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TITLE

THE DEVELOPMENT OF A MURINE MODEL FOR
ANALYZING THE Th-CELL RESPONSE TO A
BOVINE ROTAVIRUS

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**THE DEVELOPMENT OF A MURINE MODEL FOR
ANALYZING THE Th-CELL RESPONSE TO A
BOVINE ROTAVIRUS**

by

CHRISTOPHER DAVID JONES,B.Sc.(Warwick)

A thesis submitted for the degree of
Doctor of Philosophy to the
University of Warwick

All my experimental work was performed in the
Department of Biological Sciences, University
of Warwick and the World Health Organization
Laboratory, East Birmingham Hospital

Submitted in March 1993.

NUMEROUS ORIGINALS
IN COLOUR



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DECLARATION

I declare that all the work presented in this thesis is my own, although the interferon assays described in Chapter 10 were performed by Dr. Alan Morris and the ELISA's carried out in Chapter 7 were performed jointly with Dr. Graham Beards. Some of the inoculations of neonatal mice were kindly performed by Roger Jenkins.

DEDICATION

I dedicate this thesis to my mother and to the memory of my father and dear uncle Walt.

**"It has been a damned nice thing-
the nearest run thing you ever saw in your life."**

(The Duke of Wellington 1815)

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ABBREVIATIONS

Ab -	Antibody
B-lymphocyte -	Bursa-derived lymphocyte
BSA -	Bovine Serum Albumin
CD -	Cluster of Differentiation
Ci -	Curies
Con.A -	Concanavalin A
CPM -	Counts per Minute
CTL -	Cytotoxic T-lymphocyte
DC -	Dendritic Cell
ds -	Double-shelled
ds DNA -	Double-stranded Deoxyribonucleic Acid
ds RNA -	Double-stranded Ribonucleic Acid
EDTA -	Ethylenediamine tetracetic acid
EGTA -	Ethyleneglycol bis(beta-aminoethyl ether)-N,N'-tetracetic acid
ELISA -	Enzyme Linked Immunosorbent Assay
EM -	Electron Microscope
ER -	Endoplasmic Reticulum
FCS -	Foetal Calf Serum
FITC -	Fluorescein Isothiocyanate
g -	Gravitational Acceleration
HA -	Haemagglutinin
hr -	hours
IEM -	Immuno Electron Microscopy
Ig -	Immunoglobulin
IL -	Interleukin
i.m. -	intra-muscular
i.p. -	intra-peritoneal
i.v. -	intra-venous

M	Molar
mAb -	Monoclonal Antibody
mg -	Milligram
MHC -	Major Histocompatibility Complex
min. -	Minute
ml -	Millilitre
MOI -	Multiplicity Of Infection
nAb -	Neutralizing Antibody
NK -	Natural Killer
nmAb -	Neutralizing Monoclonal Antibody
PAGE -	Polyacrylamide Gel Electrophoresis
PBS -	Phosphate Buffered Saline
RER -	Rough Endoplasmic Reticulum
RPMI -	Roswell Park Memorial Institute
S.D. -	Standard Deviation
spp -	Species
ss -	Single-shelled
ss RNA -	Single-stranded Ribonucleic Acid
TCR -	T-cell Receptor
T-lymphocyte -	Thymus-Educated Lymphocyte
Th -	T-helper
Thy.1 -	Thymus-derived Antigen 1
ul -	Microlitre
ug -	Microgram

DEFINITION OF COMMON ROTAVIRUS STRAINS

EDIM -	Epizootic Diarrhoea of Infant Mice (Serotype 3)
NCDV -	Nebraska Calf Diarrhoea Virus (Serotype 6)
OSU -	Ohio State University (Porcine) (Serotype 5)
RF -	Bovine Rotavirus (Serotype 6)
RRV -	Rhesus Rotavirus (Serotype 3)

SA11 -	Simian Rotavirus (Serotype 3)
UKtc -	United Kingdom, tissue culture adapted rotavirus (Bovine) (Serotype 6)
Wa -	Human rotavirus (Serotype 1)

NUCLEOTIDE BASES

A - Adenine

T - Thymine

C - Cytosine

G - Guanine

U - Uracil

SINGLE LETTER AMINO-ACID CODE

A - Alanine

C - Cysteine

D - Aspartate

E - Glutamate

F - Phenylalanine

G - Glycine

H - Histidine

I - Isoleucine

K - Lysine

L - Leucine

M - Methionine

N - Asparagine

P - Proline

Q - Glutamine

R - Arginine

S - Serine

T - Threonine

V - Valine

W - Tryptophan

Y - Tyrosine

X - Unknown Amino-acid Replacement

SUMMARY

Rotaviruses are important human and veterinary pathogens and are responsible for some 1-2 million human deaths per annum, Worldwide. Conventional vaccine strategies for this pathogen have, on the whole been unsuccessful. Therefore, a detailed and comprehensive understanding of the immune response to rotaviruses, particularly at the cell mediated level is being sought, such that successful vaccines can be generated.

A lymphocyte proliferation assay system has been developed for examining the T_h cell response to the bovine rotavirus (BRV), UKtc. Splenocytes from adult BALB/c mice, orally inoculated with infectious BRV(UKtc) proliferated in response to *in vitro* stimulation with purified BRV(UKtc) particles.

Proliferation was (i)detected at 4 days and 8 days after primary oral inoculation, (ii)not detected in uninoculated animals, (iii)specific to the priming virus and (iv)eliminated by NH₄Cl treatment of the spleen cells. Splenocytes from animals challenged by both oral and intra-peritoneal (i.p.) routes, were more efficiently stimulated by double-shelled BRV(UKtc) particles than single-shelled particles.

Proliferation was found to be mediated by both Thy-1⁺,CD8⁻,CD4⁺ cells (*i.e.* Th cells) and Thy-1⁺,CD8⁺,CD4⁻ cells (*i.e.* cytotoxic T-cells) but was dependent on Thy-1⁺,CD8⁻,CD4⁺ cells, when mice were inoculated by either the oral or i.p. routes. A greater proportion of the splenocyte proliferative response was found to be due to Thy-1⁺,CD8⁻,CD4⁺ cells when the animals were inoculated by the i.p. route.

Viruse replication in the intestinal tract was not required for a splenocyte proliferative response to be detected and the splenocyte response was long lived. For example, significant proliferation to both double and single-shelled forms of BRV(UKtc) was detected at 144 days (oral inoculation) and 224 days (i.p.inoculation), after a single dose of virus.

BRV(UKtc) stimulated splenocytes secreted interferon-gamma, upon activation but no significant differences in titer were present between cells stimulated with double-shelled virus or single-shelled virus, in contrast to the [³H]thymidine incorporation results. Cross-challenge experiments with the porcine rotavirus OSU, showed that cross-reactivity existed at the T-cell level. However, memory splenocyte proliferative responses to this strain were not long lived following oral inoculation with BRV(UKtc).

The response of mesenteric lymph node cells to *in vitro* challenge with rotavirus was also studied at various times post oral inoculation. Of importance was the finding that proliferative responses to rotavirus were not present in mesenteric lymph node cells at 63 days post oral inoculation.

PREFACE

It was over one hundred years ago that Pasteur presented his 'germ theory' to science and since the discovery of the microbial World,great effort has been directed at the development of effective vaccines for many human and veterinary pathogens. Some vaccines such as smallpox and whooping cough have proved successful in the protection of large numbers of the human population,while others remain an elusive goal e.g.influenza,rotavirus and Human Immunodeficiency Virus (HIV).

Since empirical approaches to the development of vaccines for influenza,rotavirus and several other parasites have been unrewarding, emphasis has now shifted to a detailed analysis of these pathogens,their molecular biology,antigenicity and the role of the immune response in protection against them.

The basis of this thesis has been to gain a 'foothold' in an understanding of a single component of the immune response to rotavirus,which ultimately may increase our knowledge not only of the specific action of Th-cells to this pathogen but also of the immune response as a whole.

GENERAL INTRODUCTION

The main purpose of this thesis has been to establish a model system for analyzing the T-helper (Th)-cell immune response to the bovine rotavirus (BRV) United Kingdom,tissue culture adapted,(UKtc) strain.

The following introduction is intended to examine five distinct topics, all of which are necessary for a complete appreciation of the experimental results. First, the rotaviruses as a group will be considered, with a discussion of their major characteristics. Second, the main features of the Th-cell, its mechanism of activation and the role in the immune response, will be considered, followed by a third section on the Th-cell in Gut Associated Lymphoid Tissue (GALT). The overall immune response to rotavirus will then be examined. This fourth section will cover both the humoral and cell mediated immune responses. Finally, there is a small section covering vaccine strategies and other measures which have been undertaken to combat rotavirus infection.

CHAPTER 1

THE ROTAVIRUSES

1.1 GENERAL FEATURES/EPIDEMIOLOGY

Rotaviruses cause severe gastroenteritis and diarrhoea in infants and young children, throughout the world (Flewett & Woode, 1978; Cukor & Blacklow, 1984).

The problem of infantile gastroenteritis leading to diarrhoea and death is enormous. Acute gastroenteritis, including viral gastroenteritis is the leading cause of death in children under the age of 4 years in the Developing World (Tolia & Dubois, 1985). World Health Organization statistics indicate that diarrhoeal diseases may account for 15-34% of all annual deaths in some Third World countries (WHO, 1973). Estimates in one study suggested that between 1977 and 1978 there were 3.5 billion cases of diarrhoea and 5-10 million deaths attributable to diarrhoeal disease in Asia, Africa and Latin America (Walsh & Warren, 1979).

Rotaviruses are the single most important aetiological agents of acute gastroenteritis and diarrhoeal illness in infants and young children throughout the world (Estes *et al.*, 1983; Cukor & Blacklow, 1984; Kapikian & Chanock, 1990). They have been estimated to cause at least 873,000 deaths per annum in infants and young children between 1-4 years of age (Institute of Medicine, 1986), or even 2.5 million deaths per annum (Argarwal, 1979; Kapikian *et al.*, 1986).

Rotaviruses are ubiquitous agents, infecting virtually all children by 36 months of age (Edelman *et al.*, 1989). Children are most likely to be affected with rotavirus gastroenteritis and diarrhoea between 6 months and 2 years of age, after which the incidence of rotavirus disease declines sharply (Bryden *et al.*, 1975; Kapikian *et al.*, 1976; Bartlett *et al.*, 1987). The highest incidence of rotavirus diarrhoea, in humans, correlates with the peak period of weaning (Black *et al.*, 1982; Mata *et al.*, 1983). The shock of weaning and the increased possibility of contamination of food have been proposed to be as important as the lack of the mother's milk in the observed increase in the disease (Lecce & King, 1978; Bishop *et al.*, 1979).

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Asymptomatic rotavirus infection has also been observed before 6 months of age, since newborn infants frequently excrete rotavirus in their faeces without showing any clinical symptoms (Cameron *et al.*, 1975; Banatvala *et al.*, 1978; Champsaur *et al.*, 1984). Rotavirus carriage (*i.e.* no serologic response or diarrhoea) has also been documented (Champsaur *et al.*, 1984).

Poverty may play an important part in the susceptibility to rotavirus infection in the Third World, since several studies have shown that the poorest families have the highest diarrhoea attack rates (Guerrant *et al.*, 1983; Mata *et al.*, 1983).

There is some evidence to suggest that race correlates with susceptibility to infection. For example, in a study carried out at the Children's Hospital National Medical Centre (Washington D.C.), the age distribution of patients admitted to the hospital with rotavirus gastroenteritis was different for black and non-black patients (Brandt *et al.*, 1979). The reason for this difference may be related to crowded social conditions (Kapikian & Chanock, 1990) or it may reflect an important biological difference. This observation may be very relevant to the design of rotavirus vaccine programmes. An absence of over crowding may also account for the observation that the incidence of rotavirus infection is lower in rural areas (Schnagl *et al.*, 1979; Schoub *et al.*, 1982).

In Developed countries, gastroenteritis and diarrhoeal disease is also a problem. In the Cleveland Family Study conducted in the United States infectious gastroenteritis was the second most common disease experience, accounting for some 16% of approximately 25000 illnesses over a period of 10 years (Dingle *et al.*, 1964). Viral gastroenteritis is the second most common infectious clinical entity, second only to viral upper respiratory tract illness (Kapikian *et al.*, 1980). However, in the Developed World gastroenteritis (including rotavirus gastroenteritis) in infants and young children does not normally result in mortality but does cause extensive morbidity and a need for hospitalization (Rodriguez *et al.*, 1980; Kapikian & Chanock, 1990). Recently, rotaviruses were estimated to be responsible for over 1 million cases of severe diarrhoea per year in children from 1-4 years of age, in the USA (Ho *et al.*,

1988). Consequently, rotavirus disease is a significant economic burden to Developed Countries. In the USA, treatment for rotavirus induced dehydration has been estimated to cost 500 million to 1 billion dollars annually (Offit *et al.*, 1991).

1.2 ADULT INFECTION AND AGE SUSCEPTIBILITY

Rotavirus induced gastroenteritis also occurs in older children (Hara *et al.*, 1978) and adults (Kapikian *et al.*, 1976; Lyke *et al.*, 1978) but the incidence is very much reduced (Bartlett *et al.*, 1987). More specifically, rotavirus induced diarrhoea has been reported in staff and patients in several hospitals (Cubbitt & Holzel, 1980; Holzel *et al.*, 1980), adult traveller's (Bolivar *et al.*, 1978; Sheridan *et al.*, 1981), isolated populations (Foster *et al.*, 1980; Linharees *et al.*, 1981) and in immuno-compromised patients (Yolken *et al.*, 1982; Jarvis *et al.*, 1983). Larger outbreaks of Group B rotavirus disease in adults (see section 1.14.1) have been reported in China (Hung *et al.*, 1984; Su *et al.*, 1986).

Subclinical infection of adults with rotavirus, apparently occurs quite frequently (Kim *et al.*, 1977; Grimwood *et al.*, 1983) and it is thought that adults or older children, sub-clinically infected with rotavirus may be important in the transmission of rotavirus infection to young children (Wenman *et al.*, 1979; Grimwood *et al.*, 1983). Rotaviruses are also a problem in the elderly (Cubbitt & Holzel, 1980; Marrie *et al.*, 1982).

Together, these data suggest that there is no age resistance to infection in humans (*i.e.* discounting immunological parameters) (McNulty, 1978).

1.3 INFECTION IN ANIMALS AND AGE SUSCEPTIBILITY

Rotaviruses have a wide host range and in addition to humans, have been detected in many animal species including mice (Adams & Kraft, 1963) calves (Mebus *et al.*, 1969), piglets (Lecce *et al.*, 1976), lambs (Snodgrass & Wells, 1976), foals (Flewett *et al.*, 1975), rabbits (Bryden *et al.*, 1976), deer (Tzipori & Caple, 1976), goats (Scott *et al.*, 1978), dogs (Eugster & Sidwa, 1979), cats (Snodgrass *et al.*, 1979), apes (Ashley *et al.*, 1978) and monkeys (Stuker *et al.*,

1980). They have also been found in turkeys (McNulty *et al.*, 1979) and chickens (Jones *et al.*, 1979).

In contrast to the situation in humans, symptomatic rotaviral infections occur mainly in neonatal or very young animals (Wyatt *et al.*, 1976; McNulty, 1978; Holmes, 1979). The infection of young leads to both morbidity (resulting in animal wastage) and mortality. Mortality rates of up to 30% have been reported in some herds of cattle (Flewett & Woode, 1978). As a result, rotaviruses are a considerable economic burden in agriculture, estimated to cost the British dairy industry several million pounds each year (Johnson & McCrae, 1989).

Under experimental conditions some human rotavirus strains induce diarrhoeal illness or sub-clinical infection in the newborns of several animal species including mice (Gouvea *et al.*, 1986), gnotobiotic calves (Mebus *et al.*, 1976), piglets (Bridger *et al.*, 1975), lambs (Snodgrass *et al.*, 1977), rhesus monkeys (Wyatt *et al.*, 1976) and dogs (Tzipori, 1976).

However, it is not clear whether, under natural conditions human rotaviruses infect animals or vice-versa. Studies of the degree of genetic relatedness between human and animal rotavirus strains suggest that at least some human and animal rotaviruses exhibit significant differences (Matsuno & Nakajima, 1982; Greenberg *et al.*, 1984; Flores *et al.* 1986), suggesting that inter-species infection is unlikely under natural conditions. Field studies support this (Ryder *et al.*, 1986; Garbarge-Chenon *et al.*, 1986). However, Marrie *et al.*, (1982) suggests that animals may be a source of rotavirus infection in humans and more recently a feline rotavirus was shown to be genetically related to a human rotavirus strain (Nakagomi & Nakagomi, 1989). Several animal strains also share a neutralization antigen with strains in human serotypes 3 and 4 (see Table 1). Human and bovine serotype 8 rotaviruses have been proposed to have arisen by genome reassortment (Browning *et al.*, 1992). Several factors suggest that inter-species transfer between dogs and man is possible. For example, dogs (i) excrete relatively high levels of rotavirus in their faeces (Roseto *et al.*, 1980); (ii) they have been shown to be susceptible to human

TABLE 1 SOME COMMON GROUP A ROTAVIRUS SEROTYPES**DETERMINED BY NEUTRALIZATION**

(Subgroup antigen is shown in parentheses).

(From Kapikian & Chanock, 1990).

SEROTYPE	HUMAN ROTAVIRUS REFERENCE STRAINS	ANIMAL ROTAVIRUS REFERENCE STRAINS
1	Wa,KB,KU,D,M37,DB, RV4 (II)	None
2	DS-1,S2,KUN,390, HN 126,RV5,1076(I)	None
3	P,M,WALK,57/14,Mo, Ito,Nemoto,YO,McN, RV1,RV3(II),AU-1(I)	Simian SA11, Rhesus Monkey MMU 18006,Canine CU-1, Feline (TAKA) (I); Equine H-2 (not I or II), FI-14 (both I and II),E1 (II); Lapine C11ALA (I), R2 (II); Murine EW(EDIM),EB (not I or II); Porcine MDR-13,CRW-18 (I)
4	St Thomas No.3 & 4 Hosokawa,Hochi,VA70 (II)	Porcine SB-2 (I); Porcine Gottfried, SB-1A (II)
5	None	Porcine DSU,EE, Equine H-1 (I)
6	None	Bovine NCDV,UK,WC3 (I)
7	None	Chicken Ch-2,Turkey Ty1 (not I or II)
8	69M,B37,B38,57M(I)	
9	WI-61,F45 (II)	

rotavirus infection (Tzipori, 1976); (iii) there is a close social interaction between dogs and humans and (iv) at least one canine rotavirus strain (CU-1), shares a neutralization antigen with the human serotype 3 strain WALK 57/14 (Hoshino *et al.*, 1984). Further work may help to reveal whether there is a degree of genetic relatedness between canine and human rotavirus strains. These data suggest that rotaviruses are moderately, though certainly not absolutely, host specific in nature (Holmes, 1983).

It is not clear whether there is an age resistance to infection and disease in animals. Work with the Epizootic Diarrhoea of Infant Mice (EDIM) model of rotavirus induced gastroenteritis suggests that the susceptibility of mice to EDIM induced diarrhoea is age dependent, decreasing in concert with intestinal maturation (Wolf *et al.*, 1981; Eydelloth *et al.*, 1984). This may be due to a decrease in rotavirus receptors, with increasing age (Riepenhoff-Talty *et al.*, 1982) or to a decreased efficiency in viral protein expression (Eydelloth *et al.*, 1984). However, rabbits of different ages (up to 112 days old) clearly showed a susceptibility to infection and disease with the rabbit Ala (serotype 3) rotavirus strain (Conner *et al.*, 1988). In addition, Ward *et al.*, (1990) recently showed that mice up to 180 days old could be infected with a tissue culture (t.c.) adapted EDIM strain of rotavirus.

1.4 GEOGRAPHICAL AND TEMPORAL DISTRIBUTION

Rotaviruses have been detected throughout the world and contribute to infantile gastroenteritis in every country where the disease has been studied (Kapikian & Chanock, 1990).

In humans there is a seasonal incidence of rotavirus gastroenteritis, at least in temperate climates (McNulty, 1978) and higher levels of infection are seen during the cooler months of the year (Birch *et al.*, 1977).

The reason for the increased incidence of rotavirus induced diarrhoea during the winter months is not known, although it has been proposed that indoor crowding and low indoor relative humidity may increase aerosolization of rotavirus particles on surfaces (Brandt *et al.*, 1982). However, a correlation

between relative humidity and rotaviral disease has not been observed in all cases (Moe & Shirley, 1982; Konno *et al.*, 1983).

Outbreaks of rotavirus gastroenteritis have been reported throughout the year in South Africa, during the summer in Taiwan, during the 'small rains' in Ethiopia and most months in tropical climates (with peak periods during the cooler or drier months) (Christensen, 1989; Kapikian & Chanock, 1990).

1.5 TRANSMISSION

Current evidence suggests that rotaviruses are transmitted primarily by the foecal-oral route (Kapikian & Chanock, 1990; Bartlett *et al.*, 1987) and diarrhoea can be induced by the oral administration of rotavirus containing stool material to both animals and man (Light & Hodes, 1943; Ward *et al.*, 1986). There is, however, still speculation as to whether rotaviruses can be transmitted by the respiratory route (Gurwith *et al.*, 1981; Gordon, 1982). There is evidence to suggest that rotaviruses can (Santosham *et al.*, 1983; Prince *et al.*, 1986) and can not (Carr *et al.*, 1976; Kapikian *et al.*, 1983) be transmitted via the respiratory route. However, Prince *et al.*, (1986) showed quite clearly that rotaviruses are capable of infecting the respiratory tract epithelial mucosa and it may, therefore, be a site of primary infection, in some cases. Possible explanations for the conflicting data include the strains of rotavirus, the time of year and the locality (Christensen, 1989).

In the Developed World, water is not considered to be a major mechanism of disease transmission, despite the detection of rotaviruses in raw and treated sewage (Smith & Gerba, 1982). However, in the Developing World contaminated water is an important mode of transmission and has been attributed to several large outbreaks of rotavirus induced diarrhoea (Deetz *et al.*, 1984; Bartlett, 1987). Their ability to survive for long periods in water (Hurst & Gerba, 1980) and resistance to chlorination (Snodgrass & Herring, 1977; Tan & Schnagl, 1981) strongly suggest water as a suitable medium for transmission.

A major factor which is thought to contribute to the prevalence of rotavirus induced gastroenteritis is the resistance to physical inactivation (see section

1.9) (Kapikian & Chanock, 1990). For example, rotaviruses can survive when dried on inanimate objects (as well as human hands), and faecal matter protects the virus against inactivation, in the dried state (Ward *et al.*, 1991a).

This may account for the high incidence of nosocomial infection with rotavirus which has been reported (Middleton *et al.*, 1977; Rodriguez *et al.*, 1983). Effective disinfection (see section 1.9) and careful hand hygiene are therefore considered to be important means of control in hospital situations and in animal husbandry (Lecce *et al.*, 1978; Black *et al.*, 1981; Ward *et al.*, 1991a).

1.6 HISTORY

One of the earliest reported cases of non-bacterial diarrhoea was in 1943 when Light & Hodes transmitted diarrhoea to calves using filtrates of stools from diarrhoeic infants (Light & Hodes, 1943). Rotavirus was later shown to be present in the faecal material from one of the infected calves (Hodes, 1977).

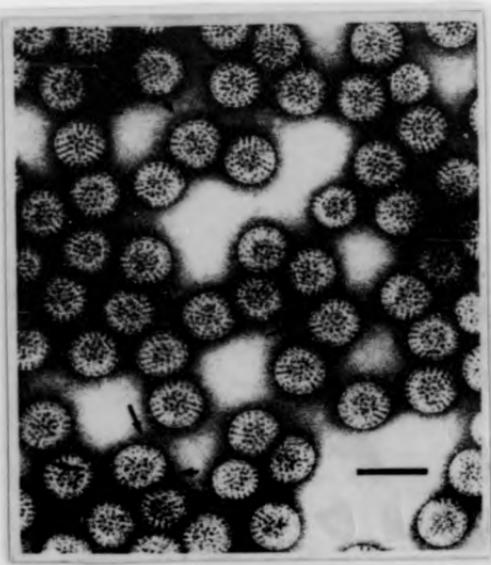
Further work of importance in the discovery of rotaviruses in animals includes Adams & Kraft, (1963) (mice), Malherbe & Strickland-Cholmley, (1967) (sheep and cattle with the 'Offal' or 'O' agent) and Mebus *et al.*, (1969) (cattle). Work by several other groups (Fernelius *et al.*, 1972; Lecatsas, 1972; Much & Zajac, 1972; Flewett *et al.*, 1974; Kapikian *et al.*, 1976), showed that several novel reovirus-like isolates possessed similar morphological and/or antigenic relatedness. Bishop *et al.*, (1973) showed that particles morphologically indistinguishable from calf, reo-like virus were present in the epithelial cells of duodenal mucosa of children with non-bacterial gastroenteritis. They were subsequently shown to be present in the faeces of many children with gastroenteritis, throughout the World (e.g. Flewett *et al.*, 1973; Holmes *et al.*, 1974; Orstavik *et al.*, 1974).

Flewett first suggested the genus name 'rotavirus' in 1974, because morphologically complete virions resemble wheels with a central 'hub', radiating 'spokes' and a circular 'rim' (Flewett *et al.*, 1974). (See Fig. 1a).

Fig.1(a)

LOW-DOSE ELECTRON MICROGRAPH OF ROTAVIRUS SA11

(From Bellamy & Both, 1990, with minor modifications)



The virus was negatively stained with uranyl formate. The preparation consists primarily of double-shelled particles and the beam-sensitive protrusions, or spikes, can be seen in "Y" (small arrows) and "lollipop" (large arrow) conformations. Bar, 0.1 μm.

1.7 CLASSIFICATION

Rotaviruses were assigned to a separate genus of the family *Reoviridae* in 1978 (Mathews, 1979). This family consists of six distinct genera: *Reovirus*, *Orbivirus*, *Rotavirus*, *Phytoreovirus*, *Fijivirus*, and *Cytoplasmic polyhedrosis virus* (McCrae, 1985; Kapikian & Chanock, 1990). Morphologically, rotaviruses resemble both reoviruses (Palmer *et al.*, 1977) and orbiviruses (Murphy *et al.*, 1971; Palmer *et al.*, 1977) but they possess a sharply defined outer capsid which contrasts with the indistinct outer capsid of the latter (Kapikian & Chanock, 1990).

1.8 PARTICLE STRUCTURE

Intact, infectious, rotavirus particles are icosahedral, have two concentric capsid layers and are 65-75nm in diameter (Woods *et al.*, 1976; McNulty, 1979) (See Fig. 1(a) & Fig. 1(b)). Such intact virions are known as double-shelled (ds) or 'smooth' particles, have a buoyant density of 1.36g/cm³ on isopycnic caesium chloride (CsCl) gradients and a sedimentation coefficient of 520 to 530S (Rodger *et al.*, 1975; Bridger & Woode, 1976; Elias, 1977).

The outer capsid of intact particles can be removed with chelating agents such as Ethylenediamine Tetracetic Acid (EDTA) (Estes *et al.*, 1979), which probably removes calcium (Ca^{2+}) ions required for the stability of ds particles (Cohen *et al.*, 1979; Shahrabadi & Lee, 1986). This procedure yields single-shelled (ss) or 'rough' particles, approximately 55-65nm in diameter, which are indistinguishable from ss particles produced in infected cells (Woode *et al.*, 1976; Elias, 1977) (see Fig. 1(c)). They have a buoyant density of 1.38g/cm³ on isopycnic CsCl gradients and a sedimentation coefficient of 380 to 400S (Newman *et al.*, 1975; Bridger & Woode, 1976; Tam *et al.*, 1976).

Infected faeces usually contain a mixture of both particle types and ss particles are produced in considerable quantities in the infected cell (McNulty, 1978; Bellamy & Both, 1990). Infectivity depends on the presence of the outer capsid (Bridger & Woode, 1976; Nandi *et al.*, 1992).

Fig.1(b) DIAGRAMMATIC REPRESENTATION OF DOUBLE-SHELL ROTAVIRUS PARTICLE

KEY:

- █ VP4]— OUTER CAPSID
- VP7
- Y CARBOHYDRATE
- ⊗ VP6 (INNER CAPSID)
- CORE PROTEINS
- || RNA SEGMENTS

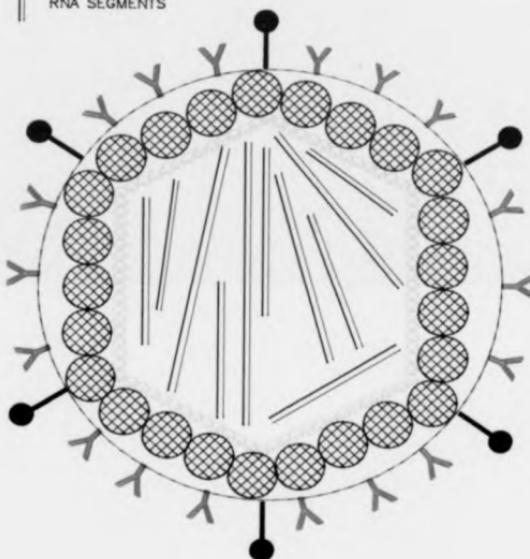
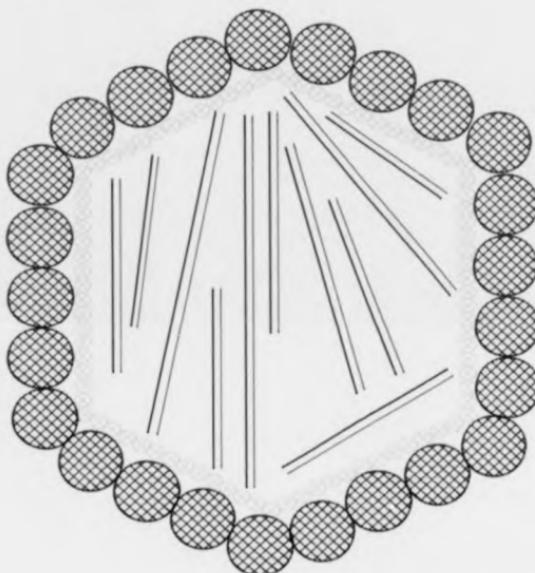


Fig.1(c) DIAGRAMMATIC REPRESENTATION OF SINGLE-SHELLED ROTAVIRUS PARTICLE

KEY: ⊗ VP6

CORE PROTEINS

|| RNA SEGMENTS



The precise arrangement of the two layers of capsomeres was recently determined by Prasad *et al.*,(1988).The inner capsomeres are arranged with a T = 13*l* (laevo) skewed icosahedral symmetry forming a surface lattice.The major surface protein of the inner capsid is VP6 (Mason *et al.*,1980;Dyall-Smith & Holmes,1981),which when dissociated from the virus tends to form multimeric (usually trimeric) aggregates (Gorziglia *et al.*,1985;Sabara *et al.*, 1987).It has been calculated that there are 780 molecules of VP6 per virion and VP6 trimers are the basic morphological units of the lattice (Bellamy & Both,1990).The binding sites for the assembly of trimers on the surface of the inner nucleocapsid,are conserved across rotavirus strains (Sandino *et al.*, 1986).The hexameric arrangement of VP6 trimers is shown in Fig.2.

Prasad *et al.*,(1988) estimate the large width channels (Fig.2(b)) to be 14nm deep and they postulate that they extend through both the inner and outer capsid layers.They suggest that nucleotide triphosphates enter the core through such pores and that mRNA is released through them.Bellamy & Both, (1990) propose that NS28 (see Table 3b) may interact with the small width channels enclosed by the VP6 trimers.

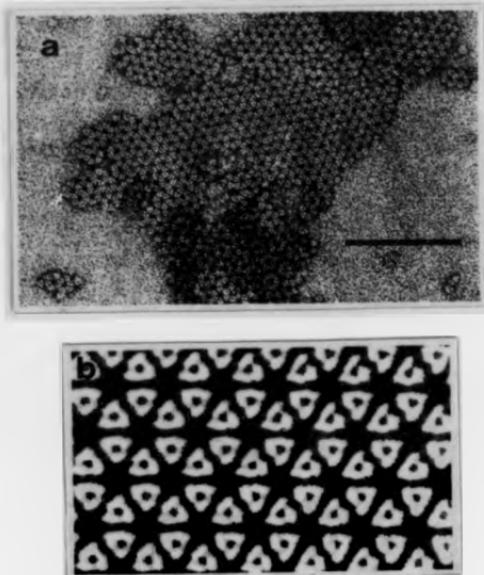
The outer layer of capsomeres is also arranged in a T=13*l* surface lattice but the surface is much smoother.This arrangement is inferred from the arrangement of surface spikes present in the outer capsid (Prasad *et al.*,1988).They are 45Å long and 35Å wide and possess a knob at the distal end.The spikes are composed of VP4 (60 copies per virion) and VP7 forms the smooth outer capsid shell (780 copies per virion) (Prasad *et al.*,1988;Prasad *et al.*, 1990) (see section 1.13.2).

Chaotropic agents such as high concentrations of CaCl_2 and potassium thiocyanate degrade ss particles to release VP6 and icosahedral,electron dense core particles of 37nm in diameter,which lack RNA polymerase activity (Bican *et al.*,1982).They have a buoyant density of 1.44g/ml in isopycnic CsCl density gradients and a sedimentation coefficient of 280S (Bican *et al.*,1982).Empty particles' (penetrated by negative stain when viewed by the EM),lacking nucleic acid within the core,have a buoyant density of 1.29g/ cm³-1.3g/cm³ in

Fig.2

HEXAMERIC ARRANGEMENT OF VP6 TRIMERS

(From Bellamy & Both, 1990, with minor modifications)



Electron micrograph of a single-layered VP6 paracrystal negatively stained with uranyl acetate (a). Regions of overlapping layers can be seen at the top and the bottom. Bar, 0.1 μ m.

Fourier noise-filtered magnification of the array, with protein shown as white areas (b). The hexagonal lattice has a periodicity of 9.8 nm.

iso-pycnic CsCl density gradients and are readily found in rotavirus preparations (Rodger *et al.*, 1975; Tam *et al.*, 1976).

1.9 STABILITY AND PHYSICOCHEMICAL PROPERTIES OF ROTAVIRUS PARTICLES

Experiments with the simian rotavirus SA11 have shown that it is stable (as defined by plaque titration), following treatment with either genetron, chloroform, repeated freeze-thawing, sonication, incubation at 37°C for 1 hr or incubation at 25°C for 24 hr (Estes *et al.*, 1979). However, it is unstable at 50°C.

Treatment of SA11 with salts such as 2M magnesium chloride ($MgCl_2$), $CaCl_2$ or sodium chloride ($NaCl$) for 15 min., at 50°C leads to almost a complete loss of viral infectivity, whereas with magnesium sulphate ($MgSO_4$) the infectivity is retained (Shirley *et al.*, 1981). Rotaviruses are less stable in 1M $MgCl_2$, tris-buffered saline or phosphate-buffered saline than in maintenance medium or water (Welch & Thompson, 1973; Estes *et al.*, 1979). Freezing SA11 in 2M $MgCl_2$ or 2M $CaCl_2$ also leads to almost a complete loss of infectivity. However, low levels of $CaCl_2$ (1.5-15mM) or strontium chloride (0.15-15mM), stabilize the infectivity of human rotavirus (Shirley *et al.*, 1981). Bovine and human rotavirus samples can retain their infectivity for many months at 4°C or 20°C when stabilized by 1.5mM $CaCl_2$ (Shirley *et al.*, 1981).

Ethanol (95% v/v) and Biogram (a chlorinated phenolic compound) (5% v/v), are more effective at inactivating SA11 than either 4% or 10% formaldehyde (Tan & Schnagl, 1983; Vaughn *et al.*, 1986) and ethanol (95% v/v) is the most efficient disinfectant for rotaviruses and exerts its effect by removing the virion outer capsid (Bishai *et al.*, 1978; Tan & Schnagl, 1981).

Hypochlorite based disinfectants show dualistic effects. They were ineffective at inactivating lamb rotavirus (Snodgrass & Herring, 1977) and SA11 (Tan & Schnagl, 1981) but they were efficient when used to inactivate human rotavirus strains (Tan & Schnagl, 1981; Tan & Schnagl, 1983). However, human rotaviruses are, in general, resistant to many chemical disinfectants (Lloyd-Evans *et al.*, 1986; Springthorpe *et al.*, 1986).

Sodium dodecyl-sulphate (SDS) at 0.1% inactivates SA11, unlike some non-ionic detergents which enhance infectivity, probably by dispersing aggregates (Ward & Ashley, 1980). The stability of EDIM virus is reduced when adventitious protein is removed from the virions (Much & Zajak, 1972).

Inherent differences in the stability of different rotavirus strains has been noted, which may be influenced by the degree of glycosylation of VP7 (see section 1.13.2.2.1). Bovine and human strains are more susceptible to outer capsid instability when grown in the presence of tunicamycin and they possess glycosylation at 2 sites (Sabara *et al.*, 1982; Suzuki *et al.*, 1984). In contrast SA11 particles are more stable when grown under similar conditions and they possess glycosylation at only 1 site (Petrie *et al.*, 1982).

The likelihood of infection of the alimentary tract is affected not only by the movement of the intestinal contents but also by the presence of acid, bile, proteolytic enzymes and mucus. Hence successful enteric pathogens must be capable of tolerating such conditions (Mims & White, 1988). Incubation of SA11 with proteolytic enzymes such as trypsin and chymotrypsin appears to enhance the infectivity of the virus (see section 1.13.2.1.) and is an obligatory requirement for the growth of human rotaviruses in tissue culture (Sato *et al.*, 1981; Kitaoka *et al.*, 1986).

Rotaviruses are readily inactivated at pH 2.0 but at pH 3.0 inhibition is much slower and at pH 4.0 it is minimal (Weiss & Clark, 1985). The gastric pH of most infants is approximately 3.2 and it remains above pH 3.0 for at least 1 hr after a meal (Weiss & Clark, 1985). Rotaviruses can, therefore, survive these conditions (Weiss & Clark, 1985).

These results suggest that rotaviruses are remarkably stable in the environment and this probably helps to explain, to some extent, the reason for the efficient transmission of the virus (Christensen, 1989).

1.10 GENOME AND ELECTROPHORETYPING

The genome of the rotaviruses consists of 11 segments (Newman *et al.*, 1975; Rodger *et al.*, 1975) of double stranded (ds) ribonucleic acid (RNA) contained

within the core structure of the virion (Welch & Thompson, 1973; Rodger *et al.*, 1975) and packaging is dependent on viral proteins (Kapahnke *et al.*, 1986). They range in molecular weight from 2×10^5 to 2.2×10^6 daltons (Newman *et al.*, 1975; Rodger *et al.*, 1975) or 667 (segment 11) to 3302 base pairs (bp) (segment 1) (Estes & Cohen, 1989). The total genome contains 18522 bp (sequence data determination (Estes & Cohen, 1989) or 18680 bp (EM measurements) (Rixon *et al.*, 1984).

The RNA segments fall into four size classes and usually migrate in polyacrylamide gels to form a characteristic pattern (see Fig. 3). There are four large segments (1-4), two medium sized (5 & 6), three smaller segments (segments 7-9) (forming a distinctive 'triplet') and two very small RNA segments (10 & 11) (Schnagi & Holmes, 1976; Todd & McNulty, 1976).

The migration patterns of RNA segments using poly-acrylamide-gel-electrophoresis (PAGE) was an important diagnostic technique, prior to their successful propagation in tissue culture (Kapikian & Chanock, 1990). Such a distinctive RNA profile was termed an electropherotype (Rodger *et al.*, 1981).

Three major types of electropherotype have been observed in human rotavirus isolates. These are designated 'long' (Kalica *et al.*, 1981a; Nakagomi *et al.*, 1987), 'short' (Espejo *et al.*, 1978) and 'super-short' (Albert *et al.*, 1987; Matsuno *et al.*, 1985) and are dependent on the migration of RNA segments 10 and 11. Most animal strains show the 'long' RNA migration profile (Kapikian & Chanock, 1990). The non-group A rotaviruses (see section 1.14.1) show distinct electropherotypes, with marked displacements of segments 7, 8 and 9 (McNulty *et al.*, 1981; Bridger *et al.*, 1982).

Electropherotyping is limited as a diagnostic tool, however, since electropherotype and serotype do not correlate (Beards, 1982; Gerna *et al.*, 1987) and gene segments with the same migration patterns do not always show sequence homology and *vice versa* (Clarke & McCrae, 1982; Flores *et al.*, 1982). Variations in RNA secondary structure may account this (Estes & Cohen, 1989). Thus, RNA profiles can not be used as the sole criterion for rotavirus classification within a specific group (*i.e.* A-E) (Pedley *et al.*, 1983; Snodgrass *et al.*, 1984) (see

Fig.3 ELECTROPHORETIC PATTERN OF HUMAN ROTAVIRUS STRAIN D

(From Kapikian & Chanock, 1990)



Polyacrylamide gel electrophoresis pattern of RNA from human rotavirus strain D (serotype 1, subgroup II). The 11 segments of double-stranded RNA comprising the rotavirus genome are identified.

section 1.14.1). However, attempts have been made to use electropherotypes as epidemiological markers (Chanock *et al.*, 1983; Garbari-Chenon *et al.*, 1985) and they provide evidence for heterogeneity within both animal and human rotaviruses (Kapikian & Chanock, 1990).

1.11 VARIATION IN THE ROTAVIRUS GENOME

Variation in the sequences of rotavirus segments may occur by three major mechanisms, which ultimately could contribute to the evolution of rotaviruses.

Antigenic SHIFT is thought to occur since intertypic or intergenogroup reassortants have been isolated *in vivo* (Midtun *et al.*, 1987; Hoshino *et al.*, 1987; Ward *et al.*, 1991). The high degree of genetic variability observed in the rotaviruses, may be due to the high frequency of gene reassortment which occurs *in vivo* (Flores *et al.*, 1982; Gombold & Ramig, 1986) and may contribute to the generation of new serotypes (Edelman *et al.*, 1989). Similarly, antigenic DRIFT may also occur (Coulson *et al.*, 1985). Both of these mechanisms have been described for influenza viruses (Air *et al.*, 1987).

Genome rearrangements within segments have also been reported (Pedley *et al.*, 1984; Tanaka *et al.*, 1988). A more detailed analysis of this latter mechanism is important, since (i) it appears to be unique to members of the Reoviridae and (ii) it may have considerable bearing on our understanding of rotavirus antigenic variation.

The electropherotypes of some group A rotaviruses show atypical profiles. The normal RNA segments are missing and are replaced by more slowly migrating concatameric forms of ds RNA, containing sequences specific to the missing RNA segments (Pedley *et al.*, 1984; Mattion *et al.*, 1988).

Rearrangements have been detected in segments 5, 6, 8 and 10 but predominantly in segment 11 (Pedley *et al.*, 1984; Hundley *et al.*, 1985; Mattion *et al.*, 1988; Tanaka *et al.*, 1988).

Detailed analysis of the rearrangements in segment 11 have been undertaken (Gonzalez *et al.*, 1989; Scott *et al.*, 1989). In two instances the rearranged segment was the result of a partial duplication event, within the segment.

Other examples of rearrangements have been found where the Open Reading Frame (ORF) has been extended or lost (Hundley *et al.*, 1985), resulting in the synthesis of a novel protein product or the loss of the protein, respectively. In other cases partial deletions and duplications have occurred with the construction of potential new ORF's (Gorziglia *et al.*, 1989). Both the 'copy choice' model (Gonzalez *et al.*, 1989; Gorziglia *et al.*, 1989) and 'transcriptional slippage' model (Scott *et al.*, 1989) have been proposed as mechanisms of genome rearrangement.

In other virus systems where genome rearrangements occur (Lazzarini *et al.*, 1981; Makino *et al.*, 1986), Defective Interference (DI) particles are produced which are dependent on helper virus for replication. However, rotavirus variants would not appear to be replication defective and rearranged segments can apparently reassort as normal RNA segments (Graham *et al.*, 1987; Mattion *et al.*, 1988). It has been proposed that segment rearrangements may not only occur during the synthesis of mRNA but also during the synthesis of negative sense strands during replication (Estes & Cohen, 1989).

That genome rearrangements have also been noted in the Orbiviruses (Ramig *et al.*, 1985; Eaton & Gould, 1987), may suggest that it has an important role in the evolution of ds RNA viruses. This mechanism of variation may have an important part to play in influencing both antigenicity (i.e. by altering virion structure) and pathogenicity (Estes & Cohen, 1989).

1.12 GENE CODING ASSIGNMENTS

Two main techniques have been used for assigning proteins to their appropriate gene segments. These include *in vitro* translation studies using viral mRNA or denatured genomic RNA (McCrae & McCorquodale, 1982; Mason *et al.*, 1983) and the use of genetic reassortants (Urasawa *et al.*, 1986; Liu *et al.*, 1988). Hybridization studies with gene specific probes (Bellinzoni *et al.*, 1989) and information from sequence data bases (Estes & Cohen, 1989) have also been used.

Rotavirus gene segments encode for six structural proteins (designated VP1,VP2,VP3,VP4,VP6 and VP7),found in the mature virion and five non-structural proteins,found only in the infected cell (Estes & Cohen,1989).

A summary of the protein coding assignments for BRV(UKtc) (as used for the studies discussed in this thesis),are shown in Table 2,together with the corresponding SA11 protein equivalents.The characteristics of the rotavirus proteins are now discussed.The SA11 protein terminology is shown in parentheses after the BRV(UKtc) term.

1.13 THE ROTAVIRUS PROTEINS

1.13.1 CORE AND INNER CAPSID PROTEINS

1.13.1.1 VP1(VP1)

VP1 is a hydrophobic,slightly basic protein found in the virion core with a molecular weight of 124847 (Cohen *et al.*,1989).It constitutes only 2% of the virion mass and the amino acid (aa) sequence contains the RNA polymerase consensus sequence:glycine-aspartate-aspartate (GDD) at aa positions 630 to 632.It also shows sequence homology with polymerases from other members of the Reoviridae (Cohen *et al.*,1989).VP1 has therefore,been proposed to have an enzymatic rather than an exclusive structural role (Estes & Cohen,1989).

Several studies suggest that it is largely inaccessible to Ab when present in the virion core (Ericson *et al.*,1982; Conner *et al.*,1988;Cohen *et al.*,1989).

1.13.1.2 VP2(VP2)

This is the most abundant structural protein found in core particles (Bican *et al.*,1982;Liu *et al.*,1988).It has an apparent molecular weight of 94000 which contrasts with the calculated molecular weight of 102431.This is probably due to the high percentage of alpha-helix predicted to be present (Estes & Cohen, 1989).VP2 is thought to be at least partially exposed on ss particles,based on ELISA reactions (Taniguchi *et al.*,1986) and iodination experiments (Novo & Esperza, 1981).It is highly immunogenic and serum Ab's to the protein are a good indicator of prior infection (Conner *et al.*,1988;Svensson *et al.*,1987).

TABLE 2 PROTEIN CODING ASSIGNMENTS OF ENY(U9c) AND SA11 ROTAVIRUSES
 (Compiled using McCrae & McCorquodale, 1982; Estes & Cohen, 1989).

SA11 SEGMENT	SA11 PRIMARY TRANSLATION PRODUCT	MATURE PROTEIN(S) (# DIFFERENT)	U9c PRIMARY TRANSLATION PRODUCT	MATURE PROTEIN(S) (# DIFFERENT)	U9c SEGMENT
1	VP1		VP1	1	1
2	VP2		VP2	2	
3	VP3		VP3	3	
4	VP4	VP8* + VP5*	VP4	4	
5	NS5.3		VP5	5	
6	VP6		VP6	6	
7	NS34		VP9	9	
8	NS35		VP8	7	
9	VP7		Vpr7	VP7 → VP7c 6	
10	NS20	NS29 → NS28	VP12	VP10 → VP10c 10	
11	NS26	28K	VP11 (NO SA11 EQUIVALENT)	VP11c 11	

Epitopes on VP2 which co-segregate with the subgroup specificity of VP6, have also been identified (Taniguchi *et al.*, 1986).

Nucleic acid binding activity is a major feature of the protein (Boyle & Holmes, 1986) and it contains a leucine 'zipper' structure capable of binding to ds nucleic acid (Landschulz *et al.*, 1988; Kumar *et al.*, 1989). This has suggested that VP2 may be a nucleocapsid protein whose function is to neutralize the charge of virion RNA, to allow packaging into the virion core.

1.13.1.3 VP3(VP3)

VP3 is a basic protein with multiple repeats of amino-acids and a predicted molecular weight of 98120 (835aa) (Estes & Cohen, 1989). It is a minor core protein (Liu *et al.*, 1988) but is thought to play a role in RNA replication (Gombold & Ramig, 1987; Pizarro *et al.*, 1991) and it is proposed to be the guanyl-transferase (Liu *et al.*, 1992). It migrates with VP4 in many gel systems (Liu *et al.*, 1988) but it is not translated very efficiently *in vitro* (McCrae & McCorquodale, 1982). VP3 is synthesised at low levels in infected cells (Liu *et al.*, 1988) but can be detected in early replication complexes (Gallegos & Patton, 1989).

1.13.1.4 VP6(VP6)

VP6 is the major structural protein of ss particles, comprising the outer capsid structure (see Fig. 1(c)) (Hofer *et al.*, 1987; Gorziglia *et al.*, 1988). It has an apparent molecular weight of 45000, is hydrophobic in nature and exists primarily as a trimer (see section 1.8) (Sabara *et al.*, 1987; Prasad *et al.*, 1988). The domains responsible for trimerization and assembly into ss particles, have been determined (Clapp & Patton, 1991).

Some evidence suggests that it may be important in RNA transcription (Bican *et al.*, 1982; Sandino *et al.*, 1986). However, it is unknown whether VP6 plays a structural or functional role in this process (Bellamy & Both, 1990).

Although overall sequence conservation in gene 6 is high, regions of sequence divergence have been identified (Both *et al.*, 1984; Hofer *et al.*, 1987;

Gorziglia *et al.*, 1988). Additionally, some rotavirus strains have been identified (e.g. FI-14) which possess a segment 6 with more than 1 initiation codon (Gorziglia *et al.*, 1988). The significance of these findings is unclear.

1.13.2 OUTER-CAPSID PROTEINS

1.13.2.1 VP4(VP4)

VP4 is the minor component of the outer capsid (Kalic *et al.*, 1983; Mason *et al.*, 1983). It was originally named VP3 in the SA11 system but was renamed (Liu *et al.*, 1988), following the identification of the product of gene segment 3 as a structural protein of the inner core (Dyall-Smith & Holmes, 1981; McCrae & McCorquodale, 1982; Holmes, 1983). It has an apparent molecular weight of 88000, is non-glycosylated (Mason *et al.*, 1980; McCrae & McCorquodale, 1982), has a net negative charge at pH7 and the amino terminal region is hydrophobic (Estes & Cohen, 1989). Secondary structure predictions suggest that many random coils are also present in the amino terminus giving rise to a globular structure. It is responsible for a number of important biological functions.

First, it is known to be the viral haemagglutinin (Kalic *et al.*, 1983; Greenberg *et al.*, 1983). The site on VP4 which binds to erythrocytes is unknown, although it may involve several regions (Greenberg *et al.*, 1983; Burns *et al.*, 1988). Glycophorin is believed to be the erythrocyte receptor for rotavirus VP4 (Mackow *et al.*, 1989).

In the presence of proteolytic enzymes such as trypsin, the VP4 of SA11 is cleaved into VP6^{*} (molecular weight ~60000) and VP8^{*} (molecular weight ~28000) (Espejo *et al.*, 1981; Estes *et al.*, 1981; Clark *et al.*, 1981). It has been proposed that proteolytic cleavage of VP4 enhances SA11 infectivity (Espejo *et al.*, 1981; Estes *et al.*, 1981). However, trypsin is not required for BRV(UKtc) infectivity. Enhanced infectivity is believed to be mediated by increased penetration but not binding of rotavirus to cells (Clark *et al.*, 1981; Fukuhara *et al.*, 1988; Kaljot *et al.*, 1988; Nandi *et al.*, 1992) and VP6^{*} has been suggested to

mediate penetration (Ruggeri & Greenberg, 1991) since it contains a putative fusogenic domain (see below).

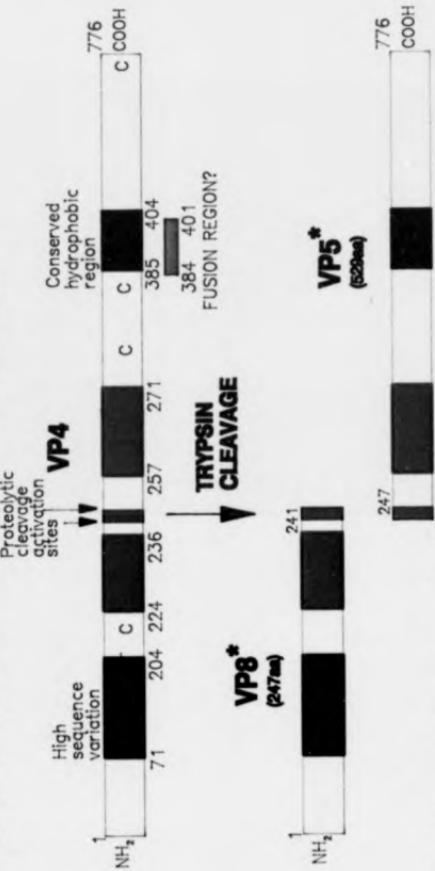
The cleavage of VP4 via proteases may also be important in plaque formation (Kalic et al., 1983) and in the restriction of growth in tissue culture cells (Greenberg et al., 1983a) and mice (Kalic et al., 1983; Offit et al., 1986a).

Two trypsin cleavage sites have been identified at arginine 241 and arginine 247 of the SA11 assortant SA11 4fm, with the latter as the preferred site (see Fig.4) (Lopez et al., 1985). Analysis of other rotavirus strains has revealed that these cleavage sites are conserved (Lopez et al., 1986; Potter et al., 1987; Mackow et al., 1988; Nishikawa et al., 1988). It has been proposed that the trypsin cleavage sites are kept accessible through disulphide bonds present at cysteine residues found at positions 203 and 216 in VP8^{*} and positions 318 and 380 in VP5^{*} (Gorziglia et al., 1988a). Certain regions flanking the cleavage sites (aa 224-235 and 257-271), are also conserved and thus are probably involved in maintaining the correct conformation for cleavage (see Fig.4).

VP4 may also be important in affecting the virulence of rotavirus strains (Offit et al., 1986a; Flores et al., 1988; Gorziglia et al., 1988a). Additional trypsin cleavage sites have been found in some human VP4 sequences, which in some instances appear to correlate with virus virulence (Lopez et al., 1985; Gorziglia et al., 1986; Gorziglia et al., 1988a). In addition, considerable aa variation occurs between and adjacent to the trypsin cleavage sites (Arg 241 and Arg 247) (Lopez et al., 1986; Nishikawa et al., 1988; Kantharidis et al., 1988) which may be important as a determinant of virulence (as it is in the influenza HA [Klenk & Rott, 1988]). However, other rotavirus genes (Nishikawa et al., 1988) or host factors (Mims & White, 1988), may also play an important part in influencing virulence.

A region of VP4 has been identified that shares some sequence similarity with the fusogenic hydrophobic domain located at the N-terminus of the E1 glycoprotein of Sindbis and Semliki Forest viruses (Garoff et al., 1980; Mackow et al., 1988). It has been postulated that this region may act alone or in conjunction with other regions, to induce membrane fusion and allow entry into

Fig. 4 STRUCTURAL FEATURES OF VP4 (From Estes & Cohen, 1989).



(Based on nucleotide sequences of different virus strains)

Symbols: █ regions of sequence conservation among different virus strains; █ region of greatest sequence variation of VP8*; █ potential fusion region; █ sites of cleavage by trypsin. Conserved cysteines (C) at aa 215, 317, 379 and 773 and the cysteine (C') at aa 203 not present in some human rotavirus strains is shown. The peptide possibly removed by trypsin cleavage at aa 241 and 247 is shown (█). The numbering shown here for VP4 is based on a protein of 776 aa as determined for SA11 4NPRV and Bo/486.VP4 of human rotavirus strains contains 775aa, lacking an aa at residue 136.

the cell by direct membrane penetration (Clark *et al.*, 1981; Fukuhara *et al.*, 1988; Kaljot *et al.*, 1988). This region is highly conserved throughout many rotavirus strains, suggesting that it has an important role in the replication cycle (Estes & Cohen, 1989). It has been suggested that the fusion region may be important in virus maturation i.e. to allow budding to occur across the Rough Endoplasmic Reticulum (RER) membrane (Estes & Cohen, 1989).

1.13.2.2 VP7(VP7)

1.13.2.2.1 VP7 IS A GLYCOPROTEIN

This is the major outer capsid protein of the rotavirus particle, has a molecular weight of 34-37000 and it is the second most abundant protein species in the virion (see Fig. 1(b)) (Liu *et al.*, 1988). It is encoded by genome segment 8 of UKtc, segment 9 of SA11 and segment 7 of RRV (McCrae & McCorquodale, 1982; Arias *et al.*, 1982).

VP7 is a glycoprotein that contains only N-linked, high mannose oligosaccharide residues which are added co-translationally as the protein enters the RER (Both *et al.*, 1983c; Kouvelos *et al.*, 1984; Kabcenell *et al.*, 1988). It has a luminal orientation and the oligosaccharides are modified by trimming in the RER (Ericson *et al.*, 1983; Kabcenell & Atkinson, 1985). The absence of complex carbohydrates suggests that VP7 remains in the RER and does not move through the Golgi apparatus (Hubbard & Ivatt, 1981). It is the only structural polypeptide which is glycosylated.

The N-linked glycosylation site N-S-T beginning at residue 69 is common to all rotavirus serotypes, except the Nebraska Calf Diarrhoea Virus (NCDV) and bovine RF strains (Gunn *et al.*, 1985; Glass *et al.*, 1985). These strains, along with some others, have additional potential glycosylation sites (with the sequence N-X-S/T) at residues 146-148 (unique to serotype 2), 238-240 (serotypes 1,2,6 and 8) and 318-320 (serotype 6) (Glass *et al.*, 1985; Gunn *et al.*, 1985; Charpilienne *et al.*, 1986; Green *et al.*, 1987). For the SA11 and porcine OSU strains, only one glycosylation site is utilized at residue 69, whereas some strains utilize 2 glycosylation sites (Kouvelos *et al.*, 1984; Sato *et al.*, 1986). However, the location of

the second site of glycosylation is not known for any strain. Some strains possess up to 3 potential glycosylation sites (Estes & Cohen, 1989). It has been proposed that the site of glycosylation in VP7 might affect the cell binding properties of the virus (Green *et al.*, 1987).

1.13.2.2.2 VP7 AS THE CELL ATTACHMENT PROTEIN?

It is not absolutely clear which of the two outer-capsid proteins (VP7 or VP4) is responsible for binding to cells, since the data is conflicting. Some experiments have shown VP7 to be important, either by Ab inhibition studies or binding studies using purified protein (Matsuno & Inouye, 1983; Sabara *et al.*, 1985; Fukuhara *et al.*, 1988).

In view of the fact that VP4 forms the spikes of the virion (Prasad *et al.*, 1988; Prasad *et al.*, 1990) (see section 1.8) it is logical to presume that this is the cell attachment protein (Yeager *et al.*, 1990; Nandi *et al.*, 1992). Similarly, VP4 is the haemagglutinin (HA) of the virus (see section 1.13.2.1) and in other systems (*e.g.* influenza [Possee *et al.*, 1982]) the HA is also the cell attachment protein. Several sialic acid containing proteins inhibit the replication of rotaviruses in MA104 cells (Yolken *et al.*, 1987; Willoughby *et al.*, 1990) and it is proposed that this is due to the inhibition of viral attachment via VP4 (Mackow *et al.*, 1989). Furthermore, Ruggeri & Greenberg, (1991) showed that mAb's to VP8* efficiently prevented the binding of radiolabelled virions to MA104 cell monolayers, whereas mAb's to VP5* and VP7, did not.

This data would appear to suggest that VP4 has a role in cell attachment. Binding studies using radiolabelled recombinant VP4 or peptides may help to elucidate the exact role of VP4 in cell binding. It is possible, that both proteins are involved.

1.13.2.2.3 VP7 AND CALCIUM BINDING

Some evidence exists to suggest that Ca^{2+} ions bind to VP7 (Shahrabadi *et al.*, 1987; Cohen *et al.*, 1979) and a putative Ca^{2+} binding region in VP7 has been identified, which is conserved throughout many rotavirus strains (Estes &

Cohen, 1989). Calcium may be important in maintaining the stability of the virus. (Cohen *et al.*, 1979; Shahrabadi & Lee, 1986).

1.13.3 NON-STRUCTURAL PROTEINS

Little is known about the non-structural proteins of the rotaviruses and their functions are poorly understood. However, sequence data have begun to yield some information on general protein structure and possible clues about function. A better understanding of these proteins is expected, as they are expressed and studied using recombinant DNA methods. Table 2 shows the nomenclature used for the SA11 and BRV(UKtc) protein equivalents and the main features of the non-structural proteins are shown in Tables 3(a) and 3(b).

1.14 ANTIGENIC CLASSIFICATION

1.14.1 GROUPS

The most general category of classification is the *group* (or *serogroup*), which is defined by the antigenic identity of the VP6 protein (Gary *et al.*, 1982; Greenberg *et al.*, 1983b; Eiden *et al.*, 1992) of the inner capsid (Woope *et al.*, 1978b; Gorziglia *et al.*, 1985). VP6 is both antigenic and highly immunogenic and is the most frequently targeted protein in diagnostic assays to detect virus particles (Gorziglia *et al.*, 1985; Svensson *et al.*, 1987). Rotaviruses sharing common group epitopes are assigned to a particular group (Pedley *et al.*, 1983). Pothier *et al.*, (1987) identified three epitopes involved in group specificity using a panel of mAb's to VP6. Most human and animal rotavirus disease, described to date, has been attributed to the group A rotaviruses (Bartlett *et al.*, 1987).

More recently, studies on the antigenic properties of rotavirus isolates have shown that group or common determinants are found on most (if not all) of the structural proteins of rotavirus particles (Estes & Graham, 1985; Brusow *et al.*, 1991).

Rotaviruses have been isolated from humans (Dimitrov *et al.*, 1983; Bridger *et al.*, 1986), animals (Bridger *et al.*, 1982; Snodgrass *et al.*, 1984) and chickens

TABLE 3(a) FEATURES OF THE NON-STRUCTURAL PROTEINS

PROTEIN NUMBER	CHARACTERISTICS	REFERENCES	PROPOSED FUNCTION	REFERENCES
VP5	Basic, with net positive charge of 9 at pH7. Detected only in infected cells. Synthesized at early times and synthesis is regulated in vivo but not in vitro. Has predicted zinc binding sites which are conserved.	Brennan et al., 1987 Ariza et al., 1982 Ericson et al., 1982 Holmes, 1983 Novo & Esperanza, 1981 Johnson & McCros, 1989	Interacts with genome segments during RNA replication or genome rearrangement.	Patton, 1986 Evona & Hollenberg, 1985 Schiff et al., 1986
VP8	Basic, detected in virophiloma and subviral particles. Recently reported as a component of viral outer-capsid and to bind to cell membranes.	McCros & McCorquodale, 1982 Mason et al., 1983 Gombold & Ramig, 1986 Petré et al., 1984 Heimbigner-Jones & Patton, 1986 Beas et al., 1990 Estes & Cohen, 1989	Involved in replication of RNA or packaging of ssRNA into sub-viral particles.	Ramig, 1983a Ramig, 1983 Heimbigner-Jones & Patton, 1986
VP9	Acidic, binds to radiolabelled nucleic acids on Western blots.	Both et al., 1984c Boyle & Holmes, 1986	Component of viral replicase.	Heimbigner-Jones & Patton, 1986 Patton & Gallegos, 1988

TABLE 2.0 FEATURES OF THE NON-STRUCTURAL PROTEINS

PROTEIN NUMBER	CHARACTERISTICS	REFERENCES	PROPOSED FUNCTION	REFERENCES
VP10c [28000]	Primary translation product is VP12 or NS20. It is glycosylated. Mature glycoprotein is an integral membrane protein of the RER. Exists as a tetramer in the RER membrane. Also observed at periphery of viroplasmic inclusion bodies. Has 3 amino terminal domains H1, H2 and H3 and shows a transmembrane orientation. Two N-linked glycosylation sites exist in H1. High mannose glycosylation is present. One of the hydrophobic domains is the signal peptide but it is not cleaved.	McCross & McCaughey, 1982 Ericson et al., 1982 Ercaen et al., 1982, 1983 Au et al., 1988 Meyer et al., 1989 Petrie et al., 1982, 1984 Kobayashi & Atkinson, 1985 Both et al., 1983a Bergmann et al., 1989 Taylor et al., 1992 Bellamy & Both, 1990	In the receptor for ss nucleic acids facilitates budding across RER membrane into the lumen. Binds to VP6. C-terminal Met is essential for ligand binding. Glycosylation of VP10c is required for removal of transient virion envelope.	Holmes, 1983 Ericson et al., 1983 Petrie et al., 1983 Au et al., 1988 Meyer et al., 1989 Taylor et al., 1992 Bellamy & Both, 1990
VP11 [28000]	Final gene product is VP11C in UK-2 which has no S711 but contains S712. Final product is VP11C which is glycoprotein. S711 is located in viroporin. S712 is located in phosphoprotein. A protein of 92aa is also expressed from gene 11 but its function is unknown. Other proteins serologically related to NS26 have also been identified. All proteins derived from gene 11 exist as a complex in infected cells.	McCaughey, 1982 Ericson et al., 1982, 1983 Mitchell & Both, 1988 Matsuno et al., 1986 Welch et al., 1989 Ward et al., 1985 Inai et al., 1985 Gonzalez & Burone, 1991 Minton et al., 1991 Eaves & Cohen, 1989 Bellamy & Both, 1990	Involved in gene re-assortment or RNA replication.	Eaves & Cohen, 1989

(McNulty *et al.*, 1981) which do not possess the common group A antigen. They are morphologically indistinguishable from other rotaviruses but they have an electropherotype that differs from that of group A viruses (Christensen, 1989). They have been termed 'antigenically distinct rotaviruses' (Eiden *et al.*, 1986), 'para-rotaviruses' (Espejo *et al.*, 1984), 'atypical rota-viruses' (Pedley *et al.*, 1983), 'novel-rotaviruses' (Hung *et al.*, 1984) and 'rotavirus-like agents' (Rodger *et al.*, 1982). Pedley *et al.*, (1983) suggested designating such viruses to groups B and C since they possess other group antigens and are genetically distinct. Group D and E rotaviruses have also been described, following analysis of atypical porcine and chicken rotaviruses (Pedley *et al.*, 1986).

In total, five distinct rotavirus groups have been described (types A-E) (Pedley *et al.*, 1983; Nakata *et al.*, 1986; Bridger, 1987). Groups A, B and C have been found in both humans and animals, whereas groups D and E have only been detected in animals/birds (Bridger, 1987).

The non-group A rotaviruses pose a major problem to on-going vaccine design, since to date, only one virus (a group C member) has been successfully cultivated in tissue culture (Saif *et al.*, 1988). Furthermore, significant protein structural differences to Group A viruses have been noted (Eiden *et al.*, 1992) and non-group A rotaviruses appear to show no antigenic cross reactivity with group A viruses (Bohl *et al.*, 1982; Qian *et al.*, 1991). However, reassortants between group A and non-group A viruses have not been found (Estes & Cohen, 1989).

1.14.2 SUBGROUPS

These are based on additional antigenic differences in the VP6 protein of group A rotaviruses (Kalica *et al.*, 1981a; Greenberg *et al.*, 1983c; Singh *et al.*, 1983). Two of the epitopes identified on VP6 have been used to designate subgroups I (S_I) and II (S_{II}) (Kalica *et al.*, 1981a; Both *et al.*, 1984). Thus, most strains (e.g. human) can be classified as either S_I or S_{II} (Greenberg *et al.*, 1983a; Kapikian & Chanock, 1990) (see Table 1). However, some (e.g. the equine

FI-14) possess both the S_I and S_{II} epitopes (Hoshino *et al.*, 1987a) and some lack the S_I and S_{II} epitopes (Hoshino *et al.*, 1984).

PAGE analysis of rotavirus genome segments (see section 1.10) has also been used in subgroup classification (Espejo *et al.*, 1980) (see Table 4a). However, electropherotyping is not a very reliable means of subgrouping, since notable exceptions to this classification scheme have been described (Kitaoka *et al.*, 1987; Steele & Alexander, 1988). Nevertheless, subgrouping using PAGE has been used in epidemiological studies (Uhnoo & Svensson, 1986) and S_{II} infections have been detected more frequently than S_I infections (White *et al.*, 1984; Nakagomi *et al.*, 1985).

1.14.3 SEROTYPES

Multiple rotavirus serotypes have been defined. Type specificity of anti-rotavirus sera was first observed in the late 1970's (Thouless *et al.*, 1977b; Bridger, 1978).

The most important technique for the determination of rotavirus serotypes was reciprocal neutralization, as measured by plaque reduction or fluorescent-focus-reduction neutralization assays using hyperimmune antisera raised to purified, infectious rotavirus particles (Wyatt *et al.*, 1982; Hoshino *et al.*, 1984). However, the mechanism of neutralization for rotaviruses remains unknown (Matsui *et al.*, 1989).

Further work demonstrated that neutralizing activity was associated with both of the outer capsid proteins (VP4 and VP7) (Hoshino *et al.*, 1985; Sabara *et al.*, 1985; Offit & Blatov, 1986). In the majority of cases, however, the predominant Ab reactivity in hyperimmune serum was against the VP7 glycoprotein (Bastardo *et al.*, 1981) and thus the serotype of the virus was reflecting different types of VP7. Studies using mAb's to particular epitopes on VP7, later confirmed this (Birch *et al.*, 1988; Urasawa *et al.*, 1988). Thus, VP7 and not VP4 is the major type specific determinant of neutralization, *in vitro* (Kalic *et al.*, 1981a; Green *et al.*, 1988; Matsui *et al.*, 1989). The reason why VP7 is the immunodominant protein is not known. However, VP4 has also been found to

TABLE 4(e) ELECTROPHORETIC CLASSIFICATION OF SUBGROUPS

		ELECTROPHORETIC PATTERN		
		SHORT	SUPER-SHORT	LONG
SUBGROUP		(Human)	(Human)	(Human) (Animal)
Kalica et al., 1981 Rodger et al., 1981 Kutsuzawa et al., 1982	Matsuno et al., 1985 Albert et al., 1987 Nakagomi et al., 1987	Kalica et al., 1981 Albert et al., 1987	Nakagomi et al., 1987	Hoshino et al., 1984 Kapikian & Chanock, 1990
REFERENCES				

be an important rotavirus antigen; Ab's directed at VP4 neutralize the virus efficiently *in vitro* (Taniguchi *et al.*, 1985; Burns *et al.*, 1988) and there is some evidence to suggest that *in vivo* VP4 may have a more important role in neutralization than *in vitro* (Shaw *et al.*, 1988; Ward *et al.*, 1988).

Serotyping studies are now regularly carried out using ELISA's incorporating mAb's to VP7 (Shaw *et al.*, 1985; Taniguchi *et al.*, 1987). They have also been applied more recently, to epidemiological studies in humans (Flores *et al.*, 1988; Georges-Courbot *et al.*, 1988), where serotype 1 was found to be the most predominant serotype.

In some cases a rotavirus strain will not react clearly in reciprocal neutralization assays. This is usually because of 2 immunologically distinct forms of VP4 (Hoshino *et al.*, 1984; Hoshino *et al.*, 1985). These can arise because both the VP4 and VP7 neutralization antigens can segregate independently (Hoshino *et al.*, 1987; Midtun *et al.*, 1987). Thus, a subtype classification system for both VP4 and VP7 has been proposed (Hoshino *et al.*, 1984; Graham & Estes, 1986). At least 8 different VP4 types are thought to exist (Estes, 1990; Snodgrass *et al.*, 1992).

The neutralization epitopes of VP4 and VP7 can therefore, be strain or serotype specific or they may be common (cross-reactive) between different virus strains (Mackow *et al.*, 1988; Burns *et al.*, 1988) and VP7 and VP4 are probably the only rotavirus proteins that directly mediate virus neutralization *in vitro* (Matsui *et al.*, 1989).

1.14.4 RESUME

Group A rotaviruses are assigned to a particular serotype and subgroup (see Table 1). In general, strains which share neutralization determinants also share subgroup determinants (Kapikian & Chanock, 1990), with one exception (Hoshino *et al.*, 1984).

Group A rotaviruses have been categorised into 14 serotypes on the basis of cross-neutralization with hyper-immune sera (Hoshino *et al.*, 1984; Matsuno *et*

al., 1985; Clark *et al.*, 1987; Ruiz *et al.*, 1988; Snodgrass *et al.*, 1990; Taniguchi *et al.*, 1990; Urasawa *et al.*, 1990; Browning *et al.*, 1991; Browning *et al.*, 1992).

Six distinct human serotypes have now been identified (see Table 1) (Wyatt *et al.*, 1983; Clark *et al.*, 1987; Matsuno *et al.*, 1985; Albert *et al.*, 1987) and more recently a seventh was described (Taniguchi *et al.*, 1990). Human serotypes 1-4 and 8 and 9 have been assigned to particular subgroups (see Table 4b).

1.15 PATHOGENESIS

1.15.1 SITE OF INFECTION AND REPLICATION

Rotaviruses infect mature villus enterocytes in the mid and upper villus epithelia, of the small intestine (Bishop *et al.*, 1973; Svensson *et al.*, 1991). The jejunum is the main site of infection but the ileum may also be involved (Davidson *et al.*, 1977; Snodgrass *et al.*, 1977a). In mice, infected intestinal cells have also been observed in the duodenum and colon (Little & Shadduck, 1982). Replication takes place primarily in the epithelial cells at the tips of the villi (Bishop *et al.*, 1973; Wyatt *et al.*, 1978).

The intestinal mucosa consists of polarized epithelial cells which are characterized by the presence of two distinct plasma membrane domains: the apical and the basolateral (Svensson *et al.*, 1991). Rotavirus (RRV) can infect these cells via either membrane domain (Svensson *et al.*, 1991) and they can also infect the liver hepatocytes of mice (Uhnoo *et al.*, 1990).

1.15.2 CYTOPATHIC EFFECT AND DETECTION OF VIRAL ANTIGEN

The main visible changes to infected cells are cytoplasmic vacuolization and the appearance of small, eosinophilic intra- cytoplasmic inclusions (Malherbe and Strickland-Chomley, 1967; McNulty *et al.*, 1977).

Viral antigen is detected more readily in the intestinal mucosa during the incubation period and virus detection correlates with the histopathological changes observed (Snodgrass *et al.*, 1977a; Eydeloth *et al.*, 1984).

TABLE 4(p) ASSIGNMENT OF HUMAN ROTAVIRUS SEROTYPES TO SUBGROUPS I AND II.

HUMAN SEROTYPES	SUBGROUP	REFERENCES
2,8	I	Thouless et al., 1982;Wyatt et al., 1983; Matsuno et al., 1985
1,3,4,8,	II	Thouless et al., 1982;Urasawa et al., 1982 Wyatt et al., 1983;Hoshino et al., 1985 Albert et al., 1987

1.15.3 HISTOPATHOLOGY

Major mucosal changes in the small intestine, following rotavirus infection, include the shortening and blunting of the villi and mononuclear cell infiltration of the lamina propria (Bishop *et al.*, 1973). Columnar epithelial cells of the villi are also replaced by cuboidal epithelium and the infected cells at the villus tips are rapidly sloughed off (Bishop *et al.*, 1973; Snodgrass *et al.*, 1977a). The cuboidal cells are thought to be immature secretory crypt cells (Tolia & Dubois, 1985) and shedding of infected cells from the villi is believed to be due to the loss of tight junctions, early in the viral replication cycle (Svensson *et al.*, 1991). Other reported characteristics of rotavirus infection include sparse and irregular microvilli, mitochondrial swelling and crypt hypertrophy (Holmes *et al.*, 1975; Davidson & Barnes, 1979).

Rotavirus infection progresses from the upper small intestine to the lower small intestine (*i.e.* in a cephalocaudal direction) (Snodgrass *et al.*, 1977a; Little & Shadduck, 1982). In children it was noted that those with the most severe mucosal lesions were more likely to become dehydrated and require intravenous therapy (Davidson & Barnes, 1979). However, mucosal damage is rapidly repaired (at least in non-malnourished patients); as early as three weeks after the onset of disease in some patients (Davidson & Barnes, 1979).

The disease process appears to vary in different species. For example, in pigs villus erosion is usually extensive (complete destruction of the villi is sometimes seen) and the disease is often lethal (Starkey *et al.*, 1990). This is in contrast to other species such as mice where villus atrophy is less severe, often patchy and associated with a self limiting diarrhoea (Starkey *et al.*, 1990).

Following infection of suckling mice with non-murine rotaviruses, multiple rounds of replication do not occur (as for murine rotaviruses) (Offit *et al.*, 1984; Greenberg *et al.*, 1986) and the mechanism of disease remains unclear (Offit & Dudzik, 1990).

1.15.4 CLINICAL SYMPTOMS

The appearance of symptoms following rotavirus infection, is variable (see section 1.1). Strains of varying virulence and host immunity factors may account, to some extent, for this observation (Wyatt *et al.*, 1983a; Christensen, 1989). The most prominent symptoms following rotavirus infection in man are vomiting and the production of a watery diarrhoea (Rodriguez *et al.*, 1977; Vesikari *et al.*, 1981). Vomiting has also been reported in pigs (Lecce *et al.*, 1976) and diarrhoeal disease occurs in most cases of homologous (*i.e.* infection of the host with a rotavirus isolated from the same species), virulent rotavirus infection of animal neonates and young (Snodgrass *et al.*, 1977a; Theil *et al.*, 1978; Little & Shaduck, 1982) (see section 1.3). However, infection of young with homologous avirulent strains may not lead to the production of diarrhoeal disease (Bridger & Oldham, 1987). The infection of adults with homologous strains of rotavirus may also result in diarrhoeal disease in some species *e.g.* rabbits (Conner *et al.*, 1988). Diarrhoeal disease in adult humans is also well documented (see section 1.2).

In animal models where heterologous rotavirus strains (*i.e.* not derived from the host species) are utilized, rotaviruses may or may not induce diarrhoea in the neonates (Ramig, 1988). Serotype 3 rotavirus strains appear to be the most infectious in the murine model (Bell *et al.*, 1987; Uhnoo *et al.*, 1990). The number of heterotypic infectious particles required to induce disease in neonatal mice is usually several orders of magnitude greater than needed for murine rotavirus strains (Greenberg *et al.*, 1986). Since non-murine rotavirus strains can enter murine enterocyte cells (Offit *et al.*, 1984) this difference can not be accounted for solely by cell surface binding and may lie at the level of virus production or virus spread (Greenberg *et al.*, 1986). Heterologous infection of children (*e.g.* with RRV) can also lead to the induction of virus shedding and fever (Losonsky *et al.*, 1986).

In adult animals heterologous rotaviruses replicate only to a very limited extent, if at all and if replication does occur then it is usually abortive and

diarrhoea is not observed (Offit *et al.*, 1986; Offit & Dudzik, 1988; Eates & Cohen, 1989).

Rotavirus diarrhoea in man is characterized by the absence of blood and the large volume which can be produced (Rodriguez *et al.*, 1977; Uhnoo & Svensson, 1986). This factor, together with vomiting can cause the patient to become dehydrated more readily than with other, non-cholera pathogens (Rodriguez *et al.*, 1977; Wyatt *et al.*, 1979; Black *et al.*, 1982). In addition to dehydration, electrolyte imbalance is another important factor which can contribute to the death of a patient (Carlson *et al.*, 1978).

In the Developing World protein-calorie malnutrition may lead to a more severe or prolonged clinical illness (Riepenhoff-Talty *et al.*, 1985). In the mouse model, malnutrition leads to a decrease in the minimal infectious dose required to induce diarrhoea, a shorter incubation period and a more severe clinical disease (Riepenhoff-Talty *et al.*, 1986). Similarly, repeated diarrhoeal illness may assist in the development of malnutrition in humans by damaging the intestinal mucosa, such that absorptive cells are compromised over an extended period (Tolia & Dubois, 1985).

1.15.4.1 DIARRHOEA

Decreased intracellular Na^+/K^+ -adenosine triphosphatase activity, an impairment of glucose-coupled Na^+ transport and an increase in plasma membrane permeability are several proposals to account for the way in which rotavirus enterocyte infection contributes to the diarrhoeal process (Tolia & Dubois, 1985; del Castillo *et al.*, 1991). All lead to an increase in the intracellular Na^+ concentration, which reduces the absorptive capacity of villus enterocyte cells. However, Spencer *et al.*, (1990) and Starkey *et al.*, (1990), propose a new concept for the mechanism of rotaviral diarrhoea induction, at least for the murine model. They suggest that stimulation of villus base cells to divide (due to infected enterocyte loss at the tip) is accompanied by the transient accumulation of intracellular Na^+ and Cl^- ions; excess NaCl is then secreted into the lumen when cell division is complete, which is the initiator of

fluid loss. It has been noted for some time that there is a faster turnover of villus epithelial cells during rotavirus infection and an impairment in the intestinal absorptive capacity (Snodgrass *et al.*, 1977a; McNulty, 1978).

Irrespective of the precise mechanism, the net result is a decrease in the absorption of NaCl and water from the intestinal lumen which leads to the initiation of diarrhoea (Kerzner *et al.*, 1977).

A reduction in disaccharidase activity in mature enterocytes is also believed to contribute to the induction of osmotic diarrhoea (Bishop *et al.*, 1973; Kerzner *et al.*, 1977). This is because many children and some animals (e.g. pigs) have a carbohydrate (usually lactose) malabsorption and intolerance problem during rotavirus gastroenteritis (Mavromichalis *et al.*, 1977). Thus, non-lactose containing diet formulae, decrease the severity of diarrhoea in many children (Hyams *et al.*, 1981).

However, in mice, lactose malabsorption would appear not to be an important mechanism in the production of diarrhoea (Collins *et al.*, 1990). Inflammation in the intestine is also proposed to assist the diarrhoeic process since it may cause an increase in peristalsis activity (McNulty, 1978).

CHAPTER 2

**THE T-HELPER CELL AND ITS
ROLE IN THE IMMUNE RESPONSE**

2.1 INTRODUCTION

After having considered the most prominent features of the rotaviruses as a group, it is perhaps now relevant to consider the T-helper (Th) cell. This will enable an appreciation of the central role of this cell in the adaptive immune response to be gained and why an understanding of its role is highly relevant to modern vaccine design.

Studies conducted over the last 20 years have resulted in the accumulation of vast amounts of information on Th-cell immunology (e.g. Stevens *et al.*, 1988; Croft & Swain, 1991). This section aims to review the salient features of the Th-cell (*i.e.* its origin, role in the immune response and mode of activation), on a general basis. It is not intended to be an exhaustive source of detailed information on Th-cell immunology. For detailed reviews of T-lymphocyte biology see Zinkernagl & Doherty, (1979); Schwartz, (1986) and Berzofsky *et al.*, (1987).

2.2 T-CELL ONTOGENY

T-lymphocytes are 'educated' and mature in the thymus of higher vertebrates *i.e.* the T-cell repertoire and self tolerance are determined (Kindred, 1979; Owen *et al.*, 1989). There is a temporal succession of gene activation in T-lymphocytes as they mature in the thymus (Farr *et al.*, 1986) and maturation leads to the appearance of characteristic T-cell markers. They include Thy.1 (Owen & Jenkinson, 1981), the cluster of differentiation (CD) 3 antigen (Compana *et al.*, 1989) and the T-cell receptor (TCR) (Kappler *et al.*, 1987).

2.3 CLASSIFICATION OF T-CELLS

T-lymphocytes are divided into 2 main subsets, termed T-helper (Th) lymphocytes and T-cytotoxic (Tc) lymphocytes (Cantor & Afoski, 1975). Th-lymphocytes were originally identified by the phenotype Thy.1⁺, Lyt.1⁺23⁻², L3T4⁺ and Tc-cells by the phenotype Thy.1⁺, Lyt.1⁻23⁺², L3T4⁻ (Cantor & Afoski, 1975; Cantor & Boyce, 1975) but the former are now identified by the

phenotype CD3⁺,CD4⁺,CD8⁻ and Tc-cells by CD3⁺,CD4⁻,CD8⁺ (Bernard *et al.*,1984).

The role of Th-cells is primarily regulatory,controlling the development and function of effector cells.By contrast Tc-cells predominantly make up the effector arm of the cell mediated response,acting to kill target cells expressing foreign antigen (Austyn,1989).

However,the rigidity of this functional definition has, and continues to become less clear.Th-cells have been shown to be capable of lysing infected target cells in an antigen specific manner (Kaplan *et al.*,1984;Fleisher *et al.*, 1985) and Tc-cells capable of secreting lymphokines and providing 'help',have been identified (Ennis,1982;Morris *et al.*,1982;Klein *et al.*,1982;Cardell *et al.*, 1991).This suggests that rather than being distinct subsets,Th and Tc-cells may represent functionally,overlapping lymphocyte subpopulations (Braciale *et al.*,1987).

A third subset of T-cells,T-suppressor (Ts) cells has also been proposed, although a definitive characterization of this subset has yet to be achieved.As a result, this area of research is controversial and will not be discussed extensively.For a review of these cells see Mitchison & Eichman, (1988).

2.4 FUNCTIONAL HETEROGENEITY OF TH-CELLS

The central role of the CD4⁺ Th-cell as 'conductor' of the immune response is reflected in the finding that at least 2 separate groups of Th-cells exist (Moermann *et al.*,1986;Cherwinski *et al.*,1987;Moermann & Moore,1991) and there is evidence that they exist *in vivo* (Taguchi *et al.*,1990).They are termed Th1 and Th2 and were originally defined on the basis of differential expression and secretion of T-cell lymphokines. Thus,Th1 cells produce interleukin (IL) 2,Interferon (Ifn) gamma,lymphotoxin,IL-3 and granulocyte-macrophage colony stimulating factor (GM-CSF) (Moermann *et al.*,1986).Th2 cells in contrast,produce IL-3,IL-4,IL-5,IL-6,IL-10 and GM-CSF but not IL-2 or Ifn-gamma (Moermann & Moore,1991).

The two cell types have been postulated as having different functional roles in the immune response (Kim *et al.*, 1985; Moermann & Coffman, 1989). Th1 cells are thought to primarily stimulate the cell-mediated 'arm' (e.g. CTL's and macrophages) and Th2 cells may preferentially stimulate the B-cell 'arm' (Betz & Fox, 1991; Scott & Kaufmann, 1991; Noelle & Snow, 1992).

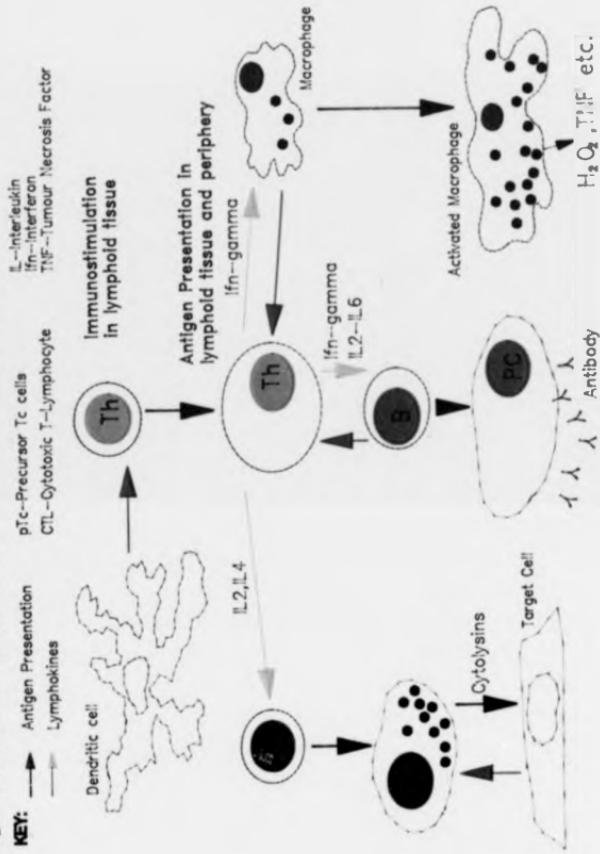
These proposals are substantiated by direct evidence that Th-cells are involved in the activation of these cell types. For example, CD4⁺ Th-cells are important in the activation of CTL's (Ashman & Mullbacher, 1979; Ciavarra, 1990; Muraoka, 1991), macrophages (Schreiber *et al.*, 1985; Paulnock, 1992) and for the growth and differentiation of B-cells (Guy & Hodes, 1989; Gascan *et al.*, 1991; Croft & Swain, 1991; Noelle & Snow, 1992). For a summary of the role of CD4⁺ Th-cells in the immune response, see Fig. 5.

Different pathogens have been found to preferentially induce one of the two Th subtypes (Yssel *et al.*, 1991; Haanen *et al.*, 1991; Yamamura *et al.*, 1991) and some pathogens influence the Th1:Th2 ratio, such that responses to other non-parasite antigens are affected (Kullberg *et al.*, 1992). These results should have a considerable influence on the development of future vaccines since it is conceivable that differential stimulation of either subset may provide a more effective immune response to a particular pathogen.

The two populations of cells are capable of regulating the responses of each other (Mosmann & Moore, 1991; Maggi *et al.*, 1992) (e.g. Ifn-gamma from Th1 cells can inhibit the generation of Th2 clones [Gajewski & Fitch, 1988]) and the frequency of Th1 and Th2 cells also varies in different tissues such as the gut (Taguchi *et al.*, 1990). In addition to negative regulation, Th1 and Th2 cells have a state of inter-dependency since although Th2 cells use IL-4 as an autocrine growth factor (Janeway, 1989) they also require Th1 derived IL-2 for activation, proliferation and growth (Mosmann & Coffman, 1989; Fernandez-Botran *et al.*, 1988).

The lymphokine environment of developing CD4⁺ cells appears to influence the development into Th1 or Th2 cells (Swain *et al.*, 1991; Chatelain *et al.*, 1992; Gajewski *et al.*, 1989) and each of the two subsets may have a different

FIG. 5 THE ROLE OF THE Th CELL IN THE IMMUNE RESPONSE (From Austyn, 1989)



antigen presenting cell (APC) requirement (Hayakawa & Hardy, 1988). Recently, a third Th subset (Th0) has been defined which has an unrestricted lymphokine pattern and release IL-2, Ifn-gamma, IL-4 and IL-5 and is considered to be the precursor to Th1 and Th2 cells (Firestein *et al.*, 1989; Rocken *et al.*, 1992). The differentiation of Th0 cells into terminal Th1 or Th2 cells is driven by antigenic stimulation (Street *et al.*, 1990) and appears to be largely influenced by the presence of IL-4 (Abehsira-Amar *et al.*, 1992).

2.5 MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) RESTRICTION OF TH-LYMPHOCYTE ANTIGEN RECOGNITION

A fundamental difference between T-lymphocytes and B-lymphocytes is that the former recognize processed foreign antigen (as peptides (see section 2.6.3)) in conjunction with molecules of the MHC (Bach *et al.*, 1976; Townsend & Bodmer, 1989; Brodsky & Guagliardi, 1991). In contrast, B-cells usually recognize conformational determinants in naturally folded molecules (Benjamin *et al.*, 1984; Male *et al.*, 1987).

One important difference between Th and Tc-cells is that Th (CD4⁺) cells recognize peptide antigen in association with MHC class II molecules (Ziegler & Unanue, 1982; Thomas *et al.*, 1981; Unanue & Allen, 1987) and Tc (CD8⁺) cells recognize peptide antigen with MHC class I molecules (Lamb *et al.*, 1982; McMichael *et al.*, 1986; Townsend *et al.*, 1986; Townsend & Bodmer, 1989), on the surface of cells. Class restriction is considered to be dependent on the binding CD4 or CD8 molecules to their respective class of MHC molecule, thereby mediating adhesion between T-cells and antigen presenting cells (APCs) (Biddison *et al.*, 1983; Doyle & Strominger, 1987). Thus, MHC molecules tend to act as 'guidance' molecules for T-cells, in the recognition of peptide antigen. The hypervariable regions of the class II MHC polypeptide chains are involved in class II mediated restriction of T-cell recognition (Folsom *et al.*, 1985; Germain *et al.*, 1985), in addition to allele specific regions (Lechler *et al.*, 1986).

The TCR is unlikely to confer MHC restriction since the variable region genes comprising the antigen receptor for class I or class II restricted T-

lymphocytes are drawn from the same variable gene pools (Kronenberg *et al.*, 1986).

2.6 REQUIREMENTS FOR CD4⁺ T-CELL ACTIVATION

2.6.1 CELL TYPES INVOLVED IN ANTIGEN PROCESSING AND PRESENTATION TO Th-CELLS

Early studies by Rosenthal & Shevach,(1973),Ellner *et al.*, (1977) and Ziegler & Unanue,(1981),suggested that in order for Th-cells to become activated, cellular processing of the protein antigen was required.

Three major cell types are primarily involved in the processing and presentation of antigen to Th-cells.They include monocytes/macrophages (Unanue & Allen,1987;Paulnock,1992),B-cells (Rock *et al.*,1984; Lanzavecchia,1985) and dendritic cells (DC's) (Steinman *et al.*,1983;Austyn, 1987).

Due to the difficulty in the isolation and culture of DC's,very little information on antigen processing and presentation in them has been obtained.However,they are potent stimulators of T-cells (King & Katz,1990; Steinman,1991) and are capable of immunostimulation *i.e.*they are able to activate unsensitized resting T-cells (Macatonia *et al.*,1989;Macatonia *et al.*, 1991),unlike other APC's (Austyn, 1989).DC's are also able to induce 'clustering' of T-cells in an antigen independent manner (Austyn,1989).

Both macrophages and B-cells are capable of processing and presenting antigen to sensitized CD4⁺ Th-cells (Brodsky & Guagliardi,1991).B-cells are considered to be important stimulators of memory Th-cell responses when they take up antigen in a specific manner,since efficient responses can be generated with very low concentrations of antigen (*i.e.* 1-10ng/ml) (Rock *et al.*,1984;Abbas *et al.*,1985;Abbas,1989).Both macrophages and DC's require at least 10³-fold more antigen for efficient Th-cell stimulation (Abbas,1989).However,using B-cells (which were not specific for a particular antigen) and macrophages,the latter were shown to be more efficient at antigen uptake and processing (Harding *et al.*,1988;Scheerlinck *et al.*,1991).These data therefore suggest that

specific mechanisms of antigen uptake and processing are probably important
in Th-cell activation.

Antigen processing and presentation to Th ($CD4^+$) cells is, therefore carried out by a rather limited number of cell types (at least in the initial stages of a $CD4^+$ mediated immune response) i.e. those capable of expressing MHC class II molecules. However, Ifn-gamma is capable of inducing MHC class II expression on a range of disparate cell types, following $CD4^+$ cell activation (Wong *et al.*, 1983; Tomkins *et al.*, 1988).

For a summary of the major antigen processing and presenting cell types used by Th ($CD4^+$) cells see Table 5.

2.6.2 ANTIGEN PROCESSING

There are two major sources of antigen for the immune system: exogenous and endogenous. Generally, $CD4^+$ Th-cells recognize peptides derived from exogenous, internalized proteins (Morrison *et al.*, 1986; Unanue & Allen, 1987). In contrast, $CD8^+$ Tc cells recognize peptides derived from the APC (i.e. endogenous cellular proteins or pathogen derived proteins) (Morrison *et al.*, 1986; Brodsky & Guagliardi, 1991). However, there are notable exceptions. For example, endogenously synthesised proteins can be presented as peptides by MHC class II molecules to Th-cells (Jin *et al.*, 1988; Jaraquemada *et al.*, 1990) and class I restricted Tc responses can be induced following internalization of exogenous protein (Gooding & Edwards, 1980; Staerz *et al.*, 1987; Moore *et al.*, 1988).

Exogenous antigens are endocytosed (McCoy *et al.*, 1989; Stossel *et al.*, 1990) and are usually proteolytically cleaved into peptides by acid proteases in endosomal compartments (Ziegler & Unanue, 1982; Guagliardi *et al.*, 1990), prior to binding with MHC molecules (en route to the cell surface) (Guagliardi *et al.*, 1990; Neeffjes *et al.*, 1990). Most of the MHC class II molecules are recycled from the cell membrane and are not newly synthesised molecules, at least in B-cells and non-adherent macrophages (Harding *et al.*, 1988). Simple disruption of

TABLE 6
CHARACTERISTICS OF CELL TYPES INVOLVED IN ANTIGEN PROCESSING AND PRESENTATION
 (From Harding et al., 1988)

FEATURE	ANTIGEN PRESENTING CELL TYPE	
	MACROPHAGE	B-CELL
MHC Class II expression	Constitutive expression in some macrophages.Increased by interferon gamma.Decreased by prostaglandin and other conditions.	Constitutive expression increased by anti-Ig and L-4.
Antigen uptake and internalization.	Via Fc receptors or by direct binding of the antigen.	By surface Ig or by direct interaction. Internalization of microorganisms may be ineffective.
Antigen Processing	Relatively fast for proteins and microbial proteins.	Effective for protein.
Cofactors	High levels of L-1 expression.L-1 expression induced by microorganisms or by interactions with T-cells.	Limited L-1 expression.L-1 can stimulate Th-1 and Th-2 clones of T-cells.

protein conformation, to expose hydrophobic residues may also be important in exogenous antigen processing (Shimonkevitz *et al.*, 1983).

Little is known about the molecular basis of antigen processing and presentation. However, recently two proteases cathepsin D and cathepsin B were identified as being important in the generation of the Th epitopes (Van Noort *et al.*, 1991). The known Th epitopes were located at the N-terminus of each different myoglobin fragment released upon processing, suggesting that some degree of protease specificity in Th epitope generation may exist.

It should be noted that endocytosis of exogenous antigens and the presence of large numbers of endosomes have not been found to be major features of DC's (Austyn, 1989) and therefore the mechanism by which these highly specialised cells process and present antigen to T-cells remains obscure. Immunostimulation by these cells, is therefore a poorly understood process.

Endogenous antigenic peptides, produced by lysosomal or non-lysosomal cytosolic proteolysis (Townsend *et al.*, 1988) are thought to be translocated to the RER or to the intermediate compartment between the RER and the Golgi where they bind to MHC molecules, prior to movement to the cell surface (Townsend *et al.*, 1989; Bennink & Yewdell, 1990).

2.6.3 PEPTIDE-MHC INTERACTIONS-THE BASIS OF ANTIGEN RECOGNITION BY T-CELLS

A variety of techniques, including equilibrium dialysis (Babbitt *et al.*, 1985) have been used successfully to show binding of antigenic peptides to MHC class II molecules. The association rate (K_a) for this process is $\sim 1M^{-1}s^{-1}$, which is very slow. Thus, it has been proposed that peptides must remain with MHC class II molecules in the same compartment for some considerable time, prior to exposure on the cell surface. However, the presence of the TCR tends to increase the amount of peptide associated with the MHC class II molecule, thereby helping to stabilize a low affinity interaction (Watts *et al.*, 1986).

The peptide binds to the MHC molecule in a groove (Bjorkman *et al.*, 1987; Brown *et al.*, 1988) and can bind to either the alpha or beta chains, depending

on the peptide. Usually, only a small number of residues is in contact with the MHC molecule and TCR (Sette *et al.*, 1987). The peptide is usually from 9-25 residues in length (and linear) and is often derived from deep within the native protein conformation (Thornton *et al.*, 1986). Generally, the peptide-MHC interaction is broad (Panina-Bordignon *et al.*, 1989), since non-conservative changes at only a few sites tends to affect binding (Sette *et al.*, 1987).

Studies of peptide-MHC interaction are important since some peptides bind to MHC molecules of certain haplotypes more efficiently than to others and this has been found to correlate with the ability to induce an immune response (Heber-Katz *et al.*, 1983; Babbitt *et al.*, 1985; Buus *et al.*, 1987). Competition for the binding sites on MHC molecules has also been reported (Maryanaki *et al.*, 1988; Bodmer *et al.*, 1989). Both findings support the concept that immune responsiveness is determined by the MHC. This is the determinant selection theory (Owen & Lamb, 1988). Determinant selection is used to explain the existence of 'high' and 'low' responders in many populations (Hedrick *et al.*, 1982; Le Meur *et al.*, 1985) and has been found to influence the effectiveness of some vaccines (Hiatae *et al.*, 1992).

However, clearly other factors are important in influencing a response since there are instances where peptides have a high affinity for MHC class II molecules *in vitro* but are non-immunogenic in individuals with the same haplotype. Quantitative expression of MHC class II molecules may be important in controlling Th-cell responses (Lechler *et al.*, 1986). 'Holes' in the T-cell repertoire may also account for the absence of a response (Kimoto *et al.*, 1981; Ishii *et al.*, 1981; Clark & Shevach, 1982).

2.6.4 ANTIGEN RECOGNITION AND THE TCR

Peptide-MHC complexes are recognized by the TCR (Hedrick *et al.*, 1984). This is a disulphide linked heterodimer (T_i) which is non-covalently associated with the CD3 polypeptide complex and zeta-zeta or zeta-n dimers (Reinherz *et al.*,

1982;John *et al.*,1989;Ashwell & Klausner,1990).However,the Ti alone is insufficient for a functional receptor (Weiss & Stobo,1984).

The dimer consists of two polypeptide chains,either Ti alpha-Ti beta (Meuer *et al.*,1983) or Ti gamma-Ti delta (Brenner *et al.*,1986) (see section 2.6.4.1).The Ti alpha-Ti beta heterodimer is known to confer clonal variability to T-cells and the CD3 complex and zeta-zeta,zeta-n dimers mediate signal transduction across the cell membrane,following interaction of peptide-MHC with the Ti dimer (John *et al.*,1989;Ashwell & Klausner,1990;Blumberg *et al.*,1991).

The TCR-alpha-beta is expressed on >95% of all peripheral T-cells and receptor expressing thymocytes (Owen & Lamb,1988) and is,therefore quantitatively the most important in mediating MHC restricted antigen recognition.The residues of the TCR which bind to the peptide comprise the paratope and the peptide residues which interact with the TCR comprise the epitope (Owen & Lamb,1988).Paratopes for several antigens have been mapped (Allen *et al.*,1987;Sette *et al.*,1987).The Ti alpha and Ti beta variable (V) regions generate an analogous binding site to that of an Ab,with the hypervariable regions forming the walls of the peptide antigen-binding pocket (Owen & Lamb,1988).The specificity of T-cell receptor recognition is influenced both by polymorphic residues in the peptide binding MHC groove (Bjorkman & Parham,1990) and by the primary sequence of the bound,antigen-derived peptide (Rothbard & Taylor,1988).

The Ti molecules share structural features with the immunoglobulins (Igs) since they are also members of the Ig supergene family (Owen & Lamb,1988).However,unlike Igs,somatic hyper-mutation does not appear to play a significant role in the diversity of Ti genes (Austyn,1989).

Of particular importance in T-lymphocyte TCR recognition of antigen is that most recognize foreign peptide antigen in the context of haplotype matched self MHC molecules (Shevach & Rosenthal,1973;Zinkernagel & Doherty, 1974;Fathman & Fitch,1982).Thus,T-cells require the expression of the same MHC gene product as during their development,for foreign antigen to be recognized (Zinkernagel & Doherty,1974).TCR's interact with the MHC in a

highly specific fashion and the residues of the TCR responsible for binding to the MHC molecule comprise the restitope and those of the MHC which interact with the TCR comprise the histotope (Hansburg *et al.*, 1983; Ronchese *et al.*, 1987).

At least some MHC-binding, T-cell stimulatory peptides have characteristic features. Some have been identified as forming an amphipathic alpha-helix (Streicher *et al.*, 1984; Allen *et al.*, 1987) and some have a sequence starting with a charged residue or glycine followed by two or three consecutive hydrophobic residues and terminating in a polar amino acid (De Lisi & Berzofsky, 1985; Rothbard & Taylor, 1988). Some have been predicted to have a beta-sheet structure (Sette *et al.*, 1987).

It is clear from this account that the genes involved in immune recognition control the the ability to mount an immune response. They therefore determine (at least in part), resistance to pathogens, susceptibility to auto-immune diseases and hyper-sensitivity.

2.6.4.1 TCR GAMMA-DELTA CELLS

TCR gamma-delta T-cells are found in large numbers at epithelial sites such as the intestine (Kuziel *et al.*, 1987) and appear to show several distinct differences to TCR alpha-beta T-cells. Generally, the function of TCR-gamma-delta cells remains speculative but some information on them has now become available.

TCR gamma-delta T-cells are of extrathymic origin (Moseley *et al.*, 1990; Banderia *et al.*, 1991) and appear to undergo thymus independent development (Poussier *et al.*, 1992). They are heterogenous and at different anatomical sites differ in TCR gene usage (Bluestone *et al.*, 1991) and in the expression of surface membrane markers (Lefrancois & Goodman, 1989).

Functionally, they have been found to have cytolytic activity and are capable of secreting lymphokines (Raulet, 1989; Bluestone & Matin, 1989). Recently, they been found to express Fc receptors for IgA, IgM and IgG upon activation, suggesting a possible role in antibody-dependent cellular cytotoxicity (Sandor

et al., 1992) and may be involved in the abrogation of oral tolerance (Fujihaashi *et al.*, 1992).

Four T-cell subsets express the CD3-TCR-gamma-delta complex. They include thymus and peripheral CD3⁺, CD4⁻, CD8⁻ cells, a Thy1⁺ epidermal cell of dendritic morphology found in the skin (Koning *et al.*, 1987) and some CD3⁺, CD4⁻, CD8⁺ intestinal intraepithelial lymphocyte's (IEL's) (Bonneville *et al.*, 1988). The latter population may be very important in immunity to rotaviruses, in animals (Bonneville *et al.*, 1988; Hein & Makay, 1991; Van Kerckhove *et al.*, 1992).

2.6.5 ACCESSORY MOLECULES IN THE ACTIVATION OF TH-CELLS

In addition to the TCR-MHC-Peptide interaction and the CD4-MHC class II interaction, other molecules on the surface of Th and APC cells are important in the activation of Th-cells and most appear to be involved in cell adhesion (Schwartz, 1990).

The presence of costimulatory molecules was first realised when transgenic mice expressing foreign MHC antigens in a tissue-specific fashion did not develop auto-immunity (Moller, 1991). Costimulatory molecules appear to be critical for the activation of T-cells. In the absence of costimulatory signals, TCR stimulation leads not to proliferation but to active induction of clonal anergy (*i.e.* a block in proliferation and antigen specific unresponsiveness) (Mueller *et al.*, 1989; Schwartz, 1990) or activation induced cell death (Liu & Janeway, 1990; Groux *et al.*, 1992).

Lymphocyte-Function-Associated-Antigen (LFA) 1 appears to be important to both T-cell proliferation and T-cell mediated lysis (Davignon *et al.*, 1981). One of the ligands for LFA-1 is the Inter-cellular Adhesion Molecule (ICAM) 1, found on the surface of APC's and another is ICAM-2 (Springer, 1990). Both ICAM-1 and ICAM-2 have been shown to augment the activation (Damle *et al.*, 1992) and proliferation (Altman *et al.*, 1989) of T-cells. ICAM-1 also binds to CD43 which enhances T-cell activation (Rosenstein *et al.*, 1991; Park *et al.*, 1991).

The T-cell surface molecule CD2 and LFA-3 on the surface of the APC have been shown to interact (Springer,1990;Bierer & Burakoff,1991) and initiate cellular conjugation prior to the peptide-MHC complex engaging the TCR (Shaw *et al.*,1986). CD2 appears to be important in T-cell activation (Bierer & Burakoff,1991) and it may also play a role in the generation of self tolerance in the thymus (Reinherz,1985).

Other accessory molecule interactions are more poorly defined. They include CD28 on T-cells and B7/BB1 on APC's (Linsley *et al.*,1990;Linsley *et al.*,1991), CTLA-4 on T-cells and B7/BB1 on APC's (Linsley *et al.*,1991a) and the heat stable antigen (HSA) on APC's and an unknown ligand on T-cells (Liu *et al.*, 1992a).Studies with vascular endothelial cells has also revealed an interaction between Vascular-Cell-Associated-Molecule (VCAM) 1 and Very-Late-Activation-Antigen (VLA) 4 on T-cells and the presence of VCAM-1 enhances the proliferation of T-cells (Damle & Aruffo,1991;Van-Seventer *et al.*,1991).

Of all the costimulatory molecules, the presence of B7 and the HSA on APC's appear to be most important in avoiding the induction of unresponsiveness or cell death and are therefore capable of regulating immune unresponsiveness (Liu & Linsley,1992).

The costimulatory molecules reviewed so far are believed to work in conjunction with the TCR peptide/MHC interaction for T-cell activation. However,there are examples where activation of sensitized T-cells occurs in the absence of TCR ligand (Linsley *et al.*,1991) and where cloned CD4⁺ and CD8⁺ T-cells received costimulatory signals from bystander cells (Otten & Germain,1991).In view of this data,it is conceivable that these mechanisms of T-cell activation could lead to autoimmunity.Thus, it has been proposed that control on the activation of costimulatory activity of APC's is mediated by products of the immune response and foreign antigen components (Kawakami *et al.*,1989;Liu & Janeway, 1990).There is evidence that lipopolysaccharides,ds RNA and influenza virions can induce the expression of B7 on APC's and the costimulatory activity of spleen cells was found to correlate with the levels of B7 induced by these agents (Linsley *et al.*,1991).

The results suggest that the signal required to induce T-cell activation and proliferation is finely regulated and a threshold level of signal induction must be achieved before activation can occur *i.e.* cells expressing both the TCR ligand and the B7 molecule were 30-80 fold more efficient at signal induction than a mixture of cells expressing either of the two molecules alone (Liu & Janeway, 1992).

Thus, the effective induction of T-cells would appear to be dependent on effective TCR/MHC peptide interaction in addition to the induction of costimulatory molecules on the surface of APC's. The reason for the presence of multiple molecules in costimulation is unknown. It has been proposed that different costimulatory molecules may have different roles at different points in the immune response *i.e.* during activation of unsensitized T-cells and sensitized T-cells (Damle *et al.*, 1992a).

2.6.6 LYMPHOKINES IN THE ACTIVATION OF Th-CELLS

So far, the discussion of Th-cell activation has focussed on the physical interaction between Th-cells and APC's. However, soluble lymphokines also play a role in (i) aiding the stimulation of Th-cells and (ii) clonal expansion.

The lymphokine interleukin (IL) 1 is involved in the activation of at least some sets of T-cells (Greenbaum *et al.*, 1988; Fernandez-Botran *et al.*, 1988). It has been detected in association with activated macrophages (Kurt-Jones *et al.*, 1985) and although it is not synthesised by DC's (Koide & Steinman, 1987; Weaver *et al.*, 1989) it acts as a potentiating lymphokine in DC antigen presentation to T-cells, since it increases the ability of DC's to cluster with T-cells (Koide *et al.*, 1987). It has been proposed that IL-1 may be required to optimize the cellular contacts between APC's and T-cells in the presence of low numbers of APC's (Koide *et al.*, 1987). Thus, IL-1 produced by APC's may have an enhancing rather than an obligatory role in Th-cell activation. However, T-cells have been shown to possess receptors for IL-1 (Dower & Urdal, 1987). Furthermore, it is possible that IL-1 has differential effects on different Th subsets. For example, IL-1 was found to co-stimulate Th2 but not

Th1 subsets of CD4⁺ T-cell clones (Cherwinski *et al.*, 1987; Lichtman *et al.*, 1988) and Th2 but not Th1 clones possess a high affinity IL-1 receptor (IL-1^r) (Greenbaum *et al.*, 1988).

The clonal expansion of Th-cells (and Tc cells) is critically dependent on the production of IL-2 and IL-2^r's (Watson, 1979; Mosmann & Coffman, 1989). The major source of IL-2 is the activated Th1 cell (Mosmann & Coffman, 1989) and IL-2 gene transcription is dependent on the activation of protein kinase C and a rise in intracellular Ca²⁺ (produced as a result of TCR-peptide-MHC interaction and costimulatory molecules) (Weis *et al.*, 1984). The IL-2 released from activated Th-cells binds to the IL-2^r and leads to clonal proliferation.

Therefore, protein kinase C activation and an increase intracellular Ca²⁺, also leads to the transcription of the 55kD IL-2^r gene (Smith & Cantrell, 1985). The 55kD IL-2^r mediates IL-2 binding and a 75kD IL-2^r mediates the signal for cell growth via tyrosine kinase activation of phospho-proteins (Damjanovich *et al.*, 1992). Both 55kD and 75kD chains comprise the high affinity IL-2^r (Wang & Smith, 1987).

The proliferative response to IL-2 is, therefore dependent on the number of receptors, the IL-2 concentration and the length of the IL-2-IL-2^r interaction (Hamblin, 1988). Removal of the T-cell stimulant, therefore results in down regulation of receptor expression and a decline in proliferation, thereby limiting the response. IL-4, as produced by activated Th2 cells also causes T-cell proliferation in IL-4^{r+} T-cells but is less potent than IL-2 (Lee *et al.*, 1986).

The results suggest that IL-2 and IL-4 are capable of inducing the proliferation of T-cells in both an autocrine and by-stander fashion which ultimately leads to an amplification of the immune response.

2.6.6.1 POSITIVE FEEDBACK MECHANISMS IN TH-LYMPHOCYTE ACTIVATION

In the course of antigen-driven Th-cell proliferation other lymphokines are generated which can, themselves feedback onto T-cells or APC's to amplify the response. For example, Ifn-gamma causes increased MHC class II gene

expression on APC's (Basham & Merigan, 1983; Scheynius *et al.*, 1986) and on macrophages the IL-1 production is enhanced by the presence of lipopolysaccharide (Adams & Hamilton, 1987). Both MHC class II and IL-1 can then serve to activate Th-cells. Tumour Necrosis Factor (TNF) receptors are also induced on activated T-cells and in the presence of TNF (from APC's e.g. macrophages) T-cells are stimulated to express more IL-2 and Ifn-gamma receptors and show enhanced IL-2 dependent Ifn-gamma production (Scheurich *et al.*, 1987).

Lymphokines such as Ifn-gamma may have effects on other non-lymphoid cells which aid the immune response. For example Ifn-gamma induces MHC class II expression on non-lymphoid cells (Tomkins *et al.*, 1988), induces ICAM-1 expression (Pober *et al.*, 1986) and induces adhesion of T-lymphocytes to endothelial cells (Yu *et al.*, 1985). Thus, Ifn-gamma is likely to facilitate localization of the immune response and so influence lymphocyte migration in general (Schattner *et al.*, 1983).

There is, therefore a complex lymphokine interaction network between APC's, endothelial cells and Th-cells during antigen dependent activation.

2.7 TH-B-CELL INTERACTION AND THE ACTIVATION OF B-CELLS BY TH-CELLS

This is an important function of Th-cells and a review of this interaction is particularly important in an understanding of the role of the Th-cell in regulation of the immune response.

The activation of Th-cells by B-cells is considered to involve at least some of the costimulatory molecules described in section 2.6.5. However, following Th-cell activation by B-cells other molecular interactions between the two cells have been identified, which lead to B-cell activation and subsequent proliferation and differentiation (Lederman *et al.*, 1992). Thus, the molecules which govern the initial interaction and formation of physical conjugates of Th and B-cells are distinct from those involved in the triggering of B-cell cycle entry and B-cell differentiation.

The activation of B-cells by Th-cells is via a class II unrestricted, antigen non-specific mechanism (Hodgkin *et al.*, 1990; Noelle *et al.*, 1991). The B-cell surface molecule CD40 has been proposed as the receptor on B-cells that is triggered by activated Th-cells (Banchereau *et al.*, 1991; Gascan *et al.*, 1991) and the ligand on the surface of activated Th-cells has been suggested to be a novel 39kD membrane protein (Noelle & Snow, 1992).

Following activation, B-cells become sensitive and responsive to Th lymphokines (e.g. IL-4), capable of stimulating progress into the G1b and S phases of the cell cycle (Hodgkin *et al.*, 1990; Noelle *et al.*, 1991). The terminal differentiation of IgA, IgE and IgG1 committed B-cells is dependent on IL-4 (and also IL-5 for IgA production) (Boom *et al.*, 1988; Coffman *et al.*, 1988; Coffman *et al.*, 1989). Thus, Th2-cell help is fundamental to the activation of these B-cell responses. However, the isotype pattern induced by Th2 cells can be modified by the intervention of Th1-cells (Noelle & Snow, 1992). For example, low levels of Th1 derived Ifn-gamma specifically inhibit IL-4 mediated enhancement of IgE and IgG1 production and high levels of Ifn-gamma also inhibit IgA synthesis (Coffman & Carty, 1986).

It is clear, therefore that Th subtypes may work in tandem and not in isolation in the regulation of the immune response, suggesting that it is a complex and intricate process.

CHAPTER 3

THE GUT ASSOCIATED LYMPHOID SYSTEM

3.1 INTRODUCTION

The existence of a localized immune system in the gut was first recognized by Besredka in (1919), who observed that rabbits were protected against fatal dysentery after immunization with killed *Shiga* bacillus, irrespective of the serum Ab titer. Later, in (1966) Tomasi *et al.*, showed that external secretions contained a unique immunoglobulin called IgA and an associated antigenic, secretory component (SC).

The importance of Gut Associated Lymphoid Tissue (GALT), in the immune system is illustrated by the finding that at least 80% of all Ig producing cells are found in this region (Brandtzaeg *et al.*, 1987). GALT is, therefore the largest source of lymphoid tissue in the mammal.

It is now generally believed that GALT forms part of the larger, Mucosal Associated Lymphoid Tissue (MALT) along with Bronchus Associated Lymphoid Tissue (BALT) (Mestecky, 1987) and cellular trafficking between these sites is apparent (Ogra *et al.*, 1989).

GALT is comprised of Peyer's patches (PP's), the appendix, colonic patches, mesenteric lymph nodes (MLN's), intra- epithelial lymphocytes (IEL's) and lymphocytes of the lamina propria (LP) (Brandtzaeg, 1989).

3.2 THE NATURE OF GALT

3.2.1 GENERALIZED MODEL OF THE PATHWAY OF ANTIGEN UPTAKE, PROCESSING AND PRESENTATION AND THE INDUCTION OF THE IMMUNE RESPONSE

It is generally accepted (Mestecky, 1987; Brandtzaeg, 1989) that gastrointestinal immune responses to protein antigens (Neutra *et al.*, 1987), bacteria (Owen *et al.*, 1986) and viruses (Wolf *et al.*, 1983; Dharakul *et al.*, 1988) are primarily initiated in PP's.

More specifically antigen is taken up by membrane (M) cells (Owen *et al.*, 1986; Neutra *et al.*, 1987), situated in the epithelium covering lymphoid follicles, which make up the PP's. Each follicle contains both B and T-cell areas (Brandtzaeg, 1989). Antigen is then passed to sub-epithelial DC's and macro-

phages, prior to presentation to T-cells. Class II MHC expressing epithelial cells in the patch dome may also be responsible for antigen processing. B-cells present in the patch dome are activated via Th-cells and become committed to secretory (Sc) IgA synthesis (Brandtzaeg, 1989). Specific antigen presentation to B-cells also takes place in follicular germinal centres by follicular DC's (Brandtzaeg & Bjerke, 1989; Austyn, 1989).

Antigen presentation to Th-cells at effector sites such as the villus epithelium occurs via MHC class II positive epithelial cells and DC's are also involved in antigen presentation in the LP (Brandtzaeg & Bjerke, 1989). Lymphokine secretion by T-cells leads to MHC class II expression on local epithelial cells (Mayer & Shlien, 1987), terminal B-cell differentiation (Mestecky & McGhee, 1987) and increased expression of SC (Solid et al., 1987), thereby promoting external transport of dimeric IgA (Brandtzaeg, 1989).

In summary, it can be seen from this overview that different regions of GALT are thought to carry out different specialized roles. It has been proposed that PP's and MLN's can be considered to be inductive sites, involved in the initiation of the immune response whereas the LP and mucosal epithelium are effector sites (James et al., 1990). This is based on the finding that virtually all the T-cells found in the LP are of the differentiated memory phenotype (James et al., 1986; James et al., 1990) and MLN's contain a high proportion of 'naive' cells (James et al., 1990).

3.2.2 LYMPHOCYTE MIGRATION IN GALT

Following antigen presentation in PP's, the T and B-cell populations expand, drain into the intestinal lymphatics and MLN's (Brandtzaeg & Bjerke, 1989; Reynolds et al., 1991) and enter the blood circulation. They are then carried to the gut where they enter the LP and IEL cell populations (Craig & Cebra, 1971). This homing process of activated or memory cells, is dependent on selective endothelial recognition mechanisms in high endothelial venules (HEV's) (Streeter et al., 1988; Picker, 1992).

Although some of the molecules involved in gut specific homing to PP's have been identified e.g. the 'selectin', leukocyte-endothelium cell adhesion molecule (LECAM) 1 (Hamann *et al.*, 1991) and the integrins, lymphocyte-Peyer's patch (HEV) adhesion molecules (LPAM) 1 and 2 (Shimizu *et al.*, 1992), it is unlikely that the same homing receptors are used for targetting to the LP. It is now clear that naive and memory lymphocytes have radically different trafficking patterns (Mackay, 1991; Lee & Vitetta, 1991; Picker, 1992) and therefore probably use different homing receptors for PP's and the LP, respectively.

Re-exposure to antigen in the LP induces T-cell activation and subsequently B-cell differentiation into Ab secreting plasma cells, with the production of sIgA (Brandtzaeg *et al.*, 1987).

In addition to specific migration, GALT cells also migrate to other mucosal sites such as the mammary glands (Goldblum *et al.*, 1975; Dahlgren *et al.*, 1987) and salivary glands (Tomasi, 1989), illustrating the degree of interaction between mucosal sites.

3.2.3 DISTRIBUTION AND FUNCTION OF CELL TYPES IN GALT

IEL's present in the gut epithelium are mostly CD3⁺, CD8⁺ T-cells, possessing the TCR gamma-delta (see section 2.6.4.1), whereas the LP contains mainly CD4⁺ Th-cells (James *et al.*, 1987; Bonneville *et al.*, 1988). The follicle associated epithelium (FAE) covering the domes of PP's also contains mainly CD4⁺ Th-cells, especially near the antigen transporting M cells (Bjerke & Brandtzaeg, 1988).

It has been suggested that many of the IEL CD8⁺, TCR gamma-delta cells are Ts cells (Trejdowsiewicz *et al.*, 1987) but it also appears that this cell population is capable of lymphokine secretion (Taguchi *et al.*, 1990; Taguchi *et al.*, 1991), suggesting a possible 'helper' function. TCR alpha-beta T-cells in the IEL population have also been claimed to have specific helper activity for B-cells (Fujihashi *et al.*, 1992).

Some groups also claim the existence of T contra-suppressor (Tcs) cells in the TCR gamma-delta populations (Fujihashi *et al.*, 1992). Both Ts and Tcs-cells

are believed to be present in PP's along with Th and B-cells (Tomasi, 1989). T_H and T_{Cs}-cells may be involved in the induction and regulation of oral tolerance (Bland & Warren, 1986; Brandtzaeg, 1989), which is beyond the scope of this review.

Recent work has attempted to analyse the frequency of Th1 and Th2 cells in GALT. Taguchi *et al.*, (1990) determined that in the mouse the Th1:Th2 ratio in the LP was 1:3. Thus, large numbers of Th2, IL-5 secreting cells occurred in IgA effector sites. Th-cells in the LP also have a higher helper activity for IgM and IgG synthesis than other sites (James *et al.*, 1990). The PP's contained smaller numbers of Ifn gamma and IL-5 secreting cells but approximately equal numbers were induced upon Con.A stimulation (Taguchi *et al.*, 1990). These data suggest that Th-cells for different functions are present in different locations in GALT.

IgA expressing B-cells are primarily found in adjacent zones in PP's and also in the LP (Craig & Cebra, 1971; Brantzseag & Bjerke, 1989). However, IgA secreting plasma cells are found predominantly in the LP (Tomasi, 1983). IgA1 secretion is more prominent in GALT than IgA2 but IgA2 is more readily expressed in GALT than at other sites (Brandtzaeg & Bjerke, 1989). IgA immunocytes in the LP are proposed to be relatively immature memory B-cell clones with characteristic high Joiner (J)-chain expression (Brandtzaeg & Bjerke, 1989).

3.3 EFFECTOR MECHANISMS OF THE GASTROINTESTINAL IMMUNE RESPONSE

3.3.1 THE INDUCTION OF IgA IN THE INTESTINE

One of the most important features of the intestinal immune system is the production of IgA (Dahlgren *et al.*, 1989). More IgA is translocated into the gut lumen every day than the total daily production of IgG (Delacroix, 1985). Therefore, in view of the importance of this Ab in intestinal immunity, its mechanism of induction will now be discussed. This will also illustrate the highly specific activity of specialized CD4⁺ Th-cells in this process.

3.3.1.1 THE REQUIREMENT FOR Th-CELLS

Th-cells have an important role in the induction and regulation of Ig synthesis (see section 2.7) and IgA is one of the most T-cell dependent of all the isotypes (Tomasi, 1989).

Th-cells that mediate the B-cell isotype switching directly from IgM to IgA expression have been cloned from murine PP's (Strober & Sneller, 1988) and Transforming Growth Factor Beta (TGF-beta) appears to be the switch factor (Coffmann *et al.*, 1989).

Work with appendiceal lymphoid cells *in vitro* showed that MHC class II molecules had a very important role in the induction of IgA and IgM production (Kawanishi, 1987). It has been proposed that the 'switch' Th-cells are autoreactive and are triggered directly by these MHC class II determinants (Brandtzaeg, 1989). However, these cells are unable to induce terminal B-cell differentiation but Fc-alpha receptor (R) positive, post-switch Th-cells favouring differentiation to plasma cells and IgA production, have been cloned (Mestecky & McGhee, 1987). These cells produce soluble IgA binding factors (IgA BF's) which are capable of suppressing or enhancing the immune response specifically for IgA. They are believed to be at least partially regulated by the level of IgA, via the Fc-alpha R on the cell surface (Tomasi, 1989) and are potentially responsible for IL-5 secretion (see below). The phenotypic definition of these highly specialized Th-cells has not been fully determined to date.

In view of these results, regulation of IgA production, by T-cells probably occurs at two sites; in PP's and in secretory tissues such as the LP (Brandtzaeg, 1989).

3.3.1.2 T-CELL LYMPHOKINES IN IgA INDUCTION

The Th2 lymphokine IL-5, is particularly important in promoting IgA production (Coffman *et al.*, 1989; McGhee *et al.*, 1989) and IL-5 also promotes IL-2^r expression on B-cells (O'Garra *et al.*, 1988). The Th1 lymphokine IL-2 is capable of promoting J chain gene transcription in B-cells (Blackman *et al.*,

1986) and potentiating the secretion of IgA (Coffman *et al.*, 1989). In contrast, Ifn-gamma secreting cells may down regulate IgA synthesis (Coffman *et al.*, 1989).

Th-cells in GALT, thus have a critical role in the induction of IgA synthesis and it is clear from this review that a detailed understanding of the inter-relationships between Th-cells and B-cells is highly relevant to the design of successful vaccines for intestinal pathogens, such as rotavirus.

3.3.1.3 THE REQUIREMENT FOR ACCESSORY CELLS

Accessory cells involved in IgA synthesis appear to be highly specialized since certain DC's from PP's were found to augment IgA production in the presence of Th-cells, whereas DC's from the spleen were not (Brandtzaeg *et al.*, 1987). Follicular DC's have been implicated in the induction of B-cell class switching from IgM to IgA (Spalding *et al.*, 1984).

3.3.1.4 B-CELLS AND IgA SECRETION

Following movement into secretory tissues such as the LP, B-cells undergo both proliferation and terminal differentiation into Ig secreting plasma cells (Brandtzaeg *et al.*, 1987) (see section 3.3.1.1). IgA producing B-cells are the predominant Ig producing cells in the intestinal mucosa, with greater than 500/mm², in some areas (Brandtzaeg, 1989).

Following synthesis, both polymeric (p) IgA (usually a dimer with a linkage via the J chain) and pentameric IgM, require the polymeric Ig receptor or secretory component (SC) for them to be transcytosed across the mucosal epithelium (Meatecky & McGhee, 1987). This process is ultimately dependent on the J chain (Brandtzaeg & Bjerke, 1989).

The ScIgA or ScIgM transcytosed and released in this way is often linked via disulphide bonds to the ectoplasmic domain of the SC which stabilizes the Ab polymer, enabling optimal antigen binding to occur (Brandtzaeg, 1989) and preventing, to some extent proteolytic digestion (Tomasi, 1989). IgA also enters

the intestinal lumen via bile (Delacroix, 1985) and blood serum can serve as a source of IgA for bile and the intestinal mucosa (Tomasi, 1989).

Th-cells and macrophages not only have a regulatory effect on IgA expression but they can also influence IgA transcytosis since both Ifn-gamma and Tumour Necrosis Factor (TNF) alpha can upregulate the expression of SC (Sollid *et al.*, 1987; Brandtzæg *et al.*, 1988). The IgA effector arm of the immune response is therefore, highly regulated.

3.3.2 INDUCTION OF CYTOTOXIC LYMPHOCYTES IN GALT

Antigen specific Tc-cells can be efficiently induced in GALT (Cebra *et al.*, 1989; Offit & Dudzik, 1989a). For example, following intra-duodenal (i.d.) inoculation of reovirus into mice, IEL's were found to be a rich source of pCTL's to the virus. Tc-cells may have an important role in prevention of disease in the intestine since CD8⁺ Tc-cells (from reovirus primed mice) were capable of preventing the development of reovirus induced histopathological lesions in severe-combined-immuno-deficient (SCID) mice (Cebra *et al.*, 1989). CD4⁺ Th-cells probably have an important role in the induction and activation of CD8⁺ CTL's in GALT (see section 2.4).

Both Natural Killer (NK) activity and Antibody Dependent Cellular Cytotoxicity (ADCC) have also been detected within the IEL population (Ernst *et al.*, 1985; Klein, 1986).

3.4 RESUME

A knowledge of GALT, its interactions and the specific functions of its individual components is critical for an understanding of immunity to gut pathogens such as the rotaviruses, such that more effective vaccine strategies can be developed.

It is clear that the CD4⁺ Th-cell plays a central role in the induction and regulation of S IgA producing B-cells and is also likely to contribute to CD8⁺ CTL activation, in the intestine.

CHAPTER 4

THE IMMUNE RESPONSE TO ROTAVIRUS

4.1 INTRODUCTION

Despite the vast amount of information on the molecular biology, replication cycle and pathogenesis of rotaviruses, the specific mechanisms responsible for immunity are poorly understood (Edelman *et al.*, 1989; Ruggieri & Greenberg, 1991; Ward *et al.*, 1992) and the results are often conflicting (Totterdell *et al.*, 1988a).

The processes involved in the induction of mucosal immunity and its role in the control and prevention of disease, are unclear (Kapikian *et al.*, 1983; Hjelt *et al.*, 1986), particularly for T-cell mediated immunity (Mestecky & McGhee, 1989). In addition, interpretation of immunological data in humans is complicated by (i) a lack of information on previous exposure to rotavirus, (ii) an inability to separate the effect of maternal Ab on protection, from active immunity and (iii) the lack of a suitable animal model which accurately mimics infection in children. In animals, a failure to perform experiments in rotavirus seronegative subjects can also lead to problems of data interpretation (Estes & Cohen, 1989; Matsui *et al.*, 1989).

However, mAb's have helped considerably in the mapping of Ab binding domains of VP4 and VP7, which may facilitate a greater understanding of the humoral immune response. A review of the humoral immune response to rotavirus is pertinent, since Th-cells have a critical role in the activation of this arm of the immune response (Icenogle *et al.*, 1986; Guy & Hodes, 1989; Noelle *et al.*, 1991; Croft & Swain, 1991; Kutubuddin *et al.*, 1992) and is central to an understanding of the basis of immunity to rotavirus. Furthermore, many Th epitopes in viral proteins have been found to lie within B-cell epitope regions (Graham *et al.*, 1989; Barnett *et al.*, 1989; Charalambos & Steward, 1990; Mathews *et al.*, 1991; Kutubuddin *et al.*, 1992; Ou *et al.*, 1992), and therefore a knowledge of the humoral response and the epitopes involved, may assist in the prediction of Th epitopes in rotavirus.

4.2 REINFECTION STUDIES AND PROTECTION

An important question in rotavirus immunology is whether primary infection can induce immunity against reinfection and disease, and if it does, then how cross-reactive is the protective response and what is its duration?

Although most reinfections in humans are asymptomatic (Kapikian & Chanock, 1990; Flores & Kapikian, 1988) it has been estimated that reinfection accounts for up to 10% of all cases of rotavirus diarrhoea (Flores & Kapikian, 1988). Symptomatic reinfections have been described (Gurwith *et al.*, 1981; Mata *et al.*, 1983) and can occur with different rotavirus serotypes (Wyatt *et al.*, 1979) or the same serotype (Black *et al.*, 1982; Ward *et al.*, 1986; De Champs *et al.*, 1991).

However, it has also been reported that natural rotavirus infection can reduce both the frequency and severity of subsequent infections (Bishop *et al.*, 1983; Chiba *et al.*, 1986; Bernstein *et al.*, 1991). Both heterotypic protection (Wyatt *et al.*, 1979a; Vesikari *et al.*, 1985; Torres & Ji-Huang, 1986; Matsui *et al.*, 1989) and homotypic protection (Woode *et al.*, 1983; Murakami *et al.*, 1986; Offit *et al.*, 1986b; Flores *et al.*, 1987) against disease have been reported. In addition, neonatal infection would appear to confer at least partial resistance to disease (Bishop *et al.*, 1983; Ward *et al.*, 1992).

Asymptomatic rotavirus infections in older children and adults (Rodriguez *et al.*, 1979; Kim *et al.*, 1977) may, in part, be due to preexisting nAb's acquired from previous exposures (Flores & Kapikian, 1988). Symptomatic reinfection may occur, therefore as a result of a 'waning' of the immune response to rotavirus (Edelman *et al.*, 1989).

These results suggest that protective immunity can, and does develop after rotavirus disease. However, the mechanism is complex and is probably associated with many interacting factors.

4.3 THE HUMORAL RESPONSE

4.3.1 IN VIVO OBSERVATIONS ON HUMORAL IMMUNITY AND PROTECTION

4.3.1.1 THE IMPORTANCE OF COLOSTRUM IN PROTECTION

Naturally occurring colostral and milk Ab's appear to be important in protection against rotavirus disease, in animals (Snodgrass *et al.*, 1980; Sheridan *et al.*, 1983; Castrucci *et al.*, 1984; Archambault *et al.*, 1988). These animal products have also been used to protect human infants from disease. For example, cattle colostrum (containing Ab's to human rotavirus), when used prophylactically reduced the incidence of rotavirus diarrhoea in infants (Ebina *et al.*, 1985). Furthermore, a reduction in the duration of rotavirus excretion and diarrhoea was noted when freeze-dried lactation milk (derived from cows immunized with human rotavirus), was given to rotavirus infected infants (Brusseau *et al.*, 1987).

4.3.1.2 FACTORS INFLUENCING THE PROTECTIVE ROLE OF ANTI-ROTAVIRUS ANTIBODY

Several factors may influence the effectiveness of rotavirus specific Ab's in protection. For example, calves fed colostrum containing rotavirus Ab's 24hr after birth, developed diarrhoea following challenge with calf rotavirus 7 days later (Woode *et al.*, 1975). In contrast, calves fed colostrum containing rotavirus Ab's 4 hr prior to challenge and 4-24hr after challenge did not develop diarrhoea within the normal incubation time (Woode *et al.*, 1975). Pre-existing Ab's to rotavirus in the intestine also appear to protect against disease (Kapikian *et al.*, 1983a; Kapikian *et al.*, 1983; Sheridan *et al.*, 1983). It would appear, therefore that rotavirus specific Ab's can prevent rotavirus disease if present at or near the time of inoculation.

The location of rotavirus specific Ab's is also relevant. Serum Ab's are seemingly unimportant in protection against disease (Snodgrass & Wells, 1976; Sheridan *et al.*, 1983; Ward *et al.*, 1986), but rotavirus specific Ab's in the intestinal mucosa and lumen are important (Woode *et al.*, 1975; McClean &

Holmes, 1981; Offit & Clark, 1985; Besser *et al.*, 1988). Hence, correlations between serum anti-rotavirus Ab's and protection against disease (Kapikian *et al.*, 1983; Hjelt *et al.*, 1987; Ward *et al.*, 1989) can be explained by the movement of serum Ab's into the intestine by transudation (Besser *et al.*, 1988; Edelman *et al.*, 1989). Alternatively, the correlation may merely reflect the localized levels of intestinal Ab (Riepenhoff-Talty *et al.*, 1981). Serum Ab's to the virus may, therefore, be useful as an indicator of localized gastrointestinal responses (Bernstein *et al.*, 1989).

The type of Ab may also be important in determining protection but the results are conflicting. Several studies suggest that IgG is important in protection (Snodgrass *et al.*, 1980; Corthier & Franz, 1981; Hess & Bachmann, 1981; Sheridan *et al.*, 1983; Bernstein *et al.*, 1989). Thus, it has been proposed by some that IgA may play a lesser role in protection against infection than IgG (Sheridan *et al.*, 1983; Hjelt *et al.*, 1987). Alternative roles for IgA have been postulated (Stals *et al.*, 1984), including reducing the severity of gastroenteritis (Sheridan *et al.*, 1983; Hjelt *et al.*, 1987) and decreasing and foreshortening the shedding of rotavirus (Wright *et al.*, 1987). However, other evidence suggests that both IgA and IgG can be important in protection against disease, depending on the route of priming (Offit & Clark, 1985).

The absence of sufficient clear data makes it impossible to state precisely which of the two Ab's is most important in protection against natural infection and it is possible that different Ab classes have different roles in different species.

4.3.1.3 HUMAN BREAST MILK AND PROTECTION

The importance of human breast milk in protection against rotavirus infection and disease is very unclear. Some evidence suggests that breast feeding is unimportant in protection against infection, since both breast-fed and bottle-fed infants can become infected and show an asymptomatic infection (Bishop *et al.*, 1979; Gurwith *et al.*, 1983). More importantly, rotavirus diarrhoea has been

noted in both breast-fed and non breast-fed infants (Cushing & Anderson, 1982; Weinberg *et al.* 1984; Glass *et al.*, 1986).

Other evidence suggests that breast-feeding confers both resistance to rotavirus infection (Chrystie *et al.*, 1978; Hjelt *et al.*, 1985) and disease (Cunningham, 1977; Pickering *et al.*, 1985).

One finding which may account, to some extent for these discrepancies is that infant serum IgG levels correlate well with those of the mother (McClean & Holmes, 1980; Hjelt *et al.*, 1985). Thus, it has been proposed that protection against disease in neonates might be mediated by high levels of maternal IgG, acquired transplacentally and not via milk Ab's (Flores & Kapikian, 1988; Matsui *et al.*, 1989; Kapikian & Chanock, 1990).

However, there is evidence to suggest that breast feeding may have some role in resistance to disease. For example, rotavirus specific Ab's have been detected in human breast milk (Totterdell *et al.*, 1983) and levels of colostral and milk Ab's can increase following maternal rotavirus infection and diarrhoea (Yolken *et al.*, 1978; McClean & Holmes, 1981).

It is possible that breast feeding may not mediate absolute protection but is more important in reducing the severity of disease and the duration of rotavirus shedding. This is supported by the work of Duffy *et al.*, (1986) where rotavirus diarrhoea was found to be milder in breast-fed infants than in bottle-fed infants.

Clearly, the role of breast feeding in protection against rotavirus disease in humans, is (and continues to be) an enigma. In reality, it is probably influenced by many closely interacting factors, including the presence of gut associated protease activity in the child (Totterdell *et al.*, 1982), the presence of cell mediated immunity (CMI) in the milk (Head *et al.*, 1977; Kohl *et al.*, 1983; Archambault *et al.*, 1988) and the virulence of the rotavirus strain (see section 1.13.2.1).

4.3.2 ROTAVIRUS PROTEINS INVOLVED IN THE INDUCTION OF PROTECTIVE IMMUNITY

The determinants of protective immunity to rotavirus infection have not been fully defined (Matsui *et al.*, 1989). However, several studies have shown that responses to both VP4 and VP7 play an important role in resistance to disease (Offit *et al.*, 1986b; Ward *et al.*, 1988; Hoshino *et al.*, 1988; Mackow *et al.*, 1990). Monoclonal Ab's to both serotype specific (Offit *et al.*, 1986) and cross-reactive epitopes (Matsui *et al.*, 1989) in these proteins induced protective immunity to disease, when passively administered to mice by the oral route. This *in vivo* protection correlated well with *in vitro* neutralization for mAb's to both VP4 and VP7. Epitopes which induce protection *in vivo* but not neutralization *in vitro* may also be present in VP4 and VP7 (Matsui *et al.*, 1989). The relative immuno-dominance of different epitopes on VP4 and VP7 following oral inoculation is not known and is clearly very relevant in recombinant vaccine design, since the determinants which induce a stronger more sustained immune response are more desirable. Neutralizing Ab's to the outer capsid proteins have also been proposed to play a role in recovery from primary infection, in addition to protection (Eates, 1990).

Although gene products other than VP4 and VP7 appear to be less important in protection (Offit *et al.*, 1986; Offit *et al.*, 1986b), more recently VP6 has also been reported to play a role (Frenchick *et al.*, 1987; Ijaz *et al.*, 1991).

4.3.3 HOMOTYPIC AND HETEROGENIC IMMUNITY

Many studies have clearly shown that following the first rotavirus infection, homotypic immunity is primarily induced which is mediated by Ab's to VP7 and VP4 (Offit & Clark, 1985; Chiba *et al.*, 1986; Brusow *et al.*, 1988; Hoshino *et al.*, 1988). However, following subsequent rotavirus infection, with the same or another serotype, the predominantly serotype specific response is expanded to include other serotypes (Snodgrass *et al.*, 1984a; Brusow *et al.*, 1988; Brusow *et al.*, 1988a; Brusow *et al.*, 1991), with convalescent sera frequently showing a broad cross-reactivity by neutralization (Holmes, 1983). This may be due to the

fact that at least some rotavirus serotypes (e.g. 1, 3 and 4) possess shared neutralization epitopes on both VP4 and VP7 (Taniguchi *et al.*, 1985; Mackow *et al.*, 1988; Mackow *et al.*, 1988a) and on repeated exposure, may become more important in Ab induction. These observations partially explain cross-protection (see section 4.2) and has important implications for vaccine design, since ideally a vaccine should protect against more than 1 serotype (Edelman *et al.*, 1989).

However, the increase in cross-reactivity of the Ab response, may be affected by the route of inoculation since following repeated parenteral immunization (a procedure which elicits a high titer of serotype specific Ab's), cross-reactive Ab's are frequently not detected (Shaw *et al.*, 1988; Matsui *et al.*, 1989). Thus, the route of inoculation may determine the specificity (*i.e.* VP7 or VP4) of the neutralizing immune response (Shaw *et al.*, 1988).

Heterologous colostral or serum Ab's, capable of neutralizing the infectivity of virus have been shown to protect animals from challenge when added to feed (Snodgrass & Wells, 1978)

4.3.4 FINE SPECIFICITY OF THE ANTIBODY RESPONSE TO ROTAVIRUS PROTEINS

4.3.4.1 VP6 AND ANTIBODY-BINDING DOMAINS

Three antigenic domains involved in group specificity have been identified using a panel of mAb's to VP6 (Pothier *et al.*, 1987) and they have been shown to detect the region between amino acids 48 and 75 (Kohli *et al.*, 1992). Another group specific epitope has also been identified in the first 80 amino acids (Gorziglia *et al.*, 1988). Group determinants are thought to be sequential (Kohli *et al.*, 1992), in contrast to subgroup epitopes which appear to be conformational (Gorziglia *et al.*, 1988).

VP6 does not appear to induce nAb's (Greenberg *et al.*, 1982; Taniguchi *et al.*, 1984).

4.3.4.2 OUTER CAPSID PROTEINS AND NEUTRALIZATION

Rotaviruses have a complex neutralization topography (Greenberg *et al.*, 1983; Taniguchi *et al.*, 1985). Competition inhibition studies and mAb selected, neutralization escape mutant mapping are the two major techniques which have been used to define the Ab binding domains of VP4 and VP7 (Mataui *et al.*, 1989).

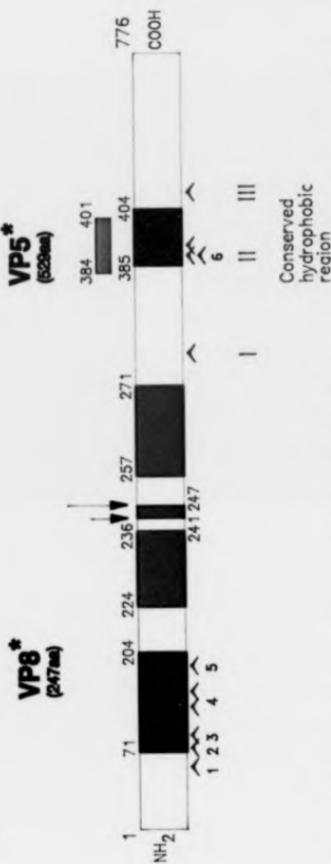
4.3.4.2.1 NEUTRALIZING DOMAINS OF VP4

Several groups have defined neutralization regions of VP4 (Shaw *et al.*, 1986a; Taniguchi *et al.*, 1987a; Burns *et al.*, 1988). The amino acid residues involved in both homologous and heterologous neutralization have been identified (Mackow *et al.*, 1988; Taniguchi *et al.*, 1988). Five type specific (homologous) regions (sites 1-5) have been identified in VP8^{*} (Shaw *et al.*, 1986a; Mackow *et al.*, 1988), the region of greatest sequence diversity in VP4 (Gorziglia *et al.*, 1986) (see Fig. 6). Mutations were observed at amino acid positions 87, 88, 89, 100, 114, 148, 150 and 188.

Several cross-reactive (heterologous) nmAb binding sites have been identified in VP5^{*} (Mackow *et al.*, 1988; Taniguchi *et al.*, 1988). Mackow *et al.*, (1988) identified one site (amino acid positions 388 and 393). Taniguchi *et al.*, (1988b) identified 3 regions; amino acid position 306 (region 1); amino acid position 393 (region 2) and amino acid position 434 (region 3). Some mAb's at region 2 also selected mutations at amino acid position 440, implying that this epitope may be conformationally dependent (Taniguchi *et al.*, 1988) (see Fig. 6), whereas region 1 is a linear epitope. Cross-reactive Ab binding sites have not been identified in VP8^{*} (Mackow *et al.*, 1988; Taniguchi *et al.*, 1988).

None of the neutralization domains correspond to the region around the cleavage site (see section 1.13.2.1). However, peptides from this region have been shown to be immunogenic (Streckert *et al.*, 1988). That neutralization escape mutants can not be isolated in this region, may be because they are lethal (Ijaz *et al.*, 1991).

Fig. 8 NEUTRALIZATION DOMAINS OF VP4 (From Estates & Cohen, 1989).



Symbols: ■, regions of sequence conservation among different virus strains; ▲, region of greatest sequence variation of VP8*; ▨, potential fusion region; ▽, sites of cleavage by trypsin. The peptide possibly removed by trypsin cleavage at aa 241 and 247 is shown (▲). The locations of neutralization domains defined by Mackow et al., 1988 are shown by ▲ and a number from 1 to 6; the epitopes defined by Taniguchi et al., 1988 are shown by ▽ and a Roman numeral (I to III). The numbering shown here for VP4 is based on a protein of 776 aa as determined for SA11 4fM, RRV and Bo/486.

4.3.4.2.2 NEUTRALIZING DOMAINS OF VP7 AND THE SIGNIFICANCE OF GLYCOSYLATION

Studies by Greenberg *et al.*,(1983) and Sonza *et al.*,(1984) showed that the neutralizing domain(s) of VP7 were made up of several distinct epitopes.A large neutralization domain was identified by Sonza *et al.*,(1984) and Shaw *et al.*,(1986a) and non-neutralizing Ab binding sites were also identified.

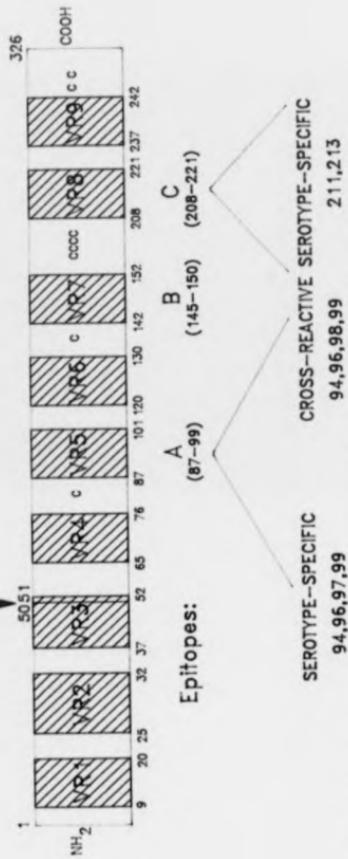
On the basis of nucleic acid sequence comparisons between serotypes,⁹ regions of linear amino acid sequence divergence have been identified in VP7;VR1-VR9 (see Fig.7) (Green *et al.*,1989).Each of these regions is highly conserved within strains of the same serotype (Green *et al.*,1987;Green *et al.*, 1989).Three regions of VP7 have been confirmed to be involved in neutralization.They occur in VR5,VR7 and VR8 and have been designated regions A,B and C,which correspond to amino acids 87-101,142-152 and 208-221,respectively (Dyall-Smith *et al.*,1986;Mackow *et al.*,1988a;Taniguchi *et al.*, 1988a) (see Fig.7).Cross-reactive epitopes are present in region A and type specific epitopes are present in regions A,B and C (Sabara *et al.*,1985;Mackow *et al.*,1988a;Taniguchi *et al.*, 1988a) (see Fig.7).Regions A and C have been proposed to lie in close proximity within the native protein,thus forming a conformational epitope (Dyall-Smith *et al.*,1986).This is supported by the finding that most nmAb's to VP7 do not react with peptides or in Western Blots of the protein (Matsui *et al.*,1989;Estes & Cohen,1989).It has been proposed that the A/C domain is the determinant of viral serotype (Green *et al.*,1988;Matsui *et al.*,1989;Nishikawa *et al.*,1991).

Antigenic 'subtypes' or 'monotypes' of rotavirus serotypes have also been described (Green *et al.*,1992).Amino acid sequence changes within region C significantly affect the neutralizing ability of hyperimmune sera,which has suggested that region C is the immunodominant antigenic site (Dyall-Smith *et al.*,1986).

Glycosylation may affect the immunogenicity of VP7 (Lazdins *et al.*,1985; Dyall-Smith *et al.*,1986;Caust *et al.*, 1987) depending on the position of the

**Fig. 7 / REGIONS OF SEQUENCE VARIATION IN VP7 AND THE NEUTRALIZING
ANTIBODY DOMAINS**

(Compiled using Estes & Cohen, 1989; Capobianco & Chanock, 1990 and Bellamy & Both, 1990).



The figure shows the regions of sequence variation (hatched) and the locations of amino acid changes in escape mutants, selected following reactivity with serotype-specific and cross-reactive neutralizing monoclonal antibodies. The peptide cleavage site is also shown (↓).

carbohydrate moiety (Mackow *et al.*, 1988a; Shaw *et al.*, 1988; Matsui *et al.*, 1989).

4.4 RESUME

These data suggest that the humoral immune response to rotavirus in the intestine is important in protection. In addition, passively acquired Ab's to rotavirus e.g. from colostrum and milk, are important in protection, at least in animals. Knowledge of the nAb epitopes in VP7 and VP4 may assist in the development of a successful sub-unit vaccine for rotavirus.

4.5 CELL-MEDIATED IMMUNITY TO ROTAVIRUS

4.5.1 INTRODUCTION

There is little detailed information on the cell-mediated immune response to rotavirus and the importance of T-lymphocytes in protection against infection (Totterdell *et al.*, 1988a). It is, however, clear that heterotypic protection against disease can not be easily explained solely by the humoral immune response (Vesikari *et al.*, 1986; Offit & Dudzik, 1988). For example, the WC3 strain protects infants and young children against disease caused by serotype 1 in the absence of serotype 1 specific Ab's in the serum (Clark *et al.*, 1988). Similarly, oral immunization of calves with rotavirus can induce protection against heterotypic challenge, even when the rotavirus specific nAb's in the serum and faeces do not correlate with a protective response (Bridger & Oldham, 1987; Woode *et al.*, 1987). A lack of correlation between Ab titers and protection was also reported by Ward *et al.*, (1992).

The majority of work on the cell mediated response to rotavirus has focussed on CTL responses, in animal models. Some work has also been directed at the Natural Killer (NK) and Th-cell responses (see Chapter 12). The following section aims to review the CTL and NK responses.

4.5.2 THE CYTOTOXIC T-CELL RESPONSE TO ROTAVIRUS

The CTL response has been studied in both gut-associated-lymphoid-tissue (GALT) cells and spleen cells, for a range of rotavirus strains.

4.5.2.1 CROSS REACTIVITY OF CTL'S AND REQUIREMENTS FOR ACTIVATION

Offit & Dudzik,(1988) defined an adult murine model system where CTL responses (mediated by CD8⁺ cells) to various heterotypic rotaviruses, were analyzed. They established that CTL's could be generated in the spleen following oral inoculation and the responses were MHC class I restricted. Significantly, rotavirus specific CTL's cross reacted with target cells infected with different human or animal rotavirus serotypes and it was proposed that heterotypic protection against challenge could be mediated by cross-reactive CTL's. Cytotoxic T-cells are clearly important in limiting viral replication and preventing disease in a number of other viral infections (Zinkernagel & Welsh, 1976; Larsen *et al.*, 1983; Kaas *et al.*, 1986).

Both serotype specific CTL's and cross reactive CTL's are present in the spleen following primary oral rotavirus inoculation (Offit & Dudzik, 1988). This finding correlates with other systems (Braciale, 1977). However, some strains of rotavirus preferentially induce cross-reactive CTL's (*e.g.* WC3) whereas others (*e.g.* RRV) primarily induce strain specific CTL's (Offit & Svoboda, 1989). This observation may be partly explained by the virulence of the rotavirus strains which were used, since serotype 3 viruses (*i.e.* RRV) generally grow better in the mouse (Ramig, 1988; Uhnoo *et al.*, 1990) and human intestine (Wright *et al.*, 1987; Clark *et al.*, 1988), than other serotypes.

Other work has shown the requirements for rotavirus specific CTL activation. *De novo* synthesis of rotavirus proteins would not appear to be essential for CTL activation (Offit *et al.*, 1989), as reported previously (Gooding & Edwards, 1980; Barnaba *et al.*, 1990). Cellular protein synthesis was also shown to be non-essential for CTL mediated lysis of rotavirus infected cells and lysis occurred before progeny virus production (Offit *et al.*, 1989). However

trypsin pretreatment of RRV rotavirus, prior to target cell infection was essential for CTL mediated cell lysis (Offit *et al.*, 1989) and oral induction of splenocyte CTL responses required infectious virus (Offit & Dudzik, 1989).

4.5.2.2 CTL ACTIVITY TO ROTAVIRUS IN GALT TISSUE

Inoculation of mice by either oral or i.p. routes with rotavirus can induce rotavirus specific CTL activity in IEL's, PP's and MLN's by 6 days p.i. (Offit & Dudzik, 1989a). This work strongly suggested that gut specific trafficking of lymphocytes occurred, as reported previously (Streeter *et al.*, 1988). By 4 weeks p.i. (oral or i.p. routes) precursor CTL's (pCTL's) for rotavirus could not be detected in the IEL population but were present in other GALT regions (Offit & Dudzik, 1989a) and it was proposed that this was due to CTL's in the gut epithelium being derived from precursors at other GALT sites e.g. PP's.

4.5.2.3 ROLE OF CTL's IN RECOVERY AND PROTECTION

A limited number of studies suggest that CD8⁺ T-lymphocytes are capable of mediating recovery from chronic rotavirus infection, in mice with severe-combined-immuno-deficiency (SCID) (Dharakul *et al.*, 1990) and protection against rotavirus gastroenteritis in suckling mice (Offit & Dudzik, 1990).

Dharakul *et al.*, (1990) reported that adoptive transfer of splenic CD8⁺ T-cells from adult mice inoculated i.p. with the EDIM-w rotavirus strain, mediated complete clearance of virus in rotavirus-chronically infected SCID mice (6-10 weeks old), in the complete absence of rotavirus specific Ab's. It was proposed that CD8⁺ T-cells derived from both the spleen and intestine may have an important role in the amelioration of acute rotavirus infection, in addition to chronic infection and they suggest that CTL's have a role in the elimination of rotavirus infected cells during primary infection. The findings in this study are clearly at variance with those of Eiden *et al.*, (1986a), who propose that recovery from rotavirus infection is independent of T-cell involvement.

Protection of neonatal mice against murine rotavirus (strain JMV) disease by splenic Thy 1⁺ CD8⁺ T-cells (derived from adult mice inoculated i.p. with either JMV or RRV), has been demonstrated (Offit & Dudzik, 1990). Protection occurred in the absence of nAb's in the sera of the suckling mice. Large numbers of lymphocytes (*i.e.* 10^7) were required for protection but this was explained by (i) the frequency of rotavirus specific CTL's in the spleen and (ii) the possible inability of rotavirus specific CTL's to bind sufficiently in the lamina propria or PP's of suckling mice due to low levels of expression of vascular addressins (Offit & Dudzik, 1990).

4.5.2.4 ROTAVIRUS PROTEINS INVOLVED IN CTL ACTIVATION

Attempts have been made to determine the proteins involved in CTL activation (Offit *et al.*, 1991). Utilizing vaccinia recombinants expressing the viral proteins VP7, VP4 and VP6 (see section 1.13), it was established that cross reactive CTL's recognize target cells expressing the outer capsid protein VP7, better than those expressing VP4 or VP6. That an outer capsid protein stimulates cross reactive CTL's more efficiently than conserved 'internal' proteins (*i.e.* nucleo- proteins) is unusual, based on reports in other virus systems (Bangham *et al.*, 1986; Yewdell *et al.*, 1986). However, it has also been reported in the Lymphocytic-Chorio-Meningitis-Virus (LCMV) system (Whitton *et al.*, 1988).

4.6 RESUME

Rotaviruses induce strong CTL responses in GALT and other lymphoid tissue. That certain rotavirus strains can preferentially induce cross-reactive CTL's more efficiently than others and that GALT CTL responses can be induced via i.p. inoculation may be particularly relevant to vaccine design.

Rotavirus specific CTL's are clearly important in both the control and prevention of rotavirus disease, at least in murine models and VP7 may play an important role in CTL mediated immunity. However, it remains to be

determined whether the observations made for CTL responses to rotavirus in animal models are consistent with those in humans.

4.7 THE NATURAL KILLER CELL RESPONSE TO ROTAVIRUS AND THE ROLE OF MACROPHAGES AND OTHER PHAGOCYTIC CELLS IN RECOVERY FROM ROTAVIRUS INFECTION

NK activity and lymphokine activated killer (LAK) cell activity are considered to constitute one of the most important mechanisms of resistance and recovery from various forms of infection (Santoli *et al.*, 1978; Yasukawa & Zarling, 1983; Froelich & Guiffaut, 1987), it is, therefore surprising that very little attention has been given to this arm of the immune response to rotaviruses.

Lymphokines appear to be important in the induction of non-MHC restricted cytotoxicity (Kohl *et al.*, 1983; Yasukawa *et al.*, 1990), therefore suggesting a strong dependency on Th- cells. Cytokine Dependent Cellular Cytotoxicity (CDCC) for SA11 infected cells was detected in the peripheral blood mononuclear (PBMC) population from lactating and non-lactating mothers but not in the colostral leukocyte population (Kohl *et al.*, 1983). Human interferon alpha was suggested to be the mediator of CDCC in this system, as reported for other systems (Santoli *et al.*, 1978).

Yasukawa *et al.*, (1990) found that human PBMC's exhibited enhanced NK and LAK (probably equivalent to the CDCC described by Kohl *et al.*, 1983) cell activity following incubation with rotavirus. They suggested that multiple subsets of lymphocytes become cytotoxic in response to stimulation with rotavirus *in vitro*, in addition to NK and LAK cells. IL-2 and interferon gamma were proposed to assist in the augmentation of the non-specific cytotoxic activity of CD16⁺ and/or NKH1A, NK lymphocytes.

Both macrophages and other phagocytic cell types have been proposed to be important in the clearance of rotavirus infected cells (Eiden *et al.*, 1986a) and adherent cells have been isolated which can lyse virally infected cells (Letvin *et al.*, 1982).

CHAPTER 5

**PREVENTION AND CONTROL OF
ROTAVIRUS INDUCED DIARRHOEA**

5.1 VACCINES

The need for immunoprophylaxis against rotavirus gastroenteritis is widely recognized in view of the contribution of this pathogen to mortality and morbidity in both humans and animals (see section 1.1) (Kapikian *et al.*, 1980). It has been estimated that an efficacious rotavirus vaccine, administered to infants under 6 months of age in Developing Countries would diminish the total number of cases of diarrhoea by more than 50 million episodes and prevent up to 1 million deaths per year (Vesikari, 1989). It would also considerably reduce infant morbidity in Developed Countries (Flores *et al.*, 1987).

Ideally a successful rotavirus vaccine should induce protection against various rotavirus serotypes (McCras & McCorquodale, 1987; Offit & Svoboda, 1989) and in humans it should prevent severe rotavirus gastro-enteritis in the first 2 years of life, when the disease is most severe (Wyatt *et al.*, 1981; Kapikian *et al.*, 1986).

The following section aims to review the approaches which have been used to develop vaccines to rotavirus and highlight the need for a more complete understanding of the immune response, in vaccine design.

5.1.1 CONVENTIONAL APPROACHES

5.1.1.1 PASSIVE PROTECTION

Passively administered Ab to rotavirus has been shown effectively to prevent disease in animal models (Snodgrass & Wells, 1978; Offit & Clark, 1985) and limit the duration and severity of diarrhoea in humans (Barnes *et al.*, 1982; Hilpert *et al.*, 1987). These results along with the ability of colostrum and milk Ab's to prevent or attenuate rotavirus illness, at least in animals (see section 4.3.1.1) have suggested that vaccination of the dam (with whole virus), before parturition may be a useful approach for the protection of neonatal animals (Woode *et al.*, 1975; Snodgrass & Wells, 1978). Vaccines based on this strategy are now used in the U.K. (Snodgrass, 1986; McNulty & Logan, 1987).

Since the role of human colostrum and milk Ab's in protection is unclear (see section 4.3.1.3) and breast feeding does not occur widely in all populations, this approach is limited to the control of animal rotavirus disease (Mackow *et al.*, 1989). Also, passive protection requires repeated immunization and this makes it somewhat impractical and expensive for human protection (Kapikian & Chanock, 1990). Thus, active immunization is the favoured route of immuno-prophylaxis in the human population.

5.1.1.2 ACTIVE PROTECTION

The induction of active immunity in the intestine of the potential host, via the inoculation of homotypic or heterotypic strains has been recognized (see section 4.2). In general, 'live' oral vaccines have been given greater attention than 'killed' vaccines since they are believed to be both more effective at stimulating local mucosal immunity (Offit & Dudzik, 1989) and to induce immunity for a longer period (Vesikari, 1985; Edelman, 1987).

5.1.1.2.1 USE OF ANIMAL STRAINS

Efforts to develop attenuated strains of human rotaviruses for vaccines have been hampered by their poor cultivability and the difficulty in determining the pathogenic potential of different strains (Kapikian *et al.*, 1986). As a result heterologous, antigenically cross-reactive rotavirus strains derived from animal sources have been developed for vaccine use in humans. There is evidence of heterotypic immunity in animal models (see section 4.2). Four candidate vaccines derived from animal strains have progressed to field trials (see Table 6). Trials with these vaccines revealed that they could confer protection but that the protection was inconsistent (Levine, 1986; Lanata *et al.*, 1989; Santosham *et al.*, 1991), when compared with the initial vaccine trials carried out in Developed Countries (Vesikari *et al.*, 1984; Vesikari *et al.*, 1985; Edelman, 1987). Another problem was the finding that the RRV vaccine induced fever and watery stools in a high proportion of volunteers (Vesikari *et al.*, 1986).

TABLE 6 THE MAJOR MONOVALENT VACCINES TO ROTAVIRUS
 (From Edelman et al., 1989).

CANDIDATE VACCINE	INVESTIGATOR	LOCATION	EFFICIENCY AGAINST DISEASE	REFERENCE
RIT 4237 (bovine, serotype 6).	Vaisakari Vaisakari Vaisakari De Mol Hanlon Santuria Santosham Lanata Yoken	Finland Finland Finland Rwanda Gambia London, Eng. Arizona, USA Peru Baltimore, USA	++ ++ 0 0 0 0 + RP	1984 1985 1987 1986 1987 1987 P P
RIT 4256 (bovine, serotype 6).	Vaisakari	Finland	SA	1987a
WC-3 (bovine, serotype 6).	Clark Albert	Philadelphia Israel	++ RP	1986, 1987a P
RRV-1 (Rhesus monkey, serotype 3).	Flores Vaisakari Gothe fors Dolin Santosham	Venezuela Finland Sweden Rochester, USA Arizona, USA	++ ++ + 0 0	1987 P P P P

EFFICIENCY KEY:

0,no protection; +,against severe diarrhoea only; ++,against all diarrhoea;
 RP,results pending; SA,safety and antigenicity trials only; P,personal communication.

The inability of the vaccine strains consistently to protect against disease has been attributed to a number of factors (see section 5.1.3). Of great importance in these studies was the finding that most of the protection conferred by animal rotavirus strains in humans, is homotypic (Flores *et al.*, 1987; Kapikian & Chanock, 1990). Therefore, if a heterotypic immune response is present then it is not protective.

The results presented strongly suggest that vaccines need to be serotype specific (Edelman *et al.*, 1989; Loosnky *et al.*, 1988) and some evidence exists to suggest that protection against rotavirus disease may be conferred by the administration of vaccines early in life (Bishop *et al.*, 1983).

5.1.1.2.2 MULTISEROTYPE VACCINES

The data discussed above suggests that if vaccines are to work consistently in all clinical settings, multivalent preparations containing several or all of the prevalent rotavirus serotypes will be necessary (Edelman *et al.*, 1989; Estes & Cohen, 1989).

5.1.1.2.2.1 REASSORTANT ROTAVIRUS VACCINES

One possible solution to this problem has been the development of reassortant rotaviruses. Ideally the gene encoding the serotype determinant (*i.e.* VP7) of an animal strain is replaced with the serotypic determinant of a human rotavirus serotype, via co-culture (Edelman *et al.*, 1989). Thus, it has been proposed that several reassortants containing different serotype determinants could be pooled in a single preparation to induce a broader protection (Edelman *et al.*, 1989; Estes & Cohen, 1989). Reassortants have been prepared for human serotypes 1, 2, 3 and 4 with RRV, UKtc and WC3 (Midtun *et al.*, 1986). Other donor strains such as naturally attenuated 'nursery' strains (see below) (Bishop *et al.*, 1983; Flores *et al.*, 1986a) are also being considered as important donors. Reassortants containing gene 4 could also be developed (see section 4.3.2) and reassortants containing VP4 and VP7 from 2 antigenically distinct rotaviruses have also been proposed (Offit *et al.*, 1986; Kapikian *et al.*, 1986).

Trials assessing the safety, antigenicity and efficacy of VP7 reassortants are being conducted.

5.1.1.2.3 ATTENUATED HUMAN ROTAVIRUS VACCINES

Naturally attenuated 'nursery' strains isolated from asymptomatic neonates (Bishop *et al.*, 1983; Gorziglia *et al.*, 1986) for each of the human serotypes have also been considered for use as vaccines either individually or as a multivalent vaccine, since neonates who experienced a subclinical rotavirus infection in a nursery during the first 14 days of life were protected against clinically significant rotavirus diarrhoea for up to 3 years (Bishop *et al.*, 1983). However, the main disadvantage of attenuation is that in many instances this correlates with a decreased ability to replicate (particularly at mucosal sites [Chanock *et al.*, 1978]) and this may result in a smaller immune response (Kapikian & Chanock, 1990). Therefore, a balance between attenuation and immunogenicity has to be achieved.

5.1.2 MODERN APPROACHES

5.1.2.1 RECOMBINANT SUB-UNIT VACCINES

The main advantage of sub-unit vaccines is the specificity of the response (McCrae & McCorquodale, 1987). Recently, attention has focussed on the expression of rotavirus proteins in prokaryotic and eukaryotic cell culture systems. RRV VP4 has been successfully expressed in a baculovirus system (Mackow *et al.*, 1989; Mackow *et al.*, 1990) and was shown to passively protect suckling mice from challenge with RRV and the murine rotavirus Eb, when inoculated into the dams (Mackow *et al.*, 1990). However, fusion proteins of VP4 have not been shown to be as effective (Arias *et al.*, 1987).

Fusion proteins of VP7 have also been generated (Arias *et al.*, 1986; McCrae & McCorquodale, 1987; Francavilla *et al.*, 1987), with varying levels of success in the induction of nAbs. For example, no nAb activity was detected by Francavilla *et al.*, (1987) whereas the N-terminal region of UKtc, when fused to beta-galactosidase of *Escherichia coli* induced a high level of nAb (McCrae &

McCorquodale, 1987). The reasons for this variation are not well understood. However, problems of correct conformation may be partly responsible (Mackow *et al.*, 1989) and the involvement of Th-cells in the induction of nAb to rotavirus proteins is not understood. Andrew *et al.*, (1987) expressed VP7 in vaccinia virus constructs and found that only low levels of nAb were induced.

Despite the somewhat disappointing results from this approach, it is proposed that recombinant VP7 may be useful in priming an immune response if administered before a live attenuated virus vaccine, or they may serve to boost an immune response if given after a live virus vaccine (Kapikian & Chanock, 1990). It is also proposed that combinations of recombinant proteins will be required to achieve the correct conformation and consequently the best immunogenicity (Estes & Cohen, 1989).

5.1.2.2 PEPTIDE VACCINES

The Ab response to peptide fragments of rotavirus proteins has been investigated. Peptides from the cleavage region of VP4 induced nAb's (Streckert *et al.*, 1988), whereas peptides of VP7 did not induce nAb's (Gunn *et al.*, 1985) and mAb's to VP7 did not bind to them (Streckert *et al.*, 1986; Taniguchi *et al.*, 1988a). It has been suggested that the conformation of the peptides is such that they do not mimic the nAb epitopes present on the native protein (Matsui *et al.*, 1989). In addition, these peptides may not stimulate Th-cells effectively. Borras-Cuesta *et al.*, (1987) showed quite clearly that the addition of Th epitopes to rotavirus peptides greatly improved the Ab titer obtained. It remains to be determined if free rotavirus peptides are capable of inducing effective CTL responses.

Recently, heterotypic passive protection in neonatal mice was induced by immunizing dams with VP4/VP7 peptide-VP6 conjugates (Ijaz *et al.*, 1991). They proposed that peptide conjugates such as these may serve as useful homotypic/ heterotypic vaccines when peptides from the highly conserved regions of either VP7 or VP4 are coupled to the rotavirus VP6 carrier.

However, there are certain fundamental questions on the efficiency of sub-unit and peptide vaccines which have not been fully addressed, to date. For example (i) is it possible to induce active protective mucosal immunity by oral vaccination with a non-replicating antigen? (ii) what is the duration of the immune response? (iii) is it possible to protect against persistent infections with these vaccines?

5.1.3 PROBLEMS IN ROTAVIRUS VACCINOLOGY

There are several fundamental problems which have to be resolved before an efficient rotavirus vaccine can be produced. Some of the major elements are shown in Table 7. There are also various intrinsic problems associated with rotavirus vaccines. They include (i) failure to swallow the vaccine, (ii) the presence of Ab from previous rotavirus infection, (iii) age related changes in cell permissiveness (which may affect the optimal age of administration), (iv) bowel transit time and (v) the route of administration, which induces the most efficient immune response (Edelman *et al.*, 1989). A rotavirus vaccine may also affect the virulence of bacterial gastrointestinal infections (Edelman *et al.*, 1988) which may have important implications in the Developing World, where bacterial gastroenteritis is also common.

A further complication in rotavirus vaccinology is the finding that the pathogenesis and epidemiology of rotavirus strains is different between and within countries (Edelman *et al.*, 1989). Another problem, is the need for alternative vaccines for the non-group A viruses, due to the lack of antigenic similarity (see section 1.14.1) (Bartlett *et al.*, 1987).

Finally any rotavirus vaccine must be cost-effective, in view of the economic situation in many Developing Countries (Kapikian *et al.*, 1986).

5.1.4 RESUME

Rotavirus vaccinology is complex (Edelman *et al.*, 1989) and there is an urgent need to define the immune response to this pathogen at a more detailed level. For example, cross-reactive epitopes in the CMI response need to be more

TABLE 7 SPECIFIC DIFFICULTIES ASSOCIATED WITH ROTAVIRUS VACCINES AND SOME POSSIBLE SOLUTIONS.

(Adapted from Edelman et al., 1989).

VACCINE ASSOCIATED PROBLEM	SOLUTIONS/IMPLICATIONS
Interference from enteroviruses e.g.Polio. Major problem in Developing World where intense transmission of enteric pathogens occurs (Mata et al., 1983).	Determine if enteroviruses inhibit the take of rotavirus vaccine or vice versa,which is of significance to polio vaccination.
Secondary transmission of vaccine virus via faecal-oral route.Will this occur?	If this does not occur then more frequent boosting is required.
Susceptibility to acid in stomach. Rotavirus is susceptible to acid (Yerikari et al., 1984g).	Effect of different gastric acid buffers on potency of rotavirus vaccine should be determined.Breast feeding may enhance vaccine potency due to buffering or inhibit the vaccine due to anti-rotavirus antibody or non-specific inhibitors.Need to establish to what extent this is true.
Optimal vaccine dose.Is a one dose vaccine a reality?	Unlikely due to rapid waning of intestinal fluid antibody (Kapikian et al., 1983).Therefore,more than one vaccine dose probably required.
Determination of the severity of clinical illness,such that efficiency of vaccine can be assessed.	Produce a clinical grading system.

thoroughly defined and a knowledge of the interaction of the humoral and CMI responses is fundamental to the design of efficient vaccines. In addition, methods of efficiently stimulating GALT immunity need to be investigated for both arms of the immune response. The proteins responsible for initiating the efficient uptake of rotaviral antigen by Peyer's Patches also need to be addressed. The role of the CMI response in protection needs to be more thoroughly investigated.

However, it is apparent from this review that great progress has already been made (particularly in animal rotavirus vaccines). Recombinant VP4 is promising as a vaccine and the VP7/VP4 peptide-VP6 conjugate is particularly encouraging, suggesting that rotavirus disease can be prevented. There may, therefore be an important role for sub-unit vaccines against rotavirus disease, in both animals and man.

5.2 ANTIROTAVIRUS AGENTS

Several agents have been studied, as a means of controlling rotavirus infection. Nucleotide analogues are effective at inhibiting rotavirus replication and this is proposed to be due to the inhibition of S-adenosylhomocysteine-hydrolase, required for the methylation of mRNA (Kitao *et al.*, 1986a). Ribavirin and 3-deazaguanine are also effective and prevent rotavirus protein synthesis, possibly via the inhibition of RNA synthesis or capping of viral RNA and 9-(S)-(2,3-Dihydroxypropyl) adenine is a good inhibitor of replication, with low toxicity (Smeé *et al.*, 1982). Clioquinol (an 8-hydroxy guanine derivative) was found to reduce infection in mice, if given at frequent intervals (Bednarz-Prashad & John, 1983). It is proposed that it may remove the virion outer-capsid.

However, the main limitations of antirotaviral therapy are the specificity of the agent, toxic side effects and expense.

5.3 ORAL REHYDRATION THERAPY

Intravenous fluid administration has been used successfully for many years in treating dehydration from diarrhoea (Kapikian & Chanock, 1990). However, due to the difficulties involved in this treatment (particularly in Developing Countries) efforts were made to assess the efficiency of oral fluid replacement therapy (Sack, 1982; Santoaham *et al.*, 1985). Oral rehydration therapy for rotavirus gastroenteritis was successful using electrolyte solutions employing either glucose or sucrose (Sack *et al.*, 1978; Nalin *et al.*, 1978).

The main advantage of this method of control is that it is theoretically relatively inexpensive and effective in the short term. However, the expectations of this simple treatment have not yet been matched in reality, due to various socio-economic factors.

CHAPTER 6

MATERIALS & METHODS

6.1 MATERIALS

6.1.1 MEDIA

RPMI 1640 medium, buffered with sodium bicarbonate (NaHCO_3) or HEPES was obtained from Sigma in 500ml aliquots and stored at 4°C .

Normal BALB/c mouse serum (M.S.) was obtained from Serotech. Ltd. and frozen at -70°C in 2ml aliquots. Foetal calf serum (FCS) was obtained from Gibco Ltd.. Prior to use, sera were thawed and heat treated for 30min. at 56°C . Batches of serum were routinely screened for the presence of anti-rotavirus Ab's by ELISA (see Chapter 7) and only rotavirus,Ab negative serum was used in these studies.

RPMI 1640 (buffered with NaHCO_3) was supplemented with 1% BALB/c mouse serum,glutamine (2mM) (Flow Laboratories Ltd.),beta-mercaptoethanol ($5 \times 10^{-5}\text{M}$) (BDH),Penicillin (100U/ml) and streptomycin (100ug/ml) (Glaxo Laboratories Ltd.) (referred to as RPMI 1640 (1% BALB/c M.S.)). Medium was also prepared (as above) with the exception that it was supplemented with either 5% FCS (RPMI 1640 (5% FCS)) or 10% FCS (RPMI 1640 (10% FCS)),rather than mouse serum.RPMI 1640 (buffered with HEPES) was supplemented with either 5% FCS or 10% FCS,in addition to the other supplements detailed above, referred to as RPMI 1640 (5% FCS) (HEPES) and RPMI 1640 (10% FCS) (HEPES),respectively.

Glasgow Modified Eagle's Medium (GMEM) was prepared by Mrs B.Wood (University of Warwick). Routinely,1L aliquots were supplemented with 5% FCS,glutamine (4mM),Penicillin (100U/ml) and streptomycin (100ug/ml) (referred to as GMEM (5% FCS)) and stored at 4°C .

6.1.2 MICE

Female BALB/c ($H-2^d$) mice,7-10 weeks old were obtained from breeding colonies within the Department of Biological Sciences,University of Warwick (origin:Banting & Kingman Labs Ltd.,Aldbrough,Hull) and housed in negative pressure isolation units (Olac,Bicester,Oxford).Mice had free access to pelleted food and water (sterile) and the animal room had a controlled temperature.

Mice were routinely screened for the presence of rotavirus Ab's by ELISA and Plaque Reduction Neutralization Assay (PRN) (See Chapter 7). Only rotavirus seronegative mice were used in these studies. Within a given experiment all mice were matched in terms of age and sex and group size was never less than 3 animals.

6.1.3 RADIOCHEMICALS

Methyl-[³H]thymidine (2.5Ci/mmol) and Methyl-[³H]uridine (2.5Ci/ mmol) were obtained from Amersham International Plc.

6.1.4 OTHER MATERIALS

Anti-BRV(UKtc) Bovine Hyperimmune Antiserum (Dr J.Bridger, Dept. Veterinary Pathology, Royal Veterinary College, University of London)
Glass Fibre Paper (Whatman)
Sucrose gradient purified Influenza Virus (A/CHR/68 H3N2)
(Dr.M.Outlaw, University of Warwick)

6.2 METHODS

6.2.1 CULTURE AND PASSAGE OF AFRICAN, GREEN MONKEY KIDNEY CELLS (BSC-1)

These cells were grown in GMEM (5% FCS) in 800cm² glass bottles and maintained at 37°C on rotating roller apparatus (Modular Cell Production, model III). When confluent, the cells were removed from the glass with a trypsin/versene mix (1:6), disaggregated and reseeded into sterile 800cm² glass bottles, routinely at a 1:3 ratio.

6.2.2 PROPAGATION OF ROTAVIRUS AND PREPARATION OF SAMPLES FOR USE IN *IN VITRO* PROLIFERATION ASSAYS

Plaque purified stocks of the bovine, Compton UKtc strain (originally obtained from M.Thouless) or the porcine OSU strain (originally obtained from L.Saif, Ohio State University) of rotavirus were routinely prepared using the method

described by McCrae,(1985) with the following modification:virus bands were concentrated by centrifugation at 50000g for 120 min. at 4⁰C, using Phosphate buffered saline (PBS) (pH7.4) as the diluent and not 50mM Tris-HCl pH8.0. The virus pellet was resuspended in a small volume of ice cold PBS (~2ml) and dialysed overnight against PBS (at 4⁰C),prior to determining the virus concentration.Both ds and ss virus particles were obtained by this method (see Fig.8 and section 1.8)

Measurment of virus yield was carried out according to the method described by Offit *et al.*,(1983);McCrae,(1985) and based on the optical density of the purified virus sample.Purified virus was stored at -70⁰C.

6.2.3 PREPARATION OF SINGLE-SHELLED ROTAVIRUS PARTICLES

Single-shelled rotavirus preparations were treated with Ethyl- eneglycol bis (beta-aminoethyl ether)-N,N'-tetracetic acid (EGTA) (5mM) for 30min. at 37⁰C,to ensure complete conversion to ss particles.Following treatment,the particles were dialysed overnight against PBS at 4⁰C to remove residual EGTA.

6.2.4 PREPARATION OF BSC-1 CELL EXTRACT AND DETERMINATION OF PROTEIN CONCENTRATION

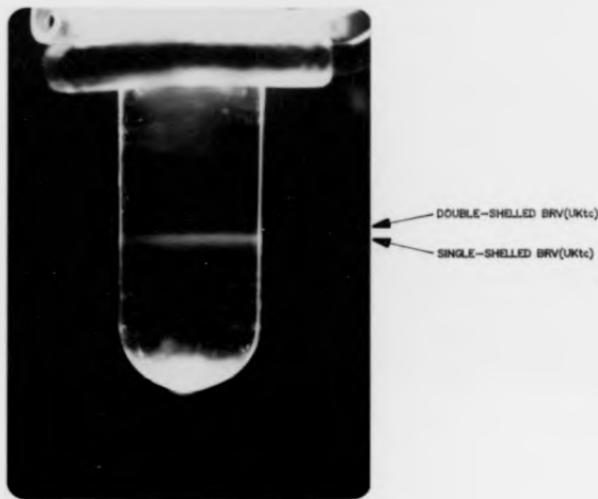
BSC-1 antigen,for use in control experiments was prepared exactly as for purified virus with the exceptions that (i)uninfected cells were used and (ii)that the concentrated Arcton aqueous extract was not purified on CsCl gradients and was resuspended in ice cold PBS.The protein concentration of this preparation was determined using the Biorad protein assay kit (BIO-RAD Laboratories,GmbH,Munich) and stored at -70⁰C.

6.2.5 PLAQUE ASSAY OF BRV(UKtc) VIRUS

The method used was that described by McCrae & Faulkner-Valle,(1981) and Offit *et al.*,(1983) with several modifications.First,cells were overlayed with

Fig.8

**CAESIUM CHLORIDE DENSITY EQUILIBRIUM BANDING
OF BRV(UK)c)**



Caesium chloride gradients were prepared as described in McCrae, 1985 and the crude virus preparation carefully layered on top. The gradients were centrifuged for 2hr at 24000 r.p.m., at 4°C in a Beckman SW28 rotor.

medium containing FCS (2%) and second, plates were incubated for 6 days at 37°C in 5% CO₂ and then fixed with formal saline (30% formaldehyde;70% PBS) overnight,followed by staining with crystal violet (0.1% in 20% ethanol).

6.2.6 BETA-PROPIO-LACTONE INACTIVATION OF BRV(UKtc)

This was carried out according to the method of Barrett *et al.*,(1984).Briefly, stocks of BRV(UKtc) were inactivated by the addition of beta-propio-lactone (grade II) (Sigma Chemical Co. Ltd,Poole,Dorset) to a final concentration of 0.01% (v/v).The mixture was incubated at 4°C overnight and then dialysed for 8hr against PBS at 4°C.Beta-propio-lactone treatment routinely caused a reduction in the titer of a stock from 10⁷/10⁸ Pfu/ml to <5 Pfu/ml.

6.2.7 MOUSE INOCULATION

Mice were usually inoculated orally with unpurified,non-trypsin treated virus of known titer (routinely 10⁷ Pfu) by proximal oesophageal intubation through a 2 inch 18 gauge metal gavage (IMS,Cheshire),following light anaesthesia with Metophane (C-Vet).

Alternatively,mice were anaesthetised,layered on their ventral side and swabbed with ethanol (70%),along the abdomen.They were then inoculated by the i.p. route,with a preparation of unpurified virus of known titer,using a 1ml hypodermic syringe and a 26^{1/2} gauge needle.

6.2.8 PREPARATION OF MOUSE INTESTINE AND FAECES FOR PLAQUE ASSAY

Mice were sacrificed by cervical dislocation.The intestine (including the ileum,caecum and large intestine) was removed and placed in ice cold PBS (3ml) and then frozen at -70°C. Faeces from the inoculated mice were also collected for the duration of the experiment and stored at -70°C.

The intestine samples were freeze/thawed five times.They were then placed in separate,sterile Dounce Homogenizers (20ml) (Jencons Ltd.),on ice and homogenized (50 strokes).The homogenates were placed in sterile universals

and sonicated for 20sec. in a Soni Bath (Townson & Mercer),followed by centrifugation at 600g for 10min.,at 4°C.The supernatants from each sample were removed and maintained at 4°C.To each supernatant sample was added Nystatin (250U/ml,final) (Sigma),Kanamycin (200ug/ml,final) (Sigma) and Ampicillin (1000ug/ml, final) (Sigma).The final volume of 4ml was made up with ice cold PBS.Serial 10 fold dilutions of each sample were then prepared in ice cold PBS and plaque assays were set up in the usual way (see above), except that Nystatin was added to the overlay medium (250U/ml,final).

A 10% suspension of each faecal sample (for each mouse) was prepared in ice cold PBS containing Nystatin (250U/ml, final), Kanamycin (200ug/ml,final) and Ampicillin (1000ug/ ml,final) and homogenized as before.Sonication of the samples,centrifugation and the plaque assays were carried out as before.

6.2.9 ISOLATION OF MESENTERIC LYMPH NODES AND SPLEENS

Mice were sacrificed by cervical dislocation.The animal was laid ventral side uppermost,the abdomen swabbed with 70% ethanol and the skin on the abdomen,removed.The region was again swabbed with 70% ethanol.An incision was made in the peritoneal membrane half way along the middle of the body cavity and the membrane carefully removed to reveal the intestine. The small and large intestines were pulled away from the body cavity and the mesenteric lymph nodes were clearly visible,embedded in large amounts of fat attached to the intestine.They were carefully teased away and placed in ice cold RPMI 1640 (10% FCS) (HEPES).

On the right hand side of the abdomen the large dark spleen was also visible and was carefully teased from its attachments to the alimentary canal.This was placed in RPMI 1640 (1% BALB/c M.S.),at room temperature.

All these procedures were performed in a biological,laminar flow cabinet (Envair UK Ltd) and strict aseptic technique was followed throughout.All dissecting equipment was heat sterilized prior to use and the dissection area was swabbed with 70% ethanol.

6.2.10 PREPARATION OF SINGLE-CELL SUSPENSIONS

6.2.10.1 SPLEEN

The spleens were washed once in RPMI 1640 (1% BALB/c M.S.) and then placed onto a sterile,200 mesh stainless steel gauze,in a sterile Petri dish.To the gauze was added 2.5ml of either RPMI 1640 (1% BALB/c M.S.) or RPMI 1640 (10 % FCS). Using the plunger from a 10ml plastic syringe,the end of one spleen was broken open and the contents teased out by gentle pressure from the plunger.This was repeated for the remaining spleens and the single-cell suspension was spun at 600g for 4 min. at room temperature.The pellet was resuspended in medium.To lyse the red blood cells an equal volume of sterile ammonium chloride (NH_4Cl) (8.3g/L) (pH 7.0) was added to the resuspended cells.Following 2min. of gentle agitation,the cells were spun (as above) and the pellet resuspended in fresh medium.This step was repeated twice and the final pellet resuspended in 2ml of medium.

6.2.10.2 MESENTERIC LYMPH NODES

The MLN's were washed at least five times in ice cold RPMI 1640 (10% FCS) (HEPES).The lymph nodes were placed onto a sterile,200 mesh stainless steel gauze (bathed in 2.5ml of ice cold RPMI 1640 (10% FCS) (HEPES)) and using a sterilized scalpel were cut open by several strokes.Using the plunger from a 10ml plastic syringe,the lymph node cells were carefully disaggregated and pushed through the mesh.The single-cell suspension was washed twice in medium (i.e.spun at 600g for 4min. at 4°C) and maintained on ice until required.

6.2.11 CELL COUNTING

An aliquot of diluted single-cell suspension (usually 10⁴ul) was mixed with an equal volume of Trypan blue (0.5%).This mix was added to a Neubauer Counting Chamber (Weber,England) and the number of viable cells determined.Cell viability,as assessed by Trypan blue exclusion,was routinely

greater than 95%.Lymphocyte cell suspensions (derived from the spleen and MLN) were adjusted to 8×10^6 cells/ml,with culture medium.

6.2.12 LYMPHOCYTE PROLIFERATION ASSAY

6.2.12.1 SPLEEN

The following method describes the final form of the assay *i.e.* when optimized.See Chapter 9 for the derivation of the assay.

Single-cell lymphocyte suspensions were prepared from inoculated mice or uninoculated controls,as described above.The cells were cultured in 96 well round bottom microtiter plates (Flow Labe Ltd.) (4×10^5 cells/well) with one of the following: BRV(UKtc),OSU,influenza,BSC-1 cell extract,Con.A (Sigma) or medium in 0.1ml of RPMI 1640 (1% BALB/c M.S.) or RPMI 1640 (10% FCS) in replicate wells (not less than 6).

Cells cultured in RPMI 1640 (1% BALB/c M.S.) were cultured for 3 days at 37°C.To each well was then added 0.1ml of RPMI 1640 (5% FCS).The cells in each well were suspended by repeated pipetting and 0.1ml was removed.The remaining cells were pulse labelled for 18hr with thymidine by the addition of 1.25uCi of [³H]thymidine (in 0.02ml of PBS),to each well. Culture was terminated by automatic cell harvest (MASH 2 cell harvester) and the [³H]thymidine incorporation determined by beta scintillation counting. Briefly,the cellular DNA incorporating [³H]thymidine in each well was retained on a glass fibre disc,which was dried under a hot lamp.Each disc was placed in a scintillation vial and 3ml of scintillation fluid was added.The level of [³H]thymidine incorporation was determined using an LKB Rackbeta scintillation counter.The mean count per minute (CPM) for each experimental group was then determined,prior to statistical analysis by the Student's t-test.

Cells cultured in RPMI 1640 (10% FCS) were incubated for 2 days at 37°C and then pulsed with [³H]thymidine for 18hr, prior to harvesting and counting.

6.2.12.2 MLN CELLS

Single-cell suspensions were prepared (as above) and the cells were cultured with the same antigens/mitogen listed above. After 3 days of incubation in RPMI 1640 (10% FCS) (HEPES) the cells were pulsed with [³H]thymidine for 18hr and the incorporation was measured.

6.2.13 ANTIBODY AND COMPLEMENT MEDIATED DEPLETION OF RESPONDING CELLS IN THE SPLENOCYTE PROLIFERATION ASSAY

Anti-Thy1.2 Ab (Sigma) was used at a dilution of 1:10 (diluted in (RPMI 1640 (5% FCS) (HEPES)). Rat antimouse-CD4 and rat antimouse-CD8 Ab's were kindly provided by Professor H. Waldmann (Cambridge) and used at a dilution of 1:100 (diluted in RPMI 1640 (5% FCS) (HEPES)).

For depletion studies, *in vitro* single-shelled BRV(UKtc) stimulated splenocyte populations (5×10^6 cells) were washed in RPMI 1640 (5% FCS) (HEPES) (see above), resuspended in 1ml of the appropriate Ab and incubated for 30min. at 4°C, with gentle agitation at 10min. intervals. The cells were then washed as before and incubated for 45min. at 37°C in 1ml of rabbit complement (Low-Tox M; Cedarlane Laboratories) (diluted 1:10 in wash medium). Controls consisted of untreated cells and cells treated with complement only. Following two further washes, the cells from each treatment were finally resuspended in an equal volume of RPMI 1640 (10% FCS) (HEPES), aliquots of 0.1ml were dispensed into 12 wells of a 96 well round bottom plate and 1.25u Ci of [³H]thymidine was added to each well. After 18hr of incubation, at 37°C, the cells were harvested and the [³H]thymidine incorporation was measured, as above.

Treatment of uninoculated mouse, splenocyte populations with these levels of Ab and rabbit complement, followed by indirect fluorescence microscopy, (using goat anti-rat and anti-mouse FITC conjugated Ab (Sigma)), revealed that in each case <6.5% of the corresponding cell population remained.

6.2.14 STIMULATION INDEX (SI)

This value enables the stimulatory activity of the antigen or mitogen, for different experiments to be compared, irrespective of the background proliferation. It is calculated as follows:

$$SI = \frac{(\text{Mean } [^3\text{H}] \text{thymidine incorporation for antigen or mitogen stimulated cells})}{(\text{Mean } [^3\text{H}] \text{thymidine incorporation for cells cultured in the absence of antigen or mitogen})}$$

SI values greater than or equal to 2.0 were considered to be significant. When a significant response was detected, these values are shown above the Standard Deviation (S.D.) bars in proliferation assay graphs.

6.2.15 STATISTICAL TESTS

The S.D. of the experimental groups was determined and the Student's t-test was performed on proliferation assay data. For the method of calculation see Parker, (1979).

6.2.16 ISOLATION OF PERIPHERAL BLOOD AND PREPARATION OF SERUM FOR PLAQUE REDUCTION NEUTRALIZATION (PRN) ASSAYS

Animals were sacrificed by cervical dislocation and the blood collected by cardiac puncture. It was maintained at room temperature for ~30min., to allow clotting to occur, the clot was then 'ringed' using a sealed Pasteur pipette and the sample placed at 4°C, overnight. Following centrifugation, the supernatant serum was aspirated from the fibrin clot and placed at -70°C.

For PRN assay, the serum sample was thawed rapidly at 37°C and then incubated at 56°C for 30min., to inactivate complement.

6.2.17 PRN ASSAY

The PRN assay used, was a modification of the technique described by Offit *et al.*, (1983). A solution of BRV(UKtc) containing 800 Pfu/ml (0.1ml) was mixed

with an equal volume of a serial 10 fold dilution of serum, or PBS (positive control). The serum-virus mixture or control was incubated at 37°C for 30min., followed by overnight incubation at 4°C. Each sample was then added to a confluent monolayer of BSC-1 cells (in duplicate) and incubated for 1hr at 37°C. The cells were overlayed, incubated and fixed as for a standard plaque assay (see above).

The neutralizing titer of the serum sample was expressed as the reciprocal of the dilution at which a 50% reduction in the mean plaque count was obtained. The titer was calculated by interpolation using the method of Reed and Muench.

6.2.18 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Standard Competitive and Indirect ELISA's (see Wreggitt & Morgan-Capner, 1990) were set up at the World Health Organization Laboratory, East Birmingham Hospital. The basis of each assay is described below.

6.2.18.1 PREPARATION OF RABBIT ANTIROTAVIRUS HYPERIMMUNE SERA, 'CAPTURE' PLATES

Polystyrene microtiter plates (96 well) (Falcon 3912) were coated with a mixture of rabbit antirotavirus hyperimmune sera as described in Beards *et al.*, (1984) and Beards, (1987).

6.2.18.2 COMPETITIVE ELISA

This was carried out according to the method of Beards & Desselberger, (1989), with several variations. The wells of the microtiter plates were emptied and 100ul of a concentrated preparation of single-shelled SA11 rotavirus particles was added to each well. The plates were incubated at 37°C for 1hr and then washed (6x) in 0.05M Tris-HCl buffered saline, pH7.2, containing 0.01% Tween 20 and 1% Bovine Serum Albumin (referred to as TBST/BSA). Serial doubling dilutions of the serum samples or mAb to VP6 (prepared as described in Beards *et al.*, 1984) were then made, down the plate (*i.e.* 8 dilutions

per sample).The negative control consisted of TBST/BSA diluent (100ul) but no serum.

Aliquots of 25ul were then removed from each well and replaced with 25ul of a 1:1000 dilution of rabbit anti-rotavirus hyperimmune,horseradish peroxidase coupled conjugate (prepared as described in Beards *et al.*,1984) and the plates were incubated for 45min. at room temperature.

After washing the plate (6x) with TBST/BSA,Citrate Phosphate Buffer (pH6.5),containing H₂O₂ (0.04%) and tetra- methyl Benzidine (TMD),(100ul), was added to each well.The plates were incubated for 3-5min.,when the reaction was terminated by the addition of 8M H₂SO₄ (50ul).The optical densities of the reaction mixes were measured using a Flow Multiscan Spectrophotometer.The degree of inhibition was expressed as a % and calculated as follows:

$$\% \text{ Inhibition} = \frac{\text{(Optical Absorbance in the Absence of serum)} - \text{(Optical Absorbance of the Test Serum)}}{\text{(Optical Absorbance in the Absence of serum)} - \text{(Optical Absorbance of the Positive Control serum)}} \times 100$$

Any sample giving a >50% inhibition was recorded as positive for antirotavirus VP6 Ab's (Beards & Desselberger,1989)

6.2.18.3 INDIRECT ELISA

This was carried out according to the method of Beards *et al.*,(1984) and Beards,(1987),with some variations.Capture plates were prepared as above and to each well was added a concentrated preparation of single-shelled SA11 and the plates were incubated for 1hr at 37°C.They were then washed six times in TBST/BSA to remove unbound virus and Ab.

Following removal of all excess wash buffer,an initial 1:50 dilution of the test serum or anti-VP6 mAb control (as above) was made in TBST/BSA and serial five fold dilutions were made across the plate.No serum was added to row A and this served as the negative control (*i.e.*TBST/BSA alone).The plates

were incubated for 2hr at 37°C and then washed six times in TBST/BSA. Goat anti-mouse,polyvalent IgG peroxidase conjugate (diluted 1:300 in TBST/ BSA) (100ul) (Sigma) was then added to each well and the platen were incubated at 37°C for 1hr.Following six washes with TBST/BSA the substrate buffer was added and the termination procedure was as for the competition ELISA.Plates were read and photographed as before.The results were plotted as absorbance vs serum dilution.

6.2.19 INTERFERON GAMMA ASSAY

This assay was carried out essentially as described in Meager,(1987),with some modifications.

The assay is based on the inhibition of the incorporation of [³H]uridine into viral ribonucleic acid.Briefly,replicate cultures of Interferon (Ifn) sensitive cells (L929) were treated with a dilution series of Ifn and left overnight.The cells were then challenged with an Ifn sensitive Togavirus, Semliki Forest Virus (SFV).The extent of replication of the virus in the cells was then determined by the incorporation of [³H]uridine into viral RNA.Cellular RNA synthesis was blocked by actinomycin (AMD) (Sigma),to which the virus is insensitive.

The mean [³H]uridine incorporation values were plotted against the log₁₀ of the dilution.The titer of the Interferon sample was then defined as the dilution,at which there was 50% incorporation.The 50% incorporation value was calculated as follows:

$$50\% \text{ Incorp.} = \frac{(\text{Virus Control (CPM)} - \text{Cell Control (CPM)})}{2} + \text{Cell Con.}$$

The dilution at which 50% incorporation occurred was determined by interpolation.The titer of a preparation was taken to be numerically equivalent to the number of Units/ml.

The titer of an Interferon-gamma standard was also calculated and compared with the defined value.If the values differed by more than 0.3 log

units, the appropriate correction was made. If the difference between the defined and calculated titers of the standard was more than 1.0 log unit then the assay was repeated. Differences in titer of less than 2 fold were not considered significant in this assay.

CHAPTER 7

ESTABLISHMENT OF THE IMMUNE RESPONSE

7.1 INTRODUCTION TO EXPERIMENTAL WORK

7.1.1 RATIONALE TO THE THESIS

The main aim of this thesis was to establish and develop a murine model system, whereby the Th-cell proliferative i.e. CD4⁺ proliferative response to rotaviruses, could be studied. In order to achieve this goal several initial objectives were defined which were considered essential to the project. First, to use only seronegative mice in these studies. Second, to determine if BRV(UKtc) was capable of inducing an immune response in adult BALB/c mice (as assessed by sero-conversion) and third, to define the ability of BRV(UKtc) to replicate in mice.

The results of these studies would then provide a suitable basis for the establishment of a lymphocyte proliferation assay and a detailed analysis of the response, if obtained.

There was no emphasis on the role of CD4⁺ Th-cells in protection against disease in this project. Rather, the studies were intended to develop a route to understanding the interaction of the Th-cell with other components of the immune response to rotavirus and ultimately towards defining the murine Th epitopes in BRV(UKtc).

The bovine rotavirus UKtc was chosen for these studies for several reasons. First, BRV(UKtc) grows well in tissue culture (i.e. when compared with other rotavirus strains) and the propagation of this strain was routinely practiced in the laboratory. Second, BRV(UKtc) is well characterized and a substantial amount of information on the molecular biology of the virus was available (e.g. McCrae & Faulkner-Valle, 1981; McCrae & McCorquodale, 1982). Finally, assessment of the T-cell proliferative response to BRV(UKtc) may have direct relevance to veterinary science, where bovine rotaviruses are a significant problem in the dairy industry (see Chapter 1). Murine strains such as EDIM were not chosen because they do not grow well in tissue culture (Greenberg *et al.*, 1986) and the molecular biology of the virus is not well characterized. However, more recently some murine strains have been adapted to grow in tissue culture (Ward *et al.*, 1990).

Adult mice (rather than neonatal mice) were chosen for this project for three major reasons. First, there are many technical difficulties involved in performing experiments in neonatal mice (Eiden *et al.*, 1986a), including the problem of an immature T-cell population (Offit & Dudzik, 1990). Since responses in GALT were also intended to be examined, technical difficulties were of particular importance. Second, adult mice provide a more well characterized system with which to generate Th-cell clones to rotavirus, for future Th-cell epitope mapping. Finally, Offit & Dudzik, (1988), reported that CTL responses to rotavirus could be induced in adult mice with heterologous rotaviruses.

7.2 RESULTS

7.2.1 DETERMINATION OF THE PRESENCE OF ANTI-ROTAVIRUS ANTIBODIES IN THE BALB/c MOUSE BREEDING COLONY

An important aim in this thesis was to determine the nature of the cross-reactive T-cell proliferative response to different rotavirus serotypes. To this end, it was decided that all mice used in the experiments should be sero-negative for anti-rotavirus Ab's, such that they were only exposed to rotavirus antigen under experimental conditions. Two techniques were chosen to determine whether natural exposure to rotavirus antigen had taken place. These were a PRN assay and an ELISA.

7.2.1.1 PLAQUE REDUCTION NEUTRALIZATION ASSAY

Adult (8 week old) female BALB/c mice were chosen at random from the breeding colony, sacrificed and the serum prepared for PRN assay. Table 8 shows a sample of the results obtained and it illustrates that the PRN titers of the sample sera were considerably lower than that of the bovine hyperimmune control sera. Sample neutralization titers were consistently <50. Offit *et al.*, (1983) and (1984) consider that animals with neutralizing Ab titers to rotavirus of >50 are positive for rotavirus neutralizing Ab. Therefore by this criterion, adult animals from the Warwick colony (Table 8) were considered to be negative for anti-BRV(UKtc) nAb's and therefore unlikely to have been exposed to BRV(UKtc) antigen, previously.

7.2.1.2 ELISA

7.2.1.2.1 COMPETITIVE ELISA

Adult, female mice were chosen at random from the breeding colony and sacrificed. Serum was then prepared and competitive ELISA's carried out. Briefly, serial doubling dilutions of the sera (or anti-VP6 mAb) were prepared in micotiter plates containing single-shelled (ss) rotavirus, bound to 'capture' Ab. To each well was added a constant volume of rabbit anti-rotavirus hyperimmune, horseradish peroxidase coupled conjugate and the plates

TABLE 8 LEGEND

Animals 1-4 and 5-7 (8 weeks old), were randomly chosen from the BALB/c mouse breeding colony, on two different occasions and assessed for the presence of anti-rotavirus antibody by plaque reduction neutralization assay.

* Antibody titer is expressed as the reciprocal of the serum dilution showing a 50% reduction in the mean plaque count for BRV(UKtc).

TABLE 8

NEUTRALIZING ANTIBODY TITERS TO SRV(URS) IN
UNINOCULATED BALB/c MOUSE SERA

COMPLEMENT INACTIVATED SERUM FROM UNINOCULATED MOUSE:	NEUTRALIZING ANTIBODY TITER WITH SRV(URS)
1	10.0
2	14.0
3	16.0
4	16.0
5	<20.0
6	<20.0
7	<20.0
BOVINE HYPERIMMUNE ANTISERA TO SRV(URS)	>2000

incubated. Substrate buffer was added and the O.D. for each well determined. The degree of inhibition was then assessed.

Table 9 shows a sample of these results. As can be seen, the uninoculated mice consistently showed percentage inhibition values of <50%, at a dilution of 1:20. In contrast the i.p. BRV (UKtc) inoculated mouse showed a percentage inhibition of 99.4%, at a dilution of 1:20. Non-complement inactivated serum showed a slightly higher level of inhibition when compared with the complement inactivated serum. Inhibition values of less than or equal to 50% were considered to be negative for rotavirus specific anti-VP6 Ab's (Beards & Desselberger, 1989; Wreggitt & Morgan-Capner, 1990). Inhibition activity was present in the i.p. inoculated mouse serum up to a dilution of at least 1:80 and in the anti-VP6 control up to a dilution of at least 1:2560 (data not shown).

Mice at 2, 4 and 6 weeks old were also screened for the presence of anti-VP6 Ab by this technique, (data not shown) and inhibition values of <50% were consistently obtained.

This technique again pointed to the conclusion that uninoculated animals were seronegative for anti-rotavirus Ab's and had not been exposed to group A rotavirus antigen, previously.

7.2.1.2.2 INDIRECT ELISA

Further analysis of the serum from young and adult uninoculated animals using an indirect technique, confirmed that anti-rotavirus Ab's to VP6 were absent (see Fig. 9 for an example of the results for adult animals). In contrast, sera from two orally inoculated adult animals and one i.p. inoculated animal contained large amounts of anti-rotavirus Ab. Interestingly, one orally inoculated animal (mouse 3), did not appear to respond.

7.2.2 ESTABLISHMENT OF THE IMMUNE RESPONSE TO BRV(UKtc)

The serum neutralizing Ab response to bovine rotavirus strains has been studied previously (Ward *et al.*, 1990) but analysis of the nAb response to BRV(UKtc) in an adult mouse model was not performed.

TABLE 9 LEGEND

Animals 1-3 and 4-6 were randomly chosen from the mouse breeding colony on different occasions, sacrificed and the serum prepared. Competitive ELISA's were set up using the samples and controls. The i.p. inoculated mouse serum was prepared from a mouse inoculated with 10^7 Pfu of BRV(UKtc) and sacrificed at 7 days p.i.. The percentage inhibition was calculated for the lowest dilution (1:20), using the method described previously (Wraghitt & Morgan-Capner, 1990).

FIGURE 9 LEGEND

Serum was prepared from both uninoculated and BRV(UKtc) inoculated adult BALB/c mice. Animals were inoculated with 10^7 Pfu by either the oral or i.p. routes and sacrificed at 8 days p.i. (oral route) or 7 days p.i. (i.p. route). Indirect ELISA's were set up to determine the relative amounts of anti-VP6 Ab in each of these samples.

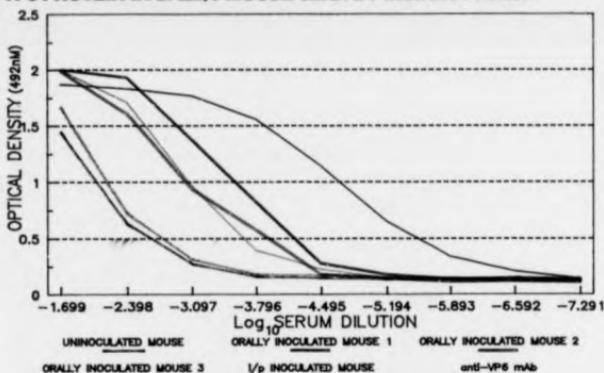
TABLE 9

DETERMINATION OF THE PRESENCE OF ANTIBODY TO ROTAVIRUS VP6 PROTEIN IN UNINOCULATED MOUSE SERA BY COMPETITION ELISA

COLLECTED SERA	% INHIBITION FOR EACH SAMPLE AT A DILUTION OF 1:20
Uninoculated Mouse 1	13.9
Uninoculated Mouse 2	16.3
Uninoculated Mouse 3	22.5
Uninoculated Mouse 4	44.3
Uninoculated Mouse 5	34.0
Uninoculated Mouse 6	42.8
I/p Inoculated Mouse	99.4
Anti-VP6 mAb	100.0
Indicator Ab Only	0.0

Fig. 9

DETERMINATION OF THE PRESENCE OF ANTIBODY TO ROTAVIRUS VP6 PROTEIN IN BALB/c MOUSE SERA BY INDIRECT ELISA



In order to ascertain if adult BALB/c mice were capable of mounting an immune response to BRV(UKtc), when administered orally (i.e. by the natural route of inoculation), a time course experiment was set up to determine the presence of serum nAb to the virus (see Fig. 10). As can be seen, no serum nAb response to BRV(UKtc) could be detected prior to 5 days post inoculation (p.i.) but a response was present at 5 and 7 days p.i.. However, it is apparent that considerable variation exists in the serum nAb titers to BRV(UKtc).

This experiment showed that a heterologous rotavirus was capable of stimulating an immune response in adult mice.

7.2.3 THE DURATION OF THE SERUM NEUTRALIZING ANTIBODY RESPONSE

Further experiments (Fig. 11) showed that a serum nAb response to BRV(UKtc) could be consistently induced at 8 days p.i., following oral inoculation of adult animals. Although considerable variation in the titers was apparent, at least 1 mouse in each experimental group responded. Serum nAb responses to BRV(UKtc) remained detectable up to 22 days p.i. (see Table 10 for samples of these results), following a single oral dose of BRV(UKtc). By 56 days p.i., serum nAb to BRV(UKtc) could no longer be detected to significant titers.

7.2.4 EFFECT OF ROUTE OF PRIMING ON VARIATION IN SERUM NEUTRALIZING ANTIBODY TITER TO BRV(UKtc)

Since oral inoculation of BALB/c mice resulted in considerable variation in the serum nAb titers to BRV(UKtc), experiments were conducted to investigate whether the route of priming affected the variation in the titer. It is clear, (Table 11) that although variation in the nAb titer was present following i.p. inoculation, it was not as great as for orally inoculated animals (Fig. 11 and Table 10). Following i.p. inoculation, serum nAb titers did not vary by 10 or 100 fold, as was apparent in the orally inoculated animals. This suggested that the i.p. route was a more efficient route for the induction of serum nAb to BRV(UKtc).

FIGURE 10 LEGEND

Adult mice were orally inoculated with 1.6×10^6 Pfu of BRV(UKtc) in groups of 3 and sacrificed at the designated time points. The serum was then prepared and PRN assays set up to determine the neutralizing antibody titer to BRV(UKtc). Antibody titers are expressed as the reciprocal of the dilution showing a 50% reduction in the mean plaque count for BRV(UKtc).

FIGURE 11 LEGEND

Adult mice (in groups of 3) were orally inoculated with 10^7 Pfu of BRV(UKtc) and were sacrificed at 8 days p.i.. The sera were then prepared and PRN assays set up, as above.

Fig. 10

**THE SERUM NEUTRALIZING ANTIBODY RESPONSE TO EBV(10⁶)
IN ORALLY INOCULATED ANIMALS OVER A SEVEN DAY PERIOD**

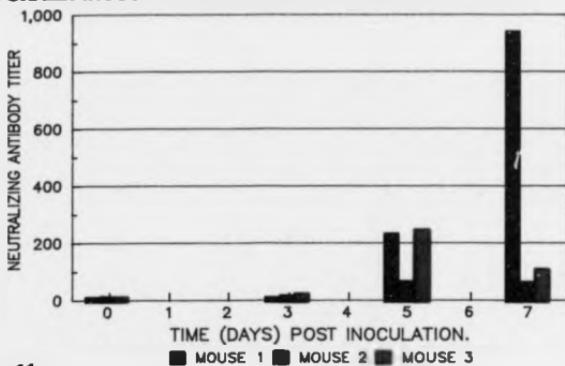


Fig. 11

**THE SERUM NEUTRALIZING ANTIBODY RESPONSE TO EBV(10⁶) AT
8 DAYS POST ORAL INOCULATION**

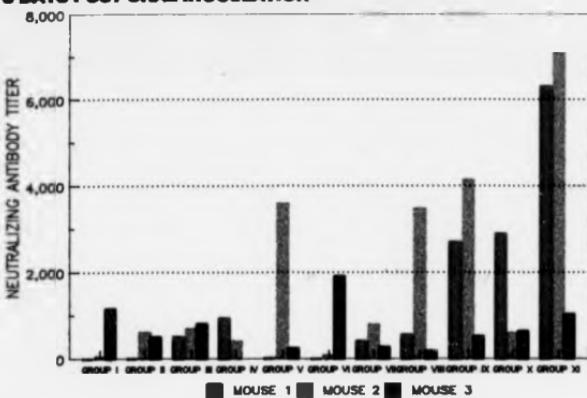


TABLE 10 LEGEND

Animals were orally inoculated with 10^7 PFU of BRV(UKtc). At various times p.i. the animals were sacrificed and PRN assays carried out on the serum samples. The neutralizing Ab titer is expressed as the reciprocal of the serum dilution showing a 50% reduction in the mean plaque count for BRV(UKtc).

TABLE 16

THE SERUM NEUTRALIZING ANTIBODY RESPONSE TO SV40(VGm) AT
VARIOUS TIMES POST ORAL INOCULATION

TIME POINT (DAYS)	MOUSE	SERUM NEUTRALIZING ANTIBODY TITER
15	1	39.0
	2	1336.7
	3	43.4
22	1	676.9
	2	8507.0
	3	947.4
56	1	36.0
	2	36.8
	3	32.8
144	1	<20.0
	2	<20.0
	3	<20.0

TABLE 11 LEGEND

Animals were inoculated with 10^7 Pfu of BRV(UKtc) by the i.p. route and sacrificed at 7 days p.i.. Serum was prepared and PRN assays were set up. The antibody titer is expressed as the reciprocal of the serum dilution showing a 50% reduction in the mean plaque count for BRV(UKtc).

TABLE 11
THE SERUM NEUTRALIZING ANTIBODY RESPONSE TO SV40(KO)
FOLLOWING INTRA-PERITONEAL INOCULATION

SERUM DERIVED FROM:	NEUTRALIZING ANTIBODY TITER
MOUSE 1	1055.0
MOUSE 2	6902.9
MOUSE 3	5260.5

7.2.5 DISCUSSION

Serum immune responses to rotavirus are a good indicator of previous infection (Conner *et al.*, 1991). The absence of serum Ab to rotavirus in uninoculated BALB/c mice from the Warwick breeding colony therefore suggested that they had not been challenged with BRV(UKtc) or any other strain of group A rotavirus, previously.

Oral inoculation of rotavirus induces both mucosal and serologic Ab's to the virus (Conner *et al.*, 1991) and as a measure of an immune response to BRV-(UKtc) in adult mice, the serum nAb response was chosen. The detection of neutralizing activity to BRV(UKtc) in the sera of orally inoculated animals indicated that an immune response had occurred and showed that the oral route was satisfactory for inducing the response. It is likely that an intestinal anti-rotavirus Ab response was also induced by this procedure (Conner *et al.*, 1991). The plasma cells producing the serum neutralizing immunoglobulin to BRV(UKtc) are most likely to be derived from GALT (Tomasi, 1989) although some may be derived from BRV(UKtc) specific spleen cells, since rotavirus antigen has been detected in APC's in the spleen following oral inoculation (Dharakul *et al.*, 1988).

Significantly, following oral inoculation, the serum nAb titers and serum anti-VP6 Ab titers showed some considerable variation. The lack of 10-100 fold variation in the titers following i.p. inoculation, suggested that this route was more efficient for stimulating serum nAb responses than the oral route. This is likely for several reasons. First, not all of the rotavirus particles inoculated by the oral route may have been taken up by M-cells in PP's (Dharakul *et al.*, 1988) such that the BRV(UKtc) antigenic load in PP's was reduced, somewhat in different mice. In i.p. inoculated animals the virus is inoculated directly into an antigen presenting environment and therefore very little antigen is lost, prior to uptake and processing by APC's. Second, variation in the serum nAb titers from orally inoculated animals may have been due to differential replication in different mice. However, this is less likely in view of the the lack of replication of other heterotypic rotaviruses reported in adult mouse models

(see section 1.15.4) and the fact that susceptibility to infection is unlikely to reside in individual animals within an inbred mouse strain. Thus the 'peculiarities' of the oral route of priming may account, to a great extent for the large degree of variation in the serum nAb titers.

The presence of serum nAb to BRV(UKtc) at 8 days p.i. and the strong dependence of GALT B-cell responses on CD4⁺ Th- cells (Tomasi, 1989) strongly suggested that Th-cells specific for BRV(UKtc) could be induced following a single oral inoculation.

7.2.6 CONCLUSIONS

The Warwick breeding colony of BALB/c mice had not been exposed to group A rotavirus antigen, previously. H2^d is a responder haplotype for BRV(UKtc) mediated Ab responses. Primary serum Ab responses to BRV(UKtc) can be induced following inoculation by both the oral and i.p. routes, (which last for up to 22 days post oral inoculation), strongly suggesting that Th-cell activation occurs. A model with which to examine murine immune responses to BRV-(UKtc), was thus established.

CHAPTER 8

**DETERMINATION OF BRV(UK_{tc}) REPLICATION
IN THE INTESTINES OF BALB/c MICE**

8.1 INTRODUCTION

Since there is considerable variation in the ability of heterologous rotaviruses to replicate in the intestines of mice (Offit *et al.*, 1984; Ramig, 1988), it was considered important to determine if BRV(UKtc) was capable of replicating in the intestines of both neonatal and adult BALB/c mice. This had not been determined previously.

8.2 REPLICATION IN NEONATAL BALB/c MICE

A litter of 5 day old BALB/c mice was orally inoculated with two doses of 2.5×10^7 Pfu of CsCl purified BRV(UKtc), given 24hr apart. Clinical symptoms were noted, which included a watery yellow opaque diarrhoea, first observed on day 2 (see Fig. 12). As can be seen, the incidence of diarrhoea reached a peak on day 4 and by day 7 diarrhoea was no longer observed. No fatalities occurred during this experiment.

Animals of an equivalent age inoculated with a BSC-1 cell extract (to the same protein concentration as the virus preparation) did not show any clinical symptoms (data not shown) and the animals remained healthy for the duration of the experiment. This suggested that the diarrhoea observed was specific to BRV(UKtc).

The presence of clinical symptoms following oral inoculation of neonatal BALB/c mice with BRV(UKtc) suggested that replication in the intestine had occurred (Ramig, 1988). Neonatal BALB/c mice were therefore susceptible to both BRV(UKtc) replication and diarrhoea.

8.3 REPLICATION IN ADULTS

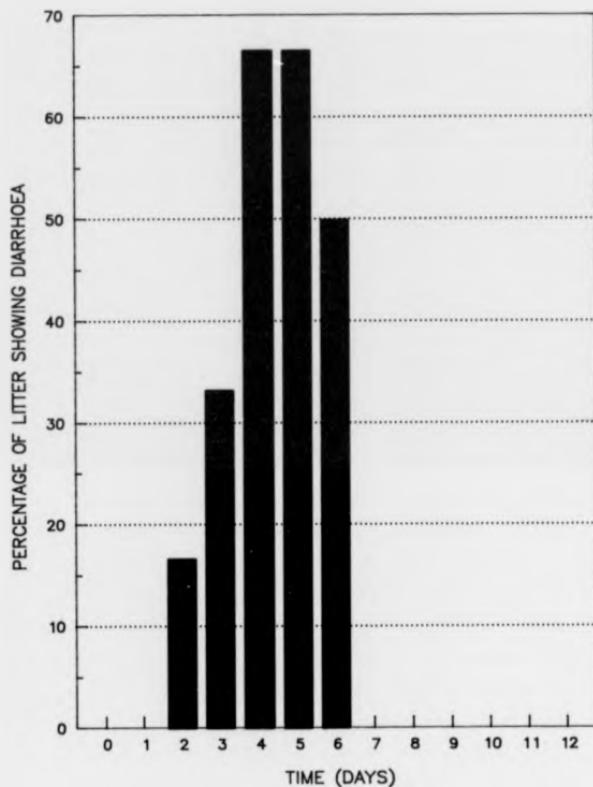
Diarrhoeal disease was not observed in adult BALB/c mice at any time following oral inoculation with 10^7 Pfu of BRV- (UKtc), in the serum Ab experiments (Chapter 7). Therefore, to ascertain if replication of BRV(UKtc) in adult mice had occurred in the absence of disease (as reported for SA11 by Ramig, 1988), animals were orally inoculated with BRV(UKtc) and at various

FIGURE 12 LEGEND

Five day old BALB/c mice were orally inoculated with 2.5×10^7 Pfu of BRV(UKtc) on day 0 and day 1. The incidence of diarrhoea in the litter was then monitored over a 12 day period.

Fig. 12

**DURATION AND INCIDENCE OF DIARRHOEA IN NEONATAL BALB/c
MICE FOLLOWING ORAL INOCULATION WITH EBV(UR3e)**



times p.i. the presence of infectious virus in the intestine and faeces was determined by plaque assay.

The experiments conducted (see Table 12) revealed that either (i)productive replication of BRV(UKtc) does not occur in the intestines of adult BALB/c mice or (ii)if it does occur,then the levels of infectious virus are beneath the limits of detection in this assay.A small increase in the number of plaque forming units detected,occurred at 8hr p.i. but this amount was at least $2 \log_{10}$ units lower than expected,if complete replication of the virus had occurred (Ramig,1988).

Control experiments (data not shown),whereby the intestines from uninoculated mice were 'spiked' with 10^7 Pfu of BRV(UKtc),clearly showed that the homogenization protocol did not affect the infectivity of the virus in these experiments.

8.4 DISCUSSION

It was apparent in this study that BRV(UKtc) was capable of fully replicating in the intestines of neonatal BALB/c mice but probably not in adult mice.The reason for adult mouse non-susceptibility to rotavirus replication and disease remains obscure and has been reported for the homologous EDIM virus (Wolf *et al.*,1981;Eydelloth *et al.*,1984) and also for SA11 (Ramig,1988).

It is unlikely that the primary mucosal immune response initiated following BRV(UKtc) inoculation of adult animals,is sufficiently large or produced at a rate that would be sufficient to control and prevent virus infection and replication.This was also proposed for the EDIM virus model (Eydelloth *et al.*, 1984).Therefore,non-susceptibility to BRV(UKtc) induced disease in adults is likely to be a function of the gut epithelial cell.Intestinal maturation has been suggested to be an important factor (Wolf *et al.*,1981) *i.e.* a decrease in the number of rotavirus receptors (Riepenhoff-Talty *et al.*,1982) and/or a decreased efficiency in rotaviral antigen expression (Eydelloth *et al.*,1984) in gut epithelial cells.It is likely that these explanations are at least partially valid (see later) for the observation with BRV(UKtc) and adult mice.

TABLE 12 LEGEND

Adult BALB/c mice were orally inoculated (in duplicate) with 10^7 Pfu of BRV(UKtc) and sacrificed at the designated time points. Homogenates of the intestines and faeces were prepared and plaque assays set up to determine the infectivity titer of these samples.

* ND-Not Determined

TABLE 12

DETECTION OF INFECTIONOUS INFLU(ON) IN ADULT BALB/c MOUSE
INTESTINES AND FAECES, OVER TIME

TIME POINT (HOURS)	NUMBER OF PFU DETERMINED/ INTESTINE		NUMBER OF PFU DETERMINED/ ml OF FAECAL HOMOGENATE (10% SUSPENSION)	
	MOUSE 1	MOUSE 2	MOUSE 1	MOUSE 2
0	10^7	10^7	<25	<25
4	<1000	<1000	<25	<25
8	<1000	<1000	200	200
12	<1000	<1000	<25	<25
24	<1000	<1000	25	<25
48	<1000	<1000	25	<25
72	<1000	<1000	*N.D.	N.D.
120	<1000	<1000	N.D.	N.D.
168	<1000	<1000	N.D.	N.D.

Although the dose of BRV(UKtc) given to the neonatal mice was 5 fold higher (*i.e.* 5×10^7 Pfu) than that given to the adult mice (1×10^7 Pfu), it is unlikely that this is the reason for the difference in pathogenesis between the two groups of mice. First, in mice where a heterologous virus (SA11) was capable of replicating and inducing disease, at doses of 8×10^6 Pfu it was also capable of replicating and inducing disease at doses of 10^2 Pfu/mouse (Ramig, 1988). Second, it has been reported that 10^7 Pfu of BRV(UKtc) can induce disease in suckling BALB/c mice, following oral inoculation (Offit *et al.*, 1986). Third, it is highly unlikely that 5×10^7 Pfu would have induced diarrhoea in adult mice, in view of the reasons for adult mouse non-susceptibility, discussed above.

Replication of BRV(UKtc) occurred in neonatal mice, as indicated by the induction of disease. However, it has been proposed by Ramig, (1988) that although some complete replication cycles do occur in epithelial cells infected with heterologous viruses in neonatal mice, the majority of these infections are abortive *i.e.* many cells are infected but produce only very low levels of virus. This is indicated by the shorter period of the replication cycle (*i.e.* when compared to the EDIM mouse model), the greater inoculum size required for heterologous rotaviruses to produce an equivalent titer to EDIM (Offit *et al.*, 1984; Ramig, 1988) and the inability to detect morphologically heterologous rotavirus structures in murine ileal enterocytes (Ramig, 1988). Thus, an extended cycle of replication by heterologous viruses does not occur in neonatal murine intestines (Offit *et al.*, 1984) in contrast to the homologous EDIM strain (Eydelloth *et al.*, 1984) and therefore replication of heterologous rotaviruses is generally poor when compared with homologous strains (Greenberg *et al.*, 1986).

A surprising feature of the infectivity results for adult animals was the observation that by 4 hr p.i. very little infectivity was apparently present in the mouse intestine. There are a number of reasons which may account for this observation.

First,M cells from the PP's may have efficiently taken up the particles and transferred them to the APC's of the patch dome (Brandtzaeg,1989).Second,the virus may have become bound to food particles and been lost from the system.Third gastric juices including trypsins and stomach acid may have led to BRV-(UKtc) inactivation.Fourth,the virus may have bound to and been internalized by intestinal epithelial cells.

In view of the variation in the serum Ab results (see Chapter 7) it is unlikely that M cell uptake is as efficient as might be expected.Therefore,it seems plausible that at least some BrV(UKtc) virus is lost from the system possibly by binding to food particles.However,if this was a major mechanism for the loss of infectious virus,then it would have been detected in the intestinal homogenate and possibly in the faeces.Although a small increase in BRV-(UKtc) infectivity was detected at 8hr p.i.,the results do not support this proposal.It is possible,therefore that binding to food particles inactivates the virus or prevents it from binding to cells,or alternatively gastric secretions are responsible for inactivating the virus.However,in view of the stability of rotaviruses to extreme pH and enzymatic conditions (see Chapter 1) it is perhaps less likely that physical inactivation plays a major role in the loss in infectivity.Hence,the majority of the virus is likely to be undetectable because it has been internalized by epithelial cells (Ramilg,1988).However,unlike neonatal mice,few if any cells produce infectious virus (Offit *et al.*,1986;Estes & Cohen,1989).

This explanation would suggest that the lack of replication and disease in adult mice infected with rotavirus,is not due primarily to a lack of receptors but to ill-defined intracellular elements which are not present,or inhibitory to rotavirus replication.

Further experiments are necessary to clarify the exact fate of BRV(UKtc) in the adult mouse intestine but it is clear that a large amplification of BRV(UKtc) antigen for presentation to immune response cells does not occur in this system.

Importantly, these results show that a heterologous rotavirus, which is not capable of replicating efficiently in the intestinal mucosa of adult mice, is capable of inducing a good immune response.

8.5 CONCLUSIONS

BRV(UKtc) can initiate diarrhoea in 5 day old BALB/c mice but not in adults. In adult mice a non-productive, abortive infection probably occurs. However, adult mice provide a good model with which to analyse murine immune responses to BRV(UKtc).

CHAPTER 9

**ESTABLISHMENT OF A LYMPHOCYTE
PROLIFERATION ASSAY FOR BRV(UKtc)**

9.1 INTRODUCTION

Several factors suggested that the spleen was a suitable tissue in which to find proliferative responses to BRV(UKtc). First, splenocytes from orally inoculated suckling mice were reported to proliferate *in vitro* when challenged with EDIM (Riepenhoff-Talty *et al.*, 1983). Second, antigen activated GALT cells are known to migrate from PP's to the LP of the gut, via the bloodstream (Brantzaeg, 1989). Third, CD8⁺ CTL responses to heterologous rotaviruses in mouse spleens had been reported (Offit & Dudzik, 1988) and since CTL responses have been shown to be dependent on CD4⁺ cells (at least in some systems) (Ciavarra, 1990), this suggested that CD4⁺ responses to BRV(UKtc) were also likely to be present. Fourth, there were many technical difficulties associated with handling GALT cells which had to be overcome before a suitable assay for these cells could be developed.

The type of assay system with which to obtain the best measure of lymphoproliferative responses to BRV(UKtc) was not known. The following results show how the assay system was derived.

9.2 RESPONSE OF SPLENOCYTES TO CONCANAVALIN A IN DIFFERENT MEDIA

As a first means of establishing an assay system with which to measure lymphoproliferation to BRV(UKtc) in murine splenocytes a series of experiments was set up using a T-cell mitogen (the lectin Concanavalin A (Con.A)), as the stimulant of proliferation.

Splenocytes were prepared from uninoculated mice, plated out with Con.A over a range concentrations in RPMI 1640 (10% FCS) and cultured for 2 days. Following pulsing with [³H]thymidine, the levels of incorporation were determined. This was considered to be a measure of DNA synthesis and therefore proliferation, in this assay system. As can be seen (Fig. 13(a)), a dose response was obtained with optimal [³H]thymidine incorporation occurring at 5ug/ml of Con.A with an SI of 32.9. Since proliferation occurred in this system it suggested that (*i*) T-cells could respond in this medium when stimulated with

FIGURE 13 LEGEND

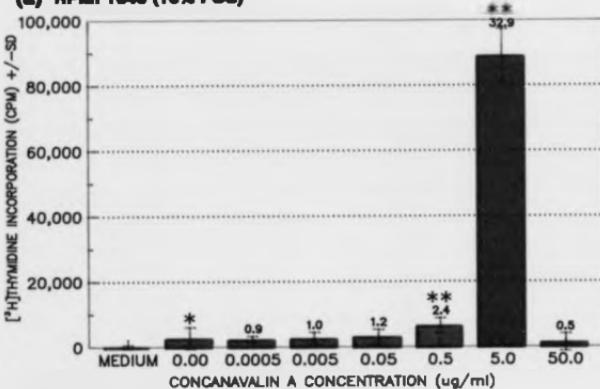
Uninoculated adult BALB/c mice (3) were sacrificed by cervical dislocation and their spleens were removed. Single cell suspensions were prepared in either RPMI 1640 (10% FCS), (a) or RPMI 1640 (1% BALB/c M.S.), (b) and plated out 5×10^5 cells/well, (a) or 4×10^5 cells/well, (b) with Con.A, over a range of concentrations (6 replicates/dilution). Splenocytes were incubated for 48hr, (a) or 72hr, (b) at 37°C in an atmosphere containing 5% CO₂. To the cells incubated in RPMI 1640 (1% BALB/c M.S.), (b) was then added 0.1ml of RPMI 1640 (5% FCS), to each well and the cells pipetted. Half the cells were then removed. Splenocytes were pulsed with 1.25uCi of [³H]thymidine for 18hr, prior to harvesting and counting, (a) and (b).

** -Denotes significant statistical difference from control,* and is shown in all subsequent Figures of this form.

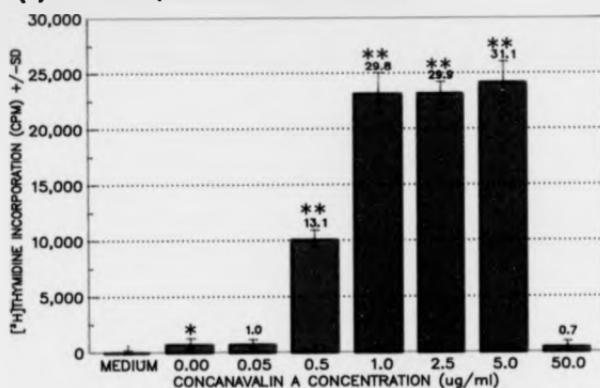
Fig.13

RESPONSE OF SPLEENOCYTES (DERIVED FROM UNINOCULATED BALB/c MICE), TO CONA, OVER A RANGE OF CONCENTRATIONS

(a) RPMI 1640 (10% FCS)



(b) RPMI 1640 (1% BALB/c MOUSE SERUM)



Con.A (Tsien *et al.*, 1982) and (ii) an APC accessory cell could also function (Gallagher *et al.*, 1986; Hirayama *et al.*, 1987).

It was noted that non-specific incorporation (*i.e.* at 0.0 µg/ml) was relatively high in this system and since the virus specific proliferative response was likely to be much lower than the response to Con.A, it was thought that this may 'mask' an antigen specific lymphoproliferative response. Consequently other medium systems were investigated.

A report by Chain *et al.*, (1987) suggested that non-specific proliferation could be significantly reduced if homologous serum was used to supplement the growth medium. Furthermore, the signal-to-noise ratio was reported to be greatly improved using this system. Experiments were set up to investigate this report.

Splenocytes from uninoculated mice were prepared and cultured with Con.A, over a range of concentrations, in RPMI 1640 (1% BALB/c M.S.). After 3 days, the medium was changed (see methods) and the cells were pulsed with [³H]thymidine. It is apparent that a dose response to the mitogen was obtained (Fig. 13(b)) but significantly the non-specific proliferation was greatly reduced. Additionally, sub-optimal responses were more clearly apparent in the RPMI 1640 (1% BALB/c M.S.) system (Fig. 13(b)).

This assay differed to that described by Chain *et al.*, (1987) in that exogenous IL-2 was not added to the 'change' medium (see methods). Incorporation values clearly showed that this did not affect the ability of murine splenocytes to respond to mitogen stimulation.

These results suggested that the assay utilizing RPMI (1640) (1% BALB/c M.S.) was more suitable for detecting responses to BRV(UKtc) antigen, where the response was likely to be weak. In addition, homologous serum systems were considered more likely to provide optimal conditions for the induction of virus specific proliferative responses. Splenocyte proliferative responses to other virus antigens were detected using this system (Rothman *et al.*, 1989), strongly suggesting its suitability for detecting proliferation to rotavirus antigen.

9.3 SPLENOCYTE PROLIFERATIVE RESPONSE TO BRV(UKtc)

The serum nAb response to BRV(UKtc) (Chapter 7) showed that a good immune response was obtained at 8 days post oral inoculation. Therefore, splenocyte proliferative responses to BRV(UKtc) were also likely to be present at this time point.

Experiments were set up to determine if splenocyte proliferation to *in vitro* BRV(UKtc) challenge, occurred using cells derived from animals orally primed 8 days previously with the virus. Fig. 14(a) shows a representative example of one of these experiments. As can be seen a clear dose response to BRV(UKtc) is present, with an optimum antigen concentration in this assay of 10ug/ml. The sensitivity of the assay is clearly revealed, since significant responses to rotaviral antigen were detectable to 0.01ug/ml.

The specificity of the BRV(UKtc) response was shown by the lack of a significant response to the influenza virus (A/CHR/68 H3N2) (concentrations were calculated from the HA titer, according to Taylor *et al.*, 1987). The results also suggest that a BRV(UKtc) specific response does not affect the response to Con.A mitogen at 8 days post oral inoculation.

As a further confirmation of the specificity of the proliferative response to BRV(UKtc), splenocytes from uninoculated animals were challenged with either BRV(UKtc), control antigen or mitogen, *in vitro* (Fig. 14(b)). It is apparent that no specific proliferative response to BRV(UKtc) or influenza virus was present. However, the viability of the cells is clearly shown by the response to Con.A mitogen, with an SI of 18.7 at 5ug/ml. Since the animals were not challenged with rotavirus, the results also suggest that primary *in vitro* responses to BRV(UKtc) can not be detected using this system. High concentrations of BRV(UKtc) antigen appeared to have an inhibitory effect in assays utilizing splenocytes from uninoculated mice since at 10ug/ml the level of [³H]thymidine incorporation was less than half the value for unstimulated cells.

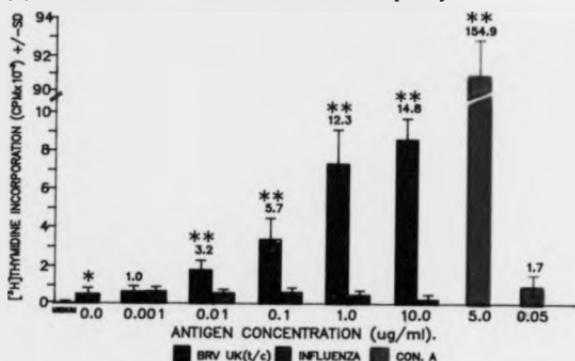
These experiments further confirmed that BALB/c mice from the Warwick breeding colony had not been exposed to rotavirus antigen previously, since no

FIGURE 14 LEGEND

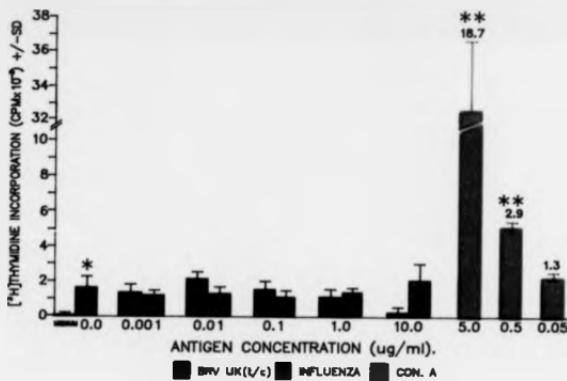
Mice (3) were each orally inoculated with 10^7 PFU of BRV-(UKtc) and 8 days later,(a) or uninoculated mice,(b),were sacrificed.Splenocytes (4×10^5 /well) (in replicates of 6) were cultured in RPMI 1640 (1% BALB/c M.S.) for 3 days with either CsCl purified BRV(UKtc),influenza or Con.A mitogen.To each well was then added 0.1ml of RPMI 1640 (5% FCS) and the cells were pipetted.Half the cells were removed and the remainder pulsed with 1.25uCi of [³H]thymidine/well for 18hr,prior to harvesting and counting.

Fig.14
RESPONSE OF SPLENOCYTES TO IN VITRO CHALLENGE WITH
BRV(UK1c),INFLUENZA OR CONA

(a) MICE ORALLY INOCULATED WITH BRV(UK1c)



(b) UNINOCULATED MICE



memory proliferative response to rotavirus antigen was detected. Offit *et al.*, (1992) propose that lymphoproliferation is a more definitive method of determining preexposure to rotavirus than the presence of serum Ab's.

Density equilibrium banding on CsCl gradients was used to purify BRV(UKtc) for *in vitro* stimulation. However, the possibility existed that cellular material carried through the purification procedure was responsible for the proliferative response observed in splenocytes, since unpurified virus was used for mouse inoculation. So as to establish if this was a correct assumption, experiments were set up in which splenocytes from BRV(UKtc) orally inoculated mice, were challenged with a range of dilutions of BSC-1 extract (*i.e.* the cells used for virus propagation). Fig. 15 shows an example of one of these experiments. It is clear that a dose response to BRV(UKtc) antigen was present and cells also responded to Con.A mitogen. No dose response was observed to BSC-1 cell extract over the same range of concentrations as viral antigen, showing that the proliferative response observed to BRV(UKtc) was not due to contaminating cellular material and was specific to the virus.

8.4 VISUAL EVIDENCE OF BRV(UKtc) INDUCED SPLENOCYTE PROLIFERATION

The splenocyte proliferative response to BRV(UKtc) antigen was also detectable by visual observation of the cells following incubation, in addition to [³H]thymidine incorporation. Fig. 16 shows a pictorial representation of splenocytes from inoculated animals stimulated with either BRV(UKtc), BSC-1 extract or Con.A mitogen. Large blast like cells are visible in 'clumps' in most wells where lymphocyte transformation was induced (*i.e.* as detected by [³H]thymidine incorporation) (see Fig. 15). Usually higher numbers of blast like cells correlated with high levels of [³H]thymidine incorporation. The relative absence of visible blasts in cells cultured in the absence of antigen or mitogen correlates well with the low levels of [³H]thymidine incorporation seen for unstimulated cells (Fig. 15).

Fig. 15

RESPONSE OF SPLENOCYTES (DERIVED FROM MICE ORALLY PRIMED WITH BRV(UK₀)) TO IN VITRO CHALLENGE WITH BRV-(UK₀), BSC-1 CELL EXTRACT AND CON.A

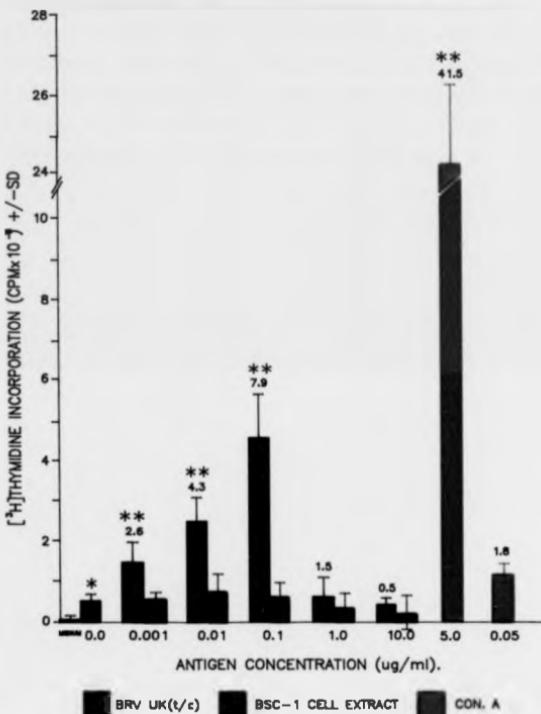
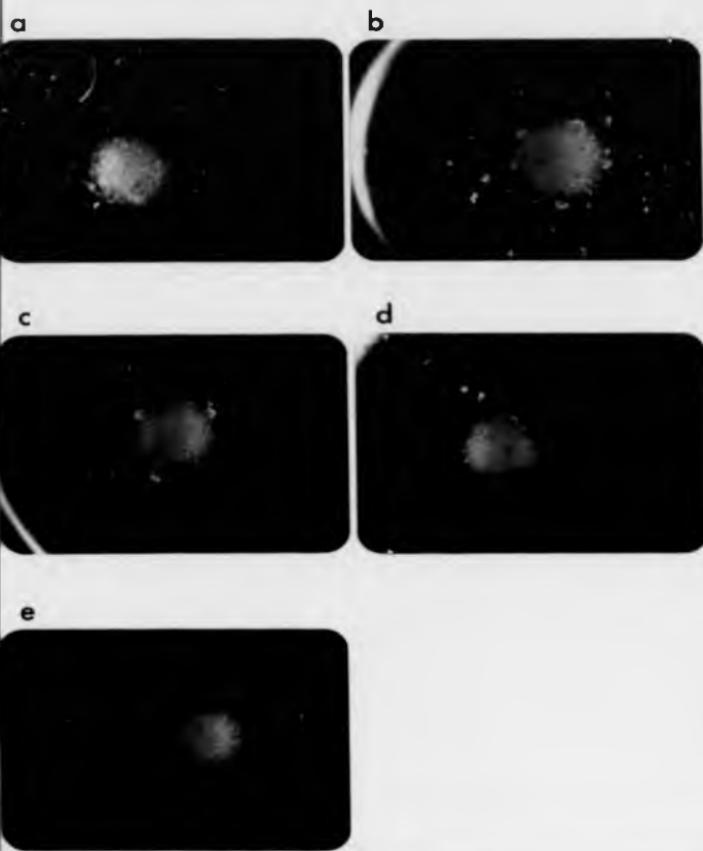


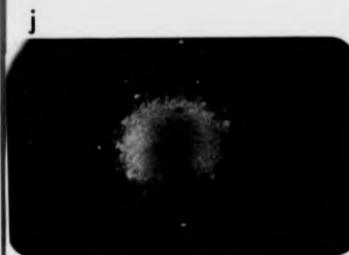
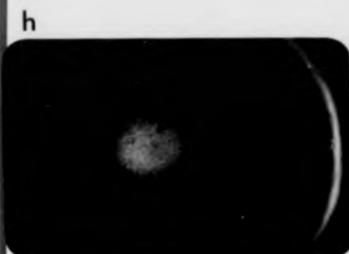
FIGURE 16 LEGEND

Prior to harvest, the cells in Fig.15 were photographed using a Nikon 35mm SLR, attached to a Nikon UFXII microscope (Optiphot). Exposure duration was regulated using a Nikon UFXIIA light box (Micro-instruments Ltd.) and Kodak Ectachrome 160, tungsten film was used. Splenocytes were cultured with either BRV(UKtc) at 1.0, 0.1, 0.01, 0.001 or 0.00 ug/ml (Photographs a,b,c,d & e, respectively), BSC-1 cell extract at 1.0, 0.1 or 0.01 ug/ml (Photographs f,g & h, respectively) or Con.A at 5.0, 0.5 or 0.05 ug/ml (Photographs i,j & k, respectively).

Fig. 16

**APPEARANCE OF SPLEENOCYTE CULTURES FOLLOWING IN VITRO
STIMULATION WITH ANTIGEN OR MITOGEN**





A proliferation assay system with which to measure lymphocyte responses to BRV(UKtc) antigen was thus established.

9.5 DISCUSSION

The results clearly showed that a murine splenocyte proliferative response to a heterologous rotavirus could be induced in adult animals, following oral priming with the virus (and in the apparent absence of complete virus replication) (Chapter 8). Splenocyte proliferation to BRV(UKtc) correlated with the serum nAb responses obtained at 8 days post oral inoculation. Since Ab responses to viral antigen are, in the main dependent upon T-cells (Scherle & Gerhard, 1986; Bunschoten *et al.*, 1989; Collen *et al.*, 1989), this suggested that the proliferative response obtained was due to activated BRV(UKtc) specific T-cells. Other evidence supported this. For example, using this assay system, the proliferative responses to dengue virus (Rothman *et al.*, 1989) and ovalbumin (Chain *et al.*, 1987) were reported to be due to T-cells. Further, there was no reported evidence that rotaviruses acted as T-independent antigens, unlike the hepatitis B core antigen (Milich & McLachlan, 1988). A murine model system with which to examine T-cell proliferative responses to heterologous rotaviruses was therefore considered to have been established.

The responses detected in the spleen are thought to be due primarily to BRV(UKtc) stimulated T-cells from PP's and MLN's, which have migrated to the spleen via the blood stream (Brandzaeg, 1989). However, since some APC's expressing rotaviral antigen have been detected in the spleen following oral inoculation of mice with rotavirus (Dharakul *et al.*, 1988), some cells may have been activated in the spleen, rather than GALT. In addition it has been proposed that rotaviruses may infect lymphocyte's in GALT (Offit & Dudzik, 1988) which then migrate to the spleen, thereby activating cells in this tissue. Other evidence suggests that this is unlikely. For example, Casali *et al.*, (1984) determined that lymphocyte infection led to an abrogation of the proliferative response. *In vitro* challenge with BRV(UKtc) (infectious double-shelled and non infectious single-shelled virus) clearly revealed that proliferation

occurred, suggesting that infection of the responding lymphocytes did not occur. In addition, rotaviruses show a relatively high degree of specificity in 'their choice' of cell receptor (Willoughby *et al.*, 1990) (*i.e.* few cell types can be infected with rotavirus) which implies that lymphocyte infection is highly unlikely.

The lack of a response to infectious influenza virus is unlikely to be due to infection of the lymphocytes since others have consistently reported the proliferation of primed T-lymphocytes in the presence of infectious influenza virus (Scherle & Gerhard, 1986; Mills *et al.*, 1986; Graham *et al.*, 1989). This suggests that the absence of proliferation in the presence of influenza virus is genuine and due to an absence of influenza specific T-cells.

Since the animals were challenged with BRV(UKtc) 8 days prior to the removal of the spleens, the T-cell population responding to *in vitro* stimulation with BRV(UKtc) is likely to consist primarily of memory T-cells *i.e.* CD45RB^{low} (Lee & Vitetta, 1991), Pgp-1⁺ (Budd *et al.*, 1987; Butterfield *et al.*, 1989) and possibly some activated blast like cells. The splenocyte population contains a range of cells capable of presenting BRV(UKtc) antigen to T-cells (see Chapter 2). At this stage in the project it was not known which cell was responsible.

Antigen specific lymphoproliferative responses have been measured successfully for a number of years using medium supplemented with heterologous serum (*e.g.* Schwartz *et al.*, 1975). Responses to viral antigens have also been detected using this form of assay (Mathews & Roehrig, 1989; Ertl *et al.*, 1989; Ou *et al.*, 1992). However, the results obtained using this type of medium system and Con.A mitogen suggested that it was unsuitable for the detection of *in vitro* proliferative responses to BRV(UKtc) antigen. The use of homologous mouse serum supplemented medium was particularly advantageous since a better signal-to-noise ratio was obtained for both antigen specific and mitogen responses, particularly at low concentrations.

9.6 CONCLUSIONS

Mouse splenocytes (most probably T-lymphocytes) are capable of proliferating in response to *in vitro* challenge with a heterologous rotavirus (BRV(UKtc)), following primary oral inoculation of the animals, 8 days previously. The response is specific to inoculated animals and to the priming virus.

RPMI 1640 supplemented with 1% BALB/c mouse serum rather than 10% FCS provides a more sensitive assay system with which to measure these responses.

CHAPTER 10

**ANALYSIS OF THE SPECIFICITY
OF THE PROLIFERATIVE
RESPONSE**

10.1 INTRODUCTION

At the commencement of this thesis very little work had been performed on the Th-cell response to rotavirus and none of this work had demonstrated specifically, the nature of the responding cells or analysed any other aspects of the respective systems in a detailed fashion.

There was a report on the response to homologous virus in mice (Riepenhoff-Talty *et al.*, 1983), two reports on the response of human PBMC's to *in vitro* rotavirus challenge (Totterdell *et al.*, 1988; Totterdell *et al.*, 1988a) and two reports on the response of bovine PBMC's to *in vitro* rotavirus challenge (Archambault *et al.*, 1988; Archambault *et al.*, 1988a).

In view of these publications and having developed an efficient proliferation assay system with which to measure responses to BRV(UKtc), a detailed analysis of this system was undertaken.

10.2 DEPENDENCY OF THE SPLENOCYTE PROLIFERATIVE RESPONSE ON ANTIGEN PROCESSING

Several observations (see Chapter 9) suggested that the splenocyte proliferative response obtained to *in vitro* rotavirus challenge was due to activated BRV(UKtc) specific T-cells. Since T-cells are dependent on processed antigen for activation to occur (Shimonkevitz *et al.*, 1983; Brodsky & Guagliardi, 1991), it was decided that this proposal could be tested by inhibiting the antigen processing pathway. Previous work using the lysosomotropic agent, NH₄Cl (Ziegler & Unanue, 1982; Jones *et al.*, 1990), showed that this was an efficient inhibitor of T-cell specific responses. It is thought to raise the intra-cellular pH of endosomal compartments in the APC (Ziegler & Unanue, 1982), thereby inhibiting the action of endosomal proteases, required for the degradation of the endocytosed antigen (Van Noort *et al.*, 1991).

Based on these reports, experiments were set up, in which splenocytes from orally challenged animals were cultured with BRV(UKtc) antigen in the

presence or absence of NH₄Cl, for 3 days. A representative example of one of these experiments is shown in Fig. 17.

As can be seen, in the absence of 10mM NH₄Cl (Fig. 17(a)) a dose response to BRV(UKtc) antigen was obtained with an SI of 4.5 at the optimum antigen concentration of 1.0ug/ml. In contrast, when splenocytes containing BRV(UKtc) primed cells were incubated in the presence of 10mM NH₄Cl and antigen (Fig. 17(b)), no dose response was obtained. However, a good dose response to Con A mitogen was present with an SI value of 92.1 at 5ug/ml. These data suggested that the splenocyte proliferative response to rotavirus antigen was dependent on endosomal processing, since the presence of NH₄Cl in the culture medium had effectively abrogated the antigen specific proliferative response.

These results suggested that (i) the response observed was dependent on T-cells since B-cells do not require processed antigen for stimulation (Benjamin *et al.*, 1984; Male *et al.*, 1987) and (ii) that BRV(UKtc) did not induce cellular proliferation as a mitogen but required endosomal protease digestion, so as to produce immunogenic peptides for presentation. The exocytic MHC class II pathway is known to intersect with the endocytic pathway of antigen degradation (Germain, 1986; Guagliardi *et al.*, 1990; Neefjes *et al.*, 1990) and therefore the results also suggested that the response was MHC class II restricted, as reported previously for *Listeria monocytogenes* (Ziegler & Unanue, 1981). Hence, the results also implied that the proliferative response was dependent on CD4⁺ Th-cells (Unanue & Allen, 1987).

Endocytic activity is reported to be poor in dendritic cells (Austyn, 1989) and consequently, these results also suggested that the majority of BRV(UKtc) antigen was presented either by B-cells, or macrophages (Scheerlinck *et al.*, 1991). On secondary exposure to antigen, B-cells (specific to that antigen) are very important presenters, particularly at low concentrations (Rock *et al.*, 1984; Abbas *et al.*, 1985).

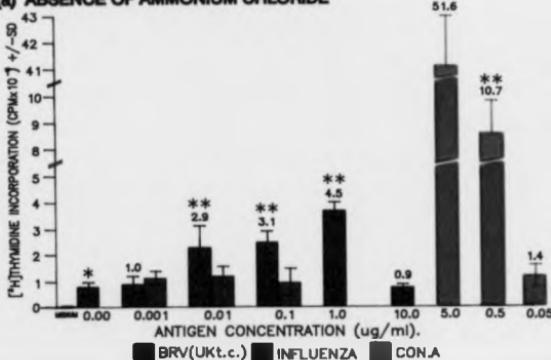
Thus, *in vitro* secondary responses to BRV(UKtc) were dependent on an endosomal mediated, antigen degradation step, strongly favouring the CD4⁺ Th-cell as the responding cell.

FIGURE 17 LEGEND

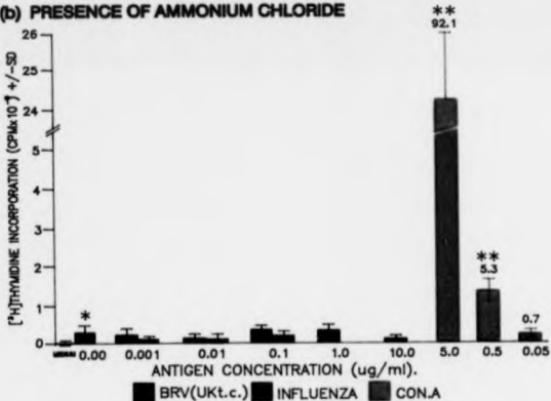
Adult animals (3) were each orally inoculated with 1.55×10^6 pfu of BRV(UKtc) and 8 days later were sacrificed. Splenocytes were cultured with either viral antigen or Con.A (Fig.17a) (using the same protocol described for Fig.14), or pre-incubated with 10mM NH₄Cl for 2hr at 37°C. Following washing, these cells were incubated with viral antigen or Con.A for 3 days, in RPMI 1640 (1% BALB/c M.S.) containing NH₄Cl (10mM) (Fig.17(b)). The medium was then changed and the cells were pulsed with [³H]thymidine for 18hr, as described for Fig.14.

Fig. 17
**EFFECT OF AMMONIUM CHLORIDE ON THE SPLENOCYTE
 PROLIFERATIVE RESPONSE TO BRV(UKt.c.)**

(a) ABSENCE OF AMMONIUM CHLORIDE



(b) PRESENCE OF AMMONIUM CHLORIDE



10.3 THE SPLENOCYTE PROLIFERATIVE RESPONSE TO DOUBLE AND SINGLE SHELLED FORMS OF BRV(UKtc)

Rotaviruses exist in two forms; as double-shelled (ds) particles (possessing the outer capsid proteins VP4 and VP7) and single-shelled (ss) particles (lacking the outer capsid proteins) (Bridger & Woode, 1976; Prasad *et al.*, 1990) (see Chapter 1). Since the optimum concentration of BRV(UKtc) varied between assays, the possibility existed that the ratio of ds:ss particles varied in the different batches of virus used for *in vitro* stimulation, affecting the optimum antigen concentrations observed. In order to establish if the *in vitro* splenocyte proliferative response was different for each particle type, ds and ss particles were prepared on CsCl gradients and removed separately. Single-shelled particles were treated with 5 mM EGTA, to ensure complete conversion to the ss form.

Splenocytes from BRV(UKtc) primed animals were challenged with either ds BRV(UKtc) or ss BRV(UKtc), along with controls. Previous experiments (data not shown) determined that the particle:Pfu ratio for BRV(UKtc) was of the order of 10^3 - 10^5 :1. Therefore, inoculated mice always received a larger dose of ss particles than ds particles.

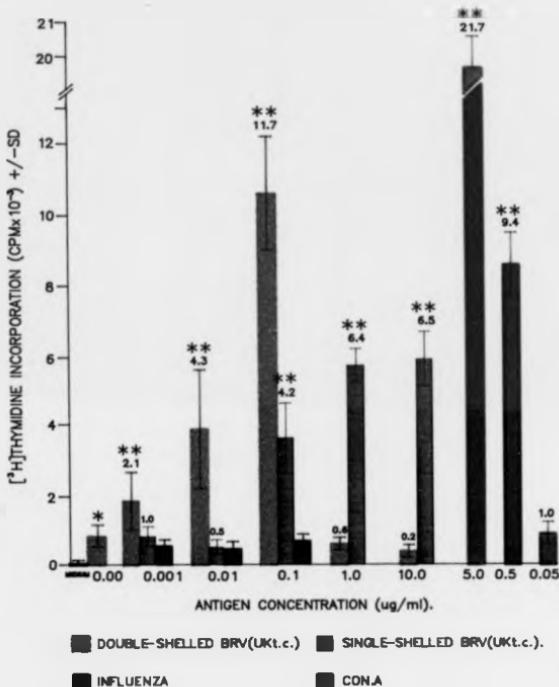
It is apparent (Fig. 18), that the splenocyte proliferative response to each particle type was distinctly different. The results were reproducible and suggested that T-cell stimulatory epitopes were present in both ss and ds forms of the virus but ds particles appeared to be more efficient as a T-cell stimulant *i.e.* 100 fold less ds virus was required to induce an optimal proliferative response when compared with ss virus. Since the infectivity of rotaviruses depends on the presence of the outer capsid proteins VP7 and VP4 (Bridger & Woode, 1976; Elias, 1977) the results also showed that non-infectious virus was capable of stimulating a proliferative response in mouse splenocytes. Furthermore, they indicated quite clearly that proteins other than VP7 and VP4 were capable of stimulating a splenocyte proliferative response and therefore a T-cell proliferative response.

FIGURE 18 LEGEND

Adult BALB/c mice were orally inoculated with 10^7 Pfu of BRV (UKtc) and 8 days later the spleens were removed. Splenocytes were challenged with either double-shelled BRV(UKtc), single-shelled BRV(UKtc), influenza or Con.A and cultured as in Fig. 14.

Fig. 18

RESPONSE OF SPLENOCYTES TO IN VITRO CHALLENGE
WITH DOUBLE AND SINGLE-SHELLED FORMS OF BRV(UKt.c.)



The question which could now be addressed was whether the differential response was due to the infectivity of the ds particle.

10.4 VIRUS REPLICATION AND THE IMMUNE RESPONSE

10.4.1 VIRUS REPLICATION AND THE PROLIFERATIVE RESPONSE

It had already been determined that BRV(UKtc) (at the doses given), did not appear to initiate a complete replication cycle in the intestines of adult mice i.e. the production of large numbers of infectious progeny virions (see Chapter 8). However, low level abortive replication with the expression of BRV(UKtc) proteins may have preferentially induced responses to different rotavirus proteins and hence responses to the two different particle types. In order to establish if replication (of any kind) affected the response obtained to ds and ss particles, animals were orally primed with beta-propio-lactone (BPL) inactivated BRV(UKtc). Infectivity assays showed that following BPL treatment, the virus titer was reduced from 10^8 PFU/ml to <5 PFU/ml (data not shown). Fig. 19 shows a representative example of one of these experiments. Clearly, a splenocyte proliferative response to BRV(UKtc) was induced when the virus used for priming was inactivated. There was a slight shift in the optimum concentration of ds virus when compared with results in Fig. 18 but it is clear that whether using non-inactivated BRV(UKtc) (Fig. 18) or inactivated BRV (UKtc) (Fig. 19) for inoculation, ds virus is a more efficient antigen. For example, the concentration of ds BRV (UKtc) required for optimal splenocyte proliferation was consistently less than the concentration of ss BRV(UKtc) required for optimal proliferation. Additionally, the SI's for ds BRV(UKtc) were at least 2 fold higher than the SI's for ss BRV(UKtc) at antigen concentrations of 0.001-0.1ug/ml (Fig. 18) and 0.001-1.0ug/ml (Fig. 19).

The efficiency of the ds particle in the induction of splenocyte proliferative responses was not, therefore associated with its ability to replicate. Furthermore, abortive replication of BRV(UKtc) in the adult mouse gut (if it occurred) did not appear to influence either (i) the induction of T-cell proliferative responses as detected in the spleen or (ii) the nature of the

FIGURE 19 LEGEND

Adult BALB/c mice (3) were each inoculated with a beta-propio-lactone inactivated preparation of BRV(UKtc) (originally containing 10^7 pfu), by the oral route and 8 days later were sacrificed. Splenocytes were cultured with either double-shelled BRV(UKtc), double-shelled OSU, single-shelled BRV(UKtc), influenza or Con.A and pulsed with [^{3}H]thymidine for 18hr, using the protocol described for Fig.14.

TABLE 13 LEGEND

Blood from the mice inoculated in Fig.19 was collected and the serum heat inactivated at 56°C for 30 min.. Plaque reduction neutralization assays using BSC-1 cells were set up to ascertain the presence of serum neutralizing antibody to BRV(UKtc). Antibody titer is expressed as the reciprocal of the serum dilution showing a 50% reduction in the mean plaque count for BRV(UKtc).

Fig. 19

SPLENOCELLULAR PROLIFERATIVE RESPONSE TO BRV(UK)c AND OSU FOLLOWING ORAL INOCULATION WITH INACTIVATED BRV(UK)c

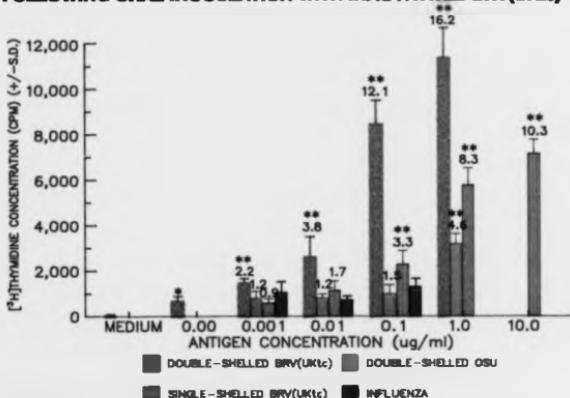


TABLE 13

SERUM NEUTRALIZING ANTIBODY RESPONSE TO BRV(UK)c FOLLOWING ORAL INOCULATION WITH INACTIVATED BRV(UK)c

MOUSE	SERUM NEUTRALIZING ANTIBODY TITER
1	<20.0
2	<20.0
3	<20.0

proliferative response obtained i.e. the magnitude of the response. These results may suggest that proliferative responses are probably initiated primarily by the viral antigen used to inoculate the animals i.e. there is very little or no amplification of the viral antigen in the intestine, by virus replication.

10.4.2 VIRUS REPLICATION AND THE SERUM NEUTRALIZING ANTIBODY RESPONSE TO BRV(UKtc)

Sera from the animals, orally inoculated with BPL inactivated virus, were screened for the presence of nAb to BRV(UKtc). Table 13 shows an example of the titers obtained and reveals that serum nAb to BRV (UKtc) could not be detected following oral inoculation with inactivated virus, in contrast to inoculation with infectious virus (see Chapter 7). Others, (Offit & Dudzik, 1989) have also reported that inactivated rotavirus (when orally inoculated into adult mice) does not induce a serum nAb response.

Oral inoculation of mice with inactivated BRV(UKtc) would appear therefore, to affect the humoral immune response to the virus but not the splenocyte proliferative response.

10.5. THE NATURE OF THE RESPONDING CELL(S) IN THE SPLENOCYTE PROLIFERATION ASSAYS

10.5.1 THE CELL TYPES RESPONDING TO SINGLE-SHELLED BRV(UKtc), FOLLOWING ORAL INOCULATION OF BRV(UKtc)

The circumstantial evidence presented in Chapter 9 and the endosomal processing inhibition experiments (section 10.2) strongly implied that T-cells were the responding cells in the proliferation assays. The type of T-cell involved in this response was therefore investigated by a series of cell depletion experiments. Splenocytes from animals challenged orally with BRV(UKtc) were cultured with as BRV(UKtc) at 1.0ug/ml, for 3 days. They were then washed in medium and treated with either complement, or a range of different T-cell specific Ab's and complement. [³H]thymidine incorporation was

determined for the cells subjected to each type of culture condition. These experiments proved difficult since (i) the number of responding cells in the splenocyte population was not particularly high and (ii) the various treatments led to a loss in cell viability. However, multiple attempts generated a series of reproducible results, an example of which is shown in Fig. 20. As can be seen, few cells were affected by the presence of complement alone. However, incubation of the splenocytes with anti-Thy1.2 and complement reduced the incorporation level by over 80%. This showed quite clearly that the splenocyte proliferative response to ss BRV(UKtc) was mediated by and dependent almost exclusively on T-cells. CD8⁺, ss BRV(UKtc) specific T-cells constituted 40% of the proliferative response detected. In the presence of anti-CD4 Ab and complement the response was reduced to the level of the anti-Thy1.2. (+ complement), treated cells. This data therefore suggested that CD4⁺ (ss BRV(UKtc) specific) T-cells also contributed ~40% of the proliferative response detected.

However, since CD8⁺ cell proliferation (i.e. 40% of the response) was not observed in the anti-CD4 and complement, treated cell population, this strongly implied that CD8⁺, ss BRV(UKtc) specific T-cells were dependent on the CD4⁺ cells for proliferation. The dependency of virus specific CD8⁺ cell activation on CD4⁺ cells has also been reported for other virus systems (Ciavarra, 1990).

These results suggested that the CD4⁺, ss BRV(UKtc) specific T-cell, was efficiently induced in GALT, upon stimulation with BRV (UKtc). CD4⁺ responses to ss BRV(UKtc) (at least) were, therefore expected to be present in GALT induction sites such as the PP's and MLN's (James *et al.*, 1990), following oral priming with the virus.

FIGURE 20 LEGEND

Adult mice (3) were orally inoculated with 10^7 Pfu of BRV-(UKtc) and 8 days later were sacrificed. Spleenocytes were cultured with single-shelled BRV(UKtc) at 1.0ug/ml for 3 days in RPMI 1640 (1% BALB/c M.S.). The cells were washed in RPMI 1640 (5% FCS) (HEPES) (1x) and then incubated with either anti-Thyl.2 mAb, anti-CD4 mAb, anti-CD8 mAb or medium alone for 30 min. at 4°C. The cells were washed as above and then incubated with either rabbit complement (low toxicity) or medium alone, for 45 min. at 37°C. The cells were then washed in RPMI 1640 (5% FCS) (HEPES) (1x) and RPMI 1640 (10% FCS) (HEPES) (1x). Finally the cells were plated out, pulsed with 1.25 uCi of [³H]thymidine and incubated for 18hr at 37°C, prior to harvesting and counting.

FIGURE 21 LEGEND

Mice (3) were inoculated with 10^7 Pfu of BRV(UKtc) by the i.p. route and 8 days later were sacrificed. Spleenocytes were cultured and treated as above with the exception that cells were initially incubated with single-shelled BRV(UKtc) (1.0ug/ml), for only 1.5 days.

Fig. 20

DEPLETION ANALYSIS OF THE RESPONDING CELLS IN THE
SPLENOCYTE POPULATION FOLLOWING ORAL INOCULATION WITH
EBV(UR3e)

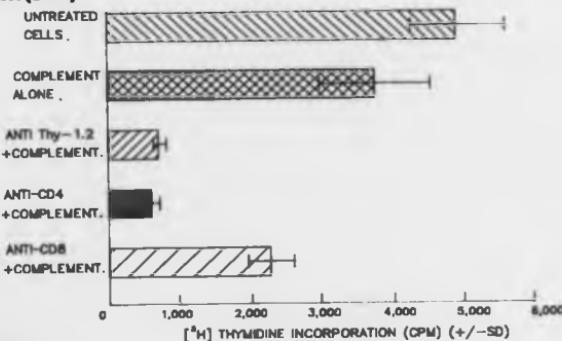
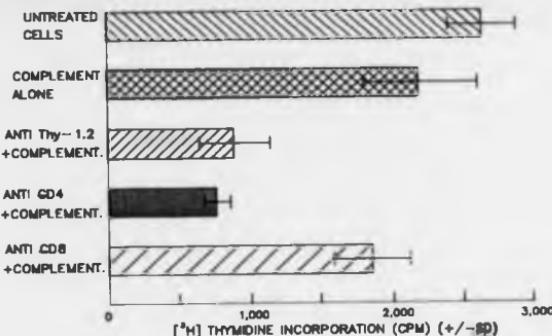


Fig. 21

DEPLETION ANALYSIS OF THE RESPONDING CELLS IN THE
SPLENOCYTE POPULATION FOLLOWING INTRA-PERITONEAL
INOCULATION WITH EBV(UR3e)



10.5.2 THE CELL RESPONDING TO SINGLE-SHELLED BRV(UKtc) FOLLOWING INTRA-PERITONEAL INOCULATION

Mice inoculated with BRV(UKtc) by the i.p. route showed a high and consistent serum nAb response to the virus (see Chapter 7). This suggested that BRV(UKtc) specific CD4⁺ cells were efficiently activated by this route.

Splenocytes derived from animals inoculated i.p. with BRV(UKtc), were cultured with ss BRV(UKtc) for 1.5 days (Fig. 21) and 3 days (data not shown) and depletions were carried out as above. A similar pattern in the depletion profiles was obtained when compared with the results for the orally challenged animals (Fig. 20). However, CD8⁺ cells contributed to only 13.9% of the response when challenged by the i.p. route. This was also seen for the 3 day cultures. The CD4⁺ contribution to the proliferative response was therefore higher (52.3%) when animals were challenged by the i.p. route than when orally challenged.

The results indicate that the CD4⁺ contribution to the proliferative response can be controlled by the route of antigen priming.

10.5.3 LYMPHOKINE SECRETION BY BRV(UKtc) SPECIFIC T-CELLS

As a further means of assessing (i) the ability of BRV(UKtc) to induce T-cell activation and (ii) the effects of BRV(UKtc) T-cell activation, the presence of lymphokines in the supernatants of splenocytes cultured in the presence of BRV(UKtc), was determined. Ifn-gamma was chosen as the marker of T-cell activation since it indicates the presence of CD4⁺ Th1 cells (Mosmann *et al.*, 1986) and possibly CD8⁺ T-cells (Morris *et al.*, 1982) and has a major role in influencing the immune response to foreign antigen (see Chapter 3).

Following *in vitro* stimulation of splenocytes (from orally primed animals), with either ds or ss BRV(UKtc) and controls, the supernatants were removed and the presence of biologically active Ifn-gamma was determined. At 8 days p.i., Con.A induced a high level of Ifn-gamma synthesis with 50 Units (U)/ml at 5ug/ml and both ds and ss BRV(UKtc) induced Ifn-gamma synthesis in the splenocyte population (Table 14(a)). In general, the levels of Ifn-gamma

TABLE 14(a) LEGEND

Animals (3) were each primed with 10^7 Pfu of BRV(UKtc) by the oral route and 4 days or 8 days later were sacrificed. Splenocytes were cultured with either double-shelled BRV-(UKtc), single-shelled BRV(UKtc), influenza or Con.A for 3 days and the supernatants (20ul), collected and stored. Interferon assays based on the inhibition of the incorporation of [3 H]uridine into Semliki Forest Virus RNA were then performed using these samples and controls.

TABLE 14(b) LEGEND

Adult, uninoculated mice were sacrificed and the splenocytes cultured with either viral antigen or mitogen, as for TABLE 14(a). Interferon assays were then set up as above.

TABLE 14

INTERFERON GAMMA PRODUCTION BY MURINE SPLEENOCYTES IN RESPONSE TO IN VITRO CHALLENGE WITH EBV(UR3)

(a) EBV(UR3) PRIMED MICE

PANTHERING (ug/ml)	INTERFERON GAMMA UNITS/ml							
	D.S. EBV(UR3)/c		S.E. EBV(UR3)/c		INFLUENZA		CONA	
	0.005 p.i.	0.01 p.i.	0.05 p.i.	0.1 p.i.	0.5 p.i.	1.0 p.i.	5.0 p.i.	30.0 p.i.
10.0	10.0	<3.0	12.6	<3.0	—	—	—	—
1.0	4.0	<3.0	4.0	<3.0	—	—	—	—
0.1	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	—	—
0.01	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	—	—
0.001	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	—	—
0.00	<3.0	<3.0						
5.0							50.1	30.0
0.5							15.9	<3.0
0.05							<3.0	<3.0

(b) UNINOCULATED MICE

PANTHERING (ug/ml)	INTERFERON GAMMA UNITS/ml				
	D.S. EBV(UR3)/c		S.E. EBV(UR3)/c		INFLUENZA
	0.005 p.i.	0.01 p.i.	0.05 p.i.	0.1 p.i.	—
10.0	<3.0	<3.0	<3.0	<3.0	—
1.0	<3.0	<3.0	<3.0	<3.0	—
0.1	<3.0	<3.0	<3.0	<3.0	—
0.01	<3.0	<3.0	<3.0	<3.0	—
0.001	<3.0	<3.0	<3.0	<3.0	—
0.00	<3.0				
5.0					30.0
0.5					<3.0
0.05					<3.0

detected were low compared with the Con.A control and could not be detected at concentrations of antigen below 1.0ug/ml.

Ifn-gamma was not detected in the supernatants of splenocytes derived from uninoculated mice, when challenged with BRV(UKtc) or influenza (Table 14(b)). This showed that the Ifn activity detected, using cells from inoculated animals, was due to Ifn-gamma and was derived from antigen activated T-cells.

The cells used for these assays were also pulsed with [³H]thymidine and the level of proliferation determined (data not shown) (producing results virtually identical to those shown in Fig.18). It was noticeable that for ss BRV(UKtc) stimulation the [³H]thymidine incorporation levels correlated with number of Ifn-gamma U's i.e. there was an increase in both moving from 1.0ug/ml to 10.0ug/ml. In contrast, the [³H]thymidine incorporation values for 10.0ug/ml of ds BRV(UKtc) were low (see Fig.18 for a reference), whereas the number of Ifn-gamma U's was approximately equal to the number for ss BRV(UKtc), at this concentration (Table 14(a)).

Ifn-gamma production was also assessed at 4 days post oral inoculation with BRV(UKtc) (Table 14(a)). Apparently, Ifn-gamma was not detected for antigen stimulated cells at any concentration but their viability was shown by the detection of 30U/ml of Ifn gamma at 5ug/ml of Con.A.

The results showed that by 8 days post oral inoculation with BRV(UKtc) Ifn-gamma could be induced in the splenocyte population, following *in vitro* challenge with either ds or ss forms of the virus. Therefore, either ds or ss forms of the virus could stimulate BRV(UKtc) specific T-cells. The results also suggested that Ifn-gamma secreting BRV(UKtc) specific T-cells were likely to be present in GALT, at 8 days p.i..

10.6 RESPONSE OF BRV(UKtc) SPECIFIC T-CELLS TO ANOTHER ROTAVIRUS SEROTYPE

Since Th-cells were reported to recognize cross-reactive epitopes in some virus systems (e.g. Uren *et al.*, 1987), in addition to serotype specific epitopes (Rothman *et al.*, 1989) it was considered important to ascertain if BRV(UKtc)

specific T-cells were capable of responding to a rotavirus of a different serotype.

Splenocytes from BRV(UKtc) primed mice were challenged *in vitro* with BRV(UKtc) (serotype 6), the porcine rotavirus OSU (serotype 5) and controls (Fig.22). As can be seen, the response to ds BRV(UKtc) was higher than the response to ds OSU, at each antigen concentration. In general the responses to ds OSU were ~2 fold less than the responses to ds BRV(UKtc). The optimum concentration of ds OSU and ds BRV(UKtc) was 1.0ug/ml with SI's of 5.5 and 9.8, respectively. Both the ds BRV(UKtc) and ds OSU particles appeared to be more efficient antigens than ss BRV (UKtc) in this experiment, since the SI's between 0.001 and 0.1 ug/ml were greater than or equal to 2 fold higher than those for the ss BRV(UKtc) particles.

These results suggest that cross-reactive T-cell proliferative epitopes are present in OSU (serotype 5) and BRV(UKtc) (serotype 6) and that cross-reactive T-cells can be induced following oral inoculation with rotavirus. Cross-reactivity between rotavirus at the CD8⁺ (CTL) level has been previously reported (Offit & Dudzik, 1988). The results also show that the serotype of the immunizing virus appears to affect the nature of the proliferative response obtained.

Inoculation of mice with inactivated BRV(UKtc) (Fig.19) did not appear to significantly affect the ability of splenocytes to respond to *in vitro* challenge with OSU, suggesting that cross-reactive T-cells can also be induced with inactivated virus in this system.

10.7. RESPONSE OF SPLENOCYTES TO *IN VITRO* CHALLENGE WITH BRV(UKtc) AT VARYING TIMES POST ORAL INOCULATION

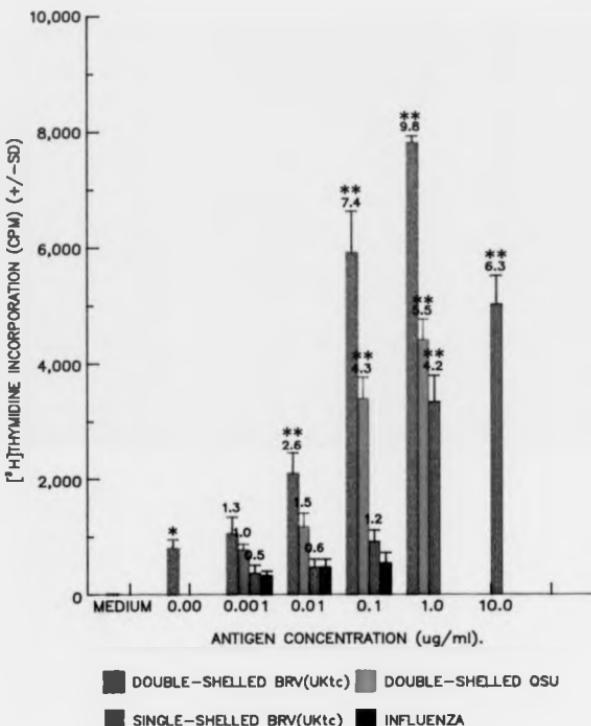
Most of the experiments conducted in the project utilized splenocytes derived from mice which were inoculated 8 days previously, by the oral route. However, it was decided that experiments should be conducted to assess the proliferative

FIGURE 22 LEGEND

Mice (3) were each orally inoculated with 5×10^6 Pfu of BRV(UKtc) and sacrificed at 8 days p.i.. Splenocytes were cultured in RPMI 1640 (lt BALB/c M.S.) with either double-shelled BRV(UKtc), double-shelled OSU, single-shelled BRV-(UKtc), influenza or Con.A (data not shown) and pulsed with [3 H]thymidine, using the method described in Fig.14.

Fig. 22

RESPONSE OF BRV(UKtc) PRIMED SPLENOCYTES TO OSU CHALLENGE



response at other time points, such that the kinetics of the T-cell proliferative response to BRV(UKtc) could be studied.

10.7.1 4 DAYS

Clearly, T-cell proliferative responses to both ds and ss BRV(UKtc) were present at 4 days post oral inoculation (Fig.23 (a)) and in general SI's for the antigen specific responses were lower at this time point when compared with those at 8 days p.i. (Fig.18 and 19). The optimum antigen concentration was the same for both ds and ss BRV(UKtc) and the SI value for the latter was higher than for ds virus.

10.7.2 15 DAYS

BRV(UKtc) specific T-cell proliferative responses were present in the spleen at 15 days p.i. (data not shown). The optimum BRV(UKtc) concentration was 10ug/ml with an SI of 18.7, possibly suggesting that the bulk of the virus used for *in vitro* stimulation was of the ss form (see Fig.18).

10.7.3 22 DAYS

A response to the priming virus was present, 3 weeks after oral inoculation (Fig.23(b)), suggesting that the T-cell proliferative response to BRV(UKtc) is long lived in adult BALB/c mice. In general the SI's were lower than those obtained at 15 days post oral inoculation and the optimum concentration for BRV(UKtc) was 0.1ug/ml.

10.7.4 56 DAYS

In the example shown in Fig.23(c), a T-cell proliferative response is present to both the ds and ss forms of the virus. The optimum concentrations of these two particle types are virtually identical to those at 8 days p.i. (Fig.18), although the SI values are lower.

FIGURE 23(a & b) LEGEND

Mice (3) were each inoculated with 10^7 Pfu of BRV(UKtc) by the oral route and 4 days later,(a) or 22 days later,(b) were sacrificed.Splenocytes were cultured in RPMI 1640 (1% BALB/c M.S.) with either double-shelled BRV(UKtc),(a),single-shelled BRV(UKtc),(a),BRV(UKtc) (pooled double and single-shelled particles),(b),influenza (a and b) or Con A (a and b) (data not shown),for 3 days.The medium was then changed and the cells were pulsed with [^{3}H]thymidine,using the method described for Fig.14.

Fig.23(a)
RESPONSE OF SPLENOCYTES TO BRV(UKc) AT 4 DAYS P.L.

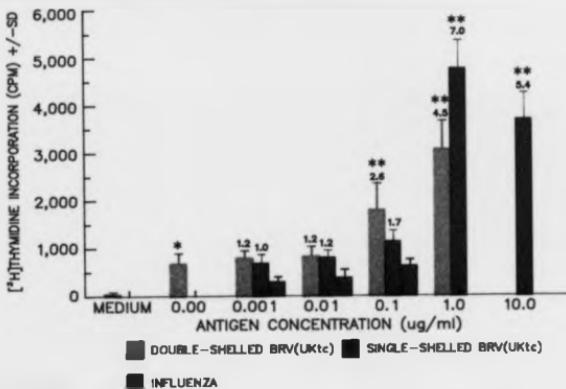


Fig.23(b)
RESPONSE OF SPLENOCYTES TO BRV(UKc) AT 22 DAYS P.L.

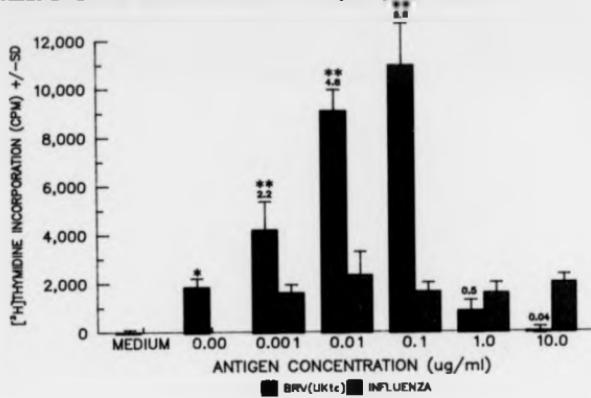


FIGURE 23(c & d) LEGEND

Mice (3) were orally inoculated with 10^7 Pfu of BRV(UKtc) and 56 days later,(c) or 144 days later,(d) were sacrificed. Splenocytes were cultured with either double-shelled BRV (UKtc) (c and d),double-shelled OSU (d alone),single-shelled BRV(UKtc) (c and d),influenza (c and d) or Con.A (c and d) (data not shown) and pulsed with [^{3}H]thymidine,using the method described for Fig.14.

Fig.23(c)

RESPONSE OF SPLENOCYTES TO BRV(UK)c AT 86 DAYS P.L.

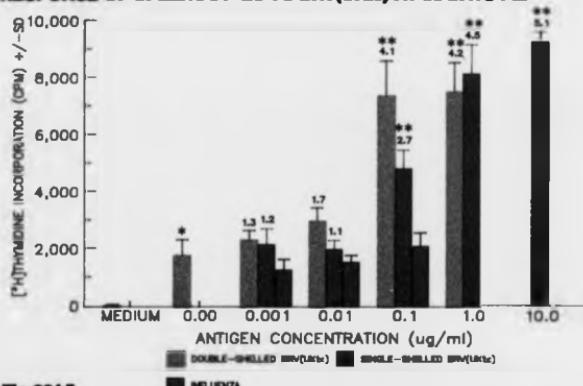
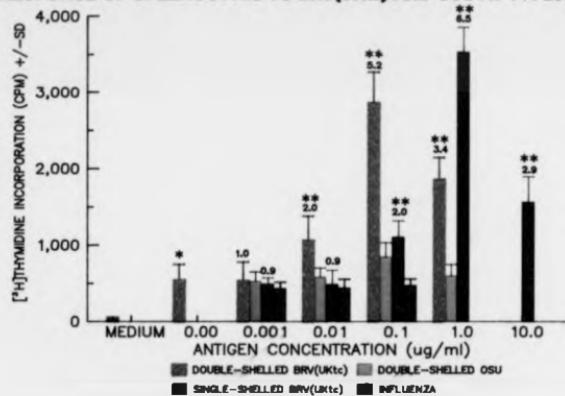


Fig.23(d)

RESPONSE OF SPLENOCYTES TO BRV(UK)c AND OSU AT 144 DAYS



10.7.5 144 DAYS

T-cell proliferative responses to BRV(UKtc) were detected at 5 months post oral inoculation (Fig.23(d)).The SI values were at a very similar level to those obtained at 2 months p.i. (Fig.23(c)) but of importance was the finding that the SI's for splenocytes challenged with ds OSU were not significant at any concentration, in contrast to the response obtained at 8 days p.i. for this antigen (see Fig.22).The optimum concentrations of ds and ss BRV(UKtc) were slightly different to those obtained at 2 months p.i.(Fig.23(c) and 8 days p.i. Fig.18).

The results obtained at 5 months p.i. confirmed that the T-cell response to BRV(UKtc) is long lived in the mouse,in contrast to the serum nAb response, which is not detectable by 56 days post oral inoculation (see Chapter 7). However,these results also showed quite clearly that T-cell responses to other rotavirus serotypes decreased with time,possibly suggesting that serotype specific determinants are more important in this response.

10.8 RESPONSE OF SPLENOCYTES TO *IN VITRO* CHALLENGE WITH BRV(UKtc) AND OSU AT 8 MONTHS POST INTRA-PERITONEAL INOCULATION

Experiments were set up to establish if splenocytes could respond to BRV(UKtc) and OSU challenge,following i.p. inoculation with BRV(UKtc),8 months previously.

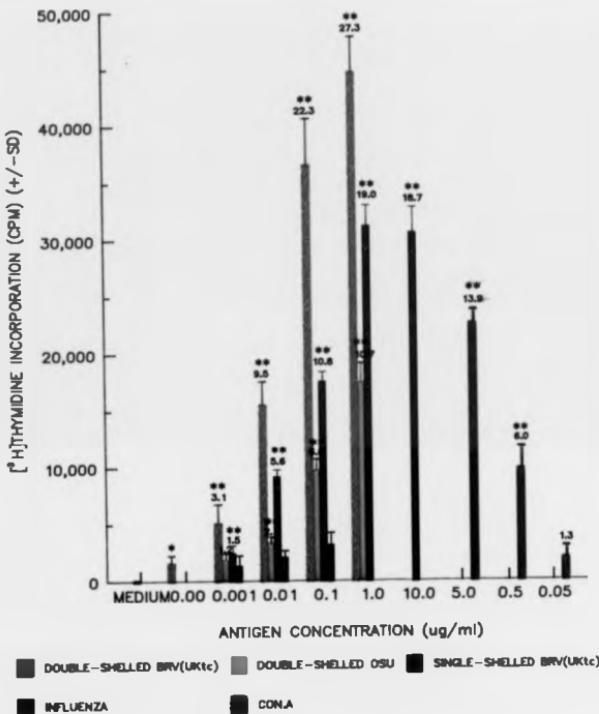
It is clear (Fig.24),that a T-cell proliferative response to both ds and ss BRV(UKtc) is present at this time point and the SI values are at least 2 fold higher than the responses at 5 months post oral inoculation (Fig.23(d)).Significantly, the response to OSU challenge was higher at 8 months post i.p. inoculation than at 5 months post oral inoculation (Fig.23(d)).Cross-reactive T-cells for OSU are thus present in significant numbers at 8 months post i.p. inoculation,in contrast to 5 months post oral inoculation (Fig.23(d)).The high SI values may be due to the large inoculum size used (4×10^7 Pfu/mouse) when compared with 10^7 Pfu given orally in most of the previous experiments.

FIGURE 24 LEGEND

Mice (3) were inoculated with 4×10^7 Pfu of BRV(UKtc) by the i.p. route and 8 months later were sacrificed. Splenocytes were cultured with either double-shelled BRV(UKtc), double-shelled OSU, single-shelled BRV(UKtc), influenza or Con.A, using the method described in Fig.14.

Fig. 24

**RESPONSE OF BRV(UK)c PRIMED SPLENOCYTES TO OSU AT 8
MONTHS POST INTRA-PERITONEAL INOCULATION**



Double-shelled BRV(UKtc) appears to more efficient at stimulating a T-cell proliferative response since the SI's for ds BRV(UKtc) are ~2 fold higher than those for ss virus at 0.001-0.1ug/ml of antigen. Therefore, irrespective of the route of priming, the ds form of BRV(UKtc) appears to be more efficient at stimulating T-cell proliferation in this system. Surprisingly, the optimal response to Con.A mitogen was less than the response to BRV(UKtc) in these experiments, which was not found in orally inoculated animals.

10.9 THE RELATIONSHIP BETWEEN SPLENOCYTE SI VALUES AND SERUM NEUTRALIZING ANTIBODY TITERS TO BRV(UKtc)

Since the presence of serum nAb was found to correlate with the T-cell proliferative response to BRV(UKtc) (at least at 8 days p.i.), the possibility existed that the level of T-cell stimulation (as determined by the SI value), correlated with the serum nAb titer to BRV(UKtc), at this and other time points. Analysis of the data (data not shown) suggested that a correlation between the two, was not apparent in this system. However, the absence of a T-cell proliferative response to the virus appeared to correlate well with the absence of a detectable serum nAb response, strongly suggesting the dependency of BRV(UKtc) specific serum nAb synthesis on T-cell stimulation.

10.10 DISCUSSION

Previous studies on the Th-cell response to rotavirus did not reveal the nature of the cell presenting rotavirus antigen. Using a complete population of splenocytes, provided a natural system in which the presentation of rotaviral antigen could occur. This system was chosen since it was not known which type of cell was responsible for the optimal presentation of rotaviral antigen, such that a T-cell response could be induced. The endosomal inhibition experiments strongly suggested that *in vitro* presentation of BRV(UKtc) antigen was mediated by B-cells and/or macrophages and the responding cells were T-cells. Thus, the nature of the stimulator and responder cell populations was revealed for secondary responses to BRV(UKtc) in the murine model.

The optimal antigen concentrations for ds BRV(UKtc) and ss BRV(UKtc) were distinctly different and this was reproducible over a range of time points and inoculation routes. The optimum ds particle concentration was consistently lower than that for ss particles, suggesting that the ds form of BRV(UKtc) was a more potent immunogen. There are two major reasons which may account for this. First, the immunogenicity of the proteins comprising the ds and ss particles may be responsible i.e. VP7 and VP4 present in the ds particles may be more readily recognized by T-cells than the proteins found in ss virus and therefore lower concentrations of ds particles are required. This may be a function of the efficiency with which the different proteins are processed (Gerhard *et al.*, 1991), the ability of the peptides to bind to MHC molecules and the efficiency with which the peptide-MHC complexes are recognized. Furthermore, proteins which may have been more readily recognized will therefore have induced a larger number of responding cells, to the proteins *in vivo*. Consequently, less antigen is probably required to stimulate their activation *in vitro*. Differences in the immunogenicity of viral proteins have been clearly illustrated with poliovirus (Kutubuddin *et al.*, 1992; Mahon *et al.*, 1992).

Second, the efficiency with which each particle type binds to and is taken up by the APC population may also account for the differential optima. Since antigen specific B-cells tend to be more efficient at antigen binding than other cells, particularly at low concentrations and on secondary exposure to antigen (Rock *et al.*, 1984; Abbas *et al.*, 1985), it is highly probable that a large proportion of the BRV(UKtc) particles are taken up and processed by BRV-(UKtc) specific B-cells. Therefore, variation in the optimal responses may reflect (i) the abundance of different B-cell populations i.e. anti-VP7, anti-VP4 and anti-VP6 specific cells and (ii) the avidity of these membrane immunoglobulins for their epitopes, in the relevant proteins. The importance of the APC in influencing T-cell responses was recently shown in a study of the response to the hepatitis B s-pre-S(2) particle (Scheerlinck *et al.*, 1991).

The variation in the optima cannot be accounted for by the responses of two different cell types since proliferation to ss BRV(UKtc) was shown to be due to both CD4⁺ and CD8⁺ cells. Similarly the ability of the ds particle to initiate replication can not account for this observation since the splenocytes from mice primed with inactivated virus showed similar differences in their optimal antigen concentrations.

A slight possibility is that B-cells may have responded to ds but not to ss particles in the splenocyte cultures. However, this is unlikely. The endosomal processing inhibition experiments and the detection of interferon-gamma, strongly suggested that the bulk of the proliferative response detected to ds particles was due to T-cells and not B-cells. Further evidence for this includes (i) the finding that VP7 induces T-cell proliferation (Andrew *et al.*, 1990) and (ii) the finding that both VP7 and VP4 induce CD8⁺ CTL responses (Offit *et al.*, 1991). Clearly T-cell epitopes are also present in proteins other than VP7 and VP4, since good CD4⁺ (and CD8⁺) proliferative responses were detected when stimulated with ss particles.

In reality the differences in optima observed for ds and ss BRV(UKtc) particles is probably influenced by both the efficiency of particle uptake and the immunogenicity of the viral proteins. Therefore, the optima are likely to be strongly influenced by both the number of BRV(UKtc) specific presenting cells and the number of BRV(UKtc) specific responder cells. Differences in the SI values at each optimum may also be, in part, a function of these two elements.

It was noted that the optimal concentration for each particle type appeared to vary at different time points (but the optimal concentration of ds virus was consistently lower than that for ss virus). In addition the response to ds OSU was lower than that for ds BRV(UKtc), using BRV(UKtc) primed animals. Both observations can be accounted for in terms of antigen specific stimulators and responders in the splenocyte population. For example, the response to ds OSU was lower because (i) ds OSU was unable to bind efficiently to BRV(UKtc) specific B-cells and/or because (ii) BRV(UKtc) derived peptides are more readily recognized by the T-cell population. However, irrespective of the

reason(s) for the lower response, the cross-serotype challenge results suggest that serotype specific determinants are particularly important in influencing the T-cell response to rotavirus. Further experiments are required to substantiate this proposal but it has been reported in other systems (Rothman *et al.*, 1989; Mathews & Roehrig, 1989).

The observation that the response to ds OSU was not significant at 5 months post oral inoculation with BRV(UKtc) was particularly interesting. These results showed that long term memory T-cell responses to rotavirus are also strongly influenced by the serotype of the priming virus. They therefore suggest that cross-reactive T-cell proliferative responses are not particularly long lived following primary oral inoculation with rotavirus. At 8 months post i.p. inoculation with BRV(UKtc) a response to ds OSU was detected but it was significantly lower than the response to ds BRV(UKtc), thus confirming the importance of serotype in influencing the T-cell proliferative response. However, it is clear that despite the importance of serotype, cross-reactive memory T-cells for different rotavirus serotypes can be efficiently induced following i.p. inoculation. The fact that responses to ds OSU were significant at 8 months post i.p. inoculation with BRV(UKtc), in contrast to 5 months post oral inoculation may have been a reflection of the number of responding cells induced by this route.

It is interesting to speculate at this point whether the 'broadening' of the Ab response to different rotavirus serotypes on repeated exposure (as reported by Brussow *et al.*, 1991) may be associated with an increased importance of these cross-reactive T-cell epitopes on repeated exposure to different serotypes. In the poliovirus system the induction of cross-reactive, polio-specific Th-cells has been proposed to enhance the primary nAb response to protective epitopes, following exposure to either a second serotype or a new virus variant (Katrak *et al.*, 1991). The importance of conserved (*i.e.* cross-reactive) epitopes in initiating Th-cell responses to virus was also recently revealed by Mahon *et al.*, (1992), in which the majority of Th-cell clones specific to poliovirus recognized conserved epitopes in the internal protein VP4. It is therefore

possible that the inner-capsid protein (VP6) of rotaviruses also possesses important Th-cell epitopes. Cross-reactive Th-cells have also been reported for two other rotaviruses; Wa (serotype 1) and NCDV (serotype 6) (Yasukawa *et al.*, 1990). A detailed understanding of the basis of T-cell cross-reactivity to rotavirus is essential for modern vaccine design.

CD4⁺ proliferative responses to BRV(UKtc) were efficiently induced in splenocytes following i.p. inoculation with the virus and this may suggest that GALT CD4⁺ cells specific for BRV(UKtc) can also be induced by this route since CD8⁺ CTL responses to rotavirus in GALT can be induced following i.p. inoculation (Offit & Dudzik, 1989a).

Memory T-cell responses to BRV(UKtc) were detected in the spleen following both oral and i.p. inoculation and this would suggest that memory T-cells specific for a particular antigen are not exclusively associated with the tissue in which priming occurred, as indicated by others (Shimizu *et al.*, 1992). However, further experiments are required to elucidate the proportion of BRV(UKtc) specific memory T-cells in different lymphoid tissues.

It was observed that interferon-gamma was readily detected in the supernatants from splenocytes, primed with BRV(UKtc) 8 days but not 4 days previously. This is somewhat puzzling since splenocyte proliferation was detected at 4 days p.i., to significant SI values. The reason for this may be due to (i) the absence of T-cell proliferation at this time point or (ii) the type of T-cell responding. It is highly unlikely that T-cell independent proliferation is responsible, since proliferation at later time points is clearly T-cell dependent. Therefore, it is possible that the proliferation detected is due to (i) non-interferon-gamma secreting CD8⁺ cells and newly activated CD4⁺ blast cells (*i.e.* not memory T-cells capable of high interferon-gamma synthesis (James *et al.*, 1990)), or (ii) simply newly activated CD4⁺ T-cells, alone. It is of course, possible that interferon-gamma is produced at 4 days p.i. but at levels below the threshold of detection. These results suggest that differences in the helper activity of the T-cell population, occur over time, irrespective of whether proliferation is detected.

At 8 days post oral inoculation, splenocytes stimulated with ds BRV(UKtc) at 10.0ug/ml did not proliferate but secreted interferon-gamma. The results of others correlate with this finding (Inoue *et al.*, 1989; Patarca *et al.*, 1991). This may suggest that lymphokine secretion is a better marker of antigen induced helper activity than proliferation. Indeed both IL-2 and IL-4 have been used as successful markers, by some (Katrak *et al.*, 1991). However, the results at 4 days p.i., suggest that both parameters give a more comprehensive picture of T-cell activation by antigen.

The fact that BRV(UKtc) specific T-cells secrete interferon-gamma may have important biological implications. For example it can upregulate MHC class II molecules on most cell types (Wong *et al.*, 1983; Scheynius *et al.*, 1986), influence the isotype of Ig produced (Coffman & Carty, 1986), increase adhesion to endothelial cell walls (Martin *et al.*, 1988), possibly via the induction of ICAM-1 expression (Pober *et al.*, 1986), upregulate the expression of SC on endothelial cells (Brandtzaeg *et al.*, 1988) and may be responsible for augmenting NK activity and other forms of non-specific cytotoxic activity (Yasukawa *et al.*, 1990), including macrophages (Paulnock, 1992). Since the T-cells responding to BRV(UKtc) in the spleen are highly likely to be derived from induction sites in GALT, this suggests that interferon-gamma secretion is also likely to occur in this tissue. In view of the effects of interferon-gamma, its presence in this tissue is probably very influential in both the induction of responses to BRV(UKtc) antigen and also in the trafficking of memory lymphocytes back to GALT.

Although the exact cell type responsible for interferon-gamma secretion was not determined, its presence may indicate that a large proportion of the CD4⁺ cells in the splenocyte cultures are Th1 cells (after 3 days in culture). Interferon-gamma preferentially inhibits the proliferation of Th2 but not Th1 cells (Gajewski & Fitch, 1988). This substantiates the proposal that B-cell activation in the *in vitro* proliferation assays is likely to be poor, since Th2 cells are primarily involved in Ab production (Moermann & Coffman, 1989). Interferon-gamma is also induced by IL-2, a Th1 product (Farrar *et al.*, 1981).

It was somewhat surprising that the SI values for the optimum Con.A responses at 8 months post i.p. inoculation were consistently less than for the antigen specific responses. This may have been due to the composition of the splenocyte population at this time point. It has been reported that memory T-cells (*i.e.* CD45RB^{low}, Pgp1⁺) show lower responses to Con.A and other lectin like mitogens than do 'naive' or virgin T-cells (James *et al.*, 1990; Miller *et al.*, 1991). It is therefore possible that the depressed response to Con.A was due to a large population of BRV(UKtc) specific memory T-cells in the spleens, which is suggested by the very high SI's obtained for BRV(UKtc) stimulated splenocytes.

An unusual observation was that BPL inactivated virus was capable of inducing a splenocyte proliferative response but not serum nAb to BRV-(UKtc), following oral inoculation. Since there is evidence that BPL can alter the structural integrity of outer-capsid proteins (Wiktor *et al.*, 1972; Offit *et al.*, 1989), it has been proposed (Offit & Dudzik, 1989) that the absence of serum nAb is due to a decreased efficiency in the ability of infectious (ds particles) to bind to villus epithelial and membranous (M) cells. However, the fact that T-cell proliferative responses can be detected in the spleen, suggests that a sufficient number of particles are able to bind to epithelial cells (for subsequent processing), to allow T-cell activation. Alternatively, these results may argue in favour of some form of productive replication in the intestinal cells of adult mice *i.e.* virus is normally produced which although non-infectious (see Chapter 8) is capable of stimulating B-cell Ab responses. Hence, when the virus used for inoculation is inactivated, this can not occur.

These results may suggest that the CD4⁺ cell contribution to the proliferative response to BRV(UKtc) was greater following inoculation with inactivated virus since Offit & Dudzik, (1989) showed that no detectable CD8⁺ (CTL) response was present in the spleen when this procedure was carried out. Further experiments would be necessary to substantiate this proposal. These findings have important implications for vaccine design since both nAb and CTL's have been shown to be important in protection against disease.

(Offit *et al.*, 1986; Offit & Dudzik, 1990; Dharakul *et al.*, 1990). A rotavirus vaccine capable of stimulating both 'arms' of the immune response is therefore desirable.

Together, these results strongly imply that a detailed analysis of the mechanisms of rotavirus uptake in the gut and the types of immune cell which are activated following different priming routes, is required as a strong basis for future vaccine strategies.

That the SI values and the serum nAb levels did not correlate exactly, is not surprising. The interferon-gamma results showed that good helper activity (lymphokine secretion) could occur in the absence of high proliferation. Furthermore, the SI values were obtained from pooled spleens. Values from individual spleens may have correlated more precisely with the serum nAb titers. These results do suggest, however, that an understanding of the efficiency of any vaccine can only be obtained by an in depth and comprehensive analysis of all the major immunological parameters. For example, the importance of T-cell activation in inducing protective Ab responses was clearly demonstrated for the Foot-and-Mouth-Disease-Virus (FMDV) (Collen *et al.*, 1989).

The data presented in this Chapter indicated that the induction of T-cell responses to rotavirus is a complex process and dependent on many closely interacting factors. The experiments described, now provide a basis with which to look more specifically at the CD4⁺ response to individual proteins of BRV-(UKtc) and to examine in detail the basis of the cross-reactive T-cell response. Preliminary experiments (data not shown) have indicated that VP7 of BRV-(UKtc), when expressed from a vaccinia construct (see Offit *et al.*, 1991), can induce T-cell proliferation. The results of Andrew *et al.*, (1990) correlate with this finding.

A heterologous system, such as described here would appear to provide an ideal system for examining the nature of the Th-cell response to rotavirus which may be important in the design of future recombinant rotavirus vaccines.

10.11 CONCLUSIONS

Oral inoculation of mice with BRV(UKtc) can induce both CD4⁺ and CD8⁺ BRV(UKtc) specific T-cells, capable of responding to the rotaviral antigen *in vitro*. Double-shelled BRV(UKtc) appears to be a more potent immunogen than ss-BRV(UKtc) in this system. *In vitro* stimulation of BRV(UKtc) primed splenocytes with either ds BRV (UKtc) or ss BRV(UKtc), induces interferon-gamma secretion. Cross-reactive epitopes exist between BRV(UKtc) (serotype 6) and OSU (serotype 5) but the cross-reactive response is not long lived following oral inoculation, in contrast to i.p. inoculation. However, the T-cell response to the serotype used for priming, is long lived following oral inoculation. The murine model provides an ideal system with which to study Th-cell responses to heterologous rotaviruses.

CHAPTER 11

**LYMPHOCYTE PROLIFERATIVE RESPONSES
IN GUT ASSOCIATED LYMPHOID TISSUE**

11.1 INTRODUCTION

Most of the work in this thesis focussed on lymphoproliferative responses to rotavirus in the spleen. An attempt was also made to establish a lympho-proliferation assay for analyzing the responses in GALT. A considerable degree of technical difficulty was encountered in working with GALT cells but since the mice were usually orally inoculated with virus it was considered important that this tissue was analyzed. Mesenteric Lymph Nodes (MLN's) (*i.e.* a GALT induction site (James *et al.*, 1980)) were chosen to look for evidence of lympho-proliferation to BRV(UKtc) in GALT.

11.2 MLN CELL PROLIFERATION TO CON.A

As a first means of assessing the proliferative responses of MLN cells to *in vitro* stimulation, MLN cells from uninoculated mice were cultured with Con.A over a range of concentrations in RPMI 1640 (1% BALB/c M.S.) using the same protocol as for splenocytes. This system proved unsuccessful since the results were inconsistent and Con.A stimulated cells frequently did not proliferate (data not shown). One possible explanation for this failure was that the mouse serum was toxic to the cells *i.e.* it either led to cell death or prevented optimal stimulation and response. Consequently, experiments were conducted in RPMI 1640 (10% FCS) HEPES (see Fig. 25). As can be seen, in this medium Con.A stimulated a good dose response with an optimum concentration of 5 μ g/ml, which correlates with the optimum for splenocytes (Fig. 13). Successful stimulation of MLN cells with Con.A strongly suggested that T-cell activation had occurred in this medium system (Tsien *et al.*, 1982) and that an APC was active (Hirayama *et al.*, 1987). However, it was clear that the signal to noise ratio was poor using this medium system.

11.3 LYMPHOPROLIFERATION TO BRV(UKtc) IN MLN'S AT 8 DAYS POST ORAL INOCULATION

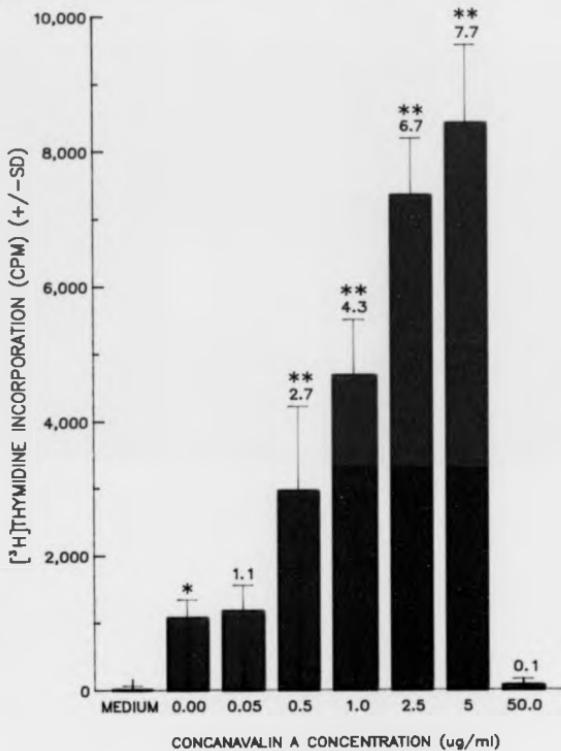
Animals were orally inoculated with infectious BRV(UKtc) and 8 days later the MLN's were removed. *In vitro* stimulation of the cells with BRV(UKtc)

FIGURE 25 LEGEND

Uninoculated adult BALB/c mice (3) were sacrificed and the mesenteric lymph nodes removed. Single cell suspensions were prepared and cells (at 4×10^5 /well) were incubated with various concentrations of Con.A, in RPMI 1640 (10% FCS) (BEPES) (in replicates of 6), for 3 days. They were then pulsed with 1.25uCi of [^{3}H]thymidine for 18hr, prior to harvesting and counting.

Fig.26

RESPONSE OF MESENTERIC LYMPH NODE CELLS TO CON.A STIMULATION



resulted in cellular proliferation (Fig.26 (a)),which was consistent and reproducible.Optimal proliferation occurred at 1.0ug/ml,with an SI of 4.0 in this assay.The specificity of the response was revealed by the absence of significant proliferation in the cells stimulated with influenza virus (A/CHR/68 H3N2).MLN cells from uninoculated mice were also challenged with *ss* BRV(UKtc) *in vitro* (data not shown) and no significant proliferative response was obtained (see Fig.14(b) for splenocytes),thus further confirming the specificity of the response.

In view of the dependency of the *ss* BRV(UKtc) specific proliferation on T-cells (using splenocytes) and the fact that 55-80% of lymph node cells are T-cells (Hunt,1987),the results strongly suggest that the proliferation detected at 8 days p.i. in the MLN's,was due to T-cells.

11.4 MLN LYMPHOPROLIFERATIVE RESPONSES TO ROTAVIRUS AT 63 DAYS POST ORAL INOCULATION

The MLN's were removed at this time point and cultured *in vitro* with a range of rotavirus antigen concentrations (2 serotypes),influenza and Con.A (Fig. 26(b)).Clearly,no antigen specific proliferative responses occurred at this time point,at any concentration.In contrast large responses to Con.A mitogen were present at 5ug/ml,indicating cell viability.The results suggest that rotavirus specific,memory T-cell proliferative responses are not present in MLN's at 63 days post oral inoculation

11.5 DISCUSSION

These are the first results showing that a heterologous rotavirus is capable of inducing GALT cell proliferative responses in mice.They strongly imply that rotavirus specific T-cells are present in MLN's at 8 days post oral inoculation.Although non-specific proliferation was high,significant SI's were obtained using the RPMI 1640 (10% FCS) medium system.The results also help to confirm the proposal in Chapter 9 that the majority of rotavirus specific T-cells in the spleen are derived from GALT induction sites.

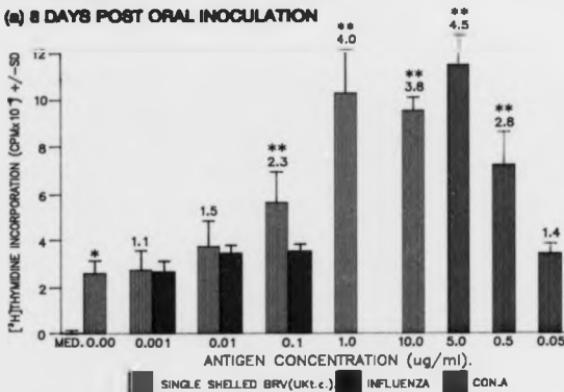
FIGURE 26(a & b) LEGEND

Mice (3) were orally inoculated with 10^7 PFU of BRV(UKtc) and 8 days later,(a) or 63 days later,(b) were sacrificed.The mesenteric lymph nodes (MLN's) were removed and single cell suspensions prepared.Cells were cultured with either double-shelled BRV(UKtc),(b),double-shelled OSU,(b),single-shelled BRV (UKtc) (a and b),influenza (a and b) or Con.A (a & b),for 3 days in RPMI 1640 (10% FCS) (HEPES).They were then pulsed and harvested as for Fig.25.

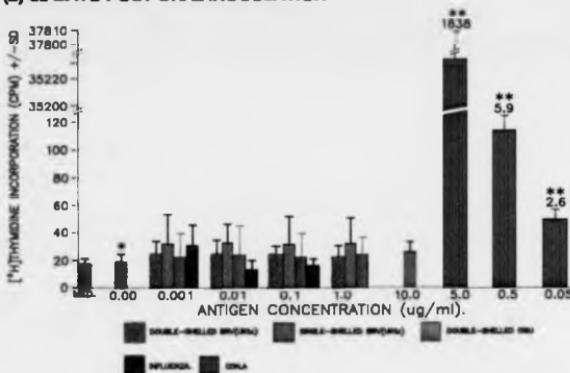
Fig.26

RESPONSE OF MESENTERIC LYMPH NODE CELLS TO BRV(UK)c AND CON.A AT:

(a) 8 DAYS POST ORAL INOCULATION



(b) 63 DAYS POST ORAL INOCULATION



The lack of an antigen specific proliferative response at 63 p.i. was an interesting result and may reflect a change in the type of cells present in this tissue. Since memory T-cell responses to BRV(UKtc) antigen were detected up to 144 days post oral inoculation in the spleen, this suggests that memory T-cells (if they are present in MLN cells) are not capable of proliferating in responses to rotavirus challenge. Since MLN's are GALT induction sites (James *et al.*, 1990), the lack of a rotavirus specific memory T-cell proliferative response is not particularly surprising. Indeed, they are proposed to consist primarily of 'naive' T-cells, in contrast to effector sites such as the lamina propria, which contains predominantly memory T-cells (James *et al.*, 1990). Responses in MLN's at 8 days p.i. are therefore possibly due to either (i) a small number of BRV(UKtc) specific memory T-cells or (ii) newly activated lymphoblasts. The high responses to Con.A mitogen and low responses to recall antigen at 63 days post oral inoculation in the MLN also helps to confirm the proposal that MLN cells at this time point are primarily naive T-cells (James *et al.*, 1990). However, it is of course possible that at this time point rotavirus specific T-cell activation has occurred *i.e.* lymphokines have been secreted but proliferation has not occurred (see Chapter 10 for interferon-gamma results).

The results clearly reveal that there is a differential response to rotavirus in different lymphoid tissues, over time.

11.6 CONCLUSIONS

Con.A mitogen can induce proliferation in MLN cells derived from uninoculated mice. BRV(UKtc) can induce MLN cell proliferation *in vitro*, following oral priming 8 days previously but not 63 days previously.

CHAPTER 12

**GENERAL DISCUSSION AND
FUTURE EXPERIMENTS**

12.1 INTRODUCTION

The results presented for BRV(UKtc) and the mouse model have clearly enhanced our understanding of both the nature of immune interaction following rotaviral inoculation and the natural biology of T-cell responses to rotavirus. The differential responses to ds and ss forms of the virus was a particularly interesting finding, as was the ability to induce different levels of CD4⁺ cell activation by different routes of priming. Of particular importance, however were the results on the nature of cross-reactive T-cell proliferative responses to rotavirus in splenocytes, following different routes of priming, over time.

It is perhaps relevant at this point to consider the work of others on Th-cell responses to rotavirus. Few detailed studies have been conducted on this aspect of the immune response to rotavirus and several of these have made use of poorly defined systems. Nevertheless, they provide useful comparisons for the work with BRV(UKtc) in mice.

12.2 LYMPHOCYTE PROLIFERATIVE RESPONSES TO ROTAVIRUS IN ANIMAL MODELS

A study by Riepenhoff-Talty *et al.*, (1983), attempted to correlate splenocyte proliferative responses with the cessation of diarrhoea, using murine rotavirus (MRV) in neonatal mice. Splenocyte proliferative responses to *in vitro* challenge with MRV were apparently demonstrated at various times over a 60 day period but were not present at 60 days post oral inoculation, unlike the response to BRV(UKtc). However, since no negative controls were shown, the specificity of the responses is uncertain. Furthermore, the proposal that the splenocyte proliferative response detected was solely responsible for the cessation of diarrhoea can not be substantiated since no data on the Ab response to the virus was provided. In general the system was poorly defined since no details of antigen purification or absolute concentrations were provided.

Studies with adult mice have shown that SA11 can induce splenocyte proliferative responses, following i.v. inoculation (Andrew *et al.*, 1990), suggesting that rotavirus can induce T-cell proliferative responses via many inoculation routes. Lymphokines from SA11 activated splenocytes were also capable of supporting proliferation of Con.A stimulated blasts, suggesting that rotavirus induced lymphokine induction is not restricted to BRV(UKtc). This paper also showed that the form in which VP7 is presented to the immune system (*i.e.* membrane bound, secreted or intracellular), strongly affects the T-cell responses. VP7 may therefore be important in the induction of Th-cell responses to rotavirus. Comparisons with other rotavirus proteins are obviously needed.

A novel method of analyzing Th-cell responses was provided in this paper (*i.e.* the infection of splenocytes with a vaccinia recombinant expressing a rotavirus protein), which overcomes the requirement for purified rotavirus proteins and may be useful for studying responses to BRV(UKtc).

Several studies have been conducted with an emphasis on veterinary applications. Archambault *et al.*, (1988a) showed that peripheral blood mononuclear cell (PBMC) proliferative responses to an attenuated BRV could be induced in 8 month old calves following several intra-muscular (i.m.) inoculations with live virus, providing the virus was emulsified with Incomplete Freund's Adjuvant (IFA). This study showed the importance of adjuvant in the induction of (T-cell) proliferative responses to rotavirus. However, no requirement for adjuvant was found when inoculating BRV(UKtc) by the i.p. route into mice, which may suggest that this is a better route for T-cell priming than the i.m. route.

The importance of inoculation route and the type of adjuvant was investigated further by Archambault *et al.*, (1988), in which proliferative responses to the Quebec BRV(17) strain, were analyzed. Inoculation of virus with various adjuvants into cattle (i.m.), induced proliferative responses in both PBMC's and colostral lymphocyte populations. In contrast oral inoculation of the virus (without adjuvant) resulted in no significant proliferation in either

PBMC's, or colostral lymphocyte's and no Ab response in the serum. This was a unusual finding (compared with the BRV(UKtc) results) and may have been due to the number of infectious particles in the inoculum or to an efficient inactivation or removal of infectious virus, in the intestine. However, more importantly, the animals were seropositive for rotavirus prior to use in this study and as a result cross-reactive Ab's in the gut may have interfered with antigen uptake.

However, clearly the results showed that i.m. challenge of rotavirus with adjuvant induced an efficient proliferative response and furthermore, showed that colostral mononuclear cells may have a role in protection against rotavirus disease.

In summary the work with cattle provided little detailed information on Th-cell responses but showed how (T) lymphocyte responses to rotavirus could be induced.

12.3 LYMPHOCYTE PROLIFERATIVE RESPONSES TO ROTAVIRUS IN HUMANS

Totterdell *et al.*, (1988) developed a lymphocyte transformation assay to rotavirus using PBMC's and lymphocytes from breast milk. Both showed proliferative responses when challenged with SA11, showing that a heterologous rotavirus can stimulate responses in humans. Circulating rotavirus specific PBMC's were shown to persist for a prolonged period, which correlates with the findings in mice. The study was primarily concerned with the establishment of a lymphoproliferation assay and no attempt was made to define the responding cell or monitor lymphokine secretion.

Totterdell *et al.*, (1988a) reported on PBMC proliferative responses to rotavirus in patients with various conditions. The results showed that rotavirus specific proliferation was reduced in most patients with some form of immunosuppression e.g. elderly patients, transplant patients.

Lymphoproliferative responses to rotavirus in whole blood were considered to be a measure of the number of circulating primed lymphocytes. It was

proposed, that susceptibility to rotavirus disease may, in part, be a function of this number. Ab responses to rotavirus were also considered to be dependent on T-cells.

Yasukawa *et al.*, (1990) showed that PBMC's from adult humans proliferated in response to U.V. inactivated Wa and NCDV but responses were not present in cord blood. It was proposed that cross-reactive epitopes between Wa and NCDV were present. However, the volunteers were seropositive for rotavirus and thus, assumptions about cross-reactivity are difficult to make (Matsui *et al.*, 1989; Estes & Cohen, 1989; Davies *et al.*, 1990).

The cells proliferating in this assay were CD3+, CD4+, CD8- i.e. Th-cells. Culture supernatants of rotavirus stimulated cells contained IL-2 and interferon-gamma, postulated to be important in the activation of NK cells and other non-specific cytotoxic lymphocytes. These results therefore correlate with the BRV (UKtc)/murine system, suggesting that the latter is an ideal model for the study of Th-cell responses to rotavirus in humans, with the distinct advantages that (i) the system is more manageable and (ii) seronegative subjects can be used. Indeed, the range of rotavirus serotypes which infect humans (see Chapter 1) makes analysis of natural Th-cell responses to rotavirus particularly difficult.

Recently, Offit *et al.*, (1992) developed a rotavirus specific PBMC transformation assay. Their results suggested that the detection of rotavirus specific proliferation in infants may more accurately determine previous exposure to rotavirus than the detection of rotavirus specific Ab's. They proposed that such an assay may be more useful for vaccine trials when trying to discriminate between active and passive immunity in infants in the first 6 months of life. This work also suggested that, like the nAb response, there is a broadening of the number of serotypes recognized by Th-cells, with increasing age. This data therefore supports the proposal in Chapter 10 that cross-reactive Th determinants may become more important on increased exposure to different rotavirus serotypes.

12.4 RESUME

Clearly lymphoproliferative responses to rotavirus can be demonstrated in a range of species and by inoculation via a variety of routes. Responses were demonstrated in splenocytes, PBMC's and colostral lymphocyte populations but no group presented data on the response of rotavirus in GALT cells. In addition, very little attention was given to (i) the use of seronegative systems, (ii) the nature of the responding cells, (iii) the response to different antigenic forms of the virus or (iv) the basis of T-cell cross-reactivity and its duration.

However, the results of others show a large degree of correlation with those presented for BRV(UKtc), which is particularly encouraging in the search for a representative model of Th-cell activation by rotaviruses.

12.5 TH-CELL RESPONSES TO OTHER VIRUSES

Th-cell responses have been studied more extensively and comprehensively in a range of other virus systems. For example, hepatitis B virus (HBV) (*Celis et al.*, 1984; *Milich et al.*, 1987; *Inoue et al.*, 1989; *Milich et al.*, 1990), influenza (*Scherle & Gerhard*, 1986; *Mills et al.*, 1986; *Graham et al.*, 1989; *Gerhard et al.*, 1991), polio (*Wang et al.*, 1989; *Katrak et al.*, 1991; *Kutubuddin et al.*, 1992; *Mahon et al.*, 1992), rabies (*Bunschoten et al.*, 1989; *Ertl et al.*, 1989), flaviviruses (*Uren et al.*, 1987; *Mathews et al.*, 1991; *Roehrig et al.*, 1992), FMDV (*Collen et al.*, 1989), measles virus (*Partidao & Steward*, 1990; *Partidao et al.*, 1991), bovine herpes virus (*Hutchings et al.*, 1990; *Hutchings et al.*, 1990a), human immunodeficiency virus (HIV) (*Hosamli et al.*, 1991; *Macatonia et al.*, 1991) and papilloma viruses (*Davies et al.*, 1990; *Tindle et al.*, 1991; *Shepherd et al.*, 1992). A brief survey of some of these findings is pertinent at this point, since they may have direct relevance to the work on Th-cell responses to rotavirus.

Of particular importance was the finding in the influenza system, that Th-cells recognize a very large number of different epitopes in the external HA molecule and are not restricted to a small number of epitopes in conserved regions (*Mills et al.*, 1986a). Many of these epitopes lay within variable Ab binding regions of the molecule. Further commonality of B and T-cell epitopes

was revealed by Barnett *et al.*,(1989),Comerford *et al.*,(1991) and Kutubuddin *et al.*, (1992) and at least some of the mutations in B-cell epitopes can affect Th-cell epitope recognition (Graham *et al.*,1989;Mahon *et al.*,1992).

The epitope recognition of several viral proteins including the HBV pre-S(2) region of the HBs antigen (Milich *et al.*,1990) and the L1 protein of human papilloma virus (HPV) 16 (Davies *et al.*,1990) has also been shown to be highly specific.Additionally,serotype specificity of Th responses has been reported for dengue virus (Rothman *et al.*,1989) and alphaviruses (Mathews & Roehrig, 1989).

In general,the results imply that the external capsid proteins play an important role in inducing Th-cell responses and therefore help,for B cell Ab production.However,'internal' viral proteins may also play a role.It has been determined that Th-cells specific for internal virus proteins can provide help for Ab production to outer capsid proteins (Russell & Liew,1980;Scherle & Gerhard,1986;Milich *et al.*,1987).This form of intrastuctural-intermolecular help may therefore be highly relevant to other virus systems such as the rotaviruses,since conserved regions of VP6 may be capable of fulfilling this role.Indeed,Katrak *et al.*,(1991) and Mahon *et al.*,(1992) have already proposed that Th-cells recognizing cross-reactive epitopes in the internal protein of poliovirus may be particularly important in stimulating B-cell responses to protective epitopes in the outer-capsid proteins,or exposure to a new virus serotype or variant.Polyclonal Th-cell responses to poliovirus are predominantly cross-reactive (Wang *et al.*,1989;Katrak *et al.*,1991) and the majority of poliovirus specific T-cell clones recognize cross-reactive epitopes on VP4 (Mahon *et al.*,1992).Conserved,cross-reactive epitopes also appear to be important in other systems (Uren *et al.*,1987;Levley *et al.*,1991).

These results show that both type specific and conserved, cross-reactive Th-cell epitopes can be important in stimulating helper responses to virus. However,the relative importance of each form of epitope in stimulating Th responses appears to vary with different virus systems.For example,intrastuctural-intermolecular help has not been demonstrated for the simian

immunodeficiency virus (SIV) (Mills *et al.*, 1991). Therefore, it is not possible to predict at this time which of the major rotavirus proteins *i.e.* VP7, VP4 (outer-capsid) or VP6 (inner-capsid) is most important in stimulating Th-cells.

It is possible that type specific and cross-reactive Th epitopes have slightly different roles *e.g.* cross-reactive epitopes (usually in inner-capsid proteins) may be more important on secondary exposure to antigen (Katrak *et al.*, 1991). The synthesis of peptides from regions of rotavirus proteins and the establishment of Th-cell clones would facilitate identification of type-specific and cross-reactive epitopes and their relative importance.

Many factors may influence the immunodominance of particular Th epitopes in viral proteins. The importance of haplotype in influencing CD4⁺ Th-cell responses has been studied (Celis & Karr, 1989; Barnett *et al.*, 1990) and it appears that the immunodominance of Th epitopes is influenced by the haplotype of the subject (Milich & McLachlan, 1988; Milich *et al.*, 1990; Alp *et al.*, 1991). It has been shown that at least some Th epitopes in viral proteins (*e.g.* the NP protein of influenza) maintain the same degree of immunodominance, irrespective of the route of antigen priming or whether infectious or non-infectious virus is used (Brett *et al.*, 1991). These findings may have relevance to the design of suitable rotavirus vaccines.

Of particular importance to the work in this thesis was the finding that Th epitopes identified in mice correlated with those in humans (Davies *et al.*, 1990; Partidos & Steward, 1990; Comerford *et al.*, 1991), thereby supporting the use of murine models for analysis of rotavirus Th epitopes. Of additional importance was the work of Gerhard *et al.*, (1991) and Tindle *et al.*, (1991) which showed that although some Th epitopes can be predicted using algorithms, many do not show typical Rothbard-Taylor motifs and can only be identified by experiment.

In the context of vaccines, various viral proteins and their epitopes have been strongly recommended as efficient inducers of Th-cell activity. They include the p148-174 region of the pre-S(2) region of HBs antigen (Milich *et al.*, 1990) (since it can bind to MHC class II molecules in a range of haplotypes) and the

HBc antigen (Milich *et al.*, 1987). An epitope (aa 48-54) has also been identified in the E7 protein of HPV 16 which is capable of providing help for Ab production to several B-cell epitopes simultaneously (Tindle *et al.*, 1991) and another peptide capable of binding to many MHC class II molecules and stimulating Th activity was identified in the E-glycoprotein of Murray-Valley Encephalitis Virus (Mathews *et al.*, 1991). A Th epitope capable of providing help for a range of heterologous B-cell epitope containing peptides, in the flavivirus system has also been identified (Roehrig *et al.*, 1992), which may have important implications in the design of peptide vaccines for other viral pathogens.

Work with potential synthetic peptide vaccines has revealed several important findings. First, peptide containing Th epitopes must be linked to the B-cell epitope to induce a response and second, the most efficient T-B epitope was obtained when the peptides were co-linearly synthesized (Roehrig *et al.*, 1992). Third, the orientation of the T-cell epitope with respect to the B-cell epitope can affect the response (Partidos *et al.*, 1991) and fourth, T-cell epitopes used for stimulating B-cell responses should also be ideally derived from the same pathogen or a very closely related pathogen as the B-cell epitope, since this provides a memory T-cell response (Partidos *et al.*, 1991; Roehrig *et al.*, 1992). The structural requirements of the peptide may reflect important antigen processing events in APC's.

Clearly synthetic peptides can and do induce efficient Ab responses to the whole virus. However, it has been found that some peptides can only prime T-cells for themselves and are incapable of stimulating a response to whole virus (Milich *et al.*, 1990). This has been proposed to be due to differences in antigen processing and other studies have shown that residues distinct from the immunodominant T-cell epitope can affect the outcome of T-cell activation (Krzycz *et al.*, 1985; Brett *et al.*, 1988). These results strongly suggest that a detailed and more comprehensive understanding of antigen processing is critical in the design of efficient recombinant and synthetic peptide vaccines.

12.6 FINAL RESUME

The results presented in this thesis have shown that Th-cell responses to heterologous rotaviruses can be effectively studied in the murine model and that at least some of the results may be relevant to vaccine strategies for this pathogen. Furthermore, they provide a firm basis for the CD4⁺ Th-cell epitope mapping of BRV(UKtc), an important element in the quest for a detailed understanding of the interaction between this pathogen and the immune response. In the light of other systems (Hoemalin *et al.*, 1991; Roehrig *et al.*, 1992; Snijders *et al.*, 1992) and the work discussed above, the development of future peptide vaccines for rotavirus will probably rest heavily on epitope mapping.

12.7 FUTURE EXPERIMENTS

Having established a successful model with which to examine Th-cell responses to rotavirus, a range of experiments could now be conducted to clarify and further the observations which were made. The following section is not intended to provide an exhaustive and detailed assessment of all the experiments which could be carried out, but is intended to give a more general appraisal of the direction in which the work could now progress.

Of primary importance now, would be to begin to analyse the response of Th (i.e. CD4⁺ cells) to individual rotavirus proteins. This could be achieved by establishing BRV(UKtc) specific CD4⁺ lines and challenging the cells with different rotavirus structural proteins. This technique has been used successfully for poliovirus (Kutubuddin *et al.*, 1992) and would indicate which of the structural proteins was most immunogenic for CD4⁺ cells. More specifically, mapping studies could be performed. Th-cell epitopes within rotavirus proteins could be predicted using algorithms such as AMPHI (Margalit *et al.*, 1987) or Cathepsin B & D cleavage sites (Van Noort *et al.*, 1991) and then peptides could be synthesised. Th-cell clones could be generated (Taylor *et al.*, 1987a) and used to determine which of these epitopes was most

immunogenic. Mahon *et al.*, (1992) have successfully mapped most of the structural proteins of poliovirus using Th-cell clones and peptides.

Due to the lack of sufficient quantities of purified rotaviral structural proteins for this work, initial assessments of the relative immunogenicity of the proteins could be assessed using vaccinia recombinants (Andrew *et al.*, 1990).

Once Th-cell epitopes were defined, their relative immunogenicity in mice of different haplotypes could then be assessed since haplotype has been found to influence both immunodominance (Milich *et al.*, 1990) and the efficiency of vaccines (Hatae *et al.*, 1992) and therefore has particular relevance to 'outbred' human populations.

It was proposed in Chapter 10 that some of the variation in the responses to rotaviral antigen may have been due to varying numbers of responders and stimulators in the splenocyte population. Therefore, a possible improvement to the assay would be to separate the two populations e.g. purify T-cells by nylon-wool chromatography (Mathews & Roehrig, 1988) and use one type of cell (e.g. adherent macrophages) for the presentation of antigen. This assay would allow both the antigen processing cell type and the haplotype to be varied.

Analysis of the importance of serotype in influencing the Th-cell response over time, is another priority for future work and is of major importance to future vaccine strategies. 'Classical switch over' experiments using two serotypes could be conducted (see Mathews & Roehrig, 1989; Rothman *et al.*, 1989) and using the assay proposed above, the importance of serotypic determinants in influencing the CD4⁺ proliferative response could be assessed. Further analysis of the nature of specificity and cross-reactivity at the Th-cell level could be achieved by identifying epitopes in BRV(UKtc) and searching sequences of other serotypes for equivalent sequences.

The Th subtype responding to different pathogens has been found to very influential in determining the type of immune response induced (see Chapter 2). In the same way therefore, the immune response to rotavirus may be particularly influenced by the ratio of Th1:Th2 cells. Using the assay described above, the lymphokine profiles of CD4⁺ cells responding to BRV(UKtc) antigen

could be assessed at different time points post inoculation. The importance of CD8⁺ cells in acting as 'helper' cells could also be determined using this technique since they have been reported to secrete both interferon-gamma (Morris *et al.*, 1982; Taguchi *et al.*, 1990) and IL-5 (Taguchi *et al.*, 1990), in other systems.

As rotaviruses infect the gut and since effective protection against rotavirus disease is mediated by GALT cells (see Chapter 4), a more detailed analysis of T-cell responses to BRV(UKtc) in GALT should now be conducted. Responses in the PP's and lamina propria could be determined using the method of Zeitz *et al.*, (1988) for the isolation of the lymphocytes. Ideally, T-cell epitope mapping of BRV(UKtc) should also be performed using cells from GALT and a comparison made with those from the spleen. The basis of Th-cell memory to rotaviruses should also be assessed in great detail for GALT cells (*i.e.* Th- subtype and the epitopes of importance) and mechanisms of enhancing the response should be investigated. In the short term, the nature of MLN responder cells for BRV-(UKtc) should be determined *i.e.* analysis of cell type and memory or naive phenotype.

Studies of the efficiency with which rotavirus stimulates Th-cell responses in GALT may be particularly important in vaccine design and may reveal mechanisms by which the response can be enhanced. The adult murine model provides an ideal system with which to analyse GALT cell responses to rotavirus, due to the difficulty of obtaining such cells from humans.

Finally, it should be remembered that experiments investigating the T-cell response to BRV(UKtc) were conducted solely in adult mice, in this thesis. Therefore, attempts could now be made to develop assays for measuring CD4⁺ responses in neonatal mice, following rotavirus inoculation. The role of CD4⁺ cells in protection, could also be addressed in this model using a similar strategy to that described for the CD8⁺ cell (Offit & Dudzik, 1990; Dharakul *et al.*, 1990). The protocols and results obtained for adult animals should provide a firm basis with which to investigate the response in neonatal mice.

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