, including PG and LTA of various streptococcal species (89) (302). Because it can facilitate responses to different bacterial structures, CD14 has been termed a pattern recognition receptor by Pugin et al. (210). Yet CD14 lacks specificity in bacterial product recognition, and interaction with a unidentified recognition/cell-activating molecule would be required for the specificity of the response (302).

The identification of the role of TLR2 in the recognition of most of these pathogens adds another layer of complexity to our understanding of the mammalian response to microbes. In contrast to CD14, TLR2 contains all of the characteristics that one would expect of a true pattern recognition receptor, including the presence of a signal-transducing intracellular domain. Although only recently described, the list of putative ligands for TLR2 is already impressively large (176, 276, 307). Of particular interest is the observation that despite the apparent interactions of TLR2 with many Gram-positive bacteria, GBS do not seem to stimulate cells through this receptor (100). In this regard, even though CD14 coexpression synergistically enhance TLR2-mediated activation, CD14-independant activation of cells by Gram-positive bacteria has also been reported (44). This highlights the fact that we cannot exclude the involvement of additional receptors, functioning either alone or as part of a receptor complex, in host responses to the microbial structures described.

3. Pathogenesis of meningitis caused by other bacteria: the example of GBS

Fifty years after the advent of antibiotics for clinical use, bacterial meningitis remains an important cause of morbidity and mortality. As such, it represents a unique human disease, because the pathophysiologic effects of disease progression and suboptimal outcomes occur despite bacteriologic cure of the infection (212). In the last two decades GBS have emerged as the leading cause of invasive bacterial infections in human neonates. The early-onset form of GBS disease typically presents in the first 24 hours of life, with fulminant pneumonia, septicemia and high mortality. The late-onset (more than 7 days of age) form of GBS disease, to which *S. suis* infections may mostly be compared, can present more indolently, with meningitis, occult bacteremia or osteoarthritis. GBS isolates from neonates with early-onset infection are well distributed among the various capsular types, whereas the majority of isolates from infants with late-onset disease (and infants with meningitis regardless of age of onset) are of serotype III (167, 247).

As for other microbial pathogens, the process of human infection by GBS is complex and multifactorial. GBS pathogenesis is perhaps best understood in the framework of an evolutionary relationship between the bacterium and its host. The pathogenesis of the meningitis (and other localized infections) caused by GBS in its late-onset form (older than one week of age), likely reflect the progressive maturation of the host immune response. GBS must establish carriage in the upper respiratory tract of the infant and gain access to the bloodstream. The predominance of serotype III GBS isolates in late-onset disease indicates a unique role of the sialylated type III CPS in resisting subsequent immune clearance or traversing the BBB (287). In contrast, early-onset disease is evenly distributed among the various GBS capsular serotypes. Bloodstream dissemination allows GBS to reach multiple body sites, where

subsequent tissue penetration results in end-organ disease manifestations (e.g. meningitis). Extracellular products such as hyaluronidase, collagenase and proteases are postulated to facilitate spread of the organism through tissue barriers (247).

In order to establish colonization of the female genital tract, GBS must successfully adhere to epithelial cells of the vagina. In comparison to other microorganisms, GBS bind very efficiently to human vaginal cells or vaginal tissue culture cells. The specific molecular interactions which mediate GBS adhesion are not well known. Isogenic mutants deficient in CPS production bind more efficiently to epithelial cells than the parent strain. One of the principal GBS surface ligand would be the cell wall LTA (255).

Newborns may be exposed to GBS in utero following ascending infection of the placental membranes and amniotic fluid. Alternatively, they may become contaminated with GBS upon passage through the birth canal (247). In order to gain access to the systemic circulation from a primary focus of infection in the lung, GBS must traverse the alveolar epithelium and the pulmonary endothelium. GBS isolates of different capsular serotypes invade epithelial cells in tissue culture, but some variation exists in the magnitude of invasion (232). The CPS itself does not appear to be essential for invasion of alveolar epithelial cells. If cellular invasion allows GBS to transit the alveolar epithelium into the pulmonary interstitial space, an additional barrier separates the organism from the circulation. GBS must encounter and penetrate the pulmonary endothelium. In fact, *in vitro* antibiotic protection assays confirm the ability of GBS to invade primary cultures of endothelial cells (106). As with epithelial monolayers, the CPS slows down GBS endothelial cell invasion (106). Thus, modulation of CPS expression during the different stages of GBS infection has been suggested.

Upon penetration of GBS into the bloodstream, an immunological response is recruited to clear the organism. The central elements of this response are host phagocytic cells of the PMN, and to a lesser extent, the monocyte-macrophage cell lines. However, as is the case with most other

pathogenic bacteria, effective phagocytosis of GBS by PMN and macrophages requires opsonization. Without the participation of specific antibodies and serum complement, phagocytosis of GBS is dramatically reduced (90, 199, 247). However, GBS possess a number of unique virulence attributes which interfere with effective opsonophagocytosis, the most important being the type-specific CPS (287). Indeed, GBS associated with disease are almost invariably encapsulated (167). In addition, direct evidence for the role of type III GBS capsule in virulence is provided by the construction of isogenic capsule-deficient mutants. These mutants were shown to be significantly less virulent in animal models of GBS infection (233, 287).

With minor exceptions, the various GBS CPS antigens are composed of the same four component monosaccharides: glucose, galactose, *N*-acetylglucosamine and sialic acid. The biochemistry and immunology of GBS capsular polysaccharide has been studied most thoroughly in serotype III organisms. The native type III CPS is a high-molecular-weight polymer composed of more than 100 repeating pentasaccharide units. Sialic acid is known to be a critical element in the epitope of type III GBS capsule which confers protective immunity (247, 286).

In fact, preliminary indications that specific antibody was important in the immunologic clearance of GBS came from Lancefield's work, since she found that multiple specific antibodies directed to polysaccharide antigens were protective. Abundant clinical and experimental data, especially on antibodies against serotype III capsule, have been accumulated to support these observations (19, 247). The interaction between GBS, specific antibody, and components of the complement system is uniquely complex and varies significantly among GBS capsule serotypes. The classical pathway of complement activation is a cascade of proteolytic cleavage and protein-binding reactions, traditionally in response to antigen-antibody complex formation, which promotes complement fixation, immune adherence and release of proinflammatory and chemoattractant mediators. As expected, participation of

the classic complement pathway maximizes specific anticapsular antibody opsonization of type III strains of GBS (175, 199).

The alternative pathway of complement activation was first described as an antibody-independent response to endotoxins; nevertheless, alternative complement pathway-mediated opsonization and phagocytosis is facilitated by specific antibody to type specific capsule antigens of GBS. Situated at the convergence of the classical and alternative pathways, deposition of C3 on the bacterial surface, with subsequent cleavage and degradation to opsonically active fragments C3b and iC3b, is a key element in host defense against invasive infections. However, the extent of C3 deposition by the alternative pathway is inversely related to the size and density of the CPS present on the surface of type III GBS strains. Indeed, isogenic mutant type III strains expressing a sialic acid-deficient capsule, or lacking CPS altogether, bind significantly more complement factor C3 and are more sensitive to phagocytic killing than the parent strain (183). Asialo- and acapsular type III GBS mutants exhibit a significant increase in LD50 in a neonatal rat model (233, 287). Thus, interference of effective C3 deposition by sialylated CPS appears to be an important virulence mechanism of GBS. On the other hand, and as mentioned above, antibody against type III polysaccharide may overcome this interference, allowing activation of C3 and conferring protection against infection.

Further discovery that macrophages can engulf GBS in the absence of immune serum, by means of C3-dependent binding (196), and C3-independent binding using the CR3 receptor (12), suggests that there is also a potential role for antibody-independent mechanisms in resistance to GBS infection. However, the recent demonstration that type III GBS ingested by macrophages in the absence of specific antibodies in fact survive within the host cell, seems to indicate that GBS, like intracellular microorganisms, could have evolved some strategies to survive intracellularly (269). This is in agreement with early studies which showed that GBS were readily ingested in the presence of normal serum. However, once ingested, these organisms were not killed and accumulated in the PMN (60). In the absence of specific immunity and low complement levels in

the neonate, the entry and survival of GBS in macrophages could play an important role in the course of invasive infections (269).

On important aspect of meningeal pathogens is their ability to induce a marked SAS inflammatory response which contributes to many of the pathophysiologic consequences of bacterial meningitis (e.g., cerebral edema and increased intracranial pressure). Despite the fact that bacterial capsule is largely responsible for intravascular and SAS survival of meningeal pathogens, capsular polysaccharides are remarkably poor inducers of inflammatory mediators (264). Intraventricular inoculation of newborn piglets with GBS results in an increase of TNF levels in the CSF, followed by PMN influx. The magnitude of the observed TNF response and inflammatory cascade is markedly increased when a nonencapsulated mutant is used, suggesting that underlying cell wall components are responsible for inducing the inflammatory response (177, 247).

Activation of the host cytokine cascade by GBS during blood dissemination may be also responsible for development of the sepsis syndrome, and in many cases profound septic shock. Indeed, increased production of proinflammatory cytokines is found in blood and CSF samples of infants with invasive GBS disease, and the ability of GBS to induce cytokine production has been demonstrated in investigations with human monocytes and whole blood cultures (247). Several GBS components can stimulate human cells to release cytokines. These components appear to be effective not only on the intact bacterial surface but also in soluble form. Both the group- and type-specific polysaccharides, as well as the cell wall LTA and PG were shown to be responsible for cytokine induction by peripheral blood mononuclear cells *in vitro*. The relative potential of each individual component varied among studies, and even though the cell wall would be the most active bacterial component, all antigens may synergize to produce inflammation *in vivo* (270, 271, 284, 294).

4. References

1. **Agarwal, K. K., S. D. Elliott, and P. J. Lachmann.** 1969. Streptococcal infection in young pigs. III. The immunity of adult pigs investigated by the bactericidal test. J. Hyg. **67:**491-503.
2. **Akira, S., T. Hirano, T. Taga, and T. Kishimoto.** 1990. Biology of multifunctional cytokines: IL-6 and related molecules (IL-1 and TNF). FASEB J. **4:**2860-2867.
3. **Albanyan, E. A., and M. S. Edwards.** 2000. Lectin site interaction with capsular polysaccharide mediates nonimmune phagocytosis of type III group B streptococci. Infect. Immun. **68:**5794-5802.
4. **Albanyan, E. A., J. G. Vallejo, C. Wayne Smith, and M. S. Edwards.** 2000. Nonopsonic binding of type III Group B streptococci to human neutrophils induces interleukin-8 release mediated by the p38 mitogen-activated protein kinase pathway. Infect. Immun. **68:**2053-2060.
5. **Albelda, S. M., and C. A. Buck.** 1990. Integrins and other cell adhesion molecules. FASEB J. **4:**2868-2880.
6. **Alexander, T.** 1995. Presented at the Allen D. Leman Swine Conference, Minnesota.
7. **Allgaier, A., R. Goethe, H. J. Wisselink, H. E. Smith, and P. Valentin-Weigand.** 2001. Relatedness of *Streptococcus suis* isolates of various serotypes and clinical backgrounds as evaluated by macrorestriction analysis and expression of potential virulence traits. J. Clin. Microbiol. **39:**445-453.
8. **Alouf, J. E., and C. Geoffroy.** 1991. The family of the antigenically-related cholesterol-binding ("sulphydryl-activated") cytolytic toxins, p. 147-186. *In* J. E. Alouf (ed.), Sourcebook of Bacterial Protein Toxins. New York Academic Press, New York.
9. **Amass, S. F.** 1997. Eradication, prevention, and treatment of meningitis in weaned pigs caused by *Streptococcus suis*. Presented at the Ann. Meet. Am. Assoc. Swine Pract., Québec, Canada.
10. **Amass, S. F., P. SanMiguel, and L. K. Clark.** 1997. Demonstration of vertical transmission of *Streptococcus suis* in swine by genomic fingerprinting. J. Clin. Microbiol. **35:**1595-1596.
11. **Amass, S. F., C. C. Wu, and L. K. Clark.** 1996. Evaluation of antibiotics for the elimination of the tonsillar carrier state of *Streptococcus suis* in pigs. J. Vet. Diagn. Invest. **8:**64-67.
12. **Antal, J. M., J. V. Cunningham, and K. J. Goodrum.** 1992. Opsonin-independent phagocytosis of group B streptococci: role of complement receptor type three. Infect. Immun. **60:**1114-1121.
13. **Arend, S. M., M. A. van Buchem, M. L. van Ogtrop, and J. Thompson.** 1995. Septicaemia, meningitis and spondylodiscitis caused by *Streptococcus suis* type 2 [letter]. Infection **23:**128.
14. **Arends, J. P., and H. C. Zanen.** 1988. Meningitis caused by *Streptococcus suis* in humans. Rev. Infect. Dis. **10:**131-137.
15. **Arnold, R., and W. Konig.** 1998. Interleukin-8 release from human neutrophils after phagocytosis of *Listeria monocytogenes* and *Yersinia enterocolitica*. J. Med. Microbiol. **47:**55-62.
16. **Athamna, A., I. Ofek, Y. Keisari, S. Markowitz, G. G. S. Dutton, and N. Sharon.** 1991. Lectinophagocytosis of encapsulated *Klebsiella pneumoniae*

mediated by surface lectins of guinea pig alveolar macrophages and human monocytes-derived macrophages. Infect. Immun. **59:**1673-1682.

17. **Baggiolini, M., B. Dewald, and B. Moser.** 1997. Human chemokines: an update. Annu. Rev. Immunol. **15:**675-705.
18. **Baggiolini, M., B. Dewald, and B. Moser.** 1994. Interleukin-8 and related chemotactic cytokines - CXC and CC chemokines. Adv. Immunol. **55:**97-179.
19. **Baker, C. J., M. S. Edwards, and D. L. Kasper.** 1981. Role of antibody to native type III polysaccharide of group B *Streptococcus* in infant infection. Pediatrics **68:**544-549.
20. **Baker, C. J., and D. L. Kasper.** 1976. Correlation of maternal antibody deficiency with susceptibility to neonatal group B streptococcal infection. New Engl. J. Med. **294:**753-756.
21. **Bauer, M. E., M. P. Goheen, C. A. Townsend, and S. M. Spinola.** 2001. *Haemophilus ducreyi* associates with phagocytes, collagen, and fibrin and remains extracellular throughout infection of human volunteers. Infect. Immun. **69:**2549-2557.
22. **Beachey, E. H.** 1981. Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. J. Infect. Dis. **143:**325-345.
23. **Beaudoin, M., J. Harel, R. Higgins, M. Gottschalk, M. Frenette, and J. I. MacInnes.** 1992. Molecular analysis of isolates of *Streptococcus suis* capsular type 2 by restriction-endonuclease-digested DNA separated on SDS-PAGE and by hybridization with an rDNA probe. J. Gen. Microbiol. **138:**2639-2645.
24. **Beaudoin, M., R. Higgins, J. Harel, and M. Gottschalk.** 1992. Studies on a murine model for evaluation of virulence of *Streptococcus suis* capsular type 2 isolates. FEMS Microbiol. Lett. **78:**111-116.
25. **Benkirane, R., M. Gottschalk, and J. D. Dubreuil.** 1997. Identification of a *Streptococcus suis* 60-kDa heat-shock protein using Western blotting. FEMS Microbiol. Lett. **153:**379-385.
26. **Benkirane, R., M. Gottschalk, M. Jacques, and J. D. Dubreuil.** 1998. Immunochemical characterization of an IgG-binding protein of *Streptococcus suis*. FEMS Immunol. Med. Microbiol. **20:**121-127.
27. **Berthelot-Hérault, F., R. Cariolet, A. Labbé, M. Gottschalk, J.-Y. Cardinal, and M. Kobisch.** 2001. Experimental infection of specific pathogen free piglets with French strains of *Streptococcus suis* capsular type 2. Can. J. Vet. Res. **65:**196-200.
28. **Berthelot-Hérault, F., M. Gottschalk, A. Labbé, R. Cariolet, and M. Kobisch.** 2001. Experimental airbone transmission of *Streptococcus suis* capsular type 2 in pigs. Vet. Microbiol. **82:**69-80.
29. **Berthelot-Hérault, F., C. Marois, M. Gottschalk, and M. Kobisch.** 2002. Genetic diversity of *Streptococcus suis* strains isolated from pigs and humans as revealed by pulsed-field gel electrophoresis. J. Clin. Microbiol. **40:**615-619.
30. **Berthelot-Hérault, F., H. Morvan, A.-M. Kéribin, M. Gottschalk, and M. Kobisch.** 2000. Epidemiological study of *Streptococcus suis* isolates from pigs in France from 1996 to 1998. Vet. Res. **31:**473-479.
31. **Berthelot-Hérault, F., H. Morvan, A.-M. Kéribin, M. Gottschalk, and M. Kobisch.** 2000. Production of muraminidase-released protein (MRP), extracellular factor (EF) and suilysin by field isolates of *Streptococcus suis* capsular types 2, 1/2, 9, 7 and 3 isolated from swine in France. Vet. Res. **31:**473-479.

32. **Bhakdi, S., F. Grimminger, N. Suttorp, D. Walmrath, and W. Seeger.** 1994. Proteinaceous bacterial toxins and pathogenesis of sepsis syndrome and septic shock: the unkown connection. Med. Microbiol. Immunol. **183:**119-144.
33. **Bhakdi, S., M. Klonisch, P. Nuber, and W. Fischer.** 1991. Stimulation of monokine production by lipoteichoic acids. Infect. Immun. **59:**4614-4620.
34. **Boetner, A. G., M. Binder, and V. Bille-Hansen.** 1987. *Streptococcus suis* infections in Danish pigs and experimental infection with *Streptococcus suis* serotype 7. Acta Pathol. Microbiol. Immunol. Scand. [B] **95:**233-239.
35. **Boye, M., A. A. Feenstra, C. Tegtmeier, L. O. Andresen, S. R. Rasmussen, and V. Bille-Hansen.** 2000. Detection of *Streptococcus suis* by in situ hybridization, indirect immunofluorescence, and peroxidase-antiperoxidase assays in formalin-fixed, paraffin-embedded tissue sections from pigs. J. Vet. Diagn. Invest. **12:**224-232.
36. **Brassard, J., M. Gottschalk, and S. Quessy.** 2001. Decrease of the adhesion of *Streptococcus suis* serotype 2 mutants to embryonic bovine tracheal cells and porcine tracheal rings. Can. J. Vet. Res. **65:**156-160.
37. **Brousseau, R., J. E. Hill, G. Préfontaine, S.-H. Goh, J. Harel, and S. M. Hemmingsen.** 2001. *Streptococcus suis* serotypes characterized by analysis of chaperonin 60 gene sequences. Appl. Environ. Microbiol. **67:**4828-4833.
38. **Brown, E. J., S. W. Hosea, and M. M. Frank.** 1983. The role of antibody and complement in the reticuloendothelial clearance of pneumococci from the bloodstream. Rev. Infect. Dis. **4:**S797-S805.
39. **Bungener, W., and R. Bialek.** 1989. Fatal *Streptococcus suis* septicemia in an abattoir worker. Eur. J. Clin. Microbiol. Infect. Dis. **8:**306-308.
40. **Busque, P., R. Higgins, S. Sénéchal, R. Marchand, and S. Quessy.** 1998. Simultaneous flow cytometric measurement of *Streptococcus suis* phagocytosis by polymorphonuclear and mononuclear blood leukocytes. Vet. Microbiol. **63:**229-238.
41. **Cain, D., F. Malouin, M. Dargis, J. Harel, and M. Gottschalk.** 1995. Alterations in penicillin-binding proteins in strains of *Streptococcus suis* possessing moderate and high levels of resistance to penicillin. FEMS Microbiol. Lett. **130:**121-127.
42. **Cantin, M., J. Harel, R. Higgins, and M. Gottschalk.** 1992. Antimicrobial resistance patterns and plasmid profiles of *Streptococcus suis* isolates. J. Vet. Diagn. Invest. **4:**170-174.
43. **Casadevall, A., and L.-A. Pirofski.** 1999. Host-pathogen interactions: Redefining the basic concepts of virulence and pathogenicity. Infect. Immun. **67:**3703-3713.
44. **Cauwels, A., E. Wan, M. Leismann, and E. Tuomanen.** 1997. Coexistence of CD14-dependent and independent pathways for stimulation of human monocytes by gram-positive bacteria. Infect. Immun. **65:**3255-3260.
45. **Cavaillon, J.-M., H. Müller-Alouf, and J. E. Alouf.** 1997. Cytokines in streptococcal infections. Adv. Exp. Med. Biol. **418:**869-879.
46. **Cerami, A.** 1992. Inflammatory cytokines. Clin. Immunol. Immunopathol. **62:**S3-S10.
47. **Chanter, N., P. W. Jones, and T. J. Alexander.** 1993. Meningitis in pigs caused by *Streptococcus suis*- a speculative review. Vet. Microbiol. **36:**39-55.
48. **Charland, N., J. Harel, M. Kobish, S. Lacasse, and M. Gottschalk.** 1998. *Streptococcus suis* serotype 2 mutants deficient in capsular expression. Microbiology **144:**325-332.

49. **Charland, N., M. Jacques, S. Lacouture, and M. Gottschalk.** 1997. Characterization and protective activity of a monoclonal antibody against a capsular epitope shared by *Streptococcus suis* serotypes 1, 2 and 1/2. Microbiology **143:**3607-3614.
50. **Charland, N., J. T. Kellens, F. Caya, and M. Gottschalk.** 1995. Agglutination of *Streptococcus suis* by sialic acid-binding lectins. J. Clin. Microbiol. **33:**2220-2221.
51. **Charland, N., M. Kobisch, B. Martineau-Doize, M. Jacques, and M. Gottschalk.** 1996. Role of capsular sialic acid in virulence and resistance to phagocytosis of *Streptococcus suis* capsular type 2. FEMS Immunol. Med. Microbiol. **14:**195-203.
52. **Charland, N., V. Nizet, C. Rubens, K. S. Kim, S. Lacouture, and M. Gottschalk.** 2000. *Streptococcus suis* serotype 2 interactions with human brain microvascular endothelial cells. Infect. Immun. **68:**637-643.
53. **Chatellier, S., M. Gottschalk, R. Higgins, R. Brousseau, and J. Harel.** 1999. Relatedness of *Streptococcus suis* serotype 2 isolates from different geographic origins as evaluated by molecular fingerprinting and phenotyping. J. Clin. Microbiol. **37:**362-366.
54. **Chatellier, S., J. Harel, Y. Zhang, M. Gottschalk, R. Higgins, L. A. Devriese, and R. Brousseau.** 1998. Phylogenetic diversity of *Streptococcus suis* strains of various serotypes as revealed by 16S rRNA gene sequence comparison. Int. J. Syst. Bacteriol. **40:**581-589.
55. **Chattopadhyay, B.** 1979. Group R streptococcal infection amongst pig meat handlers- a review. Publ. Hlth. Lond. **93:**140-142.
56. **Chau, P. Y., C. Y. Huang, and R. Kay.** 1983. *Streptococcus suis* meningitis. An important underdiagnosed disease in Hong Kong. Med. J. Aust. **1:**414-417.
57. **Chmiela, M., B. Paziak-Domanska, W. Rudnicka, and T. Wadström.** 1995. The role of heparan sulphate-binding activity of *Helicobacter pylori* bacteria in their adhesion to murine macrophages. APMIS **103:**469-474.
58. **Chotmongkol, V., J. Janma, and T. Kawamatawong.** 1999. *Streptococcus suis* meningitis: report of a case. J. Med. Assoc. Thai. **82:**922-924.
59. **Clark, L. K.** 1995. SEW: Program, problems, performances, potential profits and methods of implementation for various herd sizes. Presented at the 36th George A. Young Swine Conference, Nebraska.
60. **Cleat, P. H., and C. R. Coid.** 1982. An alternative role for specific antibody in neutrophil bactericidal activity against highly pathogenic group B streptococci. Br. J. Exp. Path. **63:**452-457.
61. **Cleveland, M. G., J. D. Gorham, T. L. Murphy, E. Tuomanen, and K. M. Murphy.** 1996. Lipoteichoic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. Infect. Immun. **64:**1906-1912.
62. **Clifton-Hadley, F. A.** 1983. *Streptococcus suis* type 2 infections. Br. Vet. J. **139:**1-5.
63. **Clifton-Hadley, F. A.** 1981. Studies of *Streptococcus suis* type 2 infection in pigs. Ph.D. thesis. University of Cambridge, Cambridge.
64. **Clifton-Hadley, F. A.** 1984. Studies of *Streptococcus suis* type 2 infection in pigs. Vet. Res. Commun. **8:**217-227.
65. **Clifton-Hadley, F. A., T. J. Alexander, and M. R. Enright.** 1986. The epidemiology, diagnosis, treatment and control of *Streptococcus suis* type 2 infection. Presented at the Ann. Meet. Am. Assoc. Swine. Pract.

66. **Clifton-Hadley, F. A., T. J. Alexander, M. R. Enright, and J. Guise.** 1984. Monitoring herds for *Streptococcus suis* type 2 by sampling tonsils of slaughter pigs. Vet. Rec. **115:**562-564.
67. **Clifton-Hadley, F. A., T. J. Alexander, I. Upton, and W. P. Duffus.** 1984. Further studies on the subclinical carrier state of *Streptococcus suis* type 2 in pigs. Vet. Rec. **114:**513-518.
68. **Colaert, J., M. Allewaert, H. Magerman, J. Vandeven, and J. Vandepitte.** 1985. *Streptococcus suis* meningitis in man. First reported observation in Belgium. Acta Clin. Belg. **40:**314-317.
69. **Couturier, C., N. Haeffner-Cavaillon, M. Caroff, and M. D. Kazatchkine.** 1991. Binding sites for endotoxins (lipopolysaccharides) on human monocytes. J. Immunol. **147:**1899-1904.
70. **Couturier, C., G. Jahns, M. D. Kazatchkine, and N. Haeffner-Cavaillon.** 1992. Membrane molecules which trigger the production of interleukin-1 and tumor necrosis factor-α by lipopolysaccharide-stimulated human monocytes. Eur. J. Immunol. **22:**1461-1466.
71. **Cruz Colque, J. I., L. A. Devriese, and F. Haesebrouck.** 1993. Streptococci and enterococci associated with tonsils of cattle. Lett. Appl. Microbiol. **16:**72-74.
72. **Curfs, J. H. A. J., J. F. G. M. Meis, and J. A. A. Hoogkamp-Korstanje.** 1997. A primer on cytokines: sources, receptors, effects, and inducers. Clin. Microbiol. Rev. **10:**742-780.
73. **De Kimpe, S. J., M. Kengatharan, C. Thiemermann, and J. R. Vane.** 1995. The cell wall components peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* act in synergy to cause shock and multiple organ failure. Proc. Natl. Acad. Sci. USA **92:**10359-10363.
74. **De Moor, C. E.** 1963. Septicaemic infections in pigs, caused by haemolytic streptococci of new Lancefield groups designated R, S, and T. Antonie van Leeuwenhoek J. Microbiol. Serol. **29:**272-280.
75. **Dee, S. A., A. R. Carlson, N. L. Winkelman, and M. M. Corey.** 1993. Effect of management practices on the *Streptococcus suis* carrier rate in nursery swine. J. Am. Vet. Med. Assoc. **203:**295-299.
76. **del Campo Sepulveda, E. M., E. Altman, M. Kobisch, S. D'Allaire, and M. Gottschalk.** 1996. Detection of antibodies against *Streptococcus suis* capsular type 2 using a purified capsular polysaccharide antigen-based indirect ELISA. Vet. Microbiol. **52:**113-125.
77. **Dentener, M. A., V. Bazil, E. J. U. Von Asmuth, M. Ceska, and W. A. Buurman.** 1993. Involvement of CD14 in lipopolysaccharide-induced tumor necrosis factor-α, IL-6 and IL-8 release by human monocytes and alveolar macrophages. J. Immunol. **150:**2885-2891.
78. **Devriese, L. A., M. Desmidt, S. Roels, J. Hoorens, and F. Haesebrouck.** 1993. *Streptococcus suis* infection in fallow deer [letter]. Vet. Rec. **132:**283.
79. **Devriese, L. A., and F. Haesebrouck.** 1992. *Streptococcus suis* infections in horses and cats. Vet. Rec. **130:**380.
80. **Devriese, L. A., F. Haesebrouck, P. De Herdt, P. Dom, R. Ducatelle, M. Desmidt, S. Messier, and R. Higgins.** 1994. *Streptococcus suis* infections in birds. Avian Pathol. **23:**721-724.
81. **Diab, A., H. Abdalla, H. Lun Li, F. Dong Shi, J. Zhu, B. Höjberg, L. Lindquist, B. Wretlind, M. Bakhiet, and H. Link.** 1999. Neutralization of macrophage inflammatory protein 2 (MIP-2) and MIP-1α attenuates neutrophil recruitment in the central nervous system during experimental bacterial meningitis. Infect. Immun. **67:**2590-2601.

82. **Dinarello, C. A.** 1994. The biological properties of interleukin-1. Eur. Cytokine Netw. **5:**517-531.
83. **Dinarello, C. A.** 1991. Interleukin-1 and interleukin-1 antagonism. Blood **77:**1627-1652.
84. **Dinarello, C. A.** 1992. Role of interleukin-1 in infectious diseases. Immunol. Rev. **127:**119-146.
85. **Dinarello, C. A., and S. M. Wolff.** 1993. The role of interleukin-1 in disease. New Engl. J. Med. **328:**106-113.
86. **Drew, T. W.** 2000. A review of evidence for immunosuppression due to Porcine Reproductive and Respiratory Syndrome Virus. Vet. Res. **31:**27-39.
87. **Duensing, T. D., J. S. Wing, and J. P. M. van Putten.** 1999. Sulfated polysaccharide-directed recruitment of mammalian host proteins: a novel strategy in microbial pathogenesis. Infect. Immun. **67:**4463-4468.
88. **Dupas, D., M. Vignon, and C. Geraut.** 1992. *Streptococcus suis* meningitis. A severe noncompensated occupational disease. J. Occup. Med. **34:**1102-1105.
89. **Dziarski, R., A. J. Ulmer, and D. Gupta.** 2000. Interactions of CD14 with components of Gram-positive bacteria. Chem. Immunol. **74:**83-107.
90. **Edwards, M. S., M. R. Wessels, and C. J. Baker.** 1993. Capsular polysaccharide regulates neutrophil complement receptor interactions with type III group B streptococci. Infect. Immun. **61:**2866-2871.
91. **Elliott, S. D., T. J. Alexander, and J. H. Thomas.** 1966. Streptococcal infection in young pigs. II. Epidemiology and experimental production of the disease. J. Hyg. Lond. **64:**213-220.
92. **Elliott, S. D., F. Clifton-Hadley, and J. Tai.** 1980. Streptococcal infection in young pigs. V. An immunogenic polysaccharide from *Streptococcus suis* type 2 with particular reference to vaccination against streptococcal meningitis in pigs. J. Hyg. Lond. **85:**275-285.
93. **Elliott, S. D., and J. Y. Tai.** 1978. The type-specific polysaccharides of *Streptococcus suis*. J. Exp. Med. **148:**1699-1704.
94. **Falkow, S., R. R. Isberg, and D. A. Portnoy.** 1992. The interaction of bacteria with mammalian cells. Annu. Rev. Cell. Biol. **8:**333-363.
95. **Fallman, M., K. Andersson, S. Hakansson, K. E. Magnusson, O. Stendahl, and H. Wolf-Watz.** 1995. *Yersinia pseudotuberculosis* inhibits Fc receptor-mediated phagocytosis in J774 cells. Infect. Immun. **63:**3117-3124.
96. **Fattal-German, M., J. Taillandier, D. Mathieu, and B. Bizzini.** 1991. Pneumococcal vaccination of elderly individuals. Vaccine **9:**542-544.
97. **Feder, I., M. M. Chengappa, B. Fenwick, M. Rider, and J. Staats.** 1994. Partial characterization of *Streptococcus suis* type 2 hemolysin. J. Clin. Microbiol. **32:**1256-1260.
98. **Field, H. I., D. Buntain, and J. T. Done.** 1954. Studies on piglet mortality. I. Streptococcal meningitis and arthritis. Vet. Rec. **66:**453-455.
99. **Finlay, B. B., and P. Cossart.** 1997. Exploitation of mammalian host cell functions by bacterial pathogens. Science **276:**718-725.
100. **Flo, T. H., O. Halaas, E. Lien, L. Ryan, G. Teti, D. T. Golenbock, A. Sudan, and T. Espevik.** 2000. Human toll-like receptor 2 mediates monocyte activation by *Listeria monocytogenes*, but not by group B streptococci or lipopolysaccharide. J. Immunol. **164:**2064-2069.
101. **Fongcom, A., S. Pruksakorn, R. Mongkol, P. Tharavichitkul, and N. Yoonim.** 2001. *Streptococcus suis* infection in northern Thailand. J. Med. Assoc. Thai. **84:**1502-1508.

102. **François, B., V. Gissot, M. C. Ploy, and P. Vignon.** 1998. Recurrent septic shock due to *Streptococcus suis*. J. Clin. Microbiol. **36:**2395.

103. **Galina, L., J. E. Collins, and C. Pijoan.** 1992. Porcine *Streptococcus suis* in Minnesota. J. Vet. Diagn. Invest. **4:**195-196.

104. **Galina, L., C. Pijoan, M. Sitjar, W. T. Christianson, K. Rossow, and J. E. Collins.** 1994. Interaction between *Streptococcus suis* serotype 2 and porcine reproductive and respiratory syndrome virus in specific pathogen-free piglets. Vet. Rec. **134:**60-64.

105. **Galina, L., U. Vecht, H. J. Wisselink, and C. Pijoan.** 1996. Prevalence of various phenotypes of *Streptococcus suis* isolated from swine in the U.S.A. based on the presence of muraminidase-released protein and extracellular factor. Can. J. Vet. Res. **60:**72-74.

106. **Gibson, R. L., M. K. Lee, C. Soderland, E. Y. Chi, and C. E. Rubens.** 1993. Group B streptococci invade endothelial cells: type III capsular polysaccharide attenuates invasion. Infect. Immun. **61:**478-485.

107. **Glabinski, A. R., and R. M. Ransohoff.** 1999. Chemokines and chemokines receptors in CNS pathology. J. Neurovirol. **5:**3-12.

108. **Glabinski, A. R., and R. M. Ransohoff.** 1999. Sentries at the gate: chemokines and the blood-brain-barrier. J. Neurovirol. **5:**623-634.

109. **Glauser, M. P.** 1996. The inflammatory cytokines. New developments in the pathophysiology and treatment of septic shock. Drugs **52, Suppl. 2:**9-17.

110. **Goosney, D. L., J. Celli, B. Kenny, and B. B. Finlay.** 1999. Enteropathogenic *Escherichia coli* inhibits phagocytosis. Infect. Immun. **67:**490-495.

111. **Gordon, S. B., G. R. B. Irving, R. A. Lawson, M. E. Lee, and R. C. Read.** 2000. Intracellular trafficking and killing of *Streptococcus pneumoniae* by human alveolar macrophages are influenced by opsonins. Infect. Immun. **68:**2286-2293.

112. **Gottschalk, M., R. Higgins, and M. Boudreau.** 1993. Use of polyvalent coagglutination reagents for serotyping of *Streptococcus suis*. J. Clin. Microbiol. **31:**2192-2194.

113. **Gottschalk, M., R. Higgins, M. Jacques, M. Beaudoin, and J. Henrichsen.** 1991. Characterization of six new capsular types (23 through 28) of *Streptococcus suis*. J. Clin. Microbiol. **29:**2590-2594.

114. **Gottschalk, M., R. Higgins, M. Jacques, M. Beaudoin, and J. Henrichsen.** 1991. Isolation and characterization of *Streptococcus suis* capsular types 9-22. J. Vet. Diagn. Invest. **3:**60-65.

115. **Gottschalk, M., R. Higgins, M. Jacques, K. R. Mittal, and J. Henrichsen.** 1989. Description of 14 new capsular types of *Streptococcus suis*. J. Clin. Microbiol. **27:**2633-2636.

116. **Gottschalk, M., R. Higgins, and S. Quessy.** 1999. Dilemma of the virulence of *Streptococcus suis* strains. J. Clin. Microbiol. **37:**4202-4203.

117. **Gottschalk, M., S. Lacouture, and L. Odierno.** 1999. Immunomagnetic isolation of *Streptococcus suis* serotypes 2 and 1/2 from swine tonsils. J. Clin. Microbiol. **37:**2877-2881.

118. **Gottschalk, M., A. Lebrun, M. Jacques, and R. Higgins.** 1990. Hemagglutination properties of *Streptococcus suis*. J. Clin. Microbiol. **28:**2156-2158.

119. **Gottschalk, M., A. Lebrun, H. Wisselink, J. D. Dubreuil, H. Smith, and U. Vecht.** 1998. Production of virulence-related proteins by canadian strains of *Streptococcus suis* capsular type 2. Can. J. Vet. Res. **62:**75-79.

120. **Gottschalk, M., S. Petitbois, R. Higgins, and M. Jacques.** 1991. Adherence of *Streptococcus suis* capsular type 2 to porcine lung sections. Can. J. Vet. Res. **55:**302-304.
121. **Gottschalk, M., and M. Segura.** 2000. The pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions. Vet. Microbiol. **75:**59-71.
122. **Gottschalk, M., P. Turgeon, R. Higgins, M. Beaudoin, and A. M. Bourgault.** 1991. Susceptibility of *Streptococcus suis* to penicillin. J. Vet. Diagn. Invest. **3:**170-172.
123. **Gottschalk, M. G., S. Lacouture, and J. D. Dubreuil.** 1995. Characterization of *Streptococcus suis* capsular type 2 haemolysin. Microbiology **141:**189-195.
124. **Haataja, S., K. Tikkanen, J. Hytonen, and J. Finne.** 1996. The Gal alpha 1-4 Gal-binding adhesin of *Streptococcus suis*, a gram-positive meningitis-associated bacterium. Adv. Exp. Med. Biol. **408:**25-34.
125. **Haataja, S., K. Tikkanen, J. Liukkonen, C. Francois-Gerard, and J. Finne.** 1993. Characterization of a novel bacterial adhesion specificity of *Streptococcus suis* recognizing blood group P receptor oligosaccharides. J. Biol. Chem. **268:**4311-4317.
126. **Haataja, S., K. Tikkanen, U. Nilsson, G. Magnusson, K. A. Karlsson, and J. Finne.** 1994. Oligosaccharide-receptor interaction of the Gal alpha 1-4Gal binding adhesin of *Streptococcus suis*. Combining site architecture and characterization of two variant adhesin specificities. J. Biol. Chem. **269:**27466-27472.
127. **Hampson, D. J., D. J. Trott, I. L. Clarke, C. G. Mwaniki, and I. D. Robertson.** 1993. Population structure of Australian isolates of *Streptococcus suis*. J. Clin. Microbiol. **31:**2895-2900.
128. **Harel, J., R. Higgins, M. Gottschalk, and M. Bigras-Poulin.** 1994. Genomic relatedness among reference strains of different *Streptococcus suis* serotypes. Can. J. Vet. Res. **58:**259-262.
129. **Hasty, D. L., I. Ofek, H. S. Courtney, and R. J. Doyle.** 1992. Multiple adhesins of streptococci. Infect. Immun. **60:**2147-2152.
130. **Hazenbos, W. L. W., B. M. van den Berg, and R. van Furth.** 1993. Very late antigen-5 and complement receptor type 3 cooperatively mediate the interaction between *Bordetella pertussis* and human monocytes. J. Immunol. **151:**6274-6282.
131. **Heath, P. J., and B. W. Hunt.** 2001. *Streptococcus suis* serotypes 3 to 28 associated with disease in pigs. Vet. Rec. **148:**207-208.
132. **Heath, P. J., B. W. Hunt, J. P. Duff, and J. D. Wilkinson.** 1996. *Streptococcus suis* serotype 14 as a cause of pig disease in the UK [letter]. Vet. Rec. **139:**450-451.
133. **Henderson, B., S. Poole, and M. Wilson.** 1996. Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. Microbiol. Rev. **60:**316-341.
134. **Henderson, B., S. Poole, and M. Wilson.** 1996. Microbial/host interactions in health and disease: who controls the cytokine network? Immunopharmacology **35:**1-21.
135. **Hersh, D., J. Weiss, and A. Zychlinsky.** 1998. How bacteria initiate inflammation: aspects of the emerging story. Curr. Opin. Microbiol. **1:**43-48.
136. **Heumann, D., C. Barras, A. Severin, M. P. Glauser, and A. Tomasz.** 1994. Gram-positive cell walls stimulate synthesis of tumor necrosis factor alpha and interleukin-6 by human monocytes. Infect. Immun. **62:**2715-2721.

137. **Higgins, R., and M. Gottschalk.** 2001. Distribution of *Streptococcus suis* capsular types in 2000. Can. Vet. J. **42:**223.
138. **Higgins, R., and M. Gottschalk.** 1999. Streptococcal diseases, p. 563-570. *In* B. E. Straw, S. D'Allaire, W. L. Mengeling, and D. J. Taylor (ed.), Diseases of swine. Iowa State University, Ames.
139. **Higgins, R., and M. Gottschalk.** 1990. An update on *Streptococcus suis* identification. J. Vet. Diagn. Invest. **2:**249-252.
140. **Higgins, R., M. Gottschalk, M. Boudreau, A. Lebrun, and J. Henrichsen.** 1995. Description of six new capsular types (29-34) of *Streptococcus suis*. J. Vet. Diagn. Invest. **7:**405-406.
141. **Higgins, R., A. Lagace, S. Messier, and L. Julien.** 1997. Isolation of *Streptococcus suis* from a young wild boar. Can. Vet. J. **38:**114.
142. **Hill, H. R., N. H. Augustine, P. A. Williams, E. J. Brown, and J. F. Bohnsack.** 1993. Mechanism of fibronectin enhancement of group B streptococcal phagocytosis by human neutrophils and culture-derived macrophages. Infect. Immun. **61:**2334-2339.
143. **Hirano, T.** 1992. Interleukin-6 and its relation to inflammation and disease. Clin. Immunol. Immunopathol. **62:**S60-S65.
144. **Hirano, T., S. Akira, T. Taga, and T. Kishimoto.** 1990. Biological and clinical aspects of interleukin 6. Immunol. Today **11:**443-449.
145. **Hitchcock, P. J., L. Leive, P. H. Makela, E. T. Rietschel, W. Strittmaster, and D. C. Morrison.** 1986. Lipopolysaccharide nomenclature - past, present, and future. J. Bacteriol. **166:**699-701.
146. **Hoepelman, A. I. M., and E. I. Tuomanen.** 1992. Consequences of microbial attachment: directing host cell functions with adhesins. Infect. Immun. **60:**1729-1733.
147. **Holt, M. E., M. R. Enright, and T. J. Alexander.** 1990. Immunisation of pigs with killed cultures of *Streptococcus suis* type 2. Res. Vet. Sci. **48:**23-27.
148. **Holt, M. E., M. R. Enright, and T. J. Alexander.** 1988. Immunisation of pigs with live cultures of *Streptococcus suis* type 2. Res. Vet. Sci. **45:**349-352.
149. **Hommez, J., L. A. Devriese, J. Henrichsen, and F. Castryck.** 1986. Identification and characterization of *Streptococcus suis*. Vet. Microbiol. **11:**349-355.
150. **Hommez, J., J. Wullepit, P. Cassimon, F. Castryck, K. Ceyssens, and L. A. Devriese.** 1988. *Streptococcus suis* and other streptococcal species as a cause of extramammary infection in ruminants. Vet. Rec. **123:**626-627.
151. **Hondalus, M. K., M. S. Diamond, L. A. Rosenthal, T. A. Springer, and D. M. Mosser.** 1993. The intracellular bacterium *Rhodococcus equi* requires Mac-1 to bind to mammalian cells. Infect. Immun. **61:**2919-2929.
152. **Iglesias, J. G., M. Trujano, and J. Xu.** 1992. Inoculation of pigs with *Streptococcus suis* type 2 alone or in combination with pseudorabies virus. Am. J. Vet. Res. **53:**364-367.
153. **Ingalls, R. R., and D. T. Golenbock.** 1995. CD11c/CD18, a transmembrane signalling receptor for lipopolysaccharide. J. Exp. Med. **181:**1473-1479.
154. **Jacobs, A. A., P. L. Loeffen, A. J. van den Berg, and P. K. Storm.** 1994. Identification, purification, and characterization of a thiol-activated hemolysin (suilysin) of *Streptococcus suis*. Infect. Immun. **62:**1742-1748.
155. **Jacobs, A. A., A. J. van den Berg, J. C. Baars, B. Nielsen, and L. W. Johannsen.** 1995. Production of suilysin, the thiol-activated haemolysin of *Streptococcus suis*, by field isolates from diseased pigs. Vet. Rec. **137:**295-296.

156. **Jacobs, A. A., A. J. van den Berg, and P. L. Loeffen.** 1996. Protection of experimentally infected pigs by suilysin, the thiol-activated haemolysin of *Streptococcus suis*. Vet. Rec. **139**:225-228.
157. **Jacques, M., M. Gottschalk, B. Foiry, and R. Higgins.** 1990. Ultrastructural study of surface components of *Streptococcus suis*. J. Bacteriol. **172**:2833-2838.
158. **Jansen, J., and C. A. van Dorssen.** 1951. Meningitis en encephalitis bij varkens door streptococcen. Tijdschr. Diergeneesk. **76**:815-832.
159. **Joh, D., E. R. Wann, B. Kreikemeyer, P. Speziale, and M. Höök.** 1999. Role of fibronectin-binding MSCRAMMs in bacterial adherence and entry into mammalian cells. Matrix Biol. **18**:211-223.
160. **Karlsson, K. A.** 1995. Microbial recognition of target-cell glycoconjugates. Curr. Opin. Struct. Biol. **5**:622-635.
161. **Kasper, D. L.** 1986. Bacterial capsule - old dogmas and new tricks. J. Infect. Dis. **153**:407-415.
162. **Katsumi, M., T. Saito, Y. Kataoka, T. Itoh, N. Kikuchi, and T. Hiramune.** 1996. Comparative preparation methods of sialylated capsule antigen from *Streptococcus suis* type 2 with type specific antigenicity. J. Vet. Med. Sci. **58**:947-952.
163. **Kay, R.** 1991. The site of the lesion causing hearing loss in bacterial meningitis: a study of experimental streptococcal meningitis in guinea-pigs. Neuropathol. Appl. Neurobiol. **17**:485-493.
164. **Keller, R., W. Fischer, R. Keist, and S. Bassetti.** 1992. Macrophage response to bacteria: induction of marked secretory and cellular activities by lipoteichoic acids. Infect. Immun. **60**:3664-3672.
165. **Keshav, S., L.-P. Chung, and S. Gordon.** 1990. Macrophage products in inflammation. Diagn. Microbiol. Infect. Dis. **13**:439-447.
166. **Kilpper-Bälz, R., and K. H. Schleifer.** 1987. *Streptococcus suis* sp. nov., nom. rev. Int. J. Syst. Bacteriol. **37**:160-162.
167. **Klegerman, M. E., K. M. Boyer, C. K. Papierniak, L. Levine, and S. P. Gotoff.** 1984. Type-specific capsular antigen is associated with virulence in late-onset group B streptococcal type III disease. Infect. Immun. **44**:124-129.
168. **Kotarsky, H., A. Thern, G. Lindahl, and U. Sjobring.** 2000. Strain-specific restriction of the antiphagocytic property of group A streptococcal M proteins. Infect. Immun. **68**:107-112.
169. **Lahrtz, F., L. Piali, K. Spanaus, J. Seebach, and A. Fontana.** 1998. Chemokines and chemotaxis of leukocytes in infectious meningitis. J. Neuroimmunol. **85**:33-43.
170. **Lalonde, M., M. Segura, S. Lacouture, and M. Gottschalk.** 2000. Interactions between *Streptococcus suis* serotype 2 and different epithelial cell lines. Microbiology **146**:1913-1921.
171. **Langford, P., A. E. Williams, and J. S. Kroll.** 1991. Superoxide dismutases of pathogenic and non-pathogenic *Streptococcus suis* type 2 isolates. FEMS Microbiol. Lett. **61**:347-350.
172. **Lapointe, L., S. D'Allaire, A. Lebrun, S. Lacouture, and M. Gottschalk.** 2002. Antibody response to an autogenous vaccine and serologic profile for *Streptococcus suis* capsular type 1/2. Can. J. Vet. Res. **66**:8-14.
173. **Leelarasamee, A., C. Nilakul, S. Tien-Grim, S. Srifuengfung, and W. Susaengrat.** 1997. *Streptococcus suis* toxic-shock syndrome and meningitis. J. Med. Assoc. Thai. **80**:63-68.

174. **Leturcq, D., A. Moriarty, G. Talbott, R. K. Winn, T. R. Martin, and R. J. Ulevitch.** 1996. Antibodies against CD14 protect primates from endotoxin-induced shock. J. Clin. Invest. **98:**1533-1538.
175. **Levy, N. J., and D. L. Kasper.** 1985. Antibody-independent and -dependent opsonization of group B *Streptococcus* requires the first component of complement C1. Infect. Immun. **49:**19-24.
176. **Lien, E., T. J. Sellati, A. Yoshimura, T. H. Flo, G. Rawadi, R. W. Finberg, J. D. Carroll, T. Espevik, R. R. Ingalls, J. D. Radolf, and D. T. Golenbock.** 1999. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. J. Biol. Chem. **274:**33419-33425.
177. **Ling, E. W. Y., F. J. D. Noya, G. Ricard, K. Beharry, E. L. Mills, and J. V. Aranda.** 1995. Biochemical mediators of meningeal inflammatory response to group B *streptococcus* in the newborn piglet model. Pediatr. Res. **38:**981-987.
178. **Liu, Y., Y. Wang, M. Yamakuchi, S. Isowaki, E. Nagata, Y. Kanmura, I. Kitajima, and I. Maruyama.** 2001. Up-regulation of Toll-like receptor 2 gene expression in macrophage response to peptidoglycan and high concentration of lipopolysacharide is involved in NF-κB activation. Infect. Immun. **69:**2788-2796.
179. **Lopez-Cortés, L. F., M. Cruz-Ruiz, J. Gomez-Mateos, P. Viciana-Fernandez, F. J. Martinez-Marcos, and J. Pachon.** 1995. Interleukin-8 in cerebrospinal fluid from patients with meningitis of different etiologies: its possible role as neutrophil chemotactic factor. J. Infect. Dis. **172:**581-584.
180. **Lutticken, R., N. Temme, G. Hahn, and E. W. Bartelheimer.** 1986. Meningitis caused by *Streptococcus suis*: case report and review of the literature. Infection **14:**181-185.
181. **Lynn, W. A., Y. Liu, and D. T. Golenbock.** 1993. Neither CD14 nor serum is absolutely necessary for activation of mononuclear phagocytes by bacterial lipopolysaccharide. Infect. Immun. **61:**4452-4461.
182. **Madsen, L. W., B. Svensmark, K. Elvestad, and H. E. Jensen.** 2001. Otitis interna is a frequent sequela to *Streptococcus suis* meningitis in pigs. Vet. Pathol. **38:**190-195.
183. **Marques, M. B., D. L. Kasper, M. K. Pangburn, and M. R. Wessels.** 1992. Prevention of C3 deposition by capsular polysaccharide is a virulence mechanism of type III group B Streptococci. Infect. Immun. **60:**3986-3993.
184. **McEver, R. P.** 1997. Selectin-carbohydrate interactions during inflammation and metastasis. Glycoconj. **14:**585-591.
185. **McNeil, N. I., and T. Gordon.** 1986. Meningitis caused by *Streptococcus suis* type II. Postgrad. Med. J. **62:**743-744.
186. **Michaud, S., R. Duperval, and R. Higgins.** 1996. *Streptococcus suis* meningitis: First case reported in Quebec. Can. J. Infect. Dis. **7:**329-331.
187. **Mogollon, J. D., C. Pijoan, M. P. Murtaugh, J. E. Collins, and P. P. Cleary.** 1991. Identification of epidemic strains of *Streptococcus suis* by genomic fingerprinting. J. Clin. Microbiol. **29:**782-787.
188. **Monter Flores, J. L., R. Higgins, S. D'Allaire, R. Charette, M. Boudreau, and M. Gottschalk.** 1993. Distribution of the different capsular types of *Streptococcus suis* in nineteen swine nurseries. Can. Vet. J. **34:**170-171.
189. **Mosser, D. M.** 1994. Receptors on phagocytic cells involved in microbial recognition, p. 99-114. *In* B. S. Zwilling and T. K. Eisenstein (ed.), Macrophage-Pathogen Interactions, vol. 60. Dekker, M., Inc., New York.
190. **Moxon, E. R., and J. S. Kroll.** 1990. The role of bacterial polysaccharide capsules as virulence factors. Curr. Top. Microbiol. Immunol. **150:**65-85.

191. **Mukaida, N., A. Harada, and K. Matsushima.** 1998. Interleukin-8 (IL-8) and monocyte chemotactic and activating factor (MCAF/MCP-1), chemokines essentially involved in inflammatory and immune reactions. Cytokine and Growth Factor Rev. **9:**9-23.

192. **Munday, J., H. Floyd, and P. R. Crocker.** 1999. Sialic acid binding receptors (siglecs) expressed by macrophages. J. Leukoc. Biol. **66:**705-711.

193. **Nielsen, B. W., N. Mukaida, and K. Matsushima.** 1994. Macrophages as producers of chemotactic proinflammatory cytokines, p. 131-142. *In* B. S. Zwilling and T. K. Eisenstein (ed.), Macrophage-Pathogen Interactions, vol. 60. Dekker, M., Inc., New York.

194. **Niven, D. F., A. Ekins, and A. A. W. Al-Samaurai.** 1999. Effects of iron and manganese availability on growth and production of superoxide dismutase by *Streptococcus suis*. Can. J. Microbiol. **45:**1027-1032.

195. **Noel, G. J., S. K. Hoiseth, and P. J. Edelson.** 1992. Type b capsule inhibits ingestion of *Haemophilus influenzae* by murine macrophages: studies with isogenic encapsulated and unencapsulated strains. J. Infect. Dis. **166:**178-182.

196. **Noel, G. J., S. L. Katz, and P. J. Edelson.** 1991. The role of C3 in mediating binding and ingestion of group B *Streptococccus* serotype III by murine macrophages. Pediatr. Res. **30:**118-123.

197. **Noel, G. J., D. M. Mosser, and P. J. Edelson.** 1990. Role of complement in mouse macrophage binding of *Haemophilus influenzae* type b. J. Clin. Invest. **85:**208-218.

198. **Norton, P. M., C. Rolph, P. N. Ward, R. W. Bentley, and J. A. Leigh.** 1999. Epithelial invasion and cell lysis by virulent strains of *Streptococcus suis* is enhanced by the presence of suilysin. FEMS Immunol. Med. Microbiol. **26:**25-35.

199. **Noya, F. J., C. J. Baker, and M. S. Edwards.** 1993. Neutrophil Fc receptor participation in phagocytosis of type III group B streptococci. Infect. Immun. **61:**1415-1420.

200. **Ofek, I., and E. H. Beachey.** 1980. General concepts and principles of bacterial adherence, p. 1-29. *In* E. H. Beachey (ed.), Receptors and recognition, series B. Bacterial adherence, vol. 6. Chapman and Hall, Ltd., London.

201. **Ofek, I., and N. Sharon.** 1988. Lectinophagocytosis: a molecular mechanism of recognition between cell surface sugars and lectins in the phagocytosis of bacteria. Infect. Immun. **56:**539-547.

202. **Okwumabua, O., O. Abdelmagid, and M. M. Chengappa.** 1999. Hybridization analysis of the gene encoding a hemolysin (suilysin) of *Streptococcus suis* type 2: evidence for the absence of the gene in some isolates. FEMS Microbiol. Lett. **181:**113-121.

203. **Oppenheim, J. J., C. O. C. Zachariae, N. Mukaida, and K. Matsushima.** 1991. Properties of the novel proinflammatory supergene "intercrine" cytokine family. Annu. Rev. Immunol. **9:**617-648.

204. **Palmer, M.** 2001. The family of thiol-activated, cholesterol-binding cytolysins. Toxicon **39:**1681-1689.

205. **Payne, N. R., and M. A. Horwitz.** 1987. Phagocytosis of *Legionella pneumophila* is mediated by human monocyte complement receptors. J. Exp. Med. **166:**1377-1389.

206. **Peetermans, W. E., B. G. Moffie, and J. Thompson.** 1989. Bacterial endocarditis caused by *Streptococcus suis* type 2 [letter]. J. Infect. Dis. **159:**595-596.

207. **Perch, B., E. Kjems, P. Slot, and K. B. Pedersen.** 1981. Biochemical and serological properties of R, S and RS streptococci. Acta Path. Microbiol. Scand. Sect. B **89:**167-171.

208. **Perch, B., P. Kristjansen, and S. K.** 1968. Group R streptococci pathogenic for man: two cases of meningitis and one fatal case of sepsis. Acta Path. Microbiol. Scand. **74:**69-76.

209. **Perch, B., K. B. Pedersen, and J. Henrichsen.** 1983. Serology of capsulated streptococci pathogenic for pigs: six new serotypes of *Streptococcus suis.* J. Clin. Microbiol. **17:**993-996.

210. **Pugin, J., D. Heumann, A. Tomasz, V. V. Kravchenko, Y. Akamatsu, M. Nishijima, M. P. Glauser, P. S. Tobias, and R. J. Ulevitch.** 1994. CD14 is a pattern recognition receptor. Immunity **1:**509-516.

211. **Pugin, J., C. C. Shurer-Maly, D. Leturcq, A. Moriarty, R. J. Ulevitch, and P. S. Tobias.** 1993. Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. Proc. Natl. Acad. Sci. USA **90:**2744-2748.

212. **Quagliarello, V., and W. M. Scheld.** 1992. Bacterial meningitis: pathogenesis, pathophysiology, and progress. New Engl. J. Med. **327:**864-872.

213. **Quessy, S., P. Busque, R. Higgins, M. Jacques, and J. D. Dubreuil.** 1997. Description of an albumin binding activity for *Streptococcus suis* serotype 2. FEMS Microbiol. Lett. **147:**245-250.

214. **Quessy, S., J. D. Dubreuil, M. Jacques, F. Malouin, and R. Higgins.** 1994. Increase of capsular material thickness following in vivo growth of virulent *Streptococcus suis* serotype 2 strains. FEMS Microbiol. Lett. **115:**19-26.

215. **Qureshi, S. T., L. Lariviere, G. Leveque, S. Clermont, K. J. Moore, P. Gros, and D. Malo.** 1999. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (TLR4). J. Exp. Med. **189:**615-625.

216. **Rabehi, L., T. Irinopoulou, B. Cholley, N. Haeffner-Cavaillon, and M.-P. Carreno.** 2001. Gram-positive and Gram-negative bacteria do not trigger monocytic cytokine production through similar intracellular pathways. Infect. Immun. **69:**4590-4599.

217. **Rainard, P.** 1993. Binding of bovine fibronectin to mastitis-causing *Streptococcus agalactiae* induces adherence to solid substrate but not phagocytosis by polymorphonuclear cells. Microb. Pathog. **14:**239-248.

218. **Ramarao, N., S. D. Gray-Owen, and T. F. Meyer.** 2000. *Helicobacter pylori* induces but survives the extracellular release of oxygen radicals from professional phagocytes using its catalase activity. Mol. Microbiol. **38:**103-113.

219. **Ramarao, N., and T. F. Meyer.** 2001. *Helicobacter pylori* resists phagocytosis by macrophages: quantitative assessment by confocal microscopy and fluorescence-activated cell sorting. Infect. Immun. **69:**2604-2611.

220. **Rasmussen, S. R., and L. O. Andresen.** 1998. 16S rDNA sequence variations of some *Streptococcus suis* serotypes. Int. J. Syst. Bacteriol. **48:**1063-1065.

221. **Read, R. C., S. Zimmerli, V. C. Broaddus, D. A. Sanan, D. S. Stephens, and J. D. Ernst.** 1996. The (α2-8)-linked polysialic acid capsule of group B *Neisseria meningitidis* modifies multiple steps during interaction with human macrophages. Infect. Immun. **64:**3210-3217.

222. **Reams, R. Y., L. T. Glickman, D. D. Harrington, T. L. Bowersock, and H. L. Thacker.** 1993. *Streptococcus suis* infection in swine: a retrospective study of 256 cases. Part I. Epidemiologic factors and antibiotic susceptibility patterns. J. Vet. Diagn. Invest. **5:**363-367.

223. **Reams, R. Y., L. T. Glickman, D. D. Harrington, H. L. Thacker, and T. L. Bowersock.** 1994. *Streptococcus suis* infection in swine: a retrospective study of 256 cases. Part II. Clinical signs, gross and microscopic lesions, and coexisting microorganisms. J. Vet. Diagn. Invest. **6:**326-334.
224. **Reams, R. Y., D. D. Harrington, L. T. Glickman, H. L. Thacker, and T. L. Bowersock.** 1996. Multiple serotypes and strains of *Streptococcus suis* in naturally infected swine herds. J. Vet. Diagn. Invest. **8:**119-121.
225. **Robertson, I. D., and D. K. Blackmore.** 1989. Occupational exposure to *Streptococcus suis* type 2. Epidemiol. Infect. **103:**157-164.
226. **Robertson, I. D., and D. K. Blackmore.** 1989. Prevalence of *Streptococcus suis* types 1 and 2 in domestic pigs in Australia and New Zealand. Vet. Rec. **124:**391-394.
227. **Robertson, I. D., D. K. Blackmore, D. J. Hampson, and Z. F. Fu.** 1991. A longitudinal study of natural infection of piglets with *Streptococcus suis* types 1 and 2. Epidemiol. Infect. **107:**119-126.
228. **Rojas, M. T., M. Gottschalk, and V. Velázquez-Ordónez.** 2001. [Evaluación de la virulencia y serotipos de *Streptococcus suis* asilados de trabajadores de rastros en el valle de Toluca, Estado de México, México]. Vet. Méx. **32:**201-205.
229. **Romani, L., P. Puccetti, and F. Bistoni.** 1997. Interleukin-12 in infectious diseases. Clin. Microbiol. Rev. **10:**611-636.
230. **Romano, M., M. Sironi, C. Toniatti, N. Polentarutti, P. Fruscella, P. Ghezzi, R. Faggioni, W. Luini, V. Van Hinsbergh, S. Sozzani, F. Bussolino, V. Poli, G. Ciliberto, and A. Mantovani.** 1997. Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. Immunity **6:**315-325.
231. **Rostand, K. S., and J. D. Esko.** 1997. Microbial adherence to and invasion through proteoglycans. Infect. Immun. **65:**1-8.
232. **Rubens, C. E., S. Smith, M. Hulse, E. Y. Chi, and G. van Belle.** 1992. Respiratory epithelial cell invasion by group B streptococci. Infect. Immun. **60:**5157-5163.
233. **Rubens, C. E., M. R. Wessels, L. M. Heggen, and D. L. Kasper.** 1987. Transposon mutagenesis of group B streptococcal type III capsular polysaccharide: correlation of capsule expression with virulence. Proc. Natl. Acad. Sci. USA **84:**7208-7212.
234. **Salasia, S. I., C. Lammler, and L. A. Devriese.** 1994. Serotypes and putative virulence markers of *Streptococcus suis* isolates from cats and dogs. Res. Vet. Sci. **57:**259-261.
235. **Sanford, S. E., and M. E. Tilker.** 1982. *Streptococcus suis* type II-associated diseases in swine: observations of a one-year study. J. Am. Vet. Med. Assoc. **181:**673-676.
236. **Schlesinger, L. S.** 1998. *Mycobacterium tuberculosis* and the complement system. Trends. Microbiol. **6:**47-50.
237. **Segers, R. P. A. M., T. Kenter, L. A. M. de Haan, and A. A. C. Jacobs.** 1998. Characterisation of the gene encoding suilysin from *Streptococcus suis* and expression in field strains. FEMS Microbiol. Lett. **167:**255-261.
238. **Serhir, B., D. Dubreuil, R. Higgins, and M. Jacques.** 1995. Purification and characterization of a 52-kilodalton immunoglobulin G-binding protein from *Streptococcus suis* capsular type 2. J. Bacteriol. **177:**3830-3836.
239. **Serhir, B., R. Higgins, B. Foiry, and M. Jacques.** 1993. Detection of immunoglobulin-G-binding proteins in *Streptococcus suis*. J. Gen. Microbiol. **139:**2953-2958.

240. **Smith, H. E., H. Buijs, R. de Vries, H. J. Wisselink, N. Stockhofe-Zurwieden, and M. A. Smits.** 2001. Environmentally regulated genes of *Streptococcus suis*: identification by the use of iron-restricted conditions in vitro and by experimental infection of piglets. Microbiology **147:**271-280.
241. **Smith, H. E., F. H. Reek, U. Vecht, A. L. Gielkens, and M. A. Smits.** 1993. Repeats in an extracellular protein of weakly pathogenic strains of *Streptococcus suis* type 2 are absent in pathogenic strains. Infect. Immun. **61:**3318-3326.
242. **Smith, H. E., M. Rijnsburger, N. Stockhofe-Zurwieden, H. J. Wisselink, U. Vecht, and M. A. Smits.** 1997. Virulent strains of *Streptococcus suis* serotype 2 and highly virulent strains of *Streptococcus suis* serotype 1 can be recognized by a unique ribotype profile. J. Clin. Microbiol. **35:**1049-1053.
243. **Smith, H. E., L. van Bruijnsvoort, H. Buijs, H. J. Wisselink, and M. A. Smits.** 1999. Rapid PCR test for *Streptococcus suis* serotype 7. FEMS Microbiol. Lett. **178:**265-270.
244. **Smith, H. E., U. Vecht, A. L. Gielkens, and M. A. Smits.** 1992. Cloning and nucleotide sequence of the gene encoding the 136-kilodalton surface protein (muramidase-released protein) of *Streptococcus suis* type 2. Infect. Immun. **60:**2361-2367.
245. **Smith, H. E., U. Vecht, H. J. Wisselink, N. Stockhofe-Zurwieden, Y. Biermann, and M. A. Smits.** 1996. Mutants of *Streptococcus suis* types 1 and 2 impaired in expression of muramidase-released protein and extracellular protein induce disease in newborn germfree pigs. Infect. Immun. **64:**4409-4412.
246. **Smith, H. E., V. Veenbergen, J. van der Velde, M. Damman, H. J. Wisselink, and M. A. Smits.** 1999. The *cps* genes of *Streptococcus suis* serotypes 1, 2, and 9: development of rapid serotype-specific PCR assays. J. Clin. Microbiol. **37:**3146-3152.
247. **Spellerberg, B.** 2000. Pathogenesis of neonatal *Streptococcus agalactiae* infections. Microbes Infect. **2:**1733-1742.
248. **Spooner, C. E., N. P. Markowitz, and L. D. Saravolatz.** 1992. The role of tumor necrosis factor in sepsis. Clin. Immunol. Immunopathol. **62:**S11-S17.
249. **Sprenger, H., A. Rösler, P. Tonn, H. J. Braune, G. Huffmann, and D. Gemsa.** 1996. Chemokines in the cerebrospinal fluid of patients with meningitis. Clin. Immunol. Immunopathol. **80:**155-161.
250. **Staats, J., P. Brandon, G. Stewart, and M. M. Chengappa.** 1999. Presence of the *Streptococcus suis* suilysin gene and expression of MRP and EF correlates with high virulence in *Streptococcus suis* type 2 isolates. Vet. Microbiol. **70:**201-211.
251. **Staats, J. J., B. L. Plattner, J. Nietfeld, S. Dritz, and M. M. Chengappa.** 1998. Use of ribotyping and hemolysin activity to identify highly virulent *Streptococcus suis* type 2 isolates. J. Clin. Microbiol. **36:**15-19.
252. **Stuart, J. G., E. J. Zimmerer, and R. L. Maddux.** 1992. Conjugation of antibiotic resistance in *Streptococcus suis*. Vet. Microbiol. **30:**213-222.
253. **Tarr, P. I., S. W. Hosea, E. J. Brown, R. Schneerson, A. Sutton, and M. M. Frank.** 1982. The requirement of specific anticapsular IgG for killing of *Haemophilus influenzae* by the alternative pathway of complement activation. J. Immunol. **128:**1772-1775.
254. **Tarradas, C., A. Arenas, A. Maldonado, I. Luque, A. Miranda, and A. Perea.** 1994. Identification of *Streptococcus suis* isolated from swine: proposal for biochemical parameters. J. Clin. Microbiol. **32:**578-580.

255. **Teti, G., F. Tomasello, M. S. Chiofalo, G. Orefici, and P. Mastroeni.** 1987. Adherence of group B streptococci to adult and neonatal epithelial cells mediated by lipoteichoic acid. Infect. Immun. **55:**3057-3064.
256. **Tikkanen, K., S. Haataja, and J. Finne.** 1996. The galactosyl-(alpha 1-4)-galactose-binding adhesin of *Streptococcus suis*: occurrence in strains of different hemagglutination activities and induction of opsonic antibodies. Infect. Immun. **64:**3659-3665.
257. **Tikkanen, K., S. Haataja, C. Francois-Gerard, and J. Finne.** 1995. Purification of a galactosyl-alpha 1-4-galactose-binding adhesin from the gram-positive meningitis-associated bacterium *Streptococcus suis*. J. Biol. Chem. **270:**28874-28878.
258. **Timmerman, C. P., E. Mattsson, L. Martinez-Martinez, L. De Graaf, J. A. G. Van Strijp, H. A. Verbrugh, J. Verhoef, and A. Fleer.** 1993. Induction of release of tumor necrosis factor from human monocytes by staphylococci and staphylococcal peptidoglycans. Infect. Immun. **61:**4167-4172.
259. **Torremorell, M., M. Calsamiglia, and C. Pijoan.** 1998. Colonization of suckling pigs by *Streptococcus suis* with particular reference to pathogenic serotype 2 strains. Can. J. Vet. Res. **62:**21-26.
260. **Torremorell, M., and C. Pijoan.** 1998. Prolonged persistence of an epidemic *Streptococcus suis* strain in a closed pig population. Vet. Rec. **143:**394-395.
261. **Tracey, K. J., and A. Cerami.** 1993. Tumor necrosis factor: An update review of its biology. Crit. Care Med. **21:**S415-S422.
262. **Tran Van Nhieu, G., and R. R. Isberg.** 1993. Bacterial internalization mediated by beta 1 chain integrins is determined by ligand affinity and receptor density. EMBO J. **12:**1887-1895.
263. **Tsutsui, O., S. Kikeguchi, K. Matsumara, and K. Kato.** 1991. Relationship of the chemical structure and immunobiological activities of lipoteichoic acid from *Streptococcus faecalis* (*Enterococcus hirae*) ATCC 9790. FEMS Microbiol. Immunol. **76:**211-218.
264. **Tunkel, A. R., and W. M. Scheld.** 1993. Pathogenesis and pathophysiology of bacterial meningitis. Clin. Microbiol. Rev **6:**118-136.
265. **Tuomanen, E. I.** 1996. Entry of pathogens into the central nervous system. FEMS Microbiol. Rev. **18:**289-299.
266. **Tuomanen, E. I., R. Austrian, and H. R. Masure.** 1995. Pathogenesis of pneumococcal infection. New Engl. J. Med. **332:**1280-1284.
267. **Ulevitch, R. J., and P. S. Tobias.** 1994. Recognition of endotoxin by cells leading to transmembrane signaling. Curr. Opin. Immunol. **6:**125-130.
268. **Unkeless, J. C., E. Scigliano, and V. H. Freedman.** 1988. Structure and function of human and murine receptors for IgG. Annu. Rev. Immunol. **6:**251-281.
269. **Valentin-Weigand, P., P. Benkel, M. Rohde, and G. S. Chhatwal.** 1996. Entry and intracellular survival of group B Streptococci in J774 macrophages. Infect. Immun. **64:**2467-2473.
270. **Vallejo, J. G., C. J. Baker, and M. S. Edwards.** 1996. Interleukin-6 production by human neonatal monocytes stimulated by type III group B streptococci. J. Infect. Dis. **174:**332-337.
271. **Vallejo, J. G., C. J. Baker, and M. S. Edwards.** 1996. Roles of the bacterial cell wall and capsule in induction of tumor necrosis factor alpha by type III group B streptococci. Infect. Immun. **64:**5042-5046.

272. **van Emmerik, L. C., E. J. Kuijper, C. A. P. Fijen, J. Dankert, and S. Thiel.** 1994. Binding of mannan-binding protein to various bacterial pathogens of meningitis. Clin. Exp. Immunol. **97:**411-416.

273. **Van Furth, A. M., J. J. Roord, and R. van Furth.** 1996. Roles of proinflammatory and anti-inflammatory cytokines in pathophysiology of bacterial meningitis and effect of adjunctive therapy. Infect. Immun. **64:**4883-4890.

274. **Van Furth, R., A. Z. Cohn, J. G. Hirsch, J. H. Humphrey, W. G. Spector, and H. L. Langevoort.** 1972. The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. Bull. Wld. Hlth. Org. **46:**845-852.

275. **Vasconcelos, D., D. M. Middleton, and J. M. Chirino-Trejo.** 1994. Lesions caused by natural infection with *Streptococcus suis* type 9 in weaned pigs. J. Vet. Diagn. Invest. **6:**335-341.

276. **Vasselon, T., and P. A. Detmers.** 2002. Toll receptors: a central element in innate immune responses. Infect. Immun. **70:**1033-1041.

277. **Vecht, U., J. P. Arends, E. J. van der Molen, and L. A. van Leengoed.** 1989. Differences in virulence between two strains of *Streptococcus suis* type II after experimentally induced infection of newborn germ-free pigs. Am. J. Vet. Res. **50:**1037-1043.

278. **Vecht, U., N. Stockhofe-Zurwieden, B. J. Tetenburg, H. J. Wisselink, and H. E. Smith.** 1997. Murine and pig models of *Streptococcus suis* type 2 infections are incompatible. Adv. Exp. Med. Biol. **418:**827-829.

279. **Vecht, U., H. J. Wisselink, M. L. Jellema, and H. E. Smith.** 1991. Identification of two proteins associated with virulence of *Streptococcus suis* type 2. Infect. Immun. **59:**3156-3162.

280. **Vecht, U., H. J. Wisselink, F. H. Reek, N. Stockhofe-Zurwieden, and H. E. Smith.** 1996. Diagnosis of several capsular serotypes of *Streptococcus suis* by phenotype and PCR and the relation with virulence for pigs. Presented at the Int. Congr. Pig. Vet. Soc.

281. **Vecht, U., H. J. Wisselink, J. E. van Dijk, and H. E. Smith.** 1992. Virulence of *Streptococcus suis* type 2 strains in newborn germfree pigs depends on phenotype. Infect. Immun. **60:**550-556.

282. **Verhoef, J., and E. Mattsson.** 1995. The role of cytokines in Gram-positive bacterial shock. Trends Microbiol. **3:**136-140.

283. **Virkola, R., K. Lähteenmäki, T. Eberhard, P. Kuusela, L. van Alphen, M. Ullberg, and K. Korhonen.** 1996. Interaction of *Haemophilus influenzae* with the mammalian extracellular matrix. J. Infect. Dis. **173:**1137-1147.

284. **Von Hunolstein, C., A. Totolian, G. Alfarone, G. Mancuso, V. Cusumano, G. Teti, and G. Orefici.** 1997. Soluble antigens from group B streptococci induce cytokine production in human blood cultures. Infect. Immun. **65:**4017-4021.

285. **Walsh, B., A. E. Williams, and J. Satsangi.** 1992. *Streptococcus suis* type 2: pathogenesis and clinical disease. Rev. Med. Microbiol. **3:**65-71.

286. **Wessels, M. R., V. Pozsgay, D. L. Kasper, and H. J. Jennings.** 1987. Structure and immunochemistry of an oligosaccharide repeating unit of the capsular polysaccharide of type III group B *Streptococcus*. A revised structure for the type III group B streptococcal polysaccharide antigen. J. Biol. Chem. **262:**8262-8267.

287. **Wessels, M. R., C. E. Rubens, V. J. Benedi, and D. L. Kasper.** 1989. Definition of a bacterial virulence factor: sialylation of the group B streptococcal capsule. Proc. Natl. Acad. Sci. USA **86:**8983-8987.

288. **Whicher, J. T., and S. W. Evans.** 1990. Cytokines in disease. Clin. Chem. **36:**1269-1281.
289. **Williams, A. E.** 1990. Relationship between intracellular survival in macrophages and pathogenicity of *Streptococcus suis* type 2 isolates. Microb. Pathog. **8:**189-196.
290. **Williams, A. E., and W. F. Blakemore.** 1990. Monocyte-mediated entry of pathogens into the central nervous system. Neuropathol. Appl. Neurobiol. **16:**377-392.
291. **Williams, A. E., and W. F. Blakemore.** 1990. Pathogenesis of meningitis caused by *Streptococcus suis* type 2. J. Infect. Dis. **162:**474-481.
292. **Williams, A. E., and W. F. Blakemore.** 1990. Pathology of streptococcal meningitis following intravenous intracisternal and natural routes of infection. Neuropathol. Appl. Neurobiol. **16:**345-356.
293. **Williams, D. M., G. H. Lawson, and A. C. Rowland.** 1973. Streptococcal infection in piglets: the palatine tonsils as portals of entry for *Streptococcus suis*. Res. Vet. Sci. **15:**352-362.
294. **Williams, P. A., J. F. Bohnsack, N. H. Augustine, W. K. Drummond, C. E. Rubens, and H. R. Hill.** 1993. Production of tumor necrosis factor by human cells in vitro and in vivo, induced by group B streptococci. J. Pediatr. **123:**292-300.
295. **Wilson, M., R. Seymour, and B. Henderson.** 1998. Bacterial perturbation of cytokine networks. Infect. Immun. **66:**2401-2409.
296. **Windsor, R. S., and S. D. Elliott.** 1975. Streptococcal infection in young pigs. IV. An outbreak of streptococcal meningitis in weaned pigs. J. Hyg. Lond. **75:**69-78.
297. **Wisselink, H. J.** 2001. *Streptococcus suis* infections in pigs. Use of virulence-associated markers in diagnostics and vaccines. Ph.D. University of Utrecht, Lelystad.
298. **Wisselink, H. J., H. E. Smith, N. Stockhofe-Zurwieden, K. Peperkamp, and U. Vecht.** 2000. Distribution of capsular types and production of muramidase-released protein (MRP) and extracellular factor (EF) of *Streptococcus suis* strains isolated from diseased pigs in seven European countries. Vet. Microbiol. **74:**237-248.
299. **Wisselink, H. J., U. Vecht, N. Stockhofe-Zurwieden, and H. E. Smith.** 2001. Protection of pigs against challenge with virulent *Streptococcus suis* serotype 2 strains by a muramidase-released protein and extracellular factor vaccine. Vet. Rec. **148:**473-477.
300. **Wong, G. G., and S. C. Clark.** 1988. Multiple actions of interleukin 6 within a cytokine network. Immunol. Today **9:**137-139.
301. **Woo, J., and E. K. Li.** 1987. *Streptococcus suis* meningitis requires prolonged treatment with penicillin [letter]. Infection **15:**129-130.
302. **Wright, S. M.** 1995. CD14 and innate recognition of bacteria. J. Immunol. **155:**6-8.
303. **Wright, S. M., R. A. Ramos, P. S. Tobias, R. J. Ulevitch, and J. C. Mathison.** 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science **249:**1431-1433.
304. **Yamamoto, Y., S. Okubo, T. W. Klein, K. Onozaki, T. Saito, and H. Friedman.** 1994. Binding of *Legionella pneumophila* to macrophages increases cellular cytokine mRNA. Infect. Immun. **62:**3947-3956.

305. **Yang, K. D., N. H. Augustine, M.-F. Shaio, J. F. Bohnsack, and H. R. Hill.** 1994. Effects of fibronectin on actin organization and respiratory burst activity in neutrophils, monocytes, and macrophages. J. Cell. Physiol. **158:**347-353.

306. **Yen, M. Y., Y. C. Liu, J. H. Wang, Y. S. Chen, Y. H. Wang, and D. L. Cheng.** 1994. *Streptococcus suis* meningitis complicated with permanent perceptive deafness: report of a case. J. Formos. Med. Assoc. **93:**349-351.

307. **Yoshimura, A., E. Lien, R. R. Ingalls, E. I. Tuomanen, R. Dziarski, and D. T. Golenbock.** 1999. Cutting Edge: Recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. J. Immunol. **163:**1-5.

308. **Zaffran, Y., L. Zhang, and J. J. Ellner.** 1998. Role of CR4 in *Mycobacterium tuberculosis*-human macrophages binding and signal transduction in the absence of serum. Infect. Immun. **66:**4541-4544.

309. **Zanen, H. C., and H. W. B. Engel.** 1975. Porcine streptococci causing meningitis and septicemia in man. The Lancet **1:**1286-1288.

310. **Zwilling, B. S., and T. K. Eisenstein.** 1994. Macrophage-Pathogen Interactions, vol. 60. Dekker, M., Inc., New York.

III. MATERIALS, METHODS AND RESULTS

ARTICLE I

Streptococcus suis and Group B *Streptococcus* differ in their interactions with murine macrophages

Mariela A. Segura, Patrick Cléroux, Marcelo Gottschalk

Groupe de Recherche sur les Maladies Infectieuses du Porc, Faculté de médecine vétérinaire, Université de Montréal, C.P. 5000, St-Hyacinthe, Québec, J2S 7C6, Canada

FEMS Immunology and Medical Microbiology. 1998. 21:189-195

Role of the candidate in conception of this article:

I was the main author of this article. I have actively participated in the conception and design of the experiments, and the standardization of techniques. I carried out all the laboratory work and also the analysis of results. Finally, I did also all the graphic conceptions and writing of the manuscript.

ABSTRACT

Streptococcus suis type 2 and Group B *Streptococcus* type III (GBS) are important encapsulated bacterial species causing meningitis. In the present study we compared quantitatively the uptake and intracellular survival of *S. suis* type 2 and GBS type III with murine macrophages in non-opsonic conditions. The role of the capsule of both pathogens was also studied using previously obtained unencapsulated isogenic mutants. Encapsulated *S. suis* wild type strain was practically not phagocyted, while the unencapsulated mutant was easily ingested by macrophages. On the other hand, the well encapsulated GBS strain, as well as its unencapsulated mutant were both phagocyted in high numbers. Even if *S. suis* unencapsulated mutant showed a higher uptake rate than the parental strain, this value was always remarkably lower than the numbers of ingested GBS strains. In addition, the intracellular survival of encapsulated and unencapsulated GBS strains was significantly higher than that of *S. suis* strains. These results suggest that interactions between GBS type III and *S. suis* type 2 with murine macrophages as well as the role of the capsule as an antiphagocytic factor are different for both bacterial pathogens.

INTRODUCTION

Streptococcus suis is an important pathogen which has been associated with a wide variety of infections in swine such as meningitis, septicemia, and pneumonia [1]. It has also been isolated from other animal species, as well as from humans [2,3]. To date, 35 different capsular types of *S. suis* have been described. *S. suis* capsular type 2 is considered to be the most virulent as well as the most prevalent capsular type in diseased pigs [4].

Group B *Streptococcus* (GBS) is an important cause of pneumonia, sepsis and meningitis in neonates. GBS associated with human disease are almost invariably encapsulated, belonging to one of the nine recognized capsule serotypes: Ia, Ib, II-VIII. Type III GBS strains account for about two-thirds of the GBS isolates associated with invasive neonatal disease [5].

S. suis and GBS present several similarities. In fact, both streptococci are well encapsulated, some capsular types are more virulent than others, they possess sialic acid in their capsule and they can cause severe meningitis. However, it is not clear whether the pathogenesis of the infection caused by both bacterial species is similar.

The capsules of a variety of bacterial species are thought to play an important role in virulence. Encapsulated organisms interact with host defense systems by several known mechanisms [6]. Among them, the sialylated polysaccharidic capsule of GBS plays a well recognized role in the resistance to phagocytosis, by modulating the alternative complement pathway, and protecting the organism from opsonophagocytic killing [7-10]. In vitro and in vivo virulence assays with unencapsulated or asialo mutants support the hypothesis that type III capsule serves as a virulent factor [11,12]. Other studies showed that well encapsulated GBS are able to enter and persist in macrophages by evading intracellular opsonin-mediated antibacterial activities [13,14]. The role of the polysaccharidic capsule in the opsonin-independent uptake is so far unknown.

S. suis is also encapsulated. In a recent work, transposon mutagenesis with the self-conjugative transposon Tn*916* was used to obtain isogenic acapsular mutants from a virulent *S. suis* type 2 strain. These mutants were shown to be avirulent for both mice

and piglets and cleared from circulation rapidly [15]. In fact, the capsule seems to be the first critical virulence factor described so far for this bacterial species. However, avirulent field strains are also encapsulated. Other putative virulence factors have been suggested, such as cell-wall and extracellular proteins, toxins, adhesins and immunoglobulin-binding proteins [16-20], but their role in the pathogenesis of the infection is unknown. As mentioned above, *S. suis* also possesses sialic acid in its capsule. However, and unlike the GBS, this sugar does not seem to be a critical component for the virulence [21]. It may be possible that interactions between *S. suis* and GBS with macrophages do differ. In fact, previous studies of interactions between encapsulated strains of *S. suis* type 2 and phagocytic cells are controversial [22].

In the present study we compare the uptake and intracellular survival of *S. suis* type 2 and GBS type III with murine macrophages in non-opsonic conditions. The role of the capsule of both pathogen is also studied using previously obtained acapsular isogenic mutants.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Two strains of *S. suis* capsular type 2 and two strains of GBS capsular type III were used: the virulent-encapsulated *S.suis* wild type strain S735-SM and its avirulent-unencapsulated isogenic transposon mutant 2A [15], as well as the highly encapsulated wild-type strain of type III GBS, COH1, and its less virulent and unencapsulated isogenic transposon mutant, COH1-13 [12]. The latter strains were kindly provided by Dr. C. E. Rubens (Children's Hospital and Medical Center, Seattle, WA).

Bacteria were maintained as stock cultures in 50% glycerol- Todd-Hewitt broth (THB; Difco Lab., Detroit, MI) at - 80°C. The THB were supplemented with 10 μg^{-1} tetracycline (Tc; Sigma-Aldrich, Oakville, Canada) for growing the mutants [15]. Bacteria were grown overnight onto bovine blood agar plates at 37°C and single colonies were used as inocula for THB or THB-Tc, which were incubated for 18 h at 37°C. Working cultures were made by inoculating 0.3 ml of these cultures in 10 ml of THB at 37°C with agitation until it reached the mid-log phase (6 h incubation-time; 540 nm-optical density of 0.4-0.5 for *S. suis* strains, and 0.7-0.8 for GBS strains). Bacteria were washed twice in phosphate-buffered saline (PBS), pH 7.4, and diluted to approximately 10^7 cfu/ml in cell culture media (without antibiotics). An accurate determination of the number of colony forming units (CFU) in the final suspension was made for each assay by plating on THB-agar.

Cell lines and cell culture

J774A1 murine (BALB/c) macrophage-like cell line (ATCC TIB 67, Rockville, Md.) was maintained in Dulbecco's Modified Eagle's medium, 1.5 g/l bicarbonate (DMEM). P388D1 murine (DBA/2) macrophage-like cell line (ATCC TIB 63) was maintained in Iscove's Modified Dulbecco's medium (IMDM). Cell media were supplemented with 10% heat-inactivated fetal bovine serum (iFBS), penicillin G (100 IU/ml), and streptomycin (100 μg/ml) (Gibco, Burlington, Canada) and cells were grown at 37°C, 5% CO_2.

For bacterial phagocytosis and survival assays, 48 h culture cells were scraped up, washed once and resuspended in antibiotic-free media at approximately 2 x 10^5 cells/ml. One ml of this suspension was distributed into 24-well tissue culture plates Falcon (VWR CanLab, Quebec,Canada) and incubated for 3 h at 37°C, 5% CO_2 to allow cell adhesion before assays.

Preparation of murine peritoneal macrophages

Peritoneal exudate macrophages (PEM) were isolated as previously described [23]. Briefly, five or six, 6-8 week-old BALB/c male mice (Charles River, St-Constant, Canada) were used per individual assay. Mice were inoculated intraperitoneally with 1.5 ml 3% (w/v) thioglycolate sterile broth (Sigma). PEM were recovered 4 days later by washing the peritoneal cavity with 5 ml Hank's Balanced Salt Solution Ca^{++}-Mg^{++}-free (HBSS, Gibco). Cells were pooled, pelleted by centrifugation 10 min at 1000 *g*, and resuspended at final concentration of 2 x 10^5 cells/ml in Minimum Essential Medium (MEM, Gibco) supplemented with 10% iFBS (MEMs) . One ml of this suspension was distributed into 24-well tissue culture plates Falcon (VWR CanLab). Cells were allowed to adhere for 1 h at 37°C, 5% CO_2, washed with warm HBSS, and reincubated overnight in MEMs before assays. Cell purity was more than 95% as determined by non-specific esterase stain and modified Wright Giemsa stain (LeukoStat, Fisher Sci., Montreal, Canada). Cell viability was more than 99% as determined by Trypan blue exclusion.

Phagocytosis assay

Phagocytosis assays were performed as already described [14] with some modifications. J774 cell-, P388 cell- and PEM- plates were infected with streptococci by removing culture media and adding 1 ml of 10^7 bacterial suspension per well (in respective cell culture media, supplemented with 10% iFBS), to obtain a ratio of about 100 bacteria per macrophage. Phagocytosis was left to proceed for 30 min and 90 min at 37°C, 5% CO_2. After incubation, cell monolayers were washed twice with warm HBSS, and reincubated for 1 h with medium containing penicillin G (5 μg/ml) and gentamicin (100 μg/ml; Sigma) to kill extracellular bacteria. It has been demonstrated that these

antibiotics do not penetrate eucaryotic cells under these conditions [14], and previous studies showed that this concentration of antibiotics was able to kill any remaining extracellular bacteria (data not shown). In addition, supernatant controls were taken in every test to confirm the activity of antibiotics. After antibiotic treatment, cell monolayers were washed three times and the medium was replaced with 1 ml of sterile distilled water to lyse the macrophages. After vigorous pipetting to ensure complete cell lysis, viable intracellular streptococci were determined by quantitative plating of serial dilutions of the lysates on THB-agar. Each test was done four times in independent experiments, and the number of CFU recovered per well (mean number ± standard deviation) was determined.

Intracellular survival assay

Survival experiments were done as described in the phagocytosis protocol, except that after 30 min of infection with streptococci, cell monolayers were washed twice with warm HBSS and antibiotic-containing medium was added and left on infected cells up to 4 h post-infection. At various intervals (60, 90, 120, 180, and 240 min), infected monolayers were washed three times and the medium was replaced with 1 ml of sterile distilled water to lyse the macrophages. After vigorous pipetting to ensure complete cell lysis, viable intracellular streptococci were determined by quantitative plating of serial dilutions of the lysates on THB-agar. Each test was done four times and intracellular killing was expressed as the percentage decrease in the initial number (100%) of viable intracellular bacteria at 60 min interval time [24].

Statistics

Differences were analyzed for significance by using Student's unpaired *t*-test (two-tailed *p*-value).

RESULTS

Phagocytosis of *S. suis* and GBS

We compared quantitatively the uptake process of *S. suis* and GBS wild type strains and the two unencapsulated avirulent mutants with two different murine phagocytic cell lines (J774 A1 and P388 D1) and with murine PEM, at 30 min and 90 min infection times.

At 30 min of phagocytosis (Fig. 1a), encapsulated S735-SM strain was practically not phagocyted, and there was not significant difference between the cell lines and PEM ($p > 0.05$). In contrast, encapsulated GBS strain COH1 was highly phagocyted. Phagocytic values were more elevated with J774A1 cells, followed by PEM, while P388D1 cells showed the lowest uptake rate ($p < 0.05$).

S. suis unencapsulated mutant 2A was highly phagocyted compared to wild type strain. Phagocytic values were more elevated with J774A1 cells than those obtained with PEM or P388D1 cells ($p < 0.05$). However, GBS unencapsulated mutant COH1-13 was slightly more phagocyted than the wild type COH1 strain by each of the three cell types. However, this difference is remarkably lower than that observed between *S.suis* unencapsulated mutant and its parental strain.

After 90 min of phagocytosis (Fig. 1b), the uptake of encapsulated S735-SM strain was not increased, with neither of the three types of phagocytic cells; and values were nearly zero. In contrast, GBS COH1 strain uptake rate was almost two log increased with PEM and nearly one log increased with both cell lines. P388D1 cells showed lower uptake degree than J774A1 cells and PEM ($p < 0.05$), while not significant difference was noted between the latter cell types ($p > 0.05$).

Unlike *S. suis* parental strain, the unencapsulated mutant 2A ingested numbers at 90 min were significant increased with respect to phagocytic values at 30 min infection time. Increase in phagocytosis was higher with J774A1 cells than with P388D1 cells or PEM ($p < 0.05$). In contrast, there were not significant differences in increasing phagocytosis between the GBS unencapsulated mutant COH1-13 and the parental strain.

Mutant COH1-13 strain was phagocyted at the same rate than parental strain by J774A1 cells and PEM ($p > 0.05$), but it was slightly more phagocyted by P388D1 cells ($p < 0.05$).

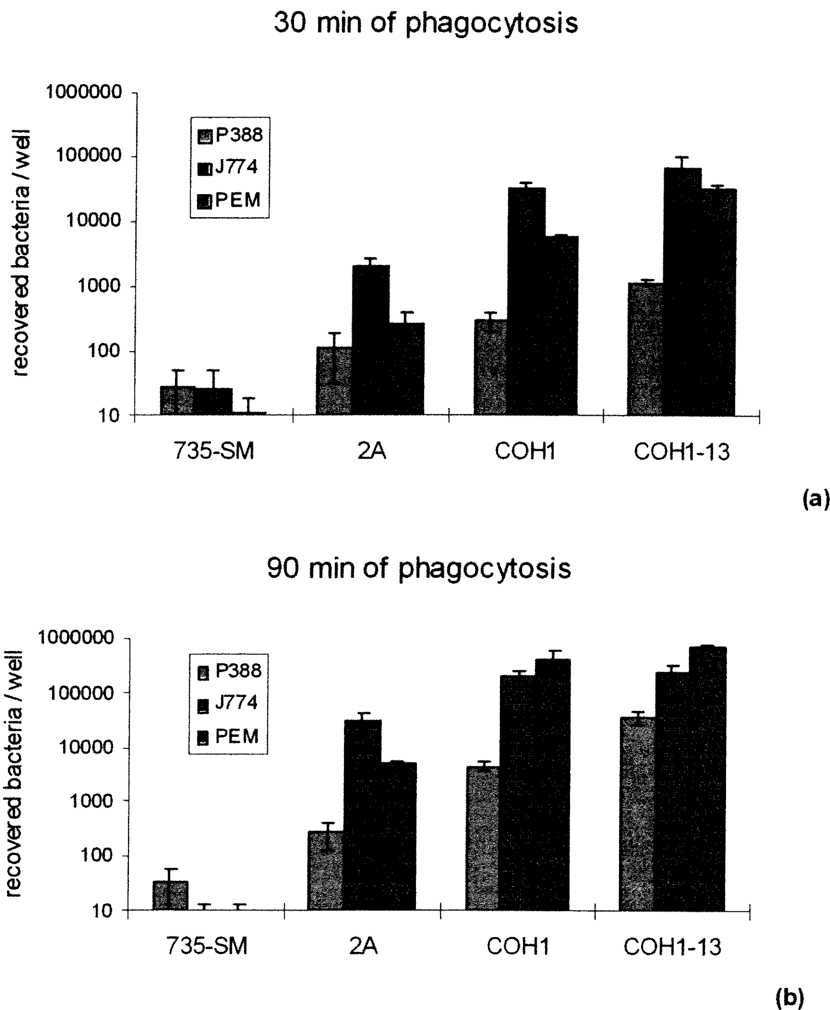


FIG. 1. Phagocytosis of *S. suis* strains (S735-SM and the unencapsulated mutant 2A) and GBS strains (COH1 and the unencapsulated mutant COH1-13) after (a) 30 min infection time and (b) 90 min infection time. Numbers of phagocytic bacteria were determined by quantitative plating after 1 h of antibiotic treatment, and results are expressed as CFU recovered bacteria per well (means ± S.D. obtained from four independent experiments).

Even if *S. suis* unencapsulated mutant showed a higher uptake rate respect to the parental strain, this value was always remarkably lower than the numbers of ingested GBS strains ($p < 0.05$). In general, for all four strains, uptake rate with J774A1 cells and PEM was higher than that shown with P388D1 cells.

Intracellular survival of *S. suis* mutant 2A compared to GBS strains

To study the intracellular survival of *S. suis* and GBS strains, we infected cells during 30 min and then samples were taken at different time points after the addition of antibiotic-containing medium. The few bacteria of *S. suis* wild type S735-SM strain, which were phagocyted, were not able to survive inside of macrophages (data not shown). Mutant 2A showed a significant linear decrease ($p < 0.05$) in the number of viable intracellular bacteria with all cell types. The time course of intracellular killing of mutant 2A with P388D1 cells was slower compared to J774A1 cells and PEM, decreasing by 90 min to 72% of that observed at 60 min (100%), and remaining nearly to 6% after 240 min post-infection time (Fig 2a). In the case of J774A1 cells and PEM (Fig 2b and 2c), the number of viable intracellular bacteria decreased rapidly to about 20% at 90 min; by 180 min, less than 4% of survival was detected, and viable intracellular bacteria were almost not detected after 240 min post-infection time.

The intracellular survival of GBS strains was significantly higher than that of *S. suis* mutant 2A. Results showed (Fig 2a, b and c) that there were not significant differences between the wild type GBS strain COH1 and the unencapsulated mutant COH1-13 (p values >0.05) with neither of the three cell types. Up to 120 min post-infection, intracellular numbers of viable GBS remained almost constant. Between 180 and 240 min post-infection, a quite significant decrease in the number of intracellular GBS was observed with P388D1 cells and PEM ($p < 0.05$), but remained constant with J774A1 cells.

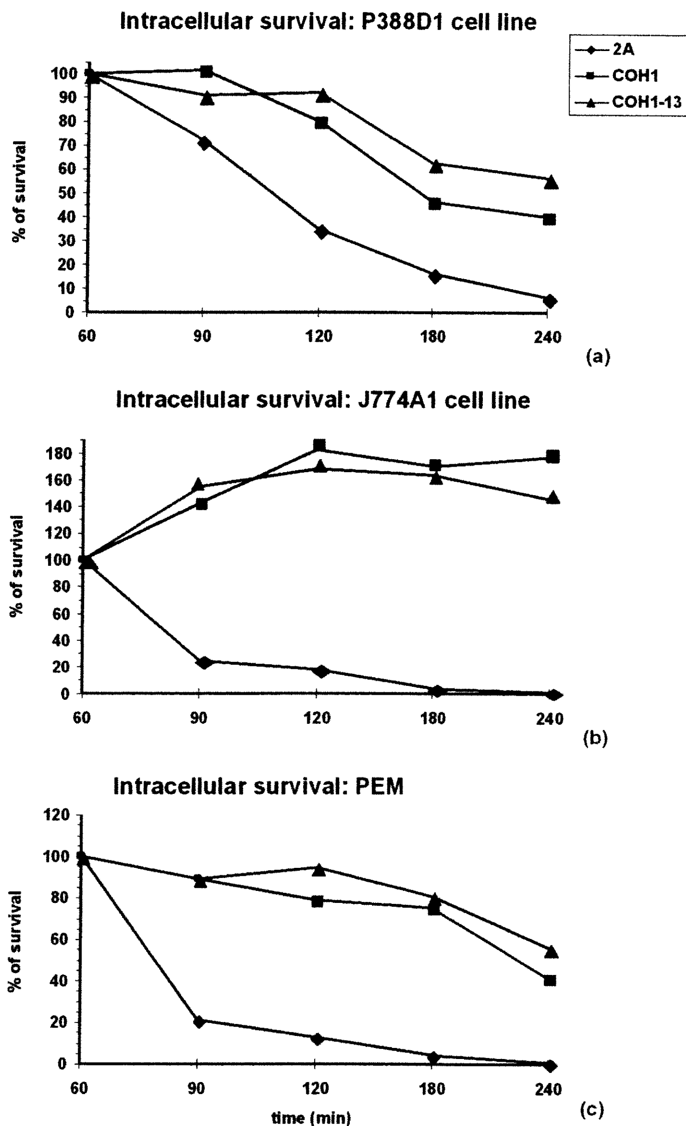


FIG. 2. Time course of intracellular survival of *S. suis* unencapsulated mutant 2A and GBS strains (COH1 and unencapsulated mutant COH1-13) within (a) P388D1 cells, (b) J774A1 cells, and (c) peritoneal exudate macrophages (PEM). Macrophages were allowed to ingest bacteria for 30 min, then antibiotics were added to kill extracellular bacteria and the numbers of viable intracellular bacteria were determined by quantitative plating at several post-infection times. Killing was expressed as the percentage decrease in the initial number (100%) of viable intracellular bacteria at 60 min post-infection time. These figures are representative of the results of four independent experiments.

DISCUSSION

Surface encapsulation is one of the most important virulence factors of several pathogenic microorganisms. Bacterial capsule, by effectively inhibiting phagocytosis and resisting complement-mediated bactericidal activity, may enhance bloodstream survival of the organism and facilitate intravascular replication. Indeed, the most common meningeal pathogens are all encapsulated [25]. However, the mechanisms of interaction of encapsulated bacteria with the host immune system are not at all known, and they are not generalized for all pathogens.

In the present study we compared quantitatively the in vitro interactions, uptake and intracellular survival, of *S. suis* capsular type 2 and GBS capsular type III with murine phagocytic cells under non-opsonic conditions. The role of their capsules in these interactions was evaluated by using unencapsulated isogenic mutants of both pathogens. Viable counting techniques for monitoring phagocytic interactions have already been used for GBS [14]. The present work is the first determination of phagocytosis and time course of intracellular survival of *S. suis* by quantitative platings.

Results in this study show that well encapsulated GBS can be ingested at high numbers by murine macrophages in the absence of complement and antibodies. Our results are similar to those described by Valentin-Weigand *et al* [14], who also showed that GBS COH1 strain is able to enter and persist efficiently in J774A1 macrophages by evading intracellular antibacterial activities. On the other hand, no differences were observed between this strain and its unencapsulated mutant, which indicate that the capsule is not necessarily an antiphagocytic factor for this bacterial species. Controversially, previous reports have assumed an anti-phagocytic role of GBS type III capsule since encapsulated bacteria were resistant to opsono-phagocytic killing by PMN in absence of specific antibodies, while unencapsulated or asialo mutants were susceptible [11,12,26]. However, in these studies, the techniques used allowed the study of bacterial killing rather than phagocytosis, since no differentiation of intracellular and extracellular bacteria was done.

Different results were obtained with *S. suis*. The well encapsulated parental strain of *S. suis* type 2 was almost not phagocytosed by murine macrophages, even after 90 min of bacteria-cell contact. Interestingly, previous in vitro studies of this bacterial species with phagocytes are contradictories. Early bacterial killing studies could not demonstrate any "phagocytic activity" with *S. suis* type 2 [27,28], whereas more recent studies showed variable percentages of phagocytosis (from 7 to 30%), using a vital staining technique [21,23,29]. These discrepancies may be due to technical differences. In fact, results of the present study represent the first data from quantitative phagocytosis using a viable counting technique. Unlike the GBS, the high percentage of phagocytosis obtained with the unencapsulated mutant of *S. suis* demonstrate the antiphagocytic role of the capsule, which confirm previous observations [15,23,30]. Similar results were obtained when porcine monocytes were used (data not shown).

The role of the capsule in the intracellular bacterial survival was also studied. Both encapsulated and unencapsulated GBS strains were able not only to enter, but also to survive efficiently inside of macrophages. To the best of our knowledge, this is the first report which demonstrate the entry and intracellular survival of an unencapsulated isogenic mutant of GBS capsular type III in murine macrophages under non-opsonic conditions. It was postulated that complement has not major effects on invasion and survival rates of well encapsulated GBS [14]. In addition, Rubens *et al* [12] showed that unencapsulated mutants will not survive in the presence of complement, which seems to indicate that GBS capsule plays a role in the resistance to opsono-intracellular killing, but it is not an antiphagocytic factor.

The few bacteria of the *S. suis* encapsulated parental strain recovered after 30 min of phagocytosis did not survive inside macrophages. In fact, after 60 min of reincubation time, no residual bacteria could be recovered (not shown). The survival of the unencapsulated mutant 2A was markedly reduced by more than 95% after 4 h of cell infection. Charland *et al* [15] demonstrated by using murine and pig models, that mutant 2A was more susceptible to early phagocyte clearance compared with the wild type strain, providing further evidence that the capsule is an antiphagocytic factor and plays an important role in virulence.

Finally, we confirmed that even if continuos murine cell lines with macrophage-like properties provide a convenient model for in vitro studies of macrophage functions, some differences may be observed with different lines. Results obtained in this study indicate that the J774A1 cell line is suitable for GBS and *S. suis* -macrophage interaction studies, since it present similar features to those of normal peritoneal macrophages [31].

In summary, results from the present work suggest that interactions between GBS type III and *S. suis* type 2 with murine macrophages as well as the role of the capsule as an antiphagocytic factor are different for both bacterial pathogens.

ACKNOWLEDGEMENTS

We thank Julie M. Trudel for excellent technical assistance. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) grant # 0680154280, and by the Fonds pour la Formation des Chercheurs et l'Aide à la Recherche du Québec (FCAR) grant # NC-1037. M.S. is a graduate scholarship holder from the Agence Canadienne de Développement International (ACDI).

REFERENCES

1. **Higgins, R. and Gottschalk, M.** 1999. Streptococcal diseases. Chapter 38. In: Diseases of swine, pp. 563-570. Iowa State University, Ames.
2. **Devriese, L.A., Sustronck, B., Maenhout, T. and Haesebrouck, F.** 1990. *Streptococcus suis* meningitis in a horse. Vet. Rec. **127:**68.
3. **Trottier, S., Higgins, R., Brochu, G. and Gottschalk, M.** 1991. A case of human endocarditis due to *Streptococcus suis* in North America. Rev. Infect. Dis. **13:**1251-1252.
4. **Higgins, R. and Gottschalk, M.** 1997. Distribution of *Streptococcus suis* capsular types in 1996. Can. Vet. J. **38:**302.
5. **Baker, C.J. and Edwards, M.S.** 1990. Group B streptococcal infections. In: Infectious Diseases of the Fetus and the Newborn. (Klein, J.S. and Klein, J.O., Eds.), pp. 742-811. The W. B. Saunders Co., Philadelphia.
6. **Kasper, D.L.** 1986. Bacterial capsule -old dogmas and new tricks. J. Infect. Dis. **153:**407-415.
7. **Edwards, M., Kasper, D., Jennings, H.J., Baker, C.J. and Nicholson-Weller, A.** 1982. Capsular sialic acid prevents activation of the alternative complement pathway by type III, group B streptococci. J. Immunol. **128:**1278-1283.
8. **Klegerman, M.E., Boyer, K.M., Papierniak, C.K., Levine, L. and Gotoff, S.P.** 1984. Type-specific capsular antigen is associated with virulence in late-onset group B Streptococcal type III disease. Infect. Immun. **44:**124-129.
9. **Wessels, M.R., Rubens, C.E., Benedi, V.J. and Kasper, D.L.** 1989. Definition of a bacterial virulence factor: sialylation of the group B streptococcal capsule. Proc. Natl. Acad. Sci. U S A **86:**8983-8987.
10. **Shigeoka, A.O., Rote, N.S., Santos, J.I. and Hill, H.R.** 1983. Assessment of the virulence factors of group B streptococci: correlation with sialic acid content. J. Infect. Dis. **147:**857-863.
11. **Wessels, M.R., Haft, R.F., Heggen, L.M. and Rubens, C.E.** 1992. Identification of a genetic locus essential for capsule sialylation in type III group B streptococci. Infect. Immun. **60:**392-400.
12. **Rubens, C.E., Heggen, L.M., Haft, R.F. and Wessels, M.R.** 1993. Identification of *cpsD*, a gene essential for type III capsule expression in group B streptococci. Mol. Microbiol. **8:**343-855.
13. **Antal, J.M., Cunningham, J.V. and Goodrum, K.J.** 1992. Opsonin-independent phagocytosis of group B streptococci: role of complement receptor type three. Infect. Immun. **60:**1114-1121.
14. **Valentin-Weigand, P., Benkel, P., Rohde, M. and Chhatwal, G.S.** 1996. Entry and intracellular survival of group B streptococci in J774 macrophages. Infect. Immun. **64:**2467-2473.
15. **Charland, N., Harel, J., Kobish, M., Lacasse, S. and Gottschalk, M.** 1998. *Streptococcus suis* serotype 2 mutants deficient in capsular expression. Microbiology **144:**325-332.
16. **Gottschalk, M., Higgins, R., Jacques, M., Dubreuil, D.** 1992. Production and characterization of two *S. suis* capsular type 2 mutants. Vet. Microbiol. **30:**59-71.

17. **Jacobs, A.A., van den Berg, A.J., Baars, J.C., Nielsen, B. and Johannsen, L.W.** 1995. Production of suilysin, the thiol-activated haemolysin of *Streptococcus suis*, by field isolates from diseased pigs. Vet. Rec. **137:**295-296.
18. **Gottschalk, M.G., Lacouture, S. and Dubreuil, J.D.** 1995. Characterization of *Streptococcus suis* capsular type 2 haemolysin. Microbiology **141:**189-195.
19. **Tikkanen, K., Haataja, S. and Finne, J.** 1996. The galactosyl-(alpha 1-4)-galactose-binding adhesin of *Streptococcus suis*: occurrence in strains of different hemagglutination activities and induction of opsonic antibodies. Infect. Immun. **64:**3659-3665.
20. **Vecht, U., Wisselink, H.J., Jellema, M.L. and Smith, H.E.** 1991. Identification of two proteins associated with virulence of *Streptococcus suis* type 2. Infect. Immun. **59:**3156-3162.
21. **Charland, N., Kobisch, M., Martineau-Doize, B., Jacques, M. and Gottschalk, M.** 1996. Role of capsular sialic acid in virulence and resistance to phagocytosis of *Streptococcus suis* capsular type 2. FEMS Immunol. Med. Microbiol. **14:**195-203.
22. **Alexander, T.** 1995. *Streptococcus suis*: pathogenesis and host response. Allen D. Leman Swine Conference (Scruton, W.C. and Cronje, R., Eds.), Vol. 22, pp. 49-53. University of Minnesota, Minnesota.
23. **Brazeau, C., Gottschalk, M., Vincelette, S. and Martineaudoize, B.** 1996. In vitro phagocytosis and survival of *Streptococcus suis* capsular type 2 inside murine macrophages. Microbiology **142:**1231-1237.
24. **Shimoji, Y., Yokomizo, Y. and Mori, Y.** 1996. Intracellular survival and replication of *Erysipelothrix rhusiopathiae* within murine macrophages: failure of induction of the oxidative burst of macrophages. Infect. Immun. **64:**1789-1793.
25. **Tunkel, A.R. and Scheld, W.M.** 1993. Pathogenesis and pathophysiology of bacterial meningitis. Clin. Microbiol. Rev. **6:**118-136.
26. **Marques, M.B., Kasper, D.L., Pangburn, M.K. and Wessels, M.R.** 1992. Prevention of C3 deposition by capsular polysaccharide is a virulence mechanism of type III group B streptococci. Infect. Immun. **60:**3986-3993.
27. **Agarwal, K.K., Elliott, S.D. and Lachmann, P.J.** 1969. Streptococcal infection in young pigs. III. The immunity of adult pigs investigated by the bactericidal test. J. Hyg. **67:**491-503.
28. **Clifton-Hadley, F.A.** 1981. Studies of *Streptococcus suis* type 2 infection in pigs. Ph. D Dissertation, Dept. Clin. Vet. Med., University of Cambridge, Cambridge.
29. **Williams, A.E.** 1990. Relationship between intracellular survival in macrophages and pathogenicity of *Streptococcus suis* type 2 isolates. Microb. Pathog. **8:**189-196.
30. **Salasia, S.I.O., Lammler, C. and Herrmann, G.** 1995. Properties of a *Streptococcus suis* isolate of serotype 2 and two capsular mutants. Vet. Microbiol. **45:**151-156.
31. **Snyderman, R., Pike, M., Fischer, D. and Koren, H.** 1977. Biologic and biochemical activities of continuous macrophage cell lines P388D1 and J774.1. J. Immunol. **119:**2060-2066.

ARTICLE II

Streptococcus suis Interactions with the Murine Macrophage Cell Line J774: Adhesion and Cytotoxicity

Mariela Segura and Marcelo Gottschalk

Groupe de Recherche sur les Maladies Infectieuses du Porc (GREMIP) and Canadian Research Network on Bacterial Pathogens of Swine, Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, Québec, Canada

Infection and Immunity. 2002. 70:4312-4322

Role of the candidate in conception of this article:

I was the main author of this article. I have actively participated in the conception and design of the experiments, and the standardization of techniques. I carried out all the laboratory work and also the analysis of results. Finally, I did also all the graphic conceptions and writing of the manuscript.

ABSTRACT

Streptococcus suis capsular type 2 is an important etiological agent of swine meningitis, and it is also a zoonotic agent. Since one hypothesis of the pathogenesis of *S. suis* infection is that bacteria enter the bloodstream and invade the meninges and other tissues in close association with mononuclear phagocytes, the objective of the present study was to evaluate the capacity of *S. suis* type 2 to adhere to macrophages. An enzyme-linked immunosorbent assay technique was standardized to simply and accurately measure the rate of bacterial attachment to phagocytic cells. Results were confirmed by plate counting. Adhesion was bacterial concentration- and incubation time- dependent, and was not affected by cytochalasin pre-treatment of macrophages. Inhibition studies showed that the sialic acid moiety of the *S. suis* capsule would be, at least in part, responsible for bacterial recognition by macrophages. Serum pre-opsonization of bacteria increased adhesion levels. Complement would be partially implicated in the serum-enhanced binding of *S. suis* to cells. Adhesion varied among different *S. suis* type 2 isolates. However, high bacterial concentrations of several isolates were cytotoxic for cells, and these cytotoxic effects correlated with suilysin production. Indeed, hemolytic strain supernatants, as well as, purified suilysin reproduced cytotoxic effects observed with live bacteria, and these effects were inhibited by cholesterol pre-treatment. Bacterial adhesion and cytotoxicity were confirmed by scanning and transmission electronic microscopy. We hypothesize that attachment of bacteria to phagocytes could play an important role in the pathogenesis of *S. suis* infection by allowing bacterial dissemination and causing a bacteremia and/or septicemia. This interaction could also be related to the activation of the host inflammatory response observed during meningitis.

INTRODUCTION

Streptococcus suis is not only one of the most important swine pathogens worldwide, but it is also a zoonotic agent. Among the serotypes described, type 2 is the serotype most frequently associated with disease (21). The most important clinical feature associated with *S. suis* is meningitis; however, other pathologies have also been described (22). Knowledge on virulence factors and the pathogenesis of *S. suis* infection is still limited. *S. suis* is transmitted via the respiratory route and remains localized in the palatine tonsils. Some animals will only be healthy carriers and will never develop disease, whereas others will, sooner or later, develop bacteremia, sometimes septicemia and finally, meningitis. Hence, in these cases, bacteria should travel and persist throughout the bloodstream and reach the central nervous system (CNS) (17). An early theory suggested uptake of bacteria by monocytes, intracellular survival and invasion of the CNS by the "Trojan horse theory" (54). This bacterial uptake could take place directly at the tonsils by macrophages or by monocytes once bacteria are in the bloodstream. However, most studies carried out during the last decade suggest that bacteria may also use (an)other mechanism(s) to disseminate (17). In fact, *S. suis* is a well encapsulated bacterium, and it has been proposed that the capsular polysaccharide (CPS) confers it antiphagocytic properties (6, 40, 43). The CPS of *S. suis* serotype 2 is composed of five sugars: glucose, galactose, N-acetylglucosamine, rhamnose and sialic acid (N-acetyl neuraminic acid) (25), this latter component being related to virulence for other bacterial agents of meningitis (29, 53). The CPS is so far the only proven critical virulence factor, based on the studies with nonencapsulated isogenic mutants. The absence of CPS correlated with increased hydrophobicity and phagocytosis using murine and porcine phagocytes. In addition, unencapsulated mutants were shown to be avirulent and cleared from circulation rapidly in both mouse and pig models of infection (6, 43). Despite the fact that the CPS seems to be a major virulence factor, most avirulent strains are encapsulated, indicating that other important virulence factors are essential.

S. suis also produces a hemolysin (suilysin), a thiol-activated toxin, which may have a role in virulence (18, 24). This protein belongs to the family of antigenically related cholesterol-binding toxins which forms transmembrane pores and possess a

"multi-hit" mechanism of action (18). Whereas, most European strains are suilysin-positive, variable production of this protein has been observed with North American strains (16, 45). Suilysin production is associated to the presence of the muramidase-released protein (MRP; 136 kDa) and the extracellular factor protein (EF; 110 kDa), suggested as virulence markers in most European strains (50). This phenotype is normally absent in virulent North American strains (16).

Thus, attributes responsible for *S. suis* bloodstream survival and dissemination leading to meningeal invasion are still not clear. Inflammation is a hallmark of *S. suis* infection, and in this regard, *S. suis* activation of cytokine release by phagocytes have been reported (38, 39). This activation was shown to be phagocytosis-independent (38). It could be hypothesized that surface adherence to phagocytes with impaired uptake, is a key step for a successful infection, as suggested for *Haemophilus influenzae* type b, another important meningeal pathogen. The type b capsule is an antiphagocytic factor and only mice infected with encapsulated bacteria, that are largely bound but not ingested by macrophages, are bacteremic (30).

Despite the fact that mononuclear phagocytes have been implicated as playing a central role in the pathogenesis of the meningitis, the interactions of *S. suis* type 2 with phagocytic cells are still controversial. Furthermore, the surface adhesion of *S. suis* to these cells have never been addressed. Since the murine model of infection has been widely used to evaluate the virulence of *S. suis* strains (4), our objectives were to evaluate the capacity of *S. suis* type 2 to adhere to murine macrophages, and to preliminary characterize this interaction.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *S. suis* capsular type 2 suilysin-positive, virulent strain 31533, originally isolated from a case of porcine meningitis (27), and previously used for evaluation of cytokine induction studies (38, 39), was used as the reference strain in this study. In some experiments, the *S. suis* capsular type 2 suilysin-negative, virulent strain 89-1591, was also used (4). *S. suis* isolates used in comparative studies are listed in Table 1. Bacteria were maintained as stock cultures in 50% glycerol-Todd-Hewitt broth (THB; Difco Lab., Detroit, MI) at -80°C. Bacteria were grown overnight onto bovine blood agar plates at 37°C and isolated colonies were used as inocula for THB, which were incubated for 18 h at 37°C. Working cultures for adhesion and cytotoxicity studies were made by inoculating 400 μl of these cultures in 10 ml of THB at 37°C with agitation until they reached the mid-log phase (4-6 h incubation-time; 540 nm-optical density of 0.4-0.5). Bacteria were washed twice in phosphate-buffered saline (PBS), pH 7.4, and appropriately diluted (see Results). An accurate determination of the CFU/ml in the final suspension was made by plating onto THB-agar.

Cell line and cell culture. J774.A1 murine (BALB/c) macrophage-like cell line (ATCC TIB 67, Rockville, Md.) was grown at 37°C, 5% CO_2 in Dulbecco's Modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (hiFBS) (Gibco, Burlington, VT). This cell line has been previously used in *S. suis* phagocytosis and cytokine stimulation studies (38, 40), and has been shown to present similar features to those of normal peritoneal macrophages (40).

Adhesion assay to J774 macrophages. The adhesion assay was adapted from Athamna and Ofek (3) and Sloan and Pistole (42). For adhesion assays, 48 h cultures of J774 cells were scraped, washed once with PBS, and resuspended at 10^6 cells/ml in DMEM 10% hiFBS (unless specified). Plates of 96-wells (Falcon, Becton Dickinson, Bedford, MA) were pre-coated overnight at 4°C with 1% bovine serum albumin (BSA; Sigma-Aldrich, Oakville, Canada) solution in PBS, and washed once with PBS before using. BSA-coated plates showed lower non-specific (background) adhesion of bacteria to the plastic surface, than untreated plates (data not shown). J774 suspension (100 μl)

was distributed in pre-coated plates and incubated 3 h at 37°C, 5% CO_2 to allow macrophage adhesion to plates. Non-adherent cells were removed by washing once with PBS just before adding bacteria. Bacterial dilutions at different concentrations (see Results) in DMEM 10% hiFBS (unless specified) were added to the macrophage-containing plates. Plates were centrifuged at 130 x *g* for 10 min to enhance the contact of bacteria with the surface of cells, and were then incubated at 37°C, 5% CO_2. At different time intervals (see Results) plates were washed four times with PBS to remove non-adherent bacteria. The plates were air-dried and fixed overnight with 50 µl-methanol 100%. Empty wells without cells served as control of background adhesion of bacteria to the plastic surface. To confirm that bacteria were extracellularly bound, cytochalasin C (from *Metarrhizium anisopliae*, Sigma-Aldrich) treatment of macrophages was performed as previously described (2 µg/ml, 30 min before and during the test). This cytochalasin concentration was shown to effectively block the uptake of a highly phagocytosed non-encapsulated strain of *S. suis* (38). Bacteria adhered to macrophages or control wells were quantified by ELISA.

ELISA. To each well, containing methanol fixed cells, was added 200 µl of 5% BSA solution in PBS, to block non-specific binding of antibodies, and 1 µg/ml of human IgG, Fc fragment (Jackson ImmunoResearch Lab., West Grove, PA), to block the Fc receptors on the macrophages. After incubation of 1 h at 37°C, plates were washed 3 times with PBS-0.05% Tween 20 (PBS-T; Sigma), followed by the addition of 100 µl/well of type specific anti-*S. suis* serotype 2 rabbit serum diluted 1/4000 in PBS-T for 1 h at 37°C, produced as previously described (23). Plates were washed 3 times with PBS-T, followed by the addition of 100 µl/well of horseradish peroxidase-labeled anti-rabbit IgG (Jackson ImmunoResearch Lab.), diluted 1/8000 in PBS-T for 1 h at 37°C. After 3 washes with PBS-T, 100 µl of the substrate 3,3',5,5'-tetramethylbenzidine (TM Blue, Intergen, St. Milford, MA) was added to each well, and the blue color was allowed to develop at room temperature. The enzyme reaction was stopped with the addition of 50 µl/well of 1N H_2SO_4. The absorbance was read at 450 nm with an ELISA plate reader (UVmax, Molecular Devices, Menlo Park, CA). Blank wells and control wells with macrophages only were included to ensure that the eukaryotic cells did not react with ELISA antibodies and substrate.

A standard curve served to derive the number of bacteria (expressed as CFU/well) adhered to macrophages. For this purpose, 100 μl of known bacterial concentrations (from 10^7 to 10^1 CFU/well) in distilled water were allowed to dry O/N in wells of a microtiter plate, followed by fixation with methanol. The ELISA test was performed on the immobilized bacteria as described above. Loss of bacteria during washes was considered negligible, as previously reported (3, 42). The ELISA values in OD_{450nm} units were plotted as a function of the number of bacteria in each well. A linear relationship between the number of immobilized bacteria and the ELISA values was obtained over the range of 10^3 to 10^5 CFU/well. The curve obtained was used to calculate the number of bacteria adhered to macrophages from the ELISA values obtained in each experiment.

Adhesion inhibition assays. For bacterial pre-treatment studies, washed organisms (grown as mentioned above) were exposed to various modifying agents: proteinase K (Boehringer-Mannheim, Laval, Qc, Canada) at 0.5 or 1 mg/ml, trypsin (Gibco) at 0.5 or 1 mg/ml, or pronase (Boehringer-Mannheim) at 50 or 100 μg/ml in PBS for 1 h at 37°C. Sialidase (from *Clostridium perfringes*; Boehringer-Mannheim) treatment was performed as previously described (8), at 0.5, 1, 2, or 5 U/ml in buffer sodium acetate (50 mM sodium acetate, 0.9% NaCl, 0.1% $CaCl_2$, pH 5.5) for 3 h at 37°C with agitation. Treated-bacteria were then washed 3 times with PBS and finally resuspended at different concentrations in DMEM 10% hiFBS for the adhesion assay. Bacterial viability and concentration after each treatment was evaluated by plating on THB-agar. In some experiments, bacteria were killed by heat-treatment at 60°C 45 min (minimal condition required to kill *S. suis*) (38), at 100°C 30 min or by treatment with formaldehyde 0.2% for 1 h at 37°C with agitation, washed and resuspended as described above. The killed cultures were subcultured on blood agar plates at 37°C for 48 h to confirm the absence of viable organisms. The different treatments did not affect bacterial recognition by the anti-*S. suis* serum in the ELISA (data not shown).

In competitive binding studies, J774 macrophages were pre-treated with the monosacharides D-galactose, D-Glucose, and *N*-acetyl-D-glucosamine (1, 10, 100 mM), D-mannose and L-rhamnose (1, 10, 100, 500 mM), *N*-acetylneuraminic acid (sialic acid; 1, 10, 100, 500 μg/ml), and the conjugate 6'-*N*-acetyl-neuramyl-*N*-acetyl-lactosamine

(α-Neu-5Ac-[2→6]-β-D-Gal-[1→4]-D-GlcNAc; 0.1, 1, 10, 100, 500 μg/ml). Different concentrations of sugars (Sigma-Aldrich) in PBS were added individually to macrophages 1 h at 37°C prior to the addition of bacterial dilution at 10^6 CFU/well in DMEM 10% hiFBS for the adhesion assay. J774 macrophages were also pre-treated 90 min at 37°C with different dilutions in PBS of purified *S. suis* CPS (at 0.5 or 1 mg/ml), purified *S. suis* cell wall (at 0.5, 1 or 2.5 mg/ml) or purified *S. faecalis* lipoteichoic acid (LTA; Sigma-Aldrich; from 0.5 to 2000 μg/ml). Purified CPS and cell wall of type 2 *S. suis* were prepared as previously described (38, 41). Since the anti-*S. suis* serum used for ELISA recognized these bacterial components, the adhesion assay was performed in these cases by the standard technique of CFU counting recovered from wells, as previously described (10). The adhesion of *S. suis* to macrophages in the absence of any treatment, served as a control for all of these studies (100% adhesion). Results were expressed as the percentage of inhibition respect to the control.

Effect of bacterial pre-opsonization on adhesion. Washed *S. suis* (grown as mentioned above) were pre-opsonized 30 min with agitation at 37°C with FBS (Gibco), or complement from mouse serum (C'MS; hemolytic titer of 1.0 CH50 Units/ml, Sigma-Aldrich) at different concentrations (0.5, 1, 5, 10, 20, or 50% v/v) in DMEM medium. In certain experiments, FBS or C'MS were inactivated by heating at 56°C 30 min to destroy the complement. Opsonized bacteria were added at 10^6 CFU/well to macrophage plates prepared as mentioned above. As a control, bacteria were pre-incubated 30 min with 5% BSA and 2% dextrose in DMEM. In fact, bacteria in DMEM alone presented high background adhesion levels to the plastic surface, as already reported (47). In our hands, a combination of BSA and dextrose considerably reduced this background (not shown).

Cytotoxicity assays. The cytotoxic effect of bacteria was evaluated in parallel with the adhesion assay, by lactate dehydrogenase (LDH) measurement using a miniaturized version of the Sigma colorimetric assay as previously described (10). Percent of cytotoxicity was calculated as $[(OD_{sample} - OD_{0\%})/(OD_{100\%} - OD_{0\%})] \times 100$, whereas $OD_{0\%}$ represents the OD_{414} of non-infected cells and $OD_{100\%}$ represents the OD_{414} of water-lysed cells. In some experiments, for comparative purposes, plates were stained to quantify the number of remained macrophages by the selective staining of

phagocyte nuclei with methylene blue as described elsewhere (42). Empty wells or wells with macrophages alone (without bacteria) served as controls to determine the percent of cytotoxicity in bacterial infected wells.

To evaluate the role of bacterial products in cytotoxicity, J774 plates were prepared as for the adhesion assay, and then incubated for 3 h at 37°C with 10^7 CFU/well of *S. suis* suilysin-positive strain 31533, or the suilysin-negative strain 89-1591 (live or heat-killed), or with 18 h-culture supernatants of either strain (supernatants were recovered by centrifugation and filtration through 0.22 µm-filters, and used immediately). Purifed suilysin (Sly, kindly provided by Dr. T. Jacobs, Intervet International, Boxmeer, The Netherlands) was also evaluated at different concentrations (1 through 5 µg/ml) in DMEM 10% hiFBS. The Sly was reactivated by addition of 1% 2-mercaptoethanol to culture medium during assays (24). Inhibition of cytotoxic activity was performed by treatment of bacterial supernatants, or Sly dilution (at 5 µg/ml), with ethanol-soluble cholesterol (Sigma-Aldrich) at a final concentration of 100 µg/ml for 1 h at 37°C (10), before adding them to the macrophage plates for LDH measurement. Non-infected cells with cholesterol in culture medium were used as control.

Electron microscopic studies. For the adhesion microscopic study, 24-well plates containing 13 mm round glass slides were used and the adhesion assay was performed as described above during an incubation period of 30 min at 37°C 5% CO_2. After 4 washes with PBS, cells were fixed for 1 h at room temperature with 2% glutaraldehyde in cacodylate buffer 0.1 M (pH 7.3), and then post-fixed for 45 min at room temperature with 2% osmium tetroxide. A modified cytotoxic test, with cells in suspension instead of the macrophage plates, was carried out to facilitate microscopic studies of damage cells. J774 cell suspension (10^6 cells/ml) was mixed with bacterial dilutions (*S. suis* strain 31533 or strain 89-1591 at 10^8 CFU/ml) in polypropylene tubes (Sarstedt, Qc, Canada) and incubated 3 h at 37°C with gently rotation. Cells were washed once with PBS, and the pellets were embedded with 4% bacteriological agar (Difco) and kept at 4°C until solidification. Agar samples were then fixed 2 h at room temperature with 2% glutaraldehyde in cacodylate buffer 0.1 M (pH 7.3), and then post-fixed overnight at 4°C with 2% osmium tetroxide. Specimens from both cytotoxic and adhesion assays were dehydrated in graded series of ethanol solutions and processed

separately for transmission (TEM) or scanning (SEM) electron microscopy, as previously described (28).

Statistical analysis. Each adhesion or cytotoxicity test was done at least in triplicate, and samples were run in quadruplicate in each plate. Results were derived from linear regression calculations from the standard curves and expressed as bound bacteria per well (as mean ± standard deviation of independent experiments). Differences were analyzed for significance by using the Student's unpaired *t*-test (two-tailed *P*-value). A P-value < 0.05 was considered significant. Differences between groups of strains (classified according to the phenotype, see Table 1), and differences among strains within the same group were analyzed for significance by using general linear models (GLM), followed by Turkey-Kramer post-hoc tests for differences between strains. The SAS v8 software (SAS, Cary, NC) was used for these analyses.

RESULTS

***S. suis* adheres to J774 macrophages.** Figure 1A shows the kinetic of adhesion of *S. suis* strain 31533 to murine J774 macrophages, with an initial infection ratio of 10 bacteria/cell (~10^6 CFU/well). Adhesion occurred rapidly and increased with incubation time, reaching a first plateau between 1 h and 2 h ($P < 0.001$ respect to 30 min-adhesion levels). A second and higher increase of adhesion is observed after 3 h of bacteria-cell contact ($P < 0.01$ respect to values observed at other incubation times). This increase in adhesion was not related to the inoculum multiplication during the adhesion assay, since the inoculum CFU counts remained constant between 2 and 3 h of incubation ($P > 0.1$). A similar kinetic of adhesion was observed with an initial infection ratio of 100 bacteria/cell (~10^7 CFU/well; data not shown). It must be noted that all data were corrected from background adhesion to plastic surface. For subsequent experiments, an incubation time of 30 min was chosen for two reasons. Firstly, low bacterial cytotoxicity was observed at 30 min (see Fig. 4), and secondly, inoculum counts were also stable between 0 and 30 min of the adhesion assay ($P > 0.1$), whereas significant differences in the inoculum growth rates were observed between 30 min and 2 h of incubation ($P < 0.01$) (Fig. 1A). When different bacterial concentrations were evaluated under these conditions, adhesion was shown to be bacterial dose-dependent (Fig. 1B). Maximal adhesion was observed at 10^7 ($P < 0.001$), and no significant increase in adhesion levels was observed with higher bacterial concentrations. Adhesion levels with less than 10^4 bacteria/well were almost not detectable. Adhesion was confirmed in selected experiments by using the viable counting technique, as previously described (data not shown) (10, 40), and by SEM and TEM as shown in Figure 2.

Since it has been previously shown that encapsulated *S. suis* is practically not ingested by J774 cells (40), determination of intracellular bacteria was not carried out. In addition, cytochalasin C pre-treatment of cells before adding bacteria (at an infection ratio of 10 bacteria/cell) did not affect adhesion (3.0×10^3 vs 2.8×10^3 CFU/well of bound bacteria to control cells respect to cytochalasin-treated cells, $P > 0.1$).

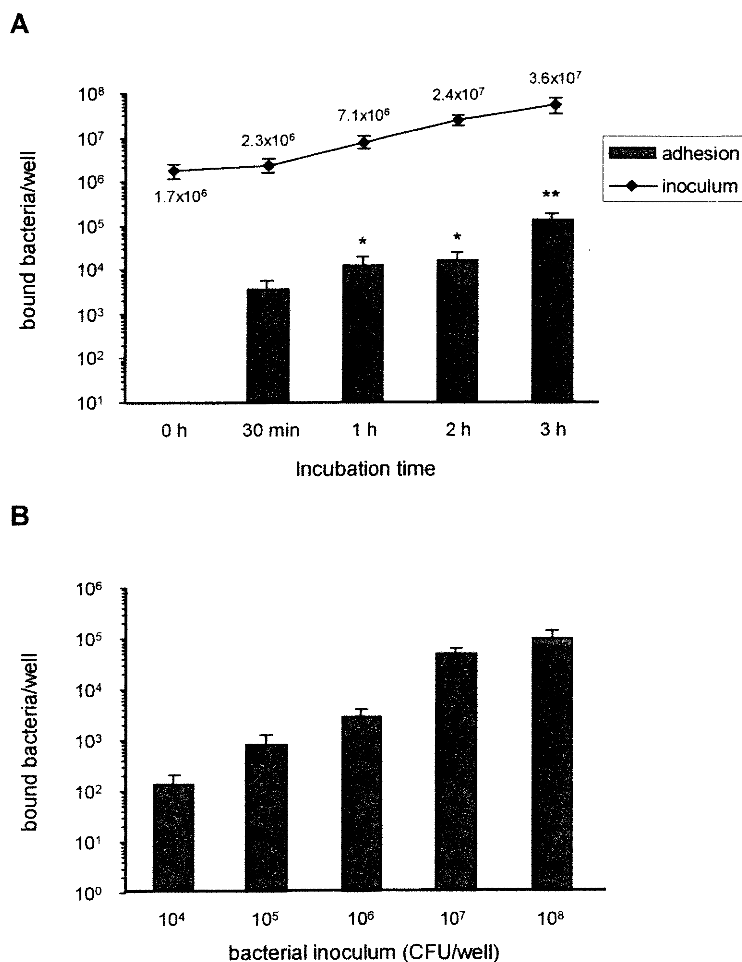


FIG. 1. (A) Kinetics of *S. suis* adhesion to J774 macrophages with an initial infection ratio of 10 bacteria/cell (~10^6 CFU/well). The kinetic of inoculum multiplication during the adhesion assay is also shown. *$P < 0.001$ respect to 30 min-adhesion levels; **$P < 0.01$ respect to values observed at other incubation times. (B) Effect of bacterial concentration on 30 min-adhesion to J774 macrophages. Data are expressed as means ± standard deviations of bound bacteria (in CFU/recovered par well).

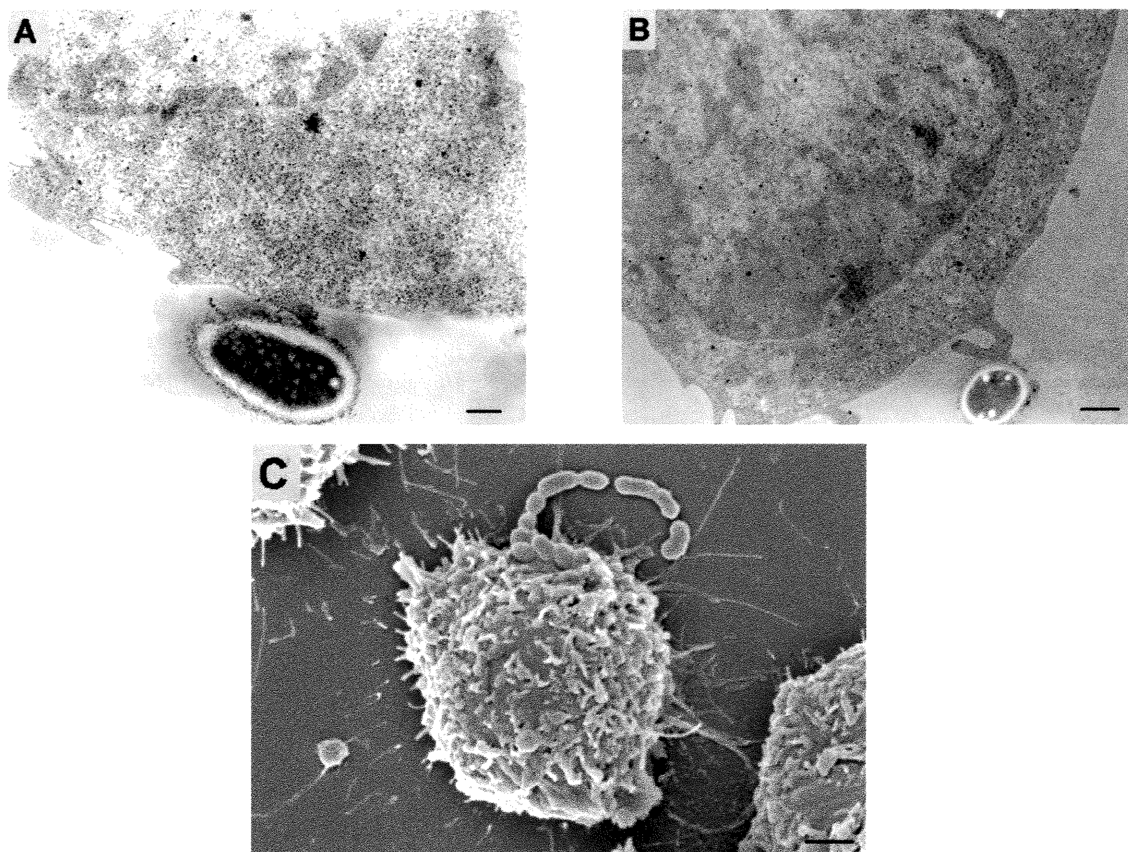


FIG. 2. Transmission electron micrographs showing *Streptococcus suis* adhesion to J774 macrophages at different parts of the plasma membrane. (A) *S. suis* adhered to the plain cell surface, bar: 250 nm; or (B) to the cell surface projections, bar: 0.5 µm. (C) Scanning electron micrograph showing a small chain of *S. suis* cocci adhering to the surface of macrophages, bar: 1 µm.

Comparative adhesion studies were done with different *S. suis* isolates, at an infection ratio of 10 bacteria/cell and 30 min of incubation time. As shown in Table 1, strains were classified into two groups according to the MRP, EF and suilysin phenotypes (11). Most European virulent strains present the suilysin+, MRP+, EF+ phenotype, whereas most of the North American strains are characterized as suilysin-, EF-, MRP variable (11, 16, 50). The analysis of the differences between the two groups of strains revealed that strains of the latter group possess significantly higher levels of adhesion than "European" phenotype group of strains ($P < 0.0001$; Table 1). On the other side, Tukey-Kramer post-hoc tests revealed several significant differences among strains. Similar results were observed at an infection ratio of 100 bacteria/cell (data not shown).

TABLE 1. Comparative studies with different *S. suis* capsular type 2 isolates

Strain	Origin	Geographic origin	Phenotype[a]			Adhesion[b] (CFU/well)	Cytotoxicity[c] (%)
			Sly	MRP	EF		
31533[d]	Diseased pig	France	+	+	+	2.5×10^3 (± 0.8)	40.6 (± 5)
S735	Diseased pig	The Netherlands	+	+	*	4.7×10^3 (± 1.1)	38.5 (± 2)
24	Diseased pig	France	+	+	+	2.0×10^3 (± 0.6)	42.8 (± 3)
166	Diseased pig	France	+	+	+	1.3×10^4 (± 0.5)	43.5 (± 4)
95-8242	Diseased pig	Canada	+	+	+	1.4×10^3 (± 0.3)	31.5 (± 6)
Reims	Human	France	+	+	+	9.1×10^2 (± 2.0)	20.5 (± 5)
			Group average:			4.1×10^3	36.2
TD10	Pig, healthy carrier	UK	-	-	-	1.0×10^4 (± 0.3)	1.0 (± 2)
94-623	Pig, healthy carrier	France	-	*	-	4.2×10^3 (± 1.0)	2.0 (± 2)
89-1591	Diseased pig	Canada	-	-	-	4.5×10^3 (± 1.1)	0.0 (± 0)
89-999	Diseased pig	Canada	-	-	-	4.4×10^3 (± 1.4)	0.0 (± 0)
AAH4	Diseased pig	USA	-	+	-	1.6×10^4 (± 0.5)	8.0 (± 5)
JL590	Diseased pig	Mexico	-	*	-	1.9×10^4 (± 0.7)	2.5 (± 2)
90-1330	Pig, healthy carrier	Canada	-	+	-	4.2×10^3 (± 1.2)	0.0 (± 0)
94-3037	Human	Canada	-	-	-	1.8×10^3 (± 0.5)	8.0 (± 3)
			Group average:			8.1×10^3	2.7

[a] Sly: suilysin; MRP: muramidase-released protein; EF: extracellular factor. *, indicates larger molecular weight variants of EF or MRP proteins.

[b] Adhesion assay was performed at an infection ratio of 10 bacteria/cell and 30 min of incubation time. Data are expressed as means ± standard deviations of adhered bacteria, n = 4.

[c] Cytotoxic assay was performed by methylene blue stain of remained macrophages after 3 h of bacteria-cell contact at an infection ratio of 100 bacteria/cell. Data are expressed as the percent of cytotoxicity (± standard deviations) in bacterial infected wells respect to control wells with macrophages alone, n = 4.

[d] Strain used as reference in the present work.

Effect of bacterial pre-treatments on adhesion. In order to further characterize the interactions between *S. suis* and J774 macrophages, the inhibitory effect of different bacterial pre-treatments was evaluated. Proteinase K, trypsin, or pronase treatment of bacteria did not significantly affect *S. suis* adhesion to macrophages (Table 2). Two different concentrations of proteases (see Materials and methods) in combination with three different concentrations of bacteria (10^7, 10^6, and 10^5 CFU/well) were evaluated, but none affected *S. suis* adhesion after 30 min of bacterial-cell contact. It must be noted that the different treatments did not affect bacterial viability (data not shown).

Since *S. suis* capsule contains sialic acid, and this sugar has been implicated in adhesion of other organisms (29), sialidase treatment was also performed. Sialidase treated-bacteria showed reduced adhesion levels compared to those of non-treated bacteria (Table 2). Increased inhibition was observed by increasing sialidase concentration up to 2 U/ml ($P < 0.01$). Higher sialidase concentrations affected bacterial viability (data not shown).

In some experiments bacteria were killed by heat-treatment at 100°C 30 min or at 60°C 45 min or by treatment with formaldehyde 0.2% for 1 h at 37°C. Killed-bacteria showed a markedly reduction in adhesion levels (~ 70% reduction of bacterial binding; $P < 0.0001$), independently of the applied treatment (Table 2).

TABLE 2. Effect of bacterial pre-treatments on adhesion

Treatments[a]	Concentration or incubation time	% of inhibition[b]	*P* value[c]
Proteinase K	0.5 mg/ml	0	> 0.1
	1 mg/ml	0	> 0.1
Trypsin	0.5 mg/ml	0	> 0.1
	1 mg/ml	0	> 0.1
Pronase	50 µg/ml	0	> 0.1
	100 µg/ml	0	> 0.1
Sialidase	0.5 U/ml	32 (± 1)	< 0.03
	1 U/ml	34 (± 5)	< 0.03
	2 U/ml	47 (± 1)	< 0.01
100°C	30 min	72 (± 6)	< 0.0001
60°C	45 min	71 (± 6)	< 0.0001
formaldehyde 0.2%	60 min	74 (± 11)	< 0.0001

[a] For different bacterial pre-treatments, washed organisms were exposed to proteinase, trypsin, and pronase for 1 h at 37°C. Sialidase (from *Clostridium perfringes*) treatment was performed in buffer sodium acetate for 3 h at 37°C with agitation. Treated-bacteria were then washed 3 times with PBS and finally resuspended in DMEM 10% hiFBS for the adhesion assay.

[b] Adhesion assay was performed at an infection ratio of 10 bacteria/cell and 30 min of incubation time. Data are expressed as means ± standard deviations.

[c] Significant inhibition of adhesion ($P < 0.05$) respect to values for bacteria without treatment (100%) as calculated by the Student's unpaired *t* test.

Competitive binding studies. J774 macrophages were pre-treated with the monosacharides galactose, glucose, *N*-acetyl-D-glucosamine, rhamnose, and *N*-acetylneuraminic acid (sialic acid), which are the five components of *S. suis* capsule. In addition, the conjugate 6'-*N*-acetyl-neuramyl-*N*-acetyl-lactosamine was also evaluated, since it contains the link α-Neu-5Ac-[2→6]-Gal, which has been hypothesized to be present in the capsule structure (8). Since mannan-binding lectin was shown to bind to *S. suis* surface (49), D-mannose was also included. Despite the fact that several concentrations of sugars were tested, only *N*-acetylneuraminic acid (sialic acid), at a concentration of 100 μg/ml, showed a significant effect on *S. suis* adhesion after 30 min of bacteria-cell contact (47 ±8 % of inhibition, $P < 0.01$). Higher concentrations of sialic acid did not increase the inhibitory effect (data not shown). In addition, J774 macrophages were also pre-treated with different concentrations of purified *S. suis* CPS or purified *S. suis* cell wall or purified *S. faecalis* LTA, before adding bacteria (at three different inoculum concentrations: 10^5, 10^6, and 10^7 CFU/well). None affected *S. suis* adhesion after 30 min of bacterial-cell contact (data not shown).

Effect of bacterial pre-opsonization on adhesion. Fig. 3 shows that pre-opsonization (30 min at 37°C) of *S. suis* with different concentrations of hiFBS resulted in significantly increased adhesion to J774 macrophages ($P < 0.001$, with respect to control bacteria which were pre-incubated 30 min with BSA-dextrose). A similar increase in adhesion was observed when different concentrations of non-inactivated FBS serum were used ($P > 0.1$, respect to hiFBS) (Fig. 3). On the other hand, when complement from mouse serum (C'MS) was used instead of FBS, the increase in adhesion was even higher ($P < 0.001$). This increase was dose-dependent, and was reduced by heating at 56°C 30 min to destroy the complement ($P < 0.01$ respect to C'MS) (Fig. 3).

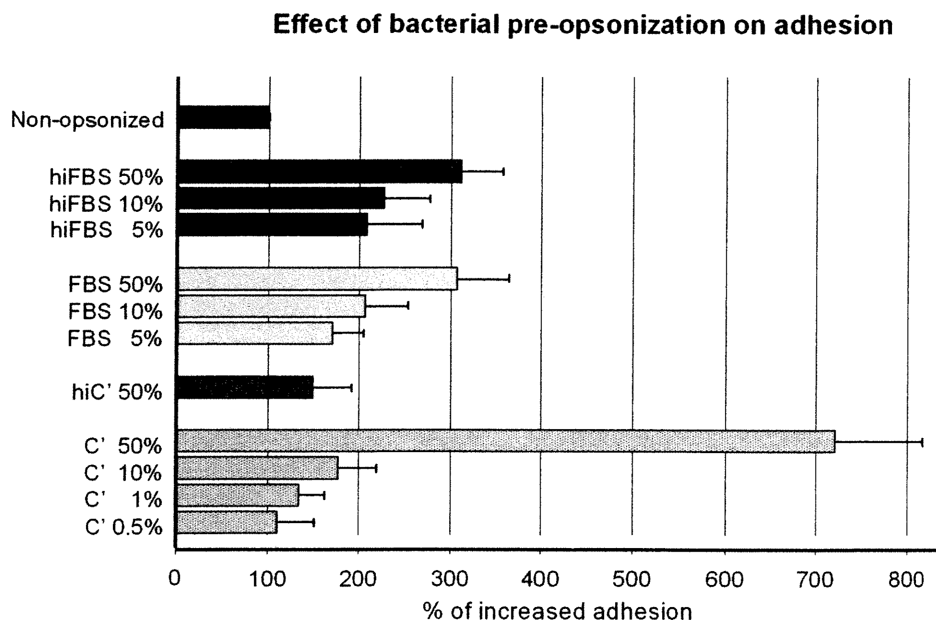


FIG. 3. Effect of bacterial pre-opsonization on 30 min-adhesion to J774 macrophages. *S suis* strain 31533 was pre-opsonized 30 min at 37°C with different concentrations of normal fetal bovine serum (FBS), heat-inactivated FBS (hiFBS), complement from mouse serum (C'), or heat-inactivated C' (hiC'). Pre-opsonized bacteria or non-opzonized control bacteria (bacteria pre-incubated 30 min with 5% BSA, 2% dextrose in DMEM) were added at 10^6 CFU/well to macrophage plates. Data are expressed as means ± standard deviations of percent of increased adhesion respect to control bacteria (100% adhesion).

S. suis **can damage J774 macrophages.** *S. suis* strain 31533 was shown to be cytotoxic to J774 cells as evaluated by LDH test and methylene blue stain. Cytotoxic effects were dose-dependent, with maximal cytotoxic levels at 10^7 and higher bacterial concentrations, after 3 h of bacteria-cell contact (data not shown, $P < 0.001$). An initial infection ratio of 10^7 bacteria/well was then chosen to study the kinetic of cytotoxicity by *S. suis*. Cytotoxicity was very low at 30 min of incubation, but increased with

incubation time, reaching a plateau between 2 h and 3 h of incubation ($P < 0.001$) (Fig. 4). Kinetics of cell damage were similar to those observed by using the methylene blue stain; although sensitivity was higher with the LDH test (Fig. 4). This difference could be due to the release of LDH by damaged but still attached macrophages.

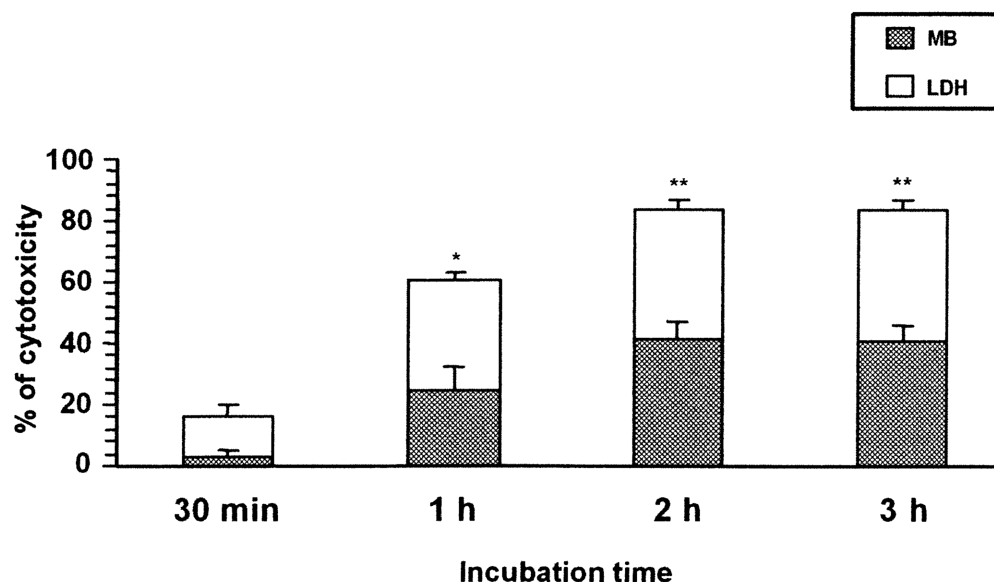


FIG. 4. Effect of incubation time in presence of *S. suis* strain 31533 on J774 injury. The cytotoxic effect of bacteria was evaluated in parallel by the lactate dehydrogenase (LDH) test and by the selective staining of cell nuclei of remaining macrophages with methylene blue (MB), after different interval times of bacteria-cell contact at an infection ratio of 100 bacteria/cell (10^7 CFU/well). Data are expressed as the percent of cytotoxicity (± standard deviations) in infected wells with respect to control wells with macrophages alone. *$P < 0.001$ respect to 30 min-incubation time; **$P < 0.001$ respect to cytotoxic levels at 30 min and 1 h of incubation time.

Suilysin-associated injury to J774 macrophages. Table 1 shows that whereas some *S. suis* isolates did not injure J774 cells, others were highly cytotoxic after 3 h of bacteria-cell contact. Interestingly, all of the cytotoxic strains produce the suilysin. It is known that suilysin is excreted in vitro in culture supernatants (18). Table 3 shows that addition of the culture supernatant from suilysin-positive strain 31533 to J774 cells induced cell injury, whereas the supernatant from suilysin-negative strain 89-1591 did

not affect the cells. Moreover, purified suilysin reproduced the observed cytotoxic effects, reaching maximal cytotoxicity at 5 μg/ml. Inhibition studies were performed to confirm the involvement of suilysin in cell damage. Firstly, to determine if cytotoxicity requires live bacteria, strain 31533 was heat-killed and the suspension was added to J774 cells. Heat-killed strain 89-1591 was used as control. Results showed that only live suilysin-positive bacteria induced J774 injury (Table 3). Cholesterol has been demonstrated to inhibit suilysin activity (10). Thus bacterial supernatants or purified suilysin (5 μg/ml) were treated with cholesterol (100 μg/ml for 1 h at 37°C), before adding them to J774 cells. Table 3 shows almost complete inhibition of cytotoxicity after cholesterol treatment.

TABLE 3. Role of bacterial products in cytotoxicity and inhibition of cytotoxic activity

Treatment	Suilysin production	% Cytotoxicity[a]
Medium alone	-	0 (± 0)
Strain 31533	Yes	73 (± 16)
Strain 89-1591	No	0 (± 0)
Strain 31533 -killed	No	3 (± 4)
Strain 89-1591 -killed	No	8 (± 7)
Strain 31533 -supernatant	Yes	72 (± 7)
Strain 89-1591 -supernatant	No	0 (± 0)
Purified suilysin	1 μg/ml	81 (± 16)
	2.5 μg/ml	90 (± 9)
	5 μg/ml	100 (± 1)
Medium alone	+ cholesterol	2 (± 2)
Strain 31533 -supernatant	+ cholesterol	0 (± 0)
Strain 89-1591 -supernatant	+ cholesterol	0 (± 0)
Purified suilysin (5 μg/ml)	+ cholesterol	9 (± 3)

[a] Cytotoxicity in cultures of infected J774 cells was determined by measurement of LDH release, after 3 h of bacteria-cell contact at an infection ratio of 100 bacteria/cell (10^7 CFU/well). Data are expressed as the percent of cytotoxicity (± standard deviations) in infected wells respect to control wells with macrophages alone, n = 4. Cholesterol inhibition: 1 h at 37°C with 100 μg/ml of ethanol soluble cholesterol.

Cytotoxic effects of *S. suis* to J774 macrophages were confirmed by TEM. Suilysin-negative strain 89-1591 did not affect cell integrity. After 3 h of bacteria-cell contact, characteristics of normal cells (similar to those seen in uninfected cells; Fig. 5A) were maintained (Fig. 5B). In contrast, 3 h-exposure of macrophages to the suilysin-positive strain 31533 resulted in cellular damage demonstrated by loss of cytoplasmic density, disruption of cytoplasmic membranes with release of cellular contents, and disappearance of the nucleus (Fig. 5C).

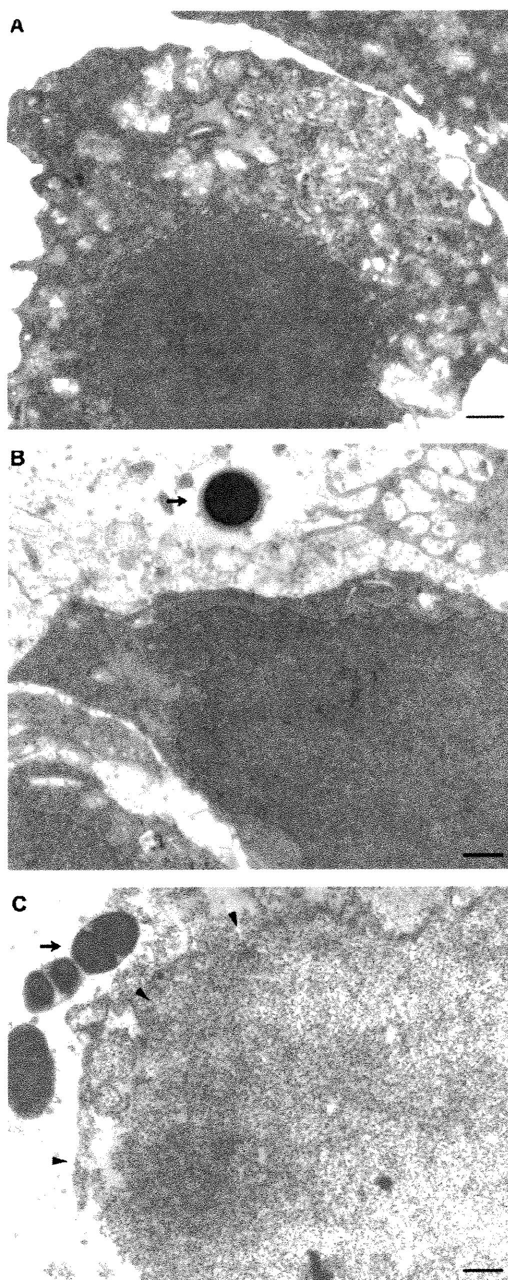


FIG. 5. Transmission electron micrographs demonstrating J774 macrophage injury after infection with 10^8 CFU/ml of *S. suis*. (A) Non-infected control cells. (B) Cells incubated for 3 h with the suilysin-negative strain 89-1591 or (C) with the suilysin-positive strain 31533. J774 integrity after 3 h incubation with strain 89-1591 was comparable to non-infected control cells. Injury was manifested by the lost of cytoplasmic density, severe disruption of cytoplasmic membranes with release of cellular contents (arrowheads), and disappearance of the nucleus (C). Bars: 0.5 μm. Arrows indicate *S. suis* cocci.

DISCUSSION

It has been clearly demonstrated by two different research groups that encapsulated *S. suis* is able to resist uptake by both murine and porcine monocytes/macrophages, and that the CPS is responsible for phagocytosis resistance (6, 40, 43). The present work reinforces the notion that *S. suis* is able to interact with macrophages and remains extracellular and largely bound to cells. Cytochalasin pre-treatment of cells had no effect in adhesion, thus confirming that *S. suis* is not phagocytosed, as previously shown by using viable counting technique after infection of J774 cells (40). In addition, we have previously shown that cytochalasin pre-treatment does not have an effect on production of tumor necrosis factor alpha (TNF) or interleukin-6 by J774 cells after *S. suis* stimulation (38). Electron microscopy analysis reveals attachment of *S. suis* to either protrusions of the cell surface or the plain plasma membrane of macrophages. Similar types of adhesion were observed in ultrastructural analysis of *Brucella suis* infected monocytes; however, for this bacterial species, attachment does result in ingestion by monocytes (36). In contrast, intracellular *S. suis* were rarely observed by TEM analysis (data not shown).

The rapid occurrence and stability of *S. suis* interactions with macrophages are similar to those reported by Albanyan et al. (1) for the association of group B *Streptococcus* (GBS) with polymorphonuclear neutrophils (PMN) (1). *S. suis* adhesion increases by increasing bacterial concentration in a stable, wash-resistant-manner after 30 min of bacterial-cell contact. It must be noted that this microorganism tends to bind with each other and then bind to macrophages in chains, a phenomenon that we observed by direct microscopy (data not shown) and electron microscopy (Fig. 2C). Similarly, this phenomenon has also been reported in GBS binding studies to PMN (1).

In order to preliminarily characterize *S. suis* components implicated in adhesion to macrophages, bacteria were subject to several pre-treatments. Despite testing several different protease digestions, *S. suis* adhesion to macrophages was shown to be protease-resistant. *S. suis* proteins that have a role as adhesins have previously been described. *S. suis* was found to recognise the disaccharide sequence Galα1-4Gal present in the trihexosylceramide, GbO_3 (20). The protease-sensitive adhesin responsible for this

interaction was named the P adhesin (48). Thus, the interactions between *S. su*is and J774 cells seem to be different to those described in the hemagglutination tests (20). Similarly, a role of the P adhesin in *S. suis* adhesion to epithelial cells could not be demonstrated (28). On the other side, heat-killed or formaldehyde-killed bacteria show a marked reduction in adhesion levels. It could be suggested that bacterial interaction with macrophages is sensitive to these treatments or, another possibility is that viable bacteria are required for binding, as already reported for other pathogens (44).

Since *S. suis* capsule contains sialic acid, and this sugar has been implicated in adhesion of other organisms (29), sialidase treatment was also performed. Sialidase-treated bacteria showed reduced adhesion levels compared to non-treated bacteria, indicating a possible role of the capsular sialic acid moiety in attachment to macrophages. In addition, competitive binding studies with *N*-acetylneuraminic acid (sialic acid) also reduced levels of adhesion and further suggest a participation of this bacterial sugar in cell attachment. None of the other sugars known to be present in the bacterial surface, nor the purified CPS, showed an effect on *S. suis* adhesion. This result is surprising, since purified CPS contains sialic acid. One possible explanation is that the extraction method could damage the sialic acid moiety being recognized by macrophages, or sialic acid concentration of purified CPS material was not enough to obtain a significant inhibition. Indeed, sialic acid concentration of *S. suis* type 2 was shown to be ~ 2-3.5 μg/mg of cells by the Warren-Aminoff method (9). Charland et al. (8) reported the agglutination of *S. suis* cells with sialic acid-binding lectins and suggested that sialic acid is the possible terminal sequence on the type 2 capsular component (7). The exact chemical structure and epitope portion of the type 2 antigen containing NeuAc, however, still remains unkown. Since the conjugate α-Neu-5Ac-[2→6]-β-D-Gal-[1→4]-D-GlcNAc was not able to interfere with *S. suis* adhesion, it could be hypothesized that either this type of link is not present in the *S. suis* CPS or is not recognized by the J774 cells.

Components of gram-positive cell wall, such as LTA and peptidoglycans, have been shown to be important adhesins which are recognized by various receptors on host cells (12, 19, 51). When purified cell wall of *S. suis* was used in competitive binding studies, no effect on bacterial adhesion to J774 cells was observed. Purified individual

sub-components of *S. suis* cell wall are not yet available, and little information about the LTA or peptidoglycan structure is found in the literature (13, 26). To further investigate the potential role of the cell wall in *S. suis* adhesion to macrophages, purified *S. faecalis* LTA was evaluated in competitive binding assay. Despite the fact that both *S. suis* and *S. faecalis* LTA react with Group D antiserum and have some structural similarities (13), *S. faecalis* LTA failed to inhibit the binding of *S. suis* to J774 cells. It has been shown that most of *S. suis* teichoic acid is lipid bound, located deep in the cell wall and hardly accessible to extraction (13). Thus, it is probably partially exposed at the bacterial surface or is less accessible for interacting with macrophage receptors. Even though the purified cell wall material from *S. suis* was shown to stimulate cytokine production by J774 cells, the presence of capsule partially masks the TNF response and thus support this hypothesis (38).

Only partial inhibition of binding was observed under the conditions used in the present study. These findings indicate that a number of recognition processes are involved in binding of non-opsonized bacteria to the phagocyte surface (52). Even though capsular sialic plays a role in *S. suis* adhesion to macrophages, it does not seem to be critical for virulence, since field strains of *S. suis* type 2 posses the same sialic acid concentration regardless of their virulence, and blocking or enzymatic removal of this sugar does not influence virulence and phagocytosis rates of *S. suis* (7-9). Since carbohydrate-specified molecular interactions may depend on extended oligosaccharide structures, in which case free monosaccharides may be poor inhibitors, a role of *S. suis* surface components, other than sialic acid, could not be excluded.

Attachment of bacteria to mammalian host cells is often mediated by sugar-lectin interactions (33). Binding of *L. monocytogenes* to murine macrophages was shown to be affected following bacterial treatment with neuraminidase and competitive inhibited by *N*-acetylneuraminic acid (29). Mannose and polysialic acid have been shown to facilitate the attachment of *Mycobacterium tuberculosis* and *Neisseria meningitidis*, respectively, to host cells (37, 46). *S. suis* was shown to bind the mannan-binding lectin (49), which recognizes mannose, *N*-acetylglucosamine and glucose (33). However, neither mannose, nor *N*-acetylglucosamine or glucose were able to inhibit the attachment of *S. suis* to macrophages under the conditions used in the present study.

It has been shown that adherence to host cells surfaces can take place by two different mechanisms: an opsonin-dependent process, in which antibody and/or complement proteins become involved in the complex interaction between bacteria and host cell; and an opsonin-independent process, in which adhesins present on the bacterial cell surface directly recognize host cell receptors (1, 15, 29, 30). It has been previously shown that complement does not affect the rate of phagocytosis of *S. suis* by macrophages (5, 9). On the other hand, it was reported in the literature that complement or other serum factors could mediate adhesion, without ingestion, of some pathogens, such as *H. influenzae* type b (30, 31). In this regard, in the present study it was demonstrated that serum pre-opsonization of *S. suis* results in a markedly increase in adhesion, and complement would be, at least in part, responsible for the increased bacterial adhesion to J774 cells, as demonstrated by using an exogenous source of mouse complement. However, other unknown serum factors would also be implicated, since heating of C'MS only partially reduced bacterial opsonization, and, in addition, no differences were observed between heat-inactivated and normal FBS in their ability to increase *S. suis* adhesion to J774 cells. Similarly, it has been reported that adhesion of *N. meningitidis* to human macrophages was significantly increased after opsonization with non-immune C5-depleted serum (35), and authors suggested that coating of meningococci with C3b and/or other serum proteins would be responsible for the increased adhesion. In the case of *S. pneumoniae*, interaction of bacteria with human alveolar macrophages in the absence of opsonization results in relatively poor binding, while pre-opsonization with complement enhances not only the binding but also the internalization and killing of pneumococci (15). Thus, the fate of *S. suis* after interaction with macrophages under both non-opsonic and opsonic conditions seems to be different from this streptococcal species (9, 15, 40).

It was demonstrated in the present study that *S. suis* not only adheres and resist phagocytosis, but also induces cytotoxic effects to macrophages as incubation time and bacterial concentrations increase. The observed cytotoxicity correlates with the proposed mode of action of suilysin, namely, a multi-hit activity by accumulation of suilysin molecules at the surface of cells (18). Indeed, many lines of evidence implicate suilysin as the bacterial component responsible for in vitro macrophage cytotoxicity. First, only

live suilysin-producing strains are toxic for macrophages. Second, the J774-cytotoxic component is present only in culture supernatants. It is known that suilysin is excreted during bacterial growth (18, 24). Third, the purified suilysin is toxic to J774 cells and cholesterol inhibits this effect. Cholesterol is required for the binding of the toxin to cell membranes, and free cholesterol acts as a competitive inhibitor (18). *S. suis* suilysin has also been shown to be cytotoxic to brain microvascular endothelial cells and epithelial cells (10, 28, 32), and thus it may facilitate bacterial dissemination (17). Recently, a defined allelic-replacement mutant of the *sly* gene, encoding the suilysin, was shown not to be toxic for J774 cells, and thus further proves that the suilysin is probably the only cytolysin produced by *S. suis* (2).

While suilysin is implicated as an important virulence factor in European *S. suis* type 2 strains, the same does not seem to be the case for North American strains. In fact, unlike European strains, most virulent field strains isolated from diseased pigs or humans in North America do not produce suilysin. Similarly, most European strains produce MRP and EF proteins (named virulence markers), whereas variable production of these proteins has been observed with North American strains (16, 45). It has been suggested that the pathogenesis of the infection caused by suilysin-positive strains ("European" phenotype) and of the infection caused by suilysin-negative strains ("North American" phenotype) is different and that different virulence factors are involved in each case (17). Indeed, in the present study, it was shown that strains presenting the "North American" phenotype adhere in higher numbers than strains presenting the "European" phenotype (Table 1). Thus, results described herein give additional evidence that the pathogenesis of the infection may differ between *S. suis* strains. In particular, it is possible that suilysin-positive strains use adherence and cell injury for dissemination and evasion of the host immune system. In contrast, suilysin-negative strains may use adherence and macrophage-dependent dissemination as a part of a complicated multistep process which leads to bacteremia and meningitis in the host (10, 17, 28). The present study demonstrates for the first time that *S. suis* is largely bound but not ingested by macrophages and thus remains extracellular. Further studies will be needed to characterize the molecule(s) which are responsible for adherence and bacterial phagocytosis resistance.

ACKNOWLEDGMENTS

We gratefully acknowledge D. Montpetit from the Centre de Recherche et Développement sur les Aliments (CRDA) for the transmission and scanning electron micrographs and S. Lacouture for technical assistance. We also wish to thank Dr. M. Jacques for critical review of the manuscript. We are also indebted to Dr. M. Kobisch (Centre National d'Études Vétérinaires et Alimentaires, Ploufragan, France), Dr. T. Alexander (University of Cambridge, England), Dr. L. Brasme (Centre Hospitalier Universitaire de Reims, France), Dr. B. Fenwick (Kansas University), and Dr. P. Norton (Institute for Animal Health, Compton, United Kingdom) for providing some of the *S. suis* type 2 strains.

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) grant # 0680154280, by the Fonds pour la Formation des Chercheurs et l'Aide à la Recherche du Québec (FCAR-équipe) grant # 99-ER-0214, and by the Canadian Research Network on Bacterial Pathogens of Swine.

REFERENCES

1. **Albanyan, E. A., J. G. Vallejo, C. Wayne Smith, and M. S. Edwards.** 2000. Nonopsonic binding of type III Group B streptococci to human neutrophils induces interleukin-8 release mediated by the p38 mitogen-activated protein kinase pathway. Infect. Immun. **68:**2053-2060.
2. **Allen, A. G., S. Bolitho, H. Lindsay, S. Khan, C. Bryant, P. M. Norton, P. N. Ward, J. A. Leigh, J. Morgan, H. Riches, S. Eastty, and D. Maskell.** 2001. Generation and characterization of a defined mutant of *Streptococcus suis* lacking suilysin. Infect. Immun. **69:**2732-2735.
3. **Athamna, A., and I. Ofek.** 1988. Enzyme-linked immunosorbent assay for quantification of attachment and ingestion stages of bacterial phagocytosis. J. Clin. Microbiol. **26:**62-66.
4. **Beaudoin, M., R. Higgins, J. Harel, and M. Gottschalk.** 1992. Studies on a murine model for evaluation of virulence of *Streptococcus suis* capsular type 2 isolates. FEMS Microbiol. Lett. **78:**111-116.
5. **Brazeau, C., M. Gottschalk, S. Vincelette, and B. Martineau-Doize.** 1996. In vitro phagocytosis and survival of *Streptococcus suis* capsular type 2 inside murine macrophages. Microbiology **142:**1231-1237.
6. **Charland, N., J. Harel, M. Kobish, S. Lacasse, and M. Gottschalk.** 1998. *Streptococcus suis* serotype 2 mutants deficient in capsular expression. Microbiology **144:**325-332.
7. **Charland, N., M. Jacques, S. Lacouture, and M. Gottschalk.** 1997. Characterization and protective activity of a monoclonal antibody against a capsular epitope shared by *Streptococcus suis* serotypes 1, 2 and 1/2. Microbiology **143:**3607-3614.
8. **Charland, N., J. T. Kellens, F. Caya, and M. Gottschalk.** 1995. Agglutination of *Streptococcus suis* by sialic acid-binding lectins. J. Clin. Microbiol. **33:**2220-2221.
9. **Charland, N., M. Kobisch, B. Martineau-Doize, M. Jacques, and M. Gottschalk.** 1996. Role of capsular sialic acid in virulence and resistance to phagocytosis of *Streptococcus suis* capsular type 2. FEMS Immunol. Med. Microbiol. **14:**195-203.
10. **Charland, N., V. Nizet, C. Rubens, K. S. Kim, S. Lacouture, and M. Gottschalk.** 2000. *Streptococcus suis* serotype 2 interactions with human brain microvascular endothelial cells. Infect. Immun. **68:**637-643.
11. **Chatellier, S., M. Gottschalk, R. Higgins, R. Brousseau, and J. Harel.** 1999. Relatedness of *Streptococcus suis* serotype 2 isolates from different geographic origins as evaluated by molecular fingerprinting and phenotyping. J. Clin. Microbiol. **37:**362-366.
12. **Courtney, H. S., J. B. Dale, and D. L. Hasty.** 1997. Host cell specific adhesins of group A streptococci. Adv. Exp. Med. Biol. **418:**605-606.
13. **Elliott, S. D., M. McCarty, and R. C. Lancefield.** 1977. Teichoic acids of group D streptococci with special reference to strains from pig meningitis (*Streptococcus suis*). J. Exp. Med. **145:**490-499.
14. **Fiani, M. L., J. Beitz, D. Turvy, J. S. Blum, and P. D. Stahl.** 1998. Regulation of mannose receptor synthesis and turnover in mouse J774 macrophages. J.

Leukoc. Biol. **64:**85-91.

15. **Gordon, S. B., G. R. B. Irving, R. A. Lawson, M. E. Lee, and R. C. Read.** 2000. Intracellular trafficking and killing of *Streptococcus pneumoniae* by human alveolar macrophages are influenced by opsonins. Infect. Immun. **68:**2286-2293.
16. **Gottschalk, M., A. Lebrun, H. Wisselink, J. D. Dubreuil, H. Smith, and U. Vecht.** 1998. Production of virulence-related proteins by canadian strains of *Streptococcus suis* capsular type 2. Can. J. Vet. Res. **62:**75-79.
17. **Gottschalk, M., and M. Segura.** 2000. The pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions. Vet. Microbiol. **75:**59-71.
18. **Gottschalk, M. G., S. Lacouture, and J. D. Dubreuil.** 1995. Characterization of *Streptococcus suis* capsular type 2 haemolysin. Microbiology **141:**189-195.
19. **Greenberg, J. W., W. Fischer, and K. A. Joiner.** 1996. Influence of lipoteichoic acid structure on recognition by the macrophage scavenger receptor. Infect. Immun. **64:**3318-3325.
20. **Haataja, S., K. Tikkanen, J. Hytonen, and J. Finne.** 1996. The Gal alpha 1-4 Gal-binding adhesin of *Streptococcus suis*, a gram-positive meningitis-associated bacterium. Adv. Exp. Med. Biol. **408:**25-34.
21. **Higgins, R., and M. Gottschalk.** 2001. Distribution of *Streptococcus suis* capsular types in 2000. Can. Vet. J. **42:**223.
22. **Higgins, R., and M. Gottschalk.** 1999. Streptococcal diseases, p. 563-570. *In* B. E. Straw, S. D'Allaire, W. L. Mengeling, and D. J. Taylor (ed.), Diseases of swine. Iowa State University, Ames.
23. **Higgins, R., and M. Gottschalk.** 1990. An update on *Streptococcus suis* identification. J. Vet. Diagn. Invest. **2:**249-52.
24. **Jacobs, A. A., P. L. Loeffen, A. J. van den Berg, and P. K. Storm.** 1994. Identification, purification, and characterization of a thiol-activated hemolysin (suilysin) of *Streptococcus suis*. Infect. Immun. **62:**1742-1748.
25. **Katsumi, M., T. Saito, Y. Kataoka, T. Itoh, N. Kikuchi, and T. Hiramune.** 1996. Comparative preparation methods of sialylated capsule antigen from *Streptococcus suis* type 2 with type specific antigenicity. J. Vet. Med. Sci. **58:**947-952.
26. **Kilpper-Bälz, R., and K. H. Schleifer.** 1987. *Streptococcus suis* sp. nov., nom. rev. Int. J. Syst. Bacteriol. **37:**160-162.
27. **Kobisch, M., M. Gottschalk, P. Morvan, R. Cariolet, G. Bénévent, and J. P. Joly.** 1995. Experimental infection of SPF piglets with *Streptococcus suis* serotype 2. Journées Rech. Porcine en France **27:**97-102.
28. **Lalonde, M., M. Segura, S. Lacouture, and M. Gottschalk.** 2000. Interactions between *Streptococcus suis* serotype 2 and different epithelial cell lines. Microbiology **146:**1913-1921.
29. **Maganti, S., M. M. Pierce, A. Hoffmaster, and F. G. Rodgers.** 1998. The role of sialic acid in opsonin-dependent and opsonin-independent adhesion of *Listeria monocytogenes* to murine peritoneal macrophages. Infect. Immun. **66:**620-626.
30. **Noel, G. J., S. K. Hoiseth, and P. J. Edelson.** 1992. Type b capsule inhibits ingestion of *Haemophilus influenzae* by murine macrophages: studies with isogenic encapsulated and unencapsulated strains. J. Infect. Dis. **166:**178-182.

31. **Noel, G. J., D. M. Mosser, and P. J. Edelson.** 1990. Role of complement in mouse macrophage binding of *Haemophilus influenzae* type b. J. Clin. Invest. **85:**208-218.
32. **Norton, P. M., C. Rolph, P. N. Ward, R. W. Bentley, and J. A. Leigh.** 1999. Epithelial invasion and cell lysis by virulent strains of *Streptococcus suis* is enhanced by the presence of suilysin. FEMS Immunol. Med. Microbiol. **26:**25-35.
33. **Ofek, I., and N. Sharon.** 1988. Lectinophagocytosis: a molecular mechanism of recognition between cell surface sugars and lectins in the phagocytosis of bacteria. Infect. Immun. **56:**539-547.
34. **Painter, R. G., J. Whisenand, and A. T. McIntosch.** 1981. Effects of cytochalasin B on actin and myosin association with particle binding sites in mouse macrophages: implications with regard to the mechanism of action of the cytochalasins. J. Cell. Biol. **91:**373-384.
35. **Read, R. C., S. Zimmerli, V. C. Broaddus, D. A. Sanan, D. S. Stephens, and J. D. Ernst.** 1996. The (α2-8)-linked polysialic acid capsule of group B *Neisseria meningitidis* modifies multiple steps during interaction with human macrophages. Infect. Immun. **64:**3210-3217.
36. **Ritting, M. G., M. T. Alvarez-Martinez, F. Porte, J.-P. Liautard, and B. Rouot.** 2001. Intracellular survival of *Brucella spp.* in human monocytes involves conventional uptake but special phagosomes. Infect. Immun. **69:**3995-4006.
37. **Schlesinger, L. S.** 1993. Macrophage phagocytosis of virulent but not attenuated strains of *Mycobacterium tuberculosis* is mediated by mannose receptors in addition to complement receptors. J. Immunol. **150:**2920-2930.
38. **Segura, M., J. Stankova, and M. Gottschalk.** 1999. Heat-killed *Streptococcus suis* capsular type 2 strains stimulate tumor necrosis factor alpha and interleukin-6 production by murine macrophages. Infect. Immun. **67:**4646-4654.
39. **Segura, M., N. Vadeboncoeur, and M. Gottschalk.** 2002. CD14-dependent and -independent cytokine and chemokine production by human THP-1 monocytes stimulated by *Streptococcus suis* capsular type 2. Clin. Exp. Immunol. **127:**243-254.
40. **Segura, M. A., P. Cléroux, and M. Gottschalk.** 1998. *Streptococcus suis* and group B *Streptococcus* differ in their interactions with murine macrophages. FEMS Immunol. Med. Microbiol. **21:**189-195.
41. **Sepulveda, E. M. D., E. Altman, M. Kobisch, S. Dallaire, and M. Gottschalk.** 1996. Detection of antibodies against *Streptococcus suis* capsular type 2 using a purified capsular polysaccharide antigen-based indirect elisa. Vet. Microbiol. **52:**113-125.
42. **Sloan, A. R., and T. G. Pistole.** 1992. A quantitative method for measuring the adherence of group B streptococci to murine peritoneal exudate macrophages. J. Immunol. Methods **154:**217-223.
43. **Smith, H. E., M. Damman, J. Van der Velde, F. Wagenaar, H. J. Wisselink, N. Stockhofe-Zurwieden, and M. A. Smits.** 1999. Identification and characterization of the *cps* locus of *Streptococcus suis* serotype 2: the capsule protects against phagocytosis and is an important virulence factor. Infect. Immun. **67:**1750-1756.

44. **St. Geme III, J. W., and S. Falkow.** 1990. *Haemophilus influenzae* adheres to and enters cultured human epithelial cells. Infect. Immun. **58:**4036-4044.

45. **Staats, J., P. Brandon, G. Stewart, and M. M. Chengappa.** 1999. Presence of the *Streptococcus suis* suilysin gene and expression of MRP and EF correlates with high virulence in *Streptococcus suis* type 2 isolates. Vet. Microbiol. **70:**201-211.

46. **Stephens, D. S., P. A. Spellman, and J. S. Swartley.** 1993. Effect of the (α2-8)-lined polysialic acid capsule on adherence of *Neisseria meningitidis* to human mucosal cells. J. Infect. Dis. **167:**475-479.

47. **Tamura, G. S., J. M. Kuypers, S. Smith, H. Raff, and C. E. Rubens.** 1994. Adherence of group B streptococci to cultured epithelial cells: roles of environmental factors and bacterial surface components. Infect. Immun. **62:**2450-2458.

48. **Tikkanen, K., S. Haataja, C. Francois-Gerard, and J. Finne.** 1995. Purification of a galactosyl-alpha 1-4-galactose-binding adhesin from the gram-positive meningitis-associated bacterium *Streptococcus suis*. J. Biol. Chem. **270:**28874-28878.

49. **van Emmerik, L. C., E. J. Kuijper, C. A. P. Fijen, J. Dankert, and S. Thiel.** 1994. Binding of mannan-binding protein to various bacterial pathogens of meningitis. Clin. Exp. Immunol. **97:**411-416.

50. **Vecht, U., H. J. Wisselink, M. L. Jellema, and H. E. Smith.** 1991. Identification of two proteins associated with virulence of *Streptococcus suis* type 2. Infect. Immun. **59:**3156-3162.

51. **Weidemann, B., J. Schletter, R. Dziarski, S. Kusumoto, F. Stelter, E. T. Rietschel, H. D. Flad, and A. J. Ulmer.** 1997. Specific binding of soluble peptidoglycan and muramyldipeptide to CD14 on human monocytes. Infect. Immun. **65:**858-864.

52. **Weir, D. M., J. Stewart, and E. Glass.** 1982. Phagocyte recognition by lectin receptors. Immunobiol. **161:**334-344.

53. **Wessels, M. R., C. E. Rubens, V. J. Benedi, and D. L. Kasper.** 1989. Definition of a bacterial virulence factor: sialylation of the group B streptococcal capsule. Proc. Natl. Acad. Sci. USA **86:**8983-8987.

54. **Williams, A. E., and W. F. Blakemore.** 1990. Pathogenesis of meningitis caused by *Streptococcus suis* type 2. J. Infect. Dis. **162:**474-481.

55. **Yamamoto, Y., S. Okubo, T. W. Klein, K. Onozaki, T. Saito, and H. Friedman.** 1994. Binding of *Legionella pneumophila* to macrophages increases cellular cytokine mRNA. Infect. Immun. **62:**3947-3956.

ARTICLE III

Heat-killed *Streptococcus suis* Capsular Type 2 Stimulates Tumor Necrosis Factor Alpha and Interleukin-6 Production by Murine Macrophages

Mariela Segura[1], Jana Stankova[2], and Marcelo Gottschalk[1]

Groupe de Recherche sur les Maladies Infectieuses du Porc (GREMIP), Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, Québec, Canada[1], and Service d'Immunologie, Université de Sherbrooke, Sherbrooke, Québec, Canada[2]

Infection and Immunity. 1999. 67: 4646-4654

Role of the candidate in conception of this article:

I was the main author of this article. I have actively participated in the conception and design of the experiments, and the standardization of techniques. I carried out all the laboratory work and also the analysis of results. Finally, I did also all the graphic conceptions and writing of the manuscript.

ABSTRACT

Streptococcus suis capsular type 2 is an important etiological agent of swine meningitis, and it is also a zoonotic agent. Since mononuclear phagocytes have been suggested to play a central role in the pathogenesis of the meningitis, the objective of the present study was to evaluate the capacity of whole killed *S. suis* type 2 to induce the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α and interleukin-6 (IL-6) by murine macrophages. Induction of cytokines was evaluated in the presence or absence of phorbol ester (PMA) co-stimulation. Results showed that *S. suis* type 2 stimulated both cytokines in a concentration- and time-dependent fashion. Although high doses of bacteria were required for maximal cytokine release, titers were similar to those obtained with the lipopolysaccharide (LPS) positive control. An increase in cytokine release was observed with both *S. suis* and LPS, in the presence of PMA co-stimulation. Experiments with cytochalasin-treated macrophages showed that cytokine stimulation was phagocytosis-independent. When macrophages were stimulated with an unencapsulated mutant an increase in TNF production was observed, but the absence of the capsule had no effect on IL-6 production. In fact, whereas purified capsular polysaccharide of *S. suis* failed to induce cytokine release, purified cell wall induced both TNF and, to a lesser extent, IL-6. IL-6 secretion probably requires some distinct stimuli which differ from TNF. Finally, the putative virulence factors suilysin and the extracellular protein (EF) showed no cytokine stimulating activity. The fact that *S. suis* is able to trigger macrophages to produce pro-inflammatory cytokines could have an important role in the initiation and development of meningitis caused by this microorganism.

INTRODUCTION

Streptococcus suis is an important pathogen which has been associated with a wide variety of infections in swine such as meningitis, septicemia, arthritis and pneumonia (22). It has also been isolated from human cases of meningitis and endocarditis (3, 49). To date, 35 different capsular types of *S. suis* have been described. *S. suis* capsular type 2 is considered to be the most virulent as well as the most prevalent capsular type in diseased pigs (21). The clinical presentation of *S. suis* infection may vary from asymptomatic bacteremia to fulminant systemic disease, resembling the clinical syndrome of gram-negative sepsis. Meningitis is the most striking feature and the most common histopathological characteristics are the presence of fibrin, edema and cellular infiltrates of the meninges and choroid plexus (9, 22). The pathogenesis of *S. suis* infections is still unclear. *S. suis* is transmitted via the respiratory route and remains localized in the palatine tonsils. From that site, bacteria may become septicemic and invade the meninges and other tissues, possibly in close association with monocytes/macrophages. Once in the central nervous system, induction of an acute inflammatory exudate increases the volume of the cerebrospinal fluid, leading to an increased intracranial pressure (22, 60).

It is now recognized that several inflammatory and infectious diseases are associated with the overproduction of cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), and IL-6. These cytokines are believed to mediate reactions associated with clinical deterioration, multiorgan system failure, and death during septic shock (6). In addition, they have been implicated in bacterial meningeal inflammation (like Group B *Streptococcus* or GBS, and *S. pneumoniae* meningitis) by alteration of the cerebrospinal fluid dynamics, brain metabolism, and cerebral blood flow (54). The cell wall of these gram positive microorganisms has been postulated as being the major modulator of the inflammatory response (51, 53). Despite the fact that mononuclear phagocytes have been implicated as playing a central role in the pathogenesis of the meningitis (22, 60), the interactions of *S. suis* type 2 with phagocytic cells and the possible induction of pro-inflammatory cytokines has not yet been studied.

Virulence factors of *S. suis* type 2 are not well characterized. Different bacterial

structures or products, such as the capsule polysaccharide (CPS), cell wall-associated (muraminidase-released protein, specific adhesins, etc.) and extracellular proteins (extracellular factor or EF, a hemolysin or suilysin) have been suggested as being implicated in the pathogenesis of the infection (22, 47). Among them, the CPS is the only critical virulence factor described so far. In a recent work, isogenic acapsular mutants of a virulent *S. suis* type 2 strain were shown to be avirulent for both mice and piglets and cleared from circulation rapidly (7). However, it is not known whether or not the capsule, as well as other bacterial components or virulence factors, may contribute to the host inflammatory response occurring during *S. suis* infection.

Since the murine model of infection has been widely used to evaluate the virulence of *S. suis* strains (4), our objectives were to evaluate the capacity of whole killed *S. suis* type 2 to induce the inflammatory cytokines TNF-α and IL-6 by murine macrophages, and to determine the relative contribution of the cell wall, the CPS, as well as the purified extracellular proteins EF and suilysin to cytokine production.

MATERIALS AND METHODS

Reagents. Cell culture media, fetal bovine serum (FBS), penicillin G (PenG) and streptomycin (Sm) were purchased from Gibco (Burlington, VT); 2-mercaptoethanol (2-ME) was obtained from Bio-Rad (Mississauga, Ont., Canada). Lipopolysaccharide (LPS) from *Escherichia coli* 0127:B8, phorbol 12-myristate 13-acetate (PMA), cytochalasin C (CyC) from *Metarrhizium anisopliae*, polymyxin B sulfate (PmB), MTT tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide; thiazolyl blue), actinomycin D from *Streptomyces sp.*, and latex beads (polystyrene, particle diameter 1.07 μm) were purchased from Sigma-Aldrich (Oakville, Ont., Canada).

Bacterial strains and growth conditions. The *S. suis* capsular type 2 virulent strain 31533, originally isolated from a case of porcine meningitis, was used as the reference strain in this study (25). The virulent-encapsulated *S. suis* wild type strain S735 was also studied together with its avirulent-unencapsulated isogenic transposon mutant 2A (7). *S. suis* type 2 strain 6860 (EF^+) used for EF purification, was kindly provided by Dr. U. Vecht (DLO Institute for Animal Sciences and Health, Lelystad, The Netherlands). Bacteria were maintained as stock cultures in 50% glycerol-Todd-Hewitt broth (THB; Difco Lab., Detroit, MI) at -80°C. The THB were supplemented with 10 μg/ml tetracycline (Sigma) for growing the mutant 2A (7). Bacteria were grown overnight onto bovine blood agar plates at 37°C and isolated colonies were used as inocula for THB, which were incubated for 18 h at 37°C. Working cultures for macrophage stimulation were made by inoculating 10 ml of these cultures in 200 ml of THB at 37°C with agitation until they reached the mid-log phase (6 h incubation-time; 540 nm-optical density of 0.4-0.5). Bacteria were washed twice in phosphate-buffered saline (PBS), pH 7.4, and diluted to approximately 2×10^9 CFU/ml in PBS. An accurate determination of the CFU/ml in the final suspension was made by plating onto THB-agar.

Preparation of killed bacteria. Bacteria were killed by heat treatment by incubating organisms at 60°C for 45 min (minimal experimental condition required for *S. suis* killing). For some experiences, bacteria were also treated at 100°C for 5 min. The

killed cultures were subcultured onto blood agar plates at 37°C for 48 h to prove that no viable organisms remained. Killed bacteria preparations were stored at 4°C and resuspended in cell culture media just before stimulation assays.

Purified bacterial components. Purification of *S. suis* cell wall, not previously reported, was adapted from Tuomanen et al. (51) and Heumann et al. (20). The unencapsulated strain 2A was grown in one-liter of THB for 12 h at 37°C with agitation to a cell concentration of ~2 x 10^8 CFU/ml. Bacteria were harvested by centrifugation (12,000 x *g*, 15 min, 4°C), washed in saline and resuspended in 2% sodium dodecyl sulfate (SDS, 200 ml). This suspension was submerged in a boiling water bath for 30 min. The denatured cells were quickly chilled in ice and disintegrated by ultrasound (Sonics & Materials- Danbury. Connecticut. USA) for 5 x 8 min (Pulsed / 80% duty cycle). The suspension was centrifuged (3,000 x *g*, 5 min) to remove unbroken cells, and the supernatant was centrifuged at 30,000 x *g* for 30 min at room temperature (RT) to sediment out the cell wall material. The pellet was resuspended in 20 ml of distilled water and subjected to a second ultrasound cycle (5 x 8 min) to assure complete cell disruption. This crude cell wall material was washed six times by centrifugation (30,000 x *g*, 30 min, RT) in distilled water and resuspended in 0.1 M Tris-HCl buffer (pH 8.0), 1 mM $MgCl_2$, and subsequently treated at 37°C with pancreatic DNase I (Sigma, 50 µg/ml) plus RNase (Sigma, 100 µg/ml) for 2 h, followed by trypsin (Gibco, 100 µg/ml) plus 10 mM $CaCl_2$ for 12 h. Cell wall was sedimented by centrifugation (30,000 x *g*, 30 min, RT) and resuspended in 5 ml of 2% SDS at 100°C in a water bath for 30 min. Detergent was removed by 10 cycles of washing, first in 1 M NaCl solution and then in distilled water, and the purified cell wall was lyophilized, weighted and stored in the dry state at RT. Purified CPS of type 2 *S. suis* S735 strain was prepared as previously described (42). Purified suilysin (Sly) from *S. suis* type 2 strain P1/7 was kindly provided by Dr. T. Jacobs (Intervet International, Boxmeer, The Netherlands). The Sly was reactivated by addition of 0.1% of 2-ME (23) to culture medium during stimulation of macrophage assays. The EF was purified from a 18 h-culture supernatant of type 2 *S. suis* 6860 strain applied to a Carbolink™ gel affinity column (Pierce, Rockford, Illinois), coated with a rabbit monospecific polyclonal anti-EF antibody. Purified material was tested by SDS-PAGE and silver nitrate stain.

Cell lines and cell culture. J774A1 murine (BALB/c) macrophage-like cell line (ATCC TIB 67) was maintained in Dulbecco's Modified Eagle's medium, 1.5 g/ml bicarbonate. P388D1 murine (DBA/2) macrophage-like cell line (ATCC TIB 63) was maintained in Iscove's Modified Dulbecco's medium. L929 murine fibroblast cell line (ATCC CCL-1) was maintained in Eagle's Minimal Essential Medium. 7TD1 C57BL/6 mouse hybridoma, IL-6-dependent (ATCC CRL-1851) was maintained in RPMI 1640, 50 mM 2-ME, 10% of IMR-90 (ATCC CCL-186) conditioned medium as a source for IL-6. All cell media were supplemented with 10% heat-inactivated FBS, PenG (100 IU/ml), and Sm (100 µg/ml) and cells were grown at 37°C, 5% CO_2.

Stimulation of macrophages. For stimulation assays, 48 h cultures of J774A1 or P388D1 cells were scraped, washed once and resuspended in culture media at 4 x 10^6 cells/ml. One ml of this suspension was distributed in polypropylene tubes (Sarstedt, Québec, Canada) and one ml of killed *S. suis* strains or purified cell wall, CPS, EF and Sly was added in appropriate dilutions made in culture media. In some experiments, macrophages were also co-stimulated with PMA (20 ng/ml). Experiments comparing cytokine production in response to the *S. suis* wild parent and mutant strains were always run concurrently. Macrophages stimulated with LPS (50 ng/ml) served as positive controls. Macrophages with medium alone served as controls for spontaneous cytokine release. In some experiments, macrophages were pretreated with CyC (2 µg/ml) for 30 min at 37°C, 5% CO_2 to block phagocytosis, then stimulated with heat killed bacteria (1 x 10^9 CFU/ml) and further incubated in the presence of CyC. As control of non-specific cytokine release, macrophages were treated with latex beads (1 x 10^9 beads/ml) in presence or absence of CyC. All cytokine induction mixtures were incubated at 37°C, 5% CO_2. At different time intervals (see Results), culture supernatants were harvested from individual tubes. The supernatants were aliquoted and frozen at -20°C until TNF and IL-6 determinations.

TNF-α bioassay. TNF activity in culture supernatants was measured by use of the L929 cytotoxicity assay, as described elsewhere (12), with minor modifications. Briefly, 5 x 10^5 L929 cells per well were incubated overnight in 96-well microtiter plates, culture medium was then removed, and samples were added in 2-fold serial dilutions. A known concentration of murine recombinant TNF-α (mrTNF-α, Sigma) was

used as a standard. Actinomycin D (5 μg/ml) was added immediately after the addition of samples or standard. The cells were further incubated for 18 h at 37°C, 5% CO_2. Supernatants were then removed and cells were stained with 0.5% crystal violet in 25% ethanol. After homogenization of stained cells with 33% acetic acid, the optical density was read in a microplate reader (UVmax, Molecular Devices, Menlo Park, CA) at 595 nm. A mrTNF-α standard curve was included in each assay. All analysis were performed at least in triplicate, and TNF concentration in samples was calculated by comparison to the standard curve. The specificity of the test was controlled by neutralization of TNF activity by a polyclonal anti-mouse TNF-α antibody (Biosource Int., Menlo Park, CA). In all cases, addition of specific antibody to macrophage supernatants reduced TNF activity by greater than 90%.

IL-6 bioassay. IL-6 activity in culture supernatants was determined by a proliferation assay with the IL-6-dependent 7TD1 mouse B-cell hybridoma cell line (31), with some modifications. Briefly, 48 h 7TD1 cells were washed twice and resuspended in IL-6 free culture medium at 6 x 10^4 cells/ml. Fifty μl of this cell suspension were added to 50 μl of 2-fold serial dilutions of macrophage supernatants in microtiter plates. After 72 h of incubation at 37°C, 5% CO_2, the number of cells was evaluated by a colorimetric method. Twenty μl of MTT (5 mg/ml in PBS solution) was added per well and incubated for 5 h. MTT precipitate in each well was then solubilized overnight with 100 μl of SDS 10%. After homogenization, the optical density was read in a microplate reader (UVmax, Molecular Devices) at 595 nm. Optical density values were corrected for background proliferation of 7TD1 cells. A standard curve of murine recombinant IL-6 (mrIL-6, Gibco) was included in each assay. All analysis were performed at least in triplicate, and IL-6 concentration in samples was calculated by comparison to the standard curve. The specificity of the test was controlled by inhibition of cell proliferation after the addition of a neutralizing rat anti-mouse IL-6 monoclonal antibody (Biosource Int.). In all cases, addition of specific antibody to macrophage supernatants reduced IL-6-stimulated growth of the 7TD1 cell line by 99%.

Enzyme-linked immunosorbent assays (ELISA) for cytokines. TNF-α and IL-6 were also measured by commercial ELISA kits (Cytoscreen™, Biosource Int.) according to the manufacturer's recommendations. The lower limit of detection is 3 and

8 pg/ml for TNF-α and IL-6, respectively. All analysis were performed in triplicate.

Endotoxin contamination. All solutions and bacterial preparations used in these experiments were tested for the presence of endotoxin by a *Limulus* amebocyte lysate (LAL) gel clot test (Pyrotell STV; Cape Cod, Falmouth, MA) with a sensitivity limit of 0.03 EU/ml. In addition, endotoxin contamination during stimulation of macrophages was controlled by parallel assays with PmB (10 μg/ml). The activity of the PmB was tested by its ability to inhibit cytokine release in response to LPS (50 ng/ml) by 99% ($P < 0.001$). In contrast, treatment with PmB did not change the levels of cytokine release induced by *S. suis* ($P > 0.1$, data not shown). Results from the LAL test confirmed data from PmB treatment protocol. Bacterial preparations contained less than 0.125 EU/ml and the cell culture medium and PMA solution each contained less than 0.03 EU/ml. Thus, endotoxin levels were determined to be always <0.025 ng/ml, below that (>0.1 ng) recognized as causing macrophage activation (28).

Cytotoxicity test. The cytotoxic effect of bacteria and bacterial products for macrophages was determined by the colorimetric MTT (tetrazolium) assay (31), with some modifications. At 24 h and 48 h interval times, 400 μl of stock MTT solution (5 mg/ml in PBS) were added to the different cytokine induction mixtures (prepared as described above) and tubes were incubated for 5 h. MTT precipitate was then solubilized overnight with 2 ml of SDS 10%. After homogenization, the optical density was read at 595 nm and the percentage of cytotoxicity was calculated. Different concentrations of bacteria or purified components tested did not cause toxic effects to mammalian cells in the experimental conditions of the present study (data not shown).

Statistical analysis. Each test of macrophage stimulation was done at least in triplicate. Results were derived from linear regression calculations and expressed in units/ml of TNF or IL-6 by comparing the reciprocals of the dilutions of TNF/IL-6-containing test samples with the 50%-endpoints of the standard curves in the bioassay systems. TNF and IL-6 values are expressed as means ± standard deviations of values from independent experiments. Differences were analyzed for significance by using the Student's unpaired *t*-test (two-tailed *P*-value). A P-value > 0.05 was considered not significant, a P-value < 0.05 was considered not quite significant, a P-value < 0.01 was considered significant, and a P-value < 0.001 was considered extremely significant.

RESULTS

Kinetics of TNF-α and IL-6 release by macrophages, triggered by whole *S. suis*. The induction of TNF and IL-6 by J774A1 and P388D1 cells was evaluated after stimulation with heat-killed whole *S. suis* organisms. Cell culture medium alone and LPS were used as controls. As shown in Fig. 1 for J774A1 cells, with a 10^9-CFU/ml dose of bacteria, the induction of these cytokines was time-dependent.

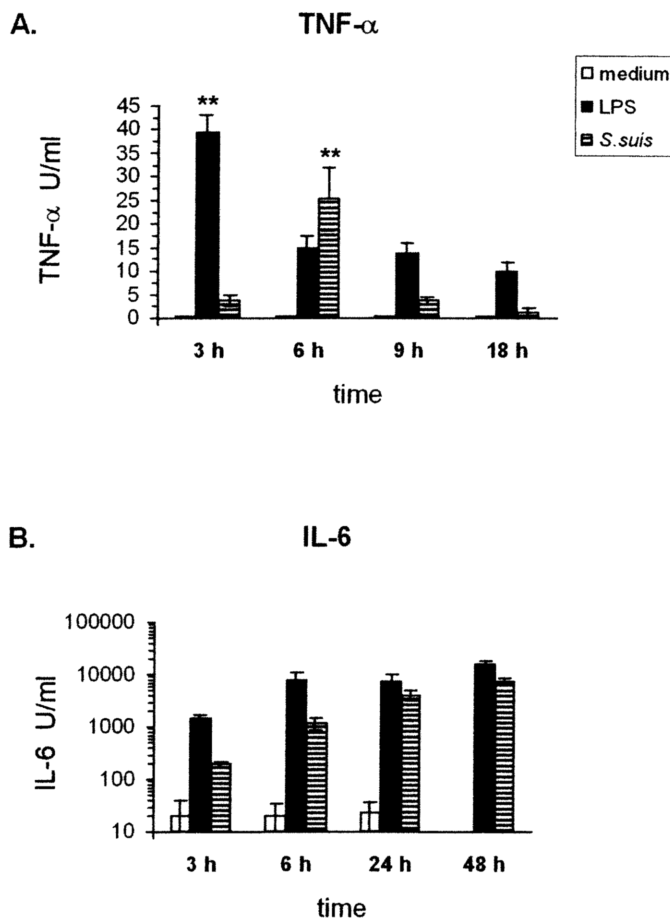


FIG. 1. Time-curse production of TNF-α (A) and IL-6 (B) by J774A1 cells stimulated with heat-killed (60°C for 45 min) *S. suis* strain 31533 (10^9 CFU/ml). Negative control: cell culture medium. Positive control: purified *E. coli* LPS (50 ng/ml). Data were collected from at least three separate experiments performed in duplicate, and are expressed as means ± SD in units/ml. **, $P < 0.001$ (versus the corresponding stimulus at each time interval).

TNF activity appearing in culture supernatants after stimulation with whole bacteria clearly peaked at 6 h of incubation ($P = 0.001$), and markedly decreased upon further incubation through 18 h. Maximum LPS induction of TNF was observed at 3 h ($P = 0.001$). In contrast, IL-6 secretion showed a progressive accumulation with higher IL-6 production observed by 48 h, for both whole bacteria ($P = 0.02$) and LPS ($P = 0.05$). IL-6 titers were higher than those measured for TNF in all cases. A similar kinetics of cytokine release was observed with P388D1 cells (data not shown). Hence, for subsequent experiments, 6 h-supernatants were used to analyze TNF induction, whereas supernatants were harvested 48 h after stimulation to measure IL-6 production.

P388D1 and J774A1 macrophages differ in cytokine release. Effect of PMA co-stimulation. In general, stimulated J774A1 cells produced significantly more cytokines than P388D1 cells (Fig. 2). Interestingly, cytokine induction by *S. suis* with J774A1 cells was similar to that obtained with 50 ng/ml of LPS, ($P = 0.09$; $P = 0.04$, for TNF and IL-6 respectively), while *S. suis* was a less potent inductor of IL-6 release by P388D1 cells ($P < 0.01$, respect to LPS values), and both, LPS and *S. suis*, showed a weak TNF-stimulating activity with this cell line.

Since macrophage activation via protein kinase C pathways (PKC) is well documented (11), cytokine induction by *S. suis* was also compared in presence of PMA, a direct stimulator of PKC (32). When macrophages were activated with PMA, in the case of J774A1 cells, the induction of both cytokines by *S. suis*, as by LPS, was significantly enhanced ($P < 0.001$). This effect was synergistic, since PMA alone was a very weak cytokine inductor to account for this enhancement. However, with P388D1 cells, this enhanced stimulation was less significant ($P < 0.01$), and not quite significant respect to PMA control ($P > 0.02$). Thus, a possible additive effect of PMA alone and stimuli alone could not be ruled out for this cell line (Fig. 2).

J774A1 is classified as one of the most mature macrophage-like cell lines, while P388D1 has an immature phenotype (33). This may account for the apparent differences in the level of enhancement of cytokine production seen with these two cell lines. Hence, due to the obtainment of a higher cytokine response, J774A1 macrophages were used to analyze cytokine induction further.

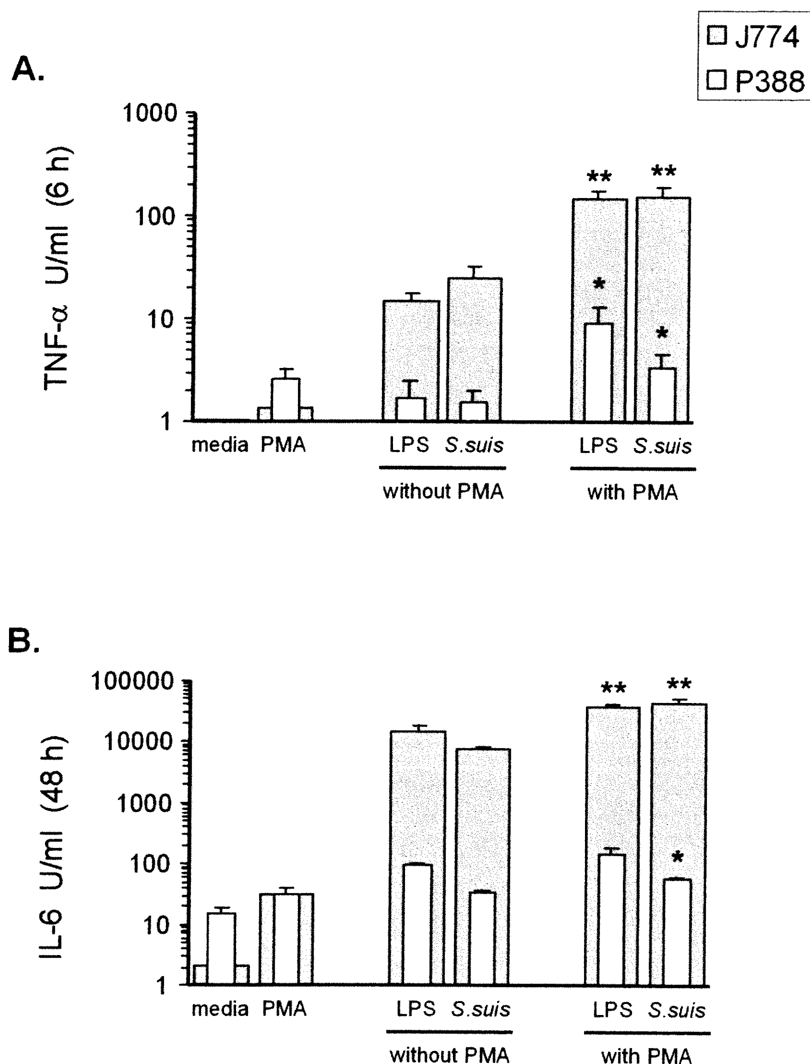


FIG. 2. Effect of PMA-costimulation in cytokine induction by *S. suis* with two macrophages cell lines, J774A1 and P388D1. J774A1 or P388D1 cells (2 x 10^6 cells/ml) were stimulated with heat-killed (60°C for 45 min) *S. suis* strain 31533 (10^9 CFU/ml) in presence or in absence of PMA (20 ng/ml). TNF-α, at 6 h-incubation time (A), and IL-6, at 48 h-incubation time (B), were measured by bioassay titration from stimulated cell supernatants. *S. suis*-cytokine induction was compared to purified *E. coli* LPS (50 ng/ml), under the same conditions. Cell culture medium and PMA alone were used as control. Data were collected from at least three separate experiments performed in duplicate, and are expressed as means ± SD in units/ml. **, $P < 0.001$ (compared to the value in the absence of PMA and to PMA control). *, $P < 0.01$ (compared to the value in the absence of PMA, but not quite significant respect to PMA control).

Bacterial concentration-dependent cytokine release. J774A1 macrophages were exposed to different concentrations of heat-killed (60°C for 45 min) *S. suis* type 2 strain 31533. A high bacterial concentration was needed for maximal TNF and IL-6 production. When bacterial titre was decreased to 10^8 CFU/ml, cytokine release decreased considerably, and almost no cytokine production was observed at bacterial concentrations lower than 10^7 CFU/ml (Fig. 3).

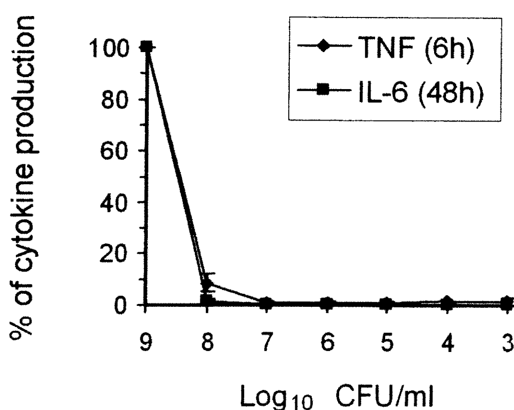


FIG. 3. Effect of bacterial concentration on the production of TNF-α and IL-6. J774A1 cells (2 x 10^6 cells/ml) were stimulated with different concentration of heat-killed (60°C for 45 min) *S. suis* strain 31533, in presence of PMA (20 ng/ml). TNF-α, at 6 h-incubation time, and IL-6, at 48 h-incubation time, were measured by bioassay titration from stimulated cell supernatants. Data were collected from at least three separate experiments performed in duplicate, and are expressed as the percentage of cytokine production respect to the maximal value (100%) obtained with 10^9 CFU/ml.

Role of bacterial up-take in cytokine release. Effect of phagocytosis inhibition by cytochalasin. Since several studies have shown, at least, a certain level of *S. suis* up-take by phagocytic cells (1), it was of interest to determine whether cytokine production was related to phagocytosis of bacteria. To address this question, experiments were performed in the presence of CyC (2 μg/ml), an inhibitor of microfilament-dependent up-take of particles by phagocytic cells. The cytochalasins

have extensively been used for inhibition of phagocytosis of many bacteria and have no detectable effect on the attachment of bacteria or on cytokine induction (10, 36, 61). Results from ELISA titration (since bioassay titration can not be performed with supernatants containing CyC) demonstrated that CyC had no effect on TNF or IL-6 production by J774A1 cells after *S. suis* stimulation ($P = 0.3$ and 0.9 respectively; Fig. 4). To confirm that cytokine release was not caused by nonspecific phagocytosis of bacteria and consequent activation of macrophages, cells were also stimulated with 10^9 latex beads of 1.07 μm. These inert particles were chosen because of their similarity in size to *S. suis* organisms. No cytokine induction was demonstrated with latex beads in presence or absence of CyC (Fig. 4). The concentration of CyC used in this study was able to effectively block phagocytosis of bacteria, and it did not cause toxic effects to mammalian cells (data not shown).

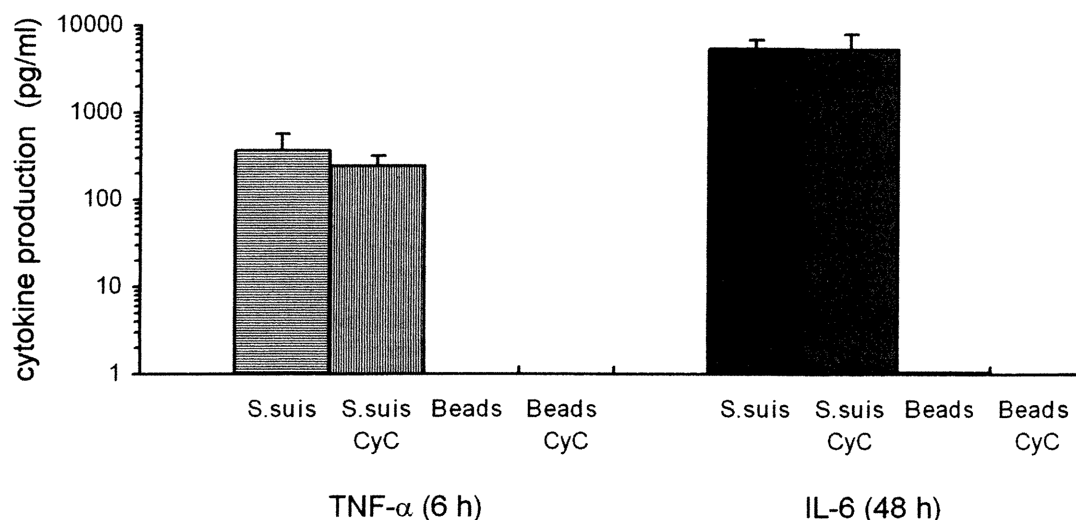


FIG. 4. Effect of phagocytosis inhibition by cytochalasin (CyC) on cytokine production. CyC (2 μg/ml) was added during stimulation of J774A1 cells, in parallel to controls without CyC. Cells were stimulated with 10^9 heat-killed (60°C for 45 min) *S. suis* strain 31533 or 10^9 latex beads/ml. TNF-α, at 6 h-incubation time, and IL-6, at 48 h-incubation time, were measured by ELISA titration from stimulated cell supernatants. Data were collected from at least three separate experiments performed in duplicate, and are expressed as means ± SD in pg/ml.

Relative role of bacterial components in cytokine production. The relative contribution of proteins, capsule polysaccharide and the *S. suis* cell wall in cytokine production was evaluated by using bacteria treated at 100°C for 5 min, an unencapsulated mutant, the purified CPS and the purified cell wall (Fig. 5). TNF and IL-6 concentrations were measured by ELISA and/or bioassay titration.

The cytokine release by *S. suis* type 2 strain 31533 treated at 100°C for 5 min, was compared to that induced by the standard bacterial suspension (60°C, 45 min heat-killed bacteria, as used above). Results showed that the induction of TNF was increased after 100°C heat-treatment of bacteria (P = 0.005; Fig. 5A), possibly due to the exposition of new denatured antigens. In contrast, no significant difference in IL-6 release was observed between 100°C heat-killed and 60°C heat-killed bacteria (P = 0.9; Fig. 5B). These results were also confirmed by bioassay titration (data not shown).

When the encapsulated strain S735 was compared to its unencapsulated isogenic mutant 2A for their ability to induce cytokine secretion, the unencapsulated mutant induced significantly more TNF than the encapsulated wild type strain ($P < 0.001$; Fig. 5A). In contrast, the induction of IL-6 was not influenced by the presence or absence of the capsule (P = 0.3; Fig. 5B). These results were also confirmed by bioassay titration (data not shown).

To study the capacity of *S. suis* type 2 CPS to induce TNF and IL-6, different concentrations of purified CPS, ranging from 0.01 to 100 μg/ml, were tested. A concentration as high as 100 μg/ml did not induce significant levels of cytokine release compared to the negative control or whole bacteria (Fig. 5). This was in agreement with the fact that an unencapsulated mutant produced the same or higher amounts of cytokines than the encapsulated parental strain.

Interestingly, when the purified cell wall (ranging from 0.1 to 500 μg/ml) was evaluated for cytokine production, a threshold of 10 μg/ml was sufficient to initiate production of both cytokines. Maximum release was achieved at concentrations of about 100 μg/ml or higher. Cell wall was roughly equivalent to whole bacteria for induction of TNF (P = 0.2; Fig. 5A), whereas it was approximately ten times less efficient than 10^9 bacterial equivalents for induction of IL-6 (Fig. 5B).

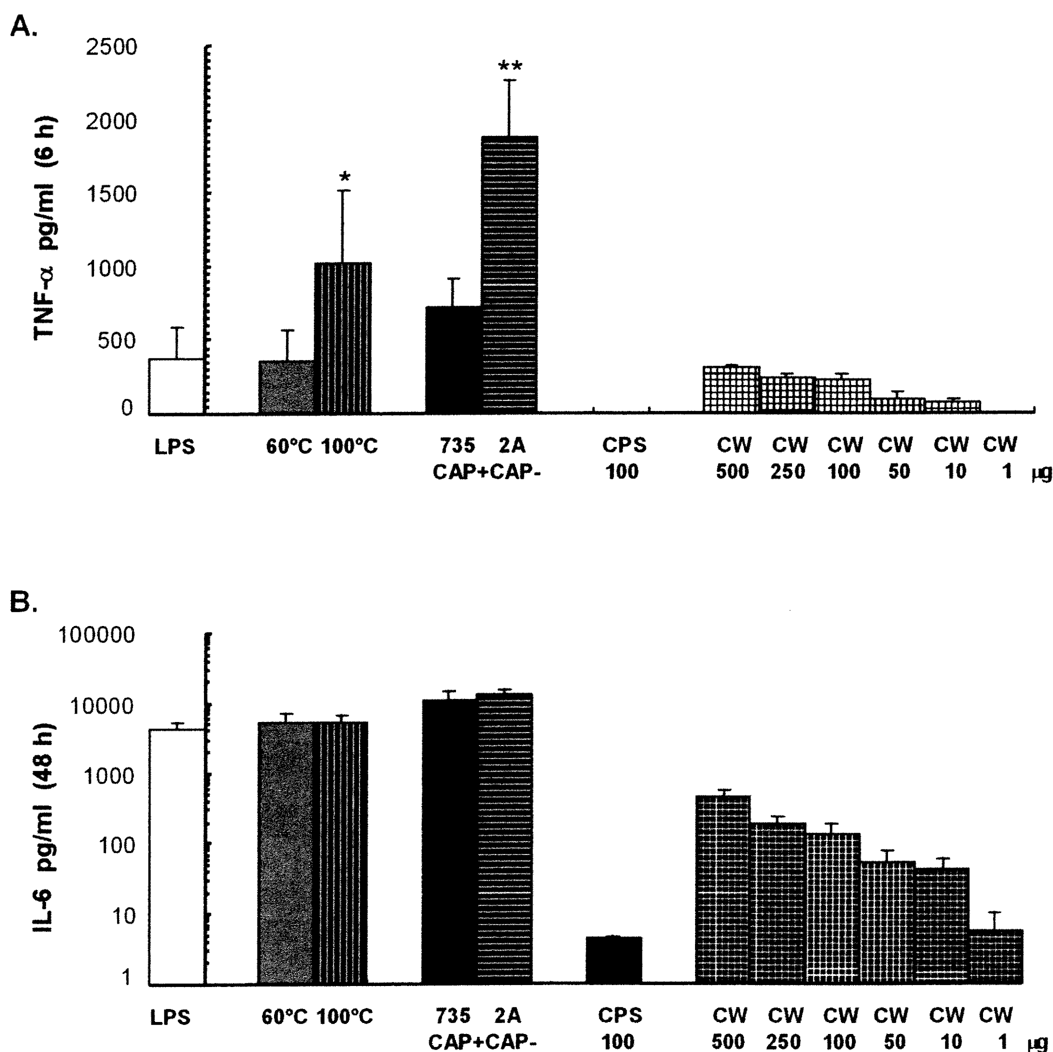


FIG. 5. Relative role of bacterial components in cytokine production. *S. suis* strain 31533 treated at 60°C for 45 min or at 100°C for 5 min, the encapsulated (CAP+) strain S735 and its unencapsulated (CAP-) mutant 2A (10^9 heat-killed bacteria), the purified capsular polysaccharide (CPS), and the purified cell wall (CW) were used for cytokine stimulation of J774A1 cells. TNF-α, at 6 h-incubation time (A), and IL-6, at 48 h-incubation time (B), were measured by ELISA titration. Purified LPS (50 ng/ml) served as positive control. Data were collected from at least three separate experiments performed in duplicate, and are expressed as means ± SD in pg/ml. *, $P < 0.01$ (compared to the value obtained with 60°C heat-killed bacteria). **, $P < 0.001$ (compared to the value obtained with the encapsulated parent strain S735).

Induction of TNF-α and IL-6 by the secreted putative *S. suis* virulence factors. When different concentrations of two secreted putative virulence factors of *S. suis* were tested, neither the purified EF factor (from 0.1 ng to 100 ng/ml) nor the purified hemolysin (from 0.01 ng to 100 ng/ml) stimulated the release of cytokines (data not shown).

DISCUSSION

The present study demonstrated for the first time that heat-killed *S. suis* stimulates mouse macrophages to release TNF-α and IL-6 in dose- and time-dependent fashions, confirming recent reports which revealed that several pathogenic gram positive cocci are powerful inducers of inflammatory cytokines (27, 40, 56). Time course studies indicated that TNF is released before IL-6 and that IL-6 levels were higher and persist longer than those of TNF. *S. suis*-induced TNF level drops much faster than that described in the literature with others gram positive bacteria (5, 8, 30). This seems to be a particular feature of *S. suis*, since similar results were observed with human monocytes (unpublished observations). TNF is known to stimulate IL-6 expression (13); however, TNF does not appear to directly induce IL-6, since an increase in TNF release after different bacterial treatments does not imply an increase in IL-6 levels (Fig. 5). These data indicate that *S. suis* is able to directly stimulate both TNF and IL-6 release. This further suggests the existence of TNF-independent mechanisms leading to IL-6 production. Other reports have already established a dissociation between TNF and IL-6 production (19, 29). However, priming of monocytes by TNF may contribute to the increased production of IL-6 observed after incubation for more than 24 h (52, 57).

The kinetics of *S.suis* cytokine induction is similar to that of LPS from *E. coli*, and to that reported during endotoxin fever (24). In addition, both *S. suis* and LPS are similarly affected by PMA co-stimulation, showing a comparable increase in cytokine release. This effect is synergistic, since PMA alone hardly stimulate cytokine production, as it has been previously shown with the same macrophage cell lines (29). PMA is an activator of PKC (32), and the PKC pathway is involved in the activation of

many cell processes, such as immune response, cell growth regulation and differentiation, as well as receptor expression (32, 34). Thus, macrophage IL-6 and TNF stimulation by *S. suis* is responsive to activation of PKC as evidenced by PMA potentiation of *S. suis*-cytokine response. This is a common feature with LPS-induced cytokines, as described herein and in previous works (28, 29, 39). Wightman et al. (59) postulated that LPS may exert its pleiotropic effects in part through activation of PKC. One common pathway shared by many gram positive bacteria and LPS is the interaction with the CD14 receptor, postulated as a pattern recognition receptor by Pugin et al. (37). In this regard, preliminary studies showed that *S. suis*-induced IL-6 release is inhibited by an anti-CD14 antibody. Further studies on the signaling mechanisms of *S. suis* cytokine induction are warranted.

High doses of heat-killed *S. suis* are required for maximal cytokine release by J774A1 macrophages. However, the observed response was as potent as 50 ng of *E.coli* LPS in the conditions used in this study. In vitro induction of TNF secretion in response to heat-killed pneumococci or staphylococci required stimulation of monocyte-macrophages by a threshold concentration of 10^6 bacteria, while maximal production was observed with more than 10^8 bacteria (44, 48). However, induction of an amount of inflammatory cytokines approximating that observed with LPS required a lower concentration of heat-killed pneumococci or GBS (5, 52, 53) than that of *S. suis*. Although a high dose of bacteria is also required for maximal cytokine release, these data suggest a greater potency for these pathogenic gram-positive bacteria compared to that of *S. suis*.

One of the major virulence factors of *S. suis* is the type 2 specific CPS (7). In an attempt to investigate the role of the capsule in cytokine release, an encapsulated and an unencapsulated type 2 *S. suis* strains were compared. The presence or absence of capsule had no effect on IL-6 production, but the absence of capsule resulted in an increased TNF production, suggesting that antigens responsible for TNF release may be partially masked by the capsule. Furthermore, purified *S. suis* CPS failed to induce cytokine release. Several in vitro and in vivo studies with purified CPS or with unencapsulated mutants failed to demonstrate a major role for this bacterial component in cytokine induction by important pathogenic gram positive cocci (35, 51-53). In addition, it has

been reported that heat-killed unencapsulated- GBS and *S. pneumoniae* strains induce higher meningeal inflammation than their respective encapsulated parent strains, suggesting that the presence of capsular material may mask the inflammatory activity of the underlying cell wall (27, 51). However, these findings do not imply that the *S. suis* capsule does not play an important role in the pathogenesis of the infection. Indeed, as demonstrated by Charland et al. (7) and Smith et al. (45), the capsule plays a critical role by protecting bacteria from in vivo clearance. Therefore, the capsule may not be necessary to induce the release of inflammatory cytokines during *S. suis* infection, but it may contribute to the progression of disease by allowing the organism to evade host defense mechanisms, such as phagocytosis. In this regard, lethal meningitis with *S. pneumoniae* could be induced despite the lack of capsular polysaccharide, but the presence of pneumococcal capsule is associated with higher and more sustained bacterial density in cerebrospinal fluid (51).

The fact that heat-killed washed organisms were able to induce cytokine production indicates that relatively heat-stable cell-associated components are probably responsible for most cytokine stimulation observed. Furthermore, a high temperature (100°C) treatment of bacteria did not decrease cytokine release, indicating a probably limited role of proteins. Nevertheless, some protein-mediated effect can not be completely ruled out, particularly with the 60°C treatment of bacteria. Heat-resistant potential candidates may include cell wall components, such as peptidoglycan or lipoteichoic acid, which have been demonstrated to be potent cytokine inductors for various gram positive cocci (20, 48). It was postulated that encapsulated bacteria produce inflammation by exposure of the underlying cell wall or by secretion of the cell wall material during growth (51). In fact, purified *S. suis* cell wall was able to induce a TNF response similar to that obtained with whole bacteria. It could also induce, to a lesser extent, IL-6. This result suggests that bacterial components other than those in the cell wall also contribute to IL-6 induction by *S. suis*, and confirms the differences observed with the unencapsulated mutant as described above. Further studies are needed to determine the exact nature of the *S. suis* components responsible for cytokine release.

Since previous studies on the pathogenesis of *S. suis* infections suggested that bacteria can be ingested, even in the presence of a capsule (1, 60), the possibility that the

release of cytokines would be a consequence of phagocytosis could not be ruled out. In fact, phagocytosis-dependence of TNF induction by GBS has already been reported (16). Results obtained in presence of CyC demonstrate that phagocytosis does not have an effect on TNF or IL-6 production by J774A1 cells after *S. suis* stimulation. In addition, non-specific stimulation with latex beads did not induce any significant increase of cytokine release under the same conditions used for *S. suis* stimulation. We conclude that cytokine release by *S. suis* is not related to phagocytosis of this microorganism. This is also confirmed by a recent study showing that, unlike GBS, well-encapsulated *S. suis* is in fact not phagocytosed (41). With a similar approach, Simpson et al. (44) also suggested that phagocytosis is not the major initiating factor for TNF synthesis in *S. pneumoniae*-stimulated macrophages. Recently, the importance of the attachment phase for cytokine induction has been shown with several bacterial species, including intracellular bacteria such as *Legionella pneumophila*, and *Listeria monocytogenes* (10, 61). Thus, the initial attachment of bacteria to macrophages may be sufficient to generate a signal for cytokine induction, and such a signal may be mediated by certain bacterial ligands and macrophage receptor interactions.

Since it has been shown that several microbial toxins may stimulate or modulate the inflammatory mediator cascade (26), two soluble proteins of *S. suis*, described as possible virulence factors, the suilysin and the EF, were analyzed (18, 55). However, we failed to demonstrate a role of these factors in cytokine induction by murine macrophages in vitro. This is in agreement with recent reports which indicate that the *S. suis* type 2 strains deficient in the production of these proteins remain virulent (17, 46). It has also been shown that a pneumolysin-deficient strain of *S. pneumoniae* caused meningeal inflammation in rabbits indistinguishable from that induced by the parent strain (15). Similarly, streptolysin O from *S. pyogenes* induced neither neutrophil influx nor significant cytokine elevations in bronchoalveolar fluids (43). These two hemolysins, like suilysin, belong to the family of the thiol-activated toxins (2). However, the role of suilysin, as well as of EF, in vivo inflammation remains to be elucidated.

Elevated levels of pro-inflammatory cytokines have been correlated with disease severity and mortality in experimental animal models, and neutralization of these cytokines improves survival in animals infected with pathogenic bacteria such as

pneumococci and *Haemophilus influenzae* type b (38, 40). The observed cytokine-inducing activity of *S. suis*, remarkably the higher IL-6 response, may have significant biologic relevance, since it has been demonstrated that this cytokine can be generated in the blood and cerebrospinal fluid during invasive meningeal infections (50, 58). Furthermore, IL-6 was recently postulated to be a possible marker for acute bacterial infection in swine (14).

In conclusion, our findings suggest that *S. suis* type 2 may trigger macrophages to produce pro-inflammatory cytokines and could therefore be implicated in the initiation and development of meningitis caused by this microorganism.

ACKNOWLEDGMENTS

We gratefully acknowledge Sonia Lacouture for excellent technical assistance. We also wish to thank Dr. Monique Doré for critical review of the manuscript.

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) grant # 0680154280, and by the Fonds pour la Formation des Chercheurs et l'Aide à la Recherche du Québec (FCAR) grant # NC-1037. M.S. holds a graduate scholarship from the Canadian International Development Agency (CIDA).

REFERENCES

1. **Alexander, T.** 1995. *Streptococcus suis*: pathogenesis and host response, p. 49-53. *In* Proceedings of the Allen D. Leman Swine Conference. University of Minessota, Minnesota.
2. **Alouf, J. E., and C. Geoffroy.** 1991. The family of the antigenically-related cholesterol-binding ("sulphydryl-activated") cytolytic toxins, p. 147-186. *In* J. E. Alouf (ed.), Sourcebook of Bacterial Protein Toxins. New York Academic Press, New York.
3. **Arends, J. P., and H. C. Zanen.** 1988. Meningitis caused by *Streptococcus suis* in humans. Rev. Infect. Dis. **10:**131-137.
4. **Beaudoin, M., R. Higgins, J. Harel, and M. Gottschalk.** 1992. Studies on a murine model for evaluation of virulence of *Streptococcus suis* capsular type 2 isolates. FEMS Microbiol. Lett. **78:**111-116.
5. **Cauwels, A., E. Wan, M. Leismann, and E. Tuomanen.** 1997. Coexistence of CD14-dependent and independent pathways for stimulation of human monocytes by gram-positive bacteria. Infect. Immun. **65:**3255-3260.
6. **Cerami, A.** 1992. Inflammatory cytokines. Clin. Immunol. Immunopathol. **62:**S3-S10.
7. **Charland, N., J. Harel, M. Kobish, S. Lacasse, and M. Gottschalk.** 1998. *Streptococcus suis* serotype 2 mutants deficient in capsular expression. Microbiology. **144:**325-332.
8. **Cleveland, M. G., J. D. Gorham, T. L. Murphy, E. Tuomanen, and K. M. Murphy.** 1996. Lipoteichoic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. Infect. Immun. **64:**1906-1912.
9. **Clifton-Hadley, F. A.** 1981. Ph.D. thesis. Studies of *Streptococcus suis* type 2 infection in pigs. University of Cambridge, Cambridge.
10. **Demuth, A., W. Goebel, H. U. Beuscher, and M. Kuhn.** 1996. Differential regulation of cytokine and cytokine receptor mRNA expression upon infection of bone marrow-derived macrophages with *Listeria monocytogenes*. Infect. Immun. **64:**3475-3483.
11. **Fan, X. D., M. Goldberg, and B. R. Bloom.** 1988. Interferon-γ-induced transcriptional activation is mediated by protein kinase C. Proc. Natl. Acad. Sci. USA. **85:**5122-5125.
12. **Flick, D. A., and G. E. Gifford.** 1984. Comparison of in vitro cell cytotoxic assays for tumor necrosis factor. J. Immunol. Methods. **68:**167-175.
13. **Fong, Y., K. J. Tracey, L. L. Moldawer, D. G. Hesse, K. B. Manogue, J. S. Kenney, A. T. Lee, G. C. Kuo, A. C. Allison, S. F. Lowry, and A. Cerami.** 1989. Antibodies to cachectin/tumor necrosis factor reduce interleukin 1β and interleukin 6 appearance during lethal bacteremia. J. Exp. Med. **170:**1627-1633.
14. **Fossum, C., E. Wattrang, L. Fuxler, K. T. Jensen, and P. Wallgren.** 1998. Evaluation of various cytokines (IL-6, INF-α, IFN-γ, TNF-α) as markers for acute bacterial infection in swine - a possible role for serum interleukin-6. Vet. Immunol. Immunopathol. **64:**161-172.
15. **Friedland, I. R., M. M. Paris, S. Hickey, S. Shelton, K. Olsen, J. C. Paton, and G. H. McCracken.** 1995. The limited role of pneumolysin in the

pathogenesis of pneumococcal meningitis. J. Infect. Dis. **172:**805-809.

16. **Goodrum, K. J., J. Dierksheide, and B. J. Yoder.** 1995. Tumor necrosis factor alpha acts as an autocrine second signal with gamma interferon to induce nitric oxide in group B *streptococcus*-treated macrophages. Infect. Immun. **63:**3715-3717.
17. **Gottschalk, M., A. Lebrun, H. Wisselink, J. D. Dubreuil, H. Smith, and U. Vecht.** 1998. Production of virulence-related proteins by canadian strains of *Streptococcus suis* capsular type 2. Can. J. Vet. Res. **62:**75-79.
18. **Gottschalk, M. G., S. Lacouture, and J. D. Dubreuil.** 1995. Characterization of *Streptococcus suis* capsular type 2 haemolysin. Microbiology. **141:**189-195.
19. **Havell, E. A., and P. B. Sehgal.** 1991. Tumor necrosis factor-independent IL-6 production during murine listeriosis. J. Immunol. **146:**756-761.
20. **Heumann, D., C. Barras, A. Severin, M. P. Glauser, and A. Tomasz.** 1994. Gram-positive cell walls stimulate synthesis of tumor necrosis factor alpha and interleukin-6 by human monocytes. Infect. Immun. **62:**2715-2721.
21. **Higgins, R., and M. Gottschalk.** 1998. Distribution of *Streptococcus suis* capsular types in 1997. Can. Vet. J. **39:**299-300.
22. **Higgins, R., and M. Gottschalk.** 1999. Streptococcal diseases. Chapter 41, p. 563-570. *In* I. S. U. Press (ed.), Diseases of swine. Iowa State University, Iowa.
23. **Jacobs, A. A., P. L. Loeffen, A. J. van den Berg, and P. K. Storm.** 1994. Identification, purification, and characterization of a thiol-activated hemolysin (suilysin) of *Streptococcus suis*. Infect. Immun. **62:**1742-1748.
24. **Jansky, L., S. Vybiral, D. Pospisilova, J. Roth, J. Dornand, E. Zeisberger, and J. Kaminkova.** 1995. Production of systemic and hypothalamic cytokines during the early phase of endotoxin fever. Neuroendocrinology. **62:**55-61.
25. **Kobisch, M., M. Gottschalk, P. Morvan, R. Cariolet, G. Bénévent, and J. P. Joly.** 1995. Infection expérimentale de porcelets par *Streptococcus suis*, serovar 2. Journées Rech. Porcine en France. **27:**97-102.
26. **König, W., S. Kasimir, T. Hensler, J. Scheffer, B. König, R. Hilger, J. Brom, and M. Köller.** 1992. Release of inflammatory mediators by toxin stimulated immune system cells and platelets. Zbl. Bakt. Suppl. **23:**385-394.
27. **Ling, E. W. Y., F. J. D. Noya, G. Ricard, K. Beharry, E. L. Mills, and J. V. Aranda.** 1995. Biochemical mediators of meningeal inflammatory response to group B *streptococcus* in the newborn piglet model. Pediatr. Res. **38:**981-987.
28. **Martin, C. A., and M. E. Dorf.** 1991. Differential regulation of interleukin-6, macrophage inflammatory protein-1, and JE/MCP-1 cytokine expression in macrophage cell lines. Cell. Immunol. **135:**245-258.
29. **Martin, C. A., and M. E. Dorf.** 1990. Interleukin-6 production by murine macrophage cell lines P388D1 and J774A.1: stimulation requirements and kinetics. Cell. Immunol. **128:**555-568.
30. **Mattsson, E., J. Rollof, J. Verhoef, H. Van Dijk, and A. Fleer.** 1994. Serum-induced potentiation of tumor necrosis factor alpha production by human monocytes in response to staphylococcal peptidoglycan: involvement of different serum factors. Infect. Immun. **62:**3837-3843.
31. **Mosmann, T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Meth. **65:**55-63.
32. **Newton, A. C.** 1995. Protein kinase C: structure, function, and regulation. J.

Biol. Chem. **270**:28495-28498.

33. **Nibbering, P. H., and R. Van Furth.** 1988. Quantitative immunocytochemical characterization of four murine macrophage-like cell lines. Immunobiology. **176**:432-439.
34. **Nishizuka, Y.** 1995. Protein kinase C and lipid signaling for sustained cellular responses. FASEB J. **9**:484-496.
35. **Orman, K. L., J. L. Shenep, and B. K. English.** 1998. Pneumococci stimulate the production of the inducible nitric oxide synthase and nitric oxide by murine macrophages. J. Infect. Dis. **178**:1649-1657.
36. **Painter, R. G., J. Whisenand, and A. T. McIntosch.** 1981. Effects of cytochalasin B on actin and myosin association with particle binding sites in mouse macrophages: implications with regard to the mechanism of action of the cytochalasins. J. Cell Biol. **91**:373-384.
37. **Pugin, J., D. Heumann, A. Tomasz, V. V. Kravchenko, Y. Akamatsu, M. Nishijima, M. P. Glauser, P. S. Tobias, and R. J. Ulevitch.** 1994. CD14 is a pattern recognition receptor. Immunity. **1**:509-516.
38. **Ramilo, O., X. Saéz-Llorens, J. Mertsola, H. Jafari, K. D. Olsen, E. J. Hansen, M. Yoshinaga, S. Ohkawara, H. Nariuchi, and G. H. McCracken Jr.** 1990. Tumor necrosis factor α/cachectin and interleukin-1β initiate meningeal inflammation. J. Exp. Med. **172**:497-507.
39. **Roberts, F. A., G. J. Richardson, and S. M. Michalek.** 1997. Effects of *Porphyromonas gingivalis* and *Escherichia coli* lipopolysaccharides on mononuclear phagocytes. Infect. Immun. **65**:3248-3254.
40. **Saukkonen, K., S. Sande, C. Cioffe, S. Wolpe, B. Sherry, A. Cerami, and E. Tuomanen.** 1990. The role of cytokines in the generation of inflammation and tissue damage in experimental gram-positive meningitis. J. Exp. Med. **171**:439-448.
41. **Segura, M. A., P. Cléroux, and M. Gottschalk.** 1998. *Streptococcus suis* and group B *Streptococcus* differ in their interactions with murine macrophages. FEMS Immunol. Med. Microbiol. **21**:189-195.
42. **Sepulveda, E. M. D., E. Altman, M. Kobisch, S. Dallaire, and M. Gottschalk.** 1996. Detection of antibodies against *Streptococcus suis* capsular type 2 using a purified capsular polysaccharide antigen-based indirect elisa. Vet. Microbiol. **52**:113-125.
43. **Shanley, T. P., D. Schrier, V. Kapur, M. Kehoe, J. M. Musser, and P. A. Ward.** 1996. Streptococcal cysteine protease augments lung injury induced by products of group A streptococci. Infect. Immun. **64**:870-877.
44. **Simpson, S. Q., R. Singh, and D. E. Bice.** 1994. Heat-killed pneumococci and pneumococcal capsular polysaccharides stimulate tumor necrosis factor-α production by murine macrophages. Am. J. Respir. Cell Mol. Biol. **10**:284-289.
45. **Smith, H. E., M. Damman, J. Van der Velde, F. Wagenaar, H. J. Wisselink, N. Stockhofe-Zurwieden, and M. A. Smits.** 1999. Identification and characterization of the *cps* locus of *Streptococcus suis* serotype 2: the capsule protects against phagocytosis and is an important virulence factor. Infect. Immun. **67**:1750-1756.
46. **Smith, H. E., U. Vecht, H. J. Wisselink, N. Stockhofe-Zurwieden, Y.**

Biermann, and M. A. Smits. 1996. Mutants of *Streptococcus suis* types 1 and 2 impaired in expression of muramidase-released protein and extracellular protein induce disease in newborn germfree pigs. Infect. Immun. **64:**4409-4412.

47. **Staats, J. J., I. Feder, O. Okwumabua, and M. M. Chengappa.** 1997. *Streptococcus suis* - past and present [Review]. Vet. Res. Commun. **21:**381-407.
48. **Timmerman, C. P., E. Mattsson, L. Martinez-Martinez, L. De Graaf, J. A. G. Van Strijp, H. A. Verbrugh, J. Verhoef, and A. Fleer.** 1993. Induction of release of tumor necrosis factor from human monocytes by staphylococci and staphylococcal peptidoglycans. Infect. Immun. **61:**4167-4172.
49. **Trottier, S., R. Higgins, G. Brochu, and M. Gottschalk.** 1991. A case of human endocarditis due to *Streptococcus suis* in North America [letter]. Rev. Infect. Dis. **13:**1251-1252.
50. **Tunkel, A. R., and W. M. Scheld.** 1993. Pathogenesis and pathophysiology of bacterial meningitis. Clin. Microbiol. Rev. **6:**118-136.
51. **Tuomanen, E., A. Tomasz, B. Hengstler, and O. Zak.** 1985. The relative role of bacterial cell wall and capsule in the induction of inflammation in pneumococcal meningitis. J. Infect. Dis. **151:**535-540.
52. **Vallejo, J. G., C. J. Baker, and M. S. Edwards.** 1996. Interleukin-6 production by human neonatal monocytes stimulated by type III group B streptococci. J. Infect. Dis. **174:**332-337.
53. **Vallejo, J. G., C. J. Baker, and M. S. Edwards.** 1996. Roles of the bacterial cell wall and capsule in induction of tumor necrosis factor alpha by type III group B streptococci. Infect. Immun. **64:**5042-5046.
54. **Van Furth, A. M., J. J. Roord, and R. van Furth.** 1996. Roles of proinflammatory and anti-inflammatory cytokines in pathophysiology of bacterial meningitis and effect of adjunctive therapy. Infect. Immun. **64:**4883-4890.
55. **Vecht, U., H. J. Wisselink, M. L. Jellema, and H. E. Smith.** 1991. Identification of two proteins associated with virulence of *Streptococcus suis* type 2. Infect. Immun. **59:**3156-3162.
56. **Verhoef, J., and E. Mattsson.** 1995. The role of cytokines in Gram-positive bacterial shock. Trends Microbiol. **3:**136-140.
57. **Von Hunolstein, C., A. Totolian, G. Alfarone, G. Mancuso, V. Cusumano, G. Teti, and G. Orefici.** 1997. Soluble antigens from group B streptococci induce cytokine production in human blood cultures. Infect. Immun. **65:**4017-4021.
58. **Waage, A., P. Brandtzaeg, A. Halstensen, P. Kierulf, and T. Espevik.** 1989. The complex pattern of cytokines in serum from patients with meningococcal septic shock. Association between interleukin 6, interleukin 1, and fatal outcome. J. Exp. Med. **169:**333-338.
59. **Wightman, P. D., and C. R. H. Raetz.** 1984. The activation of protein kinase C by biologically active lipid moieties of lipopolysaccharide. J. Biol. Chem. **259:**10048-10052.
60. **Williams, A. E., and W. F. Blakemore.** 1990. Pathogenesis of meningitis caused by *Streptococcus suis* type 2. J. Infect. Dis. **162:**474-481.
61. **Yamamoto, Y., S. Okubo, T. W. Klein, K. Onozaki, T. Saito, and H. Friedman.** 1994. Binding of *Legionella pneumophila* to macrophages increases cellular cytokine mRNA. Infect. Immun. **62:**3947-3956.

ARTICLE IV

CD14-dependent and -independent cytokine and chemokine production by human THP-1 monocytes stimulated by *Streptococcus suis* capsular type 2

Mariela Segura, Nathalie Vadeboncoeur and Marcelo Gottschalk

Groupe de Recherche sur les Maladies Infectieuses du Porc (GREMIP), Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, Québec, Canada

Clinical and Experimental Immunology. 2002. 127: 243-254

Role of the candidate in conception of this article:

I was the main author of this article. I have actively participated in the conception and design of the experiments, and the standardization of techniques. I carried out all the laboratory work and also the analysis of results. Finally, I did also all the graphic conceptions and writing of the manuscript.

SUMMARY

Streptococcus suis capsular type 2 is an important etiologic agent of swine meningitis, and it has been highlighted as a cause of occupational disease leading to meningitis and fulminant sepsis in humans. The objective of the present work was to study the ability of *S. suis* type 2 to induce the release of tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), IL-6, IL-8 and monocyte chemotactic protein one (MCP-1) by human monocytic THP-1 cells. The induction of these five cytokines was dose- and incubation time-dependent, and it was significantly enhanced by pre-treatment of cells with interferon gamma. IL-8 levels were markedly higher compared to those obtained with the other cytokines; however, elevated levels of MCP-1 and IL-6 were also observed. Levels of cytokine induced by heat-killed or live bacteria were similar. Pre-treatment of cells with anti-CD14 monoclonal antibodies suggested that this important host receptor is partially implicated in TNF, IL-1, IL-6 and MCP-1 production, while CD14-independent pathways seem to be responsible for IL-8 production after *S. suis* stimulation. In addition, blocking studies with anti-TNF and anti-IL-1 antibodies revealed that these cytokines are involved in amplification of the *S. suis*-induced cytokine cascade. When several different *S. suis* strains of human or porcine origin were compared, a very heterogeneous pattern of cytokine production was observed. Human strains did not exhibit a clear tendency to induce higher cytokine release by human THP-1 monocytes. The synergistic effect of the up-regulation of cytokines during *S. suis* meningitis may mediate many of the inflammatory reactions, including the sequestration of leukocytes at the site of infection.

INTRODUCTION

Streptococcus suis capsular type 2 is an important pathogen which has been associated with a wide variety of infections in swine such as meningitis, septicaemia, arthritis and pneumonia. Clinical presentation of *S. suis* infection may be as varied as asymptomatic bacteremia to fulminant systemic disease similar to gram-negative (G-) sepsis. Meningitis is the most striking feature, and the presence of fibrin, oedema and cellular infiltrates in the meninges and choroid plexus are the histopathological characteristics most frequently observed [1]. *S. suis* has also been isolated from human cases of meningitis, endocarditis, septicaemia and toxic-shock syndrome. *S. suis* infection in humans is considered an occupational disease of increasing importance, and is considered to be the major cause of adult meningitis in Hong Kong [2, 3].

The pathogenesis of the *S. suis* infection is still unclear although it is accepted that transmission is via the respiratory route, after which the pathogen remains localised in the palatine tonsils of pigs. From there, bacteria may invade the blood, meninges or other tissues, possibly in close association with monocytes/macrophages [4]. Once in the central nervous system (CNS), the induction of an acute inflammatory exudate increases the volume of the cerebrospinal fluid (CSF), leading to increased intracranial pressure [5].

Although the basis for the earliest steps of the innate immune response to infection with Gram-positive (G+) bacteria is poorly understood, the host response to invasion appears to share similar characteristics with the response to G- microorganisms. Invasion of the bloodstream by G+ and G- bacteria causes the sepsis syndrome characterised by the induction of cytokines and other inflammatory mediators [6, 7]. The primary immune activator of G- bacteria is lipopolysaccharide (LPS). In contrast, no clearly identifiable constituent of G+ bacteria can be linked to the sepsis syndrome. The G+ cell wall consists of peptidoglycan (PGN), lipoteichoic acids (LTA) and various proteins, any of which could be involved in the activation of host cells leading to inflammation [7-10].

CD14, in addition of being a high affinity receptor for LPS, has been proposed as the first host pattern-recognition receptor involved in recognition of most of the above-

mentioned bacterial components [9], and facilitates expression of inflammatory molecules, probably via activation of the Toll-like receptors [6, 11]. Given the common dependence on many cell wall products on CD14, similar activation pathways have been hypothesised for both G+ and G- bacteria [6]. However, blockade studies with anti-CD14 antibodies reveal that there are also CD14-independent pathways responsible for cell stimulation by G+ bacteria [12, 13].

The main cytokines involved in the regulation of inflammation are interferon gamma (IFN-γ), known as the chief macrophage-activating factor, interleukin-1 (IL-1), IL-6, and tumor necrosis factor alpha (TNF-α). Increased serum concentrations of IL-1, IL-6, and TNF-α (produced mainly by monocytes/macrophages) correlate with the severity of bacterial septicaemia [14]. In addition to these cytokines, chemokines are also involved in the initiation and propagation of inflammatory responses characterised by sequestration of leukocytes at the site of infection. IL-8 is the prototype member of the CXC chemokines which have strong neutrophil chemotactic and activating properties, whereas monocyte chemotactic protein one (MCP-1) is the prototype member of the CC chemokine family which elicit primarily mononuclear cells. Monocytes/macrophages are also a rich source of chemokines [15]. In addition to pro-inflammatory cytokines, high levels of MCP-1 and IL-8 have been observed in the CSF of patients with bacterial meningitis (i.e. *Neisseria meningitidis, Streptococcus pneumoniae, Haemophilus influenzae*), and they would be responsible for the chemotactic activity into the CNS [14, 16, 17].

Despite the well-recognised association between several infectious diseases and the overproduction of pro-inflammatory cytokines, limited studies on the role of the inflammatory response in the pathogenesis of *S. suis* infections have been reported. Only one previous study has addressed *S. suis* cytokine activation of murine macrophages, and little is known about host receptors recognised by *S. suis* [18]. Moreover, the capacity of this important zoonotic meningeal pathogen to induce cytokine production by human cells has never been studied, and the interaction between strains of *S. suis* type 2 isolated from human cases and human host cells has not been studied before. This led us to investigate the induction of proinflammatory cytokines and chemokines TNF-α, IL-1β, IL-6, IL-8 and MCP-1 in human THP-1 monocytes stimulated with *S. suis* type 2.

Since IFN-γ is the earliest cytokine detected at the site of infection and plays a critical role in cell priming and activation [19-21], the IFN-γ pre-activated cell model was compared to non-activated cells. The possible mechanism by which *S. suis* may stimulate monocytes (i.e. CD14-dependent or –independent pathways) was also addressed. In addition, we compared the ability of different *S. suis* type 2 strains of human or porcine origin to induce the release of cytokines by human THP-1 monocytes.

MATERIALS AND METHODS

Reagents

Cell culture media, foetal bovine serum (FBS), penicillin G (PenG) and streptomycin (Sm) were purchased from Gibco (Burlington, VT); 2-mercaptoethanol (2-ME) was obtained from Bio-Rad (Mississauga, Ontario, Canada). *Escherichia coli* 0127:B8 LPS, and polymyxin B sulfate (PmB), were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Recombinant human IFN-γ was purchased from R&D Systems (Minneapolis, MN).

Bacterial strains and growth conditions

The *S. suis* capsular type 2 virulent strain 31533, originally isolated from a case of porcine meningitis [22], and previously used for cytokine induction studies with murine macrophages [18], was used as the reference strain in this study. Strains of porcine or human origin used in the comparative study of cytokine production are listed in Table 1. Bacteria were maintained as stock cultures in 50% glycerol-Todd-Hewitt broth (THB; Difco Lab., Detroit, MI) at -80°C. Bacteria were grown overnight onto bovine blood agar plates at 37°C and isolated colonies were used as inocula for THB, which were incubated for 18 h at 37°C. Working cultures for monocyte stimulation were produced by inoculating 10 ml of the overnight cultures into 200 ml of THB with agitation at 37°C for 6 h until they reached the mid-log phase (540 nm-optical density of 0·4-0·5). Bacteria were washed twice in phosphate-buffered saline (PBS), pH 7·4, and diluted to approximately 2 x 10^9 CFU/ml in PBS. The final suspension was plated onto THB-agar in order to accurately determine the CFU/ml. Bacteria were then killed by heat treatment by incubating organisms at 60°C for 45 min (minimal experimental conditions required for killing *S. suis*) [18]. The killed cultures were subcultured onto blood agar plates at 37°C for 48 h to confirm the absence of viable organisms. Killed bacterial preparations were stored at 4°C and resuspended in cell culture media just before stimulation assays. In certain experiments, live bacteria (prepared as mentioned above) were used at initial infectious concentration of 10^4 CFU/ml. Heat-killed or live bacterial preparations at the concentrations indicated above did not cause cytotoxic

effects to cultured cells, as evaluated by lactate dehydrogenase (LDH) measurement as previously described (unpublished observations) [18, 23, 24].

TABLE 1. *S. suis* capsular type 2 strains of porcine and human origins used in this study

Strain	Origin	Virulence ¤	Geographic origin
31533†	Diseased pig	V	France
S735‡	Diseased pig	V	The Netherlands
D282	Diseased pig	V	The Netherlands
94-623	Pig, healthy carrier	NV	France
TD10	Pig, healthy carrier	NV	UK
89-1591	Diseased pig	V	Canada
90-1330	Diseased pig	NV	Canada
89-999	Diseased pig	V	Canada
Reims	Human; spondylodiscitis	NT	France
EUD95	Human; meningitis	NT	France
Biotype 2	Human; endocarditis	NT	France
HUD Limoge	Human; septic shock	NT	France
FRU95	Human; meningitis	NT	France
LEF95	Human; meningitis	NT	France
96-52466	Human; arthritis	NT	France
H11/1	Human; meningitis	V	UK
AR770353	Human; meningitis	NT	The Netherlands
AR770297	Human; meningitis	NT	The Netherlands
91-1804	Human; endocarditis	NT	Canada
94-3037	Human; meningitis	NT	Canada
98-3634	Human; endocarditis	NT	Canada
99-734723688	Human; septicaemia	NT	Canada

¤ As indicated in the literature by using experimental porcine models [22, 52, 53]. V: virulent; NV: non virulent; NT: never tested. Strain H11/1: P. Norton, personal communication.

† Strain used as reference in the present work, as well as in a previous work on *S. suis* cytokine stimulation [18].

‡ ATCC 43765 *S. suis* type 2 reference strain.

Cell lines and cell culture

THP-1 human monocytic cell line, derived from an acute monocytic leukaemia (ATCC TIB-202, Rockville, Md.), was maintained in RPMI 1640 medium. Cell culture

medium was supplemented with 10% heat-inactivated FBS, PenG (100 IU/ml), Sm (100 μg/ml), and 50 mM 2-ME, and cells were incubated at 37°C with 5% CO_2.

Stimulation of THP-1 monocytes

For stimulation assays, 48 h cultures of THP-1 cells were washed once, resuspended in culture media at 10^6 cells/ml, and 0·5 ml of this suspension was distributed in 24-well plates (Falcon™, Becton Dickinson, Bedford, MA). *S. suis* strains (0·5 ml), diluted appropriately in culture medium, were added and tested in duplicate wells for each stimulation assay. Pre-activation and differentiation of monocytes was afforded by pre-treatment with IFN-γ (500 U/ml) for 48 h before induction. Pre-activated cells were then washed and resuspended in fresh medium as described above. Monocytes stimulated with LPS (1 μg/ml) served as positive controls. Monocytes with medium alone served as controls for spontaneous cytokine release. Cytokine induction plates were incubated at 37°C with 5% CO_2. At different time intervals (see Results), culture supernatants were harvested from individual wells, aliquoted and frozen at -20°C until cytokine determination. Each test of monocyte stimulation was done at least in triplicate.

Enzyme-linked immunosorbent assays (ELISA) for cytokines

TNF-α, IL-1β, IL-6, IL-8 and MCP-1 were measured by ELISA, using pair-matched monoclonal antibodies (Mabs) from R&D Systems, according to the manufacturer's recommendations. Twofold dilutions of recombinant human TNF-α (3000 to 46 pg/ml), IL-1β (300 to 5 pg/ml), IL-6 (1500 to 3 pg/ml), IL-8 (600 to 5 pg/ml) or MCP-1 (500 to 8 pg/ml) were included as standard curves in each ELISA plate (Nunc™, VWR, Ville Mont Royal, Quebec, Canada). All cytokine standards were obtained from R&D Systems. Supernatant dilutions giving optical density readings in the linear portion of the appropriate standard curve were used to quantitate the levels of each cytokine in the samples. Standard and sample dilutions were added in duplicate wells to each ELISA plate. All analyses were performed at least four times for each individual monocyte-stimulation assay.

Cytokine Blockade

In selected experiments, we sought to assess whether bacterial cytokine production was the result of stimulation by other cytokines. To accomplish this objective, IFN-γ pre-activated THP-1 cells were cultured in the presence of mouse anti-human TNF-α (IgG1; 40 μg/ml), IL-1β (IgG1; 15 μg/ml), or MCP-1 (IgG2b; 5 μg/ml) Mabs, or the combination of both TNF-α + IL-1β Mabs. Mabs were obtained from R&D Systems, and were added in optimal blocking concentrations, to ensure complete neutralisation, as indicated by the manufacturer. Neutralising Mabs were added at the beginning of culture together with the bacterial stimulus (heat-killed 31533 strain at 10^9 CFU/ml) for 3 h or 24 h (depending on the cytokine being tested; see Results). Purified mouse monoclonal IgG1 and IgG2b (R&D Systems) used at similar concentrations of corresponding Mabs served as isotype controls. Values were transformed to percentage of inhibition of values obtained for bacterial stimulus without treatment (100% of production) for comparison between experiments.

CD14 blockade

To evaluate the role of the CD14 receptor in cytokine induction by *S. suis*, IFN-γ pre-activated THP-1 cells were pre-incubated for 1 h at 37°C with anti-human CD14 Mabs before adding the stimuli. Three different anti-CD14 Mabs were used: clone MY4 (IgG2b; 10 μg/ml); clone IOM2, also named RMO52 (IgG2a; 20 μg/ml; Coulter Immunology, Mississauga, ON, Canada); and clone Leu-M3 (IgG2b; 5 μg/ml; Becton Dickinson, San Jose, CA). Purified mouse monoclonal IgG2a and IgG2b (R&D Systems) used at similar concentrations of corresponding Mabs served as isotype controls. The bacterial stimulus (heat-killed 31533 strain at 10^9 CFU/ml) or LPS (1 μg/ml), used as a reference to evaluate the effect of anti-CD14 Mabs, were incubated in presence of the anti-CD14 Mabs for 3 h or 24 h (depending on the cytokine being tested; see Results). Values were transformed to percentage of inhibition of values obtained for bacteria or LPS without treatment (100% of production) for comparison between experiments.

Endotoxin contamination

All solutions and bacterial preparations used in these experiments were tested for the presence of endotoxin using a *Limulus* amebocyte lysate (LAL) gel-clot test (Pyrotell® STV, Cape Cod, Falmouth, MA) with a sensitivity limit of 0·03 EU/ml. In some experiments, endotoxin contamination during stimulation of monocytes was controlled by parallel assays with PmB (10 μg/ml). Results from the LAL test and/or data from PmB treatment demonstrated that no significant levels of endotoxin contamination could be detected in different bacterial preparations (data not shown). Cell culture medium and IFN-γ solution contained less than 0·03 EU/ml. Thus, endotoxin levels represented < 0·005 ng endotoxin per milliliter, a concentration below that known to cause macrophage activation [25].

Statistical analysis

Results were derived from linear regression calculations and expressed in pg/ml of cytokine. Results are presented as mean ± standard deviation of independent experiments. Differences were analysed for significance by using the Student's unpaired *t*-test (two-tailed *P*-value). A *P*-value < 0·05 was used as threshold for significance. Differences between the human and porcine origin group of strains and differences among strains within the same group were analysed for significance by using general linear models (GLM) followed by Tukey-Kramer post-hoc tests for differences between strains. GLM was used for analysis of variance (ANOVA) with the origin as one factor and the strain within origin as the other factor. The GLM procedure uses the method of least squares to fit general linear models. The SAS v8 software (SAS, Cary, NC) was used for these analyses.

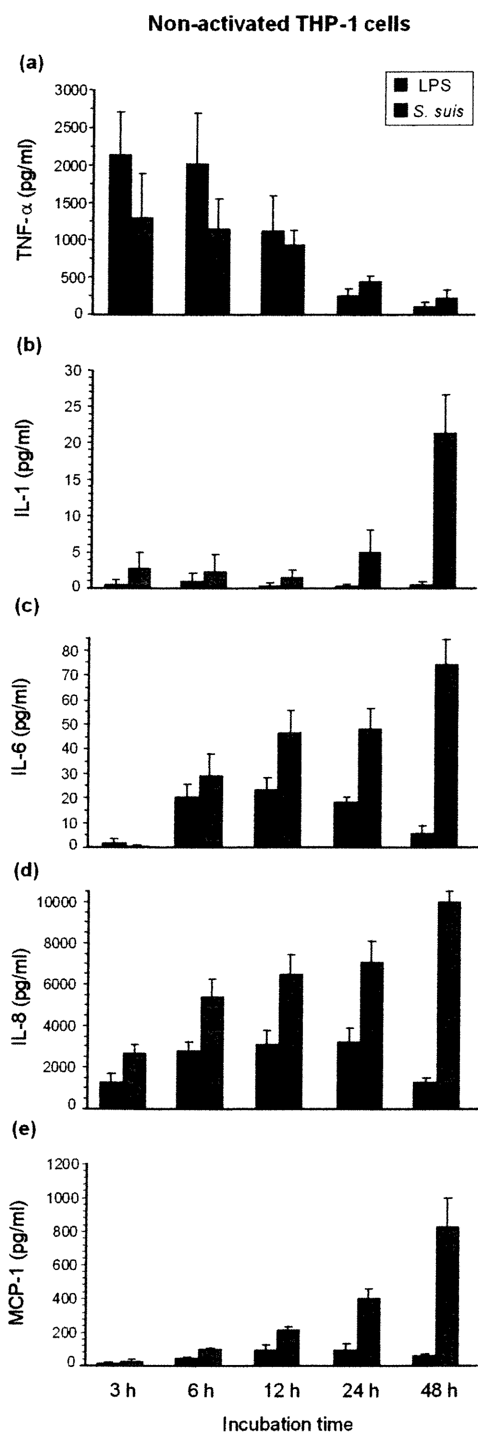
RESULTS

Kinetics of cytokine release by monocytes triggered by *S. suis*: effect of IFN-γ pre-activation of cells

Incubation of THP-1 cells without stimuli yielded a low basal level of cytokine expression. These values were used to correct data obtained after *S. suis* or LPS stimulation throughout this work. Incubation of cells with *S. suis* was associated with a time-dependent production of cytokines. TNF was the first cytokine detectable, peaking at 3 h, and gradually declining thereafter (Fig. 1a and Fig. 2a), whereas IL-1, IL-6, IL-8 and MCP-1 increased progressively and reached peak concentrations at time points between 24 h and 48 h (Fig. 1b-e and Fig. 2b-e). The kinetics of *S. suis*- induced cytokine release were similar in both non-activated and pre-activated cells. However, IFN-γ pre-activated THP-1 cells showed a markedly enhanced production of cytokines following incubation with either *S. suis* or LPS ($P < 0{\cdot}001$) (Fig. 2). IL-8 levels were significantly higher compared to observed levels of other cytokines; IL-6 and MCP-1 values were intermediate and significantly lower levels of TNF and IL-1 production were observed (Fig.1 and Fig. 2).

Comparison of LPS- and *S. suis*- stimulated cytokines with non-activated THP-1 cells showed similar kinetics for TNF production with both stimuli (Fig. 1a), whereas levels of IL-1, IL-6, IL-8 and MCP-1 production after LPS stimulation were lower and slightly less sustained, declining after 24 h of incubation, than those obtained after *S. suis* stimulation (Fig. 1b-e). In contrast to that observed with non-activated THP-1 cells, similar kinetics and cytokine levels were shown after *S. suis*- or LPS-stimulation of pre-activated THP-1 cells (Fig. 2), except that levels of IL-8 induced by *S. suis* were much higher than those induced by LPS-stimulation (Fig. 2d).

Based on these experiments, IFN-γ pre-activated THP-1 cells were chosen as a model for subsequent studies. Supernatants were harvested at 3 h after stimulation to analyse TNF production, whereas supernatants harvested at 24 h or 48 h after stimulation were used to measure induction of other cytokines.



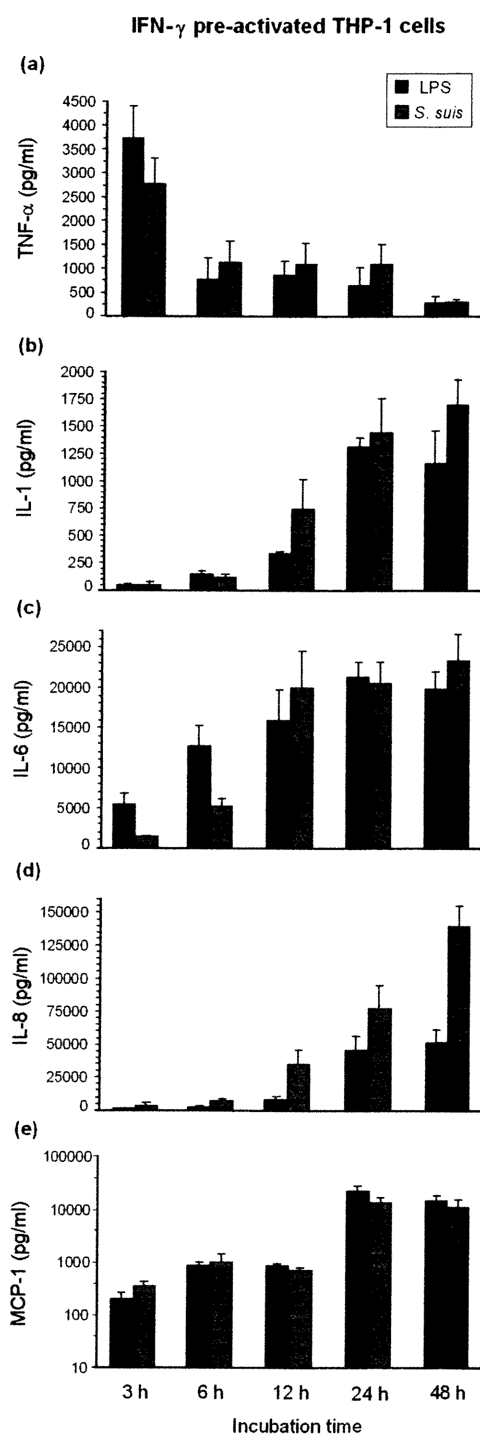


FIG. 1. Kinetics of cytokine release by non-activated THP-1 stimulated with *S. suis* (31533, 10^9 CFU) or LPS (1μg).

FIG. 2. Kinetics of cytokine release by IFN-γ-activated THP-1 stimulated with *S. suis* (31533, 10^9 CFU) or LPS (1μg).

Bacterial concentration-dependent cytokine release

IFN-γ pre-activated THP-1 monocytes were exposed to different concentrations of *S. suis* strain 31533. A high bacterial concentration was needed for maximal cytokine production. The two chemokines, IL-8 and MCP-1, showed the most sustained production. Levels first decreased at 10^8 CFU/ml, and then remained almost constant until 10^6 CFU/ml. Remarkably, IL-8 levels remain at 250 pg/ml at the lowest bacterial concentration tested in this study (5 x 10^4 CFU/ml). IL-1 and IL-6 levels gradually decreased and no production was observed at bacterial concentrations lower than 10^6 CFU/ml. TNF production quickly dropped when bacterial titre was decreased to 10^8 CFU/ml (Fig. 3).

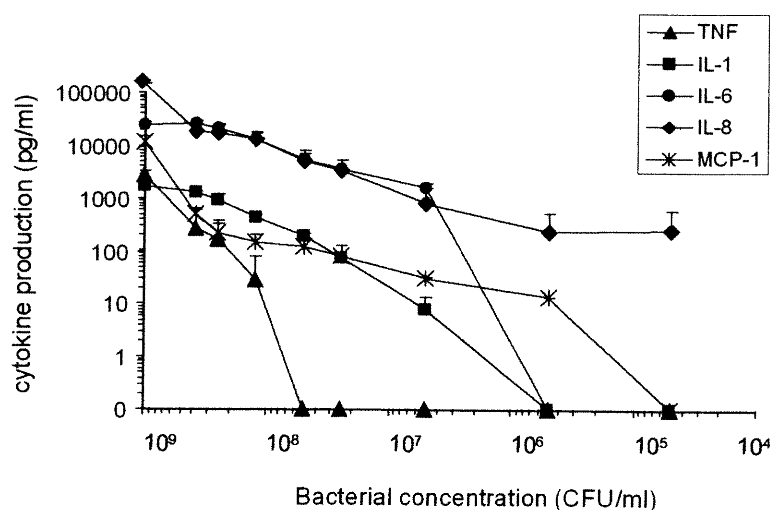


FIG. 3. Effect of bacterial concentration on cytokine production. IFN-γ pre-activated THP-1 cells were stimulated with different concentrations of heat-killed *S. suis* strain 31533. TNF-α, at 3 h of incubation, and IL-1, IL-6, IL-8 and MCP-1 at 48 h of incubation, were measured by ELISA titration of stimulated cell supernatants. Data are expressed as means ± standard deviations (in pg/ml). Values of basal expression of cytokines (cell culture medium alone, used as negative control) were corrected from data obtained after *S. suis* stimulation.

Heat-killed versus live bacteria

In certain experiments, live bacteria at an initial infectious concentration of 10^4 CFU/ml were used to induce cytokine production by THP-1 cells. Since the number of bacteria rapidly increased from 10^4 to $\sim 10^8$ CFU/ml after 3-4 h in cell culture plates, heat-killed bacteria at the latter concentration were used for comparison purposes. Under these conditions, levels of IL-6, IL-8 and MCP-1 production were similar for live and heat-killed bacteria (10739 ±870 vs_10955 ±1880; 114185 ±23220 vs 100135 ±14086; and 13523 ±3064 vs 16828 ±1431 pg/ml, respectively; $P > 0{\cdot}1$). Although live bacterial preparations stimulated cells to release slightly higher levels of IL-1 than heat-killed bacterial preparations did, the difference was not significant (3221 ±734 vs 2025 ±872, respectively; $P > 0{\cdot}05$). As indicated above, a 3 h-stimulation time was used to analyse TNF production, however TNF values were too low at the bacterial concentration used to get any conclusion. Similar results were obtained when a higher initial infectious dose was used (data not shown).

Inhibition of *S. suis*-induced cytokines by anti-cytokine Mabs: amplification pathways

In order to clarify to what extent TNF and IL-1 amplify the *S. suis*-induced cytokine network, IFN-γ pre-activated THP-1 cells were incubated with *S. suis* in the presence or absence of an excess of neutralising anti-TNF Mab, anti-IL-1 Mab or a combination of both Mabs, as indicated in Materials and Methods. Results are shown in Table 2. First, it was demonstrated that IgG isotype controls did not influence cytokine production (data not shown). Anti-TNF partially inhibited *S. suis*-induced IL-1 ($P = 0{\cdot}01$) and IL-8 production ($P = 0{\cdot}005$). The presence of the anti-IL-1 Mab had no effect on IL-8 production, and no additive effect was observed when both Mabs were used in combination. In contrast, anti-TNF or anti-IL-1 alone had no significant effect on IL-6 release, whereas the combination of both Mabs resulted in partial inhibition of IL-6 production ($P = 0{\cdot}003$). MCP-1 production was similarly inhibited by either anti-TNF or anti-IL-1 Mabs; however, the combination of both Mabs resulted in a highly significant increase of inhibition ($P = 0{\cdot}0001$) (Table 2). TNF production was not affected by the anti-IL-1 Mab (data not shown). These results suggest that TNF and IL-1 are involved in partial amplification pathways of *S. suis*-induced cytokine responses.

Other than the chemotactic properties of MCP-1, this cytokine has been shown to induce the production of IL-1 and IL-6 by monocytes, suggesting an amplification pathway which might serve to sustain an inflammatory response [15]. This hypothesis was also tested in the *S. suis*-induced response; but the anti-MCP-1 Mab did not affect the production of any cytokine tested (Table 2).

TABLE 2. Effect of anti-cytokines Mabs on *S. suis*-induced cytokine release by IFN-γ pre-activated THP-1 cells¤

	% of inhibition §			
S. suis-induced	Anti-TNF	Anti-IL-1	Anti-TNF + Anti-IL-1	Anti-MCP
IL-1	33 ± 6*	NT	NT	0 ± 0
IL-6	17 ± 3	1 ± 1	31 ± 7*	0 ± 0
IL-8	31 ± 5*	5 ± 4	28 ± 3*	6 ± 4
MCP-1	35 ± 3*	35 ± 9*	65 ± 4**	NT

¤ IFN-γ pre-activated THP-1 cells were cultured in the presence of anti-human TNF-α (40 μg/ml), IL-1β (15 μg/ml), or MCP-1 (5 μg/ml) Mabs; or the combination of both TNF-α + IL-1β Mabs. Neutralizing Mabs were added at the beginning of culture together with the bacterial stimulus (heat-killed 31533 strain at 10^9 CFU/ml) for 24 h.

§ Data are means ± SEM. NT: not tested

Significant inhibition (by the Student's unpaired *t* test) respect to values for bacterial stimulus without treatment (100% of production). $*P < 0{\cdot}01$; $**P < 0{\cdot}001$.

Role of CD14 receptor in *S. suis*-induced cytokines

We used three anti-human CD14 Mabs (MY4, IOM2 and Leu-M3) to evaluate the role of CD14 in the *S. suis*-induced cytokine response. MY4 recognises an epitope located between amino acids (aa) 34-44 [26]; the epitope recognised by IOM2 partially overlaps with that of MY4 [27], whereas that recognised by Leu-M3 is specific for aa 135-146 on the CD14 molecule [26].

IFN-γ pre-activated THP-1 cells were subjected to pretreatment with Mabs for 1 h followed by stimulation with either *S. suis* at 10^9 CFU/ml or LPS at 1 μg/ml (Fig. 4). Sub-maximal doses of bacteria (5 x 10^8, 10^8, and 5 x 10^7 CFU/ml) or LPS (50 ng/ml) were also tested to determine whether use of maximal doses of stimuli may mask

antibody blocking effects. It was first determined that the same concentration of monoclonal isotype controls had no effect on LPS- or *S. suis*-induced cytokine production (data not shown).

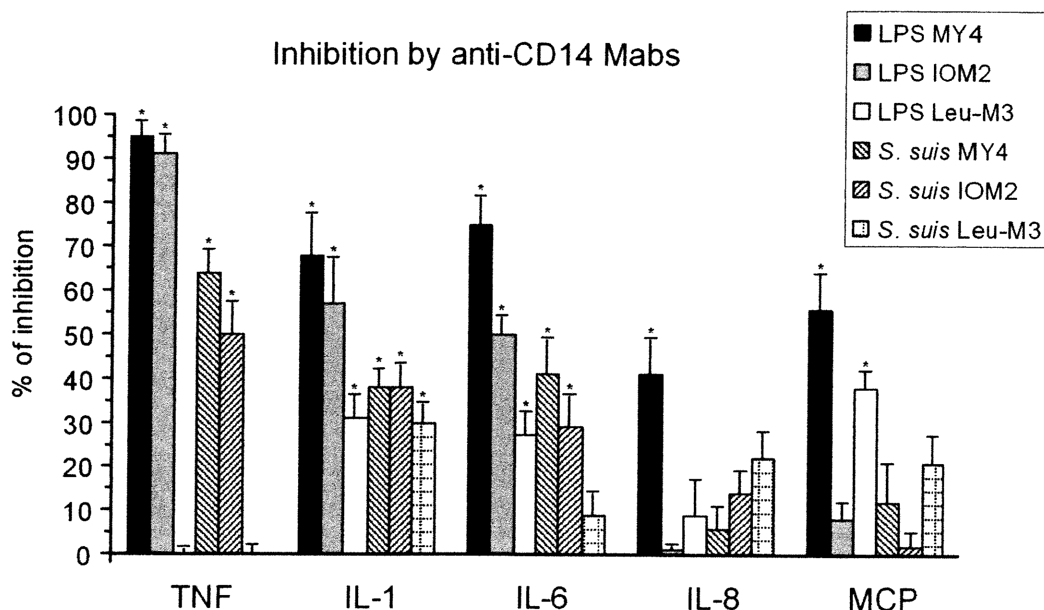


FIG. 4. Effect of CD14 blockade on cytokine production. IFN-γ pre-activated THP-1 cells were pre-incubated for 1 h at 37°C with anti-human CD14 Mab MY4 (10 μg/ml), Mab IOM2 (20 μg/ml), or Mab Leu-M3 (5 μg/ml), before adding the bacterial stimulus (heat-killed 31533 strain at 10^9 CFU/ml) or the LPS (1 μg/ml, used as a reference to evaluate the effect of anti-CD14 Mabs). TNF-α, at 3 h of incubation, and IL-1, IL-6, IL-8 and MCP-1 at 24 h of incubation, were measured by ELISA titration of stimulated cell supernatants. Data were transformed to percentage of inhibition respect to values for bacteria or LPS without treatment (100% of production), and expressed as means ± standard deviations from four separate blockade experiments. * $P < 0{\cdot}01$ (versus the corresponding stimulus without treatment).

MY4 effectively blocked LPS-induced TNF, IL-1 and IL-6 ($P < 0{\cdot}001$), and LPS-induced chemokines ($P < 0{\cdot}01$), with both maximal (Fig. 4) or sub-maximal LPS doses (not shown). IOM2 significantly blocked LPS-induced TNF, IL-1 and IL-6 production ($P < 0{\cdot}001$), but had no blocking effect on chemokine induction by a maximal LPS dose (Fig. 4). However, increased blocking effect of this Mab on IL-8 (45

±11 % inhibition) and MCP (74 ±8 % inhibition) induction by a sub-maximal LPS dose was obtained ($P < 0{\cdot}01$). When the effect of these two Mabs was evaluated on *S. suis*-induced cytokines, a significant inhibition of TNF, IL-1 and IL-6 ($P < 0{\cdot}005$) production was observed after pretreatment of cells with MY4 Mab. IOM2 also partially inhibited *S. suis*-induced TNF, IL-1 and IL-6 production ($P < 0{\cdot}01$) (Fig. 4). Similar inhibitory patterns for TNF, IL-1 and IL-6 production were observed with both Mabs after stimulation of cells with sub-maximal bacterial doses (data not shown). In contrast to that observed for LPS, neither MY4 nor IOM2 showed any effect on chemokine production by cells stimulated with *S. suis* at 10^9 CFU/ml (Fig. 4). Only MY4 was able to inhibit, to some extent, MCP-1 production by decreasing bacterial concentrations (data not shown). Maximal inhibition (38 ±8 %) was observed at a bacterial dose of 5 x 10^7 CFU/ml ($P < 0{\cdot}05$). The level of IL-8 production was not affected by any of the Mabs at any of the bacterial concentrations tested ($P > 0{\cdot}1$, data not shown).

Fig. 4 also shows results obtained with the third Mab, Leu-M3. A completely different pattern of inhibition was observed with this Mab compared to those described above. Leu-M3 partially inhibited the LPS-induced IL-1, IL-6 and MCP-1 ($P < 0{\cdot}01$), whereas it did not affect TNF or IL-8 induction. On the other hand, Leu-M3 only partially inhibited the induction of IL-1 production by *S. suis* ($P = 0{\cdot}002$).

Comparison between *S. suis* type 2 strains of porcine and human origin

Several strains of porcine or human origin (Table 1) were compared for their capacity to stimulate cytokine induction in IFN-γ pre-activated THP-1. Although levels of cytokines induced by strains of porcine origin were, in general, more homogeneous than those induced by strains of human origin, Tukey-Kramer post-hoc tests revealed significant differences between strains within each group ($P < 0{\cdot}0001$). In fact, no consistent effect on cytokine production could be attributed to the origin of the strain (Fig. 5). For example, no significant differences were observed in terms of production of IL-6 ($P = 0{\cdot}9$) or MCP-1 ($P = 0{\cdot}8$) induced by strains of different origins (Fig. 5c and Fig. 5e). While strains of human origin induced significantly higher levels of IL-1 and IL-8 production ($P < 0{\cdot}0001$) (Fig. 5b and Fig. 5d), the opposite was observed for TNF production ($P < 0{\cdot}0001$) (Fig. 5a).

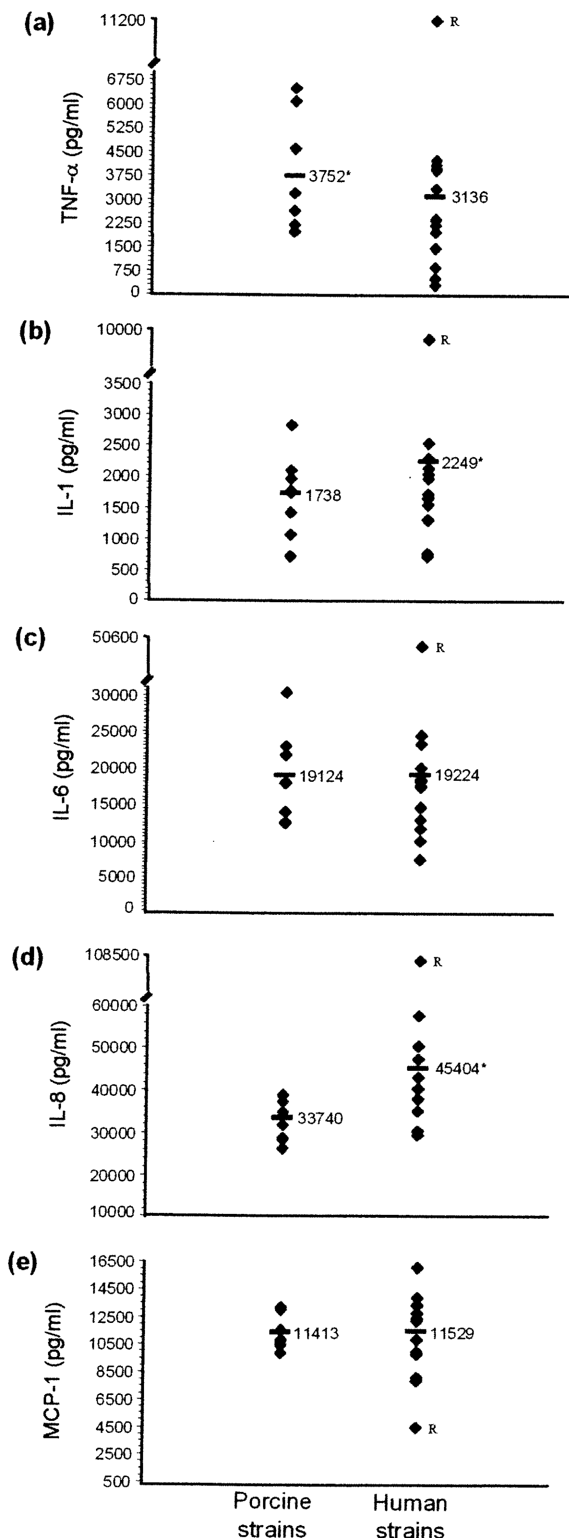


FIG. 5. Comparative study of cytokine production by different *S. suis* strains. IFN-γ pre-activated THP-1 were stimulated by heat-killed (10^9 CFU) *S. suis* strains from human or porcine origin (Listed in Table 1). TNF-α (a), at 3 h of incubation, and IL-1 (b), IL-6 (c), IL-8 (d) and MCP-1 (e) at 48 h of incubation, were measured by ELISA titration. Points represent the mean production for each individual strain (in pg/ml). Lines represent the average cytokine production of each group of strains. * $P < 0{\cdot}0001$ as calculated by the Anova test. R, indicates the *S. suis* human strain Reims [28].

Interestingly, the strain Reims, isolated from a human case of spondylodiscitis [28], induced a much higher level of TNF, IL-1, IL-6 and IL-8 production compared to all other strains ($P < 0{\cdot}0001$), but induced the lowest level of MCP-1 production ($P < 0{\cdot}001$) (Fig. 5). It must also be noted that, in general, no association was observed between the virulence of the strains and the level of cytokines produced. These results suggest individual differences in the capacity of different strains of *S. suis* type 2 to induce cytokines.

DISCUSSION

The present work demonstrates for the first time that *S. suis* serotype 2 is able to interact with monocytes of human origin inducing the release of large amounts of the pro-inflammatory cytokines TNF, IL-1, IL-6, IL-8 and MCP-1. The release of these cytokines was time- and dose- dependent. TNF is the first cytokine detected, but its levels drop much faster than those of other cytokines, that remain at high levels for at least 48 h. These findings are consistent with the kinetics of TNF and IL-6 induction previously reported in murine macrophages exposed to *S. suis* [18] and to the reported kinetics of TNF, IL-1 and IL-6 production in THP-1 monocytes stimulated with heat-killed pneumococci [12]. Cauwels *et al.* [12] found that relatively high concentrations of LPS and intact pneumococci are required to induce substantial amounts of cytokines from THP-1 cells. The authors suggest that this may be attributed to the relative immaturity of THP-1 cells, including low levels of CD14 expression. Conflicting reports do exist in the literature regarding the capacity of uninduced THP-1 to release cytokines following stimulation with LPS or bacteria, and that pre-treatment of cells with vitamin D3 or IFN-γ is, in some cases, required [9, 29]. In the present work, substantial levels of different pro-inflammatory cytokines were detected in supernatants of uninduced THP-1 cells, although IFN-γ pre-treatment of THP-1 monocytes highly increases the cytokine responses. In fact, in non-activated cells, *S. suis* induces higher cytokine levels than LPS, whereas levels are similar for both stimuli after IFN-γ pre-activation of cells. The effect of IFN-γ treatment on the expression of surface antigens (such as CD14, CD11 and CD18) and on functional activities (such as cell differentiation) may have important biological significance [30]. At the site of an immunoinflammatory response, the concentration of IFN-γ may reach very high levels. This IFN-γ may then act on infiltrating monocytes or maturing macrophages to maintain or to induce cytokine secretion in response to appropriate stimuli, such as bacterial antigens [19-21].

Important levels of IL-6, TNF and IL-1 were observed after *S. suis* stimulation of THP-1 cells; however, the release of these cytokines gradually decrease proportionately to bacterial concentrations, particularly in terms of TNF levels. This is in agreement with the reported large doses of heat-killed *S. suis* required for maximal TNF

and IL-6 release by J774A1 macrophages [18]. TNF and IL-1 may act as initiators of meningeal inflammation by modulation of the brain blood barrier [17]. *In vivo* and *in vitro* evidence support the concept that these cytokines play a positive role in local inflammatory reactions by amplifying leukocyte recruitment and by increasing the local production of chemokines by endothelial cells [17, 31]. A role for pro-inflammatory cytokines in the initiation and amplification of meningeal inflammation has been suggested in *S. suis* infection [4]. In addition, these are the key mediators of toxic shock syndrome [14, 32], and, in this regard, recurrent septic shock due to *S. suis* has already been reported [33].

S. suis also induces extremely high and sustained levels of the two chemokines, IL-8 and MCP-1, even at bacterial doses lower than 10^6 CFU/ml. Remarkably, IL-8 levels did not drop to zero even at bacterial concentrations of $5x10^4$ CFU/ml. *S. suis* experimental infection of specific pathogen-free pigs resulted in elevated bacteremia, with ~10^5 CFU/ml recuperated from blood (unpublished observations). It has also been reported that meningeal inflammation is initiated when more that 10^5 pneumococci/ml are present in the CSF [34]. Thus, the sustained levels of chemokines induced at these bacterial concentrations are relevant since chemokines are implicated in leukocyte trafficking across the vascular wall, and high levels of these chemokines are found in patients with bacterial meningitis [16]. Furthermore, IL-8 levels correlate with the number of neutrophils migrating into the CSF [17]. Neutrophils are the first leukocytes to appear at the onset of infection, but the cellular profile gradually changes to a predominantly mononuclear infiltrate [17]. The delayed and continued recruitment of monocytes mediated by chemotactic factors such as MCP-1derived from inflammatory cells, may also contribute to cellular damage [35]. In cases of bacterial meningitis caused by *N. meningitidis*, *S. pneumoniae* or *H. influenzae*, mononuclear cells are not prominent but may increase during the course of the disease [16]. Histopathologic findings from diagnosed pigs with *S. suis*-infection revealed meningeal infiltrates of a mixture of inflammatory cells. In some pigs the infiltrate was primarily neutrophilic while, in others, the infiltrates contained mostly mononuclear cells [36]. Several reports of human cases of *S. suis* meningitis indicated turbid CSF with high levels of leukocytes, and variable percentages of neutrophils and monocytes [2, 3, 37]. Unfortunately, case

reports are limited and data at later stages of the disease are not available, mainly due to the positive outcome of the patients after treatment.

It must be noted that heat-killed or viable *S. suis* induces similar levels of cytokine release in THP-1 cells. In the case of other bacterial species, such as *Listeria monocytogenes*, viable organisms are required to induce cytokine production [38], whereas this is not essential for other bacteria. For example, it has been reported that both living and heat-killed GBS induce TNF in murine macrophages [39] and that both viable and heat-killed *Ehrlichia chaffeensis* induce similar levels of IL-1β and IL-8 by human monocytes [40].

In vivo, it appears that a certain hierarchy exists wherein TNF induces IL-1; and IL-6 and a large number of other cytokines are induced by either IL-1 or TNF [14, 15, 19, 32]. In the present work, blocking experiments demonstrated that TNF and IL-1 partially amplify the *S. suis*-induced cytokine cascade. Blockade experiments of cytokine release by human monocytes in response to group B *Streptococcus* (GBS) components revealed a TNF-independent induction of IL-1. However, IL-6 levels were significantly decreased by anti-TNF but not by anti-IL-1 antibodies [41]. The interactions between these bacterial-induced pro-inflammatory cytokines seem to be different for *S. suis* and GBS. In fact, important differences in the pathogenesis of the infections caused by these two pathogens have been reported [23, 24, 42].

The similarity between the kinetics of cytokine induction by *S. suis* and LPS, as well as our previous results with J774 macrophages [18], suggests that *S. suis* and LPS may use similar macrophage-signalling pathways. Since pre-activation of THP-1 cells with IFN-γ increases cytokine production by *S. suis* (this study) and CD14 expression (unpublished observations) [29, 30], a role for CD14 in *S. suis* cytokine production may be suggested. Three anti-CD14 Mabs were used to study the interaction between human THP-1 monocytes and *S. suis*, and LPS was used to validate the effect of these Mabs. The inhibitory effect of the three anti-CD14 Mabs on production of the five cytokines under study varied, probably due to the different functional characteristics of the Mabs. In fact, conflicting reports of the ability to block binding of LPS or subsequent activation by several available anti-CD14 Mabs do exist in the literature [26, 43, 44]. It has been reported that Mabs MY4 and IOM2 inhibit both binding of LPS to CD14 (>95%) and

LPS-mediated cell activation via CD14 (90%) [27, 45]. Mab Leu-M3 does not block LPS binding to CD14, and its use was intended as a negative control, as described in previous papers [26, 27].

In the present work, *S. suis*-induced TNF, IL-1 and IL-6 release were significantly inhibited by Mabs MY4 and IOM2, independent of the bacterial concentration used as stimulus. Conversely, a partial inhibitory effect was only observed with MY4 on *S. suis*-induced MCP-1 by decreasing the bacterial concentration. No inhibition of *S. suis*-induced IL-8 release was observed, regardless of the bacterial dose used. Similarly, van Furth *et al.* [46] reported that anti-CD14 inhibition of TNF release by monocytes following stimulation with *H. influenzae* is independent of the bacterial dose used, while inhibition of IL-10 production is inversely proportional to the bacterial concentration.

The region of CD14 that recognises and binds LPS has been recently determined, and it is located in the 152 aa N-terminal region, more precisely between aa 51 and 64 [26]. Since, Mab MY4 and IOM2 epitopes partially overlap and are closely related to this region, the inhibitory effect of these two Mabs can be due to impairment of binding of LPS to CD14, as previously reported [26, 27, 45]. The region of CD14 that interacts with intact *S. suis* is not known, but results of the present work suggest similarities with the LPS-binding site. The inhibitory effect of the these anti-CD14 Mabs on *S. suis* cytokine induction could thus be explained by both impaired binding and/or reduced activation of monocytes during interaction of bacteria with CD14.

On the other hand, we confirmed that Mab Leu-M3 does not block LPS-induced TNF production [47]. However, partial inhibition of IL-1, IL-6 and MCP-1 production induced by LPS, and IL-1 production by *S. suis* stimulated THP-1 cells was observed. Although it is known that Leu-M3 does not block the binding of LPS to CD14 [26, 27], less information is available regarding the inhibition of cell activation by this Mab. In fact, the CD14 epitope that recognises Mab Leu-M3 (aa 135-146) is completely outside the LPS binding region. Thus the inhibitory effect of this Mab can be explained by a reduced activation of monocytes during interaction of stimuli with CD14. Interestingly, an anti-CD14 Mab (18E12) was also reported to inhibit LPS activation of cells (90%) without inhibiting LPS binding to CD14 [45, 46]. Furthermore, Dziarski *et al.* [26]

reported that Mab Leu-M3 inhibits soluble PGN binding to CD14 that might explain the partial inhibition of the IL-1 response in cells stimulated by *S. suis* when this Mab was present.

It has been shown previously that the cell wall of *S. suis* is the main component responsible for cytokine induction, and that the presence of the polysaccharide capsule affects IL-6 and TNF production by murine macrophages differently [18]. Binding of *S. suis* to monocytes and stimulation of cytokine production via CD14 may be mediated by PGN and LTA at the surface of the bacteria, as has been reported for other G+ bacteria, such as GBS and *S. pneumoniae* [8, 12]. Thus, it is possible that differences in the bacterial antigens may be responsible for the different cytokines induced and/or the interacting pathways used by *S. suis* to stimulate different cytokines, according to the different inhibitory patterns observed among cytokines and chemokines. Different cytokine-induction patterns have also been reported for GBS, *H. influenzae* and *S. pneumoniae* [8, 46].

Interestingly, total inhibition of cytokine production has not been achieved with anti-CD14 Mabs. This was more evident when inhibition of chemokine production was attempted. The involvement of epitopes on the CD14 receptor not recognised by the three Mabs used in the present study cannot be ruled out. On the other side, a low but still significant production of cytokines is observed in non-activated THP-1 cells, that are known to express low levels of CD14 [12]. These observations support the hypothesis that receptor(s) other than CD14 are used by *S. suis* to stimulate cytokine release by THP-1 monocytes. Since toll-like receptors have been shown to recognise several gram positive bacterial species, and co-expression of CD14 and Toll-like receptor 2 synergistically enhances cell activation [6, 11], a possible implication of toll-like receptors, either in concert with CD14 or separately, in *S. suis* cell activation remains to be elucidated.

When several *S. suis* type 2 strains from human- or porcine-origin were compared, a large variability in their capacity to stimulate cytokine induction in THP-1 cells was observed. A clear tendency for human strains to induce higher cytokine release by human THP-1 monocytes could not be demonstrated. Although the differences in IL-8 and IL-1 levels between the two groups of strains were statistically significant, the

results need to be interpreted with caution, due to the differences in cytokine induction reported for different strains. Despite the fact that pigs are of epidemiological importance to human infection [48], some cases of *S. suis* infection in persons not related to the porcine industry have also been reported [37]. Thus, the clinical relevance of potential species-specific differences in reactivity to bacterial strains still remains unclear.

The concept of "virulence" for *S. suis* has been debated in the literature [4, 49]. In this study, strains are considered as "virulent" or "non virulent" based on the development or not of clinical disease after experimental infection of piglets, respectively (Table 1). In the present work, no association could be observed between cytokine response and virulence of the strain. Interestingly, there are no observed differences between virulent and non virulent strains in adhesion to different types of host cells (unpublished observations). Unlike other important streptococcal species, information on *S. suis* virulence factors as well as molecules expressed at the surface is limited [4]. Thus, differences in otherwise unidentified surface structures may explain the observed differences between strains. Giguère and Prescott [50] have reported that virulent and non virulent *Rodococcus equi* strains induced similar levels of cytokine production in murine macrophages. They concluded that virulence is correlated to the ability to survive in macrophages, but it did not affect early cytokine release by macrophages. In this respect, using an experimental infection model, it was repeatedly observed that virulent *S. suis* strains are able to survive in circulation at high concentrations for more than 6 days, whereas non virulent strains disappear from the circulation only 48 h after infection (unpublished observations). Wilson *et al.* [51] proposed that the capacity of bacteria or bacterial components to induce overproduction of cytokine in host cells can be viewed as a factor contributing to bacterial virulence.

In conclusion, *S. suis* may induce cytokine production by THP-1 cells in both CD14-dependent and -independent manner, with TNF and IL-1 acting in amplification pathways. To the best of our knowledge, this is the first report of specific inflammatory cell receptor-interactions implicated in the recognition of *S. suis*, a poorly studied pathogen. Additional studies on the signalling mechanisms involved in *S. suis* cytokine induction are currently in progress. A complete understanding of the interacting

pathways would provide important insight into disease progression and could contribute to the development of potential therapies for *S. suis* meningitis. Although effective antibiotics are available and the risk of mortality due to *S. suis* infection is low, high rates of neurological sequelae such as permanent deafness occur in patients who survive the initial infection.

ACKNOWLEDGMENTS

We gratefully acknowledge S. Lacouture, J-M. Trudel, and G. Vanier for technical assistance, and J. Moore for having edited the manuscript. We also wish to thank Dr. M. Jacques and Dr. M. Doré for critical reviews of the manuscript. We are also indebted to Dr. M. Kobisch (Centre National d'Études Vétérinaires et Alimentaires, Ploufragan, France), Dr. U. Vecht (DLO-Institute for Animal Science and Health, Lelystad, The Netherlands), Dr. T. Alexander (University of Cambridge, England), Dr. L. Brasme (Centre Hospitalier Universitaire de Reims, France), Dr. G. Grise (Hôpital des Feugrois, Elbeuf, France), Dr. B. Cattier (Centre Hospitalier Universitaire Bretonneau, Tours, France), Dr. B. François (Dupuytren Hospital, Limoges Cedex, France), and Dr. P. Norton (Institute for Animal Health, Compton, United Kingdom) for providing some of the *Streptococcus suis* type 2 strains.

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) grant # 0680154280, by the Fonds pour la Formation des Chercheurs et l'Aide à la Recherche du Québec (FCAR-équipe) grant # 99-ER-0214, and by the Canadian Research Network on Bacterial Pathogens of Swine.

REFERENCES

1. **Higgins R, Gottschalk M.** 1999. Streptococcal diseases. In: Straw BE, D'Allaire S, Mengeling WL, Taylor DJ, eds. Diseases of swine. Ames: Iowa State University, pp 563-70.
2. **Arends JP, Zanen HC.** Meningitis caused by *Streptococcus suis* in humans. Rev Infect Dis 1988; **10**:131-7.
3. **Chau PY, Huang CY, Kay R.** *Streptococcus suis* meningitis. An important underdiagnosed disease in Hong Kong. Med J Aust 1983; **1**:414-7.
4. **Gottschalk M, Segura M.** The pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions. Vet Microbiol 2000; **75**:59-71.
5. **Chanter N, Jones PW, Alexander TJ.** Meningitis in pigs caused by *Streptococcus suis*- a speculative review. Vet Microbiol 1993; **36**:39-55.
6. **Yoshimura A, Lien E, Ingalls RR, Tuomanen EI, Dziarski R, Golenbock DT.** Cutting Edge: Recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. J Immunol 1999; **163**:1-5.
7. **Verhoef J, Mattsson E.** The role of cytokines in Gram-positive bacterial shock. Trends Microbiol 1995; **3**:136-40.
8. **Cuzzola M, Mancuso G, Beninati C *et al.*** Human monocyte receptors involved in tumor necrosis factor responses to group B Streptococcal products. Infect Immun 2000; **68**:994-8.
9. **Pugin J, Heumann D, Tomasz A *et al.*** CD14 is a pattern recognition receptor. Immunity 1994; **1**:509-16.
10. **Wright SM.** CD14 and innate recognition of bacteria. J Immunol 1995; **155**:6-8.
11. **Lien E, Sellati TJ, Yoshimura A *et al.*** Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. J Biol Chem 1999; **274**:33419-25.
12. **Cauwels A, Wan E, Leismann M, Tuomanen E.** Coexistence of CD14-dependent and independent pathways for stimulation of human monocytes by gram-positive bacteria. Infect Immun 1997; **65**:3255-60.
13. **Heumann D, Barras C, Severin A, Glauser MP, Tomasz A.** Gram-positive cell walls stimulate synthesis of tumor necrosis factor alpha and interleukin-6 by human monocytes. Infect Immun 1994; **62**:2715-21.
14. **Whicher JT, Evans SW.** Cytokines in disease. Clin Chem 1990; **36**:1269-81.
15. **Baggiolini M, Dewald B, Moser B.** Interleukin-8 and related chemotactic cytokines - CXC and CC chemokines. Adv Immunol 1994; **55**:97-179.
16. **Lahrtz F, Piali L, Spanaus K, Seebach J, Fontana A.** Chemokines and chemotaxis of leukocytes in infectious meningitis. J Neuroimmunol 1998; **85**:33-43.
17. **Sprenger H, Rösler A, Tonn P, Braune HJ, Huffmann G, Gemsa D.** Chemokines in the cerebrospinal fluid of patients with meningitis. Clin Immunol Immunopathol 1996; **80**:155-61.
18. **Segura M, Stankova J, Gottschalk M.** Heat-killed *Streptococcus suis* capsular type 2 strains stimulate tumor necrosis factor alpha and interleukin-6 production by murine macrophages. Infect Immun 1999; **67**:4646-54.

19. **Curfs JHAJ, Meis JFGM, Hoogkamp-Korstanje JAA.** A primer on cytokines: sources, receptors, effects, and inducers. Clin Microbiol Rev 1997; **10**:742-80.
20. **Haq AU, Rinehart JJ, Maca RD.** The effect of gamma interferon on IL-1 secretion of in vitro differentiated human macrophages. J Leuk Biol 1985; **38**:735-46.
21. **Kubin M, Chow JM, Trinchieri G.** Differential regulation of interleukin-12 (IL-12), tumor necrosis factor α, and IL-1β production in human myeloid leukemia cell lines and peripheral blood mononuclear cells. Blood 1994; **83**:1847-55.
22. **Kobisch M, Gottschalk M, Morvan P, Cariolet R, Bénévent G, Joly JP.** Experimental infection of SPF piglets with *Streptococcus suis* serotype 2. Journées Rech Porcine en France 1995; **27**:97-102.
23. **Charland N, Nizet V, Rubens C, Kim KS, Lacouture S, Gottschalk M.** *Streptococcus suis* serotype 2 interactions with human brain microvascular endothelial cells. Infect Immun 2000; **68**:637-43.
24. **Lalonde M, Segura M, Lacouture S, Gottschalk M.** Interactions between *Streptococcus suis* serotype 2 and different epithelial cell lines. Microbiology 2000; **146**:1913-21.
25. **Martin CA, Dorf ME.** Differential regulation of interleukin-6, macrophage inflammatory protein-1, and JE/MCP-1 cytokine expression in macrophage cell lines. Cell Immunol 1991; **135**:245-58.
26. **Dziarski R, Tapping RI, Tobias PS.** Binding of bacterial peptidoglycan to CD14. J Biol Chem 1998; **273**:8680-90.
27. **Couturier C, Haeffner-Cavaillon N, Caroff M, Kazatchkine MD.** Binding sites for endotoxins (lipopolysaccharides) on human monocytes. J Immunol 1991; **147**:1899-904.
28. **Caumont H, Gerard N, Depernet B, Brasme L, Eschard JP, Etienne JC.** *Streptococcus suis* L3-L4 spondylodiscitis in a butcher (letter). Presse Med 1996; **25**:1348.
29. **Raponi G, Ghezzi MC, Mancini C.** The release of tumor necrosis factor alpha (TNF-α) by interferon gamma (IFN-γ) induced THP-1 cells stimulated with smooth lipopolysaccharide is inhibited by Mabs against HLA-DR and CD14 receptors on the effector cell. Microbiologica 1997; **20**:1-6.
30. **Perussia B, Dayton ET, Fanning V, Thiagarajan P, Hoxie J, Trinchieri G.** Immune interferon and leukocyte-conditioned medium induce normal and leukemic myeloid cells to differentiate along the monocytic pathway. J Exp Med 1983; **158**:2058-80.
31. **Romano M, Sironi M, Toniatti C *et al.*** Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. Immunity 1997; **6**:315-25.
32. **Cerami A.** Inflammatory cytokines. Clin Immunol Immunopathol 1992; **62**:S3-S10.
33. **François B, Gissot V, Ploy MC, Vignon P.** Recurrent septic shock due to *Streptococcus suis*. J Clin Microbiol 1998; **36**:2395.
34. **Tuomanen E, Tomasz A, Hengstler B, Zak O.** The relative role of bacterial cell wall and capsule in the induction of inflammation in pneumococcal meningitis. J Infect Dis 1985; **151**:535-40.

35. **Ehrlich LC, Hu S, Sheng WS, Sutton RL, Rockswold GL, Peterson PK, Chao CC.** Cytokine regulation of human microglial cell IL-8 production. J Immunol 1998; **160**:1944-8.
36. **Sanford SE.** Gross and histopathological findings in unusual lesions caused by *Streptococcus suis* in pigs. II. Central nervous system lesions. Can J Vet Res 1987; **51**:486-9.
37. **Leelarasamee A, Nilakul C, Tien-Grim S, Srifuengfung S, Susaengrat W.** *Streptococcus suis* toxic-shock syndrome and meningitis. J Med Assoc Thai 1997; **80**:63-8.
38. **Mitsuyama M, Igarashi K, Kawamura I, Ohmori T, Nomoto K.** Difference in the induction of macrophage interleukin-1 production between viable and killed cells of *Listeria monocytogenes*. Infect Immun 1990; **58**:1254-60.
39. **Goodrum KJ, Dierksheide J, Yoder BJ.** Tumor necrosis factor alpha acts as an autocrine second signal with gamma interferon to induce nitric oxide in group B *streptococcus*-treated macrophages. Infect Immun 1995; **63**:3715-7.
40. **Lee EH, Rikihisa Y.** Absence of tumor necrosis factor alpha, interleukin-6 (IL-6), and granulocyte-macrophage colony-stimulating factor expression but presence of IL-1β, IL-8, and IL-10 expression in human monocytes exposed to viable or killed *Ehrlichia chaffeensis*. Infect Immun 1996; **64**:4211-9.
41. **Von Hunolstein C, Totolian A, Alfarone G, Mancuso G, Cusumano V, Teti G, Orefici G.** Soluble antigens from group B streptococci induce cytokine production in human blood cultures. Infect Immun 1997; **65**:4017-21.
42. **Segura MA, Cléroux P, Gottschalk M.** *Streptococcus suis* and group B *Streptococcus* differ in their interactions with murine macrophages. FEMS Immunol Med Microbiol 1998; **21**:189-95.
43. **Dentener MA, Bazil V, Von Asmuth EJU, Ceska M, Buurman WA.** Involvement of CD14 in lipopolysaccharide-induced tumor necrosis factor-α, IL-6 and IL-8 release by human monocytes and alveolar macrophages. J Immunol 1993; **150**:2885-91.
44. **Wright SM, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC.** CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 1990; **249**:1431-3.
45. **Viriyakosol S, Kirkland TN.** A region of human CD14 required for lipopolysaccharide binding. J Biol Chem 1995; **270**:361-8.
46. **Van Furth AM, Verhard-Seijmonsbergen EM, Langermans JAM, Van Dissel JT, Van Furth R.** Anti-CD14 monoclonal antibodies inhibit the production of tumor necrosis factor alpha and interleukin-10 by human monocytes stimulated with killed and live *Haemophilus influenzae* or *Streptococcus pneumoniae* organisms. Infect Immun 1999; **67**:3714-8.
47. **Landmann R, Knopf H, Link S, Sansano S, Schumann R, Zimmerli W.** Human monocyte CD14 is upregulated by lipopolysaccharide. Infect Immun 1996; **64**:1762-9.
48. **Chatellier S, Gottschalk M, Higgins R, Brousseau R, Harel J.** Relatedness of *Streptococcus suis* serotype 2 isolates from different geographic origins as evaluated by molecular fingerprinting and phenotyping. J Clin Microbiol 1999; **37**:362-6.

49. **Gottschalk M, Higgins R, Quessy S.** Dilemma of the virulence of *Streptococcus suis* strains. J Clin Microbiol 1999; **37**:4202-3.
50. **Giguère S, Prescott JF.** Cytokine induction in murine macrophages infected with virulent and avirulent *Rhodococcus equi*. Infect Immun 1998; **66**:1848-54.
51. **Wilson M, Seymour R, Henderson B.** Bacterial perturbation of cytokine networks. Infect Immun 1998; **66**:2401-9.
52. **Quessy S, Dubreuil JD, Caya M, Higgins R.** Discrimination of virulent and avirulent *Streptococcus suis* capsular type 2 isolates from different geographical origins. Infect Immun 1995; **63**:1975-9.
53. **Vecht U, Arends JP, van der Molen EJ, van Leengoed LA.** Differences in virulence between two strains of *Streptococcus suis* type II after experimentally induced infection of newborn germ-free pigs. Am J Vet Res 1989; **50**:1037-43.

IV. DISCUSSION

1. The controversy about *Streptococcus suis* phagocytosis

Surface encapsulation is one of the most important virulence factors of several pathogenic microorganisms. Bacterial capsule, by effectively inhibiting phagocytosis and resisting complement-mediated bactericidal activity, may enhance bloodstream survival of the organism and facilitate intravascular replication. Indeed, the most common meningeal pathogens are all encapsulated (96). However, the mechanisms of interaction of encapsulated bacteria with the host immune system are not at all known, and they are not generalized for all pathogens (52). In the immune-host, the presence of specific antibodies contributes to eliminate the encapsulated pathogen by antibody-mediated phagocytosis and killing. This is the case for well encapsulated pathogens, such as GBS, pneumococcus (9, 15), and also *S. suis*. Opsonizing activity has been shown for type-specific antibodies in a whole blood bactericidal test (35). In addition, a purified monoclonal antibody against the type 2 CPS was shown to significantly increase the rate of phagocytosis of *S. suis* by porcine monocytes and to activate the clearance of bacteria from the circulation in experimentally infected mice (20).

The situation may be different in the non-immune host. Under this condition, encapsulated organisms interact with the host defense systems by different mechanisms (52). For example, the sialylated polysaccharidic capsule of GBS plays a well recognized role in modulating the alternative complement pathway and protecting the organism from opsonophagocytic killing by complement and PMN in the absence of type-specific antibody (115).

The *S. suis* type 2 capsule is composed of five sugars, the third most important being sialic acid. However, it does not seem to be critical in virulence of *S. suis* serotype 2, since several field strains, differing in their virulence, showed the same low sialic acid concentration compared to GBS strains; and blocking or enzymatic removal of this sugar did not influence phagocytosis rates

by porcine monocytes or virulence in mice. Thus, these observations and previous data suggest that complement seems not to play an important role in phagocytosis of *S. suis* (14, 22, 116), and may explain why sialic acid has a pathogenic role in GBS whereas to date, no conclusive role could be drawn for sialic in *S. suis* infections. In fact, the pathogenesis of *S. suis* infections seems to be different from that reported for other streptococci also responsible for meningitis, such as GBS and pneumococci (96, 99). Furthermore, the interactions between *S. suis* and the phagocytic cells, the major effector cells of the immunoinflammatory system, are poorly characterized.

In our study we compared quantitatively the *in vitro* interactions, uptake and intracellular survival, of *S. suis* capsular type 2 and GBS capsular type III with murine phagocytic cells under non-opsonic conditions. The role of their capsules in these interactions was evaluated by using unencapsulated isogenic mutants of both pathogens (19, 78). Viable counting techniques for monitoring phagocytic interactions have already been used for GBS (102). The present work is the first determination of phagocytosis and time course of intracellular survival of *S. suis* by quantitative platings (Article I).

Results in this study show that well encapsulated GBS are ingested at high numbers by murine macrophages in the absence of complement and antibodies. Our results are similar to those described by Valentin-Weigand et al. (102), who also showed that GBS (the same strain as used in our study) is able to enter and persist efficiently in J774A1 macrophages by evading intracellular antibacterial activities. On the other hand, no differences were observed between this strain and its unencapsulated mutant, which indicate that the capsule is not necessarily an antiphagocytic factor for this bacterial species. Controversially, previous reports have assumed an anti-phagocytic role of GBS type III capsule since, as mentioned above, encapsulated bacteria were resistant to opsono-phagocytic killing by PMN in absence of specific antibodies, whereas unencapsulated or asialo mutants were susceptible (62, 78, 114). However, in these studies, the techniques used allowed the study of bacterial

killing rather than phagocytosis, since no differentiation of intracellular and extracellular bacteria was made.

Different results were obtained for *S. suis*. The well encapsulated parental strain of *S. suis* type 2 was almost not phagocytosed by murine macrophages, even after 90 min of bacteria-cell contact. Interestingly, previous *in vitro* studies of this bacterial species with phagocytes are contradictory. Early bacterial killing studies could not demonstrate any "phagocytic activity" with *S. suis* type 2, using either human or pig whole blood (1, 26). Furthermore, Upton (100) was not able to observe phagocytosis of encapsulated *S suis* type 2 by purified monocytes. However, more recent studies showed variable percentages of phagocytosis (from 7 to 30%) by porcine monocytes or murine macrophages, using a vital staining technique (14, 19, 22, 118). These discrepancies may be due to technical differences. In fact, results of the present study represent the first data from quantitative phagocytosis using a viable counting technique. Unlike the GBS, the high percentage of phagocytosis obtained with the unencapsulated mutant of *S. suis* with respect to the parent strain, demonstrates the antiphagocytic role of the capsule. Similar results were obtained when porcine monocytes were used (data not shown). Our present study (Article I) confirms and extends prior *in vitro* findings that unencapsulated isogenic mutants were more phagocytosed than the capsulated parental strain, not only with murine macrophages but also with porcine monocytes (19). Recently, Smith et al. (90) characterized the *cps* locus of *S. suis* type 2 capsule, and produced non-encapsulated isogenic mutants by insertional mutagenesis. These mutants strains were also highly sensitive to ingestion by porcine alveolar lung macrophages. Thus, the use of genetic tools lead to the production of unencapsulated isogenic mutants, and the results obtained with these mutants confirmed definitively the very early observations that well encapsulated bacteria were able to resist phagocytosis, whereas unencapsulated "variants" failed to do so (1, 14, 26, 79, 116).

At this point, it is difficult to explain how this low rate of phagocytosis with the encapsulated strains could be the pillar of the "Trojan horse theory". Williams

(118, 120) suggested that phagocytosed non-pathogenic (encapsulated) isolates were killed, whereas intracellular pathogenic organisms survived and replicated within phagosomes in the absence of anti-*S.suis* antibodies. Examination of meningeal exudate present in pigs with *S. suis* type 2 meningitis have revealed that both PMN and macrophages are capable of ingesting *S. suis* microorganisms *in vivo*, in pigs lacking specific antibodies (121). However, the outcome of the interaction differs. Multiple organisms, many showing cross-wall formation, are found within macrophages but not within PMN, indicating possible intracellular survival and replication of pathogenic bacteria within macrophages (121). Mononuclear phagocytes were thus implicated as playing a central role in the pathogenesis of bacterial meningitis (119) .

Taking into account this controversy about phagocytosis, we decided to further evaluated the role of the capsule in the intracellular bacterial survival within murine macrophages. In contrast to the observations of Williams (118), we found that the few bacteria of the *S. suis* encapsulated virulent strain recovered after 30 min of phagocytosis did not survive inside macrophages. In fact, after 60 min of re-incubation, no residual bacteria could be recovered (data not shown). In addition, the survival of the unencapsulated mutant was also markedly reduced by more than 95% after 4 h of cell infection. Charland et al. (19) demonstrated in murine and pig models that the unencapsulated mutant was more susceptible to early phagocytic clearance compared with the wild type strain, providing further evidence that the capsule is an antiphagocytic factor and plays an important role in virulence.

The antibiotic-protection assay (102) used in our study allows the elimination of all remaining extracellular bacteria and thus a more accurate evaluation of intracellular live bacteria after cell lysis and plating of intracellularly-recovered bacteria, which are protected from the antibiotic action, since antibiotics do not enter the cells. In Williams studies (118), extracellular remaining bacteria were not eliminated, and thus further phagocytosis of these organisms during the re-incubation period could account for the increased number of intracellular bacteria. Indeed, by transmission electron microscospy

(TEM) we rarely observed intracellular bacteria, and in these rare cases, bacteria in phagosomes were undergoing lytic degradation as shown in Fig. 10. This may explain why no viable bacteria were recovered in the antibiotic-protection assay.

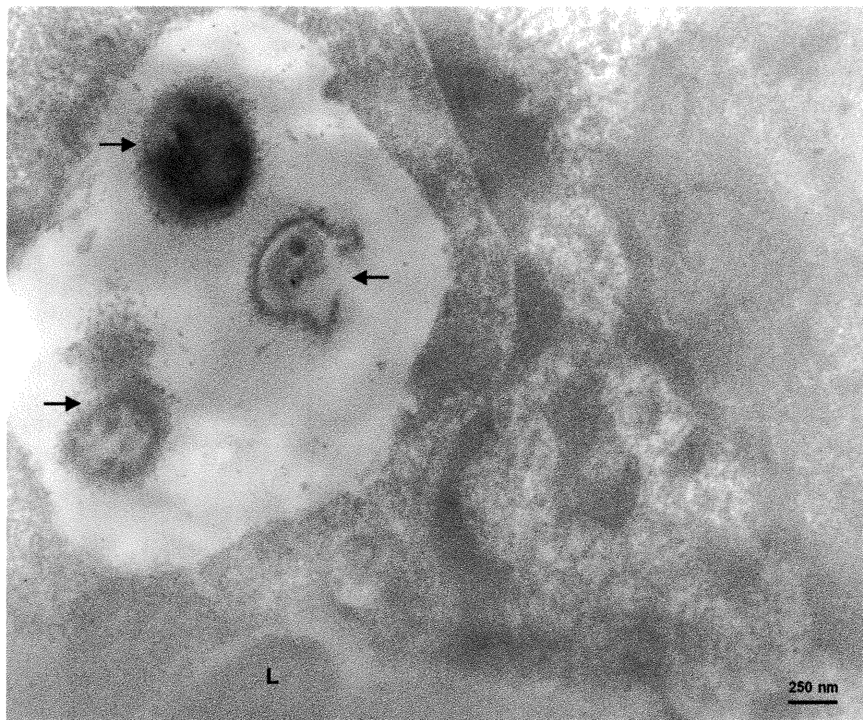


Fig. 10: Transmission electron micrograph showing *S. suis* inside the phagosome of infected J774A1 macrophages. Arrows indicate site of bacterial cell wall disruption. L, lysosomal vesicules.

The accuracy of our methodology was further demonstrated by studying the intracellular survival of GBS strains. In contrast to *S. suis*, both encapsulated and unencapsulated GBS strains were able not only to enter, but also to survive efficiently inside of macrophages. To the best of our knowledge, this is the first report which demonstrates the entry and intracellular survival of an unencapsulated isogenic mutant of GBS capsular type III in murine macrophages, under non-opsonic conditions. Similar results were later reported by Cornacchione et al. (27). It must be noted that following complement-opsonization, however, the invasion and survival rates of encapsulated GBS

does not change, whereas the unencapsulated mutant will not survive (78, 102). These observations seem to indicate that GBS capsule plays a role in the resistance to opsono-intracellular killing, but it is not an antiphagocytic factor.

Thus, results from our study (Article I) suggest that interactions between GBS type III and *S. suis* type 2 with macrophages as well as the role of the capsule as an antiphagocytic factor are different for both bacterial pathogens. A possible role of macrophages as an intracellular niche was proposed for GBS (102). In contrast, in the case of *S. suis*, our present results and previous data demonstrate that the type 2 CPS is antiphagocytic and, therefore, an intracellular phase of *S. suis* in macrophages seems not to be possible. *In vivo* experiments are needed to confirm this hypothesis.

In our phagocytosis studies (Article I), we compared two macrophage cell lines, P388D1 (55) and J774A1 (75), and mouse peritoneal exudate macrophages (PEM). Continuous cell lines with macrophage-like properties provide a convenient model for *in vitro* studies of macrophage function, since the cell lines are homogeneous and can be obtained in large numbers. P388D1 and J774A1 cells were originated from cultured murine lymphoblastoid cells from BALB/c and DBA/2 mice respectively. These two cell lines have vastly differing biologic and biochemical properties when compared to each other or to stimulated or normal peritoneal macrophages. It was described that the P388D1 cell line is deficient in phagocytic activity when compared to the J774A1 cell line (92). This observation was confirmed in this work, since the phagocytosis rate of streptococci was always lower for P388D1 cells with respect to J774A1 cells or PEM. However, in general, limited or no significant difference was seen between the two latter cells (Article I). This was in accordance with other works that established that J774A1 cells have features similar to those of normal macrophages. Furthermore, this cell line has frequently been used in GBS-macrophages interaction studies (102).

The mouse macrophage cell line, J774A1, was thus utilized to further characterize the interactions between *S. suis* and macrophages. Interestingly, it

has been reported that the number of monocytes containing bacteria in preparations from bacteremic pigs was low, less than 2% showing intracellular bacterial profiles. However, it was noted that extracellular organisms were also present (120). Since the surface adhesion of *S. suis* to these cells has never been addressed, we evaluated the capacity of *S. suis* type 2 to adhere to murine macrophages under non-opsonic conditions. An enzyme-linked immunosorbent assay technique was standardized to simply and accurately measure the rate of bacterial attachment to phagocytic cells (7, 89).

In our adhesion assays we demonstrated that *S. suis* is able to interact with macrophages and remains extracellular and largely bound to cells. Cytochalasin pre-treatment of cells has not effect in adhesion, and thus confirm that *S. suis* is not phagocytosed (19, 85, 90). In addition, inhibition of the microfilament formation by cytochalasin does not seem to affect *S. suis* adhesion. The cytochalasins have extensively been used for inhibition of phagocytosis of many bacteria and have no detectable effect on the attachment of bacteria or on cell activation, such as cytokine induction (71, 83, 123).

The high rates of *S. suis* adhesion to the surface of macrophages may explain some of the contradictory reports about bacterial phagocytosis. Busque et al. (16) reported, by flow cytometry, phagocytic rates ranging from 75% to 95% by both mononuclear and PMN cells of human or porcine origin. Similar high levels of *S. suis* uptake by PMN were reported in the literature by using fluorescent staining (79, 116). In all of these reports, quenching of extracellular bacteria were not performed. Since *S. suis* adhesion to macrophages is wash-resistant, these phagocytic rates would represent in fact total numbers of associated bacteria.

In order to preliminary characterize *S. suis* components implicated in adhesion to macrophages, bacteria were subjected to several different pre-treatments. *S. suis* adhesion to macrophages was shown to be protease-resistant, but sialidase-sensitive. In addition, competitive binding studies with *N*-acetylneuraminic acid (NeuAc or sialic acid) also reduced levels of adhesion and

further suggest a participation of this bacterial sugar in cell attachment. None of other sugars known to be present in the bacterial surface showed an effect on *S. suis* adhesion. These results indicate a possible role of the capsular sialic acid moiety in attachment to macrophages. Sialic acid has also been implicated in *Listeria monocytogenes* adhesion to murine macrophages (61). Charland et al. (21) reported the agglutination of *S. suis* cells with sialic acid-binding lectins and suggested that sialic acid is the possible terminal sequence on the type 2 capsular component (20). The exact chemical structure and epitope portion of the type 2 antigen containing sialic acid, however, still remains unknown.

Components of gram-positive cell wall, such as LTA and PG, have been shown to be important adhesins which are recognized by various receptors on host cells (28, 44, 112). When purified cell wall of *S. suis* was used in competitive binding studies, no effect on bacterial adhesion to J774A1 cells was observed. Purified individual sub-components of *S. suis* cell wall are not yet available, and poor information about the LTA or PG structure is found in the literature (36, 53). It has been shown that most of *S. suis* teichoic acid is lipid bound, located deep in the cell wall and hardly accessible to extraction (36). Thus, it is probably partially exposed at the bacterial surface or is less accessible for firm binding to macrophage receptors. Even though the purified cell wall material from *S. suis* was shown to stimulate cytokine production by J774A1 cells, the presence of capsule partially masks the TNF response and thus supports this hypothesis (Article III). Controversially, Lalonde et al. (58) reported strong inhibition of *S. suis* adhesion to epithelial cells by the purified cell wall. Thus, interactions of *S. suis* with host cells could vary for different cell types and it could be assumed that on the bacteria, the required structures may be inaccessible to the macrophage but not to the epithelial cell surface. This notion is strengthened by evidence that cell-cell interaction is dependent upon the spatial arrangement and accessibility of the ligands and receptors on the respective cells (68). Similarly, Tamura et al. (94) reported that purified LTA from GBS does not block adhesion of this bacterium to type II alveolar-like epithelial cells (A549), whereas other studies, however, have indicated that LTA is

involved in GBS adherence to neonatal buccal and vaginal origin cells (95). Thus, as for *S. suis*, the role of this cell wall component in adhesion to host cells still remains controversial.

Only partial inhibition of binding was observed under the conditions used in the present study. These findings indicate that a number of recognition processes are involved in binding of non-opsonized bacteria to the phagocyte surface (113). Even though capsular sialic acid plays a role in *S. suis* adhesion to macrophages, it does not seem to be critical for virulence, as discussed above (20-22). Since carbohydrate-specific molecular interactions may depend on extended oligosaccharide structures, in which case free monosaccharides may be poor inhibitors, a role of components of the *S. suis* surface, other than sialic acid, could not be excluded.

The receptors on the macrophage surface implicated in binding of *S. suis* were not addressed in the present work. However, it could be hypothesized that a lectin-like receptor on the macrophage surface could be responsible of sialic acid (or other surface components) recognition. Attachment of bacteria to mammalian host cells is often mediated by sugar-lectin interactions (69). Recently, sialic-acid binding receptors on the macrophages surface have been characterized and named " the siglecs" (sialic acid-binding immunoglobulin superfamily lectins). It has been suggested that recognition of sialylated ligands by these Siglecs could play an important role in the regulation of the innate immune system (29). The role of these receptors in *S. suis* interactions with macrophages is subject of further evaluation.

It has been shown that adherence to host cell surfaces can take place by two different mechanisms: an opsonin-dependent process, in which antibody and/or complement proteins become involved in the complex interaction between bacteria and host cell; and an opsonin-independent process, in which adhesins present on the bacterial cell surface directly recognize host cell receptors. (2, 40, 61, 65).

It was previously demonstrated that complement does not affect phagocytic rates of *S. suis* (14, 22). However, it has been reported in the literature that complement or other serum factors could mediate adhesion, without ingestion of some pathogens, such as *H. influenzae* type b (66, 65). To address this possibility, we evaluated the adhesion rates to macrophages of *S. suis* after 30 min-preopsonization with fetal bovine serum (FBS) or complement from mouse serum (C'MS). Pre-opsonization of *S. suis* with different concentrations of FBS resulted in markedly increased adhesion to J774A1 macrophages. On the other side, when C'MS was used instead of FBS, the increase in adhesion was even higher. This increase was dose-dependent, and was partially reduced by heating at 56°C 30 min to destroy the complement. These results suggest that complement would be, at least in part, responsible of the increased bacterial adhesion to J774A1 cells. However, other unknown serum factors would also be implicated.

Similarly, it has been reported that adhesion of *N. meningitidis* to human macrophages was significantly increased after opsonization with non-immune C5-depleted serum (77), and authors suggested that coating of meningococci with C3b and/or other serum proteins would be responsible for the increased adhesion. In the case of *S. pneumoniae*, interaction of bacteria with human alveolar macrophages in the absence of opsonization results in relatively poor binding, whereas pre-opsonization with complement enhances not only the binding but also the internalization and killing of pneumococci (40). Thus, the fate of *S. suis* after interaction with macrophages under both non-opsonic and complement-opsonic conditions seems to be different from this streptococcal species (22, 40, 85).

In addition to complement, it has been demonstrated that bacteria can utilize proteins of the host extracellular matrix and body fluids, such as fibronectin (FN), to facilitate interactions with mammalian cells (51). The interaction of bacteria with FN is believed to contribute significantly to the virulence of a number of microorganisms, including staphylococci and streptococci (51). FN is found in two major forms: plasma or soluble FN and

cellular FN. Several meningitis-associated bacteria have recently been found to bind plasma FN; these bacteria include *H. influenzae*, *N. meningitidis* and *S. pneumoniae* (51, 110). When *S. suis* was pre-opsonized 30 min at 37°C with different concentrations of soluble FN, a two-times increase in adhesion levels was observed at 500 μg/ml of FN ($P < 0.01$; data nor shown). Higher FN concentrations did not further increase *S. suis* adhesion (data not shown). Hence, with these preliminary results, it could be suggested that FN, in addition to complement, could be responsible to the serum-dependent increased adhesion of *S. suis* to cells. Binding of soluble FN may facilitate not only attachment to host cells, but also bacterial migration to distant regions of the body. In addition, coating of the microorganism with soluble FN may facilitate escape from host immune surveillance. Several bacterial surface components have been found to recognize FN (47, 51). In the case of *S. suis*, the surface components responsible for FN binding are not well known. Recently, Greeff et al. (32) cloned and characterized a FN- and fibrinogen-binding protein of *S. suis* (FBPS). However, the relative contribution of the FBPS, as well as the 60 kDa IgG-binding protein which was suggested to bind FN (10, 86), in their ability to mediate *S. suis* binding to macrophages, remains to be evaluated.

The present study demonstrates for the first time that *S. suis* is largely bound but not ingested by macrophages and thus it may remain extracellular throughout infection. This could have a direct implication in the pathogenesis of *S. suis* meningitis. Surface adherence to phagocytes with impaired uptake, could be a key step for a successful infection, as suggested for *H. influenzae* type b, another important meningeal pathogen (65). The *S. suis* type 2 CPS is clearly an antiphagocytic factor. However, further studies will be needed to characterise the molecule(s) and mechanisms which are responsible for adherence and inhibition of bacterial phagocytosis.

2. Consequences of *S. suis* interaction with phagocytes

2.1. Cytotoxic effects

During the course of our adhesion studies, we observed that under certain conditions, macrophages were subject to detrimental effects of bacteria. Cytotoxicity increased with greater incubation time and bacterial concentrations, correlating with the proposed mode of action of suilysin, namely, a multi-hit activity by accumulation of suilysin molecules at the surface of cells (43). Indeed, by using suilysin-positive or negative strains, as well as purified suilysin we clearly demonstrated that suilysin was the bacterial component responsible for *in vitro* macrophage cytotoxicity. TEM analyses further confirmed our observations. It was thus demonstrated in our present study that *S. suis* not only adheres and resists phagocytosis, but also induces cytotoxic effects to macrophages via suilysin production.

S. suis suilysin has also been shown to be cytotoxic to brain microvascular endothelial cells (BMEC) and epithelial cells (23, 58, 67), and thus it may facilitate bacterial dissemination (42). Recently, a defined allelic-replacement mutant of the *sly* gene, encoding the suilysin, was shown not to be toxic for J774.2 cells, and thus further proves that the suilysin is probably the only cytolysin produced by *S. suis* (3).

Other pore-forming toxins of several bacterial pathogens have been shown to damage leukocytes. Cell death is the most obvious and inevitable consequence of transmembrane pore formation. Death ensues because the cell is rapidly depleted of ATP, either because of efflux of nucleotides through the pores or because the cell is unable to counteract the deleterious effects of ionic disequilibrium and loss of its "milieu intérieur" which is essential for sustaining metabolic processes. For example, it has been indicated that killing of

leukocytes and monocytes by *E. coli* hemolysin cripples the local phagocytic defense system (5, 12, 72).

Whereas suilysin is implicated as an important virulence factor in European *S. suis* type 2 strains, the same does not seem to be the case for North American strains. In fact, unlike European strains, most virulent field strains isolated from diseased pigs or humans in North America do not produce suilysin. Similarly most European strains produce MRP and EF proteins (named virulence markers), whereas variable production of these proteins have been observed with North American strains (41, 93). It has been suggested that the pathogenesis of the infection caused by suilysin-positive strains (European phenotype) and that of the infection caused by suilysin-negative strains (North American phenotype) is different and that different virulence factors are involved in each case (42). Indeed, in the present study, it was shown that strains presenting the North American phenotype adhere in higher numbers than strains presenting the European phenotype, independently of the geographic origin. In fact, it was demonstrated by randomly amplified polymorphic DNA analysis (RAPD) that strain clusters are related to the phenotype defined with suilysin, MRP and EF rather than to the geographic origin of the isolates (24). Thus, results described herein give additional evidence that the pathogenesis of the infection differs between *S. suis* strains. In particular, it is possible that suilysin-positive strains use adherence and cell injury, for dissemination and evasion of the host immune system. This would be in contrast to suilysin-negative strains that may use adherence and macrophage-dependent dissemination, as a part of a complicated multistep process which leads to bacteremia and meningitis in the host (23, 42, 58).

2.2 The activation of the pro-inflammatory cytokine cascade

S. suis induces inflammatory cytokine release by murine macrophages

It is now recognized that several inflammatory and infectious diseases are associated with the overproduction of cytokines such as TNF-α, IL-1, and IL-6. These cytokines are believed to mediate reactions associated with clinical deterioration, multiorgan system failure, and death during septic shock (18, 105). Mononuclear phagocytes in the inflammatory exudate are the major origin of these pro-inflammatory cytokines. Despite the fact that *S. suis* clearly interacts with these cells, the possible induction of pro-inflammatory cytokines as a result of this interaction has never been studied before. Indeed, in the present study (Article III) we demonstrated for the first time that heat-killed *S. suis* stimulates mouse macrophages to release TNF-α and IL-6, confirming recent reports which revealed that several pathogenic gram positive cocci are powerful inducers of inflammatory cytokines (60, 81, 109).

As in our previous studies on phagocytosis (Article I), we compared the mouse macrophage cell lines, J77A1 and P388D1. Both cell lines are frequently used for cytokine induction experiments (33, 39). J774A1 cells showed a greater cytokine response after both *S. suis* or LPS stimulation, the latter being used as a positive control. Similar results were obtained by Martin et al. (63) who compared both cell lines using the same dose of LPS as used in our study. These observations indicate that the J774A1 cell line is a good model for studying *S. suis* interactions with macrophages.

The question arose as to whether the capacity of *S. suis* to induce cytokines by murine macrophages was due to small amounts of contaminating LPS. However, evidence argues against this possibility. Firstly, endotoxin was almost undetectable in our bacterial preparations as determined by the Limulus amebocyte lysate (LAL) test. Secondly, treatment with polymixin B, which is widely used for neutralization of LPS biological activity, abolished the ability of

LPS to induce cytokines but did not change the levels of cytokine induced by *S. suis*. Taken together, these data indicate that the capacity of *S. suis* to induce cytokines is an intrinsic property and is not due to the presence of small amounts of endotoxin (Fig. 11).

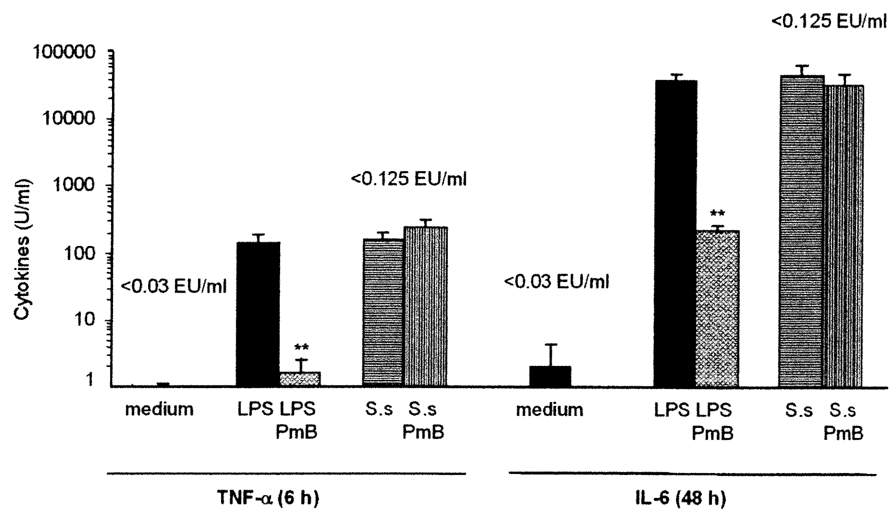


Fig. 11: *Streptococcus suis* cytokine-inducing activity is not related to endotoxin contamination. Polymixin B (PmB, 10 μg/ml) was added during stimulation of J774A1 cells, in parallel to controls without PmB. Cells were stimulated with heat-killed *S. suis* strain 31533 (S.s) at 10^9 CFU/ml. Lipopolysaccharide (LPS, 50 ng/ml), and cell culture medium alone were used as controls. ** $P < 0.001$, respect to values without PmB. Endotoxin levels (in EU/ml), measured by the LAL test, are also shown.

To further evaluate the role of *S. suis* adhesion in the induction of cytokine production by macrophages, we measured cytokine release after *S. suis* infection in presence of cytochalasin. Levels of cytokine production by cytochalasin- treated macrophages were similar to those obtained with control cells after *S. suis* stimulation. As discussed above, cytochalasin treatment has no effect on levels of *S. suis* adhesion. Thus, the release of cytokines would be

a consequence of adhesion rather than being related to bacterial phagocytosis. These results also confirm our previous study showing that, unlike GBS, well-encapsulated *S. suis* is in fact not phagocytosed (Article I). Furthermore, TNF induction by GBS has been shown to be phagocytosis-dependent (39).

Recently, the importance of the attachment phase for cytokine induction has been shown with several bacterial species, including intracellular bacteria such as *L. pneumophila*, and *L. monocytogenes* (33, 88, 123). Thus, the initial attachment of bacteria to macrophages may be sufficient to generate a signal for cytokine induction, and such a signal may be mediated by certain bacterial ligands and macrophage receptor interactions. Interestingly, *S. suis* was shown to be able to adhere to and induce the release of inflammatory cytokines by brain microvascular endothelial cells (BMEC), whereas it was not able either to adhere, nor to induce cytokines by human umbilical endothelial cells (HUVEC) (23, 101).

Since one of the major virulence factors of *S. suis* is the type 2 specific CPS, which is implicated in phagocytosis resistance and, at least in part, in adhesion to macrophages (19, 85), we decided to investigate the role of the capsule in cytokine release. An encapsulated and an unencapsulated type 2 *S. suis* strains were compared. The presence or absence of capsule had no effect on IL-6 production, but the absence of capsule resulted in an increased TNF production, suggesting that bacterial components responsible for TNF release may be partially masked by the capsule. Furthermore, purified *S. suis* CPS failed to induce cytokine release.

Several *in vitro* and *in vivo* studies with purified CPS or with unencapsulated mutants failed to demonstrate a major role for this bacterial component in cytokine induction by important pathogenic gram positive cocci (70, 97, 103, 104). Furthermore, it has been reported that heat-killed unencapsulated- GBS and *S. pneumoniae* strains induce higher meningeal inflammation than their respective encapsulated parent strains, suggesting that

the presence of capsular material may mask the inflammatory activity of the underlying cell wall (60, 97, 104). It was postulated that encapsulated bacteria produce inflammation by exposure of the underlying cell wall or by secretion of the cell wall material during growth (97). In fact, purified *S. suis* cell wall was able to induce a high TNF response. It could also induce, to a lesser extent, IL-6. This result suggests that bacterial components other than those in the cell wall also contribute to IL-6 induction by *S. suis*-activated macrophages, and confirms the differences observed with the unencapsulated mutant as described above. Similarly, the *S. suis* cell wall was shown to be the main inductor of cytokine release by BMEC (101). Since heat-treatment of bacteria does not affect their capacity to stimulate cytokine release, a limited role of proteins is suggested. Further studies are needed to determine the exact nature of the *S. suis* components responsible for cytokine release.

It must be stressed that, even though the cell wall is a major modulator of cytokine responses, encapsulated *S. suis* is not abrogated of its ability to induce the release of cytokines, as clearly shown by the potent IL-6 inducing activity. Therefore, the capsule may not be necessary to induce the release of inflammatory cytokines during *S. suis* infection, but it may contribute to the first step of adhesion to macrophages and further phagocytosis impairment, allowing other less exposed cell wall components (such as LTA) to interact with the cell surface with consequent cell activation. This may also explain why the cell wall is a poor inhibitor of adhesion (as discussed above). The concept of multiple adhesins and steps in the process of adhesion has been proposed for several pathogens, such as GAS, *S. sanguis*, *Mycobacterium avium*, and *B. pertussis* (47-49). This possibility remains to be confirmed for *S. suis* adhesion to and activation of macrophages (Fig. 12).

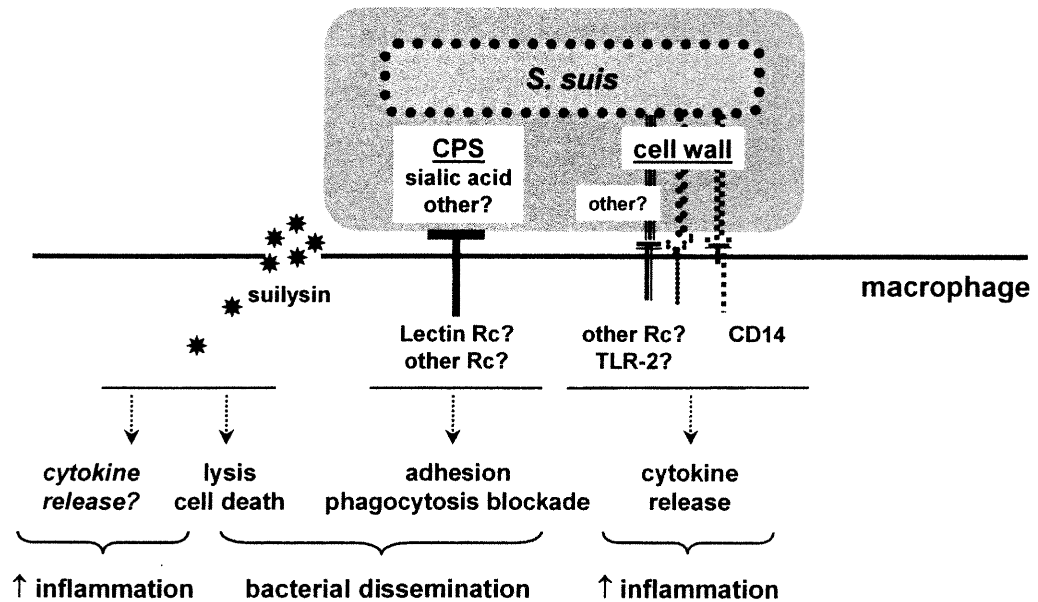


Fig. 12: Hypothetic model of *Streptococcus suis* interactions with macrophages. Firm adhesion to macrophages with consequent phagocytosis-impairment would be mediated by the sialic acid moiety of the capsular polysaccharide (CPS), as well as by other unknown bacterial components. Sialic acid-binding lectin receptor or other receptors (Rc) on the cell surface could be implicated in this step. On the other hand, cell activation with consequent cytokine release is mediated mainly by cell wall components interacting with the CD14 Rc (Article IV), as well as with other unknown Rc, for example the Toll-like receptor-2 (TLR-2). At high bacterial concentrations, suilysin secretion induces cell lysis and possible would also play a role in inflammation.

To add to the complexity of the situation, heat-killed bacteria showed a reduction in firm adhesion levels to murine macrophages, whereas they are still able to induce cytokine production by these cells. In addition, in separate experiments, no differences have been observed between non-treated (live) or heat-killed bacteria in their capacity to induce cytokine release by human monocytes (Article IV). These observations would suggest that interacting pathways implicated in firm adhesion and in cell activation may be different. To date, the only receptor described as being implicated in cytokine induction after

interaction with *S. suis* is the CD14 receptor (as discussed below; Article IV). This receptor is also known to be implicated in cytokine induction by other Gram-positive bacteria (17, 31). The role of other receptors, such as the Toll-like receptor-2, remains to be elucidated.

We mentioned above that one of the consequences of *S. suis* interaction with phagocytes is the secretion of the suilysin and consequent cell injury. Since it has been shown that several microbial toxins may stimulate or modulate the inflammatory cascade (54), the two soluble proteins of *S. suis*, described as possible virulence factors, the suilysin and the EF, were analyzed (43, 108). However, we failed to demonstrate a role of these factors in cytokine induction by murine macrophages *in vitro*. This is in agreement with recent reports which indicate that the *S. suis* type 2 strains deficient in the production of these proteins remain virulent in a pig model of infection (3, 91). It has also been shown that a pneumolysin-deficient strain of *S. pneumoniae* caused meningeal inflammation in rabbits indistinguishable from that induced by the parent strain (37). Similarly, streptolysin O from *S. pyogenes* induced neither neutrophil influx nor significant cytokine elevations in bronchoalveolar fluids (87). These two hemolysins, like suilysin, belong to the family of the thiol-activated toxins (5). In contrast to suilysin, *in vitro* reports indicated that both pneumolysin and streptolysin O, are able to stimulate the release of TNF-α and IL-1β by phagocytes (45, 50). However, they are not probably the main inflammatory mediators *in vivo*, and cytokine induction could be accomplished by other bacterial components in the case of hemolysin-deficient strains. Furthermore, it has been suggested that attack on cell membranes by streptolysin O or pneumolysin may also perpetuate local inflammation and tissue damage through an entirely different mechanism, such as the complement activation on the autologous cell membranes (13, 50).

In the case of *S. suis*, it has been shown that, even though a suilysin-negative mutant was still virulent, the total lesion scores were significantly higher in pigs infected with the wild type strains than those infected with the mutant (3). It was suggested by the authors that suilysin may have a role in increasing the

severity of clinical signs, and allowing bacterial colonization of the organs to reach higher levels. In addition, suilysin was shown not only to be cytotoxic to BMEC (23), but also to induce the release of pro-inflammatory cytokines by these cells *in vitro* (101). Thus, the role of suilysin, as well as of EF, *in vivo* inflammation remains to be elucidated.

S. suis also induces inflammatory cytokine release by human monocytes

Despite the fact that *S. suis* is an important agent of septicemia and meningitis, and in some cases fatal toxic shock in humans (6), its ability to interact with human-origin cells has not yet been addressed. This led us to investigate the induction of several proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-8 and MCP-1 by human THP-1 monocytes stimulated with several strains of *S. suis* type 2. Since IFN-γ is the earliest cytokine detected at the site of infection and plays a critical role in cell priming and activation (30, 46, 56), cells pre-activated or not with IFN-γ were compared.

Our present work (Article IV) demonstrates for the first time that *S. suis* type 2 is able to interact with monocytes of human origin inducing the release of large amounts of the pro-inflammatory cytokines TNF, IL-1, IL-6, as well as high and sustained levels of the two chemokines, IL-8 and MCP-1. The release of these cytokines was time- and dose- dependent, and it was significantly enhanced by pre-treatment of cells with IFN-γ. The human and pig origin of strains does not seem to affect the intensity of the response; indeed, a very heterogeneous pattern of cytokine and chemokine production was observed for the different strains tested in this study. Thus, the clinical relevance of potential species-specific differences in reactivity to bacterial strains still remains unclear. Similar results were observed between the two groups of strains when evaluating their capacity to induce cytokine release by BMEC (101). In addition, no association could be observed between cytokine response and virulence of the strain. Interestingly, virulent and non virulent strains do not appear to differ in

ability to adhere to different types of host cells (unpublished observations). To date, the higher macrophage-adhesion of strains with the North-American phenotype with respect to the European phenotype group of strains is the only difference observed among *S. suis* stains with respect to the interactions with host cells (Article II). Giguère and Prescott (38) have reported that virulent and non virulent *R. equi* strains induced similar levels of cytokine production in murine macrophages. They concluded that virulence is correlated to the ability to survive in macrophages, but it did not affect early cytokine release by macrophages. In this respect, as already mentioned, virulent *S. suis* strains are able to survive in the circulation at high concentrations for several days, whereas non virulent strains disappear from the circulation rapidly after infection (11).

Further studies with cells of porcine origin are currently in progress in order to confirm or clarify the results obtained with murine and human origin cells.

Decoding the S. suis induced cytokine network: the model of THP-1 cells

Several similarities observed in the cytokine responses obtained after stimulation of cells with *S. suis* or LPS, let us to hypothesize that they may use similar macrophage-signaling pathways. Firstly, the kinetics of cytokine induction by *S. suis* and LPS after stimulation of both J774A1 and THP-1 cells were similar (Article III). Secondly, it has been reported that LPS may exert its pleiotropic effects in part through activation of PKC (117). PMA is an specific activator of PKC (64), and both *S. suis* and LPS are similarly affected by PMA co-stimulation, showing a comparable increase in cytokine release by J774A1 cells (Article III). Third, pre-activation of THP-1 cells with IFN-γ increases not only CD14 expression (unpublished observations) (73, 76), but also cytokine production by *S. suis* and LPS (Article IV).

One common pathway shared by many gram positive bacteria and LPS is the interaction with the CD14 receptor (74). Preliminary studies showed that *S. suis*-induced IL-6 release by J774A1 cells is inhibited by an anti-CD14 antibody. To further evaluate the role of the CD14 receptor in *S. suis*-induced cytokine response, three anti-CD14 Mabs (MY4, IOM2, and Leu-M3) were selected for blocking experiments with human THP-1 monocytes. The inhibitory effect of the three anti-CD14 Mabs on production of the five cytokines under study varied, probably due to the different functional characteristics of the Mabs. Nevertheless, we clearly demonstrated that blocking of CD14 reduces levels of cytokine release after *S. suis* stimulation. *S. suis*-induced TNF, IL-1 and IL-6 release was significantly inhibited by anti-CD14 Mabs, independent of the bacterial concentration used as stimulus. Results were more controversial in the case of *S. suis*-induced chemokines. By stimulating cells with maximal bacterial doses, our first observation was that chemokine induction by *S. suis* was CD14-independent. However, when sub-maximal doses of bacteria were used, a partial inhibitory effect was observed with MY4 on *S. suis*-induced MCP-1. In contrast, our first observation was confirmed for *S. suis*-induced IL-8 release, and no inhibition was observed, regardless of the bacterial dose used (Fig. 13). These results suggest that *S. suis* pathways for the induction of the two chemokines seem to be, at least in part, different. Similarly, van Furth et al. (106) reported that anti-CD14 inhibition of TNF release by monocytes following stimulation with *H. influenzae* is independent of the bacterial dose used, whereas inhibition of IL-10 production is inversely proportional to the bacterial concentration.

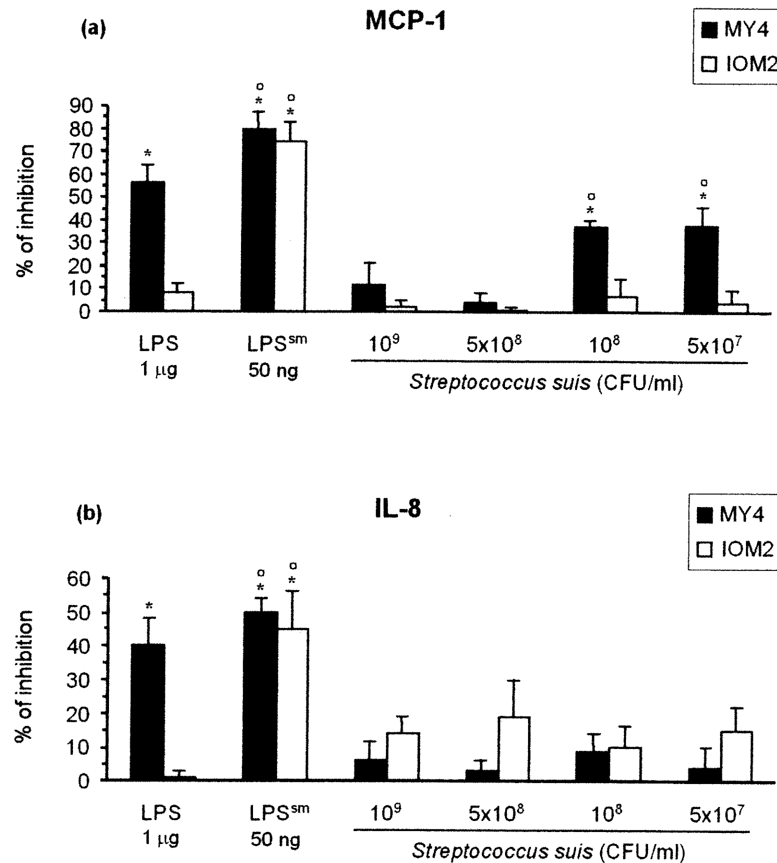


Fig. 13: Effect of CD14 blockade on MCP-1 (a) or IL-8 (b) production by cells stimulated with sub-maximal stimulus doses. IFN-γ pre-activated THP-1 cells were pre-incubated for 1 h with anti-CD14 Mab MY4 (10 μg/ml), or Mab IOM2 (20 μg/ml), before adding sub-maximal doses of *S. suis* ($5x10^8$, 10^8, or $5x10^7$ CFU/ml) or a sub-maximal dose of LPS (LPS^{sm}; 50 ng/ml) used as a reference. IL-8 and MCP-1 at 24 h of incubation were measured by ELISA titration. Data were transformed to percentage of inhibition respect to values for bacteria or LPS without treatment (100% of production). *$P < 0.05$ (versus the corresponding stimulus without treatment). ¤ $P < 0.01$ (significantly higher inhibition respect to values obtained with the corresponding maximal stimulus dose: bacteria at 10^9 CFU/ml or LPS at 1 μg/ml).

Because TNF and IL-1 are potent inducers of other cytokine synthesis in many cell types, it is important to distinguish between direct interactions between *S. suis* and the target cells, and indirect effects mediated by induction of secondary cytokines in culture (8, 18, 30). As similarly reported for other pathogens (111), in the present work, blocking experiments demonstrated that TNF and IL-1 partially amplify the *S. suis*-induced cytokine cascade. In our previous work on cytokine induction by *S. suis*-stimulated J774A1 cells (Article III), levels of produced TNF do not appear to affect those of IL-6, and thus the existence of TNF-independent mechanisms leading to IL-6 production by *S. suis* were suggested. Indeed, this preliminary observation was confirmed in our blocking experiments, since, even the combination of both anti-IL-1 and anti-TNF antibodies only partially reduces IL-6 levels. In addition, TNF and/or IL-1 also are involved in partial amplification pathways of *S. suis*-induced IL-1, IL-8, and MCP-1.

In conclusion, *S. suis* may induce cytokine production by THP-1 cells in both CD14-dependent and -independent manner, with TNF and IL-1 acting in amplification pathways. To the best of our knowledge, this is the first report of specific inflammatory cell receptor-interactions implicated in the recognition of *S. suis*, a poorly studied pathogen (Fig. 14). Additional studies on the signaling mechanisms involved in *S. suis* cytokine induction are currently in progress.

S. *suis*-induced pro-inflammatory cytokine cascade: a model for THP-1 cells

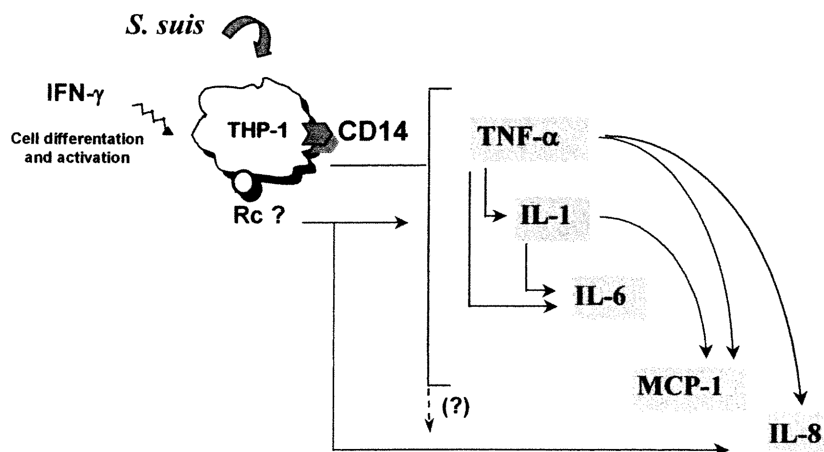


Fig. 14: Proposed model for *S. suis*-induced pro-inflammatory cytokine cascade with THP-1 human monocytes. Interferon-gamma (IFN-γ) pre-activation of THP-1 monocytes promotes cell differentiation and activation that leads to the up-regulation of several receptors, including CD14. Further activation of cells by *S. suis* directly induces the release of the pro-inflammatory cytokines: tumor necrosis factor alpha (TNF), interleukin (IL)-1, IL-6, IL-8 and monocyte chemotactic protein one (MCP-1). A second (indirect) monocyte activation would also take place, with TNF and IL-1 playing an important role in the amplification pathways of the *S. suis*-induced cytokine cascade. Interaction of *S. suis* with the CD14 receptor would be, at least in part, responsible for TNF, IL-1, IL-6 and MCP-1 release, whereas the production of IL-8 seems to be CD14-independent. Thus, other unknown receptors (Rc) on the monocyte surface would also be implicated in the *S. suis*-induced cytokine release. Finally, a possible implication of other epitopes (?) on the CD14 receptor, that are not recognized by the three antibodies used in the present work, could not be ruled out.

3. *Streptococcus suis* pathogenesis: a new hypothesis

Despite increasing amount of research being carried out in recent years, knowledge of virulence factors and the pathogenesis of the *S. suis* infection remains limited. On the basis of the present work and several other observations (4, 20, 23, 58, 82-85, 101) some hypotheses on the critical steps of *S. suis* infection, such as bacterial invasion from mucosal surfaces to the bloodstream, survival of bacteria in blood, and invasion from blood into the CNS, were postulated (42).

The first step in the pathogenesis of the infection is traversal of the mucosal barriers in the upper respiratory tract. Very few studies are available regarding the interactions between *S. suis* and epithelial cells. It has been recently reported that *S. suis* is able to adhere to several epithelial cell lines from human and different animal species, including swine (58). In addition, suilysin-positive strains are cytotoxic for these cells (58, 67). *S. suis* invasion of epithelial cells was shown to be a "rare event", and thus remains controversial (67). It has been suggested that suilysin-positive *S. suis* strains can use invasion and cell lysis as a mechanism to breach the mucosal epithelia, whereas the mechanism(s) used by suilysin-negative strains, which were shown to be non-toxic for epithelial cells, is still unknown (Fig. 15).

Once *S. suis* enters the bloodstream, it must survive to the host defense system attack and must be able to disseminate. As already discussed, an early theory suggested uptake of bacteria by monocytes, intracellular survival and invasion of the CNS by the "Trojan horse theory" (120). This bacterial uptake could take place directly at the tonsils by macrophages or once the bacteria is in the bloodstream. However, our present studies (Articles I and II) and other literature reports suggest that bacteria may also use other mechanisms to disseminate by resisting phagocytosis (19, 85, 90). Hence, it is possible that extracellular *S. suis* bacteria also travel free in circulation or, as demonstrated in our present work, bacteria can also be largely bound to the surface of

macrophages without being ingested (Article II). This possibility is named here as the "modified" Trojan horse theory (Fig. 15).

Free bacteria or cell-associated bacteria should then cross the tightly closed BBB in order to invade the CNS and cause meningitis. This barrier, responsible for maintaining biochemical homeostasis within the CNS is characterised by the presence of tight junctions, and regulates fluid, macromolecule and cell trafficking on both sides of the layer. Two kinds of cells present such tight junctions: the BMEC and the epithelial cells forming the choroid plexus (98). The choroid plexus has been largely considered as the portal of entry of several pathogens to the CNS (59). Interactions of bacteria with epithelial cells of the choroid plexus may be the consequence of pressure of high-grade constant bacteremia. In fact, disruption of the plexus brush border, with fibrin and inflammatory cell exudate present in the ventricles has been described during natural or experimentally induced *S. suis* meningitis (120). On the other side, histopathological findings indicating necrosis of vessel walls in association with inflammatory cellular aggregates have also been reported. Vessel endothelia were swollen and sometimes lumina were occluded, demonstrating inflammatory cell invasion directly from the overlying meninges (80). In addition, moderate to severe suppurative meningitis without choroid plexitis was also reported (107). Despite these observations, interactions of *S. suis* with the different cellular components of the BBB are poorly characterized. Invasion assays performed with human BMEC indicated that, unlike other meningeal pathogens, free *S. suis* organisms could adhere to but not invade this type of cells (23). However, we demonstrated that *S. suis* is able to induce high levels of the pro-inflammatory cytokines IL-6, IL-8 and MCP-1. Such factors would increase BBB permeability and leukocyte transmigration into the SAS. In addition, suilysin-positive strains are not only cytotoxic for these cells, but suilysin is also, by itself, a potent modulin of BMEC activation, and it will further increase BBB disruption and inflammation (23, 101).

If the proposed "modified" Trojan horse theory were correct, bacteria would also arrive to the BBB surface-associated with monocytes. As the CNS is

considered to be an immunoprivileged organ, normal circulation of monocytes to the CNS is still controversial. However, the local production of proinflammatory cytokines by microglial cells, endothelial cells, and migrating leukocytes upon contact with bacteria is currently regarded as the initial step of an acute infiltration of leukocytes into the SAS, which characterizes bacterial meningitis. In this regard, we demonstrated that *S. suis* is able not only to interact with monocytes and macrophages, but also to induce the release of several proinflammatory cytokines and chemokines, such as TNF-α, IL-1, IL-6, IL-8 and MCP-1 (Articles III and IV). Remarkably , the high and sustained levels of the two chemokines, IL-8 and MCP-1, at relatively low bacterial doses, are relevant since chemokines are implicated in leukocyte trafficking across the vascular wall, and high levels of these chemokines are found in patients with bacterial meningitis (57). Indeed, excellent results were obtained when cases of post-weaning meningitis were treated with both penicillin and dexamethasone, in the very early stages of the disease (25). Dexamethasone is probably one of the most potent inhibitors of C-X-C and C-C chemokine expression (8).

Several cytokines and chemokines (especially TNF-α, IL-1, IL-8) are known to up-regulate the expression of cell adhesion molecules (CAMs), such as selectins and integrins, that allow transendothelial migration of leukocytes. Some meningeal pathogens have been shown to stimulate the expression of CAMs (122). In this regard, we recently demonstrated that *S. suis* stimulates the up-regulation of the surface expression of intercellular adhesion molecule-1 (ICAM-1), CD11a/CD18 and CD11c/CD18 on human monocytes. Despite the fact that *S. suis* does not up-regulate CAM expression on endothelial cells, monocytes stimulated with *S. suis* show an increase in adherence to endothelial cells, thus providing an additional possible mechanism for some of the inflammatory features of meningitis caused by this pathogen (4).

In conclusion it seems likely that the combined actions of cell-free and cell-associated bacteria contribute to the ability of *S. suis* to invade the CNS from the bloodstream, as already suggested for *L. monocytogenes* (34) (Fig.15).

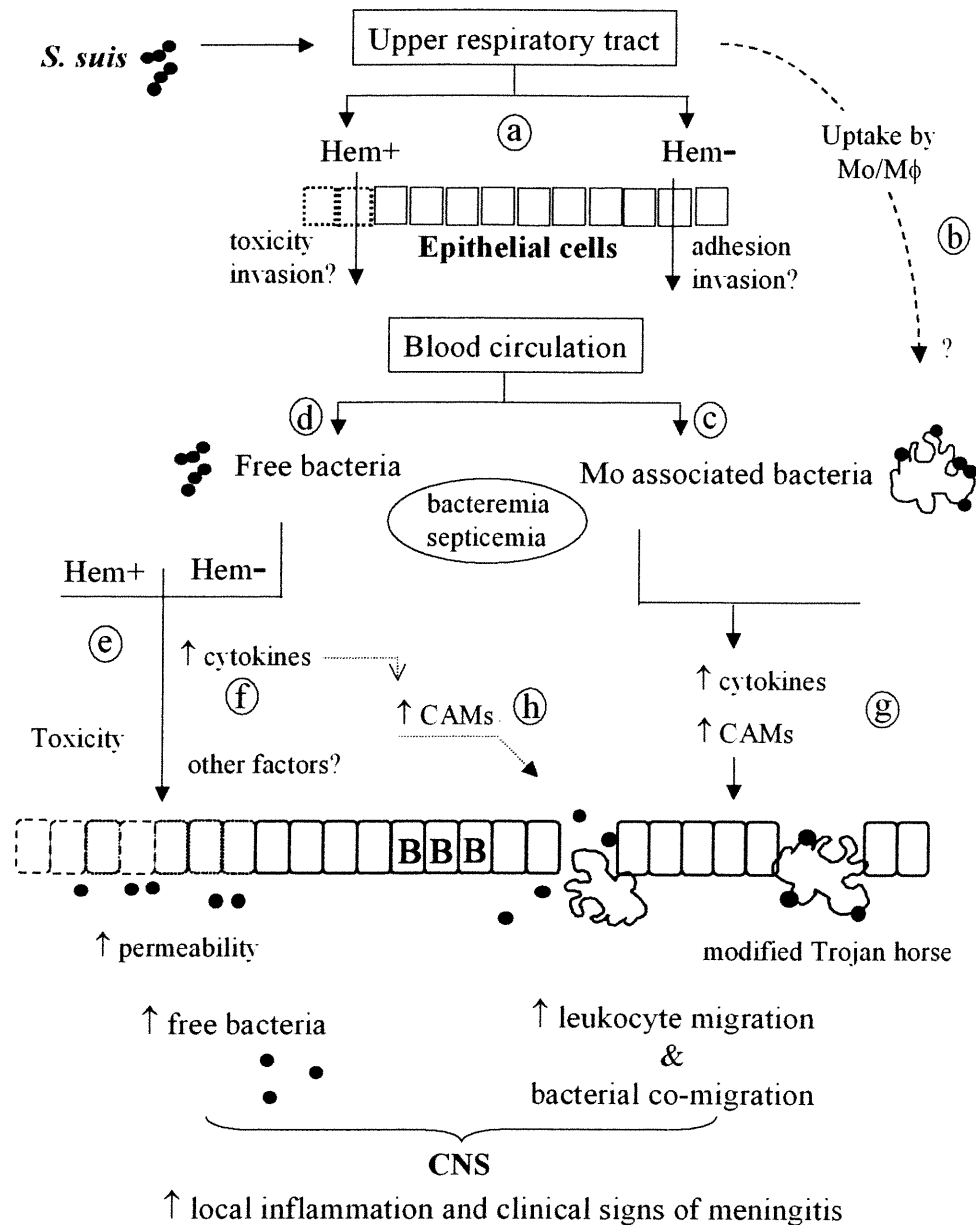


Fig. 15: Summary of the knowledge and of proposed hypotheses for the different steps involved in the pathogenesis of meningitis due to *S. suis*. Hem+: haemolysin-positive strains. Hem⁻: haemolysin-negative strains. Mo: monocytes. Mϕ: macrophages. CAMs: cell adhesion molecules. BBB: blood-brain barrier. CNS: central nervous system. Steps **a** and **b**: show *S. suis* interactions with the epithelial layers of the upper respiratory tract (colonization) and access to blood circulation. Hem+ strains may use cell disruption (toxicity) and invasion to reach the bloodstream, while the mechanisms used by adhered Hem⁻ strains are still uncertain (Step **a**). *S. suis* could also be directly uptaken by Mo/Mϕ and enter to the bloodstream within circulating cells (Step **b**). Steps **c** and **d**: describe bacterial traveling in blood. This could be as Mo-associated (bound or intracellular) bacteria (Step **c**) or as free bacteria (Step **d**), resulting in bacteremia or septicemia. Steps **e, f, h** and **g**: describe the possible mechanisms used by *S. suis* to cross the BBB. Free bacteria would enter the CNS after increasing BBB permeability, via direct cell toxicity (Hem+ strains; Step **e**), indirectly via local cytokine production (Step **f**) or via other unknown(s) mechanism(s). Local cytokine production could also increase CAMs expression and leukocyte migration, that in turn "open the door" to free bacterial trafficking (Step **h**). On the other side, Mo-associated bacteria would enter the CNS via the "Trojan horse" (bacteria inside cells) or "Modified Trojan horse" (bacteria adhered to cells) mechanism favored by activated phagocyte cytokine release (Step **g**).

4. References

1. **Agarwal, K. K., S. D. Elliott, and P. J. Lachmann.** 1969. Streptococcal infection in young pigs. III. The immunity of adult pigs investigated by the bactericidal test. J. Hyg. **67:**491-503.
2. **Albanyan, E. A., J. G. Vallejo, C. Wayne Smith, and M. S. Edwards.** 2000. Nonopsonic binding of type III Group B streptococci to human neutrophils induces interleukin-8 release mediated by the p38 mitogen-activated protein kinase pathway. Infect. Immun. **68:**2053-2060.
3. **Allen, A. G., S. Bolitho, H. Lindsay, S. Khan, C. Bryant, P. M. Norton, P. N. Ward, J. A. Leigh, J. Morgan, H. Riches, S. Eastty, and D. Maskell.** 2001. Generation and characterization of a defined mutant of *Streptococcus suis* lacking suilysin. Infect. Immun. **69:**2732-2735.
4. **Al-Numani, D., M. Segura, M. Doré, and M. Gottschalk.** 2002. Up-regulation of ICAM-1, CD11a/CD18 and CD11c/CD18 on human THP-1 monocytes stimulated by *Streptococcus suis* serotype 2. Infect. Immun.:*Submitted.*
5. **Alouf, J. E., and C. Geoffroy.** 1991. The family of the antigenically-related cholesterol-binding ("sulphydryl-activated") cytolytic toxins, p. 147-186. *In* J. E. Alouf (ed.), Sourcebook of Bacterial Protein Toxins. New York Academic Press, New York.
6. **Arends, J. P., and H. C. Zanen.** 1988. Meningitis caused by *Streptococcus suis* in humans. Rev. Infect. Dis. **10:**131-137.
7. **Athamna, A., and I. Ofek.** 1988. Enzyme-linked immunosorbent assay for quantification of attachment and ingestion stages of bacterial phagocytosis. J. Clin. Microbiol. **26:**62-66.
8. **Baggiolini, M., B. Dewald, and B. Moser.** 1994. Interleukin-8 and related chemotactic cytokines - CXC and CC chemokines. Adv. Immunol. **55:**97-179.
9. **Baker, C. J., M. S. Edwards, and D. L. Kasper.** 1981. Role of antibody to native type III polysaccharide of group B *Streptococcus* in infant infection. Pediatrics **68:**544-549.
10. **Benkirane, R., M. Gottschalk, M. Jacques, and J. D. Dubreuil.** 1998. Immunochemical characterization of an IgG-binding protein of *Streptococcus suis*. FEMS Immunol. Med. Microbiol. **20:**121-127.
11. **Berthelot-Hérault, F., R. Cariolet, A. Labbé, M. Gottschalk, J.-Y. Cardinal, and M. Kobisch.** 2001. Experimental infection of specific pathogen free piglets with French strains of *Streptococcus suis* capsular type 2. Can. J. Vet. Res. **65:**196-200.
12. **Bhakdi, S., F. Grimminger, N. Suttorp, D. Walmrath, and W. Seeger.** 1994. Proteinaceous bacterial toxins and pathogenesis of sepsis syndrome and septic shock: the unkown connection. Med. Microbiol. Immunol. **183:**119-144.
13. **Bhakdi, S., and J. Tranum-Jensen.** 1987. Damage to mammalian cells by proteins that form transmembrane pores. Rev. Physiol. Biochem. Pharmacol. **107:**147-223.
14. **Brazeau, C., M. Gottschalk, S. Vincelette, and B. Martineau-Doize.** 1996. In vitro phagocytosis and survival of *Streptococcus suis* capsular type 2 inside murine macrophages. Microbiology **142:**1231-1237.
15. **Brown, E. J., S. W. Hosea, and M. M. Frank.** 1983. The role of antibody and complement in the reticuloendothelial clearance of pneumococci from the bloodstream. Rev. Infect. Dis. **4:**S797-S805.

16. **Busque, P., R. Higgins, S. Sénéchal, R. Marchand, and S. Quessy.** 1998. Simultaneous flow cytometric measurement of *Streptococcus suis* phagocytosis by polymorphonuclear and mononuclear blood leukocytes. Vet. Microbiol. **63:**229-238.
17. **Cauwels, A., E. Wan, M. Leismann, and E. Tuomanen.** 1997. Coexistence of CD14-dependent and independent pathways for stimulation of human monocytes by gram-positive bacteria. Infect. Immun. **65:**3255-3260.
18. **Cerami, A.** 1992. Inflammatory cytokines. Clin. Immunol. Immunopathol. **62:**S3-S10.
19. **Charland, N., J. Harel, M. Kobish, S. Lacasse, and M. Gottschalk.** 1998. *Streptococcus suis* serotype 2 mutants deficient in capsular expression. Microbiology **144:**325-332.
20. **Charland, N., M. Jacques, S. Lacouture, and M. Gottschalk.** 1997. Characterization and protective activity of a monoclonal antibody against a capsular epitope shared by *Streptococcus suis* serotypes 1, 2 and 1/2. Microbiology **143:**3607-3614.
21. **Charland, N., J. T. Kellens, F. Caya, and M. Gottschalk.** 1995. Agglutination of *Streptococcus suis* by sialic acid-binding lectins. J. Clin. Microbiol. **33:**2220-2221.
22. **Charland, N., M. Kobisch, B. Martineau-Doize, M. Jacques, and M. Gottschalk.** 1996. Role of capsular sialic acid in virulence and resistance to phagocytosis of *Streptococcus suis* capsular type 2. FEMS Immunol. Med. Microbiol. **14:**195-203.
23. **Charland, N., V. Nizet, C. Rubens, K. S. Kim, S. Lacouture, and M. Gottschalk.** 2000. *Streptococcus suis* serotype 2 interactions with human brain microvascular endothelial cells. Infect. Immun. **68:**637-643.
24. **Chatellier, S., M. Gottschalk, R. Higgins, R. Brousseau, and J. Harel.** 1999. Relatedness of *Streptococcus suis* serotype 2 isolates from different geographic origins as evaluated by molecular fingerprinting and phenotyping. J. Clin. Microbiol. **37:**362-366.
25. **Clark, L. K.** 1995. SEW: Program, problems, performances, potential profits and methods of implementation for various herd sizes. Presented at the 36th George A. Young Swine Conference, Nebraska.
26. **Clifton-Hadley, F. A.** 1981. Studies of *Streptococcus suis* type 2 infection in pigs. Ph.D. thesis. University of Cambridge, Cambridge.
27. **Cornacchione, P., L. Scaringi, K. Fettucciari, E. Rosati, R. Sabatini, G. Orefici, C. von Hunolstein, A. Modesti, A. Modica, F. Minelli, and P. Marconi.** 1998. Group B streptococci persist inside macrophages. Immunology **93:**86-95.
28. **Courtney, H. S., J. B. Dale, and D. L. Hasty.** 1997. Host cell specific adhesins of group A streptococci. Adv. Exp. Med. Biol. **418:**605-606.
29. **Crocker, P. R., and A. Varki.** 2001. Siglecs in the immune system. Immunology **103:**137-145.
30. **Curfs, J. H. A. J., J. F. G. M. Meis, and J. A. A. Hoogkamp-Korstanje.** 1997. A primer on cytokines: sources, receptors, effects, and inducers. Clin. Microbiol. Rev. **10:**742-780.
31. **Cuzzola, M., G. Mancuso, C. Beninati, C. Biondo, C. Von Hunolstein, G. Orefici, T. Espevik, T. H. Flo, and G. Teti.** 2000. Human monocyte receptors involved in tumor necrosis factor responses to group B Streptococcal products. Infect. Immun. **68:**994-998.

32. **de Greeff, A., H. Buys, R. Verhaar, J. Dijkstra, v. A. L., and H. E. Smith.** 2002. Contribution of fibronectin-binding protein to pathogenesis of *Streptococcus suis* serotype 2. Infect. Immun. **70:**1319-1325.
33. **Demuth, A., W. Goebel, H. U. Beuscher, and M. Kuhn.** 1996. Differential regulation of cytokine and cytokine receptor mRNA expression upon infection of bone marrow-derived macrophages with *Listeria monocytogenes*. Infect. Immun **64:**3475-3483.
34. **Drevets, D. A.** 1999. Dissemination of *Listeria monocytogenes* by infected phagocytes. Infect. Immun. **67:**3512-3517.
35. **Elliott, S. D., F. Clifton-Hadley, and J. Tai.** 1980. Streptococcal infection in young pigs. V. An immunogenic polysaccharide from *Streptococcus suis* type 2 with particular reference to vaccination against streptococcal meningitis in pigs. J. Hyg. Lond. **85:**275-285.
36. **Elliott, S. D., M. McCarty, and R. C. Lancefield.** 1977. Teichoic acids of group D streptococci with special reference to strains from pig meningitis (*Streptococcus suis*). J. Exp. Med. **145:**490-499.
37. **Friedland, I. R., M. M. Paris, S. Hickey, S. Shelton, K. Olsen, J. C. Paton, and G. H. McCracken.** 1995. The limited role of pneumolysin in the pathogenesis of pneumococcal meningitis. J. Infect. Dis **172:**805-809.
38. **Giguère, S., and J. F. Prescott.** 1998. Cytokine induction in murine macrophages infected with virulent and avirulent *Rhodococcus equi*. Infect. Immun. **66:**1848-1854.
39. **Goodrum, K. J., J. Dierksheide, and B. J. Yoder.** 1995. Tumor necrosis factor alpha acts as an autocrine second signal with gamma interferon to induce nitric oxide in group B *streptococcus*-treated macrophages. Infect. Immun **63:**3715-3717.
40. **Gordon, S. B., G. R. B. Irving, R. A. Lawson, M. E. Lee, and R. C. Read.** 2000. Intracellular trafficking and killing of *Streptococcus pneumoniae* by human alveolar macrophages are influenced by opsonins. Infect. Immun. **68:**2286-2293.
41. **Gottschalk, M., A. Lebrun, H. Wisselink, J. D. Dubreuil, H. Smith, and U. Vecht.** 1998. Production of virulence-related proteins by canadian strains of *Streptococcus suis* capsular type 2. Can. J. Vet. Res. **62:**75-79.
42. **Gottschalk, M., and M. Segura.** 2000. The pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions. Vet. Microbiol. **75:**59-71.
43. **Gottschalk, M. G., S. Lacouture, and J. D. Dubreuil.** 1995. Characterization of *Streptococcus suis* capsular type 2 haemolysin. Microbiology **141:**189-195.
44. **Greenberg, J. W., W. Fischer, and K. A. Joiner.** 1996. Influence of lipoteichoic acid structure on recognition by the macrophage scavenger receptor. Infect. Immun. **64:**3318-3325.
45. **Hackett, S. P., and D. L. Stevens.** 1992. Streptococcal toxic shock syndrome: synthesis of tumor necrosis factor and interleukin-1 by monocytes stimulated with pyrogenic exotoxin A and streptolysin O. J. Infect. Dis. **165:**879-885.
46. **Haq, A. U., J. J. Rinehart, and R. D. Maca.** 1985. The effect of gamma interferon on IL-1 secretion of in vitro differentiated human macrophages. J. Leuk. Biol. **38:**735-746.
47. **Hasty, D. L., I. Ofek, H. S. Courtney, and R. J. Doyle.** 1992. Multiple adhesins of streptococci. Infect. Immun. **60:**2147-2152.
48. **Hayashi, T., S. P. Rao, and A. Catanzaro.** 1997. Binding of the 68-kilodalton protein of *Mycobacterium avium* to $\alpha_v\beta_3$ on human monocyte-derived

macrophages enhances complement receptor type 3 expression. Infect. Immun. **65:**1211-1216.

49. **Hazenbos, W. L. W., B. M. van den Berg, and R. van Furth.** 1993. Very late antigen-5 and complement receptor type 3 cooperatively mediate the interaction between *Bordetella pertussis* and human monocytes. J. Immunol. **151:**6274-6282.
50. **Houldsworth, S., P. W. Andrew, and T. J. Mitchell.** 1994. Pneumolysin stimulates production of tumor necrosis factor alpha and interleukin-1β by human mononuclear phagocytes. Infect. Immun **62:**1501-1503.
51. **Joh, D., E. R. Wann, B. Kreikemeyer, P. Speziale, and M. Höök.** 1999. Role of fibronectin-binding MSCRAMMs in bacterial adherence and entry into mammalian cells. Matrix Biol. **18:**211-223.
52. **Kasper, D. L.** 1986. Bacterial capsule - old dogmas and new tricks. J. Infect. Dis. **153:**407-415.
53. **Kilpper-Bälz, R., and K. H. Schleifer.** 1987. *Streptococcus suis* sp. nov., nom. rev. Int. J. Syst. Bacteriol. **37:**160-162.
54. **König, W., S. Kasimir, T. Hensler, J. Scheffer, B. König, R. Hilger, J. Brom, and M. Köller.** 1992. Release of inflammatory mediators by toxin stimulated immune system cells and platelets. Zbl. Bakt. Suppl **23:**385-394.
55. **Koren, H. S., B. S. Handwerger, and J. R. Wunderlich.** 1975. Identification of macrophage-like characteristics in a cultured murine tumor line. J. Immunol. **114:**894.
56. **Kubin, M., J. M. Chow, and G. Trinchieri.** 1994. Differential regulation of interleukin-12 (IL-12), tumor necrosis factor α, and IL-1β production in human myeloid leukemia cell lines and peripheral blood mononuclear cells. Blood **83:**1847-1855.
57. **Lahrtz, F., L. Piali, K. Spanaus, J. Seebach, and A. Fontana.** 1998. Chemokines and chemotaxis of leukocytes in infectious meningitis. J. Neuroimmunol. **85:**33-43.
58. **Lalonde, M., M. Segura, S. Lacouture, and M. Gottschalk.** 2000. Interactions between *Streptococcus suis* serotype 2 and different epithelial cell lines. Microbiology **146:**1913-1921.
59. **Levine, S.** 1987. Choroid plexus: target for systemic disease and pathway to the brain. Lab. Invest. **56:**231-233.
60. **Ling, E. W. Y., F. J. D. Noya, G. Ricard, K. Beharry, E. L. Mills, and J. V. Aranda.** 1995. Biochemical mediators of meningeal inflammatory response to group B *streptococcus* in the newborn piglet model. Pediatr. Res. **38:**981-987.
61. **Maganti, S., M. M. Pierce, A. Hoffmaster, and F. G. Rodgers.** 1998. The role of sialic acid in opsonin-dependent and opsonin-independent adhesion of *Listeria monocytogenes* to murine peritoneal macrophages. Infect. Immun. **66:**620-626.
62. **Marques, M. B., D. L. Kasper, M. K. Pangburn, and M. R. Wessels.** 1992. Prevention of C3 deposition by capsular polysaccharide is a virulence mechanism of type III group B Streptococci. Infect. Immun. **60:**3986-3993.
63. **Martin, C. A., and M. E. Dorf.** 1991. Differential regulation of interleukin-6, macrophage inflammatory protein-1, and JE/MCP-1 cytokine expression in macrophage cell lines. Cell. Immunol. **135:**245-258.
64. **Newton, A. C.** 1995. Protein kinase C: structure, function, and regulation. J. Biol. Chem **270:**28495-28498.

65. **Noel, G. J., S. K. Hoiseth, and P. J. Edelson.** 1992. Type b capsule inhibits ingestion of *Haemophilus influenzae* by murine macrophages: studies with isogenic encapsulated and unencapsulated strains. J. Infect. Dis. **166**:178-182.
66. **Noel, G. J., D. M. Mosser, and P. J. Edelson.** 1990. Role of complement in mouse macrophage binding of *Haemophilus influenzae* type b. J. Clin. Invest. **85:**208-218.
67. **Norton, P. M., C. Rolph, P. N. Ward, R. W. Bentley, and J. A. Leigh.** 1999. Epithelial invasion and cell lysis by virulent strains of *Streptococcus suis* is enhanced by the presence of suilysin. FEMS Immunol. Med. Microbiol. **26:**25-35.
68. **Ofek, I., and E. H. Beachey.** 1980. General concepts and principles of bacterial adherence, p. 1-29. *In* E. H. Beachey (ed.), Receptors and recognition, series B. Bacterial adherence, vol. 6. Chapman and Hall, Ltd., London.
69. **Ofek, I., and N. Sharon.** 1988. Lectinophagocytosis: a molecular mechanism of recognition between cell surface sugars and lectins in the phagocytosis of bacteria. Infect. Immun. **56:**539-547.
70. **Orman, K. L., J. L. Shenep, and B. K. English.** 1998. Pneumococci stimulate the production of the inducible nitric oxide synthase and nitric oxide by murine macrophages. J. Infect. Dis. **178:**1649-1657.
71. **Painter, R. G., J. Whisenand, and A. T. McIntosch.** 1981. Effects of cytochalasin B on actin and myosin association with particle binding sites in mouse macrophages: implications with regard to the mechanism of action of the cytochalasins. J. Cell. Biol. **91:**373-384.
72. **Palmer, M.** 2001. The family of thiol-activated, cholesterol-binding cytolysins. Toxicon **39:**1681-1689.
73. **Perussia, B., E. T. Dayton, V. Fanning, P. Thiagarajan, J. Hoxie, and G. Trinchieri.** 1983. Immune interferon and leukocyte-conditioned medium induce normal and leukemic myeloid cells to differentiate along the monocytic pathway. J. Exp. Med. **158:**2058-2080.
74. **Pugin, J., D. Heumann, A. Tomasz, V. V. Kravchenko, Y. Akamatsu, M. Nishijima, M. P. Glauser, P. S. Tobias, and R. J. Ulevitch.** 1994. CD14 is a pattern recognition receptor. Immunity **1:**509-516.
75. **Ralph, P., and I. Nakoinz.** 1975. Phagocytosis and cytolysis by a macrophage tumour and its cloned cell line. Nature **257:**393-394.
76. **Raponi, G., M. C. Ghezzi, and C. Mancini.** 1997. The release of tumor necrosis factor alpha (TNF-α) by interferon gamma (IFN-γ) induced THP-1 cells stimulated with smooth lipopolysaccharide is inhibited by Mabs against HLA-DR and CD14 receptors on the effector cell. Microbiologica **20:**1-6.
77. **Read, R. C., S. Zimmerli, V. C. Broaddus, D. A. Sanan, D. S. Stephens, and J. D. Ernst.** 1996. The (α2-8)-linked polysialic acid capsule of group B *Neisseria meningitidis* modifies multiple steps during interaction with human macrophages. Infect. Immun. **64:**3210-3217.
78. **Rubens, C. E., L. M. Heggen, R. F. Haft, and M. R. Wessels.** 1993. Identification of *cps*D, a gene essential for type III capsule expression in group B streptococci. Mol. Microbiol. **8:**343-855.
79. **Salasia, S. I., C. Lammler, and G. Herrmann.** 1995. Properties of a Streptococcus suis isolate of serotype 2 and two capsular mutants. Vet Microbiol **45:**151-6.
80. **Sanford, S. E.** 1987. Gross and histopathological findings in unusual lesions caused by *Streptococcus suis* in pigs. II. Central nervous system lesions. Can. J. Vet. Res. **51:**486-489.

81. **Saukkonen, K., S. Sande, C. Cioffe, S. Wolpe, B. Sherry, A. Cerami, and E. Tuomanen.** 1990. The role of cytokines in the generation of inflammation and tissue damage in experimental gram-positive meningitis. J. Exp. Med **171:**439-448.
82. **Segura, M., and M. Gottschalk.** 2002. *Streptococcus suis* interactions with the murine macrophage cell line J774: adhesion and cytotoxicity. Infect. Immun. **70:**4312-4322.
83. **Segura, M., J. Stankova, and M. Gottschalk.** 1999. Heat-killed *Streptococcus suis* capsular type 2 strains stimulate tumor necrosis factor alpha and interleukin-6 production by murine macrophages. Infect. Immun. **67:**4646-4654.
84. **Segura, M., N. Vadeboncoeur, and M. Gottschalk.** 2002. CD14-dependent and -independent cytokine and chemokine production by human THP-1 monocytes stimulated by *Streptococcus suis* capsular type 2. Clin. Exp. Immunol. **127:**243-254.
85. **Segura, M. A., P. Cléroux, and M. Gottschalk.** 1998. *Streptococcus suis* and group B *Streptococcus* differ in their interactions with murine macrophages. FEMS Immunol. Med. Microbiol. **21:**189-195.
86. **Serhir, B., D. Dubreuil, R. Higgins, and M. Jacques.** 1995. Purification and characterization of a 52-kilodalton immunoglobulin G-binding protein from *Streptococcus suis* capsular type 2. J. Bacteriol. **177:**3830-3836.
87. **Shanley, T. P., D. Schrier, V. Kapur, M. Kehoe, J. M. Musser, and P. A. Ward.** 1996. Streptococcal cysteine protease augments lung injury induced by products of group A streptococci. Infect. Immun **64:**870-877.
88. **Simpson, S. Q., R. Singh, and D. E. Bice.** 1994. Heat-killed pneumococci and pneumococcal capsular polysaccharides stimulate tumor necrosis factor-α production by murine macrophages. Am. J. Respir. Cell Mol. Biol **10:**284-289.
89. **Sloan, A. R., and T. G. Pistole.** 1992. A quantitative method for measuring the adherence of group B streptococci to murine peritoneal exudate macrophages. J. Immunol. Methods **154:**217-223.
90. **Smith, H. E., M. Damman, J. Van der Velde, F. Wagenaar, H. J. Wisselink, N. Stockhofe-Zurwieden, and M. A. Smits.** 1999. Identification and characterization of the *cps* locus of *Streptococcus suis* serotype 2: the capsule protects against phagocytosis and is an important virulence factor. Infect. Immun. **67:**1750-1756.
91. **Smith, H. E., U. Vecht, H. J. Wisselink, N. Stockhofe-Zurwieden, Y. Biermann, and M. A. Smits.** 1996. Mutants of *Streptococcus suis* types 1 and 2 impaired in expression of muramidase-released protein and extracellular protein induce disease in newborn germfree pigs. Infect. Immun. **64:**4409-4412.
92. **Snyderman, R., M. Pike, D. Fischer, and H. Koren.** 1977. Biologic and biochemical activities of continuous macrophage cell lines P388D1 and J774.1. J. Immunol. **119:**2060-2066.
93. **Staats, J., P. Brandon, G. Stewart, and M. M. Chengappa.** 1999. Presence of the *Streptococcus suis* suilysin gene and expression of MRP and EF correlates with high virulence in *Streptococcus suis* type 2 isolates. Vet. Microbiol. **70:**201-211.
94. **Tamura, G. S., J. M. Kuypers, S. Smith, H. Raff, and C. E. Rubens.** 1994. Adherence of group B streptococci to cultured epithelial cells: roles of environmental factors and bacterial surface components. Infect. Immun. **62:**2450-2458.

95. **Teti, G., F. Tomasello, M. S. Chiofalo, G. Orefici, and P. Mastroeni.** 1987. Adherence of group B streptococci to adult and neonatal epithelial cells mediated by lipoteichoic acid. Infect. Immun. **55:**3057-3064.

96. **Tunkel, A. R., and W. M. Scheld.** 1993. Pathogenesis and pathophysiology of bacterial meningitis. Clin. Microbiol. Rev **6:**118-136.

97. **Tuomanen, E., A. Tomasz, B. Hengstler, and O. Zak.** 1985. The relative role of bacterial cell wall and capsule in the induction of inflammation in pneumococcal meningitis. J. Infect. Dis. **151:**535-540.

98. **Tuomanen, E. I.** 1996. Entry of pathogens into the central nervous system. FEMS Microbiol. Rev. **18:**289-299.

99. **Tuomanen, E. I., R. Austrian, and H. R. Masure.** 1995. Pathogenesis of pneumococcal infection. New Engl. J. Med. **332:**1280-1284.

100. **Upton, I.** 1986. The immune response of the pig to infectious with *Streptococcus suis* type 2. PhD Thesis. University of Cambridge, Cambridge.

101. **Vadeboncoeur, N., M. Segura, D. Al-Numani, G. Vanier, and M. Gottschalk.** 2002. Pro-inflammatory cytokine and chemokine release by human brain microvascular endothelial cells stimulated by *Streptococcus suis* serotype 2. FEMS Immunol. Med. Microbiol.:*Submitted.*

102. **Valentin-Weigand, P., P. Benkel, M. Rohde, and G. S. Chhatwal.** 1996. Entry and intracellular survival of group B Streptococci in J774 macrophages. Infect. Immun. **64:**2467-2473.

103. **Vallejo, J. G., C. J. Baker, and M. S. Edwards.** 1996. Interleukin-6 production by human neonatal monocytes stimulated by type III group B streptococci. J. Infect. Dis. **174:**332-337.

104. **Vallejo, J. G., C. J. Baker, and M. S. Edwards.** 1996. Roles of the bacterial cell wall and capsule in induction of tumor necrosis factor alpha by type III group B streptococci. Infect. Immun. **64:**5042-5046.

105. **Van Furth, A. M., J. J. Roord, and R. van Furth.** 1996. Roles of proinflammatory and anti-inflammatory cytokines in pathophysiology of bacterial meningitis and effect of adjunctive therapy. Infect. Immun. **64:**4883-4890.

106. **Van Furth, A. M., E. M. Verhard-Seijmonsbergen, J. A. M. Langermans, J. T. Van Dissel, and R. Van Furth.** 1999. Anti-CD14 monoclonal antibodies inhibit the production of tumor necrosis factor alpha and interleukin-10 by human monocytes stimulated with killed and live *Haemophilus influenzae* or *Streptococcus pneumoniae* organisms. Infect. Immun. **67:**3714-3718.

107. **Vasconcelos, D., D. M. Middleton, and J. M. Chirino-Trejo.** 1994. Lesions caused by natural infection with *Streptococcus suis* type 9 in weaned pigs. J. Vet. Diagn. Invest. **6:**335-341.

108. **Vecht, U., H. J. Wisselink, M. L. Jellema, and H. E. Smith.** 1991. Identification of two proteins associated with virulence of *Streptococcus suis* type 2. Infect. Immun. **59:**3156-3162.

109. **Verhoef, J., and E. Mattsson.** 1995. The role of cytokines in Gram-positive bacterial shock. Trends Microbiol. **3:**136-140.

110. **Virkola, R., K. Lähteenmäki, T. Eberhard, P. Kuusela, L. van Alphen, M. Ullberg, and K. Korhonen.** 1996. Interaction of *Haemophilus influenzae* with the mammalian extracellular matrix. J. Infect. Dis. **173:**1137-1147.

111. **Von Hunolstein, C., A. Totolian, G. Alfarone, G. Mancuso, V. Cusumano, G. Teti, and G. Orefici.** 1997. Soluble antigens from group B streptococci induce cytokine production in human blood cultures. Infect. Immun. **65:**4017-4021.

112. **Weidemann, B., J. Schletter, R. Dziarski, S. Kusumoto, F. Stelter, E. T. Rietschel, H. D. Flad, and A. J. Ulmer.** 1997. Specific binding of soluble

peptidoglycan and muramyldipeptide to CD14 on human monocytes. Infect. Immun. **65:**858-864.

113. **Weir, D. M., J. Stewart, and E. Glass.** 1982. Phagocyte recognition by lectin receptors. Immunobiol. **161:**334-344.
114. **Wessels, M. R., R. F. Haft, L. M. Heggen, and C. E. Rubens.** 1992. Identification of a genetic locus essential for capsule sialylation in type III group B streptococci. Infect. Immun. **60:**392-400.
115. **Wessels, M. R., C. E. Rubens, V. J. Benedi, and D. L. Kasper.** 1989. Definition of a bacterial virulence factor: sialylation of the group B streptococcal capsule. Proc. Natl. Acad. Sci. USA **86:**8983-8987.
116. **Wibawan, I. W., and C. Lammler.** 1994. Relation between encapsulation and various properties of *Streptococcus suis*. Zentralbl. Veterinarmed. [B] **41:**453-459.
117. **Wightman, P. D., and C. R. H. Raetz.** 1984. The activation of protein kinase C by biologically active lipid moieties of lipopolysaccharide. J. Biol. Chem **259:**10048-10052.
118. **Williams, A. E.** 1990. Relationship between intracellular survival in macrophages and pathogenicity of *Streptococcus suis* type 2 isolates. Microb. Pathog. **8:**189-196.
119. **Williams, A. E., and W. F. Blakemore.** 1990. Monocyte-mediated entry of pathogens into the central nervous system. Neuropathol. Appl. Neurobiol. **16:**377-392.
120. **Williams, A. E., and W. F. Blakemore.** 1990. Pathogenesis of meningitis caused by *Streptococcus suis* type 2. J. Infect. Dis. **162:**474-481.
121. **Williams, A. E., W. F. Blakemore, and T. J. Alexander.** 1988. Observations on the pathogenesis of meningitis caused by *Streptococcus suis* type 2. Presented at the IPVS.
122. **Wilson, S., and D. A. Drevets.** 1998. *Listeria monocytogenes* infection and activation of human brain microvascular endothelial cells. J. Infect. Dis. **178:**1658-1666.
123. **Yamamoto, Y., S. Okubo, T. W. Klein, K. Onozaki, T. Saito, and H. Friedman.** 1994. Binding of *Legionella pneumophila* to macrophages increases cellular cytokine mRNA. Infect. Immun. **62:**3947-3956.

V. GENERAL CONCLUSIONS

Results from the present work demonstrate that:

✓ The *S. suis* type 2 CPS is an antiphagocytic factor, that protects bacteria from being ingested by macrophages.

✓ *S. suis* remains extracellularly bound to the surface of macrophages, and the CPS is in part responsible for this binding activity.

✓ At high bacterial concentrations, one of the consequences of *S. suis* interaction with phagocytes is cell damage due to the secretion of the suilysin.

✓ Binding of *S. suis* to the surface of phagocytes induces the release of several pro-inflammatory cytokines and chemokines, by both CD14-dependent and -independent mechanisms. Bacterial cell wall seems to be the major *S. suis* modulin.

Despite the fact that the enigma of the pathogenesis of *S. suis* meningitis remains to be solved, our findings suggest that interactions of *S. suis* type 2 with phagocytic cells may represent a key step in the pathogenesis of the infection caused by this pathogen. The result of this interaction would be bacterial dissemination and induction of an acute inflammatory response which could therefore be implicated in the initiation and development of meningitis caused by this microorganism. Invasion of the CNS may represent a synergistic event between free and associated bacteria, and the potential to establish infection relies on the capacity of an individual strain to produce a still unknown panel of virulence factors. Thus, the virulence of a strain will be the result of complex interactions between the organism and its host. A complete understanding of the interacting pathways will give important insights into disease progression. Further studies to delineate the mechanisms by which *S. suis* induces meningitis would contribute to greater knowledge and result in potential therapies to control *S. suis* infections.

VI. ANNEXE

ARTICLE V

Pro-inflammatory Cytokine and Chemokine Release by Human Brain Microvascular Endothelial Cells Stimulated by *Streptococcus suis* Serotype 2

Nathalie Vadeboncoeur, Mariela Segura, Dina Al-Numani,
Ghyslaine Vanier, and Marcelo Gottschalk

Groupe de Recherche sur les Maladies Infectieuses du Porc (GREMIP), Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, Québec, Canada

FEMS Immunology and Medical Microbiology. 2002. ***Submitted for publication***

Role of the candidate in conception of this article:

I have actively participated in the conception and design of the experiments. I have also helped the main author with the standardization of techniques, as well as the analysis of results. I have also assisted in graphic conception as well as in critical analysis of the manuscript.

ABSTRACT

Streptococcus suis serotype 2 is a world wide agent of diseases among pigs including meningitis, septicemia and arthritis. This microorganism is also recognized as an important zoonotic agent. The pathogenesis of the meningitis caused by *S. suis* is poorly understood. We have previously shown that *S. suis* is able to adhere to human brain microvascular endothelial cells (BMEC), but not to human umbilical vein endothelial cells (HUVEC). The objective of this work was to study the ability of *S. suis* serotype 2 to induce the release of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukin- (IL) 1; IL-6 and the chemokines IL-8 and monocyte chemotactic protein-1 (MCP-1) by human BMEC and HUVEC, using a sandwich ELISA. *S. suis* was able to stimulate the production of IL-6, IL-8 and MCP-1 by BMEC but not HUVEC, in a time- and concentration- dependent manner. Bacterial cell wall components were largely responsible for such stimulation. The human and pig origin of strains does not seem to affect the intensity of the response; indeed, a very heterogeneous pattern of cytokine and chemokine production was observed for the different strains tested in this study. *In situ* production of cytokines and chemokines by BMEC may be the result of specific adhesion of *S. suis* to this cell type, with several consequences such as increased recruitment of leukocytes and an increase in the blood-brain-barrier permeability.

INTRODUCTION

Streptococcus suis causes many swine diseases including meningitis, septicemia, arthritis and pneumonia. Of the 35 official serotypes described to date, serotype 2 is the most virulent and the most commonly isolated from diseased pigs. This miroorganism is also recognized as an agent of zoonosis. In fact, over 200 cases of human infection by *S. suis* have been reported, especially among persons in close contact with pigs or pig products. *S. suis* causes mainly meningitis in humans, where hearing loss is the most frequent sequela (15).

The pathogenesis of meningitis caused by *S. suis* is poorly understood and is probably a multistep process. It is not known how bacteria are able to traverse the epithelial barriers to reach the bloodstream (13). Once there, bacteria can travel inside monocytes (46) or free in circulation, as demonstrated by several studies during the last decade (4, 5, 13, 32). In fact, the presence of a polysaccharidic capsule (CPS) protects bacteria against phagocytosis (4, 32). The mechanisms by which *S. suis* traverses the blood-brain barrier (BBB) into the subarachnoid space to cause meningitis are uknown. Other meningeal pathogens including *Streptococcus pneumoniae*, *Escherichia coli* K1 and group B *Streptococcus* (GBS), are known to interact directly with the BBB as free bacteria (41). This barrier, responsible for maintaining biochemical homeostasis within the central nervous system (CNS), is characterized by intercellular tight junctions that regulate fluid, macromolecule and cell traffic across the layer (41). The BBB is composed of the arachnoid membrane, the brain microvascular endothelial cells (BMEC) and the choroid plexus. The primary site of breakdown of the BBB in most bacterial meningitis appears to be the BMEC (40).

It is generally accepted that bacterial interactions with BMEC are mainly characterized by specific attachment and consequent invasion, toxicity and increased permeability (13). *S. suis* serotype 2 has been shown to adhere to human BMEC, but unlike other meningeal pathogens, invasion does not occurs. The adhesion appears to be related to the cell type, as *S. suis* does not adhere to human umbilical vein endothelial cells (HUVEC) (6). Adhesion of *S. suis* to BMEC may have different consequences which may lead to increased permeability of the BBB. For example, some strains produce a toxin (suilysin) (12, 17), that was reported as being toxic for BMEC and other cells (6, 21). However, only European strains of *S. suis* produce

this hemolysin (12). In fact, production of toxic factors by the majority of virulent North American strains, that might lead to cell damage and BBB increased permeability, has not been described so far. This suggests that the pathogenesis of meningitis produced by European and North American strains may differ. In fact, other virulence-related proteins are produced mainly by European strains (13, 45).

Increased BBB permeability may also be induced by inflammatory mediators that might be produced following adhesion of bacteria to cells. Recent work in our laboratory show that *S. suis* is not only able to interact with monocytes/macrophages, but is also able to induce the release of several proinflammatory cytokines and chemokines, such as tumor necrosis factor alpha (TNF-α), interleukin- (IL) 1, IL-6, IL-8 and monocyte chemotactic protein-1 (MCP-1) (27, 28). However, the source of proinflammatory cytokines in cerebrospinal fluid during meningitis may be microglial and endothelial cells as well as migrating leukocytes (13, 35). Recent studies show that proinflammatory cytokines are produced by BMEC that are stimulated either by other cytokines (such as TNF-α or IL-1) or by a direct interaction with microbial pathogens (8). Cytokines and adhesion molecules expressed by endothelial cells are known to be key players in regulating the recruitment of leukocytes to the sites of inflammation (16). The objective of this work was to study the ability of *S. suis* serotype 2 to induce the release of the pro-inflammatory cytokines TNF-α, IL-1, IL-6 and the chemokines IL-8 and MCP-1, by human BMEC and HUVEC.

MATERIALS AND METHODS

Bacterial strains and growth conditions

S. suis capsular type 2 virulent strains 89-1591 and S735, from North America (Canada) and Europe (The Netherlands), respectively, were used throughout this study. The isogenic unencapsulated mutant 2A, derived from strain S735 and obtained by Tn*916* transposition, was also used (4). Other porcine and human strains of *S. suis* also used in this study are listed in Table 1.

TABLE 1. *S. suis* capsular type 2 strains of porcine and human origins used in this study

Strain	Origin	Virulence [a]	Geographic origin
31533	Diseased pig	V	France
S735 [b c]	Diseased pig	V	The Netherlands
D282	Diseased pig	V	The Netherlands
94-623	Pig, healthy carrier	NV	France
TD10	Pig, healthy carrier	NV	UK
89-1591 [b]	Diseased pig	V	Canada
90-1330	Diseased pig	NV	Canada
89-999	Diseased pig	V	Canada
Reims	Human; spondylodiscitis	NT	France
EUD95	Human; meningitis	NT	France
Biotype 2	Human; endocarditis	NT	France
HUD Limoge	Human; septic shock	NT	France
FRU95	Human; meningitis	NT	France
LEF95	Human; meningitis	NT	France
96-52466	Human; arthritis	NT	France
H11/1	Human; meningitis	V	UK
AR770353	Human; meningitis	NT	The Netherlands
AR770297	Human; meningitis	NT	The Netherlands
91-1804	Human; endocarditis	NT	Canada
94-3037	Human; meningitis	NT	Canada
98-3634	Human; endocarditis	NT	Canada
99-734723688	Human; septicaemia	NT	Canada

[a] As indicated in the literature by using experimental porcine models (19, 24, 44). V: virulent; NV: non virulent; NT: never tested. Strain H11/1: P. Norton, personal communication.
[b] Strains used as reference in the present work.
[c] ATCC 43765 *S. suis* type 2 reference strain.

Bacteria, maintained as stock cultures in 50% glycerol-Todd-Hewitt broth (THB; Difco Lab., Detroit, MI) at -80°C, were grown overnight on bovine blood agar plates at 37°C and isolated colonies were used as inocula for THB, that were incubated for 18 h at 37°C. Working cultures for endothelial cell stimulation were produced by inoculating 10 ml of these cultures in 200 ml of THB at 37°C with agitation until they reached the mid-log phase (6 h incubation-time; optical density at 540 nm of 0.4-0.5). Bacteria were washed twice in phosphate-buffered saline (PBS) pH 7.4, and diluted to approximately 10^9 CFU ml^{-1} in PBS. A more accurate determination of the CFU ml^{-1} in the final suspension was made by plating on THB-agar. Bacteria were then killed by heat treatment at 60°C for 45 min (minimal experimental condition required for killing of *S. suis*) (27). Subcultures of the heat-treated suspension on blood agar plates were incubated at 37°C for 48 h to confirm the absence of viable organisms. Killed bacterial preparations were stored at 4°C and re-suspended in cell culture media just before stimulation assays.

Cell lines and cell culture

Human brain microvascular endothelial cell line (BMEC), originating from a brain biopsy of an adult human female with epilepsy was kindly provided by Dr. K. Kim, Johns Hopkins University School of Medicine, Baltimore, MD. Cells had been immortalized by transfection with simian virus 40 large T antigen and were shown to maintain their morphologic and functional characteristics (37). Cells were grown in RPMI 1640 medium (Gibco, Burlington, VT) supplemented with 10% heat-inactivated FBS (Gibco), 10% Nu-serum IV supplement (Becton Dickinson, Bedford, MA), L-glutamin (ICN Biomedical Inc., Aurora, OH) and penicillin-streptomycin (5,000 U ml^{-1}) (Gibco). Flasks (Falcon) and 24-well tissue culture plates (Becton Dickinson) were precoated with rat tail collagen to support the cells (6). HUVEC derived from human umbilical cord were purchased from the American Type Culture Collection (ATCC CRL-1730). Cells were grown in F-12K medium (Sigma-Aldrich, Oakville, Ontario, Canada) supplemented with 10% heat-inactivated FBS, endothelial cell growth supplement (30 µg ml^{-1}) (Becton Dickinson) and penicillin-streptomycin. Flasks and 24-well tissue culture plates were pre-coated with 1% gelatin to support the cells. Both types of endothelial cells were incubated at

37°C, with 5% CO_2 in a humid atmosphere. Cells were used before passage 35 for all experiments.

Stimulation of cells

For stimulation assays, 48 h cultures of BMEC or HUVEC cells in flasks were trypsinized and diluted in culture media at 10^5 cells ml^{-1}, and 1 ml of this suspension was distributed in 24-well plates and incubated to confluence. At confluence, medium was removed and heat-killed *S. suis* strains (1 ml) was added at appropriate dilutions made in culture media. Furthermore, cytokine induction by *S. suis* strain S735 was compared to that by its unencapsulated mutant 2A (4). In separate expermients, cells were stimulated with different concentrations of purified CPS, cell wall or suilysin, purified as previously described (17, 27 ,30). Endothelial cells stimulated with lipopolysaccharide (LPS) from *E. coli* 0127:B8 (Sigma-Aldrich) (10 μg ml^{-1} for BMEC and 1 μg ml^{-1} for HUVEC) served as a positive control. Cells incubated in medium alone served as controls for spontaneous cytokine release. Cytokine induction plates were incubated at 37°C, 5% CO_2 in a humid atmosphere. At different time intervals (see below), culture supernatants were harvested from individual wells, and the supernatants aliquoted and frozen at -20°C until cytokine determinations were performed. Each test of BMEC or HUVEC stimulation was repeated at least three times. All solutions and bacterial preparations used in this study were tested for the presence of endotoxin by a *Limulus* amebocyte lysate (LAL) gel-clot test (Pyrotell STV, Cape Cod, Falmouth, MA) with a sensitivity limit of 0.03 EU ml^{-1}. In some experiments, endotoxin contamination during stimulation of endothelial cells was controlled by parallel assays with Polymixin B (PmB; 10 μg ml^{-1}). Results from the LAL test and/or data from PmB treatment demonstrated no significant levels of endotoxin contamination from different bacterial preparations (data not shown). Cell culture medium contained less than 0.03 EU ml^{-1}. Absence of cell toxicity at all bacterial concentrations as well as with purified bacterial components was confirmed by the lactate dehydrogenase cellular injury assay, as previously described (6).

Enzyme-linked immunosorbent assays (ELISA) for cytokines

IL-1, TNF-α, IL-6, IL-8 and MCP-1 were measured by sandwich ELISA, using pair matched monoclonal antibodies from R&D Systems (Minneapolis, MN), as previously described (28). Standard curves were included in each ELISA plate (Nunc, VWR, Quebec, Canada) as twofold dilutions of recombinant (R&D Systems) human IL-6 (1500 to 3 pg ml^{-1}), IL-8 (600 to 5 pg ml^{-1}), MCP-1 (500 to 8 pg ml^{-1}), IL-1 (300 to 5 pg ml^{-1}) or TNF-α (3000 to 188 pg ml^{-1}). Supernatant dilutions giving optical density readings in the linear portion of the appropriate standard curve were used to determine the level of each cytokine in the samples. Standard and sample dilutions were added in duplicate wells to each ELISA plate.

Statistical analysis

ELISA tests were performed at least four times for each individual endothelial cell stimulation assay. Results were derived from linear regression calculations and expressed in pg ml^{-1} of cytokine. Differences were analyzed for significance by using the Student's unpaired *t* test (two-tailed *P* value), with a *P* value <0.05 considered as significant. Differences between the human and porcine origin group of strains and differences among strains within the same group were analyzed for significance by using general linear models, followed by Tukey-Kramer post-hoc tests for differences between strains. The SAS software (SAS, Cary, NC) was used for these analyses.

RESULTS AND DISCUSSION

BMEC but not HUVEC produce IL-6, IL-8 and MCP-1 after *S. suis* whole cell stimulation

Previous studies have shown that stimulated endothelial cells are able to produce IL-6 and different chemokines (3, 25, 26, 34, 44, 48). Unstimulated BMEC yielded low basal levels of IL-6, IL-8 and MCP-1 expression. These basal values were substracted to correct data obtained after *S. suis* or LPS stimulation throughout this work. The production of cytokines and chemokines by BMEC stimulated with *S. suis* varied with incubation time. Both *S. suis* North American 89-1591 and European S735 strains were able to induce high levels of IL-6, low levels of MCP-1 and intermediate levels of IL-8 from stimulated BMEC and no significant difference in production was observed between the two strains ($P > 0.05$). Maximal release of IL-6 was achieved between 12 and 48 h of incubation with bacteria. On the other hand, LPS stimulation lead to a gradual increase of IL-6 with time, reaching its highest level at 48 h of incubation (Fig. 1A). Similar kinetics for IL-8 and MCP-1 production were observed with both bacteria and LPS, with maximal release observed after 48 h of incubation (Fig. 1B and C). However, cytokine levels released by cells stimulated with LPS were significantly higher ($P < 0.001$). Endothelial cells have been shown to release cytokines and chemokines with (18, 31, 33) or without (25) pre-activation with IL-1 and/or TNF-α. Since the BMEC used in this study did not produce either cytokine (see below), the observed levels of IL-6, IL-8 and MCP-1 could be considered TNF-α- and IL-1-independent. A possible amplifying role of IL-1 and TNF-α in vivo should not be ruled out. The BBB is a complex system that involves dynamic interplay of BMEC with perivascular cells such as astrocytes and macrophages. Our system focused only on the capacity of BMEC to produce proinflammatory cytokines and chemokines and did not attempt to capture the full complexity of these interactions.

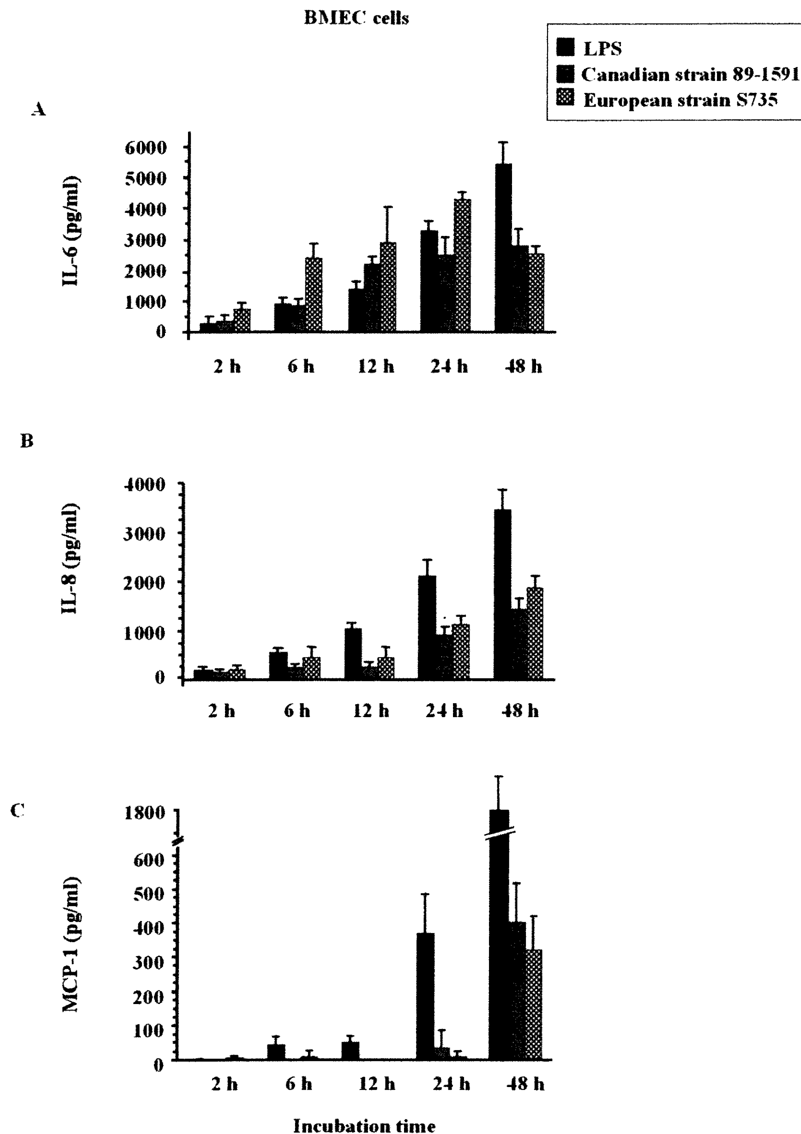


FIG. 1. Time course of production of IL-6 (A), IL-8 (B) and MCP-1 (C) by BMEC after stimulation with heat-killed *S. suis* serotype 2 (strains 89-1591 and S735; 10^9CFU/ml). Purified LPS (10 μg/ml) was used as a positive control. Culture supernatants were harvested at different time intervals and assayed for cytokine production by ELISA. Data are expressed as mean ± standard deviations (in pg/ml). Values for basal cytokine expression (cell culture medium alone) were corrected from data obtained after *S. suis* or LPS stimulation.

As done for BMEC, basal production of the cytokine and chemokines by HUVEC was subtracted from experimental results. Although a strong response was

observed with LPS, *S. suis* was not able to induce any upregulation of IL-6, IL-8 or MCP-1 from *S. suis* stimulated HUVEC (Fig. 2A-C).

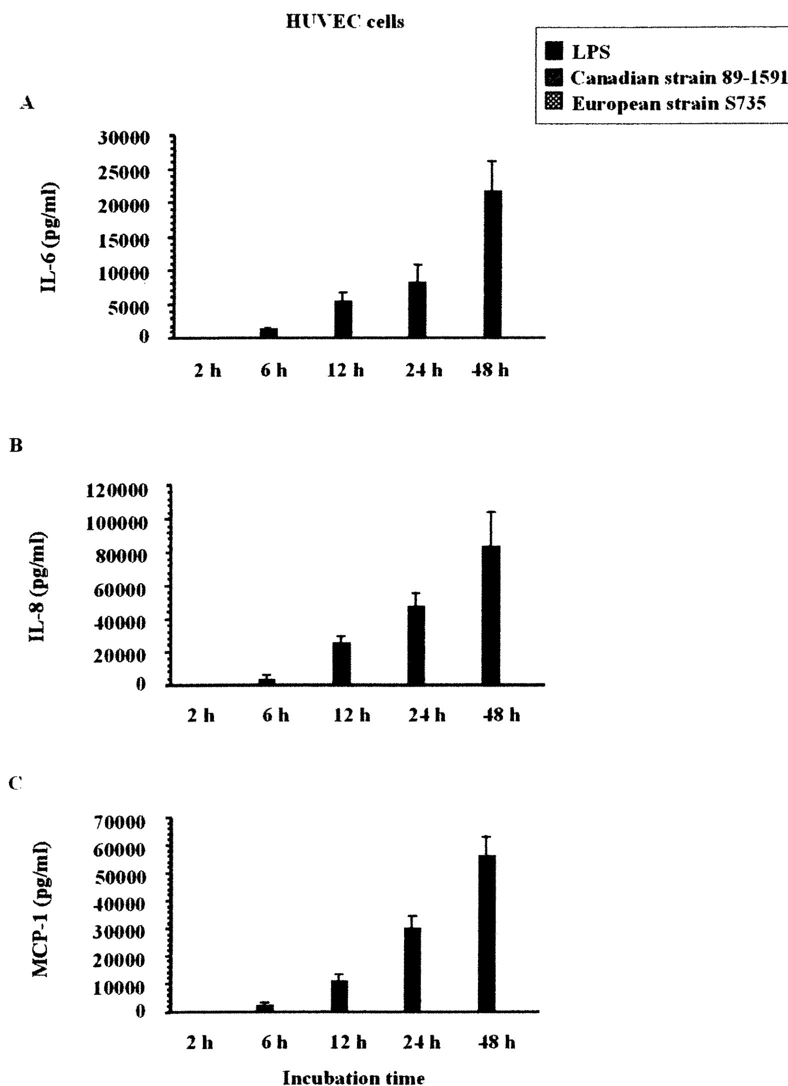


FIG. 2. Time course of production of IL-6 (A), IL-8 (B) and MCP-1 (C) by HUVEC after stimulation with heat-killed *S. suis* serotype 2 (strains 89-1591 and S735; 10^9CFU/ml). Purified LPS (1 μg/ml) was used as a positive control. Culture supernatants were harvested at different time intervals and assayed for cytokine production by ELISA. Data are expressed as mean ± standard deviations (in pg/ml). Values for basal cytokine expression (cell culture medium alone) were corrected from data obtained after *S. suis* or LPS stimulation.

HUVEC response appeared to be more sensitive to LPS than BMEC, as a much higher level of production was observed even though the concentration of LPS used for HUVEC induction was 10 times lower than that used for BMEC. A possible explanation for the sensitivity of BMEC to *S. suis* is that the induction of cytokines is the result of bacteria-cell adhesion. As indicated previously, *S. suis* is able to adhere to BMEC but not to HUVEC (6). It has already been shown for other bacterial species that adhesion is needed to stimulate cytokine production in cells (47). For example, *Streptococcus bovis* is able to induce IL-8 expression after adhesion to endothelial cells (9) and only adherent *N. meningitidis* induces the expression of TNF-α by endothelial cells (38). Similarly, the cytokine-stimulatory activity of the capsular polysaccharide of *Staphylococcus aureus* on endothelial cells resulted from ligand-receptor interactions (34). Another possible explanation is that some receptors, such as the toll-like receptor 2, which has been shown to confer responsiveness to a wide variety of Gram-positive bacterial cell wall components (43), are present in the BMEC but absent in the HUVEC tested in this study. It has been recently reported that *S. suis* is able to induce the up-regulation of pro-inflammatory cytokines from monocytes by CD14-dependent and -independent pathways (28). Receptors activated by *S. suis*, other than CD14, are presently under study in our laboratory. Since *S. suis* is not able to invade BMEC (6), cytokine activation seems to take place without cell invasion. Similarly, activation and induction of cytokine production in endothelial cells stimulated with *Listeria monocytogenes* occurs without cellular invasion (26). *S. suis* is also able to adhere to, but is not ingested, by monocytes, and this interaction induces the release of large amounts of pro-inflammatory cytokines (28).

BMEC and HUVEC tested in this study were not able to produce IL-1 or TNF-α after stimulation with high doses of LPS or *S. suis*, even if pre-stimulated with interferon-gamma (not shown). Furthermore, no mRNA signal corresponding to any of these cytokines could be detected by RT-PCR after LPS stimulation of BMEC (unpublished observations). It has been shown that oral viridans streptococci are able to induce IL-6 and IL-8, but not TNF-α or IL-1, from stimulated endothelial cells (44) and *Neisseria meningitidis*-stimulated endothelial cells induce the production of TNF-α only in the presence of monocytes (38). Other reports indicate that IL-1 production (or gene expression) occurs in endothelial cells stimulated with bacteria

or LPS (3, 23, 48). Since the cells used in this study did not produce any of these cytokines with the positive control used, no conclusion on a possible IL-1 and TNF-α induction by *S. suis* could be made. Interestingly, both cytokines are significantly up-regulated when human monocytes are activated by *S. suis* (28).

Bacterial-concentration-dependent cytokine release

The effect of bacterial concentration on cytokine production was determined. Cell-culture supernatants were harvested after 24 h of stimulation to evaluate IL-6 production, and after 48 h to measure IL-8 and MCP-1 induction and BMEC were exposed to different concentrations of heat-killed *S. suis* strains 89-1591 or S735. Cytokine induction varied directly with bacterial concentration, and only a high concentration of bacteria was able to induce cytokine production. In fact, a concentration greater than 2.5×10^8 CFU ml^{-1} was needed to obtain cytokine release (data not shown). This is in agreement with results observed with human monocytes and murine macrophages (27, 28). Interestingly, the presence of clinical signs and symptoms in diseased animals correlates with those high levels of virulent bacteria in the bloodstream (2).

Lack of relationship between the origin or virulence of the strains and cytokine induction

Despite the fact that *S. suis* serotype 2 is usually associated with severe occupational disease in humans (15), studies using strains of human origin are limited. Since cells used in this and earlier studies (21, 28) are of human origin, it was relevant to compare the ability of porcine strains to induce cytokine release with those recovered from serious cases of human disease. A very heterogeneous pattern of cytokine production was observed, with no tendency for human strains to induce higher cytokine levels (Fig. 3). Tukey-Kramer post-hoc tests revealed significant differences between strains within each group. In fact, no consistent effect on cytokine production could be attributed to the origin of the strains, specially for those of human origin. Similar observations have been reported for *S. suis* interaction with human monocytes, for oral viridans streptococci and for *S. aureus* (28, 44, 48). The observed variability may be due to the degree of exposure and/or type of components of the bacterial surface, such as bacterial cell wall, which can stimulate cytokine release from endothelial cells. Despite the epidemiological fact that pigs may be the

sole source of human infections (7, 39), cases of *S. suis* infection in individuals not associated with the porcine industry have also been reported (22). Thus, the clinical relevance of potential species-specific differences in reactivity to bacterial strains still remains unclear.

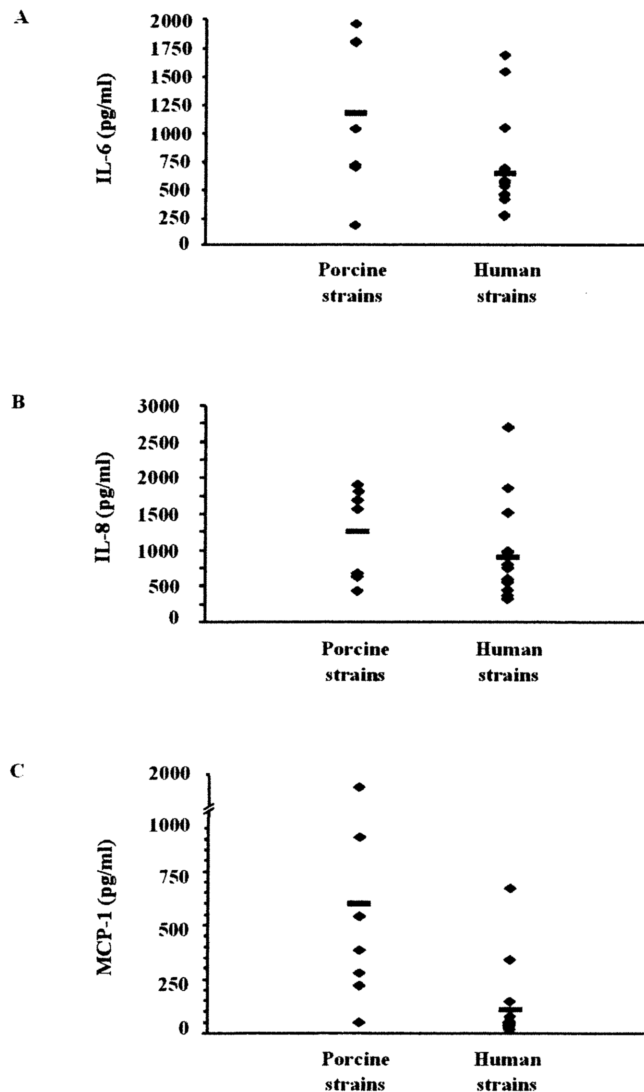


FIG. 3. Comparative study of cytokine production by different *S. suis* strains. BMEC were stimulated by heat-killed (10^9 CFU/ml) *S. suis* serotype 2 strains from human or porcine origin (Table 1). IL-6 (A), after 24 h incubation, and IL-8 (B) and MCP-1 (C) after 48 h incubation, were measured by ELISA titration of stimulated cell supernatants. Lines represent average cytokine production by each group of strains.

The ability of virulent and non virulent strains of *S. suis* to induce IL-6, IL-8 and MCP-1 was also compared. The concept of virulence for *S. suis* is currently debated in the literature (11, 13). In this study, we considered a strain as "virulent" or "non-virulent" depending on the presence or absence of clinical disease after experimental infections in piglets (Table 1). In this work, no association was observed between the cytokine response and the virulence of the strain. This is in agreement with results reported by Segura *et al.* (28). Similarly, there were no observed differences in the adhesion to different types of host cells, including BMEC, between virulent and non-virulent strains (unpublished observations). Unlike other important streptococcal species, information on *S. suis* virulence factors as well as on surface expressed molecules is limited (13). It has been shown in other bacterial species, such us *Rodococcus equi*, that virulence is not necessarily correlated with the level of cytokine production (10). In the case of *S. suis*, it has been suggested that only virulent strains are able to survive at high numbers in the bloodstream and induce disease (13). Recent research indicates that, unlike non-virulent strains, virulent *S. suis* strains are able to survive in circulation at high concentrations for more than 6 days (2).

Relative role of bacterial components in cytokine production

Different bacterial structures and products have been potentially implicated in the pathogenesis of the *S. suis* infection (13, 36), but understanding of the effect that these proposed virulence factors have on cytokine release is limited. In the present study, *S. suis* purified cell wall material was able to induce IL-6 and IL-8 production by BMEC. At a concentration of 500 $\mu g\ ml^{-1}$ of cell wall, the cytokine production level was roughly equivalent to half of that produced by whole bacteria. It has previously been shown that the cell wall of *S. suis* is the main component responsible for cytokine induction by murine macrophages (27). Similarly, antigens extracted from the cell wall of *S. bovis* and pneumocci induce pro-inflammatory cytokines from different type of cells (9, 42). The possible role of cell wall components of *S. suis* on the upregulation of IL-6 and IL-8 was confirmed by the use of an unencapsulated mutant, that was able to induce higher levels of IL-6 and IL-8, than the encapsulated parent S735 strain ($P < 0.001$) (Fig. 4 A and B). In the present study, the capacity of the unencapsulated mutant to induce higher levels of IL-6 and

IL-8 was probably not the result of greater adhesion of bacteria to cells, since both encapsulated and unencapsulated strains adhere similarly to BMEC (6).

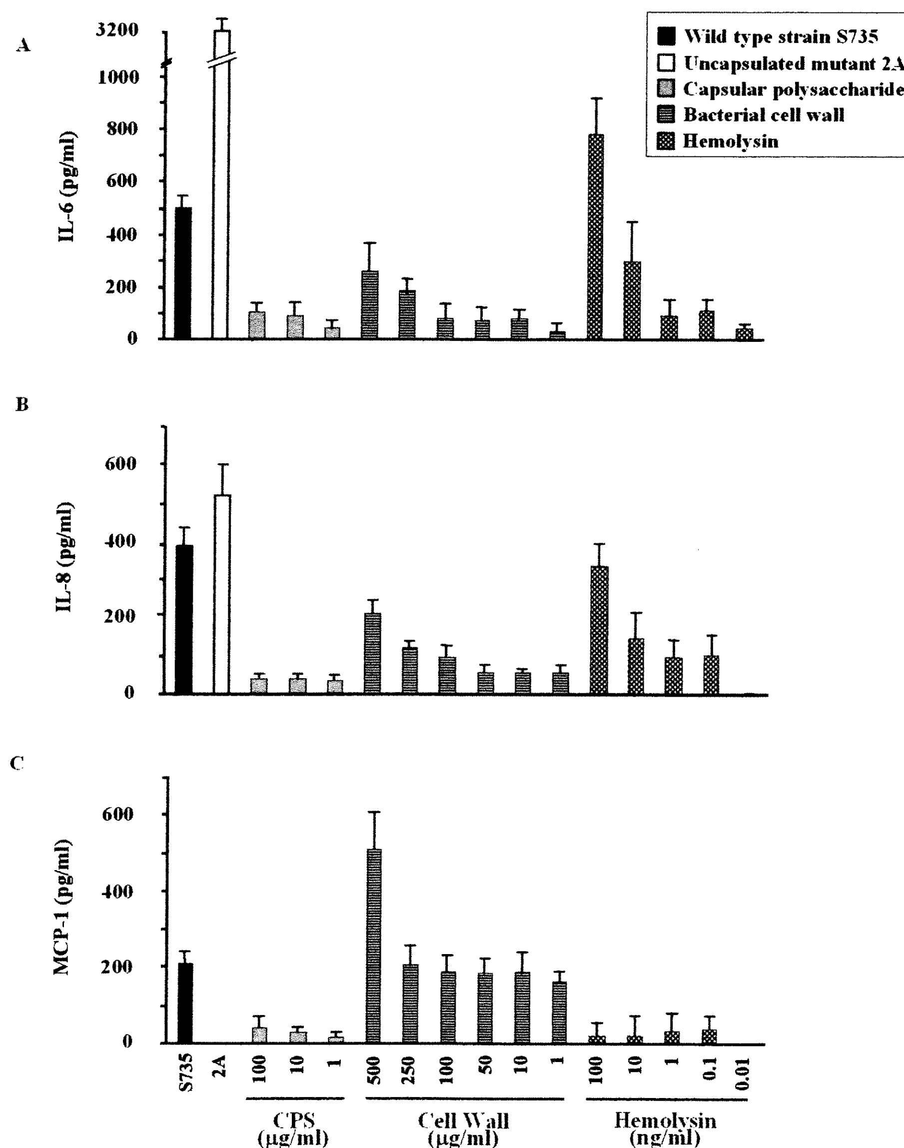


FIG. 4. Comparative study of IL-6 (A), IL-8 (B) and MCP-1 (C) production by differents proposed virulence factors for *S. suis* serotype 2. BMEC were stimulated with different concentrations of purified polysaccharide capsule, cell wall or hemolysin; in addition, the cytokine induction by *S. suis* strain S735 was compared to that obtained with its unencapsulated mutant 2A. Data are expressed as mean ± standard deviations (in pg/ml). Values for basal cytokine expression (cell culture medium alone) were corrected from data obtained after stimulation.

Results indicating that the capsule itself has no effect on the upregulation of these cytokines were confirmed by testing different concentrations of purified CPS. Concentrations as high as 100 $\mu g\ ml^{-1}$ did not induce significant levels of cytokine release compared to either negative control or whole bacteria (Fig. 4 A and B). This is in agreement with results previously observed with murine macrophages (27). Several in vitro and in vivo studies with purified CPS or with unencapsulated mutants failed to demonstrate a major role for capsular polysaccharide of pathogenic gram-positive cocci in cytokine induction (14). In the case of *S. suis*, however, the capsule may indirectly contribute to cytokine induction. In fact, the polysaccharide capsule is probably responsible for the progression of the disease by allowing *S. suis* to evade host defense mechanisms such as phagocytosis (29). As shown in this and previous works (27, 28), a high concentration of bacteria is needed to up-regulate the production of proinflammatory cytokines. Thus, only well encapsulated bacteria may be protected and survive at high concentrations in the bloodstream to reach the BBB and stimulate cells.

Since it has been shown that several toxins can stimulate or modulate the inflammatory mediator cascade (20), the cytokine induction by the extracellular hemolysin (suilysin), a possible virulence factor among European strains (13), was determined. A high IL-6 and IL-8 response was obtained with purified suilysin (Fig. 4 A and B). Rose et al. (26) have recently demonstrated that listeriolysin, a hemolysin produced by *L. monocytogenes*, is largely responsible for endothelial cytokine upregulation. The production of suilysin by European strains may contribute to a higher local inflammatory response. The fact that virulent European suilysin-positive strains present a higher virulence potential than virulent North American suilysin-negative strains has already been proposed (10). Furthermore, it has been shown that a suilysin-negative mutant was not virulent for mice and less virulent for pigs than its hemolytic parent strain (1). In the present study, heat-killed washed bacterial suspensions (free of suilysin) were used as stimuli for BMEC. Thus, the inherent capacity of suilysin-positive strains to induce cytokines by BMEC may have been underestimated.

The induction kinetics for MCP-1 production following stimulation with purified components of *S. suis* was somehow different from that obtained for IL-6 and IL-8. BMEC were insensitive to purified CPS but extremely responsive to low concentrations of cell wall (Fig. 4C). In fact, purified cell wall concentrations as low

as 1 μg induced a MCP-1 level similar to that obtained with whole bacteria. However, no activation was obtained with the unencapsulated mutant. Interestingly, purified cell wall was produced from the same mutant (4). It may be hypothesized that the surface expression of MCP-1 stimulating components has been affected in the mutant and cell wall purification methods could make these components available for BMEC stimulation. As previously observed with *S. suis* stimulated macrophages, bacterial molecules responsible for stimulating the up-regulation of different cytokines are probably different and present in the cell wall (27). Finally, no significant upregulation of MCP-1 could be observed after stimulation of cells with suilysin (Fig. 4C).

The fact that *S. suis* activated vascular endothelium expresses several different cytokines supports the contention that these active molecules act as secondary immune response modulators. Cytokines released by the BBB may act to modulate their activity or that of nearby cells, such as astrocytes and glial cells. These pro-inflammatory cytokines may play an important role in initiating changes in permeability or adhesion properties of the same BMEC that allow the immune cells to infiltrate the CNS in cases of meningitis caused by *S. suis*.

ACKNOWLEDGMENTS

We wish to thank Dr. M. Kobisch (Centre National d'Études Vétérinaires et Alimentaires, Ploufragan, France), Dr. U. Vecht (DLO-Institute for Animal Science and Health, Lelystad, The Netherlands), Dr. T. Alexander (University of Cambridge, England), Dr. L. Brasme (Centre Hospitalier Universitaire de Reims, France), Dr. G. Grise (Hôpital des Feugrois, Elbeuf, France), Dr. B. Cattier (Centre Hospitalier Universitaire Bretonneau, Tours, France), Dr. B. François (Dupuytren Hospital, Limoges Cedex, France), and Dr. P. Norton (Institute for Animal Health, Compton, United Kingdom) for providing some of the *S. suis* type 2 strains used in this study.

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) grant # 0680154280, by the Fonds pour la Formation des Chercheurs et l'Aide à la Recherche du Québec (FCAR-équipe) grant # 99-ER-0214, and by the Canadian Research Network on Bacterial Pathogens of Swine.

REFERENCES

1. **Allen, A.G., Bolitho, S., Lindsay, H., Khan, S., Bryant, C., Norton, P., et al.** 2001. Generation and characterization of a defined mutant of *S. suis* lacking suilysin. Infect. Immun. **69:**2742-2735.
2. **Berthelot-Hérault, F., Cariolet, R., Labbé, R., Gottschalk, M., Cardinal, J. and Kobisch, M.** 2001. Experimental infection of specific pathogen free piglets with French strains of *S. suis* capsular type 2. Can. J. Vet. Res. **65:**196-200.
3. **Bourdoulous, S., Bensaid A., Martinez, D., Sheikboudou, C.,Trap, Strosberg, A. and Couraud, P.** 1995. Infection of bovine brain microvessel endothelial cells with *Cowdria ruminantium* elicits IL-1 beta, -6, and -8 mRNA production and expression of an unusual MHC class II DQ alpha transcript. J. Immunol. **154:**4032-4038.
4. **Charland, N., Harel, J., Kobisch, M., Lacasse, S. and Gottschalk, M.** 1998. *Streptococcus suis* serotype 2 mutants deficient in capsular expression. Microbiology **144:**325-332.
5. **Charland, N., Kobisch, M., Martineau-Doize, B., Jacques, M., and Gottschalk, M.** 1996. Role of capsular sialic acid in virulence and resistance to phagocytosis of *Streptococcus suis* capsular type 2. FEMS Immunol. Med. Microbiol. **14:**195-203.
6. **Charland, N., Nizet, N., Rubens, C.E. Kim, K.S. Lacouture, S. and Gottschalk M.** 2000. *Streptococcus suis* serotype 2 interactions with human brain microvascular endothelial cells. Infect. Immun. **68:**637-643.
7. **Chatellier, S., Gottschalk, M., Higgins, R., Brousseau, R. and Harel, J.** 1999. Relatedness of *Streptococcus suis* serotype 2 isolates from different geographic origins as evaluated by molecular finderprinting and phenotyping. J. Clin. Microbiol. **37:**362-366.
8. **Cho, N., Seong, S., Choi, M. and Kim, I.** 2001. Expression of chemokine genes in human dermal microvascular endothelial cell lines infected with *Orientia tsutsugamushi*. Infect. Immun. **69:**1265-1272.
9. **Ellmerich, S., Djouder, N., Scholler, M. and Klein, J.P.** 2000. Production of cytokines by monocytes, epithelial and endothelial cells activated by *Streptococcus bovis*. Cytokine **12:**26-31.
10. **Giguère, S. and Prescott, J.F.** 1998. Cytokine induction in murine macrophages infected with virulent and avirulent *Rhodococcus equi*. Infect. Immun. **66:**1848-1854.
11. **Gottschalk, M., Higgins, R. and Quessy, S.** 1999. Dilemma of the virulence of *Streptococcus suis* strains. J. Clin. Microbiol. **37:**4202-4203.
12. **Gottschalk, M., Lacouture, S. and Dubreuil, J.D.** 1995. Characterization of *Streptococcus suis* capsular type 2 haemolysin. Microbiology **141:**189-195.
13. **Gottschalk, M. and Segura, M.** 2000. The pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions. Vet. Microbiol. **76:**259-272.
14. **Heumann, D., Barras, C., Severien, C., Glauser, M.P. and Tomasz, A.** 1994. Gram-positive cell walls stimulate synthesis of tumor necrosis factor alpha and interleukin-6 by human monocytes. Infect. Immun. **62:**2715-2721.
15. **Higgins, R., and Gottschalk, M.** 1998. Streptococcal diseases. *In* A. D. Leman, B. E. Straw, W. L. Mengeling, S. D'Allaire, and D. J. Taylor (ed.), Diseases of swine, 8th ed. Iowa State University Press, Ames, Iowa.

16. **Imhof, B. A. and Dunon, D.** 1995. Leukocyte migration and adhesion. Adv. Immunol. **58:**345-416.

17. **Jacobs, A. A. C., Loeffen, P.L.W., van der Berg A.J.G. and Storm, P.K.** 1994. Identification, purification, and characterization of a thiol-activated hemolysin (suilysin) of *Streptococcus suis*. Infect. Immun. **62:**1741-1748.

18. **Jirik, F. R., Podor, T.J., Hirano, T., Kishimoto, T., Loskutoff, D.J., et al.** 1989. Bacterial lipopolysaccharide and inflammatory mediators augment IL-6 secretion by human endothelial cells. J. Immunol. **142:**144-147.

19. **Kobisch, M., Gottschalk, M., Morvan, P., Cariolet, R., Bénévent, G. and Joly, J.P.** 1995. Experimental infection of SPF piglets with *Streptococcus suis* serotype 2. Journées Rech. Porcine en France **27:**97-102.

20. **Koning, W., Kasimir, S., Hensler, T., Scheffer, J., Koning, B., Hilger, J., Brom, J. and Koller, M.** 1992. Release of inflammatory mediators by toxin stimulated immune system cells and platelets. Zbl. Bakt. Suppl. 23, 385-394.

21. **Lalonde, M., Segura, M., Lacouture, S. and Gottschalk, M.** 2000. Interactions between *Streptococcus suis* serotype 2 and different epithelial cell lines. Microbiology **146:**1913-1921.

22. **Leelarasamee, A., Nilakul, C., Tien-Grim, S., Srifuengfung, S and Susaengrat, W.** 1997. *Streptococcus suis* toxic-shock syndrom and meningitis. J. Med. Assoc. Thai. **80:**63-68.

23. **Miossec, P., Cavender, D. and Ziff, M.** 1986. Production of interleukin 1 by human endothelial cells. J. Immunol. **136:**2486-2491.

24. **Quessy, S., Dubreuil, J.D., Caya, M. and Higgins, R.** 1995. Discrimination of virulent and avirulent *Streptococcus suis* capsular type 2 isolates from different geographical origins. Infect. Immun. **63:**1975-1979.

25. **Reyes, T. M., Fabry, Z. and Coe, C.L.** 1999. Brain endothelial cell production of a neuroprotective cytokine, interleukin-6, in response to noxious stimuli. Brain Res. **851:**215-220.

26. **Rose, F., Zeller, S.A., Chakraborty, T., Domann, E., Machleidt, T., Seeger, W., Grimminger, F. and Sibelius, U.** 2001. Human endothelial cell activation and mediator release in response to *Listeria monocytogenes* virulence factors. Infect. Immun. **69:**897-905.

27. **Segura, M., Stankova, J. and Gottschalk, M.** 1999. Heat-killed *S. suis* capsular type 2 strains stimulate tumor necrosis factor alpha and interleukin-6 production by murine macrophages. Infect. Immun. **67:**4646-4654.

28. **Segura, M., Vadeboncoeur, N. and Gottschalk, M.** 2002. CD14-dependant and -independent cytokine and chemokine production by human monocytes stimulated by *S. suis* capsular type 2. Clin. Exp. Immunol., **127:**243-254.

29. **Segura, M. A., Cleroux, P. and Gottschalk, M.** 1998. *Streptococcus suis* and group B *Streptococcus* differ in their interactions with murine macrophages. FEMS Immunol. Med. Microbiol. **21:**189-195.

30. **Sepulveda, E. M. D., Altman, E., Kobisch, M., Dallaire, S. and Gottschalk, M.** 1996. Detection of antibodies against *Streptococcus suis* capsular type 2 using a purified capsular polysaccharide antigen-based indirect ELISA. Vet. Microbiol. **52:**113-125.

31. **Sica, A., Wang, J., Colotta, F., Dejana, E., et al.** 1990. Monocyte chemotactic and activating factor gene expression induced in endothelial cells by IL-1 and Tumor Necrosis Factor. J. Immunol. **144:**3034-3038.

32. **Smith, H., Damman, M., van den Velde, J., Wagenaar, F., Wisselink, H.J., Stockhofe-Z., N., Smits, M.** 1999. Identification and characterization

of the *cps* locus of *S. suis* type 2: the capsule protects against phagocytosis and is an important virulence factor. Infect. Immun. **67:**1750-1756.

33. **Soderquist, B., Kallman, J., Holmberg, H., Vikerfors, T. and Kihlstrom, E.** 1998. Secretion of IL-6, IL-8 and G-CSF by human endothelial cells in response to *Staphylococcus aureus* exotoxins. APMIS **106:**1157-1164.
34. **Soell, M., Diab, M., Haan-Archipoff, G., Beretz, A., Herbelin, C., Poutrel, B. and Klein, J.** 1995. Capsular polysaccharide types 5 and 8 of *Staphylococcus aureus* bind specifically to human epithelial (KB) cells, endothelial cells, and monocytes and induce release of cytokines. Infect. Immun. **63:**1380-1386.
35. **Sprenger, H., Rosler, A., Tonn, P., Brame, H.J., Huffmann, G. and Gensa, D.** 1996. Chemokines in the cerebrospinal fluid of patients with meningitis. Clin. Immunol. Immunopathol. **80:**155-161.
36. **Staats, J. J., Feder, I., Okwumabua, O. and Chengappa, M.M.** 1997. *Streptococcus suis*: past and present. Vet. Res. Commun. **21:**381-407.
37. **Stins, M. F., Prasadarao, N.V., Zhou, J., Arditi, M. and Kim, K. S.** 1997. Bovine brain microvascular endothelial cells transfected with SV40-large T antigen: development of an immortalized cell line to study pathophysiology of CNS disease. In Vitro Cell. Dev. Biol. **33:**243-247.
38. **Taha, M. K.** 2000. *Neisseria meningitidis* induces the expression of the TNF-alpha gene in endothelial cells. Cytokine **12:**21-25.
39. **Tarradas, C., Luque, I., De Andrès, D., Abdel-Aziz Shahein, Y.E., Pons, P., Gonzalez, F., Borge, C. and Perea, A.** 2001. Epidemiological relationship of human and swine *S. suis* isolates. J. Vet. Med. B **48:**347-355.
40. **Townsend, G. C., and Scheld, W.M.** 1995. Microbe-endothelium interactions in blood-brain barrier permeability during bacterial meningitis: Although bacterial pathogens can directly disrupt the barrier, a role for host factors is still under study. ASM News **61:**294-298.
41. **Tuomanen, E.** 1993. Breaching the blood-brain barrier. Sci. Am. 268, 80-84.
42. **Tuomanen, E., Tomasz, A., Hengstler, B. and Zak, O.** 1985. The relative role of bacterial cell wall and capsule in the induction of inflammation in pneumococcal meningitis. J. Infect. Dis. **151:**535-540.
43. **Vasselon, T. and Detmers, P.** 2002. Toll receptors: a central element in innate immune responses. Infect. Immun. **70:**1033-1041.
44. **Vernier, A., Diab, M., Soell, M., Haan-Archipoff, G., Beretz, A., Wachsmann, D. and Klein, J. P.** 1996. Cytokine production by human epithelial and endothelial cells following exposure to oral viridans streptococci involves lectin interactions between bacteria and cell surface receptors. Infect. Immun. **64:**3016-3022.
45. **Vetch, U., Arends, J., van Molen, E., van Leengoed, L.** 1989. Differences in virulence between two strains of *S. suis* type II after experimentally induced infection of newborn germ-free pigs. Am. J. Vet. Res. **50:**1037-1043.
46. **Williams, A. E., and Blakemore, W.F.** 1990. Pathogenesis of meningitis caused by *Streptococcus suis* type 2. J. Infect. Dis. **162:**474-481.
47. **Wilson, S. and Drevets, D.** 1998. *Listeria monocytogenes* infection and activation of human brain microvascular endothelial cells. J. Infect. Dis. **178:**1658-1666.
48. **Yao, L., Bengualid, V., Lowy, F., Gibbons, J., Hatcher, V. and Berman, J.** 1995. Internalization of *Staphylococcus aureus* by endothelial cells induces cytokine gene expression. Infect. Immun. **63:**1835-1839.

ARTICLE VI

Up-regulation of ICAM-1, CD11a/CD18 and CD11c/CD18 on Human THP-1 Monocytes Stimulated by *Streptococcus suis* Serotype 2

Dina Al-Numani[1,2], Mariela Segura[1,2], Monique Doré[2] and Marcelo Gottschalk[1,2]

[1]*Groupe de Recherche sur les Maladies Infectieuses du Porc (GREMIP),* [2]*Département de Pathologie et Microbiologie, Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, Québec, Canada*

Infection and Immunity. 2002. ***Submitted for publication***

Role of the candidate in conception of this article:

I have actively participated in the conception and design of the experiments. I have also helped the main author with the standardization of techniques, as well as the analysis of results. I have also assisted in graphic conception as well as in critical analysis of the manuscript.

ABSTRACT

Streptococcus suis serotype 2 is known to be a major pathogen of swine, causing mainly meningitis. It is also a zoonotic agent leading predominantly to meningitis in humans working in close contact with pigs. The pathogenesis of *S. suis* infection is not well understood. During meningitis, a strong acute inflammatory response develops, in part mediated by adhesion molecules that are expressed on leukocytes and on endothelial cells. In this study, we investigated the ability of *S. suis* to up-regulate the expression of important adhesion molecules involved in inflammation, using an ELISA. The subsequent increase in monocyte adherence to endothelial cells was also measured. *S. suis* serotype 2 stimulated the up-regulation of the surface expression of intercellular adhesion molecule-1 (ICAM-1), CD11a/CD18 and CD11c/CD18 on human THP-1 monocytes, but did not change that of ICAM-1, vascular cell adhesion molecule-1 (VCAM-1) and E-selectin on endothelial cells. The up-regulation of adhesion molecules was time- and bacterial-concentration dependent, and cell wall components were largely responsible for such stimulation. In addition, a nonencapsulated mutant of *S. suis* was found to induce higher levels of up-regulated adhesion molecule expression than the wild-type strain. Stimulation of monocytes with strains of different origin showed that there was no clear tendency for human strains to induce a higher expression of adhesion molecules than strains from diseased pigs. Finally, monocytes stimulated with *S. suis* also showed an increase in adherence to endothelial cells, thus providing a possible mechanism for some of the inflammatory features of meningitis caused by this pathogen.

INTRODUCTION

Streptococcus suis is a well known swine pathogen causing a wide range of infections such as meningitis, septicemia, arthritis and pneumonia (23). *S. suis* can also cause meningitis in individuals who work in close proximity to pigs, often leading to serious sequalea like hearing loss (3). So far, 35 serotypes have been identified, of which serotype 2 is considered the most virulent and most frequently isolated from diseased animals and humans (22). Knowledge on virulence factors of *S. suis* serotype 2 is limited. So far, the only proven critical virulence factor is the polysaccharidic capsule (CPS) (10). Cell wall and extracellular proteins, including a hemolysin (named suilysin), are also associated with virulence; however most virulent North American strains do not possess these factors (18, 36, 42).

The pathogenesis of meningitis caused by *S. suis* serotype 2 is largely unknown; however, several mechanisms have been recently proposed (19). *S. suis* may be transmitted via the respiratory route, breaching the mucosal epithelia in the upper respiratory tract by yet unknown mechanisms (19). Once in the blood, bacteria come into contact with phagocytes. An early theory suggested uptake of bacteria by monocytes, intracellular survival and invasion of the central nervous system (CNS) by the "Trojan horse theory" (49, 50). However, only a low number of monocytes were shown to actually contain bacteria (< 2%), and most bacteria remained extracellular (50). In fact, recent studies using isogenic mutants defective in capsule production demonstrated the antiphagocytic properties of the CPS, since non-capsulated mutants were readily phagocytozed and destroyed (10, 41). Although *S. suis* may travel mainly as free bacteria, other alternative mechanisms may also take place. A recent study demonstrated a high level of adhesion (without phagocytosis) of *S. suis* to monocytes, which lead to the proposition of a "modified" Trojan horse theory, where bacteria may travel externally in association with monocytes (19).

Survival of *S. suis* in the bloodstream as free bacteria would lead to septicemia and invasion of the meninges and other tissues. *S. suis* has been shown to preferentially adhere to human brain microvascular endothelial cells, a single layer of specialized cells forming the blood-brain barrier (BBB) (11). One consequence of this adhesion is the up-

regulation in the production of pro-inflammatory cytokines by these cells (N. Vadeboncoeur, M. A. Segura, D. Al-Numani, M. G. Gottschalk, Abstr. 101st ASM General meeting, abstr. V9, 2001), which might be responsible for inducing an acute inflammatory exudate that increases the volume of cerebral spinal fluid (CSF), leading to increased intracranial pressure. *S. suis* is also able to stimulate the production of pro-inflammatory cytokines by both human (38) and murine (37) monocytes .

Leukocyte influx into the subarachnoid space and the increase in BBB permeability are considered hallmarks of bacterial meningitis (44). Leukocyte recruitment to sites of inflammation is mediated by several families of adhesion molecules present on the surface of leukocytes and endothelial cells (16). These include selectins (E- and P-selectin on endothelial cells, and L-selectin on leukocytes); β_2 integrins (CD11a/CD18, CD11b/CD18 and CD11c/CD18), exclusively expressed on leukocytes; and members of the immunoglobulin superfamily (IgSF), mainly intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), expressed on endothelial cells; however, ICAM-1 has been shown to be expressed on monocytes (31). These molecules work together in mediating leukocyte rolling, firm adhesion and subsequent extravasation (43), by forming multiple receptor-ligand pairs that act either in a sequential and orchestrated fashion (16) or, as more recently proposed, in parallel pathways forming bottlenecks rather than a linear process (30). The immune response has to be controlled and directed correctly, otherwise excessive trafficking of leukocytes to extravascular locations can lead to serious tissue injury and destruction (25, 32). In fact, adhesion molecules are used by various micro-organisms during their pathogenesis of infection (25). Bacteria or bacterial products can up-regulate the surface expression of adhesion molecules on leukocytes and/or on endothelial cells, which would in turn promote leukocyte adhesion (29, 31).

Hence, in this study, we examined the effect of *S. suis* serotype 2 and several of its purified components on the surface expression of ICAM-1, VCAM-1 and E-selectin by endothelial cells, and of ICAM-1, CD11a/CD18, CD11b/CD18 and CD11c/CD18 by THP-1 monocytes. The increase in THP-1 monocyte adherence to endothelial cells following stimulation with *S. suis* was also studied.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *S. suis* serotype 2 virulent strain 31533, originally isolated from a pig with meningitis, was used as the reference strain in this study (27). Seven other porcine strains and 14 strains isolated from human cases of infection were also used (Table 1), together with the avirulent, nonencapsulated isogenic transposon mutant strain 2A, derived from the wild-type strain S735 (10).

TABLE 1. Strains of *S. suis* serotype 2 used in this study

Strain	Origin	Geographical origin
31533[a]	Diseased pig	France
S735	Diseased pig	The Netherlands
D282	Diseased pig	The Netherlands
94-623	Healthy pig	France
89-1591	Diseased pig	Canada
90-1330	Diseased pig	Canada
89-999	Diseased pig	Canada
AAH4	Diseased pig	USA
Reims	Human; spondylodiscitis	France
EUD95	Human; meningitis	France
Biotype 2	Human; endocarditis	France
HUD Limoge	Human; septic shock	France
FRU95	Human; meningitis	France
LEF95	Human; meningitis	France
96-52466	Human; arthritis	France
H11/1	Human; meningitis	UK
AR770353	Human; meningitis	The Netherlands
AR770297	Human; meningitis	The Netherlands
91-1804	Human; endocarditis	Canada
94-3037	Human; meningitis	Canada
98-3634	Human; endocarditis	Canada
99-734723688	Human; septicemia	Canada

[a] Strain used as reference in this study.

Bacteria were maintained as stock cultures in Todd-Hewitt broth (THB; Difco Lab., Detroit, MI) containing 50% glycerol at –80°C. The THB was supplemented with tetracycline (10 μg/ml; Sigma-Aldrich, Oakville, Ontario, Canada) for growing mutant strain 2A (10). Bacteria were grown overnight (O/N) on bovine blood agar plates at 37°C, and isolated colonies were used as inocula for THB; these cultures were incubated for 18 h at 37°C. Working cultures for cell stimulation were made by inoculating 200 μl volumes of these cultures into 10 ml volumes of THB and incubating at 37°C with agitation until they reached the mid-log phase (6 h of incubation; final optical densities at 540nm, 0.4 to 0.5). Bacteria were washed twice in phosphate-buffered saline (PBS), pH 7.4, and diluted to approximately 2×10^9 CFU/ml in PBS. An accurate determination of the CFU per milliliter in the final suspension was made by plating onto THB agar.

Preparation of killed bacteria. Bacteria were heat killed by incubating the organisms at 60°C for 45 min, the minimal experimental condition required for *S. suis* killing (37). The killed cultures were subcultured on blood agar plates at 37°C for 48 h to confirm that no organisms remained viable. Heat-killed bacterial preparations were stored at 4°C and resuspended in cell culture medium just before stimulation assays were performed. For some experiments, live bacteria (of 6 h culture as prepared above) were used.

Purified bacterial components. CPS and bacterial cell wall were purified as previously described (37, 39). *S. suis* hemolysin, purified as previously described (24), was kindly provided by Dr T. Jacobs (Intervet, Boxmeer, The Netherlands). The hemolysin was reactivated by addition of 0.1% 2-mercaptoethanol (2-ME; Bio-Rad, Mississauga, Ontario, Canada) to cell culture medium. Concentrations used in this study were not toxic to cells (data not shown), as measured by the lactate dehydrogenase cellular injury assay, as previoulsy described (11).

Cell cultures. THP-1 monocytes were purchased from ATCC (TIB 202) and maintained in RPMI medium with L-glutamine (Gibco, Burlington, Ontario, Canada) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 0.1% 2-ME and penicillin-streptomycin (5000 U/ml) (Gibco). Cells were cultured in flasks (Sarstedt, Newton, NC) and in 96-well tissue culture plates (Becton Dickinson, Bedford, MA). Human umbilical vein endothelial cells (HUVEC) were purchased from ATCC (CRL-

1730). Cells were grown in F-12K medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS, 30 μg/ml of endothelial cell growth supplement (ECGS; Becton Dickinson) and penicillin-streptomycin (5000 U/ml). Flasks (Becton Dickinson) and 96-well tissue culture plates were pre-coated with gelatin to support these cells. All cultures of cells were incubated at 37°C, with 5% CO_2 in a humid atmosphere.

Stimulation of cells. Prior to cell stimulation, 48 h culture of THP-1 monocytes and HUVEC were plated on 96-well culture plates at 5×10^5 cells/ml and 10^5 cells/ml, respectively. Different *S. suis* strains, as well as different concentrations of purified cell wall, CPS or hemolysin that have been prepared in the respective cell culture medium, were then added to cells. At different time intervals, stimulants were removed, and cells were fixed by the addition of 50 μl of 100% ethanol (THP-1 monocytes) or 1% paraformaldehyde for 20 min (HUVEC). THP-1 cells were left to air-dry, whereas HUVEC were washed with PBS. Lipopolysaccharide (LPS) from *Escherichia coli* 0127:B8 (1 μg/ml; Sigma-Aldrich) served as a positive control for HUVEC and THP-1 monocyte stimulation assays. Cells with medium alone served as controls for the basal expression of adhesion molecules. All solutions and *S. suis* preparations used in this study were tested for the presence of endotoxin by a *Limulus* amebocyte lysate (LAL) gel-clot test (Pyrotell STV, Cape Cod, Falmouth, MA) with a sensitivity limit of 0.03 endotoxin units (EU)/ml. Parallel assays with Polymixin B (PmB, 1 μg/ml; Sigma-Aldrich) were performed during the stimulation of cells with bacteria or purified bacterial products to confirm the absence of endotoxin contamination during the test.

ELISA. Expression of ICAM-1, VCAM-1, E-selectin, CD11a/CD18, CD11b/CD18 and CD11c/CD18 was measured by an enzyme-linked immunosorbent assay (ELISA). Wells containing fixed THP-1 or HUVEC in 96-well culture plates were blocked with 1% bovine serum albumin (Boehringer mannheim, Germany) in PBS, followed by the addition of one of the following monoclonal antibodies against the adhesion molecules: anti-ICAM-1 (0.1 μg/ml) and anti-VCAM-1 (1 μg/ml), purchased from R&D Systems (Minneapolis, MN); anti-E-selectin (1.0 μg/ml), anti-CD11a/CD11c (10 μg/ml) and anti-CD11b/CD18 (15 μg/ml), kindly provided by Dr. C. Wayne Smith (Baylor College of Medicine, Houston, TX); and anti-CD11c/CD18 (10 μg/ml), purchased from BD Biosciences (Mississauga, ON, Canada). For each antibody different

concentrations were tested in order to find out the optimal one. Thereafter, plates were washed three times, and exposed to a horseradish peroxidase-conjugated polyclonal goat anti-mouse IgG and IgM (Jackson Immunoresearch laboratories, inc., West Grove, PA). Bound enzyme was detected by adding a 1:1 solution of 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide (Intergen, St. Milford, MA) for 10-20 min. Optical density (OD) was read at 450nm, using a microplate reader (UVmax; Molecular Devices, Sunnyvale, CA). Each condition was tested in triplicate, and results represent the mean of at least 3 experiments. The basal expression of each adhesion molecule was subtracted from all presented results.

Adhesion Assay. The adherence of THP-1 monocytes to HUVEC was semi-quantified by a methylene blue dye staining, as described by Oliver et al. (34). Monocytes were stimulated for 48 h with *S. suis* strain 31533 (10^9 CFU/ml) or with LPS (1 µg/ml; as a positive control) and added to non-stimulated HUVEC. In addition, non-stimulated monocytes were added to HUVEC stimulated for 24 h with strain 31533 or with LPS, or to non-stimulated HUVEC. The adherence of non-stimulated monocytes to non-stimulated HUVEC represents the basal adhesion of monocytes to HUVEC. Monocytes were allowed to adhere for 40 min at 37°C. At the end of the incubation time, wells were washed 5 times with PBS, then fixed with 100% ethanol, and left to dry. Staining of cells was carried out by adding 0.1% methylene blue (in 0.1M borate buffer, pH 8.7) for 10 min at room temperature. Wells were then washed 3 times with borate buffer (0.01M). The methylene blue dye bound by the cells was solubilized with 100 µl/well of HCL (0.1N) for 30 min at 37°C. The amount of methylene blue was determined colorimetrically by the microplate reader, at 650nm. Experiments were done at least three times, in triplicate wells. The basal adhesion of monocytes to HUVEC was systematically subtracted from all results.

Statistics. Differences were analyzed for significance by using the Student's *t*-test (two-tailed P value). A P value < 0.05 was considered significant. Differences between strains of each origin and between strains of the same group were analyzed for significance using general linear models (GLM), followed by Tukey-Kramer post-hoc tests for differences between strains. The SAS software (SAS, Cary, NC) was used for these analyses.

RESULTS

***S. suis* does not up-regulate surface expression of ICAM-1, VCAM-1 and E-selectin on HUVEC.** The basal expression of adhesion molecules was subtracted from results, thus all values obtained throughout this study represent the up-regulated expression of adhesion molecules after bacterial or LPS stimulation. ICAM-1 was present on HUVEC incubated with medium alone, whereas no expression of E-selectin and very low levels of VCAM-1 were detected (data not shown).

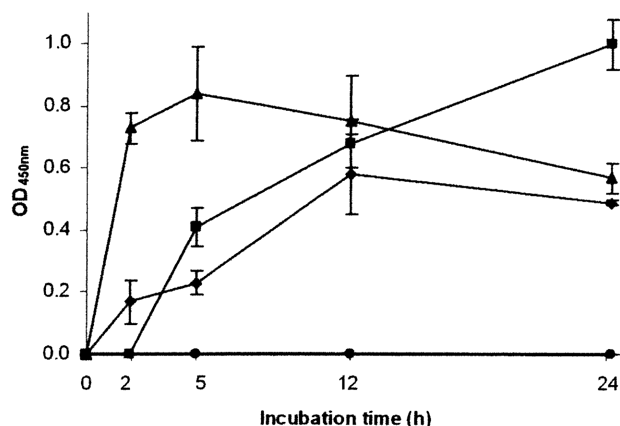


FIG. 1. Kinetics of the up-regulated expression of ICAM-1(♦), VCAM-1(■) and E-selectin (▲) on HUVEC stimulated by LPS. Results obtained after stimulation with heat-killed (10^9 CFU/ml) or live (10^8 to 10^4 CFU/ml) *S. suis* strains of porcine and human origin, included in this study (Table 1), are represented by (●) for all 3 adhesion molecules. Basal expression of adhesion molecules measured on non-stimulated cells was subtracted from all results. Data are expressed as means ± standard deviations, from at least 3 separate experiments.

Infection of HUVEC with heat-killed *S. suis* serotype 2, of porcine or human origin, did not induce the surface expression of E-selectin and VCAM-1, and did not increase the basal expression of ICAM-1 (Fig. 1). Stimulation with live bacteria of porcine or human origin also did not yield changes in adhesion molecule expression on

HUVEC. On the other hand, stimulation with LPS increased the expression of all three adhesion molecules (Fig. 1). E-selectin expression reached maximal levels at an earlier time point in comparison to the increase of ICAM-1 and VCAM-1. ICAM-1 peaked at a later time point, whereas VCAM-1 continued to increase with time. Both ICAM-1 and E-selectin expression gradually declined with time.

***S. suis* up-regulates ICAM-1, CD11a/CD18 and CD11c/CD18 on THP-1 monocytes in a time-dependent manner.** THP-1 monocytes expressed negligible levels of ICAM-1, whereas all three CD18 molecules were present on non-stimulated cells (not shown). The values for basal expression were subtracted from those following stimulation for each adhesion molecule.

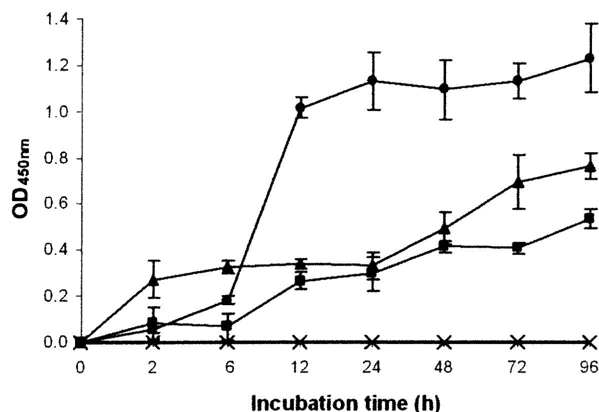


FIG. 2. Kinetics of the expression of ICAM-1(●), CD11a/CD18(■), CD11b/CD18(X) and CD11c/CD18(▲) on THP-1 monocytes stimulated by heat-killed *S. suis* type 2 strain 31533, at 10^9 CFU/ml, measured at different times of incubation. Basal expression of adhesion molecules measured on non-stimulated cells was subtracted from all results. Data are expressed as means ± SD, from at least 3 separate experiments.

Stimulation with heat-killed *S. suis* serotype 2 strain 31533 (10^9 CFU/ml) up-regulated the expression of ICAM-1, CD11a/CD18 and CD11c/CD18 in a time-dependent manner (Fig. 2). The up-regulation of CD11a/CD18 and CD11c/CD18 was gradual and lower than that observed with ICAM-1. ICAM-1 expression peaks and

levels off with time, whereas both CD11a/CD18 and CD11c/CD18 expression continue to increase with time. No up-regulation of CD11b/CD18 was detected on cells stimulated by *S. suis*. LPS-stimulated monocytes yielded similar results as *S. suis*-stimulated cells with respect to the kinetics of ICAM-1 and CD11a/CD18 expression, but not to the kinetics of CD11c/CD18 expression. Stimulation with *S. suis* results in an early peak in the up-regulation of CD11c/CD18, followed by a second burst of up-regulation; however, stimulation with LPS leads to an early peak in expression that is constant with time (data not shown). Results from the LAL test demonstrated no significant levels of endotoxin contamination in bacterial preparations. Cell culture medium contained less than 0.03 EU/ml. Data from parallel experiments in the presence of PmB to neutralize any endotoxin contamination revealed similar results (not shown).

Up-regulation of ICAM-1, CD11a/CD18 and CD11c/CD18 on THP-1 monocytes is bacterial concentration-dependent. Stimulation of THP-1 monocytes for 48 h with decreasing concentrations of heat-killed *S. suis* strain 31533 showed that the up-regulation of ICAM-1, CD11a/CD18 and CD11c/CD18 are bacterial-concentration dependent (Fig. 3).

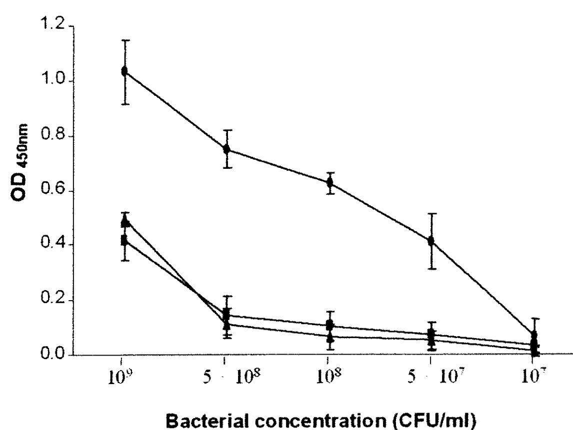


FIG. 3. Effect of decreasing concentrations of *S. suis* type 2 strain 31533 on the expression of ICAM-1(●), CD11a/CD18(■) and CD11c/CD18(▲) on THP-1 monocytes, measured at 48 h of stimulation. Basal expression of adhesion molecules measured on non-stimulated cells was subtracted from all results. Data are expressed as means ± SD, from at least 3 separate experiments.

The up-regulation of CD11a/CD18 and CD11c/CD18 expression was more sensitive to decreasing concentrations of *S. suis*, in comparison to ICAM-1 up-regulation. ICAM-1 expression decreased more gradually than the integrins, but was also not significant at bacterial concentrations lower than 10^7 CFU/ml ($P > 0.1$).

Up-regulation of ICAM-1 and integrins on THP-1 monocytes is independent on the porcine or human origin of *S. suis* strains. Strains originating from porcine or human origin were compared for their capacity to stimulate adhesion molecule expression on monocytes after 48 h stimulation. Statistical analysis revealed no significant differences in the up-regulation of ICAM-1 ($P = 0.9$), CD11a/CD18 ($P = 0.4$) and CD11c/CD18 ($P = 0.9$) induced by strains of different origins. On the other hand, there are significant differences between strains within each group ($P < 0.001$) in their ability to induce the up-regulation of adhesion molecule expression. Interestingly, strain "Reims", originating from a human case of spondylodiscitis (9), constantly showed the highest up-regulation of ICAM-1, CD11a/CD18 and CD11c/CD18 compared to all other strains (Fig. 4).

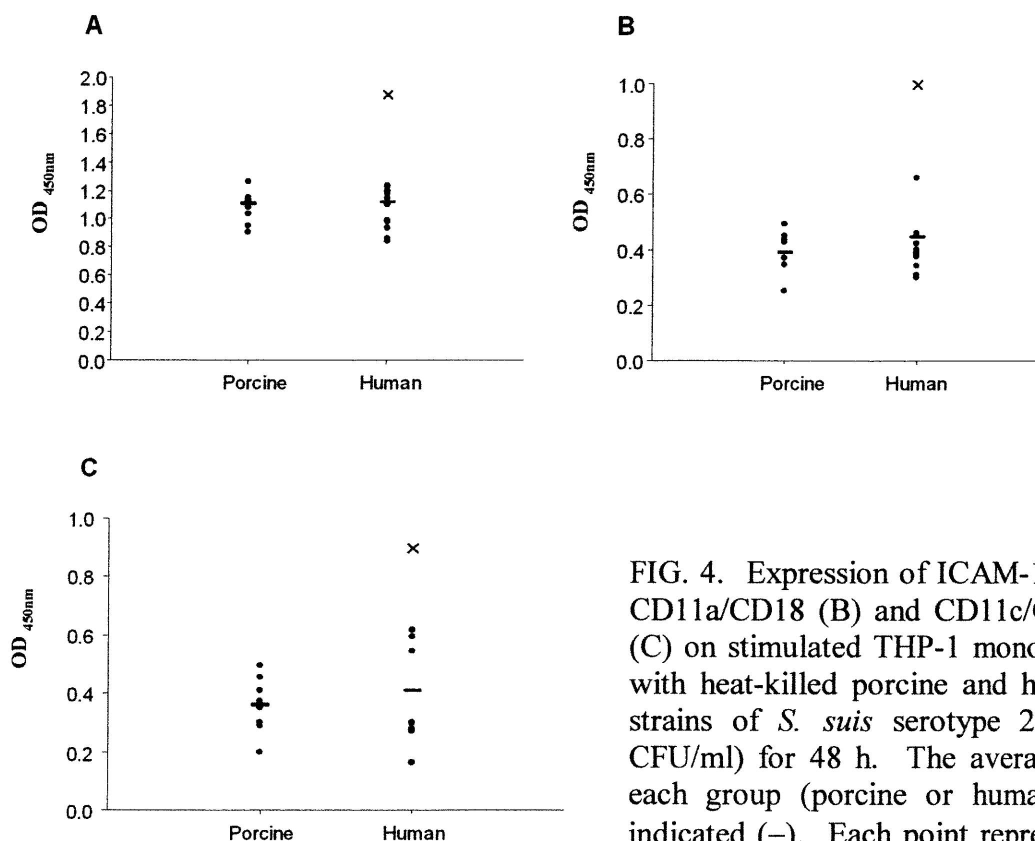


FIG. 4. Expression of ICAM-1 (A), CD11a/CD18 (B) and CD11c/CD18 (C) on stimulated THP-1 monocytes with heat-killed porcine and human strains of *S. suis* serotype 2 (10^9 CFU/ml) for 48 h. The average of each group (porcine or human) is indicated (–). Each point represents one strain, and is the average of at least three separate experiments. Human strain Reims is indicated in all three graphs as (X). Basal expression of adhesion molecules measured on non-stimulated cells was subtracted from all results.

Role of bacterial components in the up-regulation of ICAM-1, CD11a/CD18 and CD11c/CD18 on THP-1 monocytes. Purified components of *S. suis* were used to stimulate monocytes for 48 h, in order to discern which bacterial components contribute to the increase in surface expression of adhesion molecules. Stimulation of monocytes with CPS did not result in any significant up-regulation of ICAM-1, CD11a/CD18 and CD11c/C18, even when concentrations as high as 200 µg/ml were used (data not shown). On the other hand, cell stimulation with purified cell wall material resulted in a

high up-regulation of all three adhesion molecules (Fig. 5). Similarly to whole bacteria, the up-regulation was dependent on the concentration of cell wall material. The last purified component tested was the hemolysin. It is important to mention that washed heat-killed bacteria suspensions do not contain any hemolytic activity. Upon stimulation of cells with purified hemolysin, the expression of all three adhesion molecules was up-regulated (Fig. 5). The level of up-regulation was very significant especially at 1 and 0.5 μg/ml of hemolysin, but lower than that induced by the cell wall.

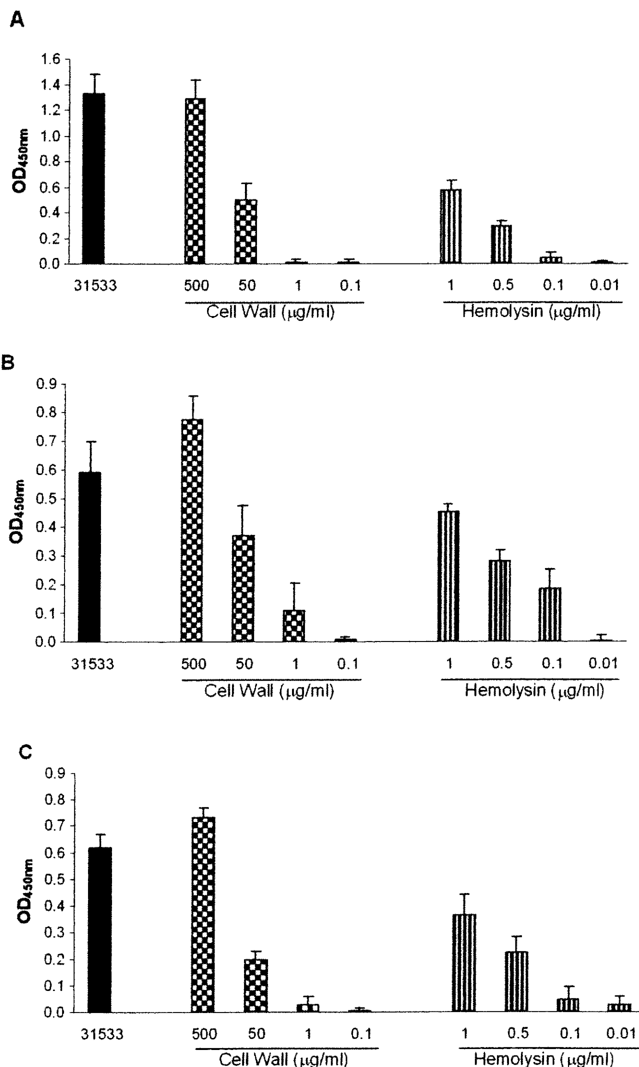


FIG. 5. The effect of different concentrations of purified cell wall (checkered bars) and hemolysin (vertical bars) of *S. suis* serotype 2 on the expression of ICAM-1 (A), CD11a/CD18 (B) and CD11c/CD18 (C) on THP-1 monocytes, at 48 h of stimulation, compared to the stimulation observed with whole bacteria (solid bar) (heat-killed strain 31533, 10^9 CFU/ml). Basal expression of adhesion molecules measured on non-stimulated cells was subtracted from all results. Data are expressed as means ± standard deviations, from at least 3 separate experiments.

Nonencapsulated mutant strain versus wild type strain. The nonencapsulated mutant strain was compared to its wild-type porcine strain S735 in its capacity to stimulate ICAM-1, CD11a/CD18 and CD11c/CD18. The mutant strain significantly induced higher up-regulation ($P < 0.001$) of all adhesion molecules in respect to the wild-type strain: ICAM-1: 1.55 ± 0.17 vs 1.03 ± 0.14; CD11a/CD18: 0.65 ± 0.08 vs 0.25 ± 0.07; CD11c/CD18: 0.66 ± 0.09 vs 0.37 ± 0.07, respectively.

***S. suis*-stimulated THP-1 monocytes increase their adherence to HUVEC.** THP-1 monocytes stimulated with heat-killed strain 31533 demonstrated an increased adherence to HUVEC ($P < 0.001$) in comparison to non-stimulated THP-1 monocytes (Fig. 6). Since *S. suis* was unable to stimulate the expression of adhesion molecules on HUVEC, it would be expected that no increase in adhesion of monocytes would be detected on *S. suis*-stimulated HUVEC. Indeed, when HUVEC were stimulated with bacteria, no significant increase in adherence of monocytes was observed ($P > 0.05$). Stimulation of THP-1 monocytes or HUVEC with LPS, as a positive control, resulted in a significant increase in adherence of monocytes ($P < 0.001$).

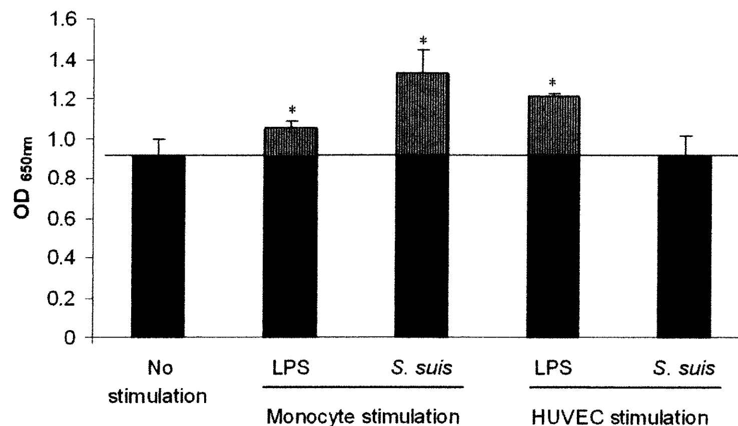


FIG 6. Adherence of THP-1 monocytes to endothelial cells following stimulation with LPS or *S. suis*. Monocyte stimulation: LPS or *S. suis* stimulated monocytes were allowed to adhere for 40 min on non-stimulated HUVEC. HUVEC stimulation: non-stimulated monocytes were allowed to adhere for 40 min on LPS or *S. suis* stimulated HUVEC. Wells were washed, fixed, and stained with methylene blue. Results were compared to the value representing basal adhesion of non-stimulated monocytes on non-stimulated HUVEC. *Increase in monocyte adherence upon stimulation ($P < 0.05$).

DISCUSSION

Pathogenesis of meningitis caused by *S. suis* is not well understood, and little is known about the role of the virulence factors that have been described so far. Recent work has shown that *S. suis* interacts with monocytes and stimulates the production of pro-inflammatory cytokines (37, 38). In this study, we further characterize this interaction by demonstrating that stimulation of THP-1 monocytes with *S. suis* serotype 2 up-regulates the expression of ICAM-1, CD11a/CD18 and CD11c/CD18. These adhesion molecules play an important role in leukocyte adherence and extravasation into inflammatory sites (16). During experimental meningitis, inflammatory leukocytes were shown to be the major cause of the BBB injury and cerebral edema (6). Blocking antibodies against CD18 or ICAM-1 reduced leukocytosis into the CSF which in turn reduced brain edema (46, 48). In addition, up-regulation of the expression of integrins has been correlated with an influx of inflammatory cells into the CSF (2, 35), and with an increase in the adherence capacity of monocytes *in vitro* (45).

The up-regulation of adhesion molecules on *S. suis*-stimulated monocytes is time- and bacterial concentration-dependent, with a specific pattern of expression for each adhesion molecule. LPS was used as a positive control since it is a well known immunomodulator that is capable of up-regulating several adhesion molecules, including ICAM-1 and β_2 integrins, on the surface of monocytes (14, 20, 21). Most previous studies report the kinetics of expression of these adhesion molecules between 0 and 24 h. In this study, we report the kinetics of adhesion molecule up-regulation by *S. suis* and LPS until 96 h of stimulation. The rapid up-regulation of CD11c/CD18 compared to ICAM-1 and CD11a/CD18 was probably due to the fact that this molecule is stored in intracellular granules, whereas ICAM-1 and CD11a/CD18 are not (7). It has been previously shown that under activation, monocytes could mobilize this pool within a few minutes, translocating CD11c/CD18 to the cell surface (33). Stimulation with LPS resulted in a different pattern of kinetics, where only a first up-regulation at 2 h was detected, and remained constant with time (data not shown), as has been previously shown (14). Finally, *S. suis* and LPS were unable to up-regulate the expression of CD11b/CD18. LPS has been previously shown to up-regulate CD11b/CD18 on fresh blood monocytes (14), thus this difference in response may be due to the relative

immaturity of THP-1 monocytes (1). Other bacteria have been shown to up-regulate the expression of adhesion molecules. For example, *Mycobacterium tuberculosis*, similarly to *S. suis*, up-regulates ICAM-1 expression in a time-dependent fashion, and does not increase the expression of CD11b/CD18 (31). On the other hand, this bacteria is unable to up-regulate the expression of CD11a/CD18 (31). Other bacteria, including *Staphylococcus aureus* and *Streptococcus pneumoniae*, do increase CD11b/CD18 expression on leukocytes (8, 28). Thus, bacterial-stimulated monocytes exhibit a specific pattern of adhesion molecule expression.

The kinetics of the three up-regulated adhesion molecules was also dependent on bacterial concentration. A high concentration of bacteria was needed to obtain high levels of adhesion molecule expression. Similarly, monocytes stimulated with *S. suis* also require a high bacterial concentration for maximal levels of cytokine release (37, 38). In fact, the presence of high levels of bacteria in the bloodstream of diseased animals is correlated with the presence of clinical signs and symptoms in these animals (4).

One consequence of the up-regulation of adhesion molecules is an increase of leukocyte rolling, firm adhesion and subsequent extravasation (43). In this study, we demonstrate a correlation between the up-regulation of adhesion molecules and the increase in adherence of monocytes to endothelial cells. *S. suis*-stimulated monocytes, expressing increased amounts of adhesion molecules, bound to HUVEC in higher numbers compared to non-stimulated monocytes. LPS-stimulated monocytes also caused an increase in monocyte adherence to HUVEC.

In order to identify possible bacterial candidates responsible for the monocyte cell adhesion molecule activation, different purified components of *S. suis* were tested. Results showed that the cell wall is largely responsible for the up-regulation of adhesion molecules. Stimulation with cell wall components results in increased ICAM-1, CD11a/CD18 and CD11c/CD18 expressions that were as high as those obtained with whole bacteria. Similarly, a cell wall component of *M. tuberculosis* up-regulated the same level of ICAM-1 expression as whole bacteria (31). The purified hemolysin also has the capability to stimulate the expression of ICAM-1, CD11a/CD18 and CD11c/CD18 on THP-1 monocytes, but with lower levels in comparison to cell wall

components. In contrast to the above purified factors, the CPS of *S. suis* does not cause any significant up-regulation of adhesion molecule expression on monocytes. These results are in agreement with other studies that have shown the important contribution of the *S. suis* cell wall to cytokine production by endothelial cells and by murine macrophages, the capability of the hemolysin to stimulate cytokine production by endothelial cells, and by the inability of CPS to stimulate any cytokine production by both endothelial cells and monocytes (37) N. Vadeboncoeur, M. A. Segura, D. Al-Numani, M. G. Gottschalk, Abstr. 101st ASM General Meeting, abstr. V9, 2001).

The nonencapsulated mutant strain stimulated a higher level of up-regulation of ICAM-1, CD11a/CD18 and CD11c/CD18 than the parent strain. The same nonencapsulated strain has been shown to stimulate a higher production of TNF-α by murine macrophages (37) in comparison to wild-type strain. Hence, the capsule seems to mask cell wall components that can contribute to the up-regulation of adhesion molecules. A nonencapsulated mutant of *Neisseria meningitidis* has also been shown to cause a different pattern in adhesion molecule expression on leukocytes in comparison to the parent strain (26).

The porcine and human origin of *S. suis* strains does not seem to influence the degree of up-regulation of adhesion molecules on cells of human origin (THP-1). Genetic comparisons between human and porcine isolates have been recently performed and have placed these isolates in the same group (5, 12). These results, along with the present study, agree with *S. suis* being a zoonotic agent. Interestingly, a strain of human origin, "Reims", induced the highest up-regulation of adhesion molecule expression in comparison to all other strains. This strain also induced higher levels of cytokine production by THP-1 monocytes (38). Ongoing studies in our laboratory are in the process of characterizing this strain. Preliminary results indicate that "Reims" is a low-capsulated strain, which would confirm results obtained with the nonencapsulated strain.

It can be argued that the up-regulation of adhesion molecules on THP-1 monocytes stimulated with *S. suis* is an indirect result of the cytokines that are produced by bacterial stimulation. In fact, this hypothesis cannot be ruled out, since we already know that *S. suis*-stimulated THP-1 monocytes induce the production of pro-inflammatory cytokines and these molecules are potent activators of cells and increase

the surface expression of adhesion molecules, including the expression of ICAM-1 and of β_2 integrins on monocytes (45, 51). For example, TNF-α has been previously shown to increase the expression of CD11a/CD18 on THP-1 monocytes, but to have not effect on the expression of CD11b/CD18 and CD11c/CD18 (45). ICAM-1 has also been shown to be up-regulated by several cytokines (40). In addition, stimulated adhesion molecules have the ability themselves of stimulating the production of cytokines (13, 47). Hence, further studies need to be done in order to understand the cause and effect of the up-regulated expression of adhesion molecules on THP-1 monocytes stimulated by *S. suis*.

Important meningeal pathogens, including *N. meningitidis*, *Listeria monocytogenes* and *S. pneumoniae*, have the capability of increasing the expression of adhesion molecules on endothelial cells (15, 17, 29). In the case of *L. monocytogenes*, this up-regulation was accompanied by an increase in leukocyte adherence (29). *S. suis*, however, was incapable of increasing the surface expression of ICAM-1, VCAM-1 and E-selectin on endothelial cells used in this study. In addition, endothelial cells stimulated by *S. suis* do not support an increase in monocyte adherence. LPS, on the other hand, increased both adhesion molecule expression and monocyte adherence to endothelial cells. This further reinforces the correlation between the up-regulation of adhesion molecules and the increase in monocyte adherence. Interestingly, preliminary work in our laboratory has shown that stimulation of endothelial cells with medium originating from a culture of THP-1 monocytes, that had previously been stimulated with *S. suis* lead to an increase in the expression of ICAM-1, E-selectin and VCAM-1 molecules (unpublished results). This indirect up-regulation may be due to cytokines released by *S. suis*-stimulated THP-1 monocytes.

In conclusion, this study demonstrates the ability of *S. suis* to up-regulate the expression of important adhesion molecules involved in inflammation. This activation may be responsible, at least in part, for the increase in adherence of monocytes to endothelial cells, thus providing a mechanism for some of the inflammatory features of meningitis caused by this pathogen.

ACKNOWLEDGEMENTS

We gratefully acknowledge Dr. M. Kobisch (Centre National d'Études Vétérinaires et Alimentaires, Ploufragan, France), Dr. U. Vecht (DLO-Institute for Animal Science and Health, Lelystad, The Netherlands), Dr. T. Alexander (University of Cambridge, England), Dr. L. Brasme (Centre Hospitalier Universitaire de Reims, France), Dr. G. Grise (Hôpital des Feugrois, Elbeuf, France), Dr. B. Cattier (Centre Hospitalier Universitaire Bretonneau, Tours, France), Dr. B. François (Dupuytren Hospital, Limoges Cedex, France), and Dr. P. Norton (Institute for Animal Health, Compton, United Kingdom) for providing some of the *Streptococcus suis* type 2 strains. We would also like to thank Dr. C. Wayne Smith for kindly providing anti-CD11a/CD18 and anti-CD11b/CD18 monoclonal antibodies.

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) grant # 0680154280, by the Fonds pour la Formation des Chercheurs et l'Aide à la Recherche du Québec (FCAR-équipe) grant # 99-ER-0214, and by the Canadian Research Network on Bacterial Pathogens of Swine.

REFERENCES

1. **Abrink, M., A. E. Gobl, R. Huang, K. Nilsson, and L. Hellman.** 1994. Human cell lines U-937, THP-1 and Mono Mac 6 represent relatively immature cells of the monocyte-macrophage cell lineage. Leukemia **8:**1579-84.
2. **Andersson, E. C., J. P. Christensen, O. Marker, and A. R. Thompson.** 1994. Changes in cell adhesion molecule expression on T cells associated with systemic virus infection. J. Immunol. **152:**1237-45.
3. **Arends, J. P., and H. C. Zanen.** 1988. Meningitis caused by *Streptococcus suis* in humans. Rev. Infect. Dis. **10:**131-137.
4. **Berthelot-Herault, F., R. Cariolet, A. Labbe, M. Gottschalk, J. Y. Cardinal, and M. Kobisch.** 2001. Experimental infection of specific pathogen free piglets with French strains of *Streptococcus suis* capsular type 2. Can. J. Vet. Res. **65:**196-200.
5. **Berthelot-Herault, F., C. Marois, M. Gottschalk, and M. Kobisch.** 2002. Genetic diversity of *Streptococcus suis* strains isolated from pigs and humans as revealed by pulsed-field gel electrophoresis. J. Clin. Microbiol. **40:**615-9.
6. **Bohr, V. A., and N. Rasmussen.** 1988. Neurological sequelae and fatality as prognostic measures in 875 cases of bacterial meningitis. Dan. Med. Bull. **35:**92-5.
7. **Carlos, T. M., and J. M. Harlan.** 1990. Membrane proteins involved in phagocyte adherence to endothelium. Immunol. Rev. **114:**5-28.
8. **Carratelli, C. R., I. Nuzzo, C. Bentivoglio, and M. Galdiero.** 1996. CD11a/CD18 and CD11b/18 modulation by lipoteichoic acid, N-acetyl-muramyl-alpha-alanyl-D-isoglutamine, muramic acid and protein A from *Staphylococcus aureus*. FEMS Immunol. Med. Microbiol. **16:**309-15.
9. **Caumont, H., N. Gerard, B. Depernet, L. Brasme, J. P. Eschard, and J. C. Etienne.** 1996. *Streptococcus suis* L3-L4 spondylodiscitis in a butcher (letter). Presse Med. **25:**1348.
10. **Charland, N., J. Harel, M. Kobish, S. Lacasse, and M. Gottschalk.** 1998. *Streptococcus suis* serotype 2 mutants deficient in capsular expression. Microbiology **144:**325-332.
11. **Charland, N., V. Nizet, C. Rubens, K. S. Kim, S. Lacouture, and M. Gottschalk.** 2000. *Streptococcus suis* serotype 2 interactions with human brain microvascular endothelial cells. Infect. Immun. **68:**637-643.
12. **Chatellier, S., M. Gottschalk, R. Higgins, R. Brousseau, and J. Harel.** 1999. Relatedness of *Streptococcus suis* serotype 2 isolates from different geographic origins as evaluated by molecular fingerprinting and phenotyping. J. Clin. Microbiol. **37:**362-366.
13. **Cuzzola, M., G. Mancuso, C. Beninati, C. Biondo, F. Genovese, F. Tomasello, T. H. Flo, T. Espevik, and G. Teti.** 2000. Beta 2 integrins are involved in cytokine responses to whole Gram- positive bacteria. J. Immunol. **164:**5871-6.
14. **Darcissac, E. C., G. M. Bahr, M. A. Parant, L. A. Chedid, and G. J. Riveau.** 1996. Selective induction of CD11a,b,c/CD18 and CD54 expression at the cell surface of human leukocytes by muramyl peptides. Cell. Immunol. **169:**294-301.

15. **Dixon, G. L., R. S. Heyderman, K. Kotovicz, D. L. Jack, S. R. Andersen, U. Vogel, M. Frosch, and N. Klein.** 1999. Endothelial adhesion molecule expression and its inhibition by recombinant bactericidal/permeability-increasing protein are influenced by the capsulation and lipooligosaccharide structure of *Neisseria meningitidis*. Infect. Immun. **67:**5626-33.
16. **Etzioni, A.** 1996. Adhesion molecules in leukocyte endothelial interaction. Adv. Exp. Med. Biol. **408:**151-7.
17. **Freyer, D., R. Manz, A. Ziegenhorn, M. Weih, K. Angstwurm, W. D. Docke, A. Meisel, R. R. Schumann, G. Schonfelder, U. Dirnagl, and J. R. Weber.** 1999. Cerebral endothelial cells release TNF-alpha after stimulation with cell walls of *Streptococcus pneumoniae* and regulate inducible nitric oxide synthase and ICAM-1 expression via autocrine loops. J. Immunol. **163:**4308-14.
18. **Gottschalk, M., A. Lebrun, H. Wisselink, J. D. Dubreuil, H. Smith, and U. Vecht.** 1998. Production of virulence-related proteins by canadian strains of *Streptococcus suis* capsular type 2. Can. J. Vet. Res. **62:**75-79.
19. **Gottschalk, M., and M. Segura.** 2000. The pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions. Vet. Microbiol. **75:**59-71.
20. **Heinzelmann, M., M. A. Mercer-Jones, S. A. Gardner, M. A. Wilson, and H. C. Polk.** 1997. Bacterial cell wall products increase monocyte HLA-DR and ICAM-1 without affecting lymphocyte CD18 expression. Cell. Immunol. **176:**127-34.
21. **Heinzelmann, M., H. C. Polk, Jr., A. Chernobelsky, T. P. Stites, and L. E. Gordon.** 2000. Endotoxin and muramyl dipeptide modulate surface receptor expression on human mononuclear cells. Immunopharmacology **48:**117-28.
22. **Higgins, R., and M. Gottschalk.** 1998. Distribution of *Streptococcus suis* capsular types in 1997. Can. Vet. J. **39:**299-300.
23. **Higgins, R., and M. Gottschalk.** 1999. Streptococcal diseases, p. 563-570. *In* B. E. Straw, S. D'Allaire, W. L. Mengeling, and D. J. Taylor (ed.), Diseases of swine. Iowa State University, Ames.
24. **Jacobs, A. A., P. L. Loeffen, A. J. van den Berg, and P. K. Storm.** 1994. Identification, purification, and characterization of a thiol-activated hemolysin (suilysin) of *Streptococcus suis*. Infect. Immun. **62:**1742-1748.
25. **Kerr, J. R.** 1999. Cell adhesion molecules in the pathogenesis of and host defence against microbial infection. Mol. Pathol. **52:**220-30.
26. **Klein, N. J., C. A. Ison, M. Peakman, M. Levin, S. Hammerschmidt, M. Frosch, and R. S. Heyderman.** 1996. The influence of capsulation and lipooligosaccharide structure on neutrophil adhesion molecule expression and endothelial injury by *Neisseria meningitidis*. J. Infect. Dis. **173:**172-9.
27. **Kobisch, M., M. Gottschalk, P. Morvan, R. Cariolet, G. Bénévent, and J. P. Joly.** 1995. Experimental infection of SPF piglets with *Streptococcus suis* serotype 2. Journées Rech. Porcine en France **27:**97-102.
28. **Kragsbjerg, P., and H. Fredlund.** 2001. The effects of live *Streptococcus pneumoniae* and tumor necrosis factor- alpha on neutrophil oxidative burst and beta 2-integrin expression. Clin. Microbiol. Infect. **7:**125-9.
29. **Krull, M., R. Nost, S. Hippenstiel, E. Domann, T. Chakraborty, and N. Suttorp.** 1997. *Listeria monocytogenes* potently induces up-regulation of

endothelial adhesion molecules and neutrophil adhesion to cultured human endothelial cells. J. Immunol. **159:**1970-6.

30. **Ley, K.** 2001. Pathways and bottlenecks in the web of inflammatory adhesion molecules and chemoattractants. Immunol. Res. **24:**87-95.
31. **Lopez Ramirez, G. M., W. N. Rom, C. Ciotoli, A. Talbot, F. Martiniuk, B. Cronstein, and J. Reibman.** 1994. *Mycobacterium tuberculosis* alters expression of adhesion molecules on monocytic cells. Infect. Immun. **62:**2515-20.
32. **Lowe, J. B., and P. A. Ward.** 1997. Therapeutic inhibition of carbohydrate-protein interactions in vivo. J. Clin. Invest. **100:**S47-51.
33. **Miller, L. J., D. F. Bainton, N. Borregaard, and T. A. Springer.** 1987. Stimulated mobilization of monocyte Mac-1 and p150,95 adhesion proteins from an intracellular vesicular compartment to the cell surface. J. Clin. Invest. **80:**535-44.
34. **Oliver, M. H., N. K. Harrison, J. E. Bishop, P. J. Cole, and G. J. Laurent.** 1989. A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors. J. Cell. Sci. **92:**513-8.
35. **Rowin, M. E., V. Xue, and J. Irazuzta.** 2000. Integrin expression on neutrophils in a rabbit model of Group B streptococcal meningitis. Inflammation **24:**157-73.
36. **Segers, R. P., T. Kenter, L. A. de Haan, and A. A. Jacobs.** 1998. Characterisation of the gene encoding suilysin from *Streptococcus suis* and expression in field strains. FEMS Microbiol. Lett. **167:**255-61.
37. **Segura, M., J. Stankova, and M. Gottschalk.** 1999. Heat-killed *Streptococcus suis* capsular type 2 strains stimulate tumor necrosis factor alpha and interleukin-6 production by murine macrophages. Infect. Immun. **67:**4646-4654.
38. **Segura, M., N. Vadeboncoeur, and M. Gottschalk.** 2002. CD14-dependent and -independent cytokine and chemokine production by human THP-1 monocytes stimulated by *Streptococcus suis* capsular type 2. Clin. Exp. Immunol. **127:**243-54.
39. **Sepulveda, E. M. D., E. Altman, M. Kobisch, S. Dallaire, and M. Gottschalk.** 1996. Detection of antibodies against *Streptococcus suis* capsular type 2 using a purified capsular polysaccharide antigen-based indirect elisa. Vet. Microbiol. **52:**113-125.
40. **Simmons, D., M. W. Makgoba, and B. Seed.** 1988. ICAM, an adhesion ligand of LFA-1, is homologous to the neural cell adhesion molecule NCAM. Nature **331:**624-7.
41. **Smith, H. E., M. Damman, J. Van der Velde, F. Wagenaar, H. J. Wisselink, N. Stockhofe-Zurwieden, and M. A. Smits.** 1999. Identification and characterization of the *cps* locus of *Streptococcus suis* serotype 2: the capsule protects against phagocytosis and is an important virulence factor. Infect. Immun. **67:**1750-1756.
42. **Smith, H. E., H. J. Wisselink, N. Stockhofe-Zurwieden, U. Vecht, and M. M. Smits.** 1997. Virulence markers of *Streptococcus suis* type 1 and 2. Adv. Exp. Med. Biol. **418:**651-5.
43. **Springer, T. A.** 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell **76:**301-14.

44. **Tang, T., P. S. Frenette, R. O. Hynes, D. D. Wagner, and T. N. Mayadas.** 1996. Cytokine-induced meningitis is dramatically attenuated in mice deficient in endothelial selectins. J. Clin. Invest. **97:**2485-90.

45. **Tiisala, S., M. L. Majuri, O. Carpen, and R. Renkonen.** 1994. Enhanced ICAM-1-dependent adhesion of myelomonocytic cells expressing increased levels of beta 2-integrins and CD43. Scand. J. Immunol. **39:**249-56.

46. **Tuomanen, E. I., K. Saukkonen, S. Sande, C. Cioffe, and S. D. Wright.** 1989. Reduction of inflammation, tissue damage, and mortality in bacterial meningitis in rabbits treated with monoclonal antibodies against adhesion-promoting receptors of leukocytes. J. Exp. Med. **170:**959-69.

47. **Walzog, B., P. Weinmann, F. Jeblonski, K. Scharffetter-Kochanek, K. Bommert, and P. Gaehtgens.** 1999. A role for beta(2) integrins (CD11/CD18) in the regulation of cytokine gene expression of polymorphonuclear neutrophils during the inflammatory response. Faseb J. **13:**1855-65.

48. **Weber, J. R., K. Angstwurm, W. Burger, K. M. Einhaupl, and U. Dirnagl.** 1995. Anti ICAM-1 (CD 54) monoclonal antibody reduces inflammatory changes in experimental bacterial meningitis. J. Neuroimmunol. **63:**63-8.

49. **Williams, A. E.** 1990. Relationship between intracellular survival in macrophages and pathogenicity of *Streptococcus suis* type 2 isolates. Microb. Pathog. **8:**189-96.

50. **Williams, A. E., and W. F. Blakemore.** 1990. Pathogenesis of meningitis caused by *Streptococcus suis* type 2. J. Infect. Dis. **162:**474-481.

51. **Yamada, A., A. Hara, M. Inoue, S. Kamizono, T. Higuchi, and K. Itoh.** 1997. Beta 2-integrin-mediated signal up-regulates counterreceptor ICAM-1 expression on human monocytic cell line THP-1 through tyrosine phosphorylation. Cell. Immunol. **178:**9-16.

ARTICLE VII

The pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions

Marcelo Gottschalk and Mariela Segura

Groupe de Recherche sur les Maladies Infectieuses du Porc, Faculté de médecine vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec, J2S 7C6, Canada

Veterinary Microbiology. 2000. 76:259-272

Role of the candidate in conception of this article:

I am co-author in this review article. I actively participated in scientific discussions and literature review. I contributed at 50% in the conception and writing of this manuscript.

ABSTRACT

Streptococcus suis is one of the most important swine pathogens worldwide. Among the serotypes described, type 2 is the serotype most frequently associated with disease. Despite increasing research in recent years, knowledge of virulence factors and the pathogenesis of the infection remains limited. This review discusses the currently available information on *S. suis* serotype 2 virulence factors and the pathogenesis of the meningitis caused by this important bacterial species. In addition, some hypotheses on the critical steps of the infection, such as bacterial invasion from mucosal surfaces to the bloodstream, survival of bacteria in blood, and invasion from blood into the central nervous system, are presented. Finally, the role that the stimulation of the immune system of animals (inflammatory reaction) could play during infection is also discussed. A complete understanding of the cell-interacting pathways that *S. suis* may follow inside the host could give important insights into the progression of disease. Further studies to delineate the mechanisms through which *S. suis* induces meningitis will contribute to the development of potential therapies for *S. suis* infections.

INTRODUCTION

Streptococcus suis infections have been considered as a major and worldwide problem in the swine industry, particularly during the past 10 years. The natural habitat of *S. suis* is the upper respiratory tract, particularly the tonsils and nasal cavities, and the genital and alimentary tracts of pigs (Higgins and Gottschalk, 1999). This bacterium has been increasingly isolated from a wide range of mammalian species (including human beings) and from birds, which suggest new concepts about some epidemiological aspects of the infection. In pigs, the most important clinical feature associated with *S. suis* is meningitis. However, other pathologies have also been described, such as arthritis, endocarditis, pneumonia, and septicaemia with sudden death (Higgins and Gottschalk, 1999). The aim of this paper is to review the most recent information on *S. suis*, focussing on the current understanding of the pathogenesis of the infection. Some

speculative theories on the different mechanisms that this bacterium may use to cause meningitis are also presented. For a more general review on *S. suis*, other publications are available (Staats et al., 1997; Higgins and Gottschalk, 1999).

1. General features of *S. suis*

S. suis is a Gram-positive, facultatively anaerobe coccus, possessing cell wall antigenic determinants related to Lancefield group D, although it is genetically unrelated to other members of this group. The original classification of *S. suis* into Lancefield groups R, S and T, which actually correspond to capsular types 2, 1 and 15, respectively (Gottschalk et al., 1989), is obsolete and should be avoided. During the last 10 years, 26 new capsular types or serotypes have been described, reaching a total of 35 serotypes in 1995 (Higgins et al., 1995).

Presumptive diagnosis of *S. suis* infections is based on clinical signs and macroscopic lesions. Confirmation of infection is achieved by the isolation of the infectious agent and the recognition of microscopic lesions in tissues. Isolation of *S. suis* from lungs has to be interpreted with caution since this organism is almost constantly present in the upper respiratory tract. Pigs may harbour a variety of *S. suis* strains or serotypes in their nasal cavities and tonsils with no relationship with a specific pathological condition. It is also possible to isolate multiple *S. suis* serotypes from diseased animals within the same herd (Higgins and Gottschalk, 1999).

Identification of *S. suis* isolates is possible with a minimum of biochemical tests, especially when they are recovered from diseased pigs and when serotyping is available. As proposed by Devriese et al. (1991), an alpha-haemolytic *Streptococcus* that produces amylase but not acetoin, can be considered as *S. suis*. For epidemiological studies as well as for eventual eradication purposes, the detection of specific serotypes or strains of *S. suis* in live animals could be attempted by the use of PCR procedures. Rasmussen and Andresen (1998) identified a *S. suis*-specific 16S RNA region that might be used for specific detection of *S. suis*. More recently, a species-specific probe (serotypes 1 through 31) targeting 16S ribosomal RNA was designed and used for fluorescent in situ

hybridization (Boye et al., 2000). Serotype-specific isolation from contaminated tissues, such as tonsils, may also be carried out using a recently reported immunocapture method (Gottschalk et al., 1999b).

With the exception of serotypes 1 through 8 (including type 1/2), no genetic analysis had been carried out on the newly described serotypes, which were originally characterised only by their phenotypic features. This was a topic of important concern, since some reference strains originated from animal species others than swine (Higgins et al., 1995). In the recent years, two independent research groups have studied the phylogenetic diversity of *S. suis* serotypes by comparison of 16 rRNA gene sequence (Chatellier et al., 1998; Rasmussen and Andresen, 1998). Results showed that 32 of 35 reference strains had a nucleotide sequence similarity which ranged between 93 and 100%, and fell into a major group comprising three clusters. Comparison with nucleotide sequence from other streptococci indicated that, with the exception of serotypes 32, 33 and 34, *S. suis* reference strains did not cluster with any other *Streptococcus* species in the genus. However, there is no indication suggesting that members of these three serotypes (32-34) should be transferred to another species.

Serotyping is an important step in the routine diagnostic procedure. Different techniques have been described, but most laboratories have adopted the coagglutination technique. Since the majority of typable isolates belong to capsular types 1-8 and 1/2, it is advisable for diagnostic laboratories to only use antiserum corresponding to these serotypes and to send untypable isolates to a reference laboratory (Higgins and Gottschalk, 1999). Some isolates react with more than one antiserum, as it is the case of serotype 1/2 and serotype 1/14 isolates, which may lead to some confusion. In the latter case, isolates of serotype 1 will usually react not only with serotype 1 antiserum but also with serotype 14 antiserum (Gottschalk et al., 1989). This seems to be a one-way reaction, since antibodies against serotype 1 will nor react with serotype 14 isolates. In fact, this explains why the reference strain of serotype 14 (strain 13730) was considered "untypable" with sera from serotypes 1 through 8 when the new serotype 14 was described (Gottschalk et al., 1989). The relationship between serotypes 1 and 14 has been recently confirmed by Smith et al. (1999b). These authors described the isolation and molecular characterisation of major parts of the *cps* loci of *S. suis* serotypes 1 and 9

as well as the identification of *cps* gene sequences specific for either serotypes 1, 2 or 9. The *cps* sequences were tested by hybridisation with chromosomal DNA of the 35 serotypes of *S. suis* and type-specific probes and PCR assays for serotypes 1, 2 and 9 were developed. In the case of serotype 1 *cps* genes, it was shown by cross-hybridisation experiments that four *cps* genes of serotype 1 (cps1F, cps1G, cps1I and cps1J) specifically hybridised with serotype 1 and 14 strains. Unfortunately, the *cps* loci for *S. suis* serotype 14 have not been characterised yet. Serotype 1/14 isolates described as highly prevalent in the United Kingdom (Heath et al., 1996), could in fact be serotype 1 isolates which cross-react with serotype 14 antiserum. Studies are presently underway to confirm this hypothesis (M. MacLennan, personal communication, 2000).

The serotype 2 has always been considered the most virulent and the most frequently isolated serotype from diseased animals. However, the situation may be different depending the geographical location and also, throughout the time. For example, distribution of *S. suis* serotypes in Canada has been changing the last years. The percentage of *S. suis* serotype 2 strains isolated from diseased animals decrease from 22% to 15% in the last 7 years (Higgins and Gottschalk, 2000). This situation is very different from that observed in some European countries, such as France, where almost 70% of isolates recovered from diseased animals belong to serotype 2 (Berthelot-Hérault et al., 2000). In this regard, it may be hypothesised that European and North American serotype 2 strains of *S. suis* possess a different virulence potential (see below). Under specific circumstances, some strains belonging to other serotypes of *S. suis* appear highly virulent, as it is the case of serotype 14 in United Kingdom (Heath et al., 1996) and serotypes 1/2 and 5 in Canada (unpublished observations). Finally, the number of untypeable isolates is in general relatively low (Higgins and Gottschalk, 2000). Most of the times, these isolates are recovered from sporadic cases of disease. It seems that there is no justification at the present time for the characterisation of new capsular types (Higgins and Gottschalk, 2000)

2. Virulence factors

Most studies on *S. suis* virulence factors have been carried out with serotype 2 strains. Although there is confusion in the description of virulence, researchers agree at least on one point: the existence of virulent and avirulent strains of *S. suis* serotype 2. However, the concept of "virulence" may differ between different groups, since experimental infection models for *S. suis* can be misleading. For example, different studies have designated field strains as virulent or avirulent based on: (1) the clinical condition of the animal from which the strain was isolated (clinically healthy or diseased animals); (2) on the presence of virulence-related proteins; or (3) different experimental infection models, using (a) different strains of mice, or (b) colostrum-deprived piglets (pre-infected or otherwise with other micro-organisms), or (c) piglets of different ages from either conventional or specific-pathogen-free herds. In fact, results from experimental infections of pigs with *S. suis* may rely, among other considerations, on the immunological status of the animals, the route of infection, the size of the inoculum and the presence of the organism as normal inhabitant of the upper respiratory tract of animals prior to the experimental infection. Caution is therefore recommended before classifying a strain as virulent or avirulent. In fact, important discrepancies exist in the literature regarding even the virulence of the same strain of *S. suis* (Gottschalk et al., 1999a; Staats et al., 1999). Different opinions or versions about the definition of the virulence of *S. suis* have contributed to hampering the studies on virulence factors of this bacterial species. It is imperative to define "virulence" for *S. suis* among the scientific community. In fact, there is a need of an international agreement on the use of an animal model, the strain(s) (well defined strain(s) and avirulent derivative(s)), the appropriate dose of micro-organisms, the conditions of bacterial growth, the route of infection, the description of symptoms (definition of an "ill" or "affected" animal), and many other aspects which may influence the definition of virulence. Presently, it is almost impossible to extrapolate results of virulence or protection from one study to another. This problem is also faced by industrial companies which intent to develop protective immunogens. This is a clearly critical problem which must be solved.

Despite the fact that knowledge on virulence factors is limited, the most important candidates in *S. suis* are the capsular polysaccharide (CPS), the virulence-related proteins, such as the muramidase-released protein and the extracellular protein factor, the haemolysin (suilysin) and the adhesins.

2.1 Capsular polysaccharide (CPS)

The CPS of *S. suis* serotype 2 is composed of five sugars, including sialic acid (N-acetyl neuraminic acid), and is so far the only proven critical virulence factor, based on the studies on nonencapsulated isogenic mutants obtained by insertional mutagenesis. The absence of CPS correlated with increased hydrophobicity and phagocytosis using murine and porcine phagocytes. In addition, unencapsulated mutants were shown to be avirulent in mice and in two different pig models of infection (Charland et al., 1998; Smith et al., 1999a). Recently, Smith et al. (1999a) have isolated and characterised the capsular locus (*cps*2 locus) of *S. suis* serotype 2 and have been described different genes potentially encoding for glucosyl-, galactosyl-, N-acetylglusoaminyl-, and rhamnosyltransferase activities. The *cps* loci of other serotypes of *S. suis* have also been identified and characterised (Smith et al., 1999b). Despite the fact that the CPS seems, based on isogenic mutational studies, to be a major virulence factor, most avirulent strains are encapsulated, indicating that other important virulence factors are essential. In addition, virulent and avirulent strains possess a capsule of similar size with similar concentrations of sialic acid (Charland et al., 1996). This latter component has already been related to virulence for other bacterial agents of meningitis (Wessels et al., 1989). Interestingly, genes involved in the synthesis of sialic acid have not so far been found. In fact, resistance to clearance from the bloodstream does not rely only on the presence of the CPS, since a well encapsulated avirulent strain is eliminated from circulation within 48 h, whereas a virulent strain can stay in relatively high numbers in the blood for more than five days (unpublished observations). Antibodies against the capsular material only partially protect against infection (Charland et al., 1997) and convalescent (and protected) animals produce low levels of these antibodies (del Campo Sepulveda et al., 1996; unpublished observations).

2.2 Muramidase-released protein and extracellular protein factor

In addition to the CPS, cell-wall and extracellular proteins have been associated with virulence of *S. suis*. Two proteins, a muramidase-released protein (MRP) and an extracellular protein factor (EF), originally associated with virulent strains, have been reported in serotype 2 strains (Smith et al., 1997). In addition, strains producing MRP and larger variants of EF (EF*) were isolated from human beings, but were non-virulent for pigs. Isogenic mutants lacking both these proteins appeared to be as virulent as the wild type strain after experimental infection of newborn germfree pigs. Similar results were obtained with isogenic MRP-EF- mutants of *S. suis* serotype 1 (Smith et al., 1997). The authors suggested that the virulence of *S. suis* is a multifactorial process in which particular functions can be fulfilled by alternative factors. They also suggested that the synthesis of these proteins may only be coincidentally associated with virulence rather than being virulence factors *per se*. However, this association of MRP and EF with virulence is observed with strains of certain countries but not with others. For example, most North American strains isolated from acute cases of septicaemia and/or meningitis (from either pig or human origin) were MRP and/or EF negative (Gottschalk et al., 1998; Chatellier et al., 1999). Interestingly, after their analysis by randomly amplified polymorphic DNA, the few MRP+EF+ North American stains were clustered in the same group than European strains which shared the same phenotype (Chatellier et al., 1999). A certain association of these proteins (specially the EF protein) with virulence seems to exist, and most isolates harbouring these factors are virulent. However, the absence of one or more of these proteins cannot necessarily be associated with a lack of virulence. Again, since the term "virulence" is poorly defined for *S. suis* it is also possible that, under standardised conditions, MRP+ EF+ strains are potentially more virulent than MRP- EF- strains.

2.3 Haemolysin (suilysin)

S. suis also produces a haemolysin (suilysin), a thiol-activated toxin, which may have a role in virulence (Jacobs et al., 1994). This protein belongs to the family of toxins known as antigenically related cholesterol-binding toxins which forms transmembrane pores and possess a "multi-hit" mechanism of action (Gottschalk et al., 1995). The gene coding for suilysin has been sequenced, revealing a relative high

similarity with the pneumolysin, a toxin produced by *Streptococcus pneumoniae* (Segers et al., 1998). Despite the fact that antibodies against the suilysin seem to protect against infection (Jacobs et al., 1994), the role of this toxin as a virulence factor has not yet been confirmed. Similarly to MRP and EF, most European strains are suilysin-positive, whereas variable production of this protein has been observed with North American strains (Gottschalk et al., 1998; Staats et al., 1999). However, since a certain role in the pathogenesis of the infection could be attributed to the suilysin (see below), this toxin may be associated with high virulence in serotype 2 strains. To the best of our knowledge, no avirulent, suilysin-positive *S. suis* serotype 2 strain has been reported. The characterisation of isogenic mutants defective in the production of such toxin will help in the comprehension of its role in the pathogenesis of the infection. Since the complete sequence of the suilysin gene is well known, these mutants will probably be obtained and characterised soon (P. Willson, VIDO, Saskatoon, personal communication, 2000). In this regard, an efficient electrotransformation system for *S. suis* has been described and several vectors replicate in *S. suis* serotype 2 (Smith et al., 1995). These tools can be (and have been) applied to this important pathogen (Smith et al., 1997; Smith et al., 1999a).

2.4 Adhesins

S. suis proteins that have a role as adhesins have been described. *S. suis* was found to recognise the disaccharide sequence Galα1-4Gal present in the trihexosylceramide, GbO_3 (a neutral glycolipid that belongs to the P blood group antigens; Haataja et al., 1996). This binding specificity is responsible of the haemagglutination properties of *S. suis*. The 18 kDa adhesin has been purified and is present in all strains so far examined. This adhesin was classified into two subtypes, P_N and P_O, based on differences in their binding specificity. Type P_O was inhibited by galactose only, whereas type P_N was inhibited by both galactose and N-acetylgalactosamine. In addition, the purified adhesin was not only shown to be highly immunogenic but also induced bactericidal activity in mice (Haataja et al., 1996). A second *S. suis* adhesin with binding activity to albumin was detected in both virulent and avirulent strains of *S. suis* serotype 2. A 39 kDa protein was responsible, at least in part, for this binding activity. Furthermore, the addition of albumin increased the

virulence of *S. suis* strains when injected in mice (Quessy et al., 1997). Since avirulent strains also possess these two binding proteins described above, it could indicate that this type of activity is not sufficient by itself to make the strains more virulent.

Finally, a 60 kDa IgG-binding protein, related to the heat shock protein 60 family, has been described (Benkirane et al., 1998). This protein represents a common antigen found in all *S. suis* serotypes tested including both virulent and avirulent strains of *S. suis* serotype 2. Hence, its role in virulence is unknown.

In summary, more studies are needed to characterise virulence factors of *S. suis* serotype 2. The presence of MRP, EF and the suilysin in European isolates represents virulence potential. Conversely, the absence of one or more of these proteins in isolates from affected animals cannot be automatically associated with a lack of virulence. It is possible that MRP-, EF-, suilysin negative virulent strains from North America are comparatively less virulent than MRP+, EF+, suilysin positive European strains. Experimental infections with a well-standardised model using several strains representative of both groups are needed to confirm this hypothesis. Finally, virulent strains can also be isolated from healthy animals and clinical disease may sometimes be the consequence of the disturbance of the immune balance due to different causes, such as other infectious diseases, management, and stress. So far, the enigma of virulence factors and/or markers for virulent North American serotype 2 strains is still unsolved. The lack of knowledge of virulence factors for other serotypes is even more marked.

3. Steps involved in the development of meningitis associated with *S. suis*

The pathogenesis of *S. suis* infections is poorly understood. Moreover, studies on this subject are limited to serotype 2 and only concern the development of meningitis. Piglets are contaminated during farrowing (vertical transmission of the infection). They also diversely and probably heterogeneously acquire the bacterium due to close contact with the sows, her faeces, and the surrounding structures (pen walls, dirty soil, etc.). Reasons that may explain why *S. suis* will successfully colonise only some piglets and not others are poorly known. Infection of newborn piglets may also take place through the respiratory route from sow to piglets and among piglets. Colonised animals will

harbour the bacteria in their tonsils. Some animals will only be healthy carriers and will never develop disease, whereas others will, sooner or later, develop bacteremia, sometimes septicaemia and finally, meningitis. Hence, in these cases, bacteria should travel throughout the bloodstream and reach the central nervous system (CNS) (*Figure 1: see page 222 of the thesis discussion*).

The first unresolved question of the pathogenesis of the infection caused by *S. suis* is how bacteria present at low levels on mucosal surfaces are able to traverse the first mucosal barriers to develop disease. In fact, bacteria would need to breach mucosal epithelia in the upper respiratory tract. Very few studies are available regarding the interactions between *S. suis* and epithelial cells. It has been recently reported that virulent *S. suis* strains can invade, to a certain extent, an epithelial cell line of human origin (Norton et al., 1999). This group also showed that suilysin-positive strains were cytotoxic for cells and that a specific monoclonal anti-suilysin antibody inhibited this toxicity. The cytotoxicity of the suilysin for epithelial cells was confirmed by Lalonde et al. (2000), using a cell line of swine origin. Norton et al. (1999) suggested that suilysin-positive *S. suis* strains can use invasion and cell lysis as a mechanism to breach the mucosal epithelia (Figure 1, "a"). Suilysin-negative strains were shown to be non-toxic for epithelial cells, but cell invasion with such strains have unfortunately not been assessed in that study (Figure 1, "a").

On the other hand, Lalonde et al. (2000) could not find any invasion of *S. suis* using several epithelial cell lines from human and different animal species, including swine. The fact that the invasion observed by Norton et al. (1999) was considered by the authors as a "rare event" and the use of different techniques may explain such differences. Interestingly, a relatively high level of adhesion was observed for different strains and different cell lines tested (Lalonde et al., 2000). Such adhesion was mediated by cell wall components and was considerably reduced in the presence of the CPS. By contrast, the CPS could not inhibit the adhesion of *S. suis* to brain microvascular endothelial cells (Charland et al., 2000). St-Geme and Cutter (1996) suggested that bacterial encapsulation may be modulated depending on the infectious stage. Encapsulation may be down-regulated during colonisation of epithelial cells (respiratory tract) and, once in the bloodstream, up-regulation of capsule production

might protect bacteria against the immune system although it does not completely prevent adhesion to other cells. It has already been shown that *in vivo* grown *S. suis* produces larger amounts of capsular polysaccharide than *in vitro* grown bacteria (Charland et al., 1996). It has also been proposed that capsule expression also influenced the accessibility of P_O and P_N adhesins (Haataja et al., 1996). These authors proposed that haemagglutination of *S. suis* undergoes spontaneous phase variation and could play a role in the different steps of *S. suis* infection. To date, however, there is no direct evidence of such encapsulation modulation for *S. suis*.

The second unresolved question is how bacteria travel in the bloodstream (dissemination). An early theory suggested uptake of bacteria by monocytes (in the absence of specific antibodies), intracellular survival and invasion of the CNS by the "Trojan horse theory" (Williams and Blackemore, 1990). Studies carried out with flow cytometry also indicated uptake of *S. suis* by swine and human phagocytes (Busque et al., 1998). This bacterial uptake could take place directly at the tonsils by macrophages (Figure 1, "b") or once the bacteria is in the bloodstream (Figure 1, "c"). However, most studies carried out during the last decade suggest that bacteria may also use (an)other mechanism(s) to disseminate. First, even though a certain level of phagocytosis is observed, most bacteria remain extracellular. In fact, the number of monocytes containing bacteria in the preparations from *S. suis* bacteremic pigs is low (less than 2%) (Williams and Blakemore, 1990). In addition, as discussed, the CPS confers antiphagocytic properties to *S. suis* and unencapsulated mutants were readily phagocytosed and destroyed (Charland et al., 1998; Smith et al., 1999a). Hence, it is possible that extracellular *S. suis* bacteria also travel free in circulation (Figure 1, "d"). Finally, a relatively high level of adhesion (without phagocytosis) of *S. suis* to phagocytic cells has recently been observed (Segura et al., 1999a), which may also suggest that bacteria can be largely bound but not ingested by macrophages, thus being responsible for persistent bacteremia and disseminated infection (a "modified" Trojan horse theory) (Figure 1, "c").

The third unresolved question is how bacteria traverse the blood-brain barrier (BBB) into the subarachnoid space (SAS). If the early theory of the Trojan horse (or the "modified" Trojan horse) were correct, bacteria would arrive to the BBB inside or

surface-associated with monocytes. In fact, Williams and Blakemore (1990) found intracellular bacteria within the choroid plexus parenchyma and also in circulating monocytes of bacteremic pigs. As the CNS is considered to be an immunoprivileged organ, normal circulation of monocytes to the CNS is still controversial. However, it is accepted that the immune-privileged status of the brain is not absolute, and the permeability to some immune cells could be modified (see below) as an adaptation to the specific local microenvironment (Wekerle, 1993).

Most meningeal pathogens, such as *S. pneumoniae*, *Escherichia coli* K1 and group B *Streptococcus* (GBS), are known to directly interact with cells of the BBB as free bacteria (Tuomanen, 1996). This barrier, responsible for maintaining biochemical homeostasis within the CNS is characterised by the presence of tight junctions, and regulates fluid, macromolecule and cell trafficking on both sides of the layer. Two kinds of cells present such tight junctions: the brain microvascular endothelial cells (BMEC) and the epithelial cells forming the choroid plexus (Tuomanen, 1996). It is generally accepted that bacterial interactions with BMEC are mainly characterised by specific bacterial attachment with consequent invasion, toxicity and/or increase of permeability. Invasion assays performed with human BMEC demonstrated that, unlike those pathogens, *S. suis* serotype 2 could adhere to but not invade this type of cells (Charland et al., 2000). It is possible that adherence of *S. suis* to BMEC plays a role in the pathogenesis of the infection and that, after adherence of *S. suis* to BMEC, bacteria secrete toxic factors which would affect the endothelial cells. Such factors could increase BBB permeability, which could lead to the development of cerebral oedema, increased intracranial pressure and cerebral blood flow blockage characteristic of bacterial meningitis. Indeed, histopathological findings indicating necrosis of vessel walls in association with inflammatory cellular aggregates have been reported. Vessel endothelia were swollen and sometimes lumina were occluded, demonstrating inflammatory cell invasion directly from the overlying meninges (Sanford, 1987). It may be pertinent, at this point, to suggest the hypothesis that suilysin-positive (European strains with probably higher virulence?) and suilysin-negative strains use different ways to induce disease. In fact, the pathogenesis of the infection may be different depending on the strain. For example, it was recently shown that suilysin can damage BMEC,

which could contribute to increased BBB permeability (Charland et al., 2000) (Figure 1, "e"). On the other hand, it is possible that the adhesion of suilysin-negative strains to BMEC may have consequences other than direct damage to the cells. One mechanism may be the stimulation of cytokine production through bacterial adherence with resultant alteration of BBB permeability (Figure 1, "f"). Preliminary studies indicate that *S. suis* can stimulate significant levels of pro-inflammatory cytokines from BMEC (Charland et al., 2000; unpublished data). In addition, the stimulation of cytokines may have other consequences, such as the presentation of new receptors on the endothelial cell surface, as described for *S. pneumoniae* (Cundell et al., 1995). The role of cytokines in the increase of BBB permeability or in the bacterial enter to the CNS in *S. suis* infections remains to be established.

On the other hand, interactions of bacteria with polarised epithelial cells of the choroid plexus (the other important cell component of the BBB) may be the consequence of pressure of high-grade constant bacteremia. In fact, disruption of the plexus brush border, with fibrin and inflammatory cell exudate present in the ventricles has been described during natural or experimentally induced *S. suis* meningitis (Williams and Blakmore, 1990). In this regard, bacteria may also use some of the mechanisms mentioned above for BMEC to traverse the BBB at the choroid plexus level. Further studies of interactions between *S. suis* and epithelial cells might bring new and relevant information.

It is now recognised that several inflammatory and infectious diseases are associated with the overproduction of cytokines and that the recruitment and activation of different leukocyte populations is a hallmark of acute inflammation (Sprenger et al., 1996). Recent works in our laboratory show that *S. suis* is able not only to interact with monocytes/macrophages but also to induce the release of several proinflammatory cytokines and chemokines (e. g. tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, IL-1, IL-8 and monocyte chemotactic protein-1) (Segura et al., 1999b; unpublished observations). The observed cytokine-inducing activity of *S. suis* may have significant biological relevance, since it has been demonstrated that these cytokines can be generated in the blood and cerebrospinal fluid during invasive meningeal infections (Sprenger et al., 1996). Acute bacterial meningitis is characterised by a migration of

leukocytes into the SAS. Although the brain is considered a "privileged" organ, with a lack of a routine immunological surveillance and containing only 1-5 leukocytes/μl CSF under normal conditions, more than 10^5 leukocytes/μl may accumulate during the acute phase of meningitis. Polymorphonuclear granulocytes have been found to be the first leukocytes at the onset of the disease, but then the cellular picture gradually changes to a mononuclear pattern consisting mainly of monocytes and lymphocytes (Sprenger et al., 1996). The exact mechanism regulating the migration of leukocytes through the tight endothelial cell barrier is still unclear. The local production of proinflammatory cytokines by microglial cells, endothelial cells, and migrating leukocytes upon contact with bacteria is currently regarded as the initial step. Several cytokines and chemokines (especially TNF-α, IL-1, IL-8) are known to up-regulate the expression of cell adhesion molecules (CAMs), such as selectins and integrins, that allow transendothelial migration of leukocytes. Some meningeal pathogens have been shown to stimulate the expression of CAMs (Wilson et al., 1998).

As a consequence, the innate immune system of animals could play an important role in the pathogenesis of the infection. For example, in the monocyte-associated theory (Trojan Horse or "modified"), cytokine release by activated phagocytes after interaction with *S. suis* might, in turn, activate endothelial cells and increase both cell and bound (or intracellular) bacterial trafficking (Figure 1, "g"). On the other side, if the free circulating bacteria theory is considered, *S. suis* could directly, or indirectly (via local cytokine release) stimulate the expression of CAMs and non-infected leukocyte transmigration across the BBB (Figure 1, "h"). In this case, migrating leukocytes "open the door" for bacterial trafficking to the CNS. These theories have not been explored, and the ability of *S. suis* to induce the up-regulation of CAMs is so far unknown.

4. Final conclusions and recommendations

Critical virulence factors (or at least virulence markers) of *S. suis* are still poorly characterised, perhaps particularly for North American strains. The use of new technologies, such as signature tagged mutagenesis, subtractive hybridisation and

differential display for *in vivo* gene expression, will probably add new and important information to clarify what is presently unknown. The enigma of the pathogenesis of *S. suis* meningitis remains to be solved. Present knowledge clearly indicates that *S. suis* interactions with the host differ from other well-characterised meningeal pathogens, such as group B *Streptococcus* (Segura et al., 1998; Charland et al., 2000; Lalonde et al., 2000). Invasion of the CNS may represent a synergistic event between free and associated bacteria, and the potential to establish infection relies on the capacity of an individual strain to produce a still unknown panel of virulence factors. Thus, the virulence of a strain will be the result of complex interactions between the organism and its host. A complete understanding of the interacting pathways will give important insights into disease progression. Further studies to delineate the mechanisms through which *S. suis* induces meningitis would contribute to greater knowledge and potential therapies to control *S. suis* infections.

At this point, it would be advisable to make some recommendations to solve most of the problems observed with the virulence of *S. suis*:

Recommendation 1: Researchers working on *S. suis* should develop and agree on an internationally standardised model by which virulence in *S. suis* is defined.

Recommendation 2: Researchers working in this field should agree to set up any study on the pathogenesis of the infection using, at least, two agreed virulent type strains as controls: an European and a North American strain, with different phenotypic characters. These two strains should be carefully kept in a reference laboratory with a minimum of in vitro passages and they should be available to any researcher upon request.

Recommendation 3: Researchers working in this field should agree to collaborate in acquiring the genome sequence of the agreed virulent type strains.

Recommendation 4: Researchers in this field should organise regular meetings (which may take place simultaneously with international meetings) to initiate and implement the above mentioned (and others if necessary) recommendations.

Acknowledgements

We would like to thank Robert Higgins for critical reviewing of the manuscript. Work in our laboratory in this area is supported by Grant 0GP0154280 from the Natural Sciences and Engineering Research Council of Canada and by Grant 99-ER-0214 from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (Québec).

References

Benkirane, R., Gottschalk, M., Jacques, M., Dubreuil, J.D., 1998. Immunochemical characterization of an IgG-binding protein of *Streptococcus suis*. FEMS Immunol. Med. Microbiol. 20, 121-127.

Berthelot-Hérault, F., Morvan, H., Kéribin, AM., Gottschalk, M., Kobisch, M., 2000. Production of muraminidase-released protein (MRP), extracellular factor (EF) and haemolysin by field isolates of *Streptococcus suis* capsular types 2, 1/2, 9, 7 and 3 isolated from swine in France. Vet. Res. 31, *in press*.

Boye, M., Feenstra, A., Tegtmeier, C., Andersen, L., Rasmussen, S., Bille-Hansen, V., 2000. Detection of *Streptococcus suis* by in situ hybridization, indirect immunofluorescence, and peroxidase-antiperoxidase assays in formalin-fixed, paraffin-embedded tissue sections from pigs. J. Vet. Diagn. Invest. 12, 224-232.

Busque, P., Higgins, R., Sénéchal, S., Marchand, R., Quessy, S., 1998. Simultaneous flow cytometric measurement of *Streptococcus suis* phagocytosis by polymorphonuclear and mononuclear blood leukocytes. Vet. Microbiol. 63, 229-238.

Chatellier, S., Gottschalk, M., Higgins, R., Brousseau, R., Harel, J., 1999. Relatedness of *Streptococcus suis* serotype 2 isolates from different geographical origins as evaluated by molecular fingerprinting and phenotyping. J. Clin. Microbiol. 37, 362-366.

Chatellier, S., Harel, J., Zhang, Y., Gottschalk, M., Higgins, R., Devriese, L., Brousseau, R., 1998. Phylogenetic diversity of *Streptococcus suis* strains of various serotypes as revealed by 16S rRNA gene sequence comparison. Int. J. Syst. Bacteriol. 48, 581-589.

Charland, N., Harel, J., Kobisch, M., Lacasse, S., Gottschalk, M., 1998. *Streptococcus suis* serotype 2 mutants deficient in capsular expression. Microbiology. 144, 325-332.

Charland, N., Jacques, M., Lacouture, S., Gottschalk, M., 1997. Characterization and protective activity of a monoclonal antibody against a capsular epitope shared by *Streptococcus suis* serotypes 1, 2 and 1/2. Microbiology. 143, 3607-3614.

Charland, N., Kobisch, M., Martineau-Doizé, B., Jacques, M., Gottschalk, M., 1996. Role of capsular sialic acid in virulence and resistance to phagocytosis of *Streptococcus suis* capsular type 2. FEMS Immunol. Med. Microbiol. 14, 195-203.

Charland, N., Nizet, V., Rubens, C., Kim, K., Lacouture, S., Gottschalk, M., 2000. *Streptococcus suis* interactions with human brain microvascular endothelial cells. Infect. Immun. 68, 637-643.

Cundell, D., Gerard, N.P., Gerard, C., Idanpaan-Helkklla, I., Tuamanen, E., 1995. *Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor. Nature 377, 435-438.

Del Campo Sepúlveda, E.M., Altman, E., Kobisch, M., D'Allaire, S., Gottschalk, M., 1996. Detection of antibodies against *Streptococcus suis* capsular type 2 using a purified capsular polysaccharide antigen-based indirect ELISA. Vet. Microbiol. 52, 113-125.

Devriese, L. A., Ceyssens, K., Hommez, J., Kilpper-Balz, R., Schleifer, K. H., 1991. Characteristics of different *Streptococcus suis* ecovars and description of a simplified identification method. Vet. Microbiol. 26, 141-150.

Gottschalk, M., Higgins, R., Jacques, M., Mittal, K.R., Henrichsen, J., 1989. Description of 14 new capsular types of *Streptococcus suis*. J. Clin. Microbiol. 27, 2633-2636.

Gottschalk, M., Higgins, R., Quessy, S., 1999a. Dilemma of the virulence of *Streptococcus suis*. J. Clin. Microbiol. 37, 4202-4203.

Gottschalk, M., Lacouture, S., Dubreuil, J.D., 1995. Characterization of *Streptococcus suis* capsular type 2 haemolysin. Microbiology. 141, 189-195.

Gottschalk, M., Lacouture, S., Odierno, L., 1999b. Immunomagnetic isolation of *Streptococcus suis* serotypes 2 and 1/2 from swine tonsils. J. Clin. Microbiol. 37, 2877-2881.

Gottschalk, M., Lebrun, A., Wisselink, H., Dubreuil, D., Smith, H., Vecht, U., 1998. Production of virulence-related proteins by Canadian strains of *Streptococcus suis* capsular type 2. Can. J. Vet. Res. 62, 75-79.

Haataja, S., Tikkanen, K., Hytönen, J., Finne, J., 1996. The Galα1-4Gal-binding adhesin of *Streptococcus suis*, a gram-positive meningitis-associated bacterium. In: Kahane, I., Ofek, I. (Eds), Toward Anti-adhesion Therapy for Microbial Diseases. Adv. Exp. Med. Biol. 408, 25-34.

Heath, P.J., Hunt, B.W., Duff, J.P., Wilkinson, J.D., 1996. *Streptococcus suis* serotype 14 as a cause of pig disease in the UK. Vet. Rec. 139, 450-451.

Higgins, R., Gottschalk, M., 1999. Streptococcal diseases. In: Straw, B.E., D'Allaire, S., Mengeling, W.L., Taylor, D.J. (Eds), Diseases of Swine, Iowa State University Press, Ames, pp. 563-570.

Higgins, R., Gottschalk, M., 2000. Distribution of *Streptococcus suis* capsular types in 1999. Can. Vet. J. 41, 414.

Higgins, R., Gottschalk, M., Boudreau, M., Lebrun, A., Henrichsen, J., 1995. Description of six new *Streptococcus suis* capsular types. J. Vet. Diagn. Invest. 7, 405-406.

Jacobs, A., Loeffen, W., van den Berg, A., Storm, P., 1994. Identification, purification and characterization of a thiol-activated hemolysin (suilysin) of *Streptococcus suis*. Infect. Immun. 62, 1742-1748.

Lalonde, M., Segura, M., Lacouture, S., Gottschalk, M., 2000. Interactions between *Streptococcus suis* serotype 2 and different epithelial cell lines. Microbiology. 146, *in press*.

Norton, P.M., Rolph, C., Ward, P.N., Bentley, R.W., Leigh, J.A., 1999. Epithelial invasion and cell lysis by virulent strains of *Streptococcus suis* is enhanced by the presence of suilysin. FEMS Immunol. Med. Microbiol. 26, 25-35.

Quessy, S., Busque, P., Higgins, R., Jacques, M., Dubreuil, J.D., 1997. Description of an albumin binding activity for *Streptococcus suis* serotype 2. FEMS Microbiol. Lett. 147, 245-250.

Rasmussen, S., Andresen, O., 1998. 16S rDNA sequence variations of some *Streptococcus suis* serotypes. Int. J. Syst. Bacteriol. 48, 1063-1065.

Sanford, S.E., 1987. Gross and histopathological findings in unusual lesions caused by *Streptococcus suis* in pigs. II. Central nervous system lesions. Can. J. Vet. Res. 51, 486-489.

Segers, R.P., Kenter, L., de Haan, A., Jacobs, A.A., 1998. Characterisation of the gene encoding suilysin from *Streptococcus suis* and expression in field strains. FEMS Microbiol. Lett. 167, 255-261.

Segura, M., Cléroux, P., Gottschalk, M., 1998. *Streptococcus suis* and group B *Streptococcus* differ in their interactions with murine macrophages. FEMS Immunol. Med. Microbiol. 21, 189-195.

Segura, M., Gottschalk, M., 1999a. Adhesion of *Streptococcus suis* capsular type 2 to phagocytic cells. In: Abstracts of the XIV Lancefield International Symposium on Streptococci and Streptococcal Diseases, abstract P8.17. Auckland, New Zealand.

Segura, M., Stankova, J., Gottschalk, M., 1999b. Heat-killed *Streptococcus suis* capsular type 2 strains stimulate tumor necrosis factor alpha and interleukin-6 production by murine macrophages. Infect. Immun. 67, 4646-4654.

Smith, H.E., Damman, M., van der Velde, J., Wagenaar, F., Wisselink, H.J., Stockhofe-Zurwieden, N., Smits, M.A., 1999a. Identification and characterization of the *cps* locus of *Streptococcus suis* serotype 2: the capsule protects against phagocytosis and is an important virulence factor. Infect. Immun. 67, 1750-1756.

Smith, H.E., Veenbergen, V., van der Velde, J., Damman, M., Wisselink, H., Smits, M.A., 1999b. The *cps* genes of *Streptococcus suis* serotypes 1, 2, and 9: development of rapid serotype-specific PCR assay. J. Clin. Microbiol. 37, 3146-3152.

Smith, H.E., Wisselink, H.J., Stockhofe-Zurwieden, N., Vecht, U., Smits, M.A., 1997. Virulence markers of *Streptococcus suis* type 1 and 2. Adv. Exp. Med. Biol. 418, 651-656.

Smith, H.E., Wisselink, H.J., Vecht, U., Gielkens, A., Smits, M.A. 1995. High-efficiency transformation and gene inactivation in *Streptococcus suis* type 2. Microbiology. 141, 181-188.

Sprenger, H., Rösler, A., Tonn, P., Braune, H. J., Huffmann, G., Gemsa, D., 1996. Chemokines in the cerebrospinal fluid of patients with meningitis. Clin. Immunol. Immunopathol. 80, 155-161.

Staats, J., Brandon, P., Stewart, G., Chengappa, M.M., 1999. Presence of the *Streptococcus suis* suilysin gene and expression of MRP and EF correlates with high virulence in *Streptococcus suis* type 2 isolates. Vet. Microbiol. 70, 201-211.

Staats, J.J., Feder, I., Okwumabua, O., Chengappa, M.M., 1997. *Streptococcus suis*: past and present. Vet. Res. Commun. 21, 381-407.

St. Geme, J. III, Cutter, D., 1996. Influence of pili, fibrils, and capsule on in vitro adherence of *Haemophilus influenzae* type b. Mol. Microbiol. 21, 21-31.

Tuomanen, E., 1996. Entry of pathogens into the central nervous system. FEMS Microbiol. Rev. 18, 289-299.

Wekerle, H., 1993. Lymphocyte traffic to the brain. In: Pardridge, W.M. (Ed), The Blood-Brain Barrier, Cellular and Molecular Biology, Raven Press, New York, pp. 67-85.

Wessels, M.R., Rubens, C.E., Benedi, V.J., Kasper, D.L., 1989. Definition of a bacterial virulence factor: Sialylation of the group B streptococcal capsule. Proc. Natl. Acad. Sci. USA. 86, 8983-8987.

Williams, A.E., Blakemore, W.E., 1990. Pathogenesis of meningitis caused by *Streptococcus suis* type 2. J. Infect Dis. 162, 474-481.

Wilson, S., Drevets, D.A., 1998. *Listeria monocytogenes* infection and activation of human brain microvascular endothelial cells. J. Infect. Dis. 178, 1658-1666.