CHARACTERIZATION OF IMMUNE RESPONSES INDUCED BY PLASMIDS ENCODING BOVINE ROTAVIRUS ANTIGENS IN THE MURINE MODEL

A Thesis
Submitted to the Faculty of Graduate Studies and Research
in Partial Fulfilment of the Requirements for the Degree of
Doctor of Philosophy
in the Department of Veterinary Microbiology
University of Saskatchewan
Saskatoon, Canada

by
Sanipa Suradhat
Spring, 1999

UNIVERSITY OF SASKATCHEWAN

College of Graduate Studies and Research

SUMMARY OF DISSERTATION

Submitted in partial fulfillment

of the requirements for the

DEGREE OF DOCTOR OF PHILOSOPHY

by

Sanipa Suradhat

Department of Veterinary Microbiology
Western College of Veterinary Medicine
University of Saskatchewan

Spring, 1999

Examining Committee:

Dr. H.G.G. Townsend	Dean's Designate, Chair College of Graduate Studies and Research
Dr. J. Gordon	Chairman of Advisory Committee, Department of Veterinary Microbiology
Dr. L. Babiuk	Supervisor, Department of Veterinary Microbiology
Dr. M. Baca-Estrada	Veterinary Infectious Disease Organization
Dr. P. Griebel	Veterinary Infectious Disease Organization
Dr. L. Qualtiere	Department of Microbiology

External Examiner:

Dr. R. Brunham
Department of Medical Microbiology
University of Manitoba
Winnipeg, Manitoba
R3E 0W3

PERMISSION TO USE

In presenting this thesis in partial fulfilment of the requirements for a postgraduate degree from the University of Saskatchewan, I agree that the libraries of this University may make it freely available for inspection. I further agree that permission for copying in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was completed. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not by allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use, which may be made of any material in my thesis.

Requests for permission to copy or make other use of material in this thesis in whole or in part should be addressed to:

Head of the Department of Veterinary Microbiology
Western College of Veterinary Medicine
University of Saskatchewan
Saskatoon, Saskatchewan
S7N 5B4 Canada

ABSTRACT

DNA immunization effectively induces humoral and cell-mediated immunity to numerous infectious diseases. To investigate the potential use of DNA-based vaccine for induction of bovine rotavirus (BRV) specific immune responses, I characterized the immune responses induced by plasmids encoding 2 important neutralizing antigens, VP4 and VP7, in a murine model. Immunization with plasmids encoding VP4 protein did not induced detectable BRV-specific antibody responses in mice. However, it induced BRV-specific cell-mediated immune responses characterized by an increased number of BRV-specific cytokine secreting cells in the spleens of immunized mice. In addition, the immunized animals were primed for both antibody and cellular immune responses following BRV boost. The priming effect was lasted, for at least 9 months, after plasmid immunization. However, plasmids encoding VP4 did not boost the BRV-specific immune responses in the BRV-primed mice. The results from this study demonstrated that although immunization with plasmids encoding VP4 did not induce detectable BRV-specific antibody response, the immune responses induced by plasmid encoding VP4, alone or following BRV boost, were biased toward Th1-like responses. This conclusion was based on the findings that the spleens of immunized mice contained predominantly BRV-specific IFN-γ and IgG2a secreting cells and that $CD4^+$ cells were the major source of IFN-γ production in the spleens.

The BRV-VP7 is also considered an important antigen for inducing protective

immunity against rotaviruses. However, this study indicated that the VP7, either by itself or in the context of viral particle, was poorly immunogenic in mice. Preliminary results indicated that plasmids encoding VP7 were less efficient for induction of immune responses than the plasmids encoding VP4. This project was then explored several approaches to enhance the immunogenicity of the VP7 protein when expressed by the plasmid, utilizing information gathered from different areas including molecular biology, virology, and immunology. These approaches included: i) enhancing the level of gene expression by modifying the expression cassette of the plasmids; ii) changing the cellular localization of the plasmid-expressed protein; iii) co-administration of plasmids encoding VP4 and VP7; iv) creating plasmids encoding a deletion mutant of VP7; and v) construction of plasmids encoding chimeric proteins of VP7 and complement C3d or bovine herpes virus-1 glycoprotein D (BHV-1 gD).

The results from these studies demonstrated that cellular localization and nature of the VP7 antigen could greatly influence the immunogenicity of the plasmid-expressed antigen and the pattern of immune responses in immunized mice. Plasmids encoding membrane-bound VP7 induced the best BRV-specific immune responses, following BRV boost or in BRV-primed mice, when compared with the authentic and the secretory versions. In addition, the plasmid encoding a deletion mutant, truncated VP7, induced predominantly BRV-specific IL-4 production, instead of the predominantly IFN-γ production previously observed following plasmid immunization.

Strategies that had previously been shown to enhance the immunogenicity of protein antigens were also examined. However, different outcomes were observed when these strategies were applied to plasmid immunization. First, addition of the C3d gene into the expression cassette which encoded antigen-C3d chimeras inhibited the induction of both humoral and cell-mediated, antigen-specific immune responses. Secondly, co-expression of the gD molecule as a gD-VP7 chimera enhanced the level

of BRV-specific antibody responses following viral boost or in the BRV-primed mice. These results with the gD protein suggested an alternative approach for improving the immunogenicity of poorly immunogenic antigens. However, findings in this study also suggested that the gD protein may have biological activities that could play a significant immunomodulatory role in immunized mice.

In conclusion, none of the plasmids used in this study induced BRV-specific antibody responses following a primary immunization. However, immunological activity was evident following BRV administration, either prior to or following plasmid immunization. Each plasmid induced a distinct pattern of immune responses in both naïve and BRV-primed mice. These investigations demonstrated specific ways that DNA immunization could be modified to modulate the outcome of BRV-specific immune responses.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Lorne A. Babiuk, and the Faculty of Veterinary Science, Chulalongkorn University who gave me the opportunity to come to Canada and learn to appreciate the fascinating world of immunology and research science. I would like to also express my gratitude to the member of my graduate committee, Drs Maria Baca-Estrada, Philip Griebel, Lou Qualtiere, John Gordon and the former members, Drs Dongwan Yoo and Henry Tabel for their advice and support during the course of my Ph.D. program.

I am specially grateful to the guidance and support, both academically and personally, from my co-supervisor, Dr. Maria Baca-Estrada. I also wish to thank Drs. Dongwan Yoo, P. Jeffrey Lewis, Dale Godson, Ralph Braun and Robert Brownlie for their academic and technical assistance. I thank Dr. Philip Griebel for his supervision, technical assistance, and "constructive" criticisms.

This project could not be accomplished without the help from the staff of VIDO, especially members of the animal care unit and GMP. I am gratefully appreciated the frequent technical assistance and advice from Kathy Ewen, Elaine Gibbon, Marlene Snider, and Terry Beskorwayne. My sincere thanks go to the graduate students in VIDO especially Dr. Zsuzsanna Papp, Dr. Joong Bok Lee and Camilo Raggo for their technical advise and friendship. Finally, I am deeply grateful to my family and friends, particularly those whom I have met in Saskatoon. Without their patience, support and encouragement, I would not be at this point.

This work was funded by grants from the Government of Thailand and the Medical Research Council of Canada.

TABLE OF CONTENTS

PERMISSION TO USE i

ABSTRACT ii

ACKNOWLEDGEMENTS v

TABLE OF CONTENTS vii

LIST OF TABLES xii

LIST OF FIGURES xiii

LIST OF ABBREVIATIONS xvi

1. INTRODUCTION 1

2. LITERATURE REVIEW 2

2.1 Rotaviruses 2

2.1.1 Rotavirus characteristics and classification 2

2.1.2 Rotavirus structure 3

2.1.3 Rotavirus genome 4

2.1.4 The rotavirus replication cycle 5

2.1.5 Rotavirus pathogenicity and virulence factor 9

2.1.6 Biology of the outer capsid proteins 10

2.1.6.1 VP4 10

2.1.6.2 VP7 11

2.1.7 Immunity to rotavirus 14

2.1.7.1 Effector mechanisms involved in the resolution of rotavirus infection and prevention of reinfection 14

2.1.7.2 Humoral immunity 17

2.1.7.3 Cell-mediated immunity 20

2.1.7.4 Strategies for development of bovine rotavirus vaccine 24

2.2 DNA immunization 27

2.2.1 Mechanism of DNA immunization 28

2.2.2 Immune responses induced by DNA immunization and factors that influence the outcome of the immune responses 38

2.2.3 The adjuvanticity of DNA 46

2.2.4 DNA immunization for induction of rotavirus-specific immunity 51

2.2.5 Enhancement of immune responses induced by DNA-based vaccines 53

2.3 The biological effects of complement protein C3d on the immune system 55

3. HYPOTHESES AND OBJECTIVES 60

4. MATERIALS AND METHODS 62

4.1 Bacterial strains, growth condition and plasmid purification 62

4.2 Plasmid construction 63

4.3 Cell lines and tissue culture reagents 74

4.4 Transient transfection 75

4.5 Radioimmunoprecipitation assay 76

4.6 Deglycosylation of glycoproteins 79

4.7 Western blotting of BRV proteins 79

4.8 Viruses 80

4.9 Plaque assay 81

4.9.1 Rotavirus plaque assay 81

4.9.2 Vaccinia virus plaque assay 82

4.10 *In vivo* procedures **82**

4.10.1 Mice 82

4.10.2 Immunization and viral inoculation protocols 83

4.11 Assessment of immune responses **83**

4.11.1 ELISA 83

4.11.2 Isolation of lymphocytes and cell culture conditions 85

4.11.3 Cytotoxicity assay 85

4.11.4 ELISPOT for detection of cytokine secreting cells (CSCs) 86

4.11.5 ELISPOT for detection of rotavirus specific-antibody secreting cells (ASCs) 87

4.11.6 Depletion of T-lymphocyte subpopulations 88

4.12 Bioassay for VP7-C3d chimeras **89**

4.12.1 Isolation of ileal Peyer's patch B cells of lambs 89

4.12.2 Binding assay for the VP7-C3d chimeras 89

4.12.3 C3d bioassay 90

4.13 Statistical analysis **91**

5. RESULTS **92**

5.1 Immune responses induced by plasmids encoding VP4 protein **92**

5.1.1 Immune responses induced by pcDNA3-VP4 92

5.1.2 Immune responses induced by pSLIA-VP4 98

5.1.2.1 *In vitro* protein expression of pSLIA-VP4 98

5.1.2.2 Immune responses induced by pSLIA-VP4 in C57Bl/6 mice 99

5.1.2.3 Immune responses induced by pSLIA-VP4 in Balb/c mice 103

5.1.2.4 Immune responses induced by pSLIA-VP4 in BRV-primed mice 106

5.2 Immune responses induced by VP7 protein and plasmids encoding VP7 proteins **108**

5.2.1 Immune responses induced by BRV-VP7 108

5.2.2 Immune responses induced by plasmid encoding BRV-VP7 108

5.3 Approaches used to alter BRV-specific immune responses by plasmid encoding VP7 or modified VP7 proteins 110

5.3.1 Improving the level of antigen expression and its compartmentalization .. 110

5.3.1.1 *In vitro* characterization of plasmids encoding VP7 and modified forms of VP7 113

5.3.1.2 Immune responses induced by plasmids encoding VP7 and modified VP7 117

5.3.1.3 Immune responses induced by plasmids encoding VP7 and modified VP7 in BRV-primed mice 120

5.3.2 Co-administration of plasmids encoding VP4 and VP7 proteins 123

5.3.3 Co-expression of the complement C3d molecule with VP7 protein 126

5.3.3.1 *In vitro* characterization of plasmids encoding the VP7-C3d chimeric proteins 126

5.3.3.2 Immune responses induced by plasmids encoding VP7-C3d chimeric proteins 130

5.3.3.3 Immune responses induced by pVP7$(C3d)_2$ in BRV-primed mice 134

5.3.3.4 BHV-1-gD specific immune responses in mice immunized with plasmids encoding tgD-C3d chimeras 134

5.3.4 Altering the VP7 molecule by deletion mutation 140

5.3.4.1 *In vitro* characterization of plasmid encoding truncated VP7 140

5.3.4.2 Immune responses induced by plasmid encoding truncated VP7 142

5.3.4.3 Immune responses induced by pVP7t in BRV-primed mice 142

5.3.5 Co-expression of the VP7 with a second antigen (gD) 146

5.3.5.1 *In vitro* characterization of plasmid encoding gD-VP7 chimeras 146

5.3.5.2 Immune responses induced by plasmid encoding gD-VP7 chimeras 148

5.3.5.3 Immune responses induced by plasmid encoding a gD-VP7 chimera in BRV-primed mice 154

6. DISCUSSION 157

6.1 Immune responses induced by plasmids encoding VP4 proteins 157

6.2 Immune responses induced by plasmids encoding VP7 proteins 163

6.2.1 Immune responses induced by plasmids encoding VP7 and modified VP7 163

6.2.2 Immune responses induced by plasmids encoding C3d chimeras 175

6.2.3 Immune responses induced by plasmids encoding truncated VP7 180

6.2.4 Immune responses induced by plasmids encoding gD-VP7 chimeras 182

6.3 General discussion 190

7. REFERENCES 195

LIST OF TABLES

Table 4.1 Plasmids used in this study 64

Table 4.2 Primers used to generate PCR amplified cDNAs in this study 67

Table 5.1.1 Effect of DNA immunization on the isotype of BRV-specific ASCs 96

Table 5.1.2 Effect of T lymphocyte subset depletion on number of BRV-specific IFN-γ secreting cells 96

Table 5.3.1 Plasmids used in this study and characteristics of their products 112

Table 6.1 Summary of the BRV-specific immune responses induced by immunizations with plasmids encoding VP7 168

LIST OF FIGURES

Figure 4.1 Diagram depicting features of the rotavirus VP7 protein and the VP7 fragments used in this study 66

Figure 4.2 Schematic diagram depicting the strategy used for cloning of pgD-VP7Sc, pTPA-VP7Anc and pgD-VP7Anc 69

Figure 4.3 Schematic diagram depicting the strategy used for cloning of pVP7(C3d) and pVP7(C3d)$_2$ 71

Figure 5.1.1 BRV-specific antibody responses in mice immunized with pcDNA3-VP4 93

Figure 5.1.2 BRV-specific cytokine responses in mice immunized with pcDNA3-VP4 95

Figure 5.1.3 BRV-specific cytotoxic T cell activity in spleen of mice immunized with pcDNA3-VP4 95

Figure 5.1.4 Effect of intron A on the level of VP4 gene expression 100

Figure 5.1.5 BRV-specific antibody responses in mice immunized with pSLIA-VP4 101

Figure 5.1.6 BRV-specific cytokine responses in mice immunized with plasmids encoding VP4 protein and then boosted with BRV 102

Figure 5.1.7 BRV-specific antibody responses in Balb/c mice immunized with pSLIA-VP4 and then boosted with BRV 104

Figure 5.1.8 BRV-specific cytokine responses in Balb/c mice immunized with pSLIA-VP4 and then boosted with BRV 105

Figure 5.2.1 Western blot analysis of sera obtained from BRV immunized mice 109

Figure 5.2.2 BRV-specific immune responses in mice immunized with plasmids encoding VP4 or VP7, with or without BRV 111

Figure 5.3.1 *In vitro* expression of plasmids encoding VP7 protein 114

Figure 5.3.2 Localization of the VP7Anc protein 115

Figure 5.3.3 Nature of carbohydrate attached to the modified VP7 proteins 116

Figure 5.3.4 BRV-specific antibody responses in mice immunized with plasmids encoding VP7 and modified VP7 and then boosted with BRV..... 118

Figure 5.3.5 Number of BRV specific cytokine secreting cells following BRV boost 119

Figure 5.3.6 BRV-specific cytotoxic T cell activity in the spleen of mice immunized with plasmids encoding VP7 and modified VP7 and then boosted with BRV 119

Figure 5.3.7 BRV-specific immune responses in BRV-primed mice boosted with plasmids encoding VP7 and modified VP7 121

Figure 5.3.8 Effect of co-administration of plasmids encoding VP4 and VP7Sc on the BRV-specific immune response of C3H mice boosted with BRV .. 125

Figure 5.3.9 Characteristics and *in vitro* expression of plasmids encoding VP7-C3d chimeras..... 127

Figure 5.3.10 Binding of VP7-C3d chimeric protein to B cells 129

Figure 5.3.11 Capacity of the VP7-C3d chimera to rescue B cells from single-signal induced apoptosis..... 131

Figure 5.3.12 BRV-specific antibody responses in mice immunized with plasmids encoding VP7-C3d chimeras and then boosted with BRV..... 132

Figure 5.3.13 BRV-specific cytokine responses in mice immunized with plasmids encoding VP7-C3d chimeras and then boosted with BRV..... 133

Figure 5.3.14 BRV-specific immune responses in BRV-primed mice boosted with plasmid encoding a VP7-C3d chimera..... 135

Figure 5.3.15 BHV-1 gD-specific antibody response in mice immunized with plasmids encoding gD-C3d chimeras..... 137

Figure 5.3.16 BHV-1 gD-specific cytokine responses in mice immunized with plasmids encoding tgD-C3d chimeras..... 138

Figure 5.3.17 Characteristics and *in vitro* expression of pVP7t..... 141

Figure 5.3.18 BRV-specific cytokine responses in mice immunized with pVP7t and then boosted with BRV 143

Figure 5.3.19 BRV-specific immune responses in BRV-primed mice boosted with pVP7t 144

Figure 5.3.20 Characteristics and *in vitro* expression of plasmids encoding gD-VP7 chimeras..... 147

Figure 5.3.21 BRV-specific antibody responses in mice immunized with plasmids encoding gD-VP7 chimeras and then boosted with BRV 149

Figure 5.3.22 BHV1-gD specific antibody responses in mice immunized with plasmids encoding gD-VP7 chimeras 150

Figure 5.3.23 BRV-specific cytokine responses in mice immunized with plasmids encoding gD-VP7 chimeras and then boosted with BRV 152

Figure 5.3.24 Ratio of BRV-specific cytokine secreting cells in mice immunized with plasmids encoding gD-VP7 chimeras and then boosted with BRV 153

Figure 5.3.25 BRV-specific immune responses in BRV-primed mice boosted with plasmids encoding gD-VP7Sc and modified VP7 155

LIST OF ABBREVIATIONS

aa.	amino acid
Ab	antibody
ABTS	2,2'-azino-di-(3-ethyl-benzthiazoline sulphonate)
APC	antigen presenting cells
ASCs	antibody secreting cells
BCR	B-cell receptor
BGH poly A	bovine growth hormone polyadenylation site
BHV-1	bovine herpesvirus type1
bp	basepair(s)
BRV	bovine rotavirus
BSA	bovine serum albumin
C3	complement cpmponent 3
C4	complement component 4
CD	clusters of differentiation, designation for cell surface markers
CD40L	CD40 ligand
cDNA	complementary deoxyribonucleic acid
CIP	calf intestinal phosphatase
CLP	core-like particles
CPM	count per minute
CSCs	cytokine secreting cells
CTL	cytotoxic T lymphocyte(s)
DC	dendritic cells
DLNs	draining lymph nodes
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay

ELISPOT	enzyme-linked immunospot
Endo H	endoglycosidase H
EP	early promoter
ER	endoplasmic reticulum
E:T	effector : target cells
FACS	fluorescence-activated cell sorter
FBS	fetal bovine serum
FDC	follicular dendritic cells
GC	germinal center
gD	glycoprotein D of bovine herpesvirus-1
GM-CSF	granulocyte-monocyte colony stimulating factor
hCMV	human cytomegalovirus
HEL	hen egg lysozyme
HEPES	N-2 hydroxylethyl piperazine-N'-2-ethanesulfonic acid
HI	haemagglutinin inhibition
hr	hour(s)
HSV-1	herpes simplex virus type 1
HVEM	herpesvirus entry mediator
i.d.	intradermal, intradermally
i.m.	intramuscular, intramuscularly
i.p.	intraperitoneal, intraperitoneally
IA	intron A
IEL	intraepithelial lymphocytes
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMBs	immunomagnetic beads
ISS	immunostimulatory sequence(s)
KDa	kilodalton
LAK	lymphokine-activated killer
LC	Langerhans cells
LPS	lipopolysaccharide
m.o.i.	multiplicity of infection
MadCAM-1	mucosal addressin cell adhesion molecule-1

MEM	Eagle's minimal essential medium
MHC	major histocompatibility complex
min	minute(s)
MPC-1	magnetic particle concentrator
mRNA	messenger ribonucleic acid
MSR	macrophage scavenger receptor
MW	molecular weight
NK	natural killer
NSP	non-structural protein
nt	nucleotide(s)
OD	optical density
ODNs	oligodeoxyribonucleotides
p.f.u.	plaque forming units
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood leukocytes
PBS	phosphate buffered saline
PBSA	Ca^{++}, Mg^{++}-free phosphate buffered saline
PCR	polymerase chain reaction
pCTL	precursor cytotoxic T lymphocytes
PBMC	peripheral blood mononuclear cells
PMSF	phenylmethylsulfonyl fluoride
PNGase F	N-glycosidase F
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
RRV	rhesus rotavirus
RSV	Rous sarcoma virus
RT	room temperature
rVV	recombinant vaccinia virus
SAP	strepavidin-alkaline phosphatase conjugate
SCID	severe combined immunodeficiency
SD	standard deviation
SDS	sodium dodecyl sulfate
sIgA	secretory IgA
ss	signal sequence

SV-40	simian virus 40
TAP	transporter associated with antigen processing
TBS	Tris buffered saline
Tc	cytotoxic T lymphocytes
tgD	truncated gD protein of bovine herpesvirus-1
Th	T helper (lymphocytes)
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TPA	tissue plasminogen activator
TRAF	TNFR-associated factor
VIDO	Veterinary Infectious Disease Organization
VLP	viral-like particles
VP	viral protein
VP7Anc	membrane anchored VP7
VP7Sc	secretory VP7
VP7t	truncated VP7
wk	week

1. INTRODUCTION

DNA immunization has been recently accepted as a novel approach for the induction of antigen-specific immunity. Recent evidence demonstrates that DNA-based vaccines can effectively induce both humoral and cell-mediated immune responses in immunized animals. Furthermore, the genes present in the plasmid vectors can be easily modified and tested, *in vivo*, within a short period of time. This offers an opportunity to explore and address basic immunological questions regarding immunogenicity of an antigen, which may improve current vaccine strategies.

Rotaviruses are the most important cause of severe gastroenteritis in young children and newborn animals (Kapikian & Chanock, 1996). The current vaccination approach in newborn animals is based on induction of lactogenic immunity in the dams (Saif & Fernandez, 1996). In addition, it is generally believed that the colostral-derived antibodies recognizing the outer capsid proteins of rotavirus, VP4 and VP7, play a significant role in protection against viral-induced diarrhea in suckling calves. Therefore, characterizing the immune responses induced by plasmids encoding the VP4 and VP7 gene was an initial step toward determining the potential use of DNA vaccine against bovine rotavirus.

2. LITERATURE REVIEW

2.1 Rotaviruses

2.1.1 Rotavirus characteristics and classification

Rotaviruses were initially recognized as agents associated with gastroenteritis in animals several years before the discovery of human rotavirus in 1973 (reviewed in Estes, 1996a). Since then, numerous epidemiological studies have shown that rotaviruses are the most important cause of severe gastroenteritis in young children and animals worldwide (Kapikian & Chanock, 1996).

Belonging to the family *Reoviridae*, rotaviruses are characterized by a segmented double-stranded RNA genome and a nonenveloped, multilayered protein capsid (Estes, 1996b). Rotaviruses have three important antigenic specificities: group, subgroup and serotype. There are at least seven groups (A-G) of rotaviruses that possess distinct RNA profiles and lack serological cross-reactivity (Kapikian & Chanock, 1996). Group A rotaviruses have been clearly associated with diarrhea in young animals. Although information on the non-group A rotaviruses is limited due to difficulties in viral culture, they have been characterized with respect to their genotypes (Estes, 1996b). Within a specific group, rotaviruses are sub-divided into subgroups by the antigenic specificity of the VP6 protein. In addition, group A rotaviruses are further classified into serotypes determined by reactivity of virus in neutralization assays (W.H.O., 1984). Neutralization assays measure the reactivity of antibodies against

the two outer capsid proteins (VP4 and VP7). Although VP4 and VP7 independently induce neutralizing antibodies (Kapikian & Chanock, 1996), in most cases the predominant antibody reactivity is against VP7 (Estes & Cohen, 1989).

The classification of group A rotavirus serotypes using a binary system recognizing the antigenicity of the VP7 (G serotype) and VP4 (P serotype) proteins is well accepted. Furthermore, since the reagents for identification of VP4 neutralization specificity are limited, an additional VP4 classification system based on nucleic acid hybridization and DNA sequence analysis has also been proposed (Estes, 1996b, Estes & Cohen, 1989). According to these classification systems, the BRV (strain C486) used in this studies belongs to group A, serotype G6, P6 (genotype P1) (Carpio *et al.*, 1981, Estes, 1996b).

2.1.2 Rotavirus structure

Rotaviruses are large, icosahedral particles with a complex structure consisting of three concentric icosahedral capsid layers surrounding the RNA genome. Each capsid layer strongly interacts with the adjacent layer. The morphologic appearance of rotavirus particles is distinctive. The mature particles resemble a wheel with short spokes and a well-define rim with a total diameter of approximately 75 nm (Estes, 1996b). The outermost layer is composed of the major capsid protein VP7 and the haemagglutinin spike protein VP4 (Prasad *et al.*, 1990, Shaw *et al.*, 1993a, Yeager *et al.*, 1990). The VP4 forms 60 homodimer-splikes that project outward from the outer VP7 shell (Prasad *et al.*, 1990, Shaw *et al.*, 1993a). The intermediate layer is composed of VP6, present in 260 trimers that interact with both VP4 and VP7 (Shaw *et al.*, 1996, Shaw *et al.*, 1993a). This implies that the VP4 may participate in maintaining the precise geometric register between the middle (VP6) and outer (VP7) capsid layers

(Estes, 1996b). The rotavirus structural proteins have intrinsic properties of self-assembly and display a high affinity for interactions among the proteins (Estes, 1996b). The strong interaction of these capsid proteins may explain the occurrence of unexpected phenotypes resulted from specific combination of VP4-VP7 or VP4-VP6 in some reassortants (Estes, 1996a). The inner capsid layer that encloses the genomic RNA is made of icosahedrally assembled dimers of VP2 (Lawton *et al.*, 1997b). The VP2 capsid layer along with the inside transcriptional enzyme complexes (VP1 and VP3) closely interacts with the genomic RNA in the core of the virion (Shaw *et al.*, 1996). This arrangement and interaction provides structural support and facilitates an orchestrated movement of the transcriptional complexes during viral transcription (Prasad *et al.*, 1996).

2.1.3 Rotavirus genome

The rotavirus genome consists of 11 segments of double-stranded RNA, each of which encodes a single protein. Of the 11 rotaviral proteins, 6 are structural (the inner core proteins VP1-3, the intermediate capsid protein VP6 and the outer capsid proteins VP4 and VP7) and 5 are nonstructural (NSP1-5, in order of decreasing size). The major features of the rotavirus genes include, lack of a polyadenylation signal, A+T rich (58-67%) and a consensus sequence at their 5'and 3' ends. These terminal sequences are thought to contain signals important for genome transcription, RNA transport, replication and possibly assembly of the viral genome segments (Estes, 1996b). The total genome contains approximately 18,522 basepairs (Estes & Cohen, 1989). The deproteinized rotavirus double-stranded RNA is not infectious, indicating that the virus particles contain RNA-dependent RNA polymerase to transcribe their own messenger RNAs (mRNAs) (Estes & Cohen, 1989).

The pattern of RNA migration using polyacrylamide gel electrophoresis (PAGE) is distinct among strains of rotaviruses. The analysis of such migration profiles (electropherotyping) has been widely used to characterize rotaviruses, isolated from stools or cell cultures, for epidemiological studies (Rodger *et al.*, 1981, Taniguchi & Urasawa, 1995). However, it should be noted that a specific serotype is not always correlated with a specific electropherotype (Kapikian & Chanock, 1996). Rotavirus electropherotypes are dynamically diverse due to genomic variations involving point mutation, reassortment, and rearrangement (Taniguchi & Urasawa, 1995). Viruses within each group are capable of genetic reassortment, but reassortment does not occur among viruses from different groups (Yolken *et al.*, 1988). In addition, since rotaviruses infect a wide range of species ranging from birds to mammals including humans, the possibility of genetic variations through interspecies transmission is also suggested (Estes, 1996a, Taniguchi & Urasawa, 1995).

2.1.4 The rotavirus replication cycle

The natural cell tropism of rotaviruses is the differentiated enterocyte in the small intestine (Estes, 1996b). Infection of these cells by rotaviruses is thought to be a multi-step process involving attachment to the specific receptor(s), entry into the cell, and uncoating to trigger viral replication. Viruses require proteolytic cleavage of VP4 into two noncovalently associated subunits, VP8* and VP5* in order to infect cells (Estes *et al.*, 1981). This process is believed to enhance membrane penetration rather than cell binding (Clarke *et al.*, 1981). In natural infection, VP4 is cleaved by proteolytic enzymes of the gastrointerstinal tract upon release from the intestinal cells (Ludert *et al.*, 1996b). For *in vitro* culture, however, trypsin is required to achieve viral infectivity (Babiuk *et al.*, 1977).

The initial viral attachment involves binding of the outer capsid VP4 to the cellular receptor (Ludert *et al.*, 1996a, Ruggeri & Greenberg, 1991). The other capsid protein, VP7, is not required for surface binding since viral-like particles lacking VP7 still bind to MA104 cells (Gilbert & Greenberg, 1997). It has been suggested that there are at least 2 binding mechanisms used by animal rotaviruses, sialic acid-dependent and independent (Mendez *et al.*, 1994), both of which involve attachment of the VP4 to the cellular receptors (Ludert *et al.*, 1996a). Most, but not all, animal rotaviruses evidently recognize sialic acid as an active receptor epitope. However, human rotavirus strains utilize a sialic acid-independent mechanism to initiate infection (Fukodome *et al.*, 1989). It is likely that the binding and entry of animal rotaviruses involves sequential binding to two different sites, initially by a sialic acid-specific receptor followed by an interaction with a second, nonsialylated receptor (Estes, 1996b).

Viral entry is likely to be mediated by direct membrane penetration, which results in permeabilization of the cell membrane. Although it is possible that rotaviruses use more than one mechanisms for entry, including endocytosis (Estes, 1996b), it is likely that both VP4 and VP7 are involved in the process. Viral entry may involve binding of both outer capsid proteins to specific intergrins on the cell surface, since all permissive cell lines that support viral growth, *in vitro*, are found to express intergrins that bind to the outer capsid proteins (Coulson *et al.*, 1997).

A mechanism of membrane penetration recently proposed suggests that penetration is mediated by fusion of the outer capsid protein to the cell membrane. This is based on the observation that, rotavirus and viral-like particles (containing both VP4 and VP7) induce syncytia of the permissive cells, in the presence of trypsin (Gilbert & Greenberg, 1997). Both VP4 and VP7 can independently induce permeabilization of the cell membrane in the presence of trypsin (Charpilienne *et al.*, 1997). Interestingly, the nonstructural protein, NSP4, also shares this permeabilization property. The NSP4

protein, however, has a more profound effect on the ER membrane than on the cell membrane (Tian *et al.*, 1996).

The low-calcium environment in the cytoplasm, at the beginning of viral infection, may mediate the uncoating of rotavirus by inducing a calcium-sensitive conformational change in the VP7 protein (Dormitzer & Greenberg, 1992). Uncoating of the triple capsid particles results in activation of the virion transcriptase (Bellamy & Both, 1990). Rotavirus replication and transcription occur exclusively in the cytoplasm and the genome transcription occurs within the double-layered particles (Cohen, 1977), in which the structural integrity of the double-layered particle is required (Prasad *et al.*, 1996). The synthesis of viral transcripts is mediated by endogenous viral RNA polymerase, which has many enzymatic activities. The transcription process is asymmetric with all transcripts which are full-length plus strands being made from the minus double stranded RNA. Viral transcripts serve as templates for both production of a minus strand and protein synthesis (Estes, 1996b). The newly synthesized single stranded mRNAs exit subviral particles via narrow channels in the protein layers, presumably to start protein synthesis within the viroplasm (Lawton *et al.*, 1997a). New double stranded RNAs are formed within newly synthesized subviral particles and, therefore, free double-stranded RNA or free minus strand single-stranded RNAs are never found in infected cells. (Estes, 1996b). Following translation, most proteins accumulate in viroplasmic inclusion bodies in the cytoplasm, except two glycoproteins, VP7 and NSP4, which are directed to the ER membrane (Bellamy & Both, 1990).

Rotavirus has a unique maturation process. Immature subviral particles assemble in the cytoplasm and then bud through the ER membrane where they acquire a transient membrane envelope (Petrie *et al.*, 1982). The strong interaction between VP4 and VP6 in the mature viral particle suggests that VP4 assembly occurs prior to that of VP7, and that VP4 interacts with the double-shelled particle before budding

(Shaw *et al.*, 1993a). At the beginning of the budding process, the NSP4 (NS28) functions as an intracellular receptor in the ER membrane which binds to the nascent subviral particles via VP4 (Au *et al.*, 1993). Subsequently, the outer capsid protein VP7, which is naturally retained as a ER integral membrane protein, repositions itself and folds into the subviral particles to form the mature viral particles (Poruchynsky & Atkinson, 1991). Once the outer capsid is formed, it is proposed that the NSP4 also plays a crucial role in removal of the transient membrane from the budding viral particles by destabilizing the transient envelope, i.e. ER membrane (Tian *et al.*, 1996).

Rotavirus maturation is a calcium-dependent process. Viruses produced in the absence of calcium are exclusively double-layered and budding of the virus through the ER membrane is inhibited. In addition, it was also found that the level of VP7 is decreased in a calcium-deprived environment, and that the decreased amount of VP7 is due to preferential degradation rather than impaired synthesis (Shahrabadi *et al.*, 1987). Since VP7 expressed in the absence of calcium does not fold properly, it is likely that calcium stabilizes or regulates folding or compartmentalization of the newly synthesized VP7 required for subsequent assembly (Dormitzer *et al.*, 1994). The requirement of calcium binding to VP7 explains the presence of calcium in the outer capsid of rotavirus (Dormitzer & Greenberg, 1992).

Once the viral maturation process is completed, viruses are released from the apical surface of infected cells through a non-conventional vesicular transport that bypasses the Golgi apparatus and lysosomes. The fact that viruses are released from the apical surface of the cells may partly explain why viral infection is usually localized and has limited tissue tropism (Jourdan *et al.*, 1997).

2.1.5 Rotavirus pathogenicity and virulence factor

Rotaviruses have a limited tissue tropism, in most cases they only infect mature enterocytes in the mid and upper villus epithelium of the small intestine. Following virus infection, the epithelial cells are killed and sloughed off, resulting in shortening of the villi (Kapikian & Chanock, 1996). Cell death is believed to be caused by the function of specific viral gene(s) on a specific target rather than from cumulative effects on host metabolism (Estes, 1996b). So far, no single gene has been shown to determine pathogenicity of rotaviruses under all circumstances. It appears that the gene(s) responsible for viral pathogenicity varies from strain to strain and host to host. Genes encoding for VP3, VP4, VP7, NSP1, NSP2, and NSP4 have been associated with rotavirus pathogenicity (reviewed in Burke & Desselberger, 1996).

Recent evidence suggests a role for NSP4 in induction of diarrhea. Free NSP4 and a peptide derived from NSP4 are able to induce dose-related diarrhea when inoculated into 6-10 day old mice. Furthermore antibodies recognizing NSP4 inhibit rotavirus-induced diarrhea (Ball *et al.*, 1996). NSP4 induces calcium mobilization from the ER of insect cells (Tian *et al.*, 1995), mouse (Ball *et al.*, 1996), and human intestinal cells (Dong *et al.*, 1997). These findings suggest that NSP4 behaves as a viral enterotoxin to potentiate transepithelial chloride secretion through a calcium-dependent signalling pathway. This effect is similar to that of the heat labile enterotoxin β of *Escherichia coli* (Ball *et al.*, 1996). The authors propose the model where two intestinal receptors are required for symptomatic rotavirus infection, i.e. diarrhea. The first receptor involves viral binding, resulting in viral entry and gene expression but is not necessary for symptomatic induction of diarrhea. The second receptor, which is NSP4-specific, interacts with newly synthesized NSP4, which then triggers the signal transduction pathway that increases the intracellular calcium level and enhances the

endogenous intestinal secretory pathway. It is speculated that NSP4 may be more effective in inducing intracellular changes in younger animals. This model may partly explain why infected adults do not develop diarrhea (Ball *et al.*, 1996).

The virulence of porcine rotavirus infection is associated with a mutation of the NSP4 gene (Zhang *et al.*, 1998). This further provides evidence that NSP4 is a virulence factor of rotavirus (Estes, 1996a). Other groups, however, report that enterotoxigenic properties of rotavirus NSP4 via intracellular calcium modulation may not be critical for induction of diarrhea, since attenuations of murine (Angel *et al.*, 1998) and human rotavirus (Ward *et al.*, 1997) are usually not associated with mutations in this gene. In addition, non-replicating, psolaren-inactivated rotavirus induces diarrhea in suckling mice, suggesting that viral attachment or entry is sufficient to induce diarrhea (Shaw & Hempson, 1996, Shaw *et al.*, 1995).

2.1.6 Biology of the outer capsid proteins

2.1.6.1 VP4

VP4 constitutes approximately 1.5% (by weight) of the virion protein. It is a product of genome segment 4. VP4 is a non-glycosylated outer capsid protein with a molecular weight (MW) of approximately 88 kilodaltons (KDa) (Estes, 1996b). BRV (strain C486) VP4-cDNA is 2,364 base pairs long, and contains one long open reading frame coding for a protein of 776 amino acids with a molecular weight of 86,516 (Potter *et al.*, 1987). VP4 has many features and functions that are important to viral biological activities, including haemagglutinin activity, protease-enhanced infectivity, virulence, putative fusion activity and cell attachment (reviewed in Estes, 1996b). In the presence

of trypsin, VP4 is cleaved into VP5* (MW 60 KDa) and VP8* (MW 28 KDa) resulting in enhancement of viral infectivity (Clarke *et al.*, 1981).

The VP4 from animal rotaviruses contains 776 amino acids, whereas VP4 from human rotavirus contains 775 amino acids. The deleted amino acid lies between amino acids 134 and 136 of the animal sequence (Kantharidis *et al.*, 1987). There are two trypsin cleavage sites (arginine 241, and arginine 247), with position 247 being the preferred cleavage site (López *et al.*, 1985). The two cleavage sites are conserved in every rotavirus VP4 sequence analyzed to date (Estes, 1996b). The VP8* subunit, located at the NH_2-terminal end of VP4, spans 247 amino acids, with great sequence diversity among rotavirus strains. It contains binding sites for serotype-specific neutralizing monoclonal antibodies (MAbs) (Estes, 1996b) and haemagglutinin activity (Fiore *et al.*, 1991). In contrast, VP5* contains sites responsible for cross-reactivity among different strains (Larralde *et al.*, 1991). Part of the VP8* domain interacts with VP6. It appears that the VP5* subunit is more externally located than is the VP8* subunit (Prasad *et al.*, 1990).

VP4 expressed in the absence of other rotavirus proteins appears to be structurally and functionally normal, indicating that expression of this gene is not dependent on other rotavirus proteins. These data also suggest that in an appropriate eukaryotic system, considerable amounts of biologically active protein should be produced (Bellamy & Both, 1990).

2.1.6.2 VP7

VP7 is an outer capsid glycoprotein that constitutes 30% of the total virion protein. It is the second most abundant protein in the virus, after the VP6, which constitutes 50% of the viral protein (Estes & Cohen, 1989). The glycosylated VP7

contains only N-linked high mannose oligosaccharides, which are processed by trimming. VP7 is an integral membrane protein of the ER with a luminal orientation. The protein is cotranslationally glycosylated as it is inserted into the ER membrane (Kabcenell & Atkinson, 1985). Gylcosylation is crucial for the subsequent formation of disulfide bonds, protein folding and ultimately for proper viral assembly (Mirazimi & Svensson, 1998). Up to three glycosylation sites in the VP7 proteins of rotaviruses have been reported (Estes, 1996b). However, glycosylation patterns of VP7 proteins vary among different strains of virus. Two glycosylation sites are used by the VP7 of BRV (C486) (Kouvelos *et al.*, 1984). The mature glycosylated VP7 of BRV (strain C486) MW is approximately 42 KDa, whereas the nonglycosylated form is approximately 35.5 KDa (Sabara *et al.*, 1982).

VP7 is encoded by either genome segment 7, 8 or 9, depending on the viral strain (Estes & Cohen, 1989). The VP7 of BRV (C486) is encoded from genome segment 8 (Dr. G. Cox, personal communication). The nucleotide sequence of the VP7 gene reveals an opening reading frame of 326 codons beginning with a weak initiation codon followed by the second in-frame initiation codon, with a strong consensus sequence, 30 codons downstream. In a natural infection, VP7 can be synthesized from both initiation codons. However, the second initiation codon is believed to be the one predominantly used by rotaviruses (Estes, 1996b). Each of these two initiation codons is followed by a region of hydrophobic amino acids (H1 and H2 domains, respectively) which function as signal peptides to direct the VP7 to the ER (Whitfeld *et al.*, 1987). Regardless of the initiation codon used, the signal peptide cleavage occurs between alanine 50 and glutamine 51, downstream of the H2 hydrophobic domain (Stirzaker *et al.*, 1987). Once the signal peptide is cleaved and the hydrophobic domains are removed, the protein translocates into the lumen of the ER (Stirzaker *et al.*, 1987). VP7 then becomes an integral membrane protein (Kabcenell &

Atkinson, 1985) with the last 36 residues present in the membrane or translocation pore of the ER, and possibly with the C-terminus protruding into the cytoplasm (Clarke *et al.*, 1995).

The mechanism by which VP7 is retained in the ER is not well understood. The protein does not contain the sequence KDEL, which is usually found in other ER-retention proteins (Munro & Pelham, 1987). It is suggested that the interaction between the cleaved H2 signal peptide and sequence within the first 111 residues is responsible for retention of the protein in the ER (Stirzaker & Both, 1989). The mechanism by which the protein remains anchored in the ER membrane in the absence of a transmembrane anchor domain is still unknown. Recent evidence suggests that the sequence between residues 290-316 is the putative membrane-binding domain (Clarke *et al.*, 1995).

Calcium is important for rotavirus replication and cytopathogenesis. The significant increase in the free cytosolic calcium concentration correlates with virus-specific protein synthesis in rotavirus-infected cells (Michelangeli *et al.*, 1991). In the absence of calcium, newly synthesized VP7 degrades quickly (Shahrabadi *et al.*, 1987). Oligomerization of viral proteins, protein rearrangement and interactions with calcium are essential for the assembly of VP7 into the outer capsid proteins (Poruchynsky *et al.*, 1991, Shahrabadi *et al.*, 1987). The recombinant VP7, expressed alone, is unstable, and does not fold properly unless it is co-expressed with other viral proteins (Dormitzer *et al.*, 1994, Dormitzer *et al.*, 1992). Interestingly, the presence of NSP4, is required for proper viral protein conformation, in particular VP7, that is necessary for subsequent viral assembly (Poruchynsky *et al.*, 1991). NSP4 increases the intracellular calcium concentration by enhancing the basal calcium permeability of the ER membrane (Dong *et al.*, 1997, Tian *et al.*, 1995), and extracellular calcium entry (Dong *et al.*, 1997). In

addition, the protein may trigger calcium immobilization by destabilizing the ER membrane (Tian *et al.*, 1996).

2.1.7 Immunity to rotaviruses

2.1.7.1 Effector mechanisms involved in the resolution of rotavirus infection and prevention of reinfection

Following oral infection, rotavirus replication occurs uniformly in the villus enterocytes of the small intestine, as well as in the epithelium overlying the Peyer's patches. Viral antigens are detected in intestinal villus epithelium from 24 hours post-infection and remain detectable for as long as 10 days post-infection, with a peak distribution at 5 days post-infection. Significant numbers of viral antigen containing cells are detected as early as 3 days post-infection in subepithelial and interfollicular areas within the Peyer's patches and mesenteric lymph nodes. The number remains constant for another 4-7 days and then declines by 20 days post-infection. No viral antigen is detected in the villus lamina propria during infection suggesting that rotavirus antigen uptake is largely limited to the Peyer's patch-associated epithelium, possibly by M cells. Viral antigen is subsequently transported to the underlying lymphoid follicles, regional lymph nodes, and spleen. A close relationship between the availability of viral antigen in the lymphoid follicles and the subsequent development of local antibody responses has been reported (Dharakul *et al.*, 1988). Rotaviruses are enteric viruses with a very short incubation period (1-4 days). Therefore, protection against infection must be mediated by immune responses at the mucosal surface. It is likely that rotavirus-specific antibodies at the intestinal mucosa, but not serum, are important in protection against disease (Offit & Clark, 1985). Whereas, rotavirus-specific memory B

and T cells located with the lamina propria are probably important for disease modulation but not in prevention, i.e. protection against disease (Offit, 1996).

Mutant strains of mice are useful for studying the role of the different arms of the immune system in clearing primary rotavirus infection and protection from reinfection. Mice that lack T cells (Franco & Greenberg, 1995), B cells (Franco & Greenberg, 1995, McNeal *et al.*, 1995), or both (Dharakul *et al.*, 1990, Franco *et al.*, 1997), exhibit delayed viral clearance or become chronically infected with murine rotavirus. Passive transfer of anti-rotavirus antibody from immunized dams to suckling pups through milk has been found to provide protection against diarrhea (Offit & Clark, 1985). Protection against rotavirus disease has also been reported following passive transfer of rotavirus-specific $CD8^+$ cells into syngenic pups (Offit & Dudzik, 1990). In addition, passive transfer of rotavirus-specific $CD8^+$ cells results in resolution of chronic rotavirus infection in SCID mice (Dharakul *et al.*, 1990). These findings suggest a role for both BRV-specific B and T cells in clearing a primary infection and protection against reinfection. A series of studies in knockout mice indicate that the presence of intestinal antibody correlates with protective immunity and that cell-mediated immunity is important in mediating virus clearance but not protection from infection (Estes, 1996a, Franco *et al.*, 1996, McNeal *et al.*, 1997). B cell functions are required for protection against reinfection and rotavirus-specific antibody may be the most important effector function involved in rotavirus immunity at the mucosal surface (Franco & Greenberg, 1995, McNeal *et al.*, 1995). Effector $CD8^+$ cells are usually short-lived (Franco & Greenberg, 1995) and are probably involved in resolution of initial infection but do not show an association with protection from long-term reinfection (McNeal *et al.*, 1995). Providing that B-cells are functional, cytotoxic T lymphocytes (CTL) may hasten viral clearance, but are not absolutely required for either clearance of a primary infection or development of immunity against reinfection (Franco & Greenberg, 1995). One study

indicated that $CD8^+$ cells in cattle also play a central role in limiting primary rotavirus infection (Oldham *et al.*, 1993). Apart from cytotoxic activity, $CD8^+$ cells can efficiently mediate clearance of rotavirus infection by a mechanism independent of perforin, fas or IFN-γ. This suggests that the $CD8^+$ population may mediate viral clearance through the production of other antiviral cytokines, including type-1 interferons and tumour necrosis factor (Franco *et al.*, 1997).

Some B-cell deficient mice that were depleted of $CD8^+$ cells, prior to rotavirus inoculation, gradually resolved viral shedding. This suggests that other immune effector function(s), possibly CD4-CTL or natural killer cell activity, may be partially involved in the clearance of viral infection (Franco & Greenberg, 1995, Franco & Greenberg, 1997, McNeal *et al.*, 1995, McNeal *et al.*, 1997). Other mechanisms, including release of anti-viral cytokines has also been suggested to play a role in viral clearance (Moser *et al.*, 1998, Ward, 1996).

A study in T cell-deficient mice and T cell-depleted mice indicates that "T cell-independent", intestinal IgA, antibody responses and other innate mechanisms exist and can contribute, to some degree, to the clearance of rotavirus infection in mice. Interestingly, T cell-independent intestinal IgA responses in these mice appears to be almost exclusively against VP6, with a smaller response against VP4 but not VP7. It is possible that the highly repetitive, oligomeric structure of the VP6 protein may be capable of inducing strong T cell-independent as well as T cell-dependent B cell responses (Franco & Greenberg, 1997). The T cell-independent antibody response is also reported in gnotobiotic calves (Oldham *et al.*, 1993).

Several studies on the contribution of gamma-delta ($\gamma\delta$) T cells to rotavirus immunity indicate that $\gamma\delta$ T cells do not have a significant role in the induction of rotavirus immunity in mice (Franco & Greenberg, 1995, Franco & Greenberg, 1997), or cattle (Oldham *et al.*, 1993).

2.1.7.2 Humoral immunity

The role of rotavirus-specific intestinal antibody in preventing disease is well accepted. Protection against disease in children is correlated with the amount of rotavirus-specific secretory IgA (sIgA) present at the intestinal mucosa (Matson *et al.*, 1993) and serum (O'Ryan *et al.*, 1994). However, the presence of viral-specific IgA at the intestinal surface is usually short-lived and cannot be detected after 1 year post-infection (Matson *et al.*, 1993). In mice orally infected with either homologous or heterologous rotaviruses, systemic antibody titres to rotavirus proteins continued to increase over time while local antibody levels peaked at week 4 and then rapidly decline (Ishida *et al.*, 1997). As noted earlier, immunization with either VP4 or VP7 protein can independently induce neutralizing antibodies in immunized animals (Estes, 1996b). Passive administration, by the oral route, of neutralizing antibodies can protect against rotavirus challenge (Matsui *et al.*, 1989, Offit *et al.*, 1986). However, several reports suggest that the presence of neutralizing antibody, either in serum or intestine, was not associated with viral protection (Bridger & Oldham, 1987, Burns *et al.*, 1996, McNeal *et al.*, 1992, Merchant *et al.*, 1991, Ward, 1996, Ward *et al.*, 1992). In addition, mucosal administration of rotavirus VP2/VP6 virus-like particles (VLP) induced protection in mice in the absence of neutralizing antibodies. Adult mice that were intranasally inoculated with VLP mixed with cholera toxin, *Escherichia coli* heat-labile toxin (LT) or a mutant version of LT, were protected from subsequent viral challenge, and the degree of protection correlated with the levels of intestinal antibody (O'neal *et al.*, 1998, O'neal *et al.*, 1997). These suggest that many viral components or factors are involved in protection from rotavirus disease.

Nine to twelve day old mice, orally infected with heterologous virus (rhesus rotavirus, RRV), develop diarrhea for a period of 2-4 days. Rotavirus-specific antibody

secreting cells (ASCs) were observed in the lamina propria of the small intestine within 4 days of infection, peaked at 3 weeks postinfection, and remained at the same level for at least 8 weeks. The magnitude of IgA-ASCs was approximately 10 times greater than IgG-ASCs and IgM-ASCs were rare (Merchant *et al.*, 1991). The later study confirms that mice, infected at a young age, produce significant levels of rotavirus-specific ASCs in the lamina propria and Peyer's patches throughout their life. Interestingly, the response, 1 year after primary infection, was dominated by antibodies recognizing VP6 protein, and the response to VP4 protein was extremely small compared to the overall antibody response in the intestinal tract (Shaw *et al.*, 1993b). The overall magnitude of the response to rotavirus was very strong. Following viral inoculation, almost 50% of ASCs found in the lamina propria are rotavirus-specific at 20-60 days postinfection. In this study, a minimal response was found in the spleen, reflecting the compartmentalization of the mucosal immune system (Merchant *et al.*, 1991). The level of intestinal IgA-ASCs was correlated with protection against subsequent challenge in a gnotobiotic pig model of human rotavirus. The level of IgG-ASCs in pigs was comparable to the IgA found in the lamina propria. In addition, the frequency of IgA-ASCs in the blood reflected the response in the gut. Thus, this parameter may be used as an indicator for intestinal immune response following viral infection and is believed to be more reliable than serum neutralizing antibody levels (Yuan *et al.*, 1996).

The significance of IgA in providing active protection against rotavirus infection was demonstrated by Burns and colleagues (Burns *et al.*, 1996). Transplanting hybridoma cells producing VP6 specific IgA monoclonal antibodies into the back of syngenic mice ("backpack tumor") conferred protection to a subsequent viral challenge. It should be noted that these antibodies did not have neutralizing activity, and that hybridomas producing VP4-specific IgA or VP7-specific IgG1 neutralizing antibodies did not confer the same effect. This model provided evidence that non-neutralizing IgA

antibody can play a role in prevention of rotavirus infection. The authors proposed that sIgA may mediate *in vivo* viral inactivation during intracellular transcytosis (Burns *et al.*, 1996).

Oral inoculation of animals with either homologous or heterologous rotavirus strains induced the production of virus-specific sIgA at the intestinal mucosa. However, the capacity to induce virus-specific IgA responses depended on the degree to which the virus was adapted to grow in the host (Feng *et al.*, 1994, Offit, 1996). The serum IgG response did not correlate with the dose of heterologous virus used for infection, but fecal IgA response appeared to be strongly related to the dose (Feng *et al.*, 1994). As previously suggested, the presence of antigen in Peyer's patches appears to determine the magnitude of the subsequent antibody response in the lamina propria (Dharakul *et al.*, 1988). Several studies suggest that viral replication, or viral load, may be crucial for induction of active immunity (Feng *et al.*, 1994, Shaw & Hempson, 1996). Humans and animals that recover from infection with virulent rotaviruses develop a high level of protective immunity, at least against the infecting serotype (Yuan *et al.*, 1996). In mice, murine rotavirus induced complete protection against subsequent challenge, and this effect was associated with the presence of enteric virus-specific IgA at the time of challenge. In contrast, non-murine rotavirus induced partial protection, and this protection occurred in the absence of mucosal, viral-specific IgA at the time of challenge and in the days immediately following challenge. This suggests that immunological mechanism, other than stimulation of B cells or CTL responses, may also be involved in the induction of protection by heterologous strains (Moser *et al.*, 1998).

Oral inoculation was shown to be more effective than parenteral inoculation for induction of virus-specific sIgA responses at the intestinal mucosal surface (Coffin *et al.*, 1994). However, several studies have shown that parenteral immunization could

induce antibody production and protection against subsequent challenge (Coffin *et al.*, 1997, Conner *et al.*, 1993, McNeal *et al.*, 1992). Protection against rotavirus shedding in rabbits, following intramuscular immunizations with inactivated rotavirus, correlated with the presence of virus-specific IgG at the intestinal mucosal surface (Conner *et al.*, 1993). Furthermore, intramuscular immunization was shown to induce antibody responses at the mucosal surface in mice. The complete protection induced by intramuscular immunization with wild-type murine rotavirus (EDIM) was associated with production of virus-specific IgA by lamina propria lymphocytes and detection of virus-specific IgA at the intestinal mucosa. Interestingly, inactivated homologous strains and heterologous strains only induced partial protection in this study (Coffin *et al.*, 1997). It is likely that the strains of the virus, doses, and host species influenced the outcome of these immunization protocols. Although strong IgG production is usually observed in parenterally immunized mice, the role of IgG for protection at the mucosal surface in mice is still unclear (Coffin *et al.*, 1997). Recent evidence suggests that although intramuscular immunization with heterologous rotavirus may increase the magnitude of the immune response, it does not change the localization of effector cells or kinetics of mucosal virus-specific IgA induction following viral challenge. Therefore, it is unlikely that this type of immunization will provide complete protection against rotavirus infection (Coffin & Offit, 1998).

2.1.7.3 Cell-mediated immunity

- **Cytotoxic T lymphocytes**

Adult mice orally inoculated with non-murine rotaviruses develop detectable viral

specific $CD8^+$ CTL activity in the spleen by 7 days post-infection (Offit & Dudzik, 1988). Oral or intraperitoneal inoculation of rhesus rotavirus into mice induced rotavirus-specific CTL in spleen and intestinal lymphoid tissues [intraepithelial lymphocytes, (IEL), mesenteric lymph nodes, Peyer's patch]. These findings suggest that expression of rotavirus antigen at the intestinal mucosa surface is not necessary to induce viral specific CTL activity in the IEL population (Offit & Dudzik, 1989). However, another study by the same group showed that footpad inoculation did not induce CTL activity in IEL and that the frequency of precursor CTL (pCTL) in Peyer's patches of these mice was 25-30 times lower than the frequency observed following oral inoculation (Offit *et al.*, 1991b). It is likely that both antigen load and localization may influence the level of CTL activity at the mucosal surface. CTL activity in IELs appeared to be short-lived with no detectable pCTL in the IEL population at 4 week after inoculation. However, pCTL were still detectable in the spleen, Peyer's patch, and lymph nodes. This indicates that CTL in the IEL compartment may arise from pCTL found in other lymphoid tissues (Offit *et al.*, 1991b, Offit & Dudzik, 1989). Nevertheless, regardless of the route of inoculation, rotavirus-specific pCTL are distributed throughout the lymphoid system 21 days after infection (Offit *et al.*, 1991b). Rotavirus-specific CTL are broadly cross-reactive with different strains of rotavirus (Offit *et al.*, 1991a, Offit *et al.*, 1994, Offit & Dudzik, 1988). Interestingly, a non-conventional antigen presentation, i.e. a direct class I loading with exogenous antigen or cross priming, may coexist in the generation of rotavirus-specific CTL. Mice primed with baculovirus-expressed VP7 protein also developed CTL recognizing target cells pulsed with VP7-derived peptide in the same way as mice primed with virus (Franco *et al.*, 1993). In addition, the adoptive transfer of $CD8^+$ T lymphocytes from mice immunized with various rotavirus serotypes or different recombinant proteins (VP1, 4, 6, or 7) could mediate viral clearance in SCID mice (Dharakul *et al.*, 1991).

A compartmentalization in the immune system during the induction of immune responses has been recently postulated. The leukocyte integrin $\alpha_4\beta_7$, a receptor for the vascular mucosal addressin cell adhesion molecule -1 (MadCAM-1), plays a crucial role in lymphocyte homing to mucosal lymphoid tissue (Rott *et al.*, 1996). This marker has been shown to define a subpopulation of memory T cells capable of homing to intestinal lymphoid tissues in mice and humans (Rott *et al.*, 1996, Williams & Butcher, 1997). Recent studies have shown that memory for rotavirus also resides mainly in the lymphocyte population that displays the mucosal homing receptor $\alpha_4\beta_7$. Splenic $CD8^+$ cells isolated from mice orally immunized with rotavirus can be divided into 3 distinct populations: i) $CD44^{hi}$ $\alpha_4\beta_7^{hi}$; ii) $CD44^{hi}$ $\alpha_4\beta_7^-$; and iii) $CD44^{lo}$. [Of note: Unlike Peyer's patch or peripheral lymphoid tissues, which display preferential homing of lymphocyte subsets, all three $CD8^+$ subsets are recruited almost equally to the spleen (Williams & Butcher, 1997)]. However, only the $\alpha_4\beta_7^{hi}$ $CD8^+$ cells efficiently cleared chronic rotavirus infection when transferred into Rag-2 deficient (T- and B-deficient) mice. In contrast, the $\alpha_4\beta_7^-$ $CD8^+$ cells cleared viral infection less inefficiently, depending on the number of transferred cells, and $CD44^{lo}$ (presumably naïve) T cells were unable to clear the virus (Rosé *et al.*, 1998). Natural rotavirus infection in humans also results in a specific memory $CD4^+$ response that is largely limited to the $\alpha_4\beta_7$ subpopulation. This phenotype is not expressed by rotavirus specific $CD4^+$ cells induced by intramuscular immunization or skin contact allergen (Rott *et al.*, 1997). Furthermore, the existence of rotavirus-specific, memory $\alpha_4\beta_7^+$ B cells, responsible for viral clearance and stool-localized IgA production, has been suggested (Rosé *et al.*, 1998). Thus, the generation of $\alpha_4\beta_7^+$ memory cells may correlate directly with protective immunity to gastrointestinal pathogens.

The VP7 protein is considered to be a major antigen recognized by both homotypic and heterotypic CTL in C57Bl/6 mice (Franco *et al.*, 1993, Offit *et al.*, 1991a, Offit *et al.*, 1994). A study, using recombinant vaccinia viruses expressing the VP7 gene from seven G serotypes, showed that VP7-specific CTL cross-reacted with the different serotypes (Heath *et al.*, 1997). The K^b-restricted CTL epitope of VP7 of rotavirus RF strain (residues 31-40) that overlaps with the H2 signal peptide of the protein (residue 38-48) has been identified as the immunodominant CTL epitope (Franco *et al.*, 1993). However, this epitope is serotype specific rather than cross-reactive (Heath *et al.*, 1997). In addition to VP7, CTL epitopes have been identified on VP2, VP3, VP6 (Franco *et al.*, 1994), and NSP-1 (Heath *et al.*, 1997).

- **T-helper (Th) cells**

Normal adult peripheral blood mononuclear cells (PBMC) contain rotavirus-specific $CD4^+$ T cells that can be stimulated *in vitro*. Upon stimulation, these cells produce several cytokines, including IL-2 and IFN-γ, which subsequently enhance non-specific cytotoxic lymphocyte activity, including NK and LAK activity (Yasukawa *et al.*, 1990). In humans, rotavirus-specific lymphoproliferative activity increases with age. This is consistent with the fact that most children are infected with rotavirus by 2 years of age (Offit *et al.*, 1992). Therefore, it has been proposed that detection of viral specific lymphoproliferative activity in infants may be a better indicator of exposure to rotavirus than detection of rotavirus-specific neutralizing antibodies (Offit *et al.*, 1992). Rotavirus specific Th cells can be detected in the spleens of suckling mice orally inoculated with murine rotavirus (Riepenhoff-Talty *et al.*, 1982), and in the PBMC of infants and children following acute rotavirus infection. Thus, circulating rotavirus-

specific T lymphocytes, together with rotavirus-specific IgA, may reflect intestinal exposure to rotavirus (Offit *et al.*, 1993).

To date, VP6 and VP7 have been reported as the Th-cell target antigens of rotavirus (Andrew *et al.*, 1990, Baños *et al.*, 1997, Bruce *et al.*, 1994). We found that VP4 can also induced serotype-specific Th activity in mice (unpublished observation). Th cell recognition of rotavirus, in both mice and humans are cross-reactive among the strains (Bruce *et al.*, 1994, Offit *et al.*, 1992, Offit *et al.*, 1993). At least two Th-epitopes on VP6 have been mapped. One of them is highly conserved among different rotavirus strains, suggesting that VP6 may be a target of highly cross-reactive Th cells (Baños *et al.*, 1997).

Th cells are important for antibody production and protection against reinfection. $CD4^+$ cells play an important role in generation of mucosal, as well as systemic antibody responses against rotavirus infection in both mice (McNeal *et al.*, 1997), and gnotobiotic calves (Oldham *et al.*, 1993). Although some studies indicate that $CD4^+$ cells are not required for clearance of the primary rotavirus infection in C57Bl/6 mice (Franco & Greenberg, 1997) and gnotobiotic calves (Oldham *et al.*, 1993). Resolution of rotavirus shedding in μMT and BALB/c mice was found to be associated with both $CD8^+$ and $CD4^+$ cell effector activities (McNeal *et al.*, 1997). The effector functions of $CD4^+$ cells may include production of antiviral cytokines, development of $CD4^+$-CTL, and providing help for antibody or CTL production (McNeal *et al.*, 1997).

2.1.7.4 Strategies for development of bovine rotavirus vaccine

Group A rotavirus infection has been associated with diarrhea in young animals, including calves and pigs younger than one month of age (Saif *et al.*, 1994). The virulence of the strains and the age of the calves at the time of infection are the two

important factors for pathogenicity of the virus. In the newborn calf, replacement of villous enterocytes is slow and the animals are susceptible to disease by strains that are moderately virulent. In older animals, there is a competition between the rate of viral replication and replacement of enterocytes, therefore the virus must be more virulent to induce diarrhea (Dodet *et al.*, 1997).

Although management practices may reduce the level of viral exposure, the high concentrations of BRV shed in feces, their long-term stability in the environment, and the low dose required for infection [<100 virions, (Dodet *et al.*, 1997)] suggest that eradication of BRV from infected herds is unlikely, if not impossible (Saif & Fernandez, 1996). The initial vaccination approach relied on vaccination of young animals with live attenuated rotavirus (reviewed in Conner *et al.*, 1994). However, these vaccines were not efficient when orally administered to calves and piglets in the field, presumably because colostral antibodies inhibited vaccine virus replication (Saif & Fernandez, 1996). Thus, the current vaccination approach for prevention of rotavirus infection in calves and pigs is focused on passive immunization by boosting lactogenic immunity and management practices that promote immediate consumption of colostrum at birth and improve sanitation to minimize the exposure level of rotavirus (Conner *et al.*, 1994, Saif & Fernandez, 1996).

It should be emphasized that there are species differences in the mechanism by which young animals acquire passive immunity that can greatly affect vaccination strategies. Unlike humans, primates, mice, and rabbits whose antibodies (primarily IgG) are transferred to the fetus across the placenta, the anatomical structure of the placenta in ungulates (pigs and ruminants) prevents transplacental transfer of antibodies (Conner *et al.*, 1994). Ungulates are born agammaglobulinemic and acquire immunoglobulins by absorption of colostral immunoglobulins from the intestine. This adsorption occurs for approximately 48 hours after birth. The predominance of the

serum-derived IgG in the colostrum results in the postpartum transfer of mainly IgG antibodies into the serum of the offspring (Saif & Fernandez, 1996). Serum-derived IgG1 remains predominant in the milk of ruminants (Conner *et al.*, 1994). In humans and monogastrics, IgA is the predominant immunoglobulin in both colostrum and milk, and only a minor portion of IgA is absorbed from these fluids (Conner *et al.*, 1994). Milk IgA of monogastrics is produced locally in the mammary gland by plasma cells originating in the intestine. This mechanism ensures that the neonates received sIgA antibodies against enteric pathogens (review in Saif & Jackwood, 1990). Regardless of the species, there is a drastic decline in the antibody concentration from colostrum to milk (Conner *et al.*, 1994).

Since the predominant BRV-specific antibodies in colostrum and milk are serum derived IgG1, parenteral vaccination of pregnant cows represents a logical approach for boosting these antibody titres in colostrum, and for prolonging secretion of milk containing high anti-rotavirus antibody titres (Saif & Fernandez, 1996). Bovine colostrum is rich in IgG1 and also contains IgM and IgA, thus conferring both systemic immunity and local protection in the intestine (Dodet *et al.*, 1997). However, the success of maternal rotavirus vaccines in the field is also influenced by vaccine dose, vaccine strain, inactivating agents, adjuvants, routes of administration and the level of environmental exposure (reviewed in Saif & Fernandez, 1996).

Although the role of neutralizing antibodies for protection in several models is controversial (see above), it appears that neutralizing antibodies, or antibodies recognizing VP4 or VP7, may be important in lactogenic immunity in cattle. Cow vaccination with one serotype of rotavirus induced serum neutralizing antibodies titres to the strain used for immunization and enhanced pre-existing serum neutralizing antibodies titres to other serotypes (Brussow *et al.*, 1988). Colostrum containing high levels of neutralizing antibodies, mainly IgG1, provided effective passive protection in a

dose dependent manner (reviewed in Saif & Fernandez, 1996). A recent study showed that cows immunized with SA 11 core-like particles (CLP) containing VP2 and VP6 had significantly increased BRV-specific IgG1, but not neutralizing antibodies, in the colostrum comparable to those vaccinated with VLP (VP2/6/4/7). However, only pooled colostrum from the VLP vaccinated cows completely protected calves from heterologous BRV-induced diarrhea whereas the colostrum from CLP-immunized cows provided only partial protection (Fernandez *et al.*, 1998).

2.2 DNA immunization

The first report describing the transfection of cells *in vivo* following injection of purified DNA was published in 1960 by Ito (Ito, 1960). However, the potential application of this finding was not realized until recently. Using direct intramuscular injection of purified DNA or RNA, Wolff and colleagues (1990) demonstrated the expression of a reporter gene in mouse skeletal muscle and demonstrated the biological activity of the encoded enzyme. The plasmid remained extrachromosomal and expressed the reporter protein for at least 19 months (Wolff *et al.*, 1992).

In 1992, Tang and colleagues (Tang *et al.*, 1992) reported the potential use of this technology for induction of antigen-specific immune responses. The plasmid encoding antigen was introduced directly into the skin of mice, using particle bombardment referred as gene gun delivery system. Induction of immune responses to other viral antigens, using the gene gun system, was described later (Eisenbraun *et al.*, 1993). In 1993, Ulmer and colleagues report that intramuscular injection of plasmids, encoding an influenza virus A nucleoprotein could effectively induce strong viral-specific antibodies and CTL that protected against a subsequent challenge with a heterologous strain of influenza A virus (Ulmer *et al.*, 1993). These early studies highlighted that

antigens, expressed *in vivo* by plasmid immunizations, effectively induced both humoral and cell-mediated immune responses. During the past few years, DNA immunization has been widely explored and it is now recognized as a promising immunological approach for the prevention and therapy of many diseases. DNA immunization, therefore, is a powerful tool for the development of strategies for immune intervention in many conditions including infectious diseases, parasitic diseases, cancer, autoimmunity, and allergy (reviewed in Donnelly *et al.*, 1997, Ertl & Xiang, 1996b, Manickan *et al.*, 1997b, Robinson & Torres, 1997). In addition to systemically inoculation, administration of DNA to mucosal surfaces showed promising results for the induction of mucosal immune responses (Chen *et al.*, 1998, reviewed in Robinson & Torres, 1997).

Plasmid vectors provide tremendous advantages as immunological tools. Besides the ease of handling, the genes present in the vectors can be easily modified without additional time for protein purification or the generation of recombinant vectors. Combining knowledge and technology from the fields of molecular biology and immunology, both antigens and vaccination regimes can be explored endlessly. This will not only give us the benefit of achieving the best vaccine strategies but, more importantly, may provide a better understanding of the fundamentals of immunological events that occur in the host.

2.2.1 Mechanism of DNA immunization

Plasmid DNA can be delivered through several routes to induce immune responses (reviewed in Manickan *et al.*, 1997b, Ulmer *et al.*, 1996b). It should be noted that the mechanism of immune induction by plasmid immunization is different from that of protein immunization (Boyle *et al.*, 1997b). Following intramuscular inoculation,

primarily muscle cells are transfected and the protein level in the muscle appears to be in the picogram range (Wolff *et al.*, 1990). The precise mechanism of DNA uptake by muscle cells is not clearly understood (Ulmer *et al.*, 1996b). Most of the transfected DNA remains episomally as a non-replicating, circular form (Wolff *et al.*, 1990). Greater stability of protein expression is obtained in slowly dividing cells, suggesting that the decreased mitotic rate of the transfected cells may enhance the stability and expression of plasmid DNA. However, other tissue specific features may also account for the enhanced stability in the muscle cells (Wolff *et al.*, 1992).

Intradermal and intramuscular immunization can effectively induce a long lasting immune response (Boyle *et al.*, 1997b, Hassett & Whitton, 1996, Raz *et al.*, 1994). Following intradermal inoculation, keratinocytes, fibrobasts, and cells with dendritic morphology appear to be transfected (Raz *et al.*, 1994). Gene gun immunization results in transfection of keratinocytes, resident Langerhans cells (LC) and possibly professional antigen presenting cells (APC), via direct entry of the DNA-coated particles through the cell membrane directly into the cytosol (Williams *et al.*, 1991). The highest level of both protein expression and antibody production following gene gun immunization correlates with delivery of DNA-coated gold particle to the epidermal layer, while delivery extending into the dermis results in decreased protein and antibody production (Eisenbraun *et al.*, 1993). Generally, intramuscular and intradermal inoculation requires 100-1000 times more DNA to induce an immune response than gene gun inoculation (Fynan *et al.*, 1993, Robinson & Torres, 1997). In fact, nanogram levels of the expressed protein is enough to effectively induce immune responses following particle bombardment (Tang *et al.*, 1992).

As reviewed by Donnelly and colleagues (Donnelly *et al.*, 1997), DNA vaccines effectively induce a full range of immune responses, including antibodies, major histocompatibility complex (MHC) class I-restricted $CD8^+$ CTL and class II restricted

$CD4^+$ Th cells. This implies that proteins expressed by the plasmids have been effectively loaded on both class I and class II MHC molecules and presented to the immune system. The observations that DNA immunization induces protective antibody responses suggests that the proteins expressed, *de novo*, retain structural conformations that are similar to the wild-type proteins. This is especially important for developing immunity against viral antigens, which are synthesized in the infected cells.

It is well established that secondary signals, i.e. costimulatory molecules, are extremely critical for the initiation of immune responses. Although local somatic cells, for example muscle cells, are the major cells transfected and expressing antigens following intramuscular inoculation of plasmids, they are not considered professional APC, i.e. lack of costimulatory molecules, and do not constitutively express MHC class II molecules (Hohlfeld & Engel, 1994). Moreover, recent evidence suggests that these somatic cells do not contribute directly to the development of immunity, since primary antibody and CTL responses can be generated following removal of the site of vaccination (Torres *et al.*, 1997). Therefore, it is unlikely that transfected somatic cells, by themselves, can efficiently stimulate the strong immunity observed following DNA immunization. Other cell types, in particular the professional APC, are probably required for the induction of immune responses following DNA immunization.

Dendritic cells (DC) are considered to be the primary professional APC for initiation of an immune response. Dendritic cells are present throughout most tissues as long-lived, immature, non-dividing cells. Upon encounter with antigen, dendritic cells in the periphery capture and process the antigen, become activated and subsequently upregulate co-stimulatory molecules. Activated DC then migrate to the draining lymph node (DLN) to initiate an immune response (reviewed in Banchereau & Steinman, 1998). Dendritic cells are very efficient in priming CTL responses. Only 100-1,000 viral-peptide pulsed DC are sufficient to induce a protective level of anti-viral CTL

(Ludewig *et al.*, 1998). The role of dendritic cells as major antigen presenting cells in the induction of antigen specific $CD8^+$ CTL and $CD4^+$ following DNA immunization has been demonstrated (Casares *et al.*, 1997, Iwasaki *et al.*, 1997b). Injection of DC, but not macrophages, transfected *in vitro* with naked DNA, leads to a significantly enhanced protective immune response, compared with injection with DNA alone. This enhanced protective immunity was related to increased Th1 $CD4^+$ T cell responses (Manickan *et al.*, 1997a). However, this finding does not rule out a possible role for the macrophage as an APC which obtains antigen from other transfected cells.

Co-injection, either intramuscular or intradermal, of plasmids encoding CD40L and protein antigen, enhanced antigen-specific antibody, CTL, and Th1-type immune responses. This suggested that CD40L-CD40 interaction was important for the immune response induced by DNA vaccine. In addition, the two plasmids had to be injected into the same site to achieve these effects. This observation supports the concept that antigen is presented by CD40 expressing APC, and the CD40-dependent signal provides a more effective cognate APC-T cell interaction (Mendoza *et al.*, 1997). The interaction of CD40 on DC with its ligand (CD40L or CD154) on T cells is critical for the maturation and function of DC, *in vivo*. In the absence of a CD40L interaction, DC fail to produce IL-12 which is critical for a subsequent immune response (Mackey *et al.*, 1998). The significance of interdigitating dendritic cells, the progeny of LC, in priming virgin T cells in the secondary lymphoid tissues has been well described by MacLennan and colleagues (MacLennan *et al.*, 1997). Although B cells also express CD40 on their surface, the naïve B cells do not efficiently present antigen to virgin T cells (Liu & Arpin, 1997). Supporting this notion, DNA-transfected dendritic cells, but not B cells, isolated from a DLN could present antigen peptides on MHC class II molecules, and subsequently activate Th cells (Casares *et al.*, 1997).

Granulocyte-monocyte colony stimulating factor (GM-CSF), interleukin-1 (IL-1), and tumour necrosis factor alpha (TNF-α) have been known to stimulate DC maturation. Once activated, DC upregulate expression of surface MHC II molecules, costimulatory molecules and synthesize high levels of IL-12 (reviewed in Banchereau & Steinman, 1998). These activation stages allow DC to interact efficiently with both B and T lymphocytes. Besides its effect on the development of Th1 and pCTL, IL-12 is a mandatory molecule that promotes the differentiation of naïve B cells into plasma cells, (Dubois *et al.*, 1998). Co-administration of GM-CSF, but not IFN-γ, with plasmid encoding a viral antigen enhanced antigen-specific immune responses (Ertl & Xiang, 1996b). Co-linear expression of antigen with B7-2, GM-CSF, and/or IL-12 significantly enhanced antigen-specific immune response against a somewhat non-immunogenic antigen. In addition, GM-CSF and IL-12 appear to act synergistically to promote early CTL induction, *in vivo* (Iwasaki *et al.*, 1997a). These results indicate that professional APC, particularly DC, are involved in the initiation of immune responses. The co-expression of costimulatory molecules and/or cytokines does not convert non-hematopoietic cells to APC in the bone marrow chimeric mouse model (Iwasaki *et al.*, 1997b). Thus, immune responses induced by DNA immunization rely on the ability to transfect and/or activate the professional APC rather than transforming transfected somatic cells into professional APC.

Although DC are superior in their antigen presenting ability, it should be noted that DC can present antigen in either a tolerogenic or stimulatory manner, and that inflammatory stimuli are required to convert the tolerogenic signal to a stimulatory signal (Finkelman *et al.*, 1996). Recent evidence suggests that plasmid DNA contributes to the induction of immunity by the presence of immunostimulatory sequences (ISS) present in the bacterial DNA (Pisetsky, 1996). This property of the plasmid DNA probably plays a crucial role in initiating the immune reaction by stimulating the

production of several cytokines, including IL-1, IL-6, IL-12, IL-18, IFNs type I, and TNF-α, from several cell types such as B cells and macrophages (reviewed in Bromberg *et al.*, 1998, Tighe *et al.*, 1998). Immunization with plasmids encoding a single class I-restricted CTL epitope can induce CTL activity. This observation suggests that ISS support the induction of an immune response in the absence of Th development (Ciernik *et al.*, 1996). In addition, IFN-α/β receptor knockout mice cannot mount immune responses following DNA immunization. This finding implies that endogenous type I interferon activity may be crucial for initiating T cell priming following DNA immunization (Ramsay *et al.*, 1997). Direct evidence was recently provided that CpG stimulated APC to produce type I IFNs, which then acted directly on T cells via type I IFN receptor (Sun *et al.*, 1998).

There appears to be a difference in the initial event of immune responses induced by different routes of plasmid inoculation. Surgical excision of the injected muscle bundle within 10 minutes of DNA inoculation did not effect the magnitude or longevity of the immune responses. Whereas, removal of the target skin up to 24 hours after gene gun immunization completely inhibited the induction of immune response. The integrity of the target skin was required for 3 days post-injection for maximal antibody responses (Torres *et al.*, 1997). This finding suggests that following intramuscular immunization, either free DNA or transfected circulating cells or APC rapidly emigrate from the injection site to initiate immune responses outside the muscle tissue. In contrast, DNA inoculation into the skin probably results in transfection of the professional APC, which then migrate later to the draining lymph nodes. Keratinocytes may also serve as an antigen factory. Following immunization, transfected keratinocytes continue to produce antigen for 2 weeks (with the maximal production within the first 3 days) while transfected DC rapidly move within 24 hours from the epidermis to the DLN (Condon *et al.*, 1996), where they initiate the immune responses

(Mor *et al.*, 1995). These migratory cells are critical for the induction of a primary T cell response and immunological memory, while non-migratory transfected cells may influence the magnitude of a primary immune response (Klinman *et al.*, 1998, Torres *et al.*, 1997).

That the initiation of an immune response can occur within the skin but not in the muscle may be explained by the fact that the skin immune system, but not skeletal muscles, has evolved a specialized function for immune surveillance (Bos & Kapsenberg, 1993). Intradermal injection induces a more rapid onset of CTL activity than intramuscular injection. Both the resident APC population and the microenvironment at the site of injection may explain these difference (Boyle *et al.*, 1997b). Although a variety of skin cells can be transfected *in vivo* with naked DNA, only LC are able to present antigen to Th cells (Casares *et al.*, 1997). Langerhans cells or epidermal dendritic cells have been shown to be potent inducers of both $CD4^+$ T cell- and $CD8^+$ CTL-mediated immunity (Celluzzi & Falo, 1997). In addition, keratinocytes, upon encounter with "stress" signals, can produced numerous pro-inflammatory cytokines including GM-CSF, IL-1, TNF-α which can activate DC (Bos & Kapsenberg, 1993).

Despite the scarcity of DC in muscle tissue, it was shown that DC (but not B cells) were transfected and presented antigen to T cells, following intramuscular immunization (Casares *et al.*, 1997). Although *in vitro* transfected macrophages do not have the same immunogenic properties as DC (Manickan *et al.*, 1997a), a role for macrophages as APC following intramuscular immunization has been suggested (Chattergoon *et al.*, 1998). Macrophages were identified as the primary transfected cell type that migrated to lymph nodes following intramuscular DNA immunization. The transfected macrophages present at the site of immunization could possibly play an important role as APC, similar to the bone marrow APC in the lymph nodes. Some of

the macrophages may also return to the blood pool and then distribute to other organs. While circulating in the blood pool, these cells are activated and are capable of priming T cell responses (Chattergoon *et al.*, 1998).

The mechanism of priming of class I-restricted CTL by DNA immunization has been examined. Several studies using the bone marrow chimeric model confirm that the priming of CTL required a bone marrow derived APC (Corr *et al.*, 1996, Fu *et al.*, 1997, Iwasaki *et al.*, 1997b, Ulmer *et al.*, 1996a). This should not be problematic if DNA is inoculated into the skin where APC are abundant and can be directly transfected following immunization. However, the scarcity of DC in muscle tissue raises the question of how CTL priming occurred following intramuscular immunization. Following intramuscular plasmid immunization, the CTL activity induced by a cytosolic antigen was lower than that induced by secreted or membrane-bound forms, whereas the induction of CTL was not affected by antigen localization following intradermal immunization (Boyle *et al.*, 1997a). This finding suggests that mechanisms of CTL induction following intramuscular, or intradermal (or gene gun) administration are somewhat different.

It has been suggested that plasmids generally persist for extended period in tissues and interstitial fluid. Following intramuscular plasmid injection, APC are recruited to the site of injection due to tissue damage, thus increasing the likelihood that APC are transfected at the site of inoculation (Chattergoon *et al.*, 1998). Co-expression of viral protein and ubiquitin, the small polypeptide that targets the linked protein to the proteosome, completely abrogated antibody responses because the protein was rapidly degraded within the cytosol of transfected cells. However, the CTL activity was improved and viral protection was induced in this model (Rodriguez *et al.*, 1997). Due to rapid degradation inside the transfected cells, the possibility of antigen secretion from myocytes is unlikely. This finding favours the idea that the direct transfection of APC

occurs, *in vivo*. It has been argued that antigen transfer from myocytes to APC may occur in the form of peptides or fragmented antigen instead of a full-length protein (Fu *et al.*, 1998).

Additional mechanisms, referred to antigen transfer, have been reported to be involved in CTL induction following intramuscular immunization (Fu *et al.*, 1997, Ulmer *et al.*, 1996a). Following immunization, the protein is synthesized, secreted from transfected muscle cells and then taken up by APC, which process and present the peptides. Using a transfected myocyte implantation model, the authors suggested that antigen synthesis by the muscle alone was sufficient to induce protective immunity and that transfection of APC was not require for priming of CTL. However, one should be cautious when interpreting these findings regarding the role of professional APC during immune induction *in vivo*. Several additional factors should be taken into consideration in these studies. The fact that the transfected myocytes were implanted intramuscularly raises the possibility of local tissue damage during injection and/or introducing dead cells to the system. The role of DC in acquiring antigen from apoptotic cells and subsequently inducing CTL priming has been shown (Albert *et al.*, 1998). In addition, the implanted cells are likely to reach the DLN, where they could deliver the antigen to APC and possibly provide additional signals required for immune activation. These events may not normally occur following the usual plasmid immunization protocol. In addition, the antigen dose given to the recipient, i.e. the high number of transfected myocytes, may not reflect the natural antigen dose obtained following DNA immunization. As pointed out by Carbone and colleagues (Carbone *et al.*, 1998), a higher dose of antigen is required for effective priming of naïve CTL by a cross-presentation mechanism (see below).

The finding that antigen transfer from myocytes to APC does occur and is involved in priming of CTL following intramuscular immunization raises an issue

regarding the pathway of antigen presentation. Generally, newly synthesized MHC class I molecules load peptides before leaving the ER and later present the peptide antigen in the context of MHC class I molecule to $CD8^+$ T cells. In this case, the loaded peptides are endogenously synthesized, cytosolic proteins that have been processed by the proteosome and transported into the ER through the specialized transporter associated with antigen processing (TAP) (reviewed in Bevan, 1995). However, the occurence of "cross priming or cross presentation", i.e. presentation of exogenous derived peptides in the context of MHC class I molecules, has been well documented (Bevan, 1995, Carbone *et al.*, 1998). The described mechanisms of antigen presentation include both noncytosolic and cytosolic pathways. The noncytosolic pathway involves digestion of antigen (mostly particulate antigen) in the phagosome and loading of the peptide onto MHC class I, possibly by regurgitation of digested materials and loading onto surface MHC class I (Pfeifer *et al.*, 1993). The described cytosolic pathway includes shunting the proteins either from phagosomes or through the process of macropinocytosis or endocytosis into the cytosol, and utilization of a heat shock protein as a chaperone to facilitate antigen entry into the cells and direct the peptide into the cytosol (reviewed in Bevan, 1995, Carbone *et al.*, 1998, Rock, 1996). It has also been recently shown that DC can ingest apoptotic cells containing antigen, and present the antigen through their class I MHC (Albert *et al.*, 1998). For the cytosolic pathway, exogenous antigen must be transported into the ER through TAP (Kovacsovics-Bankowski & Rock, 1995). Thus, peptides from either exogenous or endogenous sources ultimately follow the classical MHC class I presentation (Rock, 1996).

Both DC and a subset of macrophages have been shown to mediate cross presentation activity (reviewed in Carbone *et al.*, 1998). However, the mechanism of antigen internalization and presentation may be different depending on the nature

(soluble or particulate) and size of the antigen, and APC (DC or macrophage). DC are found to be more effective in cross presentation of soluble protein than macrophages (Norburry *et al.*, 1997). However, the heat shock protein-peptide complex may preferentially target to macrophages (as opposed to other APC) (Udono *et al.*, 1994). It is not clear which mechanism(s) is involved in the cross presentation observed following DNA immunization.

Cross presentation can lead to different outcomes (ignorance, tolerance, or immunity) depending on antigen dose, and the presence of $CD4^+$ Th (Carbone *et al.*, 1998). The requirement for $CD4^+$ cells is probably related to their ability to modify the resting APC into activated APC, a crucial step for priming of pCTL (Ridge *et al.*, 1998). It is also well documented that in the absence of Th, some immunostimulatory events such as viral infection, the presence of inflammatory cytokines or bacterial products can effectively activate DC, and induce pCTL (reviewed in Lanzavecchia, 1998). In this case, the immunostimulatory property of plasmid DNA may contribute to the induction of cross presentation. Interestingly, bone marrow derived DC cultured in the presence of GM-CSF exhibit enhanced cross presentation activity when compared with the same cells cultured in the absence of GM-CSF (Norburry *et al.*, 1997). This suggests that the level of cross priming may also depend on the activation stage of APC.

2.2.2 Immune responses induced by DNA immunization and factors that influence the outcome of the immune response

Plasmid immunization results in *in vivo* transfection, *de novo* protein expression, and subsequently induction of immune responses. Antigen expression and the activation of $CD4^+$ T lymphocytes peak and fall in a similar pattern, coincident with emerging antibody production (Chattergoon *et al.*, 1998). $CD4^+$ T lymphocytes are responsible for both IFN-γ and antibody production following plasmid immunization,

since depletion of the $CD4^+$ population prior to plasmid immunization suppresses cytokine production and totally abolishes antigen-specific antibody response (Leclerc *et al.*, 1997). Antibody responses following DNA immunization rise slowly (Ertl & Xiang, 1996a). However, unlike $CD4^-$ activity, antibody production does not diminish over time (Chattergoon *et al.*, 1998). Boosting with plasmid had little effect on the antibody titre, while restimulation with traditional vaccines revealed the priming effects of the DNA (Ertl & Xiang, 1996a). DNA immunization usually results in long-term expression of low amounts of antigen (Wolff *et al.*, 1992). This may contribute to the induction of antibody with higher affinity than that induced following protein immunization (Boyle *et al.*, 1997b). It is also suggested that antibody responses induced by DNA immunization more closely reflect that observed following a natural infection (Peet *et al.*, 1997).

DNA immunization (intradermal or intramuscular) has been shown to initiate a strong Th1-like antigen-specific immune response (Donnelly *et al.*, 1997, Raz *et al.*, 1996). Interestingly, topical ocular application of plasmid DNA also induced a Th1-like immunity (Daheshia *et al.*, 1998). This response cannot be reproduced by repeated immunization with a low dose of protein antigen (Leclerc *et al.*, 1997). Several investigators suggested that the induction of a Th1-like immune response following plasmid immunization is likely dependent on the immunostimulatory property of the plasmid DNA (Leclerc *et al.*, 1997, Leitner *et al.*, 1997, Roman *et al.*, 1997). Plasmid DNA, containing ISS, can induce IL-12 and other early inflammatory cytokine production which effectively activates the APC, and possibly the Th1 $CD4^+$ T lymphocytes (reviewed in Bromberg *et al.*, 1998). Immunization with plasmids encoding ovalbumin/IL-12 or ovalbumin/IL-1β fusion proteins induced a Th1 biased immune response, i.e. strong IFN-γ production with little IL-4 detected. The effect of IL-1β may relate to its ability to increase IL-12 production by APC (Maecker *et al.*, 1997). Co-administration of antigen with ISS-enriched plasmid DNA or ISS-oligonucleotides

effectively enhanced IgG2a and IFN-γ production, and inhibited IgE and IL-4 production (Roman *et al.*, 1997). It has also been suggested that the high level of IFN-γ induced by plasmid immunization induces switching of antibody isotype from IgG1 to IgG2a (Leitner *et al.*, 1997). Therefore, the increased IgG2a and decreased IgE production observed following plasmid immunization is probably related to the preferential induction of Th1 lymphocytes which subsequently influences isotype switching to IgG2a and, at the same time, down-regulates the Th2 development. Interestingly, immunization with DC, that are transfected *in vitro* with plasmid DNA, also induced a Th1 $CD4^+$ T cell response (Manickan *et al.*, 1997a). Thus, the early event of APC activation by the plasmid may be sufficient to induce certain factors that influence the type of immune responses.

The pattern of immune responses induced by plasmid immunization can dominate an ongoing immune response in the host. Furthermore, an immune response, previously induced by plasmid immunization, remains unchanged regardless of the boosting protocols (Feltquate *et al.*, 1997, Raz *et al.*, 1996). For example, intradermal plasmid immunization induced a Th1-like immune response, characterized by the induction of predominant IgG2a antibody, and Th1 phenotype $CD4^+$ cells (Lee *et al.*, 1997, Raz *et al.*, 1996). In contrast, immunization with the same protein antigen (in saline or alum) induced Th2-like immune responses, characterized by IgG1 and IgE antibody production and Th2 phenotype $CD4^+$ cells. The pattern of the response induced by plasmid immunization, i.e. Th1-like, remained the same even after boosting with protein (Raz *et al.*, 1996). Induction of IL-12 production by the plasmid during the priming process may have switched the Th phenotype of the memory population toward Th1-type. During secondary antigenic stimulation, not only was the memory Th1 phenotype maintained but induction of the Th2 response was also inhibited (Wynn *et al.*, 1994). On the contrary, mice that were previously immunized with protein and further boosted with plasmid switched a previously developed Th2-like to a Th1-like

immune response. Thus, DNA immunization is extremely powerful for initiating Th1-like immunity even in the presence of an ongoing Th2 type immune response (Raz *et al.*, 1996). The immune induction by plasmid immunization is somewhat different from the one induced by vaccination with protein plus IL-12 (Gurunathan *et al.*, 1997), thus the nature of antigen presentation, in addition to the immunological properties of the plasmid, may play a role in the above observations.

Many studies suggest that the route and/or method of immunization can influence the type of the immune response induced. Intradermal or intramuscular immunization with plasmid generally induce a Th1-like immune response (Feltquate *et al.*, 1997, Pertmer *et al.*, 1996, Raz *et al.*, 1996, Xiang *et al.*, 1994), whereas gene gun immunization usually elicits Th2-like immune responses (Feltquate *et al.*, 1997, Robinson & Torres, 1997). It has been suggested that both the nature of antigen and the route of delivery influences the pattern of an immune response (Pertmer *et al.*, 1996). However it has also been argued that the type of immune response can also be influenced by methods, i.e. saline injection or gene gun inoculation, and not just the route of immunization (Feltquate *et al.*, 1997, Robinson & Torres, 1997). It should be noted that the significance of DNA doses used in different protocols has never been addressed, or reasonably compared in most of these studies. As pointed out by Barry and Johnston (Barry & Johnston, 1997), the issue of the pattern of immune response induced by different routes (or methods) of delivery may be oversimplified, since several factors control the outcome of the immune responses induced by plasmid DNA. These factors include the character of the antigen, host genotype and age, method of inoculation and amount of inoculated DNA. The character of the antigen and host genotype may be the most crucial factors, whereas the method of DNA inoculation and the amount of DNA can contribute to the strength and type of the immune response (Barry & Johnston, 1997).

Generally, 10-100 μg of plasmid DNA is used for intramuscular or intradermal immunizations, whereas DNA doses for gene gun immunizations usually range between 0.4-2.5 μg. Intramuscular immunization with low or high doses of antigen induces different predominant antibody isotypes (IgG1 versus IgG2a, respectively). Similarly, gene gun delivery at a higher dose induces higher IgG2a levels (Barry & Johnston, 1997). In addition, multiple gene gun immunization promotes IgG2a production (Leitner *et al.*, 1997). The high dose of plasmid used in intramuscular or intradermal immunization can effectively induce the local production of IFN-α, β and γ as well as IL-12, IL-18, which subsequently promotes Th1-like immunity. In contrast, the low dose of plasmid DNA, used in gene gun immunization probably does not have enough local adjuvant effect to trigger Th1-like immunity (Roman *et al.*, 1997). Therefore, the induction of the type of immune response induced is likely to depend on the dose of DNA, i.e. dose of ISS.

The experiment where animals were first primed via gene gun and then boosted intramuscularly or vice versa showed that the antibody isotype was strictly determined by the conditions of the primary immunization. Interestingly, although cytokine patterns fluctuated with the number of immunizations, the antibody isotype ratios remained unchanged (Pertmer *et al.*, 1996). The fixed pattern of antibody isotype may be partly explained by the effect of IL-12. Although IL-12 clearly represents a Th1 inducer when present in the early stage of an immune response, it does not counteract and can amplify pre-existing Th2 responses (Muraille & Leo, 1998). IL-12 treatment can induce both IgG1 and IgG2a (Bliss *et al.*, 1996). The induction of high amounts of IL-12, following intramuscular immunization, may further enhance the Th2-like responses previously induced by gene gun immunization. Therefore, it may be more difficult to establish new Th1 clones in the polarlized Th2 environment. In the experiment where intramuscular priming was followed by gene gun boosting, the memory B cells

generated after intramuscular injection expressed IgG2a due to a strong Th1 inducing effect of the DNA in the primary immunization. Class switching to IgG2a is IFN-γ dependent, and B cells delete DNA encoding the γ1 heavy chain during the gene arrangement process. Thus IgG1 secreting cells must be derived from new B cell clones (Leitner *et al.*, 1997). It is unlikely that boosting with gene gun will efficiently induce these new clones, since naïve B cells may not compete effectively with the pre-existing memory B cell population.

The nature of the antigen also influences the onset, dynamics, and magnitude of the antibody response following plasmid immunization. For example, at 4-8 weeks after intramuscular immunization, plasmid encoding a secreted form of ovalbumin induced a higher antibody response than the plasmids encoding for a membrane-bound or cytosolic form of ovalbumin, although the levels of Th activity were similar (Boyle *et al.*, 1997a). In contrast, cytosolic gD which induced a low antibody response at 10 weeks post immunization, induced much higher antibody responses than the secreted and membrane-bound gD at 23 weeks post immunization (Lewis *et al.*, 1997). Immunization with plasmids encoding secreted antigens induced much higher IgG1 production than the other forms of antigen (Boyle *et al.*, 1997a, Cardoso *et al.*, 1996, Lewis *et al.*, 1997, Peet *et al.*, 1997). The greater availability of secreted antigens in DLN, i.e. higher antigen dose, may enhance B cell priming, and switching of IgG2a towards IgG1 antibody production (Boyle *et al.*, 1997a, Lewis *et al.*, 1997). The reason for high IgG1 production may be partly explained by the effect of antigen dose on IL-4 production (reviewed in Abbas *et al.*, 1996). Indeed, the level of IgG1 production following DNA immunization correlated with the detection or coadministration of IL-4 (Boyle *et al.*, 1997b, Maecker *et al.*, 1997).

The pattern of immune responses may also related to the dose of antigen presented to the immune system. Furthermore, different routes of immunization

probably produce different doses of antigen. For example, intramuscular DNA immunization induced predominantly IgG2a isotype, whereas intradermal immunization with the same dose of DNA induced higher IgG1 antibody levels than the intramuscular route (Boyle *et al.*, 1997b, Pertmer *et al.*, 1996). In addition, IL-4 was detected following intradermal but not intramuscular immunization with either protein or plasmid (Boyle *et al.*, 1997b). Since higher doses of antigen preferentially induce IL-4 production (Abbas *et al.*, 1996), it is likely that the skin associated immune system may present the antigen to the immune system more effectively than muscle. In fact, the predominant IgG1 antibody response observed following gene gun immunization may reflect a better transfection efficiency of APC that results in a higher dose of antigen for B cell priming and/or the efficient induction of IL-4, apart from the much lower dose of ISS. The issue of antigen dose may also explain the finding that different strains of mice display different dose requirements for mounting an antibody response (Haddad *et al.*, 1997) and/or switching toward predominantly IgG1 production (Leclerc *et al.*, 1997). For example, intramuscular immunization with plasmid DNA into the high responder BALB/c strain tended to induce higher IgG1 production than the low responder C57Bl/6 strain (Leclerc *et al.*, 1997).

Although antibody production, in particular IgG1, is enhanced in the presence of IL-4 (reviewed in Robinson, 1997), antibody production following DNA immunization is possibly influenced by multiple factors, since endogenous IL-4 is not crucial for the induction of an antibody response following DNA immunization (Ramsay *et al.*, 1997). The selective suppression of IgE but not IgG1 following plasmid immunization (Raz *et al.*, 1996, Roman *et al.*, 1997), also implies that other factors, which are not affected by the development of Th1 type immunity, may influence IgG1 production. For example, activated interdigitating dendritic cells could regulate the immunoglobulin class switching, from IgM to IgG1, *in vivo* (Gerloni *et al.*, 1998). Thus, the type of APC may

partly contribute to the quality of the induced immune response particularly when the amount of antigen is limited.

It should be emphasized that the immune responses induced by plasmid immunization are unique. One needs to be cautious when attempting to define the immunological profile, i.e. Th1 or Th2 immune response. Humoral and cell-mediated immunity co-exist in DNA immunized animals (Ulmer *et al.*, 1998, Pertmer *et al.*, 1996, Xiang *et al.*, 1994). Intramuscular immunization led to a Th1-like immune response, as characterized by enhanced production of IFN-γ, IgG2a level, CTL activity and the lack of IL-4 production. Gene gun immunization induced a high level of IL-4 and IgG1 production, yet at the same time, induced comparable CTL activity and a considerable amounts of IFN-γ when compared with the intramuscular route (Pertmer *et al.*, 1996). This pattern of immunity does not fit exactly the "generally believed" profile of theTh2 type biased immune response.

The induction of CTL and antibody responses by plasmid immunization are not strictly associated (Fu *et al.*, 1998, Rodriguez *et al.*, 1997). Th activation and antibody production following plasmid immunization are dose-dependent and exhibit a parallel dose-response pattern, whereas CTL can be induced at a lower dose, and does not fall into the same dose-response curve as Th and Ab (Xiang *et al.*, 1994). In some cases, CTL can be induced in the absence of an antibody response when using a very low dose of DNA (Cardoso *et al.*, 1996). In addition, CTL are induced prior to the presence of antibody and are not correlated with an antibody response (Maecker *et al.*, 1997). Interestingly, gene gun immunization with plasmid encoding a single CTL epitope resulted in the efficient induction of tumor protective CTL, suggesting that Th may not be required for the induction of CTL (Ciernik *et al.*, 1996). Furthermore, intramuscular immunization with plasmids carrying an ovalbumin/IL-4 fusion gene favoured a Th2-like immune response but, at the same time, delayed but significant CTL activity was also

induced in the immunized mice (Maecker *et al.*, 1997). In addition, although coadministration of GM-CSF and antigen encoding plasmids enhanced antigen-specific antibody production and lymphoproliferative activity, the level of CTL activity was not affected (Kim *et al.*, 1997). These results suggest that $CD4^+$ and $CD8^+$ cells are activated by different pathways (Xiang *et al.*, 1994).

The potential immunomodulatory effect of $CD8^+$ cells, i.e. Tc1 and Tc2, has been recently discussed (Mosmann & Sad, 1996). The role of $CD8^+$ cells in the immunological cascade following DNA immunization has not been explored. It is possible that the considerable amount of IFN-γ observed following DNA immunization may partly reflect $CD8^+$ cell activity. Both $CD4^+$ or $CD8^+$ T cell subsets, when passively transferred from DNA immunized mice to recipients, could suppress IgE antibody production following antigen challenge (Lee *et al.*, 1997). Furthermore, passive transfer of $CD8^+$ lymphocytes from rats immunized with plasmids encoding the Der p 5 allergen suppressed IgE production and airway hyperresponsiveness in recipient rats (Hsu *et al.*, 1996). Thus, Tc1 cells may be induced in DNA-immunized animals.

2.2.3 The adjuvanticity of DNA

Recent evidence suggests that bacterial DNA can function as an adjuvant or immunomodulator, which profoundly affects the induction of immune responses. The immunostimulatory effect of bacterial DNA was initially recognized in 1984, when Tokunaga and colleagues reported that DNA extracted from *Mycobacterium bovis* BCG exhibited antitumor activity (Tokunaga *et al.*, 1984). Further studies revealed that the immunomodulatory activity was due to a series of CpG motifs (also referred as the immunostimulatory sequence, ISS) that generally followed the sequence 5'-purine-purine-C-G-pyrimidine-pyrimidine-3' present in DNA from bacteria, viruses and

invertebrates, but not mammalian species (reviewed in Bromberg *et al.*, 1998, Tighe *et al.*, 1998).

The immunostimulatory property of the CpG motifs is possibly related to the relatively higher frequency of the CpG motifs in bacterial DNA when compared with the vertebrate genome, and/or the absence of cytosine methylation in bacterial genes (Krieg, 1996, Tighe *et al.*, 1998). Mammalian DNA exhibits CpG suppression, i.e. contains much lower numbers of CpG dinucleotides than predicted by random base utilization. In addition, the cytosines in CpG dinucleotides are highly methylated in vertebrates, but not in prokaryotes (Bird, 1987). Eliminating the CpG dinucleotides or methylation of bacterial DNA abolishes the immunostimulatory effects of the ISS, suggesting that the mammalian immune system utilizes the characteristics of DNA pattern to identify a foreign invasion (Krieg *et al.*, 1995). Bacterial DNA can behave like other foreign macromolecules and induce specific immune responses. In fact, the sera of normal human subjects contain abundant quantities of antibodies to certain bacterial DNA (Pisetsky, 1997). It has been proposed that the ability to recognize unmethylated CpG motifs was selected during vertebrate evolution so that the immune system could be rapidly activated following an encounter with microbial DNA (Krieg *et al.*, 1995). In fact, the immunomodulatory effects of CpG motifs were found to be much stronger than that of bacterial lipopolysaccharide (LPS) (Klinman *et al.*, 1996, Krieg *et al.*, 1995). Immune responses against microbial infections result from a collaborative network of rapidly activated innate immune mechanisms as well as the later development of antigen-specific immunity. Therefore, the immunostimulatory property of bacterial DNA is one of the elements that stimulates innate, non-specific, immune responses and further influences the outcome of acquired immune responses (Tighe *et al.*, 1998).

Cellular uptake of CpG motifs is required for cellular activation (Krieg, 1996). Reagents that bind to the macrophage scavenger receptor (MSR) appear to block the

immunostimulatory effects of plasmid DNA on macrophages. This suggests that MSR may be one of the receptors plasmid DNA uses for entering macrophages (Wloch *et al.*, 1998). Bacterial DNA is taken up and transported into the acidic endosomal organelles by adsorptive pinocytosis in both DC and macrophages (Sparwasser *et al.*, 1998). The mechanism of cellular activation by CpG motifs is not clearly understood. Some of the stimulatory effects of CpG motifs are found to be mediated through the activation of NF-κB (Stacey *et al.*, 1996). It has also been reported that CpG motifs activate the mitogen-activated protein kinase (MAPK) pathway and lead to activation of transcriptional factor AP-1. In fact, the activation of both NF-κB and AP-1 is required for CpG DNA-mediated cytokine production (Yi & Krieg, 1998).

Bacterial DNA can induce polyclonal B cell proliferation and antibody responses in mice (Messina *et al.*, 1991). In fact, hypomethylated DNA from various organisms including yeast, bacteria, insects, nematodes and molluscs are also strongly mitogenic for mammalian B-cells (Sun *et al.*, 1997). CpG motifs trigger a direct T-independent B cell activation that results in B-cell proliferation and secretion of IgM (Krieg *et al.*, 1995). Interestingly, CpG motifs preferentially activate B cells that simultaneously encounter an antigen through their receptors. This suggests that bacterial DNA can facilitate the induction of antigen-specific acquired immunity by providing a costimulatory signal to B cells that bind antigen (Krieg *et al.*, 1995, Yi *et al.*, 1996a). It should be noted that ISS does not promote immunoglobulin secretion from B cells that have already undergone Ig switch recombination (Davis *et al.*, 1998). The isotype switching is likely to be influenced by cytokine in the microenvironment. In the presence of IL-4, both IgG1 and IgE secretions can be induced. However, IgG2a secretion is usually observed following immunization with ISS, due to the induction of IFN-γ production by CpG motifs (see below).

Early studies suggest that the immunomodulatory effects of the ISS were mediated by their ability to activate NK cells and induce production of type I and type II IFNs. However, recent studies demonstrate that CpG motifs also induce cytokine production from many cell types including B cells, T cells, macrophages, and NK cells (reviewed in Bromberg *et al.*, 1998). Activation of B-cells by bacterial DNA induces the secretion of several cytokines including IL-6 and IL-12 (Klinman *et al.*, 1996, Yi *et al.*, 1996b). $CD4^+$ T lymphocytes produce IL-6, and IFN-γ in response to the CpG motifs (Klinman *et al.*, 1996). Both IL-6 and IFN-γ production is essential for antibody secretion from DNA-activated B-cells (Klinman *et al.*, 1996, Yi *et al.*, 1996a).

Bacterial DNA or ISS activates DC (within 1-2 hr after CpG challenge) to produce cytokines including IL-12, IL-6 and TNF-α, *in vivo* (Sparwasser *et al.*, 1998). Macrophages produce type I and type II IFNs, IL-12, IL-18, TNF-α, IL-1β, and plasminogen activator inhibitor-2 in response to ISS (Roman *et al.*, 1997, Stacey *et al.*, 1996). In the presence of IFN-γ, macrophages also produce inducible nitric oxide synthase (Stacey *et al.*, 1996). Human monocytes transfected with plasmid containing ISS transcribe large amount of IFN-α, IFN-β and IL-12 (Sato *et al.*, 1996). In contrast, direct transfection of $CD4^+$ or $CD8^+$ T cells with plasmids containing ISS does not increase IFN-γ production (Roman *et al.*, 1997). The secretion of IFN-γ from both NK and T cells is probably mediated indirectly through the production of IFN-γ inducers (such as IFN-α, IL-12, IL-18) by macrophages and monocytes (Tighe *et al.*, 1998). Furthermore, IFN-α/β knockout mice fail to mount an antibody response following immunization, indicating that endogenous type I IFN is essential for the induction of an immune response following plasmid immunization (Ramsay *et al.*, 1997). A role for type I IFN in the amplification of B cell responses to CpG motifs has also been suggested (Sun *et al.*, 1997). CpG motifs up-regulate costimulatory molecules,

including MHC class II, B7.2 (CD86) and Ly-6C, on B cells (Davis *et al.*, 1998). Bacterial DNA also induces maturation of immature DC, up-regulation of MHC class II, CD40, CD86, but not CD80, surface markers and acquisition of professional APC function (Sparwasser *et al.*, 1998). These findings suggest that DC, macrophages and monocytes are the primary target of ISS, emphasizing the importance of these cells in the CpG activated immune responses, particularly by stimulating the production of several proinflammatory cytokines and costimulatory molecules in the early stage of immune activation.

The adjuvant effects of ISS have been clearly demonstrated in several systems. Coadministration of plasmids, or oligodeoxyribonucleotides (ODNs) containing ISS with protein or viral antigens significantly improves immune responses to antigens (Davis *et al.*, 1998, Moldoveanu *et al.*, 1998, Roman *et al.*, 1997). Furthermore, administration of antigen along with adjuvant, aluminium hydroxide (alum), and CpG containing ODNs effectively induced a Th1-biased immune responses in the immunized mice. This suggests a strong synergism between CpG-ODNs and alum (Davis *et al.*, 1998). Mice immunized with antigen, either with or without alum, in the presence of CpG-ODNs exhibited strong CTL activity. In contrast, mice immunized with the antigen alone or in the presence of alum did not exhibit CTL activity (Davis *et al.*, 1998, Sparwasser *et al.*, 1998). Thus, the immunostimulatory effect induced by CpG motifs may be necessary for the antigen cross-presentation and subsequently priming of pCTL in these studies. The strong Th1-biased immune responses induced by CpG containing ODNs has recently been proposed as an effective prophylactic and/or therapeutic strategy in several models including Leishmaniasis (Zimmermann *et al.*, 1998), and asthma (Kline *et al.*, 1998).

Plasmid DNA used for immunization can be conceptually divided into 2 independent units: the transcriptional unit that directs antigen synthesis, and the

immunostimulatory unit of the plasmid backbone (Tighe *et al.*, 1998). The magnitude of the antigen specific immune response induced by different plasmids vectors appears to be related to both precise sequence and the localization of ISS within the plasmid backbone (Sato *et al.*, 1996). As previously discussed, the type of immune response, i.e. Th1-like, induced following DNA immunization is likely to be influenced by the ISS.

2.2.4 DNA immunization for induction of rotavirus-specific immunity

As discussed above, DNA immunization can effectively induce a full range of immune responses. The potential use of DNA immunization for induction of rotavirus specific immunity has been recently explored. Following gene gun immunization, plasmids encoding VP4 and VP7 induced a very low level of rotavirus-specific serum antibody titres, compared with the plasmid encoding VP6. However, all of the plasmids were able to induce CTL activity, and protection against viral challenge in an adult mouse model. The level of rotavirus-specific IgA following immunization was not addressed in these studies (Chen *et al.*, 1997, Herrmann *et al.*, 1996). It is not yet clear which immune mechanisms are important in rotavirus protection as well as which ones are induced by plasmid immunization. Previously it was thought that immunity to only VP4 and VP7 was crucial for viral protection. However, the finding that plasmid encoding VP6 protein could also induce protection in adult mice expands our appreciation of immune defences against rotavirus.

In general, the VP6 gene is expressed more efficiently than the VP4 and VP7 genes in most expression systems. Following challenge of DNA immunized mice, the level of rotavirus-specific faecal IgA in mice immunized with VP6 gene was higher than the levels found in mice immunized with VP4 and VP7, or mice previously immunized with virus (Chen *et al.*, 1997). Recently, it has been demonstrated that oral

immunization with plasmid encoding VP6, encapsulated in poly(lactide-coglycolide) microparticles, also induced both serum antibodies and intestinal IgA. Furthermore, the immunized animals were partially protected against viral challenge (Chen *et al.*, 1998). It should be noted that the studies related to immunization with plasmids encoding rotavirus genes are not all in agreement. Choi and colleagues (1997) reported that although gene gun inoculation with plasmid encoding VP6 induced high level of serum IgG, but viral specific IgA was not detected in stools and animals were not protected from viral challenge.

Although information about the efficacy of DNA immunization in rotavirus models is limited, several features have made DNA immunization an attractive approach for a rotavirus vaccine, particularly for newborn immunization. In general, rotaviruses cause disease only in newborn animals and young children. Therefore, immunization has to be done at a very early age when a high level of maternal antibody may be present. It has been thought that the susceptibility of newborn animals to viral-induced diseases is partly due to the immaturity of the immune system. However, recent studies suggest that neonates are immunocompetent, and that the immunological tolerance observed in newborn animals is actually caused by preferential induction of a Th2 type immune response (Forsthuber *et al.*, 1996, Sarzotti *et al.*, 1996). The preferential induction of Th2 type immunity may be influenced by to the dose of antigen and the type of APC that interact with naïve T cells in the newborn. An effective Th1 type immune response could also be generated in newborn mice, at a specific dose of antigen which was much lower than that used in adult mice, or in the presence of an appropriate type of adjuvant (Forsthuber *et al.*, 1996, Ridge *et al.*, 1996, Sarzotti *et al.*, 1996). Effective induction of CTL by plasmid immunization in neonatal mice has been reported (Bot *et al.*, 1996, Hassett *et al.*, 1997, Martinez *et al.*, 1997, Sarzotti *et al.*, 1997). Thus, DNA immunization can be used to overcome the interference of passive immunity and can

induce immunity in newborn animals (Hassett *et al.*, 1997, Manickan *et al.*, 1997c, Wang *et al.*, 1998). In addition, induction of immune responses, following neonatal plasmid immunization was not inhibited by the presence of hyperimmune serum (Wang *et al.*, 1998). Therefore, DNA immunization seems to be an attractive strategy for developing anti-viral immunity in the newborn. However, it should be emphasized that it is important to determine the protective mechanism prior to any immune intervention in newborn animals, since the pattern of immune response established at a young age remains throughout the life of the animals (Martinez *et al.*, 1997, Siegrist, 1997). Further studies are needed to determine whether these advantages are applicable to the rotavirus model.

2.2.5 Enhancement of immune responses induced by DNA-based vaccines

As previously discussed, DNA immunization effectively induces a wide-range of antigen-specific immune responses. However, it should be also noted that some antigens, upon expression by plasmid vectors remain immunologically silent (Ertl & Xiang, 1996b). Although the immunogenicity of the protein is likely to depend primarily on the nature and dose of protein presented to the immune system, recent findings offer an alternative strategy for enhancing the immunogenicity of the vaccine antigen through direct manipulation of the immune system itself (see below).

Plasmids encoded genes are easy to manipulate and generating plasmids encoding multiple antigens or a modified version of antigen can be done in a short period of time. Several approaches, utilizing DNA-based vaccination, have been explored to enhance the immunogenicity of the expressed antigen, without creating a drastic change in the structure of the antigen. These manipulations include construction of plasmid encoding a fusion protein of 2 antigens (Major *et al.*, 1995), co-

administration with cytokines or cytokine encoding plasmids (Ertl & Xiang, 1996b, Lewis *et al.*, 1997), co-administration with plasmid encoding CD40L (Gurunathan *et al.*, 1998, Mendoza *et al.*, 1997), and targeting the expressed antigen to the site of immune induction by a specific ligand (Boyle *et al.*, 1998). Furthermore, an undesired antigen-specific immune reaction can be down-regulated by targeting the expressed antigen to a certain cell type. For example, vaccination with plasmid encoding an immunodominant peptide of the myelin basic protein that targeted to Fc of IgG on B cells suppressed the clinical sign of autoimmune encephalomyelitis following antigen challenge (Lobell *et al.*, 1998).

2.3 The biological effects of complement protein C3d on the immune system

The regulatory role of the complement system on the induction of the adaptive immune response, in particular humoral immunity, is well established. Numerous studies has shown that the interaction of C3d fragment, the split product of C3, and its receptor (CR2 or CD21) on B cells and/or follicular dendritic cells (FDC) is crucial for induction and maintenance of a normal humoral immune response (reviewed in Carroll, 1998a, Carroll, 1998b, Carroll & Fischer, 1997, Tew *et al.*, 1997). To coligate to its receptor, CD21, and further initiate an immunostimulatory effect, C3d must first become attached to the antigen (Carroll, 1998b). This event occurs naturally in the complement activation process where the C3d fragment remains covalently attached to the surface of an antigen or immune complex. Recent studies have shown that mimicking this phenomenon by *in vitro* coupling of C3d to antigen can effectively enhance the antigen-specific immune response. Using a recombinant protein approach, hen egg lysozyme (HEL) bearing 2 and 3 copies of C3d was shown to be 1,000- and 10,000-fold more immunogenic, respectively, than HEL alone (Dempsey *et al.*, 1996). In addition, crosslinking the C3d peptide to the weakly immunogenic immunoglobulin variable domain induced a strong anti-idiotypic antibody response following immunization (Lou & Kohler, 1998). These findings suggest that the C3d molecule can profoundly influence specific immune responses and may be used to improve the immunogenicity of weakly immunogenic antigens.

The murine *Cr2* locus encodes complement receptors CD21 (CR2; 150 KDa) and CD35 (CR1; 190 KDa), which are co-expressed primarily on B lymphocytes and FDC. Both receptors bind to products of C3 and C4. CD21 binds iC3b, C3d,g, C3d, and C4d, whereas CD35 includes additional binding sites for C3b and C4b in the N-terminal region. In addition, CD35 serves as a co-factor in the inactivation of C3b by

factor I. CD21 forms a signalling complex with either CD35 or CD19 and Tapa-1, i.e. CD21/CD19/Tapa-1 complex, on the B cell surface. Studies in knockout mice indicate that complement proteins and products of the *Cr2* locus can significantly influence the induction of humoral immune responses. Mice bearing a disrupted *Cr2* locus, i.e. CD21- and CD35-deficient, and C3 or C4-deficient mice exhibited impaired antigen-specific humoral immune responses (reviewed in Carroll, 1998b, Carroll & Fischer, 1997). Enhancement of humoral immunity is mediated by engagement of C3d and its receptor CD21 on both B cells and FDC.

The finding that coligation of the CD21/CD19 coreceptor complex and the B-cell receptor (BCR) enhanced B cell signal transduction has led to the "threshold hypothesis". According to this hypothesis, naïve B cells, bearing low affinity receptors for antigen, require a coreceptor signal to lower the threshold for antigen activation (Fearon & Carter, 1995). Coligation of BCR and CD21 on B cells was shown to significantly lower both ligand concentration and the IgM:ligand affinity thresholds for B cell activation. The enhancing effect of CD21 was most obvious when the ligand, i.e. antigen, concentration was low (Mongini *et al.*, 1997). This suggests that membrane IgM and CD21 co-engagement is very important for the induction of an antigen-specific antibody responses, particularly in unprimed animals where the majority of specific B cells possess low affinity binding BCR or where the antigen concentration is low. Enhanced B cell activation through BCR-CD21 coligation may also be partly responsible for the adjuvant effect of C3d when coupled with other antigens.

Both B7-1 (CD80) and B7-2 (CD86) expression are rapidly upregulated on resting B cells within 14 hours following crosslinking of CD21/CD35 and surface IgM of B cells (Kozono *et al.*, 1998). Although cross-linking of CD21 is not a mitogenic signal, it can stimulate B cell proliferation in the presence of anti-CD40 antibodies. Thus, the signal via CD21 may also modulate the response of the B cell to later CD40 stimulation.

This property would be crucial during the early stage of B cell proliferation (Axcrona *et al.*, 1996). The CD40-CD40L interaction is also a critical step for B cell survival and directing the germinal center B cell development towards the memory B-cell pathway (Liu & Arpin, 1997).

In the spleen, naïve B cells encounter T-dependent antigens within the white pulp and then migrate into the periarteriolar lymphoid sheath region (PALS) or T cell zone where they interact with cognate T cells. As discussed above, coligation of CD21 and BCR on the B cell lowers the threshold of B cell activation and improves the interaction between T and B cells. Efficient B cell activation may lead to B cell development by two different pathways. One involves the development of extrafollicular foci in the PALS, where activated B cells, upon encounter with the cognate T cells, rapidly proliferate, and differentiate to antibody forming cells. This leads to the initial antibody production, primarily IgM. The second pathway involves entry of the activated B cells into follicles and initiation of a germinal center (GC) reaction which leads to the generation of high affinity B cells (reviewed in Carroll & Fischer, 1997, MacLennan *et al.*, 1997. Once in the follicles, the B cells (centroblasts) rapidly divide and undergo somatic hypermutation. The newly formed B cells (centrocytes) within the GC require at least 3 signals for their survival. This includes the signal from the antigen through the BCR, an antigen-independent signal from FDC through CD21, and help from T cells (Carroll, 1998b).

Follicular dendritic cells are extremely important for the initiation of GC development by trapping immune complexes via their Fc or complement receptors and transport of the antigen to the follicle (Tew *et al.*, 1997, Vora *et al.*, 1997). Expression of the CR1 and CR2 on FDC is essential for the generation and maintenance of a normal antibody response (Fang *et al.*, 1998). It was generally believed that B cells proliferated in the dark zone and affinity selection later occurred in the light, FDC-rich,

zone of the germinal center. However, recent studies suggest that proliferating B cells in the splenic GC are found in intimate contact with FDC throughout the entire GC. This suggests that both FDC, together with T cells, play a crucial role in providing antigen, and survival signals for both proliferation and for the induction of the hypermutation mechanism in the spleen (Camacho *et al.*, 1998). FDC not only provide the BCR signal to the newly produced B cells, but also deliver the second, i.e. costimulatory, signal required for proliferation and maintainance of B cell viability (Tew *et al.*, 1997, van Kooten & Banchereau, 1997). In addition, antigen provided by the FDC is essential for the acquisition of T cell help, since B cells need to endocytose, process and present antigen to the cognate T cells in order to efficiently promote B cell differentiation (Carroll & Fischer, 1997). The costimulatory or survival signal provided by FDC appears to be mediated through the C3 fragment bound to the FDC, and complement receptors on the B cells. Together with BCR ligation, an interaction between FDC-C3 fragments and B cell-CD21 is critical for an antibody response, possibly by protecting B cells from apoptosis and improving lymphocyte survival (Qin *et al.*, 1998). Supporting this concept, expression of the CD21/CD35 on the GC-B cells was found to be essential for their survival in the GC. However this survival signal is different from the signal that lowers the threshold of B cell activation in the early stage, since it cannot be overridden by high affinity BCR ligation (Fischer *et al.*, 1998).

C3 fragments have an influence on many steps of antigen processing and presentation. Covalent binding of C3b to antigen enhances antigen uptake by both antigen-specific and non-specific B cells, via their complement receptors. It also improves antigen internalization, endosomal targeting and prolongs the intracellular structural integrity of the bound antigen (Colomb *et al.*, 1996). The C3 degradation products, i.e. C3b, iC3b, C3d, covalently linked to antigen or immune complex significantly increase, and prolong the level of antigen-specific, class II restricted T cell

clones (Broackle *et al.*, 1997, Jacquier-Sarlin *et al.*, 1995). The role of iC3b and C3d in enhancing the T cell response is also highlighted by the finding that binding of complement-antigen immune complexes to CD21, but not CD35, is required for antigen presentation by B cells (Broackle *et al.*, 1997). It should be emphasized that antigen presentation is a distinct process from that involved in B cells activation. Since both antigen-specific and non-specific B cells can ingest (via the complement receptor), process, and present antigen to T cells in the context of MHC class II (Jacquier-Sarlin *et al.*, 1995, Thornton *et al.*, 1996). However, enhanced immunoglobulin synthesis occurs only in the antigen-specific B cells (Thornton *et al.*, 1996).

3. HYPOTHESES AND OBJECTIVES

DNA immunization has been recognized as a promising approach for induction of humoral and cell-mediated immunity during the past few years. When this project was initiated, it was not known if this approach could be used to induce rotavirus-specific immune responses. Colostral derived antibodies, recognizing VP4 or VP7 protein, were believed to play an important role for protection against viral-induced diarrhea in suckling calves. This suggested that plasmids encoding VP4 or VP7 were good vaccine candidates against BRV. The murine model was also useful for the design and assessment of BRV vaccines (Ijaz *et al.*, 1989, Lee *et al.*, 1995, Redmond *et al.*, 1993).

Based on the above information, we hypothesized that immunization with plasmids encoding VP4 and VP7 could induce BRV-specific immune responses in the murine model and these findings would support the potential use of DNA-based vaccine for bovine rotavirus. The studies in this thesis were directed toward the characterization of immune responses induced by plasmid immunization with the following initial objectives:

1. Generate and *in vitro* characterize plasmids encoding VP4 and VP7 genes of BRV

2. Characterize immune responses induced by immunizations with plasmids encoding the BRV genes in both naïve and BRV-primed mice

However, the results of the initial objectives clearly demonstrated that plasmids encoding VP4 and VP7 did not induce antibody responses following immunization. Therefore, the following experiments were proposed to further investigate if the lack of humoral immune responses could be altered by modifying the expression vector, or the encoded genes. Based on available knowledge, several modification strategies were designed including:

- Enhancing the level of gene expression by addition of intron A
- Changing cellular compartmentalization of the VP7
- Co-administration of plasmids encoding VP4 and VP7
- Alteration of the VP7 molecule by deletion mutation
- Co-expressions of the complement C3d, or BHV-1 gD with the VP7 protein

Following the cloning and *in vitro* characterization procedures, the impacts of these modifications on the BRV-specific immune responses were assessed.

4. MATERIALS AND METHODS

4.1 Bacterial strains, growth condition and plasmid purification

Escherichia coli strains JM109, DH5α (Sambrook *et al.*, 1989) and DH5αF'IQ [*ϕ80 lacZ*ΔM15 *endA1 recA1 hsdR17*($r_K^- m_K^+$) *supE44 thi-1*λ^- *gyrA96 relA1* Δ*(lacZYA-argF) U169/F' lacI*^{q} *proAB*$^+$ *LacZ*ΔM15 *Tn5 (Km*^{r}*)*] were from VIDO's laboratory collection (VIDO, Saskatoon, Saskatchewan). The competent cells containing plasmids encoding modified VP7 and VP7chimeric proteins were transformed and grown at 30 °C instead of 37 °C due to the toxicity of these plasmids in bacterial hosts.

Plasmids used in the pcDNA3-VP4 study were purified with anion-exchange columns (Qiagen Inc., Mississauga, Ontario) following the manufacturer's instructions. Otherwise, the plasmids were purified by CsCl gradient centrifugation twice with an additional step for removal of contaminating endotoxin using a procedure adapted from the previously described protocol (Dominico *et al.*, 1992). Briefly, the bacterial culture was grown for at least 16 hours (hr) before treatment with EDTA at the final concentration of 5mM, at room temperature (RT) for 10 minutes (min). The bacterial cells were then centrifuged at 5,000x g at 4 °C for 10 min. Supernatant was carefully discarded. The bacterial pellet was resuspended with normal saline (10 ml per 500 ml of starting culture volume) and treated with 250 μl (for every 500 ml of culture volume) of 1% Zwittergent® 3-14 detergent (Calbiochem, San Diego, CA) in 100 mM citric acids. Bacterial suspension was gently mixed and incubated at 37 °C for 10 min. The sample

was then centrifuged at 5,000x g at 4 °C for 10 min and the supernatant was discarded. The pellet was then subjected to a standard protocol for plasmid isolation using an alkaline lysis method and double cesium chloride gradient centrifugation (Sambrook *et al.*, 1989). Plasmid concentrations and purity was assessed by a spectrophotometer and by agarose gel electrophoresis of restriction digests. Plasmids were stored at -20 °C until needed.

4.2 Plasmid construction

The pTZ19R vectors containing BRV (strain C468) cDNA were kindly provided by Dr. Dongwan Yoo. DNA manipulations were done following standard techniques (Sambrook *et al.*, 1989) unless otherwise indicated. DNA markers, restriction enzymes and DNA modifying enzymes were purchased from Pharmacia (Pharmacia Biotech., Baie d'Urfé, Quebec) or New England Biolabs (NEB Ltd., Mississauga, Ontario) with the exception of shrimp alkaline phosphatase (USB, Cleveland, OH). Vent DNA polymerase (NEB Ltd.) was used in the polymerase chain reaction (PCR) for the entire study. The QIAquick gel extraction and PCR purification kits (Qiagen Inc.) were used to purify DNA from agarose gels and to clean up DNA for downstream applications. The plasmids used in this study including their relevant characteristics are listed in Table 4.1.

The plasmid pcDNA3® (Invitrogen, San Diego, CA) has an expression cassette containing the human cytomegalovirus (hCMV) immediate-early promoter and the bovine growth hormone polyadenylation site (BGH polyA). Complementary-DNA of the VP4 gene, was recovered by *Bam*H I digestion of the pTZ19R clone containing VP4-cDNA. The *Bam*H I fragment was ligated to a *Bam*H I cleaved pcDNA3® to generate pcDNA3-VP4. Plasmid pSLIA was a gift from Dr. Ralph Braun. The expression

Table 4.1 Plasmids used in this study

	Relevant characteristics (References)
pcDNA3	Expression vector with hCMV promoter and BGH polyA (Invitrogen)
pSLIA	Expression vector with hCMV-IA and BGH polyA (Braun *et al.*, 1997)
pWRG7054	Expression vector with hCMV-IA, TPA-ss and BGH polyA
pRSVmD.1	Expression vector with RSV-EP, gD, linker, transmembrane anchor sequence, SV40 polyA (Dr. Paul J. Lewis)
pSLIAmDs	pSLIA with truncated gD gene with additional linker prior to a stop codon (Dr. Ralph Braun)
pcDNA3-VP4	pcDNA3 with VP4 gene
pcDNA3-VP7	pcDNA3 with native VP7 gene
pSLIA-VP4	pSLIA with VP4 gene
pSLIA-VP7	pSLIA with native VP7 gene
pTPA-VP7Sc	pWRG7054 with TPA-ss and VP7Sc
pgD-VP7Sc	pWRG7054 with gD-ss and VP7Sc
pVP7t	pTPA-VP7Sc with destroyed *Cla* I site in the VP7Sc
pTPA-VP7Anc	pWRG7054 with TPA-ss, VP7Anc, linker and gD transmembrane anchor sequence
pgD-VP7Anc	pWRG7054 with gD-ss, VP7Anc, linker and gD transmembrane anchor sequence
pVP7(C3d)	pWRG7054 with TPA-ss, VP7Anc, linker, and C3d
pVP7(C3d)$_2$	pWRG7054 with TPA-ss, VP7Anc, linker, C3d, linker and C3d
pSLIAgD-7Sc	pSLIA with truncated gD, linker and VP7Sc
pSLIAgD-7t	pSLIAgD-7Sc with destroyed *Cla* I site in the VP7Sc gene
pSLIAtgD	pSLIA with truncated gD gene (Braun *et al.*, 1997)
pSLIAtgD(C3d)	pSLIA with truncated gD gene, intron, linker and C3d (Dr. R. Braun)
pSLIAtgD(C3d)$_2$	pSLIA with truncated gD gene, intron, linker, C3d, linker and C3d (Dr. R. Braun)

Note: ss – signal sequence

cassette of this plasmid contained the human cytomegalovirus immediate-early promoter with an intron A (hCMV-IA) and the BGH polyA (Braun *et al.*, 1997). Inserting the *Bam*H I fragment containing the VP4-cDNA into the *Bgl* II site of the plasmid pSLIA generated pSLIA-VP4. The same procedure was used to isolate and insert the cDNA of the BRV-VP7 gene into the expression vectors, creating pcDNA3-VP7 and pSLIA-VP7.

The *Bam*H I fragment containing an original VP7-cDNA, isolated from the pTZ19R cloning vector, was 1030 basepairs (bp) long. The first initiation codon of the VP7 protein started at nucleotide (nt.) 43, and the stop codon was at nt.1021 (Fig. 4.1, B). The native VP7 protein has its own signal peptide that directs the protein to the rough endoplasmic reticulum during natural viral replication (Stirzaker *et al.*, 1987). To obtain the VP7 gene without the endogenous signal sequence, the fragment of VP7-cDNA from nucleotide 166 to 1030 (Fig. 4.1, B) was amplified from pcDNA3-VP7 by PCR using the VP7Sc primers (Table 4.2). To facilitate directional cloning, the sequences of the primers were designed to contain an *Nhe* I restriction site at the 5'end immediately upstream of the signal sequence cleavage site of the VP7 protein (nt.192, Fig. 4.1) and a *Bam*H I restriction site at the 3'end. The PCR product was then digested with *Nhe* I and *Bam*H I and purified. The pTPA-VP7Sc was constructed by ligation of the 846 bp, *Nhe* I-*Bam*H I VP7Sc-fragment into *Nhe* I-*Bam*H I cleaved pWRG7054. The pWRG7054 was a gift from Dr. Jim Fuller (Agracetus, Inc., Middleton, WI). This plasmid contained the expression cassette with hCMV-IA and a BGH polyA. In addition, this plasmid also contained the sequence encoding a mouse tissue plasminogen activator (TPA) signal peptide, located downstream of the hCMV-IA element and immediately upstream of the *Nhe*I restriction site. Therefore, ligation of *Nhe* I-*Bam*H I VP7Sc-fragment to *Nhe* I-*Bam*H I digested pWRG7054 generated a plasmid encoding a fusion protein of TPA signal peptide and VP7Sc.

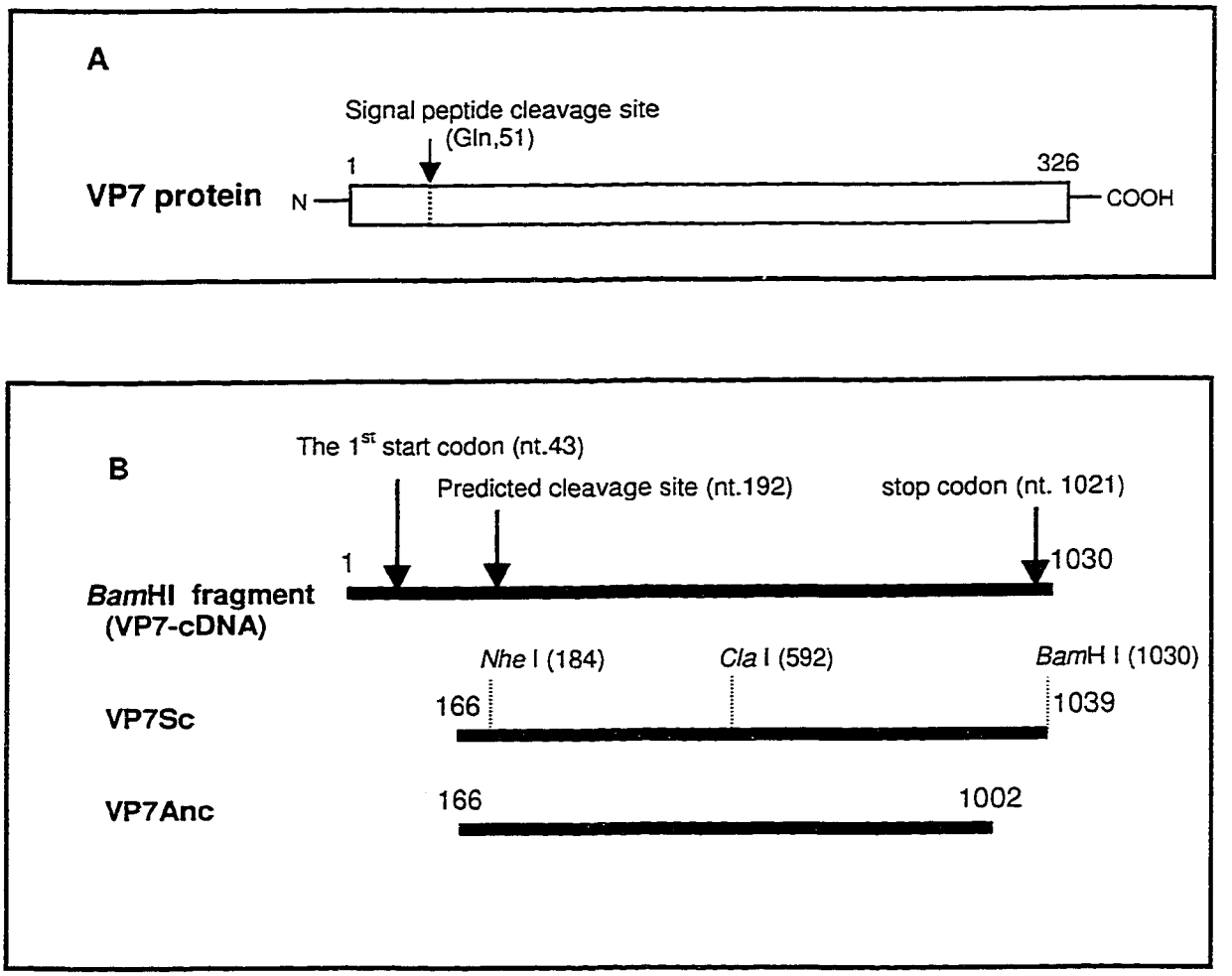


Figure 4.1 Diagram depicting features of the rotavirus VP7 protein and the VP7 fragments used in this study. (A) Feature of the native VP7 protein. Numbers represent positions of amino acids in the protein. (B) Diagram showing the PCR amplified VP7 fragments, VP7Sc and VP7Anc, in relation with the original cDNA. Numbers represent nucleotide positions (nt.).

Table 4.2 Primers used to generate PCR amplified cDNAs in this study.

Fragment		Primer Sequences	Underlined sequence
VP7Sc	S	5'-GTGATCTTGGCCACCATA*GCTAGC*GAGCAAAACTATGGAGTAAATTTGCCAATTACAGG-3'	*Nhe* I
	A	5'-CTAATC*GC*GGATCCACCTACACTCTATAGTAGAACG-3'	*Bam*H I
VP7Anc	S	5'-GTGATCTTGGCCACCATA*GCTAGC*GAGCAAAACTATGGAGTAAATTTGCCAATTACAGG-3'	*Nhe* I
	A	5'- CGACGA*GTTAAC*CGATGT*C*GATCTTTTGGACATTGTCTG-3'	*Hpa* I
gD-Nhe	S	5'-GTTGCGG*C*GAGC*GCTAGC*ACACCCGCGCCG-3'	*Nhe* I
	A	5'-TCAC*CCGCGG*CGCGGGTGT*GCTAGC*GCTC*G*CCGCAACGGCGAGCAGCGCGC-3'	*Sac* II
C3d	S	5'-CCTGATC*GTTAAC*CCCG*GACCCG*GTGGGGAACAGAACATGATTGG-3'	*Hpa* I
	A	5'-GCAGGCGAAA*CCCGGG*TGC*CTA*GCTA*GCGCT*GGGGAGGTGGAAGGACACATCC-3'	*Sma* I-Stop codon-*Eco*47 III
gD7Sc	S	5'-GTTTCG*GTTAAC*GCGCAAAACTAT-3'	*Hpa* I
	A	5'-CTAGG*GATATC*GAGGATCCACC-3'	*Eco*R V

Note : S - Sense strand
A - Antisense strand
Sequence in Italics shows nucleotides that were altered from the original DNA template for cloning purposes.
Dotted underlined sequence was the sequence changed from the original *Bgl* II site to facilitate downstream cloning.

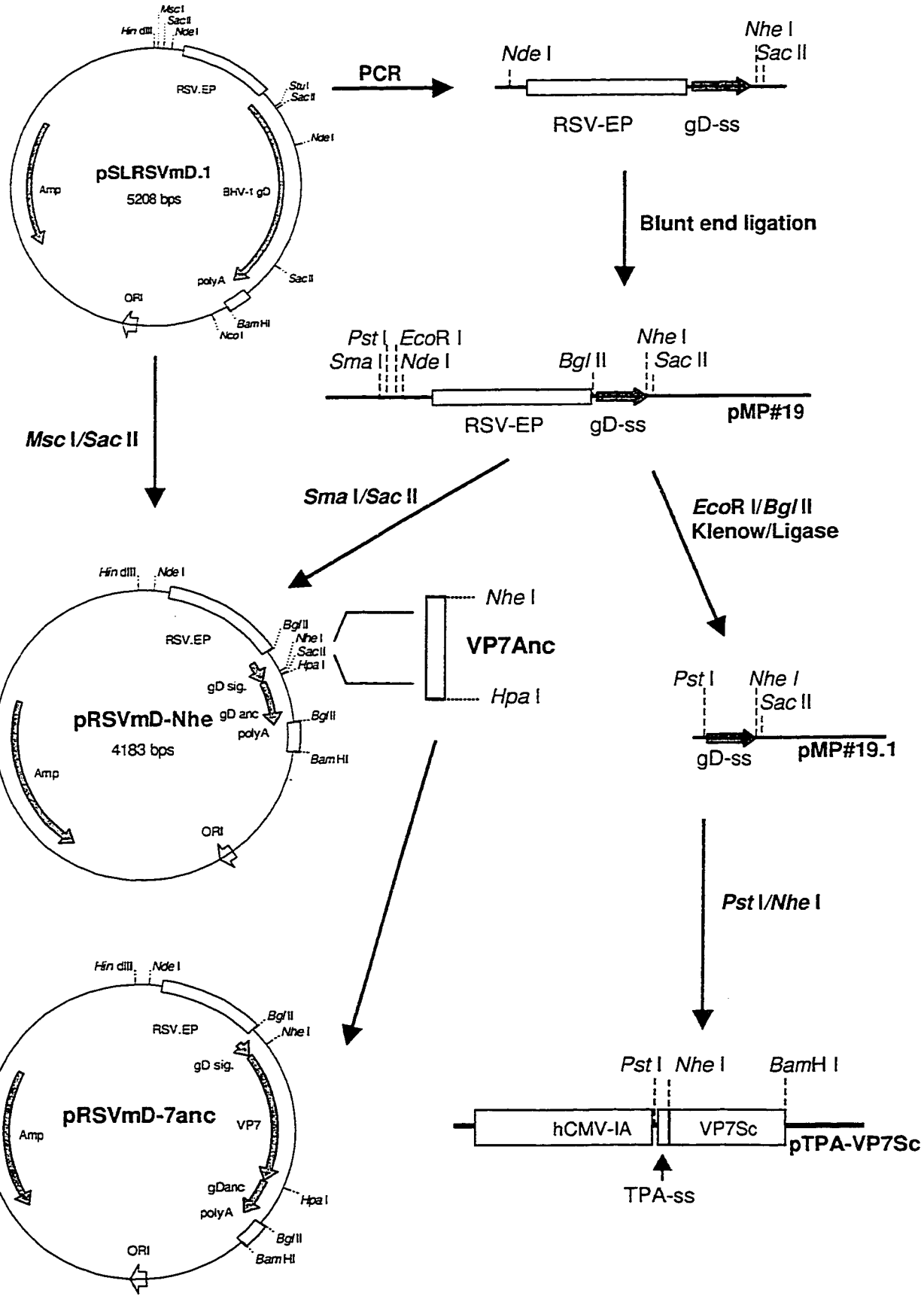
To create a plasmid encoding truncated VP7 (VP7t), pTPA-VP7Sc was digested with *Cla* I that recognized nucleotide 592 of the VP7Sc fragment (Fig. 4.1, B). The digested product was then end filled with Klenow and then religated. This gave a plasmid encoding truncated VP7 containing a frame shift mutation and stop codon located 15 amino acids downstream of the original *Cla* I site.

The plasmid pRSVmd.1 and pMP#19 were gifts from Dr. Paul J. Lewis. The pRSVmD.1 was an expression plasmid containing the authentic gD gene with the additional of a linker sequence encoding a hinge region (proline-glycine-proline-glycine) in the BHV-1 glycoprotein D(gD) immediately upstream of the transmembrane anchor sequence. To construct the plasmid expressing secretory VP7 that utilized the gD signal peptide (pgD-VP7Sc) and/or transmembrane anchor protein (pgD-VP7Anc, pTPA-VP7Anc), the modified version of the pRSVmd.1, was made (Fig. 4.2). Briefly, the sequence expanded from the RSV long terminal repeat early promoter region (RSV-EP) to the sequence downstream of the signal peptide cleavage site (*Nde* I to the first downstream *Sac* II site) was PCR-amplified using the gD-Nhe primers (Table 4.2). Within this product, the *Stu* I restriction site located immediately downstream of the gD signal sequence cleavage site of the pSLRSVmD.1 was changed to *Nhe* I. In addition, there were some changes of the nucleotides in the PCR primers around the cleavage site to ensure the optimal signal peptide cleavage as previously described (von Heijne, 1986). The PCR product was blunt end ligated to the *Eco*R V digested pBluescript® II KS (Stratagene, La Jolla, CA) to create pMP#19. The pMP#19 was digested with *Eco*R I and *Bgl* II, end filled with Klenow and then religated to remove the RSV-EP sequence and to allow the use of an upstream *Pst* I site. The *Pst* I-*Nhe* I fragment, i.e. gD-signal sequence, obtained from clone, pMP#19.1, was then ligated to the *Pst* I-*Nhe* I digested pTPA-VP7Sc to generate a plasmid containig the VP7 gene downstream of the gD signal sequence, pgDVP7Sc (Fig. 4.2).

Figure 4.2 Schematic diagram depicting the strategy used for cloning of pgD-VP7Sc, pTPA-VP7Anc and pgD-VP7Anc. (see text for detail)

The *Stu* I restriction sequence in the pSLRSVmD.1 was changed to *Nhe* I restriction sequence by PCR. The PCR fragment was blunt-end ligated to the *Eco*R V digested pBluescript® KS to generate pMP#19. Removal of the *Eco*R I-*Bgl* II fragment from pMP #19 created pMP #19.1. Replacing the TPA signal sequence (TPA-ss) in the pTPA-VP7Sc with a *Pst* I-*Nhe* I fragment containing a sequence encoded for gD signal peptide (gD-ss) creating pgD-VP7Sc.

Ligation of a *Sma* I-*Sac* II fragment from the pMP#19 to the backbone fragment of *Msc* I-*Sac* II digested pSLRSVmD.1generated pRSVmD-Nhe. Ligation of the *Nhe* I-*Hpa* I digested PCR product, VP7Anc, to an *Nhe* I-*Hpa* I cleaved pRSVmD-Nhe created the pRSVmD-7Anc. The *Nhe* I-*Bgl* II fragment from the pRSVmD-7Anc containing the sequence encoded for VP7Anc-gD transmembrane anchor protein was then used to insert into the *Nhe* I-*Bam*H I digested pTPA-VP7Sc or pgD-VP7Sc to create pTPA-VP7Anc and pgD-VP7Anc respectively.


Msc I
Sac II
Hin dIII
Nde I
RSV.EP
Stu I
Sac II
Nde I
pSLRSVmD.1
5208 bps
Amp
BHV-1 gD
polyA
Sac II
ORI
BamHI
NcoI
PCR
Nde I
Nhe I
Sac II
RSV-EP
gD-ss
Blunt end ligation
Pst I
EcoR I
Sma I
Nde I
Bgl II
Nhe I
Sac II
RSV-EP
gD-ss
pMP#19
Msc I/Sac II
Sma I/Sac II
EcoR I/Bgl II
Klenow/Ligase
Hin dIII
Nde I
RSV.EP
Bgl II
Nhe I
Sac II
Hpa I
gD sig.
gD anc
polyA
pRSVmD-Nhe
4183 bps
Amp
ORI
Bgl II
BamHI
Nhe I
VP7Anc
Hpa I
Pst I
Nhe I
Sac II
gD-ss
pMP#19.1
Pst I/Nhe I
Hin dIII
Nde I
RSV.EP
Bgl II
Nhe I
gD sig.
pRSVmD-7anc
VP7
Amp
gDanc
polyA
Hpa I
ORI
Bgl II
BamHI
Pst I
Nhe I
BamH I
hCMV-IA
VP7Sc
pTPA-VP7Sc
TPA-ss

The plasmid pMP#19 was digested with *Sma* I and *Sac* II. The 735 bp, *Sma* I-*Sac* II fragment was then purified and ligated to the backbone fragment of *Msc* I-*Sac* II digested pSLRSVmD.1 to create plasmid pRSVmD.1-Nhe (Fig. 4.2). The fragment containing VP7-cDNA from nucleotide 166 to 1002 was amplified by PCR using VP7Anc primers. To facilitate directional cloning, the primer sequences were designed to contain the *Nhe* I and *Hpa* I restriction sequences at the 5' and 3' end, respectively. In addition, the original *Bgl* II restriction site, originally existing in the VP7 gene, was mutated to facilitate the downstream cloning process (Table 4.2). The PCR product was blunt end ligated to the *Eco*R V digested pBluescript® II KS. The *Nhe* I-*Hpa* I fragment was then isolated, purified and ligated into the *Nhe* I-*Hpa* I cleaved pRSVmD.1-Nhe (Fig. 4.2). This generated a plasmid, pRSVmD-7Anc, which contained the nucleotide sequence encoding VP7Anc in conjunction with a hinge region and the transmembrane anchor protein of gD (*Nhe* I-*Bgl* II fragment). The *Nhe* I-*Bgl* II fragment was then isolated, purified and inserted into the backbone fragment of *Nhe* I-*Bam*H I digested pTPA-VP7Sc and pgD-VP7Sc to replace the secretory VP7 gene. This generated pTPA-VP7Anc and pgD-VP7Anc respectively.

The plasmid containing a cDNA of mouse complement component 3 (pMLC3/4) was purchased from the American Type Culture Collection (ATCC, Rockville, MD). The fragment containing the nucleotide sequence encoding C3d was amplified by PCR using the C3d primers (Table 4.2). The PCR product was designed to contain an *Hpa* I restriction site and a linker sequence encoding (proline-glycine-proline-glycine) at the 5'end, and an *Eco*47 III restriction site followed by a stop codon and a *Sma* I restriction site at the 3'end (Fig. 4.3). The C3d-PCR fragment was digested with *Hpa* I and *Sma* I, purified and ligated to *Eco*R V-*Sma* I digested pTPA- VP7Anc to give pVP7(C3d). Ligation of the *Hpa* I-*Sma* I C3d fragment with *Eco*47 III-*Sma* I cleaved pVP7(C3d) gave pVP7$(C3d)_2$, (Fig. 4.3).

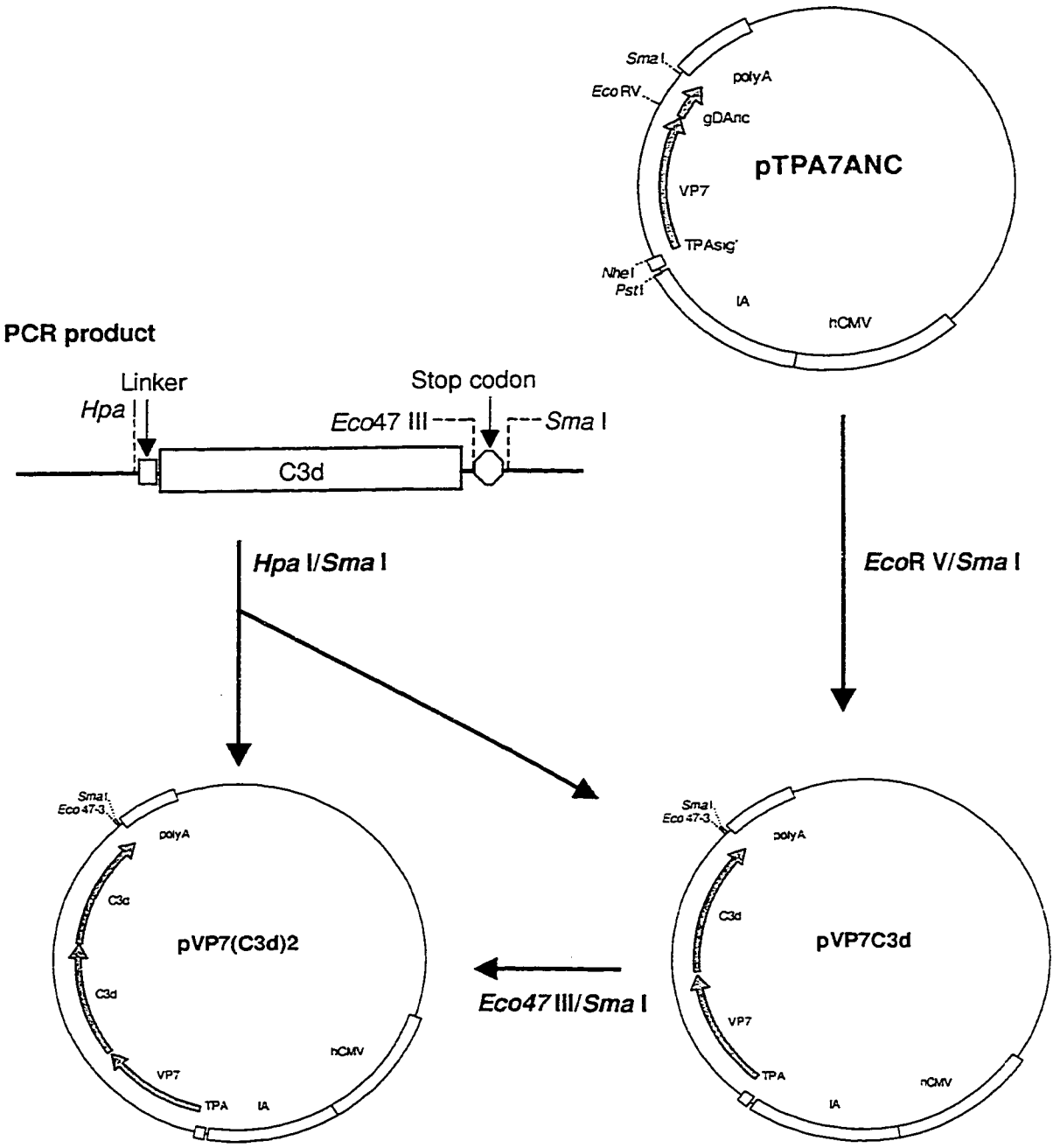


Figure 4.3 Schematic diagram depicting the strategy used for cloning of pVP7(C3d) and pVP7(C3d)$_2$, (see text).

The plasmid pSLIAmDs expressing truncated gD from the hCMV-IA was a gift from Dr. Ralph Braun. This plasmid contained the nucleotide sequence encoding truncated gD (tgD) following by a linker (proline-glycine-proline-glycine) immediately upstream of the *Eco*R V restriction site prior to a stop codon. The BRV-VP7 cDNA was amplified from a pTPA-VP7Sc by PCR using the gD7Sc primers (Table 4.2). The PCR product was then digested with *Hpa* I and *Eco*R V, purified and ligated to the *Eco*R V cleaved pSLIAmDs. This generated the plasmid, pSLIAgD-7Sc, encoding truncated gD-linker-VP7Sc chimeric protein. The pSLIAgD-7t encoding gD-linker-VP7t chimeric protein was generated by introducing a frame shift mutation at the *Cla* I site as described above.

All newly generated plasmids were confirmed by restriction analysis. In addition, transient protein expression was used to confirm protein expression by the plasmids prior to immunization studies.

In addition to the above plasmids, pSLIAtgD(C3d) and pSLIAtgD$(C3d)_2$ were constructed and *in vitro* characterized by Dr. Ralph Braun. The cloning protocol is briefly described below.

First, PCR primers were designed to amplify the C3d fragment from pVP7(C3d). The primer sequences were GCGCTCTAGACCCGGACCCGGTGGG for the 5' primer and GGCGTCTAGACTAGCTAGCGCTGGGGAGG for the 3' primer. The DNA fragment generated from the PCR reaction had *Xba* I sites at both ends and the 3' end had a *Nhe* I site, followed by a stop codon immediately upstream of the *Xba* I site. This design allowed fragments digested with *Xba* I to be inserted into an *Nhe* I site such that the original *Nhe* I site was destroyed and the internal *Nhe* I site in the PCR product was unique. Insertions of this type produced an in frame C3d addition with a stop codon. Subsequent insertions of a *Xba* I-*Nhe* I digested PCR product, which when inserted into the *Nhe* I site of the C3d segment added an additional copy of C3d in frame, but without

a stop codon. Each C3d protein segment had a proline-glycine-proline-glycine-glycine linker sequence at the 5' end.

To insert the first C3d fragment, the PCR product was digested with *Xba* I and ligated into a calf intestinal phosphatase (CIP) treated *Nhe* I site located at the 3' end of the tgD gene present on the pSLIAtgD plasmid. DNA minipreps from positive clones were digested by *Bgl* II with expected sizes being 5.2 kb fragment from the backbone and 1.3 kb fragment from a tgD gene without an insert, or a 2.2 kb fragment from a gene containing an insert. Of the 24 clones examined, only one negative clone was found, with the remainder having a single *Bgl* II site. Because the *Bgl* II sites are outside of the region of insertion, clones with only one *Bgl* II site should not occur under the designed experiment. A second investigator, using separate reagents, found all 36 clones had a single *Bgl* II site, with no patterns for either the negative or positive clones. Several clones were then examined further and restriction analysis suggested the single *Bgl* II plasmid was a result of a positive clone (i.e. C3d insertion) which had rearranged and removed a 2 kb fragment corresponding to most of the tgD gene and a large portion of the promoter. When ligation mixtures were transformed into SURE™ cells (Stratagene), which cannot rearrange DNA, no positives were found; suggesting that there was some toxicity associated with the positive clone and survival was dependent on the rearrangement of the plasmid.

Expression of genes from a eucaryotic promoter in bacteria has been documented and may account for the inability to isolate a positive tgD-C3d construct that may be toxic to bacteria. To overcome this problem an intron was inserted into the 3' end of the tgD gene. The intron contains several stop codons that would be read in bacteria, but in eucaryotic cells would be spliced out with the maturation of the mRNA. This scheme should therefore cause tgD-C3d chimeras to be expressed as full-length proteins in eucaryotic cells, but as tgD in bacteria. The intron was designed as a 140

bp PCR product from the intron present in the pCI-NEO plasmid (BIO-RAD Laboratories (Canada) Ltd., Mississauga, Ontario) using a 5' primer CGACTCTAGACAGGTAAGTAT CAAGG and a 3' primer CGACGCTAGCCACCTGTGGAGAGAAAG. The product had a 5' *Xba* I site and a 3' *Nhe* I site and when digested by *Xba* I-*Nhe* I could be inserted into a *Nhe* I site such that the 5' *Nhe* I site would be destroyed and the 3' site maintained (and thus usable for insertion of the C3d segment). As a result of intron insertion, and then removal, the final protein product converted the leucine-alanine-serine-stop sequence of tgD to a glutamine-valine-alanine-serine-stop. The insertion of a C3d fragment produced a glutamine-valine-alanine-arginine-linker amino acid section.

The PCR product from the intron PCR was digested with *Xba* I-*Nhe* I and inserted into a *Nhe* I digested, CIP treated pSLIAtgD. The positive clone (pSLIAtgDin) was then used for insertion of C3d segments as described above. Both single and double copy inserts of C3d were generated and confirmed by the expression of the desired protein product. Accurate function of the intron was presumed because of the ability to generate positive plasmids, and also the expression of proteins of expected size, which would not occur if splicing was aberrant because of stop codons in the alternate reading frames of C3d.

4.3 Cell lines and tissue culture reagents

All media and media supplements were purchased from GIBCO/BRL (Life Technologies, Burlington, Ontario) or Sigma Chemical Co. (St.Louis, MO), unless otherwise indicated. All tissue culture plasticware was purchased from Corning Inc. (Corning, N.Y.) or Costar Corporation. (Cambridge, MA).

MA-104 cells (Fetal green monkey kidney cells), CV-1 cells (African green monkey kidney cells, ATCC CCL70), NCTC clone 929 strain L (mouse connective tissue, ATCC-CCL1) and CR001 (primary bovine fibroblast, VIDO) were grown in Eagle's minimal essential medium, (MEM), (GIBCO/BRL) supplemented with 10% fetal bovine serum, (FBS), (GIBCO/BRL) and 50 μg/ml of gentamicin sulfate (Sigma Chemical Co.). COS-7 (SV40 transformed African green monkey kidney cells, ATCC CRL1650) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (GIBCO/BRL).

The SV40-T antigen transformed C57Bl/6 mouse embryo fibroblast cell line, B6/wt19 (Tevethia *et al.*, 1980), was a gift from Dr. S. Tevethia (The Pennsylvania State University, Hershey, Pennsylvania). B6/wt19 cells were grown in DMEM supplemented with 5% FBS (GIBCO/BRL), 0.075% $NaHCO_3$, 20 mM HEPES (N-2 hydroxyethyl piperazine -N'-2-ethanesulfonic acid) buffer (GIBCO/BRL), 2mM L-glutamine (GIBCO/BRL), 50 μM β-mercaptoethanol (Sigma Chemical Co.) and 50 μg/ml of gentamicin sulfate (Sigma Chemical Co.), (B6 medium).

4.4 Transient transfection

Cells were plated into a six-well tissue culture plate (Costar) at approximately 1.5 - 2 x 10^5 cells/well. Once the cells reached 70-80% confluency, cells were transfected with complexes of DNA-liposome in an Opti-MEM® reduced serum medium (GIBCO/BRL) according to the protocol insert. Briefly for each well, 2 μg of plasmid DNA diluted in 100 μl of Opti-MEM® was combined with 10 μg of Lipofectamine™ (GIBCO/BRL) in 100 μl of Opti-MEM®. The solution was incubated at RT for 30-45 min to allow formation of DNA-liposome complexes. After the incubation was completed,

800 µl of warm Opti-MEM® was added into the DNA-liposomes solution. Cell monolayers were rinsed once with warm Opti-MEM® prior to transfection. DNA-liposome solution was added to the monolayer in each well and incubated for 5-6 hr in a 37 °C, 5% CO_2 incubator. The solution was then replaced with normal growth media (DMEM, or MEM, supplemented with 10% FBS). Cell monolayers were further incubated for 18 hr, then washed once with warm methionine-free MEM (ICN Biomedicals Inc., Aurora, OH). One millilitre of methionine-free MEM (ICN Biomedicals Inc.) containing 3% dialysed FBS (GIBCO/BRL), 200 mM glutamine (GIBCO/BRL), and 50 µCi/ml of [^{35}S]-L-Methionine (Tran [^{35}S]-label™, ICN Pharmaceuticals Inc., St. Laurent, Quebec) was added to the monolayers. Labelled samples were harvested for detection of protein by an immunoprecipitation assay after a 24-hour labelling period.

4.5 Radioimmunoprecipitation assay

To assess the presence of radiolabelled protein in the supernatant, the media from labelled cultures were collected and centrifuged (16,000x g) in 1.5 ml eppendorf tubes for 5 min to remove cell debris. Supernatants were collected to a clean tube and either used immediately or stored at -20 °C until needed. Immunoprecipitation of the BRV protein was carried out by mixing 800 µl of [^{35}S]-labelled media with 200 µl of 5x binding buffer [750 mM NaCl, 200 mM Tris-HCl (pH 7.6), 10 mM EDTA (pH 8.0), 0.5% NP-40 and 5 mM phenylmethylsulfonyl fluoride (PMSF)] and 2 µl of rabbit anti-BRV antiserum. For detection of gD protein, samples were incubated with 2 µl of 1.5:10 dilution of anti-gD pooled monoclonal antibodies (9D6, 3D9, 10C2, 2C8, PB136, 3E7, 3C1 and 4C1) instead of anti-BRV antiserum. Samples were incubated at RT for 1 hr. After incubation was complete, 100 µl of 100 mg/ml protein A Sepharose CL-4B

(Pharmacia), in RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1% sodium deoxycholate, 1% TritonX-100, 0.5% sodium dodecyl sulfate (SDS)] was added. Samples were further incubated at 4 °C for at least 3 hr on an Adams nutator (Model 1105, Clay Adams, Parssipany, NJ).

To assess the presence of radiolabelled protein in the cells, the monolayer were washed once with 1 ml of phosphate buffered saline (PBS) after removal of the supernatant. Cells were scraped off and the cell suspension was collected into an eppendorf tube. Samples were centrifuged at 16,000x g for 2 min and the supernatants were discarded. Cell pellets from each transfected well were resuspended in 100 µl of ice-chilled dissociation buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP-40, 0.5% TritonX-100]. Samples were incubated on ice for 10 min and centrifuged at 16,000x g for 5 min. The supernatants, referred to as cell lysates were transferred to clean tubes and either used immediately or stored at -20 °C until needed. Immunoprecipitation for the BRV protein from the cell lysates was carried out by mixing 100 µl of the [^{35}S]- labelled cell lysate with 50 µl of cold cell lysate, 3 µl of rabbit anti-BRV antiserum and 147 µl of RIPA buffer. For detection of gD protein, the samples were incubated with 3 µl of a 1:30 dilution of pooled anti-gD monoclonal antibodies instead of the anti-BRV antiserum. The samples were incubated for 1 hr at RT, then 400 µl of RIPA buffer and 100 µl of 100 mg/ml protein A Sepharose CL-4B (equilibrated in RIPA buffer) were added into each reaction. Samples were further incubated at 4 °C for at least 3 hr on a nutator.

For detection of surface antigen, media was removed from the transfected monolayer after a 24-hour labelling period. Monolayers were washed once with PBS, then 500 µl of a 1:100 dilution of rabbit anti-BRV in PBS was added into each well. Cells were incubated with antibody at 37 °C for 1 hr in a 5% CO_2 incubator. The

monolayers were washed gently 3 times with PBS to remove excess antibodies, 500 µl of dissociation buffer was added, cells were scraped off and collected to an eppendorf tube. The cell suspension was incubated on ice for 10 min followed by centrifugation at 16,000x g for 5 min. The supernatant was collected to a clean tube. The immunoprecipitation reaction was carried out by mixing 500 µl of labelled sample with 200 µl of RIPA buffer and 100 µl of 100 mg/ml protein A Sepharose CL-4B (equilibrated in RIPA buffer). Samples were further incubated at 4 °C for at least 3 hr on a nutator.

After incubation, the immunoprecipitates were harvested by centrifugation at 16,000x g for 30 seconds. The supernatants were discarded and pellets were washed with 800 µl of RIPA buffer at least 3 times. Following the final wash, 30 µl of 1x reducing SDS-polyacrylamide (SDS-PAGE) loading buffer [500 mM Tris-HCl (pH 6.8), 10% glycerol, 10% SDS, 5% β-mercaptoethatnol, 0.05% bromophenol blue] was added to each samples. Samples were boiled for 5 min prior to loading on SDS-PAGE gels. Electrophoresis was carried out at 60 volts for overnight. Gels were fixed (40% methanol, 10% acetic acid) for 15 min followed by a 30 min treatment with a fluorographic agent (Amplify™, Amersham, Oakville, Ontario). Gels were dried under vacuum at 80 °C for 2 hr (Model 583 gel dryer, BIO-RAD) and exposed to Kodak X-OMAT™ AR diagnostic film at -70 °C.

In some experiments, MA104 cell monolayers were infected with BRV at a multiplicity of infection (m.o.i.) of 10. Cells were incubated with virus for 1 hr and then further incubated at 37 °C for 8 hr in a 5% CO_2 incubator. Infected cell monolayers were labelled with 50 µCi/ml of [^{35}S]-L-Methionine in methionine-free media at 37 °C for 24 hr in a 5% CO_2 incubator. Cell lysates were then harvested using the protocol described above and used as a positive control in an immunoprecipretation assay.

4.6 Deglycosylation of glycoproteins

Radiolabelled samples were immunoprecipitated as described above. After the final wash, proteins were eluted from protein A Sepharose beads by boiling with 75 µl of 0.5% SDS for 3 min. The samples were centrifuged for 30 seconds and supernatants were collected and used for deglycosylation studies. Deglycosylation with endoglycosidase H (Endo H) was carried out by mixing 25 µl of labelled sample with 50 µl of 125 mM sodium citrate (pH 5.5), 5 µl of 10 mM PMSF (in isopropanol), 5 µl of 20% β-mercaptoethanol and 15 µl of Endo H (Boehringer Mannheim, Laval, Quebec). N-Glycosidase F (PNGase F) treatment was carried out by mixing 25 µl of sample with 40 µl of 550 mM sodium phosphate (pH 8.5), 5 µl of 20% β-mercaptoethanol, 10 µl of 10% NP-40, 5 µl of 500 mM EDTA and 15 µl of PNGase F (Boehringer Mannheim). Control reactions containing 25 µl of the samples mixed with reagents used for PNGase F treatment excluding the enzyme, were also included. All reactions were incubated at 37 °C for 18 hr. Proteins were precipitated by the addition of 1 ml of cold acetone and centrifugation at 16,000x g for 15 min. Supernatants were discarded. Pellets were air-dried in a fume hood, resuspended with 40 µl of 1x reducing SDS-PAGE loading buffer and loaded on an SDS-PAGE gel (4% stacking, 12% separating). Electrophoresis was carried out at 60 volts overnight followed by gel treatment as described above.

4.7 Western blotting of BRV proteins

Antibodies recognizing individual BRV proteins were analysed by a western blot assay slightly modified from the previous protocol (Ijaz *et al.*, 1990). Two hundred micrograms of BRV was solubilized in reducing SDS-PAGE loading buffer and

separated on a 10% SDS-polyacrylamide gel using a one millimetre-thick 2-D comb (Mini-Protean II system, BIO-RAD). Proteins were transferred to a 0.45 μm nitrocellulose membrane (BIO-RAD) by electroblotting at 250 mA for 1 hr in a transfer buffer [20mM Tris-HCl (pH 8.3), 190 mM glycine, 20% methanol]. Non-specific reactions were blocked by soaking the nitrocellulose membrane in 3% skim milk in 100 mM Tris buffered saline (TBS) at RT for 1 hr. The membrane was washed 3 times with 0.05% Tween 20 in TBS (TTBS), 10 min for each wash with gentle agitation, and then cut into strips. Strips were incubated, overnight, with 600 μl of a 1:20 dilution of pooled antibodies in TTBS at RT on a nutator. Positive control antiserum, mouse anti-BRV, at 1:100 dilution was also included. Strips were washed 3 times with TTBS to remove excess antibodies and incubated with a 1:2,000 dilution of biotinylated horse anti-mouse immunoglobulin G (Vector Laboratories Inc., Burlingame, CA) in TTBS at RT for 1 hr. After 3 washes with TTBS, strips were incubated with a 1:1000 dilution of strepavidin-alkaline phosphatase conjugate, (SAP), (BIO/CAN Scientific, Mississauga, Ontario) in TTBS at RT for 1 hr and then washed. Colour development was done by addition of substrate solution, Sigma Fast^{R} BCIP/NBT (Sigma Chemical Co.). The reactions were stopped by extensive washing with tap water.

4.8 Viruses

Bovine rotavirus strain C486 was propagated in confluent MA-104 cells in the presence of 1 μg/ml of trypsin (Difco Laboratories, Detroit, MI) in the absence of FBS. Once extensive cytopathic effects were evident, cells and supernatants were harvested together and the cellular debris was removed by centrifugation (1000x g for 20 min). The clarified supernatant was used as the viral stock. The viral titres were determined by plaque assay. Alternatively, the supernatant was concentrated and partially purified

by centrifugation through a 40% sucrose cushion at 112,700x g (25,000 rpm) in a sw28 rotor (Beckman Instruments (Canada) Inc., Mississauga, Ontario) at 15 °C for 2.5 hr. The virus pellet was resuspended in sterile distilled water and the amount of viral protein was estimated by spectophotometry using a formula; $183(A_{230})-75.8(A_{260}) \times 800 = \mu g/ml$ where A_{230}, and A_{260} are the optical density values at 230 and 260, respectively (Ijaz *et al.*, 1987).

Recombinant vaccinia viruses expressing BRV (C486)-VP4 protein (rVV-VP4) previously constructed and characterized by Zhao (Zhao, 1992), were propagated in CV-1 cells. Infected cells were collected by centrifugation (1000x g for 20 min), resuspended in a minimum volume and subjected to 3 freeze-thaw cycles to release the viral particles. The viral suspension was centrifuged at 1000x g for 20 min and the supernatant was collected and referred as the clarified crude stock. The viral titres were determined by plaque assay. Viruses were kept at -70 °C until needed.

4.9 Plaque assay

4.9.1 Rotavirus plaque assay

Plaque assays for bovine rotavirus titration were performed by the method described previously (Aha & Sabara, 1990). Briefly, 12-well tissue culture plates (Costar) containing confluent MA-104 cells were washed twice with MEM. Cell monolayers were inoculated with 200 µl of serial 10-fold dilutions of BRV diluted in MEM. After 1 hr incubation at 37 °C in a CO_2 incubator, cells were washed once with MEM and overlaid with 1 ml of medium containing MEM, 2% Sephadex G-75 beads (Pharmacia), 20 mM L-glutamine (GIBCO/BRL) and 25 µg/ml of pancreatin (GIBCO/BRL). The plates were incubated at 37 °C in a CO_2 incubator for 48 hr. The

overlay medium was removed and the plates were stained (0.5% crystal violet, 80% methanol in PBS) at RT for 20 min. The stained monolayers were gently washed with tap water, air-dried, and the plaque numbers were enumerated.

4.9.2 Vaccinia virus plaque assay

Six-well tissue culture plates (Costar) containing 60-80% confluent CV-1 cells were infected with 200 µl of serial 10-fold dilutions of clarified crude stock of rVV-VP4 in MEM supplemented with 10% FBS (GIBCO/BRL). Virus was incubated with the cell monolayers at 37 °C in a CO_2 incubator for 1 hr. After incubation, viruses were removed, cells were washed once and overlaid with 5 ml of 0.5% agarose (Pharmacia) in MEM supplemented with 5% FBS (GIBCO/BRL). Plates were incubated for 48-72 hr and plaque numbers were enumerated.

4.10 *In vivo* procedures

4.10.1 Mice

Six to eight weeks old (rotavirus-free) C57Bl/6, C3H/HeNCrlBR and BALB/c mice were purchased from Charles River Laboratories (St.Constante, Quebec). Prior to immunization, mice were bled and confirmed as seronegative for anti-BRV (C486) antibodies by ELISA. Animals were maintained in isolation units (Rotavirus-free condition) throughout the study or until they were boosted with the virus.

4.10.2 Immunization and viral inoculation protocols

For intramuscular immunization (i.m.), plasmids were injected into both quadriceps muscles (50 μl for each side). For intradermal immunization (i.d.), mice were intradermally injected into 2 separated sites either at the tail base or a newly shaved skin on the back of anaesthetized mouse. For immunization with a gene gun, plasmids were co-precipitated with 1.6 μm gold particles (BIO-RAD) at a 2:1 ratio of DNA:gold particle following the Helios™ gene gun system manufacturer's instructions (BIO-RAD). DNA doses and delivery conditions were previously optimized by Dr. Ralph Braun (personal communication). Mice were anaesthetized and shaved at the abdominal area. Plasmids (1 μg/dose) were delivered to the epidermal layer by the gene gun using pressurized helium at 180 pound per inch^2.

For viral inoculation, each mouse was intraperitoneally, or in some experiment intradermally, injected with 50 μg of partially purified BRV or 10^6 plaque forming units (p.f.u.) of rVV-VP4 diluted in PBS.

4.11 Assessment of immune responses

4.11.1 ELISA

Detection of BRV-specific antibodies

The protocol for ELISA was modified from one previously described (Ijaz *et al.*, 1990). Briefly, 96-well microtiter plates (Immulon 2, Dynatech Laboratories, Alexandria, VA) were coated with 0.5 μg/well of partially purified BRV (C486) diluted in 50 mM carbonate bicarbonate buffer, pH 9.6 (coating buffer) at 4 °C, overnight. After

incubation, the plates were washed with distilled water 4 times and then blocked with 0.1% gelatin (Sigma Chemical Co.) in PBS, at RT for 1 hr. Plates were washed 4 times with distilled water before adding 100 µl of serum, serially 2-fold-diluted in 0.05%Tween 20 (Sigma Chemical Co.) in PBS (PBST) into each well. Plates were incubated at RT for 2 hr and then washed 4 times with PBST. One hundred microlitres of biotinylated goat anti-mouse IgG (Zymed Laboratories Inc., San Francisco, CA), diluted 1:5,000 in PBST, was added into each well and incubated at RT for 1 hr. The plates were washed and then incubated with strepavidin-horseradish peroxidase conjugate (Zymed Laboratories Inc.), diluted 1:2,000 in PBST, at RT for 1 hr. Wells were washed before addition of 100 µl of the substrate; 0.15 mg/ml of 2,2'-azino-di-(3-ethyl-benzthiazoline sulphonate), (ABTS, Boehringer Mannheim) in 0.055 M citric acid, pH 4.0 (Sigma Chemical Co.) and incubated at RT for 20 min. The reaction was terminated by the addition of 100 µl of 10% sodium dodecyl sulfate (SDS) (Sigma Chemical Co.). The optical density (OD) values were determined at 405 nm by an ELISA-reader (BIO-RAD).

Detection of gD-specific antibodies

Immunolon 2 (Dynatech Laboratories) plates were coated, overnight at 4 °C, with purified recombinant tgD (VIDO) in coating buffer at a concentration of 0.05 µg/well. Plates were washed 4 times with PBST prior to addition of 100 µl of serial 4-fold dilutions of serum diluted in 0.5% gelatin PBST (PBST-g). After a 2-hour incubation at RT, plates were washed and 100 µl of biotinylated goat anti-mouse IgG (Zymed Laboratories Inc.) diluted 1:10,000 in PBST-g were added to each wells. After 1 hr of incubation, plates were washed and 100 µl of SAP (BIO/CAN Scientific) diluted 1:5,000 in PBST-g were added into each wells and further incubated for an hour. After 4 washes, colour was developed by the addition of 100 µl of 0.01 M p-nitrophenyl

phosphate (Sigma Chemical Co.) in substrate buffer (0.104 M Diethanolamine, 0.5 mM $MgCl_2$, (Sigma Chemical Co.) to each well. The optical density (OD) values were determined at 405 nm by an ELISA-reader (BIO-RAD).

4.11.2 Isolation of lymphocytes and cell culture condition

Spleens and draining lymph nodes were aseptically removed from euthanized mice. Single-cell suspensions were obtained by mincing and then teasing the tissue through a nylon mesh. Red blood cells were lysed by exposure of the cell suspension to 0.75% ammonium chloride in TRIS buffer for 1 minute. Cells were washed twice with MEM (GIBCO/BRL) and resuspended in RPMI 1640 medium (Sigma Chemical Co.) supplemented with 10% FBS (Sigma Chemical Co.), 2 mM L-glutamine (GIBCO/BRL), 100 μM non-essential amino acids (GIBCO/BRL), 1mM sodium pyruvate (GIBCO/BRL), 50 μM β-mercaptoethanol (Sigma Chemical Co.), 100 U/ml of penicillin and 100 μg/ml of streptomycin (Sigma Chemical Co.) (complete medium).

All of the following *in vitro* cell cultures were done at 37 °C, in a 5% CO_2 incubator.

4.11.3 Cytotoxicity assay

Pooled splenocytes, at a concentration of $6x10^6$ cells/ml/well, were cultured in 24-well plates (Costar) for 4 days in the presence of BRV at 0.3 m.o.i. After *in vitro* stimulation, viable lymphocytes were isolated with density separation medium, Lympholyte®-M (Cedarlane Laboratories Ltd., Hornby, Ontario), following the manufacturer's instructions. Ten thousand B6/wt19 target cells per well were labelled

with 1 μCi of $Na_2{}^{51}CrO_4$ (Amersham, Oakville, Ontario) in a 96-well round-bottomed plate by an overnight incubation. Cells were then washed twice with MEM and infected with BRV at 50 m.o.i in a 50 μl volume and incubated at 37 °C for 1 hr. Mock-infected cells were incubated with media. Cell monolayers were washed once with MEM and further incubated for 3 hr in 100 μl of complete medium. Effector cells in a 100 μl volume were added to triplicate wells of target cells at ratios of 100:1, 50:1 and 25:1. Effector cells were incubated with target cells for 4 hr. Supernatants from each well were harvested and measured for radioactivity (count per minute) using a gamma radiation counter (Gamma 5500, Beckman Instruments Inc., Fullerton, CA). Target cells incubated with complete medium only (spontaneous release) or 5% Triton X-100 (Sigma Chemical Co.) (total release) were included in the assay. Spontaneous release/total release ratios were less than 30%. Percent specific release was calculated using the formula; %specific lysis = [(experimental release - spontaneous release) / (total release - spontaneous release)] x 100.

4.11.4 ELISPOT for detection of cytokine secreting cells (CSCs)

Cells ($6x10^6$ cells/ml/well) were cultured in 24-well plates for 18-24 hr in the presence of partially purified BRV (5 μg/ml) for the pcDNA3-VP4 study (section 5.1.1). For the rest of the experiments, cells were cultured with lived BRV stock ($2x10^6$ p.f.u. /ml). For detection of gD specific CSCs, cells were incubated with purified gD protein (VIDO) at a final concentration of 0.4 μg/ml instead of BRV. After incubation was complete, cells were washed twice and resuspended to the appropriate concentrations in complete medium. Nitrocellulose-backed, 96-well microtiter plates (Millipore, Bedford, MA) were coated, overnight at 4 °C, with 2 μg/ml of purified anti-mouse

interleukin-4 (IL-4) (11B11) or 5 μg/ml of purified anti-mouse interferon-gamma (IFN-γ) (R4-6A2) (Pharmingen, San Diego, CA), diluted in coating buffer. Plates were washed 4 times with PBS containing 0.1% Tween 20 (0.1% PBST) followed by 4 washes with PBS. This washing protocol was used in all the following washing steps. Plates were incubated with 50 μl/well of complete medium at 37 °C for 1 hr or at RT for at least 2 hr to block non-specific reaction. Cells at appropriate concentrations were then added into triplicate wells. After an overnight incubation, cells were discarded and plates were quickly washed once with distilled water prior to PBST/PBS washes. One hundred microlitres of 2 μg/ml of biotinylated antibodies specific to mouse IL-4 (BVD6-24G2) or IFN-γ (XMG1.2) (Pharmingen) diluted in diluent buffer [1% bovine serum albumin (BSA, Sigma Chemical Co.) in 0.1% PBST] were added to each of the respective wells and incubated at RT for 2 hr. Plates were washed and 100 μl of SAP (BIO/CAN Scientific), diluted 1:1000 in diluent buffer, was added into each well. After 2 hr of incubation at RT, the plates were washed and 100 μl of the substrate reagent; Sigma Fast^{R} BCIP/NBT (Sigma Chemical Co.) was added to each well. Plates were incubated with the substrate reagent for 20-40 min at RT and then washed in distilled water in order to stop the enzymatic reaction. Plates were air-dried and spots were counted with the aid of an Olympus™ stereoscope.

4.11.5 ELISPOT for detection of rotavirus specific-antibody secreting cells (ASCs)

Freshly isolated cells were counted and resuspended in complete medium to the appropriate concentrations. Cells were added to triplicate wells of nitrocellulose plates (Millipore) which were previously coated with 5 μg per well of partially purified BRV, diluted in coating buffer, and blocked with complete medium (see above). Cells were

incubated for 6 hours or overnight at 37 °C and then washed off. One hundred microlitres of biotinylated horse anti-mouse IgG diluted 1:2000 (Vector Laboratories Inc., Burlingame, CA) or biotinylated goat anti-mouse IgG1, IgG2a (Caltag Laboratories, San Francisco, CA) or mouse IgM (Vector Laboratories Inc.), diluted 1:5,000 in diluent buffer, were added to each well. Antibodies were incubated at 4 °C overnight or at RT for 2 hr. Colour development was performed as described above.

4.11.6 Depletion of T-lymphocyte subpopulations

Splenocytes were cultured with 5 μg/ml of partially purified BRV using the same conditions as described for the ELISPOT assay for detecting of cytokine secreting cells. Cells were harvested and resuspended at $4x10^7$ cells/ml in 0.1% BSA in PBS and incubated with a 1:100 dilution of rat monoclonal antibodies (MAb) GK1.5 (anti-CD4) (Becton Dickinson, Bedford, MA, USA) and/or MAb 53-6.7 (anti-CD8) (Pharmingen). After 30 min incubation on ice with occasional mixing, cells were washed twice with 0.1%BSA in PBS to remove excess antibodies and then resuspended in 0.1% BSA in PBS. Positive cells were depleted from the cell suspensions by using immunomagnetic beads coupled with sheep anti-rat IgG (IMBs) (Dynal Inc., Oslo, Norway) at a 50:1 ratio (IMB : target cells). The final concentration of the cell/IMB mixture was adjusted to 3-$5x10^6$ cells/ml. This mixture was incubated at 4 °C for 1 hr on a rocking plate before the IMBs were removed with a magnetic particle concentrator (MPC-1) (Dynal Inc.), for 5 min. The separation process was repeated 3 times to ensure complete depletion. The depleted population was counted and resuspended to appropriate concentrations in complete medium and subjected to ELISPOT, for detection of the IFN-γ secreting cells.

Prior to, and after the depletion procedure, aliquots of cells were collected and the cell phenotype was analyzed by indirect immunocytometry. Briefly, cells were

stained with antibodies used in the depletion procedures (see above). The positive cells were detected by addition of an FITC-goat anti-rat IgG conjugate (Caltag Laboratories). Viable cells were analyzed with a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA), using the Lysis II program. Less than 4% of $CD4^+$ cells and less than 0.5% of $CD8^+$ cells were detected in the CD4 or CD8 depleted populations, respectively.

4.12 Bioassay for VP7-C3d chimeras

4.12.1 Isolation of ileal Peyer's Patch B cells of lambs

Cells (>95% $sIgM^+$, immature B-cells) were isolated from lymphoid follicles of the ileal Peyer's patch from new-born lambs by a protocol described by Griebel (1997). The isolated cells were resuspended in AIM V medium (GIBCO/BRL) supplemented with 2% FBS and 50 μM β-mercaptoethanol (Sigma Chemical Co.) referred as AIM V medium.

4.12.2 Binding assay for the VP7-C3d chimeras

Immature B-cells express higher level of C3d-cellular receptors (CD21) than mature cells (Dr. P. Griebel, personal communication). To assess the ability of the C3d molecule in VP7-C3d chimeric proteins to bind with CD21 on B-cells, approximately $2x10^6$ cells of ileal Peyer's patch cells resuspended in 50 μl of Ca^{++}, Mg^{++}-free PBS (PBSA) were incubated with 150 μl of supernatant harvested from COS-7 monolayers transfected with plasmids encoding secretory VP7, VP7(C3d) or $VP7(C3d)_2$. Ileal Peyer's patch cells were incubated with the supernatants containing the putative

chimeric proteins for 30 min at 4 °C in a 96-well tissue culture plate (Costar) and then washed 3 times with PBSA. To detect the presence of cell-bound chimeric proteins, in particular VP7 which was co-expressed with the C3d, 100 μl of a 1:100 dilution of rabbit anti-BRV in FACola [10 mM PBS (pH 7.2), 0.2% gelatin, 0.03% sodium azide] was added to each well and incubated for 30 min at 4 °C. Cells were washed 3 times with FACola followed by incubation with 100 μl of a 1:100 dilution of FITC conjugated goat anti-rabbit IgG (Caltag Laboratories) in FACola at 4 °C for 30 min. Viable cells were analyzed with a FACScan (Becton Dickinson Immunocytometry Systems), using the Lysis II program.

4.12.3 C3d bioassay

Interaction of B cells with C3d molecules has been proposed as a crucial co-stimulatory signal for B-cell survival and activation (Tew *et al.*, 1997). To assess if the VP7-C3d chimeric protein retained the biological activity of the C3d molecule in rescuing immature B-cells from single-signal induced cell death, ileal Peyer's patch B-cells, approximately $4x10^5$ in 100 μl of AIM V medium were plated into each well of a 96-well tissue culture plate along with 100 μl of AIM V media or 1:10 dilution, in AIM V medium, of the transfected supernatants containing the chimeric proteins in the presence or absence of an $F(ab')_2$ rabbit anti-sheep Ig (Cappel Laboratories, West Chester, PA) for 96 hr. Cell proliferation was measured by the addition of 0.4 μCi/well of [^{3}H]-thymidine (Amersham) into the culture during the last 6 hr of incubation. Incorporation of [^{3}H]-thymidine was assessed by harvesting cells onto glass fibre filter mats (Sakatron, Sterling, VA) and determining the radioactivity using a β-scintillation counter (Beckman). Data represents means of CPM from six replicates.

4.13 Statistical analysis

Statistical analysis of the data was performed using analysis programs in GraphPad Prism software, (GraphPad Software Inc., San Diego, CA).

5. RESULTS

5.1 Immune responses induced by plasmids encoding VP4 protein

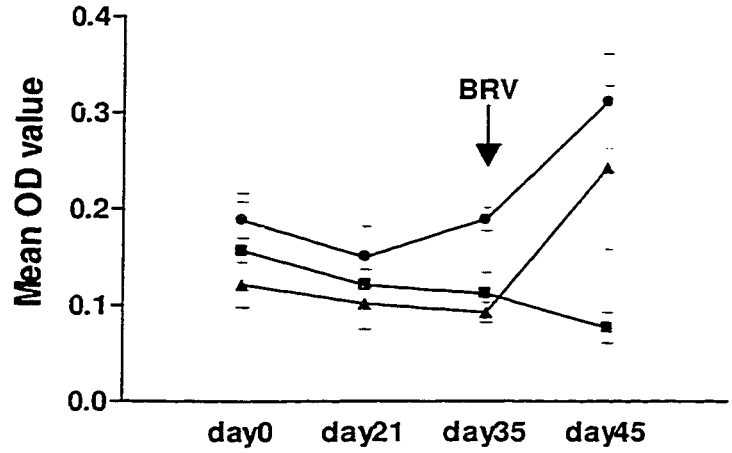
5.1.1 Immune responses induced by pcDNA3-VP4

To characterize the immune responses induced by a plasmid encoding the VP4 protein of BRV (pcDNA3-VP4), groups of 10 C57Bl/6 mice were intramuscularly immunized twice (day 0, day 21) with 200 μg of pcDNA3-VP4. Plasmids were resuspended in PBS in a total volume 100 μl and injected into both quadriceps muscles (50 μl per side). The control group was injected with an equal volume of PBS. At day 31, 5 mice from each group were sacrificed and splenocytes were collected for various immunological tests. The remaining animals were intraperitoneally inoculated with 50 μg of partially purified bovine rotavirus at day 35. In some experiments, mice were injected with rVV-VP4 (10^6 p.f.u. per mouse). The animals were then sacrificed for immunological assays, 10 days after viral inoculation (day 45).

Immunizations with the plasmid pcDNA3-VP4 did not induce detectable BRV-specific antibody responses in the serum nor in the splenocyte population. However, following BRV administration, the pcDNA3-VP4 immunized mice had a higher number of BRV-specific ASCs in the spleen than the group immunized with PBS (Fig.5.1.1).

To investigate the pattern of cytokine production induced following pcDNA3-VP4 immunization, the numbers of BRV-specific IFN-γ and IL-4 secreting cells in spleens

A.



B.

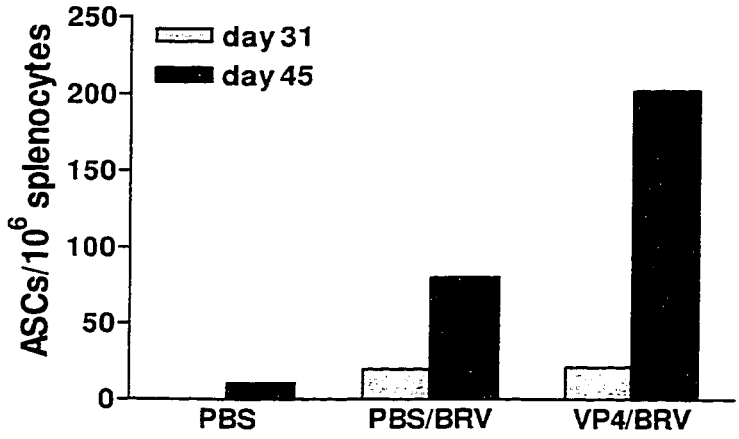


Figure 5.1.1 BRV-specific antibody responses in mice immunized with pcDNA3-VP4.

A. Mice were intramuscularly immunized with 200 μg of pcDNA3-VP4 (day 0, day 21) and later inoculated with 50 μg of BRV at day 35 (●). The control groups were injected with PBS and then boosted with PBS (■) or BRV (▲) on day 35. Data represent BRV-specific serum antibody level at 1:50 serum dilution (mean OD ± SD of 5 mice) measured by ELISA.

B. BRV-specific IgG-ASCs in spleens before (day 31) and after BRV boost (day 45) determined by ELISPOT assay. Data represent the mean (of triplicate wells) number of ASC per 10^6 cells from pooled splenocytes of 5 mice. Data are representative of three separate experiments.

were assessed. Mice immunized with pcDNA3-VP4 had higher numbers of BRV-specific cytokine secreting cells than the control mice both prior to and after BRV boost (Fig. 5.1.2). Splenocytes from pcDNA3-VP4 immunized mice contained higher levels of BRV-specific IFN-γ secreting cells than IL-4 secreting cells, and the stronger polarization toward IFN-γ production was observed after BRV boost (Fig. 5.1.2).

The priming effect of pcDNA3-VP4 was further characterized in another separate experiment. To further investigate the antibody isotypes induced by pcDNA3-VP4 immunization, mice were injected with the plasmid using the same protocol as above and then boosted with rVV-VP4 (10^6 p.f.u. per mouse, i.p.) on day 35 to ensure that the induced immune response was solely due to the VP4 protein. The animals that received rVV-VP4 exhibited increased number of BRV-specific IgG1. However, only animals that received pcDNA3-VP4 developed BRV-specific IgG2a ASC (Table 5.1.1).

To characterize the lymphocyte subpopulation responsible for IFN-γ production in pcDNA3-VP4 immunized animals following rVV-VP4 boost, splenocytes were collected 10 days after the viral boost and *in vitro* cultured with BRV according to a standard ELISPOT protocol for detection of CSCs. Lymphocyte subpopulations ($CD4^+$ and/or $CD8^+$) were depleted, using immunomagnetic beads, prior to detection of cytokine secreting cells. The data indicate that $CD4^+$ cells were a major source of IFN-γ. However, the $CD4^-CD8^-$ population also produced a fraction of IFN-γ in DNA-immunized mice (Table 5.1.2).

Following the BRV boost, we found that the DNA-immunized mice exhibited higher cytotoxic activity than the non-immunized group that received the same amount of BRV (Fig. 5.1.3).

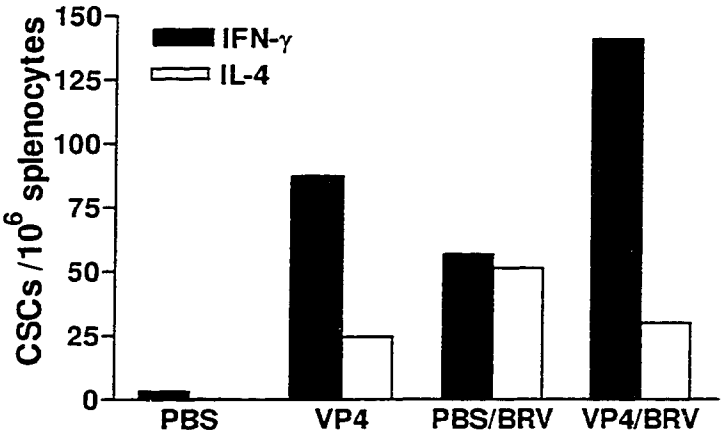


Figure 5.1.2 BRV-specific cytokine responses in mice immunized with pcDNA3-VP4.

The frequency of BRV-specific IFN-γ and IL-4 secreting cells determined by ELISPOT assay. Mice were intramuscularly immunized twice with 200 μg of pcDNA3-VP4 (VP4) at a 3-week interval (day 0, 21), and then boosted with BRV, at day 35 (VP4/BRV). Spleens were collected before (day 31) or after (day 45) the BRV boost. Control groups included naïve mice injected with PBS at the time of immunization (PBS) or mice injected with PBS at the time of immunization and boosted with BRV (PBS/BRV). Data represent the mean (from triplicate wells) of number of CSCs per 10^6 splenocytes from pooled spleen samples of 5 mice. Data are representative of three separate experiments.

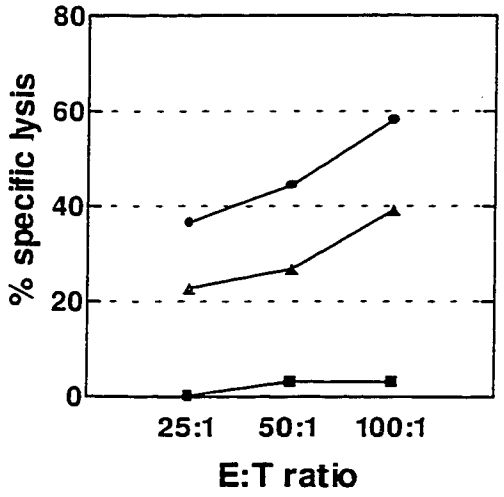


Figure 5.1.3 BRV-specific cytotoxic T cell activity in spleen of mice immunized with pcDNA3-VP4.

Splenocytes were collected, 10 days after the BRV boost, from mice immunized twice with pcDNA3-VP4 and boosted with BRV (●), mice that received only BRV (▲), and naïve mice (■). Cells were *in vitro* stimulated with BRV for 72 hr. prior to testing for BRV-specific cytotoxic activity to BRV-infected target cells. Data represent the mean of % specific lysis (from triplicate wells) from pooled spleen cells of 5 mice. The average % specific lysis of mock infected cells at E:T ratio of 100:1 was 7.5%. The spontaneous release was less then 15%.

Table 5.1.1 Effect of DNA immunization on the isotype of BRV-specific ASCs

Treatment	IgG1[a]	IgG2a[a]
pcDNA3-VP4 + rVV-VP4	12.6	14.0
PBS + rVV-VP4	16.6	<1
PBS + control medium	2.5	<1

[a] Number of BRV-specific ASCs per 10^6 splenocytes. Mice were immunized twice with pcDNA3-VP4 or PBS and then inoculated with rVV-VP4 (10^6 p.f.u. per mouse, i.p.) or CV-1 cell-derived supernatant medium (control medium), 2 weeks after the second immunization. Bovine rotavirus specific ASC were detected, 10 days after viral inoculation. Data represent the mean (of triplicate wells) from pooled splenocytes of 5 mice.

Table 5.1.2 Effect of T lymphocyte subset depletion on number of BRV-specific IFN-γ secreting cells[a]

Depleted lymphocyte subpopulations	Immunization protocol		
	pcDNA3-VP4+rVV-VP4[b]	PBS+rVV-VP4[b]	PBS+Cont.Med[b]
None	80	30	22
$CD4^+$	35	20	nd[c]
$CD8^+$	133	43	nd
$CD4^+$ and $CD8^+$	46	10	nd

[a] Mice were immunized with the same protocol as in table 5.1.1. Pooled splenocytes were cultured in the presence of 5 μg/ml of BRV for 24 hours prior to depletion of lymphocyte subpopulations by immunomagnetic beads.
[b] Number of BRV-specific IFN-γ secreting cells per 10^6 cells. Data represent the mean (of triplicate wells) from pooled splenocytes of 5 mice.
[c] Not determined

Conclusion

Intramuscular immunization with pcDNA3-VP4 induced cell-mediated immunity characterized by the enhancement of BRV-specific cytokine secreting cells in the spleens of immunized mice. The plasmid did not induce a detectable BRV-specific antibody response after 2 immunizations. However, the immunized mice were primed for both antibody and cell-mediated immune responses, including enhanced antigen-specific cytokine production and BRV-specific cytotoxic activity as seen by the increased response, following BRV boost. The predominant secretion of IFN-γ characterized immune responses induced by plasmid immunization, both prior to and after the viral boost. The $CD4^+$ population was a major source of IFN-γ produced in the splenocytes of immunized mice.

5.1.2 Immune responses induced by pSLIA-VP4

It was very difficult to demonstrate *in vitro* transient protein expression of BRV genes using plasmid vectors, which utilized either an RSV or hCMV promoter (data not shown). This suggested that the level of expression of BRV genes was very poor in these systems. It was previously reported that intron A of the immediate early gene of human cytomegalovirus, when incorporated with an hCMV immediate early enhancer/promoter, had a strong positive regulatory effect on the level of gene expression (Chapman *et al.*, 1991). It was postulated that the addition of intron A into the hCMV- driven expression cassette enhanced the level of protein expression from cDNA derived from RNA virus genes, possibly by overriding the internal RNA processing signals (Lehman *et al.*, 1993).

Intradermal immunization with plasmid DNA was also found to be an effective delivery route for induction of an immune response (Raz *et al.*, 1994). In addition, intradermal immunization was shown to induce a stronger antibody response than intramuscular immunization (Braun *et al.*, 1997). Based on these observations, the objective of this part of the project was to investigate whether the immune responses induced by plasmid encoding BRV-VP4 protein could be enhanced by the addition of the intron A element to the expression cassette and by changing the route of immunization from intramuscular to intradermal.

5.1.2.1 *In vitro* protein expression of pSLIA-VP4

The plasmid pSLIA-VP4 was cloned by insertion of the VP4 gene into an expression vector, pSLIA, in which gene expression was driven by the hCMV-intron A promoter/enhancer element. The levels of protein expression induced by pSLIA-VP4

and pcDNA3-VP4 were compared by *in vitro* transient protein expression assay. Figure 5.1.4 demonstrates that the pSLIA-VP4 induced higher amount of VP4 protein in transfected cells than did pcDNA3-VP4.

5.1.2.2 Immune responses induced by pSLIA-VP4 in C57Bl/6 mice

To assess the immune responses induced by immunization with pSLIA-VP4. C57Bl/6 mice were immunized twice with 10 μg of plasmid at a 3-week-interval. Ten micrograms of plasmid was diluted in 20 μl of PBS and intradermally injected into the tail base area of a mouse (2 sites of 10 μl). Since there was no seroconversion observed after two immunizations, all mice were boosted with BRV (50 μg, i.p.) at wk16 and then sacrificed for immunological tests 10 days after.

Although pSLIA-VP4 did not induce seroconversion after 2 immunizations, it primed the mice for an antibody response following viral boost (Fig. 5.1.5). The antibody response induced by viral boost following immunization was significantly higher than the other groups, as judged by one-way ANOVA followed with Tukey's test ($p<0.05$), (Fig.5.1.5, A). In contrast, intradermal immunization with pcDNA3-VP4 did not prime for an antibody response. Further analysis of antibody isotypes by an ELISPOT assay showed higher numbers of ASCs, of every tested isotype, in the spleens of pSLIA-VP4 immunized mice than spleens from mice of the other experimental groups (Fig.5.1.5, B).

The pattern of cytokine responses, induced following viral boost was determined by ELISPOT assay. The groups that received plasmids encoding VP4 exhibited a higher number of IFN-γ secreting cells, compared with IL-4 secreting cells, (Fig.5.1.6).

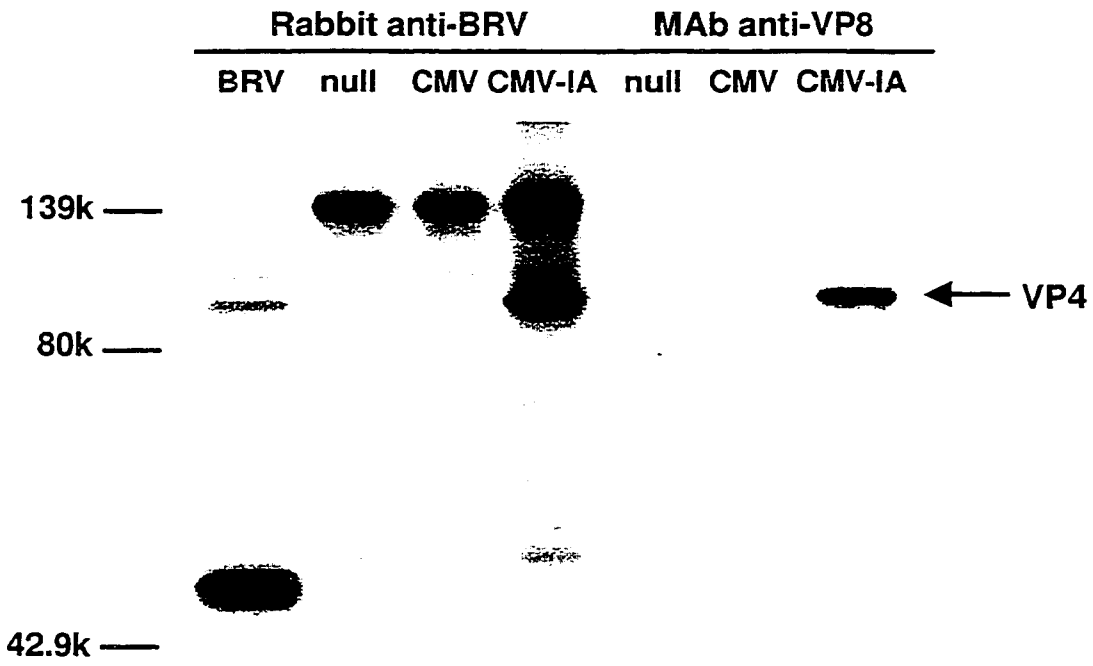
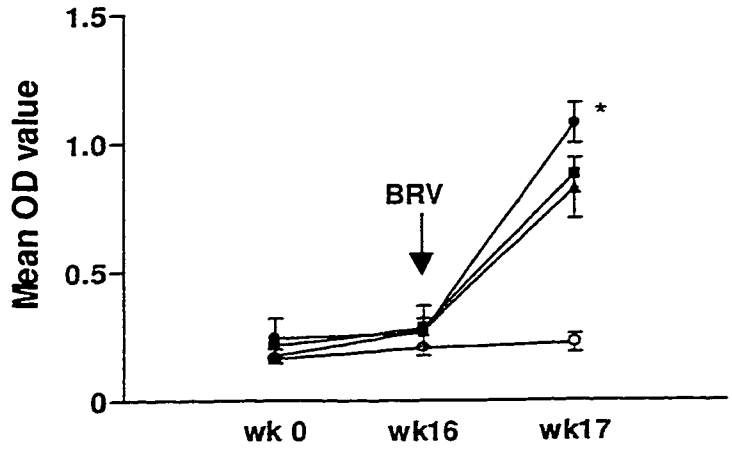


Figure 5.1.4 Effect of intron A on the level of VP4 gene expression.

COS-7 cells were transfected with pSLIA (null), pcDNA3-VP4 (CMV) or pSLIA-VP4 (CMV-IA) and then labelled with [^{35}S]-methionine. Cell lysates were immunoprecipitated with rabbit anti-BRV antibody or VP8-specific MAb and separated with SDS-PAGE. Radiolabelled cell lysate from BRV-infected MA-104 cells was included as a positive control (BRV).

A.



B.

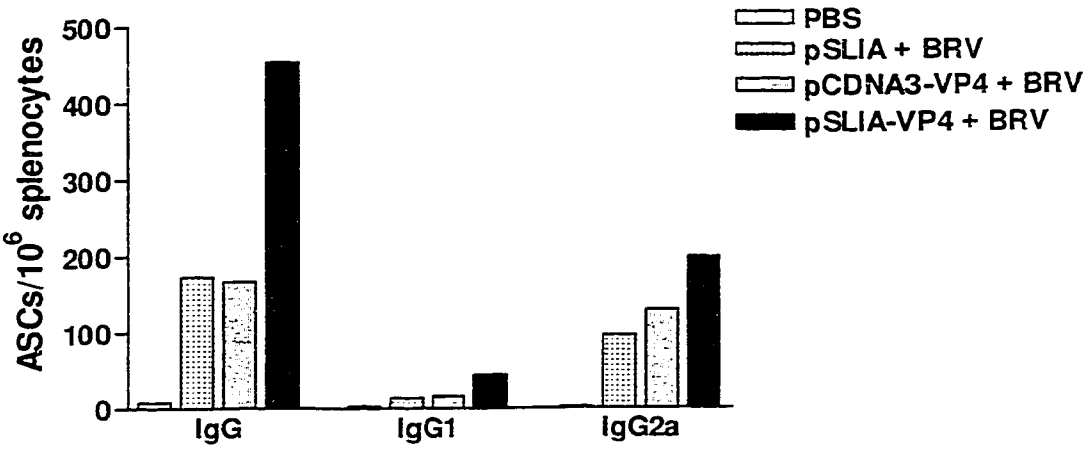


Figure 5.1.5 BRV-specific antibody responses in mice immunized with pSLIA-VP4.

A. Mice were intradermally immunized twice with 10 μg of pSLIA-VP4 (●), pcDNA3-VP4 (▲), pSLIA (■) and then boosted with 50 μg of BRV, i.p., at wk 16. Control naïve mice received only PBS (O). Data represent the BRV-specific serum antibody level at 1:50 serum dilution (mean OD ± SD from 5 mice) measured by ELISA. *Indicates significant differences between experimental groups ($p<0.05$).

B. BRV-specific ASCs in the spleen after BRV boost determined by ELISPOT assay. Data represent the mean number of ASCs per 10^6 cells from pooled splenocytes of 5 mice.

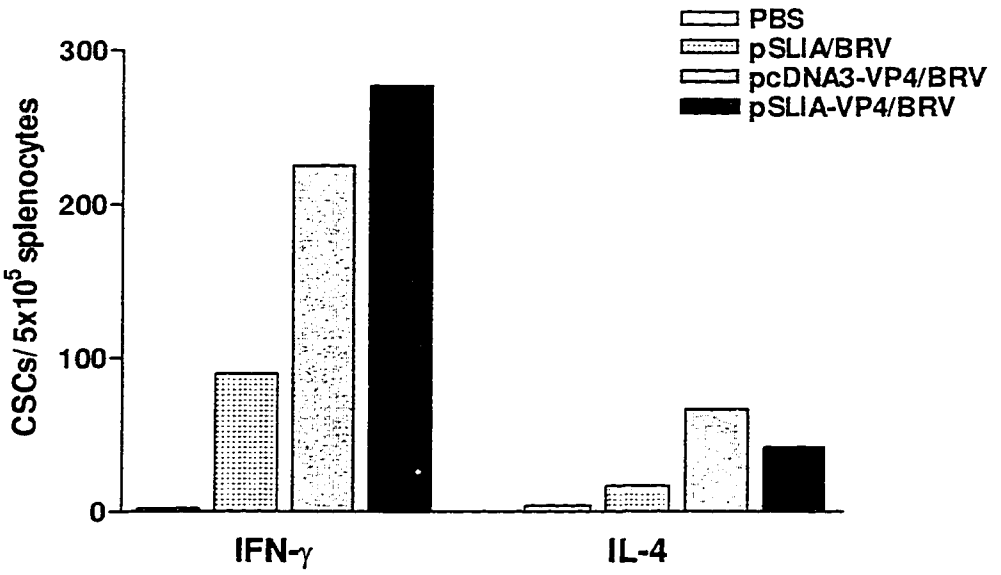


Figure 5.1.6 BRV-specific cytokine responses in mice immunized with plasmids encoding VP4 protein and then boosted with BRV.

The frequency of BRV-specific IFN-γ and IL-4 secreting cells was determined by an ELISPOT assay. Mice were intradermally immunized twice with 10 μg of plasmids as indicated, and then boosted with 50 μg of BRV, i.p. Control group received only PBS at the time of immunization (PBS). Data represent the mean (of triplicate wells) of the number of CSCs per $5x10^5$ splenocytes from pooled spleen samples of 5 mice.

5.1.2.3 Immune responses induced by pSLIA-VP4 in Balb/c mice

To address whether the genetic background of mice influenced the outcome of immune responses induced by DNA immunization, particularly the ability to mount an antibody response, Balb/c mice were immunized, either i.d. or i.m., twice at a 3-week interval with different doses of pSLIA-VP4. No seroconversion was observed after 2 immunizations. Mice were kept in a rotavirus-free environment for 9 months without detectable seroconversion. At this point all mice were boosted with 50 μg of BRV, i.p., and then sacrificed, ten days after boosting. Serum samples and spleens were collected for immunological tests.

Although seroconversion was not observed in mice immunized with pSLIA-VP4, priming of BRV-specific immune responses was still apparent in DNA immunized mice after 9 months. The priming for antibody production was route and dose-dependent (Fig.5.1.7). Intradermal injection of pSLIA-VP4, using 10-100 μg of plasmids, induced higher numbers of antibody producing cells in the spleens than plasmid injected intramuscularly (Fig.5.1.7, A-B). However, the antibody titres measured by ELISA were not significantly different from the control group (Fig.5.1.7, C).

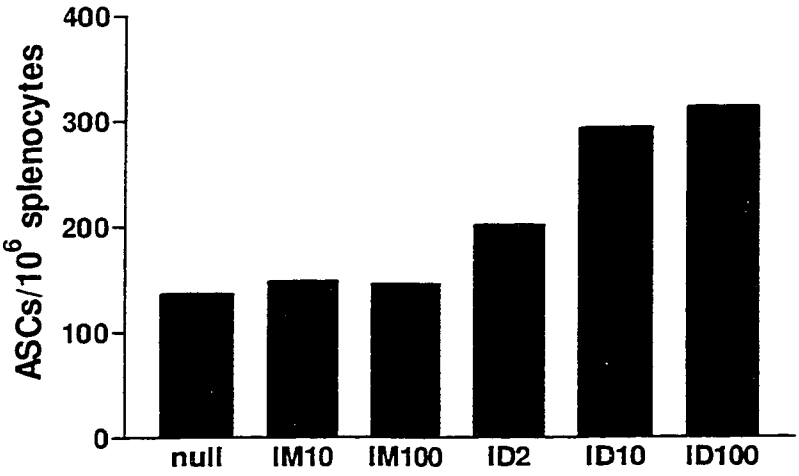
Following a viral boost, mice that received pSLIA-VP4 had a higher number of IFN-γ secreting cells in the spleen than the group that received null plasmid (Fig.5.1.8, A). Additionally, the number of IL-4 secreting cells in the spleen was affected by plasmid dose and route of immunization (Fig.5.1.8, B). A lower dose, with the same route of immunization, tended to induce lower numbers of IL-4 secreting cells. Groups immunized intradermally with 10 and 100 μg of pSLIA-VP4 had a significantly higher number of IFN-γ and IL-4 secreting cells than the control group as judged by one-way ANOVA followed by the Dunnett's test ($p<0.05$), (Fig. 5.1.8, A-B). The IFN-γ/IL-4 ratios

Figure 5.1.7 BRV-specific antibody responses in Balb/c mice immunized with pSLIA-VP4 and then boosted with BRV.

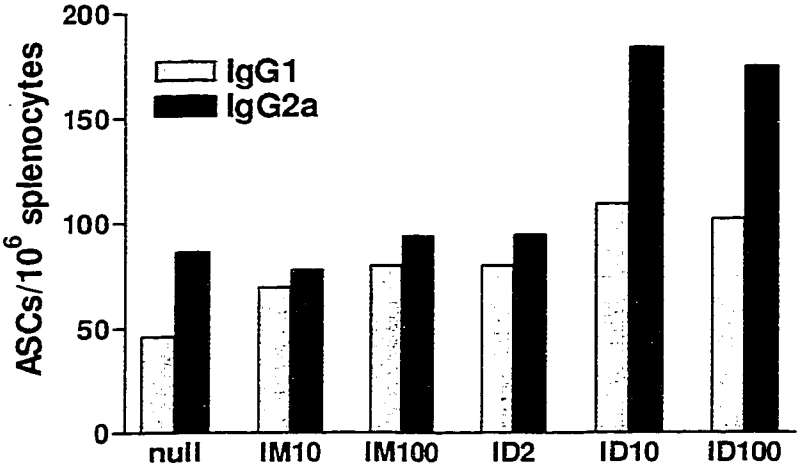
Balb/c mice were immunized with pSLIA-VP4 twice at a 3-week interval. The routes of immunization, intramuscular (IM) or intradermal (ID), and doses (μg/dose) are indicated in the figures. The control group (null) was intradermally immunized with 100 μg of pSLIA. All mice were boosted with 50 μg of BRV, i.p, at 9 months after the second DNA immunization.

Numbers of BRV-specific IgG (A), IgG1 and IgG2a (B) secreting cells in spleen determined by ELISPOT assay. Data represent the mean (of triplicate wells) number of ASC per 10^6 cells from pooled splenocytes of 5 mice. (C) BRV-specific antibody titres of individual mice determined by ELISA. Solid line represents mean of BRV-specific serum antibody titre from each group.

A.


400
300
200
100
0
ASCs/10^6 splenocytes
null
IM10
IM100
ID2
ID10
ID100

B.


200
150
100
50
0
ASCs/10^6 splenocytes
IgG1
IgG2a
null
IM10
IM100
ID2
ID10
ID100

C.

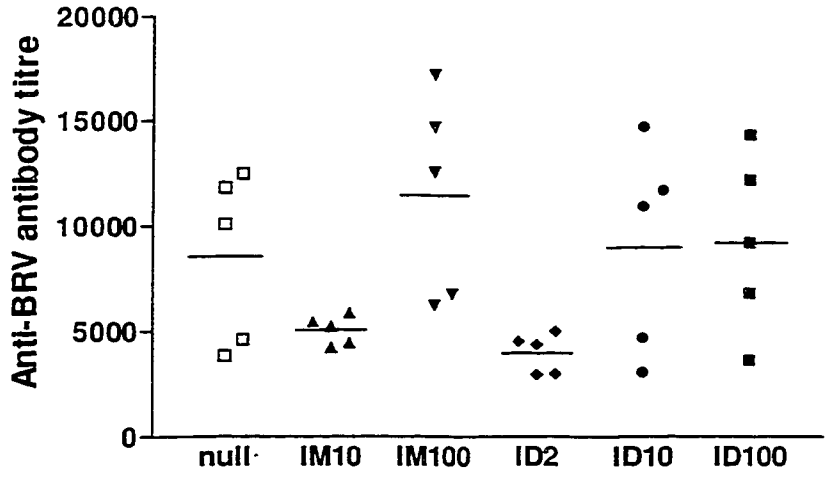
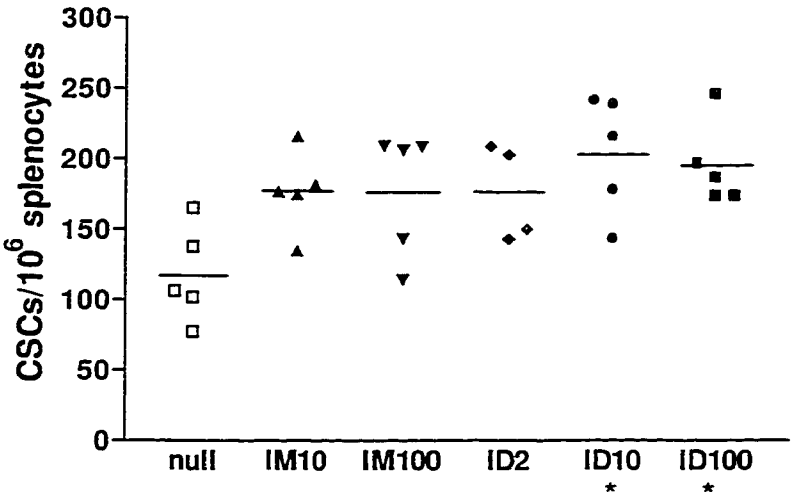

20000
15000
10000
5000
0
Anti-BRV antibody titre
null
IM10
IM100
ID2
ID10
ID100

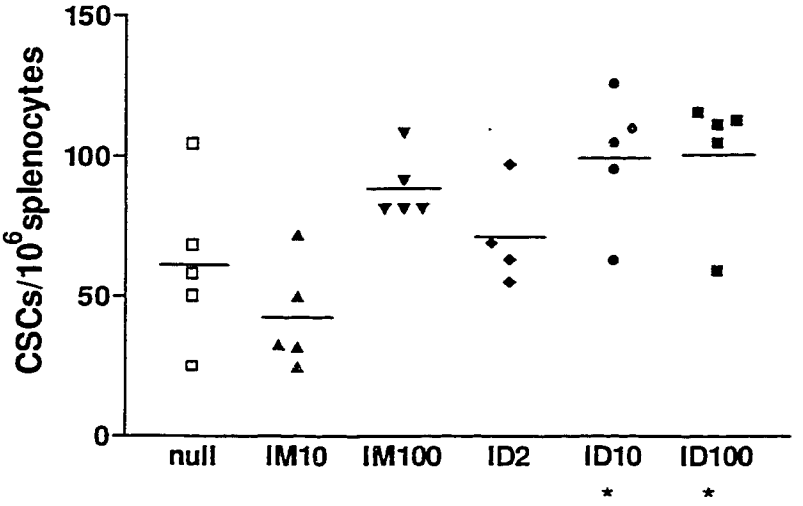
Figure 5.1.8 BRV-specific cytokine responses in Balb/c mice immunized with pSLIA-VP4 and then boosted with BRV.

Mice were immunized with the same protocol as Fig.5.1.7. Numbers of BRV-specific IFN-γ (A), and IL-4 secreting cells (B) were determined by ELISPOT assay. Data represent the number of CSCs per 10^6 splenocytes or ratio of CSCs (C) from individual mice. Solid line represents the mean value from each group. * Indicates significant difference from the control group ($p<0.01$).

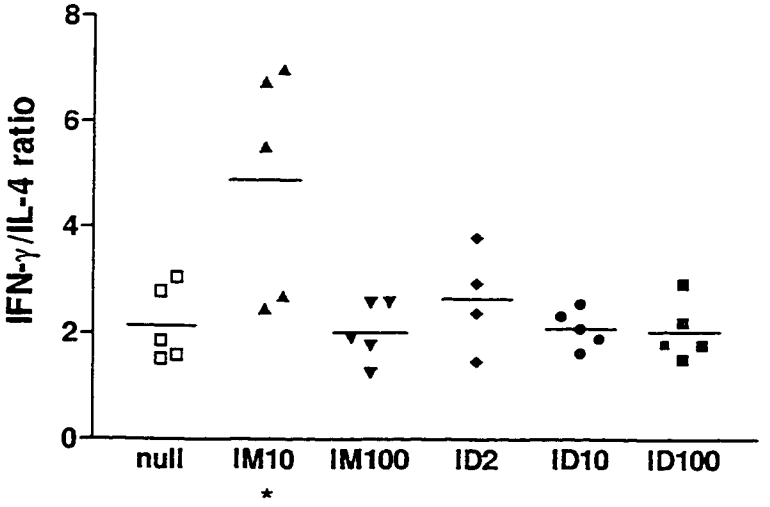
A.


CSCs/10^6 splenocytes
300
250
200
150
100
50
0
null
IM10
IM100
ID2
ID10
ID100
*
*

B.


CSCs/10^6splenocytes
150
100
50
0
null
IM10
IM100
ID2
ID10
ID100
*
*

C.


IFN-γ/IL-4 ratio
8
6
4
2
0
null
IM10
IM100
ID2
ID10
ID100
*

of treatment groups were not statistically different from the control, except the group intramuscularly immunized with 10 μg of pSLIA-VP4 (Fig.5.1.8, C).

5.1.2.4 Immune responses induced by pSLIA-VP4 in BRV-primed mice

The ability of pSLIA-VP4 to boost immune responses in BRV-primed C57Bl/6 mice was determined using the following immunization protocol. All mice were intraperitoneally injected with 50 μg of BRV, and then boosted, i.d., with 10 μg of plasmid 4 weeks after receiving BRV. The intradermal injection was done on the back (dorsal area) of the mice. This injection site facilitated collection of cells from the draining lymph nodes (inguinal and axillary) for further immunological tests. Mice were sacrificed 12 days after boosting. Spleen and draining lymph node cells were collected for various tests, including ELISA, ELISPOT for ASCs and CSCs. We found that pSLIA-VP4 did not boost antibody or cell-mediated immune responses (data not shown).

Conclusion

Addition of the intron A into the hCMV-driven expression cassette improved *in vitro* gene expression and the induction of BRV-specific immune responses following a viral boost. The plasmid pSLIA-VP4 did not induce antibody responses after 2 immunizations but primed the mice for both antibody and cytokine production, particularly IFN-γ secretion. The pSLIA-VP4 was significantly more efficient in priming an antibody response than the pcDNA3-VP4. The immune response induced by the

VP4 gene following viral boost was characterized by a predominance of IFN-γ production.

Immunizations with plasmid encoding VP4 did not induce antibody responses in either C57Bl/6 or Balb/c mice, suggesting that the lack of antibody production after immunizations was not due to the genetic background of the mice. The doses and routes of immunization affected the level of immune responses following viral boost. Intradermal immunization was more efficient in priming of antibody and cytokine responses than intramuscular immunization. In addition, the dose of the intradermally injected plasmid required to induce maximal priming effect could be reduced to 10 μg.

The plasmid encoded VP4 protein did not have a boosting effect on immune responses of BRV-primed mice.

5.2 Immune responses induced by VP7 protein and plasmids encoding VP7 proteins

5.2.1 Immune response induced by BRV-VP7

The VP7 protein of rotavirus is considered to be one of the most important antigens for inducing protective immunity against rotavirus (Paul & Lyoo, 1993). However, several studies indicate that VP7 is weakly immunogenic (Svensson *et al.*, 1987, Ward, 1996, Ward *et al.*, 1988). Immunization with recombinant VP7 has been of limited success to date. Immunization with baculoviral expressed-recombinant BRV-VP7 protein did not induce BRV-specific antibody responses in mice, unless given in the context of viral-like particles (Redmond *et al.*, 1993). To assess the immunogenicity of BRV-VP7 in mice, C57Bl/6 mice were inoculated with 50 μg of partially purified BRV either intradermally or intraperitoneally. Serum samples were collected at 2, 6, and 8 week after viral inoculation and then used in a western blot assay to detected BRV proteins. Figure 5.2.1 showed that BRV induced a poor VP7-specific antibody response in mice, when compared to other viral proteins, particularly VP4.

5.2.2 Immune responses induced by plasmid encoding BRV-VP7

To assess the potential use of plasmids carrying the VP7 gene for induction of BRV-specific immune responses, groups of 10 C57Bl/6 mice were immunized intramuscularly twice (day 0, day 21) with 200 μg of pcDNA3-VP7 using the same protocol as in section 5.1.1. At day 31, 5 mice from each group were sacrificed and splenocytes were collected for various immunological assays. The rest of the mice in each group were intraperitoneally boosted by injecting 50 μg of partially purified BRV on

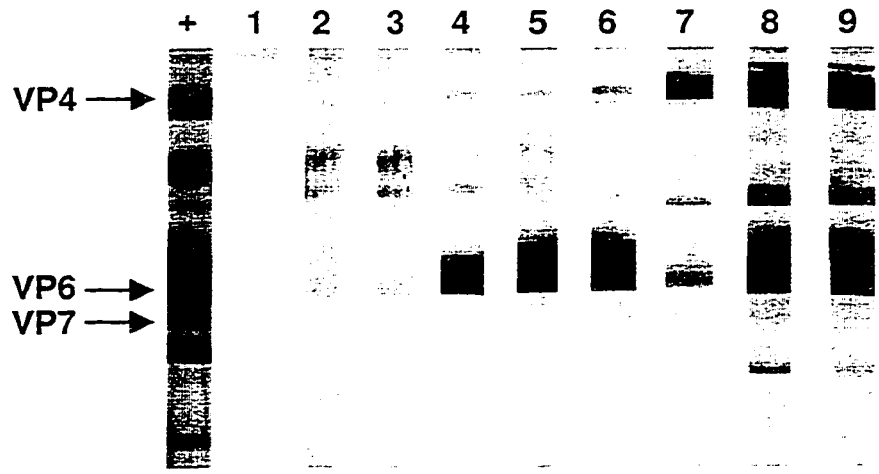


Figure 5.2.1 Western blot analysis of sera obtained from BRV immunized mice.

Bovine rotavirus proteins from whole virus were separated by SDS-PAGE, transferred onto a blotting membrane and detected by using pooled anti-sera collected from C57Bl/6 mice that were intradermally inoculated with PBS (lane 1-3), or 50 μg of partially purified BRV (lane 4-6), or intramuscularly inoculated with the same amount of virus (lane 7-9) at 2, 6 and 8 week respectively. Pooled serum from mice immunized several times with BRV was used as a positive control in the (+) lane.

day 35. All animals were then sacrificed for immunological tests on day 45.

The results from this preliminary trial indicated that the BRV-VP7 gene in the pcDNA3 vector induced neither antibody response nor cell-mediated immunity following 2 immunizations. In contrast to the VP4 gene, the pcDNA3-VP7 did not prime for cytokine production (Fig.5.2.2, A). A slight increase in the number of BRV-specific ASCs was observed (Fig.5.2.2, B) but there was no difference in antibody titres when compared with the control group that received only BRV. In addition, the plasmid did not prime for BRV-specific cytotoxic activity as was previously observed for mice immunized with the pcDNA3-VP4 (data not shown).

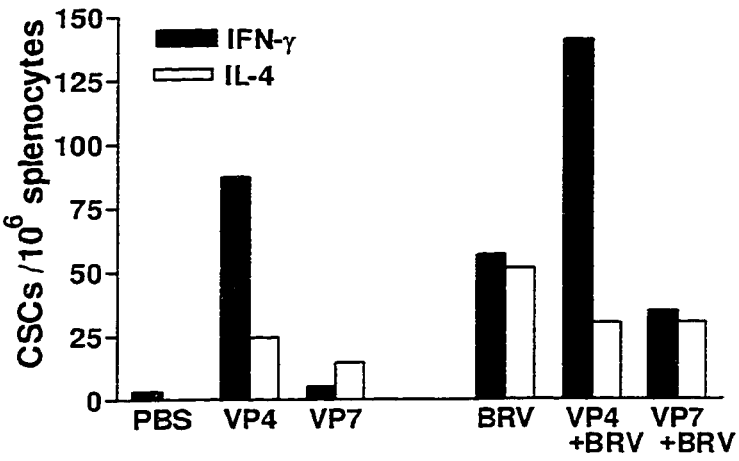
5.3 Approaches used to alter BRV-specific immune responses by plasmids encoding VP7 or modified VP7 proteins

5.3.1 Improving the level of antigen expression and its comparmentalization

It is, in my experience, very difficult to obtain a good level of protein expression in mammalian cells when using rotavirus genes, which are derived from an RNA genome. This may be due to the potential internal RNA processing sequences that reside in the gene (Lehman *et al.*, 1993). Although these sequences do not interfere with protein synthesis in the cytoplasm of viral-infected cells, they may potentially obstruct protein synthesis in the transfected cells where mRNAs are synthesized within the nucleus and have to be transported into the cytoplasm for protein translation.

In a natural infection, the newly synthesized rotavirus VP7 protein contains a signal peptide that directs the protein to the ER. As a result, the protein is retained there as a membrane-associated protein for later viral assemble process (Poruchynsky *et al.*, 1985). In addition, the VP7 must bind to calcium to stabilize its structural integrity during the viral maturation process (Poruchynsky *et al.*, 1991, Ruiz *et al.*, 1996).

A.



B.

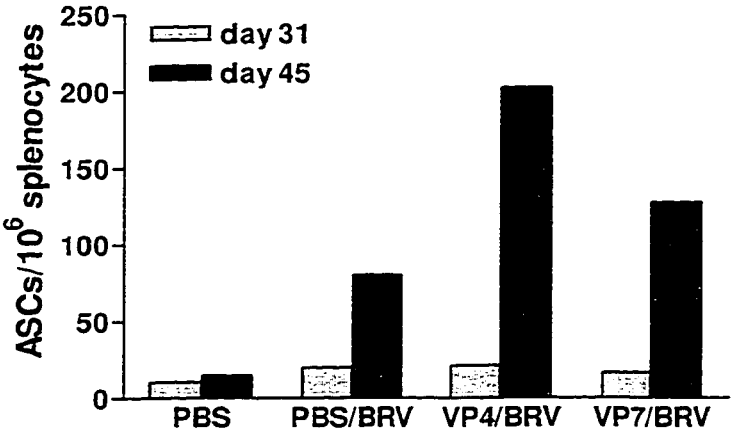


Figure 5.2.2 BRV-specific immune responses in mice immunized with plasmids encoding VP4 or VP7, with or without BRV.

C57Bl/6 mice were intramuscularly immunized with 200 μg of pcDNA3-VP4 (VP4) or pcDNA3-VP7 (VP7) twice at 3-week-interval (day 0, day 21). The control group received PBS. On day 31, half of the animals in each group were sacrificed, the remaining animals were boosted with 50 μg of BRV and then sacrificed on day 45.

A. The frequency of BRV-specific IFN-γ and IL-4 secreting cells was determined by ELISPOT assay. Data represent the mean (from triplicate wells) of numbers of CSCs per 10^6 splenocytes from pooled splenocytes of 5 mice.

B. BRV-specific IgG-ASCs in the spleen before (day 31) and after BRV boost (day 45) as determined by ELISPOT assay. Data represent the mean (of triplicate wells) number of ASC per 10^6 cells from pooled splenocytes of 5 mice.

In the absence of other viral protein or high calcium environment, VP7 is unstable (Dormitzer *et al.*, 1994). Consequently, recombinant VP7 expressed as an intracellular antigen is poorly immunogenic, and the secreted form of VP7 does not show enhanced immunogenicity when compared with the wild-type protein (Andrew *et al.*, 1990, Andrew *et al.*, 1992, Dormitzer *et al.*, 1994). However, addition of a surface anchor protein to the carboxyl terminal end of VP7 effectively enhanced the immunogenicity of the protein in several systems, including vaccinia virus and adenovirus recombinant vectors (Andrew *et al.*, 1990, Andrew *et al.*, 1992, Both *et al.*, 1993, Dormitzer *et al.*, 1994). This suggests that changing the protein structure to possibly increase protein stability may enhance the immunogenicity of the VP7.

To explore if increasing the level of gene expression and/or changing protein localization enhanced the immunogenicity of the BRV-VP7, several plasmids encoding various modified forms of VP7 were designed and cloned. First, an intron A was added into the expression cassette to enhance the level of gene expression as previously shown with the pSLIA-VP4 plasmid. Second, the original VP7 signal sequence was replaced with a TPA or gD signal sequence to generate plasmids that expressed secretory VP7 proteins (VP7Sc). Finally, a transmembrane anchor sequence from the gD gene of BHV-1 was added to the carboxyl terminal end of the VP7 fragment to generate a sequence encoding a membrane bound VP7 (VP7Anc). The plasmids and their important characteristics are summarized in table 5.3.1.

Table 5.3.1 Plasmids used in this study and characteristics of their products.

Plasmid	Characteristics of the expression cassette	Fate of VP7
pSLIA-VP7	authentic VP7 gene	ER
pTPA-VP7Sc	TPA-ss + VP7Sc	secreted
pgD-VP7Sc	gD-ss + VP7Sc	secreted
pTPA-VP7Anc	TPA-ss + VP7Anc + gD transmembrane anchor sequence	cell surface
pgD-VP7Anc	gD-ss + VP7Anc + gD transmembrane anchor sequence	cell surface

5.3.1.1 *In vitro* characterization of plasmids encoding VP7 and modified forms of VP7

COS-7 cells transfected with plasmids encoding VP7 proteins were radiolabelled with [^{35}S]-methionine and BRV proteins were recovered by immunoprecipitation using rabbit anti-BRV antiserum. The protein products were of predicted molecular sizes (Fig. 5.3.1). The plasmids utilizing either TPA or gD signal peptides exhibited the same protein profiles. Interestingly, there was some secreted VP7 detected in the culture medium of cells transfected with pSLIA-VP7. The secreted VP7 in the medium from pSLIA-VP7, pTPA-VP7Sc and pgD-VP7Sc exhibited shifting molecular sizes when compared with the VP7 associated with cells, suggesting that these proteins had gone through post-translation modification after leaving the ER. The VP7 proteins from cells transfected with pTPA-VP7Anc and pgD-VP7Anc were expressed on the cellular surface of the cells as expected (Fig. 5.3.2). In addition, protein expression from these plasmids was also confirmed in cell lines derived from other species including L929 (murine), and CR001 (bovine) cells (data not shown).

Deglycosylation studies were done to further characterized the proteins expressed from these plasmids (Fig. 5.3.3). The intracellular VP7 from pSLIA-VP7 transfected cells was sensitive to both Endo H and PNGase F. The protein, in particular the higher band, from pgD-VP7Anc transfected cells was Endo H resistant, indicating that this protein had left the ER. The lower band seen in the gel was probably the precursor of the top band (Fig. 5.3.1, 5.3.2, 5.3.3, A). The deglycosylation studies of secreted protein from cells transfected with pSLIA-VP7 and pgD-VP7Sc indicated that these proteins had actually left the ER (Endo H resistant), and the 2 bands found in the media originated from the same precursor protein (Fig. 5.3.3, B).

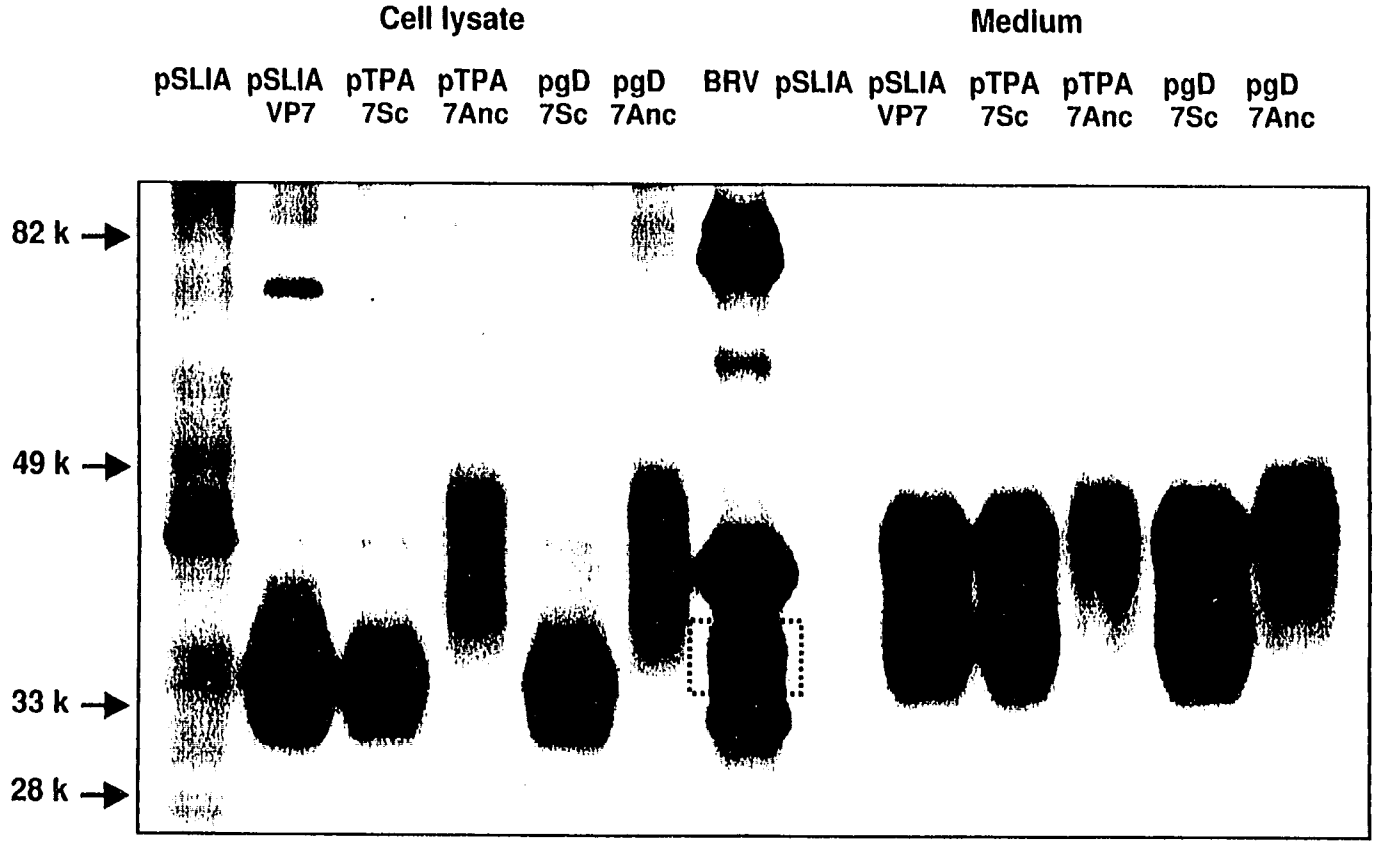


Figure 5.3.1 ***In vitro*** **expression of plasmids encoding VP7 proteins.**

COS-7 cells transfected with plasmids encoding VP7 proteins were radiolabelled with [^{35}S]-methionine. Radiolabelled proteins in cell lysates and media were recovered by immunoprecipitation using rabbit anti-BRV antiserum. Radiolabelled cell lysate from BRV-infected MA104 cells was included as a positive control (BRV). Dotted square showed the native-VP7 protein of BRV.

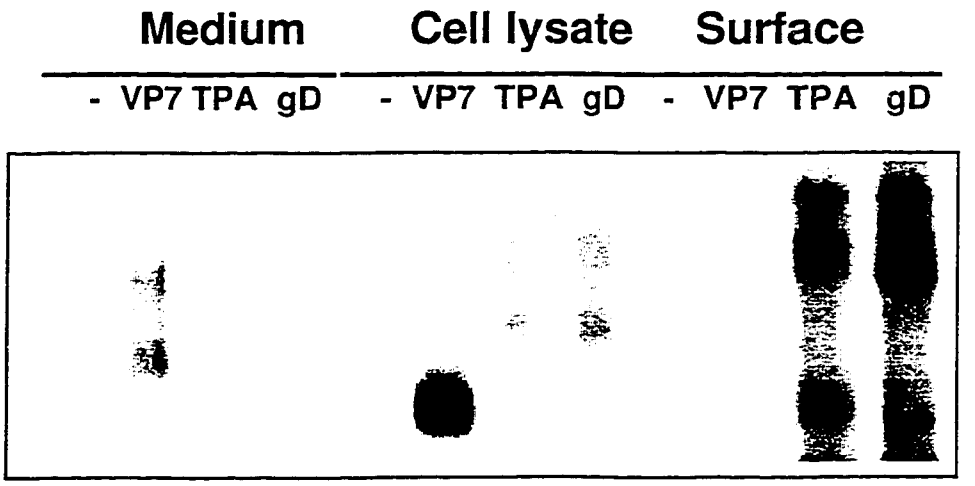


Figure 5.3.2 Localization of the VP7Anc protein.

Radiolabelled samples from COS-7 cells transfected with pSLIA (-), pSLIA-VP7 (VP7), pTPA-VP7Anc (TPA), pgD-VP7Anc (gD) were recovered by immunoprecipitation, using rabbit ant-BRV antiserum, and then separated by SDS-PAGE. The VP7 gene products in the cell lysates were detected by solubilizing transfected cells before immunoprecipitation. The cell surface-expressed VP7 was detected by incubating the transfected cells with rabbit anti-BRV antiserum prior to cellular solubilization.

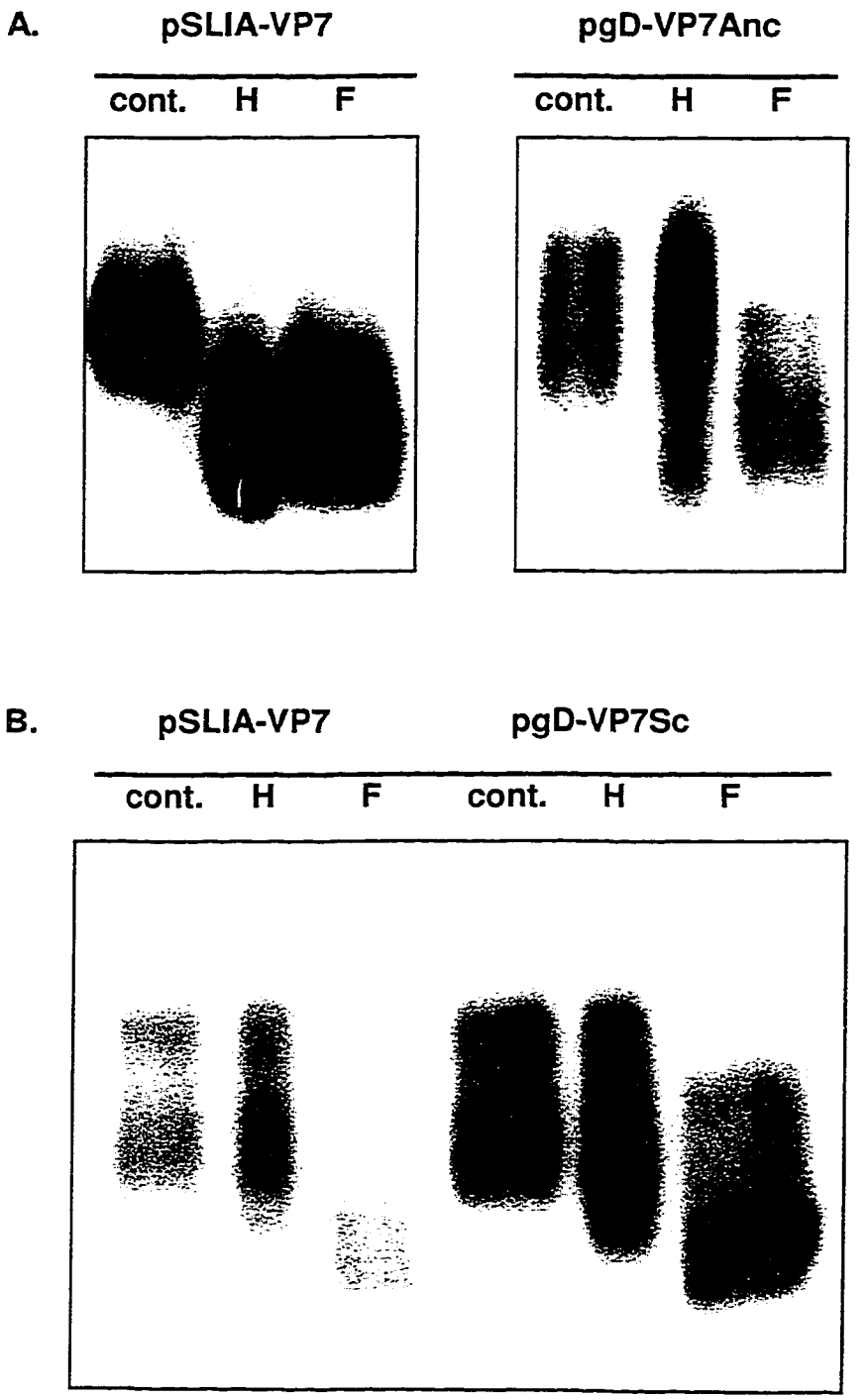


Figure 5.3.3 Nature of carbohydrates attached to the modified VP7 proteins.

Proteins expressed in transfected COS-7 cells (A) or secreted into the medium (B) were recovered by immunoprecipitation, digested with Endo H (H) or PNGase F (F) and then separated by SDS-PAGE. The protein which was only incubated with a buffer used for the PNGase F reaction was included as a control (cont.).

5.3.1.2 Immune responses induced by plasmids encoding VP7 and modified VP7

Having established that all the plasmids could transfect cells and express protein *in vitro*, they were tested for the capacity to induce immune responses *in vivo*. To achieve this goal, C57Bl/6 mice were immunized with 10 μg of plasmids twice at 3-week interval. Ten micrograms of plasmids was diluted in 20 μl of PBA and injected intradermally into the tail base area of mice (2 sites of 10 μl). No seroconversion was observed after 2 immunizations. Mice were then boosted with 50 μg of BRV intraperitoneally at week 7 and sacrificed for serum and spleen collections, 10 days after the boost.

Although seroconversion was not observed after 2 immunizations with any of the plasmids, there was slight priming of the serum antibody responses of mice immunized with pTPA-VP7Anc (Fig. 5.3.4, A). The pSLIA-VP7 primed the immunized mice for antibody production in the spleen, but not in the serum (Fig. 5.3.4, B).

Cytokine production in the spleen was predominantly IFN-γ (Fig. 5.3.5). Immunization with pTPA-VP7Sc primed mice for IFN-γ production. Slight priming of IFN-γ production was also observed in mice immunized with plasmids encoding authentic VP7 and VP7Anc (Fig. 5.3.5). Although the CTL activity detected in this experiment was somewhat low, the modified VP7 induced higher CTL activity when compared with the original VP7 construct. In addition, the plasmids encoding secretory VP7 that utilized the TPA signal peptides induced higher cytotoxic activity than those utilizing the gD signal peptides (Fig. 5.3.6).

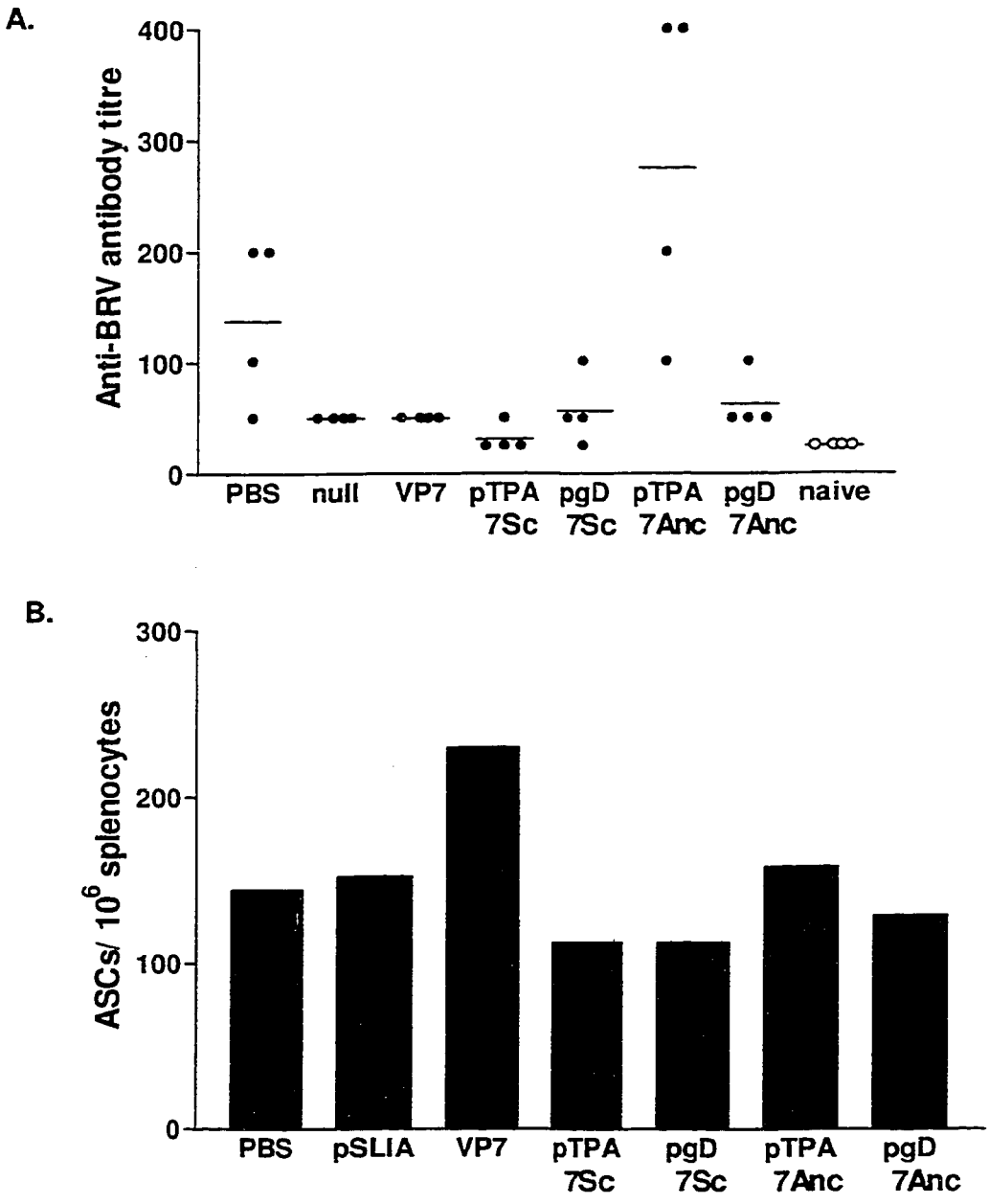


Figure 5.3.4 BRV-specific antibody responses in mice immunized with plasmids encoding VP7 and modified VP7 and then boosted with BRV.

Mice were intradermally immunized with PBS or 10 μg of indicated plasmids twice at a 3-week interval, and then boosted with 50 μg of BRV intraperitoneally at week 16.

A. BRV-specific antibody titre of individual mice measured by ELISA. The solid line represents the mean of the titre from each group. Naïve mice were included as a negative control.

B. BRV-specific IgG-ASCs in spleens measured by ELISPOT assay. Data represent the mean (of triplicate wells) number of ASC per 10^6 pooled splenocytes from 5 mice.

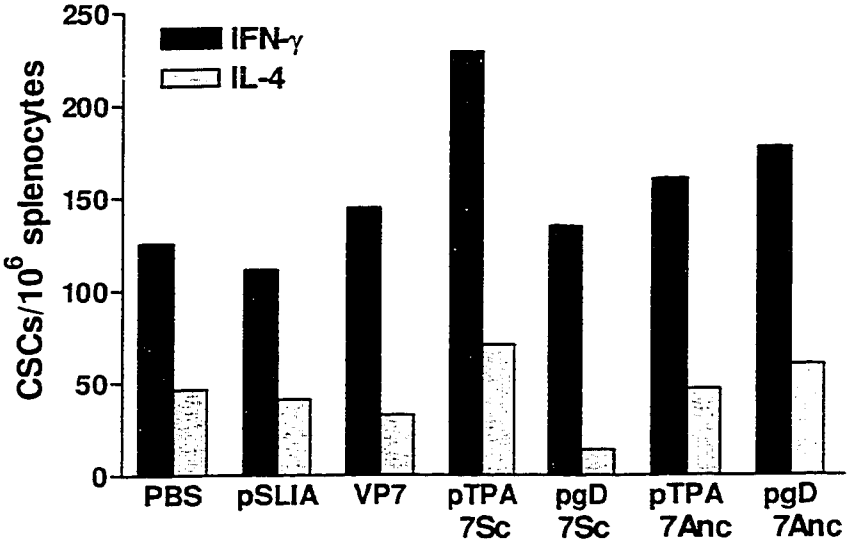


Figure 5.3.5 Number of BRV-specific cytokine secreting cells following BRV boost.

Mice were immunized with the same protocol as fig. 5.3.4. The frequency of BRV-specific IFN-γ and IL-4 secreting cells was determined by ELISPOT assay. Data represent the mean (of triplicate wells) number of CSCs per 10^6 pooled splenocytes from 5 mice.

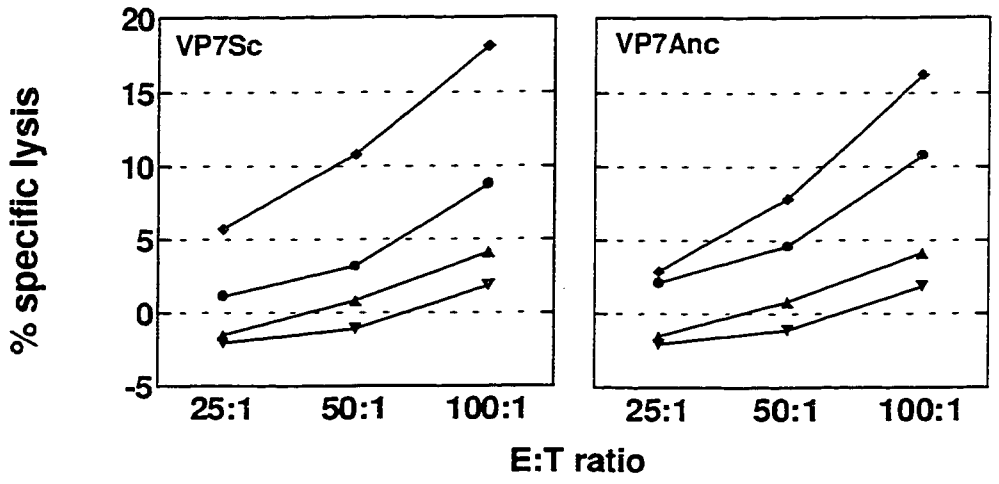
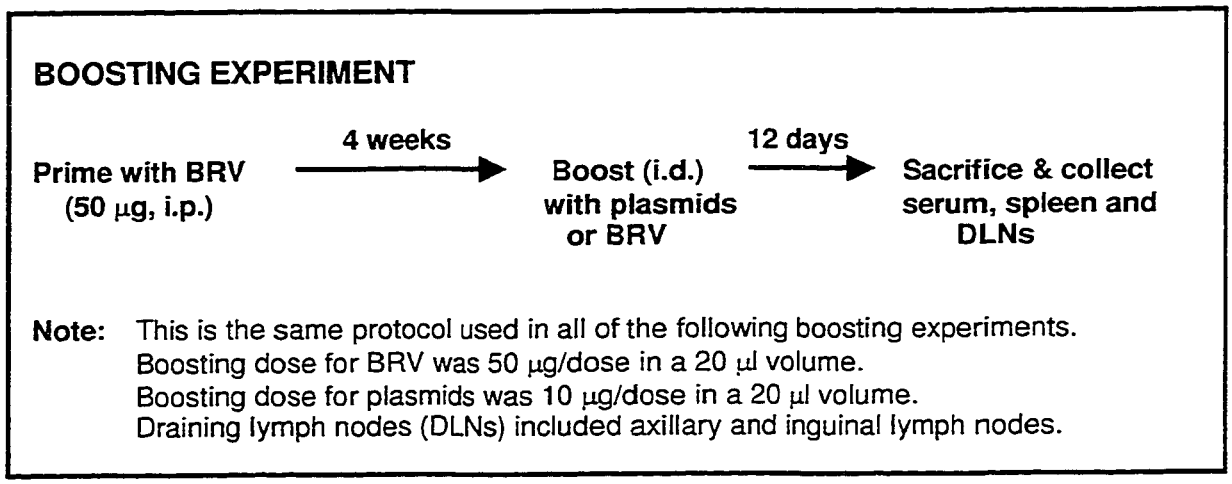


Figure 5.3.6 BRV-specific cytotoxic T cell activity in the spleen of mice immunized with plasmids encoding VP7 and modified VP7 and then boosted with BRV.

Mice were immunized with pSLIA (▲), pSLIA-VP7 (▼), pTPA-VP7Sc (◆, left panel), pgD-VP7Sc (●, left panel) or pTPA-VP7Anc (◆, right panel), pgD-VP7Anc (●, right panel) with the same protocol as in fig. 5.3.4. Splenocytes were in vitro stimulated with BRV for 72 hours prior to the assay. Data represent the mean (from triplicate wells) of % specific lysis from pooled splenocytes of 5 mice.

5.3.1.3 Immune responses induced by plasmids encoding VP7 and modified VP7 in BRV-primed mice

The ability of the plasmids encoding VP7 and modified VP7 to boost the immune responses in BRV-primed C57Bl/6 mice was determined by the following immunization protocol.

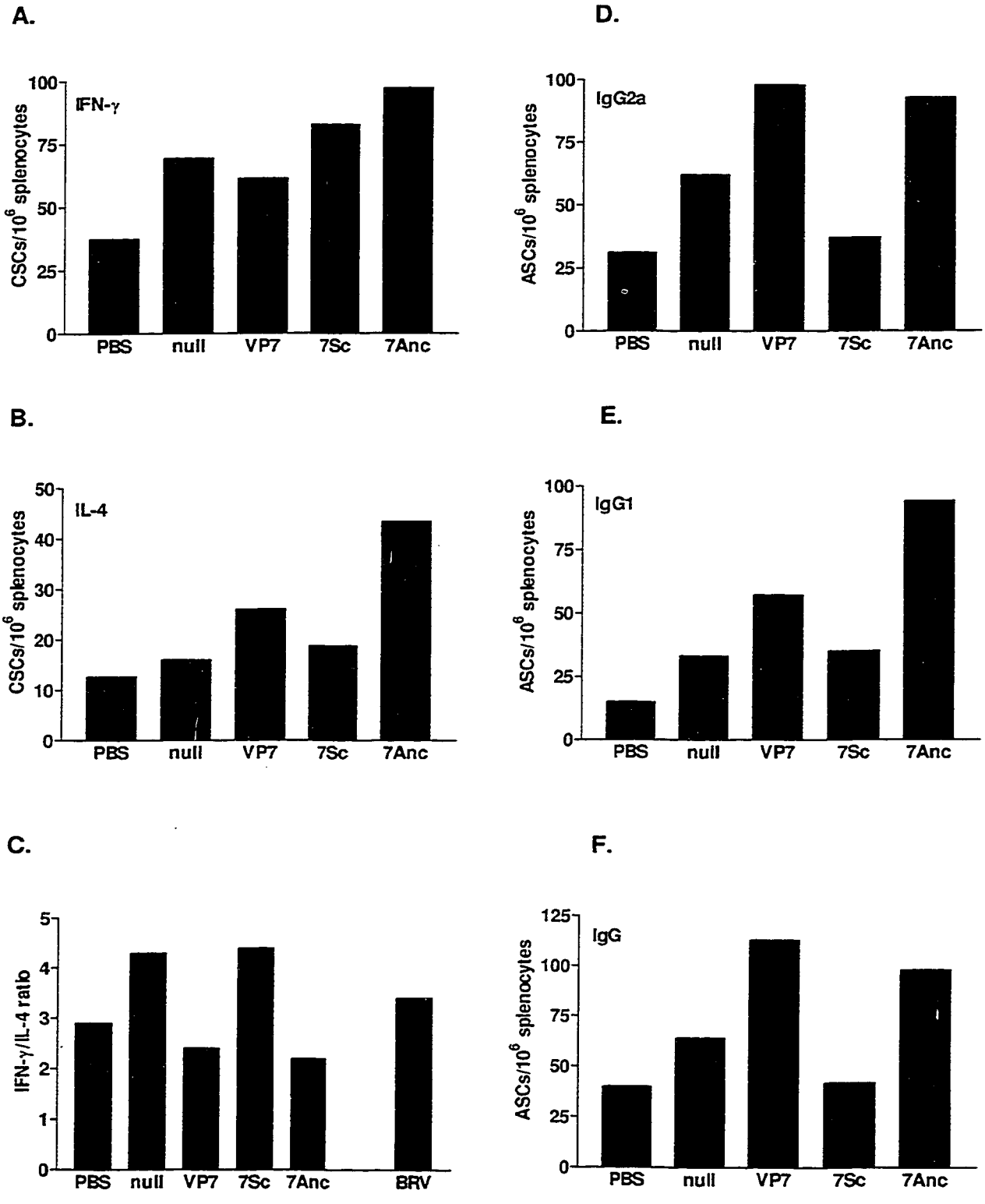


Following plasmid boost, pSLIA-VP7 and pTPA-VP7Anc induced higher numbers of ASCs for all Ig isotypes and more IL-4 secreting cells in the spleen than the control groups and pTPA-VP7Sc (Fig. 5.3.7, D-F, Fig. 5.3.7, B). Additionally, pTPA-VP7Anc also induced a higher number of IFN-γ secreting cells in the spleens. The secretory VP7 induced high IFN-γ (Fig. 5.3.7, A) but did not show any boosting effect on antibody responses. In fact, there were lower numbers of ASCs with secretory VP7 when comparing with the group receiving null plasmid (Fig. 5.3.7, D, F). It should be noted that different plasmids induced different patterns of cytokine secretion and different IFN-γ/IL-4 ratios (Fig. 5.3.7, C). Among these plasmids, the pTPA-VP7Anc induced a strong boosting effect on both antibody and cytokine responses. It should be noted that the plasmid backbone might partly contribute to the boosting activity since

Figure 5.3.7 BRV-specific immune responses in BRV-primed mice boosted with plasmids encoding VP7 and modified VP7.

C57Bl/6 mice were intraperitoneally injected with 50 μg of BRV. At week 4, mice were intradermally boosted with 20 μl of PBS, or 10 μg of pSLIA (null), pSLIA-VP7 (VP7), pTPA-VP7Sc (7Sc), pTPA-VP7Anc (7Anc), or 50 μg of BRV (BRV). All mice were sacrificed for immunological assays 12 days after the boost.

Numbers of BRV-specific IFN-γ (A), IL-4 (B), IgG2a (D), IgG1 (E) and IgG (F) secreting cells determined by ELISPOT assay. Data represent the mean (of triplicate wells) number of cytokine or antibody secreting cells per 10^6 pooled splenocytes. (C) The ratio of the number of IFN-γ/IL-4 from each group.


A.
IFN-γ
CSCs/10^6 splenocytes
100
75
50
25
0
PBS
null
VP7
7Sc
7Anc
D.
IgG2a
ASCs/10^6 splenocytes
100
75
50
25
0
PBS
null
VP7
7Sc
7Anc
B.
IL-4
CSCs/10^6 splenocytes
50
40
30
20
10
0
PBS
null
VP7
7Sc
7Anc
E.
IgG1
ASCs/10^6 splenocytes
100
75
50
25
0
PBS
null
VP7
7Sc
7Anc
C.
IFN-γ/IL-4 ratio
5
4
3
2
1
0
PBS
null
VP7
7Sc
7Anc
BRV
F.
IgG
ASCs/10^6 splenocytes
125
100
75
50
25
0
PBS
null
VP7
7Sc
7Anc

the null plasmid (pSLIA) also enhanced responses, particularly IFN-γ production (Fig. 5.3.7, A), when compared with the group that received PBS at the time of boosting. There was no increased serum antibody titre in any group except the group that received BRV at the time of boosting (data not shown). Results from the boosting experiment also indirectly indicated that the VP7 proteins expressed by these plasmids were recognized by the BRV-primed immune system, suggesting that the proteins were expressed, *in vivo*. There were no differences among groups when assaying the immune responses present in the draining lymph nodes. The fact that mice were primed and boosted by different routes may have affected cell trafficking and compartmentalization of the immune cells.

Conclusion

Based on previous findings and publications, it was hypothesized that the poor immunogenicity of the BRV-VP7 protein may involve several factors including poor gene expression, improper antigen localization, and instability of the newly synthesized VP7 in the absence of other viral proteins. In an attempt to circumvent these problems, several plasmids were constructed. The plasmids were shown to express the appropriate VP7 and modified VP7 proteins, *in vitro*. Unfortunately, none of the plasmids induced antibody responses in mice after 2 immunizations. However, pSLIA-VP7 and pTPA-VP7Anc primed the immunized mice, and boosted a BRV-primed humoral response. Some plasmids also primed immunized mice for IFN-γ production and cytotoxic activity. The boosting experiments suggest that the proteins expressed by these plasmids were recognized by the BRV-primed immune system. Each form of antigen induced a different pattern of antibody and cytokine responses.

In summary, the level of antigen expression, stability, and cellular compartmentalization appears to affect the immunogenicity of the VP7 protein. However, none of the plasmids were able to induce BRV-specific immune response following 2 immunizations.

5.3.2 Co-administration of plasmids encoding VP4 and VP7 proteins

Based on previous findings, I tried to enhance the antigenicity of VP7 through several genetic modifications. However, none of the plasmids induced detectable immune response following two immunizations. We have previously shown that plasmid encoding VP4 protein could prime mice for both antibody and cell-mediated immunity, possibly by activating T helper cells (Suradhat *et al.*, 1997). In the following experiment, we addressed if plasmid encoding VP4 could provide "help" and subsequently improve the BRV-specific immune response by co-administration of plasmids encoding VP4 and VP7 at the time of immunization.

Although pTPA-VP7Sc did not induce or prime for an antibody response, it was chosen because of its priming ability for cell-mediated immunity. In addition, it had less toxicity on the bacterial hosts and induced a high level of protein expression in mammalian cells (personal observation). C3H mice were used in this experiment to verify that the effect of plasmid encoding VP7 on the immune system was not strain specific. In addition, C3H mice had been shown to give a stronger antibody response to plasmid immunization when compared to other strains, at least in the gD system (Dr. P. J. Lewis, personal communication).

Mice were intradermally immunized twice at a 3-week interval using a mixture of 10 μg each of pSLIA-VP4 and pTPA-VP7Sc. Mice were then boosted, intraperitoneally at week 11, with 50 μg of BRV and then sacrificed for immunological assays 10 days

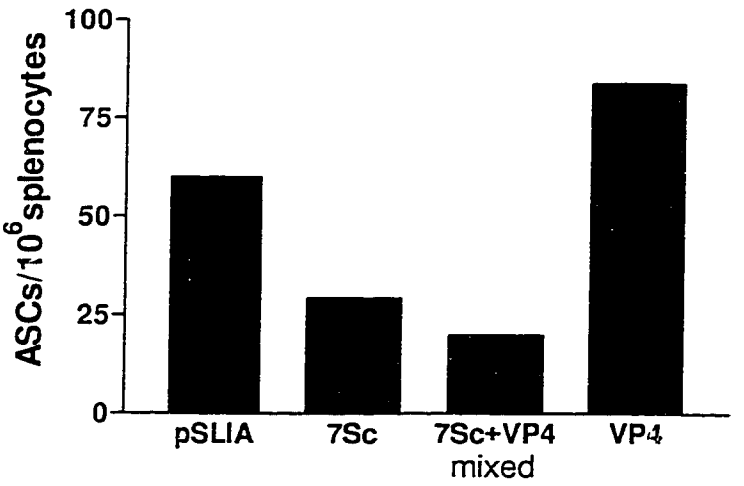
later. No seroconversion was observed in any group after two immunizations. Furthermore, the number of BRV-specific ASCs in the spleen of animals immunized with both plasmids was the lowest among the groups receiving BRV boost. In contrast, the mice immunized with pSLIA-VP4 were still primed for an antibody response (Fig. 5.3.8, A). There was no conclusive result of the cytokine production due to high variation within the same group.

The second experiment was repeated using the same immunization protocol as above. In addition, a group was immunized with the two plasmids at two separate areas. This group was included to rule out the possibility that there was inhibition of plasmid uptake with the 2 plasmids. Again, there was no seroconversion observed after immunization. Both groups immunized with two plasmids contained a lower number of BRV-specific ASCs following viral boost (Fig. 5.3.8, B). The priming of cytokine production was inconclusive due to a high variation. It should be noted that in the BRV system, C3H mice had a higher serum background and high variation within the groups when compared with the C57Bl/6 or Balb/c strains.

Conclusion

Co-administration of plasmids encoding VP4 and VP7Sc proteins did not enhance the level of BRV-specific antibody response. In addition, it ablated the priming ability of the plasmid encoding VP4 protein. The C3H mice exhibited high variations within group and did not give a better response when compared with the C57Bl/6 or Balb/c strains. Nevertheless, the results obtained from these experiments indicated that the effects of plasmids that encoded for the VP4 and VP7Sc proteins on the immune system were not strain specific.

A.



B.

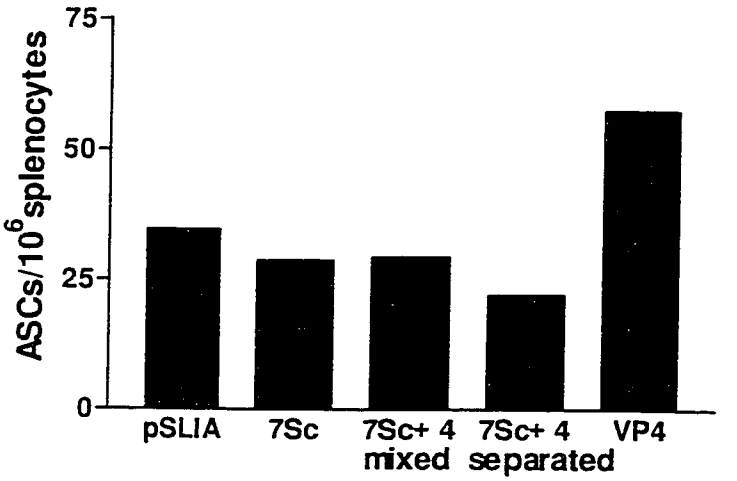


Figure 5.3.8 Effect of co-administration of plasmids encoding VP4 and VP7Sc on the BRV-specific immune response of C3H mice boosted with BRV.

C3H mice were intradermally immunized twice at a 3-week interval with 10 μg of pSLIA, pTPA-VP7Sc (7Sc), pSLIA-VP4 (VP4), or a combination of 10 μg of pSLIA-VP4 and 10 μg of pTPA-VP7Sc (7Sc+VP4 or 7Sc+4). All mice were then intraperitoneally boosted with 50 μg of BRV at week 11, and then sacrificed for immunological assays 10 days later. Figures A and B were results from 2 separate experiments. Data represent the mean (of triplicate wells) number of BRV-specific IgG-ASC per 10^6 cells from pooled splenocytes of 5 mice.

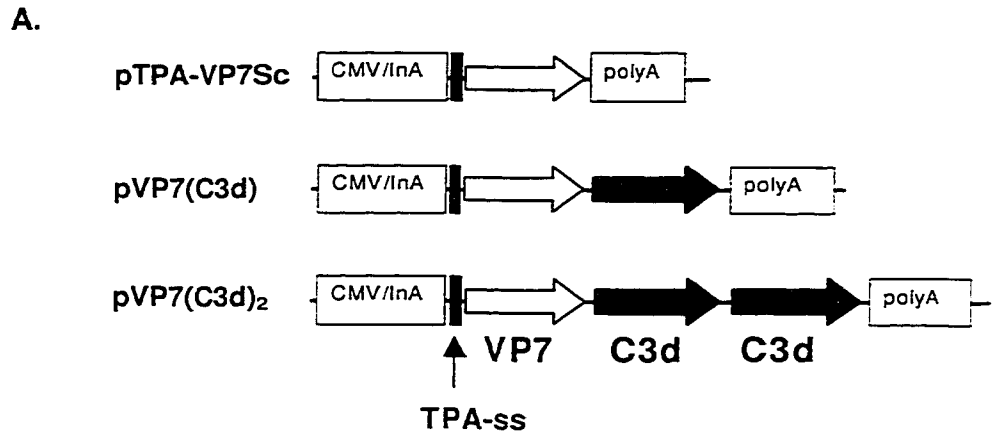
5.3.3 Co-expression of the complement C3d molecule with VP7 protein

The complement protein C3d has been shown to have a strong adjuvant effect, possibly by targeting the antigen to cells that express the C3d receptor, in particular B-cells and follicular dendritic cells. Recombinant hen egg lysozyme (HEL) bearing two and three copies of C3d is 1,000- and 10,000- fold more immunogenic, respectively, than HEL alone (Dempsey *et al.*, 1996). Additionally, C3d-conjugated anti-idiotypic antibodies effectively increased the immunogenicity of an anti-idiotype vaccine in mice (Lou & Kohler, 1998). In this study, we assessed if the C3d molecule could enhance the immunogenicity of the VP7 protein when co-expressed as chimeric proteins by the same plasmid.

5.3.3.1 *In vitro* characterization of plasmids encoding VP7-C3d chimeric proteins

The clone containing the C3d-cDNA, previously described by Wetsel and co-workers (Wetsel *et al.*, 1984), was purchased from ATCC. The details for plasmid construction are described in the Materials and Methods. Figure 5.3.9 (A) depicts a simplified diagram of the expression cassettes of plasmids encoding the chimeric proteins, pVP7(C3d) and pVP7$(C3d)_2$. The expression of the chimeric proteins was confirmed by transfection of COS-7 cells with the plasmids followed by radiolabelling and immunoprecipitation using rabbit anti-BRV antiserum. An autoradiograph revealed that both plasmids expressed chimeric proteins of the expected molecular weights, and the proteins were secreted into the medium as predicted (Fig. 5.3.9, B).

The biological activities of the C3d molecule on the chimeric proteins were tested with two separate bioassays. To test if the C3d molecule of the chimeric proteins could still bind to the cellular receptor (CD21), ileal Peyer's patch cells (>95% immature



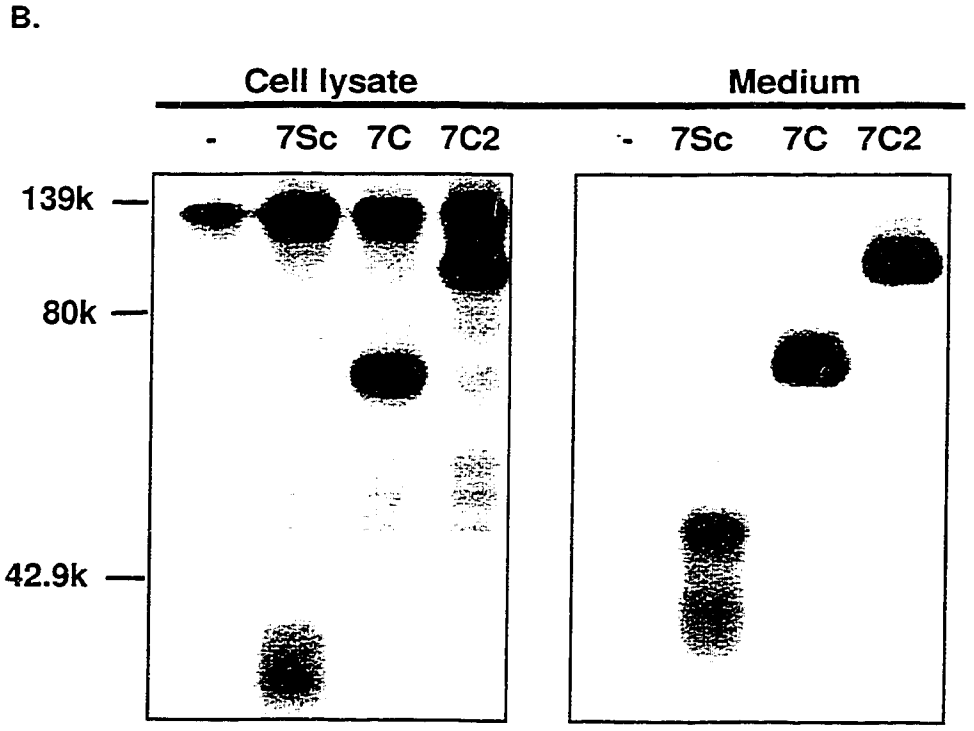


Figure 5.3.9 Characteristics and *in vitro* expression of plasmids encoding VP7-C3d chimeras.

A. Diagram depicted the expression cassettes of pTPA-VP7Sc, pVP7(C3d) and pVP7(C3d)$_2$

B. COS-7 cells transfected with pSLIA (-), pTPA-VP7Sc (7Sc), pVP7(C3d); (7C), pVP7(C3d)$_2$; (7C2) were radiolabelled with [^{35}S]-methionine. Radiolabelled proteins were recovered by immunoprecipitation using rabbit anti-BRV antiserum.

B-cells) were incubated with supernatants from COS-7 cells transfected with plasmids encoding the chimeric proteins. To assess whether the chimeric protein VP7-C3d or VP7-$(C3d)_2$ bound to the cellular receptor through the C3d molecule, an antibody recognizing the VP7 protein was used to detect bound VP7-C3d and VP7-$(C3d)_2$. Briefly, after the cell incubation was completed, cells were washed and incubated with rabbit anti-BRV antiserum followed by FITC conjugated goat anti-rabbit IgG antibodies. Cell labelling was detected by flow cytometry. It should be noted that the binding affinity of C3d with CD21 is very low, and usually could not be detected by flow cytometer (Dr. W. R. Hein, personal communication). However, by co-expressing one or two copies of the C3d molecule in conjunction with the VP7 protein, we hoped to be able to increase the binding affinity and detect the presence of the proteins bound on the cell surface. Figure 5.3.10 shows the flow cytometric analysis from the above experiment. We found an increased percentage of FITC-labelled cells in the population that were incubated with the supernatant from pVP7$(C3d)_2$ transfected cells, but not with pVP7(C3d) or control supernatant. This indicates that the chimeric protein was bound to a cellular receptor and that chimeric protein, containing two copies of the C3d molecule, bound to the cell surface with higher affinity.

The crucial role of C3d-CD21 binding to the survival of immature B cells, possibly by providing a second co-stimulatory signal, is well documented (Carroll & Fischer, 1997, Tew *et al.*, 1997). Based on this knowledge, we sought to determine if C3d molecules, on the chimeric proteins, still retained the ability to rescue immature B cells from single-signal induced apoptosis. To achieve this objective, sheep ileal Peyer's patch cells were cultured for 96 hr with media from COS-7 cells, transfected with the plasmids encoding VP7-C3d chimeras, and co-stimulated with $F(ab')_2$ rabbit anti-sheep Ig. The $F(ab')_2$ rabbit anti-sheep Ig was used as the first signal for polyclonal B cell activation *in vitro*. Therefore, the survival of B cells in this system would rely on

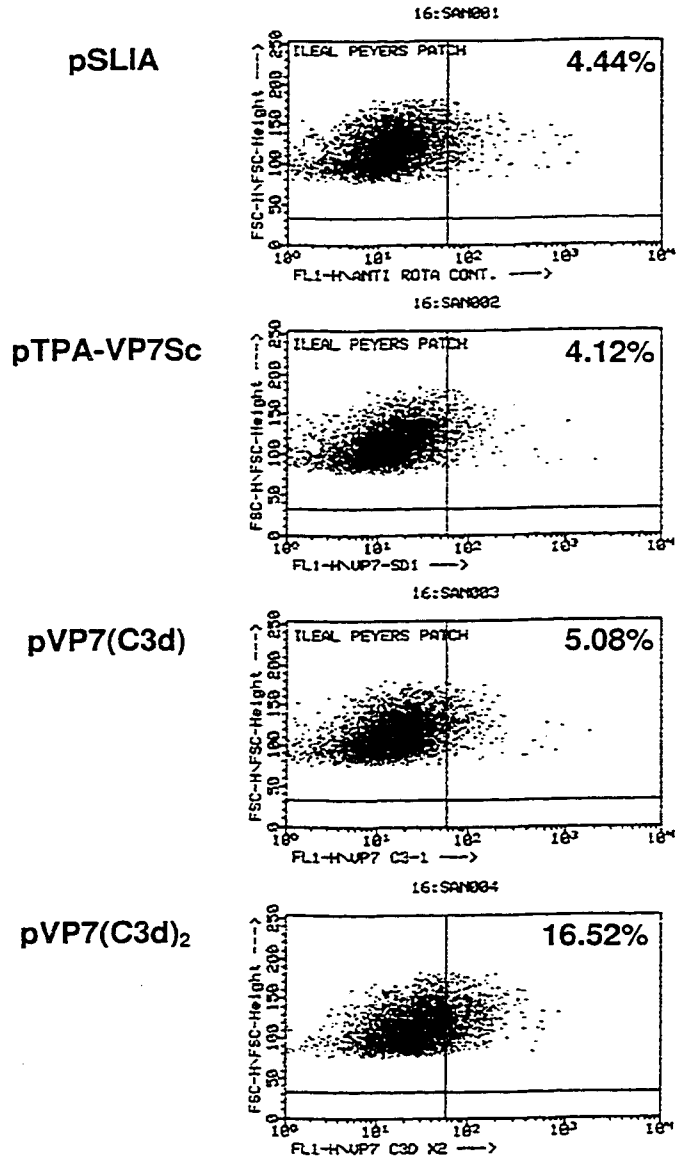


Figure 5.3.10 Binding of VP7-C3d chimeric protein to B cells

Ileal Peyer's patch cells (>95% B-cells) were incubated with supernatants from COS-7 cells transfected with pSLIA, pTPA-VP7Sc, pVP7(C3d), or pVP7(C3d)$_2$. After incubation was completed, cells were washed and incubated with rabbit anti-BRV antiserum followed by FITC conjugated goat anti-rabbit IgG antibodies. The labelled cells were then analysed with the flow cytometer. The number on the top right corner represents the % of positive cells.

the availability of the co-stimulatory signal to rescue them from single-signal induced apoptosis. The lymphoproliferative activity of cells incubated with VP7-C3d was significantly different from that of the control group ($p<0.05$, one-way ANOVA followed by Tukey's test), suggesting that the product from the pVP7(C3d) was able to rescue a proportion of the cells from apoptosis (Fig. 5.3.11). Interestingly, the VP7-$(C3d)_2$ chimera, which displayed better affinity binding in the previous assay (Fig. 5.3.10) did not induce better cell survival than the control VP7Sc (Fig. 5.3.11).

5.3.3.2 Immune responses induced by plasmids encoding VP7-C3d chimeric proteins

Following *in vitro* characterization, the ability of the VP7-C3d chimeric proteins to induce and/or enhance the BRV-specific immune responses was tested in the following study. C57Bl/6 mice were immunized twice (i.d. or by gene gun) at a 3-week interval. The dose for intradermal immunization was 10 μg/dose. For gene gun immunization, mice were immunized with a 1 μg/dose. Mice were then boosted with 50 μg of BRV at week 7 and sacrificed 10 days later.

No seroconversion was observed after 2 immunizations, regardless of the immunization method. Following the viral boost, an enhanced antibody response was not observed in animals immunized by either route (Fig. 5.3.12). Furthermore, no priming of cytokine production was observed. In fact, the number of IFN-γ producing cells was significantly lower in the spleens of mice immunized with pVP7$(C3d)_2$ than those immunized with the control plasmid or pTPA-VP7Sc, ($p<0.05$), as judged by one-way ANOVA followed by Tukey's test, (Fig. 5.3.13).

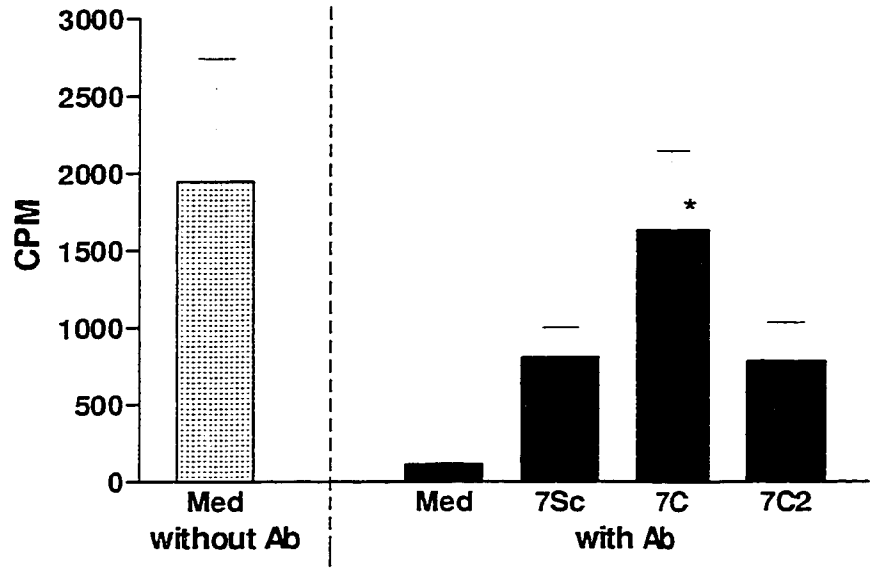
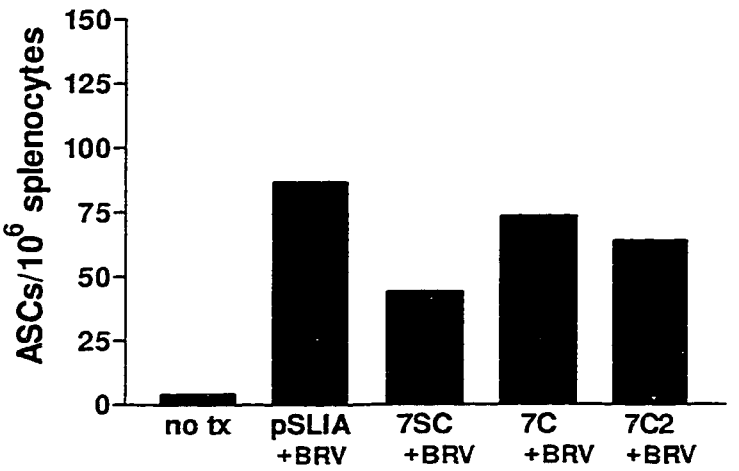


Figure 5.3.11 Capacity of the VP7-C3d chimera to rescue B cells from single-signal induced apoptosis.

Ileal Peyer's patch cells were cultured for 96 hr with medium (Med) or supernatant from COS-7 cells transfected with pTPA-VP7Sc (7Sc); pVP7(C3d) (7C); or pVP7(C3d)$_2$ (7C2) in the absence (without Ab), or presence of the F(ab')$_2$ rabbit anti-sheep Ig (with Ab). Cell proliferation was assessed by [^{3}H]-thymidine incorporation. Data represent the mean of CPM (± SEM) from 6 replicate wells. * Indicates significant difference from the control (medium with rabbit anti-sheep Ig).

A.



B.

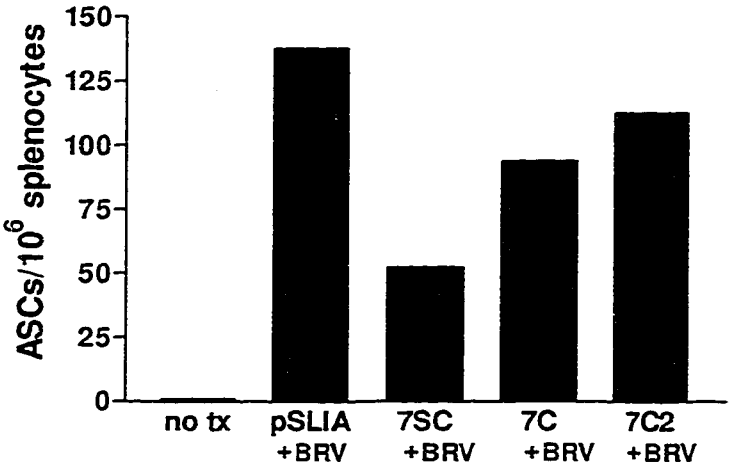
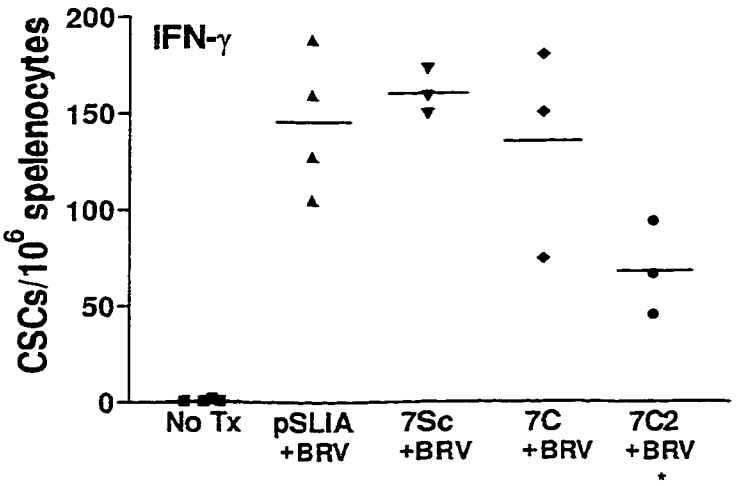


Figure 5.3.12 BRV-specific antibody responses in mice immunized with plasmids encoding VP7-C3d chimeras and then boosted with BRV.

C57Bl/6 mice were immunized twice at a 3-week interval with 10 μg/dose, intradermally (A) or 1 μg/dose by gene gun (B). The plasmids used for immunizations included pSLIA, pTPA-VP7Sc (7Sc), pVP7(C3d) (7C), and pVP7(C3d)$_2$ (7C2). At week 7, mice were boosted with 50 μg of BRV intraperitoneally. Naïve mice were included as a negative control (no tx).

BRV-specific IgG-ASCs in spleens after BRV boost determined by ELISPOT assay. Data represent the mean (of triplicate wells) number of ASCs per 10^6 cells from pooled splenocytes of 4 mice.

A.



B.

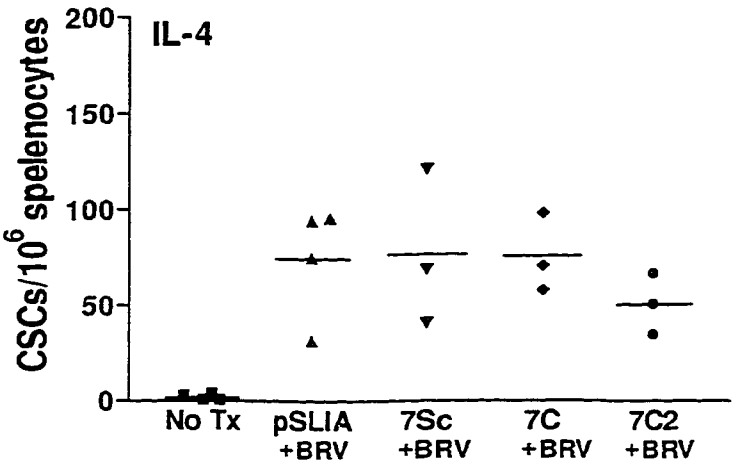


Figure 5.3.13 BRV-specific cytokine responses in mice immunized with plasmids encoding VP7-C3d chimeras and then boosted with BRV.

C57Bl/6 mice were intradermally immunized twice at a 3-week interval with 10 μg/dose of pSLIA, pTPA-VP7Sc (7Sc), pVP7(C3d) (7C), or pVP7(C3d)$_2$ (7C2). At week 7, mice were boosted with 50 μg of BRV intraperitoneally. Naïve mice were included as a negative control (no tx).

Number or BRV-specific IFN-γ (A), and IL-4 (B) secreting cells determined by ELISPOT assay. Data represent the number of CSCs per 10^6 splenocytes from individual mice. The solid line represents the mean value from each group. * Indicates significant difference from the control and 7Sc groups ($p<0.05$).

5.3.3.3 Immune responses induced by pVP7(C3d)$_2$ in BRV-primed mice

Since the plasmids encoding VP7-C3d chimeras did not induce a primary BRV-specific immune response, their ability to induce a secondary immune response in BRV-primed mice was assessed. The boosting protocol described in section 5.3.1.3 was used. The pVP7(C3d)$_2$ did not boost the immune response in the BRV-primed mice (Fig. 5.3.14). In addition, the number of IFN-γ secreting cells in the mice immunized with the plasmid was suppressed when compared with the control group (Fig. 5.3.14, B).

5.3.3.4 BHV-1-gD specific immune responses in mice immunized with plasmids encoding tgD-C3d chimeras

To determine if the above finding was specific to VP7, the effect of C3d on the immune response to gD of BHV-1 was investigated. The plasmids, pSLIAtgD(C3d) and pSLIAtgD(C3d)$_2$, were gifts from Dr. Ralph Braun. The pSLIAtgD(C3d) and pSLIAtgD(C3d)$_2$ encoded chimeric proteins of truncated gD (tgD) in conjunction with a one or two copies of the C3d molecule, respectively. The plasmids were shown to express comparable amounts of the expected protein products in transfected COS-7 cells, and the proteins were secreted into the media (Dr. R. Braun, personal communication).

C57Bl/6 mice were intradermally immunized, in the dorsal area, twice at a 3-week interval with 5 μg of plasmids. Serum samples were collected at wk 0,3, 5 and 7. Spleen and draining lymph nodes were collected at the time of killing (wk 7).

Although immunized mice displayed anti-gD antibody responses after the first immunization (wk 3), the groups immunized with plasmids encoding the chimeric proteins, tgD(C3D) and tgD(C3d)$_2$, had significantly lower serum antibody titres

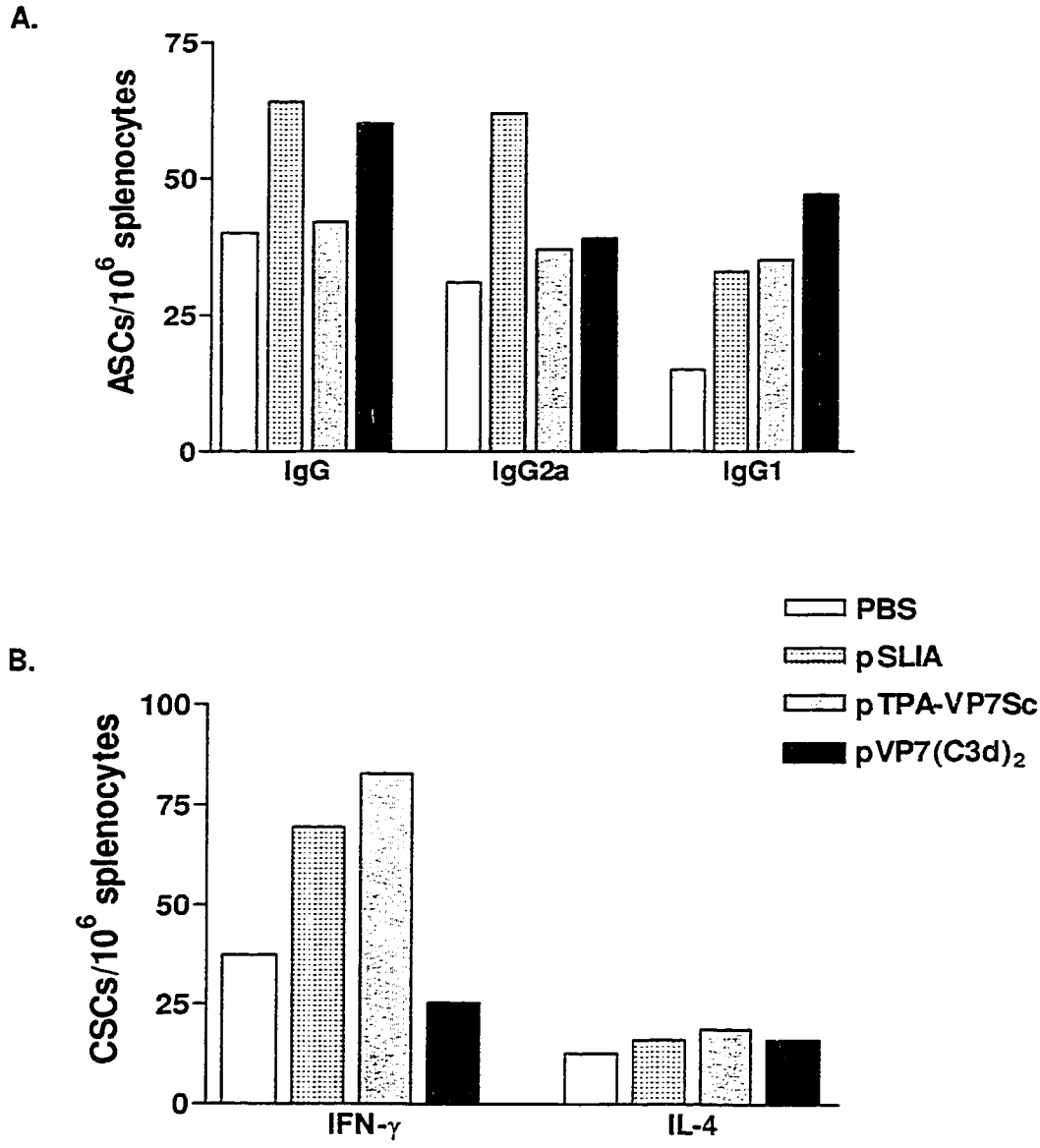


Figure 5.3.14 BRV-specific immune responses in BRV-primed mice boosted with plasmid encoding a VP7-C3d chimera

C57Bl/6 mice were intraperitoneally injected with 50 μg of BRV. At week 4, mice were intradermally boosted with 20 μl of PBS, or 10 μg of plasmids as indicated in legends. Mice were sacrificed for immunological tests 12 days after the boost.

Number of BRV-specific ASCs (A), or CSCs (B) determined by ELISPOT assay. Data represent the mean (of triplicate wells) number of antibody or cytokine secreting cells per 10^6 pooled splenocytes.

($p<0.01$) as judged by one-way ANOVA followed by Tukey's test (Fig. 5.3.15). Following the second immunization, wk 5 and 7, the antibody responses were improved but still significantly lower than that of pSLIAtgD immunized group, as judged by the above tests ($p<0.05$), (Fig. 5.3.15). This finding indicated that addition of the C3d molecule significantly delayed and inhibited the anti-gD antibody production. In addition, there was no difference between the antibody responses induced by pSLIAtgD(C3d) and pSLIAtgD$(C3d)_2$.

Cytokine production in the spleen and draining lymph nodes following 2 immunizations were assessed at 4 weeks after the second immunization (wk 7) by ELISPOT assay. Plasmids encoding gD-C3d chimeras, pSLIAtgD(C3d) and pSLIAtgD$(C3d)_2$, slightly inhibited cytokine responses in spleens of immunized mice when comparing to mice immunized with pSLIAtgD (Fig. 5.3.16, A-B). Furthermore, the number of gD-specific cytokine secreting cells in the DLN of these mice were also lower than those from mice immunized with pSLIAtgD (Fig. 5.3.16, C). Similar results were obtained in a separate experiment (data not shown). The data from this experiment clearly indicated that addition of the C3d molecule to the gD antigen did not enhance antigen-specific immune responses following DNA immunization.

Conclusion

The plasmids encoding VP7-C3d chimeras were cloned and characterized. Both pVP7(C3d) and pVP7$(C3d)_2$ expressed the chimeric protein with the expected molecular sizes. In addition, the C3d-bioassays suggested that the chimeric proteins retained the biological activity of the C3d molecule. Results from the *in vivo* experiments indicated that the plasmids did not induce, prime or boost a BRV-specific immune response. In addition, suppression of the number of BRV-specific IFN-γ

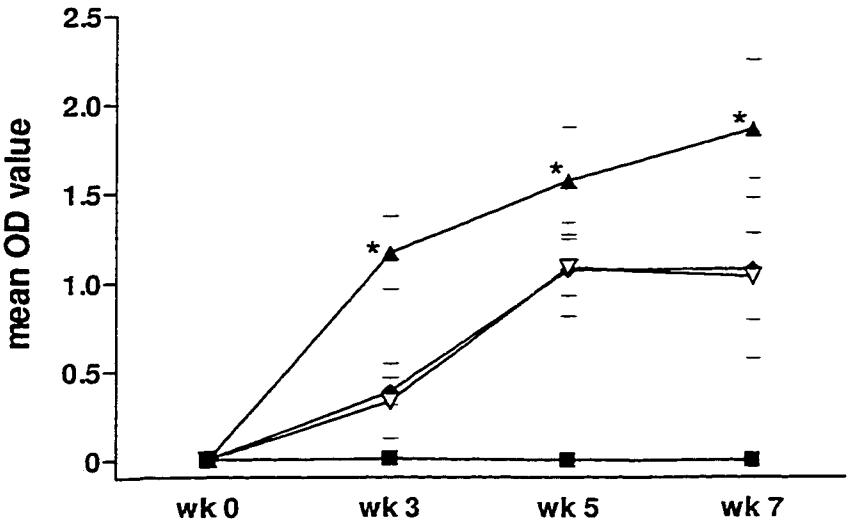


Figure 5.3.15 BHV-1 gD-specific antibody response in mice immunized with plasmids encoding gD-C3d chimeras.

C57Bl/6 mice were intradermally immunized twice (wk 0, wk 3) with 5 μg of pSLIA (■), pSLIAtgD (▲), pSLIAtgD(C3d);(▽), or pSLIAtgD(C3d)$_2$; (◆). Data represent the gD-specific serum antibody levels at 1/640 dilution (mean ± SD of 5 mice) measured by ELISA. * Indicates significant difference from other groups (see text).

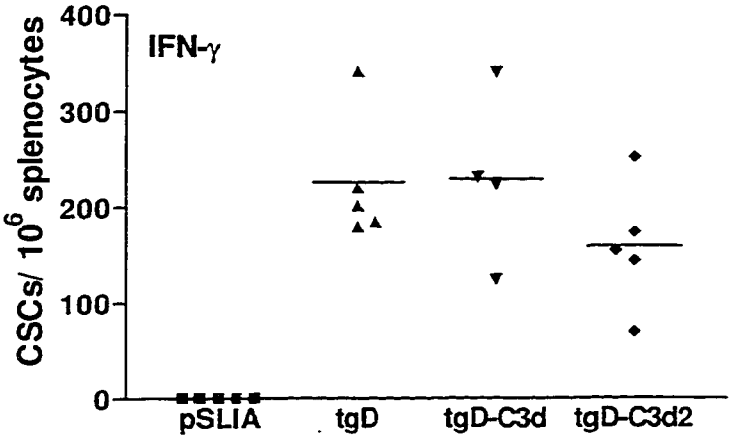
Figure 5.3.16 BHV-1 gD-specific cytokine responses in mice immunized with plasmids encoding gD-C3d chimeras.

C57Bl/6 mice were intradermally immunized twice (wk 0, wk 3) with 5 μg of pSLIA, pSLIAtgD (tgD), pSLIAtgD(C3d) (tgD-C3d), or pSLIAtgD$(C3d)_2$ (tgD-C3d2). Mice were sacrificed one month after the second immunization (wk 7).

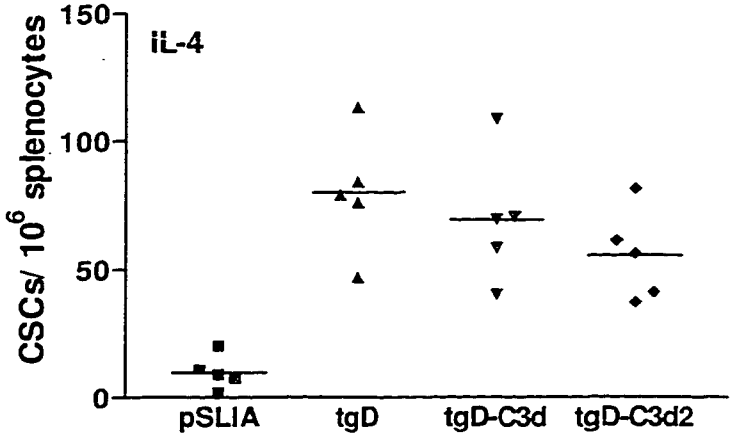
A, B. gD-specific CSCs in spleens determined by ELISPOT assay. Data represent the number of IFN-γ (A), and IL-4 (B) secreting cells per 10^6 splenocytes from individual mouse. Solid line represents the mean value from each group.

C. gD-specific CSCs in draining lymph nodes determined by ELISPOT assay. Data represent the mean (of triplicate wells) number of CSCs per 10^6 cells from pooled lymphocytes isolated from draining lymph nodes (5 mice/group).

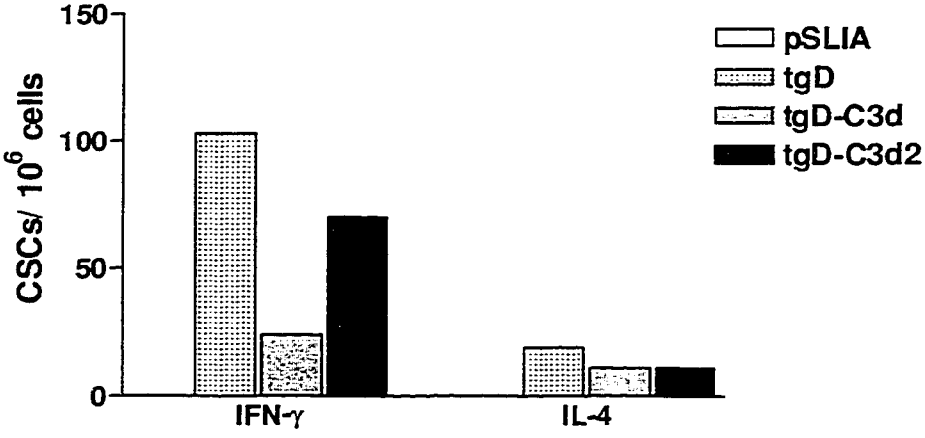
A.


IFN-γ
CSCs/ 10^6 splenocytes
400
300
200
100
0
pSLIA
tgD
tgD-C3d
tgD-C3d2

B.


iL-4
CSCs/ 10^6 splenocytes
150
100
50
0
pSLIA
tgD
tgD-C3d
tgD-C3d2

C.


CSCs/ 10^6 cells
150
100
50
0
IFN-γ
IL-4
pSLIA
tgD
tgD-C3d
tgD-C3d2

secreting cells was consistently observed in both experiments.

In a separate system, plasmids encoding gD-C3d chimeras inhibited gD-specific antibody production, and did not enhance gD-specific cytokine responses in immunized mice. Therefore, co-expression of the C3d molecule with antigen did not enhance but rather suppressed antigen-specific immune responses.

5.3.4 Altering the VP7 molecule by deletion mutation

Deletion mutation is one of several approaches that have been used for investigating the functional properties and immunogenicity of proteins. Differences between the immune responses induced by the wild type and mutant proteins may help identify part(s) of the protein that are critical in influencing the induction of antigen-specific immune response. Although several strategies were employed in this study to enhance immune responses to VP7 expressing plasmids, we were not able to effectively induce a humoral immune response using these approaches. The following experiments addressed whether alteration of the VP7 molecule by deletion mutation could influence the outcome of an immune response in immunized animals.

5.3.4.1 *In vitro* characterization of plasmid encoding truncated VP7

A plasmid encoding truncated VP7 (pVP7t) was cloned by altering the *Cla* I site located in the middle of the VP7 gene in pTPA-VP7Sc (see Materials and Methods). The pVP7t contained almost the same DNA sequence when compared with pTPA-VP7Sc but expressed a shorter version of the VP7 protein due to a frame-shift mutation. The VP7t protein contained the first 132 amino acid from the original VP7 protein (275 amino acids long), with an additional 14 amino acids that resulted from the frame-shift prior to the first available stop codon. A simplified diagram of the construction process is shown in Figure 5.3.17, A. Transient protein expression was confirmed by transfection of the plasmid pVP7t in COS-7 cells followed by radiolabelling and immunoprecipitation using the rabbit anti-BRV antiserum. The autoradiograph revealed that pVP7t expressed the truncated protein with the expected molecular weight (Fig. 5.3.17, B). Although similar amounts of protein were seen in the cellular

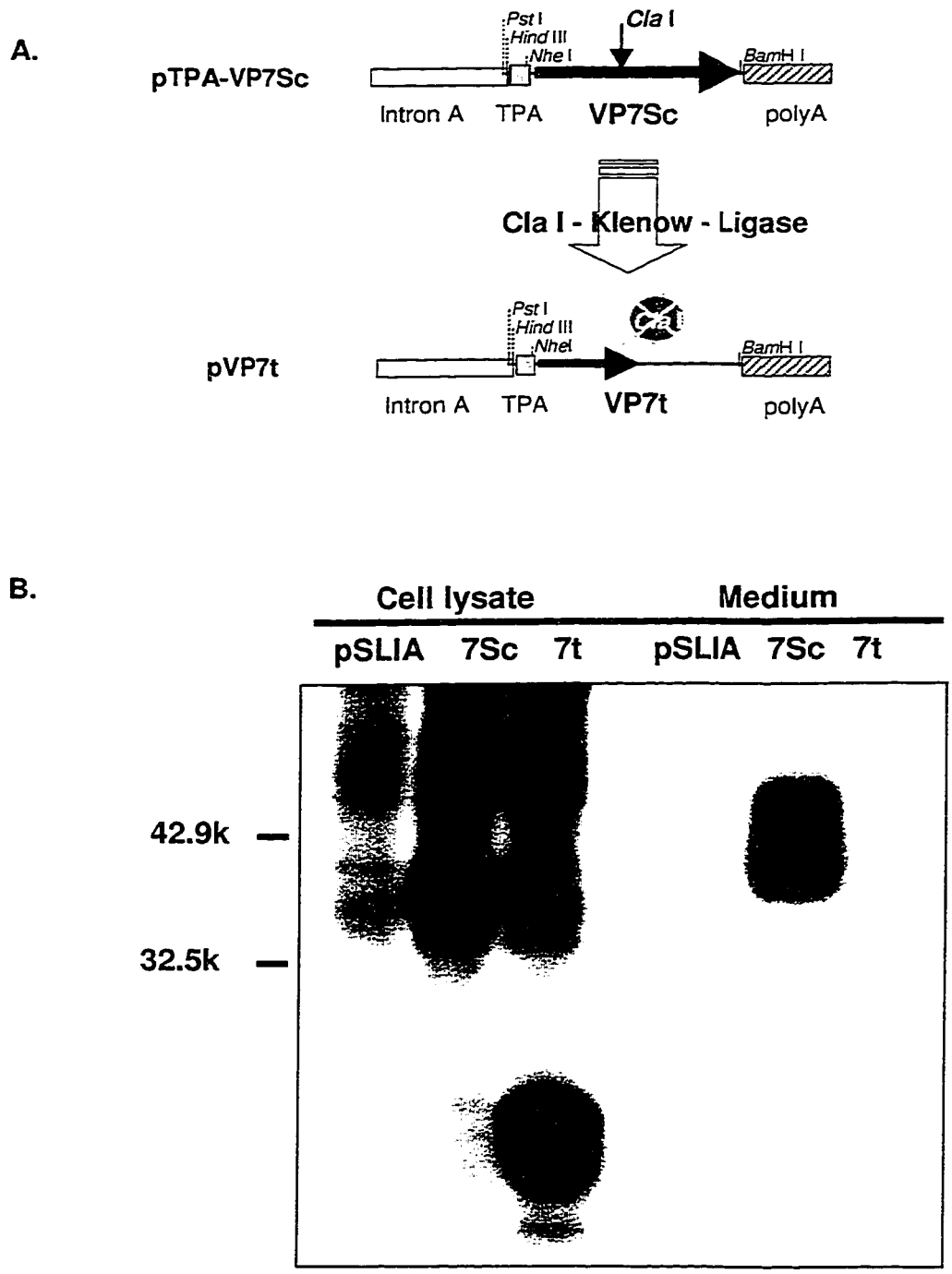


Figure 5.3.17 Characteristics and *in vitro* expression of pVP7t

A. Diagram depicting the cloning process for pVP7t (see text)

B. COS-7 cells transfected with pSLIA, pTPA-VP7Sc (7Sc) and pVP7t (7t) were radiolabelled with [^{35}S]-methionine. Radiolabelled proteins from cell lysates and media were then recovered by immunoprecipitation using rabbit anti-BRV antiserum.

compartment, there was much less VP7t secreted into the media, when compared with the VP7Sc. The reason for this reduced secretion may be due to a decreased stability of the smaller protein, with more rapid degradation. Alternatively, the protein may not be efficiently transported through the secretory compartment of the transfected cells.

5.3.4.2 Immune responses induced by plasmid encoding truncated VP7

To determine if pVP7t could induce rotavirus-specific immune responses, C57Bl/6 mice were intradermally immunized with 10 μg of pVP7t twice at a 3-week interval. Mice were then intraperitoneally boosted with 50 μg of BRV at week 10 and sacrificed 10 day later. No seroconversion was observed after 2 plasmid immunizations. Furthermore, priming of the antibody response was not observed after the viral boost (data not shown). However, the spleens from mice immunized with pVP7t contained a significantly higher number of IL-4 secreting cells than other groups (Fig. 5.3.18, A) and as a result, a significantly lower IFN-γ/IL-4 ratio (Fig. 5.3.18, B), as judged by one-way ANOVA followed by Tukey's test ($p<0.05$). The average IFN-γ/IL-4 ratio from the group immunized with pVP7t was less than 1. Such a low ratio was not observed following immunization with other constructs.

5.3.4.3 Immune responses induced by pVP7t in BRV-primed mice

The ability of plasmids encoding VP7t to boost the BRV-primed immune system was assessed using the protocol described in section 5.3.1.3. Although the pVP7t did not induce or prime the antibody response, pVP7t exhibited a strong boosting effect on both antibody (Fig. 5.3.19, A), and cytokine, in particular IL-4, responses (Fig. 5.3.19,

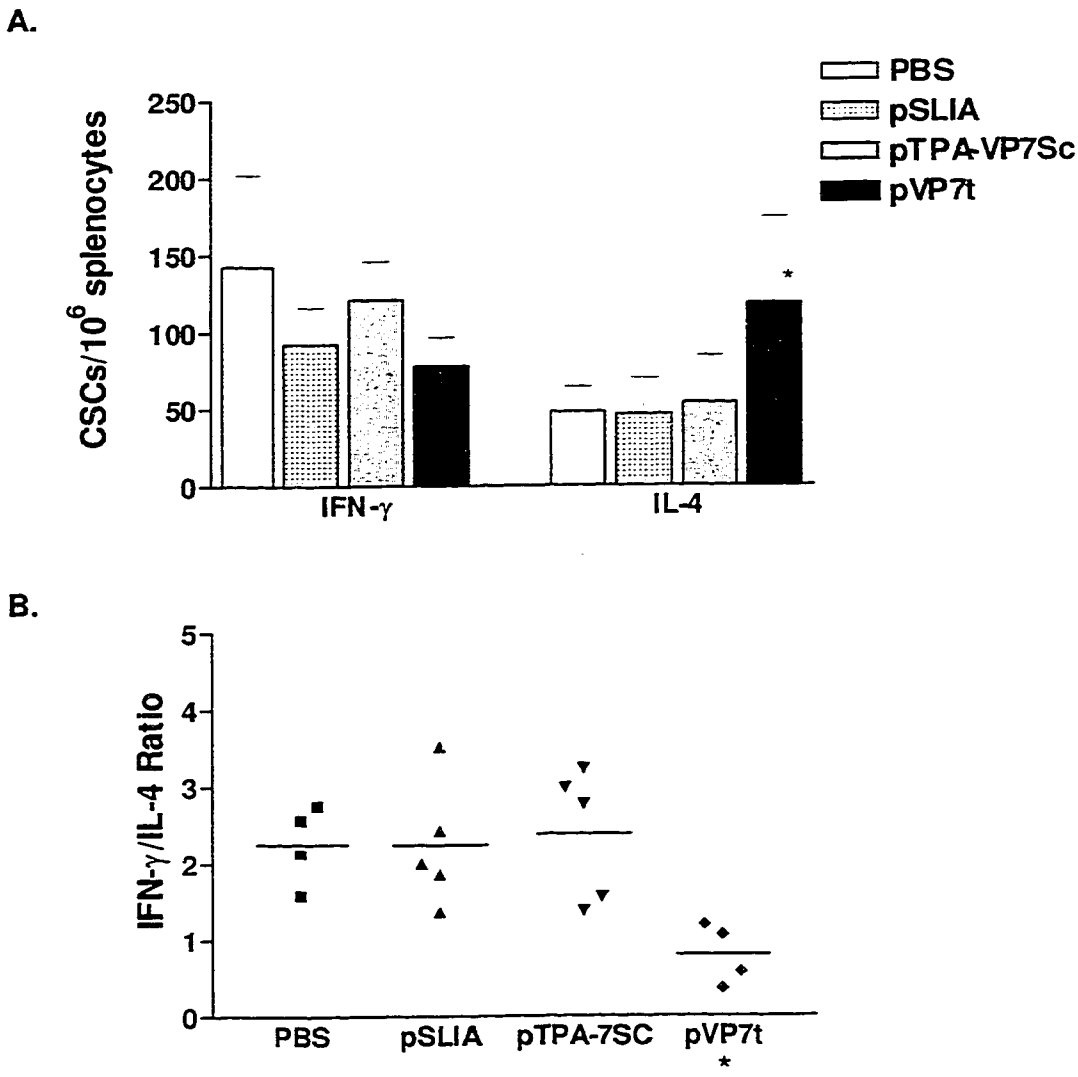


Figure 5.3.18 BRV-specific cytokine responses in mice immunized with pVP7t and then boosted with BRV.

C57Bl/6 mice were intradermally immunized twice at a 3-week interval with PBS, or 10 μg of pSLIA, pTPA-VP7Sc, pVP7t. Mice were intraperitoneally boosted with 50 μg of BRV at week 10 and then sacrificed 10 days after.

A. Number of BRV-specific CSCs determined by ELISPOT assay. Data represent the mean (±SD) number of CSCs per 10^6 splenocytes from individual mice (4-5 mice/group). * Indicates significant difference from other groups ($p<0.05$).

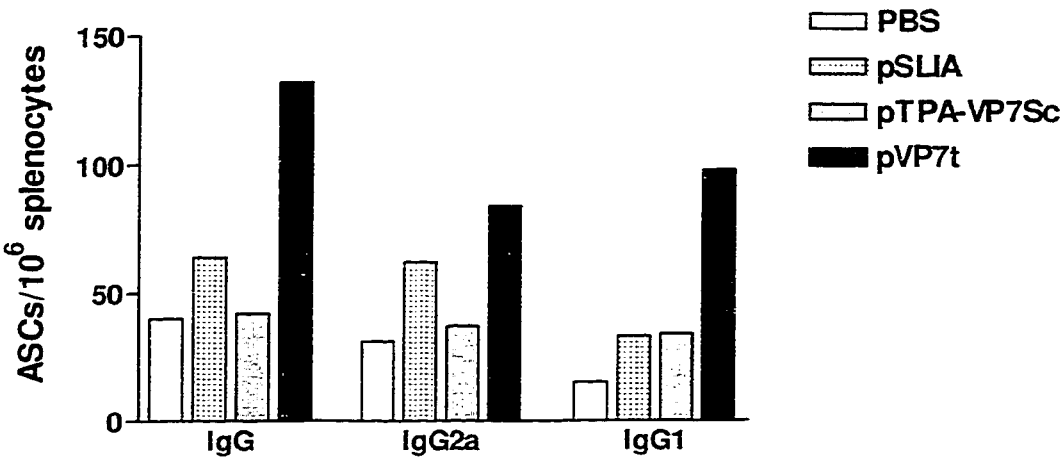
B. The ratio of IFN-γ secreting cells/IL-4 secreting cells from individual mice. * Indicates significant difference from other groups ($p<0.05$).

Figure 5.3.19 BRV-specific immune responses in BRV-primed mice boosted with pVP7t.

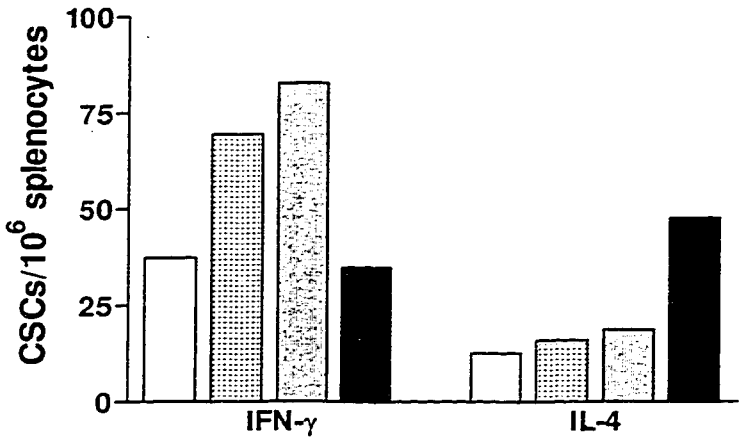
C57Bl/6 mice were intraperitoneally injected with 50 μg of BRV. At week 4, mice were intradermally boosted with 20 μl of PBS, or 10 μg of plasmid as indicated in the legends. Mice were sacrificed for immune assays 12 days after the boost.

Number of BRV-specific ASCs (A), or CSCs (B) determined by ELISPOT assay. Data represent the mean (of triplicate wells) number of antibody or cytokine secreting cells per 10^6 pooled splenocytes. (C) The ratio of the number of IFN-γ/IL-4 secreting cells from BRV-primed mice which were boosted, i.d., with 20 μl of PBS, 10 μg of pSLIA, pTPA-7Sc (7Sc), pVP7t (7t) or 50 μg of BRV.

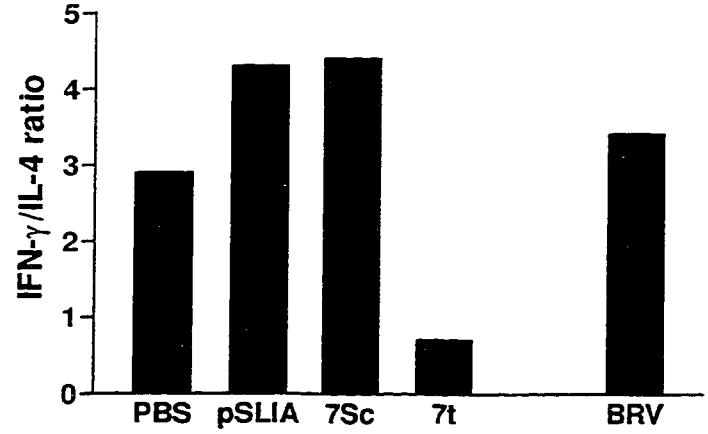
A.


PBS
pSLIA
pTPA-VP7Sc
pVP7t
ASCs/10^6 splenocytes
150
100
50
0
IgG
IgG2a
IgG1

B.


CSCs/10^6 splenocytes
100
75
50
25
0
IFN-γ
IL-4

C.


IFN-γ/IL-4 ratio
5
4
3
2
1
0
PBS
pSLIA
7Sc
7t
BRV

B). In fact, this plasmid induced the highest numbers of IgG-ASCs and IL-4 secreting cells among the plasmids tested in the boosting experiment (Fig. 5.3.25). It should be noted that the plasmid also induced a low number of IFN-γ secreting cells (Fig. 5.3.19, B) and a very low IFN-γ/IL-4 ratio when compared to other groups (Fig. 5.3.19, C). This effect was also observed in the priming experiment (Fig. 5.3.18, B).

Conclusion

The plasmid encoding truncated VP7 (pVP7t) was cloned and characterized. The pVP7t expressed truncated VP7 with a predicted molecular size but the protein was largely retained within the cellular compartment rather than secreted. The *in vivo* experiments showed that the plasmid did not induce an immune response following 2 immunizations but did prime animals for IL-4 production. In addition, pVP7t strongly boosted the BRV-primed immune response, particularly the number of IL-4 secreting cells and the antibody response. The reversed IFN-γ/IL-4 ratios, in comparison with those induced by other plasmids or BRV, were observed in both priming and boosting experiments.

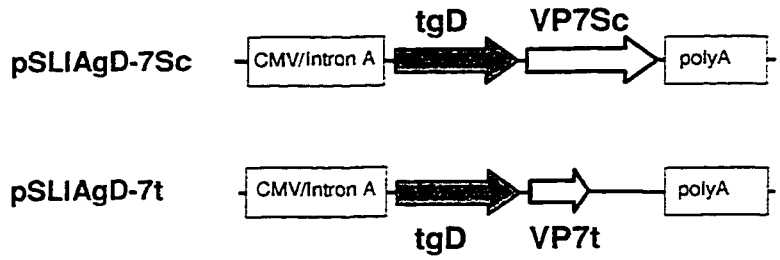
5.3.5 Co-expression of the VP7 with a second antigen (gD)

The BHV-1 gD protein is known to be an excellent antigen regardless of whether it is presented as a single protein or in the context of a whole virus (Babiuk *et al.*, 1987). Furthermore, plasmids encoding authentic gD and a secreted version of gD (truncated gD; tgD) effectively induced BHV-1 specific immune responses in various species (Babiuk *et al.*, 1995, Cox *et al.*, 1993, Lewis *et al.*, 1997, van Drunen Littel-van den Hurk *et al.*, 1998). The finding that tgD always induced a strong antibody response suggested that tgD may be a strong B cell inducer. In addition, the level of gD expression is known to be one of the highest of all the viral genes ever studied at VIDO (Dr. L. A. Babiuk, personal communication). Therefore, it was speculated that co-expression of tgD and VP7 as a chimeric protein would not only help increase the level of expression of VP7 but also improve the BRV-specific immune response by the additional "help" from gD epitopes. This part of the project addressed whether the anti-BRV immune response can be enhanced by co-expression of the VP7 protein and tgD.

5.3.5.1 *In vitro* characterization of plasmid encoding gD-VP7 chimeras

Figure 5.3.20 (A) depicts a simplified diagram of the expression cassettes of plasmids encoding chimeric proteins: tgD-VP7Sc (pSLIAgD-7Sc) and tgD-VP7t (pSLIAgD-7t). Details of plasmid construction are described in the Materials and Methods. Protein expression was confirmed by *in vitro* transient protein expression. Both plasmids expressed the chimeric protein with the expected molecular weights. The chimeric proteins were recognized by both rabbit anti-BRV antiserum and pooled anti-gD monoclonal antibodies, indicating that both proteins retained their antigenic determinants (Fig. 5.3.20, B-C). In addition, comparable amounts of the chimeric

A.



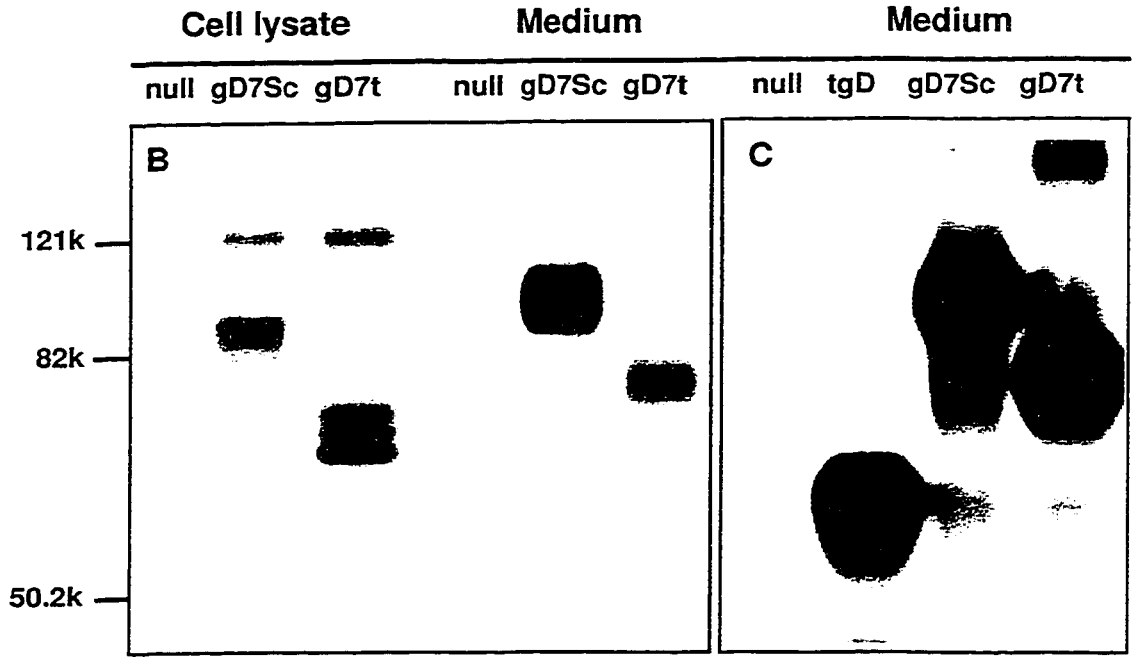


Figure 5.3.20 Characteristics and *in vitro* expression of plasmids encoding gD-VP7 chimeras.

A. Diagram depicts simplified expression cassettes of pSLIAgD-7Sc and pSLIAgD-7t

B,C. COS-7 cells transfected with pSLIA (null), pSLIAgD-7Sc (gD7Sc), pSLIAgD-7t (gD7t) or pSLIAtgD (tgD) were radiolabelled with [^{35}S]-methionine. Radiolabelled proteins in cell lysates and media were recovered by immunoprecipitation using rabbit anti-BRV antiserum (B), or pooled anti-gD monoclonal antibodies (C).

proteins, and tgD were secreted into the media (Fig. 5.3.20, C).

5.3.5.2 Immune responses induced by plasmids encoding gD-VP7 chimeras.

C57Bl/6 mice were intradermally immunized twice with 10 μg of the plasmids, at a 3-week interval. All mice were boosted, intradermally, with 50 μg of BRV at week 8 and then sacrificed for immunological assays one week later. Since the animals were intradermally immunized and boosted in the same area (dorsal skin), draining lymph nodes (axillary and inguinal) were also included in the assays.

No seroconversion was observed after 2 immunizations. After the viral boost, there was no different in the serum antibody titres among the groups. Priming of BRV-specific antibody response, in both spleen and lymph nodes, was observed in the groups immunized with the gD-VP7 chimeras (Fig. 5.3.21). The pSLIAgD-7Sc induced better antibody priming than did pSLIAgD-7t. In addition, some differences in the pattern of antibody responses between spleen and lymph node were observed (Fig.5.3.21). Draining lymph nodes contained higher numbers of BRV-specific ASCs than spleens. It should also be noted that the distribution of the antibody isotype in each organ was different. Spleens contained predominantly IgG2a-ASCs whereas IgG1 was a predominant isotype in draining lymph nodes (Fig.5.3.21, B, C). In addition, the gD-specific serum antibody titres were determined. The plasmids encoding gD-VP7Sc and gD-VP7t induced considerable levels of gD-specific antibody, and the presence of VP7 in the chimeric protein had no significant effect on the gD-specific antibody responses (Fig. 5.3.22).

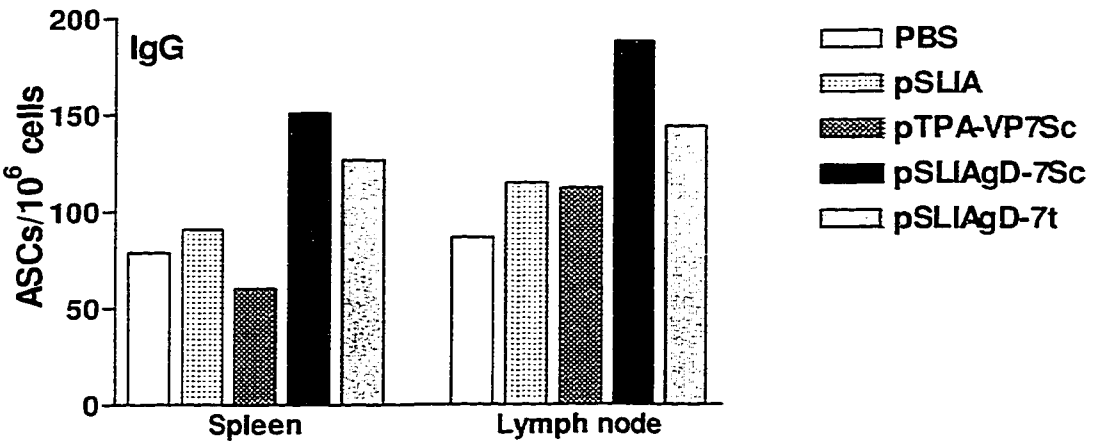
The patterns of BRV-specific BRV cytokine response following viral boost, in the spleens and DLN indicated that IFN-γ was the predominant cytokine produced in this

Figure 5.3.21 BRV-specific antibody responses in mice immunized with plasmids encoding gD-VP7 chimeras and then boosted with BRV.

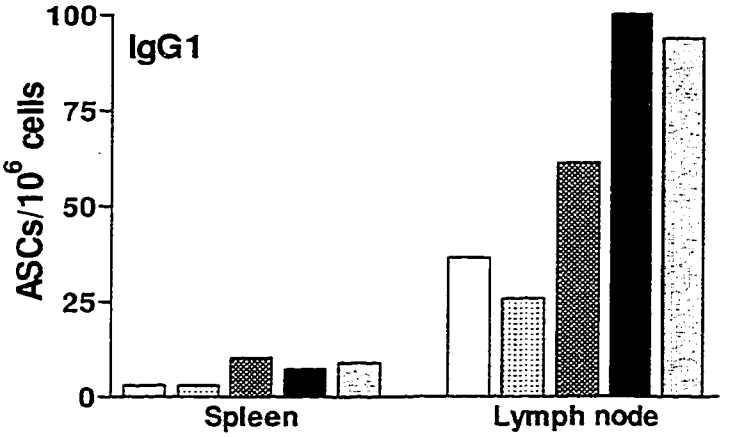
C57Bl/6 mice were intradermally immunized twice at a 3-week interval with 10 μg of the plasmids as indicated in the legends. All mice were boosted, intradermally, with 50 μg of BRV at week 8 and then sacrificed a week after the viral boost.

BRV-specific IgG (A), IgG1 (B), IgG2a (C) antibody secreting cells were determined by ELISPOT assay. Data represent the mean (of triplicate wells) number of ASCs per 10^6 cells from pooled splenocytes or lymphocytes from draining lymph nodes (5 mice/group).

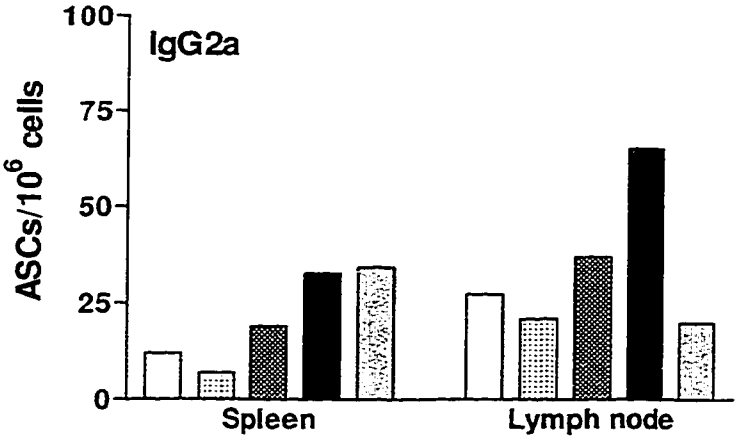
A.


IgG
ASCs/10^6 cells
200
150
100
50
0
Spleen
Lymph node
PBS
pSLIA
pTPA-VP7Sc
pSLIAgD-7Sc
pSLIAgD-7t

B.


IgG1
ASCs/10^6 cells
100
75
50
25
0
Spleen
Lymph node

C.


IgG2a
ASCs/10^6 cells
100
75
50
25
0
Spleen
Lymph node

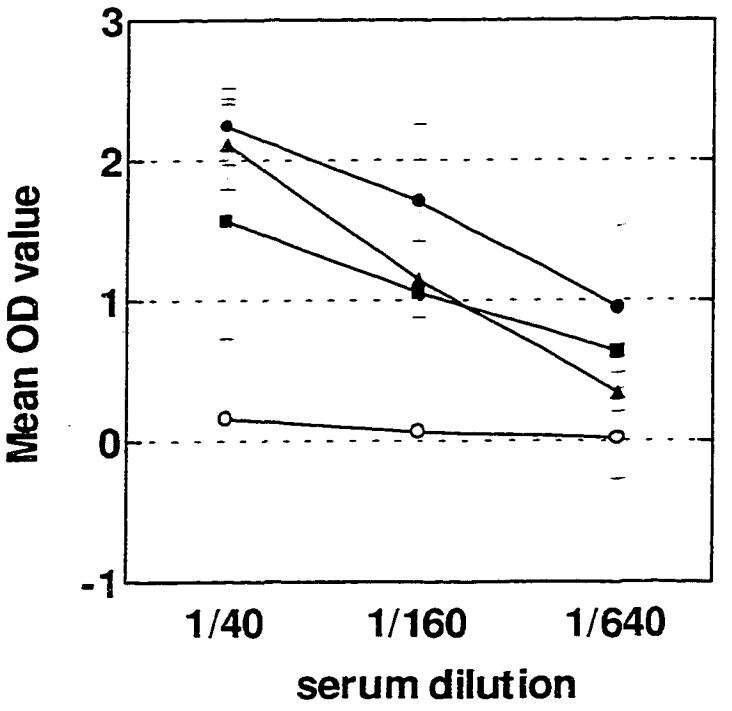


Figure 5.3.22 BHV1-gD specific antibody responses in mice immunized with plasmids encoding gD-VP7 chimeras.

C57Bl/6 mice were intradermally immunized, twice at a 3-week interval with 10 μg of pSLIAtgD (●), pSLIAgD-7Sc (■), pSLIAgD-7t (▲) or pTPA-VP7Sc (O). Serum samples were collected 2 weeks after the second immunization. Data represent the mean of gD-specific serum antibody level (± SD) from 5 mice per group. Antibody titre was measured by ELISA.

system. In addition, the plasmids induced somewhat similar cytokine responses in both tissues. No enhancement of BRV specific cytokine responses was observed following the BRV boost. However, the chimeric proteins exhibited different cytokine responses when compared with the VP7Sc (Fig. 5.3.23-24). Spleens from mice immunized with pSLIAgD-7Sc and pSLIAgD-7t contained significantly lower numbers of BRV-specific IFN-γ secreting cells than those from pTPA-VP7Sc immunized mice, $p<0.05$, as judged by one-way ANOVA followed by Tukey's test (Fig. 5.2.23, A). There was no significant difference in IL-4 production observed among the groups (Fig. 5.2.23, B). As a result, the ratios of BRV-specific IFN-γ/IL-4 secreting cells from mice immunized with pSLIAgD-7Sc and pSLIAgD-7t were significantly lower than that of pTPA-VP7Sc immunized mice, as judged by the same test (Fig. 5.3.24, A). This effect was also observed in the DLN (Fig. 5.3.24, B). There were some discrepancies between cytokine production in DLN and spleens (Fig. 5.3.23, C). However, the group that received pSLIAgD-7t contained lower numbers of IFN-γ secreting cells than the other groups (Fig. 5.3.23, C). This may have contributed to the lower number of IgG2a-ASCs detected in the draining lymph nodes (Fig. 5.3.21, C). It should also be noted that although pVP7t induced a lower IFN-γ/IL-4 ratio, by enhancing IL-4 production (Fig. 5.3.18, B), this effect was not observed following immunization with pSLIAgD-7t.

The number of cytokine secreting cells observed in this experiment was very high compared with other experiments. The fact that the mice were immunized and boosted at the same site in this experiment may have contributed to the magnitude and type of immune response in each immune compartment.

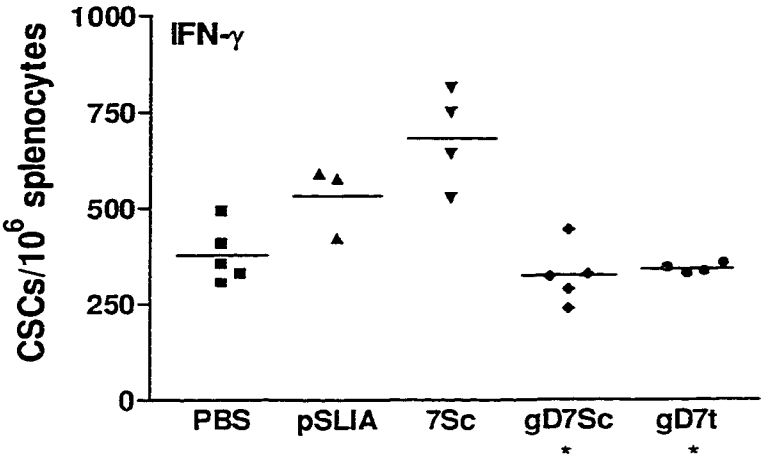
Figure 5.3.23 BRV-specific cytokine responses in mice immunized with plasmids encoding gD-VP7 chimeras and then boosted with BRV.

C57Bl/6 mice were intradermally immunized, twice at a 3-week interval with PBS or 10 μg of pSLIA, pTPA-VP7Sc (7Sc), pSLIAgD-7Sc (gD7Sc) or pSLIAgD-7t (gD7t). All mice were boosted, intradermally, with 50 μg of BRV at week 8 and then sacrificed a week after.

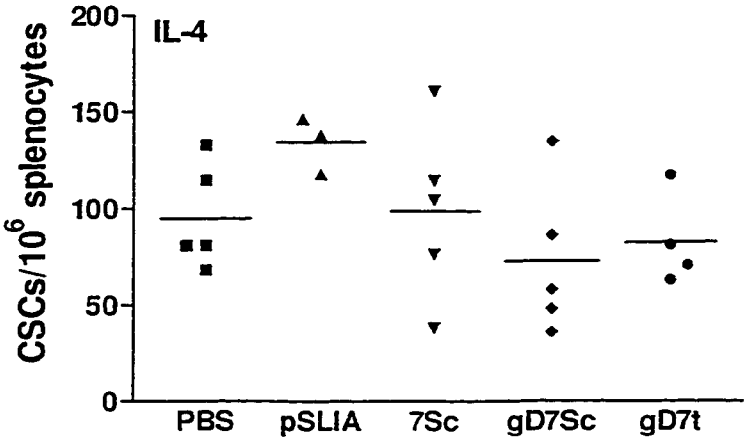
A, B. BRV-specific CSCs in spleens determined by ELISPOT assay. Data represent the number of IFN-γ (A), or IL-4 (B) secreting cells per 10^6 splenocytes from individual mice. Solid line represents the mean value from each group. * Indicates significantly different from 7Sc ($p<0.05$).

C. BRV-specific CSCs in draining lymph nodes determined by ELISPOT assay. Data represent the mean (of triplicate wells) number of CSCs per 10^6 cells from pooled lymphocytes isolated from the draining lymph nodes (4-5 mice/group).

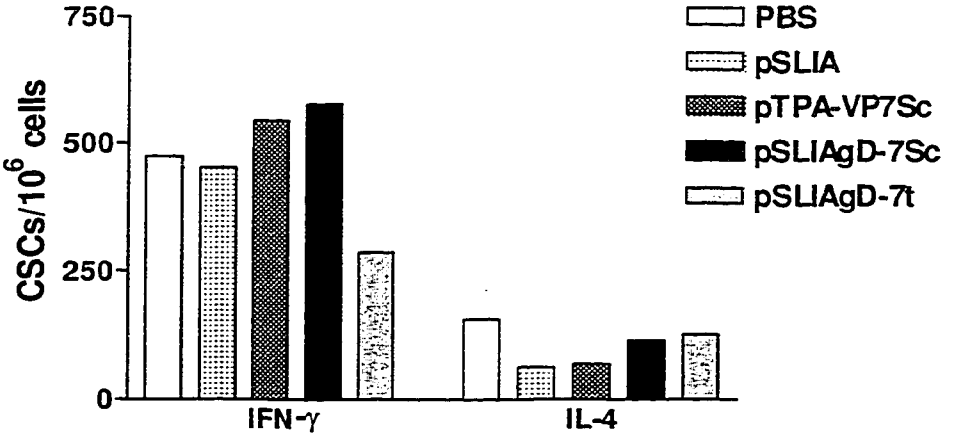
A.


IFN-γ
CSCs/10^6 splenocytes
1000
750
500
250
0
PBS
pSLIA
7Sc
gD7Sc
gD7t
*
*

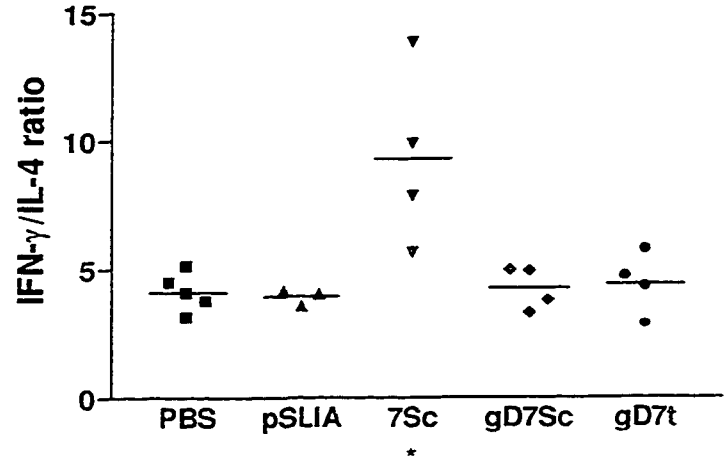
B.


IL-4
CSCs/10^6 splenocytes
200
150
100
50
0
PBS
pSLIA
7Sc
gD7Sc
gD7t

C.


CSCs/10^6 cells
750
500
250
0
IFN-γ
IL-4
PBS
pSLIA
pTPA-VP7Sc
pSLIAgD-7Sc
pSLIAgD-7t

A.



B.

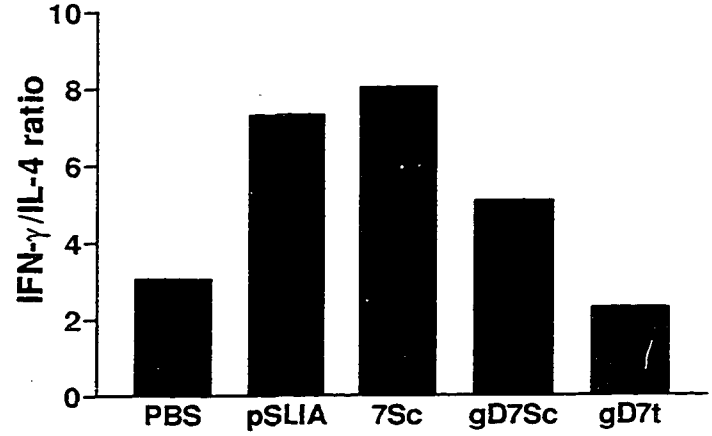


Figure 5.3.24 Ratio of BRV-specific cytokine secreting cells in mice immunized with plasmids encoding gD-VP7 chimeras and then boosted with BRV.

Mice were immunized with the same protocol described in Fig. 5.3.23. Ratio of CSCs (IFN-γ/IL-4) from the spleen of individual mice (A), or pooled draining lymph nodes (B) were obtained from data in Fig. 5.3.23. Solid line represents the mean of IFN-γ/IL-4 ratios for each group.
* Indicates significant difference from other groups ($p<0.05$).

5.3.5.3 Immune responses induced by plasmid encoding a gD-VP7 chimera in BRV-primed mice.

The ability of pSLIAgD-7Sc to boost immune responses in BRV-primed mice was assessed using the protocol described in section 5.3.1.3. Figure 5.3.25 shows the results from the boosting experiment. In addition to data from groups of mice receiving pSLIAgD-7Sc, the data from the group boosted with pTPA-VP7Anc and pVP7t was included in this figure for later discussion.

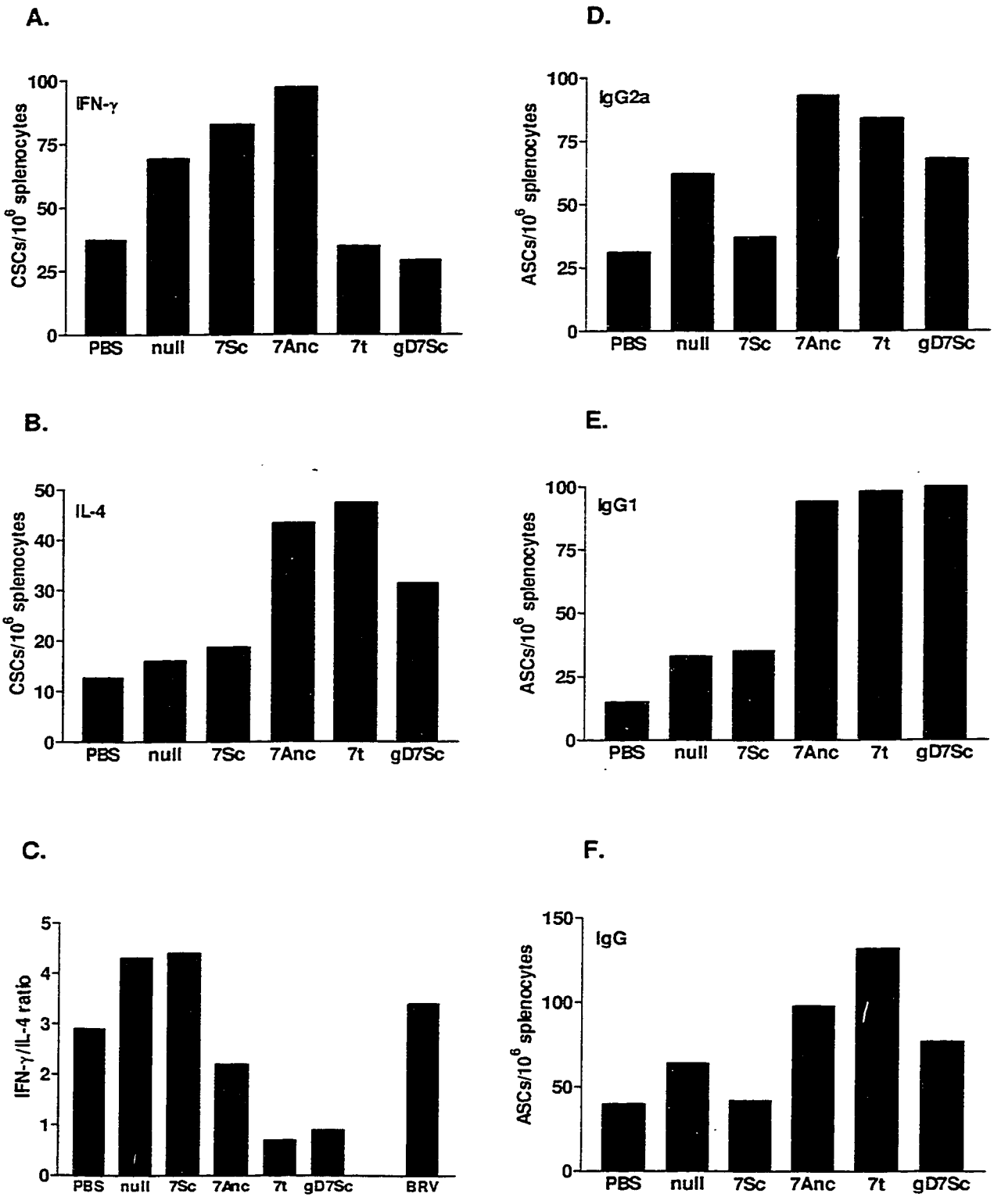
The group boosted with pSLIAgD-7Sc exhibited a lower number of IFN-γ secreting cells compared to that obtained from the pVP7t-boosted group (Fig. 5.3.25, A). This was also consistent with the finding from the priming experiment. The number of IL-4 secreting cells from mice boosted with pSLIAgD-7Sc was higher than the plasmid control but not better than the pTPA-VP7Anc or pVP7t (Fig. 5.3.25, B). The group that received pSLIAgD-7Sc exhibited a low IFN-γ/IL-4 ratio, similar to the one that received pVP7t (Fig. 5.3.25, C). The pSLIAgD-7Sc had a boosting effect on the number of IgG1-ASCs, but the effect was minimal for the IgG- and IgG2a-ASCs (Fig. 5.3.25, D-F).

Although pTPA-VP7Sc did not enhance the BRV-specific antibody response, pSLIAgD-7Sc did enhance the antibody responses in both priming and boosting experiments. Furthermore, pTPA-VP7Sc enhanced IFN-γ production whereas the pSLIAgD-7Sc inhibited IFN-γ production in both experiments. The fact that the same VP7 molecule was expressed by these 2 plasmids suggests that the addition of tgD to the chimeric protein contributed to the outcome of BRV-specific immune responses.

Figure 5.3.25 BRV-specific immune responses in BRV-primed mice boosted with plasmids encoding gD-VP7Sc and modified VP7.

C57Bl/6 mice were intraperitoneally injected with 50 μg of BRV. At week 4, mice were intradermally boosted with PBS, or 10 μg of pSLIA (null), pTPA-VP7Sc (7Sc), pTPA-VP7Anc (7Anc), pVP7t (7t), pSLIAgD-7Sc, or 50 μg of BRV (BRV). All mice were sacrificed for immunological assays 12 days after the boost.

Numbers of BRV-specific IFN-γ (A), IL-4 (B), IgG2a (D), IgG1 (E) and IgG (F) secreting cells determined by ELISPOT assay. Data represent the mean (of triplicate wells) number of cytokine or antibody secreting cells per10^6 pooled splenocytes. (C) The ratio of the number of IFN-γ/IL-4 secreting cells from each group.


A.
IFN-γ
CSCs/10^6 splenocytes
100
75
50
25
0
PBS
null
7Sc
7Anc
7t
gD7Sc
D.
IgG2a
ASCs/10^6 splenocytes
B.
IL-4
CSCs/10^6 splenocytes
50
40
30
20
10
E.
IgG1
ASCs/10^6 splenocytes
C.
IFN-γ/IL-4 ratio
5
4
3
2
1
BRV
F.
IgG
ASCs/10^6 splenocytes
150

Conclusion

The plasmids encoding the chimeric gD-VP7 protein, pSLIAgD-7Sc and pSLIAgD-7t, were cloned and characterized. The plasmids expressed chimeric proteins with predicted molecular weights and the proteins were recognized by antibodies recognizing both BRV and gD proteins. After 2 immunizations, the plasmids did not induce a BRV-specific antibody response. In contrast, gD-specific antibody responses were induced by these plasmids at levels comparable to that of pSLIAtgD. Following BRV boost, the mice immunized with pSLIAgD-7Sc and pSLIAgD-7t were primed for antibody responses in spleens and draining lymph nodes. However, a decreased number of IFN-γ secreting cells was observed and the ratios of IFN-γ/IL-4 in spleen cells were lower than those immunized with pTPA-VP7Sc. The boosting experiment showed somewhat similar results to the priming experiment. The gD-VP7 chimeras inhibited IFN-γ production, induced lower IFN-γ/IL-4 ratios, but still enhanced the level of antibody response, particularly the IgG1-ASCs in spleens. These findings indicate that the gD protein, co-expressed in conjunction with VP7, can enhance BRV-specific humoral immune responses, but inhibits BRV-specific IFN-γ production. The pattern of immune responses induced by the gD-VP7 chimeras was unique and different from those induced by plasmids encoding VP7Sc, or VP7t.

6. DISCUSSION

6.1 Immune responses induced by plasmids encoding VP4 protein

Immunization with plasmids encoding the BRV-VP4 protein (pcDNA3-VP4) primed immunized mice for both humoral and cell-mediated immune responses. Following a BRV boost, the number of BRV-specific ASCs from the DNA-immunized group was higher than the control group, but the serum antibody titre was not increased (Fig. 5.1.1). The ELISPOT assay allowed us to accurately enumerate the frequency of antigen-specific ASCs in local tissues. However, a discrepancy between the numbers of ASCs and the ELISA titre was observed in several experiments in this study (see also Fig. 5.3.4), and by other investigators (Merchant *et al.*, 1991). In this study, differences in antibody responses were usually observed in the ELISPOT assay rather than ELISA. Therefore, most of the results reported in this study were obtained by ELISPOT assay. It may be that the kinetics of antibody production at the time samples were collected may have contributed to the differences between the 2 assays. In addition, the fact that ELISPOT plates were coated with 10 times more BRV than that used for ELISA plates may have made this assay more sensitive.

It should also be noted that VP4 represents only 1.5% of the total virion protein (Estes, 1996b). Therefore, the amount of VP4 protein was substantially limited by coating plate with the whole BRV. In addition, the purified BRV preparation used for inoculation, plate coating and *in vitro* stimulation in this study may have contained

immature viral particles, which do not have the outer capsid proteins. Therefore, measuring BRV-specific antibody titres, ASCs, and CSCs may not always correlate with assays measuring the immune responses induced by the VP4 protein alone. One of the main limitations in this study was the lack of purified VP4 protein for *in vitro* evaluation of the immune responses.

The addition of intron A into the expression cassette (pSLIA-VP4) significantly improved the level of VP4 expression in transfected cells compared with the pcDNA3-VP4 (Fig. 5.1.4). This finding is in agreement with other investigators who demonstrated the effectiveness of the intron A for improving the expression of microbial cDNAs (Chapman *et al.*, 1991, Robinson & Torres, 1997). However, immunization with pSLIA-VP4 still did not induce detectable BRV-specific antibody response, although priming for antibody responses was observed (Fig. 5.1.5). The inability to induce antibody responses following immunization with plasmids encoding VP4 was observed in at least 3 strains of mice, namely C57Bl/6, Balb/c, and C3H. This suggests that the genetic background of the mice did not account for this failure. Although several investigators suggest that intradermal immunization is more effective for inducing immune responses than the intramuscular route (Braun *et al.*, 1997, Yokoyama *et al.*, 1997), neither intradermal nor intramuscular immunization with plasmids encoding VP4 induced antibody production. Furthermore, no antibody response was induced, even with 100 μg of plasmid. This finding indicates that the failure to induce immune responses was not due to the route or amount of plasmids used.

Compartmentalization of expressed antigens within the cells can influence the kinetics of immune responses induced by plasmid immunization. It has been suggested that antigen retained within the cytosolic compartment may exhibited a delayed induction of immune responses when compared with responses induced by secreted or membrane bound antigen (Lewis *et al.*, 1997). VP4 does not contain a secretory signal

peptide and, therefore, is expressed as a cytosolic protein. However, mice immunized with pSLIA-VP4 remained seronegative for at least 9 months following plasmid immunization. This suggests that the failure to measure BRV-specific antibody responses was not due to delayed antibody production.

As reviewed earlier, the VP4 protein consists of 2 subunits, VP5* and VP8* (Estes, 1996b). The recombinant VP8* has been shown to be highly immunogenic in several species (Lee *et al.*, 1995). Early in this study, it was originally thought that the lack of antibody response might be due to the relatively large size of the VP4 molecule, which might affect the level of protein expression *in vivo*, and/or induction of immune responses. However, plasmids encoding BRV VP5* and VP8* subunit, also failed to induce an antibody response (data not shown). This finding suggests that the failure to induce an antibody response was not due to the size of the protein.

Although it is difficult to determine the level of DNA-expressed VP4 *in vivo*, one of the explanations of why the plasmids did not induce an antibody response may relate to the nature of the DNA-expressed protein. In rotavirus, VP4 is present on the outer capsid in the form of a homodimer that strongly interacts with other structural proteins, particularly VP6 and VP7 (Shaw *et al.*, 1996, Shaw *et al.*, 1993a). The finding that the haemagglutinating inhibition (HI) titre of VLP, containing VP 6, 4 and 7, was significantly higher than that of the unassembled VP4 (Redmond *et al.*, 1993), suggests that other viral protein(s) may be required for formation of the functional domain or conformational epitopes of VP4 during a natural viral infection. It is possible that the *in vivo* expressed recombinant VP4 may not contain all the conformational epitopes of the VP4 in the viral particle. However, the VP4 from transfected cells was recognized by both rabbit anti-BRV and a VP8-specific MAb, suggesting that the plasmid-expressed protein still retained some antigenicity. Furthermore, recombinant vaccinia virus expressing VP4 efficiently induced BRV-specific antibody responses (Zhao, 1992).

The more likely explanation for the failure of VP4 encoding plasmids to induce an antibody response is that plasmid immunization probably induced a suboptimal dose of antigen for antibody production. Generally, very high or very low doses of antigen favor the induction of cell-mediated immunity, whereas intermediate doses of antigen favor the induction of antibody responses (Parish, 1996). This notion is also true for plasmid immunization, since much less antigen is required to induce a cellular immune response than an humoral immure response (Barry & Johnston, 1997). A low dose of plasmid-expressed VP4 may have been able to activate and prime the cell-mediated immune response (Fig. 5.1.2). However the amount of protein was probably insufficient to induce an antibody response. The finding that plasmid encoding VP4 did not have a boosting effect on either antibody or cytokine production in BRV-primed mice is also consistent with the hypothesis that the level of expressed VP4 was insufficient to activate a BRV-primed immune response. This may also be due to the fact that antibody responses induced by rotavirus are directed primarily toward VP6 and VP2 proteins. In fact, antibodies recognizing VP4 represent an extremely small portion (less than 2%) of the overall antibody response following inoculation with a heterotypic strain (Shaw *et al.*, 1991). Therefore, it was unlikely that the very small amount of VP4 expressed by a plasmid could effectively boost the BRV-primed immune response, when the frequency of VP4-specific B and T cells was low.

The cellular immune response following plasmid immunization was characterized by an increase in the number of BRV-specific IFN-γ secreting cells (Fig. 5.1.2). The polarization towards IFN-γ production was maintained after BRV boost (Fig. 5.1.2), and seemed to correlate with the level of protein expression (Fig. 5.1.6). It should be noted that BRV itself also induced a higher number of IFN-γ than IL-4 secreting cells. However, the magnitude and the difference in these frequencies were not as great as observed for the plasmid immunized group. The priming effect was

observed even when immunized animals were boosted with rVV-VP4, indicating that the VP4, and not other BRV components, was responsible for the enhanced frequency of BRV-specific IFN-γ secreting cells in DNA-immunized mice. The depletion experiment confirmed that $CD4^+$ T lymphocytes were the primary source of IFN-γ (Table 5.1.2). This finding was consistent with other reports demonstrating that $CD4^+$ cells were responsible for the antigen-specific IFN-γ production following DNA immunization (Leclerc *et al.*, 1997, Sjölander *et al.*, 1998, Ulmer *et al.*, 1998). It should also be noted that IgG2a was the dominant antibody isotype detected in plasmid immunized mice (Table 5.1.1, Fig. 5.1.5, 5.1.7). Thus, the antibody isotype seemed to correlate well with the dominance of IFN-γ in this system. The above findings indicate that immunization with plasmid encoding VP4 induces a Th1-like immune response. Since $CD4^+$ cells are essential for the induction of both IFN-γ secretion and antibody production following plasmid immunization (Leclerc *et al.*, 1997), the findings from this study also emphasized the significance of VP4 as an inducer of Th, that could effectively enhance both humoral and cell-mediated immune responses following the viral boost. It should also be noted that the priming effect of plasmid encoding VP4 persisted for a long period, at least 9 months after immunization (section 5.1.2.3).

The priming effect of plasmid immunization on BRV-specific cytotoxic activity is somewhat interesting. It is generally believed that VP7 is the major target of rotavirus-specific CTL (Franco *et al.*, 1993, Offit *et al.*, 1991). Several reports also suggest that VP4 is not a CTL inducer (Heath *et al.*, 1997, Offit *et al.*, 1991, Offit *et al.*, 1994). In fact, CTL epitopes of rotaviruses have never been found on VP4 (Franco *et al.*, 1994, Franco *et al.*, 1993, Heath *et al.*, 1997). Recombinant vaccinia virus expressing VP4 did not induce rotavirus-specific CTL activity (Offit *et al.*, 1994). In this study, the CTL priming induced by plasmid encoding VP4 was observed only when animals were

boosted with BRV (Fig. 5.1.3), but not the rVV-VP4 (data not shown). Therefore, CTL priming induced by plasmids encoding VP4 may have been mediated through the induction of a Th population that subsequently enhanced the overall CTL activity, rather than VP4-specific CTL, following BRV boost.

A pattern of Th1-like immune responses is commonly observed following intramuscular or intradermal plasmid immunizations (reviewed in Donnelly *et al.*, 1997, Robinson & Torres, 1997). Accumulating information suggests that the major factor that influences Th1-like development is the immunostimulatory property of the DNA itself (Leclerc *et al.*, 1997, Leitner *et al.*, 1997, Roman *et al.*, 1997). In fact, administration of ISS with protein antigen induced a Th1-biased response, suggesting that ISS are a powerful tool for the induction of Th1-like immune responses (Davis *et al.*, 1998). As reviewed in section 2.2.3, bacterial DNA contains immunostimulatory sequences that can induce the secretion of several cytokines that influence the development of a Th1 phenotype. Although the immunostimulatory property of the plasmid DNA is likely to be the primary factor that influences the pattern of Th development, this does not exclude the significance of the antigen dose. The plasmid pcDNA3-VP4 and pSLIA-VP4 contained the same number of ISS (GACGTT) reported to be optimal for immune activation (Krieg *et al.*, 1995), however pSLIA-VP4 exhibited greater priming ability (Fig. 5.1.5-6), suggesting that the higher level of antigen expression resulted in more efficient B and T cell priming. Therefore, both the ISS content in the plasmid and antigen dose may have contributed to the induction of immune responses observed in this study.

Immunization with a low dose of antigen can result in a true Th1 polarization, which favors the development of cell-mediated immunity in the absence of an antibody response, i.e. immune deviation (Bretscher *et al.*, 1992). As reviewed earlier, plasmid immunization effectively induces a Th1 phenotype with strong antibody responses

(Pertmer *et al.*, 1996, Ulmer *et al.*, 1998, Xiang *et al.*, 1994). Furthermore, the mechanism of immune induction by plasmid immunization differs from repeated immunizations with low doses of antigen (Leclerc *et al.*, 1997). Although plasmids encoding VP4 induced cell-mediated immune responses, in the absence of an antibody response, the priming effect on both cytokine and antibody production was observed when immunized mice were boosted with rVV-VP4 (Table 5.1.5), or BRV (Fig. 5.1.7). The present findings, together with previous observations, suggest that a Th1 polarlization or immune deviation did not occur in this system.

6.2 Immune responses induced by plasmids encoding VP7 protein

6.2.1 Immune responses induced by plasmids encoding VP7 and modified VP7

Although the outer capsid protein VP7 is generally believed to be the major neutralization antigen of rotaviruses (Kapikian & Chanock, 1996), many observations indicate that VP7 itself is poorly immunogenic. Among the structural proteins, VP2 and VP6 are immunodominant proteins recognized by convalescent sera from infected children (Svensson *et al.*, 1987). The poor immunogenicity of VP7, relative to the other neutralizing antigen VP4, has been reported in several studies (Giammarioli *et al.*, 1996, Svensson *et al.*, 1987, Ward *et al.*, 1988). Furthermore, VP4 appears to be more broadly immunogenic among different strains of rotaviruses than VP7 (Taniguchi *et al.*, 1987). In addition to the above findings, most attempts to induce rotavirus-specific immune responses using VP7 expressed from several recombinant systems have had limited success (Dormitzer *et al.*, 1994).

Western blot analysis demonstrated that both mouse hyperimmune serum and sera from mice immunized once with whole BRV reacted poorly with the VP7 protein

when compared to other BRV proteins, particularly VP4 (Fig. 5.2.1). It was possible that there was not sufficient VP7 on the blotting membrane for antibody binding. In a separate western blot assay, rabbit hyperimmune serum recognized the VP7 of the same BRV sample with higher band intensity than sera from mice, and the profile of band intensity among the viral proteins remained the same (data not shown). This suggested that there was a sufficient VP7 on the blotting membrane, but that the VP7 might not be highly immunogenic in mice. Viral purification separates and purifies mainly infectious viral particles containing the triple capsid structure. However, this technique may also allow some degree of contamination with cellular debris and/or immature viral particles that lack the outer capsid shell. It should be noted that VP7 is the second most abundant protein in the viral virion, and represents 30% of the total virion protein whereas VP4 represents only 1.5% of the total virion protein (Estes & Cohen, 1989). Although the exact quantity of each viral protein in the purified BRV preparation was not known in this study, theoretically there should be more VP7 than VP4 present in the BRV preparation. Furthermore, each viral preparation had a high BRV titre, suggesting that many infectious particles were present after the purification process (data not shown).

As discussed earlier, the effect of ISS in the gene sequence is an important issue, particularly in the plasmid immunization approach. Theoretically, more ISS should result in a more efficient induction of immune responses (Sato *et al.*, 1996). The VP7 gene contains one optimal ISS, GACGTT, whereas none are found in the VP4 gene sequence. Nevertheless, plasmids encoding VP7 were less effective for inducing BRV-specific immune responses than the plasmid, with the same backbone, carrying the VP4 gene (Fig. 5.2.2).

The early work in this study focused on improving the immunogenicity of the VP7 protein by improving the level of gene expression and modifying the protein itself.

Several factors were thought to be responsible for the poor immunogenicity of VP7, including the low level of protein expression and the unusual nature of the nascent protein. Demonstration of *in vitro* transient protein expression using a plasmid containing the CMV promoter (pcDNA3-VP7) had never been achieved (data not shown). Addition of intron A into the enhancer/promoter element significantly improved the BRV-VP7 gene expression (Fig. 5.3.1). Interestingly, although the VP7 gene contains its own signal peptide, which directs the protein to the ER (Poruchynsky *et al.*, 1985), a considerable amount of secreted VP7 was found in the media. Furthermore, the secreted VP7 from pSLIA-VP7 transfected cells exhibited a molecular weight shift similar to VP7Sc (Fig. 5.3.1) and was resistant to Endo H treatment (Fig. 5.3.3). These findings rule out the possibility of cellular leakage and indicate that the protein had actually left the ER and was secreted through the secretory compartment. Intron A can significantly enhance the expression and secretion of several glycoproteins containing the secretory signal peptide (Chapman *et al.*, 1991). However, it is not known whether it can enhance the secretion of proteins which do not contain the secretory signal peptide.

Antigen localization can influence the induction of antibody responses. Antibody responses following DNA immunization are suboptimal when antigen is cytoplasmic (Boyle *et al.*, 1997a). The endogenous signal peptide leads the nascent VP7 to the ER, which may result in the inefficient presentation of VP7 to B cells. This study showed that the BRV-VP7 could be efficiently secreted by replacing the original signal sequence with the TPA or BHV-1 gD signal sequence (pTPA-VP7Sc, pgD-VP7Sc, respectively). These plasmids could be used to investigate if the secretion of VP7 product enhanced antibody responses. The other important factor responsible for the immunogenicity of VP7 protein is likely to be the unstable nature of the nascent protein. As previously reviewed, calcium and/or other rotavirus proteins are crucial for the stability of the newly

produced VP7 and the formation of VP7 neutralizing epitopes (Dormitzer & Greenberg, 1992, Dormitzer *et al.*, 1992, Shahrabadi *et al.*, 1987). An approach that proved to be effective in enhancing the immunogenicity of VP7 was creating a surface anchored VP7 by replacing the VP7 signal peptide with the secretory signal peptide and c-terminal addition of the membrane anchor and cytoplasmic domains from the influenza virus haemagglutinin gene (Andrew *et al.*, 1990). This result can also be achieved by using different signal and anchor sequences, as shown in this study (pTPA-VP7Anc and pgD-VP7Anc). The VP7Anc was expressed and localized on the cellular surface as expected (Fig. 5.3.1-2). Both TPA and gD signal could be used in combination with the addition of the gD transmembrane anchor sequence. Although the plasmid containing a gD signal peptide (pgD-VP7Anc) yielded higher levels of VP7 than pTPA-VP7Anc, the plasmid was highly toxic to the bacterial host and transfected mammalian cells. Thus, in the later animal experiment, pTPA-VP7Anc was chosen for the ease of plasmid preparation.

In the attempt to characterize and improve BRV-specific immune responses induced by plasmid immunization at the beginning of this project, many VP7 encoding plasmids were designed and constructed based on available biological and immunological knowledge. To be more efficient in the screening process, several plasmids were tested at the same time. Therefore, some of the assays were performed using pooled samples of splenocytes or lymph node cells for practical reasons. It became apparent, at the end of the project, that none the approaches could induce antibody responses in immunized mice. However, along with this failure, these plasmids induced unique patterns of immune responses, which I found to be fascinating. I realized that results obtained from the assays using pooled samples limited the statistical comparisons of responses among the groups. However, the reproducibility from repeated experiments allowed me to draw several important

conclusions in this study. In addition, the fact that the same pattern of immune responses were observed in both priming and boosting experiments provided convincing evidence that the impact of these plasmids on the immune system was a consistent phenomenon.

All *in vivo* experiments in the VP7 study were performed using the pSLIA vector as a null plasmid control, however, not all of the plasmids were derived from pSLIA. This raised the question whether different numbers of ISS in each backbone might induce different levels of background cytokine secretion, in particular IFN-γ production, and thus complicate a comparison of the immune responses induced by different plasmid backbones. It should be noted that pSLIA contains more CpG motifs, 5'-purine-purine-C-G-pyrimidine-pyrimidine-3', (24 versus 21) and one more ISS, reported to be the optimal sequence for immune activation, GACGTT (Krieg *et al.*, 1995), than the pWRG7054. Thus, in some experiments when different plasmids were compared, it would have been more appropriated to use pSLIA, rather than pWRG7054, as a baseline for plasmid-induced background. (The numbers of the optimal ISS found in each plasmid encoding VP7 are provided in Table 6.1.) It should be noted that the null plasmid also contributed, to some extent, to the induction of immune responses. This effect was more obvious in the boosting experiment where samples were collected shortly following the plasmid boost, when the stimulatory effect of the ISS was still prominent. It is possible that bacterial DNA may enhance the ongoing BRV-specific immune response through its non-specific immunostimulatory properties.

Following two immunizations, pcDNA3-VP7 did not induce Th1-like immune responses as did the pcDNA3-VP4. Furthermore, following BRV boost, the number of both IFN-γ and IL-4 secreting cells from pcDNA3-VP7 immunized animals were lower than the those from the BRV control group and did not exhibit the predominance of IFN-γ (Fig. 5.2.2). The pSLIA-VP7 exhibited enhanced IFN-γ production, and higher

Table 6.1 Summary of the BRV-specific immune responses induced by immunizations with plasmids encoding VP7

Plasmid	No. of ISS[c]	Protein	Induction of antibody response	Priming effect[a]		Boosting effect[b]	
				Cytokines	Antibody	Cytokines	Antibody
pSLIA-VP7	3	authentic VP7	No	+/-	Yes	IL-4	Yes
pTPA-VP7Sc	2	secreted VP7	No	IFN	⇩	IFN	⇩
pgD-VP7Sc	2	secreted VP7	No	+/-	No	ND	ND
pTPA-VP7Anc	3	membrane-bound VP7	No	IFN	Yes	IFN & IL-4	Yes
pgD-VP7Anc	3	membrane-bound VP7	No	IFN	No	ND	ND
pVP7(C3d)	2	secreted VP7-C3d	No	No	No	ND	ND
pVP7(C3d)$_2$	2	secreted VP7-(C3d)$_2$	No	⇩ IFN	No	⇩ IFN	No
pVP7t	2	truncated VP7	No	IL-4	No	IL-4	Yes
pSLIAgD-7Sc	3	secreted tgD-VP7Sc	No	⇩ IFN	Yes	⇧ IL-4, ⇩ IFN	Yes
pSLIAgD-7t	3	secreted tgD-VP7t	No	⇩ IFN	Yes	ND	ND

ND Not determined

a C57Bl/6 mice (or C3H mice in some experiments) were intradermally immunized with 10 μg of plasmids, twice at 3-week interval, prior to the BRV boost (50 μg, i.p. or i.d.).

b C57Bl/6 mice were primed with BRV (50 μg, i.p.) and then boosted with plasmids (10 μg,i.d).

c Number of the optimal ISS, GACGTT

IFN-γ/IL-4 ratios than the pcDNA3-VP7 (Fig. 5.3.5). The enhanced IFN-γ production and priming of the antibody response by switching from pcDNA3 to pSLIA was also observed earlier in the VP4 work. Although the cytokine profile was IFN-γ predominant, pSLIA-VP7 induced a higher frequency of IL-4 secreting cells than the plasmid control, in the boosting experiment (Fig. 5.3.7).

The level of VP7 protein expression was significantly improved in pSLIA-VP7, but immunizations with the plasmid still did not induce an antibody response. However, enhancement of antibody production was observed in both priming and boosting experiments. This finding is consistent with other studies using recombinant vaccinia virus as a delivery system. Recombinant vaccinia virus expressing wild-type VP7 induced a weak rotavirus-specific antibody response, but could be used to boost the antibody response in rotavirus primed mice (Andrew *et al.*, 1992). Together, these findings suggest that although VP7 was not highly immunogenic, T and/or suboptimal B cell activation were probably induced in plasmid-immunized animals. Additionally, the boosting effect on antibody production suggested that the memory cells in the BRV-primed mice recognized the VP7 protein expressed by the plasmid. Since the plasmid was given following BRV inoculation, it is also possible that the strong boosting effect of pSLIA-VP7 resulted from a capacity to boost BRV-primed Th cells. This Th activation could enhance the ongoing BRV-specific antibody response, in addition to a direct activation of VP7-specific B cells. Although a considerable amount of secreted VP7 was observed in transient protein expression *in vitro*, the immune responses induced by pSLIA-VP7 were clearly different from those induced by pTPA-VP7Sc or pgD-VP7Sc, in particular the induction of CTL and antibody responses. This suggests that the VP7 protein expressed *in vivo* by pSLIA-VP7 behaved differently from the secreted VP7.

The plasmids encoding VP7Sc did not induce or enhance BRV-specific antibody responses in any experiment. The cytokine responses induced by plasmids encoding

VP7Sc were similar to those induced by the null plasmid (Fig. 5.3.7, 5.3.13, 5.3.18), except in one experiment (Fig. 5.3.24A). The predominance of IFN-γ production was likely due to the immunostimulatory effect of bacterial DNA and not antigen-specific. However, both pTPA-VP7Sc and pgD-VP7Sc primed BRV-specific CTL activity, indicating that the protein was expressed *in vivo*. Although the secreted VP7 retained antigenicity, as detected by hyperimmune serum *in vitro*, immunizations with plasmids encoding VP7Sc did not induce or prime an antibody response. This finding indicated that the VP7Sc did not induce B cell activation. It is also possible that the VP7Sc was not efficient in Th priming. Other investigators have also noted the poor immunogenicity of secreted VP7 for both T and B cell activation. Recombinant vaccinia virus, expressing secretory VP7, weakly stimulated proliferative responses in splenocytes from mice previously inoculated with virus (Andrew *et al.*, 1990). In addition, recombinant vaccinia expressing secreted VP7 induced poor rotavirus-specific antibody responses, lower than those induced by vaccinia virus expressing wild-type VP7 protein (Andrew *et al.*, 1992). Therefore, construction of a secreted VP7 was not a successful strategy to enhance BRV-specific antibody responses .

The finding that plasmid encoding VP7Sc did not perform as well as the plasmid encoding authentic VP7 is interesting, considering that VP7Sc should contain all the Th epitope in their authentic configuration. One factor that contributes to the induction of an antibody response is probably the nature of the protein itself. It is possible that the secreted protein may be unstable and is degraded quickly after its secretion. The pattern of immune responses induced by this plasmid may also be related to the different APC used by the immune system. It has been suggested previously that different physical forms of the protein can target different types of APC, which then influence the outcome of the immune response (Parish, 1996). The secreted, unstable protein may be internalized and presented to the immune system by macrophages or

dendritic cells prior to being recognized by antigen specific B cells. Although both plasmids pTPA-VP7Sc and pgD-VP7Sc exhibited the same level of protein expression *in vitro* (Fig. 5.3.1), the two plasmids exhibit different levels of toxicity in the transfected cells. The immune responses induced by these plasmids were somewhat different. Therefore, other factors including cellular toxicity induced by each plasmid may also contribute to the outcome of immune responses.

Interestingly, immunization with plasmids encoding VP7Sc resulted in decreased BRV-specific antibody responses. Although the statistical significance could not be analysed, due to the pooled samples, there was a tendency in different experiments for antibody responses induced by pTPA-VP7Sc to be slightly lower than the control groups (Fig. 5.3.4, 5.3.8, 5.3.19). This was also observed following gene gun immunization (Fig. 5.3.12). Boosting with pTPA-VP7Sc also inhibited the induction of an antibody response in BRV-primed mice when compared with the null plasmid (Fig. 5.3.7). Furthermore, co-administration of pTPA-VP7Sc with pSLIA-VP4 also inhibited the antibody responses (Fig. 5.3.8). This suggests that immunization with plasmids encoding VP7Sc could override the immune responses induced by the BRV or plasmid encoding VP4.

Results from this study indicate that immunization with plasmids encoding VP7Sc induced predominantly IFN-γ production, without efficient B cell activation. In addition to the fact that plasmid immunization preferentially induced Th1-like immune responses, immunization with conformationally unstable antigen usually favours the development of cell-mediated responses in the absence of antibody production (Parish, 1996). These factors may contribute to a true polarlization toward cell-mediated immune response, which subsequently inhibit the antibody responses observed in mice immunized with pTPA-VP7Sc. It is also possible that other unidentified factors, such as

cytokines, are induced by immunization with plasmids encoding VP7Sc and may influence the inhibition of BRV-specific antibody response.

Plasmids encoding membrane bound VP7, particularly pTPA-VP7Anc primed the immunized mice for the BRV-specific cell-mediated and humoral immune responses (Fig. 5.3.4-6). The plasmid encoding VP7Anc induced strong IFN-γ production, and also enhanced CTL activity following viral boost. Although pgD-VP7Anc yielded better *in vitro* protein expression (Fig. 5.3.1-2), it did not prime the immune response as well as pTPA-VP7Anc. This is likely due to the high toxicity of the protein product of this plasmid in the transfected cell *in vivo*, since higher cellular toxicity was always observed during the *in vitro* transient transfection with pgD-VP7Anc. In the boosting experiment, pTPA-VP7Sc influenced both cytokine (both IFN-γ and IL-4) and antibody responses. The strong priming and boosting effects on antibody responses suggests that the protein retained epitopes of the native protein, and the addition of a gD anchor sequence did not interfere with protein folding or antigenicity. The enhanced immunogenicity of the membrane-bound recombinant VP7 protein has been previously reported. Recombinant vaccinia virus expressing surface-anchored VP7 induced more than a 100-fold greater rotavirus-specific neutralizing antibody titre than wild-type VP7. It also induced higher lymphoproliferative activity in splenocytes from immunized mice (Andrew *et al.*, 1990). Furthermore, female mice immunized with recombinant virus, expressing surface-anchored VP7, conferred lactogenic immunity that protected suckling pups from rotavirus-induced diarrhoea (Andrew *et al.*, 1992, Both *et al.*, 1993). Together, these reports and the finding from this study demonstrate that the surface-anchored VP7 efficiently induced both humoral and cell-mediated immunity. More importantly, the increased neutralizing antibody titre indicated that the surface-anchored VP7 retained the conformational epitope required for induction of neutralizing antibodies.

It is unlikely that the better immune responses observed following immunization with plasmid encoding VP7Anc resulted from a higher level of protein expression than other plasmids. The *in vitro* transient protein expression did not indicate any obvious differences in protein expression by plasmids encoding VP7Anc. In addition, the induction of cytokine and CTL responses by the plasmid encoding VP7Anc was not higher than that induced by pTPA-VP7Sc (Fig. 5.3.5-6), suggesting that the levels of *in vivo* protein expression from these plasmids were comparable. It is also unlikely that the enhanced immune responses observed following immunization with pTPA-VP7Anc resulted from an increased number of ISS present in the plasmid. Although, plasmids encoding VP7Anc contained more optimal ISS than the plasmid encoding VP7Sc (3 versus 2, respectively), they contained the same number of ISS as pSLIA-VP7 (Table 6.1). These plasmids, however, exhibited different characteristics for induction of immune responses.

The fact that VP7Anc contained almost the exact protein sequence as VP7Sc with the addition of the gD anchor sequence suggests that the interaction of VP7Anc with the cellular surface was crucial for enhancing the immunogenicity of the protein. The mechanism by which surface anchoring mediates enhanced immunogenicity is not understood. It was previously shown that the neutralizing domain on surface-anchored VP7 was more stable than wild-type VP7 (Dormitzer *et al.*, 1994). It is possible that interactions between VP7 and the cell surface may stabilize the protein or, alternatively, may increase B cell binding affinity and/or crosslinking of the membrane immunoglobulins. Antigen recognition and subsequently presentation by B cells can result in enhanced production of IL-4 and subsequently switching toward the Th2 phenotype (Stockinger *et al.*, 1996). In fact, the enhanced IgG1 and IL-4 production following boosting of BRV-primed mice with plasmids encoding VP7Anc is consistent with the possibility that in BRV-primed mice, VP7-specific B cells recognize the VP7Anc

better than other forms of VP7 and therefore, are more efficiently activated. The lack of antibody response following immunization in this study may reflect the production of a suboptimal antigen dose rather than the poor immunogenicity of the protein.

Plasmid-expressed antigens that were targeted to different cellular localizations exhibited different induction times and magnitudes of antibody responses (Lewis *et al.*, 1997). It has been previously reported that secreted antigen induced better antibody production than other forms, including membrane-bound antigen (Boyle *et al.*, 1997a). It has been suggested that antigen availability (i.e. dose) for B cell priming within the lymphoid tissues is crucial for induction of an antibody response and B cell switching toward an IgG1 isotype (Boyle *et al.*, 1997a, Robinson, 1997). In fact, secretory antigens tended to induce high levels of IgG1 following plasmid immunization (Boyle *et al.*, 1997a, Cardoso *et al.*, 1996, Lewis *et al.*, 1997, Peet *et al.*, 1997). The results from this study differed from the above reports and further emphasized the significance of cellular compartmentalization for the immunogenicity of VP7 and the induction of an immune response following plasmid immunization. Plasmids encoding membrane-bound VP7 induced the highest immune response among the plasmids encoding VP7s. In contrast, immunizations with secretory VP7 tended to inhibit the antibody responses. Nevertheless, it should also be noted that the conclusions drawn from this study are likely to be specific to the BRV-VP7 protein and should not be extrapolated as a general concept.

The induction of CTL activity following plasmid immunization is unique and occurs independently of B and Th cell activation (see section 2.2.2). The finding that modified forms of VP7 induced better CTL priming than the original version (Fig. 5.3.6) suggest that antigen compartmentalization could influence the priming of CTL activity following plasmid immunization. This is in contrast with the study demonstrating that recombinant vaccinia virus expressing secretory VP7 did not enhance CTL activity,

suggesting that transport of VP7 out of the ER is not required for induction of CTL (Offit *et al.*, 1994). Although the overall level of CTL activity was somewhat lower when compared with the CTL activity induced by plasmid encoding VP4, this study indicates that the secretory and membrane-bound forms of VP7, but not the intracellular form, enhanced the priming of CTL. These results are consistent with the finding that an intracellular antigen does not induce CTL activity as well as other antigenic forms following plasmid immunization (Boyle *et al.*, 1997a). As reviewed earlier, the mechanism of CTL priming following plasmid immunization is different from the classical MHC class I antigen presentation. The presentation of exogenous antigen on the MHC class I molecules, i.e. cross presentation, by the professional APC appears to play a significant role following DNA immunization. It is likely that the priming of CTL in this study was also mediated by the cross-presentation of antigen by the non-transfected professional APC. Thus, secreted or membrane-bound antigen was likely to be more efficiently taken up, processed, and presented by the professional APC than the authentic form, which remained intracellular.

Despite the fact that VP7 is reported to contain the major CTL epitopes of rotavirus (Franco *et al.*, 1993, Offit *et al.*, 1991, Offit *et al.*, 1994), plasmid immunization with plasmids encoding VP7 did not prime for CTL activity as well as the plasmid encoding VP4. These findings suggest that other viral proteins may be required for efficient induction of CTL in natural infection, possibly through the activation of viral-specific Th populations.

6.2.2 Immune responses induced by plasmids encoding C3d chimeras

Immunization with protein antigen fused with the C3d, or peptide derived from C3d remarkably enhanced antigen-specific immune responses (Dempsey *et al.*, 1996,

Lou, 1998 #29). However, the results from this study demonstrated that plasmids encoding C3d chimeras inhibited antigen specific immune responses in two separate systems. *In vitro* characterization indicated that the chimeric proteins retained the biological activities of the C3d molecule. The VP7-$(C3d)_2$ chimera bound to B cells and could be detected by flow cytometry (Fig. 5.3.10). The biological activity of VP7-C3d was demonstrated in a B cell survival study (Fig. 5.3.10). Although the chimeric proteins exhibited different levels of B cell binding and differed in their capacity to prevent apoptosis, it should be emphasized that these assays were established for *in vitro* characterization purposes, as anti-C3d antibodies were not available. In addition, immature B cells were used to ensure that most of the cells expressed CD21.

Plasmids encoding VP7-C3d chimeras did not enhance BRV-specific immune responses in any experiment (compared with the group, which receiving null plasmid), (Fig. 5.3.12, 5.3.14A). This suggests that fusion with the C3d molecule was not sufficient to enhance BRV-specific antibody responses. Furthermore, BRV-specific cytokine production, in particular IFN-γ, was significantly suppressed in the group immunized or boosted with plasmid encoding VP7-$(C3d)_2$ (Fig. 5.3.13, 5.3.14B). This suggested that the addition of two copies of the C3d molecule interfered with the induction of cell-mediated immune responses. The fact that the VP7-$(C3d)_2$ bound to the receptor on B cells with higher affinity (Fig. 5.3.10), might contribute to the stronger inhibitory effect, compared with VP7-C3d in our system. The finding from this study is different from an earlier report in which protein fusion with two copies of C3d significantly enhanced the induction of antigen-specific antibody responses. The same study also indicated that a fusion containing one copy of C3d tended to suppress the antibody response (Dempsey *et al.*, 1996). Unfortunately, cell-mediated immune responses were not assayed in this study. The lower number of IFN-γ secreting cells, compared with the control group, following boosting with plasmid encoding VP7-$(C3d)_2$

chimera suggested that the responses induced by the plasmid dominated the BRV-induced immune responses.

Considering that VP7Sc, by itself, was not immunogenic (see above), the inhibitory effect of the C3d fusion protein, following plasmid immunization, was questionable. Therefore, this observation was tested with a second antigen (BHV-1 gD) which induces a strong antibody response following plasmid immunization (Babiuk *et al.*, 1995, Braun *et al.*, 1997). Following plasmid immunization, the gD-specific antibody responses in mice immunized with plasmids encoding gD-C3d chimeras were significantly delayed and lower than the responses in the group receiving plasmid encoding tgD (Fig. 5.3.15). Furthermore, antigen-specific cytokine production in both spleen and DLNs were also lower, although this difference was not statistically significant. It should be noted that the inhibitory effect was not related to the method of immunization or dose of DNA, since gene gun immunization with plasmids encoding VP7-C3d chimeras did not enhance the antibody response either (Fig. 5.3.12). In addition, gene gun immunization with plasmid encoding the gD-C3d chimera also significantly inhibited gD-specific antibody responses (Dr. R. Braun, personal communication). These findings clearly demonstrated the inhibitory effect of the C3d molecule on antigen-specific immune responses following plasmid immunization.

The inhibitory effect on antigen-specific immune responses, following immunization with plasmids encoding C3d chimeras was most likely mediated through the biological properties of the C3d molecule and an interaction with the APC. As reviewed earlier, the C3d molecule can target the chimeric protein to cells expressing the C3d receptor (CD21), including follicular dendritic cells and B cells (Carroll & Fischer, 1997). Generally, both DC and B cells can present, to a similar extent, class II-associated peptides derived from soluble protein in the extracellular fluid. However, B cells are more abundant than DC, therefore it is more likely that antigen will encounter a

B cell before a DC (Zhong *et al.*, 1997). It should be noted that B cells can bind antigen through the complement receptors, internalize, process and present the antigen in the context of MHC class II, regardless of their BCR specificity (Jacquier-Sarlin *et al.*, 1995, Thornton *et al.*, 1996). Therefore, any $CD21^+$ B cell could bind the C3d fusion protein and subsequently act as an APC. Unlike protein immunization, plasmid immunization yields very low amounts of protein antigen *in vivo*. Therefore, a possible explanation for the inhibition of antibody response in this study may be the direct competition, for a limited amount of antigen, between lower frequency, antigen-specific B cells and the rest of the $CD21^+$ B cell population. Furthermore, CD21-mediated enhancement of B cell activation is highly dependent on the presence of IL-4 (Mongini *et al.*, 1997). The fact that plasmid immunization generally induces IFN-γ suggests that the C3d molecule may have no effect on B cell activation even when the antigen is targeted to the antigen-specific B cells. This may be one explanation for the different results obtained with protein and plasmid immunizations that used antigen-C3d chimeras, since protein immunization usually induces production of higher levels of IL-4 (Raz *et al.*, 1996).

The inhibitory effect of the C3d molecule may also be explained by the effect of C3d on T cell activation. Since CD21 expression is downregulated on activated B cells, the population that expresses CD21 is likely to be naïve resting B cells (Tedder *et al.*, 1997, Young *et al.*, 1997). B cells are not considered professional APC and the initial antigen presentation by B cells results in deletion or inactivation of naïve T cells (Eynon & Parker, 1992, Fuchs & Matzinger, 1992). This is likely to be to due to the inefficient induction of costimulatory molecules required for B-T cell interactions. In contrast to DC or pre-activated B cells, antigen presentation by resting B cells, lacking accessory molecules, induces very little CD40L expression on naïve T cells. This, in turn, is insufficient to promote APC costimulatory activities on the B cells that are required for effective T cell activation and survival (Croft *et al.*, 1997, Jaiswal & Croft, 1997).

Furthermore, efficient B cell and APC activation also requires signaling through CD40 (reviewed in van Kooten & Banchereau, 1997). Thus, an inadequate CD40L-CD40 interaction profoundly affects the subsequent induction of T cell-dependent immune responses. Although cross-linking of CD21/CD35 on resting B cells can substantially upregulate the expression of both B7-1 and B7-2, both surface IgM and CD21/CD35 coligation are required for effective T cell activation (Kozono *et al.*, 1998).

Theoretically, some of the chimeric antigens may be trapped by FDC. It should be noted that FDC are different from ordinary DC, and are not bone-marrow-derived (Banchereau & Steinman, 1998). In the primary immune response where immune complexes are not present, follicular dendritic cells could pick up the C3d fusion protein through their CD21 receptors. These cells then move to the FDC network in the germinal center to interact with centroblasts and centrocytes (Camacho *et al.*, 1998). However, all 3 signals, i.e. antigen, CD21 ligation, and T cell help, are essential for successful B cell activation in the GC (Camacho *et al.*, 1998, Carroll & Fischer, 1997). As discussed earlier, non-professional APC do not effectively activate the Th population. Therefore, without sufficient Th, the newly differentiated B cells would not survive the activation and selection process in the GC.

As demonstrated by others investigators, antigen presentation by B cells during the priming phase inhibited induction of T-cell dependent tumor immunity (Qin *et al.*, 1998). Targeting peptide antigens to B cells resulted in the induction of Th cell unresponsiveness (MacDonald *et al.*, 1997). Immunization with plasmid encoding myelin basic protein targeted to B cells induced tolerance in the experimental autoimmune encephalomyelitis model. Interestingly, IFN-γ production was also significantly reduced in DNA immunized animals, whereas IL-4 and IL-10 production remained unchanged (Lobell *et al.*, 1998). The suppression of IFN-γ production was similar to the result observed in this study.

6.2.3 Immune responses induced by plasmid encoding truncated VP7

The plasmid encoding truncated VP7 (pVP7t) induced an interesting pattern of immune responses. Although the plasmid did not induce or prime for antibody production, it significantly increased the BRV-specific IL-4 production in both priming and boosting experiments (Fig. 5.3.18-19), and enhanced antibody responses in BRV-primed mice. In fact, this is the only plasmid that enhanced IL-4 production rather than IFN-γ production.

One possible explanation for the priming and boosting effect of pVP7t is may be due to the availability of B cell epitopes on the protein. Based on neutralizing antibody studies, 3 major neutralizing epitopes have been mapped on the VP7 molecule; region A (aa. 87-99), B (aa. 145-150) and C (aa. 208-221). Region C is reported to be the dominant antigenic site of the VP7 protein (reviewed in Estes & Cohen, 1989, Kapikian & Chanock, 1996). The VP7t spans aa. 51-183 of the original VP7 and, therefore, does not contain region C, the immunodominant epitope. (Although, the possibility of other non-neutralizing epitopes on the VP7t should not be excluded, only the major epitopes will be addressed in this discussion.) VP7t protein was produced during an *in vitro* transient protein expression assay and an enhanced BRV-specific antibody response was observed in the BRV-primed immune system. These findings suggested that the VP7t protein retained at least some of the B cells epitopes. Immunization with plasmid encoding VP7t possibly resulted in priming of both region A and B specific Th and B cells. Following BRV boost, the primed Th cells were activated resulting in enhanced BRV-specific IL-4 production. However, the BRV-VP7 should activate primarily B cells that recognized region C, rather than A and B, due to its immunodominant properties. Therefore the plasmid-primed, region A and B-specific, B cells should not be effectively activated. Consistent with this hypothesis is an inability to boost antibody responses in

pVP7t immunized mice following viral boost. In the BRV-primed animals, it is likely that all regions of VP7 induced both T and B cell responses, although region C was still predominantly recognized by BRV-activated B cells. Thus, boosting with pVP7t enhanced not only region A and B-specific immune responses, but the strong IL-4 response induced by plasmids could also enhance the overall BRV-specific immune responses.

The finding that VP7t, the deletion mutant of the VP7Sc, enhanced the immunogenicity of VP7 is somewhat intriguing. As discussed above, VP7Sc was not immunogenic and did not induce any significant immune responses. The truncated VP7 was much shorter than the VP7Sc and did not contain the immunodominant epitope of VP7, yet it influenced the induction of BRV specific immune responses. It is possible that the effect of pVP7t on the immune system relied on its ability to induce strong BRV-specific IL-4 production. In general, the predominance of antigen-specific IL-4 following intradermal plasmid immunization is not commonly observed. However, pVP7t enhanced antigen-specific IL-4 production in both priming and boosting experiments (Fig. 5.3.18-19). It should be noted that although both BRV-specific IgG1 and IgG2a antibody isotypes were enhanced, the enhancement of IgG1 was more prominent. This suggests a direct influence by IL-4 on the BRV-specific antibody responses following plasmid boost. These findings also demonstrated that the IL-4 inducing effect of pVP7t dominated over the BRV or ISS effect of the plasmid. Differences in DNA sequences cannot explain the unique pattern of immune responses induced by pVP7t, since pVP7t contained almost exactly the same DNA sequence as the pTPAVP7Sc. This suggests that strong IL-4 induction was most likely due to the VP7t protein itself.

It has been suggested that antigen presentation by B cells can enhance IL-4 production, whereas dendritic cells and macrophages induce IFN-γ production from the

Th population (Stockinger *et al.*, 1996). Results from the transient protein expression assay indicate that the VP7t remained primarily intracellular with a small amount of protein secreted into the media (Fig 5.3.17). Thus it is unlikely that the VP7t would be mainly presented by B cells following plasmid immunization. However, the situation might be different in the boosting experiment in which the number of antigen-specific B cells had already increased.

The Th epitopes on VP7 have never been studied. It is not known if VP7t contains any Th epitope which, for some reasons, favors IL-4 production. In addition, the truncated VP7 contains an additional 14 amino acids, resulted from the frame shift reading. It is not known whether those extra amino acids can influence the induction of immune responses. Thus, more intricate plasmid constructs and experimental designs are required to answer these questions. In fact, generating a number of plasmids encoding deletion mutants (containing the exact sequence of VP7) will be needed to address the question regarding the induction of IL-4 by VP7t.

6.2.4 Immune responses induced by plasmids encoding gD-VP7 chimeras

A chimeric vector expressing a fusion protein of two distinct viral antigens has been shown to induce immune responses to an antigen that is not immunogenic by itself (Major *et al.*, 1995). Therefore, it was speculated that the addition of the BHV-1 truncated gD into the expression cassette might enhance the immunogenicity of the VP7 protein. Plasmids encoding gD-VP7Sc, and gD-VP7t were cloned and characterized. The protein expressed *in vitro* retained its antigenicity as it was recognized by anti-BRV hyperimmune serum and gD-specific monoclonal antibodies (Fig. 5.3.20). A plasmid encoding the switched orientation of the proteins, VP7Sc-gD, was also successfully cloned, but the plasmid did not express protein *in vitro*. Other

investigators have also observed that plasmids containing genes upstream of the tgD gene did not express the chimeric protein *in vitro* (Dr. R. Braun, personal communication). The reason for this is not known.

Immunization with the plasmids, pSLIAgD-7Sc and pSLIAgD-7t, did not induce but did prime for antibody responses following BRV boost. The group immunized with pSLIAgD-7Sc exhibited better priming than the group receiving pSLIAgD-7t. Therefore, pSLIAgD-7Sc was used in the boosting experiment where it also exhibited a boosting effect in BRV-primed mice. The number of BRV-specific ASCs following viral boost was found to be higher in the DLNs than in spleens (Fig. 5.3.21). This was probably due to the fact that mice were immunized and boosted intradermally, at the same site in this particular experiment. Interestingly, the antibody responses, following plasmid boost were observed only in the spleens but not in DLNs (data not shown). These findings suggest that antibody responses to the recall antigen occurred primarily at the initial antigen-draining site. Following BRV boosting, spleens and DLNs from plasmid immunized mice exhibited a different pattern of antibody isotypes (Fig. 5.3.21). Draining lymph nodes contained higher BRV-specific IgG1 ASCs, whereas spleens contained higher IgG2a isotype. The antibody isotype in spleens was also predominantly IgG1 in the boosting experiment. The difference in the antibody isotype possibly reflects cellular trafficking and/or the influence of gD on anti-BRV immune responses. It was commonly observed that plasmids encoding tgD induced primarily IgG1 antibody in the serum, despite that fact that the cytokine production in the spleens was predominantly IFN-γ (Braun *et al.*, 1997, Lewis *et al.*, 1997). Previous observations also indicate that the predominant cytokine following intramuscular immunization with plasmid encoding gD can also be different in spleens and DLNs (IFN-γ and IL-4, respectively), (Lewis, 1998). Anti-gD antibodies induced by plasmids encoding gD-VP7 chimeras were also found to be predominantly IgG1 (data not shown).

Following BRV boost, pSLIAgD-VP7Sc primed for IFN-γ production, whereas the pSLIAgD-7t induced lower IFN-γ production in the DLNs (Fig. 5.3.23). This is consistent with the strong IFN-γ production that was observed following viral boost in the group immunized with pTPA-VP7Sc but not pVP7t. Again, the site of antigen delivery influenced the level of cytokine production, since a different pattern of cytokine production was observed in the spleen. Spleens from mice immunized with the plasmids encoding gD-VP7 chimeras contained a significantly lower number of BRV-specific IFN-γ secreting cells than spleens from mice immunized with plasmid encoding VP7Sc (Fig. 5.3.23). A decreased IFN-γ production was also observed in the boosting experiment (Fig. 5.3.25). Generally, plasmids encoding gD-VP7 chimeras induced lower IFN-γ/IL-4 ratios when compared with those encoding VP7Sc (Fig. 5.3.24-25). The lower cytokine ratio induced by the pSLIAgD-7t was due to a lower IFN-γ production rather than an enhancement of IL-4 production.

Plasmids encoding VP7Sc and VP7t never induced or primed an antibody response in this study, but the addition of tgD significantly enhanced the immunogenicity of the BRV-VP7 in the chimeric proteins. The mechanism of enhancement of the immunogenicity of VP7 by gD may be related to the preservation of the structural integrity of the chimeric protein. The gD-VP7 chimeras may be more stable than the original VP7Sc and, therefore effectively enhanced the antibody responses. The role of antigen stability was clearly demonstrated in the boosting experiment where antigen conformation was required for recognition by the BRV-primed B cells. In fact, the enhanced IL-4 and IgG1 observed following plasmid boost might partly reflect the induction of IL-4 through antigen presentation by B cells. The reason why pSLIAgD-7Sc induced better antibody priming than pSLIAgD-7t may be due to the fact that VP7Sc contained most of the VP7 molecule whereas VP7t contained

only the amino terminal half of the protein. However, the concept that VP7 structural integrity was enhanced by the gD chimeras was not completely confirmed when comparing the immune responses induced by pTPA-VP7Anc and pSLIAgD-VP7Sc. Previous findings from the pTPA-VP7Anc study suggested that enhancing the structural integrity of the VP7 could enhance both humoral immune response and cytokine production. The pTPA-VP7Anc induced strong IFN-γ production in both priming and boosting experiments, whereas the pSLIAgD-7Sc exhibited decreased IFN-γ production. Thus, increased protein stability could not exclusively account for the outcome of immune responses induced by the gD-VP7 chimeric protein. It should not be excluded that gD might behave as a carrier and enhance the induction of BRV-specific antibody responses through a linked recognition, since the same APC could present both gD- and VP7-derived peptides on class II MHC molecules. However, this does not completely explain the decreased IFN-γ production that was consistently observed in both priming and boosting experiments. Thus, BRV-specific immune responses induced by the gD-VP7 chimera were influenced by the immunomodulatory property of the gD.

The BHV-1 gD is an immunodominant antigen among BHV-1 proteins. It induces strong neutralizing antibodies and its immunodominant epitopes are recognized by both $CD4^+$ and $CD8^+$ T cells. Immunization with recombinant gD induces strong antibody and cell-mediated immune responses, and more importantly, protection against viral challenge. Therefore gD appears to be the best vaccine candidate for BHV-1 (reviewed in Tikoo *et al.*, 1995a). It should also be noted that the patterns of humoral and cell-mediated immune responses induced by tgD does not fall into a typical dose-response curve (Baca-Estrada *et al.*, 1996), suggested earlier by Parish (Parish, 1996). Despite the recognition of gD by both $CD4^+$ and $CD8^+$ cells in immune animals (Denis *et al.*, 1993, Hutchings *et al.*, 1990), the induction of CTL following

immunization with gD has never been demonstrated in any animal model. Furthermore, attempts to induce CTL by immunization with plasmids encoding gD have not been successful (Dr. P.J. Lewis, personal communication).

Recent reports suggest that herpes simplex virus-1 (HSV-1) gD protein may have a significant biological role in modulating the induction of immune responses. It should be pointed out that the BHV-1 gD has high homology with the HSV-1 gD, at both the amino acid level and in terms of biological functions (Tikoo *et al.*, 1990). Therefore, it is likely that BHV-1 gD possess the same characteristics as HSV-1 gD. HSV-1 gD binds to the cellular receptor called herpesvirus entry mediator (HVEM) and facilitates viral entry during the infection process (Montgomery *et al.*, 1996, Whitbeck *et al.*, 1997). In addition, gD also binds to another structurally unrelated receptor, the poliovirus receptor-related protein 1 (PRR1, HveC) which belongs to the immunoglobulin superfamily (Krummenacher *et al.*, 1998). HVEM (also called HveA or TR2) is a member of the tumour necrosis factor receptor (TNFR) superfamily, and it has been suggested that an interaction between gD and HVEM results in immunomodulatory effects (Harrop *et al.*, 1998, Marsters *et al.*, 1997, Montgomery *et al.*, 1996). HVEM is expressed predominantly in lymphocyte rich tissues as well as peripheral blood leukocytes (PBL), (Kwon *et al.*, 1997, Marsters *et al.*, 1997). HVEM is constitutively expressed on the surface of all freshly isolated lymphocyte populations, including T, B, and NK cells (Harrop *et al.*, 1998).

Engagement of TNFR family members can induce a variety of immunomodulatory effects including cell proliferation, differentiation, and/or apoptosis depending on the type of ligands and the microenvironment. The members of this family include, TNFR-1, Fas, Lymphotoxin-β receptor, CD27, CD40 etc. (reviewed in Gravestein & Borst, 1998). HVEM binds to two cellular ligands, secreted lymphotoxin-α (LTα) and LIGHT. LIGHT, a homolog of lymphotoxins, is a type II transmembrane

protein produced by activated T cells (Mauri *et al.*, 1998). LIGHT mRNA is also highly expressed in splenocytes, activated PBL, $CD8^+$ tumor infiltrating lymphocytes, granulocytes, and monocytes. Interestingly, treatment of PBL expressing HVEM with LIGHT resulted in increased IFN-γ release. In contrast, LIGHT could also trigger apoptosis of various tumor cells that expressed both lymphotoxin beta receptor and HVEM. Therefore, LIGHT induces distinct biological responses based on the patterns of receptor expression on target cells (Zhai *et al.*, 1998). HSV-1 gD can inhibit the interaction of HVEM and LIGHT suggesting that gD may behave as a membrane-bound virokine, inducing a wide spectrum of biological activities (Mauri *et al.*, 1998).

BHV-1 has been reported to inhibit IL-2 dependent bovine T lymphocyte proliferation. This inhibition was not associated with viral replication or expression of viral proteins, but rather by viral induced apoptosis (Griebel *et al.*, 1990). In fact, both live and inactivated BHV-1 were capable of inducing apoptosis in mitogen-stimulated peripheral blood mononuclear cells (Hannon *et al.*, 1996). A recent study indicated that the interaction of virion associated gD, but not purified form, with its ligand was a necessary step for the induction of apoptosis in B cells (Hannon *et al.*, submitted). Therefore, the association of gD with virion or cellular membrane may be required for the induction of cellular apoptosis. It should be noted that LIGHT which can induce apoptosis, is also a membrane bound protein (Mauri *et al.*, 1998). These findings suggest a possible immunomodulatory role for gD during natural infection. The newly produced gD is expressed on the surface of infected cells prior to the release of mature viral particles. Therefore, it is possible that herpesviruses use the gD-HVEM interaction to induce apoptosis of HVEM expressed cells.

Recent evidence suggests that engagement of HVEM results in an inhibition of T cell activation. Antibodies to HVEM inhibited $CD4^+$ T cells proliferation in response to stimulation by immobilized CD3 or CD3 plus CD28 MAbs. Cytokine production

including TNF-α, IFN-γ, IL-2, IL-4, and expression of costimulatory ligands and receptors including CD40L, CD25 (IL-2Rα), CD30, CD69, CD71 and OX40 were also inhibited. Furthermore, antibodies to HVEM inhibited the proliferation in a three-way mixed lymphocyte reaction (MLR), and a response to recall antigen (Harrop *et al.*, 1998). These findings may explain the inhibitory effect of gD on IFN-γ production observed in this study. The inhibition of IFN-γ production following immunization with plasmids encoding gD-VP7 chimeras might be mediated through the interaction of truncated gD to its ligand(s) on T cells.

Although both affinity purified gD and truncated gD efficiently induce humoral and cell-mediated immunity, the authentic form appears to be superior possibly due to its ability to form oligomers. High doses of tgD induce a strong antibody response but inhibit lymphoproliferative responses (Baca-Estrada *et al.*, 1996). Interestingly, although tgD contains the immunogenic epitopes similar to the authentic version, the protein is less efficient in mediating *in vitro* stimulation of primed mouse spleen cells (Baca-Estrada *et al.*, 1996), or peripheral blood mononuclear lymphocytes from immune cattle (Tikoo *et al.*, 1995b). A possible explanation may be that monomeric tgD is not presented efficiently by APC as the authentic gD (Baca-Estrada *et al.*, 1996). However, it is also possible that tgD may have stronger inhibitory effects on T cell activation via binding through HVEM. Authentic gD oligomers might not bind as efficiently to HVEM as the monomeric form, and are more likely to be internalized by monocytes or macrophages.

The successful induction of CTL requires an interaction between pCTL and APC, which are previously activated by Th, possibly through the CD40-CD40L interactions (Bennett *et al.*, 1998, Ridge *et al.*, 1998, Schoenberger *et al.*, 1998). The lack of CTL priming observed following immunization with tgD might reflect an inhibition

of T cell activation by gD, and subsequently inefficient activation of APC and pCTL. The induction of CTL is probably not completely inhibited in a natural virus infection, since it has been shown that viral infection of APC can provide sufficient signal(s), replacing the requirement for CD40-CD40L interaction, and effectively activate the APC for CTL priming (Lanzavecchia, 1998),

The effect of gD on B cells may be different from those observed on T cells. It was clearly shown that BHV-1 gD can bind to B cells (Hannon *et al.*, 1996). Furthermore, overwhelming evidence indicates that gD can induce a strong B cell response. It is possible that gD may be targeted to B cells and capable of inducing antibody responses from antigen-specific B cells in the absence of Th. This may also partly explain the finding that the immunity induced by the gD subunit vaccines is short-lived (Babiuk *et al.*, 1998). Since HVEM is highly expressed in the lymphoid tissue, gD may primarily target and remain within the DLNs, increasing the opportunity for an interaction with lymphoid cells. It should be noted that although cross-reactivity between extracellular ligands for proteins in the TNFR family is not common, the intracellular ligands seem to be much more promiscuous (van Kooten & Banchereau, 1997). HVEM is linked via several TNFR-associated factors (TRAF) to signal transduction pathways that can activate immune responses (Marsters *et al.*, 1997). Both CD40 and HVEM shared the intracellular ligands TRAF-2, 3, and 5 (Gravestein & Borst, 1998, Marsters *et al.*, 1997). Theoretically, it is also possible that signalling via HVEM may have effect(s) on B cells similar to those of CD40. Interestingly, one of the effects induced by signalling through CD40 on B cells is an induction of immunoglobulin switching to IgG1 (Lin *et al.*, 1998). As stated earlier, immunization with plasmids encoding tgD preferentially induced an IgG1 antibody isotype. This finding may relate to the immunomodulatory effect of gD on B cells. At present, there is no available information regarding the effect of HVEM or gD on B cell activation. Nevertheless, the

above information strongly suggests that the gD protein may not be simply an antigen, but in fact, a viral protein with immunomodulatory properties that can influence the induction of antigen-specific immune responses.

6.3 General discussion

Immune protection against BRV-induced diarrhea in young calves appears to depend on the level of BRV-specific colostral antibodies, derived from serum antibodies of the dam. Using a mouse model, the original objective of this study was to investigate a potential vaccination strategy based on the hypothesis that immunization with plasmids, encoding BRV genes, could induce immune responses that may provide a foundation for further protection studies. Plasmids encoding BRV genes were cloned and characterized for their ability to induce immune responses. However, none of the original genes induced satisfactory levels of specific immunity, particularly antibody. Various constructs were then systematically designed with the aim of overcoming this problem. After several attempts, it was clear that none of plasmids could induce antibody responses in immunized mice. Results obtained from this study are not in agreement with others. Gene gun immunization with plasmids encoding the murine rotavirus EDIM, VP4, 6, or 7, induced strong rotavirus-specific antibody in mice (Chen *et al.*, 1997, Choi *et al.*, 1997, Herrmann *et al.*, 1996). In addition, gene gun immunizations with plasmid encoding VP4, 6 or 7 induced rotavirus specific CTL activity (Chen *et al.*, 1997). However, in this study, none of BRV genes, including VP4, 6, and 7, induced antibody responses following gene gun immunizations (data not shown). Thus, it is unlikely that the difference between results is determined solely by the method of immunization. Other strategies used, but not discussed in this thesis included co-administration of plasmids encoding cytokine, GM-CSF or IL-4, with

plasmids encoding BRV proteins. This strategy also failed to induce a BRV-specific immune response (data not shown). Differences in the immunogenicity of the bovine and murine rotavirus protein in mice may provide some explanation for these discrepancies. It should be noted that although the plasmids by themselves did not induce detectable antibody responses following immunization, the influence of these plasmids on BRV-specific immune responses, i.e. boosting or priming effect, could be clearly observed.

Although the immune responses induced by plasmids encoding BRV genes were disappointing, the data obtained from the *in vivo* experiments provided useful information regarding the immune responses to BRV proteins and plasmid immunization. This study highlights the relationships among virion proteins that can influence the induction of an immune response during natural infection. Although advanced molecular biology and recombinant DNA technology offer an opportunity to dissect and explore each individual viral component, nevertheless several factors appear to be involved in the induction of antigen-specific immune responses *in vivo*. Information obtained from studies, utilizing whole virus as an antigen, does not always correlate to the immunogenicity of individual viral proteins, when used as an antigen. Therefore, the significance of host-virus relationships and the mechanisms of immune activation cannot be overlooked, particularly when developing vaccine strategy. Considering that viruses have to survive within the host, for their productive infection, it is likely that viral components or immune evasion strategies have been developed and evolved along with the selection pressure from the immune system. In addition, the induction of efficient immune responses is likely based on how the immune system sees the viral antigen during the natural infection. It is, therefore, not surprising that the two neutralizing antigens of BRV, particularly VP7, are not highly immunogenic when delivered individually. The finding from this study also emphasized that the results from

different delivery protocols, for example protein versus plasmid immunization, may not always correlate. Results from each delivery protocol provide useful information. However, these results may not be broadly applied to different delivery systems. For example, it was clearly shown in the C3d-chimera study that protein and DNA immunization gave contradictory results.

Most of the studies to date indicate that there is a preferential induction of IFN-γ following intradermal or intramuscular plasmid immunization. This may be due to the adjuvanticity of bacterial DNA (Feltquate *et al.*, 1997, Leclerc *et al.*, 1997, Raz *et al.*, 1996). The results obtained from this study clearly demonstrate that antigen dose and the intrinsic property of each antigen may also determine the pattern of immune responses induced following plasmid immunization. Higher protein expression increased the magnitude of the immune response. This study also demonstrated that immunization with plasmids encoding truncated VP7 could actually induce a switched pattern of cytokine responses, i.e. predominantly IL-4. Therefore, each of the proteins exhibited a unique characteristic, which had a great impact on the induction of immune responses. This is in agreement with a previous report demonstrating that the level of antigen expression and the immunogenicity of the antigen could be more crucial than the immunostimulatory effect of ISS for the induction of immune responses (Grifantini *et al.*, 1998).

The BRV-specific immune responses induced by plasmids encoding VP7 in both priming and boosting experiments seemed to correlate well. In other words, the pattern of cytokine profiles and induction of antibody response from animals primed with plasmid can be used, in most cases, to predict the boosting effect on the immune response in BRV-primed mice (Table 6.1). Although BRV generally induced more IFN-γ than IL-4 when inoculated into mice, the patterns of cytokine profiles appeared to be influenced by the plasmid used prior to the viral boost. Furthermore, the same pattern

of responses was observed even when plasmids were used to boost BRV-primed mice. This finding demonstrates the strong immunomodulatory capacity of plasmid immunization which can dominate the virus itself, regardless of the immunization protocol. It has been also observed by others that the type of immune responses induced following plasmid immunization can dominate an ongoing or subsequent immune response (Feltquate *et al.*, 1997, Raz *et al.*, 1996).

The work presented here demonstrates that molecular alterations of the encoded antigen can determine the course of BRV-specific immune responses. These alterations included increasing the level of antigen expression, and molecular modifications that influenced antigen stability and cellular compartmentalization. In addition, this study also demonstrated that targeting the expressed antigen to certain cell populations and additional immunomodulatory effects from co-expressed antigen also influenced the BRV-specific immune responses following plasmid immunization. However, none of these approaches induced BRV-specific antibody. I have discussed the possible explanations for this result in light of current knowledge. However, I have not been able to identify a clear reason for the inability of these constructs to induce detectable humoral immune responses.

Several observations obtained from the work with plasmids encoding VP7 and modified VP7 remain puzzling. For example, the deletion mutant, truncated VP7, effectively primed and boosted the IL-4 and antibody responses, whereas the secretory VP7 inhibited antibody production following viral boost. It would be interesting to determine if the protein contains immunomodulatory domain(s). This may allow us to improve the immunogenicity of the protein and would certainly be useful for rotavirus vaccine development. In this case, the plasmid immunization approach would significantly shorten the time and cost required for testing and modifying the VP7 fragments.

The finding that the gD protein from plasmid encoding gD-VP7 chimera can enhance the induction of BRV-specific immune responses could offer a possible solution for poorly immunogenic antigens. However, this study also implied that gD might have an immunomodulatory role that influences the outcome of the antigen-specific immune responses, in particular interfering with T cell activation. It would be interesting to further investigate the mechanisms responsible for this phenomenon. Further analysis of the impact of the gD molecule on the immune system may help us to better understand the unique characteristic of immune responses induced by the gD protein. This, in turn, may allow further improvement of the immunogenicity of gD itself, in particular the ability to enhance cell-mediated immunity.

As stated earlier, it is not known whether the immunogenicity of the expressed BRV-proteins in mice is similar to that in cattle. Although none of the plasmids induced detectable antibody responses in immunized mice, it remains possible that these plasmids may yield different outcomes when introduced into cattle. More studies are needed before conclusions can be made regarding this issue. In fact, since rotaviruses are ubiquitous agents (Dodet *et al.*, 1997), and most adult animals have anti-BRV immunity. It may be interesting to investigate whether these plasmids may be useful for boosting BRV-specific immune responses in adult BRV-primed cattle. The results from this study imply that a cocktail of plasmids encoding different proteins may be required to achieve the same level of immune activation as that induced by natural viral infection. Since neutralizing antibodies appear to be critical for lactogenic immunity against BRV infection, boosting with plasmid encoding neutralizing antigen may enhance the induction of neutralizing antibody that can be transferred to the suckling offspring.

7. REFERENCES

Abbas, A. K., Murphy, K. M. & Sher, A. (1996). Functional diversity of helper T lymphocytes. *Science* **383**, 787-793.

Aha, P. M. & Sabara, M. I. (1990). Development of a rotavirus plaque assay using Sephadex G-75. *J. Virol. Methods* **28**, 25-32.

Albert, M. L., Sauter, B. & Brardwaj, N. (1998). Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* **392**, 86-89.

Andrew, M. E., Boyle, D. B., Coupar, B. E. H., Reddy, D., Bellamy, A. R. & Both, G. W. (1992). Vaccinia-rotavirus VP7 recombinants protect mice against rotavirus-induced diarrhoea. *Vaccine* **10**, 185-191.

Andrew, M. E., Boyle, D. B., Whitfeld, P. L., Lockett, L. J., Anthony, I. D., Bellamy, A. R. & Both, G. W. (1990). The immunogenicity of VP7, a rotavirus antigen resident in the endoplasmic reticulum, is enhanced by cell surface expression. *J. Virol.* **64**, 4776-4783.

Angel, J., Tang, B., Feng, N., Greenberg, H. B. & Bass, D. (1998). Studies of the role for NSP4 in the pathogenesis of homologous murine rotavirus diarrhea. *J. Infect. Dis.* **177**, 455-458.

Au, K. S., Mattion, N. M. & Estes, M. K. (1993). A subviral particle binding domain on the rotavirus nonstructural glycoprotein NS28. *Virology* **194**, 665-673.

Axcrona, K., Gray, D. & Leanderson, T. (1996). Regulation of B cell growth and differentiation via CD21 and CD40. *Eur. J. Immunol.* **26**, 2203-2207.

Babiuk, L. A., Lewis, P. J., Cox, G., van Drunen Littel-van den Hurk, S., Baca-Estrada, M. & Tikoo, S. K. (1995). DNA immunization with bovine herpes virus-1 genes. In *DNA vaccine: a new era in vaccinology*, pp. 50-56. Edited by M. Liu. New York: New York Academy of Science.

Babiuk, L. A., Lewis, P. J., van Drunen Littel-van den hurk, S., Tikoo, S. & Liang, X. (1998). Nucleic acid vaccines: veterinary applications. *Curr. Topics Microbiol. Immunol.* **226**, 90-105.

Babiuk, L. A., L'Italien, J., van Drunen Littel-van den Hurk, S., Zamb, T. J., Lawman, M. J. P., Hughes, G. & Gifford, G. A. (1987). Protection of cattle from bovine herpesvirus type-1 (BHV-1) infection by immunization with individual glycoproteins. *Virology* **159**, 57-66.

Babiuk, L. A., Mohammed, K., Spence, L., Fauvel, M. & Petro, R. (1977). Rotavirus isolation and cultivation in the presence of trypsin. *J.Clin.Microbiol.* **6**, 610-617.

Baca-Estrada, M. E., Snider, M., Tikoo, S. K., Harland, R., Babiuk, L. A. & van Drunen Littel-van den Hurk, S. (1996). Immunogenicity of bovine herpesvirus 1 glycoprotein D in mice: effect of antigen form on the induction of cellular and humoral immune responses. *Viral Immunol.* **9**, 11-22.

Ball, J. M., Tian, P., Zeng, C. Q. Y., Morris, A. P. & Estes, M. K. (1996). Age-dependent diarrhea induced by a rotaviral nonstructural glycoprotein. *Science* **272**, 101-104.

Banchereau, J. & Steinman, R. M. (1998). Dendritic cells and the control of immunity. *Nature* **392**, 245-252.

Baños, D. M., Lopez, S., Arias, C. F. & Esquivel, F. R. (1997). Identification of a T-helper cell epitope on the rotavirus VP6 protein. *J. Virol.* **71**, 419-426.

Barry, M. A. & Johnston, S. A. (1997). Biological features of genetic immunization. *Vaccine* **15**, 788-791.

Bellamy, A. R. & Both, G. W. (1990). Molecular biology of rotaviruses. *Adv. Virus Res.* **38**, 1-43.

Bennett, S. R. M., Carbone, F. R., Karamalis, F., Flavell, R. A., Miller, J. F. A. P. & Heath, W. R. (1998). Help for cytotoxic-T-cell responses is mediated by CD40 signaling. *Nature* **393**, 478-480.

Bevan, M. J. (1995). Antigen presentation to cytotoxic T lymphocytes in vivo. *J. Exp. Med.* **182**, 639-641.

Bird, A. P. (1987). CpG islands as gene markers in the vertebrate nucleus. *Trends Genet.* **3**, 342-346.

Bliss, J., Van Cleave, V., Murray, K., Wiencis, A., Ketchum, M., Maylor, R., Haire, T., Resmini, C., Abbas, A. K. & Wolf, S. F. (1996). IL-12, as an adjuvant, promotes a T helper 1 cell, but does not suppress a T helper 2 cell recall response. *J Immunol.* **156**, 887-894.

Bos, J. D. & Kapsenberg, M. L. (1993). The skin immune system: progress in cutaneous biology. *Imm. Today* **14**, 75-78.

Bot, A., Bot, S., Garcia-Sastre, A. & Bona, C. (1996). DNA immunization of newborn mice with a plasmid-expressing nucleoprotein of influenza virus. *Viral Immunol.* **9**, 207-210.

Both, G. W., Lockett, L. J., Janardhana, V., Edwards, S. J., Bellamy, A. R., Graham, F. L., Prevec, L. & 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 recombinant adenovirus expressing rotavirus VP7sc. *Virology* **193**, 940-950.

Boyle, J. S., Brady, J. L. & Lew, A. M. (1998). Enhanced responses to a DNA vaccine encoding a fusion antigen that is directed to sites of immune induction. *Nature* **392**, 408-411.

Boyle, J. S., Koniaras, C. & Lew, A. M. (1997a). Influence of cellular location of expressed antigen on the efficacy of DNA vaccination: cytotoxic T lymphocyte and antibody responses are suboptimal when antigen is cytoplasmic after intramuscular DNA immunization. *Inter. Immunol.* **9**, 1897-1906.

Boyle, J. S., Silva, A., Brady, J. L. & Lew, A. M. (1997b). DNA immunization : induction of higher avidity antibody and effect of route on T cells cytotoxicity. *Proc. Natl. Acad. Sci. USA* **94**, 14626-14631.

Braun, R., Babiuk, L. & van Drunen Littel-van den Hurk, S. (1997). Enhanced immune response to an intradermally delivered DNA vaccine expressing a secreted form of BHV-1 gD. *Vaccine Res.* **6**, 151-163.

Bretscher, P. A., Wei, G., Menon, J. N. & Bielefeldt-Ohmann, H. (1992). Establishment of stable, cell-mediated immunity that make "susceptible" mice resistant to *Leishmania major. Science* **257**, 539-542.

Bridger, J. C. & Oldham, G. (1987). Avirulent rotavirus infections protect calves from disease with and without inducing high levels of neutralizing antibody. *J. Gen. Virol.* **68**, 231-2317.

Broackle, S. A., Holers, V. M. & Karp, D. R. (1997). CD21 augments antigen presentation in immune individuals. *Eur. J. Immunol.* **27**, 12-130.

Bromberg, J. S., Debruyne, L. A. & Qin, L. (1998). Interaction between the immune system and gene therapy vectors: bidirectional regulation of response and expression. *Adv. Immunol.* **69**, 353-409.

Bruce, M. G., Campbell, I., Xiong, Y., Redmond, M. & Snodgrass, D. R. (1994). Recognition of rotavirus antigens by mouse L3T4-positive T helper cells. *J. Gen. Virol.* **75**, 1859-1866.

Brussow, H., Walther, J., Fryder, V., Sidoti, J. & Bruttin, A. (1988). Cross-neutralizing antibodies induced by single serotype vaccination of cows with rotavirus. *J. Gen. Virol.* **69**, 1647-1658.

Burke, B. & Desselberger, U. (1996). Rotavirus pathogenicity. *Virology* **218**, 299-305.

Burns, J. W., Siadat-Pajouh, M., Krishnaney, A. A. & Greenberg, H. B. (1996). Protective effect of rotavirus VP6-specific IgA monoclonal antibodies that lack neutralizing activity. *Science* **272**, 104-107.

Camacho, S. A., Kosco-Vilbois, M. H. & Berek, C. (1998). The dynamic structure of the germinal center. *Immunol. Today* **19**, 511-514.

Carbone, F. R., Kurts, C., Bennett, S. R. M., Miller, F. A. P. & Heath, W. R. (1998). Cross-presentation : a general mechanism for CTL immunity and tolerance. *Immunol. Today* **19**, 368-373.

Cardoso, A. I., Blixenkrone-Moller, M., Fayolle, J., Liu, M., Buckland, R. & Wild, T. F. (1996). Immunization with plasmid DNA encoding for the measles virus haemagglutinin and nucleoprotein leads to humoral and cell-mediated immunity. *Virology* **225**, 293-299.

Carpio, M., Bellamy, J. E. C. & Babiuk, L. A. (1981). Comparative virulence of different bovine rotavirus isolates. *Can. J. Comp. Med.* **45**, 38-42.

Carroll, M. C. (1998a). CD21/CD35 in the B cells activation. *Sem. Immunol.* **10**, 279-286.

Carroll, M. C. (1998b). The role of complement and complement receptors in induction and regulation of immunity. *Annu. Rev. Immunol.* **16**, 545-568.

Carroll, M. C. & Fischer, M. B. (1997). Complement and the immune response. *Curr. Opinion Immunol.* **9**, 64-69.

Casares, S., Inaba, K., Brumeanu, T.-D., Steinmen, R. M. & Bona, C. A. (1997). Antigen presentation by dendritic cells after immunization with DNA encoding a major histocompatibility complex class II-restricted viral epitope. *J. Exp. Med.* **186**, 1481-1486.

Celluzzi, C. M. & Falo, L. D. (1997). Epidermal dendritic cells induce potent antigen-specific CTL mediated immunity. *J. Invest. Dermatol.* **108**, 716-720.

Chapman, B. S., Thayer, R. M., Vincent, K. A. & Haigwood, N. L. (1991). Effect of intron A from human cytomegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells. *Nucleic Acids Res.* **19**, 3979-3986.

Charpilienne, A., Abad, M. J., Michelangeli, F., Alvarado, F., Vasseur, M., Cohen, J. & Ruiz, M. C. (1997). Solubilized and cleaved VP7, the outer glycoprotein of rotavirus, induces permeabilization of cell membrane vesicles. *J.Gen.Virol.* **78**, 1367-1371.

Chattergoon, M. A., Robinson, T. M., Boyer, J. D. & Weiner, D. B. (1998). Specific immune induction following DNA-based immunization through in vivo transfection and activation of macrophages/antigen presenting cells. *J. Immunol.* **160**, 5707-5718.

Chen, S. C., Fynan, E. F., Robinson, H. L., Lu, S., Greenberg, H. B., Santoro, J. C. & Herrmann, J. E. (1997). Protective immunity induced by rotavirus DNA vaccines. *Vaccine* **15**, 899-902.

Chen, S. C., Jones, D. H., Fynan, E. F., Farrar, G. H., Christopher, J., Clegg, S., Greenberg, H. B. & Herrmann, J. E. (1998). Protective immunity induced by oral immunization with a rotavirus DNA vaccine encapsulated in microparticles. *J. Virol.* **72**, 5757-5761.

Choi, A. H.-C., Knowlton, D. R., McNeal, M. M. & Ward, R. L. (1997). Particle bombardment-mediated DNA vaccination with rotavirus VP6 induces high levels of serum rotavirus IgG but fails to protect mice against challenge. *Virology* **232**, 129-138.

Ciernik, F. I., Brezofsky, J. A. & Carbone, D. P. (1996). Induction of cytotoxic T-lymphocytes and antitumour immunity with DNA vaccines expressing single T-cell epitopes. *J. Immunol.* **156**, 2369-2375.

Clarke, S. M., Roth, J. R., Clarke, M. L., Barnett, B. B. & Spendlove, R. S. (1981). Trypsin enhancement of rotavirus infectivity: mechanism of enhancement. *J.Virol.* **39**, 816-822.

Clarke, M. L., Lockett, L. J. & Both, G. W. (1995). Membrane binding and endoplasmic reticulum retention sequences of rotavirus VP7 are distinct: role of carboxy-terminal and other residues in membrane binding. *J. Virol.* **69**, 6473-6478.

Coffin, S. E., Klinek, M. & Offit, P. A. (1994). Induction of rotavirus-specific antibody production by lamina propria lymphocytes following intramuscular inoculation with rotavirus. *J. Infect. Dis.* **172**, 874-878.

Coffin, S. E., Moser, C. A., Cohen, S., Clark, H. F. & Offit, P. A. (1997). Immunologic correlates of protection against rotavirus challenge after intramuscular immunization in mice. *J. Virol.* **71**, 7851-7856.

Coffin, S. E. & Offit, P. A. (1998). Induction of mucosal B-cell memory by intramuscular inoculation of mice with rotavirus. *J. Virol.* **72**, 3479-3483.

Cohen, J. (1977). Ribonucleic acid polymerase activity associated with purified calf rotavirus. *J. Gen. Virol.* **36**, 395-402.

Colomb, M. G., Villiers, C. L., Villiers, M.-B., Gabert, F. M., Santoro, L. & Rey-Millet, C. A. (1996). The role of antigen bound C3b in antigen processing. *Res. Immunol.* **147**, 75-82.

Condon, C., Watkins, S. C., Celluzzi, C. M., Thompson, K. & Falo, L. D. (1996). DNA-based immunization by *in vivo* transfection of dendritic cells. *Nature Med.* **2**, 1122-1128.

Conner, M. E., Crawford, S. E., Barone, C. & Estes, M. K. (1993). Rotavirus vaccine administered parenterally induces protective immunity. *J. Virol.* **67**, 6633-6641.

Conner, M. E., Matson, D. O. & Estes, M. K. (1994). Rotavirus vaccines and vaccination potential. *Curr. Topics Microbiol. Immunol.* **185**, 285-337.

Corr, M., Lee, D. J., Carson, D. A. & Tighe, H. (1996). Gene vaccination with naked plasmid DNA : mechanism of CTL priming. *J. Exp. Med.* **184**, 1555-1560.

Coulson, B. S., Londrigan, S. L. & Lee, D. J. (1997). Rotavirus contains intergrin ligand sequence and a disintergrin-like domain that are implicated in virus entry to the cells. *Proc. Natl. Acad. Sci. USA* **94**, 5389-5394.

Cox, G. J. M., Zamb, T. J. & Babiuk, L. A. (1993). Bovine herpesvirus 1 : immune responses in mice and cattle injected with plasmid DNA. *J. Virol.* **67**, 5664-5667.

Croft, M., Joseph, S. B. & Miner, K. T. (1997). Partial activation of naive CD4 T cells and tolerance induction in response to peptide presented by resting B cells. *J. Immunol.* **159**, 3257-3265.

Daheshia, M., Kuklin, N., Manickan, E., Chun, S. & Rouse, B. T. (1998). Immune induction and modulation by topical ocular administration of plasmid DNA encoding antigens and cytokines. *Vaccine* **16**, 1103-1110.

Davis, H. L., Weeranta, R., Waldschmidt, T. J., Tygrett, L., Schorr, J. & Krieg, A. M. (1998). CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. *J. Immunol.* **160**, 870-876.

Dempsey, P. W., Allison, M. E. D., Akkaraju, S., Goodnow, C. C. & Fearon, D. T. (1996). C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science* **271**, 348-350.

Denis, M., Slaoui, M., Keil, G., Babiuk, L. A., Ernst, E., Pastoret, P.-P. & Thiry, E. (1993). Identification of different target glycoproteins for bovine herpes virus type 1-specific cytotoxic T lymphocytes depending on the method of *in vitro* stimulation. *Immunology* **78**, 7-13.

Dharakul, T., Labbe, M., Cohen, J., Bellamy, A. R., Street, J. E., Mackow, E. R., Fiore, L., Rott, L. & Greenberg, H. B. (1991). Immunization with baculovirus-expressed recombinant rotavirus protein VP1, VP4, VP6 and VP7 induces $CD8^+$ T lymphocytes that mediate clearance of chronic rotavirus infection in SCID mice. *J. Virol.* **65**, 5928-5932.

Dharakul, T., Riepenhoff-Talty, M., Albini, B. & Ogra, P. L. (1988). Distribution of rotavirus antigen in intestinal lymphoid tissues: potential role in development of the mucosal immune response to rotavirus. *Clin. exp. Immunol.* **74**, 14-19.

Dharakul, T., Rott, L. & Greenberg, H. B. (1990). Recovery from chronic rotavirus infection in mice with severe combined immunodeficiency: virus clearance mediated by adoptive transfer of immune $CD8^+$ T lymphocytes. *J. Virol.* **64**, 4375-4382.

Dodet, B., Heseltine, E. & Saliou, P. (1997). Rotaviruses in human and veterinary medicine. *Trends in Microbiol.* **5**, 176-178.

Dominico, P., Marx, J. L., Schoch, P. E. & Cunha, B. A. (1992). Rapid plasmid DNA isolation from mucoid gram-negative bacteria. *J. Clin. Microbiol.* **30**, 2859-2863.

Dong, Y., Zeng, C. Q. Y., Ball, J. M., Estes, M. K. & Morris, A. P. (1997). The rotavirus enterotoxin NSP4 mobilizes intracellular calcium in human intestinal cells by stimulating phospholipase C-mediated inositol 1,4,5-triphosphate production. *Proc. Natl. Acad. Sci. USA* **94**, 3960-3965.

Donnelly, J. J., Ulmer, J. B., Shiver, J. W. & Liu, M. A. (1997). DNA vaccines. *Annu. Rev. Immunol.* **15**, 617-648.

Dormitzer, P. R., Both, G. W. & Greenberg, H. B. (1994). Presentation of neutralizing epitopes by engineered rotavirus VP7's expressed by recombinant vaccinia viruses. *Virology* **204**, 391-402.

Dormitzer, P. R. & Greenberg, H. B. (1992). Calcium chelation induces a conformational change in recombinant herpes simplex virus-1-expressed rotavirus VP7. *Virology* **189**, 828-832.

Dormitzer, P. R., Ho, D. Y., Mackow, E. R., Mocarski, E. S. & Greenberg, H. B. (1992). Neutralizing epitopes on recombinant herpes simplex virus-1-expressed VP7 require interaction with other rotavirus proteins. *Virology* **187**, 18-32.

Dubois, B., Massacrier, C., Vanbervliet, B., Fayette, J., Brière, F., Banchereau, J. & Caux, C. (1998). Critical role of IL-12 in dendritic cell-induced differentiation of naive B lymphocytes. *J. Immunol.* **161**, 2223-2231.

Eisenbraun, M. D., Fuller, D. H. & Haynes, J. R. (1993). Examination of parameters affecting the elicitation of humoral immune responses by particle bombardment-mediated genetic immunization. *DNA and Cell Biol.* **12**, 791-797.

Ertl, H. C. J. & Xiang, Z. (1996a). Novel vaccine approaches. *J. Immunol.* **156**, 3579-3582.

Ertl, H. C. J. & Xiang, Z. Q. (1996b). Genetic immunization. *Viral Immunol.* **1**, 1-9.

Estes, M. K. (1996a). Advances in molecular biology: Impact on rotavirus vaccine development. *J. Inf. Dis.* **174(Suppl 1)**, S37-46.

Estes, M. K. (1996b). Rotaviruses and their replication. In *Fields Virology*, 3 edn, pp. 1625-1655. Edited by B. N. Fields, D. M. Knipe, P. M. Howley & e. al. Philadelphia: Lippincott-Raven Publishers.

Estes, M. K. & Cohen, J. (1989). Rotavirus gene structure and function. *Microbiol. Rev.* **53**, 410-449.

Estes, M. K., Graham, D. Y. & Mason, B. B. (1981). Proteolytic enhancement of rotavirus infectivity: molecular mechanisms. *J.Virol.* **39**, 879-888.

Eynon, E. E. & Parker, D. C. (1992). Small B cells as antigen-presenting cells in the induction of tolerance to soluble protein antigens. *J. Exp. Med.* **175**, 131-138.

Fang, Y., Xu, C., Fu, Y.-X., Holers, V. M. & Molina, H. (1998). Expression of complement receptor 1 and 2 on follicular dendritic cells is necessary for the generation of a strong antigen-specific IgG response. *J. Immunol.* **160**, 5273-5279.

Fearon, D. T. & Carter, R. H. (1995). The CD19/CR2/TAPA-1 complex of B lymphocytes: linking natural to acquired immunity. *Annu. Rev. Immunol.* **13**, 127-149.

Feltquate, D. M., Heaney, S., Webster, R. G. & Robinson, H. L. (1997). Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *J. Immunol.* **158**, 2278-2284.

Feng, N., Burns, J. W., Bracy, L. & Greenberg, H. B. (1994). Comparison of mucosal and systemic humoral immune responses and subsequent protection in mice orally inoculated with a homologous or a heterologous rotavirus. *J. Virol.* **68**, 7766-7773.

Fernandez, F. M., Conner, M. E., Hodgins, D. C., Parwani, A. V., Nielson, P. R., Crawford, S. E., Estes, M. K. & Saif, L. J. (1998). Passive immunity to bovine rotavirus in newborn calves fed colostrum supplements from cows immunized with recombinant SA 11 rotavirus core-like particles (CLP) or viral-like particle (VLP) vaccines. *Vaccine* **16**, 507-516.

Finkelman, F. D., Lees, A., Birnbaum, R., Gause, W. C. & Morris, S. C. (1996). Dendritic cells can present antigen in vivo in a tolerogenic or immunogenic fashion. *J. Immunol.* **157**, 1406-1414.

Fiore, L., Greenberg, H. B. & Mackow, E. R. (1991). The VP8 fragment of VP4 is the rhesus rotavirus haemagglutinin. *Virology* **181**, 553-563.

Fischer, M. B., Goerg, S., Shen, L., Prodeus, A. P., Goodnow, C. C., Kelsoe, G. & Carroll, M. C. (1998). Dependence of germinal center B cells on expression of CD21/CD35 for survival. *Science* **280**, 582-585.

Forsthuber, T., Yip, H. C. & Lehmann, P. V. (1996). Induction of Th1 and Th2 immunity in neonatal mice. *Science* **271**, 1728-1730.

Franco, M. A., Feng, N. & Greenberg, H. B. (1996). Molecular determinants of immunity and pathogenicity of rotavirus infection in the mouse model. *J. Infect. Dis.* **174 (suppl 1)**, S47-50.

Franco, M. A. & Greenberg, H. B. (1995). Role of B cells and cytotoxic T lymphocytes in clearance of and immunity to rotavirus infection in mice. *J. Virol.* **69**, 7800-7806.

Franco, M. A. & Greenberg, H. B. (1997). Immunity to rotavirus in T cell deficient mice. *Virology* **238**, 169-179.

Franco, M. A., Lefevre, P., Willems, P., Tosser, G., Lintermanns, P. & Cohen, J. (1994). Identification of cytotoxic T cell epitopes on the VP3 and VP6 of rotavirus proteins. *J. Gen. Virol.* **75**, 589-596.

Franco, M. A., Prieto, I., Labbé, M., Poncet, D., Borras-Cuesta, F. & Cohen, J. (1993). An immunodominant cytotoxic T cell epitope on the VP7 rotavirus protein overlaps the H2 signal peptide. *J. Gen. Virol.* **74**, 2579-2586.

Franco, M. A., Tin, C., Rott, L. S., VanCott, J. L., McGhee, J. R. & Greenberg, H. B. (1997). Evidence for $CD8^+$ T-cell immunity to murine rotavirus in the absence of perforin, Fas and gamma interferon. *J. Virol.* **71**, 479-486.

Fu, T.-M., Guan, L., Friedman, A., Ulmer, J. B., Liu, M. A. & Donnelly, J. J. (1998). Induction of MHC class I-restricted CTL response by DNA immunization with ubiquitin-influenza virus nucleoprotein fusion antigens. *Vaccine* **16**, 1711-1717.

Fu, T.-M., Ulmer, J. B., Caulfield, M. J., Deck, R. R., Friedman, A., Wang, S., Liu, X., Donnelly, J. J. & Liu, M. A. (1997). Priming of cytotoxic T lymphocytes by DNA vaccine : requirement for professional antigen presenting cells and evidence for antigen transfer from myocytes. *Mol. Medicine* **3**, 362-371.

Fuchs, E. J. & Matzinger, P. (1992). B cells turn off virgin but not memory T cells. *Science* **258**, 1156-1159.

Fukodome, K., Yoshie, O. & Konno, T. (1989). Comparison of human, simian, and bovine rotaviruses for requirement of sialic acid in haemagglutination and cell adsorption. *Virology* **172**, 196-205.

Fynan, E. F., Webster, R. G., Fuller, D. H., Haynes, J. R., Santoro, J. C. & Robinson, H. L. (1993). DNA vaccine: Protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Immunology* **90**, 11478-11482.

Gerloni, M., Lo, D. & Zanetti, M. (1998). DNA immunization in *rel*B-deficient mice discloses a role for dendritic cells in IgM→IgG1 switch *in vivo*. *Eur. J. Immunol.* **28**, 516-524.

Giammarioli, A. M., Mackow, E. R., Fiore, L., Greenberg, H. B. & Ruggeri, F. M. (1996). Production and characterization of murine IgA monoclonal antibodies to the surface antigens of rhesus rotavirus. *Virology* **225**, 97-110.

Gilbert, J. M. & Greenberg, H. B. (1997). Viral-like particle-induced fusion from without in tissue culture cells: role of outer-layer protein VP4 and VP7. *J.Virol.* **71**, 4555-4563.

Gravestein, L. A. & Borst, J. (1998). Tumor necrosis factor receptor family members in the immune system. *Sem. Immunol.* **10**, 423-434.

Griebel, P. J. (1997). Isolation of lymphoid follicles from Peyer's patches. In *Immunology methods manual: the comprehensive sourcebook of techniques*, pp. 2079-2082. Edited by I. Lefkovits. San Diego: Academic press.

Griebel, P. J., Bielefeldt Ohmann, H., Lawman, M. J. P. & Babiuk, L. A. (1990). The interaction between bovine herpesvirus type 1 and activated bovine T lymphocytes. *J. Gen. Virol.* **71**, 369-377.

Grifantini, R., Finco, O., Bartolini, E., Draghi, M., Giudice, G. D., Kocken, C., Thomas, A., Abrignani, S. & Grandi, G. (1998). Multi-plasmid DNA vaccination avoids antigenic competition and enhances immunogenicity of a poorly immunogenic plasmid. *Eur. J. Immunol.* **28**, 1225-1232.

Gurunathan, S., Irvine, K. R., Wu, C.-Y., Cohen, J. I., Thomas, E., Prussin, C., Restifo, N. P. & Seder, R. A. (1998). CD40 ligand/trimer enhances both humoral and cellular immune responses and induces protective immunity to infectious and tumor challenge. *J. Immunol.* **161**, 4563-4571.

Gurunathan, S., Sacks, D. L., Brown, D. R., Reiner, S. L., Charest, H., Glaichenhaus, N. & Seder, R. A. (1997). Vaccination with DNA encoding the immunodominant LACK parasite antigen confers protective immunity to mice infected with *Leishmania major*. *J. Exp. Med.* **186**, 1137-1147.

Haddad, D., Liljeqvist, S., Ståhl, S., Andersson, I., Perlmann, P., Berzins, K. & Ahlborg, N. (1997). Comparative study of DNA-based immunization vectors: effect of secretion signals on the antibody responses in mice. *FEMS Immunol. and Med. Microbiol.* **18**, 193-202.

Hannon, E., Keil, G., van Drunen Littel-van den Hurk, S., Griebel, P., Vanderplasschen, A., Babiuk, L. A. & Pastoret, P.-Y. , P. Role for glycoprotein D in bovine herpesvirus 1-induced apoptosis cell death.(submitted).

Hannon, E., Vanderplasschen, A., Lyaku, J., Keil, G., Denis, M. & Pastoret, P. -P. (1996). Inactivated bovine herpesvirus 1 induces apoptotic cell death of mitogen-stimulated bovine peripheral bloods mononuclear cells. *J. Virol.* **70**, 4116-4120.

Harrop, J. A., Reddy, M., Dede, K., Brigham-Burke, M., Lyn, S., Tan, K. B., Silverman, C., Eichman, C., DiPrinzio, R., Spampanato, J., Porter, T., Holmes, S., Young, P. R. & Truneh, A. (1998). Antibodies to TR2 (Herpesvirus entry mediator), a new member of the TNF receptor superfamily, block T cell proliferation, expression of activation markers, and production of cytokines. *J. Immunol.* **161**, 1786-1794.

Hassett, D. E. & Whitton, J. L. (1996). DNA immunization. *Trends Microbiol.* **4**, 307-312.

Hassett, D. E., Zhang, J. & Whitton, J. L. (1997). Neonatal DNA immunization with a plasmid encoding an internal viral protein is effective in the presence of maternal antibodies and protects against subsequent viral challenge. *J. Virol.* **71**, 7881-7888.

Heath, R. R., Stagg, S., Xu, F. & McCrae, M. A. (1997). Mapping of the target antigens of the rotavirus-specific cytotoxic T cell response. *J. Gen. Virol.* **78**, 1065-1075.

Herrmann, J. E., Chen, S. C., Fynan, E. F., Santoro, J. C., Greenberg, H. B., Wang, S. & Robinson, H. L. (1996). Protection against rotavirus infections by DNA vaccination. *J. Inf. Dis.* **174 (Suppl 1)**, S93-S97.

Hohlfeld, R. & Engel, A. G. (1994). The immunobiology of muscle. *Immunol. Today* **15**, 269-74.

Hsu, C.-H., Chua, K.-Y., Tao, M.-H., Lai, Y.-L., Wu, H.-D., Huang, S.-K. & Hsieh, K.-H. (1996). Immunoprophylaxis of allergen-induced immunoglobulin E synthesis and airway hyperresponsiveness *in vivo* by genetic immunization. *Nat. Med.* **2**, 540-544.

Hutchings, D. L., van Drunen Littel-van den Hurk, S. & Babiuk, L. A. (1990). Lymphocyte proliferative responses to separated bovine herpesvirus 1 proteins in immune cattle. *J. Virol.* **64**, 5114-5122.

Ijaz, M. K., Dent, D. & Babiuk, L. A. (1990). Neuroimmunomodulation of in vivo anti-rotavirus humoral immune response. *J. Neuroimmunol.* **26**, 159-171.

Ijaz, M. K., Dent, D., Haines, D. & Babiuk, L. A. (1989). Development of a murine model to study the pathogenesis of rotavirus infection. *Exp. Molec. Pathol.* **51**, 186-204.

Ijaz, M. K., Sabara, M. I., Frenchich, P. J. & Babiuk, L. A. (1987). Assessment of intestinal damage in rotavirus infected neonatal mice by a D-xylose absorption test. *J. Virol. methods* **18**, 153-157.

Ishida, S., Feng, N., Gilbert, J. M., Tang, B. & Greenberg, H. B. (1997). Immune responses to individual rotavirus proteins following heterologous and homologous rotavirus infection in mice. *J. Infect. D.* **175**, 1317-1323.

Ito, Y. (1960). A tumor-producing factor extracted by phenol from papillomatous tissue (Shope) of cottontail rabbits. *Virology* **12**, 596-601.

Iwasaki, A., Stiernholm, B. J. N., Chan, A. K., Berinstein, N. L. & Baber, B. H. (1997a). Enhanced CTL responses mediated by plasmid DNA immunogens encoding costimulatory molecules and cytokines. *J. Immunol.* **158**, 4591-4601.

Iwasaki, A., Torres, C. A., Ohashi, P. S., Robinson, H. L. & Baber, B. H. (1997b). The dominant role of bone marrow-derived cells in CTL induction following plasmid DNA immunization at different sites. *J. Immunol.* **159**, 11-14.

Jacquier-Sarlin, M. R., Gabert, F. M., Villiers, M. B. & Colomb, M. G. (1995). Modulation of antigen processing and presentation by covalently linked complement C3b fragment. *Immunology* **84**, 164-170.

Jaiswal, A. I. & Croft, M. (1997). CD40 ligand induction on T cell subsets by peptide-presenting B cells. *J. Immunol.* **159**, 2282-2291.

Jourdan, N., Maurice, M., Delautier, D., Quero, A. M., Servin, A. L. & Trugnan, G. (1997). Rotavirus is released from the apical surface of cultures human intestinal cells through nonconventional vesicular transport that bypasses the golgi apparatus. *J.Virol.* **71**, 8268-8278.

Kabcenell, A. K. & Atkinson, P. H. (1985). Processing of the rough endoplasmic reticulum membrane glycoproteins of rotavirus SA11. *J. Cell Biol.* **101**.

Kantharidis, P., Dyall-Smith, M. L. & Holmes, I. H. (1987). Marked sequence variation between segment 4 genes of human RV-5 and simian SA11 rotaviruses. *Arch. Virol.* **93**, 111-121.

Kapikian, A. Z. & Chanock, R. M. (1996). Rotaviruses. In *Fields Virology*, 3 edn, pp. 1657-1708. Edited by B. N. Fields, D. M. Knipe, P. M. Howley & e. al. Philadelphia: Lippincott-Raven Publishers.

Kim, J. J., Ayyavoo, V., Bagarazzi, M. L., Chattergoon, M. T., Dang, K., Wang, B., Boyer, J. D. & Weiner, D. B. (1997). In vivo engineering of cellular immune response by coadministratin of IL-12 expression vector with a DNA immunogen. *J. Immunol.* **158**, 816-826.

Kline, J. N., Waldschmidt, T. J., Businga, T. R., Lemish, J. E., Weinstock, J. V., Thorne, P. S. & Krieg, A. M. (1998). Cutting edge: Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J. Immunol.* **160**, 2555-2559.

Klinman, D. M., Sechler, J. M. G., Canover, J., Gu, M. & Rosenberg, A. S. (1998). Contribution of cells at the site of DNA vaccination to the generation of antigen-specific memory. *J. Immunol.* **160**, 2388-2392.

Klinman, D. M., Yi, A.-K., Beaucage, S. L., Conover, J. & Krieg, A. M. (1996). CpG motifs present in bacterial DNA rapidly induce lymphocytes to secret interleukin 6, interleukin 12 and interferon γ. *Proc. Natl. Acad. Sci.* **93**, 2879-2883.

Kouvelos, K., Petric, M. & Middleton, P. J. (1984). Comparison of bovine, simian and human rotavirus structural glycoproteins. *J. Gen. Virol.* **65**, 1211-1214.

Kovacsovics-Bankowski, M. & Rock, K. L. (1995). A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science* **267**, 243-246.

Kozono, Y., Abe, R., Kozono, H., Kelly, R. G., Azuma, T. & Holers, V. M. (1998). Cross-linking CD21/CD35 or CD19 increases both B7-1 and B7-2 expression on murine splenic B cells. *J. Immunol.* **160**, 1565-1572.

Krieg, A. M. (1996). Lymphocyte activation by CpG dinucleotide motifs in prokaryotic DNA. *Trends Microbiol.* **4**, 73-77.

Krieg, A. M., Yi, A.-K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretzky, G. A. & Klinman, D. M. (1995). CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546-549.

Krummenacher, C., Nicola, A. V., Whitbeck, J. C., Lou, H., Hou, W., Lambris, J. D., Geraghty, R. J., Spear, P. G., Cohen, G. H. & Eisenberg, R. J. (1998). Herpes simplex virus glycoprotein D binds to poliovirus receptor-related protein 1 or herpesvirus entry mediator, two structurally unrelated mediators of virus entry. *J. Virol.* **72**, 7064-7074.

Kwon, B. S., Tan, K. B., Ni, J., Lee, K. O., Kim, K. K., Kim, Y. J., Wang, S., Gentz, R., Yu, G. L., Harrop, J., Lyn, S. D., Siiverman, C., Porter, T. G., Truneh, A. & young, P. R. (1997). A newly identified member of the tumor necrosis factor receptor superfamily with a wide tissue distribution and involvement in lymphocyte activation. *J. Biol. Chem.* **272**, 14272-14276.

Lanzavecchia, A. (1998). Licence to kill. *Nature* **393**, 413-414.

Larralde, G., Li, B., Kapikian, A. Z. & Gorziglia, M. (1991). Serotype specific epitope(s) present on the VP8 subunit of rotavirus VP4. *J.Virol.* **65**, 3213-3218.

Lawton, J. A., M.K., E. & Prasad, B. V. (1997a). Three dimensional visualization of mRNA release from actively transcribing rotavirus particles. *Nat. Struc. Biol.* **4**, 118-121.

Lawton, J. A., Zeng, C. Q. Y., Mukherjee, S. K., Cohen, J., Estes, M. K. & Prasad, B. V. V. (1997b). Three-dimensional structural analysis of recombinant rotavirus-like particles with intact and amino-terminal deleted VP2: Implications for the architecture of the VP2 capsid layer. *J.Virol.* **71**, 7353-7360.

Leclerc, C., Dériaud, E., Rojas, M. & Whalen, R. G. (1997). The preferential induction of a Th1 immune response by DNA-based immunizations is mediated by the immunostimulatory effect of plasmid DNA. *Cell. Immunol.* **179**, 97-106.

Lee, D. J., Tighe, H., Corr, M., Roman, M., Carson, D. A., Spiegelberg, H. L. & Raz, E. (1997). Inhibition of IgE antibody formation by plasmid DNA immunization is mediated by both $CD4^+$ and $CD8^+$ T cells. *Int. Arch. Allergy Immunol.* **113**, 227-230.

Lee, J., Babiuk, L. A., Harland, R., Gibbons, E., Elazhary, Y. & Yoo, D. (1995). Immunological response to recombinant VP8* subunit protein of bovine rotavirus in pregnant cattle. *J. Gen. Virol.* **76**, 2477-2483.

Lehman, D. J., Roof, L. L., Brideau, R. J., Aeed, P. A., Thomsen, D. R., Elhammaer, A. P., Wathen, M. W. & Homa, F. L. (1993). Comparison of soluble and secreted forms of human parainfluenza virus type 3 glycoproteins expressed from mammalian and insect cells as subunit vaccines. *J. Gen. Virol.* **74**, 459-469.

Leitner, W. W., Sequin, M. C., Ballou, W. R., Seitz, J. P., Schultz, A. M., Sheehy, M. J. & Lyon, J. A. (1997). Immnue responses induced by intramuscular or gene gun injection of protective deoxyribonucleic acid vaccines that express the circumsporozoite protein from *Plasmodium berghei* malaria parasites. *J. Immunol.* **159**, 6112-6119.

Lewis, P. J. (1998). Thesis: Characterization and modulation of immune responses in mice to a DNA-based vaccine. In *Department of Veterinary Microbiology*, pp. 279. Saskatoon: University of Saskatchewan.

Lewis, P. J., Cox, G. J. M., van Drunen Littel-van den Hurk, S. & Babiuk, L. A. (1997). Polynucleotide vaccines in animals: enhancing and modulating responses. *Vaccine* **15**, 861-864.

Lin, S.-C., Wortis, H. H. & Stavnezer, J. (1998). The ability of CD40L, but not lipopolysaccharide, to initiate immunoglobulin switching to immunoglobulin G1 is explained by differential induction of NF-κB/Rel protein. *Mol. Cell. Biol.* **18**, 5523-5532.

Liu, Y.-J. & Arpin, C. (1997). Germinal center development. *Immunol. Rev.* **156**, 111-126.

Lobell, A., Weissert, R., Storch, M. K., Svanholm, C., de Graaf, K. L., Lassmann, H., Andersson, R., Olsson, T. & Wigzell, H. (1998). Vaccination with DNA encoding an immunodominant myelin basic protein peptide targeted to Fc of immunoglobulin G suppresses experimental autoimmune encephalomyelitis. *J. Exp. Med.* **187**, 1543-1548.

López, S., Arias, C. F., Bell, J. R., Strauss, J. H. & Espejo, R. T. (1985). Primary structure of the cleavage site associated with trypsin enhancement of rotavirus SA11 infectivity. *Virology* **144**, 11-19.

Lou, D. & Kohler, H. (1998). Enhanced molecular mimicry of CEA using photoaffinity crosslinked C3d peptide. *Nat. Biotechnol.* **16**, 458-462.

Ludert, J. E., Feng, N. G., Yu, J. H., Broom, R. L., Hoshino, Y. & Greenberg, H. B. (1996a). Genetic mapping indicates that VP4 is the rotavirus cell attachment protein *in vitro* and *in vivo*. *J.Virol.* **70**, 487-493.

Ludert, J. E., Krishnaney, A. A., Burns, J. W., Vo, P. T. & Greenberg, H. B. (1996b). Cleavage of rotavirus VP4 *in vivo*. *J. Gen. Virol.* **77**, 391-395.

Ludewig, B., Ehl, S., Karrer, U., Odermatt, B., Hengartner, H. & Zinkernagel, R. M. (1998). Dendritic cells efficiently induce protective antiviral immunity. *J. Virol.* **72**, 3812-3818.

MacDonald, C. M., Bolton, E. M., Jaques, B. C., Walker, K. G. & Bradley, J. A. (1997). Reduction of alloantibody response to class I major histocompatibility complex by targeting synthetic allopeptides for presentation by B cells. *Transplantation* **63**, 92-932.

Mackey, M. F., Gunn, J. R., Maliszewski, C., Kikutatani, H., Noelle, R. J. & Barth Jr, R. J. (1998). Cutting edge: Dendritic cells require maturation via CD40 to generate protective antitumor immunity. *J. Immunol.* **161**, 2094-2098.

MacLennan, I. C. M., Gulbranson-Judge, A., Toellner, K.-M., Casamayor-Palleja, M., Chan, E., Sze, D. M.-Y., Luther, S. A. & Orbea, H. A. (1997). The changing preference of T and B cells for partners as T-dependent antibody responses develop. *Immunol. Rev.* **156**, 53-66.

Maecker, H. T., Umetsu, D. T., Dekruyff, R. H. & Levy, S. (1997). DNA vaccination with cytokine fusion constructs biases the immune response to ovalbumin. *Vaccine* **15**, 1687-1696.

Major, M. E., Vitvitski, L., Mink, M. A., Schleef, M., Whalen, R. G., Trépo, C. & Inchauspé, G. (1995). DNA-based immunization with chimeric vectors for the induction of immune responses against the hepatitis C virus nucleocapsid. *J. Virol.* **69**, 5798-5805.

Manickan, E., Kanangat, S., Rouse, R. J. D., Yu, Z. & Rouse, B. T. (1997a). Enhancement of immune response to naked DNA vaccine by immunization with transfected dendritic cells. *J. Leukoc. Biol.* **61**, 125-132.

Manickan, E., Karem, K. L. & Rouse, B. T. (1997b). DNA vaccine-A modern gimmick or a boon to vaccinology. *Critical Rev. Immunol.* **17**, 139-154.

Manickan, E., Yu, Z. & Rouse, B. T. (1997c). DNA immunization of neonates induces immunity despite the presence of maternal antibody. *J. Clin. Invest.* **100**, 2371-2375.

Marsters, S. A., Ayres, T. M., Skubatch, M., Gray, C. L., Rothe, M. & Ashkenazi, A. (1997). Herpesvirus entry mediator, a member of the tumor necrosis factor receptor (TNFR) family, interacts with member of the TNFR-associated factor family and activates the transcription factors NF-kappa B and AP-1. *J. Biol. Chem.* **272**, 14029-14032.

Martinez, X., Brandt, C., Saddallah, F., Tougne, C., Barrios, C., Wild, F., Dougan, G., Lambert, P.-H. & Siegrist, C.-A. (1997). DNA immunization circumvents deficient

induction of T helper type 1 and cytotoxic T lymphocyte responses in neonates and during early life. *Proc. Natl. Acad. Sci. USA* **94**, 8726-8731.

Matson, D. O., O'Ryan, M. L., Herrera, I., Pickering, L. K. & Estes, M. K. (1993). Fecal antibody responses to symptomatic and asymptomatic rotavirus infections. *J. Infect. Dis.* **167**, 557-583.

Matsui, S. M., Offit, P. A., Vo, P. T., Mackow, E. R., Benfield, D. A., Shaw, R. D., Padilla-Noriega, L. & Greenberg, H. B. (1989). Passive protection against rotavirus-induced diarrhea by monoclonal antibodies to the heterotypic neutralization domain of VP7 and the VP8 fragment of VP4. *J. Clin. Microbiol.* **27**, 780-782.

Mauri, D. N., Ebner, R., Montgomery, R. I., Kochel, K. D., Cheung, T. C., Yu, G.-A. , L., Ruben, S., Murphy, M., Eisenberg, R. J., Cohen, G. H., Spear, P. G. & Ware, C. F. (1998). LIGHT, a new member of the TNF superfamily, and lymphotoxin α are ligands for herpesvirus entry mediator. *Immunity* **8**, 21-30.

McNeal, M. M., Barone, K. S., Rae, M. N. & Ward, R. L. (1995). Effector functions of antibody and $CD8^+$ cells in resolution of rotavirus infection and protection against reinfection in mice. *Virology* **214**, 387-397.

McNeal, M. M., Rae, M. N. & Ward, R. L. (1997). Evidence that resolution of rotavirus infection in mice is due to both CD4 and CD8 cell-dependent activities. *J. Virol.* **71**, 8735-8742.

McNeal, M. M., Sheridan, J. F. & Ward, R. L. (1992). Active protection against rotavirus infection of mice following intraperitoneal immunization. *Virology* **191**, 150-157.

Mendez, E., Arias, C. F. & López, S. (1994). Binding of sialic acids is not an essential step for the entry of animal rotaviruses to epithelial cells in culture. *J.Virol.* **67**, 5253-5259.

Mendoza, R. B., Cantwell, M. J. & Kipps, T. M. (1997). Cutting edge: Immunostimulatory effects of a plasmid expressing CD40 ligand (CD154) in gene immunization. *J. Immunol.* **159**, 5777-5781.

Merchant, A. A., Groene, W. S., Cheng, E. H. & Shaw, R. D. (1991). Murine intestinal antibody response to heterologous rotavirus infection. *J. Clin. Microbiol.* **29**, 1693-1701.

Messina, J. P., Gilkeson, G. S. & Pisetsky, D. S. (1991). Stimulation of in vitro murine lymphocyte proliferation by bacterial DNA. *J. Immunol.* **147**, 1759-1764.

Michelangeli, F., Ruiz, M. C., Del Castillo, J. R., Ludert, J. E. & Liprandi, F. (1991). Effect of rotavirus infection on intracellular calcium homeostasis in cultured cells. *Virology* **181**, 520-527.

Mirazimi, A. & Svensson, L. (1998). Carbohydrates facilitate correct disulfide bond formation and folding of rotavirus VP7. *J. Virol.* **72**, 3887-3892.

Moldoveanu, Z., Love-Homan, L., Huang, W. Q. & Krieg, A. M. (1998). CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. *Vaccine* **16**, 1216-1224.

Mongini, P. K. A., Vilensky, M. A., Highet, P. F. & Inman, J. K. (1997). The affinity threshold for human B cell activation via the antigen receptor complex is reduced upon co-ligation of the antigen receptor with CD21 (CR2). *J. Immunol.* **159**, 3782-3791.

Montgomery, R. I., Warner, M. S., Lum, B. J. & Spear, P. G. (1996). Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* **87**, 427-436.

Mor, G., Klinman, D. M., Shapiro, S., Hagiwara, E., Sedegah, M., Norman, J. A., Hoffman, S. L. & Steinberg, A. D. (1995). Complexity of the cytokine and

antibody response elicited by immunizing mice with *Plasmidium yoelii* circumsporozoite protein plasmid DNA. *J. Immunol.* **155**, 2039-2046.

Moser, C. A., Cookinham, S., Coffin, S. E., Clark, H. F. & Offit, P. A. (1998). Relative importance of rotavirus-specific effector and memory B cells in protection against challenge. *J. Virol.* **72**, 1108-1114.

Mosmann, T. R. & Sad, S. (1996). The expanding universe of T-cells subsets: Th1, Th2 and more. *Immunology Today* **17**, 138-146.

Munro, S. & Pelham, H. R. B. (1987). A C-terminal signal prevent secretion of luminal ER proteins. *Cell* **48**, 899-907.

Muraille, E. & Leo, O. (1998). Revisiting the Th1/Th2 paradigm. *Scand. J. Immunol.* **47**, 1-9.

Norburry, C. C., Chambers, B. J., Prescott, A. R., Ljunggren, H.-G. & Watts, C. (1997). Constitutive macropinocytosis allows TAP-dependent major histocompatibility complex class I presentation of exogenous soluble antigen by bone marrow-derived dendritic cells. *Eur. J. Immunol.* **27**, 280-288.

Offit, P. A. (1996). Host factors associated with protection against rotavirus disease: the skies are clearing. *J. Infect. Dis.* **174 (Suppl 1)**, S59-S64.

Offit, P. A., Boyle, D. B., Both, G. W., Hill, N. L., Svoboda, Y. M., Cunningham, S. L., Jenkins, R. J. & McCrae, M. A. (1991a). Outer capsid glycoprotein VP7 is recognized by cross-reactive, rotavirus-specific, cytotoxic T lymphocytes. *Virology* **184**, 563-568.

Offit, P. A. & Clark, H. F. (1985). Protection against rotavirus induced gastroenteritis in a murine model by passively acquired gastrointestinal but not circulating antibodies. *J. Virol.* **54**, 58-64.

Offit, P. A., Coupar, B. E. H., Svoboda, Y. M., Jenkins, R. J., McCrae, M. A., Abraham, A., Hill, N. L., Boyle, D. B., Andrew, M. E. & Both, G. W. (1994). Induction of rotavirus-specific cytotoxic T lymphocytes by vaccinia virus recombinants expressing individual rotavirus genes. *Virology* **198**, 10-16.

Offit, P. A., Cunningham, S. L. & Dudzik, K. L. (1991b). Memory and distribution of virus-specific cytotoxic T lymphocytes (CTLs) and CTL precursors after rotavirus infection. *J. Virol.* **65**, 1318-1324.

Offit, P. A. & Dudzik, K. I. (1990). Rotavirus-specific cytotoxic T lymphocytes passively protect against gastroenteritis in suckling mice. *J. Virol.* **64**, 6325-6328.

Offit, P. A. & Dudzik, K. L. (1988). Rotavirus-specific cytotoxic T lymphocytes cross-react with target cells infected with different rotavirus serotypes. *J. Virol.* **62**, 127-131.

Offit, P. A. & Dudzik, K. L. (1989). Rotavirus-specific cytotoxic T lymphocytes appear at the intestinal mucosa surface after rotavirus infection. *J. Virol.* **63**, 3507-3512.

Offit, P. A., Hofenberg, E. J., Pia, E. S., Panackal, P. A. & Hill, N. L. (1992). Rotavirus-specific helper T cell responses in newborns, infants, children, and adults. *J. Inf. Dis.* **165**, 1107-1111.

Offit, P. A., Hoffenberg, E. J., Santos, N. & Gouvea, V. (1993). Rotavirus-specific humoral and cellular immune response after primary, symptomatic infection. *J. Inf. Dis.* **167**, 1436-1440.

Offit, P. A., Shaw, R. D. & Greenberg, H. B. (1986). Passive protection against rotavirus induced diarrhea by monoclonal antibodies to surface proteins of VP3 and VP7. *J. Virol.* **58**, 700-703.

Oldham, G., Bridger, J. C., Howard, C. J. & Parsons, K. R. (1993). In vivo role of lymphocyte subpopulations in the control of virus excretion and mucosal antibody responses of cattle infected with rotavirus. *J. Virol.* **67**, 5012-5019.

O'neal, C. M., Clements, J. D., Estes, M. K. & Conner, M. E. (1998). Rotavirus 2/6 virus-like particles administered intranasally with cholera toxin, *Escherichia coli* heat-labile toxin (LT), and LT-R192G induce protection from rotavirus challenge. *J. Virol.* **72**, 3390-3393.

O'neal, C. M., Crawford, S. E., Estes, M. K. & Conner, M. E. (1997). Rotavirus virus-like particles administered mucosally induce protective immunity. *J. Virol.* **71**, 8707-8717.

O'Ryan, M. L., Matson, D. O., Estes, M. K. & Pickering, L. K. (1994). Anti-rotavirus G type-specific and isotype-specific antibodies in children with natural rotavirus infection. *J. Infect. Dis.* **169**.

Parish, C. R. (1996). Immune deviation: a historical perspective*. *Immunol. Cell Biol.* **74**, 449-456.

Paul, P. S. & Lyoo, Y. S. (1993). Immunogens of rotaviruses. *Vet. Microbiol.* **37**, 299-317.

Peet, N. M., McKeating, J. A., Ramos, B., Klonnisch, T., De Souza, J. B., Delves, P. J. & Lund, T. (1997). Comparison of nucleic acid and protein immunization for induction of antibodies specific for HIV-1 gp120. *Clin. Exp. Immunol.* **109**, 226-232.

Pertmer, T. M., Roberts, T. & Haynes, J. R. (1996). Influenza virus nucleoprotein-specific immunoglobulin G subclass and cytokine responses elicited by DNA vaccination are dependent on the route of vector DNA delivery. *J. Virol.* **70**, 6119-6125.

Petrie, B. L., Graham, D. Y., Hanssen, H. & Estes, M. K. (1982). Localization of rotavirus antigens in infected cells by ultrastrutural immunocytochemistry. *J.Gen.Virol.* **63**, 457-467.

Pfeifer, J. D., Wick, M. J., Roberts, R. L., Findlay, K., Normark, S. J. & Harding, C. V. (1993). Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature* **361**, 359-362.

Pisetsky, D. S. (1996). The immunologic properties of DNA. *J. Immunol.* **156**, 421-423.

Pisetsky, D. S. (1997). Specificity and immunochemical properties of antibodies to bacterial DNA. *Methods* **11**, 55-61.

Poruchynsky, M. S. & Atkinson, P. H. (1991). Rotavirus protein rearrangement in purified membrane-enveloped intermediate particles. *J.Virol.* **65**, 4720-4727.

Poruchynsky, M. S., Maas, D. R. & Atkinson, P. H. (1991). Calcium depletion blocks the maturation of rotavirus by altering the oligomerization of virus-encoded proteins in the ER. *J. Cell. Biol.* **114**, 651-661.

Poruchynsky, M. S., Tyndall, C., Both, G. W., Sato, F., Bellamy, A. R. & Atkinson, P. A. (1985). Deletions into an NH2-terminal hydrophobic domain result in secretion of rotavirus VP7, a resident endoplasmic reticulum membrane glycoprotein. *J. Cell. Biol.* **101**, 2199-2209.

Potter, A. A., Cox, G., Parker, M. & Babiuk, L. A. (1987). The complete nucleotide sequence of bovine rotavirus C486 gene 4 cDNA. *Nucleic Acids Res.* **15**, 4361.

Prasad, B. V. V., Burns, J. W., Marietta, E., Estes, M. K. & Chiu, W. (1990). Localization of VP4 neutralization sites in rotavirus by three-dimensional cryo-electron microscopy. *Nature* **343**, 476-479.

Prasad, B. V. V., Rothnagel, R., Zeng, C. Q. Y., Jakana, J., Lawton, J. A., Chiu, W. & Estes, M. K. (1996). Visualization of ordered genomic RNA and localization of transcriptional complexes in rotavirus. *Nature* **382**, 471-473.

Qin, D., Wu, J., Carroll, M. C., Burton, G. F., Szakal, A. K. & Tew, J. G. (1998a). Evidence for an important interaction between a complement-derived CD21

ligand on follicular dendritic cells and CD21 on B cells in the initiation of IgG responses. *J. Immunol.* **161**, 4549-4554.

Qin, Z., Richter, G., Schüler, T., Ibe, S., Cao, X. & Blankenstein, T. (1998b). B cells inhibit induction of T-cell dependent tumor immunity. *Nat. Med.* **4**, 627-630.

Ramsay, A. J., Leong, K. H. & Ramshaw, I. A. (1997). DNA vaccination against virus infection and enhancement of antiviral immunity following consecutive immunization with DNA and viral vectors. *Immunol. and Cell. Biol.* **75**, 382-388.

Raz, E., Carson, D. A., Parker, S. E., Parr, T. B., Abai, A. M., Aichinger, G., Gromkowski, S. H., Singh, M., Lew, D., Yankauckas, M. A., Baird, S. M. & Rhodes, G. H. (1994). Intradermal gene immunization: The possible role of DNA uptake in the induction of cellular immunity to viruses. *Proc. Natl. Acad. Sci. USA* **91**, 9519-9523.

Raz, E., Tighe, H., Sato, Y., Corr, M., Dudler, J. A., Roman, M., Swain, S. L., Spiegelberg, H. L. & Carson, D. A. (1996). Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. *Proc. Natl. Acad. Sci. USA* **93**, 5141-5145.

Redmond, M. J., Ijaz, M. K., Parker, M. D., Sabara, M. I., Dent, D., Gibbons, E. & Babiuk, L. A. (1993). Assembly of recombinant rotavirus proteins into virus-like particles and assessment of vaccine potential. *Vaccine* **11**, 273-281.

Ridge, J. P., Fuchs, E. J. & Matzinger, P. (1996). Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. *Science* **271**, 1723-1726.

Ridge, J. P., Rosa, F. D. & Matzinger, P. (1998). A conditioned dendritic cell can be a temporal bridge between a $CD4^+$ T-helper and T-killer cell. *Nature* **393**, 474-478.

Riepenhoff-Talty, M., Suzuki, H. & Ogra, P. L. (1982). Characteristics of the cell-mediated immune response to rotavirus in suckling mice. *Dev. Biol. Stand.* **53**, 263-268.

Robinson, H. L. (1997). Nucleic acid vaccines : an overview. *Vaccine* **15**, 785-787.

Robinson, H. L. & Torres, C. A. (1997). DNA vaccines. *Sem. in Immunol.* **9**, 271-283.

Rock, K. L. (1996). A new foreign policy : MHC class I molecules monitor the outside world. *Immunol. Today* **17**, 131-137.

Rodger, S. M., Bishop, R. F., Birch, C., McLean, B. & Holmes, I. H. (1981). Molecular epidemiology of human rotaviruses in Melbourne, Australia from 1973 to 1979, as determined by electrophoresis of genome ribonucleic acid. *J.Clin.Microbiol.* **13**, 272-278.

Rodriguez, F., Zhang, J. & Whitton, J. L. (1997). DNA immunization: ubiquitination of a viral protein enhanced cytotoxic T-lymphocyte induction and antiviral protection but abrogates antibody induction. *J. Virol.* **71**, 8497-8503.

Roman, M., Martin-Orozco, E., Goodman, J. S., Nguyen, M.-D., Sato, Y., Ronaghy, A., Kornbluth, R. S., Richman, D. D., Carson, D. A. & Raz, E. (1997). Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat. Med.* **3**, 849-854.

Rosé, R. J., Williams, M. B., Rott, L. S., Butcher, E. C. & Greenberg, H. B. (1998). Expression of the mucosal homing receptor $\alpha_4\,\beta_7$ correlates with the ability of $CD8^+$ memory T cells to clear rotavirus infection. *J. Virol.* **72**, 726-730.

Rott, L. S., Briskin, M. J., Anrew, D. P., Berg, E. L. & Butcher, E. C. (1996). A fundamental subdivision of circulating lymphocytes defined by adhesion to mucosal addressin cell adhesion molecule-1. *J. Immunol.* **156**, 3727-3736.

Rott, L. S., Rosé, J. R., Bass, D., Williams, M. B., Greenberg, H. B. & Butcher, E. C. (1997). Expression of mucosal homing receptor alpha4beta7 by circulation $CD4^+$ cells with memory for intestinal rotavirus. *J. Clin. Invest.* **100**, 1204-1208.

Ruggeri, F. M. & Greenberg, H. B. (1991). Antibodies to the trypsin cleavage peptide VP8* neutralize rotevirus by inhibiting binding of virions to target cells in culture. *J.Virol.* **65**, 2211-2219.

Ruiz, M. C., Charpilienne, A., Liprandi, F., Gajardo, R., Michelangeli, F. & Cohen, J. (1996). The concentration of Ca^{2+} that solubilizes outer capsid proteins from rotavirus particles is dependent on the strain. *J. Virol.* **70**, 4877-4883.

Sabara, M., Babiuk, L. A., Gilchrist, J. & Misra, V. (1982). Effect of tunicamycin on rotavirus assembly and infectivity. *J. Virol.* **43**, 1082-1090.

Saif, L. J. & Fernandez, F. M. (1996). Group A rotavirus veterinary vaccines. *J. Infect. Dis.* **174 (Suppl. 1)**, S98-S106.

Saif, L. J. & Jackwood, D. L. (1990). Enteric virus vaccine: theoretical considerations, current status, and future approaches. In *Viral diarrheas of man and animals*, pp. 313-329. Edited by L. J. Saif & K. W. Theil. Boca Raton, FL: CRC Press.

Saif, L. J., Rosen, B. & Parwani, A. (1994). Animal rotaviruses. In *Virus infections of the gastrointestinal tract*, 2nd edn, pp. 279-367. Edited by A. Z. Kapikian. New York: Marcel-Dekker.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). Plasmid vectors. In *Molecular cloning: a laboratory manual*, pp. 1.1-1.110. Edited by C. Nolan. New York: Cold Spring Harbor Laboratory press.

Sarzotti, M., Dean, T. A., Remington, M. P., Ly, C. D., Furth, P. A. & Robbins, D. S. (1997). Induction of cytotoxic T cell responses in newborn mice by DNA immunization. *Vaccine* **15**, 795-797.

Sarzotti, M., Robbins, D. S. & Hoffman, P. M. (1996). Induction of protective CTL responses in newborn mice by a murine retrovirus. *Science* **271**, 1726-1728.

Sato, Y., Roman, M., Tighe, H., Lee, D., Corr, M., Nguyen, M.-D., Silverman, G. J., Lotz, M., Carson, D. A. & Raz, E. (1996). Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273**, 352-354.

Schoenberger, S. P., Toes, R. E. M., van der Voort, E. I. H., Offringa, R. & Melief, C. J. M. (1998). T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* **393**, 480-483.

Shahrabadi, M. S., Babiuk, L. A. & Lee, P. W. K. (1987). Further analysis of the role of calcium in rotavirus morphogenesis. *Virology* **158**, 103-111.

Shaw, A. L., Rothnagel, R., Zeng, C. Q. Y., Lawton, J. A., Ramig, R. F., Estes, M. K. & Prasad, B. V. V. (1996). Rotavirus structure: interactions between the structural proteins. *Arch. Virol.* **12 (Suppl.)**, 21-27.

Shaw, A. L., Rothnagel, R., Zeng, C. Q. Y., Ramig, R. F., Chiu, W. & Prasad, B. V. V. (1993a). Three-dimensional visualization of the rotavirus hemagglutinin structure. *Cell* **74**, 693-701.

Shaw, R. D., Groene, W. S., Mackow, E. R., Merchant, A. A. & Cheng, E. H. (1991). VP4-specific intestinal antibody response to rotavirus in a murine model of heterotypic infection. *J. Virol.* **65**, 3052-3059.

Shaw, R. D. & Hempson, S. J. (1996). Replication as a determinant of the intestinal response to rotavirus. *J. Inf. Dis.* **174**, 1328-1331.

Shaw, R. D., Hempson, S. J. & Mackow, E. R. (1995). Rotavirus diarrhea is caused by nonreplication viral particles. *J.Virol.* **69**, 5946-5950.

Shaw, R. D., Merchant, A. A., Groene, W. S. & Cheng, E. H. (1993b). Persistence of intestinal antibody response to heterologous rotavirus infection in a murine model beyond 1 year. *J. Clin. Microl.* **31**, 188-191.

Siegrist, C.-A. (1997). Potential advantages and risks of nucleic acid vaccines for infant immunization. *Vaccine* **15**, 798-800.

Sjölander, A., Baldwin, T. M., Curtis, J. M. & Handman, E. (1998). Induction of a Th1 immune response and simultaneous lack of activation of a Th2 response are required for generation of immunity to Leishmaniasis. *J. Immunol.* **160**, 3949-3957.

Sparwasser, T., Koch, E.-S., Vabulas, R. M., Heeg, K., Lipford, G. B., Ellwart, J. W. & Wagner, H. (1998). Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol.* **28**, 2045-2054.

Stacey, K. J., Sweet, M. J. & Hume, D. A. (1996). Macrophages ingest and are activated by bacterial DNA. *J. Immunol.* **157**, 2116-2122.

Stirzaker, S. C. & Both, G. W. (1989). The signal peptide of the rotavirus glycoprotein VP7 is essential for its retention in the ER as an integral membrane protein. *Cell* **56**, 741-747.

Stirzaker, S. C., Whitfeld, P. L., Christie, D. L., Bellamy, A. R. & Both, G. W. (1987). Processing of rotavirus glycoprotein VP7: implication for the retention of the protein in the endoplasmic reticulum. *J. Cell Biol.* **105**, 2897-2903.

Stockinger, B., Zal, T., Zal, A. & Gray, D. (1996). B cells solicit their own help from T cells. *J. Exp. Med.* **183**, 891-899.

Sun, S., Beard, C., Jaenisch, R., Jones, P. & Sprent, J. (1997). Mitogenicity of DNA from different organisms for murine B cells. *J. Immunol.* **159**, 3119-3125.

Sun, S., Zhang, X., Tough, D. F. & Sprent, J. (1998). Type I interferon-mediated stimulation of T cells by CpG DNA. *J. Exp. Med.* **188**, 2335-2342.

Suradhat, S., Yoo, D., Babiuk, L. A., Griebel, P. & Baca-Estrada, M. (1997). DNA immunization with a bovine rotavirus VP4 gene induced a Th1-like immune response in mice. *Viral Immunol.* **10**, 117-127.

Svensson, L., Sheshberadaran, H., Vene, S., Norrby, E., Grandien, M. & Wadell, G. (1987). Serum antibody responses to individual viral polypeptides in human rotavirus infection. *J. Gen. Virol.* **68**, 643-651.

Tang, D., DeVit, M. & Johnston, S. A. (1992). Genetic immunization is a simple method for eliciting an immune response. *Nature* **356**, 152-154.

Taniguchi, K., Morita, Y., Urasawa, T. & Urasawa, S. (1987). Cross-reactive neutralization epitopes on VP3 of human rotavirus: analysis with monoclonal antibodies and antigenic variants. *J. Virol.* **61**, 1726-1730.

Taniguchi, K. & Urasawa, S. (1995). Diversity in rotavirus genomes. *Sem. Virol.* **6**, 123-131.

Tedder, T. F., Inaoki, M. & Sato, S. (1997). The CD19-CD21 complex regulates signal transduction thresholds governing humoral immunity and autoimmunity. *Immunity* **6**, 107-118.

Tevethia, S. S., Greenfield, R. S., Flyer, D. C. & Tevethia, M. J. (1980). SV40 transplantation antigen: relationship to SV40-specific proteins. *Cold Spring Habor Symp.* **44**, 235-242.

Tew, J. G., Wu, J., Qin, D., Helm, S., Burton, G. F. & Szakal, A. K. (1997). Follicular dendritic cells and presentation of antigen and costimulatory signals to B cells. *Immunol. Rev.* **156**, 39-52.

Thornton, B. P., Vetvicka, V. & Ross, G. D. (1996). Function of C3 in a humoral response: iC3b/C3dg bound to an immune complex generated with natural antibody and primary antigen promotes antigen uptake and the expression of co-stimulatory molecules by all B cells, but only stimulates immunoglobulin synthesis by antigen specific B cells. *Clin. Exp. Immunol.* **104**, 531-537.

Tian, P., Ball, J. M., Zeng, C. Q. Y. & Estes, M. K. (1996). The rotavirus nonstructural glycoprotein NSP4 possesses membrane destabilization activity. *J. Virol.* **70**, 6973-6981.

Tian, P., Estes, M. K., Hu, Y., Ball, J. M., Zeng, C. Q. Y. & Shilling, W. P. (1995). The rotavirus nonstructural glycoprotein NSP4 mobilizes Ca^{2+} from the endoplasmic reticulum. *J. Virol.* **69**, 5763-5772.

Tighe, H., Corr, M., Roman, M. & Raz, E. (1998). Gene vaccination: plasmid DNA is more than just a blueprint. *Immunol. Today* **19**, 89-97.

Tikoo, S. K., Campos, M. & Babiuk, L. A. (1995a). Bovine herpesvirus 1 (BHV-1): biology, pathogenesis and control. *Adv. Virus Res.* **45**, 191-223.

Tikoo, S. K., Campos, M., Popowich, Y. I., van Drunen Littel-van den Hurk, S. & Babiuk, L. A. (1995b). Lymphocyte proliferative responses to recombinant bovine herpes virus type 1 (BHV-1) glycoprotein gD (gIV) in immune cattle: identification of a T cell epitope. *Viral Immunol.* **8**, 19-25.

Tikoo, S. K., Fitzpatrick, D. R., Babiuk, L. A. & Zamb, T. J. (1990). Molecular cloning, sequencing and expression of functional bovine herpesvirus 1 glycoprotein gIV in transfected bovine cells. *J. Virol.* **64**, 5132-5142.

Tokunaga, T., Yamamoto, H., Shimada, S., Abe, H., Fukuda, T., Fujisawa, Y., Furutani, Y., Yano, O., Kataoka, T., Sudo, T., Makiguchi, N. & Suganuma, T. (1984). Antitumor activity of deoxyribonucleic acid fraction from *Micobacterium bovis* BCG. I. Isolation, physiochemical characterization, and antitumor activity. *J. Natl. Cancer Inst.* **72**, 955-962.

Torres, C. A. T., Iwasaki, A., Baber, B. H. & Robinson, H. L. (1997). Differential dependence on target site tissue for gene gun and intramuscular immunizations. *J. Immunol.* **158**, 4529-4532.

Udono, H., Levey, D. L. & Srivastava, P. K. (1994). Cellular requirements for tumor-specific immunity elicited by heat shock proteins: tumor rejection antigen gp96 primes $CD8^+$ T cells *in vivo*. *Proc. Natl. Acad. Sci. USA* **91**, 3077-3081.

Ulmer, J. B., Deck, R. R., Dewitt, C. M., Donnelly, J. J. & Liu, M. A. (1996a). Generation of MHC class I-restricted cytotoxic lymphocytes by expression of a viral protein in muscle cells: antigen presentation by non-muscle cells. *Immunology* **89**, 59-67.

Ulmer, J. B., Donelly, J. J., Parker, S. E., Rhodes, G. H., Felgner, P. L., Dwarki, V. J., Gromkowski, S. H., Deck, R. R., DeWitt, C. M., Friedman, A., Hawe, L. A., Leander, K. R., Martinez, D., Perry, H. C., Shiver, J. W., Montgomery, D. L. & Liu, M. A. (1993). Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* **259**, 1745-1749.

Ulmer, J. B., Donelly, J. J., Shiver, J. W. & Liu, M. A. (1996b). Prospects for induction of mucosal immunity by DNA vaccines. In *Mucosal vaccines*, pp. 119-127. Edited by H. Kiyono, J. R. McGhee & P. L. Ogra. San Diego, CA: Academic press.

Ulmer, J. B., Fu, T.-M., Deck, R. R., Friedman, A., Guan, L., DeWitt, C., Liu, X., Wang, S., Liu, M. A., Donnelly, J. J. & Caulfield, M. J. (1998). Protective $CD4^+$ and $CD8^+$ T cells against influenza virus induced by vaccination with nucleoprotein DNA. *J. Virol.* **72**, 5648-5653.

van Drunen Littel-van den Hurk, S., Braun, R. P., Lewis, P. J., Karvonen, B. C., Baca-Estrada, M. E., Snider, M., McCartney, D., Watts, T. & Babiuk, L. A. (1998). Intradermal immunization with a bovine herpesvirus-1 DNA vaccine induces protective immunity in cattle. *J. Gen. Virol.* **79**, 831-839.

van Kooten, C. & Banchereau, J. (1997). Functions of CD40 on B cells, dendritic cells, and other cells. *Curr. Opin. Immunol.* **9**, 330-337.

von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* **14**, 4683-4691.

Vora, K. A., Ravetch, J. V. & Manser, T. (1997). Amplified follicular immune complex deposition in mice lacking the Fc receptor γ-chain does not alter maturation of the B cell response. *J. Immunol.* **159**, 2116-2124.

W.H.O. Steering committee of the scientific working group on viral diarrheas. (1984). Nomenclature of human rotaviruses: designation of subgroups and serotypes. *Bull. WHO* **62**, 501-503.

Wang, Y., Xiang, Z., Pasquini, S. & Ertl, H. C. (1998). Effect of passive immunization or maternal transferred immunity on the antibody response to a genetic vaccine to rabies virus. *J. Virol.* **72**, 1790-1796.

Ward, R. L. (1996). Mechanisms of protection against rotavirus in humans and mice. *J. Infect. Dis.* **174 (Suppl 1)**, S51-S58.

Ward, R. L., Knowlton, D. R., Schiff, G. M., Hoshino, Y. & Greenberg, H. B. (1988). Relative concentrations of serum neutralizing antibody to VP3 and VP7 proteins in adults infected with human rotavirus. *J. Virol.* **62**, 1543-1549.

Ward, R. L., Mason, B. B., Bernstein, D. I., Sander, D. S., Smith, V. E., Zandle, G. A. & Rappaport, R. S. (1997). Attenuation of a human rotavirus vaccine candidate did not correlate with mutations in the NSP4 protein gene. *J.Virol.* **71**, 6267-6270.

Ward, R. L., McNeal, M. M. & Sheridan, J. F. (1992). Evidence that active protection following oral immunization of mice with live rotavirus is not dependent on neutralizing antibody. *Virology* **188**, 57-66.

Wetsel, R. A., Lundwall, A., Davidson, F., Gibson, T., Tack, B. F. & Fey, G. H. (1984). Structure of murine complement component C3. *J. Biol. Chem.* **259**, 13857-13862.

Whitbeck, J. C., Peng, C., Lou, H., Xu, R., Willis, S. H., Ponce-de-Leon, M., Peng, T., Nicola, A. V., Montgomery, R. I., Warner, M. S., Soulika, A. M., Spruce, L. A., Moore, W. T., Lambris, J. D., Spear, P. G., Cohen, G. H. & Eisenberg, R. J. (1997). Glycoprotein D of herpes simplex virus (HSV) binds directly to HVEM, a member of the tumor necrosis factor receptor superfamily and a mediator of HSV entry. *J. Virol.* **71**, 6083-6093.

Whitfeld, P. L., Tyndall, C., Stirzaker, S. C., Bellamy, A. R. & Both, G. W. (1987). Location of signal sequences within the rotavirus SA11 glycoprotein VP7 which direct it to the endoplasmic reticulum. *Mol. Cell. Biol.* **7**, 2491-2497.

Williams, M. B. & Butcher, E. C. (1997). Homing of naive and memory T lymphocyte subsets to Peyer's patches, lymph nodes and spleen. *J. Immunol.* **159**, 1746-1752.

Williams, R. S., Johnston, S. A., Riedy, M. & Devit, M. J. (1991). Introduction of foreign genes into tissue of living mice by DNA-coated microprojectiles. *Proc. Natl. Acad. Sci. USA* **88**, 2726-2730.

Wloch, M. K., Pasquini, S., Ertl, H. C. J. & Pisetsky, D. S. (1998). The influence of DNA sequence on the immunostimulatory properties of plasmid DNA vectors. *Hum. Gene Ther.* **9**, 1439-1447.

Wolff, J. A., Ludtke, J. J., Acsadi, G., Williams, P. & Jani, A. (1992). Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Human Molec. Gen.* **1**, 363-369.

Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A. & Felgner, P. L. (1990). Direct gene transfer into mouse muscle in vivo. *Science* **247**, 1465-1468.

Wynn, T. A., Eltoum, I., Oswald, I. P., Cheever, A. W. & Sher, A. (1994). Endogenous interleukin 12 (IL-12) regulates granuloma formation induced by eggs of

Schistosoma mansoni and exogenous IL-12 both inhibits and prophylactically immunizes against egg patholcgy. *J. Exp. Med.* **179**, 1551-1561.

Xiang, Z. Q., Spitalnik, S., Tran, M., Wunner, W. H., Cheng, J. & Ertl, H. C. J. (1994). Vaccination with a plasmid vector carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus. *Virology* **199**, 132-144.

Yasukawa, M., Nakagomi, O. & Kobayashi, Y. (1990). Rotavirus induces proliferative response and augments non-specific cytotoxic activity of lymphocytes in humans. *Clin. Exp. Immunol.* **80**, 49-55.

Yeager, M., Dryden, K. A., Olsen, N. H., Greenberg, H. B. & Baker, T. S. (1990). Three-dimensional structure of rhesus rotavirus by cryoelectron microscopy and image reconstruction. *J.Cell.Biol.* **110**, 2133-2144.

Yi, A. K., Chace, J. H., Cowdery, J. S. & Krieg, A. M. (1996a). IFN-γ promotes IL-6 and IgM secretion in response to CpG motifs in bacterial DNA and oligodeoxynucleotides. *J. Immunol.* **156**, 558-564.

Yi, A.-K., Klinman, D. M., Martin, T. L., Matson, S. & Krieg, A. M. (1996b). Rapid immune activation by CpG motifs in bacterial DNA : systemic induction of IL-6 transcription through an antioxidant-sensitive pathway. *J. Immunol.* **157**, 5394-5402.

Yi, A.-K. & Krieg, A. M. (1998). Cutting edge: Rapid induction of mitogen-activated protein kinases by immune stimulatory CpG DNA. *J. Immunol.* **161**, 4493-4497.

Yokoyama, M., Hassett, D. E., Zhang, J. & Whitton, L. (1997). DNA immunization can stimulate florid local inflammation, and the antiviral immunity induced varies depending on injection site. *Vaccine* **15**, 553-560.

Yolken, R., Arango-Jaramillo, S., Eiden, J. & Vonderfecht, S. (1988). Lack of genomic reassortment following infection of infant rats with group A and group B rotaviruses. *J. Infect. Dis.* **158**, 1120-1123.

Young, A. J., Marston, W. L., Dessing, M., Dudler, L. & Hein, W. R. (1997). Distinct recirculating and non-circulating B-lymphocyte pool in the peripheral blood are defined by coordinated expression of CD21 and L-selectin. *Blood* **90**, 4865-4875.

Yuan, L., Ward, L. A., Rosen, B. I., Thanh, L. T. & Saif, L. J. (1996). Systemic and intestinal antibody-secreting cell responses and correlates of protective immunity to human rotavirus in a gnotobiotic pig model of disease. *J. Virol.* **70**, 3075-3083.

Zhai, Y., Guo, R., Hsu, T. L., Yu, G. L., Ni, J., Kwon, B. S., Jiang, G. W., Lu, J., Tan, J., Ugustus, M., Carter, K., Rojas, L., Zhu, F., Lincoln, C., Endress, G., Xing, L., Wang, S., Oh, K. O., Gentz, R., Ruben, S., Lippman, M. E., Hsieh, S. L. & Yang, D. (1998). LIGHT, a novel ligand for lymphotoxin beta receptor and TR2/HVEM induces apoptosis and suppresses in vivo tumor formation via gene transfer. *J. Clin. Invest.* **102**, 1142-1151.

Zhang, M., Zeng, C. Q. Y., Dong, Y., Ball, J. M., Saif, L. J., Morris, A. P. & Estes, M. K. (1998). Mutations in rotavirus nonstructural glycoprotein NSP4 are associated with altered virus virulence. *J. Virol.* **72**, 3666-3672.

Zhao, D. (1992). Thesis: Molecular characterization and humoral immunity of bovine rotavirus VP4, VP6 and VP7. In *Department of Veterinary Microbiology*, pp. 138. Saskatoon: University of Saskatchewan.

Zhong, G., Sousa, C. R. & Germain, R. N. (1997). Antigen-unspecific B cells and lymphoid dendritic cells both show extensive surface expression of processed antigen-major histocompatibility complex class II complexes after soluble protein exposure in vivo or in vitro. *J. Exp. Med.* **186**, 673-682.

Zimmermann, S., Egeter, O., Hausmann, S., Lipford, G. B., Röcken, M., Wagner, H. & Heeg, K. (1998). Cutting edge: CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine Leishmaniasis. *J. Immunol.* **160**, 3627-3630.