Université de Montréal

Recombinant adenovirus and fowlpox: new approach in bovine viral diarrhea virus vaccination

par

Seyyed Mehdy Elahi

Département de microbiologie et immunologie

Faculté de médecine

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

Janvier, 1999

Université de Montréal
Faculté des études supérieures

Cette thèse intitulée:

Recombinant adenovirus and fowlpox: new approach in bovine viral diarrhea virus vaccination

présentée par:

Seyyed Mehdy Elahi

a été évalué par un jury composé des personnes suivantes:

Dr. David W. Silversides, président rapporteur
Dr. Youssef Elazhary, directeur de recherche
Dr. Shi-Hsiang Shen, codirecteur de recherche
Dr. Eric Frost, examinateur interne
Dr. Alaka Mullick, examinateur externe
Dr. Yves Langelier, représentant du doyen de la FES

Thèse acceptée le.....99-04-30.....

SUMMARY

Bovine viral diarrhea virus (BVDV) is classified in the *pestivirus* genus of the *Flaviviridae* family and is a ubiquitous pathogen of cattle causing important economic losses. BVDV infection has a wide range of clinical manifestations ranging from subclinical infection to a hemorrhagic syndrome. The hemorrhagic syndrome of bovine viral diarrhea appeared in Ontario and Quebec during the 1993 outbreak.

In chapter II, as the first objective, we studied the antigenic variation among BVDV isolates of Quebec before and during the 1993 outbreak and compared them to reference strains and American isolates by a peroxidase-linked antibody assay (PLA assay) and a neutralization test (NT). We demonstrated that the BVDV isolates from the 1993 outbreak in Quebec are antigenically different from reference strains and from isolates existing in Quebec before 1993. In addition, we have shown that 2 internationally used fixation-methods (acetone and formalin-fixation methods) in PLA assay give different results.

The modified-live and inactivated vaccines presently available have not been successful in eliminating the BVDV. To control BVDV infections, there is a need for better and safer vaccines. The role of the individual BVDV protein in cellular immunity has not been clearly established. In the majority of previous works the humoral and cellular immunity were studied after immunization of animals with experimental or commercial modified-live or inactivated vaccines. The first step in the construction a recombinant vaccine is to determinate the proteins responsible for the induction of a humoral and cellular immune response (second objective, Chapter III to VI).

We used two different approaches for gene delivery: adenovirus and fowlpox. Different recombinant adenoviruses and fowlpox expressing the major viral proteins of BVDV were constructed. The coding region for the E2 (gp53) protein, the NS3

(p80) and finally the nucleocapsid (p14) of BVDV were used as transgenes. All these recombinant viruses expressed the transgene *in vitro* in different cell lines and also induced a humoral immune response in mice immunized by these recombinants. In the case of recombinant adenoviruses and fowlpox expressing the BVDV/E2 protein, the antibodies neutralized the BVDV infection. The role of each protein in induction of cell immunity was also demonstrated by a lymphoproliferation test and detection of cytokines (IFN-γ, IL-2 and IL-4) in supernatant of stimulated lymphocytes *in vitro* by two genotypes of BVDV (type 1 and 2). We demonstrated the lymphoproliferative response to homologous E2 protein as well as homo- and heterologous C protein of BVDV by recombinant adenoviruses. Also the production of IFN-γ by all the recombinant viruses were demonstrated.

In conclusion, our data suggest a major implication of the C, E2 and NS3 protein of BVDV in the induction of humoral and cellular immune responses in a mouse model although no conclusions can be made about the role of these responses in protection against the virus until the work is repeated in cattle. Our data encourage further study to evaluate these recombinant viruses as vaccines in cattle, the natural host for BVDV.

RÉSUMÉ

Le virus de la diarrhée virale bovine ("bovine viral diarrhea virus" ou BVDV) appartient au genre pestivirus, dans la famille des *Flaviviridae*, tout comme le virus de la peste porcine classique ("classical swine fever virus" ou "hog cholera virus") et le virus de la maladie des frontières ("border disease") chez le mouton (Wengler *et al.*, 1991).

Le BVDV est un virus enveloppé de symétrie icosahédrique, à ARN monocaténaire de polarité positive. Comme chez les flavivirus, les protéines de structure du BVDV sont codées par le premier tiers du génome à l'extrémité 5′ , tandis que les protéines non-structurales sont codées par les 2/3 restants (Baker, 1995). Les différentes protéines virales seraient produites suite à une série de clivages réalisés par des protéases d'origine virale ou cellulaire (Baker, 1995).

Les symptômes d'infection par le BVDV chez les bovins sont très variables. Ils varient d'une maladie inapparente jusqu'à la forme mortelle de la maladie des muqueuses (Bolin, 1993). Depuis 1993, la diarrhée virale bovine (BVD) se manifeste par des symptômes inhabituels comme des problèmes respiratoires et digestifs sévères. Dans certains cas, des hémorragies internes et externes avec leucopénie, anémie et thrombocytopénie y sont observées (Pellerin *et al.*, 1994; Ridpath *et al.*, 1994). Cette forme virulente de BVDV se distingue par des symptômes de la maladie des muqueuses par son taux de morbidité très élevé (plus de 30%).

Les études génomiques et antigéniques de ces isolats démontrent une variation antigénique importante en particulier en ce qui concerne la protéine E2 (gp53) versus les souches de références. La variation génomique observée entre ces groupes de BVDV a permis aux chercheurs de proposer une nouvelle classification pour BVDV (Harpin *et al.*, 1995; Pellerin *et al.*, 1994; Ridpath *et al.*, 1994), selon laquelle, les isolats de BVDV se divisent en deux génotypes. Les souches de références font maintenant parti du génotype 1 de BVDV. Les isolats de type 2 ont une homologie de séquence d'environ 71.5%

(Harpin *et al.*, 1995) avec le type 1. Les symptômes caratéristiques d'une infection par des isolats de type 2 sont des hémorragies, une leucopénie et d'une thrombocytopénie.

Même avant de l'apparition du BVDV de type 2, le virus a toujours été considéré comme un agent pathogène majeur pour le bétail. Ce virus est très répandu en Europe et en Amérique du Nord (36 à 88% des bovins possèdent des anticorps dirigés contre ce virus) ainsi qu'en Afrique et en Australie. L'apparition du type 2 renforce son importance économique (Houe, 1995).

Présentement il y a deux formes de vaccin disponibles, les vaccins atténués et les vaccins inactivés. Les vaccins atténués peuvent causer des maladies post-vaccinales si l'agent pathogène n'est pas bien inactivé. La contamination de la lignée cellulaire utilisée pour la fabrication du vaccin atténué avec les souches non cytopathogène de BVDV a déjà été rapportée (Bolin *et al.* 1991; Nuttall *et al.*, 1997). Il n'est pas recommandé d'utiliser les vaccins atténués dans la période de gestation à cause du risque élevé d'avortement et de malformation de foetus (Liess *et al.*, 1984). L'utilisation de vaccins atténués peut causer l'immunosuppression et augmenter la pathogénèsité des autres pathogènes (Schultz, 1993). L'efficacité de ces vaccins peut être aussi réduite due à la présence d'anticorps maternels.

Les vaccins inactivés démontrent aussi des désavantages. Ils sont beaucoup plus cher à produire que les vaccins atténués. Ils ont besoin de plus de temps pour donner une immunité à l'animal. Les réactions inflammatoires localisées au site d'injection, le choc anaphylaxique et la baisse de production laitière ont été rapportées après leur utilisation. Les désavantages majeurs pour les vaccins inactivés sont le manque d'immunité cellulaire et l'inactivation à cause de la présence d'anticorps maternels (Schultz, 1993).

L'utilisation d'un vaccin recombinant peut résoudre une grosse partie des désavantages des vaccins atténués et inactivés. Un vaccin recombinant peut donner une bonne immunité humorale et cellulaire. Il est compatible avec les anticorps maternels et

les risques du retour à la forme virulente, les maladies post-vaccinal et d'immunosopression sont éliminés, en plus il est utilisable pendant toute la durée de la gestation.

Premier objectif- A cause de l'importance économique du BVD et des problèmes rencontrés pendant la vaccination avec les vaccins atténués et inactivés et l'inefficacité de ces vaccins à contrer une infection de BVDV en particulier contre le BVDV de type 2, la première partie de mon projet de doctorat visait à étudier la variation antigénique entre les souches de références et les isolats québécois de BVDV avant et durant l'épidémie de 1993 au Québec. En résumé la variation antigénique entre 13 isolats québecois de BVDV, 4 souches de référence et 2 isolats des États Unis ont été étudiées par les tests d'immunopéroxidase (IP) et de neutralisation (NT). Les isolats québecois consistaient en 3 isolats d'avant 1993 et 10 isolats provenant de l'épidémie de 1993. Deux différentes méthodes de fixation (acétone et formaline) ont été utilisées pour le test d'IP. La fixation avec l'acétone nous permettait de classifier nos isolats et les souches de référence en 6 groupes. Tous les isolats du Québec étaient différents des souches de référence, de plus les isolats d'avant 1993 appartenaient à un groupe différent d'isolats du Québec durant l'épidémie de 1993. La fixation à la formaline ne démontre pas cette différence. Le test de NT, en utilisant 2 anticorps polyclonaux et six anticorps monoclonaux, nous permettaient de classifier respectivement nos virus en 4 groupes et 7 sous-groupes. En conclusion, nous avons démontré que les isolats de BVDV de l'épidémie de 1993 du Québec sont antigéniquement différents des souches de référence et des isolats provenant d'avant 1993. En plus nous avons démontré que selon la technique de fixation utilisé dans le test d'IP, les résultats sont différents.

Deuxième objectif- Depuis d'épidémie de 1993 au Québec la nécessité de fabriquer un nouveau vaccin contre de la BVD est devenue plus importante. Le première étape pour arriver à cet objectif à long terme est l'étude de l'immunité humorale et cellulaire contre les protéines virales exprimées par des vecteurs viraux. Entre les

différents vecteurs viraux pour la construction d'un vaccin recombinant, nous avons choisi les virus de fowlpox et l'adénovirus humain de type 5.

Pour cette deuxième partie de mon projet nous avons décidé d'exprimer 3 protéines virales du BVDV et d'étudier l'immunité humorale et cellulaire contre ces protéines. Les 3 protéines virales qui nous intéressent sont la p14 (nucléocapside), la région de génome qui code pour cette protéine est très conservée entre les différents pestivirus. Il n'y a jusqu'à présent aucune études sur cette nucléoprotéine. La gp 53 est une glycoprotéine d'enveloppe virale. Elle est très immunogène et elle est la cible des anticorps neutralisants (Baker, 1995; Bolin, 1993). La p80 est une protéine non-structuralle. Elle a une activité de protéase et d'hélicase et elle est responsable du clivage entre les différentes protéines non structurales (Wiskerchen & Collett, 1991).

Dans tous les expériences, l'expression des gènes d'intérêt sont vérifiées dans les cellules appropriées par les test de la radioimmunoprécipitation. Les protéines recombinantes sont précipitées en utilisant des anticorps monoclonaux contre la protéine d'intérêt. Nos résultats démontrent que tous les virus recombinants ont exprimés les protéines de BVDV *in vitro*.

L'immunité humorale est démontrée par la présence des anticorps contre les protéines exprimées *in vivo* après l'immunisation de souris avec les virus recombinants. La présence des anticorps neutralisant contre le BVDV sont démontrés par un test de neutralisation virale.

L'immunité cellulaire est démontrée par le test de prolifération de lymphocytes dans laquelle les lymphocytes des souris immunisées sont stimulés *in vitro* avec le BVDV type 1 ou type 2. En plus la présence des cytokines (IFN-γ, IL-2 et IL-4) dans les surnagent des cellules stimulées a aussi été mesurée par ELISA. Les résultats de ces expériences sont montrés dans les chapitres III à VI.

Dans le chapitre III, un virus de Fowlpox recombinant qui exprime la glycoprotéine E2 (gp53) de BVDV (nommé rFPV/E2) est étudié. Les souris immunisées avec rFPV/E2 ont développé une immunité humorale qui a été identifié par la présence des anticorps contre la protéine E2 de BVDV dans les tests d'ELISA et de NT. En plus, les lymphocytes des ces souris produisent 7 fois plus d'IFN-γ que les lymphocytes des souris immunisées avec le virus parent. Ce qui signifie une activation des cellules Th1.

Dans le chapitre IV, trois adénovirus expriment la protéine E2 de BVDV sous le contrôle de deux promoteurs constitutifs (major later promoter, et promoteur de CMV) et un promoteur inductible sont étudiés. Les trois adenovirus recombinants induisent une réponse humorale très forte contre la protéine E2 de BVDV détectable dans les tests d'ELISA et de NT. En plus, une réponse proliférative et aussi la production d'IFN-γ ont été démontrées par les lymphocytes des souris stimulées *in vitro* avec le BVDV type 1.

Dans le chapitre V, les résultats de construction de deux adenovirus recombinants exprimant la protéine NS3 (p80) de BVDV sont montrés. Les capacités d'expression du promoteur principal de la phase tardive de transcription d'adénovirus ("major late promoter", le virus recombinant nommé rAdBM5/E2) et du CMV sont comparés dans les différentes lignées cellulaire et aussi *in vivo* chez les souris pour induire les anticorps contre la NS3 de BVDV après immunisation par voies intramusculaire et intranasale. Dans la deuxième étape, les groupes de souris sont immunisées avec rAdBM5/E2 par la voie intramusculaire. Dans les surnagents, les lymphocytes des souris immunisées avec le virus recombinant ont permi la sécrétion d'une forte concentration d'IFN-γ après stimulation homologue (BVDV type 1) et hétérologue (BVDV type 2). Ces résultats démontrent une activation de lymphocyte de type Th1.

Finalement dans le chapitre VI, les résultats de construction d'un adenovirus recombinant exprimant la nucléocapside (C) de BVDV ont été démontrés. On a utilisé un système inductible pour l'expression de la protéine C de BVDV. Ce virus recombinant a produit une forte réponse humorale chez les souris immunisées. En plus, les lymphocytes

de souris immunisées ont aussi démontré une prolifération cellulaire significative après une stimulation avec les BVDV type 1 et type 2 et aussi une production d'IFN-γ. Ce qui signifie qu'une réponse d'immunité cellulaire homologue et hétérologue contre la nucleocapside de BVDV chez les souris.

En conclusion, notre résultats publiés dans cette thèse démontrent pour la première fois l'efficacité des vecteurs fowlpox et d'adenovirus pour exprimer les différents gènes de BVDV. Nos résultats démontrent une grande implication des protéines de la nucléocapside, la E2 et la NS3 de BVDV dans l'immunité humorale et cellulaire chez la souris. Mais on peut pas faire aucune conclusion concernant le rôle des ces protéines au niveau de la protection contre une infection par le BVDV puisque ces expériences n'ont pas été étudiées chez le bovin, l'hôte naturel du BVDV. Une combinaison d'adénovirus qui exprimeraient ces trois gènes d'intérêt pourrait constituer un vaccin très efficace pour induire une protection.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

CHAPTER III

CHAPTER IV

CHPTER V

CHPATER VI

LIST OF ABBREVIATIONS

Ad:	adenovirus
BDV:	border disease virus
BVDV:	bovine viral diarrhea virus
CEF:	chicken embryo fibroblast
CES:	chicken embryo skin
CP:	cytopathic
CPV:	canarypox
CSFV:	classical swine fever virus
CTL:	cytotoxic T lymphocyte
FPV:	fowlpox virus
gpt:	xanthine-guanine phosphoribosyltransferase
HA:	hemagglutinin
HCV:	hog cholera virus
IL:	interleukin
ITR:	inverted terminal repeats
NCP:	noncytopathic
Mab:	monoclonal antibody
Mabs:	monoclonal antibodies
MD:	mucosal disease
MHC:	major histocompatibility complex
MIP:	major later promoter
PI:	persistently infection
rFPV	recombinant fowlpox virus
tk:	thymidine kinase
tTA:	tetracycline trans-activating

In the name of God, Most Gracious, Most Merciful	**Au nom d'Allah, le Tout Miséricordieux, le Très Miséricordieux.**
Read in the name of your Sustainer,	**Lis, au nom de ton Seigneur qui a créé,**
Who has created man out of a germ-cell !	**qui a créé l'homme d'une adhérence.**
Read, for your Sustainer is the most Bountiful One,	**Lis ! Ton Seigneur est le Très Noble,**
Who has taught man the use of the pen,	**qui a enseigné par la plume [le calame],**
taught man what he did not know !	**a enseigné à l'homme ce qu'il ne savait pas.**
Nay, verily, man becomes grossly	**Prenez-garde ! Vraiment l'homme devient rebelle,**
overweening whenever he believes himself to be self-sufficient:	**dès qu'il estime qu'il peut se suffire à lui-même (à cause de sa richesse).**
for, behold, unto your Sustainer all must return.	**Mais, c'est vers ton Seigneur qu'est le retour.**
Qur'an 96/1-8	**Le Coran 96/1-8**

To: My father, My mother,

My family, my most valuable treasure.

Acknowledgments

First, I would like to extend my sincere thanks to my research supervisor, Dr. Youssef Elazhary for his generous support, encouragement and invaluable help during all the years of my studies. I am very lucky to be one of his students. I express my profound gratitude to him for the financial support during the last few years and also for a great oppurtunity to expand my knowledge by allowing me to work in different laboratories.

I would like to express my gratitude to Dr. Shi-Hsiang Shen, my co-directors, for suggestions, discussion and help in my studies. I am also thankful to have giving me the chance to work in his laboratory for more than 4 years.

My highest thanks to Dr. Brian Talbot, for his continuous collaboration and support with valuable advice throughout the work and also for the help in preparation of all the manuscripts.

I would like to thank Dr. Bernard Massie, Dr. Éva Nagy and Dr. Estela Cornaglia for their kind collaboration and for fruitful discussions during the different steps of this project.

Special thanks and best regards to my partner in Dr. Elazhary's laboratory Serge Harpin for his collaborations and friendship.

Deep thanks are conferred to Dr. Amer Silim for his benevolent enthusiasm, guidance and helpful advice during the work with fowlpox virus.

I am also deeply grateful to Dr. David Silversides, Dr. Eric Frost, Dr. Alaka Mullick and Dr. Yves Langelier who accepted becoming members of my jury and also for comments and corrections of this thesis.

I would like to express my sincere appreciation and gratitude to the Ministry of Culture and Higher Education of the Islamic Republic of Iran for the financial support provided during my stay and education in Canada.

I would like to thank Claude Paquet, Brigitte Bousquet, Diane Frenette, Michelle Levesque and Normand Jolicoeur for skillful technical assistance.

INTRODUCTION

Bovine virus diarrhea (BVD) is an economically important disease in cattle. The extensive variability of clinical signs and lesions from subclinical disease to hemorrhagic syndrome, following infection with bovine virus diarrhea virus (BVDV) in cattle has been well documented (Bolin, 1995; Carman *et al.*, 1998; Corapi *et al.*, 1990; Pellerin *et al.*, 1994; Perdrizet *et al.*, 1987; Rebhum *et al.*, 1989). The hemorrhagic syndrome of BVD appeared in 1993 in Ontario and Quebec in epidemic form (Baker, 1995). In Quebec the mortality rate among veal calves increased four times and was estimated at 31.5% for grain-fed calves and 17.1% for milk-fed calves (Pellerin *et al.*, 1994). As the first objective, we studied the antigenic variation among Quebec BVDV strains before and during 1993 outbreak with different reference strains. Our results confirm the presence of antigenic diversity among the BVDV strains.

The serologic cross-reactivity between BVDV strains used in commercial vaccines (which contain only type I of BVDV) and BVDV type II isolates was relatively low (Pellerin *et al.*, 1994) which may indicate a need to include BVDV type II isolates in vaccines. Several modified-live and inactivated vaccines are commercially available. Modified vaccines are considered to be efficacious, but their safety is controversial (Liess *et al.*, 1984; Orban *et al.*, 1983). The available inactivated vaccines are safe, but their efficacy does not seem to be satisfactory (Zimmer *et al.*, 1996). To control BVDV infections, there is need for better and safer vaccines.

Our knowledge about the ability of BVDV proteins in induction of cellular immunity is very limited. Cellular immunity was demonstrated in the majority of cases only after immunization with BVDV and the role of individual proteins was not investigated. For the construction of the recombinant or subunit vaccines only proteins that can induce a humoral (especially neutralizing antibodies) and/or cellular immune response are attractive. Identification of these kinds of proteins is the first

step toward the construction of a new BVDV vaccine. In consequence, as the second objective we studied the ability of fowlpox and adenovirus recombinants to express the BVDV genes *in vitro* and to induce humoral and cellular responses *in vivo*.

The results published in this thesis will be the first report to demonstrate the efficacy of recombinant fowlpox and adenovirus vectors as a BVDV recombinant immunogen to express different BVDV genes *in vitro* in different cell lines and also *in vivo* in a mouse model. Further study to evaluate its use as a vaccine in cattle, the natural host for BVDV is encouraged.

CHAPTER I

LITERATURE REVIEW

1. BOVINE VIRAL DIARRHEA VIRUS (BVDV)

1. 1. Classification and virus properties

Bovine viral diarrhea virus (BVDV) is an economically important pathogen of cattle that is distributed worldwide. Currently, BVDV is classified as a member of the genus *pestivirus*. There are three pestiviruses: classical swine fever virus (CSFV) (also called Hog cholera virus, HCV), bovine virus diarrhea virus (BVDV) (also called mucosal disease virus, MD) and border disease virus (BDV). They were named after the important diseases from which they were first isolated; specifically a systemic haemorrhagic disease of pigs in the USA, an enteric disease of cattle in the USA and a congenital disease of sheep in the border region between England and Wales. The viruses are classified in the family *flaviviridae* along with the flaviviruses and human hepatitis C virus (Wengler *et al.*, 1991).

Two biotypes of BVDV exist, noncytopathic (NCP) and cytopathic (CP), which are differentiated by their effect in cultured cells. The cytopathic viral biotype induces cytoplasmic vacuolation and cell death in susceptible cell cultures (Gillespie *et al.*, 1960). The noncytopathic viral biotype has little effect on cultured cells (Lee & Gillespie, 1957) and readily establishes a persistent infection at the culture level. There is not, however, a correlation between viral biotype and virulence in cattle. Either separately or in combination, the two viral biotypes induce dieases that range from clinically mild to fatal (Baker, 1990; Brownli, 1990).

1. 2. Virus structure

Pestiviruses are enveloped, spherical particles approximately 50 nm diameter containing single-stranded positive sense RNA approximately 12.5 kb in length. Early studies showed that naked genomic RNA extracted from virion, if transfected into

bovine cells, gives rise to infectious viral progeny (Diderholm & Dinter, 1966). No RNA molecules of subgenomic size are found in virus preparations of infected cells (Purchio *et al.*, 1984). The BVDV consists of a single open reading frame (ORF), encoding a large polyprotein comprised of approximately 4,000 amino acids (Deng & Brock, 1992). Currently, 11 BVDV proteins have been identified as products co- and post-translationally processed by either host or viral proteases. In the hypothetical polyprotein, the proteins are arranged in the order N^{pro}-C-E^{ms}-E1-E2-NS23(NS2-NS3)-NS4A-NS4B-NS5A-NS5B (Akkina, 1991; Collett *et al.*, 1988*b*). The ends of the RNA flanking the ORF are called the 5′ and 3′ untranslated regions (UTR). The BVDV genome RNA molecule seems to lack 5′ cap and 3′ poly A structures (Brock *et al.*, 1992; Collett *et al.*, 1998*a*). The 5′ UTR is 385 nucleotides in length, whereas the 3′ UTR is 226 nucleotides in length. UTRs may be functional equivalents of the 5′ cap and poly A, controlling translation and RNA stability, respectively (Drummond *et al.*, 1985; Iizuka *et al.*, 1994; Zingg *et al.*, 1988). Because the 5′ UTR is highly conserved among pestivirus species, it has been proposed that sequences from this region can be used to differentiate among the member viruses (Boye, *et al.*, 1991; Harpin *et al.*, 1995; Ridpath *et al.*, 1993; Ridpath *et al.*, 1994).

1. 3. Genomic comparison of BVDV

Nucleotide sequence comparisons among different pestivirus isolates show an overall conservation of the sequence (Collett *et al.*, 1988*a*; Collett *et al.*, 1989; Deng & Brock, 1992). Sequence conservation is not uniform along the genome of pestiviruses. For example, there is a high degree of variability in the structural glycoprotein E2 (gp53), with values of amino acid identities as low as 80% within one species and 60% between all three species (Becher *et al.*, 1994); these values are comparable to the ones obtained for NS2 (p52). There is only one less-conserved pestivirus-encoded protein, namely p7, for which the degree of amino acid identity between species can be as low as 43% (Elbers *et al.*, 1996). In contrast, NS3 (p80) represents the most conserved protein among pestiviruses (Meyers *et al.*, 1989), with

more than 90% amino acid identity among pestiviral species. The most conserved nucleotide sequence blocks can be found in 5' UTR and least conserved sequences are insertions of host cell-derived genetic information into NS23 (p125) of some isolates of cytopathic BVDV. Excepting these, four hypervariable regions are found among standard BVDV genomes (Deng & Brock 1992). Two hypervariable sequences are found in a region encoding for the major surface glycoprotein, gp53/E2 and two other are located in the nonstructural polypeptides NS23 (p125) and p58/NS5A (see Figure 1).

The highly conserved 5′ UTR is particularly useful to identify pestiviruses by PCR. Moreover, nucleotide sequencing of this short region seems to provide sufficient information for their differentiation (Harasawa & Tomiyama, 1994; Harpin *et al.*, 1995; Hofmann *et al.*, 1994; Pellerin *et al.*, 1994; Ridpath *et al.*, 1994). Ridpath *et al.* (1994) have shown that two distinct genotypes of BVDV can be identified. Comparison of the nucleotide sequences among classical laboratory strains (NADL, Osloss and SD-1), genotype I, shows a sequence homology of nearly 78% to 88% for the entire genome. Conserved regions such as the 5′ UTR of these viruses have a homology ranging from 86% to 93%. Newly described BVDV isolates, genotype II have 5′ UTR sequences only with 75% homology to the classical strains, and are more than 90% homologous when compared with each other (Pellerin *et al.*, 1994; Ridpath *et al.*, 1994).

On the basis of the 5′ UTR sequence of several BVDV isolates from the severe Quebec outbreak we suggested dividing the BVDV strains in two genotypes (Harpin *et al.*, 1995). Sequences revealed the loss, for the BVDV type II isolates, of an internal *Pst*I restriction site, which is present in all known BVDV type I 5′ UTR sequences. A single restriction enzyme digestion (*Pst*I) of an aliquot of PCR product allowed us to differentiate BVDV type I and BVDV type II.

Recently the comparison of E2 (gp53) amino acid sequences has been used to demonstrate variation among pestiviruses (Becher *et al.*, 1994). The latter study led to identification of two groups of ovine pestiviruses, namely BVDV-like strains and

"true" BDV strains. Recently a third group of sheep-derived pestiviruses has been identified by PCR and nucleotide sequencing of the 5′ UTR as well as of the N^{pro} and C coding regions (Becher *et al.*, 1995). This third group of ovine pestiviruses has a high degree of similarity with the viruses which are classified by Ridpath as genotype II.

In this thesis to avoid confusion, the classification of Ridpath *et al* (1994) and Harpin *et al.*, (1995) in which the different BVDV isolates where divided into genotypes I and II, was used. The BVDV isolates which cause a hemorrhagic syndrome are placed in genotype II.

1. 4. Viral proteins encoded by BVDV

Viral gene expression is believed to occur via synthesis of a polyprotein and subsequent proteolytic processing mediated by cellular and viral proteases (Collett *et al.*, 1988*b*; Wiskerchen & Collett, 1991). The final products of the ORF of BVDV are represented diagrammatically in Figure 1 chapter I. The first cleavage event is due to an autoproteloytic activity of the N-terminal protease, N^{pro} (p20) that is responsible for cleavage between non-structural protein N^{pro} (p20) and the nucleocapsid protein (C or p14) (Stark *et al.*, 1993; Wiskerchen *et al.*, 1991).

Pestiviruses encode four structural proteins, which are represented by the nucleocapsid protein C (p14) and three glycoproteins $E0^{ms}$ (gp48) E1 (gp25) and E2 (gp53), in the order of their arrangement in the polyprotein (Collett *et al.*, 1998*b*; Strark *et al.*, 1990). In the past, pestiviral proteins have mostly been named according to their apparent molecular weights, and thus homologous proteins from different pestiviral strains and isolates have different names.

C (p14) protein is well conserved across different pestiviruses. The C (p14) protein presumably is located in the cytoplasm of infected cells. It is not known whether it migrates to other compartments. The function of the protein is to package the genomic RNA and to provide necessary interactions for the formation of the enveloped virion. Domains involved in these processes have not been identified. The

poor immunogenicity of the BVDV capsid in cattle contrasts with the abundance of antibody to the hepatitis C capsid protein in infected human sera (Donis & Dubovi, 1987*a*; Khudyakov *et al.*, 1993).

$E0^{rns}$ (gp48) forms homodimers covalently linked by disulfide bonds after its translocation to the lumen of the endoplasmic reticulum (ER). The function of $E0^{rns}$ is not very clear; it is a putative component of the virion, although the absence of a hydrophobic membrane anchor region suggests a loose interaction with the envelope. RNase activity has been detected in purified preparations of hog cholera virus $E0^{rns}$. A similar activity is predicted for the BVDV conterpart, $E0^{rns}$ (Hulst *et al.*, 1994; Schneider *et al.*, 1993). The significance of this enzymatic activity is unknown. The $E0^{rns}$ induces considerable levels of antibodies in infected cattle, but these antibodies have limited virus-neutralizing activity (Boulanger *et al.*, 1991; Xue *et al.*, 1990) The E1 (p25) has a predicted mass of 21.6 kDa. After post-translational modification it migrates in SDS-PAGE as 25 kDa. The E1 is found in virions covalently linked to E2 by disulfide bonds. Convalescent cattle serum does not contain significant levels of antibody to E1 (Donis & Dubovi, 1987*a*).

Two different forms of E2 protein, E2 (53 kDa) and E2p7 (60 kDa), have been found in infected cells depending on the different cleavage patterns in their C-terminal tail (Elbers *et al.*, 1996). The glycoprotein is likely to be found in the virion envelope as homodimers and as heterodimers with E1. The C-terminus of E2 is anchored in the lipid envelope by a transmembrane region. The E2 is very antigenic and elicits the production of neutralizing antibodies in the host after infection or vaccination with live or killed vaccines (Donis *et al.*, 1988). One of the hypervariable sequence regions found in the viral genome is present in this polypeptide (Potgieter, 1995).

The C-terminal two thirds of the ORF encode exclusively nonstructural proteins (Figure. 1. Chapter I). Downstream of p7, the first cleavage product is represented by NS23 (p125), which for most pestiviruses is partially processed to yield NS2 (p54) and NS3 (p80) (Collett *et al.*, 1988*b*; Meyers *et al.*, 1991). There are,

however, remarkable exception: after infection with NCP BVDV strains, only NS23 but no respective processing products can be detected (Donis & Dubovi, 1987*b*; Donis & Dubovi, *1987c; Pocock et al.*, 1987). In addition CP BVDV strains express NS3 from a duplicated genomic region and their NS23 in not cleaved. NS2 is a hydrophobic protein and NS3 is a hydrophilic protein (Wiskerchen & Collett, 1991). The NS3 protein is a trypsin-like serine protease related to the NS3 protein of flaviviruses. It is responsible for the cleavage between non-structural proteins (Bazan & Fletterick, 1989). It also possesses an RNA helicase activity (Warrener & Collett, 1995).

Our knowledge about the non-structural proteins, NS4A-NS4B-NS5A-NS5B, are limited. Only the NS5B function has been determined as a putative viral RNA-dependent-RNA polymerase (Donis, 1995).

1. 5. Antigenic variation among pestiviruses

These is antigenic and genetic diversity within the pestiviruses, but all members cross-react serologically to various extents. Virus transmission between different ruminant species is documented (Carlsson, 1991; Carlsson & Belák, 1994). The monoclonal antobodies (Mabs) directed against NS3 generally recognize conserved epitopes and their reactivity pattern is considered panpestivirus-specific (Edward *et al.*, 1988; Peters *et al.*, 1986). In contrast, Mabs against $E0^{ms}$ and E2 have been used to discriminate between pestivirus species as well as between strains of one species (Cay *et al.*, 1989; Elahi *et al.*, 1997, chapter II ; Kosmidou *et al.*, 1995; Weiland *et al.*, 1992; Wensvoort *et al.*, 1989). Mabs against E2 have also been used to map respective epitopes by using competitive binding assay, antigen capture assays, virus neutralization assays, deletion mutants, and neutralization escape mutants (Paton *et al.*, 1992; van Rijn *et al.*, 1993; Wensvoort, 1989). Although E2

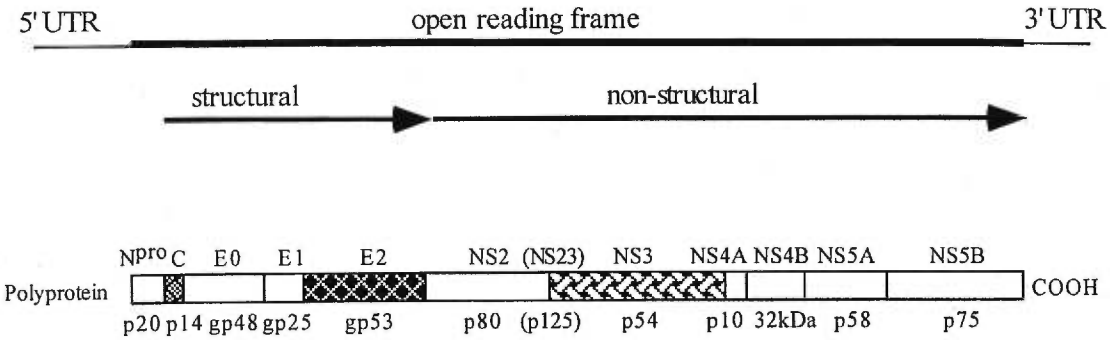

Fig. 1. Schematic representation of the BVDV genome and protein coding domains. For designation of the individual structural and non-structural viral proteins, see text. Polypeptides in parenthesis are not present in all virus isolates. The 32 kDa polypeptide has not been identified unambiguously.

appears to represent the major target of virus neutralizing antibodies, Mabs against $E0^{ms}$ also mediate virus neutralization (Bolin *et al.*, 1988; Donis *et al.*, 1988; Greiser-Wilke *et al.*, 1990; Wensvoort *et al.*, 1989). BVDV isolates shows a greater heterogeneity than do classical swine fever viruses (CSFVs). Although there are reports of apparently BVDV-specific Mabs (Edwards & Paton, 1995), studies that have included extensive collections of bovine pestiviruses have failed to confirm this (Bolin *et al.*, 1988; Corapi *et al.*, 1990; Deregt *et al.*, 1990; Edwards *et al.*, 1988; Paton *et al.*, 1991; Xue *et al.*, 1990). Those scientists with access both to panels of Mabs and to extensive collections of virus isolates have attempted to subdivide BVDV strains into antigenic groups, either by immune binding assays (using indirect fluoresent or enzymatic lables) or by neutralization. Different workers have proposed 3, 4, 6, 7, 9, or 32 distinct groups within BVDV (Bolin *et al.*, 1988; Corapi *et al.*, 1990; Deregt *et al.*, 1990; Edwards *et al.*, 1988; Magar *et al.*, 1988; Paton *et al.*, 1991; Xue *et al.*, 1990). None of these classifications seems to have universal applicability, and they are mostly based on Mabs raised against CP reference strains of the virus. Antigenic diversity is important between different pestiviruses for diagnosis and for control by vaccinations. Recently we demonstrated that recombinant adenovirus expressing the BVDV/E2 protein of NADL strain produced a lymphocyte proliferation response only after stimulation with homologous virus (Chapter IV). Harpin *et al.* (1998, submitted manuscript) also demonstrated the same results after immunization of calves with DNA plasmid encoding the BVDV/E2 protein. Antigenic cross-reactivity also has an impact on differential diagnostic procedures, particularly in swine for differentiated between the CSFV and BVDV.

1. 6. Humoral and cellular immune responses to BVDV

Upon either natural or experimental infection with BVDV and subsequent recovery, a serum antibody response is generated. Immunity following natural infection is not lifelong. The implication arising from this observation is that

immunity will also be of limited duration following vaccination with live virus, which can be regarded as simply an artificial infection (Howard, 1990).

Immunization with live or inactivated virus elicits antibodies to numerous viral proteins (Bolin & Ridpath, 1989; Bolin & Ridpath, 1990). The majority of these antibodies are against the E2 and NS3 proteins. The majority of natural neutralizing antibodies against BVDVs are directed against the E2 protein (Bolin *et al.*, 1988; Donis *et al.*, 1988). The role of the E2 protein of BVDV in cellular immunity has not been clearly established. However, in the case of CSFV, another pestivirus, the E1 protein (E2 homologue in BVDV) is not a major T-cell antigen (Kimman *et al.* 1993). Recently Harpin *et al* (1998, submitted manuscript) demonstrated a protection in calves vaccinated with DNA plasmid encoding the E2 protein of BVDV/NADL strain after challenge with BVDV type I. In this study, animals vaccinated with naked DNA (N-DNA) or DNA in cationic liposomes (L-DNA) were presented neutralizing antibodies before and after challenge (at 16 wk). N-DNA-vaccinated animals also showed virus-specific lymphocyte proliferation responses to type I, live BVDV *in vitro.* Also, N-DNA-vaccinated calves were protected from viral challenge.

Two other glycoproteins of BVDV, $E0^{ms}$ and E1, did not elicit antibodies that efficiently neutralize the BVDV (Bolin, 1993, Boulanger *et al.*, 1991, Xue *et al.*, 1990). Antibodies to $E0^{ms}$ were present in cattle vaccinated with killed or modified-live virus or subunit vaccination or following natural infection (Kweon *et al.*, 1997)

Using the ewe as a model, Carlsson *et al.* (1991) demonstrated that the immunostimulating complex (ISCOM) subunit vaccine designed to contain the envelope proteins of a Danish CP BVDV had the potential of eliciting high virus neutralization titers as well as protecting fetuses against transplacental infection after challenge with a virulent BVDV isolate.

The several mutant envelope glycoprotein E2 of CSFV (formerly called E1 of CSFV) expressed in insect cells protects swine from classical swine fever (van Rijn *et al.* 1996). In protection experiments performed by Konig *et al.* (1995) only swine

vaccinated with recombinant vaccinia virus expressing E0 and/or E2 resisted a lethal challenge infection with CSFV.

The correlation between antibody titers and protection against virus replication is not very clear. The results of an E2 vaccination study in calves showed that the homologous challenge strain Singer likely did not replicate in calves with a neutralizing antibody titer ≥ 512. Virus replication occurred in all calves challenged with a heterologous BVDV strain, regardless of neutralizing antibody titer (Bolin & Ridpath, 1996). However, Bruschke, *et al.* (1997) could not find a correlation between antibody titers and protection.

Between non-structural proteins, antibodies against the NS3 protein, after infection or vaccinated animal with live vaccine, are immunodominant. However there is no evidence that these antibodies have the capacity for neutralization (Bolin & Ridpath, 1989; Cortese, 1994). Some of the epitopes on this protein are not conformation-dependent, and many are highly conserved among all pestiviruses (Deregt *et al.*, 1990; Kamstrup *et al.*, 1991; Paton *et al.*, 1992). Four antigenic domains were defined with monoclonal antibodies (Paton *et al.*, 1992). Lambot *et al.* (1997) demonstrated a proliferative response after vaccination of cattle with cytopathic and non-cytopathic BVDV and stimulation *in vitro* with a NS3 recombinant protein.

The direct role of cell-mediated responses is more difficult to assess, particularly in outbred cattle where adoptive transfer of cells in not possible. To overcome this problem one approach has been the investigation of the potential role of cytotoxic T-cells, in recovery from infection with BVDV, by specific depletion of lymphocyte sub-populations. This was achieved *in vivo* by inoculation of calves with murine monoclonal antibodies directed against the bovine CD4 or CD8 antigens and determination of the effect of this treatment on infection (Howard *et al.*, 1989). Depletion of BoCD8+ lymphocytes had no effect on infection but following depletion of BoCD4+ lymphocytes viramia was prolonged. Thus, no evidence was obtained to

indicate that MHC class I restricted cytotoxic T-cells play a pivotal role in the resolution of infection.

1. 7. Clinical aspects and pathogenesis of BVDV infection

The clinical aspects of BVDV is complex , and multiple and diverse clinical manifestations ranging from subclinical infection to a highly fatal form known as mucosal disease (MD) may occur in cattle infected with the virus. In recent years, another form of acute infection has been recognized in immunocompetent cattle, which is known as the hemorrhagic syndrome. Fever, pneumonia, diarrhea, and sudden death occuring in all age groups of cattle was reported (Carman *et al.*, 1998). Thrombocytopenia and hemorrhagic syndrome has been reported in adult cattle and in veal calves (Corapi *et al.*, 1990; Pellerin *et al.*, 1994; Rebhun *et al.*, 1989). The BVDV type II seems to be associated with this hemorrhagic syndrome (Pellerin *et al.*, 1994; Ridpath *et al.*, 1994).

Infection of seronegative cattle with non-cytopathic BVDV during the first 120 days of pregnancy can result in the birth of a immunotolerant calf, persistently infected (PI) with BVDV. These BVDV carriers continuously shed high amounts of virus into their environmet and are the most important source of virus spread (Bruschke *et al.*, 1997). In preliminary experiments in Denmark, it was found that eradication in herds could be based on the identification and removal of PI animals. Also BVDV is also considered an important teratogen in calves and can result in numerous congenital defects (Bolin, 1995) .

Pathogenesis of MD is complex and remains somewhat obscure. It is clear that the MD only occur in PI animals. At some point in the life of such an animal, a mutational event occurs in the RNA of a single noncytopathic virus and creates a cytopathic virus. The immune system does not react against the cytopathic BVDV because the structural proteins of that virus are similar antigenically to the resident noncytopathic BVDV spread in the host. The animal develops diarrhea, becomes

dehydrated, and dies by MD. Another way to develop MD is superinfection of a PI animal with homologous cytopathic BVDV (Bolin, 1995).

In this chapter only different aspects of BVDV that are important to the understanding of this thesis were explained. For more information about BVDV, I refer you to volume 11 of "The Veterinary Clinics of North America, Food animal practice" (Baker & Houe, 1995), which is completely dedicated to BVDV.

2. RECOMBINANT FOWLPOX VIRUS VECTORS

2. 1. Molecular biology of poxviridae

The virus family, *Poxviridae*, is divided into two subfamilies, *Chordopoxviridae* and *Entomopoxviridae*. The subfamily, *Chordopoxviridae*, is further divided into a number of genera based on the chordate species they infect, as well as antigenic and genomic similarities (Moss, 1995). An important characteristic of *Avipox* genus such as fowlpox and canarypox is their host restriction for replication to avian species. This is in contrast to the vaccinia virus, prototype *Othopoxvirus* , which has a broad vertebrate host-range (Esposito, 1991). As a family, poxviruses are a group of large, enveloped viruses that contain a linear double-stranded DNA genome (130-300 kb). These viruses have unique biochemical characteristices: they are the only DNA-containing viruses that replicate in the cytoplasm of infected cells. Poxviruses encode most of the functions necessary to replicate in host cells, including the enzymatic functions required for DNA replication and RNA metabolism (Moss, 1995). Based on the biological properties of the these viruses, several considerations for their development as eukaryotic expression vectors are relevant: (1) the use of vaccinia virus promoters is necessary to ensure gene expression because the virus encoded DNA-dependent RNA polymerase does not recognize exogenous promoters; (2) to date, splicing of poxvirus-derived mRNAs has not been documented; and (3) due to the cytoplasmic site of replication and the obligatory requirement for virion-associated enzymatic functions to initiate the replicative cycle, naked poxvirus DNA is non-infectious (Perkus *et al.*, 1995).

2. 2. Advantage of fowlpox virus

The fowlpox virus is suitable candidate as a vaccine vector. The virus has a safe and effective history as a vaccine strain for protection against the disease of fowlpox. The virus is extremly stable, not requiring a cold chain for storage, and is inexpensive to produce. The cytoplasmic expression, eliminating special requirements

for nuclear processing and transport of RNA, and relatively high expression levels are the others advantage of fowlpox virus. Also large quantities of foreign DNA can be stably integrated into the genome. Smith and Moss (1983) demonstrated that 25,000 bases of genetic information could be inserted into the vaccinia genome. This should be higher for avipox viruses as their genomes are 150% longer than vaccinia virus. The generation of viable deletion mutants provides still more space for packaging foreign DNA. This also suggests the possibility of elimiating viral DNA sequences that are not essential for viral replication but may lead to unnecessary virulance. The ability to incorporate large amounts of genetic information into avipox viruses provides two economic and practical advantages; (1) construction of multivalent vaccines expressing the appropriate antigens of many serotypes (2) ability to vaccinate against multiple diseases with one dose (Taylor & Paoletti, 1988).

2. 3. Limit of fowlpox virus

As the fowlpox virus replicates only in some avian cells like primary or secondery Chicken embryo skin (CES) cells or Chicken embryo fibroblasts (CEF) cells, the preparation of these kinds of cells is time consuming and needs specific equipments such as an egg incubator.

2. 4. Choice of promoter

The time and level of gene expression is dependent on the promoter chosen. An early poxvirus promoter will allow expression to occur before DNA replication, whereas expression will be delayed until after DNA replication if a late promoter is used. The strongest promoters belong to the late class and will generally provide higher levels of protein. The most extensively used promoter, p7.5, is a compound promoter with both early and late transcription start sites, thereby providing for continued expression throughout the growth cycle (Cochran *et al.*, 1985). The late p11 promoter (Bertholet *et al.*, 1985) usually provides higher levels of expression than p7.5. A bidirectional promoter element with early/late and late functions in opposite orientaion was describled for FPV by Kumar and Boyle (1990).

2. 5. Construction of transfer vector

To facilitate the construction of recombinant viruses, different transfer plasmid were developed (Nazerian & Dhawale, 1991; Letellier, 1993; Parks *et al.*, 1994; Elahi *et al.*, 1999*a*, Chapter III). These vectors contain a segment of fowlpox virus DNA (homologe with parental virus) within an expression cassette, consisting of a vaccinia virus promoter followed by one or more unique restriction endonuclease sites for gene insertion. In some constructs selection marker has been placed in an expression casette. The FPV DNA flanking the cassette serves to guide recombination to a nonessential site in the genome.

2. 6. Insertion site in the FPV genome

As previously mentioned, the foreign gene must be inserted into a nonessential site if the recombinant virus is to retain infectivity. Many such sites have been identified for vaccinia virus by sequence analysis of spontaneous deletion mutants or by making deletions or insertions (Kotwal & Moss, 1988; Perkus *et al.*, 1986). For FPV several nonessential sites were identified and used for insertion of foreign genes (Calvert *et al.*, 1993; Ogawa *et al.*, 1993). The most popular site is the *tk* gene (Elahi *et al.*, 1999, Chapter III; Letellier, 1993; Nazerian & Dhawale, 1991; Parks *et al.*, 1994).

2. 7. Generation of recombinant FPV (rFPV)

Protocols describing methods for generation of recombinant avipox virus have been published previously (Boyle & Coupar, 1988; Nazerian & Dhawale, 1991; Ogawa *et al.*, 1993; Parks *et al.*, 1994). In general, the CEF or CEF previously infected with FPV at a low multiplicity of infection (0.01-0.1) were transfected with transfer vector by using the electroporation or calcium phosphate precipitation methods. Two to three days later progeny FPV were recoverd. The products of recombination in vaccinia virus infected cells have been thoroughly investigated (Spyropoulos *et al.*, 1988) and it has been shown (Falkner & Moss, 1990) that the

most frequently generated initial recombinations occure as a result of a single cross-over event leading to the integration of the entire plasmid. The analysis of random recombinant events involving the *tk* region of FPV was demonstrated as with vaccinia virus, and the frequency of reciprocal exchange of plasmid and virus DNA mediated by a double cross-over is significantly lower then plasmid integration resulting from a single cross-over event (Nazerian & Dhawale, 1991). The single cross-over events between the insertion vector and viral DNA are unstable due to target sequence duplications, and frequently undergo secondary recombinational events resulting in a loss of the foreign gene in at least a portion of the viral population.

2. 8. Selection and screening of recombinant virus plaques

The frequency of generating FPV recombinants is low (0.01-0.1%, Boursnell *et al.*, 1990; Nazerian & Dhawale, 1991; Parks *et al.*, 1994) and several approaches have been utilized to aid the identification of rFPV. The first and still most popular selection method is negative *tk* selection. The plasmid insertion vector contains the FPV *tk* gene within which the expression casette has been placed. When recombination occurs, the foreign DNA will recombine into the *tk* locus and interrupt the *tk* gene. Recombinant FPV can then be distinguished from parental tk^+ virus by their tk^- phenotype. The basis for the selection is the lethal effect of incorporation of nucleoside analogs, such as 5-bromodeoxyuridine (BUdR), into the viral genome. Incorporation of the analog depends on its intracellular phosphorylation. For vaccinia virus the tk^- cell lines (e.g, tk^- 143) must be employed so that phosphorylation is dependent on the *tk* of vaccinia virus. Therefore, tk^- viurs will form BUdR-resistant plaques whereas tk^+ virus will not. Unfortunately the CES and CEF are not tk^- but it was shown that confluent CEF cells have 5 to 10 time less *tk* mRNA than the same cell in logarithmic phase (Groudine *et al.*, 1984). In the mixed population arising from the transfection, this would cause the selective amplification of the tk^+ virus. Disadvantages of this method, however, include requirement for (i) inactivation of the viral *tk* gene which attenuates virus infectivity (Buller *et al.*, 1985), (ii) use of special

agents, e.g., 5-bromodeoxyuridine. In addition, spontaneous *tk*$^-$ mutants arise at a high frequency (about 1:10,000). Therefore, it is necessary to screen the *tk*$^-$ plaques. This can be done by infecting mini-well monolayers of cells with virus from plaques and then performing dot blot hybridization with extracted total DNA (Hammermueller *et al.*, 1991).

Another approach for selection involves the cotransfer of a dominant selectable marker along with the desined foreign gene. This is accomplished by using FPV DNA to flank both the marker gene and the desired foreign gene. The *E. coli* xanthine-guanine phosphoribosyltransferase (*gpt*) have been employed for this purpose. In a *gpt* selection scheme, *de novo* purine synthesis is blocked by mycophenolic acid (MPA) and aminopterin. High concentrations of hypoxanthine are used to inhibit the conversion of guanine to guanosine monophosphate by the cellular enzyme hypoxanthine-guanine phosphoribosyltransferase. Xanthine, a precursor to guanosine monophosphate, is added as a substrate for *gpt*. Only cells expressiong *gpt* survive the inhibitors (Boyle & Coupar, 1988; Elahi, *et al.,* 1999*a*, Chapter III).

In the case of vaccinia virus, a modification of *gpt* gene seclection procedure termed "transient dominant selection" (Falkner & Moss, 1990) has been successfully employed. In this system, the *gpt* gene is in the same plasmid as the desired foreign gene but is not flanked by vaccinia virus DNA. Nevertheless, the *gpt* gene (as well as the remainder of the plasmid) will be transiently incorporated into the vaccinia virus genome as a result of a single crossover event and mycophenolic acid-resistent plaques can be isolated. Because the recombinant viruses are unstable, upon replaquing without selection, a mixture of wild-type and recombinant plaques are obtained. Thus, it is necessary to use either DNA hybridization or PCR to identify the recombinants. As a dominant selectable marker, the prokaryotic neomycine-resistance which provides resistance to G418 was also used (Franke *et al.*, 1985). Deletion and replacement of a vaccinia gene modulates its host range in cultured human cells. This finding was the basis for a host-range plaque selection system (Perkus *et al.*, 1989).

Several screening methods have been developed for picking recombinant virus plaques. One convenient method involves the cotransfer of the β-galactosidase gene with the desired foreign gene. Recombinant plaques turn blue upon staining with a chromogenic β-galactosidase substrate (Calvert *et al.*, 1993; Nazerian & Dhawale, 1991; Parks *et al.*, 1994). Insertion into the vaccinia hemagglutinin (HA) gene also provides a screening method since HA^+ plaques appear red upon addition of chicken erythrocytes whereas HA^- plaques do not (Shida *et al.*, 1987). Another screening method makes use of the observation that deletion of a gene encoding a 14 kDa protein leads to the generation of small plaques (Dallo *et al.*, 1987). By incorporation of the wild-type version of this gene into the insertion vector, recombinants can be distinguished by their large plaque size (Rodriguez & Esteban, 1989).

2. 9. Using of Avipoxviruses in veterinary vaccines

Avian poxviruses were initially considered as vectors for birds (Taylor *et al.*, 1988; Taylor *et al.*, 1990) since they do not replicate in mammals. Subsequent studies, however, indicated that expression of recombinant genes occurs in mammalian cells and that immune responses are induced in mammals (Taylor *et al.*, 1991; Taylor *et al.*, 1992*a*). Recombinant FPV and CPV are capable of protecting animals against the important veterinary diseases. A recombinants FPV expressing the rabies G protein protected mice, cat, and dogs from lethal rabies challenge. A similar CPV-based recombinant expressing the rabies G protein was approximately 100 times more efficacious than the FPV-based recombinant in protecting mice against a lethal challenge with rabies virus (Taylor *et al.*, 1991). The protective efficacy of the nonreplicating CPV-based rabies recombinants in mice was equivalent to the protective efficacy of a replication-competent, thymidine kinase-deficient vaccinia -based rabies recombinant (Tartaglia *et al.*, 1993; Taylor *et al.*, 1991). In the target species a single dose (5.0 $\log_{10}$ $TCID_{50}$) of the CPV-based rabies recombinant protected cats and dogs fully, despite low or undetectable antibody to rabies in some animals (Taylor *et al.*, 1991).

The efficacy of FPV-based recombinant expressing the F antigen of measles virus was investigated in mice (Wild *et al.*, 1992). A single intraperitoneal injection of an FPV recombinant expressing the measles virus F antigen protected mice from intracerebral challenge with measles virus. Also, dogs inoculated with CPV-based recombinants expressing measles virus with hemagglutinin and F antigens were protected from lethal canine distemper virus challenge (Taylor *et al.*, 1992*a*).

The CPV-based recombinant expressing the different proteins of Japanese encephalitis virus (Konishi *et al.*, 1992), feline leukemia virus (Tartaglia *et al.*, 1993) and equine influenza virus (Taylor *et al.*, 1992*b*) were constructed. These recombinant CPVs could protect completely or partially the susceptible host after challenge with the appropriate virus.

3. RECOMBINANT ADENOVIRUS VECTORS

3. 1. Molecular biology of adenoviruses

Adenoviruses belong to the *Adenoviridae* family and can be isolated from organisms varying from mammals (*Mastadenovirus* genus) to birds (*Aviadenovirus* genus). Different serotypes of adenoviruses have been isolated from humans and have been grouped into six subgenera (A-F) according to their structural, biological (oncogenic potential) and immunological characteristics (Ginsberg *et al.*, 1966; Shenk, 1995). The human adenoviruses, particulary serotypes 2 and 5 (subgenus C), 7 (subgenus B), and 12 (subgenus A), have been extensively characterized. Types 2 and 5, especially, have served as valuable tools in the study of the molecular biology of DNA replication, transcription, RNA processing, and protein synthesis in mammalian cells (see: Acsadi *et al.*, 1995; Ginsberg & Prince, 1994; Graham & Prevec, 1995, for a general review in molecular biology of adenoviruses).

The nonenveloped adenovirus (Ad) is about 140 nm in diameter and consists of capsid proteins (252 capsomers, 240 of which are hexons and 12 of which are pentons) and a nucleoprotein core with one cpoy of a double-stranded, linear DNA molecule of 36 kb, conventionally divided into 100 map unit (mu) (Graham & Prevec, 1992). Each DNA strand has inverted terminal repeats of 100-140 bp in length, depending on the serotype. These inverted terminal repeats are necessary for viral replication (Hay, 1985).

The replication cycle of the virus can be divided into two phases: early, corresponsding to events occurring before the onset of viral DNA replication; and late, corresponding to the period after initiation of DNA replication. There are six early (E) regions, E1A (1.3-4.5 mu), E1B (4.6-11.2), E2A (67.9-61.5 mu), E2B (29-14.2 mu), E3 (76.6-86.2 mu) and E4 (96.8-91.3 mu), that are transcribed from individual promoters (except for the E2A-E2B regions) during the early phase before DNA synthesis begins (Graham & Prevec, 1992). Transcription of E1A strarts shortly after infection and is followed by sequential activation of the E1B, E3, E4 and E2

regions. E1A encoded proteins are involved in transactivation of the majority of other viral genes. The E1B products play a role in viral DNA replication and in viral and cellular mRNA metabolism and protein synthesis late in infection. E1 is not required for viral replication in human 293 cells, a human embryonic kidney cell line that is transformed by Ad type 5 DNA and thereby constitutively express the E1A and E1B gene products (Ghosh-Choudhury, *et al.*, 1987). The E2 region encodes three major proteins involved in DNA replication. E3, though not required for viral replication in cultured cells, probably plays a role in modulating the host immune reponse to virus infections *in vivo*. One of the E3 gene products, a 14.7 kDa polypeptide, inhibits cytolysis of the infected cells by tumor necrosis factor (Gooding, *et al.*, 1988). Another gene product, a 19 kDa glycoprotein, gp19k, has been shown to bind to major histocompatibility complex (MCH) class I antigens and prevent their transport to the surface of virus-infected cells. Since cytotoxic T-lymphocyte (CTL) lysis of Ad-infected cells depends on recognition of viral peptides presented on the cell surface as complexes with MHC class I antigen, the effect of E3 gp19k synthesis is to interfere with CTL lysis (Rawle *et al.*, 1989). E4 region encodes several proteins which generally seem to function in viral DNA replication and in enhancing the efficiency of late viral gene expression by inhibiting the transport of cellular mRNA (Herisse *et al.*, 1981). During the late phase, early promoters become less active as the majority of the transcription originates at the major late promoter (MLP), generating 5 groups of polycistronic transcripts that are processed by alternative splicing pathways resulting in different mRNA species with an identical 5′ untranslated leader sequence, the tripartite leader (for general review please see Acsadi *et al.*, 1995; Graham & Prevec, 1992).

3. 2. Available sites for construction of recombinant adenoviruses

Three regions of the viral genome, E1, E3 and upstream of E4, can be used for foreign DNA cloning. Adenoviruses are able to package efficiently a maximum of 105% of their total genome length (Byers, *et al.*, 1991). This upper limit allows for approximately 2 kbp of foreign DNA without prior deletions. To incorporate larger DNA segments, it is necessary to compensate by deleting appropriate amounts of viral DNA. One of the most useful deletions is made by collapsing the two naturally occurring *Xba* I sites within E3 to remove 1.9 kb of viral DNA. This results in replicative vectors (helper-independent vector) having a capacity of about 4 kb of foreign DNA without impairing viral growth (Acsadi *et al.*, 1995; Graham & Prevec, 1992).

The E1 gene products are not required for viral replication in human 293 cell. Deletion of up to 3.2 kbp can be made in E1 without compromising the ability of the virus to grow in 293 cells (Grahame *et al.*, 1977). Combined deletions in E1 and E3 should allow insertion of almost 8 kbp of foreign DNA into vectors that can be replicated in a helper-independent fashion in 293 cells.

The third region of Ad that may also be used for cloning is located at the right end of the viral genome in the E4 region. Insertions of foreign DNA between the begining of the E4 transcription start site and the right invert terminal repeat have no apparent compromising effect on viral viability. E4 deleted or E3 and E4 double deleted vector can also be used (Weinberg & Ketner, 1983; Chanda *et al.*, 1990).

For cloning fragments that exceed the capacity of the helper-independent vectors we can use helper-dependent vectors in which additional regions of the viral DNA were removed. As a consequence, all these vectors are defective for growth, and they must be propagated with an intact adenovirus as a helper to provide essential functions. However, the utilization of helper-dependent vectors as vaccines is not suitable because the composition of mixtures of helper and vector is variable, because

of the ability of the helper/vector combinations to be expressed efficiently and because reproducibly *in vivo* is uncertain (Graham & Prevec, 1992).

The most common technique for introducing functional DNA sequences into the adenovirus genome is *in vivo* homologous recombination. Recombination was performed between shuttle a plasmid, containing the gene of interest flanked by adenovirus (Ad) E1 or E3 sequences, and the restricted viral DNA of the appropriate parental adenovirus (with deletion in E1 or E3 or both), after cotranfection into 293 cells by the calcium phosphate coprecipitation method (Graham & Van der Eb, 1973). Cleavage of viral DNA reduces the infectivity of the parental viral DNA and enhances the efficiency of isolation of recombinant vectors resulting from *in vivo* recombination. *In vitro* ligation to reconstitute a complete viral DNA molecule prior to cotransfection is also an option (Haj Ahmad & Graham, 1986).

3. 3. Advantages of adenovirus vactors

3.3.1. Safety of Ad vectors-Adenoviruses (Ads) are a common cause of upper respiratory disease in humans. Ad infection are normally without significant or severe clinical symptoms. Nevertheless, some Ad serotypes can be the causative agents of mild or severe respiratory diseases. Since 1969, the U.S. military has administered an oral vaccine consisting of live enteric-coated capsules containing unattenuated Ad4 and Ad7 in an attempt to prevent Ad4- and Ad7-induced respiratory diseases (Top *et al.*, 1971). To date, no significant side effects or illnesses related to the use of these vaccines have been reported. No significant infant mortality has been attributed to Ad5, despite its widespread distribution. Thus, Ad5 would be an efficient and safe vector even in unattenuated form. The Ad genome rarely integrates into its host DNA, but rather persists extrachromosomally. This minimizes the risks of insertional oncogenesis and cellular gene activation. Nevertheless, the number of copies of viral genome were gradual decreased during cell division. Adenovirus can infect postmitotic cells. In post-mitotic cells such as myofibres and neurons long-term expression is observed (Jewtoukoff & Perricaudet, 1995).

3.3.2. Ad manipulation, administration and immune response induction- There are several major advantages of adenovirus vectors making it a particularly attractive cloning vehicle. Ad can be produced in 293 cells at high titer (10^{12}-10^{13} virus particles per ml). Ad is stable and is easy to manipulate. Purified Ad may by kept for many years at -80°C. Furthermore, lyophylized Ad preparations do not require refrigeration. Ad can easily be administrated by oral, intranasal, intratracheal, intraperitoneal, intravenous, subcutaneous, or intramuscular, routes. Consequently, Ad based live vaccines are able to induce not only a systemic immunity, but also a good mucosal response (Acsadi *et al.*, 1995; Graham & Prevec, 1992).

3.3.3. Efficiency of Ad expression vectors- The helper-dependent vectors require a complementing wild type Ad to overcome their replication defect. Therefore, the Ad helper-dependent system is suitable for large DNA cloning, but not for vaccine use because of the requirement for the helper/vector mixture. Furthermore, expression for a helper-dependent vector is less efficient than from a helper-independent vector. For helper-independent Ad with deletions in E1 (AdE1-) or E4 (AdE4-) a transcomplementing cell line (293 for E1 deleted and W162 for E4 deleted viruses) are required. The AdE1- or AdE1-E3- do not replicate in other cells except 293, however the early expression of antigen within the infected cells of the animal is sufficient to establish the humoral and cellular immune responses in these animal (different examples will be given later in this text). Non-defective Ad (with a deletion in E3 region) induce the protection even when low doses of viruses are used. Nevertheless, the biosafety of such constructs is questionable, as the recombinant virus can be excreted into the environment (Oualikene *et al.*, 1994).

3. 4. Limit of Ad vectors

Some of the major limitation of the adenovirus vector system for use as vaccines and also in gene therapy are vector induced inflammation, the transient expression of transgenes, and the development of Ad specific neutralizing antibodies, which hinder repeat administration of the vector.

Adenovirus induced inflammatory response is characterized by the infiltration of inflammatory cells and the local release of TNF-α, and interleukin (IL)-1β, IL-6 and IL-8, and precedes the cytotoxic T lymphocyte (CTL) response (Ginsberg *et al.*, 1989; Wilmott *et al.*, 1996). The decline in transgene expression is due in part to the activation of adenovirus- and transgene-specific $CD8^+$ CTLs. These cells perform the major effector function and limit transgene expression in a major histocompatibility complex (CMH) class I restricted fashion (Yang *et al.*, 1994). Activation of $CD4^+$ lymphocytes by adenovirus capsid proteins leads to the upregulation of MHC class I molecules on the target cells and thus contributes to the clearing of adenovirus infected cells by CTLs (Yang, *et al.*, 1995). In addition, $CD4^+$ cell activation is required for the production of neutralizing antibodies by B cells, which block repeat administration of adenovirus vector (Yang, *et al.*, 1996*a*). Because neutralizing antibodies directed against one adenovirus serotype do not block infection by another adenovirus, one approach to achieving successful repeat administration is to alternate the serotype of Ad vector used for booster vaccination or gene therapy. Use the non-replicating adenovirus has the advantage of a reduced immune response against the capsid protein, allowing multiple immunizations against the desired antigen (Mastrangeli, *et al.*, 1996). The other problem is that of potential oncogenicity and pathogenicity of some Ad (Ad4, 7, 11, 21, 37) especially in infants and immunodeficient subjects (Graham, 1984).

3. 5. Adenovirus vectors as vaccines

Efficacy of the recombinant Ad for induction of humoral or cellular immune responses and also protection against many viral diseases has already been investigated. The following viral genes were already expressed by adenovirus for vaccination proposes: the surface antigen (HBsAg) of the Hepatitis B virus (Chengalvala *et al.*, 1991; Chengalvala *et al.*, 1997); gB of Herpes Simplex virus (Hanke *et al.*, 1991; McDermott *et al.*, 1989); rabies glycoprotein; fusion (F) and the attachement (G) glycoprotein of Respiratory Syncytial Viurs (Hsu *et al.*, 1994),

Rotavirus VP7sc (Both *et al.*, 1993), spike, nucleoprotein and membrane protein of Murine Hepatitis Virus (Wesseling *et al.*, 1993); glycoprotein of Vesicular stomatitis Virus (Prevec *et al.*, 1989; Yoshida *et al.*, 1997), nucleocapsid of Measles virus (Fooks *et al.*, 1995) non-structural protein of Tick-Borne Encephalitis Virus (Jacobs *et al.*, 1992; Jacobs *et al.*, 1994); and envelope protein of Human Immunodeficiency Virus (HIV) (Dewar *et al.*, 1989; Lubeck *et al.*, 1997), glycoprotein of rabies virus (Charlton *et al.*, 1992; Prevec *et al.*, 1990) are among the recombinant adenoviruses already studied.

3. 6. Adenovirus vectors for gene therapy

The objective of gene therapy is to deliver a functional gene to the tissues where the respective gene activity is missing or defective. While the number of human diseases (genetic or deficiencies) that potentially can be treated in such a manner is large, the common point is the need for a vector to efficiently deliver the respective therapeutic gene to the affected tissues and/or organs. There are a variety of vectors currently being studied for potential use *in vivo*. Recombinant adenovirus can be used as an efficient delivery system in a broad range of host cells. Because this aspect of adenoviruses is not the subject of this thesis, for more information about the utilization of adenovirus in gene therapy please see the following recent reviews: Chang & Leiden, 1996; Davidson & Bohn, 1997; Eisensmith & Woo, 1996; Hermens & Verhaagen, 1998; Horellou & Mallet, 1997; Kiwaki & Matsuda, 1996; Kovesdi *et al.*, 1997; Pasi, 1996; Petrof, 1998; Southern, 1996; Walter & High, 1997.

3. 7. Different promoters for the expression of target genes

Several different promoters can be used for the expression of foreign genes, such as: the cytomegalovirus (CMV) immediate-early (IE) promoter (Massie *et al.*, 1998*a*; Xu *et al.*, 1995), the major late promoter (MLP) (Massie *et al.*, 1995; Xu *et al.*, 1995), the SV40 early and late promoters and the β-actin promoter (Xu *et al.*,

1995). However, the CMV and MLP are the most commonly used because they are stronger than the others. Recently, to add more control to the system and also to avoid any interference between the recombinant protein and adenovirus replication, inducible promoters have been used to regulate the expression of the transgenes. The tetracycline-controllable transactivator system is becoming the most widely used in mammalian cells in cultures (Gossen *et al.*, 1995; Gossen & Bujard, 1992; Mosser *et al.*, 1997; Massie *et al.*, 1998*a*; Massie *et al.*, 1998*b*) and in transgenic mice (Schultze *et al.*, 1996). This system makes use of a *trans*-acting factor (tTA) formed by the fusion of the activation domain of herpes simplex virus (HSV) protein VP16 to the *Escherichia coli* tetracycline repressor protein (Gossen & Bujard, 1992). In this system, the expression of a target gene, placed under the control of a promoter containing the tetracycline operator sequence (*tetO*), can be induced by the tetracycline-regulated trans-activator protein (tTA). The tTA protein can be supplied by using the 293-tTA cell line (a stable 293 cell which constitutively expresses the tTA protein, Massie *et al.*, 1998*a*) or by co-infection with a recombinant virus such as Ad5CMV-tTA (Massie *et al.*, 1998*a*). The transcription of the tTA protein can be prevented by adding tetracycline at a concentration that is not toxic for eukaryotic cells (Gossen, *et al.*, 1994). A modified tTA (rtTA) which interacts with *tetO* only when certain tetracycline analogs are present has also been developed (Gossen *et al.*, 1995).

CHAPTER II

Antigenic variation among bovine viral diarrhoea virus (BVDV) strains and the role of different cell fixation methods in immunoassays

S. M. Elahi[1], S. Harpin[1], E. Cornaglia[1], B. Talbot[2], Y. Elazhary[1]*

Can J Vet Res 1997, 61: 34-38

[1]Virology Section, Faculty of Veterinary Medicine, University of Montreal, P. O. Box 5000, Saint-Hyacinthe, Que. J2S 7C6 (Canada).

[2]Department of Biology, Faculty of Sciences, University of Sherbrooke, Que. J1K 2R1 (Canada)

*Author to whom correspondence should be addressed.

Phone: (450) 773-8521 ext. 8201

Fax: (450) 778-8106

E-mail: elazhary@medvet.umontreal.ca

ABSTRACT

Antigenic variation among 13 Quebec isolates of bovine viral diarrhoea virus (BVDV), four reference strains and two American isolates were studied by peroxidase-linked antibody assay (PLA assay) and neutralization test (NT). The Quebec strains consisted of three isolates before 1993 and ten isolates from 1993. In the PLA assay, we compared two different fixatives, acetone and formalin. Acetone-fixation allowed us to identify six groups from amongst the viruses tested. All the Quebec isolates were different from the reference strains. In addition, antigenic variation was detected between Quebec isolates obtained before and during 1993. However, PLA assays performed after formalin fixation did not detect these antigenic variations. Neutralisation tests were carried out with two polyclonal antibodies (PAb) and six monoclonal antibodies (MAb). They were used to classify BVDV strains and isolates into four groups and seven subgroups respectively. In conclusion, we demonstrated that the BVDV isolates from the 1993 outbreak in Quebec are antigenically different from reference strains and from isolates existing in Quebec before 1993. In addition, we have shown that two internationally used fixation-methods in PLA assay give different results. The usefulness of each method is discussed.

Key words: Bovine viral diarrhoea virus, acetone, formalin, fixation, antigenic variation.

INTRODUCTION

Bovine viral diarrhoea virus (BVDV) is an enveloped positive strand RNA virus (1). It is currently classified as a member of the genus Pestivirus in the Flaviviridae family (2). Both cytopathic (cp-BVD) and non-cytopathic (ncp-BVDV) strains of BVDV have been identified in tissue culture (3). BVDV infection in cattle can range from clinically inapparent to fatal mucosal disease (4). Recently, in Quebec, the disease has manifested with lymphopenia, thrombocytopenia, high morbidity and mortality (31.5% for grain-fed calves and 17.1% for milk-fed calves) (5) and occasionally severe haemorrhage.

There appears to be considerable antigenic variation among BVDV isolates and strains. Xue et al.(6) classified 40 cp and ncp BVDV isolates into six groups by neutralization test (NT) using 5 MAbs specific for gp48 or gp53. In a recent study, Pellerin et al. (5) classified 15 Canadian isolates into two groups by NT using six polyclonal antibodies (PAb) and analysis of the sequences of the 5` untranslated regions.

The detection of viral antigens is usually carried out by peroxidase-linked antibody assay (PLA assay), or by immunofluorescence (IF). The most common substances used for cell fixation are acetone (7-9), or formalin (10-11, and Shannon et al., personal communication, 1994). Yet nothing has been reported on the comparison of these two compounds for detection of BVDV.

The present work describes the antigenic variation of Quebec isolates obtained before and during the 1993 outbreak and at the same time identifies the potentially confusing effects of acetone and formalin as fixatives on the PLA assay.

MATERIAL AND METHODS

Viruses and cells

Cytopathic BVDV , NADL, Singer and C24V-Oregon strains and the non-cytopathic New York-1 (NY-1) strain were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland). In this study, 13 field isolates were isolated in our laboratory from different geographic regions. Three field isolates from before the 1993 outbreak in Quebec and 10 field isolates during the 1993 outbreak were used. The field isolates 93-1, 93-3, 93-61 and 93-619 were isolated from calves with haemorrhagic syndrome. All these calves (ranging from two days to three months old) presented different degree of watery and sometimes bloody diarrhoea, respiratory disorder, internal and external hemorrhages, leukopenia, anemia, and thrombocytopenia. In the infected herd at least 30% of animals manifested clinical signs. All the isolates were cloned by three cycles of plaque purification. Two isolates (87-2552 and 86-7061) were obtained from Dr. H. C. Minocha (Kansas State University, Manhattan, Kansas, 66506).

Madin-Darby Bovine Kidney (MDBK) cells free of BVDV (ATCC) were grown in Earle's minimum essential medium (MEM, Gibco Canada Inc.), supplemented with 2.2 gm/L sodium bicarbonate, 10% of fetal bovine serum (FBS) (Gibco, Grand Island, New York) and antibiotics. All viruses were propagated in MDBK cells in the presence of 2% FBS.

Polyclonal and monoclonal antibodies

Specific bovine BVDV polyclonal antisera (PAb-1) were raised in calves by immunization with NADL and Singer strains. The animals seronegative for BVDV, infectious bovine rhinotracheitis virus, parainfluenza virus a and respiratory syncytial virus were kept individually in negative pressure rooms. The BVD strains NADL and Singer were propagated in MDBK cells and purified by ulteracentrifugation over a sucrose cushion. The virus was inactivated and mixed

with alhydrogel and Quil A as discribed by Bikour et al. (1994) (12). Three mL of inactivated BVDV (10 $^{6.5}$ TCID $_{50}$ /mL) were injected intramuscularly at three different sites. The calves were immunized with the same quantity of antigen at 3 and 5 weeks. Animals with a low antibody titer received another dose at 7 weeks. Two weeks after the last injection the calves were bled. In an indirect IF assay using the homologous viral strains this polyclonal antibody had a IF titre of 1: 1280. Polyclonal antisera (PAb-2) with IF titer of 1: 1280 were collected from clinical cases of BVD during the 1993 outbreak in Quebec.

MAbs supernatant fluid; 2NB2, P1H11, P4G11, P4A11, P3C12, P1D8, 1NB5, N2B12, P3D10, P3F6, O2A1, 40B4 and N4B4 were obtained from Dr. A. D. Shannon (Elizabeth Macarthur Agricultural Institute, Camden, New South Wales, Australia). These antibodies were produced against a total of five BVDV isolates, including the reference strain Oregon-C24V, two cp and two ncp New South Wales isolates. Ascites fluid of the MAbs D89, C17, E15, F15, (NADL-specific), D11 and B7 (field isolate 87-2552-specific) were obtained from Dr. H.C. Minocha. Characterization of MAbs is presented in Table I.

Peroxidase-linked antibody assay (PLA assay)

The MDBK cells were grown in 96 well tissue culture plates and were infected with each of the BVDV strains or isolates. After 3-4 days, two different fixation methods were used in order to compare the results. In the first method, the plates were washed with PBS-Tween (0.05% Tween 20, Sigma, Mississauga, Ont), fixed by the addition of 100 uL/well of acetone (20%) for 15 min, and dried at 30 C for 3-4 h. In the second method, the plates were fixed, without removing the medium, with 100 uL of formalin (5.5% formaldehyde) . The plates were then washed with PBS-Tween and dried at room temperature (RT). Both sets were blocked with 100 uL/well of 5% skim-milk powder in PBS and incubated for 30 min at 37 C. Washing steps throughout the test were performed with PBS-Tween

(0.05% Tween 20) and the plates were dried at RT. The plates were incubated with 100 uL of each MAb for 90 min at 37 C. Ascites fluids were diluted 1/100, the hybridoma supernatants were not diluted. Each MAb was tested in triplicate. For the detection of bound MAbs, 100 uL/well of Goat Anti-Mouse IgG (H+L) HRP conjugate (BIO-RAD, Mississauga, Ont.) containing 1% gelatin, were added and incubated for 90 min at RT. The substrates were 3-amino-9-ethylcarbazole (Sigma, Mississauga, Ont.) and hydrogen peroxide in 0.05 M acetate buffer (pH 5). After 15 min incubation, the plates were read by microscopic examination. All the negative results were checked twice.

Neutralization test (NT)

NT was performed in 96 well tissue culture plate. The MAbs D89, C17, E15, F15, D11 and B7 and 2 PAbs (PAb-1 and PAb-2) were diluted 1/125 followed by serial fourfold dilution. Fifty uL of each antibody was incubated for 1 h at 37 C with 100 uL of virus suspension containing 100 $TCID_{50}$. Then, 100 uL of medium containing 3 x 10^4 MDBK cells was dispensed per well. Each antibody dilution was tested in quadruplicate. The plates were kept at 37 C in 5% CO_2 for 4 days. The highest dilution that inhibited the cytopathic effect in at least 50% of the cultures was considered as the virus neutralization titer for each antibody. For testing the noncytopathic virus, after four days the plates were stained by PLA assay, as described above, except that PAb-1 (1/50 in PBS-Tween 0.1%) and rabbit anti-bovine IgG HRP-conjugate were used. The neutralization titer of the antibodies against ncp virus was considered as the highest dilution that inhibited the virus detection by PLA assay in at least 50% of the culture.

RESULTS

Peroxidase-linked antibody assay (PLA assay)

The binding of the 19 MAbs to 19 different BVDV strains and isolates was determined by a PLA assay to ascertain the pattern and extent of antigenic variation among viruses. At the same time, two different fixation methods were compared. Using the acetone fixation, BVDV strains and isolates could be divided into six groups (Table II). All the Quebec BVDV isolated before and the during 1993 outbreak were classified into two distinct groups. NADL as NY-1 strain showed a private MAb reactivity pattern. C24V-Oregon strain and 87-2552 isolates were the only viruses to react with MAbs O2A1 and 40B4. Nevertheless, when the same BVDV preparations were fixed with formalin, different results were observed (Table II). Only the cytopathic strain C24V-Oregon and American isolate 87-2552 reacted with all of the MAbs used. The rest of the viruses showed the same reactivity pattern, they reacted with all of the MAbs except O2A1 and 40B4.

Neutralization test (NT)

Separate neutralization experiments were carried out to test PAb and MAb against 19 BVDV strains and isolates. A neutralization titer of 8000 or more was considered as a "strong reaction" and a neutralization titer of 2000 or less as a "weak reaction". Based on cross-reactions with two PAbs, the BVDV strains and isolates were classified into four groups (Table III). Group I included all strains and isolates that reacted strongly with PAb-1 and weakly with PAb-2. Group II included those strains which manifested a strong reaction with both PAbs. Group III included strains or isolates that had weak reactions with PAb-1 and reacted strongly with PAb-2. Finally, Group IV is was represented by Quebec isolates ; 93-1, 93-3, and 93-619 and one US isolate (87-2552) which reacted weakly with

both PAbs. In a second series of experiments based on the use of MAbs, group I and group IV BVDV described above could be subdivided into three and two groups, respectively (Table III). The viruses in group Ia were neutralized by MAbs D89, C17, E15 and F15. In group Ib, viruses were not neutralized by any MAb. In Ic and IVa groups, viruses were neutralized by MAbs D89 and F15. All viruses in groups Ic, II, III, and IVa (66.7%) had the same pattern of reactivity with the MAbs. Group IVb represents one isolate, 87-2552, which only reacted with MAbs D11 and B7. None of the MAbs neutralized all the viruses at the dilutions used in this study. This result confirmed the existence of widespread antigenic variation among BVDV.

DISCUSSION

The PLA assay is widely used for the detection or classification of BVDV. The cell fixation methods commonly used employed either acetone or formalin (7-9, Shannon et al. personal communication, 1994). In our study, the PLA assay showed considerable differences depending on which of these two common fixatives were used. The acetone-fixation method allowed us to classify BVDV isolates into separate groups from before and during the 1993 Quebec outbreak. When formalin fixation was used, all of the MAbs except O2A1 and 40B4 reacted with all of the BVDV. This indicates a high degree of conservation of the three proteins p80, gp53 and gp48, at least for MAbs used in this study but a lower degree of conformational conservation than was previously envisaged. In our assays, the acetone must have affected some of the conformational epitopes that we were attempting to identify. Seven MAbs lost their binding with some or all of BVDV. This may be attributed to the conformation-dependant nature of some BVD viral epitopes (13) which renders them sensitive to the denaturing effect of fixation. However, this effect of acetone-fixation allowed us to demonstrate previously undetected differences between the viruses. Using the formalin-fixation method, the results indicate that all strains and isolates have the same epitopes.

This property allowed us to tentatively classify BVDV on the basis of their stability to acetone-fixation. Our formalin-fixation result in PLA assay confirmed a report by Shannon et al. (personal communication, 1994) who found that the same MAbs detected 95 to 100% of 115 strains even though two MAbs, O2A1 and 40B4, were specific for C24V-Oregon. It is known that cellular structure is better preserved by formalin than by acetone fixation (14). Boecke et al., (1994) and Perez et al, (1995) compared two fixation methods using formaldehyde or acetone for quantitative and qualitative cytomegalovirus antigenemia assay respectively. They showed that formaldehyde fixation is superior to acetone fixation.

The use of NT with two PAbs allowed us to classify strains and isolates into four groups. In addition, these strains and isolates were classified into seven subgroups using six MAbs. Of these groups, group IV, (which reacted weakly with both PAbs) is interesting because it is composed of three out of the four viruses that were isolated from animals with haemorrhagic syndrome (isolates 93-1, 93-3 and 93-619). This result confirms the finding of Pellerin et al. (5) who classified two haemorrhagic syndrome associated isolates into a different group from classical BVDV. In the same way, Ridpath et al. (15) demonstrated that all the 32 BVDV isolated from haemorrhagic syndrome cases belong to a different group from the reference strains. This study was based on the sequences of the 5′ untranslated region and the DNA region which codes for the viral polypeptide p125 kDa, However, our isolate 93-61, which was isolated from a calf with haemorrhagic syndrome was classified in group I along with all the reference strains. This result is difficult to explain unless the original isolate come from a mixed-infection from which one of the viruses disappeared during passage in the cells.

It is difficult to establish a correlation between PLA assay and NT results. However, if we evaluate the results of the PLA assay (acetone fixation) and NT

obtained with the six common MAbs used in both techniques, all the Quebec isolates, with exception of 89-E770 isolate (in table III), form one group. This group is different from the reference strains.

Radioimmunoprecipitation assay (RIPA) was also performed on seven reference strains as well Quebec isolates. No difference between molecular weight of viral polypeptides could be detected (data not shown).

In conclusion, our results show that observations obtained after acetone or formalin fixation may be used for different purposes. We propose that formalin fixation should be used for detection of BVDV and that acetone fixation be used for the study of the BVDV antigenic variation. In addition to these results, significant antigenic differences among isolates, associated or not with haemorrhagic syndrome, was demonstrated by two PAbs used in this study, and the MAbs used in neutralisation tests were not able to demonstrate this variation. Production of MAbs against isolates from animals with a history of haemorrhagic syndrome would be very useful to identify the changed epitopes. We can also conclude that both PLA assay and NT can be used separately for the analysis of antigenic variation.

REFERENCES

1. HERMODSSON, S. and DINTER, Z. Properties of the bovine virus diarrhea virus. Nature 1962; 19: 893-894.

2. FRANCKI, R. I., FAUQUET, C. M., KNUDSON, D. L. and BROWN, F. Classification and Nomenclature of Viruses. Fifth Report of the International Committee on the Taxonomy of Viruses. Arch Virol 1991; 2(Suppl.): 228-229.

3. LEE, K. M. and GILLESPIE, J. H. Propagation of virus diarrhea virus of cattle in tissue culture. Am J Vet Res 1957; 18: 952-953.

4. BOLIN, S. R. Immunogens of bovine viral diarrhea virus. Vet Microbiol 1993; 37: 263-271.

5. PELLERIN, C., VAN DEN HURK, J., LECOMTE, J. and TUSSEN, P. Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology 1994; 203: 260-268.

6. XUE, W., BLECHA, F. and MINOCHA, H. C. Antigenic variations in bovine viral diarrhea viruses detected by monoclonal antibodies. J Clin Microbiol. 1990; 28: 1688-1693.

7. AFSHAR, A., DULAC, G. C. and BOUFFARD, A. Application of peroxidase labelled antibody assay for detection of porcine IgG antibodies to hog cholera and bovine viral diarrhea viruses. J Virol Methods 1989; 23: 253-262.

8. CAY, B., CHAPPUIS, G., COULIBALY, C., DINTER, Z., EDWARDS, S., GREISER-WILKE, I., GUNN, M., HAVE, P., HESS, G., JUNTTI, N., LIESS, B., MATEO., A., MCHUGH, P., MOENNIG, V., NETTLETON, P. and WENSVOORT, G. Comparative analysis of monoclonal antibodies against

pestiviruses: Report of an international workshop. Vet Microbiol 1989; 20: 123-129.

9. EDWARDS, S., MOENNIG, V. and WENSVOORT, G. The development of an international reference panel of monoclonal antibodies for the differentiation of hog cholera virus form other pestiviruses. Vet Microbiol 1991; 29: 101-108.

10. BOECKH, M., WOOGERD, P. M., STEVEN-AYERS, T., RAY, C. G. and BOWDEN R. A. Factors influencing detection of quantitative cytomegalovirus antigenemia. J Clin Microbiol 1994; 32: 832-834.

11. PEREZ, J.,L., DE ONA, M., NIUBO, J., VILLAR, H., MELON, S., GARCIA, A. and MARTIN, R. Comparison of several fixation methods for cytomegalovirus antigenemia assay. J Clin Microbiol 1995; 33: 1646-49.

12. BIKOUR, M., H., CORNAGLIA, E. and ELAZHARY, Y. Comparative study of immunostimulatory properties of different adjuvants administered with an inactivated influenza virus vaccine and evaluation of passive immunity in pigs. Immunol Infect Dis 1994; 4: 166-172.

13. MOENNIG, V. Pestiviruses: a review . Vet Microbiol 1990; 23: 35-54.

14. JUNQUEIRA, L. C., CARNEIRO, J., KELLEY, R. O. (Editors). Basic Histology, 6. 1989 Appleton & Lange,pp. 1.

15. RIDPATH, J., F., BOLIN, S., R. and DUBOVI, E., J. Segregation of bovine viral diarrhea virus into genotypes. Virology 1994; 205: 66-74.

TABLE I. Characterization of monoclonal antibodies

MAbs	subisotype	specificity*	differenciation#
2NB2	IgG2a	p125/80	Group specific
P1H11	IgG2b	p125/80	Group specific
P4G11	IgG2b	p125/80	Group specific
P4A11	IgG2b	p125/80	Group specific
P3C12	IgG2b	p125/80	Group specific
P1D8	IgG1	p125/80	Bovine specific
1NB5	IgG2a	gp53	Bovine specific
N2B12	IgG2a	gp53	Bovine specific
P3D10	IgG2b	gp53	Ruminant specific
P3F6	IgG2a	gp53	Ruminant specific
02A1	IgG2a	gp53	C24V-Oregon specific
40B4	IgG2a	gp53	C24V-Oregon specific
D89	IgG2a	gp53	ND
C17	IgG1	gp53	ND
E15	IgG2a	gp53	ND
N4B4	IgG2b	gp48	BVD+ CSFV[a] specific
F15	IgG3	gp48	ND
D11	IgG1	gp48	ND
B7	IgG3	gp48	ND

*; Detected by immunoprecipitation

#; Capacity of differentiation among pestivirus

ND; Not done

CSFV[a]; Classical swine fever virus or hog cholera virus

Characterization of MAbs; D89, C17, E15, D11, B7 and F15 was done by Xue et al. (6) and the rest of MAbs by Shannon et al. (1994, personal communication).

TABLE II. BVDV antigenic classification and Comparison of two fixation methods in peroxidase-Linked antibody assay: acetone and formalin.

		monoclonal antibodies																		
		p 125/80 kDa					gp 53 kDa										gp 48 kDa			
Group*	Virus	2NB2	P1H11	P4G11	P4A11	P3C12	P1D8	1NB5	N2B12	P3D10	P3F6	O2A1	40B4	D89	C17	E15	N4B4	F15	D11	B7
1	NADL	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	+
2	C24V-Oregon	-/+	+	+	+	+	-/+	+	+	+	-/+	+	+	+	+	+	-/+	+	+	+
	87-2552	-/+	+	+	+	+	-/+	+	+	+	-/+	+	+	+	+	+	-/+	+	+	+
3	NY-1	+	+	+	+	+	+	-/+	-/+	+	-/+	-	-	+	+	+	-/+	+	+	+
4	Singer	-/+	+	+	+	+	-/+	+	+	+	-/+	-	-	+	+	+	-/+	+	+	+
	86-7061	-/+	+	+	+	+	-/+	+	+	+	-/+	-	-	+	+	+	-/+	+	+	+
5†	88-88	-/+	+	+	+	+	-/+	-/+	-/+	+	-/+	-	-	+	+	+	-/+	-/+	-/+	+
	89-E770	-/+	+	+	+	+	-/+	-/+	-/+	+	-/+	-	-	+	+	+	-/+	-/+	-/+	+
	91-Domar	-/+	+	+	+	+	-/+	-/+	-/+	+	-/+	-	-	+	+	+	-/+	-/+	-/+	+
6‡	93-1	+	+	+	+	+	-/+	+	+	+	-/+	-	-	+	+	+	-/+	-/+	-/+	+
	93-3	+	+	+	+	+	-/+	+	+	+	-/+	-	-	+	+	+	-/+	-/+	-/+	+
	93-8	+	+	+	+	+	-/+	+	+	+	-/+	-	-	+	+	+	-/+	-/+	-/+	+
	93-9	+	+	+	+	+	-/+	+	+	+	-/+	-	-	+	+	+	-/+	-/+	-/+	+
	93-10	+	+	+	+	+	-/+	+	+	+	-/+	-	-	+	+	+	-/+	-/+	-/+	+
	93-11	+	+	+	+	+	-/+	+	+	+	-/+	-	-	+	+	+	-/+	-/+	-/+	+
	93-15	+	+	+	+	+	-/+	+	+	+	-/+	-	-	+	+	+	-/+	-/+	-/+	+
	93-61	+	+	+	+	+	-/+	+	+	+	-/+	-	-	+	+	+	-/+	-/+	-/+	+
	93-619	+	+	+	+	+	-/+	+	+	+	-/+	-	-	+	+	+	-/+	-/+	-/+	+
	93-671	+	+	+	+	+	-/+	+	+	+	-/+	-	-	+	+	+	-/+	-/+	-/+	+

* classification is based on acetone-fixation method, + positive in both methods, - negative in both methods, -/+ negative in aceton-fixation and positive in formalin-fixation method, † Quebec isolates before 1993, ‡ Quebec isolates in 1993

TABLE III. BVDV antigenic characterisation by neutralization test.

Group	virus	Polyclonal antibodies		Monoclonal antibodies					
		PAb-1	PAb-2	D89	C17	E15	F15	D11	B7
Ia	NADL	8000	500	8000	8000	32000	32000	<125*	<125
	Singer	8000	500	8000	2000	8000	32000	<125	<125
	NY-1	8000	500	<125	<125	<125	<125	<125	<125
Ib	C24V-Oregon	8000	500	<125	<125	<125	<125	<125	<125
	89-E770	8000	2000	<125	<125	<125	<125	<125	<125
	93-11	32000	2000	8000	<125	<125	8000	<125	<125
	93-61	8000	500	2000	<125	<125	2000	<125	<125
Ic	93-671	8000	500	2000	<125	<125	8000	<125	<125
	86-7061	8000	125	8000	<125	<125	500	<125	<125
	88-88	8000	8000	2000	<125	<125	8000	<125	<125
II	91-Domar	8000	8000	32000	<125	<125	32000	<125	<125
	93-15	8000	8000	8000	<125	<125	32000	<125	<125
	93-8	2000	8000	8000	<125	<125	32000	<125	<125
III	93-9	2000	8000	8000	<125	<125	32000	<125	<125
	93-10	500	8000	2000	<125	<125	2000	<125	<125
	93-1	2000	500	2000	<125	<125	2000	<125	<125
IVa	93-3	125	500	8000	<125	<125	8000	<125	<125
	93-619	500	500	8000	<125	<125	8000	<125	<125
IVb	87-2552	2000	500	<125	<125	<125	<125	2000	2000

* All the negative reactions in this study were presented as <125

CHAPTER III

Induction of humoral and cellular immune responses in mice by a recombinant fowlpox virus expressing the E2 protein of bovine viral diarrhea virus

Seyyed Mehdy Elahi[1], Jean Bergeron[2] , Éva Nagy[3], Brian Geoffrey Talbot[4], Serge Harpin[1], Shi-Hsiang Shen[2], Youssef Elazhary[1]*

FEMS Microbiol. Lett. (1999) 171, 107-114

[1]Virology Section, Faculty of Veterinary Medicine, University of Montreal, P. O. Box 5000, Saint-Hyacinthe, Quebec. J2S 7C6, Canada

[2]Biotechnology Research Institute, National Research Council, Montreal, Quebec, Canada

[3]Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Canada

[4]Department of Biology, Faculty of Sciences, University of Sherbrooke, Quebec, Canada

*Author to whom correspondence should be addressed.

Phone: (450) 773-8521 ext. 8201

Fax: (450) 778-8106

E-mail: elazhary@medvet.umontreal.ca

Keywords: bovine viral diarrhea virus; recombinant fowlpox virus; humoral and cellular immune responses; IFN-γ.

Abstract

A recombinant fowlpox virus (rFPV/E2) expressing the E2 protein of bovine viral diarrhea virus (BVDV) was constructed and characterized. Mice were immunized with recombinant virus and both humoral and cellular immune responses were studied. The rFPV/E2 induced BVDV specific antibodies which were detected by ELISA. In addition, mouse sera were shown to neutralize BVDV. A cytokine ELISA assay revealed that mice vaccinated with the rFPV/E2 induced 7-fold more IFN-γ than parental fowlpox virus.

1. Introduction

Bovine viral diarrhea virus (BVDV) is classified in the pestivirus genus of the flaviviridae family [1] and is a ubiquitous pathogen of cattle causing important economic losses. Vaccines presently available have not been successful in eliminating this pathogen.

BVDV contains a single-stranded positive-sense RNA molecule of approximately 12.5 kb which has a single open reading frame (ORF) that encodes a number of structural and non-structural polypeptides that are co-translationally and post-translationally cleaved from polyprotein precursors [2]. Three structural glycoproteins E^{ms} (gp48), E1 (gp25) and E2 (gp53) are encoded in the first third of the ORF. The E2 (gp53) protein is a major viral glycoprotein which is highly antigenic and elicits the production of neutralizing antibodies in the host after infection or vaccination with live or killed vaccines. The majority of BVDV neutralizing antibodies are directed against the E2 protein [3-4]. Two different forms of E2 protein, E2 (53 kDa) and E2p7 (60 kDa), can be found in infected cells depending on the different cleavage patterns in their C-terminal tail [5]. The role of the E2 protein of BVDV in cellular immunity is not very well known. However, in

the case of hog cholera virus, another pestivirus, the E1 protein (E2 homologous in BVDV) was shown not to be a major T-cell antigen [6].

Fowlpox virus (FPV) is a member of the avipox genus of the orthopoxvirus family. In contrast to vaccinia virus, which has a very broad vertebrate host range, other members of the family such as the avipox genus are naturally restricted for productive replication to avian species [7]. Nevertheless, avipox vectors, like FPV and canarypox, engineered to express extrinsic antigens can elicit protective immune responses to viral pathogens in non-avian species [8-10]. Fowlpox-base recombinant vaccines possess several advantages as vaccine vectors. The virus is safe since it was used previously in protection against the poultry disease. The virus is also extremely stable and inexpensive to produce. It is capable of initiating an abortive infection in non-avian cells and expresses antigens on the infected cell surface. Consequently it can induce both cellular and humoral immune responses [8-10].

In this paper we used recombinant fowlpox virus to study the role of the E2 protein of BVDV in the induction of both humoral and cellular immune responses.

2. Materials and Methods

2.1. Cells and viruses- Primary chicken embryo fibroblast (CEF) cell cultures were prepared from 9-11 day old specific pathogen-free embryos essentially by the method of Salomon [11]. CEF cells were grown in DMEM/F12 medium supplemented with 4% fetal bovine serum (FBS) and antibiotics. The vaccine strain of FPV, Poxine, (Solvay Animal Health, Inc., Mendota Heights, MN 55120-1139 USA) was used as the parental virus to construct a recombinant virus (rFPV/E2). The parental FPV (pFPV) and rFPV/E2 were grown and titrated in CEF cells. The NADL strain (BVDV type I) and Madin-Darby kidney (MDBK) cells were obtained from the American Type Culture Collection (Rockville, MD). The 125 strain (BVDV type II) was obtained from USDA (Ames, Iowa, USA).

2.2. Construction of plasmid vectors- Molecular cloning procedures were essentially as described by Maniatis [12]. The intermediate plasmid pFPVtkgpt was constructed by cloning the 1.1 kb PCR product of the *tk* gene [13] in *Eco*R I / *Hin*d III digested pUC18 (Gibco BRL) and the subsequent insertion of a synthetic bi-directional promoter element with early/late and late functions [14], flanked by a synthetic multiple cloning site (MCS) in the blunt-ended *Xba* I site of the *tk* gene (Fig. 1). The *E. coli* gpt gene flanked by the P7.5 promoter of the vaccinia virus from pTKgptF1s [15], was cloned as a *Bam*H I/*Cal* I fragment in pBCKS (Stratagene) and removed as a *Bam*H I/*Xho* I fragment for subsequent cloning into pFPVtkBi. Also, the GFP gene from pS65T [16] was digested with *Bam*H I/*Xba* I and, following filling-in, was inserted in the *Sma* I site of the pFPVtkBi. The vector, pFPVtkBigptGFP, contains a unique *Bam*H I site for the cloning of the target gene. The construction of pcDNA/gp53 which contains the E2 (gp53) fragment of NADL strain of BVDV (nucleotides 2414-3725) was described previously [17]. Plasmid pcDNA/gp53 *Bg*l II was derived from pcDNA/gp53 by adding two annealed oligonucleotides 5′-CTAGCA GATCTG-3′ and 5′-GTCTAGACGATC-3′ to generate a *Bg*l II site at the *Xba* I site of the MCS. The resulting plasmid, was digested with *Bam*H I/*Bg*l II and the E2 fragment was cloned into the *Bam*H I site of pFPVtkBigptGFP. The final plasmid containing the E2 gene of BVDV was designated pFPVtkBigptGFP/E2 (Fig. 1).

2.3. Generation and purification of rFPV/E2- Procedures for transfection of FPV-infected cells with the transfer vector DNA, and the generation of rFPV were described previously [18]. The progeny virus was passaged three times on CEF cells under two selective conditions. Briefly, 5 hours (h) prior to infection of the 60 mm dish of CEF cells with 10-20% of progeny virus (in 0.5-1 ml of medium), the medium (DMEM with 2% v/v FBS) was replaced with medium containing 20 $\mu g\ ml^{-1}$ of 5-bromo-2′-deoxyuridine (BUdR) or MXHAT (10 $\mu g\ ml^{-1}$ mycophenolic acid, 250 $\mu g\ ml^{-1}$ xanthine, 13.6 $\mu g\ ml^{-1}$ hypoxanthine, 2 $\mu g\ ml^{-1}$ aminopterin and 8 $\mu g\ ml^{-1}$

thymidine). After adsorbtion, the selective media were replaced and the conditions maintained until 4-5 days post infection (p.i.). The parental virus failed to grow in medium containing BUdR or MXHAT, while the rFPV/E2 was enriched under these conditions. The progeny virus was subjected to three rounds of plaque purification on CEF cells. At 5 days p.i. the lysis plaques were picked up and placed into 300 µl of medium (EMEM with 2% calf serum). Recombinant viruses were identified by dot-blot hybridization of infected CEF cells. The methods for alkaline lysis of cells and membrane preparation were described previously [19]. The probe was labelled with the digoxigein (DIG) (Boehringer Mannheim) and the detection kit from the same company was used according to the manufacture's instructions.

2.4. Characterization of rFPV/E2- One-step growth curves to compare the intracellular and extracellular viruses for both of pFPV and rFPV/E2 were done as described previously [20]. The viral DNA of rFPV/E2 and pFPV were analysed by Southern blotting as described by Parks *et al.* [21].

2.5. Radioimmunoprecipitation analysis-The protocols for metabolic labelling and immunoprecipitation of antigen from CEF cells infected by rFPV/E2 or pFPV and MDBK cells infected by NADL were previously described [4, 21] Monoclonal antibody (Mab) D89 to the BVDV/E2 protein provided kindly by Dr. H. C. Minocha (Kansas State University, Manhattan, Kansas, 66506) was used for immunoprecipitation of E2 protein.

2.6. Immunization of mice- Four groups of 10 inbred BALB/c mice were inoculated in the footpad with 50-100 µl of a range of dilutions 4 X 10^5 to 4 X 10^7 p.f.u. of rFPV/E2 or 4 X 10^7 p.f.u. of pFPV. Booster immunizations were given at 3 and 6 weeks (wk) after priming injection. Bleeding was performed from orbital puncture at 0, 3, 6, 9, and 12 wk post injection (p.i.) (Table I).

2.7. Enzyme-Linked Immunosorbant Assay (ELISA)-The MDBK cells infected with the NADL strain of BVDV were re-suspended in carbonate, bicarbonate buffer ($NaHCo_3$ 35 mM, $Na2Co_3$ 15 mM, NaN_3 0.02%, pH 9.6). After three freeze-thaw cycles, the supernatant was treated with N-octylglucoside (2% final concentration) and the plates were coated by diluted antigens in carbonate, bicarbonate buffer at a concentration of 1 µg in 50 µl. After blocking with 5% skim milk (1 h at 37°C), diluted mouse sera (1:160) were added to the wells in duplicate, and the plates were incubated for 30 min at 37°C. HRP-goat anti-mouse IgG (1:8000, Bio-Rad) was used as the second antibody (30 min at 37 °C) and Tetra-Methylbenzidine as the substrate (10 min at room temperature). The positive cut-off O.D. was considered as the mean A_{450} values for each sample greater than the mean A_{450} value obtained from mice in group I plus two times the standard deviation (O.D. ≈ 0.18).

2.8. Neutralization test (NT)- After decomplementation of mouse sera at 56°C for 30 min, 50 µl of virus solution containing 100 $TCID_{50}$ of BVDV (NADL strain) was added to 50 µl of serial twofold serum dilutions (starting at 1: 10) and the plates were incubated for 1 h at 37°C. Then, 100 µl of medium containing 3 X 10^4 MDBK cells was added to each well. Each serum dilution was tested in quadruplicate. Plates were incubated at 37°C in 5% CO_2 for 5 days. The highest serum dilution that inhibited the cytopathic effect in at least 50% of the cultures was considered as the virus neutralization titer.

2.9. Proliferation response of murine mononuclear cells (MNC)- At 15 wk p.i. splenocytes of 5 mice from group I and IV were pooled and suspended at a concentration of 3 X 10^6 MNC ml^{-1} in RPMI-1640 supplemented with 10% FBS, 2 mM sodium pyruvate, 2 mM glutamine and 50 µg ml^{-1} gentamycin and 5 X 10^{-5} M of 2-mercaptoethanol. Murine MNC were added to 96-well flat bottom plates (100 µl) and incubated with 100 µl of diluted BVDV (NADL or 125 strain) to reach final dilutions of 1/10, 1/20 and 1/40. The titers of the original NADL and 125 strains were

10^7 and $10^{6.2}$ $TCID_{50}$ ml^{-1} respectively. The test was performed in triplicate for each dilution. After 5 days, the wells were pulsed for 6 hours with 0.2 μCi of [^{3}H] thymidine (6.6 Ci / mM, ICN) and harvested with a Skatron semiautomatic cell harvester (Flow Laboratories, Rockville MD). Stimulation index (SI) was calculated by the following formula: SI = average counts per minute in antigen stimulated wells / average counts per minute in wells containing only cells with medium.

2.10. Cytokine detection assay- 1.5 X 10^6 murine MNC were stimulated with BVDV (NADL and 125 strains) at a final dilution of 1/10 or mock stimulated. Three days later 100 μl of supernatants in duplicate were used in the cytokine ELISA assay (PharMingen) for detection of IL-2, IL-4 and IFN- γ according to manufacture's instructions.

3. Results and Discussion

3.1. Generation of rFPV/E2- The transfer vector, pFPVtkBigptGFP/E2, was constructed by interupting the FPV *tk* gene by inserting 3 elements into the *tk* gene of FPV: (1) The *E. coli* gpt gene was cloned under the control of the P7.5 promoter, thus allowing the selection of recombinant viruses in the presence of medium containing MXHAT. (2) The GFP gene was cloned under the control of the late function of the bi-directional promoter of FPV. (3) The expression of the BVDV E2 was controlled by the early/late function of the bi-directional promoter. Because the expression of the *tk* gene was interrupted, the rFPV/E2 was *tk*$^-$, and therefore only rFPV/E2 could be amplified in the presence of BUdR. The transfer vector pFPVtkBigptGFP/E2 was used to transfect CEF cells previously infected with pFPV. After three rounds of amplification in the presence of BUdR and/or MXHAT, the putative recombinant viruses were plaque purified four times in the absence of selection medium. The progeny viruses were identified by dot blot hybridization. Both selection media were effective for enrichment of rFPV. However, better results were observed in the presence of BUdR (data non shown). During BUdR selection, only recombinant

viruses with double cross-over will be *tk*$^-$ and can grow. In the presence of MXHAT, both single and double cross-over recombinant viruses can express the gpt gene and can grow in the selection medium. The rFPV/E2 with a single cross-over is unstable and undergoes subsequent rounds of recombination, leading to the formation of both stable recombinant (double cross-over) and parental viruses.

Screening on the basis of GFP expression was efficacious when cells in 96 well plates were infected by eluted plaques during the plaque purification step. Supernatants of the wells that were positive within 2-3 days p.i. by GFP were used for subsequent plaque purification, without waiting for dot blot results. This saved 4-5 days for each cycle and also reduced the number of dot blot assays performed. Expression of GFP was also used as an indicator of the presence of rFPV/E2. A decrease in the number of GFP$^+$ positive cells in subsequent passages was the first sign of the presence of pFPV in the viral population. Finally, Fluorescent Activated Cell Sorter (FACS) or other appropriate equipment for the isolation of GFP$^+$ cells could be used to enrich rFPV/E2 by isolation of GFP$^+$ cells. However, enrichment the rFPV/E2 by using the BUdR or MXHAT selection media considerably increased the percentage of GFP$^+$ cells and thus facilitated the task. In our case however, plaque screening on the basis of GFP in highly confluent cells was not recommended because GFP was hard to detect in these conditions.

3.2. Characterization of rFPV/E2- The presence of one copy of the E2 gene in 5.5 kb *Hin*d III digested fragment of rFPV/E2 was confirmed by Southern blot (data not shown). Titration of the both the virus in culture medium and the cell-associated viruses revealed that the rFPV/E2 produced 2.4-fold fewer infectious extracellular and 1.8-fold fewer infectious intracellular viruses than pFPV at 72 h p.i. This observation between the two viruses was apparent as early as 18 h p.i. and remained roughly constant throughout the course of infection course (Fig. 2). This difference could be due to the interruption of the *tk* gene in rFPV/E2. Letellier [23] has also

demonstrated that tk^+ recombinant pigeonpox virus has a growth advantage over tk^- recombinant virus.

3.3. Expression of the E2 protein by rFPV/E2- The D89 Mab was used to precipitate the E2 protein (53 kDa) in BVDV-infected MDBK cells (Fig. 3, line 2). A protein of about 60 kDa was also co-precipitated. This protein could be the E2p7 protein which was reported previously [5]. However, in CEF cells infected with rFPV/E2, the size of the E2p7 protein was slightly lower (57 kDa) because our construct lacked 22 amino acids at the C-terminal end of the E2p7 protein (Fig. 3, line 4). However, no specific proteins were observed when lysates of pFPV-infected cells were immunoprecipitated with the D89 Mab (Fig. 3, line 3).

3.4. Humoral immune reponse to rFPV/E2- Mouse sera were assayed for BVDV specific antibodies in an indirect ELISA. Table I shows that the humoral immune response to BVDV E2 protein was dose-dependent. When mice were injected with 4 X 10^6 and 4 X 10^7 p.f.u. of rFPV/E2 (group III and IV) all the mice seroconverted at 6 wk p.i. But only 20 % of mouse sera immunized by 4 X 10^5 p.f.u. of rFPV/E2 (group II) were seropositive by ELISA at the same time. Neutralization antibodies at a minimal titer of 10 were also detected in 20% and 60% of mice in group II and III respectively at 9 wk p.i. (Table I). 60% of mice in group IV possed antibodies that neutralized the BVDV/NADL strain after 6 wk p.i. Both the number of mice positive for the presence of neutralization antibodies against BVDV and also the titer of neutralizing antibodies were dose-dependent. The immune response increased from 2 mice with titers of 10 ± 0 in group II to 8 mice with titers of 21 ± 12 in group IV at 12 wk p.i. (Table I). To demonstrate that the route of injection had no effect on neutralization titers, a separate group of mice were injected by the intramuscular route with 4 X 10^7 p.f.u. of rFPV/E2, following the same protocol used for group IV. No differences were observed in the neutralizing titer (data not shown).

3.5. Cellular immunity responses- ELISA assays for IFN-γ revealed the production of 2200 ± 160 pg ml^{-1} of IFN-γ by the murine MNC immunized with rFPV/E2 (group IV) and 284 ± 23 pg ml^{-1} by the murine MNC immunized with pFPV (group I), after stimulation with the BVDV/NADL strain. No IFN-γ was detected after stimulation with BVDV/125 strain. This significant increase in IFN-γ concentration (7-fold) in the supernatant of murine MNC immunized with the rFPV/E2 compared to the group immunized with the pFPV could be caused by the activation of specific Th1 cells. No increases were observed in the production of IL-2 and IL-4. This is not surprising since IFN-γ is an indicator of the activation of Th1 cells and consequently the absence of IL-4 by Th2 cells is easy explainable. However it is more difficult to explain why no proliferative responses were observed after stimulation of murine MNC with BVDV/NADL or 125 strains. This is more surprising considering that there was the strong non-specific proliferation response to pFPV. The stimulation index for mice immunized with pFPV was between 2.8 to 13 (depending on the dilution of BVDV/NADL) compared with 2.6 to 4.5 for rFPV/E2. This non-specific proliferation may have masked specific T-cell responses to BVDV by rFPV/E2. Another possible explanation for the absence of specific lymphoproliferation in response to BVDV stimulation, could be the effect of the high levels of IFN-γ and/or prostaglandins produced via activated macrophages [24-25-26]. As part of a separate study, we have investigated the effect of indomethacin, an inhibitor of prostaglandin E2, on the restoration of proliferative responses. Using a recombinant adenovirus expressing the nucleocapsid of BVDV we found that the proliferation response to the recombinant adenovirus was detectable only in the presence of indomethacin (data non shown). This result derives from the potent ability of prostaglandins to inhibit T-cell mitogenesis and IL-2 production [27].

In conclusion, mice immunized with the recombinant fowlpox virus, rFPV/E2, induced a neutralizing humoral immune response. In addition, the BVDV E2 protein induced high levels of IFN-γ which suggests the activation of Th1 cells.

This study demonstrated the efficacy of rFPV/E2 as a BVDV recombinant immunogen, and encourages further study to evaluate its use as a vaccine in cattle, the natural host for BVDV.

Acknowledgements

We acknowledge the technical assistance of D. Frenette for contributing to the production of pFPV and Dr. Y. Chorfi for collecting the murine MNC. We thank Dr. R. Tsien and Dr. B. Moss for providing the plasmid encoding the S65T mutant GFP gene and pTKgptF1s respectively and also Dr. H. C. Minocha for providing the D89 Mab. Dr. S. Mehdy Elahi is supported in part by a scholarship from the Islamic Republic of Iran government.

References

[1] Wengler, G., Bradley, D.W., Collett, M.S., Heinz, F.X., Schesinger, R.W. and Strauss, J. (1995) Flaviviridae, p. 415-427. In Murphy, F.A., Fauquet, C.M., Bishpo, D.H.L., Ghabrial, S.A., Jarvis, A.W., Martelli, G.P., Mayo, M.A. and Summers, M.D. (ed), Virus taxonomy. Sixth report on the International Committee on Taxonomy of Viruses. Springer-Verlag. New York.

[2] Collett, M.S., Larson, R., Belzer, S.K. and Retzel, E. (1988) Proteins encoded by bovine viral diarrhea virus: the genomic organization of a pestivirus. Virology 165, 200-208.

[3] Donis, R.O., Corapi, W. and Dubovi, E.J. (1988) Neutralizing monoclonal antibodies to bovine viral diarrhea virus bind to the 56K to 58k glycoprotein. J. Gen. Virol. 69, 77-86.

[4] Xue, W., Blecha, F. and Minocha, H. C. (1990) Antigenic variations in bovine viral diarrhea viruses detected by monoclonal antibodies. J. Clin. Microbiol. 28, 1688-1693.

[5] Elbers, K., Tautz, N., Becher, P., Stoll, D., Rumenapf, T. and Thiel, H-J. (1996) Processing in the Pestivirus E2-NS2 region: Identification of proteins p7 and E2p7. J. Virol. 70, 4131-4135.

[6] Kimman T.G., Bianchi, A.T., Wensvoort, G., de Bruin, T.G. and Meliefstec, C. (1993) Cellular immune response to hog cholera virus (HCV): T cell of immune pigs proliferate in vitro upon stimulation with live HCV, but the E1 envelope glycoprotein is not a major T-cell antigen. J. Virol. 167, 2922-2927.

[7] Somogyi, P., Frazier, J. and Skinner, M.A. (1993) Fowlpox virus host range restriction: gene expression, DNA replication, and morphogenesis in nonpermissive mammalain cells. Virology 197, 439-444.

[8] Taylor, J. and Paoletti, E. (1988) Fowlpox virus as a vector in non-avian species. Vaccine 6, 466-468.

[9] Baxby, D. and Paoletti, E (1992) Potential use of non-replicating vectors as recombinant vaccines. Vaccine. 10, 8-9.

[10] Pincus, S., Tartaglia, J. and Paoletti, E. (1995) Poxvirus-based vectors as vaccine candidates. Biologicals. 23, 156-164.

[11] Solomon, J. J. (1975). Preparation of avian cell cultures. Tissue Culture Association. 1, 7-11.

[12] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1990) Molecular cloning. a laboratory manual. Cold Spring Harbor Laboratoru, Cold Spring Harbor, N.Y.

[13] Boyle, D.B., Coupar, B.E.H., Gibbs, A.J., Seigman, L. J. and Both, G.W. (1987) Fowlpox virus thymidine kinase: Nucleotide sequence and relationships to other thymidine kinases. Virology 156, 355-365.

[14] Kumar, S. and Boyle, D.B. (1990) A poxvirus bi-directional promoter element with early/late and late fuctions. Virology 179, 151-158.

[15] Falkner, F.G. and Moss, B. (1988) Escherichia coli gpt gene provides dominant selection for vaccinia virus open reading frame expression vectors. J. Virol. 62, 1849-1854.

[16] Heim, R., Cubitt, A.B. and Tsien, R.Y. (1995). Improved geen fluorescence. Nature. 373, 663-664.

[17] Harpin, S., Talbot, B., Mbikay, B. and Elazhary, Y. (1997) Immune response to vaccination with DNA encoding the bovine viral diarrhea virus major glycoprotein gp53 (E2). FEMS Microbiol. Lett. 146, 229-234.

[18] Calvert, J.G., Nazerian, K., Witter, R. and Yanagida, N. (1993) Fowlpox virus recombinants expressing the envelope glycoprotein of a avian reticuloendotheliosis retrovirus induce neutralizing antibodies and reduce viremia in chickens. J. Virol. 67, 3069-3076.

[19] Hammermueller, J.D., Krell, P.J., Derbyshire, J.B. and Nagy, É. (1991) An improved dot-blot method for virus detection in chicken embryo fibroblast cultures. J. Virol. Methods. 31, 47-56.

[20] Calvert, J.G., ogawa, R., Yanagida, N. and Nazerian, K. (1992) Identification and functional analysis of the fowlpox virus homolog of the vaccinia virus p37k major envelope antigen gene. Virology 191, 783-792.

[21] Parks, R.J., Krell, P.J., Derbyshire, J.B. and Nagy, É. (1994) Studies of fowlpox virus recombinat in the generation of recombinant vaccines. Virus Res. 32, 283-297.

[22] Letellier, C., Burny, A. and Meulemans, G. (1991) Construction of a pigeonpox virus recombinant: expression of the Newcastle diseases virus (NDV) fusion glycoprotein and protection of chickens against NDV challenge. Arch. Virol. 118, 43-56.

[23] Letellier, C. (1993) Role of the TK+ phenotype in the stability of pigeonpox virus recombinant. Arch. Virol. 131, 431-439.

[24] Liew, F.V. (1995) Interaction between cytokines and nitric oxide. Adv. Neuroimmunol. 5, 201-209.

[25] Schleifer, K.W. and Mansfield, J.M. (1993) Suppressor macrophages in African trypanosomiasis inhibit T cell proliferative responses by nitric oxide and prostaglandins. J. Immunol. 151, 5492-5503.

[26] Fu, Y. and Blankenhorn, E.P. (1992) Nitric oxide-induced anti-mitogenic effects in high and low responder rat strains. J. Immunol. 148, 2217-2222.

[27] Phipps, R. P., Stein, S.H. and Roper R.L. (1991) A new view of prostaglandin E regulation of the immune response. Immuno. Today 12, 349-352.

Table I. Serum antibody response of mice following immunization with parental (pFPV) or recombinant fowlpox virus (rFPV/E2).

Group	Number	virus	Dose* (p.f.u)	Route	Vaccination time	ELISA†			Neutralisation test‡		
						6 wk§	9 wk	12 wk	6 wk	9 wk	12 wk
I	10	pFPV	4×10^7	Footpad	0-3-6 wk p.i.	0/10	0/10	0/10	0/10 (<10)	0/10 (<10)	0/10 (<10)
II	10	rFPV/E2	4×10^5	Footpad	0-3-6 wk p.i.	2/10[b]	2/10[a]	6/10[b]	0/10 (<10)	2/10 (10±0)	2/10 (10±0)
III	10	rFPV/E2	4×10^6	Footpad	0-3-6 wk p.i.	10/10[c]	10/10[c]	10/10[c]	0/10 (<10)	6/10 (10±0)	6/10 (10±0)
IV	10	rFPV/E2	4×10^7	Footpad	0-3-6 wk p.i.	10/10[c]	10/10[c]	10/10[c]	6/10 (18±4)	8/10 (16±5)	8/10 (21±12)

Table I. Serum antibody response of mice following immunization with parental (pFPV) and recombinant fowlpox virus (rFPV/E2).

Bleeding was performed by orbital plexus puncture at 0, 3, 6, 9 and 12 weeks (wk) postinfection (p.i.). The mouse sera were tested in an Enzyme-linked immunosorbant assay (ELISA) and a Neutralization test (NT) for detection of BVDV specific antibody and neutralization antibody against E2 protein of BVDV, respectively. In both tests, the numbers of positive mice / number of total mice in each experiment were shown in this table.

*Dose expressed as plaque forming unit (p.f.u.) / mice

†In ELISA the mouse sera with optical density higher than 0.18 at dilution 1/160 were considered as positive. Difference between optical density of group II, III and IV with group I was significant in pear t test (two tails). a; $p<0.05$, b; $p<0.005$, c; $p<0.0005$

‡In NT the mouse sera neutralized BVDV/NADL strain at minimal dilution of 1/10 were considered as positive. Average of neutralizing antibodies for positive samples ± standard deviation was shown in parenthesis. The mouse sera with titer <10 were considered as negative.

§ wk; weeks post-infection, No positive samples were detected before 6 wk p.i.

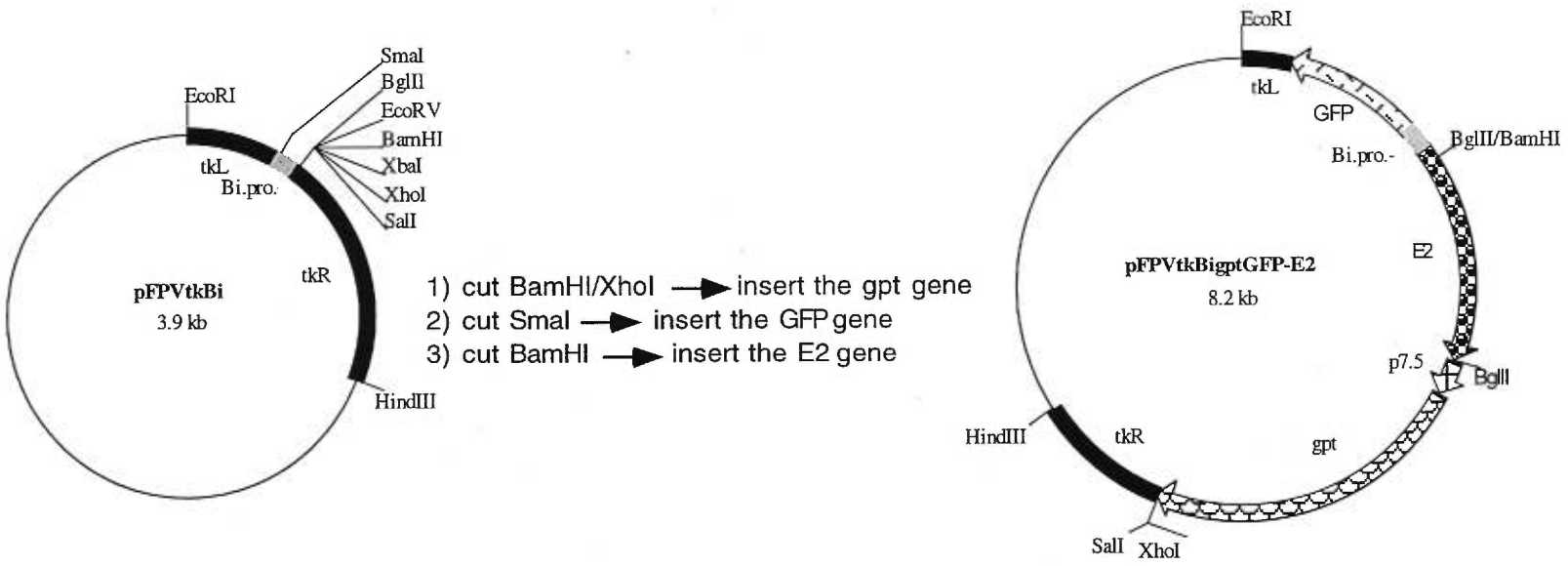

Fig. 1. Construction of the transfer vector pFPVtkBigptGFP/E2 from pFPVtkBi. The pFPVtkBi was constructed by insertion of the gene in pUC18 and a synthetic bi-directional promoter (Bi.pro.) in Xba I site of tk gene as described in Materials and Methods. Then, (1) the gpt gene (BamH I /Xho I) from pBCKSgpt was inserted in BamH I /Xho I of pFPVtkBi. (2) the GFP gene (*BamH* I/*Xho* I- end filled) from pS65T was inserted in *Sma* I site, and (3) the E2 gene of BVD (*BamH* I/*Bgl* II) from pcDNAgp53 *Bgl* II was inserted into the *BamH* I site to generate the transfer vector pFPVtkBigptGFP/E2.

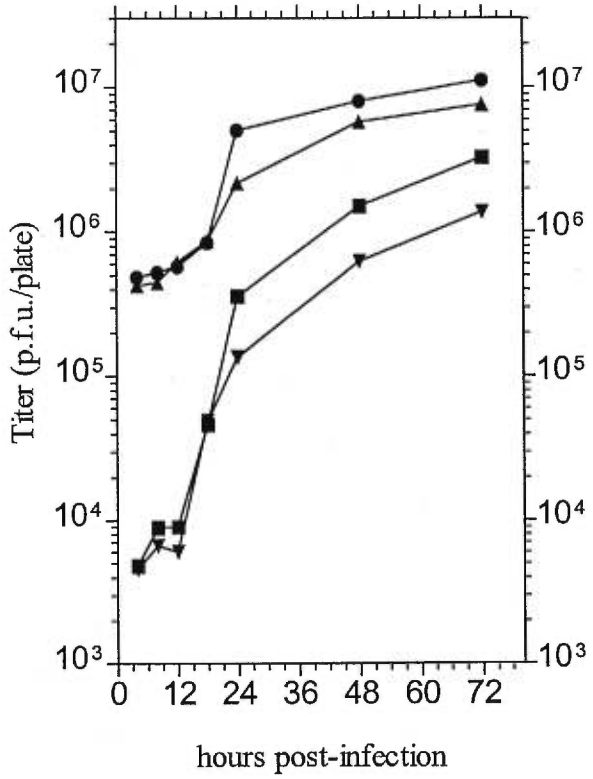

Fig. 2. One step growth curves.

The production of intracellular and extracellular viruses of pFPF and rFPV/E2 were investigated at various times after infection. The data points represent the average of two experiments, each of which was performed in duplicate. pFPV (● intra-, ■ extracellular virus), rFPV/E2 (▲ intra-, ▼ extracellular virus).

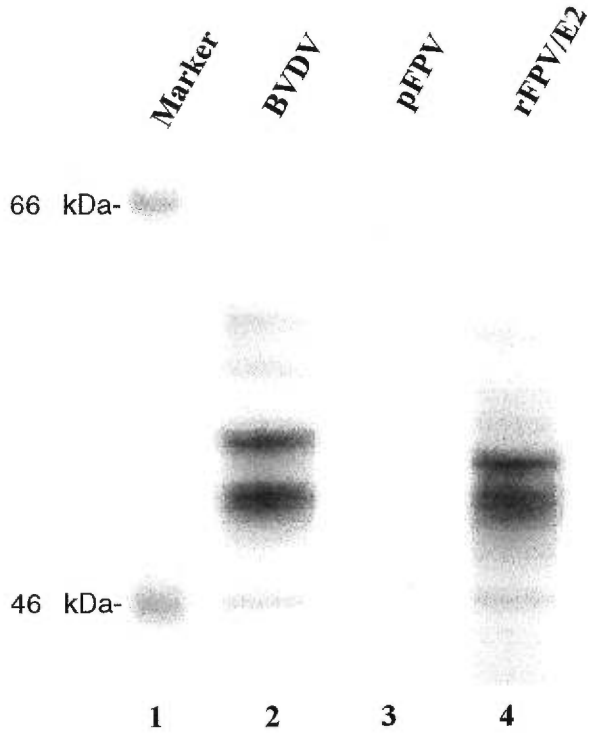

Fig. 3. *In vitro* expression of rFPV/E2- After metabolic labelling of BVDV- infected MDBK cells (line 2) and CEF cells pFPV-infected (line 3) or rFPV/E2-infected (line 4), cells extracts were immunoprecipitated by the D89 Mab. The markers for protein molecular weights are shown on line 1.

CHAPTER IV

Recombinant adenoviruses expressing the E2 protein of bovine viral diarrhea virus induce humoral and cellular immune responses

Seyyed Mehdy Elahi[1], Shi-Hsiang Shen[2], Brian Geoffrey Talbot[3], Bernard Massie[2], Serge Harpin[1], Youssef Elazhary[1]*

FEMS Microbiol. Lett. "Submitted"

[1]Virology Section, Faculty of Veterinary Medicine, University of Montreal, P. O. Box 5000, Saint-Hyacinthe, Quebec, J2S 7C6, Canada

[2]Biotechnology Research Institute, National Research Council, Montreal, Quebec, H4P 2R2, Canada

[3]Department of Biology, Faculty of Sciences, University of Sherbrooke, Quebec, J1K 2R1, Canada

*Author to whom correspondence should be addressed.

Phone: (450) 773-8521 ext. 8201

Fax: (450) 778-8105

E-mail: elazhary@medvet.umontreal.ca

Main text; 3642 words

Summary; 83 words

1 table & 4 figures

Keyword: Bovine viral diarrhea virus; recombinant adenoviruses; humoral and cellular immune responses; Cytokines

Summary

The E2 protein of bovine viral diarrhea virus (BVDV) is a major viral glycoprotein and an attractive target for BVDV vaccines. Three replication defective recombinant adenoviruses expressing the BVDV/E2 protein (rAds/E2) were constructed. Two contain a constitutive promoter, and one an inducible promoter. All three recombinant adenoviruses induced very strong BVDV specific antibody responses in a mouse model as detected by ELISA and neutralization tests. A proliferation response and the production of IFN-γ were observed in BVDV-stimulated mononuclear cells from the immunized-rAds/E2 mice.

1. Introduction

Bovine viral diarrhea virus (BVDV) is a world-wide important pathogen of cattle. It is classified in the pestivirus genus of the *Flaviviridae* family [1] and BVDV strains are divided in two genotypes (group I and II) [2-3]. The majority of natural neutralizing antibodies against BVDVs are directed against the E2 protein [4-5] which is a major viral glycoprotein. Two different forms of E2 protein, E2 (53 kDa) and E2p7 (60 kDa), have been found in infected cells depending on the different cleavage patterns in their C-terminal tail [6]. The role of the E2 protein of BVDV in cellular immunity has not been clearly established. In the case of hog cholera virus, another pestivirus, the E1 protein (E2 homologous in BVDV) is not a major T-cell antigen [7]. However, we recently demonstrated a Th1 response to BVDV/E2 protein after immunization of mice with a fowlpox recombinant expressing the BVDV/E2 protein [8].

Defective adenoviruses containing an E1 deletion have been developed as vectors for gene therapy and vaccinations [9-14]. Human adenovirus type 5 is the common adenovirus used for this purpose for several reasons: Its genome has been well characterized, it has a very wide host range and also a very stable virion that can

be produced in large quantities. These viruses are unable to replicate in cells which do not complement the defective E1 gene but they are still able to induce the synthesis of very high levels of the foreign protein [9, 11-14].

Several different promoters can be used for expression of foreign genes in adenovirus vectors such as: the cytomegalovirus (CMV) immediate-early (IE) promoter [12, 14], the major late promoter (MLP) [11, 14], the SV40 early and late promoters and the B-actin promoter [14]. The CMV and MLP are the most commonly used because they are stronger than the others. Recently, a tetracycline regulatable system (tTA-responsive promoter) has been developed to regulate the expression of the transgenes [12-13,15]. In this system, the expression of a target gene is placed under the control of a promoter containing the tetracycline operator sequence (tet O) that can be induced by the tetracycline-regulated trans-activator protein (tTA). The tTA protein can be supplied by using the 293-tTA cell line (a stable 293 cell which constitutively expresses the tTA protein, [12] or by co-infection with a recombinant virus such as Ad5CMV-tTA [12]. The transcription of the tTA protein can be prevented by adding tetracycline at a concentration that is not toxic for eukaryotic cells [16].

In this paper we used the coding region of the BVDV/E2 protein to construct three recombinant adenoviruses (rAds). Two rAds contained constitutive promoters, BM5 and CMV5, the modified forms of MLP and CMV promoter respectively. One rAd contained an inducible promoter designated as TR5 promoter (tTA-responsive promoter). With these rAds, we investigated the role of the BVDV/E2 protein in induction of humoral and cellular immune responses in mice as part of a program to develop recombinant viral vectors for viral immunization.

2. Materials and Methods

2.1. Cultures and Viruses- The conditions for culture of human 293 cells, either the original anchorage-dependant 293A line [17] or 293S (obtained from Cold Spring Harbor Laboratories), an anchorage-independent clone, were as described previously [11, 18]. Madin-Darby bovine kidney (MDBK) cells (free of BVDV) were obtained from the American Type Culture Collection (Rockville , MD) and grown in Dulbecco's Modified Eagle medium, (Gibco), supplemented with 5% of fetal bovine serum (FBS), [free of antigen and antibody against BVDV, (Gibco)]. The NADL strain of BVDV (type 1) and 125 strain (BVDV type 2) were obtained from ATCC and USDA (Ames, Iowa, USA) respectively and were propagated in MDBK cells in the presence of 2% FBS. Human adenovirus type-5 with deletions in the E1 and E3 regions (Ad5/ΔE1ΔE3) [19], and Ad5CMV-tTA [12] were amplified in 293S cells.

2. 2. Construction of recombinant adenoviruses- The construction of pcDNA/gp53 plasmid which contained the E2 (gp53) fragment of the BVDV/NADL strain (nucleotides 2414-3725) was described previously [20]. The E2 fragment was excised by restriction enzymes *Bam*H I and *Xba* I, blunt-ended with klenow enzyme, and ligated into *Bam*H I digested, klenow polymerase treated pAdBM5 [11] and pAdCMV5 [12] to yield pAdBM5/E2 and pAdCMV5/E2 respectively. The E2 fragment was also cloned in pAdTR5-DC/GFP plasmid [13] with the same strategy, except that the *Bg*l II site of the plasmid was used as the cloning site. The resulting construction was named pAdTR5-DC/E2-GFP.

The 293A cells were co-transfected separately with the viral DNAs of Ad5/ΔE1ΔE3 and one of the shuttle plasmids pAdBM5/E2, pAdCMV5/E2 and pAdTR5-DC/E2-GFP as described previously [12]. Viral plaques appeared approximately 7 to 10 days later. Twenty four plaques were picked and amplified by infecting 293A cells in 24-well plates. When the shuttle plasmid pAdTR5-DC/E2-GFP was used for co-transfection, the progency recombinant adenoviruses also co-

expressed GFP protein. In consequence the recombinant plaques were green and could be rapidly identified by fluorescence microscopy. Expression from the TR5 promoter in 293A cells was sufficient to identify green plaques in the uninduced state. Recombinant adenoviruses (rAdBM5/E2, rAdCMV5/E2 and rAdTR5-DC/E2-GFP, were referred to as rAds/E2) were plaque purified four times and each time the presence of the BVDV/E2 gene was analyzed by PCR. In addition, the expression of the E2 protein was detected by radioimmunoprecipitation (RIPA) using an MAb against BVDV as described later. In the case of rAdTR5-DC/E2-GFP the GFP co-expression was also monitored by fluorescence microscopy. The rAds/E2 were purified by two successive discontinuous and continuous ultracentrifugations on CsCl gradients. Viral stocks were titrated on 293A cells using a plaque assay [21].

2. 3. Metabolic radiolabeling and electrophoretic analysis- 293A cells were metabolically radiolabeled using [^{35}S] methionine/ [^{35}S]cysteine (Amersham; 100 μCi per 60 mm dish) 16-18 h after infection with either Ad5/ΔE1ΔE3 + Ad5CMV-tTA (negative control) or rAds/E2 at a multiplicity of infection (m.o.i.) of 20 for each virus. The rAdTR5-DC/E2-GFP was co-infected with Ad5CMV-tTA. The MDBK cells were also infected with the BVDV at a m.o.i. of 5 and labeled between 16 to 18 h p.i. The proteins were precipitated using MAb D89 [22] (donated by Dr. Minocha, Kansas State University, Manhattan, Kansas) and Protein A Sepharose before being analyzed by SDS-PAGE as described previously [23].

2. 4. Immunization experiments- Four groups of inbred mice (BALB/c) were injected three times with 10^9 plaque forming units (p.f.u.) from each purified adenovirus as follow : Group I (negative control); Ad5/ΔE1ΔE3 + Ad5CMV-tTA, group II; rAdBM5/E2, group III; rAdCMV5/E2, and group IV; rAdTR5-DC/E2-GFP + Ad5CMV-tTA at 0, 3, and 6 wk post-infection (p.i.) by intramuscular (i.m.) injections. The blood was collected by orbital plexus puncture at 3 week intervals until week 12 p.i.

2. 5. Detection of humoral immunity- The presence of IgG antibodies and neutralizing antibodies against the BVDV/E2 protein was detected in mouse sera by an Enzyme-Linked Immunosorbant Assay (ELISA) and Neutralization test (NT) respectively as described previously [8] with only one modification. The NT was performed by using both genotypes of BVDV, BVDV/NADL (type 1) and BVDV/125 strain (type 2).

2.6. Detection of cellular immunity- At 15 wk p.i. the murine mononuclear cells (MNC) of 5 mice from group I, II and III were stimulated with BVDV/NDAL (type 1) or BVDV/125 (type 2) *in vitro* and after 5 days the proliferative response was measured [8]. Also the concentration of IFN-γ, a key regulators of Th1 differentiation, [24-25] and IL-4, a key regulator of Th2 differentiation [24], were measured in the supernatant of stimulated cells after 3 days [8]. Finally, a memory response was demonstrated by injection of 10^7 $TCID_{50}$ of BVDV/NADL in the foodpad of 5 mice in group II and III at 15 wk p.i.

3. Results and Discussion

3. 1. Construction of rAds/E2- It has already been demonstrated that the expression of some foreign genes interferes with adenovirus replication or produces cytotoxic effects [12]. To avoid these possibilities, a rAd with an inducible promoter (TR5) was constructed. In case of BVDV/E2 protein our results demonstrated that this protein had no cytotoxic effect or any interferance with adenovirus replication (data not shown). However, during the plaque purification, the co-expression of GFP by rAdTR5-DC/E2-GFP simplified the cloning selection, since GFP fluorescence can be easily visualized in live cells with a standard fluorescence microscope. The inconvenience of this system *in vivo* is its dependence on the second adenovirus (such as Ad5CMV-tTA) for expression of the tTA protein. This increases the manipulation time and causes the production of more anti-adenovirus antibodies. The problem could be resolved by constructing a rAd which expressed the tTA and

foreign protein together. This vector has already been constructed (unpublished results). We also constructed two rAds which express the BVDV/E2 protein under the control of two constitutive promoters, BM5 and CM5 which are modified MLP and CMV promoters, respectively.

3. 2. In vitro expression of the E2 protein of BVDV by rAds/E2- The cytoplasmic extracts of cells infected by rAds/E2 or BVDV were precipitated with the D89 MAb (Fig. 1). In addition to the E2 protein (53 kDa) in MDBK cells infected by BVDV (Fig. 1, lane 2), a protein of approximately 60 kDa was also precipitated. This protein was probably the E2p7 protein previously reported by Elbers *et al.* [4]. In 293A cells infected with either rAdTR5-DC/E2-GFP + Ad5CMV-tTA, rAdBM5/E2, or rAdCMV5/E2, the E2 protein was shown to have an identical molecular mass. However, the molecular mass of the E2p7 protein is slightly lower because the construct lacked 22 amino acids at the C-terminus (Fig. 1, lanes 4-6). No other proteins were precipitated from cells infected with Ad5/ΔE1ΔE3 + Ad5CMV-tTA (Fig. 1, lane 3). The three rAds/E2 appeared to express a similar level of the E2 protein of BVDV in 393A cells which was clearly higher than the BVDV/E2 protein expressed in MDBK cells.

To demonstrate that the immunized mouse sera contain specific antibodies for the BVDV/E2 protein, the cytoplasmic extracts of cells infected with BVDV were precipitated by a pool of (12 wk p.i.) mouse sera from each group. The E2 protein of BVDV could be precipitated by sera from groups II, III, and IV but not by sera from group I (data not shown). This data showed that the expressed proteins *in vivo* and *in vitro* were identical (or at least immunologically very similar) to the BVDV/E2 protein.

3. 3. Humoral immune response to rAds/E2- In this experiment, we investigated whether the constitutive and inducible replication-deficient rAds/E2, were capable of inducing an immune response to the BVDV/E2 protein. The mice

were immunized with rAds/E2 as described in "Materials and Methods". The sera were assayed for BVDV specific antibodies at a dilution of 1/160 by indirect ELISA (Table 1). BVDV specific antibodies could be detected in only one mouse at 3 wk p.i. in group II and IV whereas 6 mice in group III seroconverted. However, at 6 wk p.i. almost all the mice in group II to IV were seropositive to BVDV. No positive results were detected in group I.

The average optical density (O.D.) in each group of mice was used to evaluate the kinetics of the BVDV-specific antibody responses. The mice immunized by rAdCMV5/E2 (group III) showed a higher O.D. than groups II and IV (P value < 0.05, data not shown).

BVDV neutralizing antibodies against the homologous strain (BVDV/NADL strain) were detected in all mice in groups II, III and IV at 3 wk p.i. and the average neutralization titer increased following the subsequent injections to reach maxima of 1086 ± 601 (mean ± standard deviation), 1022 ± 612 and 1086 ± 601 respectively at 9 wk p.i. (Fig. 2). No significant differences in the BVDV neutralizing antibody titer between the three rAds/E2 viruses were observed during the experiment (P value > 0.05). No BVDV neutralizing antibodies were detected in group I (Ad5/ΔE1ΔE3 + Ad5CMV-tTA). The mouse sera did not neutralize the heterologous strain (BVDV/125) at minimal dilution 1/10 until the challenge at 12 wk p.i.

At the early stages of immunization (3 wk p.i.) the NT was more sensitive than ELISA at detecting the response against the BVDV/E2 protein. Neutralizing antibodies were detected in 100% (30/30) of the mice immunized with rAds/E2 at the minimal dilution 1/160 (data not shown). At the same dilution in ELISA, only 26% of samples (8/30) were seropositive (Table 1). Taken together, these results indicated that, in general, the NT was a more appropriate method than ELISA when recombinant or subunit vaccines, expressing the BVDV/E2 protein, are being studied.

3. 4. Cellular Immunity- The MNC from mice immunized with rAdBM5/E2 (group II) and rAdCMV5/E2 (group III) showed a proliferative response after stimulation with BVDV/NADL strain *in vitro* (Fig. 3), whereas no proliferative response was seen among Ad5/ΔE1ΔE3 + Ad5CMV-tTA (group I). The proliferative response was dose-dependent (data not shown). The stimulation index (SI = average counts per minute in antigen stimulated wells / average counts per minute in wells containing only cells with medium) for mice immunized with rAdBM5/E2 and rAdCMV5/E2 were 19.2 ± 8.1 (mean ± standard deviation), and 10 ± 6.1, respectively compared with 1 ± 0.6 for mice in the negative group (P value < 0.05). The data for group IV are not available. No significant proliferative response (P value > 0.05) was observed after stimulation with BVDV/125 strain (type 2).

The type of T cell response induced was further characterized by quantification of IFN-γ and IL-4 produced during the T cell proliferation responses. IL-12 and cytokines that modulate the effectiveness of IL-12, such as IFN-γ and IFN-α , are key regulators of Th1 differentiation [24-25], while IL-4 is a key regulator of Th2 differentiation [24]. MNC from mice immunized with rAdCMV5/E2 produced 10-fold more IFN-γ than those immunized with rAdBM5/E2 (836 ± 140 pg ml^{-1} compare with 74 ± 0 pg ml^{-1}, Fig. 4) when stimulated by the NADL strain. The difference between rAdBM5/E2 and rAdCMV/E2 in the production of IFN-γ is not easy to explain. Perhaps the different capacity of these two promoters for expression in different cell types, present at the injection site played a role. The greater production of IFN-γ (a Th1-type cytokine) by rAdCMV5/E2 compared with Ad5/ΔE1ΔE3 (P value < 0.05) could be an indicator of a potential specific Th1 response to the BVDV/E2 protein. However, this activation of Th1 was limited to the homologous virus (BVDV type 1) which was used for the construction of rAds/E2. No production of IL-4 (a Th2-type cytokine) was observed after stimulation of murine MNC with BVDV/NADL and 125 strain.

In a similar series of studies we recently constructed a recombinant fowlpox expressing the BVDV/E2 protein [8]. In this case the recombinant fowlpox induced a Th1 response which was demonstrated by the production of IFN-γ after stimulation of MNC with BVDV/NADL. However, no antigen specific proliferation responses were observed. This demonstrates that the recombinant vector plays an important role in the generation of the immune response against its added protein. This was further illustrated during experiments using DNA vaccine vectors for E2. In this case the recombinant adenoviruses expressing the BVDV/E2 protein produced neutralizing antibodies at least 60 times higher than a DNA plasmid expressing the same gene in the mouse model [20].

The presence of memory response to the BVDV/E2 protein was investigated with 5 mice from groups II and III after footpad injection of BVDV/NADL at 15 wk p.i. There was a significant increase in neutralization antibody titer in sera from groups II and III (P value < 0.05). During the two weeks following the challenge, an initial titer of 560 ± 160 (mean $\pm$ standard deviation) for both groups increased to 3840 ± 1478 (6.85-fold) and 4160 ± 1920 (7.42-fold), respectively. This suggested the presence of a strong memory response to the BVDV/E2 protein. A combination of a recombinant adenovirus vaccine followed by a traditional BVDV vaccine would seem to be a very effective strategy. The mouse sera after challenge also neutralized the BVDV type 2 (BVDV/125 strain) *in vitro* with a titer 48 ± 17 and 40 ± 0 for groups II and III respectively. These heterotypic immune responses are interesting because an efficient vaccine against BVDV should neutralize viruses belonging to both genotypes of BVDV. However, as expected, the best results were observed with homologous virus (BVDV/NADL strain).

In conclusion, the mice immunized with rAds/E2 produced a strong humoral immune response against the BVDV/E2 protein. In addition, the proliferation of murine MNC after stimulation by the NADL strain is an indicator of a cell-mediated

response. We observed an increase in T-helper cell proliferation in murine MNC vaccinated by rAdBM5/E2 and rAdCMV/E2, and also an increase in the production of INF-γ in supernatant of stimulated murine MNC immunized by rAdCMV/E2. In addition, we have shown that a recombinant adenovirus with an inducible promoter not only can express a gene *in vivo* but is just as efficient as a consecutive promoter for induction of humoral immunity against BVDV/E2 protein.

Acknowledgements

We thank Dr. H. C. Minocha for providing the D89 MAb. We also thank Dr. Y. Chorfi and S. St-Onge for their contributions to lymphoproliferation test.

References

[1] Wengler, G., Bradley, D.W., Collett, M.S., Heinz, F.X., Schesinger, R.W. and Strauss, J. (1995) Flaviviridae, p. 415-427. In Murphy, F.A., Fauquet, C.M., Bishpo, D.H.L., Ghabrial, S.A., Jarvis, A.W., Martelli, G.P., Mayo, M.A. and Summers, M.D. (ed), Virus taxonomy. Sixth report on the International Committee on Taxonomy of Viruses. Springer-Verlag. New York.

[2] Harpin, S., Elahi, S.M., Cornaglia, E., Yolken, R.H. and Elazhary, Y. (1995) The 5′-untranslated region sequence of a potential new genotype of bovine viral diarrhea virus. Arch. Virol. 140, 1285-1290.

[3] Pellerin, C., Van Den Hurk, J., Lecomte, J. and Tijssen, P. (1994) Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology 203, 260-268.

[4] Bolin, S., Moenning, V., Gourley, N.E.K. and Ridpath, J. (1988) Monoclonal antibodies with neutralizing activity segregate isolates of bovine viral diarrhea virus into groups. Arch. Virol. 99, 117-123.

[5] Donis, R.O., Corapi, W. and Dubovi, E.J. (1988) Neutralizing monoclonal antibodies to bovine viral diarrhea virus bind to the 56K to 58k glycoprotein. J. Gen. Virol. 69, 77-86.

[6] Elbers, K., Tautz, N., Becher, P., Stoll, D., Rumenapf, T. and Thiel, H-J. (1996) Processing in the Pestivirus E2-NS2 region: Identification of proteins p7 and E2p7. J. Virol. 70, 4131-4135.

[7] Kimman, T.G., Bianchi, A.T., Wensvoort, G., de Bruin, T.G. and Meliefstec, C. (1993) Cellular immune response to hog cholera virus (HCV): T cell of immune pigs proliferate in vitro upon stimulation with live HCV, but the E1 envelope glycoprotein is not a major T-cell antigen. J. Virol. 167, 2922-2927.

[8] Elahi, S.M., Bergeron, J., Nagy, É., Talbot, B.G., Harpin, S., Shen, S.H. and Elazhary, Y. (1999) Induction of humoral and cellular immune responses in mice by a recombinant fowlpox virus expressing the E2 protein of bovine viral diarrhea virus. FEMS Microbiol. Lett. 171, 107-114.

[9] Eloit, M. and Adam, M. (1995) Isogenic adenoviruses type 5 expressing or not expressing the E1A gene: efficiency as virus vectors in the vaccination of permissive and non-permissive species. J. Gen. Virol. 76, 1583-1589.

[10] Gerard , R.D. and Meidell, R.S. (1993) Adenovirus-mediated gene transfer. Trends in Cardiovascular Medicine. 3, 171-177.

[11] Massie, B., Dionne, J., Lamarche, N., Fleurent, J. and Langelier, Y. (1995) Improved adenovirus vector provides herpes simplex virus r ibonucleotide Reductase R1 and R2 subunits very efficiently. Biotechnology (NY) 13, 602-608.

[12] Massie, B., Couture, F., Lamoureux, L., Mosser, D.D., Guilbault, C., Jolicoeur, P., Bélanger, F. and Langelier, Y. (1998*a*) Inducible overexpression of toxic protein by an adenovirus vector with a tetracyclin-regulatable expression cassette. J. Virol. 72, 2289-2296.

[13] Massie, B., Mosser, D.D., Koutroumanis, M., Vitte-Mony, I., Lamoureux, L., Couture, F., Paquet, L., Guilbault, C., Dionne, J., Chahla, D., Jolicoueur, P. and Langelier, Y. (1998*b*) New adenovirus vectors for protein production and gene transfer. Cytotechnology 28, 53-64. "In press"

[14] Xu, Z.Z., Krougliak, V., Pervec, L., Graham, F.L. and Both, W. (1995) Investigation of promoter function in animal cells infected with human recombinant adenoviruses expressing rotavirus antigen VP7sc. J. Gen. Virol. 76, 1971-1980.

[15] Mosser, D.D., Caron, A.W., Bourget, L., Jolicoeur, P. and Massie, B. (1997) Use of a dicistronic expression cassette encoding the green-fluorescent protein for the screening and selection of cells expressing inducible gene products. Bio-Techniques 22, 150-161.

[16] Gossen, M., Bonin, A.L., Freundlieb, S. and Bujard, H. (1994) Inducible gene expression systems for higher eukaryotic cells. Current Opinion In Biotechnology 5, 516-520.

[17] Graham, F.L., Smiley, J., Russell, W.C. and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. 36, 59-74.

[18] Garnier, A., Coté, J., Nadeau, I., Kamen, A. and Massie, B. (1994) Scale-up of the adenovirus expression system for the production of recombinant protein in human 293S cells. Cytothechnology 15, 145-155.

[19] Gluzman, Y., Reichl, H. and Solnick, D. (1982) Helper-free adenovirus type 5 vectors, p. 187-192. In Y. Gluzman (ed), Eucaryotic viral vectors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

[20] Harpin, S., Talbot, B., Mbikay, B. and Elazhary, Y. (1997) Immune response to vaccination with DNA encoding the bovine viral diarrhea virus major glycoprotein gp53 (E2). FEMS Microbiol. Lett. 146, 229-234.

[21] Mittereder, N., March, K. and Trapnell, B.C. (1996) Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. J. Virol. 70, 7498-7509.

[22] Xue, W., Blecha, F. and Minocha, H.C. (1990) Antigenic variations in bovine viral diarrhea viruses detected by monoclonal antibodies. J. Clin. Microbiol. 28, 1688-1693.

[23] Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

[24] Bardley, L.M., Yoshimoto, M.K. and Swain, S.L. (1995) The cytokines IL-4, IFN- , and IL-12 regulate the development of subsetes of memory effector helper T-cells in vitro. J. Immunol. 155, 1713-1724.

[25] Reiner, S.L. and Seder, R.A. (1995) T helper cell differentiation in immune response. Curr. Opin. Immunol. 7, 360-366.

Group	Virus	Dose	Route	BVDV specific antibody				
				0 wk†	3 wk	6 wk	9 wk	12 wk
I	Ad5/ΔE1ΔE3 + Ad5CMV-tTA	$1 \times 10^9 + 1 \times 10^9$	i.m.	‡0/10	0/10	0/10	0/10	0/10
II	rAdBM5/E2	1×10^9	i.m.	0/10*	1/10**	9/10***	10/10***	10/10***
III	rAdCMV5/E2	1×10^9	i.m.	0/10*	6/10***	10/10***	10/10***	10/10***
IV	rAdTR5-DC/E2-GFP + Ad5CMV-tTA	$1 \times 10^9 + 1 \times 10^9$	i.m.	0/10*	1/10*	10/10***	10/10***	10/10***

Table 1. Serum antibody responses of mice following administration of parental or recombinant adenoviruses

Groups of mice were immunized three times at 0, 3 and 6 weeks post-infection (wk p.i.) with the different purified parental or recombinant adenoviruses at 10^9 plaque forming unite (p.f.u)/mice for each virus by intramuscular (i.m.) route. Mice were bled at the times indicated and the sera were diluted 1/160 and tested for BVDV specific antibodies by ELISA. In ELISA the mouse sera with optical density higher than 0.2 at dilution 1/160 were considered as positive. The difference between optical density of group II, III and IV with group I since 3 wk p.i. was significant in a Pearson's *t*-test (two tailed). *P > 0.05 (non-significant), **P < 0.005, ***P < 0.0005

† Time post-infection

‡Number of positive mice/ number of total mice

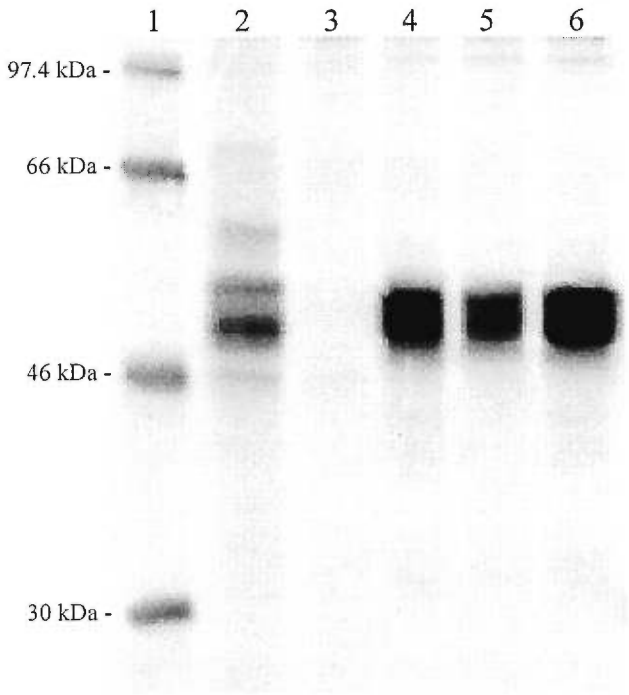

Fig. 1. Expression of BVDV/E2 protein by various promoters.
The MDBK infected cells with BVDV/NADL (lane 2), and 293A cells infected by Ad5/ΔE1ΔE3 + AdCMV5-tTA (lane 3), rAdBM5/E2 (line 4), rAdCMV5/E2 (lane 5) or rAdTR5-DC/E2-GFP + Ad5CMV-tTA (line 6) were radiolabelled for 2 h using [^{35}S] methionine/ [^{35}S]cysteine from 16 to 18 h p.i. The E2 protien was precipitated by D89 Mab. All lanes are from same autoradiograph. The markers for protein molecular weights are shown in lane 1.

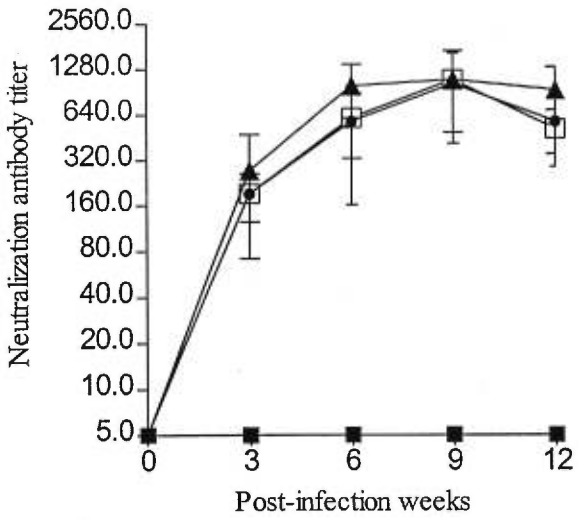

Fig. 2. The kinetics of neutralizing antibody titers to BVDV/NADL strain in post vaccination mouse sera.

Serial two-fold dilutions (starting with 1: 10) of the mouse sera were tested in a neutralizing test for detection of neutralizing antibodies against the E2 protein of BVDV. Data are presented as group means ± standard deviation. ■; group I (Ad5/ΔE1ΔE3 + Ad5CMV-tTA), ; group II (rAdBM5/E2), ●; group III (rAdCMV5/E2), ▲; group IV (rAdTR5-DC/E2-GFP + Ad5CMV-tTA). A Neutralizing titer of 5 in this figure represents all the mouse sera with a neutralizing titer of less than 10 (minimum dilution used in this study) . No BVDV neutralizing antibodies were detected in group I (Ad5/ΔE1ΔE3 + Ad5CMV-tTA). No significant difference in the BVDV neutralizing antibody titer between the three rAds/E2 viruses was observed during the experiment (P value > 0.05).

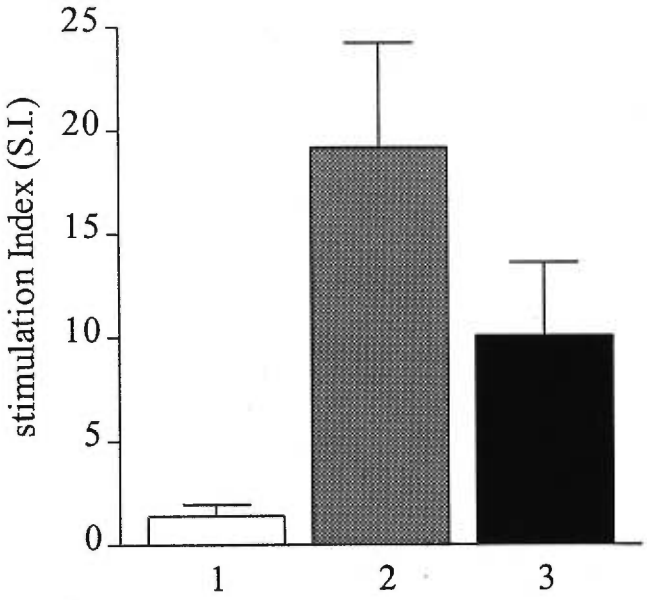

Fig. 3. Proliferation response of murine mononuclear cells stimulated with BVDV/NADL strain.

Murine mononuclear cells of mice in group I, II and III stimulated *in vitro* by BVDV/NADL strain (type 1). The Stimulation index (SI) for each group was calculated by the following formula: SI = average counts per minute in antigen stimulated wells / average counts per minute in wells containing only cells with medium. 1; group I (Ad5/ΔE1ΔE3 + Ad5CMV-tTA), 2; group II (rAdBM5/E2), 3; group III (rAdCMV5/E2). Only the results of stimulation with optimal dilution were presented in this figure. Results are the mean ± standard deviation and represent three experiments. The difference between the groups II and III with group I was significant (P value < 0.05).

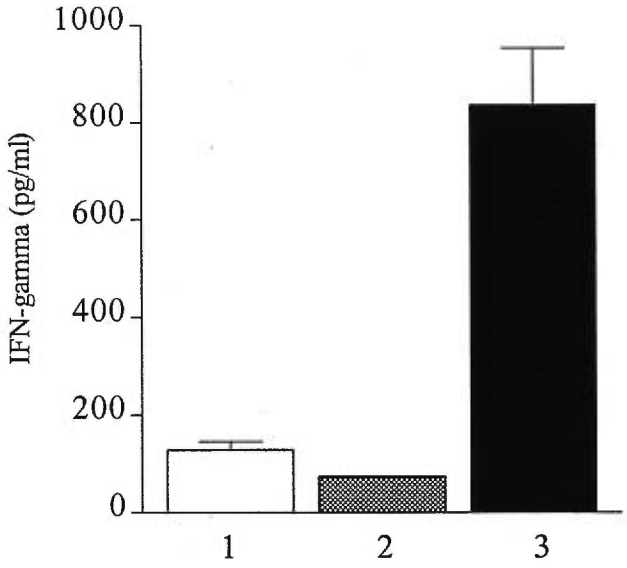

Fig. 4. INF-γ production from murine mononuclear cells stimulated by BVDV/NADL.

The concentration of the IFN-γ was shown as gp/ml^{-1} of supernatant. 1; group I (Ad5/ΔE1ΔE3 + Ad5CMV-tTA), 2; group II (rAdBM5/E2), 3; group III (rAdCMV5/E2). Results are the mean ± standard deviation and represent two experiments. The difference between the groups III with groups I and II was significant (P value < 0.05).

CHAPTER V

Investigation of the immunological properties of the Bovine Viral Diarrhea Virus protein NS3 expressed by an adenovirus vector in mice

Seyyed Mehdy Elahi[1], Shi-Hsiang Shen[2], Serge Harpin[1], Brian Geoffrey Talbot[3] and Youssef Elazhary[1]*

Archives of Virology "in press"

[1]Virology Section, Faculty of Veterinary Medicine, University of Montreal, P. O. Box 5000, Saint-Hyacinthe, Quebec, J2S 7C6, Canada

[2]Biotechnology Research Institute, National Research Council, Montreal, Quebec, H4P 2R2, Canada

[3]Department of Biology, Faculty of Sciences, University of Sherbrooke, Quebec, J1K 2R1, Canada

*Author to whom correspondence should be addressed.

Phone: (450) 773-8521 ext. 8201

Fax: (450) 778-8105

E-mail: elazhary@medvet.umontreal.ca

Running title: Role of the NS3 protein of BVDV in cellular immunity

Summary

Two replication-defective human adenovirus recombinants encoding the NS3 protein (p80) of bovine viral diarrhea virus (BVDV) under the control of a modified adenovirus major later promoter (BM5), rAdBM5/NS3, and human cytomegalovirus promoter (CMV5), rAdCMV5/NS3, were constructed. These two recombinant adenoviruses were tested for their expression of the NS3 protein *in vitro* in three different cell lines and also *in vivo* for the induction of BVDV-specific immune responses in mice. The recombinant adenoviruses containing two different promoters induced different levels of humoral responses to the NS3 protein. The rAdBM5/NS3 was used to vaccinate mice in order to evaluate the ability of the NS3 protein in the induction of cellular immune responses. The rAdBM5/NS3 did not cause a stimulation of cell proliferation but caused a very strong increase in production of IFN-γ in murine mononuclear cells stimulated *in vivo* by BVDV strains of genotype 1 and 2.

Introduction

The bovine viral diarrhea virus (BVDV) is a small enveloped RNA virus belonging to the pestivirus and it has become one of the most important viral pathogens of cattle. This genus, together with the genus flavivirus and the hepatitis C virus group, forms the family *Flaviviridae* [37]. The BVDV genome consists of a single open reading frame of approximately 12.5 kb which is translated into a precursor polyprotein. Proteolytic cleavage of the polyprotein results in the formation of the core, envelope and non-structural proteins in the following order, NS2-3-NS4A-NS4B-NS5A-NS5B [35]. Field isolates of BVDV can be divided into cytopathic (cp) and noncytopathic (ncp) biotypes according to their ability to induce cytopathology in bovine cell culture. On the basis of recent data, cp BVDV strains develop from ncp BVDV strains as a consequence of alterations in their genome such as host-cell derived insertions and genome rearrangements. Sometimes these are

combined with large duplication or deletions of viral sequences [26]. Cp BVDV strains express NS3 (p80) which is regarded as a molecular marker for cp BVDV. However, ncp viruses express NS2-3 (p125), which is also present in cells infected with cp BVDV [6-7]. In addition, BVDV strains are separated into two genotypes, type 1 and type 2, on the basis of differences in the viral genome and antigenic differences [16, 28, 31]. An efficient vaccine has to protect the animal against all types of virus.

The flavivirus NS3 proteins are trypsin-like serine proteases which are responsible for the cleavage of non-structural precursor proteins [3]. They are the major and highly immunogenic viral proteins found in virus-infected cells [4]. Studies with other flaviviruses have shown that the non-structural proteins such as NS3 are strong stimulators of cellular immunity and the envelope proteins are relatively weak [17, 23, 32]. Monoclonal antibodies to NS3 of dengue 1 virus (a flavivirus) induce a passive protection in mice [34] and NS3 of hepatitis C virus induce a strong T-lymphocyte response [8]. Recently a proliferative response to the NS3 protein of BVDV was reported after vaccination by BVDV and stimulation *in vitro* with a recombinant NS3 protein [21]. These observations from other members of the *Flaviviradea*, encouraged us to evaluate the ability of BVDV/NS3 protein to stimulate humoral and cellular immune responses. The present report describes the immune responses to recombinant NS3 in mice using a human adenovirus expression vector [13]. Two recombinant adenoviruses were constructed expressing the NS3 protein of BVDV under the control of two different promoters. These recombinant adenoviruses were evaluated *in vitro* in different cell lines for the expression of the NS3 protein. The constructs were also evaluated for their ability to induce humoral and cellular immune responses in mice.

This is the first of a series of studies leading to the development of recombinant viral vectors for BVDV vaccination. The experiments to examine the immune responses to such recombinant proteins were carried out in mice because the

immune mechanisms are well known, significant numbers of animals can be used and inbred lines are available. Despite the differences in immune responses between the two mammals, almost all the data on T and B cell epitopes has been obtained using monoclonal constructs which were the result of the murine immune response to BVDV proteins. These have proved to mimic very well the bovine response.

Materials and Methods

Cultures and Viruses: Human 293 cells were used, either as the original anchorage-dependent 293A line [15], or as the anchorage-independent clone 293S, as described previously [12, 24]. Madin-Darby Bovine Kidney (MDBK) cells (free of BVDV), and HeLa cells were obtained from the American Type Culture Collection (ATCC, Rockville , MD) and grown in Dulbecco's Modified Eagle medium, supplemented with 5% fetal bovine serum (FBS, free of antigen and antibody against BVDV). The NADL strain of BVDV (type 1) from the ATCC and the 125 strain (BVDV type 2) from the USDA (Ames, Iowa, USA) were propagated in MDBK cells in the presence of 2% FBS. Human adenovirus type-5 with deletions in the E1 and E3 regions, Ad5/ΔE1ΔE3, [14] was obtained from Dr. B. Massie (Biotechnology Research Institute, Montreal, Canada) and was amplified in 293S cells.

RNA extraction, cDNA synthesis and PCR: BVDV-RNA was extracted according to the acid guanidinium thiocyanate/ phenol-chloroform extraction method of Chomcyzynski and Sacchi [5]. cDNA synthesis was carried out using the SuperScript pre-amplification system kit (GibcoBRL). The target sequence, 2227 bp (nucleotide 5501-7738), was amplified with forward primer, 5′-TAGATCTACCATG GGGATCATGCCAAGGGGGAC TAC-3′, and reverse primer, 5′-TAGATCTCTAG GATTTACCAAACTCCAGTCC-3′. To facilitate the cloning of the PCR product, a *Bg*l II restriction site was added to both primers. The forward primer contained an in-frame translational start codon and the reverse primer contained an in-frame 3′ translation stop codon.

Construction of plasmids: The purified cDNA fragments were cloned in a pGEM-T vector (Promega) generating pGEM-T/NS3. The *Bg*l II / *Bg*l II NS3 fragment was excised from pGEM-T/NS3 and inserted into unique *Bam*H I sites in pAdBM5 [24] and pAdCMV5 [25]. The resulting constructs were named pAdBM5/NS3 and pAdCMV5/NS3 respectively. This manipulation placed the NS3 gene under the control of the MLP or CMV promoters, respectively.

Construction of recombinant adenoviruses: The shuttle plasmids were linearized at the unique *Cla*I site and rescued separately into the genome of Ad5/ΔE1ΔE3 by homologous recombination in 293A cells, as described [2]. Upon co-transfection, virus plaques were isolated, amplified in 293A cells, and analyzed for the presence of the transgene by PCR. The NS3 protein expression by the positive viral plaques was confirmed by immunoprecipitation. Finally, for each construct, one recombinant adenovirus was subjected to three consecutive rounds of plaque purification and viral stocks were prepared from 293S cells. The recombinant adenoviruses rAdBM5/NS3 and rAdCMV5/NS3 (together referred to as rAds/NS3) were purified on CsCl gradients. Viral stocks were titrated on 293A cells using a plaque assay [27].

Radio-labeling and preparation of cellular extracts: Confluent monolayers of 293A cells, MDBK cells or HeLa cells were infected with rAdBM5/NS3, rAdCMV5/NS3 or Ad5/ΔE1ΔE3 at an m.o.i. of 20 p.f.u./cell for 293A cells and 100 p.f.u. for MDBK cells and HeLa cells. The MDBK cells also were infected with the NADL strain of BVDV at a multiplicity of 5 p.f.u./cell. At 4, 8, 12, 16, 20 h post-infection (h.p.i.) for the 293A cells and at 16 h. p.i. for the MDBK cells and HeLa cells they were labeled with [^{35}S] methionine/ [^{35}S]cysteine (Amersham; 100 μCi per dish) for 2 h. The proteins were immunoprecipitated with the BVDV-NS3 specific monoclonal antibody (MAb) WB-1 (Dr. A. D. Shannon, Elizabeth Agriculture Institute, Camden, New South Wales, Australia), using Protein A Sepharose before being analyzed by SDS-PAGE [20].

Immunization experiments: In the first series of experiments, five groups of 10 inbred mice (BALB/c) were vaccinated with 5 X 10^8 p.f.u. of purified Ad5/ΔE1ΔE3 (group I), rAdBM5/NS3 (group II & IV) or rAdCMV5/NS3 (group III & V). Two routes of immunizations were used; intramuscular (i.m.; for groups I, II & III) and intranasal (i.n.; for groups IV & V) (Table 1). The booster immunization were performed with the same protocol at 3, 6 and 12 wk p.i. Blood was collected by orbital plexus puncture at 0, 3, 6, 9, 12, 15 and 18 wk p.i. On the basis of the results from these groups, a second experiment was carried out by vaccinating i.m. two groups of 6 BALB/c mice with 5 X 10^8 p.f.u. purified, Ad5/ΔE1ΔE3 (group VI) and rAdBM5/NS3 (group VII) at 0 and 4 wk p.i.

Enzyme-Linked Immunosorbant Assay (ELISA)- The detection of BVDV specific antibodies in mouse sera involved an indirect ELISA technique based on coating 96 well microplates with lysate from MDBK cells infected by the NADL strain of BVDV as described previously [10].

Proliferation response of murine mononuclear cells (MNC)- At 7 wk p.i., the murine MNC immunized by Ad5/ΔE1ΔE3 (group VI) or rAdBM5/NS3 (group VII) were stimulated *in vitro* by non-purified BVDV strains of NADL or 125 as described previously [10] with one modification. The stimulated murine MNC were kept in the presence or absence of 30 μM of indomethacin, an inhibitor of prostaglandin E2 [33]. The stimulation index (SI) was calculated as follows: SI = average counts per minute in antigen stimulated wells / average counts per minute in wells containing only cells with medium.

NS3 ELISA : As the NS3 protein is a non-structural protein, the concentration of this protein in viral stocks was compared by a double sandwich ELISA (a commercial kit which was developed in our laboratory and was approved by Food and Agriculture Canada) in which polyclonal anti-NS2-3 is used for antigen capture and monoclonal anti-NS2-3 is the second antibody.

Cytokine production assay: 1.5 X 10^6 murine MNC were stimulated with non-purified BVDV (NADL strain and/or 125 strains) or non-stimulated [10], in the presence of 30 μM of indomethacin. Three days later, 100 μl of supernatant was used in a cytokine ELISA assay (PharMingen) for detection of IL-2 and IFN-γ (Th1-type cytokines) and IL-4 (a Th2-type cytokine).

Results

Construction of rAds/NS3: On the basis of previously (1993) published results [36], the presumed NS3 coding region was amplified by PCR and subsequently cloned in pGEM-T to be finally transferred into shuttle plasmids. According to the recent studies [18, 38], our construct lacked the first 26 amino acids of the NS3 protein and contained the whole NS4A protein region including the first 25 amino acids of the NS4B protein. The rAdBM5/NS3 and rAdCMV5/NS3 were constructed by co-transfection of Ad5/ΔE1ΔE3 viral DNA and the shuttle plasmids in 293A cells. The expression of BVDV/NS3 protein in rAdBM5/NS3 and rAdCMV5/NS3 was under the control of the BM5 and the CMV5 promoters respectively.

In vivo expression of the NS3 protein of BVDV by rAds/NS3: Three cell lines were used to compare the ability of the MLP (BM5) and CMV (CMV5) to express the BVDV-NS3 protein. The 293A cell line was permissive for ΔE1ΔE3 adenovirus expression, the other two non-permissive lines were the BVDV target cell line MDBK and the human Hela cell line. The NS3 protein appeared as an 80 kDa polypeptide in immunoprecipitated lysates of MDBK cells infected with BVDV (Fig 1*a*. & Fig 1*b*. lane 2). In contrast, the recombinant NS3 protein expressed by rAds/NS3 appeared as two bands. The higher molecular weight band of about 87 kDa is the estimated size for the uncleaved polypeptide product expressed by the constructs. It contained the truncated NS3 protein (77 kDa), the NS4A protein (7.1 kDa) and the 25 first amino acids of NS4B (2.7 kDa). The lower band of 77 kDa was the NS3 protein after a clivage event (e.g. Fig 1*a*. lanes 4-5 and 7-8). There was no

band of similar size in cells which were infected by the parental adenovirus (Fig 1*a*. lanes 3, 6 & 9). A difference was observed between the level of expression induced by the BM5 promoter and the CMV5 promoter in different cells. In the human 293A cells, which support the replication of the adenovirus, expression from the BM5 promoter in rAdBM5/NS3 was higher than the CMV5 promoter in rAdCMV5/NS3 (Fig. 1*a*, lanes 4-5). However, in the MDBK cells, which are non-replicative for the E1E3 deleted adenoviruses, expression levels from rAdBM5/NS3 and rAdCMV5/NS3 were similar to each other but lower than 293A cells (Fig. 1*a*, lanes 7-8). In HeLa cells, expression from any of the rAds/NS3 was barely detectable (Fig. 1*a*, lanes 10-11).The kinetics of NS3 protein expression under the control of the BM5 or CMV5 promoter were also investigated. The 293A cells after 2 h of metabolic labeling at different times post-infection were lysed and BVDV/NS3 protein was immunoprecipitated. Expression from the BM5 promoter in rAdMB5/NS3 was low at 10 h p.i. (Fig. 1*b*. lane 4), peaked at 18 h. p.i. and then declined slightly at 22 h. p.i. (Fig. 1*b*. lanes 5-7). In contrast, expression from the CMV5 promoter in rAdCMV5/NS3 was always lower than that from the BM5 constructs, yet it was detectable as early as 6 h. p.i., and increased to a maximum from 6-18 h, (Fig. 1*b*., lanes 8-12).

Humoral immune response to rAds/NS3: In this experiment, the replication-deficient rAds/NS3 were studied for their ability to induce an immune response to the BVDV/NS3 protein. Groups of 10 mice (BALB/c) were immunized with rAds/NS3 or Ad5/ΔE1ΔE3 by i.m. or i.n. routes. The sera were assayed for the presence of BVDV specific antibodies at a dilution of 1/160 by indirect ELISA. Table 1 shows that the humoral response to the NS3 protein following i.m. immunization with rAdBM5/NS3 (group II) was detectable as early as 3 wk p.i. in 8 out of 10 mice. The i.n. administration (group IV) was less efficient than i.m. In fact, only one mouse had seroconverted at 6 wk p.i. However, at 18 wk p.i. 7 out of 10 mice were seropositive. When rAdCMV5/NS3 was used for i.m. immunization (group III), only 3 mice were

seropositive at 3 wk p.i. this increased to 8 at 18 weeks. Intranasal immunization with rAdCMV5/NS3 (group V) gave similar results to rAdBM5/NS3.

The kinetics of BVDV-specific antibody responses in mouse sera were also monitored at various times p.i. by ELISA. The average optical density (O.D.) in each group of mice was used for evaluation of the kinetics of the BVDV specific antibody responses. The results shown in figure 2 indicate that there were significant differences ($p < 0.05$) in the pattern of O.D. changes for mouse sera in group II (rAdBM5/NS3) and group III (rAdCMV5/NS3) after i.m. injection. However, no significant difference was observed between these two recombinant adenoviruses after i.n. injection (group IV and V), ($p > 0.05$).

Cellular Immunity- The T-cell response was further characterized by quantification of IL-2, IFN-γ and IL-4 produced during T-cell proliferative responses from MNC stimulated by homologous and heterologous virus. IL-12 and cytokines that modulate the effectiveness of IL-12, such as, IFN-γ and IFN-α , are key regulators of Th1 differentiation, while IL-4 is a key regulator of Th2 differentiation [1, 30]. The MNC from mice immunized by rAdBM5/NS3 (group VII) produced 3726 ± 73 (mean ± standard deviation) pg/ml of IFN-γ after stimulation by the NADL (type 1) strain and 1180 ± 10 pg/ml after stimulation by the 125 (type 2) strain (Fig. 3). The concentration of IFN-γ in the supernatant of non-stimulated cells was not significantly different between groups VI and VII ($p > 0.05$). The concentration of IL-2 also appeared to increase in the supernatant of MNC of mice immunized with rAdBM5/NS3 but, due to the variability of the results, the differences with the negative group (Ad5/ΔE1ΔE3) were not significant ($p > 0.05$, data was not shown).

No increase in IL-4 was observed in the supernatants of stimulated murine MNC from mice immunized by rAdBM5/NS3 (group VII). The MNC also did not show a proliferative response after stimulation with NADL or the 125 strain of

BVDV *in vitro*. Nor was the proliferative response of MNC restored by *in vitro* treatment with indomethacin (data not shown).

Discussion

In vivo expression of NS3 protein: The NS3 protein expressed by rAds/NS3 appeared as a mixture of 77 and 87 kDa polypeptides after immunoprecipitation and SDS-PAGE. These bands correspond to the cleaved and uncleaved proteins expressed by the virus and suggest that the NS3 protease was partially inactivated in these constructs. This is in contrast to results reported by Kümmerer *et al.* [18] which demonstrated that constructs containing N-terminal deletions of the NS2-3-NS4A-NS4B, completely retained their protease activity for cleavage at the NS3-NS4A site. The additional deletion in our constructs of 81 amino-acids (55 from the C-terminal of NS2 and 26 from the N-terminal end of NS3) was probably responsible for the partial loss of the protease activity of NS3 protein and suggested that protein integrity may be necessary for the complete activation of NS3.

In a previous study we have shown that there was no difference in the ability of the BM5 and CMV promoters to drive the expression of the E2 BVDV protein (unpublished data). The present results however, show that in 293A cells, the expression of NS3 protein by the BM5 promoter was higher than that of the CMV promoter. Since NS3 is a protease, it is possible that the earlier expression of NS3 by rAdCMV5/NS3 in 293A cells (as demonstrated in Fig. 1*b*) interfered with viral replication and then down-regulated the expression of the NS3 protein. This hypothesis is supported by the observation that the rAds/NS3 always generated at least a 10 fold lower titer of virus stocks compared with those of the original Ad5/ΔE1ΔE3 and our recombinant adenovirus vectors which express the BVDV structural protein E2. However, the determination of the exact interaction of NS3 with adenovirus replication is beyond the scope of this article.

Induction of humoral immune response: The mouse sera were analyzed by an indirect ELISA for BVDV/NS3 antibodies in order to determine which of the promoters would be best to use in immune response studies. The two recombinant adenoviruses induced different levels of humoral responses to the NS3 protein. A comparison of the antibody produced by i.m. and i.n. immunization shows that the rAdBM5/NS3 was more efficient than rAdCMV5/NS3 for production of BVDV specific antibodies after i.m. injection. The lack of a strong immunization after i.n. injection could be a consequence of our injection method. For i.n. injection the viral solution was delivered drop-wise. A major portion of the virus could have been lost by swallowing. This hypothesis was supported by measuring the anti-adenovirus neutralizing antibodies in mouse sera in group III and V. We detected a considerable amount of the anti-adenovirus neutralizing antibodies as early as 3 wk p.i. in mice sera after i.m. vaccination with rAdCMV5/NS3 and the titer rose after each immunization. In the mice immunized i.n. with same virus, the titer of anti-adenovirus neutralization antibodies was lower than those immunized by the i.m. route. (data not shown). The rAdBM5/NS3 construct was used for later experiments because the overall results were better than rAdCMV5/NS3.

Induction of cellular immune response: NS3 is acknowledged to be responsible for the production of a high titer non-neutralizing antibody during BVDV infection [4]. Since this is an intracellular protein which is synthesized during viral replication, it is likely that it stimulates a cellular response specific for infected cells. An extra-cellular humoral response would not be expected to have any effect on the virus. The results demonstrate however, a remarkable focus of the immune response to the recombinant NS3. There was a considerable increase in IFN-γ production by virus-stimulated MNC from mice vaccinated with rAdBM5/NS3 which would suggest the activation of a Th1 response. This is supported by the lack of production of IL-4 by stimulated MNC. These results confirm those of Diepolder *et al.* [8], who demonstrated that Hepatitis C virus NS3-specific T-cell clones produced considerable

amounts of IFN-γ and variable amounts of IL-4. In our study no proliferative responses were observed after stimulation of murine MNC with BVDV/NADL or 125 strains. This was unexpected since Lambot *et al.* [21] demonstrated a proliferative response after vaccination of cattle with cp and ncp BVDV and stimulation *in vitro* with an NS3 recombinant protein. However, the action of IFN-γ and prostaglandins (produced via activated macrophages) as inhibitors of cell proliferation are very well documented [22, 29, 33] and explain our results. By including indomethacin in our assays we could exclude the intervention of prostaglandin E in the inhibition of cell proliferation [29, 33] and assume that it was caused by the IFN-γ. This cytokine release appears to be NS3 specific since only slight increases (about 296 pg/ml after stimulation with BVDV/NADL) were demonstrated in mice immunized by Ad5/ΔE1ΔE3. It remains to be determined whether or not the IFN-γ stimulated by the NS3 protein has a "bystander" effect on the immune response to other BVDV proteins. The results could also be interpreted as being applicable only at the levels of stimulating protein used in our assays. It is quite possible that as the virus infection increases the direction of the immune response changes.

The lack of a significant increase in IL-2 concentration could be caused by the IFN-γ since it is a powerful inducer of inducible nitric oxide synthesis which can inhibit production of IL-2 by Th1 cells [22].

Heterologous activation- The heterologous activation of MNC after stimulation by type 2 BVDV (125 strain) could be a consequence of the homology of this protein between these two genotypes and suggests a partial cross-reactivity at the T-cell level. However, as expected, the best results were observed after homologous stimulation. We excluded the hypothesis that the difference in IFN-γ induction between two BVDV types was caused by the differences in concentration of NS3 in virus preparation by comparing the concentration of NS3 in viral stocks. The BVDV/NADL and BVDV/125 strains at the dilution 1/10 (final dilution for murine

MNC stimulation in our experiment) in a double sandwich ELISA had very similar O. D. (≈ 3.4 and ≈ 3.2 respectively). These data showed that the two virus stocks contained similar quantities of NS3. However, if the differences in IFN-γ production were dependent on such small differences in concentration of NS3, as were demonstrated by ELISA, then we should have expected that the heterologous response would have been stronger than that which we observed.

Our construct also contained the coding sequence for NS4A. According to the available data about the role of the non-structural proteins of *Flaviviridae* in the induction of humoral and cellular immune responses [8, 19, 32] it is unlikely that the NS4A protein has any significant effect. It is thought that NS3 is the major immunogenic protein in our vector.

Overall our data show that the NS3 protein can be efficiently produced by an adenovirus recombinant vector under the control of different promoters. The results of immunization with this vector implicate NS3 in the alteration of the cytokine environment during BVDV infections. The addition of the NS3 protein in subunit or recombinant vaccines which contain only the structural protein or in inactivated vaccines would be an interesting asset in the induction of immune responses against BVDV in cattle.

Acknowledgments

The authors wish to thank Dr. B. Massie for his gift of the Ad5/ΔE1ΔE3 and the shuttle plasmids, pAdBM5 and pAdCMV5, and especially for his invaluable support and criticism. We acknowledge the technical assistance of N. Jolicoeur for contributing to the construction of plasmid pGEM-T/NS3. We thank Dr. A. D. Shannon for providing the MAb WB-1. Dr. S. Mehdy Elahi is supported in part by a scholarship from the Islamic Republic of Iran.

References

[1] Abbas AK, Murphy KM, Sher A (1996) Functional diversity of helper T lymphocytes. Nature 383: 787-793.

[2] Acsadi G, Jani A, Massie B, Simoneau M, Holland P, Blaschuk K, Karpati G (1994) A differential efficiency of an adenovirus-mediated *in vivo* gene transfer into skeletal muscle cells of different maturity. Hum. Mol. Genet. 3: 579-584.

[3] Bazan JF, Fletterick RJ (1989) Detection of a trypsin-like serine protease domain in flaviviruses and pestiviruses. Virology 171: 637-639.

[4] Bolin SR, Ridpath JF (1989) Specificity of neutralizing and precipitating antibodies induced in healthy calves by monovalent modified-live bovine viral diarrhea virus vaccines. Am. J. Vet. Res. 50: 817- 821.

[5] Chomcyzynski P, Sacchi N (1987) A Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156-159.

[6] Collett MS, Larson R, Belzer SK, Retzel E (1988) Proteins encoded by bovine viral diarrhea virus: the genomic organization of a pestivirus. Virology 165: 200-208.

[7] Corapi WV, Donis RO, Dubovi EJ (1988) Monoclonal antibody analyses of cytopathic and noncytopathic viruses from fatal bovine viral diarrhea virus infections. J. Virol. 62: 2823-2827.

[8] Diepolder HM, Zachoval R, Hoffmann RM, Wierenga EA, Santantonio T, Jung MC, Eichenlaub D, Pape GR (1995) A possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. Lancet 346: 1006-1007.

[9] Donis RO, Dubovi EJ (1987) Differences in virus-induced polypeptides in cells infected by cytopathic and non-cytopathic biotypes of bovine virus diarrhea-mucosal disease virus. Virology 158: 168-173.

[10] Elahi SM, Bergeron J, Nagy É, Talbot BG, Harpin S, Shen SH, Elazhary Y (1998) Induction of humoral and cellular immune responses in mice by a recombinant

fowlpox virus expressing the E2 protein of bovine viral diarrhea virus. FEMS Microbiol. Lett. 171: 107-114. "in press".

[11] Fu Y, Blankenhorn EP (1992) Nitric oxide-induced anti-mitogenic effects in high and low responder rat strains. J. Immunol.. 148: 2217-2222.

[12] Garnier A, Côte J, Nadeau I, Kamen A, Massie B (1994) Scale-up of the adenovirus expression system for the production of recombinant protein in human 293S cells. Cytotechnology 15: 145-155.

[13] Gerard RD, Meidell RS (1993) Adenovirus-mediated gene transfer. Trends Cardiovasc. Med. 3: 171-177.

[14] Gluzman Y, Reichl H, Solnick D (1982) Helper-free adenovirus type 5 vectors, p. 187-192. In: Eucaryotic viral vectors. Edited by Gluzman Y (Ed.). Cold Spring Harbor Laboratory, NY.

[15] Graham FL, Smiley J, Russell, WC, Nairn R (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. 36: 59-74.

[16] Harpin S, Elahi SM, Cornaglia E, Yolken RH, Elazhary Y (1995) The 5′-untranslated region sequence of a potential new genotype of bovine viral diarrhea virus. Arch. Virol. 140: 1285-1290.

[17] Hill AB, Mullbacher A, Parrish C, Coia G, Westaway EG, Blanden RV (1992) Broad cross-reactivity with marked fine specificity in the cytotoxic T cell response to flaviviruses. J. Gen. Virol. 73: 1115-1123.

[18] Kümmerer BM, Stoll D, Meyers G (1998) Bovine viral diarrhea virus strain Oregon: a novel mechanism for processing of NS2-3 based on point mutations. J. Virol. 72: 4127-4138.

[19] Kurane I, Bukowski JF, Lai C-J, Brinton M, Bray M, Falgout B, Zhao B, Ennis FA (1989) Dengue virus-specific human cytotoxic T lymphocytes (CTL). In 2nd International symposium on Positives Strand RNA Viruses. Vienna, Austria.

[20] Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, London 227: 680-685.

[21] Lambot M, Douart A, Joris E, Letesson JJ, Pastoret PP (1997) Characterization of the immune response of cattle against non-cytopathic and cytopathic biotypes of bovine viral diarrhea virus. J. Gen. Virol. 78: 1041-1047.

[22] Liew FV (1995) Interaction between cytokines and nitric oxide. Adv. Neuroimmunol. 5: 201-209.

[23] Lobigs M, Arthur CE, Mullbacher A, Blanden RV (1994) The flavivirus nonstructural protein NS3 is a dominant source of cytotoxic T cell peptide determinants. Virology 202:195-201.

[24] Massie B, Dionne J, Lamarche N, Fleurent J, Langelier Y (1995) Improved adenovirus vector provides herpes simplex virus ribonucleotide reductase R1 and R2 subunits very efficiently. Biotechnology (NY) 13: 602-608.

[25] Massie B, Couture F, Lamoureux L, Mosser DD, Guilbault C, Jolicoeur P, Bélanger F, Langelier Y (1998) Inducible over-expression of toxic protein by an adenovirus vector with a tetracyclin-regulatable expression cassette. J. Virol. 72: 2289-2296.

[26] Meyer G, Thiel HJ, (1996) Molecular characterization of pestiviruses. Adv. Virus Res. 47: 53-118.

[27] Mittereder N, March K, Trapnell BC (1996) Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. J. Virol. 70: 7498-7509.

[28] Pellerin C, Van Den Hurk J, Lecomte J, Tijssen P (1994) Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology 203: 260-268.

[29] Phipps RP, Stein SH, Roper RL (1991) A new view of prostaglandin E regulation of the immune response. Immunol. Today 12: 349-352.

[30] Reiner SL, Seder RA (1995) T helper cell differentiation in immune response. Curr. Opin. Immunol. 7: 360-366.

[31] Ridpath JF, Bolin SR, Dubovi EJ (1994) Segregation of bovine viral diarrhea virus into genotypes. Virology 205: 66-74.

[32] Rothman AL, Kurane I, Lai CJ, Bray M, Falgout B, Men R, Ennis FA (1993) Dengue virus protein recognition by virus-specific murine CD8+ cytotoxic T lymphocytes. J. Virol. 67: 801-806.

[33] Schleifer KW, Mansfield JM (1993) Suppressor macrophages in African trypanosomiasis inhibit T cell proliferative responses by nitric oxide and prostaglandins. J. Immunol. 151: 5492-5503.

[34] Tan CH, Yap EH, Singh M, Deubel V, Chan YC (1990) Passive protection studies in mice with monoclonal antibodies directed against the non-structural protein NS3 of dengue 1 virus. J. Gen. Virol. 71: 745-749.

[35] Thiel H-J, Plagemann W, Moenning V (1996) Pestiviruses, p.1059-1073. In Fields, Fields BN, Knipe DM, Howley PM (ed), Virology, 3rd. Lippincott-Raven Publishers, Philadelphia.

[36] Vanderheijden N, De Moerlooze L, Vandenbergh D, Chappuis G, Renard A, Lecomte C (1993) Expression of the bovine viral diarrhea virus Osloss p80 protein: its use as ELISA antigen for cattle serum antibody detection. J. Gen. Virol. 74: 1427-1431.

[37] Wengler G, Bradley DW, Collett MS, Heinz FX, Schesinger RW, Strauss J (1995) Flaviviridae, p. 415-427. In Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MA, Summers MD (ed), Virus taxonomy. Sixth report on the International Committee on Taxonomy of Viruses. Springer-Verlag. New York.

[38] Xu J, Mendez E, Caron PR, Lin C, Murcko MA, Collett MS, Rice CM (1997) Bovine viral diarrhea virus NS3 serine proteinase: polyprotein cleavage sites, cofactor requirements, and molecular model of an enzyme essential for pestivirus replication. J. Virol. 71: 5312-5322.

Group	Number	Adenovirus	Route	Vaccination time	BVDV specific antibody						
					0 wk	3 wk	6 wk	9 wk	12 wk	15 wk	18 wk
I	10	Ad5/ΔE1ΔE3	i.m.	0-3-6-12 wk p.i.	0/10[†]	0/10	0/10	0/10	0/10	0/10	0/10
II	10	rAdBM5/NS3	i.m.	0-3-6-12 wk p.i.	0/10[a]	8/10[c]	9/10[c]	10/10[c]	9/10[c]	10/10[d]	10/10[d]
III	10	rAdCMV5/NS3	i.m.	0-3-6-12 wk p.i.	0/10[a]	3/10[b]	7/10[b]	7/10[c]	7/10[c]	8/10[c]	8/10[c]
IV	10	rAdBM5/NS3	i.n.	0-3-6-12 wk p.i.	0/10[a]	0/10[a]	1/10[b]	2/10[b]	3/10[a]	5/10[b]	7/10[c]
V	10	rAdCMV5/NS3	i.n.	0-3-6-12 wk p.i.	0/10[a]	0/10[a]	0/10[a]	4/10[a]	4/10[b]	7/10[b]	7/10[c]

Table 1. Serum antibody responses of mice following administration of parental or recombinant adenoviruses.

Groups of mice were immunized with the different recombinants and parental adenoviruses, at 5 X 10^8 plaque forming unit, i.m. or i.n. Mice were bled at 0, 3, 6, 9, 12, 15 and 18 weeks (wk) post-infection (p.i.) and the sera were diluted 1/160. The mouse sera were tested in an indirect ELISA for detection of BVDV/NS3 specific antibodies. The mouse sera with optical densities higher than 0.2 at dilution 1/160 were considered as positive. Differences between optical densities of mouse sera in groups II, III, IV and V were compared with group I and analyzed with a Pear "t" test (two tails).

a; non-significant; b; $p < 0.05$, c; $p < 0.005$, d; $p < 0.0005$.

†The numbers of positive mice / number of total mice in each experiment were shown in this table.

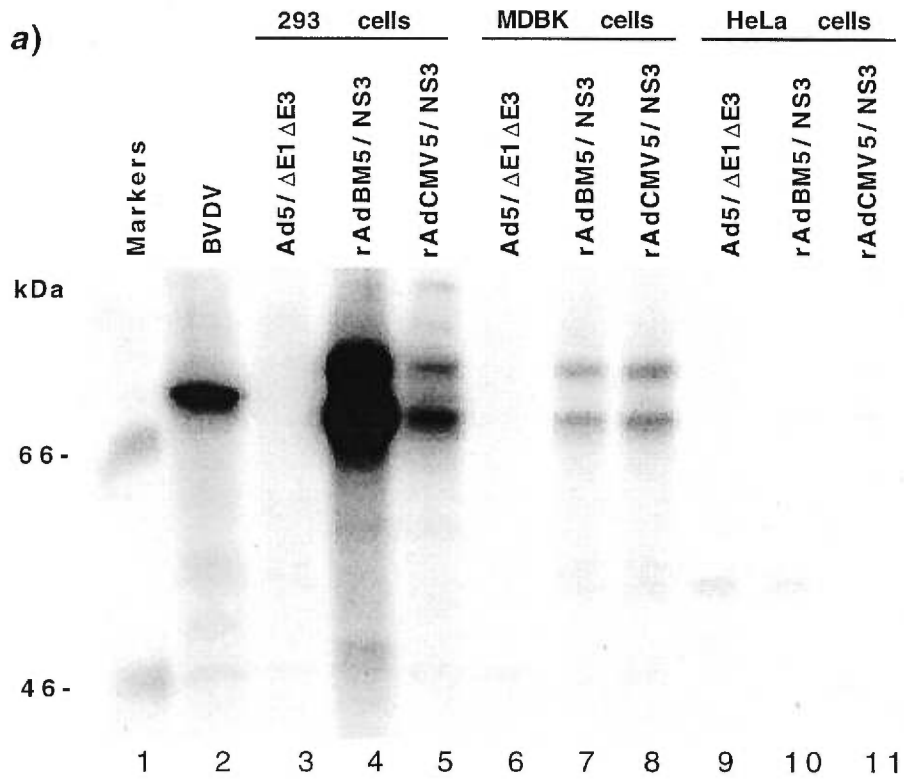

Fig. 1*a*. Expression of NS3 protein by various promoters in 293A, MDBK and HeLa cells.

Infected cells were metabolically labeled with [^{35}S] methionine/ [^{35}S]cysteine, and BVDV/NS3 protein was immunoprecipitated with MAb WB-1 as described in Materials and Methods. The following cell lines and viruses were used: lane 2; MDBK cells infected with BVDV, lanes 3-5; 293A cells, lanes 6-8; MDBK cells, and lanes 9-11; HeLa cells. The cells were infected with Ad5/(E1(E3 (lanes 3, 6 & 9) , rAdBM5/NS3 (lanes 4, 7 & 10) and rAdCMV5/NS3 (lanes 5, 8 & 11) respectively. All lanes are from the same autoradiograph. The ^{14}C-labeled molecular weight markers are shown on lane 1.

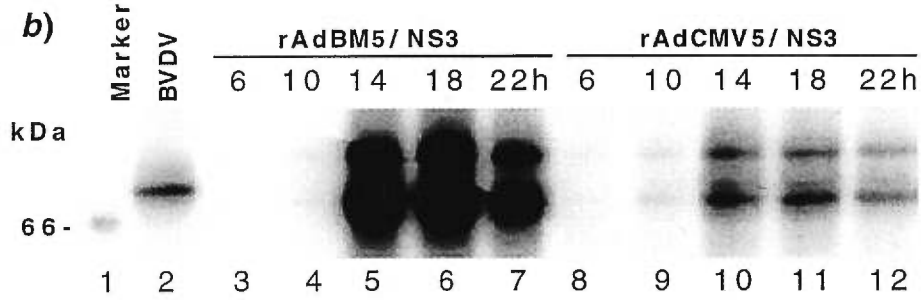

Fig. 1*b*. Kinetics of the expression of NS3 protein by the BM5 and the CMV5 promoters in 293 cells infected with recombinant adenoviruses.

The 293A cells were infected with rAdBM5/NS3 (lanes 3-7) and rAdCMV5/NS3 (lanes 8-12). After different time post-infection (4 to 20 h), the infected cells metabolically labeled for 2 h. At the times indicated above (for lanes 3-12) the lysis buffer was added and the BVDV/NS3 protein was immunoprecipitated with MAb WB-1 as described in Materials and Methods. Lane 2; MDBK cells infected with BVDV. All lanes are from the same autoradiograph. The 14C-labeled molecular weight marker (66 kDa) shown on lane 1.

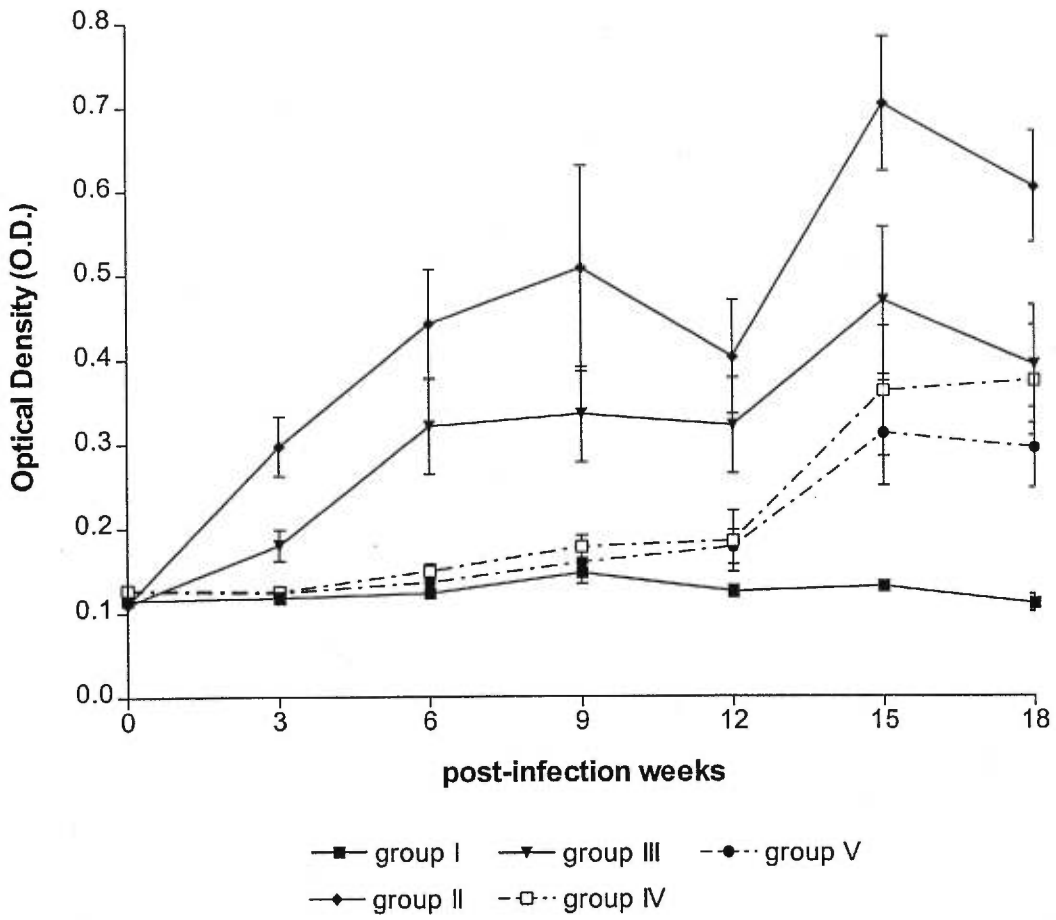

Fig. 2. The kinetics of BVDV specific antibodies. Mice were immunized and individual sera were tested by ELISA for the presence of BVDV specific antibodies as described in Materials and Methods. Data are presented as group means ± standard errors of the mean. The cutoff level was fixed at O.D.= 0.2. group I; mice immunized with Ad5/ΔE1ΔE3 i.m. , group II; mice immunized with rAdBM5/NS3 i.m., group III; mice immunized with rAdCMV5/NS3 i.m., group IV; mice immunized with rAdBM5/NS3 i.n., group V; mice immunized with rAdBM5/NS3 i.n. All groups of mice immunized by recombinant adenoviruses, (groups II-V) showed significant difference from mice immunized by parental adenovirus (group I, p value < 0.05). However, the differences between group IV and V were not significant (p value > 0.05).

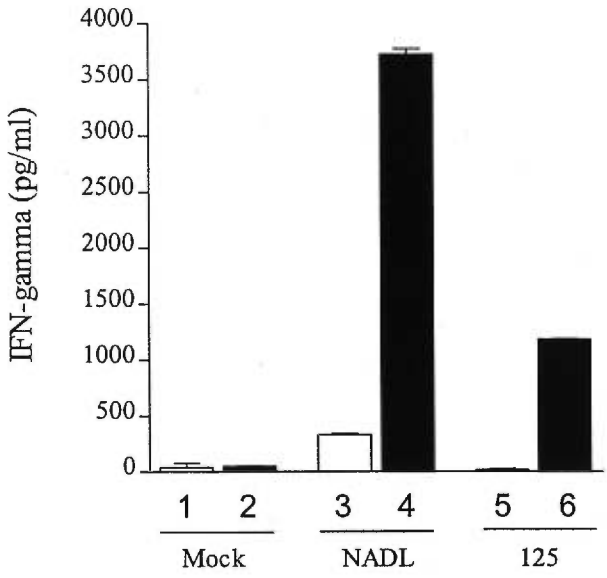

Fig. 3. IFN-γ production in supernatants from mononuclear cells after stimulation by BVDV.

The murine mononuclear cells of mice immunized with Ad5/ΔE1ΔE3 (group VI, open bar) or AdBM5/NS3 (group VII, black bar) were mock stimulated (lanes 1 & 2) or stimulated by BVDV/NADL (lanes 3 & 4) or BVDV/125 strains (lanes 5 & 6). The concentrations of IFN-γ were determined in the supernatant of the murine MNC by ELISA and expressed as pg/ml. Results are the mean ± standard deviation from two experiments.

CHAPTER VI

Induction of humoral and cellular immune responses against the nucleocapsid of bovine viral diarrhea virus by an adenovirus vector with an inducible promoter

Seyyed Mehdy Elahi[1], Shi-Hsiang Shen[2], Brian Geoffrey Talbot[3], Bernard Massie[2], Serge Harpin[1], Youssef Elazhary[1]*

Virology "resubmitted"

[1]Virology Section, Faculty of Veterinary Medicine, University of Montreal, P. O. Box 5000, Saint-Hyacinthe, Quebec. J2S 7C6, Canada

[2]Biotechnology Research Institute, National Research Council, Montreal, Quebec, H4P 2R2, Canada

[3]Department of Biology, Faculty of Sciences, University of Sherbrooke, Quebec, J1K 2R1 Canada

*Author to whom correspondence should be addressed.

Phone: (450) 773-8521 ext. 8201

Fax: (450) 778-8105

E-mail: elazhary@medvet.umontreal.ca

Keywords: bovine viral diarrhea virus; nucleocapsid; recombinant adenoviruses; tetracycline-regulatable promoter; humoral and cellular immune responses

Abstract

A new recombinant adenovirus was constructed which expressed the nucleocapsid (C protein or p14) of the bovine viral diarrhea virus (BVDV) under the control of a tetracycline-regulatable promoter. Mice co-vaccinated with this recombinant adenovirus, accompanied by another recombinant adenovirus expressing the trans-activator protein (tTA protein) induced a strong humoral immune response to the BVDV/C protein as detected by ELISA. Splenocytes from mice immunized with the recombinant adenovirus showed a specific proliferation response to both genotypes (type 1 and 2) of BVDV. High levels of IFN-γ were detected in the supernatant of murine mononuclear cells of mice immunized by the recombinant adenovirus when stimulated *in vitro* byboth genotypes of BVDV. These results indicate that this recombinant adenovirus is highly immunogenic and stimulates both cellular and humoral immune responses against the nucleocapsid of BVDV.

Introduction

Bovine viral diarrhea virus (BVDV) belongs to the pestivirus genus. This genus, with the genus flavivirus and the hepatitis C virus group, forms the family *Flaviviridae*. The virions of pestiviruses consist of a positive-stranded RNA and four structural proteins. These are; the nucleocapsid C protein (p14) and the three envelope glycoproteins E0 (gp48), E1 (gp25) and E2 (gp53) (28). Recently, BVDV strains have been divided into two genotypes (type 1 and 2) (9, 22). The C protein (p14) of BVDV is highly conserved among many different pestiviruses yet in contrast to hepatitis C capsid protein, BVDV/C protein does not appear to be immunogenic since sera from convalescent cattle do not contain antibodies to C protein (1, 10, 11). In this report we expressed a fragment of the BVDV genome containing the coding region of the BVDV/C protein in a recombinant adenovirus (rAdTR5-DC/C-GFP) under the control of the tetracycline-regulatable promoter. In this system, the expression of a target gene, placed under the control of the promoter containing the tetracycline

operator sequence (tet O), can be induced by the tetracycline-regulated trans-activator protein (tTA). The tTA protein can be supplied by using the 293-tTA cell line (a stable 293 cell line that constitutively expresses the tTA protein) or by co-infection with a recombinant virus such as Ad5CMV-tTA (16). The transcription of the tTA protein can be prevented by adding tetracycline at a concentration that is not toxic for eukaryotic cells (7). Previously, we have demonstrated that murine mononuclear cells of mice immunized by a recombinant fowlpox virus (2) or by two Adenoviruses (unpublished results) expressing the envelope E2 protein of BVDV can produce a Th1 response only after homologous stimulation with BVDV type 1. An efficient vaccine has to protect the animal against all types of viruses and determination of immune responses to the more conserved proteins of BVDV, such as the nucleocapsid, is a subject of interest.

In this study we demonstrated that the BVDV/C protein induced both humoral and cellular immune responses in a mouse model. This report also shows that a recombinant adenovirus with a tetracycline-regulatable promoter can express a foreign gene *in vivo* when it is induced by another adenovirus which supplies the trans-activator protein (tTA protein).

Results and discussion

Construction of rAdTR5-DC/C-GFP- At time of start this study the position of the nucleocapsid protein C and the glycoprotein E0 had only been determined for C and E0 of classical swine fever virus (CSFV) by amino acid sequencing (23, 26). In our cloning strategy, we included the conserved sequence situated upstream and downstream of the C protein. These sequence are conserved between several pestiviruses. A fragment of the BVDV/NADL genome containing the presumed BVDV/C protein was amplified by PCR and subsequently cloned into pGEM-T to be finally transferred into the shuttle plasmid (pAdTR5-DC/GFP). By comparing the

NADL sequence with other pestivirus (18) , our construct was found to contain 21 amino acid of N^{pro} at the N-terminal and 12 amino acids of E0 at the C-terminal.

Homologous recombination between transfer vector (pAdTR5-DC/GFP) (17) and Ad5/ΔE1ΔE3 (6) resulted in rAdTR5-DC/C-GFP in which the expression of BVDV/C gene was under the control of the tetracycline-regulatable promoter (TR5). This recombinant adenoviruses also co-expressed the GFP protein so that the recombinant plaques could be rapidly identified by fluorescence microscopy. The expression of GFP by the TR5 promoter in 293A cells was sufficient to identify green plaques in the uninduced state. The rAdTR5-DC/C-GFP was defined by performing PCR and visualizing the co-expression of GFP gene (data not shown). One of the positive plaques was subjected to three consecutive rounds of plaque purification and the expression of the BVDV/C protein was detected by radioimmunoprecipitation using a monospecific antiserum as described below.

We used a dicistronic inducible system for the following reasons: Firstly, co-expression of GFP simplified the cloning selection since GFP fluorescence can be easily visualized in live cells with a standard fluorescence microscope. Secondly, this system allowed the expression of the transgene in the presence of tTA protein and could be completely inactivated at tetracycline concentrations that are not toxic for eukaryotic cells (7). These two characteristics allow the generation of recombinant adenoviruses in which the production of transgene may be toxic and they permit the control of the transgene expression *in vitro* (16) and *in vivo* (25). Previously, we demonstrated that recombinant adenovirus (with a constitutive promoter) expressing the NS3 of BVDV (a viral protease) has a titer about 10 times lower than parental adenovirus, probably due to the effect of the recombinant protein (unpublished results). Finally, previous observations in our laboratory have demonstrated that this system can express the E2 protein of BVDV *in vivo* and *in vitro* as efficiently as the constitutive promoter (unpublished results). Thus the use of an inducible promotor

appears to be the best choice for an uncharacterized protein (such as BVDV/C protein).

In vitro expression of BVDV/C protein- SDS-PAGE analysis of the MBP-BVDV/C fusion protein (the result of fusion between maltose binding protein and BVDV/C protein as described in Materials and Methods) showed a protein band of approximately 60 kDa. The maltose-binding protein was responsible for 42.7 kDa of this and 17 kDa (predicted molecular mass for our construct) was the BVDV/C expressed protein (data not shown). The Figure 1 shows that the "anti-C antibody" precipitated a 17 kDa protein from 293-tTA cells infected by rAdTR5-DC/C-GFP (Fig. 1*a*, lane 2). There was no band of similar size in cells which were infected by the Ad5/ΔE1ΔE3 (Fig. 1*a*, lane 1). We also detected a band of about 23 kDa in 293-tTA cells infected by rAdTR5-DC/C-GFP (Fig. 1*a*, lane 2). This band could be a post-translation modification of the recombinant protein. However, the BVDV/C precipitated from MDBK cells infected by BVDV was at the lower limits of detection (Fig. 1*a*, lane 3). This maybe because the "anti-C antibody" had a low titer and in both cases had to be used at high concentrations (10-15% of cell lysate) in order to detect the expression of the BVDV/C protein. In 293A cells, expression of the recombinant protein was possible after co-infection of rAdTR5-DC/C-GFP with Ad5CMV-tTA (Fig. 1*b* lane 2). However, the level of expression in 293A cells was lower than 293-tTA cells. These observations were not surprising because in 293A cells, the expression of BVDV/C protein depends on co-infection of both viruses in the same cell.

Demonstration of the Specificity of "anti-C antibody"- The "anti-C antibody" reacted with the fusion protein (MBP-BVDV/C protein) in our ELISA assay and also in our ELISA assay in which the plates were coated with non-purified BVDV (2). In addition, none of the adenovirus proteins or 293A, 293-tTA and MDBK cells (up to 55 kDa) were precipitated by this antibody (Fig 1*a* & Fig. 1*b*). The only BVDV protein which was precipitated by this antibody was BVDV/C (Fig 1*a* & Fig. 1*b*, lane

3) in MDBK cells infected with the virus. The N-terminal and C-terminal extremities of the recombinant proteins did not produce any antibody against N^{pro} or E0. We did not find any neutralizing antibodies against the E0 protein in rabbit or mouse sera after vaccination with recombinant protein and rAd/C respectively (data not shown). These data confirm that the BVDV/C protein in our construct is the only important protein for induction of humoral immunity against the BVDV.

Humoral immune response against BVDV/C protein- Two groups of 6 mice were immunized by Ad5/ΔE1ΔE3 + Ad5CMV-tTA (referred to as parental adenovirus or group I) or rAdTR5-DC/C-GFP + Ad5CMV-tTA (referred as rAd/C or group II). At 7 weeks (wk) post-infection (p.i.) mouse sera were tested by ELISA for BVDV specific antibody. The average of the optical density (O.D.) for both groups, starting at a dilution of 1:20 up to 1: 20480 are shown in Fig. 2. The difference between the two groups was extremely significant (pear *t* tests, P value = 0.0005). Cut-off level (the O.D. that represents a positive result) for each dilution was established at three time the O.D. average of mice in group I. The end point dilutions for mice in group II, were between 1:320 to 1:10240. The number of mice with positive titers for each dilution are also shown in Fig. 2. All mice in group II were positive at 1:320. However, the end point dilution for two immunized rabbits at 13 wk p.i. was only 1:40 and 1: 80. None of the antibodies against BVDV/C protein produced in mice or rabbits neutralized the BVDV/NADL strain *in vitro* (data not shown).

However, other authors (1) have not found any antibodies to BVDV/C protein after natural or experimental infection in cattle. Yet in our study, we were successful in inducing a significant BVDV/C antibody response in mouse sera after immunization with rAd/C. Previous studies indicated that the first-generation of recombinant adenoviruses (E1-deleted) elicited destructive class I-restricted cytotoxic T lymphocytes to both viral and transgene proteins (such as *Lac*Z). These recombinant adenoviruses also induced an inflammatory reaction at the site of

injection (29, 30). This phenomenon was not detected when a recombinant adeno-associated virus was used alone for expression of the lacZ gene (4). This "adjuvant effect" of adenovirus protein to stimulate an immune response to transgene products could explain our success in the production of humoral and cellular immune responses against the BVDV/C protein. The lack of a strong humoral immune response in rabbits following immunization with the MBP-BVDV/C protein lends weight to this hypotheses. The mouse sera were also positive by ELISA when native non-purified BVDV (NADL strain) was used to coat the plate (2) although the average end point dilutions decreased about 4 fold (data not shown). The proteins expressed by adenovirus probably did not have exactly the same configurations as the native protein and consequently the mouse sera reacted better with the recombinant protein than native form.

Previously, we carried out *in vitro* studies with a recombinant adenovirus expressing the GFP protein under the control of tetracycline-regulatable promoter. We found that the best level of expression of GFP protein in non-permissive cells (e.g. HeLa cells) for defective-adenovirus was obtained after co-infection with a ratio 3:1 of rAdCMV-tTA to the recombinant adenovirus which expressed the GFP and which had been infected at a very high m.o.i. (total of 1500, data not shown). In the present work, for *in vivo* studies we chose the same ratio with a dose of 5×10^8 p.f.u. for rAdTR5-DC/C-GFP and 1.5×10^9 p.f.u. for Ad5CMV-tTA. The dose of 10^8 to 10^9 is used currently for vaccination of laboratory animal by recombinant adenovirus. In the experiments where difference doses were administrated, the immune response appeared to be dose-dependant (3, 19). However, dose-dependency was not determined in the present study.

Cellular immunity- T cell responses were measured as antigen-dependent cell proliferation and production of cytokines following *in vitro* stimulation with BVDV strains. In the presence of indomethacin, an inhibitor of prostaglandin E2 (24), the murine mononuclear cells (MNC) of mice immunized by rAd/C (group II) showed a

clear class-II restricted stimulation with both types of BVDV strain (NADL, type 1 and 125 type 2) (Fig. 3). The cross-genotypic proliferative response in MNC of mice immunized by rAd/C which expressed the BVDV/C protein of NADL strain (type 1) to BVDV/125 strain (type 2) could be a consequence of the homology of this protein between these two genotypes and this suggests a substantial crossreactivity at the T-cell level. The proliferation response to both strains was completely abolished in the absence of indomethacin (data not shown). These results are not surprising since it is known that prostaglandins inhibit T-cell mitogenesis (19).

The production of IL-2, IL-4 and IFN-γ was also monitored in the supernatants of murine MNC immunized with the rAd/C or parental adenovirus. IL-12 and cytokines that modulate the effectiveness of IL-12 such as IFN-γ and IFN-α, are key regulators of Th1 differentiation, while IL-4 is a key regulator of Th2 differentiation (21). The murine MNC, immunized with the rAd/C (group II), produced 4249 ± 164 (mean ± standard deviation) pg/ml of IFN-γ following stimulation by BVDV/NADL strain and 2249 ± 153 pg/ml of IFN-γ by BVDV/125 strain (Fig. 4). This remarkable production of IFN-γ in mice immunized with rAd/C could be an indicator of the activation of specific Th1 cells. IFN-γ was detected at much lower levels (593 ± 3 pg/ml) in supernatants of murine MNC from mice immunized with parental adenovirus and stimulated with BVDV/NADL. Between the two groups of mice, there was no detectable difference in the production of IL-2 or IL-4 from murine MNC stimulated with both BVDV strains (data not shown). The lymphoproliferation responses and production of IFN-γ are indicators of Th1 activation and explain the absence of IL-4 production by Th2 (20). The lack of IL-2 could be caused by the IFN-γ since it is a powerful inducer of inducible nitric oxide synthesis which can inhibit production of IL-2 by Th1 cells (13).

Our construct also contained the non-C protein sequences. The role of these two short sequence in humoral immunity should be negligible as discussed

previously. However, we were not able to positively exclude these two peptides from cellular immune responses. However, the present published data about the N^{pro}, C and E0 proteins of other *Flaviviraidae,* particularly HCV and CSFV, suggests that BVDV/C is the active protein in our constructs (10, 11, 27).

This study showed that a recombinant adenovirus with an tetracycline-regulatable promoter can express a immunogenic foreign gene *in vivo* when it is induced by another adenovirus which supplies the trans-activator protein (tTA protein). In previous studies we have shown that this inducible promoter can drive the expression of the BVDV E2 protein as efficiently as a constitutive promoter (unpublished results). In spite of this , we did not find any significant toxicity or differences between the virus titers of recombinant adenovirus and parental virus. Our results confirm that such a tetracycline-regulatable promotor can be used for expression of an uncharacterized gene.

Recently, Liu *et al.*, (14) demonstrated that the recombinant nucleocapsid protein of CSFV can act as transcriptinal regulator. This protein activated the promoter of human heat shock protein 70 gene, and suppressed the SV40 early promoter. Since the nucleocapsid proteins of pestiviruses are highly conserved, this justifies the use of an inducible promoters for expression of BVDV/C. However, the activity of BVDV/C as a transcriptional regulator still remains to be determined.

This manuscript also reports the induction of both humoral and cellular immune responses to the nucleocapsid of BVDV in a mouse model. The experiments to examine the immune responses to such recombinant proteins were carried out in mice because the immune mechanisms are well known, significant numbers of animals can be used and inbred lines are available. Despite the differences in immune responses between mice and cattle, the normal host for the virus, almost all the data on T and B cell epitopes has been obtained using monoclonal constructs which were the result of the murine immune response to BVDV proteins. These have proved to

mimic very well the bovine response. The ability of rAd/C to stimulate the immune response to the more conserved core proteins has important implications in the development of vaccines against BVDV.

Materials and Methods

Cultures and Viruses- Madin-Darby bovine kidney (MDBK) cells (free of BVDV) were obtained from the American Type Culture Collection (Rockville , MD) and grown in Dulbecco's Modified Eagle medium, (Gibco), supplemented with 5% of fetal bovine serum (FBS), [free of antigen and antibody against BVDV, (Gibco)]. The NADL strain of BVDV (type 1) and 125 strain (BVDV type 2) were obtained from ATCC and USDA (Ames, Iowa, USA) respectively and were propagated in MDBK cells in the presence of 2% FBS. The conditions for culture of human 293 cells, either the original anchorage-dependent 293A line (8) or 293S (obtained from Cold Spring Harbor Laboratories), an anchorage-independent clone, were as described previously (5, 15). Human adenovirus type-5 with deletions in the E1 and E3 regions Ad5/ΔE1ΔE3 (6) and Ad5CMV-tTA (16) were amplified in 293S cells.

Construction of the transfer vector- The nucleotides 827 to 1232 of BVDV/NADL strain were amplified after RNA extraction and cDNA synthesis by using, a forward primer, 5′-GAGATCTACCATGTACCAAAGGGTGTTCAGGTG G-3′and reverse primer 5′-TAGATCTCTACCCATTATCTTGTAGGTTCCA-3′. To facilitate the cloning of the PCR product, a *Bg*l II restriction site was added to both primers. The forward primer also contains an in-frame translational start codon and the reverse primer contains an in-frame translational stop codon for termination of expression at the 3′ end. The PCR product was cloned into the pGEM-T vector (Promega) and the coding region for BVDV/C protein was then excised from pGEM-T/C by *Bg*l II digestion and cloned in the *Bg*l II site of the pAdTR5-DC/GFP plasmid (17) to generate pAdTR5-DC/C-GFP.

Construction of recombinant adenovirus- The transfer vector, pAdTR5-DC/C-GFP, was linearized at the unique *Fes* I site and co-transfected with *Cla* I digested Ad5/ΔE1ΔE3 (6) into 293A cells as described previously by Massie *et al.* (16). The recombinant adenovirus which expressed the BVDV/C protein was designated as rAdTR5-DC/C-GFP.

Generation of the fusion protein and monospecific antisera- The recombinant BVDV/C protein was also expressed in a bacterial system after the coding sequence had been cloned at the *Bam*H I site of pMAL-c2 plasmid (New England Biolabs). The fusion protein (Maltose binding protein and BVDV/C protein, referred as MBP-BVDV/C protein) was purified using the maltose-binding protein's affinity for maltose according to the manufacturer's instructions (New England Biolabs). A monospecific antisera against the BVDV/C protein, "anti-C antibody", was generated in two female rabbits (New-Zealand) by intramuscular (i.m.) injection of 100 μg of purified MBP-BVDV/C fusion protein accompanied by complete Freund's adjuvant for the first immunization and incomplete Freund's adjuvant for subsequent immunizations at 3, 6, and 10 wk after the first immunization.

Radioimmunoprecipitaton (RIPA) - Approximately 2 X 10^6 293-tTA cells in a 60 mm dish were infected at m.o.i. of 20 p.f.u /cell with rAdTR5-DC/C-GFP or Ad5/ΔE1ΔE3. The expression of recombinant protein also was also investigated in 293A cells by co-infection of two viruses; rAdTR5-DC/C-GFP or Ad5/ΔE1ΔE3 with Ad5CMV-tTA (m.o.i. of 20 for each virus). At 16 (hours) h post infection, after 1 h of starvation in DMEM lacking methionine and cysteine, cells were labeled in the same medium for 2 h in the presence of [^{35}S] methionine/ [^{35}S] cysteine (Amersham; 100 (Ci per dish). Metabolic labeling of MDBK cells infected by BVDV/NADL strain was as described previously (2). The proteins were precipitated by using "anti-C antibody" and Protein A Sepharose before being analyzed by SDS-PAGE (12).

Immunization protocol- Two groups of 6 BALB/c mice were immunized with 5 X 10^8 p.f.u. of Ad5/ΔE1ΔE3 + 1.5 X 10^9 p.f.u. of Ad5CMV-tTA (group I) or 5 X 10^8 p.f.u. of rAdTR5-DC/C-GFP + 1.5 X 10^9 p.f.u. of Ad5CMV-tTA (group II) by subcutaneous (s.c.) injection. The booster immunization was performed at 4 wk post injection (p.i.) with same dose. The blood samples were collected at 0, 4 ,and 7 wk p.i. by orbital plexus puncture.

Enzyme-Linked Immunosorbant Assay (ELISA)- We developed an ELISA detection system by using the purified MBP-BVDV/C protein at 250 pg/well for coating the plates. After blocking with 5% skim milk in Tris-buffered saline (12.5 mM trizma hydrochloride, 46.4 mM NaCl, 0.005% thimerosal and 0.05% Tween-20, pH 7.0, 1 h at 37 °C, twofold serum dilutions starting at 1: 20 up to 1: 20480 were added to the wells in duplicate, and the plates were incubated for 30 min at 37 °C. HRP-goat anti-mouse IgG (1:8000, Bio-Rad) was used as the second antibody (30 min at 37 °C) and Tetra-Methylbenzidine as the substrate. Color was allowed to develop for 10 min at room temperature at which time absorbance was determined at 450 nm.

Proliferation response of murine mononuclear cells (MNC)- At 7 wk p.i. splenocytes of mice from group I (immunized with parental adenoviruses) and II (immunized with rAd/C) were stimulated *in vitro* by non-purified BVDV strains of NADL or 125 as described previously (2) with one modification. The stimulated murine MNC were kept in the presence or absence of 30 μM of indomethacin, an inhibitor of prostaglandin E2 (24) The stimulation index (SI) was calculated as follows: SI = average counts per minute in antigen stimulated wells / average counts per minute in wells containing only cells with medium.

Cytokine production assay- 1.5 X 10^6 murine MNC were stimulated with BVDV (NADL and 125 strains) (2) in the presence of 30 μM of indomethacin . Three days later, 100 μl of supernatant was used in duplicate in a cytokine ELISA assay

(PharMingen) for detection of IL-2 and, IFN-γ (Th1-type cytokines) and IL-4 (a Th2-type cytokine) according to manufacturer's instructions.

References

1. Donis, R. O., and Dubovi, E. J. (1987). Molecular specificity of the antibody responses of cattle naturally and experimentally infected with cytopathic and noncytopathic bovine viral diarrhea virus biotypes. *Am. J. Vet. Res.* 48, 1549-1554.

2. Elahi, S. M., Bergeron, J., Nagy, É., Talbot, B. G., Harpin, S., Shen, S. H., and Elazhary, Y. (1998). Induction of humoral and cellular immune responses in mice by a recombinant fowlpox virus expressing the E2 protein of bovine viral diarrhea virus. *FEMS Microbiol. Lett.* 171, 107-114.

3. Eloit, M., and Adam, M. (1995). Isogenic adenoviruses type 5 expressing or not expressing the E1A gene: efficiency as virus vectors in the vaccination of permissive and non-permissive species. *J. Gen. Virol.* 76, 1583-1589.

4. Fisher, K. J, Jooss, K., Alston, J., Yang, Y., Haecker, S. E., High, K., Pathak, R., Raper, S. E., and Wilson, J.M. (1997). Recombinant adeno-associated virus for muscle directed gene therapy. *Nat. Med.* 3, 306-312

5. Garnier, A., Coté, J., Nadeau, I., Kamen, A., and Massie, B. (1994). Scale-up of the adenovirus expression system for the production of recombinant protein in human 293S cells. *Cytothechnology* 15, 145-155.

6. Gluzman, Y., Reichl, H., and Solnick, D. (1982). Helper-free adenovirus type 5 vectors, p. 187-192. In Y. Gluzman (ed), Eucaryotic viral vectors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

7. Gossen, M., Bonin, A. L., Freundlieb, S., and Bujard, H. (1994). Inducible gene expression systems for higher eukaryotic cells. *Curr. Opin. Biotechnol.* 5, 516-520.

8. Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 36, 59-74.

9. Harpin, S., Elahi, S. M., Cornaglia, E., Yolken, R. H., and Elazhary, Y. (1995). The 5′-untranslated region sequence of a potential new genotype of bovine viral diarrhea virus. *Arch. Virol.* 140, 1285-1290.

10. Inchauspe, G., Major, M. E., Nakano, I., Vitvitski, L., and Trepo, C. (1997). DNA vaccination for the induction of immune responses against hepatitis C virus proteins. *Vaccine* 15, 853-856.

11. Inchauspe, G., Major, M. E., Nakano, I., Vivitski, L., Maisonnas, M., and Trepo, C. (1998). Immune responses against hepatitis C virus structural proteins following genetic immunisation. *Dev. Biol. Stand.* 92, 163-168.

12. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

13. Liew, F. V. (1995). Interaction between cytokines and nitric oxide. *Adv. Neuroimmunol.* 5, 201-209.

14. Liu, J. J., Wong, M. L., and Chang, T. J. (1998). The recombinant nucleocapsid protein of classical swine, fever virus can act as a transcriptional regulator. *Virus. Res.* 53, 75-80.

15. Massie, B., Dionne, J., Lamarche, N., Fleurent, J., and Langelier, Y. (1995). Improved adenovirus vector provides herpes simplex virus ribonucleotide Reductase R1 and R2 subunits very efficiently. *Biotechnology (NY)* 13, 602-608.

16. Massie, B., Couture, F., Lamoureux, L., Mosser, D. D., Guilbault, C., Jolicoeur, P., Bélanger, F., and Langelier, Y. (1998*a*). Inducible overexpression of toxic protein by an adenovirus vector with a tetracyclin-regulatable expression cassette. *J. Virol.* 72, 2289-2296.

17. Massie, B., Mosser, D. D., Koutroumanis, M., Vitte-Mony, I., Lamoureux, L., Couture, F., Paquet, L., Guilbault, C., Dionne, J., Chahla, D., Jolicoueur, P., and Langelier, Y. (1998*b*). New adenovirus vectors for protein production and gene transfer. *Cytotechnology* 28, 53-64. "In press"

18. Meyer, G., and Thiel, H. –J. (1996). Molecular characterization of pestiviruses. *Adv. Virus Res.* 47, 53-118.

19. Morin, J. E., Lubeck, M. D. , Barton, J. E., Conley, A. J., Davis, A. R., and Hung, P. P. (1987). Recombinant adenovirus induces antibody response to hepatitis B virus surface antigen in hamsters. *Proc. Natl. Acad. Sci. USA* 84, 4626-4630.

20. Phipps, R. P., Stein, S. H., and Roper R. L. (1991). A new view of prostaglandin E regulation of the immune response. *Immuno. Today* 12, 349-352.

21. Reiner, S. L., and Seder, R. A. (1995). T helper cell differentiation in immune response. *Curr. Opin. Immunol.* 7, 360-366.

22. Ridpath, J. F., Bolin, S. R., and Dubovi, E. J. (1994). Segregation of bovine viral diarrhea virus into genotypes. *Virology* 205, 66-74.

23. Rumenapf, T., Unger, G., Strauss, J. H., and Thiel, H. –J. (1993). Processing of the envelope glycoproteins of pestiviruses. *J. Virol.* 67, 3288-3294.

24. Schleifer, K. W., and Mansfield, J. M. (1993). Suppressor macrophages in African trypanosomiasis inhibit T cell proliferative responses by nitric oxide and prostaglandins. *J. Immunol.* 151, 5492-5503.

25. Schultze, N., Burki, Y., Lang, Y., Certa, U., and Bleuthmann, H. (1996). Efficient control of gene expression by single step integration of the tetracycline system in transgenic mice. *Nat. Biotechnol.* 14, 499-503.

26. Stark, R., Meyers, G., Rumenapf, T., and Thiel, H. –J. (1993). Processing of pestivirus polyprotein: cleavage site between autoprotease and nucleocapsid protein of classical swine fever virus. *J. Virol.* 67, 7088-7095.

27. Tratschin, J. D., Moser, C., Ruggli, N., and Hofmann, M. A. (1988). Classical swine fever virus leader proteinase Npro is not required for viral replication in cell culture. *J. Virol.* 72, 7681-7684.

28. Wengler, G., Bradley, D. W., Collett, M. S., Heinz, F. X., Schesinger, R. W. and Strauss, J. (1995). *Flaviviridae*, p. 415-427., In "Virus taxonomy. Sixth report on the International Committee on Taxonomy of Viruses. " (F. A. Murphy, , C. M. Fauquet, D. H. L. Bishpo, S. A. Ghabrial, A. W. Jarvis, G. P. Martelli, M. A. Mayo, and M. D. Summers, Eed) Springer-Verlag. New York.

29. Yang, Y., Su, Q., and Wilson, J. M. (1996). Role of viral antigens in destructive cellular immune responses to adenovirus vector-transduced cells in mouse lungs. *J. Virol.* 70, 7209-7212.

30. Yang, Y., Jooss, .K. U., Su, Q., Ertl, H. C., and Wilson, J. M. (1996). Immune responses to viral antigens versus transgene product in the elimination of recombinant adenovirus-infected hepatocytes in vivo. *Gene. Ther.* 3, 137-144.

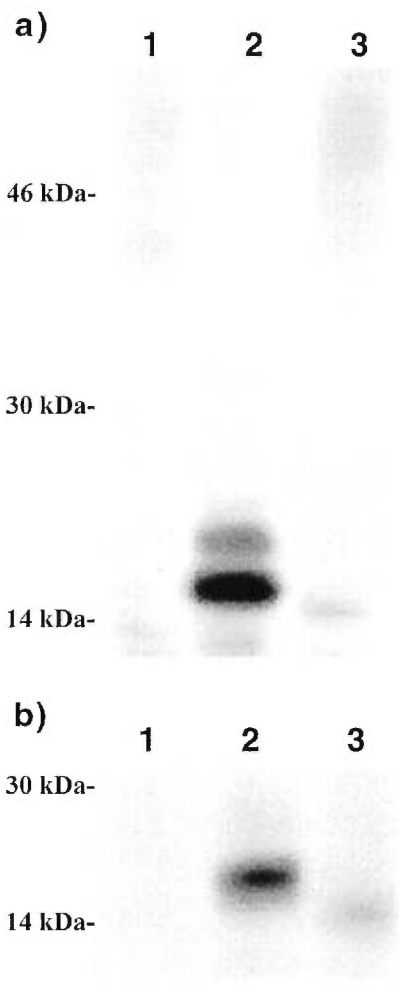

FIG. 1. In vivo expression of rAdTR5-DC/C-GFP in 293-tTA (Fig. 1a) and 293A (Fig. 1b) cells.

The 293-tTA cells were infected with Ad5/(E1(E3 (Fig. 1a, lane 1) or rAdTR5-DC/C-GFP (Fig. 1a, Lane 2). The 293A cells were infected with Ad5/(E1(E3 + Ad5CMV-tTA (Fig. 1b, lane 1) and rAdTR5-DC/C-GFP + Ad5CMV-tTA (Fig. 1b. lane 2). The MDBK cells were infected by BVDV/NADL (Fig. 1a & Fig. 1b, lane 3). After metabolic labelling, the cell lysates were precipitated with the "anti-C antibody" as described in Materials and Methods. The molecular weights markers are shown on the left side.

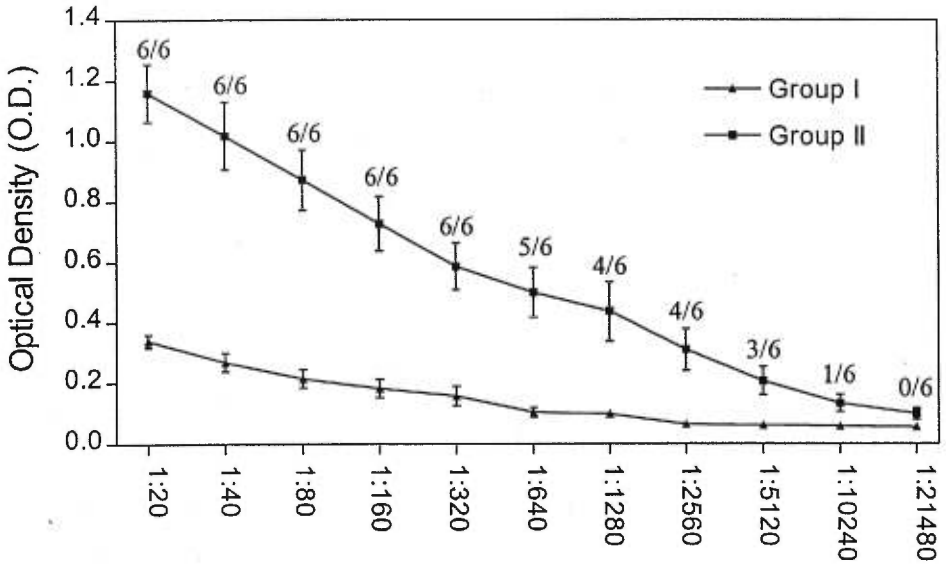

FIG. 2. Humoral immune response to recombinant adenovrirus expressing the nucleocapsid of BVDV.

At 7 weeks post-infection the mouse sera, diluted 1: 20 to 1: 21480, were tested in an ELISA assay as described in Materials and Methods. The mouse sera with optical density higher than three time those of mice in the group I for each dilution were considered as positive. The number of positive sera over the total number of sera for each dilution is shown above each average point. Group I; mice immunized by Ad5/ΔE1ΔE3 + Ad5CMV-tTA. Group II; mice immunized by rAdTR5-DC/C-GFP + Ad5CMV-tTA. Results are the mean ± standard deviation. The difference between the two groups was extremely significant (P value = 0.0005).

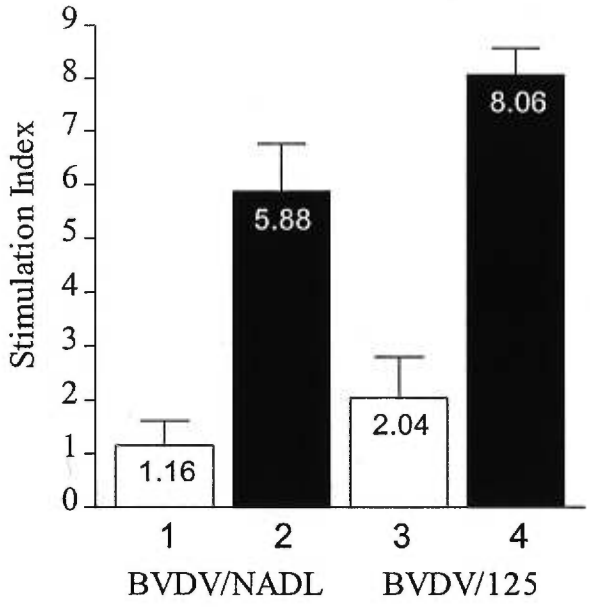

FIG 3. Proliferation responses of murine mononuclear cells stimulated with BVDV/NADL or BVDV/125 strains.

The murine mononuclear cells of mice in the group I (Ad5/ΔE1ΔE3 + Ad5CMV-tTA, open bar) and the group II (rAdTR5-DC/C-GFP + Ad5CMV-tTA, black bar) stimulated *in vitro* with BVDV/NADL strain (lines 1 & 2) or BVDV/125 strain (lines 3 & 4). The Stimulation index (SI) for each group was calculated by the following formula: SI = average counts per minute in antigen stimulated wells / average counts per minute in wells containing only cells with medium. Only the results of stimulation with optimal dilution for each BVDV strain were presented in this figure. Results are the mean ± standard deviation and representative of three experiments. The difference between the two groups was significant (P value < 0.05).

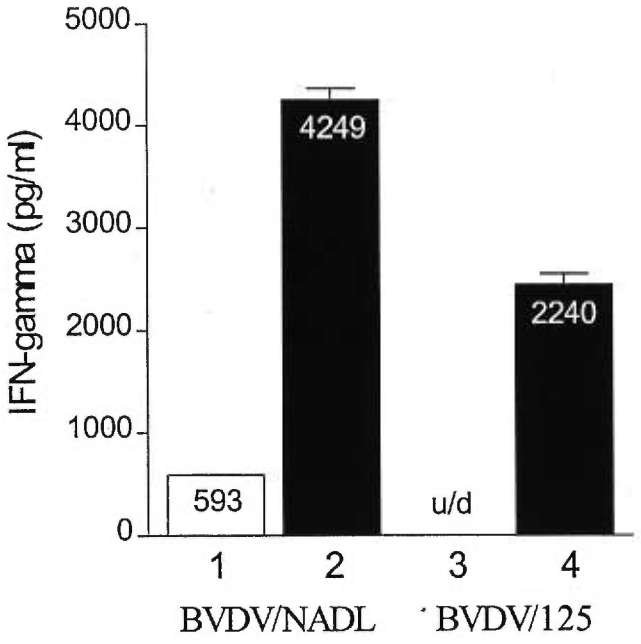

FIG. 4. Detection of IFN-γ in the supernatant of vaccinated murine mononuclear cells after stimulation by BVDV strains.

The murine mononuclear cells of mice immunized with Ad5/ΔE1ΔE3 + Ad5CMV-tTA (group I, open bar) or rAdTR5-DC/C-GFP + Ad5CMV-tTA (group II, black bar) were stimulated by BVDV/NADL (lines 1 & 2) or BVDV/125 strains (lines 3 & 4). The concentrations of IFN-γ were determined in the supernatant of the stimulated cells by ELISA and expressed as pg/ml. Results are the mean ± standard deviation and representative of two experiments. u/d; undetectable. The difference between the two groups was significant (P value < 0.05).

CHAPTER VII

DISCUSSION AND CONCLUSIONS

In 1993, the bovine industry in the province of Quebec (Canada) experienced a severe outbreak of BVDV-associated disease. There was a panic atmosphere about the inefficacy of traditional vaccines available at that time against BVDV. To characterize the BVDV responsible for this epidemic during 1993 and 1994, my initial work concentrated on studying the antigenic variation among Quebec BVDV strains before and during the outbreak as compared with different reference strains. The results are shown in Chapter 2 (Elahi *et al.*, 1997). In this report, all the Quebec isolates before and during the 1993 outbreak were classified into two distinct groups by PLA assay (acetone-fixation method). However, the two fixation methods (acetone and formalin) gave different results and it appears that acetone-fixation affects some epitopes since different isolates displayed different sensitivities to this fixation method. This variation in sensitivity was used as the basis of our classification. However, when formalin fixation was used, all of the MAbs except O2A1 and 40B4 reacted with all of the BVDV. This indicates a high degree of conservation of the three proteins NS3 (p80), E2 (gp53) and E^{ms} (gp48), at least for MAbs used in this study. Our formalin-fixation results by PLA assay confirmed a report by Shannon *et al.* (personal communication, 1994) who found that the same MAbs detected 95 to 100% of 115 strains even though two MAbs, O2A1 and 40B4, were specific for C24V-Oregon. Also, the neutralizing Mabs used in this study were not able to differentiate between Quebec isolates obtained before and during the 1993 outbreak, probably due to recognition of the conserved epitope in E^{ms} (gp48) and E2 (gp53) proteins. The use of NT with two polyclonal antibodies (PAbs) allowed us to classify strains and isolates into four groups. None of the Quebec isolates from the 1993 outbreak showed the same pattern as the reference strains (Chapter II or Elahi *et al.*, 1997).

The second part of the study concerned the development of recombinant vaccine vectors for use with BVDV. We used the reference strain of NADL to study humoral and cellular immune responses for the following reasons: (1) New data demonstrated the presence of cross-protection between BVDVs type 1 and 2 (Potgieter, 1995); (2) The nucleotide sequence of BVDV type 2 strains was not available; (3) There was no data available about immunization with BVDV type 2. Use of the NADL strain would allow us (if we were able to use these constructs in cattle) to more effectively compare the efficacy of our approach with results that had already been published concerning subunit and traditional vaccines. At present the only data available for comparison of the different approaches to BVDV vaccination in the mouse (DNA, Fowlpox and adenovirus) are the results of Harpin *et al.*, (1997), Elahi *et al.*, (Chapter III or Elahi *et al.*, 1999*a*,) and Elahi *et al.*, (Chapter IV). In these publications the mice were vaccinated by DNA, rFPV and recombinant adenoviruses (rAd) expressing the E2 protein of BVDV/NADL (rAds/E2).

We constructed three recombinant adenoviruses expressing one protein from each category of BVDV protein: (1) the C protein (p14), as the only nucleocapsid protein of BVDV, (2) the E2 (gp53) protein, as the major glycoprotein of the viral envelop, and (3) NS3 (p80) protein, as the immunodominant non-structural protein of BVDV.

To construct the recombinant fowlpox virus, three different regions of the BVDV genome, the E2 coding region, the NS3 coding region and the first third of the BVDV genome encoding the N^{pro}-C-E^{rns}-E1-E2 (p20-p14-gp48-gp25-gp53 proteins), were cloned into the transfer vector. Unfortunately, for the two recombinant fowlpox vectors encoding the NS3 and N^{pro}-C-E^{rns}-E1-E2, we were unable to obtain a stable recombinant after 4 rounds of plaque purification. In consequence, these constructs were withdrawn from subsequent studies. It appeared that as the size of the fragment cloned into the vector was increased, the recombination frequency as well as the stability of the recombinant was decreased. (data not shown).

The E2 coding region of the BVDV/NADL strain was expressed by three adenoviruses and one fowlpox virus thus providing tools to compare these two different approaches for BVDV vaccination. In Chapter III (Elahi *et al.* ,1999*a*) we demonstrated that the humoral immune response to BVDV/E2 protein after vaccination of mice with rFPV/E2 was dose-dependant. With the higher dose (4 X 10^7 pfu), all the mice seroconverted in ELISA after 6 wks p.i. In mice immunized by rAdBM5/E2 and rAdTR5-DC/E2-GFP, ELISA could detect BVDV specific antibodies in only one mouse at 3 wks p.i., whereas 6 mice vaccinated with rAdCMV5/E2 seroconverted at this time. However, at the end of experiment all the mice were seropositive to BVDV (Table 1, Chapter VII; Table 1, Chapter IV).

The rFPV/E2 produced homologous neutralizing antibodies at very low titer (maximum of 21 ± 12 at 12 wk p.i.) (Table 1, Chapter VII; Table 1, Chapter III or Elahi *et al.*, 1999*a*). The number of mice positive for the presence of homologous neutralizing antibodies against BVDV and also the titer of homologous neutralizing antibodies were dose-dependent (Table 1, Chapter III or Elahi *et al.*, 1999*a*). However, the mice immunized by rAds/E2 produced neutralizing antibodies at a minimum titer of 1022 ± 601 at 9 wk p.i. No significant difference in the BVDV neutralizing antibody titer was observed between the three rAds/E2 during the experiment (Table 1, Chapter VII; Table 1, Chapter IV). The immunization dose for rFPV/E2 was at least 25 times lower than mice immunized by rAds/E2 which could explain, in part, the low level of neutralization antibodies induced by rFPV/E2. However, we used the same dose as previously report by Taylor *et al.* (1991) in which recombinant fowlpox expressing the glycoprotein of the rabies virus successfully protected mice against rabies virus challenge. The injection of rFPV/E2 at titers higher than 4 X 10^7 in 100 µl, was not technically possible, because the titer of the purified virus stocks was not higher than 4 X 10^8 / ml. The procedure for purification significantly decreased the infectious titer of the virus stock (data not shown). On the

other hand, titres of 10^{11} to 10^{12} / ml can readily be obtained with recombinant adenoviruses and purification does not decrease the infectious titre significantly.

Recently, genetic vaccination for BVDV was reported by Harpin *et al.* (1997). In a first experiment using the mouse as an experimental model, they were able to detect neutralizing antibodies against only BVDV type 1 strain (NADL) after two immunizations with a pcDNA/gp53 (E2) vector. Neutralizing antibody titers induced by plasmid DNA immunization ranged from 4 to 32 after i.m. injection. This compared favorably with the titers induced by immunization with the virus alone (Table 1, Chapter VII or Harpin *et al.*, 1997). Neutralizing antibodies were not detected to the type 2 virus.

We detected both homologous and heterologous neutralizing antibodies in mouse sera after injection of BVDV/NADL in mice vaccinated with rAdBM5/E2 and rAdCMV5/E2. During the two weeks following this "challenge", the initial (vaccine response) homologous neutralizing antibody titer of 560 ± 160 (mean ± standard deviation) for both groups increased to 3840 ± 1478 (6.85-fold) and 4160 ± 1920 (7.42-fold), respectively. This suggested the presence of a strong memory response to the BVDV/E2 protein.. The mouse sera after challenge also neutralized the BVDV type 2 (BVDV/125 strain) *in vitro* with a titer 48 ± 17 and 40 ± 0 respectively. Yet no heterologous neutralizing antibodies were observed in mice immunized by rFPV/E2. Our results and also results of Harpin *et al.*, (1999) showed that the homologous neutralizing antibody titer is at least 60 to 100 times higher than the heterologous titer. In this case the adenovirus vectors were more efficient than the fowlpox vector, which was equivalent to the DNA vector. A combination of a recombinant adenovirus vaccine followed by a traditional BVDV vaccine would seem to be a potentially effective strategy.

Induction of an overall cellular immune response against the BVDV/E2 protein was also studied using the two rAds and one rFPV. The mononuclear cells (MNC) from mice immunized with rAdBM5/E2 and rAdCMV5/E2 showed a

homologous proliferative response after stimulation with the BVDV/NADL strain *in vitro* (Table 2, Chapter VII; Fig. 3. Chapter IV). The stimulation index (SI = average counts per minute in antigen stimulated wells / average counts per minute in wells containing only cells with medium) for mice immunized with rAdBM5/E2 and rAdCMV5/E2 were 19.2 ± 8.1 (mean ± standard deviation), and 10 ± 6.1, respectively compared with 1 ± 0.6 for mice in the negative group. Unlike the humoral response, no significant proliferative response was observed after stimulation with the BVDV/125 strain (BVDV type 2, Table 2, Chapter VII). In the case of MNC of mice immunized with rFPV/E2, no specific proliferative response was observed under the same conditions. However, a strong non-specific proliferative response was observed in mice immunized by parental fowlpox virus. This could have masked specific T-cell responses to BVDV by rFPV/E2 (Table 1, Chapter VII).

On the basis of our results (Chapter III or Elahi *et al.*, 1999*a*; Chapter IV) and previous published results (Pellerin *et al.*, 1994; Ridpath *et al.*, 1994), we suggest that the BVDV/E2 of type 2 should be included in future subunit or recombinant vaccines against BVDV. Construction of an adenovirus that expresses the BVDV/E2 protein of both types under control of the same promoter should be feasible by fusing two genes or by using the dicystronic approach (e.g. by replacing the GFP gene in pAdTR5-DC-E2/GFP construct with the BVDV/E2 type 2).

The type of T-cell response induced was characterized by quantification of, IL-2, IFN-γ (Th1-type cytokines) and IL-4 (Th2-type cytokines) produced during the T cell proliferation responses. Production of IFN-γ by homologous stimulation of murine MNC vaccinated with rFPV/E2 was higher than mice immunized with rAdCMV5/E2 (2200 ± 160 compared with 836 ± 140, mean ± standard deviation). The strong production of IFN-γ by these recombinant viruses could be an indicator of a Th1 response to the BVDV/E2 protein. However, as for the humoral response, this activation of Th1 was limited to the homologous virus (BVDV/NADL strain) used for the construction of these recombinant viruses (Table 2, Chapter VII).

Harpin *et al.* (1999) in their second experiment immunized calves with plasmid DNA expressing the BVDV/E2 protein. They showed neutralizing antibody titers against the BVDV type 1 (Singer strain) of about 8 for naked DNA and 32 for DNA in cationic liposomes until 16 wk p.i. (challenge time). It is not possible to perform a direct comparison between the results presented in Chapter IV and the results reported by Harpin *et al* (1999) using cattle. In the mouse model used for the present experiments, we clearly show the superiority of an adenovirus approach over DNA plasmid vaccination. Considering that DNA plasmid vaccination induces protection in cattle, in spite of producing low neutralizing antibody titers in mouse and low titers before challenge in cattle (Harpin *et al.*, 1999), it is quite conceivable that our adenoviruses expressing a similarly immunogenic BVDV/E2 also could induce protection in cattle.

An efficacious vaccine for BVDV should induce a protective immune response against both BVDV types. However, the role of cellular immunity in protection is still not clear despite investigations by several groups (Bolin & Ridpath, 1996; Bruschke, *et al.*, 1997; Carlsson *et al.*, 1991; Howard *et al.*, 1989). The use of only BVDV/E2 protein of the type 1 virus did not induce a cellular immune response against BVDV type 2 in mice (Chapter III or Elahi *et al.*, 1999*a*; Chapter IV) or in cattle (Harpin *et al.*, 1999). In an attempt to induce a cellular immune response against both BVDV types, we developed different adenoviruses that express the more conserved NS3 and C (nucleocapsid) proteins. The MNC of mice immunized by rAdBM5/NS3 after stimulation by both types of BVDV (NADL and 125 strains) produced 3726 ± 73 pg/ml (mean ± standard deviation) of IFN-γ after homologous stimulation and 1180 ± 10 pg/ml after heterologous stimulation (Table 2, Chapter VII; Fig 3, Chapter V or Elahi *et al.*, 1999*b*). This remarkable increase in IFN-γ production could indicate the activation of specific Th1 cells and clearly shows a cross-reactivity at the T-cell level.

One of the most intriguing findings in this thesis was the demonstration of both humoral and cellular immune responses against the nucleocapsid of BVDV expressed by a recombinant adenovirus. We detected a considerable amount of BVDV/C antibody in mouse sera after two immunizations with a mixture of the adenovirus containing an inducible promoter (rAdTR5-DC/C-FGP) and a recombinant adenovirus expressing the tTA protein (AdCMV5/tTA). Also we demonstrated a BVDV-specific MNC cell proliferation in mice after homo- and heterologous stimulation (Table 2, Chapter VII; Fig. 3, Chapter VI or Elahi *et al.*, 1999*c*). At the T cell level strong cross-reaction between two genotypes of BVDV could be a consequence of the homology of this protein between these two genotypes. A part of the response can probably be attributed to the "adjuvant effect" of adenovirus (Yang *et al.*, 1996*a*; Yang *et al.*, 1996*b*). This was the first study to show that a recombinant adenovirus with an tetracycline-inducible promoter can express a foreign gene *in vivo* when it is induced by another adenovirus which supplies the trans-activator protein (tTA protein). In following studies (Chapter IV) we have shown that this inducible promoter could drive the expression of the BVDV/E2 protein as efficiently as a constitutive promoter.

The levels of IL-2 and IL-4 in all the experiments were either undetectable or the variations were not significant between supernatants of MNC from mice immunized by recombinant viruses compared with those immunized by parental viruses (data not shown).

In summary, the major achievements of our experiments are mentioned below:

1- All the recombinant viruses expressed the BVDV genes *in vitro*.

2- ELISA assays were developed to detect antibodies against different BVDV proteins.

3- In the case of recombinant adenoviruses and fowlpox expressing the BVDV/E2 protein, the antibodies were able to neutralize BVDV infection.

2- ELISA assays were developed to detect antibodies against different BVDV proteins.

3- In the case of recombinant adenoviruses and fowlpox expressing the BVDV/E2 protein, the antibodies were able to neutralize BVDV infection.

4- Detection of lymphoproliferative responses to homologous E2 protein as well as homo- and heterologous C protein of BVDV by recombinant adenoviruses.

5- Demonstration of the BVDV specific stimulation of IFN-γ by almost all the recombinant viruses.

In conclusion, our data suggest a major implication of the C, E2 and NS3 protein of BVDV in the induction of humoral and cellular immune responses in a mouse model although no conclusions can be made about the role of these responses in protection against the virus until the work is repeated in cattle. A combination of recombinant adenoviruses expressing these proteins could increase the efficacy of any future vaccine. Our data encourage further studies to evaluate these recombinant viruses as vaccines for cattle; the natural host for BVDV.

The new data in this thesis also demonstrated the possibility of using the FPV and adenovirus for induction of humoral and cellular immune responses against BVDV proteins. However, much work still remains to demonstrate the validity of this approach for an efficient vaccine for cattle. In order to achieve this goal several avenues of investigation are mentioned below;

1- Investigate other Avipox viruses such as canarypox, as the expressing vector.

2- Carry out protection studies in cattle using the rFPV/E2. Taylor *et al.*, (1991) showed that a low dose of a recombinant canarypox can give protection in cats and dogs against rabies even in the presence of low or undetectable antibody levels in some animals. In spite of low neutralization antibody produced by rFPV/E2 the

capacity of this recombinant virus to protect against BVDV is not excluded and remains to be determined.

3- Develop a cytotoxic T-cell test in the mouse model. Mouse cell lines cannot be infected by BVDV. This is the biggest obstacle to the development of a cytotoxic T-cell test for BVDV in the mouse model. We can stimulate murine MNC *in vivo* by different approaches for gene delivery such as the adenovirus, FPV or DNA plasmid and express the different BVDV gene in target cell lines by using a recombinant adenovirus or FPV expressing the same BVDV protein. It is important to mention that the approaches used for *in vivo* stimulation and for expression of BVDV protein in mouse cell lines have to be different. Construction of an adenovirus expressing the first third of the BVDV genome encoding the N^{pro}-C-E^{ms}-E1-E2 (p20-p14-gp48-gp25-gp53 proteins) or an even longer fragment could be very helpful for this purpose.

4- Initiate challenge studies in cattle using the recombinant adenoviruses which gave the best results in the mouse model, i.e. rAdCMV5/E2, rAdBM5/NS3 and rAdTR5-DC/C-GFP separately or in combination. In the booster immunization the same adenovirus or a traditional BVDV vaccine could be used.

5- Construction of new adenovirus containing the BVDV/E2 protein type 2 or construction of new adenovirus containing both genes as a fusion gene or in a dicystronic system with inducible or constructive promoters.

6- Study use of bovine adenovirus for BVDV gene delivery.

Table 1. *In vitro* expression and humoral immune responses to recombinant FPV and adenoviruses in comparison with DNA vaccines.

Chapter [1]	Virus [2]	In vitro [3]	Immunization [4]	ELISA [5]		NT [6]
				3wk	End	
III	rFPV/E2	+	footpad	0/10	10/10	21 ± 12
III	Poxine	-	footpad	0/10	0/10	u/d
IV	rAdBM5/E2	+	i.m.	1/10	10/10	1086 ± 601
IV	rAdCMV5/E2	+	i.m.	6/10	10/10	1022 ± 612
IV	rAdTR5/DC/E2-GFP	+	i.m.	1/10	10/10	1086 ± 601
IV	Parental Ads	-	i.m.	0/10	0/10	u/d
V	rAdBM5/NS3	+	i.m.	8/10	10/10	u/d
V	rAdCMV5/NS3	+	i.m.	3/10	8/10	u/d
V	rAdBM5/NS3	+	i.n.	0/10	7/10	u/d
V	rAdCMV5/NS3	+	i.n.	0/10	7/10	u/d
V	Parental Ad	-	i.m.	0/10	0/10	u/d
VI	Ad/C	+	s.c.	6/6	6/6	u/d
VI	Parental Ads	-	s.c.	0/6	0/6	u/d
Harpin	pcDNA/gp53	+	i.m.	n/d	n/d	4 -32
Harpin	PcDNA/control	-	i.m.	n/d	n/d	u/d

[1] For more information about the Materials and Methods, please refer to the appropriate chapter.

[2] The identification of parental and recombinant viruses used in this thesis are as indicated: rFPV/E2; recombinant fowlpox virus expressing the BVDV/E2 protein, Poxine; parental Fowlpox virus, rAdBM5/E2 and rAdCMV5/E2; recombinant adenoviruses expressing the BVDV/E2 protein under control of the constitutive BM5 and CMV5 promoters respectively, rAdTR5-DC/E2-GFP; recombinant adenovirus expressing the BVDV/E2 protein under control of the inducible promoter (TR5) plus recombinant adenovirus expressing the tTA promoter (AdCMV5/tTA), Parental Ads;

parental adenovirus with deletion in E1 and E3 region Ad5/ΔE1ΔE3) + recombinant adenovirus expressing the tTA promoter (AdCMV5/tTA), rAdBM5/NS3 and rAdCMV5/NS3; recombinant adenoviruses expressing the BVDV/NS3 protein under control of the BM5 and CMV5 promoter respectively. Parental Ad; parental adenovirus with deletion in E1 and E3 region (Ad5/ΔE1ΔE3), rAd/C; recombinant adenoviruses expressing the BVDV/C protein (rAdTR5-DC/C-GFP) + tTA protein (AdCMV5/tTA), pcDNA/gp53; plasmid DNA expressing the BVDV/E2 protein (Harpin *et al.*, 1997), pcDNA3/control; plasmid control (Harpin *et al.*, 1997).

[3] *In vitro* expression of BVDV proteins were investigated in different cell lines by Radioimmunoprecipitation assay as described in Materials and Methods of Chapters III to VI or by indirect immunofluorescence (Harpin *et al.*, 1997).

[4] Immunization; mice immunized 2 to 4 times with recombinant or parental Fowlpox or Adenoviuses by footpad, intramuscular (i.m.), intranasal (i.n.) or subcutaneous (s.c.) route.

[5] ELISA; The presence of BVDV antibodies in mouse sera was detected by different Enzyme-linked immunosorbent assays as explained in the appropriate chapter. In this table only the results of ELISA at 3 week post-infection and at the end of the experiment were demonstrated.

[6] NT; neutralization test, the maximum titer of homologous neutralizing antibodies were demonstrated in this table.

u/d; undetected at the minimal dilution (1/10) used in this study

n/d; not-determined

Table 2. Cellular immune responses to recombinant FPV and adenoviruses.

Chapter [1]	Virus [2]	Ind [3]	Stimulation Index (S.I.) [4]		IFN-gamma (pg/ml) [5]	
			NADL	125	NADL	125
III	rFPV/E2	-	4.48 ± 1.2	0.83 ± 0.17	2200 ± 160	50 ± 22
III	Poxine	-	13.1 ± 5.8	1.4 ± 0.2	284 ± 23	u/d
IV	rAdBM5/E2	-	19.2 ± 8.1	1.21 ± 0.28	74 ± 0	u/d
IV	rAdCMV5/E2	-	10 ± 6.1	1.27 ± 0.27	836 ± 140	u/d
IV	Parental Ads	-	1.41 ± 0.55	0.31 ± 0.07	129 ± 17	u/d
V	rAdBM5/NS3	+	1.162 ± 0.22	1.79 ± 0.53	3726 ± 73	1180 ± 10
V	Parental Ad	+	1.32 ± 0.48	0.52 ± 0.13	329 ± 7	13 ± 14
VI	Ad/C	+	5.88 ± 0.89	8.06 ± 0.51	4249 ± 164	2249 ± 153
VI	Parental Ads	+	1.16 ± 0.45	2.04 ± 0.76	593 ± 3	u/d

[1] For more information about the Materials and Methods, please refer to the appropriate chapter.

[2] The identification of parental and recombinant viruses used in this thesis are as indicated in legend of Table 1.

[3] Ind; The murine mononuclear cells of mice stimulated *in vitro* in the presence (+) or absence (-) of indomethacin (an inhibitor of prostaglandin E2).

[4] The murine mononuclear cells of mice vaccinated with different parental or recombinant viruses stimulated *in vitro* by BVDV/NADL (type 1, homologous) or BVDV/125 strain (type 2, heterologous). The Stimulation Index (S.I.) was calculated as follows: S.I. = average counts per minute in antigen stimulated wells / average counts per minute in wells containing only cells with medium. Results are the mean ± standard deviation.

[5] The concentration of IFN-γ, in the supernatant of murine mononuclear cells stimulated by BVDV/NADL (type 1, homologous) or BVDV/125 strain (type 2, heterologous) as estimated with a cytokine ELISA assay. Results are the mean ± standard deviation.

u/d; undetected

References

Acsadi, G., Jani, A., Massie, B., Simoneau, M., Holland, P., Blaschuk, K. and Karpati, G. (1995) A differential efficiency of adenovirus-mediated in vivo gene transfer into skeletal muscle cells of different maturity. Hum Mol Genet 3, 579-584.

Akkina, RK. (1991) Pestivirus bovine viral diarrhea virus polypeptides: identification of new precursor proteins and alternative cleavage pathways. Virus Res 19, 67-81.

Baker, JC. (1990) Clinical aspects of bovine virus diarrhoea virus infection. Rev Sci Tech 9, 25-41.

Baker, JCB. (1995) The clinical manifectations of bovine viral diarrhea infection. North Am Food Anim Pract 11, 425-445.

Baker, JC. and Houe, H. (1995) "The Veterinary Clinics of North America, Food animal practice" Bovine viral diarrhea virus. 11, 393-641.

Bazan, JF. and Fletterick, RJ. (1989) Detection of trypsin-like serine protease domain in flaviviruses and pestiviruses. Virology 171: 637-639.

Becher, P., Shannon, AD., Tautz, N. and Thiel, HJ. (1994) Molecular characterization of border disease virus, a pestivirus from sheep. Virology 198, 542-551.

Becher P, Konig M, Paton DJ. and Thiel HJ. (1995) Further characterization of border disease virus isolates: evidence for the presence of more than three species within the genus pestivirus. Virology 209, 200-206.

Bertholet, C., Drillien, R. and Wittek, R. (1985) One hundred base pairs of 5' flanking sequence of a vaccinia virus late gene are sufficient to temporally regulate late transcription. Proc Natl Acad Sci U S A 82, 2096-2100.

Bolin, SR. (1993) Immunogens of bovine viral diarrhea virus. Vet Microbiol 37, 263-271.

Bolin, SR (1995) The pathogenesis of mucosal disease. Vet Clin North Am Food Anim Pract 11, 489-500.

Bolin, S., Moenning, V., Gourley, NEK. and Ridpath, J. (1988) Monoclonal antibodies with neutralizing activity segregate isolates of bovine viral diarrhea virus into groups. Arch Virol 99, 117-123.

Bolin, SR. and Ridpath, JF. (1989) Specificity of neutralizing and precipitating antibodies induced in healthy calves by monovalent modified-live bovine viral diarrhea virus vaccines. Am J Vet Res 50, 817-821.

Bolin, SR. and Ridpath JF. (1990) Range of viral neutralizing activity and molecular specificity of antibodies induced in cattle by inactivated bovine viral diarrhea virus vaccines. Am J Vet Res 51,703-707.

Bolin, SR. and Ridpath, JF. (1996) Glycoprotein E2 of bovine viral diarrhea virus expressed in insect cells provides calves limited protection from systemic infection and disease. Arch Virol 141, 1463-1477.

Bolin, SR., Matthews, PJ. and Ridpath, JF. (1991) Methode for frequency of contamination of fetal calf serum with bovine viral diarrhea virus and antibodies against bovine viral diarrhea virus. J Vet Diagn Invest 1991; 3: 199-203.

Boulanger, D., Waxweiler, S., Karelle, L., Loncar, M., Mignon, B., Dubuisson, J., Thiry, E. and Pastoret, PP. (1991) Characterization of monoclonal antibodies to bovine viral diarrhoea virus: evidence of a neutralizing activity against gp48 in the presence of goat anti-mouse immunoglobulin serum. J Gen Virol 72, 1195-1198.

Boursnell, ME., Green, PF., Campbell, JI., Deuter, A., Peters, RW., Tomley, FM., Samson, AC., Chambers, P., Emmerson, PT. and Binns, MM. (1990) Insertion of the fusion gene from Newcastle disease virus into a non-essential region in the terminal repeats of fowlpox virus and demonstration of protective immunity induced by the recombinant. J Gen Virol 71, 621-628.

Both, GW., Lockett, LJ., Janardhana, V., Edwards, SJ., Bellamy, AR., Graham, FL., Prevec, L., and Andrew, M.E. (1993) Protective immunity to rotavirus-induced diarrhoea is passively transferred to newborn mice from naive dams vaccinated with a single dose of a recombinant adenovirus expressing rotavirus VP7sc. Virol 193, 940-950.

Boye, M., Kamstrup, S. and Dalsgaard, K. (1991) Specific sequence amplification of bovine virus diarrhea virus (BVDV) and hog cholera virus and sequencing of BVDV nucleic acid. Vet Microbiol 29, 1-13.

Boyle, DB. and Coupar, BE. (1988) Construction of recombinant fowlpox viruses as vectors for poultry vaccines. Virus Res 10, 343-356.

Brock, V., Deng, R. and Riblet, SM. (1992) Nucleotide sequencing of 5' and 3' termini of bovine viral diarrhea virus by RNA ligation and PCR. J Virol Methods 38, 39-46.

Brownlie, J. (1990) The pathogenesis of bovine virus diarrhoea virus infections. Rev Sci Tech 9, 43-59.

Bruschke, CJ., Moormann, RJ., van Oirschot, JT. and van Rijn, PA. (1997) A subunit vaccine based on glycoprotein E2 of bovine virus diarrhea virus induces fetal protection in sheep against homologous challenge. Vaccine 15,1940-1945.

Buller, RM., Smith, GL., Cremer, K., Notkins, AL. and Moss, B. (1985) Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. Nature 317, 813-815.

Byers, TJ., Kunkel, LM. and Watkins, SC. (1991) The subcellular distribution of dystrophin inn mouse skeletal cardiac and smooth muscle. J Cell Biol 115, 411-421.

Calvert, JG., Nazerian, K., Witter, RL. and Yanagida, N. (1993) Fowlpox virus recombinants expressing the envelope glycoprotein of an avian reticuloendotheliosis retrovirus induce neutralizing antibodies and reduce viremia in chickens. J Virol 67, 3069-3076.

Carlsson, U. (1991) Border disease in sheep caused by transmission of virus from cattle persistently infected with bovine virus diarrhoea virus. Vet Rec 128, 145-147.

Carlsson, U. and Belák, K. (1994) Border disease virus transmitted to sheep and cattle by a persistently infected ewe: epidemiology and control. Acta Vet Scand 35, 79-88.

Carlsson, U., Alenius, S. and Sundquist, B. (1991) Protective effect of an ISCOM bovine virus diarrhoea virus (BVDV) vaccine against an experimental BVDV infection in vaccinated and non-vaccinated pregnant ewes. Vaccine 9, 577-580.

Carman, S., van Dreumel, T., Ridpath, J., Hazlett, M., Alves, D., Dubovi, E., Tremblay, R., Bolin, S., Godkin, A. and Anderson, N. (1998) Severe acute bovine viral diarrhea in Ontario, 1993-1995. J Vet Diagn Invest 10, 27-35.

Cay, B., Chappuis, G., Coulibaly, C., Dinter, Z., Edwards, S., Greiser-Wilke, I., Gunn, M., Have, P., Hess, G., Juntti, N, *et al.* (1989) Comparative analysis of monoclonal antibodies against pestiviruses: report of an international workshop. Vet Microbiol 20, 123-129.

Chanda, PK., Natuk, RJ., Mason, BB., Bhat, M., Greenberg, L., Dheer, SK., Molnar-Kimber, KL., Mizutani, S., Lubeck, MD., Davis, AR. and Hung, PP. (1990) High level expression of the envelope glycoproteins of the human immunodeficiency virus type I in presence of rev gene using helper-independent adenovirus type 7 recombinants. Virol 175, 535-547.

Chang, MW. and Leiden, JM. (1996) Gene therapy for vascular proliferative disorders. Semin Interv Cardiol 1, 185-193.

Charlton, KM., Artois, M., Prevec, L., Campbell, JB., Casey, GA., Wandeler, AI. and Armstrong, J. (1992) Oral rabies vaccination of skunks and foxes with arecombinant human adenovirus vaccine. Arch. Virol 123, 169-179.

Chengalvala, M., Lubeck, MD., Davis, AR., Mizutani, S., Molnar-Kimber, K., Morin, J. and Hung, PP. (1991) Evaluation of adenovirus type 4 and type 7 recombinant hepatitis B vaccines in dogs. Vaccine 9, 485-490.

Chengalvala, MV., Bhat, BM., Bhat, RA., Dheer, SK., Lubeck, MD., Purcell, RH. and Murthy, KK. (1997) Replication and immunogenicity of Ad7-, Ad4-, and Ad5-hepatitis B virus surface antigen recombinants, with or without a portion of E3 region, in chimpanzees. Vaccine 15, 335-339.

Cochran, MA., Puckett, C. and Moss, B. (1985) In vitro mutagenesis of the promoter region for a vaccinia virus gene: evidence for tandem early and late regulatory signals. J Virol 54, 30-37.

Collett, MS., Anderson, DK. and Retzel, E. (1988*a*) Comparisons of the pestivirus bovine viral diarrhoea virus with members of the flaviviridae. J Gen Virol 69, 2637-2643.

Collett, MS., Larson, R., Belzer, SK. and Retzel, E. (1988*b*) Proteins encoded by bovine viral diarrhea virus: the genomic organization of a pestivirus. Virol 165, 200-208.

Collett, MS., Moennig, V. and Horzinek, MC. (1989) Recent advances in pestivirus research. J Gen Virol 70, 253-266.

Corapi, WV., Donis, RO. and Dubovi, EJ. (1990) Characterization of a panel of monoclonal antibodies and their use in the study of the antigenic diversity of bovine viral diarrhea virus. Am J Vet Res 51, 1388-1394.

Cortese, VS. (1994) No cause for alarm: Current BVD vaccines appear to cross-protect against virulent type 2 strains. Topics Vet Med 5, 10-14.

Dallo, S., Rodriguez, JF. and Esteban, M. (1987) A 14K envelope protein of vaccinia virus with an important role in virus-host cell interactions is altered during virus persistence and determines the plaque size phenotype of the virus. Virology 159, 423-432.

Davidson, BL. and Bohn, MC. (1997) Recombinant adenovirus: a gene transfer vector for study and treatment of CNS diseases. Exp Neurol 144, 125-130.

Deng, R. and Brock, KV. (1992) Molecular cloning and nucleotide sequence of a pestivirus genome, noncytopathic bovine viral diarrhea virus strain SD-1. Virol 191, 867-869 .

Deregt, D., Masri, SA., Cho, HJ. and Bielefeldt Ohmann, H. (1990) Monoclonal antibodies to the p80/125 and gp53 proteins of bovine viral diarrhea virus: their potential use as diagnostic reagents. Can J Vet Res 54, 343-348.

Dewar, RL., Natarajan, V., Vasudevachari, MB. and Salzman, NP. (1989) Synthesis and processing of human immunodeficiency virus type 1 envelope proteins encoded by a recombinant humain adenovirus. J Virol 63, 129-136.

Diderholm, H. and Dinter, Z. (1966) Infectious RNA derived from bovine virus diarrhoea virus. Zentralbl Bakteriol 201, 270-272.

Donis, RO. (1995) Molecular biology of bovine viral diarrhea virus and its interactions with the host. Vet Clin North Am Food Anim Pract 11, 393-423.

Donis, RO. and Dubovi, EJ. (1987*a*) Molecular specificity of the antibody responses of cattle naturally and experimentally infected with cytopathic and noncytopathic bovine viral diarrhea virus biotypes. Am J Vet Res 48, 1549-1554.

Donis, RO. and Dubovi, EJ. (1987*b*) Differences in virus-induced polypeptides in cells infected by cytopathic and noncytopathic biotypes of bovine virus diarrhea-mucosal disease virus. Virology 158, 168-173.

Donis, RO. and Dubovi, EJ. (1987*c*) Molecular specificity of the antibody responses of cattle naturally and experimentally infected with cytopathic and noncytopathic bovine viral diarrhea virus biotypes. Am J Vet Res 48, 1549-1554.

Donis, RO., Corapi, W. and Dubovi, EJ. (1988) Neutralizing monoclonal antibodies to bovine viral diarrhea virus bind to the 56K to 58k glycoprotein. Journal of General Virology 69, 77-86.

Drummond, DR., Armstrong, J. and Colman, A. (1985) The effect of capping and polyadenylation on the stability, movement and translation of synthetic messenger RNAs in Xenopus oocytes. Nucleic Acids Res 13, 7375-7394.

Edwards, S. and Paton, D. (1995) Antigenic differences among pestiviruses. Vet Clin North Am Food Anim Pract 11, 563-577.

Edwards, S., Sands, JJ. and Harkness, JW. (1988) The application of monoclonal antibody panels to characterize pestivirus isolates from ruminants in Great Britain. Arch Virol 1988;102(3-4):197-206

Eisensmith, RC. and Woo, SL. (1996) Gene therapy for phenylketonuria. Eur J Pediatr 155, Suppl 1: S16-S19.

Elahi, SM., Harpin, S., Cornaglia, E., Talbot, B. and Elazhary, Y. (1997) Antigenic variation among bovine viral diarrhea virus (BVDV) strains and the role of different cell fixation methods in immunoassays. Can J Vet Res 61, 34-38.

Elahi, SM., Bergeron, J., Nagy, É., Talbot, BG., Harpin, S., Shen, SH. and Elazhary, Y. (1999*a*) Induction of humoral and cellular immune responses in mice by a recombinant fowlpox virus expressing the E2 protein of bovine viral diarrhea virus. FEMS Microbiol Lett "in press"

Elahi, SM., Shen, SH., Harpin, S., Talbot BG. and Elazhary, Y. (1999*b*) Investigation of the immunological properties of the Bovine Viral Diarrhea Virus protein NS3 expressed by an adenovirus vector in mice. Arch Virol "in press"

Elahi, SM., Shen, SH., Talbot BG., Massie, B., Harpin, S. and Elazhary, Y. (1999*c*) Induction of humoral and cellular immune responses against the nucleocapsid of bovine viral diarrhea virus by an adenovirus vector with an inducible promoter. Virology "in press" .

Elbers, K., Tautz, N., Becher, P., Stoll, D., Rumenapf, T. and Thiel, HJ. (1996) Processing in the pestivirus E2-NS2 region: identification of proteins p7 and E2p7. Virol 70, 4131-4135.

Esposito, JJ. (1991) Poxviridae. In *Classification and Nomenculature of Viruses* (Francki, RIB., Fauquet, CM., Knudson, DL., and Brown, F. eds.) Springer-Verlag, New York, 91-102.

Falkner, FG. and Moss, B. (1990) Transient dominant selection of recombinant vaccinia viruses. J Virol 64, 3108-3111.

Fooks, AR., Schadeck, E., Liebert, UG., Dowsett. AB., Rima, BK., Steward, M., Stephenson, JR. and Wilkinson, GW. (1995) High-level expression of the measles virus nucleocapsid protein by using a replication-deficient adenovirus vector: induction of an MHC-1-restricted CTL response and protection in a murine model. Virol 210, 456-465.

Franke, CA., Rice, CM., Strauss, JH. and Hruby, DE. (1985) Neomycin resistance as a dominant selectable marker for selection and isolation of vaccinia virus recombinants. Mol Cell Biol 5, 1918-1924.

Ghosh-Choudhury, G., Haj-Ahmad, Y. and Graham, FL. (1987) Protein IX, a minor component of the human adenovirus capsid, is essential for the packaging of full length genomes. EMBO J 6, 1733-1739.

Gillespie, JH., Baker, JA. and McEntt, K. (1960) A cytopathogenic strain of virus diarrhea virus. Cornell Vet 50, 73-79.

Ginsberg, HS., Pereira, HG., Valentine, RC. and Wilcox, WC. (1966) A proposed terminology for the adenovirus antigens and virion morphology. Virol 28, 782-783.

Ginsberg, HS. and Prince, GA. (1994) The molecular basis of adenovirus pathogenesis. Infect Agents Dis 3, 1-8.

Ginsberg, H., Lundholm-Beauchamp, U., Horswood, R., Pernis, B., Wold, W., Chanock, R. and Prince, G. (1989) Role of early region 3 (E3) in pathogenesis of adenovirus disease. Pro Natl Acad Sci USA 86, 3823-3827.

Gooding, LR., Elmore, LW., Tollefson, AE., Brady, HA. and Wold, WSM. (1988) A 14700 MW protein from the E3 region of adenovirus inhibits cytolysis by tumor necrosis factor. Cell 53, 341-346.

Gossen, M. and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters Proc Natl Acad Sci USA 89, 5547-5551.

Gossen, M., Bonin, AL., Freundlieb, S. and Bujard, H. (1994) Inducible gene expression systems for higher eukaryotic cells. Curr Opin Biotechnol 5, 516-520.

Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W. and Bujard, H. (1995) Transcriptional activation by tetracyclines in mammalian cells. Science 268, 1766-1769.

Graham FL and van der, Eb AJ. (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. Virol 52, 456-467.

Graham, FL., Smiley, J., Russell, WC. and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol 36, 59-74.

Graham, FL. (1984) In the adenoviruses (Ginsberg, HS., ed.), pp. 339-398, Plenum Press, New York.

Graham, FL. and Prevec, L. (1992) Adenovirus-based expression vectors and recombinant vaccines. In: Ellis, R.W. (ed) Vaccines: New approaches to immunological problems. Butterworth-Heinemann, Boston, pp 363-389.

Greiser-Wilke, I., Moennig, V., Coulibaly, CO., Dahle, J., Leder, L. and Liess, B. (1990) Identification of conserved epitopes on a hog cholera virus protein. Arch Virol 111, 213-225.

Groudine, M. and Casimir, C. (1984) Post-transcriptional regulation of the chicken thymidine kinase gene. Nucleic Acids Res 12, 1427-1446.

Haj-Ahmad, Y. and Graham, FL. (1986) Development of a helper-independent human adenovirus vector and its use in the transfer of the herpes simplex virus thymidine kinase gene. J Virol 57, 267-274.

Hammermueller, JD., Krell, PJ., Derbyshire, JB. and Nagy, É. (1991) An improved dot-blot method for virus detection in chicken embryo fibroblast cultures. J Virol Methods 31, 47-56.

Hanke, T., Graham, FL., Rosenthal, KL. and Johnson, DC. (1991) Identification of an immunodominant cytotoxic T-lymphocyte recognition site in glycoprotein B of herpes simplex virus by using recombinant adenovirus vectors and synthetic peptides. J Virol 65, 1177-1186.

Harasawa, R. and Tomiyama, T. (1994) Evidence of pestivirus RNA in human virus vaccines. J Clin Microbiol 32, 1604-1605.

Harpin, S., Elahi, SM., Cornaglia, E., Yolken, .H. and Elazhary, Y. (1995) The 5′-untranslated region sequence of a potential new genotype of bovine viral diarrhea virus. Arch Virol 140, 1285-1290.

Harpin, S., Talbot, B., Mbikay, B. and Elazhary, Y. (1997) Immune response to vaccination with DNA encoding the bovine viral diarrhea virus major glycoprotein gp53 (E2). FEMS Microbiol Lett 146, 229-234.

Harpin, S., Hurley, DJ., Mbikay, M., Talbot, B. and Elazhary, Y. (1999) Vaccination of cattle with a DNA plasmid encoding the bovine viral diarrhea virus major glycoprotein E2. Harpin, S., Etude d'une nouvelle méthode vaccinale contre le virus de la diarrhée virale bovine (BVD): la vaccination par ADN. Thèse de doctorat, Université de Montréal.

Hay, RT (1985) The origin of adenovirus DNA replication: minimal DNA sequence requirement in vivo. EMBO J 4, 421-426.

Herisse, J., Rigolet, M., de Dinechin, SD. and Galibert, F. (1981) Nucleotide sequence of adenovirus 2 DNA fragment encoding for the carboxylic region of the fiber protein and the entire E4 region. Nucleic Acids Res 9, 4023-4042.

Hermens, WT. and Verhaagen, J. (1998) Viral vectors, tools for gene transfer in the nervous system Prog Neurobiol 55, 399-432.

Hofmann, MA., Brechtbuhl, K., Stauber, N. (1994) Rapid characterization of new pestivirus strains by direct sequencing of PCR-amplified cDNA from the 5' noncoding region. Arch Virol 139, 217-229.

Horellou, P. and Mallet, J. (1997) Gene therapy for Parkinson's disease. Mol Neurobiol 15, 241-256.

Howard, CJ., Clarke, MC. and Brownlie, J. (1989) Protection against respiratory infection with bovine virus diarrhoea virus by passively acquired antibody. Vet Microbiol 3, 195-203.

Howard, CJ. (1990) Immunological responses to bovine virus diarrhoea virus infections. Rev Sci Tech 9, 95-103.

Houe, H. (1995) Epidemiology of bovine viral diarrhea virus. Vet Clin North Am Food Anim Pract 11, 521-547.

Hsu, KH., Lubeck, M.D., Bhat, BM., Bhat, RA., Kostek, B., Selling, BH., Mizutani, S., Davis, AR. and Hung, PP. (1994) Efficacy of adenovirus-vectored respiratory syncytial virus vaccines in a new ferret model. Vaccine 12, 607-612.

Hulst, MM., Himes, G., Newbigin, E. and Moormann, RJ. (1994) Glycoprotein E2 of classical swine fever virus: expression in insect cells and identification as a ribonuclease. Virology 200, 558-565.

Iizuka, N., Najita, L., Franzusoff, A. and Sarnow, P. (1994) Cap-dependent and cap independent translation by internal initiation of mRNAs in cell extracts prepared from Saccharomyces cerevisiae. Mol Cell Biol 14, 7322-7330.

Jacobs, SC., Stephenson, JR. and Wilkinson, GW. (1992) High-level expression of the tick-borne encephalitis virus NS1 protein by using an adenovirus-based vector: protection elicited in a murine model. J Virol 66, 2086-2095.

Jacobs, SC., Stephenson, JR. and Wilkinson, GW. (1994) Protection elicited by a replication-defective adenovirus vector expressing the tick-borne encephalitis virus non-structural glycoprotein NS1. J Gen Virol 75, 2399-2402.

Jewtoukoff, VR. and Perricaudet, M. (1995) Recombinant adenovirus as vaccines. Biologicals 23, 145-157.

Kamstrup, S., Roensholt, L. and Dalsgaard, K. (1991) Immunological reactivity of bovine viral diarrhea virus proteins after proteolytic treatment. Arch Virol Suppl 3, 225-230.

Khudyakov, YE., Fields, HA., Favorov, MO., Khudyakova, NS., Bonafonte, MT. and Holloway, B. (1993) Synthetic gene for the hepatitis C nucleocapsid protein. Nucleic Acids Res 21, 2747-2754.

Kimman, TG., Bianchi, AT., Wensvoort, G., de Bruin, TG. and Meliefstec, C. (1993) Cellular immune response to hog cholera virus (HCV): T cell of immune pigs proliferate in vitro upon stimulation with live HCV, but the E1 envelope glycoprotein is not a major T-cell antigen. J Virol 167, 2922-2927.

Kiwaki, K. and Matsuda, I. (1996) Gene therapy for ornithine transcarbamylase deficiency. Acta Paediatr 38, 189-192.

Konig, M., Lengsfeld, T., Pauly, T., Stark, R., and Thiel, HJ. (1995) Classical swine fever virus: independent induction of protective immunity by two structural glycoproteins. J Virol 69, 6479-6486.

Konishi, E., Pincus, S., Paoletti, E., Shope, RE., Burrage, T. and Mason, PW. (1992) Mice immunized with a subviral particle containing the Japanese encephalitis virus prM/M and E proteins are protected from lethal JEV infection. Virol 188, 714-720.

Kosmidou, A., Ahl, R., Thiel, HJ. and Weiland, E. (1995) Differentiation of classical swine fever virus (CSFV) strains using monoclonal antibodies against structural glycoproteins. Vet Microbiol 47, 111-118.

Kotwal, GJ. and Moss, B. (1988) Analysis of a large cluster of nonessential genes deleted from a vaccinia virus terminal transposition mutant. Virol 167, 524-537.

Kovesdi, I., Brough, DE., Bruder, JT. and Wickham, TJ. (1997) Adenoviral vectors for gene transfer. Curr Opin Biotechnol 8, 583-589.

Kumar, S. and Boyle, DB. (1990) A poxvirus bidirectional promoter element with early/late and late functions. Virol 179, 151-158.

Kweon, CH., Yoon, YD., An, SH. and Lee, YS. (1997) Expression of envelope protein (E2) of bovine viral diarrhea virus in insect cells. J Vet Med Sci 1997 59, 415-419.

Lambot, M., Douart, A., Joris, E., Letesson, JJ. and Pastoret, PP. (1997) Characterization of the immune response of cattle against non-cytopathic and cytopathic biotypes of bovine viral diarrhoea virus. J Gen Virol 78, 1041-1047.

Lee, KM. and Gillespie, JH. (1957) Propagation of virus diarrhea virus of cattle in tissue culture. Am J Vet Res 18, 952-955.

Letellier, C. (1993) Role of the TK+ phenotype in the stability of pigeonpox virus recombinant. Arch Virol 131, 431-439.

Liess, B., Orban, S., Frey, HR., Trautwein, G., Wiefel, W., Blindow, H. (1984) Studies on transplacental transmissibility of a bovine virus diarrhoea (BVD) vaccine virus in cattle. II. Inoculation of pregnant cows without detectable neutralizing antibodies to BVD virus 90-229 days before parturition (51st to 190th day of gestation). Zentralbl Veterinarmed [B] 3, 669-681.

Lubeck, MD., Natuk, R., Myagkikh, M., Kalyanm N., Aldrich, K., Sinangil, F., Alipanah, S., Murthy, SC., Chanda, PK., Nigida, SM. Jr., Markham, PD., Zolla-Pazner, S., Steimer, K., Wade, M., Reitz, MS. Jr., Arthur, LO., Mizutani, S., Davis, A., Hung, PP., Gallo, RC., Eichberg, J. and Robert-Guroff, M. (1997) Long-term protection of chimpanzees against high-dose HIV-1 challenge induced by immunization. Nat Med 3, 651-658.

Magar, R., Minocha, HC., Montpetit, C., Carman, PS. and Lecomte, J. (1988) Typing of cytopathic and noncytopathic bovine viral diarrhea virus reference and Canadian field strains using a neutralizing monoclonal antibody. Can J Vet Res 52,42-45.

Massie, B., Couture, F., Lamoureux, L., Mosser, DD., Guilbault, C., Jolicoeur, P., Bélanger, F. and Langelier, Y. (1998*a*) Inducible overexpression of toxic protein by an adenovirus vector with a tetracyclin-regulatable expression cassette. J Virol 72, 2289-2296.

Massie, B., Mosser, DD., Koutroumanis, M., Vitte-Mony, Lamoureux, L., Couture, F., Paquet, L., Guilbault, C., Dionne, J., Chahla, D., Jolicoueur, P. and Langelier, Y.

(1998*b*) New adenovirus vectors for protein production and gene transfer. Cytotechnology 28, 53-64. "in press"

Mastrangeli, A., Harvey, BG., Yao, J., Wolff, G., Kovesdi, I., Crystal, RG., Falck-Pedersen, E. (1996) "Sero-switch" adenovirus-mediated in vivo gene transfer: circumvention of anti-adenovirus humoral immune defenses against repeat adenovirus vector administration by changing the adenovirus serotype. Hum Gene Ther 7, 79-87.

McDermott, MR., Graham, FL., Hanke, T. and Johnson, DC. (1989) Protection of mice against lethal challenge with herpes simplex virus by vaccination with an adenovirus vector expressing HSV glycoprotein B. Virology 169, 244-247.

Meyers, G., Rumenapf, T. and Thiel, HJ. (1989) Molecular cloning and nucleotide sequence of the genome of hog cholera virus. Virology 171, 555-567.

Meyers, G., Tautz, N., Dubovi, EJ. and Thiel, HJ. (1991) Viral cytopathogenicity correlated with integration of ubiquitin-coding sequences. Virology 180, 602-616.

Moss, B. (1995) Poxviridae and their replication. In virology (BN. Field, ed), Raven Press, Ltd., New York, 2637-2671.

Mosser, DD., Caron, AW., Bourget, L., Jolicoeur, P. and Massie, B. (1997) Use of a dicistronic expression cassette encoding the green-fluorescent protein for the screening and selection of cells expressing inducible gene products. Bio-Techniques 22, 150-161.

Nazerian, K. and Dhawale, S. (1991) Structural analysis of unstable intermediate and stable forms of recombinant fowlpox virus. J Gen Virol 72, 2791-2795.

Nuttal, PA., Luther, PD. and Stott, EJ. (1977) Viral contamination of bovine feotal serum and cell culture. Nature 266: 835-837.

Ogawa, R., Calvert, JG., Yanagida, N. and Nazerian, K. (1993) Insertional inactivation of a fowlpox virus homologue of the vaccinia virus F12L gene inhibits the release of enveloped virions. J Gen Virol 74, 55-64.

Orban, S., Liess, B., Hafez, SM., Frey, HR., Blindow, H. and Sasse-Patzer, B. (1983) Studies on transplacental transmissibility of a Bovine Virus Diarrhoea (BVD) vaccine virus. I. Inoculation of pregnant cows 15 to 90 days before parturition (190th to 265th day of gestation). Zentralbl Veterinarmed [B] 30, 619-634.

Oualikene, W., Gonin, P. and Eliot, M. (1994) Short and long term dissemination of deletion mutants of adenovirus in permissive (cotton rat) and non-permissive (mouse) species. J Gen Virol 75, 2765-2768.

Parks, RJ., Krell, PJ., Derbyshire, JB. and Nagy, É. (1994) Studies of fowlpox virus recombinat in the generation of recombinant vaccines. Virus Res 32, 283-297.

Pasi, KJ. (1996) Gene therapy for haemophilia. Baillieres. Clin Haematol J 9, 305-317.

Paton, DJ., Ibata, G., Edwards, S. and Wensvoort, G. (1991) An ELISA detecting antibody to conserved pestivirus epitopes. J Virol Methods 31, 315-324.

Paton, DJ., Lowings, JP. and Barrett, AD. (1992) Epitope mapping of the gp53 envelope protein of bovine viral diarrhea virus. Virology 190, 763-772.

Pellerin, C., Van Den Hurk, J., Lecomte, J. and Tijssen, P. (1994) Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology 203, 260-268.

Perdrizet, JA., Rebhun, WC., Dubovi, EJ. and Donis, RO. (1987) Bovine virus diarrhea clinical syndromes in dairy herds. Cornell Vet 77, 46-74.

Perkus, ME., Limbach, K. and Paoletti, E. (1989) Cloning and expression of foreign genes in vaccinia virus, using a host range selection system. J Virol 63, 3829-3836.

Perkus, ME., Tartaglia, J. and Paoletti, E. (1995) Poxvirus-based vaccine candidates for cancer, AIDS, and other infectious diseases. J Leukoc Biol 58, 1-13.

Peters, W., Greiser-Wilke, I., Moennig, V. and Liess, B. (1986) Preliminary serological characterization of bovine viral diarrhoea virus strains using monoclonal antibodies. Vet Microbiol 12, 195-200.

Petrof, BJ. (1998) Respiratory muscles as a target for adenovirus-mediated gene therapy. Eur Respir J 11, 492-497.

Pocock, DH., Howard, CJ., Clarke, MC. and Brownlie, J. (1987) Variation in the intracellular polypeptide profiles from different isolates of bovine virus diarrhoea virus. Arch Virol 94, 43-53.

Potgieter, LN. (1995) Immunology of bovine viral diarrhea virus. Vet Clin North Am Food Anim Pract 11, 501-520.

Prevec, L., Schneider, M., Rosenthal, KL., Belbeck, LW, Derbyshire, JB. and Graham, FL. (1989) Use of human adenovirus-based vectors for antigen expression in animals. J Gen Virol 70, 429-434.

Prevec, L., Campbell, JB., Christie. BS., Belbeck, L. and Graham, FL. (1990) A recombinant human adenovirus vaccine against rabies. J Infect Dis 161, 27-30.

Purchio, AF., Larson, R., Torborg, LL. and Collett, MS. (1984) Cell-free translation of bovine viral diarrhea virus RNA. J Virol 52, 973-975.

Rawle, FC., Tollefson, AE., Wold, WS. and Gooding, LR. (1989) Mouse anti-adenovirus cytotoxic T lymphocytes. Inhibition of lysis by E3 gp19K but not E3 14.7K. J Immunol 143, 2031-2037.

Rebhun, WC., French, TW., Perdrizet, JA., Dubovi, EJ., Dill, SG. and Karcher, LF. (1989) Thrombocytopenia associated with acute bovine virus diarrhea infection in cattle. J Vet Intern Med 3, 42-46.

Ridpath, JF., Bolin, SR. and Katz, J. (1993) Comparison of nucleic acid hybridization and nucleic acid amplification using conserved sequences from the 5' noncoding region for detection of bovine viral diarrhea virus. J Clin Microbiol 31, 986-989.

Ridpath, JF., Bolin, SR. and Dubovi, EJ. (1994) Segregation of bovine viral diarrhea virus into genotypes. Virol 205, 66-74.

Rodriguez, JF. and Esteban, M. (1989) Plaque size phenotype as a selectable marker to generate vaccinia virus recombinants. J Virol 63, 997-1001.

Schneider, R., Unger, G., Stark, R., Schneider-Scherzer, E. and Thiel, HJ. (1993) Identification of a structural glycoprotein of an RNA virus as a ribonuclease. Science 261, 1169-1171.

Schultz, RD. (1993) Certain factors to consider when designing a bovine vaccination program. In proceedings of the 26th Annual Convention of the American Association of Bovine Practitioners, Albuquerque, NM, 19.

Schultze, N., Burki, Y., Lang, Y., Certa, U. and Bluethmann, H. (1996) Efficient control of gene expression by single step integration of the tetracycline system in transgenic mice. Nat Biotechnol 14, 499-503.

Shenk, T. (1995) Adenoviridae: The virus and their replication. In: Field, BN., Knipe, DM. *et al.*, (eds). Virology, 3th ed. New York: Reven Press, 2111-2148.

Shida, H., Tochikura, T., Sato, T., Konno,T., Hirayoshi, K., Seki, M., Ito, Y., Hatanaka, M., inuma, Y., Sugimoto, M., *et al.,* (1987) Effect of the recombinant

vaccinia viruses that express HTLV-I envelope gene on HTLV-I infection. EMBO J 6, 3379-3384.

Smith, GL. and Moss, B. (1983) Infectious poxvirus vectors have capacity for at least 25000 base pairs of foreign DNA. Gene 1, 21-28.

Southern, KW. (1996) Gene therapy for cystic fibrosis: current issues. Br J Hosp Med 55, 495-499.

Spyropoulos, DD., Roberts, BE., Panicali, DL. and Cohen, LK. (1988) Delineation of the viral products of recombination in vaccinia virus-infected cells. J Virol 62, 1046-1054.

Stark, R., Rumenapf, T., Meyers, G. and Thiel, HJ. (1990) Genomic localization of hog cholera virus glycoproteins. Virology 174, 286-289.

Stark, R., Meyers, G., Rumenapf, T. and Thiel, HJ. (1993) Processing of pestivirus polyprotein: cleavage site between autoprotease and nucleocapsid protein of classical swine fever virus. J Virol 7088-7095.

Tartaglia, J., Jarrett, O., Neil, JC., Desmettre, P. and Paoletti, E. (1993) Protection of cats against feline leukemia virus by vaccination with a canarypox virus recombinant, ALVAC-FL. J Virol 67, 2370-2375.

Taylor, J. and Paoletti, E. (1988) Fowlpox virus as a vactor in non-avian species. Vaccine 6, 466-468.

Taylor, J., Weinberg, R., Kawaoka, Y., Webster, RG. and Paoletti, E. (1988) Protective immunity against avian influenza induced by a fowlpox virus recombinant. Vaccine 6, 504-508.

Taylor, J., Edbauer, C., Rey-Senelonge, A., Bouquet, JF., Norton, E., Goebel, S., Desmettre, P. and Paoletti, E. (1990) Newcastle disease virus fusion protein expressed in a fowlpox virus recombinant confers protection in chickens. J Virol 64, 1441-1450.

Taylor, J., Trimarchi, C., Weinberg, R., Languet, B., Guillemin, F., Desmettre, P. and Paoletti, E. (1991) Efficacy studies on a canarypox-rabies recombinant virus. Vaccine 9, 190-193.

Taylor, J., Weinberg, R., Tartaglia, J., Richardson, C., Alkhatib, G., Briedis, D., Appel, M., Norton, E. and Paoletti, E. (1992*a*) Nonreplicating viral vectors as potential vaccines: recombinant canarypox virus expressing measles virus fusion (F) and hemagglutinin (HA) glycoproteins. Virology 187, 321-328.

Taylor, J., Tartaglia, J., Moran, T., Webster, RG., Bouquer, J-F., Quimby, FW., Holmes, D., Laplace, E., Mickle, T. and Paoletti, E. (1992*b*) The role of poxvirus vectors in influenza vaccine development. *In proceeding of the Third International Symposium on Avian Influenza.* University of Wisconsin-Madison. Extension Duplicating Services, 311-335.

Top, FH., Grossman, RA., Bartelloni, PJ., Segal, HE., Dudding, BA., Russel, PK. and Buescher, EL. (1971) Immunization with live type 7 and 4 adenovirus vaccines. I. Safety, infectivity, antigenicity, and potency of adenovirus type 7 vaccine in humans. J Infect Dis 124, 148-154.

van Rijn, PA., van Gennip, HG., de Meijer, EJ. and Moormann, RJ. (1993) Epitope mapping of envelope glycoprotein E1 of hog cholera virus strain Brescia. J Gen Virol 74, 2053-2060.

van Rijn, PA., Bossers, A., Wensvoort, G. and Moormann, RJ. (1996) Classical swine fever virus (CSFV) envelope glycoprotein E2 containing one structural antigenic unit protects pigs from lethal CSFV challenge. J Gen Virol 77,2737-2745.

Walter, J. and High, KA. (1997) Gene therapy for the hemophilias. Adv Vet Med 40, 119-134.

Warrener, P. and Collett, M. (1995) Pestivirus NS3 (p80) protein posseses RNA helicase activity. J Virol 69, 1720-1726.

Weiland, E., Ahl, R., Stark, R., Weiland, F. and Thiel, HJ. (1992) A second envelope glycoprotein mediates neutralization of a pestivirus, hog cholera virus. J Virol 66, 3677-3682.

Weinberg, D. and Ketner, G. (1983) A cell line that supports the growth of a defective early region 4 deletion mutant of human adenovirus type 2. Proc Natl Acad Sci USA 80, 5383-5386.

Wengler, G., Bradley, DW., Collett, MS., Heinz, FX., Schesinger, RW. and Strauss, J. (1995) Flaviviridae, p. 415-427. In Murphy, FA., Fauquet, CM., Bishpo, DH. L., Ghabrial, SA., Jarvis, AW., GP. Martelli, Mayo, MA. and Summers, MD. (ed), Virus taxonomy. Sixth report on the International Committee on Taxonomy of Viruses. Springer-Verlag. New York.

Wensvoort, G., Terpstra, C. and de Kluyver, EP. (1989) Characterization of porcine and some ruminant pestiviruses by cross-neutralization. Vet Microbiol 20, 291-306.

Wesseling, JG., Godeke, GJ., Schijns, VE., Prevec, L., Graham, FL., Horzinek, MC. and Rottier, PJ. (1993) Mouse hepatitis virus spike and nucleocapsid proteins expressed by adenovirus vectors protect mice against a lethal infection. J Gen Virol 74, 2061-2069.

Wild, TF., Bernard, A., Spehner, D. and Drillien, R. (1992) Construction of vaccinia virus recombinants expressing several measles virus proteins and analysis of their efficacy in vaccination of mice. J Gen Virol 73, 359-367.

Wilmott, RW., Amin, RS., Perez, CR., Wert, SE., Keller, G., Boivin, GP., Hirsch, R., De Inocencio, J., Lu, P., Reising, SF, Yei, S., Whitsett, JA. and Trapnell, BC. (1996) Safety of adenovirus-mediated transfer of the human cystic fibrosis transmembrane conductance regulator cDNA to the lungs of nonhuman primates. Hum Gene Ther 7, 301-318.

Wiskerchen, M. and Collett, MS. (1991) Pestivirus gene expression: protein p80 of bovine viral diarrhea virus is a proteinase involved in polyprotein processing. Virology 184, 341-350.

Wiskerchen, M., Belzer, SK. and Collett, MS. (1991) Pestivirus gene expression: the first protein product of the bovine viral diarrhea virus large open reading frame, p20, possesses proteolytic activity. J Virol 65, 4508-4514.

Xu, ZZ., Krougliak, V., Pervec, L., Graham, FL. and Both, W. (1995) Investigation of promoter function in animal cells infected with human recombinant adenoviruses expressing rotavirus antigen VP7sc. J Gen Virol 76, 1971-1980.

Xue, W., Blecha, F. and Minocha, HC. (1990) Antigenic variations in bovine viral diarrhea viruses detected by monoclonal antibodies. J Clin Microbiol 28, 1688-1693.

Yang, Y., Ertl, HC. and Wilson, JM. (1994) MHC class I-restricted cytotoxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. Immunity 1, 433-442.

Yang, Y., Xiang, Z., Ertl, HC. and Wilson, JM. (1995) Upregulation of class I major histocompatibility complex antigens by interferon gamma is necessary for T-cell-mediated elimination of recombinant adenovirus-infected hepatocytes in vivo. Proc Natl Acad Sci U S A 92, 7257-7261.

Yang, Y., Jooss, .KU., Su, Q., Ertl, HC., and Wilson, JM. (1996*a*) Immune responses to viral antigens versus transgene product in the elimination of recombinant adenovirus-infected hepatocytes in vivo. Gene Ther 3, 137-144.

Yang, Y., Su, Q. and Wilson, JM. (1996*b*) Role of viral antigens in destructive cellular immune responses to adenovirus vector-transduced cells in mouse lungs. J Virol 70, 7209-7212.

Yoshida, Y., Emi, N. and Hamada, H. (1997) VSV-G-pseudotyped retroviral packaging through adenovirus-mediated inducible gene expression. Biochem Biophys Res Commun 232, 379-382.

Zimmer, GM., Wentink, GH., Brinkhof, J., Bruschke, CJM., Westenbrink, F., Crauwels, APP. and de Goey, I. (1996) Model for testing efficacy of bovine viral diarrhoea virus vaccines against intrauterine infection. In: Proceedings of the XIX World Buiatrics Congress, pp. 217-220.

Zingg, HH., Lefebvre, DL. and Almazan, G. (1988) Regulation of poly(A) tail size of vasopressin mRNA. J Biol Chem 263, 11041-11043.