Université de Montréal

The effect of *Streptococcus suis* serotype 2 on the surface expression of adhesion molecules on human monocytes and endothelial cells.

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Ce mémoire intitulé :

The effect of *Streptococcus suis* serotype 2 on the surface expression of adhesion molecules on human monocytes and endothelial cells.

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Résumé

Les infections occasionnées par *Streptococcus suis* sont un problème d'ordre mondial. Elles affectent l'industrie du porc ainsi que les humains qui travaillent dans un environnement à proximité des porcs ou de ses produits. La méningite est l'une des pathologies les plus commune causée par une infection de ce pathogène. La pathogenèse des infections causées par *S. suis* est encore peu comprise. Durant la méningite, une réponse inflammatoire aiguë se manifeste et les molécules d'adhésion exprimées à la surface des leucocytes et des cellules endothéliales joueraient un rôle important dans ce processus. Le but de cette etude était de determiner si *S. suis* était capable d'induire une augmentation de l'expression de molécules d'adhésion. De plus, nous voulions investiguer la capacité d'adhérence des monocytes aux cellules endothéliales suite à une exposition à *S. suis*. Nous avons trouvé que *S. suis* sérotype 2 peut induire l'expression d'ICAM-1, de CD11a/CD18 et de CD11c/CD18 à la surface des monocytes humains THP-1. Par contre, les cellules endothéliales humaines stimulées avec *S. suis* n'ont pas démontré d'augmentation dans leur expression de ICAM-1, VCAM-1 et E-selectin. Les composantes de la paroi cellulaire de *S. suis* contribueraient principalement à l'induction de ces molécules d'adhésion. D'ailleurs, un mutant non encapsulé de *S. suis* est capable d'induire des niveaux d'expression de molécules d'adhésion plus élevés que la souche sauvage. En testant des souches humaines et porcines de *S. suis*, nous n'avons trouvé aucune différence entre les souches dans le niveau

d'expression des molécules d'adhésion. Nos résultats démontrent que l'augmentation de l'expression des molécules d'adhésion à la surface des monocytes accompagne une augmentation de l'adhérence des monocytes aux cellules endothéliales. Nous pensons qu'il s'agit d'un processus contribuant à expliquer les caractéristiques observées lors de la réaction inflammatoire durant la méningite.

Mots clés: *Streptococcus suis*, molécules d'adhésion, méningite, humain, monocytes, cellules endothéliales.

Summary

Streptococcus suis infections are a major problem world-wide in the swine industry, and also affect humans who work in close proximity to pigs or pig products. Meningitis is the most common manifestation of infection due to this pathogen. 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. We investigated the ability of *S. suis* to up-regulate the expression of important adhesion molecules involved in inflammation. Subsequent adherence of monocytes to endothelial cells was also measured. We found that *S. suis* serotype 2 is capable of up-regulating the surface expression of intercellular adhesion molecule -1 (ICAM-1), CD11a/CD18 and CD11c/CD18 on human THP-1 monocytes, in a time- and bacterial-concentration dependent manner. On the other hand, stimulation of endothelial cells of human origin by *S. suis* did not induce an increased expression of ICAM-1, vascular cell adhesion molecule -1 (VCAM-1) and E-selectin. The up-regulation of adhesion molecules in monocytes was mainly due to cell wall components of *S. suis*. 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. There was no clear tendency for human strains to induce a higher expression of adhesion molecules than strains from diseased pigs. A consequence of this increase in adhesion molecule expression on monocytes stimulated by *S. suis* was an increase in monocyte adherence to endothelial cells, thus providing a possible

mechanism for some of the inflammatory features of meningitis caused by this pathogen.

Key words: *Streptococcus suis*, adhesion molecules, meningitis, human, monocytes, endothelial cells.

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List of acronyms and abbreviations

BBB:	Blood-brain barrier
CNS:	Central nervous system
CPS:	Polysaccharide capsule
EF:	Extracellular factor
GBS:	Group B Streptococci
ICAM-1:	Intercellular adhesion molecule – 1
IgSF:	Immunoglobulin superfamily
IL:	Interleukin
MCP-1:	Monocyte chemotactic protein –1
MRP:	Muramidase-released protein
PECAM-1:	Platelet-endothelial cell adhesion molecule – 1
TNF-α:	Tumor necrosis factor alpha
VCAM-1:	Vascular cell adhesion molecule – 1

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Dedication

To my parents….

Introduction

Streptococcus suis infections have been a major and world-wide problem in the swine industry, especially in the past ten years, causing a variety of infections including meningitis, arthritis, serositis, endocarditis and pneumonia. This organism is also associated with disease in other animal species, and more importantly, is considered a zoonotic agent, causing mainly meningitis in humans who work in close proximity to pigs. The pathogenesis of infection leading to meningitis is not well understood, and the virulence factors of *S. suis* that play an important role in the pathogenesis of disease are not well described. Most studies have focussed on serotype 2, since it is the serotype most commonly isolated from diseased animals.

It is generally accepted that *S. suis* infection is transmitted via the respiratory route, after which bacteria remain localised in the palatine tonsils of pigs. From there, *S. suis* may invade the blood, meninges or other tissues, possibly in close association with monocytes. Leukocyte influx into the subarachnoid space and the increase in blood-brain barrier permeability are considered hallmarks of bacterial meningitis. The pavementing of leukocytes on the vascular endothelium is critical for the recruitment of these circulating cells to sites of inflammation. This pavementing process is exaggerated as a result of increased expression of various cell adhesion molecules on leukocytes and endothelial cells. These adhesion molecules include selectins (E-, P-, and L-selectin), β_2 integrins (CD11a/CD18, CD11b/CD18 and CD11c/CD18) and members of the immunoglobulin superfamily (IgSF),

mainly intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). They work together in mediating leukocyte rolling, firm adhesion and subsequent extravasation, by forming multiple receptor-ligand pairs that act either in a sequential and orchestrated fashion or, as more recently proposed, in parallel pathways forming bottlenecks rather than a linear process. Some of these adhesion molecules have been shown to be up-regulated on pathogen infected cells, resulting in an increased adhesion of leukocytes to endothelial cells. This might be a means for recruiting leukocytes to the CNS.

Hence, we investigated the ability of *S. suis* serotype 2 to modulate the surface expression of ICAM-1, VCAM-1 and E-selectin on endothelial cells, and of ICAM-1, CD11a/CD18, CD11b/CD18 and CD11c/CD18 on THP-1 monocytes. We also examined the adhesiveness of THP-1 monocytes to endothelial cells following *S. suis* stimulation of one or both of the two cell types.

Literature Review

1. *Streptococcus suis*

1.1. General aspects of *S. suis*

S. suis infections date back to the early 1950's, when outbreaks of meningo-encephalitis in pigs of 1-6 months of age were documented (Field et al., 1954; Jensen and van Dorsenn, 1951). *S. suis* was officially designated as a new bacterial species in 1987 by Kilpper-Balz and Schleifer (Kilpper-Balz and Schleifer, 1987). *S. suis* is now known as a worldwide cause of a variety of porcine infections, resulting in substantial economic losses to the pig industry, in all countries where the swine industry is important. It has been isolated from cases of arthritis, pericarditis, bronchopneumonia, endocarditis, polyserositis, septicemia, rhinitis, and abortion (Higgins and Gottschalk, 2001; Perch et al., 1983; Sihvonen et al., 1988; Touil et al., 1988), but the most important clinical feature associated with *S. suis* is meningitis. This bacterium has also been increasingly isolated from a wide variety of animal species, most importantly from humans (Devriese and Haesebrouck, 1992; Halaby et al., 2000; Higgins et al., 1997; Keymer et al., 1983).

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. 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 observation of typical microscopic lesions in tissues (Higgins and Gottschalk, 1999).

Usually, infections due to *S. suis* have a favourable outcome with appropriate therapy, such as treatment with penicillin (Durand et al., 2001). However, control of the disease by vaccines and antimicrobials has generally been ineffective, partly because of increased resistance to antimicrobials, and partly because of a paucity of information on its protective antigens and virulence factors (Staats et al., 1997).

1.2. Description and classification

S. suis is a gram positive, hemolytic, facultative anaerobe. It appears as non-motile small ovoid cocci, occurring 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 (Kilpper-Balz and Schleifer, 1987).

S. suis was first biochemically and serologically characterized in the Netherlands by de Moor between 1956 and 1963, erroneously placing the identified strains in new Lancefield groups S, R, RS and T (de Moor, 1963). In 1966, Elliott found that de Moor's group S was similar to his PM *Streptococcus* that caused meningitis in piglets (Elliott, 1966). He suggested the name *Streptococcus suis* capsular type 1. Other porcine streptococci that corresponded to de Moor's group R were later isolated and were named *S. suis* capsular type 2 (Windsor and Elliott, 1975). In contrast to *S. suis* capsular type 1, *S. suis* capsular type 2 infected pigs of all ages (Windsor and Elliott, 1975). Later studies showed that both capsular types have a common

cell wall antigen, that is a lipid-bound teichoic acid closely related to that found in group D streptococci (Elliott et al., 1977), and are thus identified as Lancefield group D. However, DNA homologies and 16S RNA typing show that *S. suis* forms a group distinct from group D and other streptococci (Brousseau et al., 2001; Chatellier et al., 1999; Rasmussen and Andresen, 1998).

S. suis is now subdivided into serotypes by the antigenic specificity of its capsular polysaccharide. Thirty-five different serotypes have been identified to date, consisting of serotypes 1 to 34 and serotype 1/2 (Gottschalk et al., 1991; Gottschalk et al., 1991; Higgins and Gottschalk, 1995; Perch et al., 1983). Serotyping is an important step in the routine diagnostic procedure. Different techniques have been described, but most laboratories have adopted the coagglutination technique (Gottschalk and Segura, 2000).

1.3. Serotype prevalence and geographic distribution

Most *S. suis* isolated from diseased pigs belong to a limited number of capsular types, often being types 1 to 8 (Chatellier et al., 1999; Higgins and Gottschalk, 1999). Some capsular types, such as 17, 18, 19 and 21, are generally recovered from clinically healthy animals (Gottschalk et al., 1989). 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 on the geographical location, and also changes with time. For example, the proportion of *S. suis* strains of serotype 2 isolated from diseased animals in Canada decreased from 32% to 16% during the last

10 years (Higgins and Gottschalk, 2001). 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 Scandinavia, serotype 7 predominated for several years, but serotype 2 has become more prominent during the last years (Higgins and Gottschalk, 1999). Finally, the number of untypeable isolates is in general relatively low (Higgins and Gottschalk, 2000). Mostly, these isolates are recovered from sporadic cases of disease (Higgins and Gottschalk, 2000).

1.4. S. *suis* infection and transmission

1.4.1. In pigs

Pigs may develop infection when they first encounter *S. suis*, or they may become tonsillar carriers and may develop the clinico-pathological syndrome at some later date, usually following some form of environmental stress such as mixing, overcrowding, temperature changes, or rise in slurry gas (Alexander, 1995). Pigs can carry the organism subclinically for long periods, the main carrier site being the tonsils, but the organism can also be found in the genital and alimentary tracts (Clifton-Hadley, 1981; Robertson and Blackmore, 1989). Pigs may harbor a variety of *S. suis* strains or serotypes with no relationship to any specific pathological condition. It is also possible to isolate different serotypes from diseased animals within the same herd (Higgins and Gottschalk, 1999).

Thus, pigs carrying pathogenic *S. suis* serotypes and/or strains are known to be the source of infection for naive herds. Piglets born to sows with uterine and/or vaginal infections are exposed during the birth process and while suckling (Robertson and Blackmore, 1989). Infection of newborn piglets may also take place through the respiratory route from sow to piglets and among piglets (Berthelot-Herault et al., 2001; Higgins and Gottschalk, 1999). Thus, both vertical and horizontal transmission are involved in the spread of disease.

The most persistent clinical signs reported are neurological signs, including opisthotonus, lateral recumbency, paddling, convulsions, and ataxia. Sudden death, without premonitory signs, is also reported (Reams et al., 1994).

1.4.2. In humans

S. suis infections in humans have been associated with intensive contact with pigs or pig products (Arends and Zanen, 1988; Dupas et al., 1992). Individuals have also been infected following close contact with wild boars (Halaby et al., 2000) or from eating raw meat (Fongcom et al., 2001). The most common manifestation is meningitis, followed by septicemia and endocarditis (Arends and Zanen, 1988; Trottier et al., 1991). Individuals recovering from meningitis often suffer from hearing loss, which has been reported more frequently in these patients than in patients with meningitis due to other bacteria (Arends and Zanen, 1988). Skin injuries or minor abrasions

might represent the route of entry in most of the cases (Dupas et al., 1992); however, other routes of entry are possible.

Serotype 2 is responsible for the majority of *S. suis* infections in humans (Arends and Zanen, 1988; Durand et al., 2001). Types 4 and 14 are also associated with human infection. Prevalence of positive serum antibody titres to *S. suis* serotype 2 may be as high as 10% in meat inspectors and 20% in pig farmers (Robertson and Blackmore, 1989). The epidemiological importance of pigs to human infection was further established by DNA fingerprinting, using random amplified polymorphic (RAPD) analysis, which showed common RAPD patterns in isolates of human and pig origin (Chatellier et al., 1999). These observations were recently confirmed by a study of genetic diversity of *S. suis* strains using pulsed-field gel electrophoresis, where human strains were very homogenous and were statistically clustered in the same group of strains as those isolated from diseased pigs (Berthelot-Herault et al., 2002). Interestingly, another recent study found that although human and pig isolates were similar in serotype and phenotype, they differed in their ribotype pattern (Tarradas et al., 2001).

1.5. Virulence factors of *S. suis* serotype 2

A virulence factor denotes a bacterial product or strategy that contributes to virulence or pathogenicity (Salyers and Whitt, 1994). So far, *S. suis* serotype 2 has been described as having proposed virulence factors, since 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, whereas other strains devoid of the same virulence factor are still virulent. To complicate this even further, many discrepencies are present in the literature with respect to definition of strains as virulent or non-virulent (Gottschalk et al., 1999). Eliminating these discrepencies will facilitate the study of the virulence factors of *S. suis*. The main proposed virulence factors of *S. suis* serotype 2 are the polysaccharide capsule (CPS), extracellular factor (EF), muramidase-released protein (MRP), hemolysin, and adhesins. Other proteins are also implicated, and are briefly mentioned in this review.

1.5.1. The polysaccharide capsule (CPS)

Capsulated bacteria are responsible for causing some of the most serious invasive infections, including septicemia, pneumonia, septic arthritis, and meningitis (Moxon and Kroll, 1990). The role of capsules in bacterial virulence is to protect the bacteria from the host's inflammatory response, i.e. complement activation and phagocyte-mediated killing (Salyers and Whitt, 1994). The three most common bacteria causing meningitis in humans are *Neisseria meningitidis*, *Haemophilus influenzae* type b, and *Streptococcus pneumoniae*, and all three produce polysaccharide capsules (Salyers and Whitt, 1994). The capsule of *S. suis* is the only virulence factor of this bacterium that has been shown, to date, to be of critical importance (Gottschalk and Segura, 2000).

The capsule of *S. suis* serotype 2 is composed of five different polysacharides (Katsumi et al., 1996). One of these five sugars is N-acetyl-

neuraminic acid (NANA), or sialic acid (Elliott and Tai, 1978), which is a common component of host cell glycoproteins (Salyers and Whitt, 1994). Acting as a virulence factor in some bacteria, such as Group B *streptococci* (GBS), sialic acid inhibits the activation of the alternative complement pathway, thus permitting bacteria to avoid being engulfed by phagocytic cells (Edwards et al., 1982). However , sialic acid does not seem to be a critical virulence factor for *S. suis* serotype 2, since different strains possess a similar concentration of NANA, regardless of their virulence (Charland et al., 1996). In addition, a monoclonal antibody generated against a sialic acid epitope of *S. suis* serotype 2 could only partially protect mice from a lethal dose of bacteria (Charland et al., 1997).

The production of non-capsulated mutant strains of *S. suis* serotype 2 helped to further understand the function of the capsule as a virulence factor. An isogenic mutant defective in capsular production (2A) was produced from strain S735 using transposon mutagenesis (Charland et al., 1998). In porcine and murine models, the virulence of this non-capsulated mutant was significantly less than that of the wildtype strain, showing that the presence of the capsule was important in causing disease, and especially important in the survival of *S. suis* in blood, since the non-capsulated mutant was readily eliminated from the circulation, whereas the wild-type strain persisted for more than 48 hours (Charland et al., 1998). In fact, the capsule has been shown to be an anti-phagocytic factor. Studies using the same non-capsulated mutant, 2A, showed that whereas the capsulated wild-type strain was poorly phagocytosed, the non-capsulated mutant was rapidly ingested by

macrophages (Charland et al., 1998; Segura et al., 1998). Other non-capsular mutants constructed by insertional mutagenesis were also shown to be highly ingested by porcine macrophages (Smith et al., 1999). Nevertheless, most non-virulent strains are capsulated, indicating the importance of other factors, in addition to the capsule, in virulence (Gottschalk and Segura, 2000).

1.5.2. MRP and EF

Muramidase-released protein (MRP) is a 136 kDa cell wall-associated protein, also found in the culture supernatant (Vecht et al., 1989), whereas extracellular factor (EF) is a 110 kDa extracellular protein (Vecht et al., 1991). Variants of these 2 proteins were later described (Vecht et al., 1996). Both EF and MRP were first identified as potential virulence factors of *S. suis* serotype 2, since most disease-causing strains were positive for both proteins (Vecht et al., 1989; Vecht et al., 1991). However, the association of these two proteins with virulence is observed in strains of certain countries but not withose of other countries. Most virulent North American strains of *S. suis* serotype 2 are MRP^- EF^-, and most virulent European strains are MRP^+ EF^+ (Gottschalk et al., 1998). Mutants that lacked EF and MRP still behaved as virulent strains, suggesting that the proteins themselves do not contribute to the pathogenicity of *S. suis*, but that their synthesis is coincidentally associated with pathogenicity (Smith et al., 1996). An alternative explanation, however, is that the virulence associated with *S. suis* is a multifactorial process in which particular functions can be fulfilled by redundant or

alternative factors. This would mean that, in the absence of MRP and EF, other virulence factors can take over their functions (Smith et al., 1996), as is exemplified by the filamentous hemagglutinin and pertussis toxin of *Bordetella pertussis* (Relman et al., 1990).

To date, the function of these two proteins in the pathogenesis of *S. suis* infections remains unclear. Although vaccines containing MRP and EF protected pigs against challenge with *S. suis* serotype 2 of the phenotype $MRP^{+}EF^{+}$ (Wisselink et al., 2001), this kind of vaccine is not useful in North America, since most virulent strains lack these proteins.

1.5.3. Hemolysin

Hemolysins have often been implicated as virulence factors (Rose et al., 2001). A 54 kDa hemolysin, also known as suilysin, has been identified in *S. suis* serotype 2 by Jacobs et al. (Jacobs et al., 1994). Gottschalk et al. also described a 65 kDa hemolysin (Gottschalk et al., 1995). These two proteins were shown to be the same toxin and the molecular mass variation was in fact related to purification methods (Gottschalk et al., 1995). The hemolysin of *S. suis* belongs to the family of toxins known as thiol-activated toxins (Gottschalk et al., 1995). The gene encoding suilysin is *sly*, and very limited genetic diversity is present in *sly* genes found in different isolates (King et al., 2001). There is an apparent correlation between the absence of *sly* and association of isolates with pneumonia (Allgaier et al., 2001; King et al., 2001). Studies on mutations in the *sly* gene have demonstrated that suilysin is not required for the infection of the meninges but, may aid in the

development of disease (Allen et al., 2001). Suilysin has been shown to be cytotoxic for endothelial cells (Charland et al., 2000) and epithelial cells (Lalonde et al., 2000; Norton et al., 1999).

As seen for MRP and EF, most virulent European strains of *S. suis* serotype 2 are Hem^+ (Jacobs et al., 1995), whereas most virulent North American strains are Hem^- (Chatellier et al., 1999; Gottschalk et al., 1995; Segers et al., 1998).

The Hem+ phenotype is, most of the time, very much associated with high virulence, and strains of this phenotype also express EF and MRP (King et al., 2001; Staats et al., 1999). A vaccine containing purified hemolysin protected pigs against a lethal homologous challenge of *S. suis* serotype 2 (Jacobs et al., 1994); however, this kind of vaccine is also not useful in North America, since most strains are Hem^-.

Thus, hemolysin, EF and MRP are highly associated with virulence only in some parts of the world and thus should not be universally used to determine virulence of *S. suis* (Berthelot-Hérault et al., 2000; Norton et al., 1999).

1.5.4. Adhesins

Adhesins are important virulence factors which mediate the attachment of pathogenic bacteria to host cells (Ofek, 1980). *S. suis* was found to possess hemagglutinating properties (Gottschalk et al., 1990). 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 (Haataja et al., 1993). More specifically, *S. suis* recognizes the disaccharide sequence Galα1-4Gal that is present in the trihexosylceramide GbO_3, a neutral glycolipid belonging to the P blood group antigens (Haataja et al., 1993). 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 (galactose and N-acetylgalactosamine or galactose only, respectively) (Haataja et al., 1993). The adhesin was later purified and shown to be 18 kDa in size (Tikkanen et al., 1995). The purified adhesin was shown to be highly immunogenic, and also induced bactericidal activity in mice (Haataja et al., 1996). Both virulent and non-virulent strains of *S. suis* possessed this adhesin (Haataja et al., 1996).

A second *S. suis* adhesin with albumin binding activity was detected in virulent and non-virulent strains of serotype 2 (Quessy et al., 1997). A 39 kDa protein was shown to be responsible, in part, for this binding activity (Quessy et al., 1997). A more greatly capsulated strain showed a higher percentage of albumin binding, suggesting that capsular material could be involved in this binding activity. Virulence was increased when albumin was present in an *S. suis* infection in mice. Mutant strains that lacked this 39 kDa protein showed a significant reduction in adherence to porcine and bovine tracheal rings (Brassard et al., 2001).

Since both of the 2 adhesins mentioned above are found in virulent and non-virulent strains, their activity seems not to be sufficient by itself to make strains more virulent (Gottschalk and Segura, 2000).

1.5.5. Other virulence factors

Other proteins that could be associated with virulence are fimbriae, IgG binding proteins, and a 44 kDa protein.

Fimbriae were detected on the reference strains of several *S. suis* serotypes, including serotype 2 (Jacques et al., 1990). In comparison to other known fimbriae, they are thin and short. Fimbriae are generally involved in mediating attachment of bacteria to host surfaces; however, in the case of *S. suis*, this is yet to be studied. The role of fimbriae in hemagglutination and as a virulence factor has not been demonstrated.

A 60 kDa IgG-binding protein, related to the heat shock protein 60 family, was purified and characterized from *S. suis* serotype 2. It is commonly present in all serotypes (Benkirane et al., 1997; Serhir et al., 1995). 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 bacterial lysis (Boyle, 1990). The role of this specific IgG-binding protein is yet to be studied.

Finally, a 44 kDa cell wall protein is thought to be involved in virulence. A mutant strain devoid of this protein was non-virulent in mice, whereas the parent strain was highly virulent (Gottschalk et al., 1992). The presence of antibodies against this protein in an antiserum were necessary to obtain complete protection; however, the actual function of this cell wall protein is not known.

1.6. Pathogenesis of meningitis caused by *S. suis* serotype 2

The pathogenesis of meningitis caused by *S. suis* is poorly understood, and most of the studies have been done using only serotype 2. Colonized animals will harbor the bacteria in their tonsils. Some animals will be healthy carriers only and will never develop disease, whereas others will at some point develop bacteremia, possibly septicemia and, finally, meningitis. Hence, in the latter cases, bacteria would travel via 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.

1.6.1. Route of entry

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* serotype 1 up the nose and into the throat (Elliott, 1966; Field et al., 1954). Later, typical syndromes were also reproduced experimentally using *S. suis* serotype 2 (Clifton-Hadley, 1981; Windsor and Elliott, 1975). Serotypes 1 and 2 were thus shown to be primary pathogens capable of producing disease unaided. This has been confirmed subsequently with a few other serotypes (e.g. serotype 7) (Boetner et al., 1987). More recent work has also shown the respiratory route to be the portal of entry (Berthelot-Herault et al., 2001; Madsen et al., 2001).

Acute bacterial meningitis is characterized by a migration of leukocytes into the subarachnoid space. Polymorphonuclear granulocytes have been

found to be the first leukocytes to appear at the onset of the disease, but then the cellular picture gradually changes to a mononuclear pattern consisting of mainly monocytes and lymphocytes (Sprenger et al., 1996). Recent studies on the interaction of *S. suis* with epithelial cells, leukocytes and brain microvascular endothelial cells (BMEC) have brought some more insight into the understanding of the pathogenesis of disease (see below).

1.6.2. Interaction of *S. suis* with epithelial cells

It is as yet unclear how *S. suis*, present at low levels on mucosal surfaces, is able to traverse the mucosal epithelial lining in the upper respiratory tract to develop disease. Recent work has investigated the interaction of *S. suis* with epithelial cells. This bacteria has been shown to adhere to porcine and human epithelial cells, which is an important step in the colonization of the host (Lalonde et al., 2000). The observed adhesion was mediated by cell wall components, and was considerably reduced in the presence of the CPS. Hemolysin-positive strains were shown to be cytotoxic for epithelial cells (Lalonde et al., 2000; Norton et al., 1999), and this cytotoxicity was inhibited in the presence of a monoclonal antibody specific to the hemolysin of *S. suis* (Norton et al., 1999). Invasion of cells was also observed for the hemolysin-positive strains, hence the authors of this study suggested that hemolysin-positive *S. suis* strains can use invasion and cell lysis as a mechanism to breach the mucosal epithelia (Norton et al., 1999). In the same study, hemolysin-negative strains were shown to be non-toxic for epithelial cells, although their cell invasion capacity was not assessed.

On the other hand, Lalonde et al. (2000) could not find any invasion of cells by *S. suis* in several epithelial cell lines originating from human and different animal species, including swine. In fact, the authors of the previous study considered invasion as a 'rare event' (Norton et al., 1999).

1.6.3. Interaction of *S. suis* with leukocytes

A high-level persistent bacteremia usually precedes the onset of bacterial meningitis. The mononuclear phagocytes in the liver and spleen play a major role in the clearance of circulating organisms from the blood (Moxon et al., 1980). Thus, the outcome of the interaction between bacteria and these phagocytes is crucial for the development of a persistent bacteremia and meningitis. So far, studies have shown contradictory results in regard to the interaction of *S. suis* with phagocytes.

Earlier work showed that murine macrophages phagocytosed pathogenic and non-pathogenic *S. suis* strains, in the absence of antibodies and/or complement, *in vitro* (Williams, 1990). This resulted in the killing of non-pathogenic strains, whereas the pathogenic ones survived and even replicated inside the macrophages (Williams, 1990). This lead to the idea, referred to as the "Trojan horse theory", that *S. suis* could be carried into the CNS by traveling within monocytes, (Williams and Blakemore, 1990). Another study has demonstrated the ability of pathogenic *S. suis* to survive in macrophages (Brazeau et al., 1996). However, in both studies, only a small percentage of macrophages contained intracellular bacteria. Busque et al.

(1998) demonstrated the ability of human and swine leukocytes to phagocytose *S. suis*, but did not assess intracellular bacterial survival.

More recently, it has been demonstrated that virulent strains are not phagocytosed by murine macrophages (Segura et al., 1998). In fact, as discussed earlier, the CPS confers antiphagocytic properties to *S. suis*, and noncapsulated mutants were readily phagocytosed and destroyed (Charland et al., 1998; Segura et al., 1998; Smith et al., 1999). Hence, capsulated bacteria would travel free in the circulation. In addition, a relatively high level of adhesion of *S. suis* to monocytes, without phagocytosis, has recently been observed (Segura and Gottschalk, 1999). Thus, a modified Trojan horse theory has been proposed, where *S. suis* is actually bound to, and not ingested by, macrophages (Gottschalk and Segura, 2000).

Not only does *S. suis* bind to monocytes, but it also stimulates cells to produce pro-inflammatory cytokines. *S. suis* has been shown to stimulate the production of tumor necrosis factor (TNF) -α and interleukin (IL) -6 by murine macrophages (Segura et al., 1999), and of TNF-α, IL-6, IL-1, and the chemokines IL-8 and monocyte chemotactic protein (MCP) -1 by human THP-1 monocytes (Segura et al., 2002). TNF-α 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 hours. TNF-α and IL-1 may act as initiators of meningeal inflammation by modulation of the brain-blood barrier (BBB). *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 (Romano et al., 1997; Sprenger et al., 1996). Cytokines have been implicated in bacterial meningeal inflammation (GBS and *S. pneumoniae* meningitis) by alteration of the cerebrospinal fluid dynamics, brain metabolism, and cerebral blood flow (van Furth et al., 1996).

1.6.4. Interaction of *S. suis* with human brain microvascular endothelial cells (BMEC)

Since the presence of the capsule inhibits phagocytosis, *S. suis* circulating in the bloodstream would come in contact with BMEC (Charland et al., 2000). The tight junctions of BMEC, which constitute microvessels, form the anatomical basis of the BBB. The BBB is an anatomically and functionally unique barrier, separating the brain from the intravascular compartment and maintaining the homeostasis of the CNS environment (Pardridge, 1999). During meningitis, the permeability of the BBB is increased, due to separation of intercellular tight junctions and to increased pinocytosis (Quagliarello and Scheld, 1992). In the case of *S. suis*, it is unknown how bacteria traverse the BBB.

A recent study has shown that, as free bacteria, *S. suis* preferentially adheres to human BMEC (Charland et al., 2000). Both capsulated and noncapsulated *S. suis* strains adhered similarly to BMEC (Charland et al., 2000). As mentioned previously, noncapsulated strains bound to epithelial cells at a higher level than capsulated ones (Lalonde et al., 2000). This might indicate that the degree of capsulation of *S. suis* may be modulated

depending on the infectious stage. Encapsulation may be down-regulated during colonization of epithelial cells, and once bacteria are in the bloodstream, up-regulation of capsule production would protect them against the immune system (Charland et al., 2000). This has been previously suggested as a model for infection by *Haemophilis influenzae* type b (St Geme and Cutter, 1996). Other virulence factors would be expressed while *S. suis* is in the blood allowing the adherence of *S. suis* to endothelial cells, by means of adhesins that are not masked by the capsule. This hypothesis is supported by the fact that the absence of the capsule did not lead to an increase in adherence.

One consequence of adhesion of *S. suis* to BMEC is the up-regulation in the production of pro-inflammatory cytokines by these cells (Vadeboncoeur et al., 2001). *S. suis* is capable of stimulating the production of IL-6, IL-8 and MCP-1 by BMEC. IL-8 and MCP-1 are chemoattractants for neutrophils and monocytes, respectively. Hence, *S. suis* has the capability of stimulating the production of cytokines and chemokines by both monocytes and endothelial cells. This cytokine production might be responsible for inducing an acute inflammatory exudate at the BBB level, which increases the volume of CSF, leading to increased intracranial pressure.

S. suis does not invade human BMEC, unlike other pathogens such as GBS (Charland et al., 2000). In the same study, only hemolysin-positive strains were shown to be toxic for BMEC, and cytotoxicity was inhibited by adding anti-hemolysin antibodies (Charland et al., 2000). It is possible that the pathogenesis of the infection caused by hemolysin-positive strains and

that of the infection caused by hemolysin-negative strains are different, and that different virulence factors are involved in each case.

2. Adhesion Molecules

2.1. Adhesion Cascade

The multistep model of leukocyte binding to endothelium, commonly referred to as the adhesion cascade, is composed of three general steps (Figure 1) (Luscinskas and Gimbrone, 1996; Springer, 1994). The first step is rolling of leukocytes on endothelium which mainly involves the selectin family of adhesion molecules, that comprises L-, P- and E-selectin. The second step is the firm adhesion of leukocytes on endothelium, which is mainly mediated by β_2 integrins and members of the immunoglobulin superfamily (IgSF). The third and final step of the cascade is the transendothelial migration of leukocytes, involving the β_2 integrins and members of the IgSF.

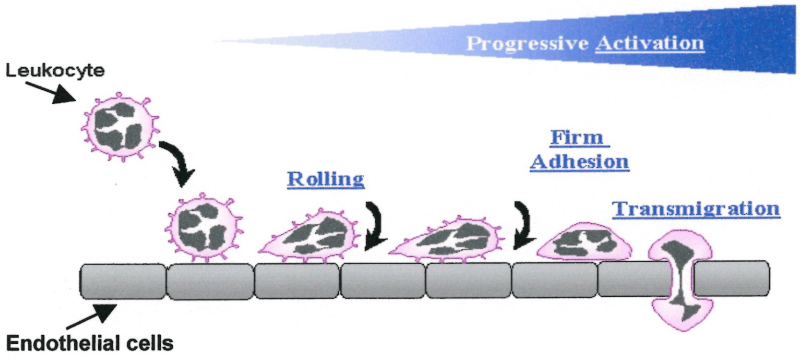

Figure 1. The inflammatory adhesion cascade. (source: University of Virginia, Department of Biomedical Engineering, Dr. Klaus Ley, website: http://hsc.virginia.edu/medicine/basic-sci/biomed/ley/index.html)

Recently, it has been proposed that instead of a linear process, the inflammatory adhesion cascade behaves more like a web of events, where groups of molecules define bottlenecks that restrict the inflammatory response (Ley, 2001). The following is a description of the 3 major events (rolling, firm adhesion and transmigration) and of the adhesion molecules that mediate these steps.

2.1.1. Rolling

Rolling is when leukocytes attach loosely to the endothelium of blood vessels through weak and reversible interactions (Springer, 1994). This allows for sampling of the local environment for signs of inflammation (Butcher, 1991). Using *in vivo* (Bosse and Vestweber, 1994; Dore et al., 1993; Kishimoto et al., 1991; Ley et al., 1991) and *in vitro* (Luscinskas et al., 1996) models, L-, E- and P-selectin were shown to be essential in mediating leukocyte rolling. Additionaly, lines of knock-out mice, deficient in various selectins, also demonstrated the importance of these molecules. For instance, L-selectin-deficient mice showed an impaired leukocyte rolling (Arbones et al., 1994) and P-selectin-deficient mice showed a total absence of leukocyte rolling (Mayadas et al., 1993). On the other hand, E-selectin-deficient mice showed limited defects in leukocyte rolling (Labow et al., 1994). Nevertheless, double mutated mice, deficient in both E- and P-selectin, displayed more severe defects in comparison to P-selectin-null mice (Labow et al., 1994).

A β_1 integrin, very late antigen (VLA) -4, can also mediate rolling, by binding to its ligand vascular cell adhesion molecule (VCAM) -1 (Berlin et al., 1995). The shedding of L-selectin has also been shown to mediate leukocyte rolling, as inhibition of L-selectin shedding resulted in adhesion of leukocytes on the endothelium for a longer period (Hafezi-Moghadam and Ley, 1999).

2.1.2. Firm adhesion

When leukocytes are activated, they become more adhesive and bind firmly to endothelium. This increased adhesive capacity is mediated through both qualitative (i.e. conformational) and quantitative (i.e. up-regulation of surface expression) changes in the β_2 integrins that are expressed on leukocytes (Etzioni, 1996). The integrins expressed on leukocytes normally have low-ligand capacity. Upon stimulation with chemokines, other chemoattractants, or lipid mediators (e.g. platelet activating factor), integrins are activated and bind to their ligands in a high affinity and avidity manner, resulting in firm and stable arrest of leuckoytes (Shimizu et al., 1999). For example, MCP–1 activates leukocytes by increasing the adherence capacity of β_2 integrins to their main ligand, intercellular adhesion molecule (ICAM) -1 (Lawrence and Springer, 1991). Also, the binding of VLA-4 to VCAM-1 (Chan et al., 2000) and L-selectin binding to its ligands (Simon et al., 1999) increase the adherence capacity of β_2 integrins to ICAM-1. The expression of ICAM-1 and VCAM-1 is up-regulated on inflamed blood vessels, thus also contributing to an increase in adherence (Springer, 1995). ICAM-1 is also expressed on

leukocytes (Schulz et al., 1988), and has been shown to be up-regulated by MCP-1 (Audran et al., 1996). Activated endothelial cells are a source of chemoattractants. Thus, inflamed (or activated) endothelium would activate rolling leukocytes to adhere firmly through integrin-mediated mechanisms, leading to transendothelial migration of leukocytes (Luscinskas and Gimbrone, 1996).

2.1.3. Transmigration

Transmigration (diapedesis) of leukocytes across endothelial cells of blood vessels is a complex event that involves several families of adhesion molecules. They include the β_2 integrins (CD11a/CD18, CD11b/CD18, CD11c/CD18), $\alpha_v\beta_3$ (a β_3 integrin) and members of the IgSF, namely ICAM-1 and platelet-endothelial cell adhesion molecule (PECAM) -1. All of the three β_2 integrins bind to ICAM-1 (Shimizu et al., 1999), whereas PECAM-1 binds in a homophilic way (Muller and Randolph, 1999) and can bind in a heterophilic way to $\alpha_v\beta_3$ (Buckley et al., 1996).

Studies using monoclonal antibodies against each of the three β_2 integrins demonstrated the importance of these molecules in diapedesis (Arnaout et al., 1988; Hakkert et al., 1990; Meerschaert and Furie, 1995). PECAM-1 has been shown to be largely involved in transmigration (Muller et al., 1993; Thompson et al., 2001).

Continuous migration requires cycles of adhesion and detachment. Whereas early adhesive interactions stimulate further integrin adhesion,

activation of integrins late in the adhesion cascade negatively feedback to promote detachment of the upstream adhesion (Worthylake and Burridge, 2001). Indeed, this can be seen when the adherence of VLA-4 to VCAM-1 is downregulated after the stimulation of β_2 and β_3 integrins (Imhof et al., 1997; Porter and Hogg, 1997). Furthermore, stimulation of β_3 integrins decreases the adhesiveness of β_2 integrins for ICAM-1 (Weerasinghe et al., 1998).

The following is a description of the 3 families of adhesion molecules mentioned above. The specific adhesion molecules (E-selectin, ICAM-1, VCAM-1, CD11a/CD18, CD11b/CD18 and CD11c/CD18) studied in this project are explained in more details. Please refer to Table 1 for a summary of characteristics of the 3 different families of adhesion molecules.

2.2. Adhesion molecule families

2.2.1. Selectins

Selectins mediate leukocyte rolling on endothelial cells of the blood vessel walls (Vestweber and Blanks, 1999). They are carbohydrate-binding (lectin) proteins, also known as lectin cell adhesion molecules, or Lec-CAMS, that comprise a class of cell adhesion molecules that are expressed on the surface of endothelial cells, leukocytes and platelets (Elangbam et al., 1997). There are three members in the selectin family, L-selectin, E-selectin and P-

Table I : Summary table of the characteristics of the adhesion molecules belonging to the selectin family, immunoglobulin superfamily, and the β_2 integrin family.

Adhesion molecule family	Members	Synonym	Distribution	Ligand
Selectins	E-selectin	CD62E, ELAM-1	Endothelial cells	PSGL-1, ESL-1, sialylated Lewis^x and Lewis^a glycolipids
	P-selectin	CD62P, GMP140, PADGEM	Platelets, endothelial cells	PSGL-1, GlyCAM-1
	L-selectin	CD62L, LAM-1, MEL-14	Leukocytes	GlyCAM-1
Immunoglobulin super family	ICAM-1	CD54	Monocytes, epithelial and endothelial cells, fibroblasts	CD11a/CD18, CD11b/CD18
	ICAM-2	CD102	Endothelial cells, leukocytes	CD11a/CD18
	ICAM-3	CD50, ICAM-R	Endothelial cells, leukocytes, Langerhans cells	CD11a/CD18
	ICAM-4	ICAM-LW, LW blood group glycoprotein	Erythrocytes	CD11a/CD18, CD11b/CD18
	ICAM-5	Telencephalin	Telencephalic neurons	?
	VCAM-1	CD106	Endothelial cells	VLA-4
	PECAM-1	CD31	Endothelial cells, leukocytes	CD31
β_2 integrin family	CD11a/CD18	LFA-1, $\alpha_L\beta_2$	Leukocytes	ICAM-1, ICAM-2, ICAM-3
	CD11b/CD18	Mac-1, $\alpha_M\beta_2$, CR3	Leukocytes	C3bi, ICAM-1, fibrinogen
	CD11c/CD18	gp150,96; $\alpha_x\beta_2$, CR4	Leukocytes	C3bi
	CD11d/CD18	$\alpha_D\beta_2$	Leukocytes	ICAM-3 ?

selectin, for which the designated prefixes were chosen according to the cell type where the molecules were first identified.

The selectins are composed of an extracellular domain, a transmembrane domain, and a cytoplasmic domain (Figure 2).

The extracellular domain itself is composed of three domains: a lectin domain, an epidermal growth factor (EGF)-like domain and 2 to 9 concensus repeats (CR) similar to sequences found in complement regulatory proteins (Vestweber and Blanks, 1999).

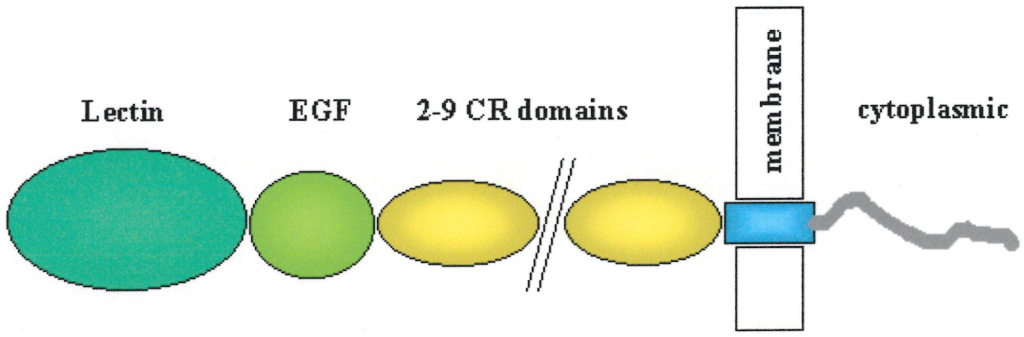

Figure 2 : Schematic representation of the structure of selectins. (source: University of Virginia, Department of Biomedical Engineering, Dr. Klaus Ley, website: http://hsc.virginia.edu/medicine/basic-sci/biomed/ley/index.html)

The lectin and the EGF domains play a crucial role in mediating the binding of selectins to their ligands (Varki, 1994). Unlike most other cell adhesion molecules that bind to their ligands on the basis of protein-protein interactions, the ligands of selectins contain a scaffold protein, or a lipid carrier molecule, which is modified by certain carbohydrates. Thus, the carrier molecule is not sufficient to define a selectin ligand; it needs to be expressed in the right cellular background that provides the necessary

repertoire of glycosylation enzymes which confer selectin-binding activity to the carrier molecule (Vestweber and Blanks, 1999).

L-selectin (LAM-1, MEL-14 or CD62L) is expressed on all circulating leukocytes except for a subpopulation of memory lymphocytes. It is exposed on the tips of microvilli. In addition to its role during rolling, L-selectin functions in the continuous process of lymphocyte homing. This molecule is rapidly shed from cells stimulated by TNF-α or IL-8 (among other activating factors). This shedding might facilitate detachment of leukocytes from endothelial cells as they start migrating through the endothelial cell layer (Elangbam et al., 1997).

P-selectin (GMP140, PADGEM or CD62P) is stored preformed in the Weibel-Palade bodies of endothelial cells and the α granules of platelets. In response to inflammatory mediators, such as thrombin or histamine, P-selectin is rapidly mobilized to the plasma membrane to bind neutrophils and monocytes (Elangbam et al., 1997).

2.2.1.1. E-selectin

E-selectin is a 112 kDa glycoprotein, that was first identified on cultured endothelial cells stimulated by TNF-α and IL-1β (Bevilacqua et al., 1987; Bevilacqua et al., 1989). Since then, several other cytokines, e.g. IL-3 and IL-10, have been shown to stimulate the surface expression of E-selectin on endothelial cells (Brizzi et al., 1993; Vora et al., 1996). E-selectin is synthesized *de novo* by cytokine-activated endothelial cells where the surface

expression peaks within 3-4 hrs and decreases until it is almost absent at 24 hrs (Scholz et al., 1996). E-selectin has also been found to be expressed on stimulated monocytes (Prieto et al., 1994). There are numerous ligands of E-selectin, the main ones being:

1. Sialyl Lewis^{x}- and Lewis^{a}- carrying glycolipids, found on all circulating myeloid cells. They support rolling of leukocytes (Alon et al., 1995).
2. E-selectin ligand (ESL) -1, also found on myeloid cells (Levinovitz et al., 1993). Blocking of ESL-1 has been shown to partially inhibit leukocyte binding via E-selectin (Steegmaier et al., 1995).
3. P-selectin glycoprotein ligand (PSGL) –1, broadly expressed on cells of myeloid, lymphoid and dendritic lineage cells. Until now, there has been no direct evidence for its relevance in cellular interactions with E-selectin (Asa et al., 1995; Lenter et al., 1994).
4. L-selectin. Even though it was found that L-selectin binds with high affinity to E-selectin, its role as a ligand during leukocyte adherence to endothelial cells is still questionable (Patel and McEver, 1997; Zollner et al., 1997).

The importance of selectins in leukocyte recruitment is revealed by the hereditary disease leukocyte adhesion deficiency (LAD) syndrome II, where there is a poorly characterized genetic defect(s) in fucose metabolism, resulting in failure to synthesize selectin ligands, such as sialyl Lewis^{x} and related carbohydrate determinants. Leukocytes from LAD II patients have impaired rolling interactions with activated endothelial monolayers *in vitro* (Etzioni et al., 1992).

2.2.2. Immunoglobulin super family (IgSF)

This family of adhesion molecules exhibits a broad range of structural and functional diversity. Structurally, these proteins are classified together because they contain one or more common Ig-like repeats that are characterized by two cysteines separated by 55 to 75 amino acids. Members of this family of adhesion molecules that play a critical role in firm adhesion and transendothelial migration of leukocytes are the ICAMs, of which there are now five members, VCAM-1 and PECAM-1 (Petruzzelli et al., 1999).

2.2.2.1. ICAM-1

ICAMs are cell surface glycoproteins expressed on a wide variety of cell types, including endothelial cells and leukocytes, with distinct patterns of gene regulation and effector functions (Hayflick et al., 1998). ICAM-1 is the most extensively studied of the 5 ICAM molecules because of its wide distribution and specific regulation.

ICAM-1 (or CD54) is a surface glycoprotein that has five extracellular immunoglobulin-like domains (Figure 3), with a molecular mass ranging from 80 to 114 kDa depending on the degree of glycosylation, which varies with cell type (Hayflick et al., 1998; Roebuck and Finnegan, 1999). It is expressed constitutively at low levels on vascular endothelial cells and on some lymphocytes and monocytes. Stimulation with IL-1, TNF-α, IFN-γ or LPS has been documented to increase ICAM-1 expression (Roebuck and Finnegan, 1999). The stimulatory effects of these mediators can be tempered by the

anti-inflammatory cytokines transforming growth factor (TGF) -β, IL-4 and IL-10, and by glucocorticoids, which interfere with the signal transduction pathways of transcription factors critical for the induction of ICAM-1 expression (Roebuck and Finnegan, 1999). ICAM-1 is also expressed on stimulated leukocytes (Prieto et al., 1994). ICAM-1 binds to β_2 integrins that are expressed exclusively on leukocytes. A motif within domains 1 and 3 of ICAM-1 has been shown to be critical for this binding (Hayflick et al., 1998). Mice deficient in ICAM-1 expression have numerous inflammatory response abnormalities (Hayflick et al., 1998).

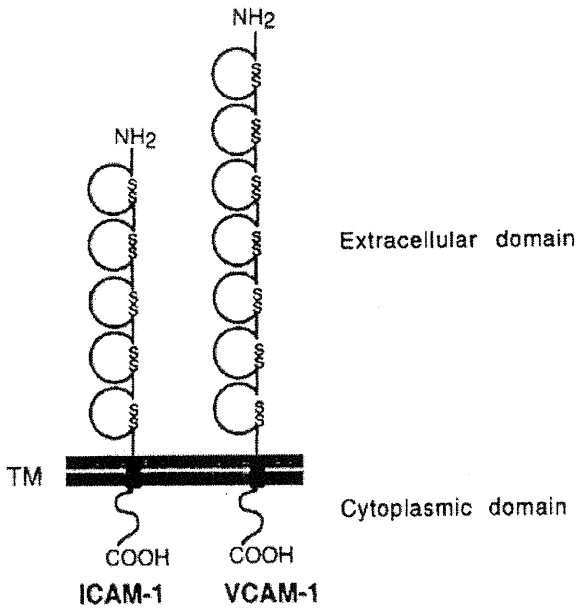

Figure 3. Schematic representation of ICAM-1 and VCAM-1. The extracellular domain of each domain of each molecule consists of several immunoglobulin-like repeats folded into a loop by disulfide bonds, followed by a transmembrane domain (TM) and a short cytoplasmic tail. (source: Schnapp, Lynn. Elmer's glue, Elsie and You: Clinical applications of adhesion molecules. The Mount Sinai Journal of Medicine, Vol. 65 (3), 1998).

ICAM-1 is utilized by several pathogens to infect host cells and evade immune detection. It was identified as the major cell-surface receptor for rhinovirus and *Plasmodium falciparum* (Berendt et al., 1989; Staunton et al., 1990). HIV particles have been found to incorporate ICAM-1 into the viral envelop during the budding process, resulting in increased infectivity of cells (Fortin et al., 1997).

2.2.2.2. VCAM-1

VCAM-1 (CD106) is a 100-110 kDa glycoprotein, originally characterized as an alternative ligand on cytokine activated endothelial cells for integrin-mediated lymphocyte adhesion and was subsequently found to bind VLA-4. Although cDNA that was initially sequenced had six immunoglobulin (Ig) domains, the majority of VCAM-1 transcripts have an extra Ig domain which provides an additional VLA-4 binding. VCAM-1 has also been shown to bind to the integrin $\alpha_4\beta_7$ (Petruzzelli et al., 1999).

This adhesion molecule is expressed on normal endothelium at very low levels, but can be up-regulated by inflammatory cytokines such as IL-1β and TNF-α, and to a lesser extent, by IL-4 (Petruzzelli et al., 1999).

2.2.3. β_2 Integrins

β_2 Integrins mediate firm adhesion and transendothelial migration of leukocytes. They share a common beta subunit (CD18) of 94 kDa that can associate noncovalently with one of three different alpha subunits,

α_L(CD11a), α_M (CD11b) and α_X (CD11c) with molecular masses of 180 kDa, 155 kDa, and 150 kDa, respectively (Figure 4) (Arnaout, 1990; Luscinskas and Gimbrone, 1996). They are also known as LFA-1 (CD11a/C18), Mac-1 (CD11b/CD18) and p150,96 (CD11c/CD18) (Luscinskas and Gimbrone, 1996). A fourth CD18 molecule (α_D subunit) has been discovered, although it has not been well characterized (Danilenko et al., 1995). These molecules are expressed exclusively on leukocytes, and hence are also referred to as leukointegrins (Dib, 2000). The clinical relevance of these molecules is highlighted by the manifestation of β_2 chain (CD18) deficiency (LAD I) in which each of the 3 β_2 integrins are either absent or markedly reduced (Arnaout, 1990).

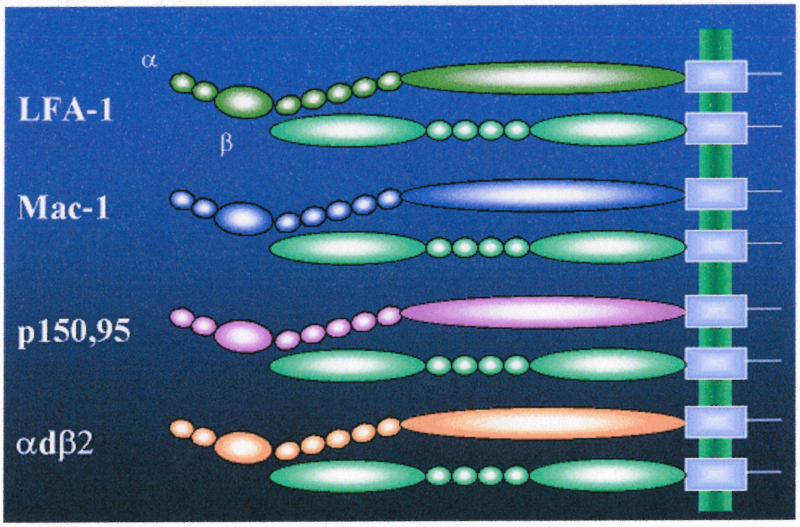

Figure 4. Schematic representation of the four β_2 integrins, showing the common β subunit shared by the four molecules, and the unique α unit of each. (source: University of Virginia, Department of Biomedical Engineering, Dr. Klaus Ley, website: http://hsc.virginia.edu/medicine/basic-sci/biomed/ley/index.html)

In such patients neutrophils are unable to migrate from the vasculature to sites of tissue injury, with a subsequent marked reduction in inflammatory response and enhanced susceptibility to bacterial infection.

The divalent cations Ca^{2+} and Mg^{2+} are essential to the stabilization and function of the $\alpha\beta$ complex (Arnaout, 1990). CD11a/CD18 is present on all leukocytes. Expression of CD11b/CD18 and CD11c/CD18 is more restricted, being observed on monocytes, macrophages, neutrophils, and natural killer cells. CD11c/CD18 is also expressed on some B-cell lines such as in hairy cell leukemia and certain cloned cytotoxic T lymphocytes (Arnaout, 1990).

The relative quantity of CD11/CD18 varies depending on the cell type and the state of cell activation and differentiation. Activated granulocytes express far more CD11b/CD18 than the other two antigens (Arnaout et al., 1984). In resting monocytes, the relative abundance of these antigens is CD11a/CD18 $\geq$ CD11b/CD18 > CD11c/CD18. In tissue macrophages, the relative amounts expressed are CD11c/CD18 > CD11a/CD18 > CD11b/CD18 (Freyer et al., 1988; Miller et al., 1986).

Significant intracellular storage pools for CD11b/CD18 and CD11c/CD18 exist in circulating granulocytes and monocytes. These pools are present in the secondary and tertiary granules in granulocytes and in intracellular vesicles and in peroxidase-negative granules in monocytes (Bainton et al., 1987; Freyer et al., 1988). Little or no CD11a/CD18 is stored intracellularly (Arnaout et al., 1984; Freyer et al., 1988). On activation by

PMA, granulocytes and monocytes increase their cell surface expression of CD11b/CD18 and CD11c/CD18 in minutes (Bainton et al., 1987).

The first well-characterized ligand was iC3B, a 175 kDa complement C3 fragment generated by cleavage of C3b (Arnaout et al., 1983). This ligand binds only to CD11b/CD18 and CD11c/CD18. However, the predominant cellular ligands for the β_2 integrins are the ICAMs (Gahmberg et al., 1997).

2.3. Adhesion molecules involved during meningitis

The cellular immune system is a potent force which, if controlled and directed correctly, is effective against an enemy and forms an appropriate host defense; however, if uncontrolled, it is ineffective against the enemy and can actually be harmful to the host itself. Therefore, leukocyte action must be held in check in the circulation, and discharged at the site of infection (Kerr, 1999). As mentioned above, integrins, selectins and IgSF members of adhesion molecules mediate leukocyte adhesion and transendothelial migration.

In experimentally induced bacterial meningitis, inflammatory leukocytes were shown to be the major cause of BBB injury and cerebral edema, and their number in the CSF was correlated with the clinical outcome (Bohr and Rasmussen, 1988). This was demonstrated by a reduction in leukocytosis following addition of blocking antibodies against CD18 or ICAM-1 (Saez-Llorens et al., 1991; Tuomanen et al., 1989; Weber et al., 1995). The inhibition of leukocyte adhesion has proved effective in reducing brain edema, reducing intracranial pressure and regional cerebral blood flow, and reducing

leukocyte count in the subarachnoid space in experimental meningitis (Weber et al., 1997).

Activated lymphocytes, macrophages and certain types of metastatic cells can cross the intact BBB (Rubin and Staddon, 1999). In fact, exposure to activated leukocytes, or inflammatory agents, lead to a significant up-regulation of ICAM-1 and VCAM-1 expression on cultured BMEC (Rubin and Staddon, 1999). Immunohistochemical studies of frozen normal brain tissues show minimal to non-detectable levels of expression of various integrin-binding adhesion molecules, including ICAM-1, VCAM-1 and PECAM-1 in the BBB. However, during activation of immune responses in the CNS, there is a significant up-regulation of the expression of ICAM-1, VCAM-1 and PECAM-1, in the brain microvessels (Rubin and Staddon, 1999). These findings suggest that the increased penetration of leukocytes into the CNS during activated or inflammatory conditions may be the result of increased expression of integrins on leukocytes or of integrin-binding proteins on endothelial cells (Rubin and Staddon, 1999).

The selectins also appear to be involved in leukocyte crossing of the BBB. Studies on the involvement of selectins in the BBB during normal and inflammatory conditions are however contradictory (Engelhardt et al., 1997). In experimental autoimmune encephalomyelitis (EAE), where inflammatory cells cross the BBB and gain access to the CNS, anti-E- and P-selectin antibodies did not influence emigration of inflammatory cells across the BBB nor the development of EAE (Engelhardt et al., 1997). However, use of P- and E-selectin knock-out mice in a cytokine-induced meningitis model,

resulted in a nearly complete inhibition of leukocyte migration and BBB permeability (Tang et al., 1996).

Thus, alterations in the expression of selectins, integrins and members of the IgSF affect the penetration of leukocytes across the BBB (Rubin and Staddon, 1999). Micro-organisms have been shown to utilize these adhesion molecules in the pathogenesis of disease (Kerr, 1999).

3. Pathogenesis of meningitis caused by bacteria other than *S. suis*

The principal pathogens of human bacterial meningitis are *N. meningitidis*, *S. pneumoniae*, GBS and *H. influenzae*. In immunocompromised patients, *L. monocytogenes* is also an important pathogen. Bacterial meningitis, like many other disease states, increases the permeability of the BBB. The increased BBB permeability may result from separation of the intercellular junctions of BMEC, from increased pinocytosis, from both alterations, or from other as yet unknown processes. It is unclear how bacteria increase BBB permeability (Tunkel and Scheld, 1993). Nevertheless contact of bacteria with cells of the BBB has been studied in order to elucidate bacterial entry mechanisms. Adhesion molecules seem to play an important part in this bacteria-host interaction, since their cell surface expression is modulated upon bacterial stimulation.

3.1. Group B *streptococci*

GBS affects neonates, causing meningitis that develops following bacteremia (Nizet et al., 1997). GBS associated with human disease are almost invariably encapsulated. Serotypes Ia, Ib, II, III and V are commonly associated with bloodstream infections, and each may produce meningitis; however, type III strains cause almost all GBS meningitis (Adderson et al., 2000). The polysaccharide capsule was previously shown to have an important role in the resistance to phagocytosis. Isogenic mutants defective in capsule expression were more susceptible to opsonophagocytosis and exhibited decreased virulence in animal models (Martin et al., 1992). However, a different study showed no difference in phagocytosis between wild-type GBS and its noncapsulated mutant, in the absence of complement and antibodies (Segura et al., 1998). GBS capsule is also sialylated, and has been shown to inhibit direct activation of the alternative complement pathway (Edwards et al., 1982). Loss of capsular sialic acid was associated with loss of virulence (Wessels et al., 1989).

GBS has the ability to stimulate human monocytes to produce TNF-α, IL-1 and IL-6 (Cuzzola et al., 2000). Bacterial components responsible for cytokine stimulation include the type- and group- specific polysaccharides (CHOs) and cell walls. CD18 and CD14 (LPS receptor) expressed on the surface of monocytes have been shown to be important in the induction of TNF-α, since blocking of either molecule decreased cell wall-induced TNF-α release (Medvedev et al., 1998).

TNF-α levels were also shown to be increased in the CSF of newborn piglets inoculated intraventricularly with GBS (Ling et al., 1995). An isogenic noncapsulated mutant resulted in a markedly increased TNF-α response, suggesting that a component of the underlying GBS cell wall, and not capsule, is responsible for inducing the inflammatory response (Ling et al., 1995). Treatment with anti-TNF-α antibodies was found to be beneficial in neonatal rats infected with GBS (Givner et al., 1995).

GBS are able to invade (or transcytose) BMEC, and at high bacterial concentrations, significant injury to BMEC is correlated with β-hemolysin expression by the organism (Nizet et al., 1997). The correlation between cell injury and β-hemolysin expression has already been observed with lung epithelial cells *in vitro* (Nizet et al., 1996). Characteristic features of cell injury were disruption of the cytoplasmic membrane and loss of cytoplasmic density consistent with hypo-osmotic damage due to water influx (Nizet et al., 1997). GBS invasion of BMEC may be a primary step in the pathogenesis of neonatal meningitis, allowing bacteria access to the CNS by trancytosis or by injury and disruption of the BBB (Nizet et al., 1997). GBS serotype III was most invasive compared to other GBS serotypes (Nizet et al., 1997).

The noncapsulated isogenic mutant strain invaded BMEC more efficiently than its capsulated parent strain, even though the level of adherence was similar (Charland et al., 2000; Nizet et al., 1997). It is possible that the polysaccharide capsule produces steric interference of certain receptor-ligand interactions important in the invasion process, or that

repulsive forces are generated between negatively charged sialic acid residues on the capsule and the BMEC surface (Nizet et al., 1997).

Another study has shown the involvement of integrins in GBS meningitis. In a rabbit model of GBS meningitis, extravasated CSF neutrophils showed a marked increase in β_1 and β_2 integrin expression compared to concurrent expression on circulating neutrophils (Rowin et al., 2000). Since integrins are involved in the adhesion and transmigration of leukocytes across endothelial cells, their up-regulation might increase leukocyte adhesion, and subsequent extravasation.

3.2 *Streptococcus pneumoniae*

S. pneumoniae is a gram-positive bacterium that causes pneumonia, sepsis and is the most common cause of meningitis in adults. Pneumococcal meningitis causes the highest rates of mortality and morbidity among common meningeal pathogens (Schlech et al., 1985). Although invasive disease is severe, some 40% of individuals harbor the pneumococcus in the nasopharynx asymptomatically (Cundell et al., 1995).

S. pneumoniae usually enters the host via the nasopharynx, where they can attach to the epithelial cells. This organism has been shown to also adhere to HUVEC, although no entry is observed. Invasion is promoted when cells are stimulated with cytokines, such as TNF-α, which increases the amount of surface-expressed platelet-activating factor (PAF) receptor that in

turn binds the phosphorylcholine component of the pneumococcal cell wall (Cundell et al., 1995).

Studies on the interaction of *S. pneumoniae* with cells of the BBB demonstrated the capability of this bacteria to invade cells, via transcytosis. Cytokine activation of BMEC resulted in increased invasion, but the effect is less than that seen with HUVEC stimulation (Ring et al., 1998). Invasion of BMEC was partially inhibited by PAF antagonists (Ring et al., 1998).

Several studies have looked at the involvement of ICAM-1, integrins and selectins during *S. pneumoniae* infection. Treatment with antibodies against ICAM-1 in a rabbit model of early meningitis that was induced with pneumococcal cell wall, significantly reduced intracranial pressure, brain edema, and leukocyte entry into the CSF (Weber et al., 1995). However, another study showed that antibodies against ICAM-1 did not cause a significant decrease in CSF pleocytosis (Zysk et al., 1995). In addition, when using ICAM-1-deficient mice, the incidence of bacteremia, meningitis and meningeal inflammatory response was similar between ICAM-1-deficient mice and wild-type mice (Gerber et al., 2001; Tan et al., 1995). The authors of the latter study suggested that, in most forms of brain pathology associated with *S. pneumoniae*, there was a redundancy for cell adhesion molecules.

Nevertheless, the situation is different with CD18 and P- and E-selectin. In order to define the specific role of CD11a/CD18 and CD11b/CD18, mice deficient in either one of these molecules were inoculated intraperitoneally with *S. pneumoniae* (Prince et al., 2001). An increase in mortality was observed in comparison to wild-type mice, with early (first 48

hours) mortality in CD11b/CD18-deficient mice, and late (> 72 hours) mortality in CD11a/CD18-deficient mice (Prince et al., 2001). The increased early mortality in CD11b/CD18-deficient mice was secondary to overwhelming sepsis. CD11b/CD18 serves as a complement receptor, thus the increase in number of bacteria may be due to a CD11b/CD18-dependent defect in phagocytosis or intracellular killing of pneumococci by leukocytes. The late mortality of CD11a/CD18-deficient mice was associated with meningitis and meningoencephalitis, along with an increased incidence of otitis media (Prince et al., 2001). The authors of this study did not look for the emigration of leukocytes into the CSF, but rather emigration into the peritoneal cavity. The percentage of leukocytes was significantly reduced compared with wild-type mice. The absence of CD11a/CD18 may reduce both leukocyte migration and bacterial phagocytosis, as binding of CD11a/CD18 to ICAM-1 on endothelial cells was shown to be required for full phagocytic function to occur (Schnitzler et al., 1999).

Increased mortality associated with impaired leukocyte emigration following pneumococcal challenge is not specific to CD11/CD18 integrins. Mice genetically deficient in one or both of E- and P-selectin, following inoculation with *S. pneumoniae*, showed more morbidity, substantially increased mortality, persistent bacteremia, and a higher number of bacteria in the CSF compared with wild-type mice (Munoz et al., 1997). These results, along with observations of increased susceptibility to infections in patients with leukocyte adhesion deficiency syndromes types I and II, provide clear

evidence of the importance of integrins and selectins, respectively, in the host defense against bacterial infections (Anderson et al., 1995).

S. pneumoniae has been shown to be capable of up-regulating the expression of ICAM-1 on BMEC, and to induce the production of TNF-α by these cells. Neutralizing antibodies against TNF-α completely inhibited the up-regulation of ICAM-1 (Freyer et al., 1999). Interestingly, *Plasmodium falciparum* also induces the up-regulation of ICAM-1 on HUVEC, however, it does not induce TNF-α (Esslinger et al., 1994). This might reflect different mechanisms used by the two cell types in expressing ICAM-1, or that micro-organisms use different mechanisms to cause an up-regulated expression of ICAM-1. *S. pneumoniae* has also been shown to stimulate human leukocytes to increase expression of CD11b/CD18 (Kragsbjerg and Fredlund, 2001).

3.3. *Listeria monocytogenes*

L. monocytogenes is a gram-positive facultative intracellular bacterium that causes severe disease in both animals and humans. Primarily immunocompromised individuals, such as pregnant woman, neonates, and elderly people, become infected and undergo bacteremia, sepsis, abortion, meningitis, or encephalitis (Schuchat et al., 1991). The normal route of entry into the host is the gut via listeria-contaminated food (Farber and Peterkin, 1991). During the course of infection, *L. monocytogenes* encounters different cell types, including epithelial cells, fibroblasts, hepatocytes, macrophages, and endothelial cells, which are all, at least *in vitro*, readily infected. Once

inside the host cell, bacteria rapidly lyse the phagosomal membrane in order to escape into the host cell cytoplasm, where intensive intracellular multiplication and intracellular movement occur. Direct transfer from one cell to another allows the bacteria to enter neighbouring cells without an extracellular phase (Greiffenberg et al., 1998).

A number of listerial virulence determinants have been characterized. A family of internalins was discovered (Gaillard et al., 1991). InlA and InlB have been shown to be necessary for triggering bacterial uptake by several cell types (Braun et al., 1998; Gaillard et al., 1991). A sulfhydryl-activated pore-forming cytolysin called listeriolysin is required, along with two phospholipases, for lysis of the phagosome (Drevets, 1998). ActA, a listerial cell wall protein, promotes F-actin-driven intracellular movement (Kocks et al., 1992). The expression of most of these virulence factors is controlled in a complex manner by the positive regulatory factor PrfA (Chakraborty et al., 1992).

The main feature of lesions seen in experimental murine infection with *L. monocytogenes* is the recruitment of inflammatory cells, especially macrophages and neutrophils, to the subarachnoid and ventricular space causing meningitis and choroiditis (Prats et al., 1992). In the same model of infection, an initial generalized up-regulation of ICAM-1 expression and P-selectin induction in the CNS, liver and spleen occurred on the first day of infection (Prats et al., 1992). This suggests a systemic nonspecific endothelial activation that may allow leukocytes to scavenge the organism to find the inflammatory stimulus. When leptomeningeal inflammation was

evident, it correlated with a stronger local up-regulation of ICAM-1 and P-selectin expression. This indicates that both molecules may play a key role in the recruitment of leukocytes and development of meningitis during experimental murine listeriosis (Prats et al., 1992).

L. monocytogenes has been shown to invade endothelial cells directly as free bacteria, or by cell-to-cell spread from adherent monocytes which were previously infected by this bacterium (Drevets et al., 1995). Thus monocytes could be vectors by which intracellular bacteria avoid host defenses and enter into the CNS (Drevets et al., 1995). Internalins of listeria were shown to be important in invasion of HUVEC, but not of BMEC (Drevets et al., 1995; Wilson and Drevets, 1998). Invasion of both cell types (and epithelial cells) was prevented by cytochalasin D, indicating an absolute dependence of invasion on actin polymerization and rearrangement of actin filaments (Drevets, 1998).

Infection of endothelial cells with *L. monocytogenes* elicits an up-regulation in the expression of adhesion molecules. HUVEC stimulation by bacteria caused an up-regulation of P-selectin, E-selectin, ICAM-1 and VCAM-1 (Krull et al., 1997). BMEC stimulation resulted in an up-regulated expression of E-selectin, ICAM-1, and to a lesser extent, VCAM-1 (Wilson and Drevets, 1998). This change in surface expression of adhesion molecules resulted in an increase in adhesion of neutrophils to HUVEC and BMEC that were stimulated with *L. monocytogenes* (Krull et al., 1997; Wilson and Drevets, 1998). Addition of anti-CD18 antibodies blocked this adhesion; however, anti-E-selectin antibodies only blocked leukocyte adhesion on

HUVEC, but not BMEC, which had been stimulated by bacteria (Krull et al., 1997; Wilson and Drevets, 1998). It is possible that HUVEC and BMEC differentially produce other factors, such as IL-8 and IL-1, important for leukocyte binding that result in this difference (Wilson and Drevets, 1998).

The importance of CD18-mediated binding of leukocytes to BMEC activated by *L. monocytogenes* has also been demonstrated using *in vivo* studies, showing that anti-CD18 antibodies block leukocyte migration into the CSF in experimental meningitis (Tuomanen et al., 1989).

L. monocytogenes infection of human and porcine brain endothelial cells also stimulated monocyte adhesion (Drevets, 1999). Thus, activation of microvascular cells during bacteremic *L. monocytogenes* infection may facilitate binding and/or transmigration of parasitized monocytes and consequently promote CSN infection. Both cell-free and cell-associated bacteria, and the potential of either to establish infection, contribute to the ability of this organism to invade the CNS from the bloodstream (Drevets, 1999).

Methodology

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 (Kobisch et al., 1995). Seven other porcine strains and 14 strains isolated from human cases of infection were also used (Refer to Table 1 of article), together with the avirulent, nonencapsulated isogenic transposon mutant strain 2A, derived from the wild-type strain S735 (Charland et al., 1998). 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 (Charland et al., 1998). 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 of these cultures into 10 ml of THB and incubating at 37°C with agitation until 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 (Segura et al., 1999). 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 (from 6 h cultures as prepared above) were used.

Purified bacterial components. CPS and bacterial cell wall were purified as previously described (Segura et al., 1999; Sepulveda et al., 1996). *S. suis* hemolysin, purified as previously described (Jacobs et al., 1994), 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 previously described (Charland et al., 2000).

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). Four different types of endothelial cells were used: 1) Human umbilical vein endothelial cells (HUVEC), 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. 2) Immortalized human brain microvascular endothelial cells (iHBMEC1), kindly provided by Dr. K. S. Kim, Division of Infectious Diseases, Children's Hospital Los Angeles, Los Angeles, CA, were immortalized using SV40 Large T antigen (Stins et al., 1994). Cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated FBS, 10% Nu-Serum IV supplement (Becton Dickinson), L-glutamin, and penicillin-streptomycin. Flasks and 96-well tissue culture plates were pre-coated with rat tail collagen to support these cells. 3) Immortalized human BMEC (iHBMEC2), kindly provided by Dr. D. Stanimirovic, National Research Council of Canada, Ottawa, Ontario, were also immortalized using SV40 Large T antigen (Muruganandam et al., 1997). Cells were cultured in M199 medium (Gibco), supplemented with 10% heat-inactivated FBS, 5% human serum (Wisent Inc.), 10 μg/ml of ECGS and penicillin-streptomycin (5,000 U/ml). Flasks and 96-well tissue culture plates were pre-coated with gelatin to support the cells. 4) Primary HBMEC (pHBMEC) was purchased from Cell Systems (ACBRI 376; Kirkland, WA). Cells were cultured in Growth Medium (Cell Systems, 4Z0-500), in flasks pre-coated with Attachment Factor (Cell Systems, 4Z0-210). 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 cultures 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. In some experiments, THP-1 monocytes were prestimulated for 2 or 3 days with phorbol 12-myristrate 13-

acetate (PMA; 100 ng/ml; Sigma), or Vitamin D_3 (100nM; Sigma) or MCP-1 (10 ng/ml; R&D Systems) or IFN-γ (500 U/ml; Bender MedSystems, Vienne, Austria). Different *S. suis* strains, as well as different concentrations of purified cell wall, CPS or hemolysin that had been prepared in the appropriate 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 Polymyxin 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 cells in 96-well culture plates were blocked with 1% bovine serum albumin (Boehringer mannheim, Germany) in PBS, and the following monoclonal

antibodies against the adhesion molecules were added to different wells: 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 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 following methylene blue dye staining, as described by Oliver et al. (Oliver et al., 1989). 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) followed by incubation 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 HCL (0.1N) per well 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 diferences between strains. The SAS software (SAS, Cary, NC) was used for these analyses.

Article

Al-Numani, D., Segura, M., Doré, M., Gottschalk, M. Up-regulation of ICAM-1, CD11a/CD18 and CD11c/CD18 on Human THP-1 Monocytes Stimulated by *Streptococcus suis*.

Up-regulation of ICAM-1, CD11a/CD18 and CD11c/CD18 on Human THP-1 Monocytes Stimulated by *Streptococcus suis* Serotype 2

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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 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 the surface expression 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 sequelea such as hearing loss (3). To date, 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 of virulence factors of *S. suis* serotype 2 is limited. To date, 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 as yet unknown mechanisms (19). Once in the blood, bacteria would 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 the 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). 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 of these cultures into 10 ml of THB and incubating at 37°C with agitation until 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 (from 6 h cultures 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 previously 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 cultures 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 had been prepared in the appropriate 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 cells

in 96-well culture plates were blocked with 1% bovine serum albumin (Boehringer mannheim, Germany) in PBS, and the following monoclonal antibodies against the adhesion molecules were added to different wells: 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 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 following 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) followed by incubation 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 HCL (0.1N) per well of 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). Infection of HUVEC with heat-killed bacteria of *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 *S. suis* serotype 2 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 in expression 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 (data not shown). The values for basal expression were subtracted from those following stimulation for each

adhesion molecule. Stimulation with heat-killed bacteria of *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 to *S. suis*-stimulated cells with respect to the kinetics of ICAM-1 and CD11a/CD18 expression, but not to the kinetics 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 which PmB was present to neutralize any endotoxin contamination, revealed similar results (data 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 bacteria of *S. suis* strain 31533 showed that the up-regulation of ICAM-1, CD11a/CD18 and CD11c/CD18 were bacterial concentration-dependent (Fig. 3). 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 of the porcine or human origin of *S. suis* strains. Strains of porcine or human origin were compared for their capacity to stimulate adhesion molecule expression on monocytes following 48 h of 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 were 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).

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 contributed 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 or 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). As observed for whole bacteria, the up-regulation was dependent on the concentration of cell wall material. 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. It is important to mention that washed heat-killed bacteria suspensions do not contain any hemolytic activity.

Nonencapsulated mutant strain versus wild type strain. The nonencapsulated mutant strain was compared to its wild-type porcine strain S735 with respect to 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 bacteria of *S. suis* were 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 ($P < 0.001$).

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 to date. 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 in 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 bacterium 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 resulted 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 was able 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 from our laboratory that have shown the important contribution of the *S. suis* cell wall to cytokine production by endothelial cells and by murine macrophages, the capacity of the hemolysin to stimulate cytokine production by endothelial cells, and 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 focus on the characterization of 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. 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 induce an increased expression of CD11a/CD18 on THP-1 monocytes, but to have no 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 themselves have the ability to stimulate 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 is 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.

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Legends

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 mean ± standard deviation, and represent at least 3 separate experiments.

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 bacteria of *S. suis* serotype 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 ± standard deviations, from at least 3 separate experiments.

FIG. 3. Effect of different concentrations of bacteria of heat-killed *S. suis* serotype 2 strain 31533 on the expression of ICAM-1(●), CD11a/CD18(■) and CD11c/CD18(▲) on THP-1 monocytes, measured following 48 h of stimulation. Basal expression of the appropriate adhesion molecule measured

on non-stimulated cells was subtracted from each result. Data are expressed as mean ± standard deviation, and represent at least 3 separate experiments.

FIG. 4. Expression of ICAM-1 (A), CD11a/CD18 (B) and CD11c/CD18 (C) on THP-1 monocytes stimulated with heat-killed bacteria of 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.

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, following 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 mean ± standard deviation, and represent at least 3 separate experiments.

FIG. 6. Adherence of THP-1 monocytes to endothelial cells following stimulation with LPS (1 μg/ml) or bacteria of *S. suis* (10^9 CFU/ml) strain 31533. Monocyte stimulation: LPS- or *S. suis*- stimulated THP-1 monocytes

were incubated for 40 min with non-stimulated HUVEC. HUVEC stimulation: non-stimulated THP-1 monocytes were incubated for 40 min with LPS- or *S. suis*- stimulated HUVEC. Wells were washed, fixed, and stained with methylene blue. HCL was added to solubilize the stain, and the OD was read at 650 nm. Results were compared to the value representing basal adhesion of non-stimulated THP-1 monocytes on non-stimulated HUVEC. Increase in monocyte adherence upon stimulation, in comparison to non-stimulated cells ($P < 0.05$), is represented by an *. Data are expressed as mean ± standard deviation, and represent 3 separate experiments.

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.

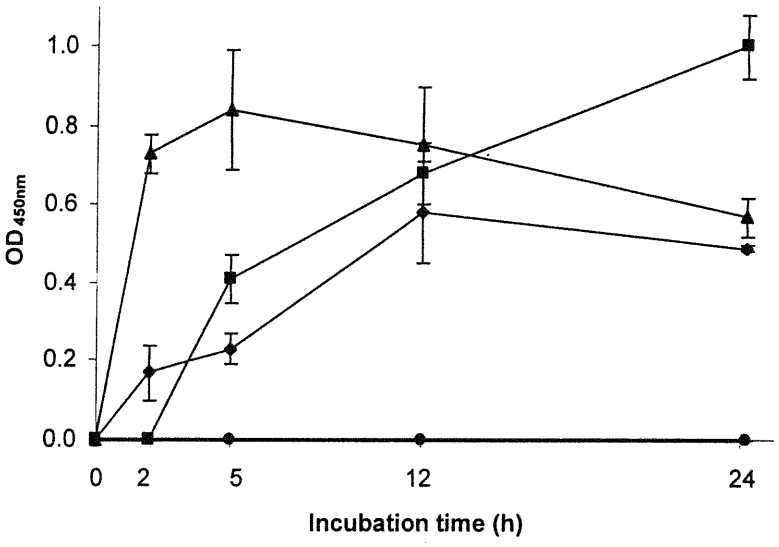

Figure 1

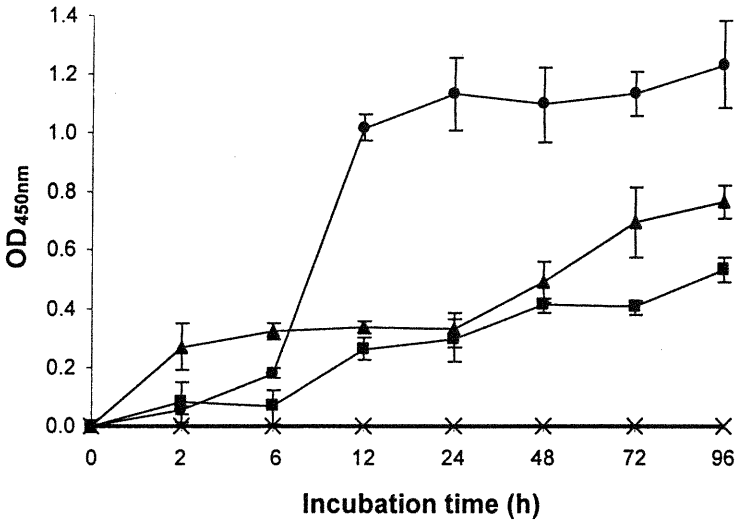
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD450nm
0
2
6
12
24
48
72
96
Incubation time (h)

Figure 2

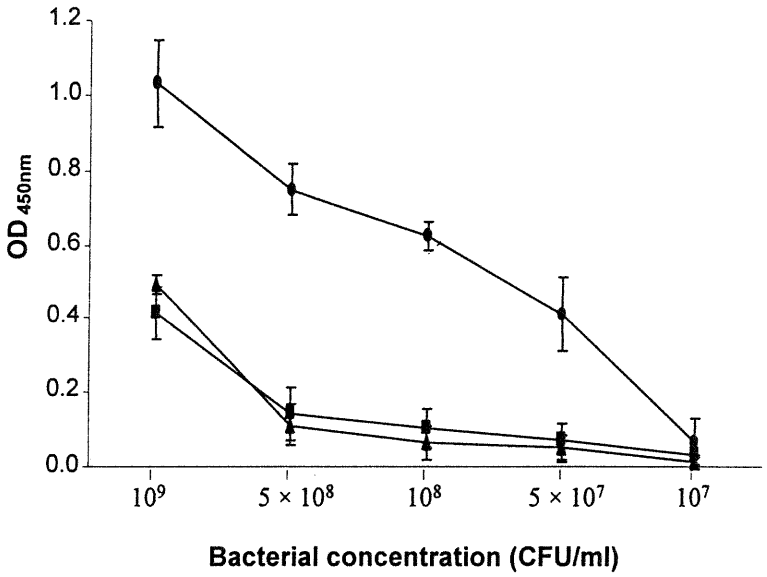
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD 450nm
10⁹
5 × 10⁸
10⁸
5 × 10⁷
10⁷
Bacterial concentration (CFU/ml)

Figure 3

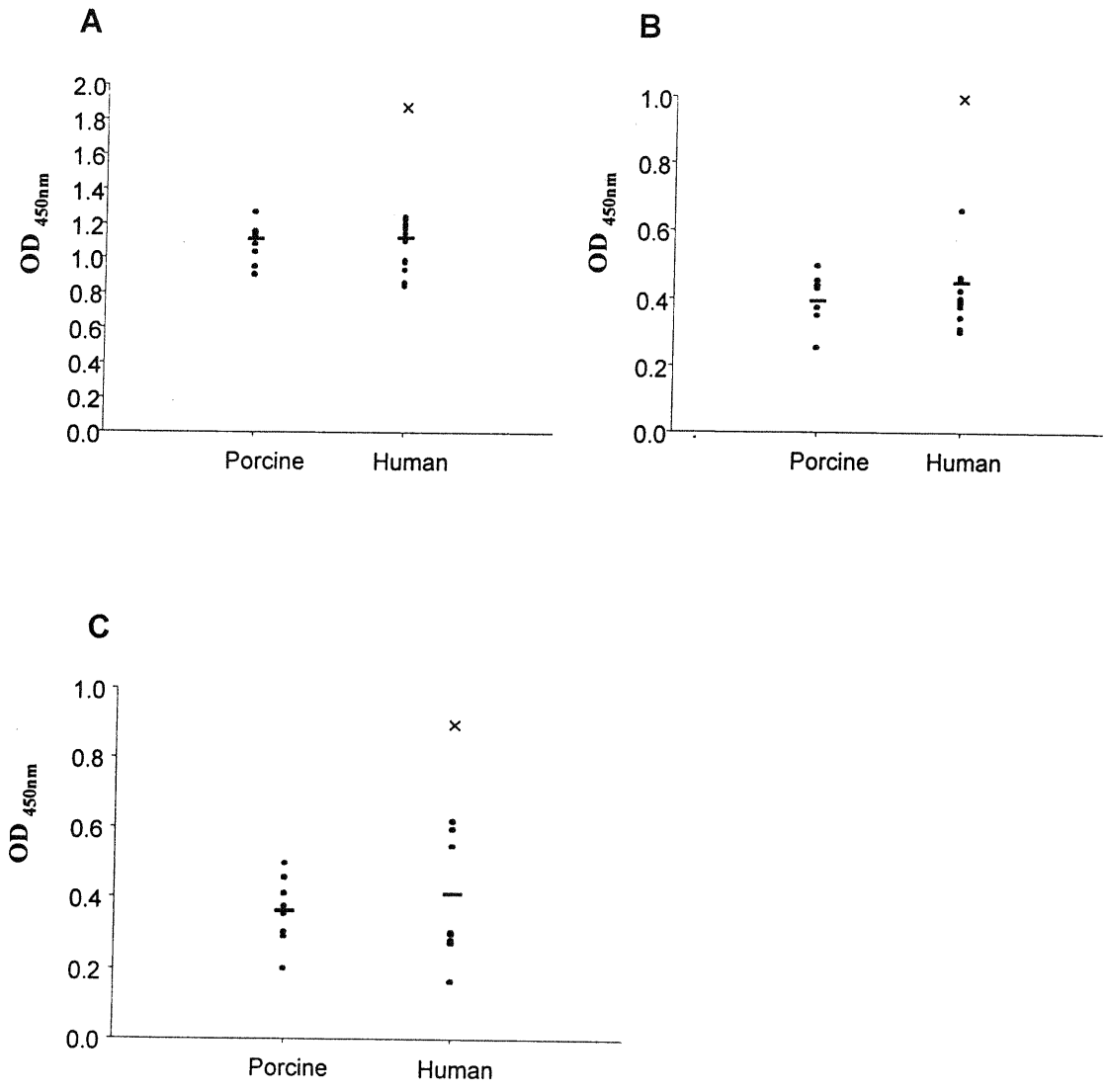
A
OD 450nm
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Porcine
Human
B
OD 450nm
1.0
0.8
0.6
0.4
0.2
0.0
Porcine
Human
C
OD 450nm
1.0
0.8
0.6
0.4
0.2
0.0
Porcine
Human

Figure 4

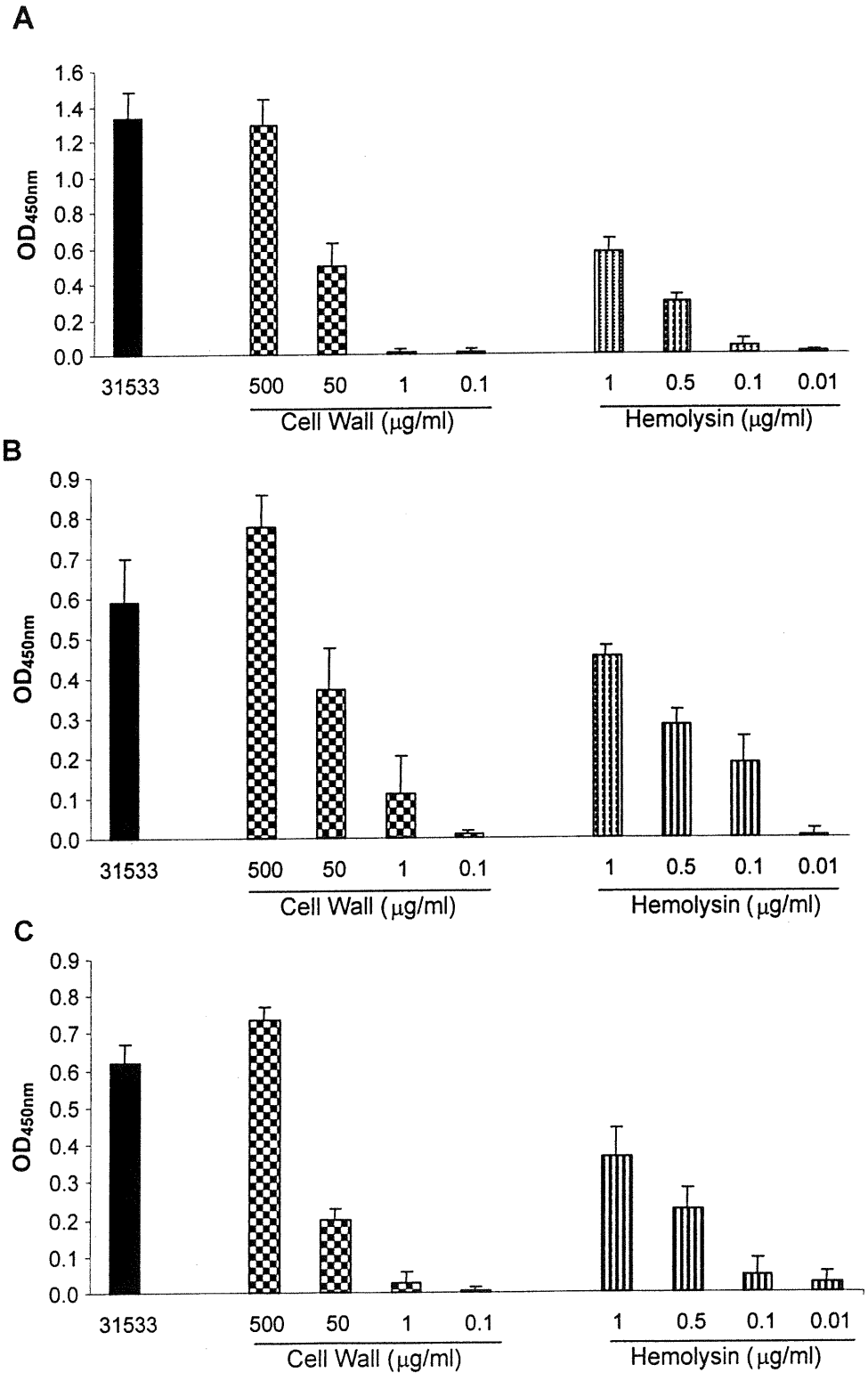
A
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD450nm
31533
500
50
1
0.1
Cell Wall (μg/ml)
1
0.5
0.1
0.01
Hemolysin (μg/ml)
B
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
OD450nm
31533
500
50
1
0.1
Cell Wall (μg/ml)
1
0.5
0.1
0.01
Hemolysin (μg/ml)
C
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
OD450nm
31533
500
50
1
0.1
Cell Wall (μg/ml)
1
0.5
0.1
0.01
Hemolysin (μg/ml)

Figure 5

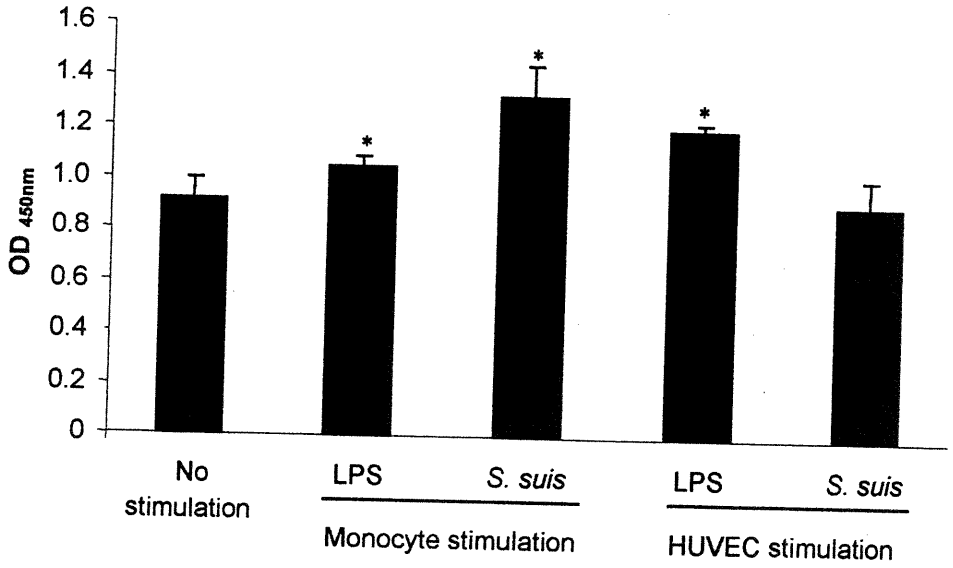
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
OD 450nm
*
*
*
No stimulation
LPS
S. suis
Monocyte stimulation
LPS
S. suis
HUVEC stimulation

Figure 6

Discussion

The interaction between pathogens and host cells can initiate an inflammatory response and contribute to host defense as well as to tissue damage. Previous work has shown that *S. suis* serotype 2 stimulates the production of pro-inflammatory cytokines by both monocytes and endothelial cells (Segura et al., 1999; Segura et al., 2002; Vadeboncoeur et al., 2001). Both cytokines and adhesion molecules are key players that mediate leukocyte adherence and transendothelial migration during an inflammatory response. The leukocyte-endothelial interaction is increased upon the up-regulation of the surface expression of adhesion molecules involved in inflammation (Nagahata et al., 1995; Osborn, 1990). This study investigated the ability of *S. suis* serotype 2 to up-regulate adhesion molecule expression on human THP-1 monocytes and on different types of human endothelial cells. Throughout this study, 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 (Darcissac et al., 1996; Heinzelmann et al., 1997; Heinzelmann et al., 2000).

We found that *S. suis* is capable of up-regulating the surface expression of ICAM-1, CD11a/CD18 and CD11c/CD18 on THP-1 monocytes, in a time- and concentration-dependent manner. Most previous studies report the kinetics of expression of these adhesion molecules between 0 and 24 h. In this study, we have extended cell stimulation to 96 h. *S. suis* induced a

maximal ICAM-1 expression by 12 h, and this high expression was maintained even after 96 h of stimulation. LPS produced a similar ICAM-1 kinetic to that observed with *S. suis*. *S. suis*- and LPS- stimulated monocytes also exhibit a significant increase in CD11a/CD18 expression at 12 h, and continue to increase with time. On the other hand, CD11c/CD18 was up-regulated as early as 2 h, probably due to the fact that this molecule is stored in intracellular granules, whereas ICAM-1 and CD11a/CD18 are not (Carlos and Harlan, 1990). It has been previously shown that, upon activation, monocytes could mobilize this pool within a few minutes, translocating CD11c/CD18 to the cell surface (Miller et al., 1987). Stimulation with LPS resulted in a different pattern of kinetics for this adhesion molecule, where only a first up-regulation at 2 h was detected, and remained constant with time (data not shown), as has been previously observed (Darcissac et al., 1996). 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 (Darcissac et al., 1996). Thus, this difference in response may be due to the relative immaturity of the THP-1 monocytes (Abrink et al., 1994).

In some experiments, THP-1 monocytes were prestimulated with one of these 4 molecules : PMA, Vitamin D_3, MCP-1 and IFN-γ for 2 or 3 days before stimulating with *S. suis*. The prestimulation of cells did not result in an increase in adhesion molecule up-regulation, following the *S. suis* stimulation (data not shown).

Other bacteria have been shown to up-regulate the expression of adhesion molecules. However, the specific pattern of adhesion molecule expression is different for each pathogen. For example, *M. tuberculosis*, similarly to *S. suis*, up-regulates ICAM-1 expression in a time-dependent fashion, and does not increase the expression of CD11b/CD18 (Lopez Ramirez et al., 1994). On the other hand, this bacteria is unable to up-regulate the expression of CD11a/CD18 (Lopez Ramirez et al., 1994). Other bacteria, including *S. aureus* and *S. pneumoniae*, do increase CD11b/CD18 expression on leukocytes (Carratelli et al., 1996; Kragsbjerg and Fredlund, 2001).

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 required a high bacterial concentration for maximal levels of cytokine release (Segura et al., 1999; Segura et al., 2002). 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 (Berthelot-Herault et al., 2001).

A consequence of the up-regulation of adhesion molecules is an increase of leukocyte adhesion to vascular endothelium (Springer, 1994). Hence, we measured the adherence to HUVEC of *S. suis*-stimulated monocytes in comparison to non-stimulated monocytes. Indeed, *S. suis*-stimulated monocytes bound to HUVEC in higher numbers compared to non-

stimulated monocytes. LPS stimulation also caused an increase in monocyte adherence to HUVEC.

In order to identify candidate bacterial components 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 resulted in increased ICAM-1, CD11a/CD18 and CD11c/CD18 expression, as high as that obtained with whole bacteria. Similarly, a cell wall component of *M. tuberculosis* up-regulated the same level of ICAM-1 expression as whole bacteria (Lopez Ramirez et al., 1994). The purified hemolysin also had the capability to stimulate the expression of ICAM-1, CD11a/CD18 and CD11c/CD18 on THP-1 monocytes, but at lower levels in comparison to cell wall components. In contrast to the above purified factors, the CPS of *S. suis* did not cause any significant up-regulation of adhesion molecule expression on monocytes. These results are in agreement with our 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 the inability of CPS to stimulate any cytokine production by both endothelial cells and monocytes (Segura et al., 1999; Vadeboncoeur et al., 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 (Segura et al., 1999) 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 *N. meningitidis* has also been shown to cause a different pattern in adhesion molecule expression on leukocytes in comparison to the parent strain (Klein et al., 1996).

The porcine and human origin of *S. suis* strains did 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 (Berthelot-Herault et al., 2002; Chatellier et al., 1999). These results, along with the present study, agree with the hypothesis that *S. suis* is 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 (Segura et al., 2002). Ongoing studies in our laboratory focus on the characterization of this strain. Preliminary results indicate that "Reims" is a low-capsulated strain, which would confirm results obtained with the nonencapsulated mutant.

S. suis-stimulated THP-1 monocytes have been previously shown to produce increased amounts of pro-inflammatory cytokines and chemokines, for e.g. TNF-α and MCP-1 (Segura et al., 2002). Some of these cytokines have been shown to increase the surface expression of ICAM-1 and β_2-

integrins on monocytes (Tiisala et al., 1994; Yamada et al., 1997). For example, TNF-α has been previously shown to increase the expression of CD11a/CD18 on THP-1 monocytes, but not the expression of CD11b/CD18 and CD11c/CD18 (Tiisala et al., 1994). ICAM-1 has also been shown to be up-regulated by several cytokines (Simmons et al., 1988). An inversion in roles has been demonstrated, where the stimulation (or activation) of adhesion molecules caused an increase in the production of cytokines (Cuzzola et al., 2000; Walzog et al., 1999). In the case of *S. suis*, it is not clear whether the increase in adhesion molecule expression is a result of cytokine production or a direct effect of bacteria on cells, hence, further studies need to be done to clarify this point.

Important meningeal pathogens, including *N. meningitidis*, *L. monocytogenes* and *S. pneumoniae*, have the capability of increasing the expression of adhesion molecules on endothelial cells (Dixon et al., 1999; Freyer et al., 1999; Krull et al., 1997). In the case of *L. monocytogenes*, this up-regulation was accompanied by an increase in leukocyte adherence (Krull et al., 1997). In this study, we investigated the effect of *S. suis* on the surface expression of adhesion molecules on two different cell types, HUVEC and human BMEC. For BMEC, we used primary cells (pHBMEC) and two immortalized cell lines (iHBMEC1 and iHBMEC2). In our initial experiments, we used iHBMEC1 cell line; however, we found that this cell line was not ideal for adhesion molecule studies, since we were unable to up-regulate at high levels the expression of ICAM-1, VCAM-1 and E-selectin using different positive controls (Table 1; all tables referred to in this section are presented in

the annex). In fact, LPS, IL-1β, TNF-α, IL-18 and a mix of IL-1β and TNF-α were all tested at different concentrations. As expected, results with *S. suis* were negative. Using iHBMEC2 cell line, we demonstrated an up-regulation of the expression of ICAM-1 and VCAM-1, but not of E-selectin (Table 2). A mixture of TNF-α and IL-1β caused the highest adhesion molecule up-regulation. *S. suis* did not cause an increase in ICAM-1 and VCAM-1 expression on this cell line. Since neither of the cell lines were capable of up-regulating E-selectin, we could not conclude that *S. suis* did not up-regulate the expression of this adhesion molecule. We then tested primary BMEC (pHBMEC). We observed an up-regulation of the expression of these adhesion molecules (that of VCAM-1 was low in comparison to iHBMEC2) upon stimulation with a mixture of TNF-α and IL-1β (Table 3). However, *S. suis* was unable to up-regulate the expression of any of these adhesion molecules. *S. suis* -stimulated HUVEC also did not show any increase in ICAM-1, VCAM-1 and E-selectin expression (Table 4). All three adhesion molecules on HUVEC were up-regulated by LPS. We did not show results obtained with BMEC cell types in the article, since non of them were able to express all three adhesion molecules.

Since *S. suis* was not able to up-regulate adhesion molecule expression on endothelial cells, we expected that *S. suis*-stimulated HUVEC would not support an increase in monocyte adherence, in comparison to non-stimulated HUVEC. Indeed, this was the case. This further reinforces the correlation between the up-regulation of adhesion molecules and the increase

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in monocyte adherence. We chose to perform our adherence study with HUVEC cells, and not BMEC cells, because we had demonstrated the ability of HUVEC to express all three adhesion molecules that are important in monocyte adherence to endothelial cells.

Interestingly, when endothelial cells were stimulated with medium originating from a culture of THP-1 monocytes that had previously been stimulated with *S. suis* (referred to as monocyte conditioned medium, or MCM), endothelial cells demonstrated an increase in the expression of ICAM-1, E-selectin and VCAM-1 molecules (Tables 2, 3 and 4). This indirect up-regulation may be due to cytokines released by *S. suis*-stimulated THP-1 monocytes, since the production of both TNF-α and IL-1β is increased by *S. suis*, and we showed that a mixture of these two cytokines does increase adhesion molecule expression on endothelial cells.

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.

Conclusion

In conclusion, *S. suis* serotype 2 has the ability to up-regulate the surface expression of ICAM-1, CD11a/CD18 and CD11c/CD18 on human THP-1 monocytes, leading to an increase in the adhesion of monocyte to endothelial cells. On the other hand, *S. suis*-stimulated endothelial cells did not demonstrate any increase in ICAM-1, VCAM-1 and E-selectin expression, and did not support an increase in monocyte adhesion. *S. suis* has been previously shown to increase the production of pro-inflammatory cytokines and chemokines by monocytes. Cytokines are known to be potent activators of cells, and have been shown to increase the expression of adhesion molecules on monocytes. Hence, future studies could be done using monoclonal antibodies that block these cytokines, to investigate whether *S. suis* directly up-regulates adhesion molecules, or whether this up-regulation is a secondary effect to cytokine production. It would be interesting to test whether treatment using antibodies which block adhesion molecules found to be involved in the *S. suis* interaction with monocytes would result in a more rapid recovery or in less severe effects on the infected host.

Understanding the mechanisms utilized by *S. suis* during the infection process is crucial in order to establish efficacious methods for protection of animals and humans from infection. The finding that adhesion molecules are involved in the course of the *S. suis* infection increases our understanding of the pathogenesis of the disease caused by this pathogen.

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Annex

Table 1: Summary table of results obtained with iHBMEC1 cell line.

Treatment	ICAM-1[3]			VCAM-1[3]			E-selectin[3]			
Incub time (hrs) :	12	24	48	12	24	48	5	12	24	48
LPS 0.1 µg/ml	0	0	ND[2]	0	0	-	0	ND	ND	ND
LPS 0.5 µg/ml	0	0	ND	0	0	-	0	ND	ND	ND
LPS 1.0 µg/ml	0	0.11 ± 0.03	0.16 ± 0.01	0	0	0	0	ND	ND	ND
LPS 5.0 µg/ml	0	0.14 ± 0.02	0.13 ± 0.02	0	0	0	0	ND	ND	ND
LPS 10 µg/ml	0	0.11 ± 0.02	0.22 ± 0.03	0	0	0	0	ND	ND	ND
LPS 50 µg/ml	0	0.15 ± 0.03	0.23 ± 0.05	0	0	0	0	0	0.1 ± 0.02	0.11 ± 0.01
IL-1β 0.5 ng/ml	0.14 ± 0.02	ND	ND	0	ND	ND	ND	ND	ND	ND
IL-1β 1.0 ng/ml	0.25 ± 0.1	0.25 ± 0.06	0.33 ± 0.06	0	0	0	0	ND	ND	ND
IL-1β 10 ng/ml	0.23 ± 0.01	0.2 ± 0.04	0.32 ± 0.02	0	0	0	0	ND	ND	ND
IL-1β 50 ng/ml	0.22 ± 0.05	0.24 ± 0.02	0.34 ± 0.03	0	0.16 ± 0.02	0	0	0	0.13 ± 0.02	0
TNF-α 0.5 ng/ml	0	0	ND	0	0	ND	0	ND	ND	ND
TNF-α 1.0 ng/ml	0.13 ± 0.07	0.13 ± 0.02	0.21 ± 0.02	0	0	0	0	ND	ND	ND
TNF-α 10 ng/ml	0.15 ± 0.02	0.16 ± 0.01	0.27 ± 0.04	0	0.1 ± 0.02	0	0	ND	ND	ND
TNF-α 50 ng/ml	0.22 ± 0.06	0.26 ± 0.03	0.32 ± 0.05	0	0.1 ± 0.01	0	0	0	0	0
IL-8 1 ng/ml	0	ND	0	0	0	0	0	ND	ND	ND
IL-8 10 ng/ml	0	ND	0	0	0	0	0	ND	ND	ND
IL-8 50 ng/ml	0	0	0	0	0	0	0	0	0	0
TNF-α 50 ng/ml + IL-1β 10 ng/ml	0.4 ± 0.04	0.21 ± 0.06	ND	0	0	ND	0.09 ± 0.02	0.12 ± 0.02	0.06 ± 0.03	ND
Heat-killed *S. suis* (10^9 CFU/ml)[1]	0	0	0	0	0	0	0	0	0	0

[1]Different *S. suis* serotype 2 strains were used. Strains are listed in Table 1 of article.
[2]Not done.
[3]Basal expression of the three adhesion molecules was subtracted from all results.

Table 2: Summary table of results obtained with iHBMEC2 cell line.

Treatment		ICAM-1[4]		VCAM-1[4]		E-selectin[4]
	Incub time (hrs):	12	24	12	24	5
LPS 0.1 μg/ml		0.24 ± 0.06	0.43 ± 0.08	0.40 ± 0.11	0.33 ± 0.03	0
LPS 1.0 μg/ml		0.19 ± 0.00	0.29 ± 0.07	0.44 ± 0.08	0.29 ± 0.03	0
TNF-α 50 ng/ml		0.14 ± 0.01	0.3 ± 0.02	0	0	0
IL-1β 1 ng/ml		0.19 ± 0.08	0.47 ± 0.05	0.27 ± 0.02	0.22 ± 0.03	0
IL-1β 10 ng/ml		0.24 ± 0.01	0.31 ± 0.02	0	0.14 ± 0.01	0
TNF-α 50 ng/ml + IL-1β 10 ng/ml		0.35 ± 0.04	0.45 ± 0.07	0.38 ± 0.06	0.4 ± 0.05	0
Heat-killed *S. suis* (10^9 CFU/ml)[1]		0	0	0	0	0
MCM[2]		0.37 ± 0.06	0.37 ± 0.03	0.27 ± 0.02	0.13 ± 0.00	ND[3]

[1]Different *S. suis* serotype 2 strains were used. Strains are listed in Table 1 of article.
[2]Monocyte conditioned medium: originating from the medium of monocytes that have been stimulated with *S. suis* for 24 hrs.
[3]Not done.
[4]Basal expression of the three adhesion molecules was subtracted from all results.

Table 3: Summary table of results obtained with primary HBMEC.

Treatment	ICAM-1[3]			VCAM-1[3]			E-selectin[3]		
Incub time (hrs) :	5	12	24	5	12	24	5	12	24
LPS 0.1 μg/ml	0.11 ± 0.05	0.18 ± 0.03	0.20 ± 0.08	0.00 ± 0.01	0.00 ± 0.03	0.00 ± 0.02	0.24 ± 0.12	0.04 ± 0.03	0.00 ± 0.05
LPS 1.0 μg/ml	0.23 ± 0.04	0.24 ± 0.06	0.20 ± 0.07	0.00 ± 0.01	0.00 ± 0.03	0.00 ± 0.02	0.39 ± 0.14	0.15 ± 0.05	0.00 ± 0.04
TNF-α 50 ng/ml + IL-1β 10 ng/ml	0.47 ± 0.11	0.75 ± 0.12	0.79 ± 0.12	0.16 ± 0.06	0.17 ± 0.05	0.07 ± 0.02	0.80 ± 0.13	0.68 ± 0.09	0.26 ± 0.07
Live *S. suis* (10^4-10^8 CFU/well)[1]	0	0	0	0	0	0	0	0	0
Heat-killed *S. suis* (10^9 CFU/well)[1]	0	0	0	0	0	0	0	0	0
MCM[2]	0.63 ± 0.09	0.65 ± 0.06	0.68 ± 0.07	0.16 ± 0.04	0.03 ± 0.01	0.00 ± 0.00	0.813 ± 0.12	0.52 ± 0.07	0.42 ± 0.03

[1]Different *S. suis* serotype 2 strains were used. Strains are listed in Table 1 of article.

[2]Monocyte conditioned medium: originating from the medium of monocytes that have been stimulated with *S. suis* for 24 hrs.

[3]Basal expression of the three adhesion molecules was subtracted from all results.

Table 4: Summary table of results obtained with HUVEC cells.

Treatment	ICAM-1[4]				VCAM-1[4]				E-selectin[4]			
Incub time (hrs):	2	5	12	24	2	5	12	24	2	5	12	24
LPS 0.1 µg/ml	0.17 ± 0.07	0.23 ± 0.04	0.58 ± 0.13	0.49 ± 0.01	0	0.41 ± 0.06	0.68 ± 0.08	1.0 ± 0.08	0.73 ± 0.05	0.84 ± 0.15	0.75 ± 0.15	0.57 ± 0.04
Live *S. suis* (10^4 - 10^8 CFU/well)[1]	ND[3]	0	0	0	ND	0	0	0	ND	0	ND	ND
Heat-killed *S. suis* (10^9 CFU/well)[1]	ND	0	0	0	ND	0	0	0	ND	0	ND	ND
MCM[2]	ND	ND	ND	0.86 ± 0.05	ND	ND	ND	ND	ND	ND	ND	ND

[1]Different *S. suis* serotype 2 srains were used. Strains are listed in Table 1 of article.
[2]Monocyte conditioned medium: originating from the medium of monocytes that have been stimulated with *S. suis* for 24 hrs.
[3]Not done.
[4]Basal expression of the three adhesion molecules was subtracted from all results.

Abstracts

1. Vadeboncoeur, N., Segura, M., **Al-Numani, D.**, and Gottschalk, M. *Streptococcus suis* serotype 2 stimulated the release of pro-inflammatory cytokines by human brain microvascular endothelial cells. Presented at the 101st conference of the American Society of Microbiology, 2001, Orlando, Florida.

2. **Al-Numani, D.**, Segura, M., Doré, M., and Gottschalk, M.Interaction of *Streptococcus suis* serotype 2 with human THP-1 monocytes and endothelial cells. Presented at the conference of the Canadian Society of Microbiology, 2001, Waterloo, Ontario.

3. Vadeboncoeur, N., Segura, M., **Al-Numani, D.**, Vanier, G., and Gottschalk, M. Pro-inflammatory cytokine release by human brain microvascular endothelial cells stimulated by *Streptococcus suis* serotype 2. Presented at the conference of the Research Workers in Animal Disease, 2000, Chicago, Illinois.