INDUCTION AND CHARACTERISATION OF THE OVINE MUCOSAL IMMUNE RESPONSE TO ROTAVIRUS

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DECLARATION

I hereby declare that the work presented in this thesis is my own work, carried out entirely by myself except where specifically stated in the acknowledgement.

Laurens A.H. van Pinxteren

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DEDICATION

To my parents

For their love, support, advice, and patience.

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ABSTRACT

The local and systemic immune response to rotavirus was investigated, both in previously exposed adult sheep and gnotobiotic lambs.

Vaccines were used parenterally to boost the local and systemic humoral immune response of adult sheep. The response was evaluated by assays for rotavirus (RV)-specific IgA and IgG antibodies and virus neutralising activity (VNT) in serum, nasal secretions, and intestinal scrapings. Sheep vaccinated (n=12) parenterally with a standard rotavirus vaccine (IFA and inactivated rotavirus lysate (low dose)) showed a significant ($p<0.05$) increase in RV IgA antibodies in nasal secretions and intestinal scrapings 2 weeks after vaccination. Sheep vaccinated (n=4) parenterally with different adjuvants and antigen doses showed both dose-related and adjuvant-related effects in RV IgG antibodies in serum and nasal secretions 2 weeks after vaccination. At a low virus dose, ISCOMs and IFA induced a significantly ($p<0.05$) higher humoral immune response compared with microspheres, however at a high dose (inactivated purified virus), ISCOMs and microspheres induced a significantly ($p<0.05$) higher humoral immune response compared with IFA. The immune response after parenteral vaccination is dose- and adjuvant-dependent.

Sheep vaccinated (n=4) orally with live virus, with different adjuvants and antigen doses showed no significantly increased humoral immune response. No dose effect or adjuvant effect was observed.

More extensive techniques were used to characterise the primary immune response in gnotobiotic lambs after infection with a lamb-passaged rotavirus strain K923. Lambs (n=10) were infected at 6 days of age and killed over a 7 week time interval together with controls (n=6). RV antibodies and VNT were determined in serum, nasal secretions, and intestinal scrapings. RV antibody producing cells were enumerated in blood. Lymphocyte populations in blood and GALT were analysed. Lymphocyte proliferations were determined in blood and GALT and cytokine expression was analysed in JPPs and MLNs.

Infected lambs cleared the virus by 8-9 days post-infection without showing any clinical signs. RV IgA antibody-secreting cells (ASC) in blood and RV IgA antibodies in serum and nasal secretions were detected from 7 days after infection

followed at 10 days after infection by RV IgG ASC and antibodies. RV IgA antibodies dominated after an infection with rotavirus. RV IgA antibodies were not detected in intestinal scrapings in the first 10 days after infection, however at 52 days after infection rotavirus-specific IgA antibodies were observed. Lymphocyte proliferation was seen in JPPs at 52 days after infection. No significant changes were observed in lymphocyte sub-populations. IFNγ transcripts were expressed in JPPs and MLNs in both groups and infection had no effect on the expression of IFNγ. IL-4 transcripts increased with time but the infected group showed a higher expression at 3 and 52 days after infection. The first evidence of an immune response was seen in the increased level of IL-4 3 days after infection, which preceded the presence of RV IgA and IgG antibodies in serum and mucosal surfaces. IL-6 transcripts were expressed and increased with time in the infected groups. No clear evidence for $CD8^+$ T cell or rotavirus-specific secretory antibody involvement in viral clearance was found suggesting that other mechanisms may play a role.

Vaccines composed of either rotavirus mixed with ISCOMs or recombinant VP6 incorporated into ISCOMs were used to examine if one oral dose could induce a mucosal immune response and protection against subsequent challenge. Gnotobiotic lambs were vaccinated orally either with PBS/ISC, inactivated rotavirus (IRV)/ISC, recombinant VP6/ISC, or IRV alone, challenged 3 weeks later with a live lamb-passaged strain, and killed 8-9 days after challenge. The immune response was measured as described above. The rotavirus-vaccinated groups had RV IgG ASC and antibodies in blood from 7 and 11 days respectively. After challenge, rotavirus-vaccinated groups cleared the virus in a reduced period (7.0 days vs 9.0 days), however this was only significant ($p<0.05$) in the IRV/ISC group. RV IgA antibodies were observed in serum and nasal secretions from 4 days after challenge. In intestinal scrapings, these were significantly ($p<0.05$) higher in the IRV/ISC and IRV groups. RV IgG antibodies were significantly ($p<0.05$) increased in nasal secretions and intestinal scrapings in the IRV/ISC and IRV groups. Lymphocytes from JPPs proliferated significantly against rotavirus in the IRV/ISC and IRV groups. $CD45R^+$ cells were significantly increased in blood in the IRV/ISC group before challenge, however no differences were found in other lymphocyte sub-populations either in blood or GALT. A down-regulation of IFNγ transcripts was observed in JPPs and

MLNs in the IRV/ISC group while the VP6/ISC group had a higher expression compared with the PBS/ISC and IRV groups. IL-4 transcripts were low in MLNs in all groups but in JPPs the rotavirus-vaccinated groups had a higher expression. IL-6 transcripts in JPPs were higher in the IRV/ISC and VP6/ISC groups but in MLNs all rotavirus-vaccinated groups had a higher level of expression. One oral dose of inactivated rotavirus alone, mixed with ISCOMs, or recombinant VP6 incorporated into ISCOMs can induce priming and partial protection. These results suggest also that different immunological mechanisms take place when different vaccination protocols are used.

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CHAPTER 1

INTRODUCTION - REVIEW OF THE LITERATURE

1.1 INTRODUCTION

Diarrhoeal diseases have a world-wide impact on both human and animal health and are a major cause of morbidity and mortality. Analyses indicate that diarrhoeal diseases are responsible for a large proportion of the total reported childhood deaths in developing countries, accounting for as many as 15-34% of all deaths annually among children younger than 5 years of age in certain countries (World Health Organization, 1973; Martines *et al,* 1991). It was estimated that the annual toll in Asia, Africa, and Latin America was 3-5 billion cases of diarrhoea and 3.0-3.2 million deaths associated with diarrhoea (Bern and Glass, 1994; Glass *et al,* 1996).

In farm animals, diarrhoeal diseases are responsible for up to 18% of the deaths in pigs and calves under 3 weeks of age. Economic losses in North America alone due to neonatal diarrhoea were estimated at $700 million annually in calves and suckling pigs (Saif, 1990). In Wyoming (North America), diarrhoeal diseases were responsible for 10-25% of the deaths in shed-lambs under 4 weeks of age (Theil *et al,* 1996). On a world-wide basis, this translates into considerable economic losses in animal production.

Diarrhoea can be caused by many different agents including viruses such as rotavirus, coronavirus, adenovirus, astrovirus, calicivirus, and other small round structured viruses (Norwalk viruses), bacteria such as *Escherichia coli*, *Campylobacter jejuni*, *Vibrio*, *Yersina*, *Shigella*, and *Salmonella* species, parasites like *Entamoeba histolytica* and *Giardia lamblia*, and toxins produced by *Vibrio*, *Escherichia coli*, and *Shigella* species (Bern and Glass, 1994). This thesis will focus on diarrhoeal diseases caused by viruses and by rotaviruses in particular.

1.2 VIRAL ENTERIC INFECTIONS

1.2.1 History

The search for important aetiologic agents of severe viral diarrhoea was unrewarding until the 1970s. This frustration ended in 1972 with the discovery of a 27-nm small round virus (Norwalk virus) and its association with gastroenteritis in older children and adults followed by the discovery in 1973 of the human 70-nm rotavirus, and its association with severe diarrhoea in infants and young children (Kapikian *et al,* 1972; Bishop *et al,* 1973; Flewett *et al,* 1973). It soon became apparent that the 70-nm particle was an important aetiologic agent of infantile diarrhoea, causing 35-50% of hospital admissions for this condition during the first two years of life (Estes *et al,* 1983; Holmes, 1983; Kapikian *et al,* 1989). However, the first work on what was probably rotavirus gastroenteritis was done in 1943. Filtrates of stools from infants with gastroenteritis produced diarrhoea in newborn calves (Light and Hodes, 1943; 1949).

Although the human rotaviruses were discovered in 1973, it should be noted that in 1957 and 1963, virus-like particles were detected in intestinal tissue of mice infected with epizootic diarrhoea of infant mice (EDIM) (Kraft, 1957; Adams and Kraft, 1967; Bishop *et al,* 1973). In the 1960s, what came to be recognised as rotavirus strains SA-11 (simian agent 11) and O (Offal) were isolated from a rectal swab from a healthy vervet monkey and from the mixed washings of intestines of cattle and sheep respectively (Malherbe and Harwin, 1963; Malherbe and Strickland-Cholmley, 1967; Stuker *et al,* 1979;1980). In 1969, 70-nm virus particles were detected in calves with diarrhoea and this agent could be passaged serially in calves with the production of disease (Mebus *et al,* 1969; Woode *et al,* 1974). In 1971, the Nebraska calf diarrhoea virus (NCDV) was successfully cultivated in primary bovine cell cultures (Mebus *et al,* 1971a,b). NCDV was found to resemble the reoviruses morphologically but to be distinct antigenically (Fernelius *et al,* 1972). The murine, simian, O, and bovine agents were later found to share a group antigen with other rotaviruses (Lecatsas, 1972; Much and Zajac, 1972; Flewett *et al,* 1974a,b; Kapikian *et al,* 1974).

Rotaviruses have also been isolated from many other mammalian and avian species including foals (Flewett *et al,* 1975), sheep (Snodgrass *et al,* 1976a,b, 1977), goats (Scott *et al,* 1978), rabbits (Bryden *et al,* 1976), pigs (Lecce *et al,* 1976), deer (Tzipori *et al,* 1976), pronghorn antelopes (Reed *et al,* 1976), apes (Ashley *et al,* 1982), impala, Thompson's gazelles, and addax (Eugster *et al,* 1978), dogs (Eugster and Sidwa, 1979), kittens (Snodgrass *et al,* 1979), turkeys (Bergeland *et al,* 1977), chickens (Jones *et al,* 1979), and pigeons (Minamoto *et al,* 1988).

1.2.2 Viruses associated with diarrhoeal diseases

Rotavirus is the most common endemic agent of severe diarrhoea in children and young animals world-wide. Virtually all children and animals are infected in the first 5 years and few weeks of life respectively and repeated infection is common, but repeated episodes of disease are not. Most rotavirus infections are mild, but one-third of gastroenteritis hospitalisations world-wide are due to rotavirus and an estimated 870,000 children die from rotavirus diarrhoea each year (Kapikian and Chanock, 1990; Saif *et al,* 1994).

Enteric adenovirus is also a cause of endemic gastroenteritis in children younger than 2 years and in young animals. It is detected at a frequency of about one-quarter to one-third that of rotavirus. In developed countries, enteric adenovirus is responsible for 4-12% of diarrhoea in hospitalised children and in 2-7% of hospitalised children in developing countries (Wadell *et al,* 1994).

Astrovirus and classical calicivirus are endemic agents of gastroenteritis, infecting every child in the first few years of life and young animals. Both viruses appear to cause predominantly mild disease in childhood, with detection rates of less than 5% in inpatient studies. However, immunity to these agents appears to wane with age and both have been implicated as agents of epidemic gastroenteritis among the institutionalised elderly (Cubitt, 1994; Kurtz, 1994).

Norwalk virus, a calicivirus, is an epidemic viral agent. In developed countries, immunity is acquired later in life and outbreaks generally involve older children and adults. In developing countries, however, it may play a role as a cause

of endemic childhood diarrhoea, as antibodies are acquired in the first 4 years of life (Kapikian, 1994).

Coronaviruses are important intestinal pathogens in newborn animals and mortality is variable between animal species and time of infection. In humans, coronavirus is as common in healthy children as in children with diarrhoea (Caul, 1994; Pensaert *et al,* 1994).

1.3 ROTAVIRUS

1.3.1 Rotavirus structure and classification

The rotaviral genome consists of 11 segments of double-stranded RNA (dsRNA) and is contained within the virus core capsid. These 11 genome segments encode for structural and non-structural proteins. The structural proteins can be divided into core and inner capsid proteins viral protein (VP)1, VP2, VP3, and VP6 and outer capsid proteins VP4 and VP7. Rotaviruses comprise seven distinct (common VP6 protein) serological groups (A-G). Group A rotaviruses have been clearly established as causing significant diarrhoea in the young. Further serological sub-classification of group A rotavirus is based on the surface proteins VP4 and VP7. Rotavirus types based on VP4 have been designated as P types, whereas types based on VP7 have been referred to as G types. The current serotype classification scheme distinguishes over 20 different P types and 14 different G types. Some G types are associated with a certain P type, for example human strains of G 1, 3, and 4 specificity belong to P serotype 1A and those of G 2 specificity belong to P serotype 1B (Estes and Cohen, 1989; Snodgrass *et al,* 1992; Prem and Lyoo, 1993; Hoshino and Kapikian, 1994; Midthun and Kapikian, 1996).

1.3.2 Pathogenicity, pathology, and clinical signs

Rotavirus in the homologous host infects mature small intestinal villous epithelial cells in mid and upper small intestine. Crypt cells are not infected. The virus has also been found in goblet cells, epithelial endocrine cells, and macrophages in the lamina propria (Suzuki and Konno, 1975; Philips, 1981; 1989) but it is a strictly mucosal infection with no evidence of systemic spread. Rotaviruses selectively infect mature villous absorptive cells through the binding with specific receptors on the cell surface. Animal rotaviruses have been shown to bind to sialic acid-containing compounds but specifically recognise the neutral glycosphingolipid gangliotetraosylceramide (asiolo-GM1 or GA1) (Fukudome *et al,* 1989; Willoughby *et al.,* 1990). A previous study has shown that GA1 is not the critical receptor but that several sialoglycoproteins like sialoglycoprotein glycophorin A also mediate viral attachment to epithelial cells (Méndez *et al,* 1993). VP4 has been shown to be the viral attachment protein (Bass *et al,* 1991). Rotavirus infections destroy mature villous absorptive cells, resulting in loss of their normal functions relating to digestion and absorption. The destroyed mature villous epithelial cells are replaced by immature squamous to cubical cells lacking a brush border (Saif, 1990). These cells are not fully functional but they lead to the regeneration of a normal villous epithelium and the end of the disease.

After an incubation period of 11-96 hours, dependent on the host species, the predominant signs of rotavirus infection present as depression, occasional vomiting in infants and piglets followed shortly by the onset of watery diarrhoea, and dehydration. Fever is common in the majority of children but not in animals (Flewett, 1982).

1.3.3 Epidemiology

All humans are infected frequently during the entire course of their lives with their first encounter with rotavirus in the first 5 years of life (Martines *et al,* 1991). In a five year study (1990-1994) in England and Wales, 88% of identified rotavirus infections were in children <5 years old (Ryan *et al,* 1996).

Bovine rotaviruses have a world-wide distribution and have been consistently detected in a high percentage (30-83%) of scouring calves, 1-3 weeks old, on both beef and dairy farms (Snodgrass *et al,* 1986; Fijtman *et al,* 1987; Bellinzoni *et al,* 1987; 1989).

Porcine rotaviruses are enzootic in pig herds around the world. Antigen detection methods have indicated that 60% or more of swine herds are infected and serologically nearly 100% of the swine herds have been infected (Bohl *et al,* 1978; Rubenstein and Miller, 1982; Bohl *et al,* 1984).

Ovine rotaviruses are ubiquitous enteropathogens and persist in the environment. In a survey in Scotland, ovine rotavirus was detected in 25% of the diarrhoeic lambs and 56% of neonatal lambs had rotavirus-specific antibodies (Snodgrass *et al,* 1977).

Canine rotaviruses have been detected in 2-22% of diarrhoeic dogs and the percentage of positive serum samples ranged between 42-84% (McNulty *et al,* 1978; Hammon and Timoney, 1983; Mochizuli and Ata, 1986).

Equine rotaviruses have been reported all over the world. Rotavirus shedding has been detected in 11-62% of the foals with diarrhoea (Dwyer *et al,* 1988; Browning *et al,* 1991). In a three year study (1991-1994) in the United Kingdom, diarrhoea was associated with rotavirus in 17% of all foals (Netherwood *et al,* 1996).

1.4 THE MUCOSAL IMMUNE SYSTEM

The gastrointestinal, upper respiratory, and urogenital tracts are all lined by mucosal tissues. The total mucosal surface in humans is an area larger than $400m^2$ (compared with $1.8m^2$ of the skin). This represents the primary site of entry of most viral, bacterial, parasitic pathogens, and of potentially harmful antigenic substances from the environment (Brandtzaeg *et al,* 1989; McGhee and Kiyono, 1993; Shalaby, 1995). Mucosal lymphoid tissues contain more immunocytes such as B and T lymphocytes, plasma cells, and monocytes/macrophages than any other tissue in humans and animals (Beagley and Elson, 1992). In humans, the small intestine contains 10^{10} plasma cells per meter and makes this organ the richest lymphoid tissue in the body. The human gastrointestinal tract produces more immunoglobulin (Ig)

than the bone marrow, spleen, and lymph nodes together (Brandtzaeg *et al,* 1989; Childers *et al,* 1989). The predominant immunoglobulin class (60-100%) produced in the gastrointestinal tract and in other mucosal tissues is secretory IgA (Tomasi and Bienenstock, 1968; Brandtzaeg *et al,* 1989; Childers *et al,* 1989; Beagley and Elson, 1992; McGhee and Kiyono, 1993).

Environmental antigens are most commonly encountered by either inhalation or ingestion and are taken up by specialised lymphoreticular tissues in the upper respiratory (bronchus-associated lymphoid tissues (BALT)) and gastrointestinal tract (gut-associated lymphoid tissues (GALT)) (Craig and Cebra, 1971; Rudzik *et al,* 1975; Mestecky, 1987). This thesis will focus on the gut-associated lymphoid tissues (GALT).

1.4.1 History

The first evidence of a mucosal immune system was reported in rabbits after oral infection/immunisation with enterobacteriaceae (killed *Shiga* bacillus). The experimental animals were protected against fatal dysentery by "coproantibodies" (local immunity) that seemed to appear independently of serum antibodies (systemic immunity) and this was of great importance in the general resistance of the animal to infections originating in the gastrointestinal tract and at other mucosal sites (Besredka, 1919). In 1922, Davies obtained evidence that specific antibodies could be found in the faeces of patients suffering from dysentery caused by *Bacillus dysenteriae*; he noted that bacterial agglutinins could appear in dysentery stools earlier than in peripheral blood (Davies, 1922).

In the late 1940s, Burrows and co-workers demonstrated a correlation between coproantibody and protection against experimental infection with cholera in guinea pigs. Studies in irradiated animals further highlighted the independent behaviour of serum and faecal antibodies by demonstrating that the faecal antibody response to cholera vaccine was inhibited whereas serum antibody levels remained essentially unchanged (Burrows *et al,* 1947; Burrows and Havens, 1948; Burrows *et al,* 1950a,b).

The independence of serum and local antibodies in other secretions such as in saliva and mucus has also been demonstrated (Bull and McKee, 1929; Sugg and Neill, 1931; Walsh and Cannon, 1936; Fazekas de St. Groth, 1951).

The recognition of the existence of a secretory system at the different mucosal sites has depended on the recognition of the heterogenecity of antibodies and the definition of various immunoglobulins. Most important was the discovery of the γA class of immunoglobulins (antibodies) which is now identified as the predominant class in secretions (Heremans *et al,* 1959). The observation that many different external secretions like saliva, milk, tears, bronchial mucus, and intestinal mucus contain a marked predominance of γA, now called secretory IgA or sIgA (Chodirker and Tomasi, 1963). This together with the finding of a predominance of γA-containing plasma cells locally in secretory glands (Tomasi *et al,* 1965) formed the basis of the evidence for a more or less distinct immunological system characteristic of external secretions.

1.4.2 Gut-associated lymphoid tissues

The lymphoid cells in the gut are present in organised lymphoid aggregates such as Peyer's patches (PP) and mesenteric lymph nodes (MLNs) or in non-organised elements in the epithelium like intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) (Holmgren *et al,* 1992; Abreu-Martin and Targan, 1996).

These lymphoid tissues can be divided into inductive and effector sites. Inductive sites such as the PP are where immune cells first encounter environmental antigens and initial B and T cell triggering occurs. After this, both B and T cells drain via lymphatics to the regional MLNs where further clonal expansion and differentiation occurs. Then, they are transported through the thoracic duct into the blood circulation (Holmgren *et al,* 1992). Many of these cells will return eventually to the effector sites such as the LP where the terminal differentiation to effector cells (plasma cells and cytotoxic T-lymphocytes (CTL) etc.) takes place and the production of antibody resulting in local immune protection (Beagley and Elson, 1992).

1.4.2.1 Peyer's patches

Peyer's patches were first described by Johann Peyer in 1677, and have a follicular structure. In monogastric animals, PP occur with increased frequency more distally in the small intestine. There are relatively fewer PP in the distal duodenum and proximal jejunum, with more and larger PP in the ileum, especially in the terminal ileum. In humans, there are usually between 20-30 PP in the intestine. They are oval, usually 1-4 cm long and 1-2 cm wide. They contain an average of 10-60 follicles but have been noted to contain several hundred in the terminal ileum of man. The size of individual follicles does not vary greatly among species, but the number of follicles aggregated within each patch increases with the size of the animal. The mouse for example, has only 4-6 follicles per patch (Carlson and Owen, 1987).

In ruminants, separate jejunal and ileal PP exist (Plate 1.1-1.4). However, the ileal PP involute at a young age while the jejunal PP persist in adult animals (Carlens, 1928; Reynolds and Morris, 1983). Jejunal PP have long interfollicular areas, and their follicles are small and pearshaped compared to the long sac-like follicles of the ileal PP. Jejunal PP have the same shape as PP from monogastrics but the ileal PP is constituted by one continuous long PP (Landsverk *et al,* 1991).

The PP in monogastric animals and the jejunal PP in ruminants are seen as secondary lymphoid organs, while the ileal PP in ruminants is seen as a primary lymphoid organ where B cell lymphopoiesis occurs in the early stages of life (Larsen and Landsverk, 1986; Hein *et al,* 1989; Landsverk *et al,* 1991).

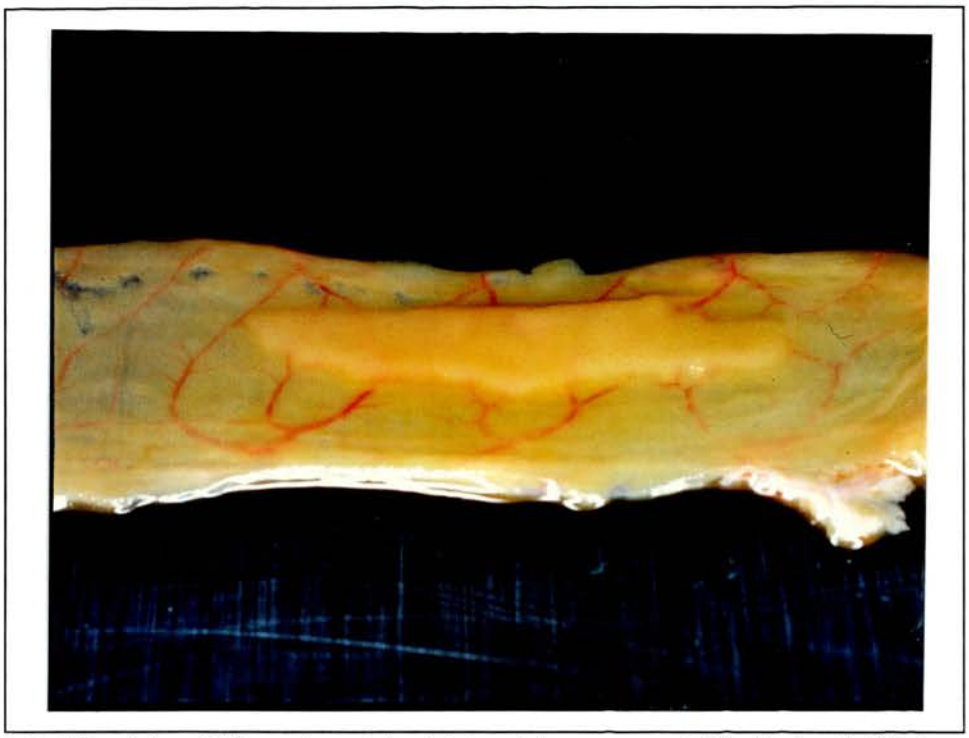


Plate 1.1: The jejunal Peyer's patch obtained from a gnotobiotic lamb 37 days after infection with rotavirus.

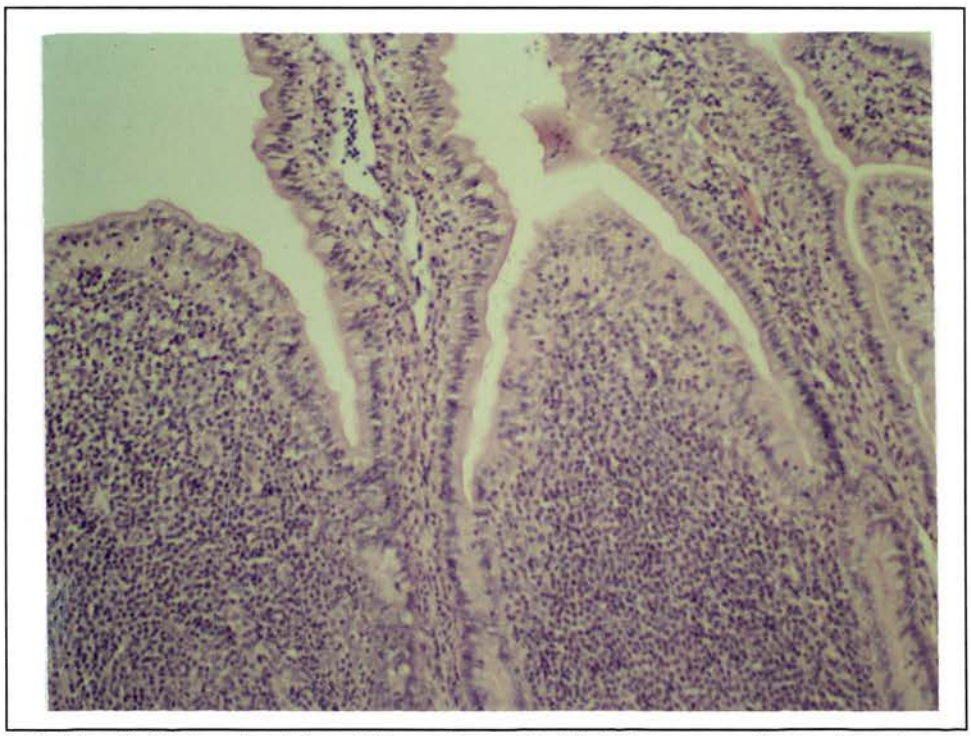


Plate 1.2: The follicle of a jejunal Peyer's patch (j) (Haematoxylin and eosin (H&E); ×100).

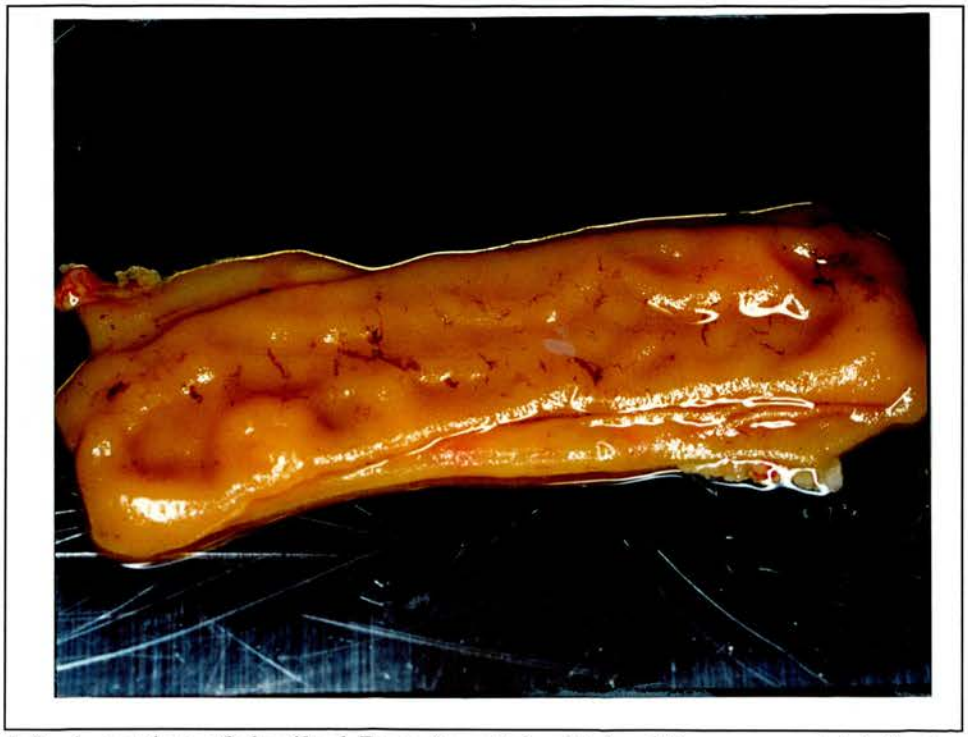


Plate 1.3: A portion of the ileal Peyer's patch obtained from a gnotobiotic lamb 37 days after infection with rotavirus.

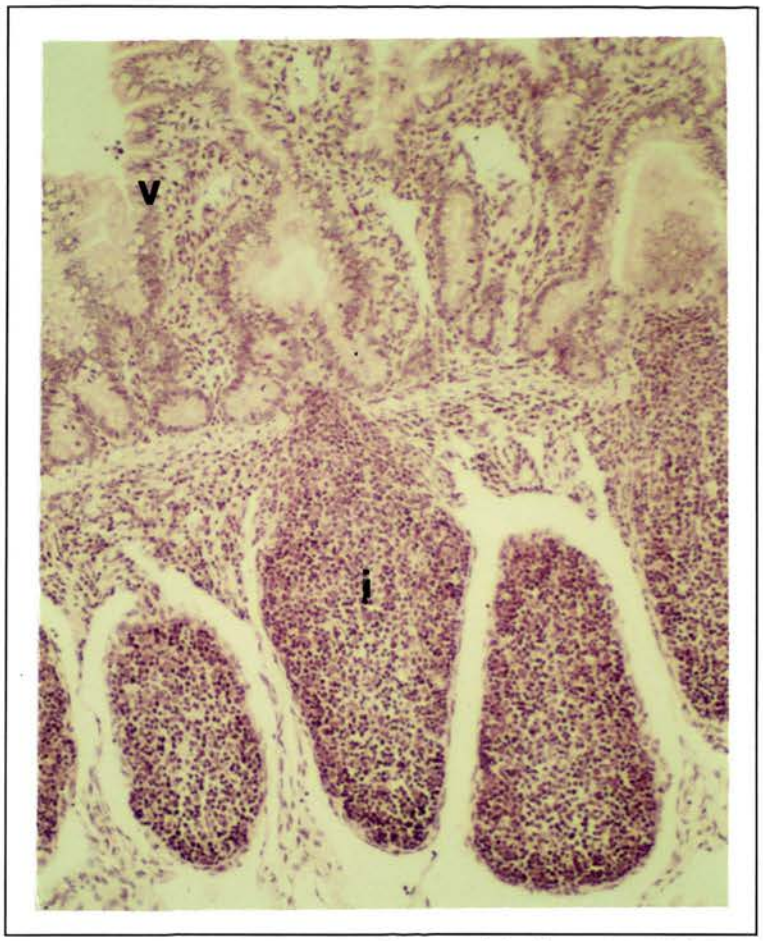


Plate 1.4: The ileal Peyer's patch (i) and villous epithelium (v) (H&E; ×100).

The PP follicles can be divided into three distinct regions: the specialised dome, the B cell zone, and the parafollicular T cell zone.

The dome epithelium consists of a specialised follicle-associated epithelium (FAE) (Plate 1.5) that has a cell composition distinct from the villous epithelium: mucus-producing goblet cells are rare, but absorptive cuboidal epithelial cells are present, as are specialised phagocytic (antigen-sampling) cells called microfold (M) cells. The proportion of M cells in the FAE ranges from 10% in humans and mice, to 50% in rabbits and 100% in pigs and calves (Siebers and Finlay, 1996). These M cells are located between the absorptive cells in the follicle-associated epithelium and migrate from the adjacent crypt epithelium (Keren, 1992). In ruminants, the ileal PP lack M cells while the jejunal PP have M cells (Landsverk *et al,* 1991).

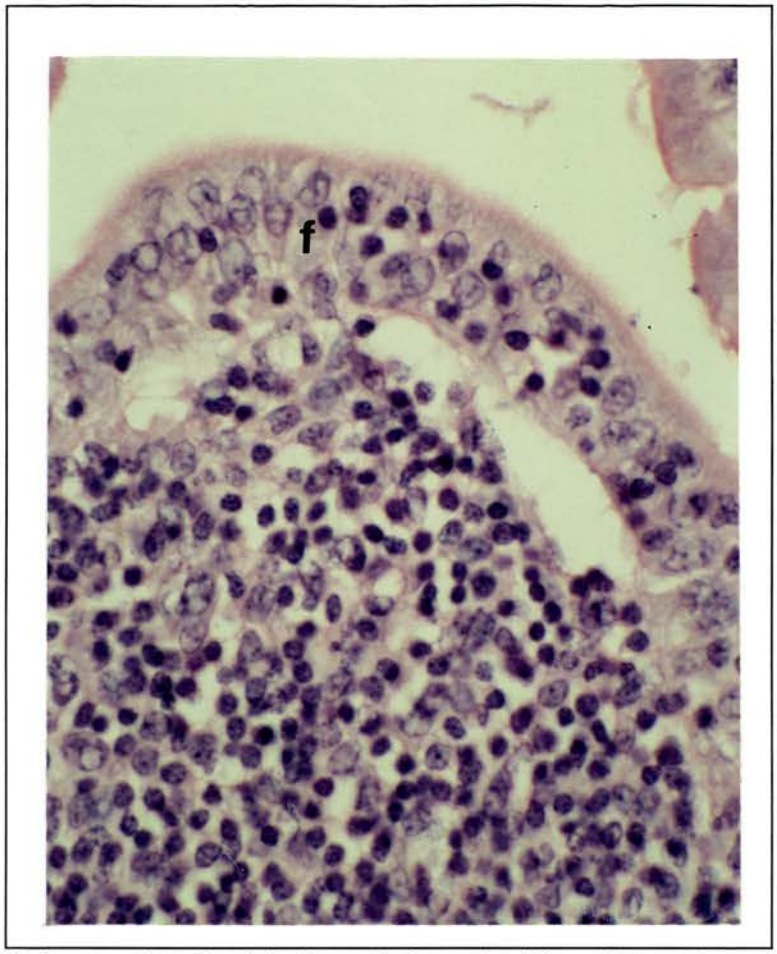


Plate 1.5: Follicle-associated epithelium (f) from the jejunal Peyer's patch obtained from a gnotobiotic lamb 37 days after infection with rotavirus (H&E; ×400).

Morphologically, M cells are characterised by the absence of overlying mucus, a poorly developed glycocalyx (the surface coat of glycoproteins and glycolipids), sparse microvilli that are irregular in size, shape, and arrangement, lack an organised terminal web of microfilaments, and the presence of an invaginated basolateral membrane containing T lymphocytes, B lymphocytes, macrophages, and dendritic cells (Neutra *et al,* 1996). Most M cells lack the long regular microvilli of the adjacent absorptive cells (Carlson and Owen, 1987; Keren, 1992; Giannasca and Neutra, 1994).

The structural specialisation of M cells brings the apical and basolateral membrane to within a few microns and shortens the distance that transcytotic vesicles must travel to cross the epithelial barrier. Endocytic or phagocytic uptake of foreign antigens or particles is followed by rapid transcytosis to the intraepithelial pocket, with little or no retention in M cell lysosomes (Neutra *et al,* 1996). It is

thought that endocytosis does not result in significant antigen digestion presumably due to the lack of fusion with lysosomes (Neutra and Kreahenbuhl, 1992). Recent studies in rats suggest that some level of processing may be taking place due to the presence of lysosomal markers on apical vesicles and the expression of major histocompatibility complex (MHC) class II determinants on endosomal-lysosomal compartments on the basolateral membrane (Allan *et al,* 1993). In contrast to absorptive cells, soluble antigens and even whole viruses (reovirus types 1 and 3, poliovirus, and rotavirus) and bacteria (*Escherichia coli*-RDEC, *Vibrio cholerae*, *Shigella*, and *Salmonella*) can pass relatively intact through the M cells (Wolf *et al,* 1981; Inman and Cantey, 1983; Kohbata *et al,* 1986; Owen *et al,* 1986; Dharakul *et al,* 1988; Wassef *et al,* 1989; Sicinski *et al,* 1990).

The adjacent absorptive epithelial cells synthesise a 60kDa glycoprotein called secretory component (SC). This is an important molecule that is present on the basal-lateral surfaces of the absorptive epithelial cells. SC combines through disulfide bridges with polymeric IgA (pIgA) that is secreted by the plasma cells in the lamina propria and is collected into vesicles, which aggregate at the apical border of the cell. Polymeric IgA consists of dimers of IgA molecules joined with each other by the J (joining) chain through disulfide bridges (Mestecky and McGhee, 1987). These polymeric IgA-SC complexes are then secreted into the intestinal lumen. The J chain is not essental for IgA transport but is necessary for the stable association of pIgA with SC (Hendrickson *et al,* 1996). In the intestine, the SC is thought to protect the pIgA from digestive enzymes. M cells do not make SC, nor do they express SC to bind pIgA. As a result, the follicle-associated epithelium does not transport IgA into the intestinal lumen (Brandtzaeg and Bjerke, 1990). This may be an important mechanism to facilitate transport of antigens into the dome area of the GALT. Since secretory IgA is thought to bind to and prevent uptake of luminal antigens, the lack of such a mechanism over the dome regions should facilitate attachment of antigens to these sites (Keren, 1992).

Since M cells are clearly responsible for the initial uptake of antigens by the intestinal lumen, it would be logical to believe that they also present the antigen but this is likely to be limited to their physical isolation from the majority of the T cells. However, the adjacent follicle-associated epithelium does express surface MHC class

II, as does the absorptive villous epithelium (Bjerke and Brandtzaeg, 1988). Thus, the role of M cells may merely be the initial antigen uptake and transport to the underlying follicle, or to adjacent B lymphocytes within the epithelium (Jarry *et al,* 1989). B lymphocytes are known to be capable of antigen processing and presentation as they bind soluble antigens through their cell-surface immunoglobulin. They internalise the antigens bound by their surface immunoglobulin receptors and then display peptide fragments of these antigens as peptide-MHC Class II complexes on their surface where they can interact with $CD4^+$ T cells. However, the role of B cells in priming naïve T cells in natural immune responses is still unclear (Ashwell, 1988; Kurt-Jones *et al,* 1988; Panja and Mayer, 1994).

Although M cells are known to be the major antigen-sampling cell of the intestine, other cells have been implicated in this function as well. One of the more intriguing candidates is the Paneth cell (Erlandsen and Chase, 1972; Kern *et al,* 1987). These are poorly characterised cells found in the base of crypts in the small intestine. Although their location at the base of the crypt does not suggest an important role in sampling luminal antigens, these cells have phagocytic capabilities *in vivo* and *in vitro*. Further, as with other phagocytic cells they contain lysozyme. However their role in processing antigens for a mucosal immune response is still unclear.

After the initial uptake of antigens by M cells, the antigens are transported into the underlying follicular areas, where antigen-presenting cells (APCs) such as macrophages and dendritic cells determine which antigens are presented to T and B lymphocytes. Dendritic cells are not unique in the PP being found in other lymphoid organs. They are also found in the skin where they are called Langerhans cells. In the murine PP they are found in the sub-epithelial dome, just beneath the FAE, and throughout the follicle sparing the germinal centres (Kelsall and Strober, 1996). Dendritic cells are stellate and irregularly shaped and occur at a frequency of less than 1%. They are capable of antigen uptake and processing these antigens into peptides. These peptides are presented with MHC Class I or Class II molecules on their surface where they can interact with $CD8^+$ and $CD4^+$ T cells respectively (Steinman and Nussenzweig, 1980; Mayrhofen *et al,* 1983; Spalding *et al,* 1983; Liu and McPherson, 1994; Panja and Mayer, 1994; Kelsall and Strober, 1996).

APCs internalise exogenous antigens through phagocytosis. Within the endosome, proteolysis takes place resulting in antigen fragments. When these antigen fragments bind with MHC Class II molecules (produced in the Golgi-system) in multivesicular bodies, they are targeted to the surface to interact with $CD4^+$ T lymphocytes. Endogenous antigens (e.g., viral proteins) are processed in the cytoplasm. MHC Class I molecules formed in the endoplasmic reticulum (ER) associate with cytoplasmic peptides that are carried to the ER by peptide transporters. These MHC Class I-peptide complexes traffic to the membrane, where they can be recognised by TcRs on $CD8^+$ T cells. MHC Class I molecules are expressed on virtually all nucleated cells (Panja and Mayer, 1994).

Macrophages and dendritic cells when stimulated by antigens presented by M cells produce a T cell-stimulating factor (TSF) or interleukin (IL)-12. IL-12 has a stimulating effect on natural killer (NK) cells as well as on $CD4^+$ and $CD8^+$ T cells (Germann and Rüde, 1995; Orange and Biron, 1996; Heufler *et al,* 1996).

The B-cell zone, below the dome, contains germinal centres believed to be the site where B-cells receive triggering signals from the antigen to develop into IgA precursors and affinity maturation (Butcher *et al,* 1982; Weinstein and Cebra, 1991). The B-cell activation is induced by T helper cells and these T cells express a typical phenotype $CD3^+$, $CD4^+$, $CD5^+$, and $CD8^-$. In mice, based on *in vitro* work with T cell clones, these T helper cells can be divided into Th1- and Th2-cells, however these subsets have never been identified in sheep (Mosmann *et al,* 1986). Th1- T cells can produce IL-2, interferon gamma (IFNγ), and lymphotoxin (tumor necrosis factor-β) (TNFβ). Th2- T cells can produce IL-4, IL-5, IL-6, and IL-10 (Mosmann *et al,* 1986; Mosmann and Coffman, 1989). Th1- T cells provide help for the elimination of intracellularly infected host cells via activation of macrophages and direct cytolytic activity through activation of cytotoxic $CD8^+$ T cells. In addition, Th1- T cells regulate delayed-type hypersensitivity (DTH) responses and downregulate Th2- cell function. Maximal B cell help is provided by Th2- T cells although Th1- T cells can also provide helper activity through IL-2. Th2- T cells inhibits Th1-cells through IL-10 (Mosmann and Moore, 1991; Street and Mosmann, 1991).

Cognate interactions between B cells and T helper cells then leads to immunoglobulin isotype switching in B cells. Soluble factors do not mediate this

effect (Kawanishi *et al,* 1983a; 1983b; 1985). Transforming growth factor-β (TGFβ) is an important cytokine for isotype switching to IgA. TGFβ enhances the IgA production by lipopolysaccharide-stimulated murine B lymphocytes (Coffman *et al,* 1989). TGFβ causes a switch in the immunoglobulin heavy-chain-constant region genes from mainly IgM^+ cells to IgA^+ cells. IL-2 and/or IL-5 can increase secretion of IgA, which is important for clonal expansion. IL-2 and IL-5 cause the terminal differentiation of IgA-committed B cells (Coffman *et al,* 1989; Sonoda *et al,* 1989; Lebman *et al,* 1990). The IgA enhancing activity of IL-5 is further increased by IL-4 or IL-2 (Lebman *et al,* 1987; Murray *et al,* 1987). IL-6 induces levels of IgA two- to threefold higher than IL-5, suggesting that IL-6 plays an important role in the terminal differentiation of IgA^+ cells and other isotypes. It would appear that both IL-5 and IL-6 are involved in the regulation of IgA synthesis and the terminal differentiation of IgA^+ B cells. This fit well with the finding that gut epithelial cells secrete high levels of IL-6 (Beagley *et al,* 1989; Beagley and Elson, 1992). Thus, the predominance of IgA in mucosal secretions could be explained by a higher predominance of Th2-type cells in mucosal sites.

Adjacent to the B-cell zone is the T-cell dependent areas in which both $CD4^+$ T cells and $CD8^+$ T cells can be detected. After antigen presentation in these two regions B cells undergo partial differentiation and proliferation, followed by transport to the regional MLNs via lymphatics. More then 95% of lymphocytes that leave the PP do so via the lymph (Reynolds and Pabst, 1984). While it is believed that when T cells migrate out of the PP, they are fully differentiated and primed to perform their regulatory tasks, B cells require further differentiation in the MLNs. It is believed that up to 20% of PP-derived cells home to mucosal tissue outside the intestine (Butcher, 1986).

1.4.2.2 Mesenteric lymph nodes

MLNs lie within the mesentery and drain the lymphatics, which originate from the intestinal mucosa and PP. They are structurally identical to peripheral lymph nodes, with well-defined thymus-dependent areas (TDA), primary or secondary follicles, germinal centres, and medullary structures (Plate 1.5).

Unlike PP (T cells 20-35%; B cells 40-50%), T cells predominate in MLNs (60-70%; B cells 30-40%), but, similarly to PP, show the same preponderance of cells with the helper T cell phenotype (60-70% of all T cells). T cells with Fc receptors for IgA are relatively common and many B cells carry IgA on their surface (Arnaud-Battandier *et al,* 1980; Durkin *et al,* 1981; Stevens *et al,* 1982; Kraal *et al,* 1983; Gibson *et al,* 1984).

The lymphocytes primed in the PP may lodge in the MLNs for a period before continuing their migration to the different mucosae. In the MLNs, a further differentiation of mainly B lymphocytes occurs. The MLNs contain helper T cells, mainly of the Th2-type which can induce terminal differentiation of B cells previously committed to IgA production following interaction with isotype-specific PP T cells (Kawanishi *et al,* 1983a).

After further differentiation in the MLNs, the lymphocytes enter the systemic circulation via the thoracic duct and preferentially "home" to sites within the secretory immune system. It is postulated that disseminated lymphocytes possess surface proteins such as the $\alpha_4\beta_7$ ligand that can act as homing receptor and interact with adressins (e.g., MAdCAM-1) on endothelial cells in mucosal glandular and lymphoid tissues (Kagnoff, 1996). Within the intestine, lymphocytes enter the lamina propria and the inter-epithelial spaces. B and mainly T helper cells migrate to the lamina propria and suppressor/cytotoxic T cells migrate to the epithelium, where they are known as intraepithelial lymphocytes (IELs). B cells in the lamina propria undergo the final differentiation into IgA-secreting plasma cells.

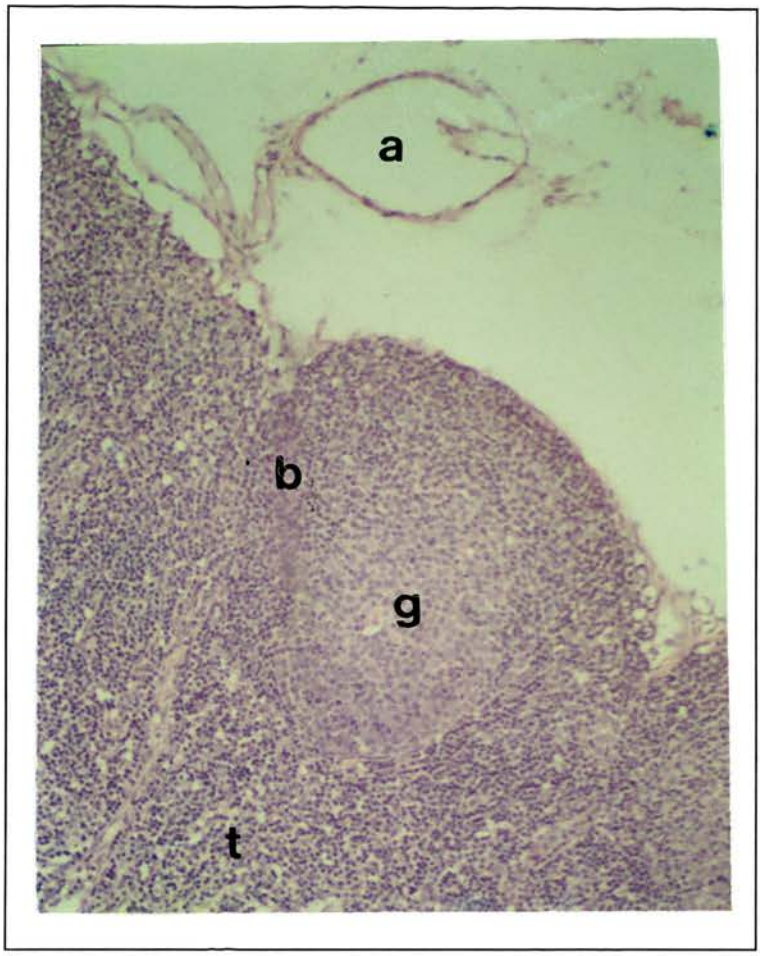


Plate 1.5: The mesenteric lymph node obtained from a gnotobiotic lamb 37 days after infection with rotavirus showing a B cell follicle (b), a germinal centre (g), a T cell area (t), and an afferent lymphatic (a) (H&E; ×100).

1.4.2.3 Lamina propria

The lamina propria (LP) (Plate 1.6) is heavily infiltrated by a wide variety of lymphoid cells including lymphocytes, plasma cells, macrophages, eosinophils, basophils and mast cells.

Plate 1.6: The intestine obtained from gnotobiotic lamb 37 days after infection with rotavirus showing enterocytes (e), intraepithelial lymphocytes (→), and the lamina propria (lp) (H&E; ×400).

The LP contains both T and B lymphocytes. The B cell compartment of the LP in mice, rat, and cattle constitutes 20-40% of all the cells (Lyscom and Brueton, 1982; Tseng, 1982; Nagi and Babiuk, 1987) and comprises small lymphocytes and terminally differentiated plasma cells, the majority of which are committed to IgA synthesis. Most IgA antibody commited "conventional" B cells derive from PP precusors which enter the blood circulation from intestinal lymphatics and relocate in the LP (Husband and Gowans, 1978). However a small population of B cells arise from B cell precusors orginating in the peritoneal cavity, the so-called B-1 cells (Kroese *et al,* 1989; Solvason *et al,* 1991). B-1 cells have a distinctive surface phenotype (possess the pan-T-cell glycoprotein Ly-1 (CD5 in humans), a unique anatomical localisation (enrichment in the peritoneal cavity), and an extensive

capacity for self-renewal compared to "conventional" B cells which are activated in the PP. They preferentially produce IgM of low affinity and broad specificity for polysaccharides, lipids, and proteins compared to "conventional" B cells, which produce IgA of high affinity and narrow specificity. However, TGFβ, IL-4, IL-5, and IL-10 can activate B1-cells and induce class switching from IgM to IgA. After invasion of the mucosa by a pathogen they immediately produce low-affinity IgM and, subsequently, with the help of Th2- cells, generate high affinity IgA (Murakami and Honjo, 1995).

The LP is the only tissue in which large numbers of plasma cells are present continuously under normal conditions (Mowat, 1987). IgA plasma cells account for 70-90% of the total with most of the remainder being IgM-producing cells. IgG and IgE plasma cells are found less often while IgD plasma cells are extremely rare (Lamm, 1976). Most of the small B lymphocytes are predominantly IgM bearing cells ($sIgM^+$). Thus, the LP contains not only actively secreting IgA plasma cells, but also the precursors of these cells.

The significance of the local production of other Ig isotypes is a matter of some controversy. Polymeric IgM bound to secretory component can be transported across the epithelium in the same way as IgA, and in individuals with selective IgA deficiency the mucosa is populated with IgM plasma cells (Hobbs and Hepner, 1968). IgG plasma cells frequently increase in number as a result of intestinal inflammation.

T lymphocytes constitute 40-60% of the LP population in mice, rats, humans, and cattle. 60% of these T cells are $CD4^+$ (both Th1- and Th2-type), and one third are $CD8^+$ (Selby *et al,* 1981; Lyscom and Brueton, 1982; Parrot *et al,* 1982; Nagi and Babiuk, 1987; Beagley and Elson, 1992; Staats *et al,* 1994). Macrophages, eosinophils, and mucosal mast cells make up approximately 10%, 5%, and 1-3% respectively of the LP (Bull and Bookman, 1979; Staats *et al,* 1994).

The LP is a major site of T cell-dependent B cell differentiation. T lymphocytes in the LP are enriched for Th2- T cells. Thus, the predominance of IgA plasma cells can be explained by a predominance of the Th2- T cells, which produce IL-5 and IL-6, relative to Th1- T cells (Taguchi *et al,* 1990).

1.4.2.4 Intraepithelial lymphocytes

The epithelium is the layer of the intestine in closest contact with antigen and might therefore be expected to have a wide range of immune effector cells to deal with the constant antigenic exposure. The IELs reside within this epithelium (Plate 1.7).

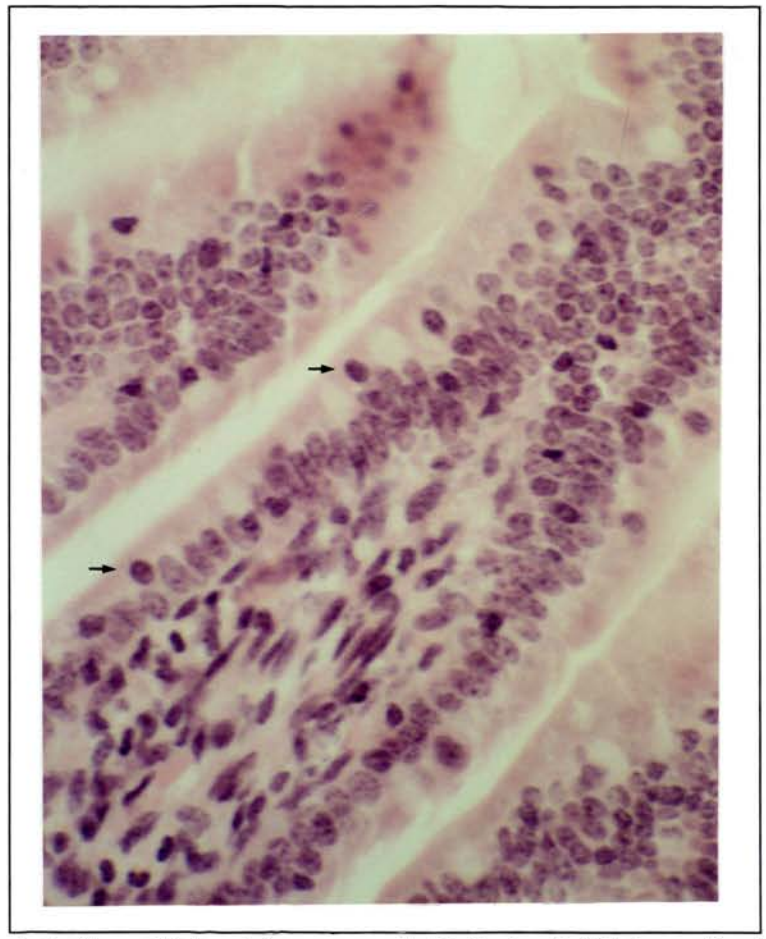


Plate 1.7: The intestine obtained from a gnotobiotic lamb 37 days after infection with rotavirus showing intraepithelial lymphocytes (→) (H&E; ×400).

IELs in mice, guinea-pigs, cattle, and humans make up approximately 5-20% of lymphocytes in the normal intestinal epithelium (Arnaud-Battandier *et al,* 1980; Selby *et al,* 1981; 1983; Nagi and Babiuk, 1987; Goodman and Lefrancois, 1988). 80-90% of IELs can be subdivided into two classes based on their T cell receptor (TcR): those expressing αβTcRs (known to recognise foreign antigen presented in the context of class I and II MHC molecules) and those expressing γδ TcRs (shown

to have cytotoxic activity usually of a non-MHC restricted nature) (Goodman and Lefrancois, 1988). 80% of IELs bear the CD8 surface marker while 5-15% express the CD4 surface marker irrespective of their TcR expression in mice and humans (Goodman and Lefrancois, 1989; Cerf-Bensussan and Guy-Grand, 1991). This is quite different from the CD4:CD8 ratio found in the LP (Mowat, 1990). In ruminants, a higher percentage of $CD4^-$ $CD8^-$ $\gamma\delta^+$ T cells are present within the epithelium compared to mice and humans (Clevers *et al,* 1990; Hein and MacKay, 1991). The precise functions of this cell population are still unclear. IELs participate in the defense of the epithelium through either cytotoxic activity or the secretion of lymphokines. In a study in mice, cytotoxic IELs protected the mucosa against reovirus by eliminating infected epithelial cells through lysis (London *et al,* 1989). IELs produce IFNγ, TNFα, IL-2, IL-5, and TGFβ but the role of these cytokines in the mucosal immune response is still unclear. IFNγ could be a cell growth inhibitor and TNFα could be involved in recruitment and activation of lamina propria macrophages and neutrophils able to release cytotoxic factors (Cerf-Bensussan and Guy-Grand, 1991; Lefrancois, 1994).

1.5 MUCOSAL IMMUNITY TO VIRUSES

Mucosal immunity to viruses involves the cellular and humoral immune system acting to limit the extent of virus replication, to clear virus, and to prevent reinfection. The secretory IgA system provides the main protection of mucosal surfaces, however significant contributions are also made by the cellular arm of the immune system and through diffusion of antiviral IgG antibodies onto mucosal surfaces (Murphy, 1994).

1.5.1 Cellular antiviral immune mechanisms at mucosal surfaces

From the cellular immune system natural killer (NK) cells, MHC class I-restricted cytotoxic $CD8^+$ T cells, and MHC class II-restricted helper $CD4^+$ T cells can all act as antiviral effector cells.

1.5.1.1 Natural killer cells

NK cells are a population of T cell-receptor-negative ($CD3^-$) lymphocytes that spontaneously mediate the lysis of virus-infected cells. Morphologically, they are similar to large granular lymphocytes. NK cells do not have to recognise special receptors on the infected cell unlike cytotoxic $CD8^+$ cells.

The important early role of NK cells in limiting the extent of certain mucosal viral infections was shown in humans with an inherited deficiency in NK cells who experienced more severe herpesvirus infection then normal individuals (Biron *et al,* 1988; Abraham and Ogra, 1994).

1.5.1.2 Cytotoxic $CD8^+$ T cells

Cytotoxic $CD8^+$ T cells appear to be the major T cell effector with antiviral activity (Offit and Dudzik, 1990). Cytotoxic $CD8^+$ T cells recognise a short viral peptide produced endogenously, together with the MHC class I β2-microglobulin heterodimer which is expressed on the surface of every infected cell (Madden *et al,* 1991).

Since viral infection of a cell is generally required for antigen presentation by the MHC class I β2-microglobulin heterodimer, cytotoxic $CD8^+$ T cells which are located close to epithelial cells cannot prevent infection but can eliminate cells already infected or restrict virus replication in infected cells. The net effect is the prevention of further spread of virus (MacKenzie *et al,* 1989; Godson *et al,* 1992).

Cytotoxic $CD8^+$ T cells are most efficient during primary infection when a sufficient antibody response is still lacking. Immunisation with antigens that induce predominantly cytotoxic $CD8^+$ T cell activity in the absence of antibody is less

successful in restricting replication of virus and preventing illness than immunisation that induces a sustained antibody response (Andrew *et al,* 1987; Webster *et al,* 1991).

Cytotoxic $CD8^+$ T cells kill the infected target cells by lysis and two lymphocytotoxicity mechanisms are involved. The secretory lytic pathway when pore-forming protein (perforin) and a series of granule serine proteinases (granzymes) are released into the localised environment of the effector and target cell, causing lysis of the target cell, but sparing the effector cell. The non-secretory lytic pathway when apoptosis-inducing cell-surface molecules, such as Fas molecules, interact with FasL molecules on the effector cell and could crosslink the intracellular "death domain" of Fas and initiate intracellular signals that culminate in apoptosis of the target cell (Berke, 1995).

1.5.1.3 Helper $CD4^+$ T cells

Helper T cells provide help to B cells, but in addition may have a direct antiviral activity cells (Taylor and Askonas, 1986; McDermott *et al,* 1987). They are less effective than cytotoxic $CD8^+$ T cells. This is due to the more limiting distribution of its restricting element, the MHC class II $\alpha\beta$ heterodimer, which is present predominantly on B cells and antigen presenting cells such as macrophages and dendritic cells (Taylor and Askonas, 1986; McDermott *et al,* 1987).

1.5.2 Humoral antiviral immune mechanisms at mucosal surfaces

1.5.2.1 Mucosal IgA antibodies

Although cellular immune mechanisms and passively acquired IgG antibodies contribute to the mucosal immune response to viruses, the major mediators of resistance to viral infection on mucosal surfaces are IgA antibodies. IgA antibodies play a significant role in the clearance of viral infections, modification of the severity of disease on reinfection, and the prevention of infection on re-exposure to virus (Murphy, 1994; Maxson *et al,* 1995). Effector mechanisms of IgA are: 1) immune exclusion, preventing viral attachment and penetration; 2) intra-epithelial

cell neutralisation; and 3) excretion of antigens from the lamina propria (Mazanec *et al,* 1993).

The major viral antigens that induce a protective antibody response are the surface glycoproteins of viruses. IgA recognises the same viral antigens as IgG antibodies. The specificity of the neutralising activity of IgA and IgG antibodies for antigenically related variant viruses appears to be similar; the mucosal IgA antibody response possesses similar breadth of specificity as the systemic IgG antibody response. The main advantage of IgA antibodies in protecting mucosal surfaces appears to be their ability to be transported selectively across mucosal surfaces, and not an inherently greater antiviral activity (Renegar and Small, 1991). In contrast to this, protection of piglets against transmissible gastroenteritis (TGE) virus is more effective by milk/colostral IgA than IgG (Bohl and Saif, 1975; Saif and Bohl, 1979; Bohl *et al,* 1972; Saif *et al,* 1972).

The mucosal IgA response to viral infections (e.g., Sendai virus) is rapid after first infection and can be detected as early as day 3 following infection (Blandford and Heath, 1972). The primary response peaks within 6 weeks and can decrease to a low often barely detectable level by 3 months. Reinfection results in a secondary response, indicating immunological memory characterised by a rapid rise in IgA titre to a higher peak titre and maintenance of detectable levels over a longer period (Bishop *et al,* 1990; Coulson *et al,* 1990; 1992). Frequent reinfection causes an active mucosal antibody (IgA) response and stimulates memory IgA plasma cells as these are short-lived.

Evidence for the existence of a common mucosal immune system comes from the observation that immunisation of a mucosal site often leads to detectable immune responses at distant mucosal sites (Mestecky, 1987). For example, sows first infected with the respiratory variant of TGEV, porcine respiratory coronavirus (PRCV) which is antigenically related, are protected against intestinal TGEV (De Diego *et al,* 1994). This seeding of distant mucosal sites is likely to be the result of trafficking of locally stimulated mucosal B lymphocytes to distant sites, where they reside as IgA plasma cells actively producing antibody or as memory B cells (Rudzik *et al,* 1975; Czerkinsky *et al,* 1987; Mestecky, 1987). Although this common mucosal system exists, it appears to be relatively inefficient at protecting sites not directly stimulated

with antigen (Ogra and Karzon, 1969; Nedrud *et al,* 1987). The local nature of the mucosal antibody response comes from the finding that the concentration of virus-specific IgA producing B cells at the site of antigenic stimulation is much higher than that at more distant sites (Dharakul *et al,* 1988).

1.5.2.2 IgG antibodies

The majority of IgG antibodies present at mucosal surfaces are derived from plasma through passive diffusion. IgG antibodies produced by the mucosa also contribute to the total antiviral activity in mucosal secretions (Ogra *et al,* 1974). Further, in IgA-deficient patients, mucosal production and secretion of IgG and IgM antiviral antibodies can compensate for the deficiency of production and secretion of IgA antibodies (Ogra *et al,* 1974).

The activity of IgG antibodies against viruses that replicate at mucosal surfaces is derived from three different sources: 1) antibodies produced by the host at the mucosal site (Ogra *et al,* 1974); 2) maternally derived antibodies through the feeding of colostrum (Puck *et al,* 1980; Snodgrass *et al,* 1980; Reuman *et al,* 1983; 1987); and 3) passively transferred polyclonal antibodies through the transudation of serum antibodies (Offit *et al,* 1986; Besser *et al,* 1988).

The impact of the IgG antiviral antibodies varies at different mucosal surfaces. In the lung, serum antibodies can diffuse readily across the alveolar wall and therefore have an impact against respiratory viruses (Prince *et al,* 1985). Diffusion of IgG across the gastrointestinal tract is poorer and the hostile luminal environment and the dilution effect further limit the effectiveness of antibodies of this class. The antiviral activity of IgG antibodies *in vivo* is related to direct neutralisation of virus infectivity.

Many viruses infect mucosal surfaces in the first few months of life when maternal IgG antibodies may still be present. These maternal IgG antibodies can significantly modify the immune response to viruses that replicate at mucosal surfaces. The magnitude of the mucosal and systemic antibody response to a viral infection, in the presence of maternal IgG antibodies, can be reduced significantly despite a high level of virus replication (Kimman *et al,* 1987). However, a secondary

IgG response is seen when rechallenged with virus (Reuman *et al,* 1983). Individuals in which the IgG response is suppressed by passively derived IgG antibodies still show a well developed IgA antibody response (Kimman and Westenbrink, 1990).

1.6 MUCOSAL IMMUNITY TO ROTAVIRUS

1.6.1 Cellular antirotavirus immune mechanisms at mucosal surfaces

In monogastric animals, a primary rotavirus infection produces increases in both rotavirus-specific $CD4^+$ T helper cells (Offit *et al,* 1992; 1993) and cytotoxic $CD8^+$ T cells (Offit and Dudzik, 1990). An increase in cytotoxic T cells is also found after oral infection with vaccinia virus recombinants expressing rotavirus outer capsid protein VP7 (Offit *et al,* 1994). Shedding of rotavirus was ablated after passive transfer of $CD8^+$ lymphocytes to mice with severe combined immunodeficiency (SCID mice) (Dharakul *et al,* 1990). Rotavirus-specific cytotoxic T cells seem to be important in the lysis of rotavirus-infected villous epithelial cells, restricting rotavirus growth, and in protection against reinfection (Offit and Dudzik, 1989; Offit *et al,* 1989; Dharakul *et al,* 1990).

β_2-microglobulin knockout mice (deficient in MHC-class I-restricted $CD8^+$ T cells), either treated with anti-CD8 antibody, or not treated, shed the virus for 1-4 days longer than normal control mice did but they completely resolved the primary infection and they all were resistant to reinfection. Clearance of rotavirus infection in these knockout mice correlated with the development of an intestinal rotavirus-specific IgA response. This suggests that cytotoxic T lymphocytes mediate rotavirus clearance but are not essential for this function and that they are not necessary for the development of immunity to rotavirus reinfection.

J_HD knockout mice (B-cell-deficient) had similar virus-shedding curves compared to normal control mice and completely resolved infection. However, when treated with anti-CD8 antibody they became chronically infected after primary infection. Upon rechallenge, J_HD mice who had cleared the primary infection were all susceptible to reinfection but shed the virus in smaller quantities and for fewer days than naive mice. This suggests that B cells also play a role in clearance of

primary infection but are absolutely necessary for development of immunity against rotavirus reinfection and that $CD8^+$ T cells can actively mediate almost complete short-term and partial long-term protection from reinfection (Franco and Greenberg, 1995; Franco *et al,* 1997a).

μMT knockout mice (B-cell-deficient) gradually resolved primary infection and when depleted of $CD8^+$ T cells no difference was observed in viral clearance. This suggests that other effector mechanisms may operate like cytotoxic $CD4^+$ T cells or NK cells which play protective roles in other viral infections (Yasukawa and Zarling, 1984; Karupiah *et al,* 1990; McNeal *et al,* 1995).

Mechanisms involving the release of antiviral cytokines such as IFNγ and TNF-α may also play roles in resolution of virus shedding (Ramsay *et al,* 1993). Type 1 interferons have been shown to be produced in response to infection with rotavirus and could mediate the antirotavirus effect (La Bonnardiere *et al,* 1981; Lecce *et al,* 1990). J_HD knockout mice become chronically infected when depleted of $CD8^+$ T cells. When J_HD knockout mice are depleted of IFNγ they clear the primary infection like normal control mice. IFNγ knockout mice clear primary infection efficiently but this is delayed when depleted of $CD8^+$ T cells. This suggests the antirotaviral activity of $CD8^+$ T cells is not dependent on killing of the host-infected cells by the secretion of IFNγ (Franco *et al.* 1997b). Non-immunological mechanisms such as depletion of villous epithelial cells susceptible to rotavirus infection could also play a role in the resolution of viral shedding (McNeal *et al,* 1995).

A similar picture is seen in ruminants infected orally with rotavirus. The number of cytotoxic T cells is increased in the epithelium (IEL) and lamina propria in the mid and lower small intestine (the site of rotavirus infection) (Parsons *et al,* 1993). In $CD8^+$ T cell-depleted gnotobiotic calves significantly increased levels of virus excretion were detected and no effect on serum and faecal antibody levels was seen. In $CD4^+$ T cell-depleted gnotobiotic calves reduced levels of faecal and serum antibody levels were found and no effect on virus excretion was seen (Oldham *et al,* 1993).

1.6.2 Humoral antirotavirus immune mechanisms at mucosal surfaces

1.6.2.1 Monogastrics

In monogastric animals, after a primary rotavirus infection, increased levels of serum antirotavirus IgM followed by antirotavirus IgA and IgG were found. In the duodenal fluid, saliva, and faeces a similar picture was seen for secretory IgM and IgA (Hjelt *et al,* 1985a; Grimwood *et al,* 1988; Conner *et al,* 1991; Coulson *et al,* 1992). This intestinal antirotavirus IgA is found only when an animal is infected orally. Because the antirotavirus IgM response is short-lived, serum IgA and IgG are more sensitive markers of a previous rotavirus infection (Grimwood *et al,* 1988).

After reinfection, antirotavirus faecal (intestinal) IgA is increased which is a more reliable marker of reinfection than seroconversion. With frequent reinfection of rotavirus, an increased faecal IgA antibody level was observed. Frequent reinfection causes an active mucosal antibody (IgA) response and stimulates memory IgA plasma cells (Merchant *et al,* 1991; Coulson *et al,* 1992; Shaw *et al,* 1993). Colostrum derived from women previously exposed to rotavirus showed a predominant antirotavirus IgA response produced in the breast tissue (Hjelt *et al,* 1985b; Rahman *et al,* 1987).

The mechanism of protection by mucosal antibody has not been determined, but it may not be through classic neutralisation. Passively transferred monoclonal IgA antibodies (non-neutralising) against the VP6 protein of rotavirus were capable of preventing primary and resolving chronic murine rotavirus infection. This could support the hypothesis that a possible mechanism of host defense to rotavirus infection is the intracellular inactivation of rotavirus by secretory IgA during transcytosis (Burns *et al,* 1996).

1.6.2.2 Ruminants

In ruminants a similar picture is seen for both the primary and secondary response except that IgA persists for a much shorter period (1-3 weeks) in serum than in faeces and nasal secretions (Vonderfecht and Osburn, 1982; Saif, 1987).

Colostrum derived from dams previously exposed to rotavirus showed a predominant antirotavirus IgG which diffused from serum and selectively concentrated in the mammary gland (Wells *et al,* 1978; Newby *et al,* 1982).

1.7 VACCINE STUDIES

Many attempts are being made to vaccinate animals in order to develop protection against pathogens in the environment. Because mucosal surfaces are the most frequent portals of entry for common viral, bacterial, and parasitic infectious diseases, it is therefore clearly important to try to stimulate the mucosal immune system.

If successfully manipulated, GALT could confer long-term mucosal as well as systemic immunity against a variety of toxins and pathogens (Holmgren, 1991; Shalaby, 1995). There are several potential routes of vaccination but this thesis will focus on the oral and parenteral vaccination routes.

1.7.1 Oral vaccination

Oral vaccines are often more desirable than parenteral types due to their ease of production and quality control. They are also easier (and safer) to administer to large numbers of recipients because they do not require medically trained personnel or sterile supplies, and the protective barrier of the skin will not be breached during administration (e.g., poliovirus vaccine). With oral vaccination the antigen delivery can be at the site of natural infection (Holmgren, 1991). However orally administered vaccines are still limited due to the poor long-term efficacy and the requirement for multiple doses to stimulate and maintain a mucosal immune response. Furthermore repeated oral vaccination could induce oral tolerance which could result in a non-responsiveness of the immune system with a subsequent infection (Mowat, 1994).

There are three general barriers that may influence the effectiveness of oral vaccination. Firstly, the harsh environment of enzymatic digestion and pH changes (e.g., gastric acidity) can alter the antigenic structure which could result in markedly decreased immunogenicity due to loss of critical epitopes. Conformational changes

may result in precipitation in the gut lumen, loss of binding affinity to M cell surfaces, or presentation of inappropriate epitopes to the PP. Conformational changes can arise in both freely dispersed or surface bound macromolecules (surface molecules on bacteria or viral vaccines). So feeding during oral vaccination can be important in buffering the harsh environment to minimise the loss of important epitopes.

Secondly, variation in contact exposure time between antigens and PP could influence the efficacy of oral vaccines. The variability in contact exposure time is attributed to the gastric-emptying time which in humans can be as short as 2 hours (fasted state) or as long as 16 hours (fed state) (Mojaverian *et al,* 1985). Prolonged residence in the stomach could have significant drawbacks in terms of proteolytic digestion by pepsin or protein denaturation due to low pH. The residence time in the small intestine is less variable ranging from 3 to 5 hours irrespective of feeding state (Davies *et al,* 1986). Oral vaccines which empty as a bolus from the stomach, such as in a fasted state, will have a shorter period of contact with the PP than in a fed state where the vaccine is released from the stomach in conjunction with the meal. So feeding conditions are important while giving an oral vaccine. Whether a brief contact exposure time is sufficient to stimulate an immune response will depend on the dose and on the third general barrier which is the efficiency of antigen uptake by the PP. In any event, changes in the total exposure time and the adhesion between the antigen and PP add an element of variability which could influence overall efficacy (Shalaby, 1995).

The ideal vaccine would be one that would provide 90% efficacy within a few weeks of a single administration. Furthermore, the mucosal protection would be of long duration without the need for reimmunising on a regular basis. The vaccine would be low cost, safe, cause no adverse side reactions such as immunosuppression, no interference with immunity to other vaccines given simultaneously, and be genetically and thermally stable (Babiuk and Campos, 1993).

1.7.2 Parenteral vaccination

Parenteral vaccines do not have the advantage of antigen delivery at the site of natural infection; they can have possible side effects, and are less easy to give to large numbers of people or animals.

Parenteral vaccines with various antigens usually do not stimulate a secretory IgA immune response in mucosal secretions but can stimulate a systemic immune response consisting primarily of IgM and IgG antibodies. Serum or sIgA is infrequently induced. However in individuals previously exposed to the antigen by the mucosal route (e.g., by natural infection), a secretory IgA response could be detected in external secretions (Mestecky, 1987; Ogra *et al,* 1976; Mestecky *et al,* 1986).

In a previous study on cholera vaccine, in unexposed individuals (lactating Swedish women) parenteral immunisation with cholera vaccine did not consistently induce secretory IgA antibodies in either saliva or milk. In contrast, parenteral immunisation in previously exposed individuals (lactating Pakistani women) to *Vibrio cholerae* induced specific IgA antibodies in both these external secretions (Svennerholm *et al,* 1980)

An increase in secretory IgA by parenteral immunisation is therefore dependent on the initial mucosal exposure (Mestecky, 1987). A systemic immune response is always triggered by parenteral immunisation.

1.7.3 Current trends in vaccine development

Chemical modification of antigens has been studied in an effort to improve immunogenicity through enhanced uptake by PP. Certain molecules such as cholera toxin-binding subunit B (CTB) are taken up preferentially by M cells. Both CTB and the heat-labile toxin (LT) of *E.coli* exhibit increased affinity largely because of binding to the GM-1 ganglioside receptor on M cells and enterocytes (Holmgren, 1991; Frey *et al,* 1996). Furthermore, these molecules are resistant to enzyme-catalysed hydrolysis. Thus, chemical modification with CTB or LT could improve

immunogenicity either by facilitating uptake by M cells or by providing a steric barrier to enzymatic degradation (Shalaby, 1995).

Certain bacteria and viruses have high specificity for PP, and in some cases can colonise PP without mucosal invasion. This would enable the recombinant vectors of these bacteria or viruses to provide sustained expression of endogenous as well as foreign antigens to underlying follicles. Vectors which have been studied include *Salmonella typhi*, *Salmonella typhimurium, Yersinia*, *Vaccinia,* Bacillus Calmette-Guerin (BCG), and adenovirus (Holmgren, 1991; Dogget and Curtiss, 1992; Sutter and Moss, 1992; Stover *et al,* 1992; 1993; Shalaby, 1995). However, this area is still in its infancy with regard to practical human vaccine application and must overcome a number of obstacles such as safety of the proposed vector, pre-existing immunity to vector, and development of appropriate formulations to preserve the viability and immunogenicity of the vector (Holmgren, 1991).

Mucosal adjuvants have been used to enhance the immunogenicity of certain antigens. One potent adjuvant is cholera toxin (CT) that has a high binding affinity for M cells and intestinal epithelium. CT also appears to enhance the antigen-presenting capacity of macrophages, modulate growth of B cells (promotion of isotype differentiation leading to increased IgA formation), and T cells (increased levels of $CD4^+$ T cells against CT-induced disease) (Lycke and Holmgren, 1986; Owen *et al,* 1986; Bromander *et al,* 1991; Hörnqvist *et al,* 1991; Umesaki and Setoyama, 1992; Vjady and Lycke, 1993; Holmgren *et al,* 1993). LT seems to have a similar effect to CT (Holmgren *et al,* 1993).

A unique feature of polymeric delivery systems is that they can be designed to meet the specific physical, chemical, and immunogenic requirements of a particular antigen. The delivery system can be manipulated to incorporate variable amounts of antigen and/or adjuvants, used to minimise antigen digestion by enzymes, chemically modified at the surface to improve site specificity, designed to be biodegradable, and finally, to provide selective uptake by the PP and sustained antigen release following uptake by the PP. Thus, many of the immunological and physiological barriers encountered with oral vaccine development may be addressed using polymeric delivery systems. Several different polymeric antigen delivery systems which can be taken up by PP and enhance mucosal immune response are

being studied. These include microspheres made from a variety of biodegradable polymers such as poly (lactic)/glycolic acid (PLGA), polyphosphazene, and polyanhydrides (Eldridge *et al,* 1989; 1990; McGhee and Kiyono, 1993; Nakaoka *et al,* 1995; Shalaby, 1995; Singh *et al,* 1997), liposomes which are made from biodegradable materials such as phospholipids (Haan *et al,* 1995; Zhou *et al,* 1995), and immunostimulating complexes (ISCOMs) which are non-covalently bound complexes of Quil-A adjuvant, cholesterol and amphipathic antigen (Hoglund *et al,* 1989; Morein *et al,* 1990; Morein and Åkerblom, 1992; McGhee and Kiyono, 1993; Sundquist *et al,* 1996). However, further study is required to optimise these polymeric matrixes (especially microspheres and liposomes) because the uptake in PP is still not efficient enough (Shalaby, 1995). Current studies involve addition of CT or CTB to their surfaces to enhance uptake in the gastrointestinal tract.

In the last few years, research has been initiated on DNA vaccination. Direct inoculation of plasmid DNAs encoding for specific viral proteins allows the expression of immunising proteins by the host cells and presentation to MHC class I molecules, eliciting $CD8^+$ T cell responses. DNA vaccines have been administered by several routes including intramuscular, intravenous, and intradermal inoculations, and by gene-gun delivery of DNA-coated particles into the epidermis. Inoculation of DNA has generated immune responses and protective immunity for influenza A virus in mice and ferrets, lymphocytic choriomeningitis virus and rabies virus in mice, and rotavirus in mice (Fynan *et al,* 1993; Ulmer *et al,* 1993; Webster *et al,* 1994; Xiang *et al,* 1994; Zarozinski *et al,* 1995; Herrmann *et al,* 1996; Chen *et al,* 1997).

1.7.4 Vaccination against rotavirus

A safe and effective oral rotavirus vaccine for young children has yet to be achieved but progress with live oral reassortant vaccines may lead to the first licensed vaccine. Previous work has been done on vaccinating infants with heterologous rotavirus strains using bovine NCDV (Lincoln [RIT 4237]) (serotype 6), bovine WC3 (serotype 6), or rhesus MMU18006 (serotype 3). Although neutralising antibodies were detected after vaccination they were specific only against 1 or 2 human rotavirus strains (serotypes). Vaccination with NCDV, WC3, or

rhesus MMU18006 was protective against human rotavirus serotype 1, 1 and 3, and 3 respectively (Vesikari *et al,* 1983; 1985; Anderson *et al,* 1986; Clark *et al,* 1986; Kapikian *et al,* 1989; 1991; Vesikari, 1993; Ukae *et al,* 1994; Ward and Bernstein, 1994; Midthun and Kapikian, 1996). A previous natural rotavirus infection or rotavirus vaccination, regardless of serotype, is necessary to induce a heterotypic response (Ukae *et al,* 1994).

In developed countries, these vaccines had efficacy against rotavirus diarrhoea, however in developing countries a lack in efficacy was reported using NCDV (DeMol *et al,* 1986; Hanlon *et al,* 1987). Reasons for vaccine failure could be the following: higher rotavirus challenge doses may be common in developing countries overwhelming vaccine-induced immunity; other enteric micro-organisms present in the gut may interfere with vaccine replication; higher levels of breast milk or placentally derived antibodies in infants may neutralise vaccine virus and thereby prevent replication; and vaccine-induced immunity may be poor in malnourished infants (Saif and Jackwood, 1990).

The difference in protection following natural infection with human rotavirus versus vaccination with heterologous strains could be due to differences in epitopes or to the relatively inefficient replication of heterologous rotaviruses in humans which could translate into generally poor immune responses. Therefore new approaches have been tried such as human×animal reassortant vaccines containing 10 genes derived from animal rotavirus (bovine or rhesus rotavirus) and one gene (encoding for VP7) from a human rotavirus (strain DS-1) or quadrivalent vaccines consisting of mixtures of serotype 1, 2, and 4 reassortants and rhesus rotavirus. (Perez-Schael *et al,* 1990; Kapikian *et al,* 1989; 1991; Vesikari *et al,* 1992; Bishop, 1993; McNeal *et al,* 1994).

Trials have been conducted using a quadrivalent vaccine composed of three rhesus-human (DS-1) reassortants, each with 10 rhesus rotavirus genes and 1 human rotavirus gene that encodes VP7 serotype 1, 2, or 4 specificity and the rhesus rotavirus itself provides coverage for VP7 serotype 3. The quadrivalent vaccine has been evaluated in two trials in the USA and was 80-82% protective against severe diarrhoea and 49-57% protection against any rotavirus diarrhoea (Bernstein *et al,* 1995; Kapikian *et al,* 1996).

Trials have also been conducted using a multivalent bovine rotavirus (WC3) reassortant vaccine composed of bovine rotavirus genes and at least one human rotavirus gene for either of the virion surface proteins (VP4 or VP7). The monovalent WC3 reassortant (WI79-9) containing VP7 serotype 1 of a human rotavirus was 64-100% protective in three trials against all rotavirus disease, with no clinical events (Clark *et al,* 1990; Treanor *et al,* 1995; Clark *et al,* 1996a,b). A multivalent vaccine consisting of three reassortants of serotype 1, 2, and 3 combined with a separate reassortant bearing the VP4 of a human rotavirus P8 yielded 67% protection (Clark *et al,* 1996a,b).

Some animal rotavirus vaccines are available, and successful vaccination has been achieved by maternal parenteral vaccination with subsequent passive transfer of immunity via milk to the offspring. This has been particularly successful in cattle stimulating the secretion of specific antibodies in colostrum and milk to take advantage of the fact that rotavirus antibody present in the lumen of the neonatal intestine is an effective mediator of protection.

In monogastrics with primary rotavirus immunisation, an antirotavirus IgG antibody response in serum and milk was detected after parenteral vaccination (Offit and Clark, 1985; Conner *et al,* 1993). Intestinal antirotavirus IgG was also found but this is mainly through transfer of circulating serum IgG into the lumen (Yolken *et al,* 1990). However, in the case of a secondary immunisation in monogastrics previously exposed to rotavirus, a significant IgA increase in milk was found. In serum there was a predominantly IgG response with some IgA detected (McGuire and Crawford, 1973; Browning *et al,* 1991; Snodgrass *et al,* 1995).

In ruminants after a primary or secondary parenteral immunisation, an increase in IgG antibodies was found in milk and serum (Wells *et al,* 1978; Snodgrass *et al,* 1980; Fahey *et al,* 1981; Saif *et al,* 1983; Crouch, 1985; van Zaane *et al,* 1986).

Development of animal vaccines must take account of differences in mucosal immunity between monogastric animals and ruminants, particular in their different antibody response in milk.

1.8 AIMS OF THIS STUDY

No detailed characterisation of the mucosal immune response against a pathogen is yet available. Rotavirus is used as model to investigate the mucosal immune response. This study aims:

I) to establish assays to monitor the immune response at humoral, cellular, and cytokine level (chapter 2).

II) to study if sheep vaccinated parenterally with a standard rotavirus vaccine can boost the immune response to rotavirus (chapter 3).

III) to compare the effect of different adjuvants and antigen doses in parenteral vaccines on the immune response in sheep (chapter 4).

IV) to compare the effect of different adjuvants and antigen doses in oral vaccines on the immune response in sheep (chapter 5).

V) to characterise the primary immune response to rotavirus in gnotobiotic lambs (chapter 6).

VI) to induce immunological priming and protection to a subsequent challenge in gnotobiotic lambs using ISCOMs as a mucosal adjuvant (chapter 7).

CHAPTER 2

MATERIALS AND METHODS

2.1 CELLS

In this study, MA104 cells were used for virus growth, virus titration, and virus neutralisation tests. MA104 cells are an epithelial-like continuous cell line, derived from embryonic rhesus monkey kidney (Stormont, Ireland). The cells were prepared in our tissue culture unit by Mrs. K. Hall and Mrs. K. Quinn. They were grown in Iscove's Modified Dulbeco Medium (IMDM) (Hyclone, UK) containing 1% (v/v) 0.1M glutamine and 10% (v/v) inactivated Foetal Bovine Serum (FBS) (Sigma, UK) (FBS was inactivated by a 30 minutes incubation at 56°C). For investigative purposes (virus titration and virus neutralisation tests) cells were seeded at a concentration of 2×10^5/ml, with 10% inactivated FBS.

2.2 VIRUS, GROWTH, PURIFICATION, INACTIVATION, AND TITRATION

2.2.1 Virus

In this study two different strains of rotavirus were used. These were a bovine strain UK G6P5 (Bridger and Woode, 1975) and an ovine strain K923 G10P14 (Snodgrass *et al,* 1976a; Fitzgerald *et al,* 1995). Both strains were plaque-purified and adapted to *in vitro* propagation in MA104 cells. K923 was also used as a gnotobiotic lamb passaged isolate.

2.2.2 Virus growth

Virus lysate was treated with trypsin (Sigma, UK) for 1 hour at 37°C at a concentration of 10µg/ml. Flasks ($150cm^2$) with confluent monolayers of MA104 cells were washed twice with PBS and 1ml of inoculum was added to the MA104 cells and incubated for 1 hour at 37°C. After incubation, 50mls of 199 M/M medium which was treated for 1 hour at 37°C with 1 µg/ml of trypsin was added to each flask and incubated at 37°C on a roller. When there was a full cytopathic effect (between 1-5 days), flasks were stored at -70°C to lyse the cells and release the virus. Flasks

were thawed and virus lysate was collected and stored at -20°C. When used for *in vivo* studies, rotavirus lysate was extracted with 1,1,2-trichlorotrifluoroethane (arcton) (BDH, UK). This was mixed together in equal amounts, shaken vigorously for 5 minutes, and centrifuged for 10 minutes at 250×g (Beckman TJ-6 centrifuge). The top layer was collected and stored at -20°C.

2.2.3 Virus purification

After virus growth, virus lysate was centrifuged at 17,000×g (Beckmann J6; SW40 rotor) for 1 hour to pellet cell debris. Supernatant fluids were collected and spun at 100,000×g (Beckman J6; SW40 rotor) to pellet the virus. Virus pellets were resuspended in 3mls of a 20mM Tris-buffer pH 7.5 and layered on 4mls of a 40% (w/v) sucrose solution and spun down for 2 hours at 100,000×g (Beckman J6, SW40 rotor). Pellets were resuspended in 10mls of sterile PBS and stored at -20°C until further use.

2.2.4 Virus inactivation

Virus was inactivated by three different methods:

a) by addition of 1.25% (v/v) of a formaldehyde solution (38% w/v) (Fisons, UK) to the virus lysate or the purified virus pellet and incubated for 1 hour at 37°C and then left overnight at 4°C. This method was used to prepare virus for parenteral vaccination. As this is a standard method of rotavirus inactivation (Fontaine *et al,* 1974), the effectiveness of the method was not confirmed.

b) by addition of 5% (v/v) binary ethylene imine (BEI). 2-bromideethylamine hydrobromide salt (0.1M) (Sigma, UK) was dissolved in a pre-warmed 0.2 N sodium hydroxide solution and kept for 1 hour at 37°C (Girard *et al,* 1977), and added to the virus lysate or the purified virus pellet and left at 37°C for 24 hours. After inactivation with BEI at 5% (v/v), virus was passaged three times through MA104 cells and no virus was detected (Table 2.1). This method of inactivation was used when the virus was given by oral vaccination.

Table 2.1: Infectivity of inactivated bovine rotavirus strain UK (arcton extracted) with 1% (v/v) and 5% (v/v) of BEI.

Material	Time	ffu[1]	Material	Time	ffu
Virus + 1% (v/v) BEI	0h	$10^{5.8}$/ml	Virus + 5% (v/v) BEI	0h	$10^{6.8}$/ml
	2h	ND		2h	$10^{5.3}$/ml
	4h	ND		4h	$10^{4.3}$/ml
	6h	$10^{5.8}$/ml		6h	$10^{3.3}$/ml
	8h	ND		8h	$10^{2.3}$/ml
	10h	ND		10h	$10^{1.3}$/ml
	12h	$10^{5.8}$/ml		12h	$<10^{1.3}$/ml
	14h	ND		14h	$<10^{1.3}$/ml
	16h	ND		16h	$<10^{1.3}$/ml
	18h	$10^{5.3}$/ml		18h	$<10^{1.3}$/ml
	20h	ND		20h	$<10^{1.3}$/ml
	22h	ND		22h	$<10^{1.3}$/ml
	24h	$10^{4.8}$/ml		24h	$<10^{1.3}$/ml

ND=Not done; [1] ffu = fluorescent focus units

c) by addition of 5×10^{-3}mg of psoralen to 10mls of purified virus suspension and exposing this for 20 minutes to UVB-light at a distance of 7.5cm (Groene and Shaw, 1992). Before further use the protein concentration was determined by a BCA™ protein assay (Pierce, USA). This method was used to inactivate purified virus for use in *in vitro* assays. Psoralen itself had no influence on the proliferation of mouse lymphocytes *in vitro* (Bruce *et al,* 1994)

2.2.5 Virus titration

Each passage of every rotavirus strain was titrated in MA104 cell monolayers grown in 96-well plates (Nunc, Denmark) which were infected with 10-fold dilutions of the virus in duplicate. Sealed plates were centrifuged for 1 hour at 150×g (Beckmann J-6 M/E centrifuge) at 20°C. After virus inoculation, plates were incubated overnight at 37°C. After incubation, the cells were fixed with diluted acetone for 2-5 minutes. Plates were washed twice with PBS and 50µl of a lamb anti-rotavirus strain UK serum (Lab. No. #3625) diluted 1/200 in PBS was added to the wells and incubated for 25 minutes at 37°C. Plates were washed twice with PBS and 50µl of a donkey anti-sheep/goat IgG fluorescein-labelled, (FITC) (SAPU, UK) diluted 1/320 in PBS was added to the wells. Plates were incubated for a further 25

minutes at 37°C, then washed twice with PBS. Air dried plates were examined for fluorescence by UV microscopy (Ortholux 2, Leitz, Germany). The titre of the virus was calculated by the Kärber formula (Kärber, 1931, cited by Cruickshank R. *et al.* 1965).

2.3 ANIMALS

Animals used in this study for a ruminant model of rotavirus infection, were multiparous mature crossbred sheep and gnotobiotic male and female cross-Suffolk lambs. Sheep were chosen as the supply of sheep and availability of reagents were greater than for cattle. The sheep were purchased from the Moredun flock, housed in pens and fed *ad libitum* on hay and water with a regulated supply of concentrates. Gnotobiotic lambs were delivered aseptically by hysterectomy, held in positive-pressure plastic film isolators (Plate 2.1) (isolators were provided by Moredun Isolators) and fed evaporated milk (Carnation®; Nestle, UK), diluted 1:3 in sterile water, according the following feeding regime (Table 2.2). This reduced regime was used as gnotobiotic lambs developed diarrhoea when fed normally. The amount of milk given to these gnotobiotic lambs was 50% reduced compared to the normal diet. This study would like to investigate if rotavirus was able to induce diarrhoea and not influenced by diet. Lambs increased in weight with time when fed with this reduced regime (no weight graphs shown).

Table 2.2: Feeding regime for gnotobiotic lambs

Day	first feed	second feed	third feed	fourth feed
Day of birth	60mls glucose	150mls milk	150mls milk	200mls milk
Day 1 - Day 4	240mls milk	240mls milk	240mls milk	X
Day 5 - Day 11	350mls milk	300mls milk	350mls milk	X
Day 12 - termination	450mls milk	300mls milk	450mls milk	X

Plate 2.1: Isolator with three gnotobiotic lambs.

2.4 SAMPLES

2.4.1 Nasal secretions

Nasal secretions were collected by tampons (Lil-lets, Smith & Nephew Ltd., UK), size 2cm × 0.5cm, which were inserted into the ventral nasal meatus, withdrawn after 10 minutes and squeezed out with a syringe. The tampons were carefully examined and those with blood present were excluded from the assays. The nasal secretions were stored at -20°C until use.

2.4.2 Blood

Blood (10mls) was collected from the jugular vein in vacutainers with or without 200µl preservative-free heparin (1000units/ml) (Sigma, UK) for the collection of peripheral blood lymphocytes (see 2.7.2) or serum respectively.

Blood samples were left overnight at room temperature to coagulate. The next day, blood samples were centrifuged for 5 minutes at 250×g (Beckman TJ-6 centrifuge) and the serum fractions were collected. The serum samples were stored at -20°C until use.

2.4.3 Saliva

Saliva (1ml) was collected from under the tongue with a syringe. The saliva was centrifuged for 5 minutes at maximum speed with a micro-centrifuge (Damon, IEC CentraM-centrifuge, USA). The saliva supernatants were harvested and stored at -20°C until use.

2.4.4 Faeces

Faeces was collected from the rectum with a swab (lambs) or by manual stimulation (sheep). Faecal swabs were stored in bijoux for viral RNA extraction and examination.

Faeces were also collected for the detection of rotavirus-specific and total antibody levels. 4mls of a 50mM ethylenediaminetetraacetic acid (dihydrate) (EDTA) (Sigma) and 0.1mg/ml of trypsin inhibitor Type II-S: Soybean (Sigma) solution was added to 0.5g of faeces. This suspension was processed in a Colwarth Stomacher (Seward Laboratory, London) for 2 minutes and centrifuged for 5 minutes at maximum speed with a micro-centrifuge. The samples were kept on ice during processing. After centrifugation supernatants were collected and stored at -70°C until use.

2.4.5 Intestinal scrapings

The intestinal secretions were collected by scraping gently along the length of a longitudinally incised piece (20cm) of small intestine with the edge of a glass microscope slide. The scraping was placed into universals (Sterilin) containing 5mls of an enzyme inhibition buffer. This contained 1mM phenyl methyl-sulphonyl fluoride (PMSF) (Sigma), 1mM iodoacetate (sodium salt) (Sigma), 0.1mg trypsin inhibitor (soybean type I-S) (Sigma), and 10mM EDTA dissolved in 100mls of distilled water. A few drops of 95% ethanol were required to dissolve the PMSF. The suspension was sonicated for 30 seconds at amplitude of 20 microns (MSE) and then centrifuged for 5 minutes at maximum speed with a micro-centrifuge. The supernatants were collected and stored at -20°C until use.

2.5 ASSAYS

2.5.1 Rotavirus-specific IgA antibody ELISA

Maxisorp plates (Nunc, Denmark) were coated, except column 1 for blanking the ELISA-reader (Dynatech 500), with 100µl monoclonal mouse anti-rotavirus VP6 UK/1 (Lab No. #5328) diluted 1/5000 in ELISA coating buffer and incubated overnight at 4°C in a humid box. The ELISA coating buffer contained 0.795g disodium carbonate (Fisons, UK) and 1.465g sodium hydrogen carbonate (Fisons, UK) dissolved in 500mls of distilled water and adjusted to pH 9.6. Plates were washed three times with PBS containing 0.05% (v/v) polyoxyethylene-sorbitan monolaurate (Tween-20) (Sigma) (wash buffer). Rows A, C, E, and G were incubated with 100µl of a rotavirus strain K923, diluted 1/10 in PBS containing 0.05% (v/v) Tween-20, and 2% (v/v) dried skimmed milk (Marvel, UK) (PTS) and rows B, D, F, and H with negative MA104-cells, diluted 1/10 in PTS as a negative control. Plates were incubated for 1 hour at 37°C in a humid box. Plates were washed 3 times with wash buffer and 100µl of samples and standards, 8 doubling dilutions of a bovine colostral whey (Lab. No. W3640/3) diluted in PTS were added in duplicate to positive and negative wells. Plates were incubated for 1 hour at 37°C in a humid

box. Plates were washed three times with wash buffer and 100µl of monoclonal mouse IgG α bovine IgA-horseradish peroxidase (HRP) (Lab. No. B/84/1/5) (monoclonal mouse IgG anti-bovine IgA was a gift from Dr. C. Stokes, University of Bristol and this was purified and conjugated to HRP by Mr. D. Harkins, Moredun Research Institute) diluted 1/1000 in PTS was added. Plates were incubated for 1 hour at 37°C in a humid box. After incubation plates were washed three times with wash buffer and 100µl of the substrate σ-phenylenediamine dihydrochloride (OPD) (Sigma) was added. Colour development was stopped with 50µl of 2M H_2SO_4. Plates were read with a Dynatech500 ELISA-reader at 492nm. OD values of negative wells were subtracted from positive wells. OD means of each duplicate were compared to the OD values of standards and expressed as units/100mls or units/ml. The standard was assigned an arbitrary value of 50,000 units.

2.5.2 Total immunoglobulin A ELISA

Maxisorp plates (Nunc, Denmark) were coated, except column 1 for blanking ELISA-reader (Dynatech MR500), with 100µl of pig anti-sheep IgA serum (gift from Dr. W.D. Smith, Moredun Research Institute), diluted 1/4000 in ELISA coating buffer and incubated overnight at 4°C in a humid box. Plates were washed three times with PBS containing 0.05% (v/v) Tween-20. 100µl of samples and standards, 8 doubling dilutions of standard IgA, this was purified from clarified lung fluid of a sheep infected with ovine retrovirus (Jaagsiekte) (JSRV) (gift from Ms. P. Dewar and purified by Mr. D. Harkins, Moredun Research Institute), diluted in PTS and were added in duplicate to rows A, C, E, and G. In rows B, D, F, and H 100µl of PTS was added as a negative control. Plates were incubated for 1 hour at 37°C in a humid box. Plates were washed three times with wash buffer and 100µl of monoclonal mouse IgG anti-bovine IgA-horseradish peroxidase diluted 1/1000 in PTS was added. After an incubation of 1 hour at 37°C, plates were treated and developed in the same way as described in the rotavirus-specific IgA ELISA. OD means were expressed in mg/100mls.

2.5.3 Rotavirus-specific IgG antibody ELISA

The assay is similar to the rotavirus-specific ELISA except specific reagents were: a hyperimmune lamb anti-rotavirus serum (Lab. No. #3625) was used as the standard and the detection conjugate was a donkey anti-sheep IgG HRP (The Binding Site, UK) diluted 1/1000. The standard was assigned an arbitrary value of 800,000 units.

2.5.4 Total immunoglobulin G ELISA

The assay is similar to the total IgA ELISA except specific reagents were: rabbit anti-sheep immunoglobulins (Ig) (DAKO, UK) diluted 1/2000 was used as the coating antibody, a hyperimmune lamb anti-rotavirus serum (Lab. No. #3625) with 9,600mg IgG/L (determined by a sheep IgG calibration kit (Serotec, UK)) was used as the standard, and the detection conjugate was a rabbit anti-sheep IgG-HRP (Pierce, USA) diluted 1/20,000.

2.5.5 Virus neutralisation assay

Neutralisation tests were performed using the microtitre fluorescent focus reduction method (Ojeh *et al,* 1984). MA104 cells monolayers were prepared by addition of 100µl of 2×10^5 MA104 cells/ml diluted in IMDM containing 10% (v/v) inactivated FBS, 1% (v/v) 0.1M glutamine, 1% (v/v) 2.5M hepes (Sigma, UK), and 1M NaOH (pH 7.0) to a 96 well plate (Nunc, Denmark). Plates were sealed (Greiner, UK) carefully and incubated overnight at 37°C.

All sera and other body fluids were diluted in 199 M/M media to 1/10 and inactivated at 56°C for 30 minutes before use. Using transfer plates (Nunc, Denmark), doubling two-fold dilutions of sera were made in 199 M/M. The stock antigen was diluted to a titre of approximately 50 fluorescent focus forming units per well and equal amounts were added to every well except cell controls. For antigen control, virus was added to 199 M/M. The loaded transfer plates were sealed and incubated at 37 °C for 1 hour. After incubation, the MA104 cells monolayers were

inoculated with the virus/serum mixture, plates were sealed (Greiner, UK) and centrifuged at 150×g for 1 hour at 20°C.

After an overnight incubation at 37°C, plates were treated the same as described in virus titration (see 2.2.5). Titres were expressed as the reciprocal of the highest serum dilution giving a 60% reduction of fluorescent foci when compared to the virus control wells. As the quality control, a rabbit anti-rotavirus strain UK serum (Lab. No. #5877) was used.

2.6 VIRAL RNA EXTRACTION AND DETECTION

Analysis for the presence of viral RNA in faeces was based on a previous described method (Herring *et al,* 1982). Double-stranded RNA was extracted from the faeces by placing the faecal swabs in a plastic tube with 0.75mls of SDS-extraction buffer (appendix) and 1ml of Tris saturated phenol/chloroform (Sigma, UK). This was mixed using the vortex mixer and centrifuged for 10 minutes at 250×g (Beckman, TJ-6 centrifuge). The top aqueous phase was collected and stored at -20°C. Samples were run on a 7.5% polyacrylamide continuous gel for 1.5 hours and double-stranded RNA was visualised by silver nitrate staining.

2.7 LYMPHOID TISSUE AND LYMPHOCYTES

2.7.1 Collection of gut-associated lymphoid tissue

Sheep or lambs were killed with 20mls or 10mls of pentibarbitone 200mg/ml respectively (Euthatal) (Rhône Mérieux Limited) by inoculation into the jugular vein. The gut (from pylorus to rectum) was removed aseptically for collection of mesenteric lymph nodes (MLNs), jejunal and ileal Peyer's patches (JPPs and IPPs), and small intestine. MLNs, JPPs, IPPs, and small intestine were placed into a Hanks Balanced Salts solution without calcium and magnesium (Gibco, BRL) containing 100units/ml benzyl penicillin (sodium) (Britannia), and 0.1mg/ml streptomycin sulphate (Gibco, BRL) until future use.

Plate 2.2: Gut obtained from a gnotobiotic lamb 37 days after infection with rotavirus.

2.7.2 Isolation of lymphocytes

2.7.2.1 Peripheral blood lymphocytes

Blood was centrifuged for 10 minutes at 250×g to collect buffy-coat cells. These were resuspended and washed twice with HBSS; viable cells were counted by the trypan blue dye exclusion technique and resuspended at the appropriate cell concentration in IMDM containing 100units/ml penicillin, 0.1mg/ml streptomycin, 1% (v/v) 0.1M glutamine, and 10% (v/v) inactivated FBS until further use.

2.7.2.2 Mesenteric lymph nodes and Peyer's patches

The collection of lymphocytes from MLNs and PPs was based on previously described methods (Entrican *et al,* 1992; Bruce *et al,* 1995). Fat and surrounding gut tissues were trimmed off. A cell suspension was prepared by chopping the MLNs or the PPs with scissors in HBSS (Gibco, BRL) containing 100units/ml benzyl penicillin (sodium) (Britannia), 0.1mg/ml streptomycin, 0.5µg/ml gentamicin (Gibco, BRL), and 2% (v/v) inactivated foetal bovine serum (FBS) (Sigma, UK). The cell suspension was processed for 2 minutes with a Colwarth Stomacher and passed through two layers of sterile lens tissue (Whatman Paper Ltd., Maidstone UK). Cells were washed twice with HBSS and treated as above (see 2.7.2.1). The average yield of lymphocytes obtained from a piece of MLN, a JPP, and a 5-10cm piece of IPP was >> 250×10^6, 25-45×10^6, and >>250×10^6 cells respectively.

2.7.2.3 Small intestine

The collection of lamina propria (LPLs) and intraepithelial lymphocytes (IELs) was based on a previously described method (Nagi and Babiuk, 1987). Fat was trimmed off and the collected small intestine was cut into pieces of 10cm to 15cm length (Plate 2.3). Each piece was inverted so that the mucosal surface was exposed, filled with HBSS without magnesium and calcium and ends were tied together with a non-biological string.

These pieces of small intestine were placed into a glass beaker filled with 100mls of HBSS containing 2mM EDTA, to release epithelial cells (including IELs) (Plate 2.4). The pieces were stirred gently at 37°C. After 30 minutes, the first cell suspension was collected and discarded. The beaker was filled with 100mls of fresh EDTA solution and stirred for 45 minutes. The second cell suspension was centrifuged for 10 minutes at 250×g (Beckman TJ-6 centrifuge) and collected. The beaker was filled again with 100mls of fresh EDTA-solution for the collection of the third cell suspension. The second and third batches of cells with IELs were pooled for further processing.

The fragments of small intestine were then washed for 30 minutes with a 37°C solution of RPMI containing 100units/ml penicillin, 0.1mg/ml streptomycin, 1% (v/v) 0.1M glutamine, and 10% (v/v) inactivated FBS (this to remove the EDTA). Wash medium was decanted and the beaker was filled with 75mls of RPMI containing 80units/ml collagenase XI (Sigma, UK), 0.1mg/ml deoxyribonuclease type V (Sigma, UK), 100units/ml penicillin, 0.1mg/ml streptomycin, 1% (v/v) 0.1M glutamine, and 10% (v/v) inactivated FBS to remove LPLs (Plate 2.5). The beaker contents were stirred gently for 1 hour at 37°C and the cell suspension was collected by centrifugation. The average yield of lymphocytes obtained from a 10cm piece of small intestine was 25-40$\times 10^6$ IELs and 25-50$\times 10^6$ LPLs.

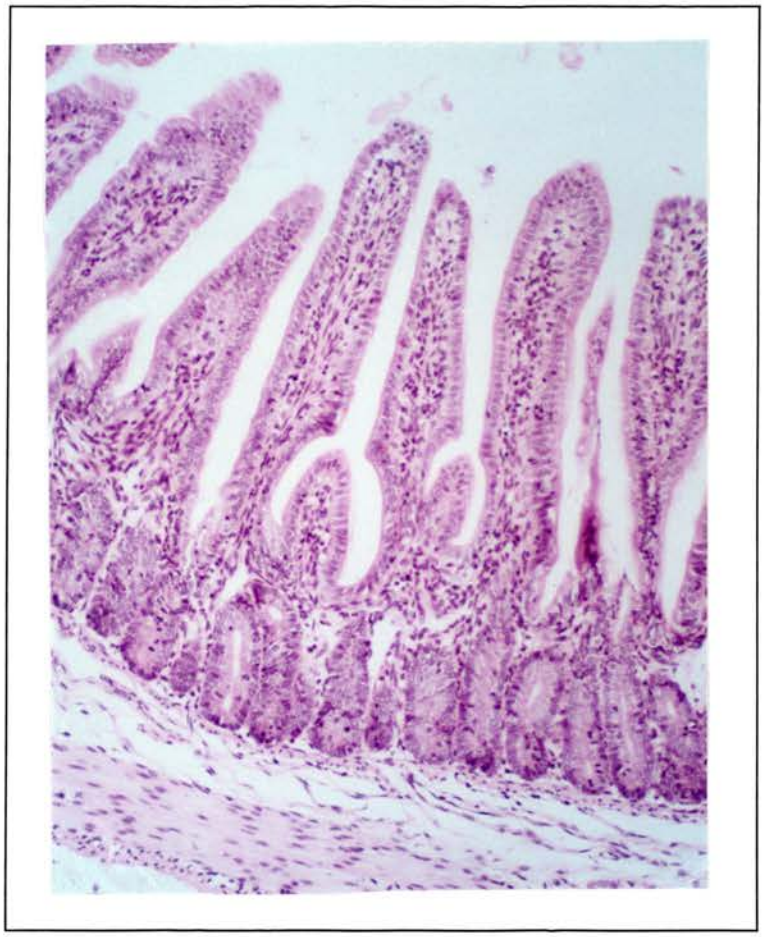


Plate 2.3: Small intestine before treatment (H&E; ×100).

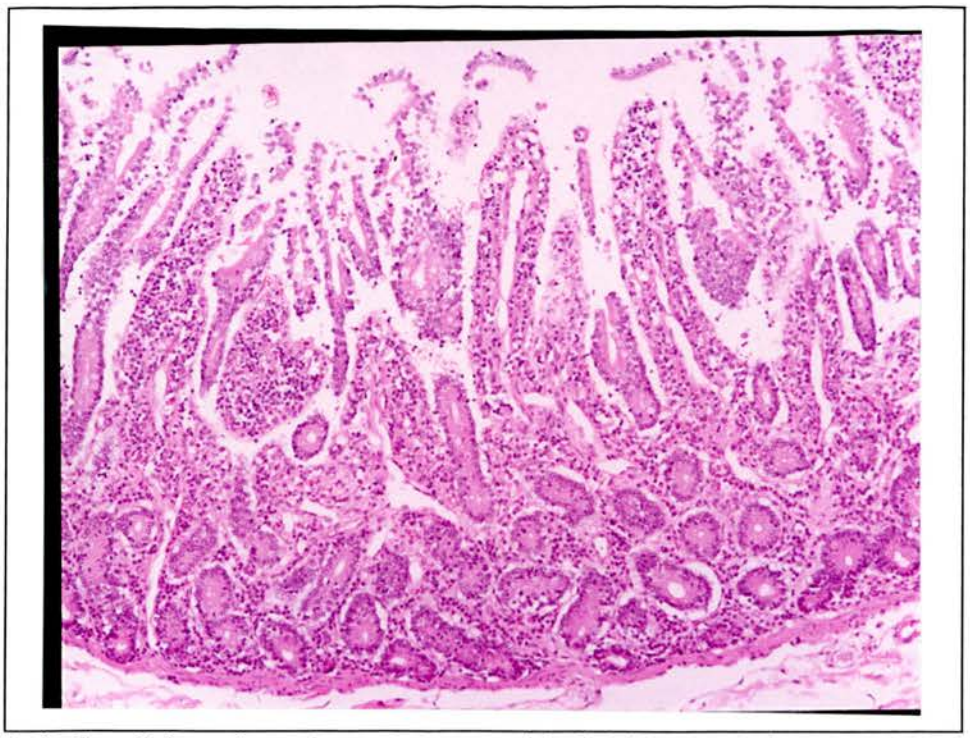


Plate 2.4: Small intestine after treatment with HBSS containing 2mM EDTA to obtain IELs (H&E; ×100).

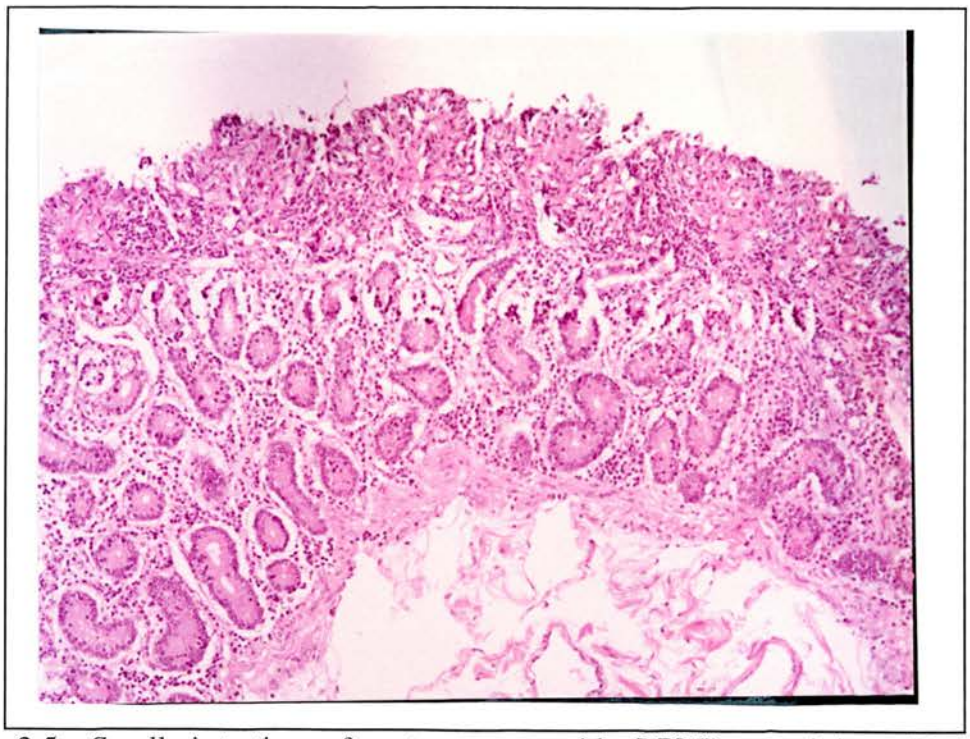


Plate 2.5: Small intestine after treatment with RPMI containing 80units/ml collagenase XI to obtain LPLs (H&E; ×100).

Sterile glasswool columns in 10 or 20mls syringes were washed through with HBSS until a neutral pH was obtained. The cell suspensions with IELs were passed through the 20mls syringe and cell suspensions with LPLs through the 10mls syringe. Columns were washed with HBSS. These cell suspensions were put on an equal volume of Lymphoprep™ (Nycomed, Pharma AS, Norway) and centrifuged for 1 hour at 250×g (Beckmann TJ-6 centrifuge). The top layers with IELs and LPLs were collected and washed twice with HBSS and treated as described above (see 2.7.2.1).

2.8 LYMPHOCYTE ASSAYS

2.8.1 Lymphocyte proliferation assay

200µl of a lymphocyte suspension at a concentration of 1×10^6/ml in IMDM containing 100units/ml penicillin, 0.1mg/ml streptomycin, 1% (v/v) 0.1M glutamine, and 10% (v/v) inactivated FBS was added to each well of a 96-well (U-bottomed) microtiter plate (Nunc, Denmark). 20µl of inactivated purified rotavirus antigen at a concentration of 10µg/ml, 4µg/ml, and 0.4µg/ml was added to the test wells in triplicate. Controls, all in triplicate, included unstimulated cultures (20µl of medium only), cultures stimulated with 20µl of 10µg/ml concanavalin A (ConA) (Sigma, UK), cultures stimulated with 20µl of 2.5µg/ml pokeweed mitogen (PWM; lectin from *Phytolacca americana*) (Sigma, UK), and cultures stimulated with 4µg/ml ovalbumin (OVA) (Sigma, UK) as proliferation and specificity controls. Plates were placed in a humid box and kept at 37°C and in an atmosphere of 5% CO_2.

Lymphocytes were cultured for four days and then pulsed with 0.1µCi ^3H thymidine (Amersham) for a further 18 hours. Cultures were then harvested with a Micromate™ 196 (Packard, USA) and thymidine uptake was determined by dry scintillation counting with a Matrix™ 96 (Packard, USA).

Mean counts per minute (cpm) were determined for replicate wells and a stimulation index (SI) was calculated as the mean cpm in stimulated cultures divided by the mean cpm in cultures with medium only.

2.8.2 Immunofluorescent labelling

100µl of a lymphocyte suspension at 5×10^6cells/ml was added to round-bottomed test tubes and centrifuged at 250×g for 3 minutes (Beckman CS 6R centrifuge). Cell pellets were resuspended in 500µl of wash buffer (PBS containing 0.01M sodium azide and 2% (v/v) inactivated FBS) and centrifuged at 250×g for 3 minutes. Wash buffer was discarded and 50µl of wash buffer was added to the first two test tubes (controls). 50µl of the appropriate monoclonal mouse anti-sheep cell surface marker (Table 2.3) (monoclonal antibodies were provided by Mr. D. Deane, Moredun Research Institute) diluted in wash buffer was added to the remaining test tubes and incubated for 30 minutes at 4°C.

After incubation cells were washed with 500µl of wash buffer. After last wash, 50µl of wash buffer was added to the first test tube (control) and to the remaining test tubes 50µl of fluorescein-labelled rabbit anti-mouse IgG (DAKO, UK) diluted 1/50 in wash buffer was added and incubated for 30 minutes at 4°C.

After incubation cells were washed twice with wash buffer and resuspended in 200 µl of a 4% (w/v) paraformaldehyde solution (BDH) and stored at 4°C until cells were scanned and counted with a FACScan® (Becton-Dickinson Ltd). Analyses were based on 5000-10000 cells. FACS profiles of the different monoclonal antibodies used on the different lymphocyte populations are shown in the appendix.

Table 2.3: Monoclonal antibodies (Mab) used for FACS analysis

Mab	Isotype	Specificity	Reference
17D	IgG_1	Ovine CD4	(Maddox *et al,* 1985)
7C2	IgG_{2a}	Ovine CD8	(Maddox *et al,* 1985)
86D	IgG_1	Ovine WC2 (γδ T cells)	(MacKay *et al,* 1989)
VPM8	IgG_1	Ovine light chain Ig (B cells)	(Puri *et al,* 1985)
73B	IgG_1	Ovine CD45R (B cells and naïve T cells)	(MacKay *et al,* 1990)

2.8.3 ELISPOT

Maxisorp plates (Nunc, Denmark) were coated with 100µl of the monoclonal rabbit anti-rotavirus antibody (Lab. No. #5722) diluted 1/2000 in ELISA coating buffer and incubated for 1 hour at 37°C in a humid box. Plates were washed four times with PBS. Plates were coated with 100µl of 5µg/ml purified bovine rotavirus (strain UK) in PBS. Plates were incubated overnight at 4°C in a humid box, and then washed four times with PBS. Plates were incubated for 1 hour at 37°C and 5% CO_2 with 200µl of IMDM containing 10% foetal calf serum (FCS). After incubation, medium was discarded from the plates and 100µl of a suspension containing 0.5×10^6 peripheral blood lymphocytes/ml diluted in IMDM containing 10%FCS, was added to the wells. Plates were incubated overnight at 37°C in an atmosphere of 5% CO_2. The next day, plates were washed four times with PBS/0.05% Tween-20 and 100µl of monoclonal mouse IgG anti-bovine IgA-HRP (Lab. No. B/84/1/5) or donkey anti-sheep IgG-HRP affinity purified (The Binding Site, UK), diluted 1/1000 in PBS/0.05% Tween-20 was added. Plates were incubated overnight at room temperature and left in the dark. After overnight incubation, plates were washed four times with PBS/0.05% Tween-20 and 100µl of the precipitating substrate aminoethylcarbazole (AEC) was added to each well. Plates were left until spots were visible (Plate 2.6 and 2.7). Washing the plates with distilled water stopped the reaction. Spots were counted and expressed as antibody secreting cells (ASC)/1,000,000 lymphocytes.

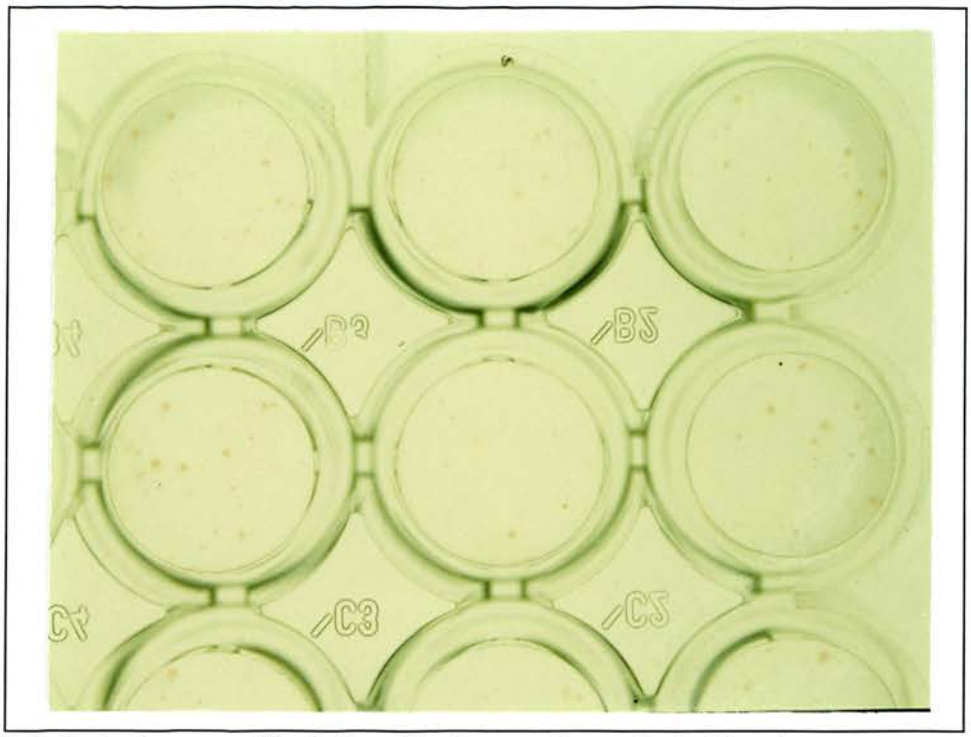


Plate 2.6: Rotavirus-specific IgA antibody secreting cells in blood.

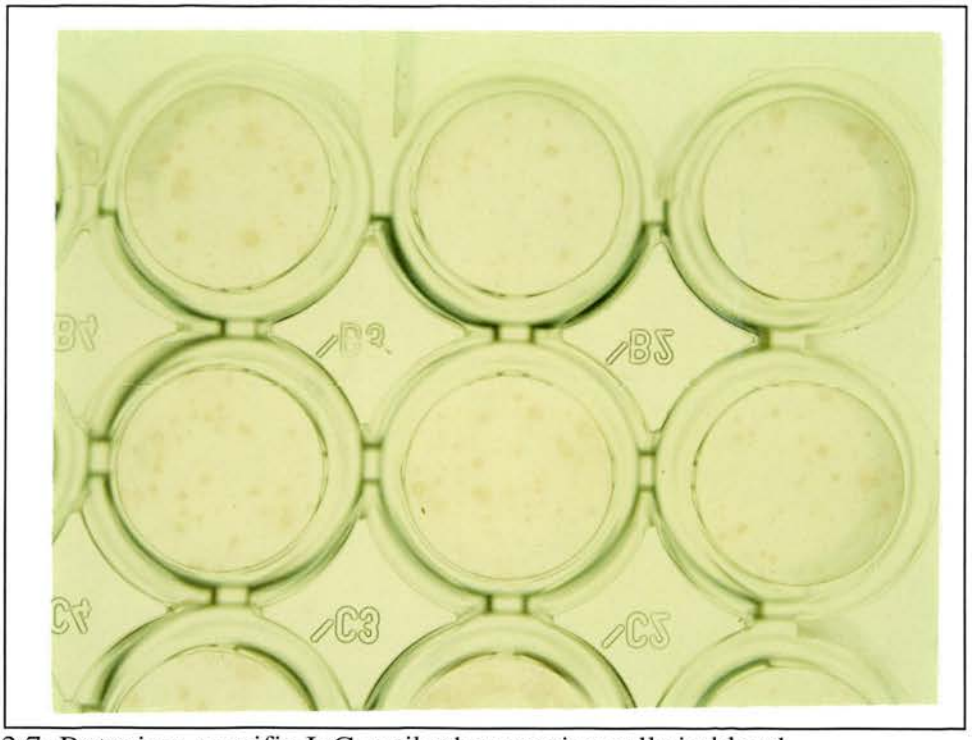


Plate 2.7: Rotavirus-specific IgG antibody secreting cells in blood.

2.9 RNA EXTRACTION, REVERSE TRANSCRIPTASE, POLYMERASE CHAIN REACTION, AND HYBRIDISATION

2.9.1 RNA extraction

RNA extraction was based on a previously described method (Chomczynski and Sacchi, 1987). 1×10^8 lymphocytes were homogenised in 5mls of extraction buffer (appendix). After homogenising, 0.5ml of a sterile 3M sodium acetate solution pH 4.5 was added and mixed, followed by 5mls of phenol (BDH) and 1ml of chloroform, this mixture was shaken vigorously for 5 minutes.

After shaking, the mixture was left on ice for 10 minutes and centrifuged at maximum speed for 10 minutes with a micro-centrifuge. After centrifugation, the aqueous phase was transferred to a sterile centrifuge tube and an equal volume of isopropanol (propan-2-ol) (BDH) was added and mixed. This was left at -20°C for at least 1 hour before centrifugation at maximum speed for 20 minutes with a micro-centrifuge. The supernatant was discarded and the pellet was washed with 70% ethanol and centrifuged at maximum speed for 10 minutes with a micro-centrifuge. Finally, the pellet was resuspended in 200µl of sterile distilled water (per 1×10^8 starting cells).

The RNA sample were checked on a northern gel for RNA integrity and contaminating DNA (genomic DNA runs as a distinct band above the RNA). 0.4g agarose was dissolved in 40mls of a 1×MOPS buffer (appendix) using a microwave and once cooled below 50°C, 2mls of a 37% formaldehyde solution was added before pouring the gel. To 2µl of RNA sample, 8µl of RNA sample loading buffer (appendix) was added. This was heated at 65°C for 10 minutes and then chilled on ice. Sample was loaded on the gel and run for 30-60 minutes at 80V. Running buffer was a 1×MOPS buffer. RNA bands were visualised with UV-light.

RNA was quantified with a spectrophotometer at ODs of 260nm and 280nm used as an internal control (absorption at 260nm/280nm ≈ 2.0). 1 absorption unit at 260nm = 40µg of RNA and final concentration was expressed as µg/ml.

2.9.2 Reverse transcriptase (RT)

cDNA was prepared from RNA by using the following protocol. RNA was heated at 65°C for 10 minutes and cooled on ice. 1μg of RNA was reverse transcribed in a 20μl reaction mixture containing 4.0μl 5×RT buffer (Boehringer), 2.0μl 10×dNTPs (2mM) (Promega), 2.0μl 10×random primer pd$(N)_6$ (62.5units/ml) (Pharmacia Biotech), 0.25μl (5 units) of AMV reverse transcriptase (gene product of avian myeloblastosis virus) (Boehringer), and made up with sterile distilled water. The AMV reverse transcriptase was added to each reaction and mixed gently. This was incubated at 37°C in a water bath for 90 minutes. After incubation, samples were vortexed, centrifuged and 80μl of sterile distilled water was added to each sample making a total of 100μl. Samples were stored at -20°C if not used straight away.

2.9.3 Polymerase chain reaction (PCR)

DNA amplification was performed by the following method. 10μl of each of the resulting reverse transcriptase reactions was used as a template in a 20 μl PCR consisting of 2.0μl 10×PCR buffer (Boehringer), 2.0μl 10×dNTPs (2mM) (Promega), 2.0μl of the specific upstream primer (10μM), 2.0μl of the specific downstream primer (10μM), 0.2μl (1 unit) Taq DNA Polymerase (Boehringer), and 1.8μl sterile distilled water.

Cytokine-specific primers (Table 2.4) were chosen since they spanned the coding region of the cytokine transcripts based on the published sequences for bovine IFNγ (Cerretti *et al,* 1986a), bovine IL-2 (Cerretti *et al,* 1986b), ovine IL-4 (Seow *et al,* 1993), and ovine IL-6 (Ebrahimi *et al,* 1995). The glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH), based on the human sequence, was used as a non-specific control (Tokunaga *et al,* 1987).

Table 2.4: Nucleotide sequence of upstream (sense) and downstream (antisense) PCR primers for target genes

Cytokine		Oligonucleotide	Predicted product (bp)
GAPDH	sense	5'CATAACACAGGAGCTCAAGA3'	646
	anti-sense	5'TGCCAAGTTGGACCCTGAGA3'	
IFNγ	sense	5'CATAACACAGGAGCTACCGA3'	550
	anti-sense	5'TGCCAAGTTGGACCCTGAGA3'	
IL-2	sense	5'ATGTACAAGATACAACTC3'	465
	anti-sense	5'GTCATTGTTGAGTAGATG3'	
IL-4	sense	5'ATGGGTCTCACCTCCCAGCTGATC3'	405
	anti-sense	5'ACACTTTGAGTATTTCTCCCTCAT3'	
IL-6	sense	5'ATGAACTCCCTCTTCACAAGCGCC3'	627
	anti-sense	5'ACTTCATCCGAATAGCTCTCAGGC3'	

The PCR mixture was quickly centrifuged and overlayed with 20µl of sterile mineral oil. Cycling parameters for all primer sets were 30 cycles of 95°C for 1 minute, 50°C for 2 minutes, and 72°C for 2 minutes.

4µl of loading buffer, 0.25% (w/v) bromophenol blue in a 60% glycerol solution, was added to each PCR and analysed on a 1.5% agarose gel. Agarose was dissolved in 1×TBE (10×TBE buffer: 1M Tris, 1M boric acid, and 10mM EDTA, pH 8.0) using a microwave and once cooled below 50°C, ethidium bromide (10mg/ml) (5µl per 40mls) was added before pouring the gel. 5 µl of each sample was loaded, including 5µl of a 1Kb DNA ladder (Gibco BRL) on the gel and run for 30-60 minutes at 80V in a running buffer of 1×TBE buffer. DNA bands were visualised with UV light.

2.9.4 Southern blotting

Denaturation of the gel was performed by soaking in a 0.5M sodium hydroxide (Fisher Scientific) and 1.5M sodium chloride solution (Fisher Scientific). After 15 minutes, the solution was poured off and a 1M ammonium actetate (BDH) and 0.02M sodium hydroxide (Fisher Scientific) solution was added to the gel. After 15 minutes the solution was changed and replaced for a last soak of 30-60 minutes.

The Southern blot was set up by placing cling film on the bench followed by the gel (wells side down). The gel was covered with a wet Hybond-N nylon

membrane (Amersham) avoiding air bubbles, one piece of wet 3MM blotting paper, and followed by two pieces of blotting paper. This was covered with layers of blue paper roll and a glass plate and left overnight. The next day, glass plate, blue roll, and blotting paper were removed to place nylon membrane on a piece of blue roll to let it air-dry. When dry, DNA was fixed by exposing the nylon membrane for 3 minutes to UVB light.

2.9.5 Hybridisation

Hybridisation was based on a protocol provided with the DIG DNA labelling and detection kit (Boehringer Mannheim, Cat. No. 1093657).

Membranes were pre-hybridised in a glass tube for 30 minutes at 55°C with 5-10mls of a pre-hybridisation buffer containing 5×SSC buffer (20×SSC buffer: 3M sodium chloride and 300mM sodium citrate, adjusted to pH 7.0 and autoclaved), 1.0% of a 10×blocking stock solution (blocking reagent provided in kit), 0.1% N-laurolsylsacosine, and 0.02% sodium dodecyl sulphate (SDS). Membranes were hybridised overnight at 55°C with 10mls of pre-hybridisation buffer containing 6µl of the specific cytokine probe.

The probe was made, using the DIG oligonucleotide tailing kit (Boehringer Mannheim, Cat. No. 1417231). In short, 4µl of tailing buffer, 4µl of cobalt chloride solution, 100pmol of oligonucleotide (Table 2.5), 1µl of DIG-dUTP solution, 1µl dATP solution, 50 units (≈1µl) terminal transferase was made up with sterile water to 20µl in an eppendorf on ice. This was incubated for 15 minutes at 37°C and then placed on ice.

Table 2.5:Nucleotide sequence of upstream (sense) and downstream (antisense) hybridisation probes

Cytokine		Oligonucleotide
GAPDH	sense	5'CTCATGACCACAGTCCATGCCATCACTGCC3'
IFNγ	sense	5'CTGTGTGCTTTTGGGTTTTTCTGGTTCTTATGGCCAGGG3'
	anti-sense	5'GAAGTCCTCCAGTTTCTCAGAGCTGCCGTTCAAGAACTT3'
IL-2	sense	5'GGTGCACCTACTTCAAGCTCTACGGGGAACACAATGAAAG3'
	anti-sense	5'AGTTAAATGTATGCATCCTGGAGAGCTTGAGGTTCTCGGG3'
IL-4	sense	5'GACTGGAATTGAGCTTAGGCGTATCTACAGGAGCCACATG3'
	anti-sense	5'CAAGAGGTATCTCAGCGTACTTGTACTCGTCTTGGCTTCA3'
IL-6	sense	5'TGCTTCCAATCTGGGTTCAATCAGGCGATT3'

After overnight incubation, membranes were washed twice with a solution containing 0.1% SDS and 2XSSC for 5 minutes at room temperature and washed twice with a solution containing 0.1% SDS and 0.2×SSC for 15 minutes at 55°C. Membranes were washed for 1 minute at room temperature with DIG-buffer 1 (DIG-buffer 1: 0.1M maleic acid and 0.15M sodium chloride, adjusted to pH 7.5 and autoclaved) followed by 30 minutes at room temperature with DIG-buffer 2 (DIG-buffer 2: 10×blocking stock solution diluted 1/10 in DIG-buffer 1). Membranes were incubated for 30 minutes at room temperature with anti-DIG-AP conjugate diluted 1/5000 in DIG-buffer 2 in sealed plastic bags. After incubation, membranes were washed twice with DIG-buffer 1 for 15 minutes followed by a 2 minutes wash with DIG-buffer 3 (DIG-buffer 3: 0.1M Tris-HCl, 0.1M sodium chloride, and 50mM magnesium chloride, pH 9.5). Membranes were developed in the dark by adding 10mls of DIG-buffer 3 containing 45µl of NBT-solution and 35µl of X-phosphate solution in sealed plastic bags. Colour precipitate started to form within a few minutes and the reaction was usually completed after 16 hours. Washing the membranes with distilled water stopped the reactions.

2.10 STATISTICS

Results were analysed using Minitab 9.2 (Minitab Inc.) and Genstat 5 (IACR) software. Group differences between rotavirus-specific antibody titres and total antibody concentrations in serum and nasal secretions were analysed using a model for repeated measurement (Genstat 5). Group differences in rotavirus-specific antibody titres and total antibody concentrations in intestinal scrapings, virus neutralising titres (^{10}log-values), and rotavirus-specific antibody secreting cells in blood were analysed with analysis of variance (ANOVAs) (Minitab 9.2). The percentage of lymphocyte sub-populations in blood and GALT were compared to pre-vaccination levels and control group respectively with analysis of variance (ANOVAs) (Minitab 9.2). Lymphocyte proliferation results (^{10}log-values) were analysed using a 2-tailed t-test (Minitab 9.2). Cytokine expressions were analysed as positive (1) versus negative (0) using a 1-sample t-test (Minitab 9.2).

CHAPTER 3

STIMULATION OF MUCOSAL ROTAVIRUS-SPECIFIC IMMUNITY IN SHEEP BY A STANDARD PARENTERAL VACCINATION PROTOCOL

3.1 INTRODUCTION

Parenteral administration of inert non-replicating antigens tends to induce a poor immune response, which can be enhanced with adjuvants. Inactivated adjuvanted rotavirus dam vaccines are used in cattle and are based on oil-adjuvantation.

Freunds incomplete adjuvant (IFA), an oil-based adjuvant, has been used as a successful carrier for antigen. Increased rotavirus-specific IgG antibodies were found in serum and colostrum when vaccinating the dam parenterally in cattle and sheep (Wells *et al,* 1978; Snodgrass *et al,* 1980; Castrucci *et al,* 1984; Saif *et al,* 1984; Archambault *et al,* 1988; Möstl and Bürke, 1988; Bellinzoni *et al,* 1989). The majority of these rotavirus-specific IgG antibodies present in colostrum are derived from serum (Saif *et al,* 1984). The maternally derived neutralising antibodies present in the intestine through the feeding of colostrum protect the calves and lambs against rotavirus infection.

In general, parenterally administered vaccines are not effective at inducing a mucosal immune response especially in naive animals. Mucosal immune responses are not seen after parenteral vaccination in unexposed humans. In contrast, in previously exposed humans parenteral vaccination can induce or boost an immune response at mucosal surfaces (Svennerholm *et al,* 1980; Pickering *et al,* 1995). Naive sows vaccinated parenterally with live attenuated TGE virus showed a virus-specific IgG response in serum and milk (Bohl *et al,* 1975), however a mucosal response (e.g., virus-specific secretory IgA in milk) was only seen in naturally infected sows (Saif and Bohl, 1980).

Currently it is not clear if this standard rotavirus vaccination of the dam can boost rotavirus-specific antibodies at mucosal surfaces other than from serum transudation.

The aim of this chapter was to study whether parenteral vaccination using a standard parenteral rotavirus vaccine can boost the mucosal humoral immune response and to make a comparison with the systemic humoral immune response.

3.2 EXPERIMENTAL DESIGN

3.2.1 Animals

Non-lactating multiparous crossbred sheep were used. All animals had serum neutralising antibodies to rotavirus measured by virus neutralising assay, indicating previous natural intestinal exposure.

In this study, two groups of sheep (n=12 for each group) were used and vaccinated parenterally either with PBS or with inactivated rotavirus lysate (RV) each adjuvanted with IFA (PBS/IFA and RV/IFA respectively).

3.2.2 Vaccines

Bovine rotavirus strain UK was used as a cell culture lysate (infectivity titre of $10^{6.8}$ ffu/ml) (see 2.2.2) and inactivated by formaldehyde before use (see 2.2.4).

The RV/IFA group was vaccinated with 2mls each of inactivated rotavirus lysate emulsified (1:1) with IFA (DIFCO, UK). The PBS/IFA group was vaccinated with 2mls each of PBS emulsified (1:1) with IFA. All sheep were vaccinated in equal volumes in each back leg by deep intramuscular injection.

3.2.3 Sample collection

Blood, nasal secretions, saliva, and faeces were collected at intervals and intestinal scrapings were collected at necropsy. Sheep were sampled at vaccination (pre-bleed) and at 2, 4, 6, 8, 12, and 16 weeks after vaccination. 7 out of 12 sheep from each group were killed 2-3 weeks after vaccination and the remaining sheep were killed 16-18 weeks after vaccination.

Serum, nasal secretions, saliva, faeces, and intestinal scrapings were tested for rotavirus-specific antibodies and total IgA and IgG concentrations by ELISA. Virus neutralising titres were determined in serum and intestinal washings by virus neutralising assay.

3.3 RESULTS

3.3.1 Standardisation of ELISAs

Each ELISA was standardised on an 8-point curve. The rotavirus-specific antibody IgA and IgG standard curves with sample curves are shown in Fig 3.1 and 3.2 respectively. The total IgA and IgG standard curves with sample curves are given in Fig. 3.3 and 3.4 respectively.

Fig. 3.1: Rotavirus-specific IgA ELISA

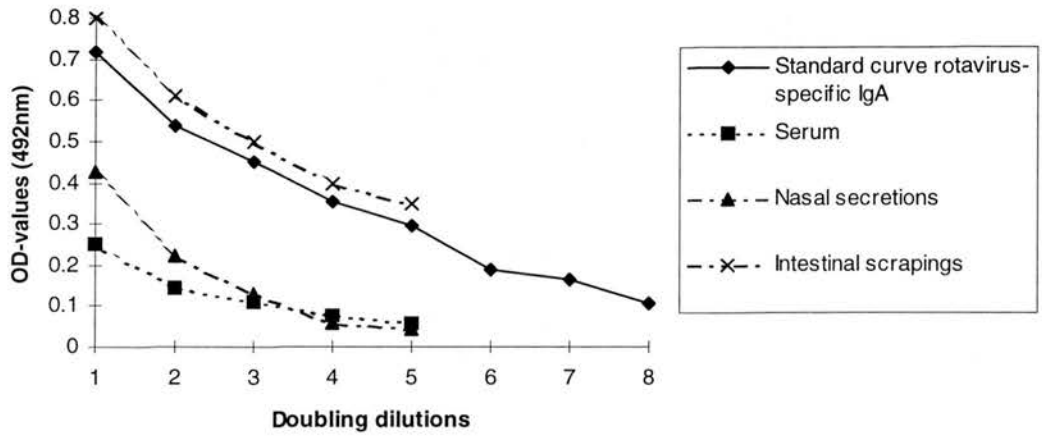


Fig. 3.2: Rotavirus-specific IgG ELISA

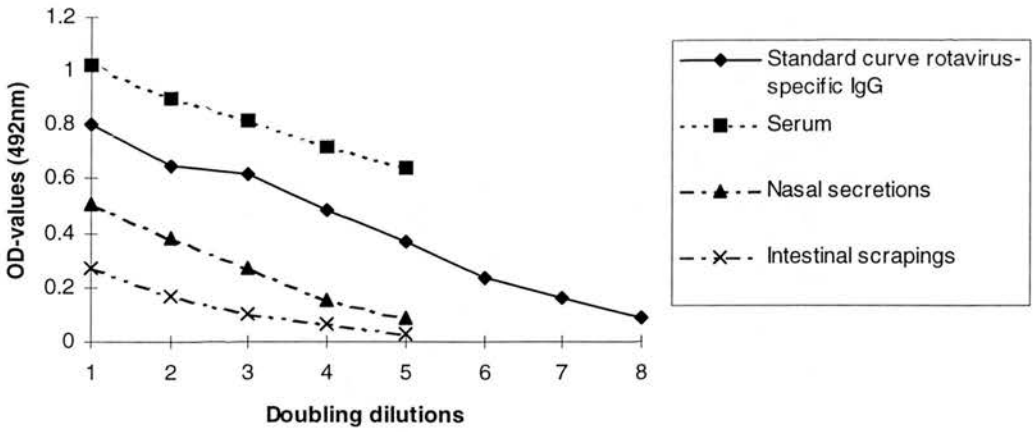


Fig. 3.3: Total immunoglobulin A ELISA

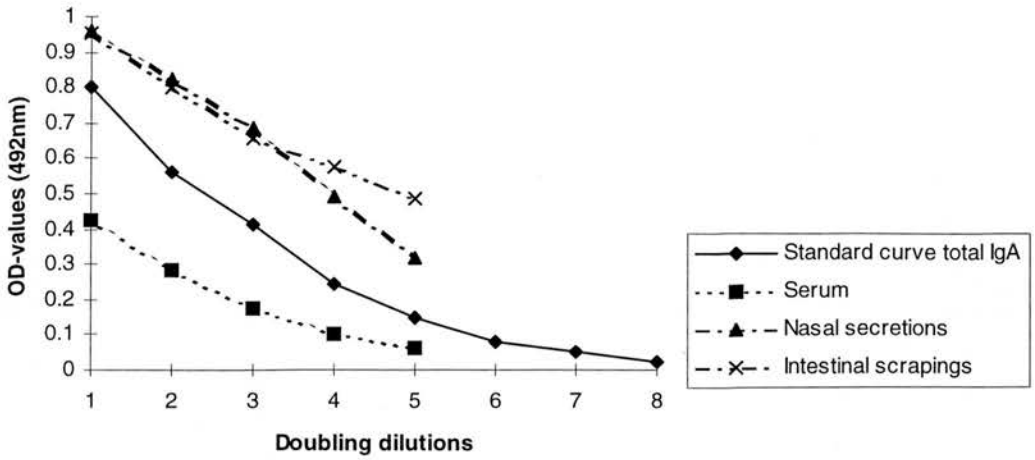
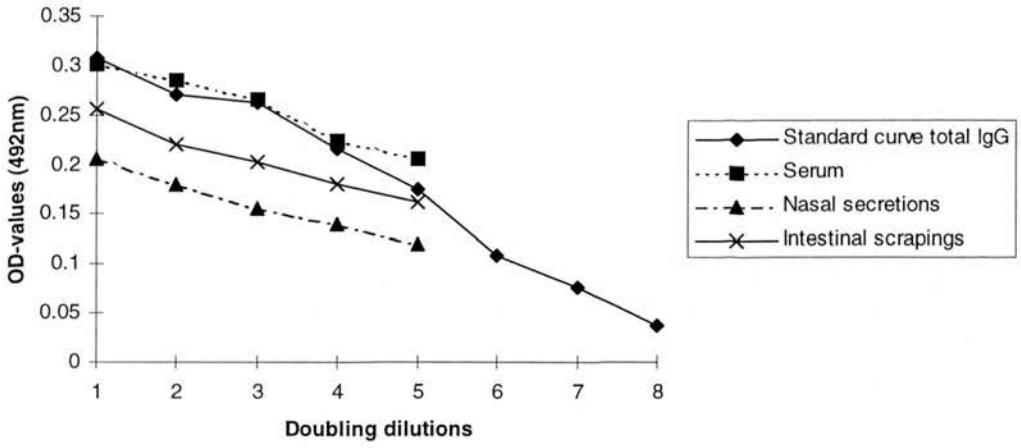


Fig. 3.4: Total immunoglobulin G ELISA



Each standard curve was reproducible with a linear/linear fit of r^2 >0.95. When serial dilutions of test samples produce results parallel to the standard curve, it indicates that antibody values can be compared at a single test dilution with a standard curve generated by serial dilutions of the standard sample.

Sample curves in all four ELISAs from serum, nasal secretions, and intestinal scrapings were parallel to standard curves. The faecal and saliva samples were not further analysed because faecal samples had no detectable levels of antibodies and the sample curve of saliva was not parallel to the standard curve.

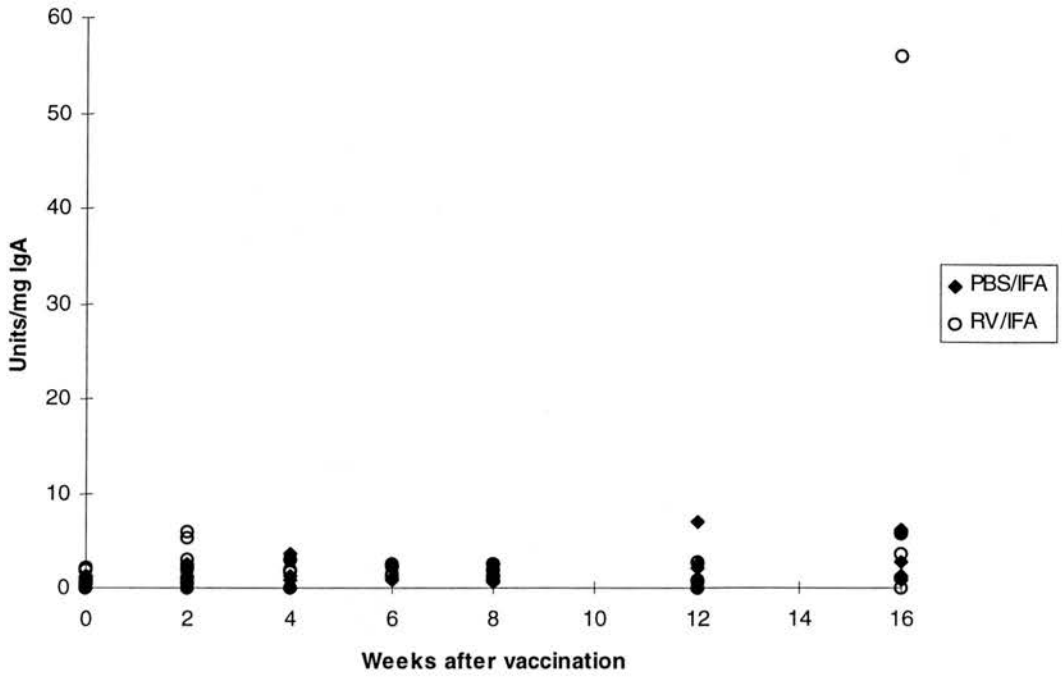
3.3.2 Antibody responses after parenteral vaccination

3.3.2.1 Rotavirus-specific antibodies

The RV/IFA group showed a significant ($p<0.05$) but transient increase in rotavirus-specific IgA antibodies (Fig.3.5 and Table 3.1) in nasal secretions 2 weeks after vaccination together with a significant ($p<0.05$) difference in rotavirus-specific IgA antibodies at the intestine at time of killing 2-3 weeks after vaccination. No differences were observed in intestinal scrapings 16-18 weeks after vaccination (Fig. 3.6 and Table 3.2). No increase in rotavirus-specific IgA antibodies was seen in serum at any time.

A significant increase ($p<0.05$) in rotavirus-specific IgG antibodies was observed in serum (Fig. 3.7 and Table 3.3) and nasal secretions (Fig. 3.8 and Table 3.4) in the RV/IFA group from 2 weeks after vaccination. The RV/IFA group had a significantly ($p<0.05$) higher level of rotavirus-specific IgG antibodies in intestinal scrapings 2-3 weeks after vaccination but not at 16-18 weeks after vaccination (Fig. 3.9 and Table 3.5).

Fig. 3.5: Rotavirus-specific IgA antibody responses in nasal secretions



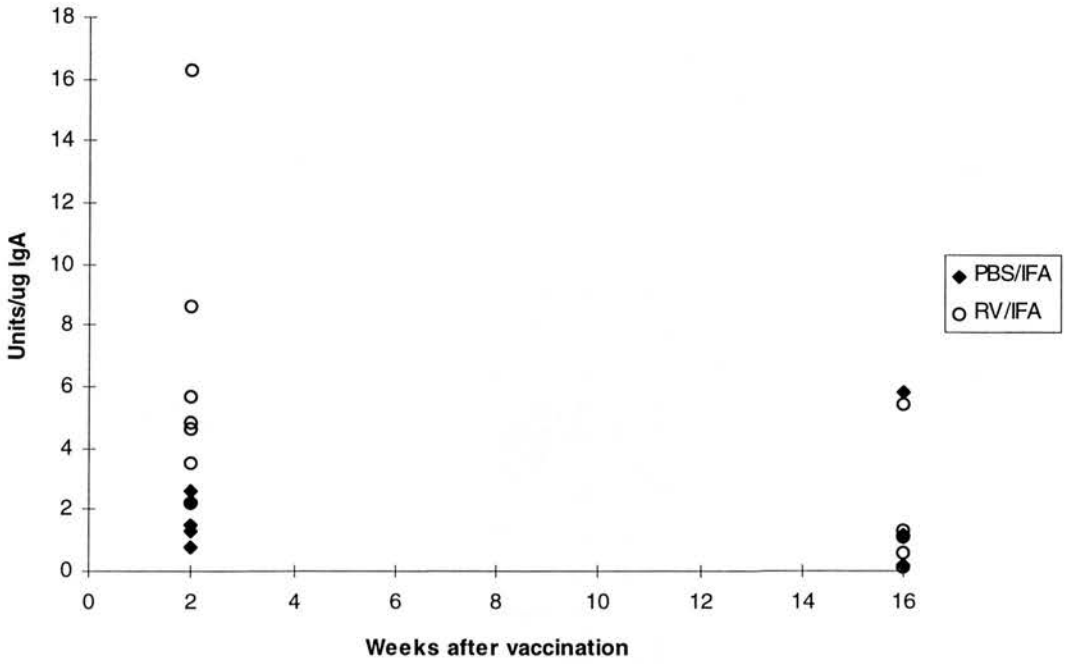
Rotavirus-specific IgA antibody responses in nasal secretions of each animal after parenteral vaccination with RV/IFA and PBS/IFA. Results expressed in units/mg IgA.

Table 3.1: Mean rotavirus-specific IgA antibody responses in nasal secretions

Group/Weeks after vaccination	0	2	4	6	8	12	16
PBS/IFA	0.6	1.1	2.2	1.5	1.2	3.8	3.4
	(0.6)	(0.8)	(1.2)	(0.7)	(0.8)	(2.7)	(2.5)
RV/IFA	0.7	2.4*	2.2	1.8	1.5	1.3	17
	(0.5)	(1.8)	(0.7)	(0.6)	(0.7)	(1.0)	(26)

Results given as mean with (SD) and expressed in units/mg IgA; * $p<0.05$ (RV/IFA vs PBS/IFA)

Fig. 3.6: Rotavirus-specific IgA antibody responses in intestinal scrapings



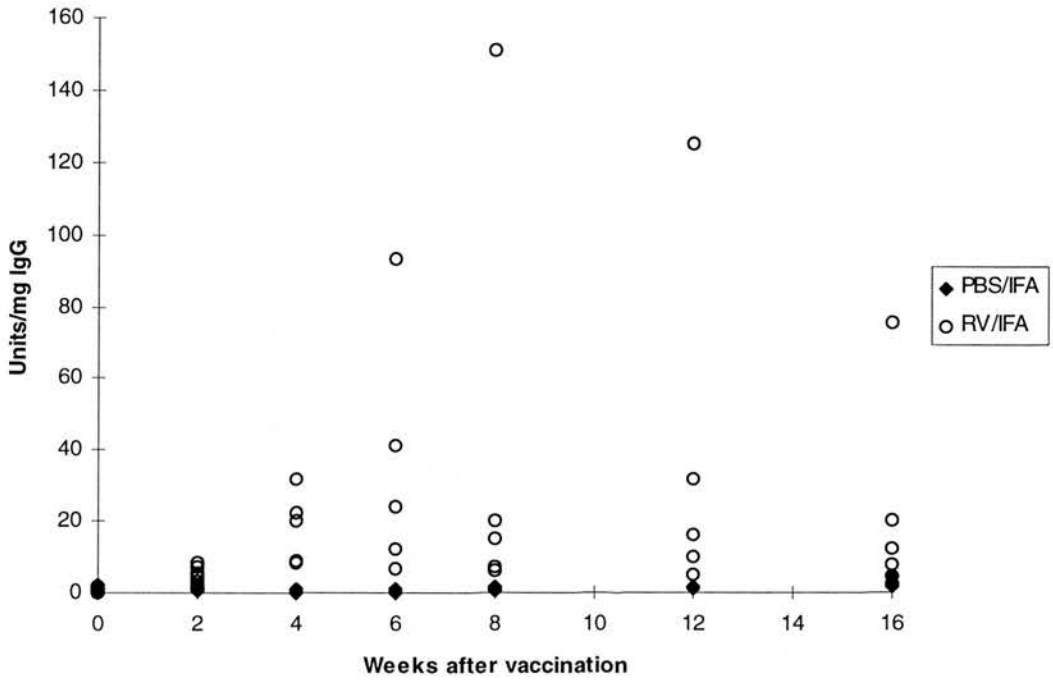
Rotavirus-specific IgA antibody responses in intestinal scrapings of each animal after parenteral vaccination with RV/IFA and PBS/IFA. Results expressed in units/µg IgA.

Table 3.2: Mean rotavirus-specific IgA antibody responses in intestinal scrapings

Isotype	Group/Weeks after vaccination	2-3	16-18
IgA	PBS/IFA	1.7 (0.7)	2.2 (2.2)
	RV/IFA	6.5 (4.7)*	1.7 (2.1)

Results given as mean with (SD) and expressed in units/µg IgA; * $p<0.05$ (RV/IFA vs PBS/IFA)

Fig. 3.7: Rotavirus-specific IgG antibody responses in serum



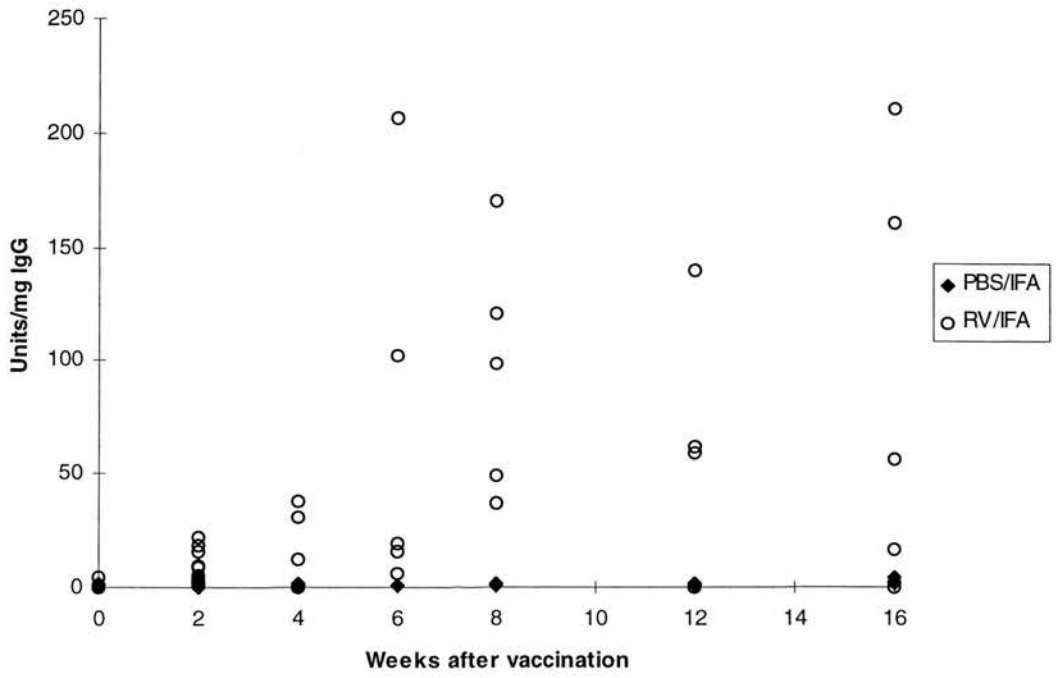
Rotavirus-specific IgG antibody responses in serum of each animal after parenteral vaccination with RV/IFA and PBS/IFA. Results expressed in units/mg IgG.

Table 3.3: Mean rotavirus-specific IgG antibody responses in serum

Group/Weeks after vaccination	0	2	4	6	8	12	16
PBS/IFA	1.5	0.7	0.6	0.6	0.9	1.0	0.9
	(0.5)	(0.4)	(0.3)	(0.4)	(0.3)	(0.4)	(0.3)
RV/IFA	1.2	4.2*	18**	35	40	38	40
	(0.6)	(2.7)	(9.7)	(35)	(63)	(50)	(63)

Results given as mean with (SD) and expressed in units/mg IgG; * $p<0.05$; ** $p<0.01$ (RV/IFA vs PBS/IFA)

Fig. 3.8: Rotavirus-specific IgG antibody responses in nasal secretions



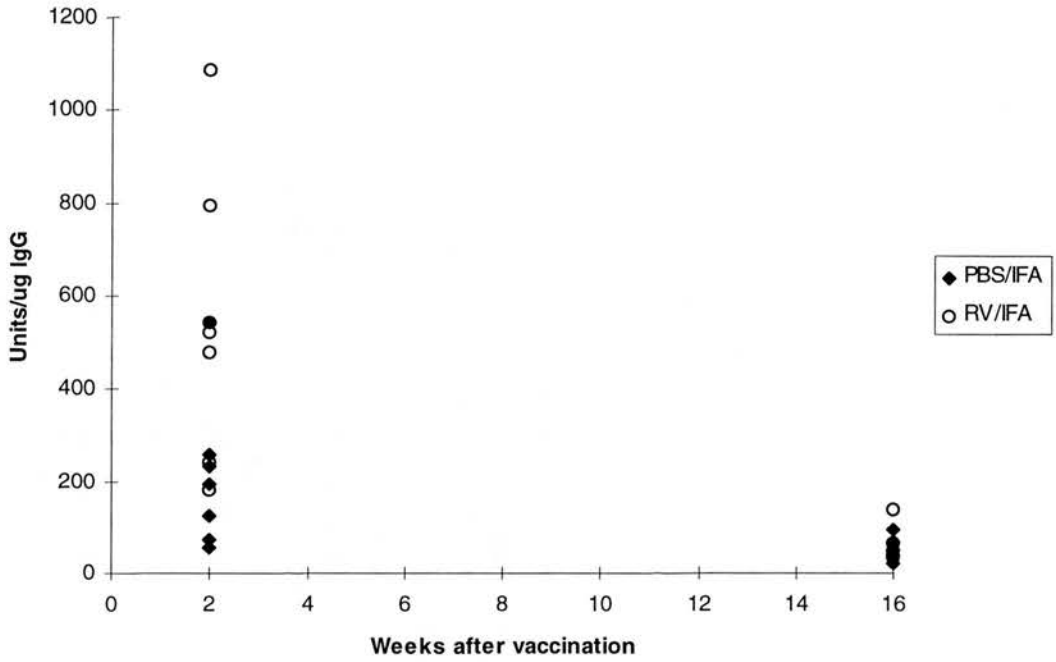
Rotavirus-specific IgG antibody responses in nasal secretions of each animal after parenteral vaccination with RV/IFA and PBS/IFA. Results expressed in units/mg IgG.

Table 3.4: Mean rotavirus-specific IgG antibody responses in nasal secretions

Group/Weeks after vaccination	0	2	4	6	8	12	16
PBS/IFA	1.0	0.9	1.0	1.0	1.1	1.5	2.8
	(1.1)	(0.6)	(0.5)	(0.3)	(0.3)	(0.4)	(0.9)
RV/IFA	1.3	8.3*	27**	70	95**	87**	111**
	(1.5)	(7.0)	(14)	(86)	(54)	(45)	(90)

Results given as mean with (SD) and expressed in units/mg IgG; * $p<0.05$; ** $p<0.01$ (RV/IFA vs PBS/IFA)

Fig. 3.9: Rotavirus-specific IgG antibody responses in intestinal scrapings



Rotavirus-specific IgG antibody responses in intestinal scrapings of each animal after parenteral vaccination with RV/IFA and PBS/IFA. Results expressed in units/µg IgG.

Table 3.5: Mean rotavirus-specific IgG antibody responses in intestinal scrapings

Isotype	Group/Weeks after vaccination	2-3	16-18
IgG	PBS/IFA	213 (166)	56 (29)
	RV/IFA	550 (312)*	65 (42)

Results given as mean with (SD) and expressed in units/µg IgG; * $p<0.05$ (RV/IFA vs PBS/IFA)

3.3.2.2 Neutralising titres against rotavirus

All animals had pre-existing serum neutralising antibodies against rotavirus. After vaccination, significant ($p<0.05$) increases were observed in the RV/IFA group from 2 weeks after vaccination which lasted until the termination of the experiment (Table 3.6). Intestinal scrapings had a low level of neutralising activity against rotavirus (maximum VNT=10) but no changes were observed between the groups (data not shown).

Table 3.6: Mean neutralising titres in serum

Group/Weeks after vaccination	0	2	4	6	8	12	16
PBS/IFA	80 (20-320)	80 (40-320)	80 (40-160)	80 (20-160)	80 (40-160)	80 (40-160)	40 (40-80)
RV/IFA	80 (20-320)	2560* (640-20480)	10240** (2560-20480)	10240** (5120-10240)	5120** (2560-10240)	5120** (2560-10240)	10240** (5120-10240)

Results given as mean with (range); * $p<0.05$; ** $p<0.01$ (RV/IFA vs PBS/IFA)

3.3.2.3 Total immunoglobulin A and G concentrations

Individual IgA concentrations varied markedly. No significant differences were observed in serum (range: 5-168 mg/100mls), nasal secretions (range: 96-1140 mg/100mls) (Table 3.7), and intestinal scrapings (range: 1.2-40 mg/100mls) (data not shown) after vaccination.

Table 3.7: IgA concentrations in serum and nasal secretions

Sample	Group/Weeks after vaccination	0	2	4	6	8	12	16
Serum	PBS/IFA	25 (13)	21 (10)	20 (13)	18 (12)	18 (14)	24 (10)	29 (16)
	RV/IFA	29 (16)	22 (8.7)	27 (11)	42 (50)	34 (35)	60 (61)	51 (45)
Nasal	PBS/IFA	275 (181)	357 (302)	212 (134)	204 (94)	251 (99)	174 (154)	180 (106)
	RV/IFA	310 (210)	324 (168)	161 (95)	185 (99)	176 (84)	277 (64)	169 (122)

Results given as mean with (SD) and expressed in mg IgA/100mls

Individual IgG concentrations varied markedly. No significant differences were observed in serum (range: 2181-11776 mg/100mls), nasal secretions (range: 17-140 mg/100mls) (Table 3.8), and intestinal scrapings (range: 0.01-0.1 mg/100mls) (data not shown) after vaccination.

Table 3.8: IgG concentrations in serum and nasal secretions

Sample	Group/Weeks after vaccination	0	2	4	6	8	12	16
Serum	PBS/IFA	4765 (1530)	4513 (1330)	5675 (1087)	5290 (676)	4479 (1232)	4864 (1942)	6197 (2466)
	RV/IFA	4578 (1401)	4317 (1598)	4249 (898)	6287 (2320)	5381 (2458)	4495 (830)	3761 (789)
Nasal	PBS/IFA	74 (26)	84 (52)	44 (4)	55 (33)	49 (18)	34 (16)	20 (5)
	RV/IFA	67 (23)	81 (47)	54 (27)	95 (44)	34 (12)	58 (50)	19 (2)

Results given as mean with (SD) and expressed in mg IgG/100mls

3.4 DISCUSSION

In this study, different ELISAs were used to measure rotavirus-specific antibody and total IgA and IgG levels in serum, nasal secretions, saliva, faeces, and intestinal scrapings. Doubling dilutions of samples were compared to an 8-point doubling dilution standard curve. The use of measurement by single dilution was justified when sample curves were parallel to standard curves. Serum, nasal secretions, and intestinal washings could be tested by single dilutions.

Saliva could not be tested, as the sample curve was not parallel to the standard curve. This could be due to degradation by endogenous proteolytic enzymes present in saliva during storage (Coulson *et al,* 1989), the method of sampling by taking saliva from under the tongue and not at the parotid gland, or the time of feeding which could alter the dilution factor of antibodies. Faecal samples had no detectable antibodies. This may be due to the high dilution factor in faeces.

The standard vaccine with IFA and inactivated rotavirus lysate was successful in stimulating rotavirus-specific IgG antibodies and neutralising activity in serum as previously observed in animals vaccinated parenterally with rotavirus lysate (live or inactivated) and IFA (Snodgrass *et al,* 1980; Castrucci *et al,* 1984; Saif *et al,* 1984; Bellinzoni *et al,* 1989).

Significantly increased levels of rotavirus-specific IgG antibodies were also found in nasal secretions from 2 weeks after vaccination. Rotavirus-specific IgG antibodies in intestinal scrapings were significantly increased 2-3 weeks but not at 16-18 weeks after vaccination. Rotavirus-specific IgG antibodies detected in nasal secretions and intestinal scrapings in cattle and rabbits are assumed to be derived from serum by transudation (Besser *et al,* 1988; Conner *et al,* 1993). A study on influenza virus in humans indicated that virus-specific IgG antibodies in saliva and nasal secretions were derived from the circulation (Murphy and Clements, 1989). Although, 5-15% of plasma cells in mucosal tissues produce IgG but the relative importance of this locally produced IgG in mucosal immunity is still unclear (McGhee and Kiyono, 1993).

In this study, high rotavirus-specific IgG antibody levels were detected in serum from 2-16 weeks after vaccination. However, this was not reflected in high rotavirus-specific IgG antibody levels in the intestine. The significant difference between the PBS/IFA and RV/IFA group observed in intestinal rotavirus-specific IgG antibodies 2-3 weeks after vaccination could be related to the effect of stimulated local rotavirus-specific IgG production or it could be due to transudation. However, rotavirus-specific IgG antibodies were higher at 16 weeks than at 2 weeks after vaccination. In a study with specific-pathogen (rotavirus)-free rabbits, a similar

suggestion was made that parenteral vaccination could stimulate locally produced rotavirus-specific IgG antibodies (Conner *et al,* 1993).

However, of greater significance were the observations that a single parenteral vaccination with RV/IFA stimulated an increase in rotavirus-specific IgA antibodies in nasal secretions and in the intestine, providing evidence that the common mucosal immune system operates in sheep

Several possible mechanisms exist whereby parenteral vaccination in previously exposed animals induces production of virus-specific intestinal or respiratory antibodies. First, circulating antigen-presenting cells (APCs) carrying rotavirus antigen can traffic to the GALT and trigger memory B cells (McGhee *et al,* 1992). Secondly, circulating APCs can trigger memory B cells present in blood these may disseminate to the GALT (Coffin *et al,* 1995).

No rotavirus-specific IgA antibodies were detected in serum. Most work in parenterally vaccinated ruminants has concentrated on the antibody response in colostrum and milk, which is predominantly an IgG response (Saif *et al,* 1984). This is in contrast to monogastrics in which the IgA response is predominant. In sheep vaccinated parenterally with killed *Salmonella typhimurium*, the contribution of IgA in serum was minimal while IgG was predominant (Mukkur *et al,* 1995). However, rotavirus-specific IgA antibody levels were detected in the intestine and nasal secretions. It is likely that these antibodies were produced by plasma cells in the GALT and not derived from serum. In a previous study on influenza and cholera in naturally exposed humans, IgA antibodies increased in serum and milk respectively after parenteral immunisation (Svennerholm *et al,* 1980; Moldoveanu *et al,* 1995).

The significant difference seen in rotavirus-specific IgA antibodies in the intestine 2 weeks after vaccination correlated with the significant increase seen in rotavirus-specific IgA antibodies in nasal secretions suggesting that nasal secretions could be an accurate reflection and a good marker for intestinal mucosal immunity. However, more data is needed about the levels of rotavirus-specific IgA antibodies in the intestine at other time points after vaccination. In a previous study in humans salivary rotavirus-specific antibodies were found to be a good indicator for intestinal rotavirus-specific antibody (Ward *et al,* 1992a), but this was not the case in this study.

No significant increases were found in total immunoglobulin levels in serum, nasal secretions, and intestinal scrapings. Significant increases in rotavirus-specific antibodies do not result in increases in total immunoglobulin levels. The total immunoglobulin levels found in this study are in general higher, except for total IgA levels in serum, than observed in a previous study in which immunoglobulin levels were measured (Smith *et al,* 1975). The difference could be explained by the different methods (ELISA vs single radial immunodiffusion) used or the history and age of the animals.

This study showed that rotavirus-specific antibody levels in nasal secretions could be a good reflection of intestinal anti-rotavirus antibody levels. Collecting nasal secretions is simple and non-invasive, and could have potential in screening and monitoring the kinetics of the mucosal immune response for example in vaccine studies.

This study also demonstrates that parenteral vaccination can boost rotavirus-specific IgA antibodies in the intestine and related mucosae in naturally exposed sheep using incomplete Freunds adjuvant. Such a response should be expected to correlate with increased protection against infection but no challenge system in adults was available to test this.

Stimulation of IgA antibodies in the intestine and other mucosal surfaces such as in the respiratory tract could increase protection against reinfection by a mucosal pathogen. Finally, if mammary gland IgA and IgG antibody levels could also be increased then this could improve passive immunity transfer to offspring in diseases such as TGE in pigs and rotaviral enteritis in man and animals.

CHAPTER 4

EFFECTS OF DIFFERENT ADJUVANTS AND ANTIGEN DOSES IN PARENTERAL VACCINES ON ROTAVIRUS-SPECIFIC IMMUNITY IN SHEEP

4.1 INTRODUCTION

IFA has been used successfully as an antigen carrier in rotavirus dam vaccines for cattle, increasing rotavirus-specific IgG antibodies in serum and colostrum, neutralising antibodies in serum (Snodgrass *et al,* 1980, Fahey *et al,* 1981), and rotavirus-specific IgA antibodies at mucosal surfaces (see chapter 3).

Due to the possible side effects of IFA such as the formation of granulomas or even abscesses at the injection site and the difficulty of injection through its high viscosity, other adjuvants are being developed and tested (Edelman, 1980; Osebold, 1982).

ISCOMs and biodegradable poly(lactic)/glycolic acid (PLGA) microspheres have been used as an adjuvant and were selected for use in this study. Increased rotavirus-specific antibodies were found in colostrum in baboons when vaccinating the mother intramuscularly with a mixed rotavirus/ISCOM vaccine (Snodgrass *et al,* 1995). Increased specific antibody levels were observed when rhesus macaques were vaccinated intramuscularly with micro-encapsulated vaccines containing either staphylococcal enterotoxin B, whole formalin-inactivated simian immune deficiency virus (SIV) or tetanus toxoid (Eldridge *et al,* 1991; Marx *et al.* 1993; Singh *et al,* 1997).

Most rotavirus dam vaccines available use an oil-based adjuvant (e.g., IFA) emulsified with virus antigens in the form of tissue culture lysates. These commercial vaccines tend to use a dose of virus antigen that can be grown in cell culture without further concentration, which in practice incorporates a titre of approximately $10^{6.0\text{-}6.5}$ ffu/ml per dose (Snodgrass, personal communication). No studies have been done to compare the effect of purified rotavirus or a higher dose of rotavirus antigen, on the immune response.

The aim of the work described in this chapter was to study the effect of different adjuvants (IFA, ISCOMs and microspheres) and antigen doses (lysate and purified) in parenteral vaccines on the local and systemic humoral immune response.

4.2 EXPERIMENTAL DESIGN

4.2.1 Animals

Non-lactating multiparous crossbred sheep were used and all had serum neutralising antibodies to rotavirus.

Sheep were divided into 9 groups of n=4 and vaccinated intramuscularly with either inactivated rotavirus lysate (RV) (low antigen dose) or inactivated purified rotavirus (PRV) (high antigen dose) adjuvanted with incomplete Freunds (IFA) (RV/IFA; PRV/IFA), ISCOMs (RV/ISC; PRV/ISC), or microspheres (RV/MSPH; PRV/MSPH). One group received no treatment.

4.2.2 Vaccines

The rotavirus used was the bovine strain UK either as a cell culture lysate (infectivity titre of $10^{6.8}$ ffu/ml) (see 2.2.2) or sucrose-purified (infectivity titre of $10^{8.8}$ ffu/ml) (see 2.2.3). Both preparations were inactivated by the formaldehyde method (see 2.2.4). Protein concentrations of rotavirus lysate and purified rotavirus were determined with a BCA™ protein assay.

The RV/IFA group was vaccinated with 2mls each of inactivated rotavirus lysate emulsified (1:1) with IFA (DIFCO, UK). The RV/ISC group received 2mls each containing 1ml inactivated rotavirus lysate, 500μg ISCOM matrix (Iscotec, Sweden), and PBS making a total volume of 2mls. The RV/MSPH group received 10mg each of microspheres (gift from Dr. A. Coombes, University of Nottingham) adsorbed with 1.49mg of lysate protein (≈28% of the amount of lysate protein used in the RV/IFA and RV/ISC groups). 40mg of microspheres were incubated overnight at 37°C with 4mls of inactivated rotavirus lysate (protein concentration was 5.35mg/ml). After incubation, suspension was centrifuged for 5 minutes at maximum speed with a micro-centrifuge and washed twice with PBS. Protein concentration was determined in supernatants to measure the adsorption of protein to the microspheres. Adsorption percentage is (the total amount of protein adsorbed to the

microspheres/ the total amount of protein used) ×100%. The adsorption percentage was (4×1.49/21.4) ×100% =27.8%.

The PRV/IFA group received 2mls each of 100μg of inactivated sucrose-purified rotavirus emulsified (1:1) with IFA. The PRV/ISC group was vaccinated with 2mls containing 100μg of inactivated sucrose-purified rotavirus, 500μg ISCOM matrix, and PBS making a total volume of 2mls. The PRV/MSPH group received 10mg each of microspheres adsorbed with 136μg of inactivated sucrose-purified rotavirus. 40mg of microspheres were incubated overnight at 37°C with 3mls of purified rotavirus (protein concentration was 482μg/ml). After incubation, microspheres were treated the same as above. Adsorption percentage was 37.6%.

All sheep were vaccinated with equal volumes of inocula in each back leg by deep intramuscular injection.

4.2.3 Sample collection

Blood and nasal secretions were collected at initial vaccination and at 1 and 2 weeks after vaccination. At necropsy, 2-3 weeks after vaccination, intestinal scrapings were collected.

Serum, nasal secretions, and intestinal scrapings were tested for rotavirus-specific antibodies and total IgA and IgG concentrations by ELISA. Virus neutralising titres were determined in serum and intestinal scrapings by virus neutralising assay.

4.3 RESULTS

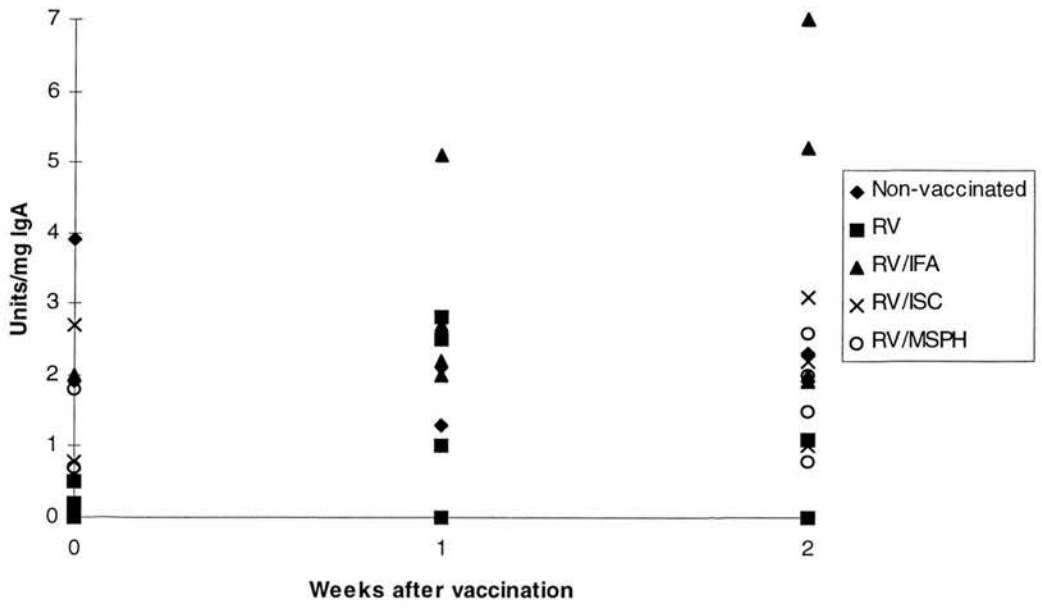
4.3.1 Antibody responses after a low dose of rotavirus antigen

4.3.1.1 Rotavirus-specific antibodies

Rotavirus-specific IgA antibodies were present in nasal secretions from all animals before initial vaccination. None of the vaccinated groups showed any significant increase at 1 and 2 weeks after vaccination, although in the RV/IFA group 2 of 4 animals had marked individual increases (Fig. 4.1 and Table 4.1). Rotavirus-specific IgA antibodies were not detected in serum at any stage (data not shown). Rotavirus-specific IgA antibodies were present in intestinal scrapings taken at necropsy in all animals (range: 1.0-27 units/μg IgA), but no difference in response was detected between the groups (data not shown).

All animals had rotavirus-specific IgG antibodies in serum and nasal secretions at initial vaccination. A significant ($p<0.05$) increase in serum antibody responses was observed in the RV/IFA and RV/ISC groups 2 weeks after vaccination (Fig. 4.2 and Table 4.2). In nasal secretions, the RV/IFA (4 of 4) and RV/ISC (3 of 4) groups had marked individual increases in rotavirus-specific IgG antibodies, but the overall response was not significant (Fig. 4.3 and Table 4.3). Rotavirus-specific IgG antibodies were observed in intestinal scrapings (range: 57-796 units/μg IgG), but no significant differences were observed between the groups (data not shown).

Fig. 4.1: Rotavirus-specific IgA antibody responses in nasal secretions



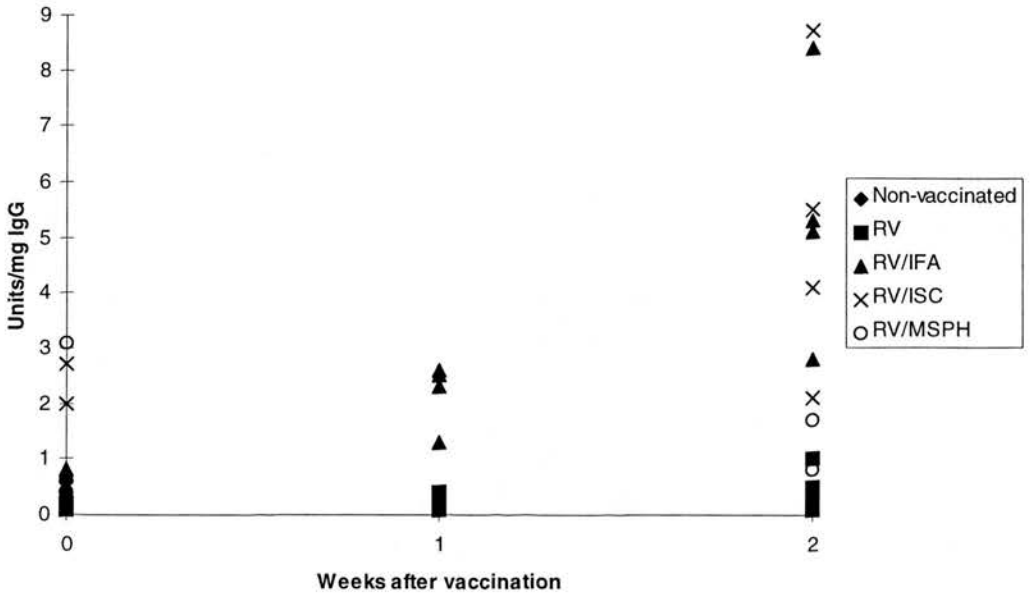
Rotavirus-specific IgA antibody responses in nasal secretions of each animal after parenteral vaccination with a low antigen dose. Results expressed in units/mg IgA.

Table 4.1: Mean rotavirus-specific IgA antibody responses in nasal secretions

Group/Weeks after vaccination	0	1	2
Non-vaccinated	2.9 (1.4)	1.5 (0.6)	2.3[1]
RV	0.4 (0.2)	2.1 (1.0)	1.1[1]
RV/IFA	0.7 (1.1)	3.0 (1.4)	4.0 (2.5)
RV/ISC	1.2 (1.0)	ND	1.8 (1.0)
RV/MSPH	0.8 (0.7)	ND	1.7 (0.8)

Results given as mean with (SD) and expressed in units/mg IgA; [1] one animal; (vaccinated vs non-vaccinated); ND = not done

Fig. 4.2: Rotavirus-specific IgG antibody responses in serum



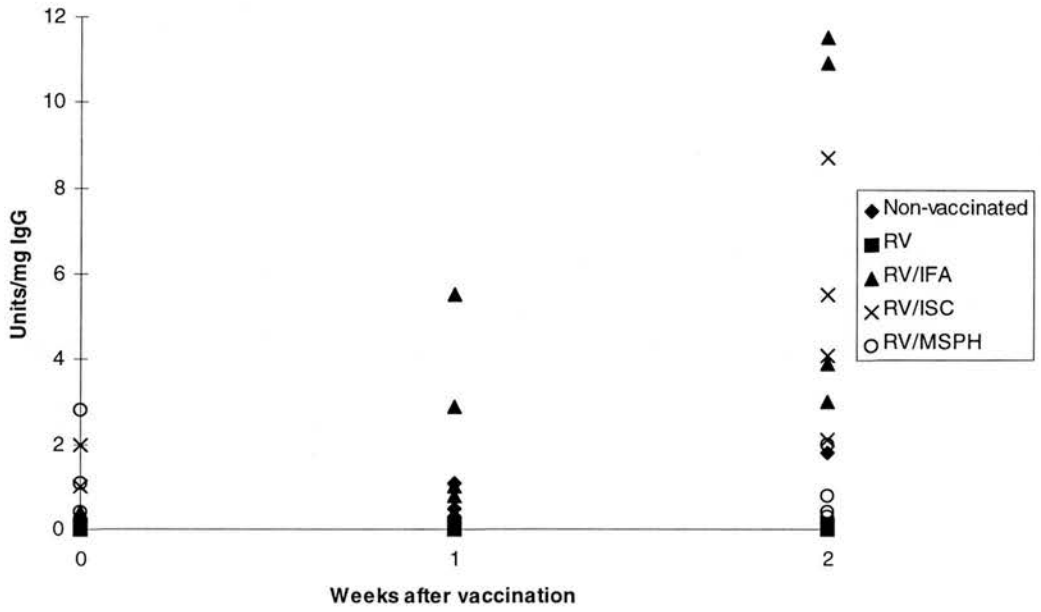
Rotavirus-specific IgG antibody responses in serum of each animal after parenteral vaccination with a low antigen dose. Results expressed in units/mg IgG.

Table 4.2: Mean rotavirus-specific IgG antibody responses in serum

Group/Weeks after vaccination	0	1	2
Non-vaccinated	0.3 (0.2)	0.1 (0.1)	0.2 (0.1)
RV	0.1 (0.1)	0.2 (0.1)	0.5 (0.4)
RV/IFA	0.6 (0.2)	2.2 (0.6)	5.4 (2.3)**
RV/ISC	1.2 (1.3)	ND	5.1 (2.8)**
RV/MSPH	1.1 (1.4)	ND	0.8 (0.6)

Results given as mean with (SD) and expressed in units/mg IgG; ** $p<0.01$ (vaccinated vs non-vaccinated); ND = not done

Fig.4.3: Rotavirus-specific IgG antibody responses in nasal secretions



Rotavirus-specific IgG antibody responses in nasal secretions of each animal after parenteral vaccination with a low antigen dose. Results expressed in units/mg IgG.

Table 4.3: Mean rotavirus-specific IgG antibody responses in nasal secretions

Group/Weeks after vaccination	0	1	2
Non-vaccinated	0.2 (0)	0.6 (0.4)	1.8[1]
RV	0.1 (0)	0.1 (0)	0.1[1]
RV/IFA	0.3 (0.1)	2.6 (2.2)	7.2 (4.3)
RV/ISC	0.8 (0.9)	ND	5.1 (2.8)
RV/MSPH	1.1 (1.2)	ND	0.9 (0.8)

Results given as mean with (SD) and expressed in units/mg IgG; [1] one animal; (vaccinated vs non-vaccinated); ND = not done

4.3.1.2 Neutralising titres against rotavirus

All animals had rotavirus neutralising responses in serum at initial vaccination. After vaccination, the RV/IFA and the RV/ISC group showed a significant ($p<0.05$) increase of neutralising antibodies in serum. (Table 4.6). The RV group showed smaller increases but these were not statistically significant.

Table 4.6: Mean neutralising titres in serum

Group/Weeks after vaccination	0	1	2
Non-vaccinated	80	80	80
	(40-160)	(40-160)	(40-160)
RV	40	160	320
	(10-80)	(40-160)	(160-640)
RV/IFA	40	1280	5120
	(10-80)	(320-2560)*	(640-10240)**
RV/ISC	80	ND	5120
	(20-640)		(640-10420)**
RV/MSPH	80	ND	80
	(40-160)		(40-160)

Results given as mean with (range); * $p<0.05$; ** $P<0.01$ (vaccinated vs non-vaccinated); ND = not done

4.3.1.3 Total immunoglobulin A and G concentrations

Individual IgA concentrations varied markedly. No significant changes were observed in serum (range: 6-101 mg/100mls), nasal secretions (range: 73-1310 mg/100mls) (Table 4.4), and intestinal scrapings (range: 0.1-2.4 mg/100mls) (data not shown) between the groups after vaccination.

Table 4.4: IgA concentrations in serum and nasal secretions

Sample	Group/Weeks after vaccination	0	1	2
Serum	Non-vaccinated	31 (15)	25 (14)	30 (15)
	RV	54 (32)	40 (22)	36 (30)
	RV/IFA	22 (8)	22 (15)	26 (16)
	RV/ISC	16 (9)	ND	11 (4)
	RV/MSPH	20 (9)	ND	27 (17)
Nasal	Non-vaccinated	839 (86)	1069 (364)	388[1]
	RV	729 (376)	572 (392)	248[1]
	RV/IFA	201 (36)	363 (281)	297 (201)
	RV/ISC	284 (53)	ND	112 (30)
	RV/MSPH	369 (195)	ND	279 (99)

Results given as mean with (SD) and expressed in mg IgA/100mls; [1] one animal; (vaccinated vs non-vaccinated); ND = not done

Individual IgG concentrations also varied markedly. No significant differences were found in serum (range: 1851-6758 mg/100mls), nasal secretions (range: 14-169 mg/100mls) (Table 4.5), and intestinal scrapings (0.01-0.2 mg/100mls) (data not shown) between the groups after vaccination.

Table 4.5: IgG concentrations in serum and nasal secretions

Sample	Group/Weeks after vaccination	0	1	2
Serum	Non-vaccinated	3534 (1016)	3060 (619)	4753 (1263)
	RV	4067 (1057)	4313 (1277)	4886 (2115)
	RV/IFA	2579 (1203)	2636 (688)	3299 (1355)
	RV/ISC	4544 (1190)	ND	5059 (938)
	RV/MSPH	4167 (206)	ND	5086 (1024)
Nasal	Non-vaccinated	45 (21)	95 (66)	27[1]
	RV	72 (69)	47 (21)	87[1]
	RV/IFA	90 (46)	69 (37)	61 (20)
	RV/ISC	64 (9)	ND	32 (21)
	RV/MSPH	74 (37)	ND	89 (37)

Results given as mean with (SD) and expressed in mg IgG/100mls; [1] one animal; (vaccinated vs non-vaccinated); ND = not done

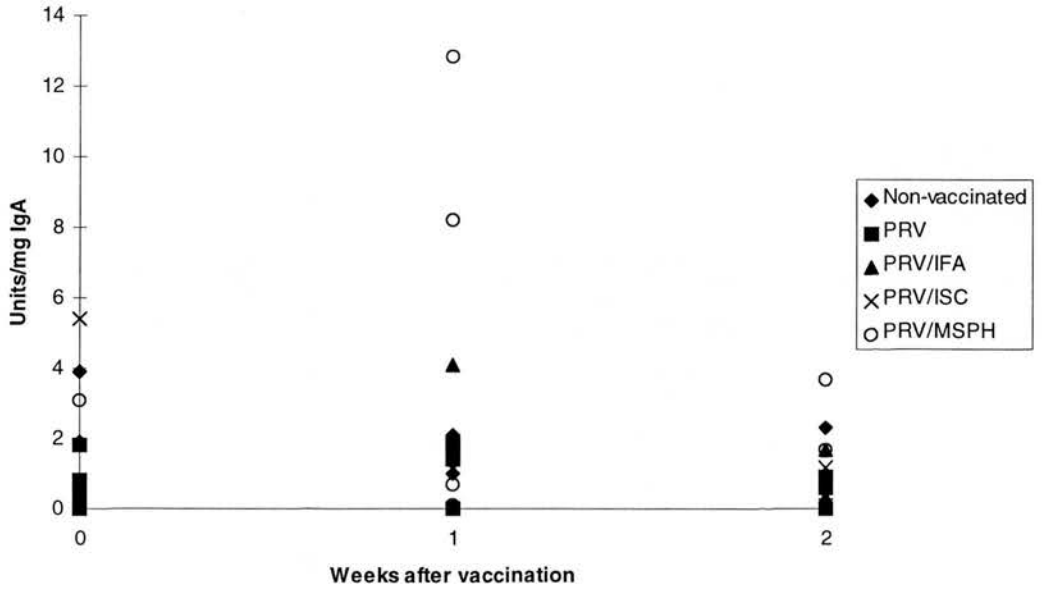
4.3.2 Antibody responses after a high dose of rotavirus antigen

4.3.2.1 Rotavirus-specific antibodies

Rotavirus-specific IgA antibodies were present in all animals before initial vaccination. No significant increase was observed in the vaccinated groups, although in the PRV/MSPH group 2 of 4 sheep had marked individual increases 1 week after vaccination (Fig. 4.4 and Table 4.7). Rotavirus-specific IgA antibodies were not seen in serum at any stage, but were present in intestinal scrapings (range: 1.0-15 units/μg IgA) with no significant differences between the groups (data not shown).

Rotavirus-specific IgG antibodies were found in serum and nasal secretions in most animals at initial vaccination. Marked individual increases in serum rotavirus-specific IgG antibodies were observed in the PRV/IFA (3 of 4), PRV/ISC (2 of 4), and PRV/MSPH (2 of 4) groups, however this was only significant ($p<0.05$) in the PRV/IFA group 2 weeks after vaccination (Fig. 4.5 and Table 4.8). None of the vaccinated groups showed a significant increase in rotavirus-specific IgG antibodies in nasal secretions, although in the PRV/ISC and PRV/MSPH groups 2 and 4 animals respectively had marked individual increases (Fig. 4.6 and Table 4.9). No significant differences were observed in intestinal scrapings (range: 18-564 units/μg IgG) at necropsy (data not shown).

Fig. 4.4: Rotavirus-specific IgA antibody responses in nasal secretions



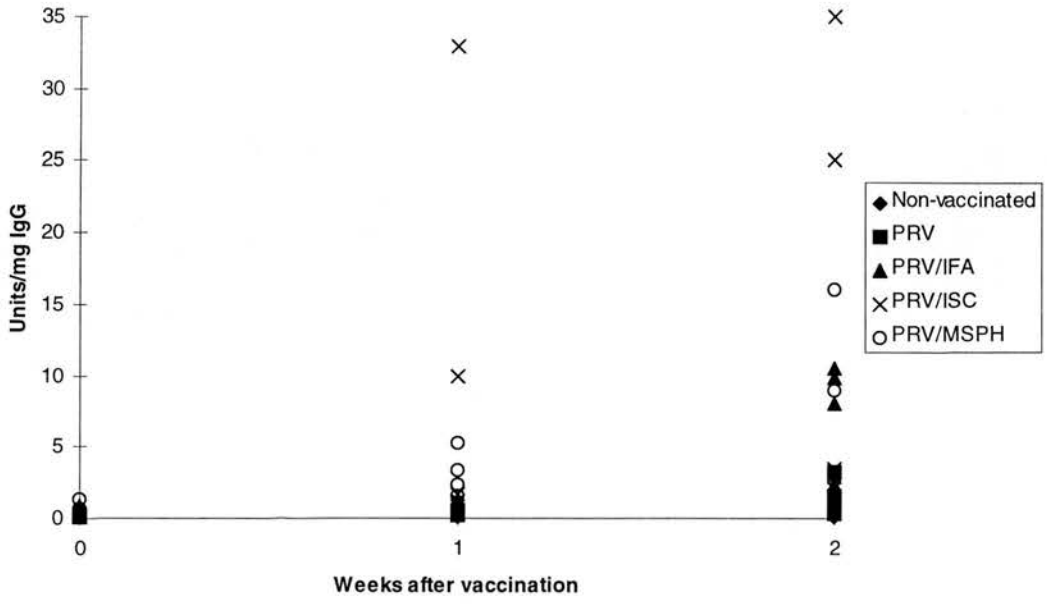
Rotavirus-specific IgA antibody responses in nasal secretions of each animal after parenteral vaccination with a high antigen dose. Results expressed in units/mg IgA.

Table 4.7: Mean rotavirus-specific IgA antibody responses in nasal secretions

Group/Weeks after vaccination	0	1	2
Non-vaccinated	2.9 (1.4)	1.5 (0.6)	2.3[1]
PRV	1.0 (0.7)	1.7 (0.3)	0.8 (0.2)
PRV/IFA	0.2 (0.1)	2.1 (2.8)	0.9 (0.6)
PRV/ISC	2.1 (2.0)	ND	0.7 (0.8)
PRV/MSPH	1.1 (1.7)	5.5 (2.8)	1.4 (1.7)

Results given as mean with (SD) and expressed in units/mg IgA; [1] one animal; (vaccinated vs non-vaccinated); ND = not done

Fig 4.5: Rotavirus-specific IgG antibody responses in serum



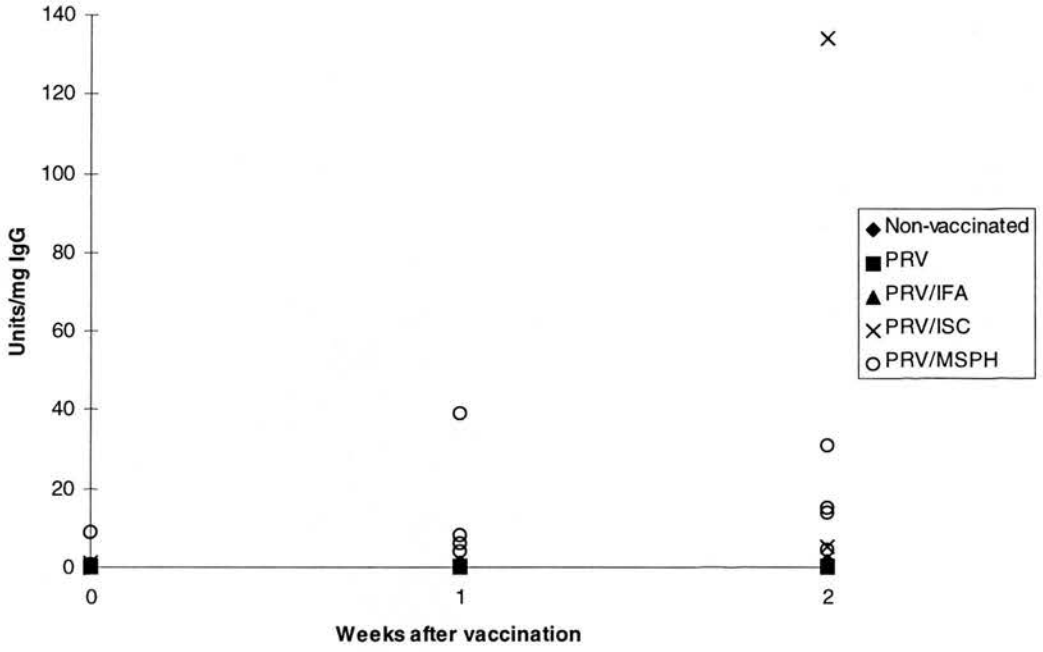
Rotavirus-specific IgG antibody responses in serum of each animal after parenteral vaccination with a high antigen dose. Results expressed in units/mg IgG.

Table 4.8: Mean rotavirus-specific IgG antibody responses in serum

Group/Weeks after vaccination	0	1	2
Non-vaccinated	0.3 (0.2)	0.1 (0.1)	0.2 (0.1)
PRV	0.1 (0)	0.4 (0.2)	1.4 (1.3)
PRV/IFA	0.3 (0.4)	1.0 (0.5)	7.7 (3.7)**
PRV/ISC	0.2 (0.1)	12 (14.9)	17 (16)
PRV/MSPH	0.6 (0.5)	3.1 (1.5)	7.3 (6.7)

Results given as mean with (SD) and expressed in units/mg IgG; ** $p<0.01$ (vaccinated vs non-vaccinated)

Fig. 4.6: Rotavirus-specific IgG antibody responses in nasal secretions



Rotavirus-specific IgG antibody responses in nasal secretions of each animal after parenteral vaccination with a high antigen dose. Results expressed in units/mg IgG.

Table 4.9: Mean rotavirus-specific IgG antibody responses in nasal secretions

Group/Weeks after vaccination	0	1	2
Non-vaccinated	0.2 (0)	0.6 (0.4)	1.8[1]
PRV	0.1 (0)	0.2 (0.2)	0.4 (0.4)
PRV/IFA	0.2 (0.1)	0.1 (0)	0.1(0)
PRV/ISC	0.7 (0.6)	ND	70 (91)
PRV/MSPH	3.5 (4.8)	14 (17)	16 (11)

Results given as mean with (SD) and expressed in units/mg IgG; [1] one animal; (vaccinated vs non-vaccinated); ND = not done

4.3.2.2 Neutralising titres against rotavirus

All animals had serum neutralising responses at initial vaccination. After vaccination, significant ($p<0.05$) increased levels of neutralising responses were observed in the PRV/ISC and the PRV/MSPH vaccinated groups (Table 4.10).

Table 4.10: Mean neutralising responses in serum

Group/Weeks after vaccination	0	1	2
Non-vaccinated	80	80	80
	(40-160)	(40-160)	(40-160)
PRV	80	80	80
	(20-320)	(40-160)	(40-160)
PRV/IFA	80	40	80
	(40-160)	(20-160)	(40-160)
PRV/ISV	80	1280	1280
	(40-160)	(320-5120)*	(640-5120)*
PRV/MSPH	80	320	640
	(20-160)	(80-640)*	(320-1280)*

Results given as mean with (SD); * $p < 0.05$ (vaccinated vs non-vaccinated)

4.3.2.3 Total immunoglobulin A and G concentrations

Individual IgA concentrations varied markedly. No significant changes were observed in serum (range: 12-160 mg/100mls), nasal secretions (range: 155-1588 mg/100mls) (Table 4.11), and intestinal scrapings (range: 2.0-32 mg/100mls) (data not shown) between the groups after vaccination.

Table 4.11: IgA concentrations in serum and nasal secretions

Sample	Group/Weeks after vaccination	0	1	2
Serum	Non-vaccinated	31 (15)	25 (14)	30 (15)
	PRV	84 (53)	51 (20)	38 (5.5)
	PRV/IFA	54 (18)	30 (12)	31 (9.6)
	PRV/ISC	17 (3.1)	19 (5.9)	17 (3.6)
	PRV/MSPH	36 (30)	31 (23)	35 (21)
Nasal	Non-vaccinated	839 (86)	1069 (364)	388[1]
	PRV	547 (102)	483 (163)	435 (213)
	PRV/IFA	899 (124)	1018 (811)	354 (56)
	PRV/ISC	253 (166)	ND	406 (314)
	PRV/MSPH	549 (407)	891 (721)	384 (84)

Results given as mean with (SD) and expressed in mg IgA/100mls; [1] one animal; (vaccinated vs non-vaccinated); ND = not done

Individual IgG concentrations also varied markedly. No significant differences were found in serum (range: 2038-9846 mg/100mls), nasal secretion (range: 16-169 mg/100mls) (Table 4.12), and intestinal scrapings (range: 0.01-0.5 mg/100mls) (data not shown) between the groups after vaccination.

Table 4.12: IgG concentrations in serum and nasal secretions

Sample	Group/Weeks after vaccination	0	1	2
Serum	Non-vaccinated	3449 (1016)	3310 (575)	4753 (1263)
	PRV	5572 (480)	6044 (1608)	4352 (2647)
	PRV/IFA	4641 (1543)	5178 (1920)	5418 (2224)
	PRV/ISC	5180 (2569)	3564 (694)	6519 (2855)
	PRV/MSPH	5105 (2427)	5719 (1837)	3861 (1091)
Nasal	Non-vaccinated	45 (21)	95 (66)	27[1]
	PRV	63 (7.0)	59 (21)	65 (33)
	PRV/IFA	43 (12)	59 (9.2)	54 (18)
	PRV/ISC	45 (25)	ND	76 (21)
	PRV/MSPH	79 (25)	79 (54)	52 (28)

Results given as mean with (SD) and expressed in mg IgG/100mls; [1] one animal; (vaccinated vs non-vaccinated); ND = not done

4.3.2.4 Summarised results of rotavirus-specific antibody responses

Summarised results of rotavirus-specific antibody responses after parenteral vaccination are shown in Table 4.13.

Table 4.13: Summarised results of rotavirus-specific antibody responses after parenteral vaccination

	Rotavirus-specific antibody responses				
Groups	Serum IgA	Serum IgG	Nasal IgA	Nasal IgG	Serum VNT
RV	-	-	-	-	-
RV/IFA	-	+	-	-	+
RV/ISC	-	+	?	-	+
RV/MSPH	-	-	-	-	-
PRV	-	-	-	-	-
PRV/IFA	-	+	-	-	-
PRV/ISC	-	-	?	-	+
PRV/MSPH	-	-	-	-	+
Non-vaccinated	-	-	-	-	-

+ = significant increase; - = no response; ? = no clear observation due to missing data

4.4 DISCUSSION

In general, parenteral vaccination was successful in raising rotavirus-specific IgG antibodies or virus neutralising activity in serum and rotavirus-specific IgA antibodies at mucosal surfaces in previously exposed animals. Similar responses have been reported in pigs against transmissible gastroenteritis virus (TGEV) and in humans against influenza virus following parenteral vaccination (Crouch, 1985; Moldoveanu *et al,* 1995).

Vaccination with virus lysate or purified virus alone was unsuccessful in stimulating an immune response to rotavirus. Similar observations were seen with parenteral vaccination of inactivated (Salk) poliovirus (Ogra *et al,* 1980).

After vaccination, serum rotavirus-specific IgA antibodies were not detected in any of the vaccinated groups at any stage. In parenterally vaccinated ruminants, the antibody response is predominantly an IgG response (Saif *et al,* 1984). A similar observation was made in chapter 3.

Significant increases in serum rotavirus-specific IgG antibodies were observed in the RV/IFA, PRV/IFA, and RV/ISC vaccinated animals. Smaller but non-significant increases were also observed in the PRV/ISC and PRV/MSPH vaccinated groups.

IFA was successful in boosting rotavirus-specific IgG antibodies, but no difference in response was observed between the two antigen doses. Vaccination with ISCOMs increased serum rotavirus-specific IgG antibodies. Similar increased levels of virus-specific IgG antibodies were seen in mice and baboons vaccinated parenterally with an influenza/ISCOM and rotavirus/ISCOM vaccine respectively (Lövgren, 1988; Lövgren *et al,* 1990; Snodgrass *et al,* 1995). A dose effect was observed and a high antigen dose correlated with an increased rotavirus-specific IgG antibodies in serum compared to the response seen in animals given the lower antigen dose. Microspheres were successful in boosting serum rotavirus-specific IgG antibodies only when they were given with the higher antigen dose. The lack of response at the low antigen dose is probably due to the small amount of rotavirus antigen adsorbed to the microspheres. Similar observations were made in mice vaccinated intramuscularly with microspheres and free rotavirus (Moser *et al,* 1996). In previous studies on *Mycobacterium tuberculosis* and tetanus toxoid, purified antigens encapsulated within microspheres were able to stimulate specific serum antibodies after parenteral vaccination (Vordermeier *et al,* 1995; Singh *et al,* 1997).

Increased levels of rotavirus-specific IgA antibodies in nasal secretions were observed in the RV/IFA, PRV/IFA, and PRV/MSPH vaccinated groups, but these increases were not significant. This is probably due to the limitation of group size.

The study described in chapter 3 showed significantly increased rotavirus-specific IgA antibodies in nasal secretions using a standard parenteral rotavirus vaccine (RV/IFA). No conclusion can be drawn for the ISCOM vaccinated animals due to missing data. However, in previously exposed baboons rotavirus-specific IgA antibodies were increased in milk after parenteral vaccination with a rotavirus/ISCOM vaccine (Snodgrass *et al,* 1995).

Increased levels of rotavirus-specific IgG antibodies in nasal secretions were observed in the RV/IFA, RV/ISC, PRV/ISC, and PRV/MSPH vaccinated animals. Vaccination with IFA and a high antigen dose did not increase rotavirus-specific IgG antibodies in nasal secretions while an increase in serum was seen, which could suggest that no diffusion of antibodies from serum to nasal secretions takes place and that these IgG antibodies are locally produced at the mucosal site. A similar suggestion was made in chapter 3. The lack of response seen in the animals vaccinated with the microspheres and the lower antigen dose is probably due to the small amount of antigen adsorbed to the microspheres. A dose effect was also seen when vaccinated with ISCOMs, a high antigen dose correlated with an increased level of rotavirus-specific antibodies.

Significantly increased serum neutralising antibodies were detected in the RV/IFA, RV/ISC, PRV/ISC, and PRV/MSPH vaccinated groups. The increase in neutralising activity was observed with the standard rotavirus vaccine (RV/IFA), while no increase in neutralising activity was seen in PRV/IFA group. This might be due to the loss of presentation of neutralising epitopes when purified rotavirus is emulsified with IFA and that the rotavirus antigens present in the cell culture lysate are protected by other proteins. This could suggest that the significant increase observed in rotavirus-specific IgG antibodies in serum in the PRV/IFA group is due to an increase in non-neutralising VP6-specific antibodies. Increased neutralising activity was seen when purified virus was used with ISCOMs or microspheres suggesting that the purification process does not have an effect on the antigenic structure.

Total immunoglobulin levels were similar to those observed in chapter 3, no significant changes occurred. Total immunoglobulin levels were in general higher, except for serum IgA, than previously described (Smith *et al,* 1975).

This study demonstrates that parenteral vaccines can boost a rotavirus-specific antibody response in serum and at mucosal surfaces. However different adjuvants and antigen doses influence the immune response, with a low antigen dose, IFA and ISCOMs cause significant increases in rotavirus-specific and neutralising antibodies, while no response is observed with microspheres. However with a high antigen dose, ISCOMs and microspheres induce increases in rotavirus-specific and neutralising antibodies compared to IFA. The small number of animals in each group had an influence on the outcome of the results. The standard parenteral rotavirus vaccine (RV/IFA) as described in chapter 3 and in this study is efficient in inducing circulating and mucosal rotavirus-specific antibodies and neutralising antibodies in previously exposed animals.

CHAPTER 5

EFFECTS OF DIFFERENT ADJUVANTS AND ANTIGEN DOSES IN ORAL VACCINES ON ROTAVIRUS-SPECIFIC IMMUNITY IN SHEEP

5.1 INTRODUCTION

Oral rotavirus vaccines are being developed for young children. The most promising vaccines are a quadrivalent vaccine composed of three rhesus-human genetic reassortants and a rhesus rotavirus strain, and a multivalent bovine rotavirus genetic reassortant vaccine composed of bovine rotavirus genes and human rotavirus genes (Bernstein *et al,* 1995; Clark *et al,* 1996a,b; Kapikian *et al,* 1996). However, in animals, rotavirus vaccination is based on maternal vaccination with inactivated rotavirus emulsified with an oil-based adjuvant (Snodgrass *et al,* 1980; Saif *et al,* 1984). Aimed at a passive transfer of maternal rotavirus neutralising antibodies via colostrum and milk to the offspring. These maternal antibodies present in the lumen of the neonatal intestine are an effective mediator of protection.

Oral vaccination has the advantage that the antigen is delivered at the site of infection and a specific immune response at mucosal surfaces is induced. Several studies have shown that oral vaccination can induce a mucosal immune response in naive animals. Cholera toxin (CT) and ISCOMs have successfully been used as oral adjuvants in increasing specific antibodies both systemically and locally in mice against tetanus toxoid and influenza (Jackson *et al,* 1993; Ghazi *et al,* 1995). Biodegradable microspheres have been reported to be a successful carrier for oral antigen. For example one study, toxin-specific antibodies in serum and in mucosal secretions were induced when mice were vaccinated orally with staphylococcal enterotoxin B toxoid encapsulated in microspheres respectively (Eldridge *et al,* 1989; 1990). However, no evidence is available that oral vaccination in previously exposed animals can boost the mucosal immune response.

The aim of the experiments described in this chapter was to study whether oral vaccination can boost immune responses at mucosal surfaces. Two doses of antigen (lysate and purified) were compared and three mucosal adjuvants: CT, ISCOMs, and microspheres.

5.2 EXPERIMENTAL DESIGN

5.2.1 Animals

Non-lactating multiparous crossbred sheep were used and divided into groups. All animals in these groups had serum neutralising antibodies to rotavirus measured by virus neutralising assay, indicating previous natural intestinal exposure.

Sheep were divided into 8 groups (n=4 for each group) and vaccinated orally with either inactivated rotavirus lysate (RV) (low antigen dose) or inactivated purified rotavirus (PRV) (high antigen dose) adjuvanted with CT (RV/CT), ISCOMs (RV/ISC; PRV/ISC) or microspheres (RV/MSPH; PRV/MSPH). Three groups were used as controls, receiving either live rotavirus lysate (LRV), live passaged ovine rotavirus isolate (LK923) or no treatment at all.

5.2.2 Vaccines

Rotavirus used was bovine strain UK or ovine strain K923. The bovine strain was used either as a cell culture lysate (infectivity titre of $10^{6.8}$ ffu/ml) (see 2.2.2) or sucrose-purified (infectivity titre $10^{8.8}$ ffu/ml) (see 2.2.3). The cell culture lysate was used as a live rotavirus dose (LRV), or inactivated by the BEI method (see 2.2.4) and adjuvanted. Protein concentrations of rotavirus lysate and purified rotavirus were determined with a BCA™ protein assay. The ovine strain of rotavirus, K923 was contained in faeces from the first gnotobiotic lamb passage of the Moredun strain of lamb rotavirus (Snodgrass *et al,* 1976a).

The LRV and LK923 group received 5mls of rotavirus lysate and 1g of faeces diluted in 5mls PBS respectively. The RV/CT group received 500µg of CT (Sigma, UK) mixed with 5mls of inactivated rotavirus. The RV/ISC group received 500µg ISCOM matrix (Iscotec, Sweden) mixed with 5mls of inactivated rotavirus. The RV/MSPH group received 10mg each of microspheres (gift from Dr. A. Coombes, University of Nottingham) adsorbed with 1.05mg of lysate protein. 40mg of microspheres were incubated overnight at 37°C with 4mls of virus lysate (protein concentration was 4.45mg/ml). After incubation, the suspension was centrifuged for

5 minutes at maximum speed with a micro-centrifuge and washed twice with PBS. Protein concentration was determined in supernatants to measure the adsorption of protein to the microspheres. Adsorption percentage was 23.6%.

The PRV/ISC group was vaccinated with 100μg of inactivated purified rotavirus, 500μg ISCOM matrix, and PBS making a total volume of 5mls. The PRV/MSPH group received 10mg each of microspheres adsorbed with 136μg of purified rotavirus protein. 40mg of microspheres were incubated overnight at 37°C with 3mls of purified rotavirus (protein concentration was 482μg/ml). After incubation, microspheres were treated the same as above. Adsorption percentage was 37.6%.

5.2.3 Sample collection

Blood and nasal secretions were collected at initial vaccination, and then at 1 and 2 weeks post vaccination. The sheep were killed at 2-3 weeks after vaccination and at necropsy intestinal scrapings were collected.

Serum, nasal secretions, and intestinal scrapings were tested for rotavirus-specific antibodies and total IgA and IgG concentrations by ELISA. Virus neutralising titres were determined in serum and intestinal scrapings by virus neutralising assay.

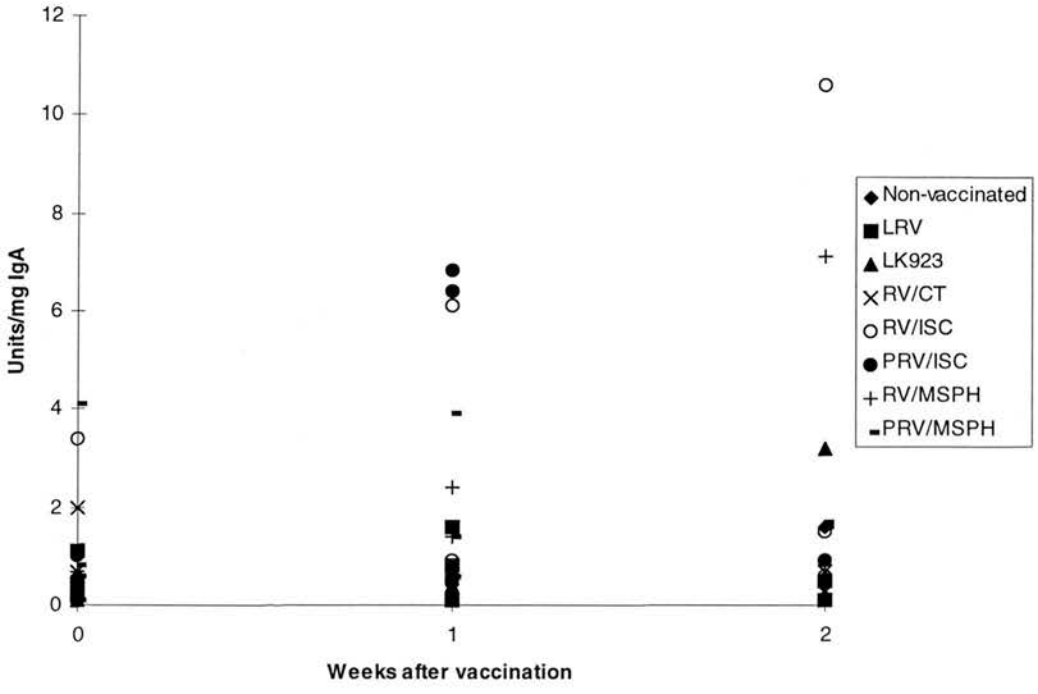
5.3 RESULTS

5.3.1 Rotavirus-specific antibodies

Rotavirus-specific IgA antibodies were present in nasal secretions at initial vaccination. None of the vaccinated groups showed a significant increase although in each of the RV/ISC, PRV/ISC, and RV/MSPH groups 1, 2, and 1 animal respectively had marked individual increases (Fig 5.1 and Table 5.1). Rotavirus-specific IgA antibodies were not observed in serum at any stage (data not shown) but were present in intestinal scrapings (range: 1.0-61 units/μg IgA) with no differences between the groups (data not shown).

Rotavirus-specific IgG antibodies were present in serum (Fig. 5.2 and Table 5.2), nasal secretions (Fig. 5.3 and Table 5.3), and intestinal scrapings (range: 4-354 units/μg IgG) (data not shown) but no significant differences were found between the groups.

Fig. 5.1: Rotavirus-specific IgA antibody responses in nasal secretions



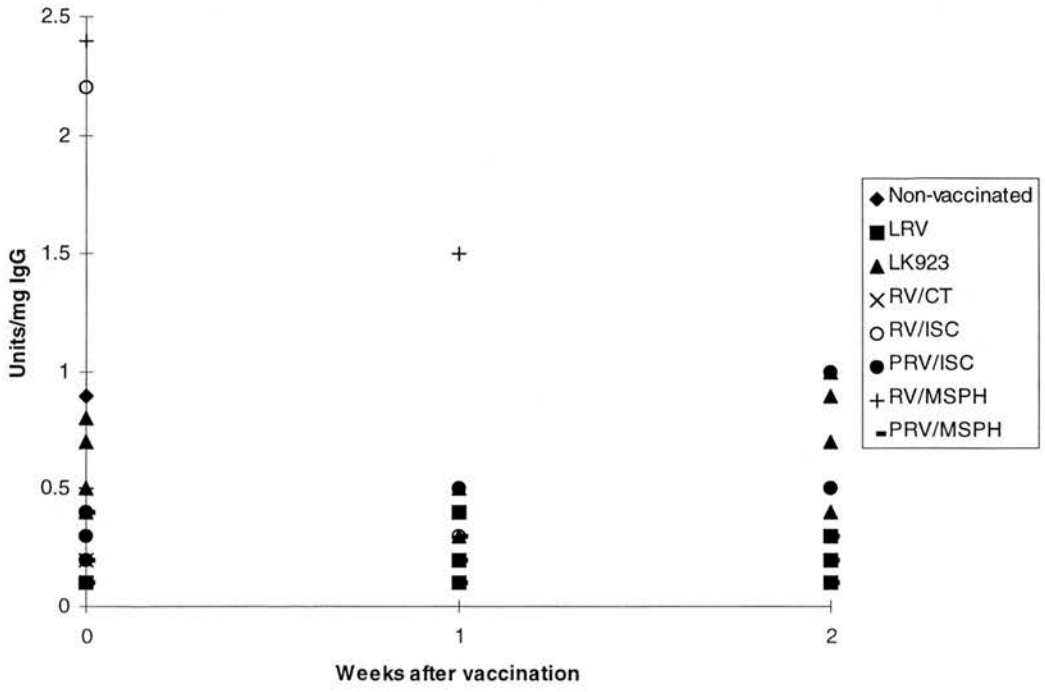
Rotavirus-specific IgA antibody responses in nasal secretions of each animal after oral vaccination. Results expressed in units/mg IgA.

Table 5.1: Mean rotavirus-specific IgA antibody responses in nasal secretion

Group/Weeks after vaccination	0	1	2
Non-vaccinated	0.5 (0.1)	0.7 (0.1)	0.7 (0.8)
LRV	0.5 (0.4)	0.4 (0.4)	0.3 (0.3)
LK923	0.1 (0)	0.2 (0.2)	1.1 (1.8)
RV/CT	0.9 (0.9)	0.4 (0.2)	0.5 (0.3)
RV/ISC	1.6 (1.6)	2.1 (2.7)	4.2 (5.5)
PRV/ISC	0.3 (0.5)	3.5 (3.6)	0.3 (0.4)
RV/MSPH	0.5 (0.4)	1.0 (1.1)	2.7 (3.9)
PRV/MSPH	1.4 (1.8)	2.0 (1.7)	1.7 (0.1)

Results given as mean with (SD) and expressed as units/mg IgA; (vaccinated vs non-vaccinated)

Fig. 5.2: Rotavirus-specific IgG antibody responses in serum



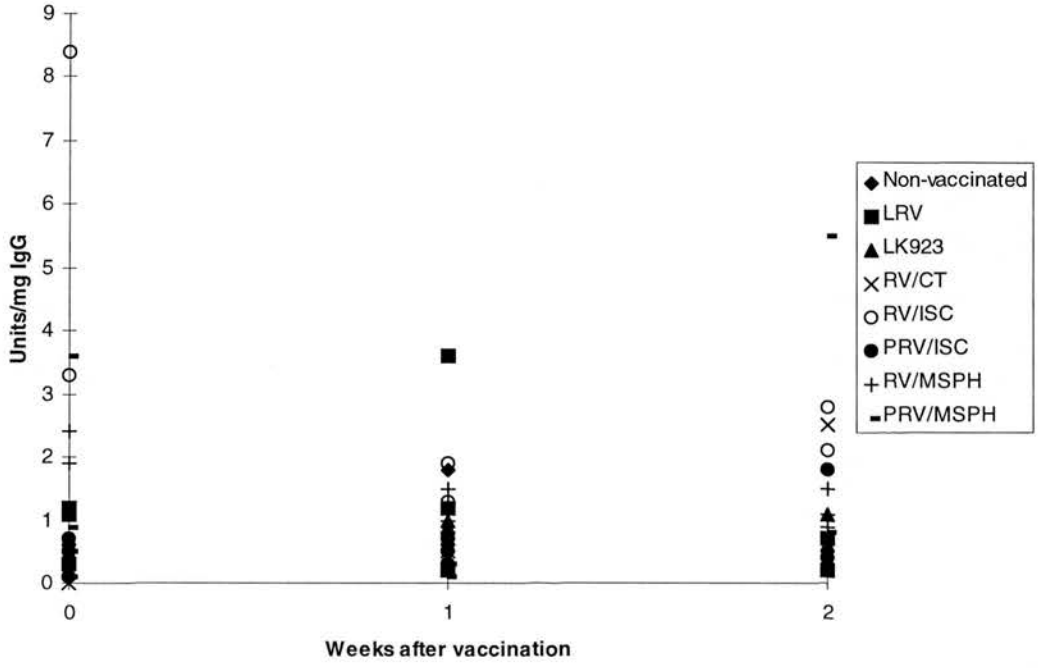
Rotavirus-specific IgG antibody responses in serum of each animal after oral vaccination. Results expressed in units/mg IgG.

Table 5.2: Mean rotavirus-specific IgG antibody responses in serum

Group/Weeks after vaccination	0	1	2
Non-vaccinated	0.3 (0.4)	0.5 (0.7)	0.1 (0.1)
LRV	0.1 (0)	0.2 (0.1)	0.2 (0.1)
LK923	0.6 (0.2)	0.4 (0.1)	0.8 (0.4)
RV/CT	0.1 (0.1)	0.1 (0.1)	0.2 (0.1)
RV/ISC	0.7 (1.0)	0.3 (0.1)	0.3 (0.2)
PRV/ISC	0.3 (0.1)	0.2 (0.2)	0.3 (0.2)
RV/MSPH	1.0 (1.2)	0.6 (0.6)	0.4 (0.4)
PRV/MSPH	0.2 (0.1)	0.2 (0.1)	0.3 (0.2)

Results given as mean with (SD) and expressed as units/mg IgG; (vaccinated vs non-vaccinated)

Fig. 5.3: Rotavirus-specific IgG antibody responses in nasal secretions



Rotavirus-specific IgG antibody responses in nasal secretions of each animal after oral vaccination. Results expressed in units/mg IgG.

Table 5.3: Mean rotavirus-specific IgG antibody responses in nasal secretions

Group/Weeks after vaccination	0	1	2
Non-vaccinated	0.4 (0.2)	1.0 (0.7)	0.4 (0.1)
LRV	0.7 (0.5)	1.3 (1.6)	0.5 (0.4)
LK923	0.6 (0.2)	0.7 (0.3)	0.8 (0.3)
RV/CT	0.4 (0.1)	0.6 (0.2)	1.5 (1.5)
RV/ISC	4.1 (4.0)	1.3 (0.6)	1.8 (1.2)
PRV/ISC	0.4 (0.3)	0.6 (0.5)	0.7 (0.7)
RV/MSPH	2.2 (0.4)	1.2 (0.3)	1.2 (0.3)
PRV/MSPH	1.3 (1.6)	0.2 (0.1)	3.2 (3.3)

Results given as mean with (SD) and expressed as units/mg IgG; (vaccinated vs non-vaccinated)

5.3.2 Neutralising titres against rotavirus

No significant differences were observed in serum (Table 5.4) between the groups. In intestinal scrapings a low neutralising activity (VNT=10) (data not shown) that was not affected by vaccination was observed.

Table 5.4: Mean neutralising titres in serum

Group/Weeks after vaccination	0	1	2
Non-vaccinated	80 (40-320)	80 (20-160)	80 (40-320)
LRV	40 (20-160)	40 (20-160)	80 (40-160)
LK923	40 (20-40)	40 (20-40)	80 (40-160)
RV/CT	160 (80-320)	80 (20-320)	80 (20-160)
RV/ISC	80 (40-320)	80 (40-320)	80 (40-320)
PRV/ISC	160 (80-640)	160 (80-640)	160 (80-640)
RV/MSPH	160 (20-640)	160 (20-640)	160 (20-640)
PRV/MSPH	320 (80-2560)	320 (80-2560)	320 (80-2560)

Results given as mean with (range); (vaccinated vs non-vaccinated)

5.3.3 Total immunoglobulin A and G concentrations

Individual IgA concentration varied markedly. No significant differences were found in serum (range: 9-87 mg/100mls) (Table 5.4) and intestinal scrapings (0.7-51 mg/100mls) (data not shown) between the groups. Increased IgA levels were observed in the PRV/ISC and PRV/MSPH groups in nasal secretions (range: 110-1977 mg/100mls) (Table 5.5) 2 weeks after vaccination, but these levels were not significant.

Table 5.5: IgA concentrations in serum and nasal secretions

Sample	Group/Weeks after vaccination	0	1	2
Serum	Non-vaccinated	59 (18)	54 (24)	55 (17)
	LRV	34 (35)	37 (27)	33 (23)
	LK923	17 (5.0)	15 (2.0)	17 (3.0)
	RV/CT	33 (19)	33 (28)	27 (18)
	RV/ISC	43 (9.0)	32 (11)	35 (14)
	PRV/ISC	29 (10)	19 (6)	32 (15)
	RV/MSPH	37 (9.0)	36 (8)	33 (13)
	PRV/MSPH	33 (2.0)	21 (3)	36 (13)
Nasal	Non-vaccinated	288 (95)	344 (265)	278 (130)
	LRV	522 (221)	646 (171)	279 (250)
	LK923	273 (62)	819 (790)	349 (78)
	RV/CT	234 (116)	418 (174)	651 (579)
	RV/ISC	335 (9)	447 (191)	328 (199)
	PRV/ISC	772 (211)	718 (518)	1166 (683)
	RV/MSPH	633 (596)	467 (229)	512 (456)
	PRV/MSPH	800 (221)	1412 (657)	1297 (909)

Results given as mean with (SD) and expressed in mg IgA/100mls; vaccinated vs non-vaccinated

Individual IgG concentrations also varied markedly. No significant differences were found in serum (range: 1484-13563 mg/100mls) and intestinal scrapings (range: 0.1-1.3 mg/100mls) (data not shown) between the groups. Significant increases were observed in nasal secretions (range: 28-225 mg/100mls) (Table 5.6) in the PRV/ISC and PRV/MSPH groups 1-2 weeks after vaccination.

Table 5.6: IgG concentrations in serum and nasal secretions

Sample	Group/Weeks after vaccination	0	1	2
Serum	Non-vaccinated	4190 (1193)	4432(781)	5909(1537)
	LRV	4901 (1926)	6725(1385)	6683(1272)
	LK923	1846 (473)	1451(492)	1548(646)
	RV/CT	6475 (1998)	6235(1427)	7583(1372)
	RV/ISC	4650 (1177)	6170(2766)	5548(1117)
	PRV/ISC	3912 (856)	4631(3836)	4265(2556)
	RV/MSPH	4879 (930)	5601(2521)	6546(2391)
	PRV/MSPH	4586 (498)	2588(676)	3956(432)
Nasal	Non-vaccinated	48 (14)	29 (14)	33 (4)
	LRV	48 (14)	54 (19)	60 (20)
	LK923	34 (18)	48 (32)	37 (5)
	RV/CT	65 (25)	32 (7)	43 (9)
	RV/ISC	31 (62)	32 (5)	47 (21)
	PRV/ISC	35 (6.0)	217 (191)*	168 (77)*
	RV/MSPH	40 (11)	32 (9)	52 (29)
	PRV/MSPH	41 (10)	234 (82)*	91 (29)

Results given as mean with (SD) and expressed in mg IgG/100mls; * $p<0.05$ (vaccinated vs non-vaccinated)

5.4 DISCUSSION

Oral vaccination was not successful in significantly boosting rotavirus-specific antibodies and neutralising activity in serum, nasal secretions, and intestinal scrapings. Significantly increased levels of total IgG were observed in nasal secretions after vaccination with a high antigen dose either mixed with ISCOMs or bound to microspheres and no response was observed with the low antigen dose.

No immune response was seen when animals received live rotavirus orally either as a cell culture lysate or a lamb passaged strain. This finding is in accordance with other studies where no antibody response occured after oral administration of previously exposed humans with influenza and *Salmonella typhi* Ty21a respectively

(Forrest, 1992; Moldoveanu *et al,* 1995). A low level of rotavirus-specific antibodies was present before vaccination in serum, nasal secretions, and presumptively in intestinal scrapings as nasal secretions are a good marker for the antibody status of intestinal scrapings as described in chapter 3.

The reasons for the lack of detectable humoral immune response could be that the rotavirus-specific antibodies present at mucosal surfaces particularly in the intestinal lumen could be capable of preventing infection without a detectable immune response to rotavirus. The antigenic structure of the virus could be changed due to the harsh environment of enzymatic digestion and pH changes together with a prolonged exposure of antigens to this environment especially in ruminants resulting in a decreased immunogenicity (loss of critical epitopes) (Shalaby, 1995). The dilution factor in ruminants compared to monogastric animals could influence the concentration of antigens at the intestinal mucosa. The time of monitoring could be critical and weekly sampling could miss an earlier humoral immune response or the repeated exposure to rotavirus antigen could induce a state of unresponsiveness (oral tolerance) (Mowat, 1994).

CT is reported to be a mucosal adjuvant inducing both mucosal and serum antibodies after oral administration in naive animals (McGhee *et al,* 1992). The high-affinity binding of CT, specifically the B subunits of CT to GM1 gangliosides on intestinal epithelial cells and M cells, appears to contribute to its strong immunogenicity (Lycke *et al,* 1985). CT has also the ability to serve as an adjuvant for enhancement of mucosal and serum antibody responses to co-administered antigens such as tetanus toxoid, bacterial proteins such as the surface fibrillar protein of *Streptococcus mutans*, ovalbumin, influenza virus, sendai virus, and keyhole limpet hemocyanin (KLH) (Lycke and Holmgren, 1986; Liang *et al,* 1988; Chen and Quinnan, 1989; Dertzbaugh and Elson, 1991; Van der Heijden *et al,* 1991; Jackson *et al,* 1993; Wu and Russell, 1994).

However, in this study CT had no effect on mucosal and serum antibody responses after oral vaccination with inactivated rotavirus lysate. This lack of a humoral immune response could be due to the same reasons as described when animals were vaccinated with virus alone.

ISCOMs have been demonstrated to be a mucosal adjuvant inducing strong antibody responses and cellular immunity in naive animals (Lövgren, 1988; Fossum *et al,* 1990). ISCOMs have successful been used intranasally in inducing protection against influenza virus and generated high antibody levels in mice and horses (Hannant *et al,* 1988; Sundquist *et al,* 1988; Lövgren *et al,* 1990). Ovalbumin given with ISCOMs induced a wide spectrum of humoral and cellular immunity instead of a state of oral tolerance when given alone (Mowat and Donachie, 1991; Mowat *et al,* 1991). However, no studies have been conducted if ISCOMs can enhance immune responses in previously exposed animals by oral vaccination.

There was no evidence that ISCOMs had an effect on rotavirus-specific antibody responses although a transient increase in rotavirus-specific IgA antibodies in nasal secretions was observed. However, IgG concentrations were significantly increased together with elevated IgA concentrations in nasal secretions when animals were vaccinated with purified rotavirus and ISCOMs.

In this study the antigen was mixed with the ISCOMs, whereas incorporating the antigen into the ISCOMs could improve the antigen uptake by the PP and enhance the specific immune response (Ghazi *et al,* 1995). When mixed there is an uncertainty as to whether the antigen is taken up by the PP together with the ISCOMs or taken up by epithelial cells.

The increased levels of total IgA and IgG concentrations in nasal secretions observed is probably stimulated by the presence of a high level of rotavirus protein and not caused by the presence of ISCOMs as no increase was seen when ISCOMs were given with the rotavirus lysate.

Biodegradable microspheres have been used as a mucosal adjuvant to enhance mucosal responses to antigens such as ovalbumin and staphylococcal enterotoxin B in naive animals (Eldridge *et al,* 1989; 1990; Challacombe *et al,* 1992). No studies have been conducted in previously exposed animals.

Microspheres adsorbed with virus lysate or purified virus and given orally had no significant effect on rotavirus-specific antibody responses in serum, nasal secretions, and intestinal scrapings. However, a moderate increase in rotavirus-specific IgA antibodies was observed in nasal secretions. A non-specific effect was

seen in animals vaccinated with purified virus and microspheres, they had a transient increase in IgA concentrations together with significant increased IgG concentrations in nasal secretions. A similar observation was noted with ISCOMs and purified virus.

This study demonstrates that it is difficult to enhance the virus-specific immune response in previously exposed animals after oral vaccination possibly due to reasons described earlier. An adjuvant effect was seen as ISCOMs and microspheres vaccinated animals had a transient increase in rotavirus-specific IgA antibodies in nasal secretions while CT vaccinated animals showed no response. The small number of animals in each group was likely to influence the significance of the outcome of the results. A dose effect was only observed in the total immunoglobulin levels in nasal secretions in the ISCOMs and microspheres vaccinated animals. These were increased in animals given the higher antigen dose. In conclusion, these results show that there are particular difficulties in using oral vaccines to boost mucosal and systemic responses in previously exposed sheep. Parenteral vaccines remain more successful.

CHAPTER 6

CHARACTERISATION OF THE PRIMARY LOCAL AND SYSTEMIC IMMUNE RESPONSE TO ROTAVIRUS IN GNOTOBIOTIC LAMBS

6.1 INTRODUCTION

Natural infections of infants and young animals with rotavirus have been correlated with protection against subsequent rotavirus disease. The immune mechanisms responsible for resolution of the infection and protection against subsequent challenge are not well understood. Several isolated mechanisms have been described in chapter 1 but no clear picture has been established.

The importance of rotavirus-specific antibodies, cytotoxic T lymphocytes, and T helper lymphocytes has been reported in several studies. The majority of these have been conducted in mice and rabbits and some in lambs and calves (Offit and Dudzik, 1988; 1989; Conner *et al,* 1991; Coulson *et al,* 1992; Oldham *et al,* 1993; Bruce *et al,* 1995).

Mice inoculated with rotavirus had high antibody responses to rotavirus and measurable cytotoxic T lymphocyte activity against syngeneic rotavirus-infected target cells (Offit and Dudzik, 1988; 1989; McNeal and Ward, 1995; McNeal *et al,* 1995). These mechanisms have also been described in studies in which rotavirus-specific antibodies passively transferred through breast milk, or passive administration of cytotoxic T lymphocytes resulted in protection and resolution of infection respectively (Offit and Clark, 1985; Offit *et al,* 1986; Dharakul *et al,* 1990; Offit and Dudzik, 1990). In calves, similar observations of increased rotavirus-specific antibodies and cytotoxic T lymphocytes after infection have been observed. In $CD8^+$ and $CD4^+$ T cell-depleted gnotobiotic calves, increased levels of virus excretion and reduced levels of faecal and serum antibody respectively were detected (Oldham *et al,* 1993; Parsons *et al,* 1993). The importance of either effector arm in active immunity against rotavirus has not been clarified.

This chapter aims to characterise the immune mechanisms involved after a primary rotavirus infection in gnotobiotic lambs. Many studies on the mechanisms of the primary immune response to rotavirus have been conducted in mice. To study immunity to rotavirus in ruminants we used a lamb model. Gnotobiotic lambs were used to get a clear picture of the primary immune mechanisms involved against rotavirus and not influenced by other pathogens found in a normal environment.

The reduced bacterial flora present in gnotobiotic lambs will affect the immune response to orally administered antigens. Bacterial lipopolysaccharide (LPS) affects the gut-associated lymphoid tissues resulting in enlargement of Peyer's patches and significant expansion in the B cell zones of the follicle, with the subsequent development of germinal centres, and thus influences the host response to naturally encountered antigens (Pollard and Sharon, 1970, Ferguson and Parrott, 1972). Germ-free mice showed reduced humoral and cellular immune responses to sheep erythrocytes given orally or parenterally compared to conventional mice (MacDonald and Carter, 1979; Wannemuehler *et al,* 1982).

6.2 EXPERIMENTAL DESIGN

6.2.1 Animals

Gnotobiotic cross-Suffolk lambs were delivered aseptically by hysterectomy and maintained in gnotobiotic isolator units. The lambs were divided into an infected group and a control group.

The infected group (n=10) were infected orally at 5-6 days of age (day 0) with 1 gram of lamb faeces containing $10^{8.5}$ ffu of a live lamb passaged ovine rotavirus strain (K923) which was suspended in 5mls of PBS. The control group (n=6) was given no treatment.

6.2.2 Samples

Blood and nasal secretions were sampled at regular intervals and faecal swabs were taken daily. In the infected group, 2, 3, 2, and 3 lambs were killed at 3, 8, 10, and 52 days after infection respectively. In the control group, 2 lambs were killed at each of 3, 28, and 52 days after infection. At necropsy, MLNs, JPPs, IPPs, small intestine, and intestinal scrapings were taken. Faeces were examined for the presence of viral RNA. Blood, nasal secretions, and intestinal scrapings were tested for rotavirus-specific antibodies and total IgA and IgG levels were measured by ELISA. Rotavirus-specific antibody secreting cells were determined in PBLs by ELISPOT.

Lymphocyte sub-populations and proliferation were determined in PBLs, MLNs, JPPs, IPPs, IELs, and LPLs by FACS-analysis and lymphocyte stimulation tests respectively. RT-PCR and hybridisation measured the expression of cytokines (IFNγ, IL-2, IL-4, and IL-6) in MLNs and JPPs.

6.3 RESULTS

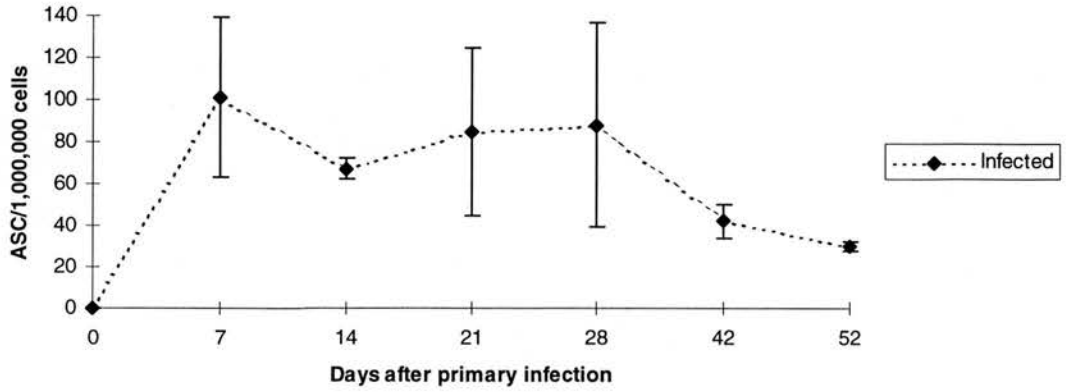
6.3.1 Viral clearance

All lambs in the infected group excreted virus within 24-48 hours, cleared the virus after 8-9 days, and showed no clinical signs of disease while in the control group no viral RNA was detected at any stage.

6.3.2 Rotavirus-specific antibody secreting cells and antibodies

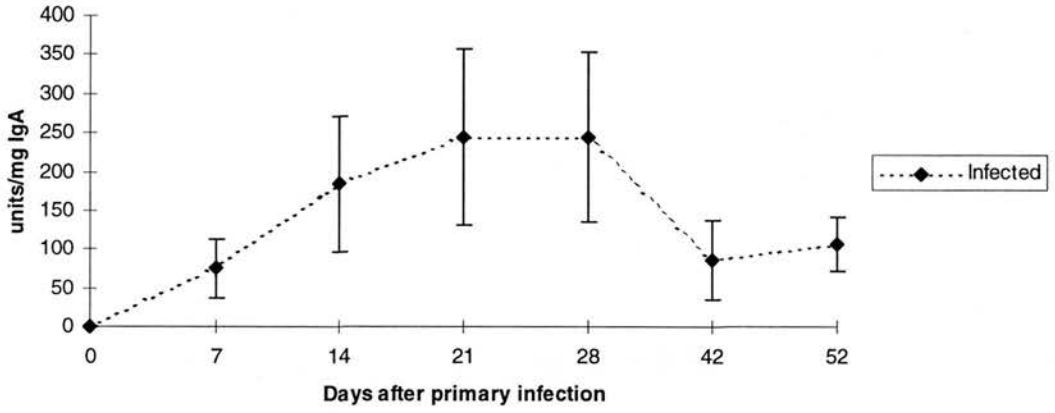
Increased numbers of rotavirus-specific IgA ASC were observed in blood from 7 days after infection until the end of the experiment. Maximum levels were reached 7 days after infection and declined slowly from 4 weeks after infection (Fig. 6.1). Rotavirus-specific IgA antibodies in serum were also detected from 7 days after infection until the end of the experiment, reaching maximum levels 3-4 weeks after infection (Fig. 6.2). In nasal secretions, rotavirus-specific IgA antibodies were seen from 7 days after infection and showed a transient peak at 28-42 days after infection (Fig. 6.3). In intestinal scrapings, no rotavirus-specific IgA antibodies were found in the first 10 days after infection, however at 52 days after infection rotavirus-specific IgA antibodies were observed in the infected group (Fig. 6.4). In the control group, no rotavirus-specific IgA antibodies were found in serum, nasal secretions or intestinal scrapings.

Fig. 6.1: Mean numbers of rotavirus-specific IgA secreting cells in blood.



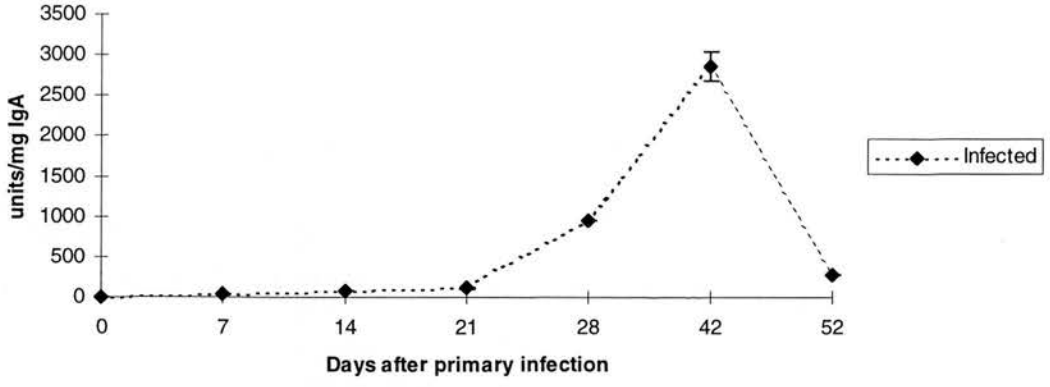
Numbers of rotavirus-specific ASC in blood after rotavirus infection.
Results expressed in ASC/10^6 peripheral blood and given as mean with SD.

Fig. 6.2: Mean rotavirus-specific IgA antibody responses in serum



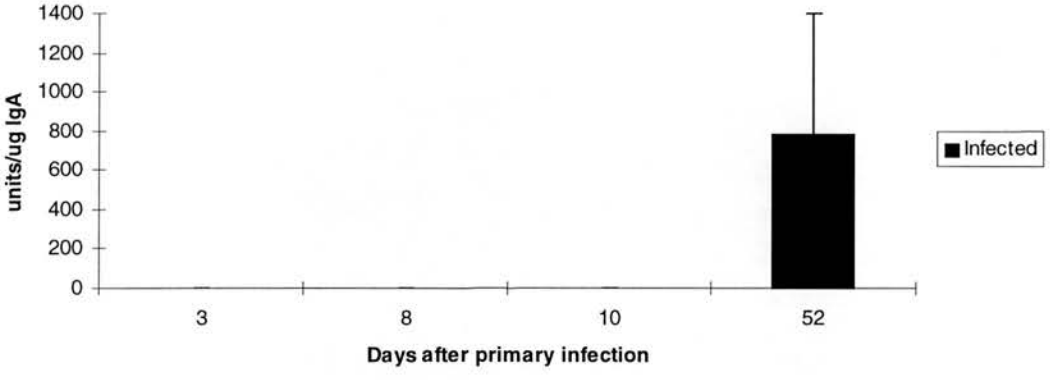
Rotavirus-specific IgA antibody responses in serum after rotavirus infection.
Results expressed in units/mg IgA and given as mean with SD.

Fig. 6.3: Mean rotavirus-specific IgA antibody responses in nasal secretions



Rotavirus-specific IgA antibody responses in nasal secretions after rotavirus infection. Results expressed as units/mg IgA and given as mean with SD.

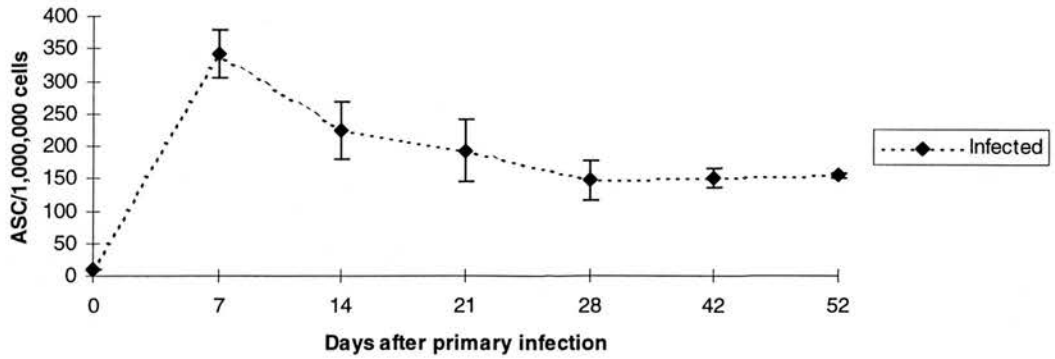
Fig. 6.4: Mean rotavirus-specific IgA antibody responses in intestinal scrapings



Rotavirus-specific IgA antibody responses in intestinal scrapings after rotavirus infection. Results expressed in units/μg IgA and given as mean with SD.

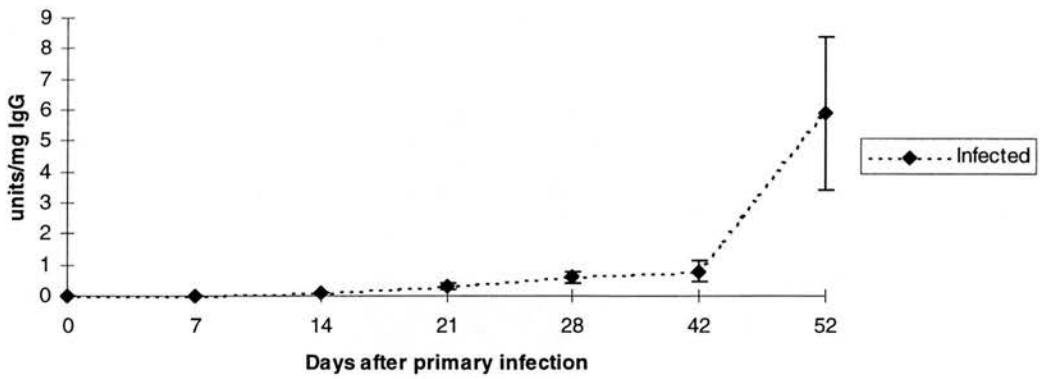
Rotavirus-specific IgG ASC were detected in blood at maximum levels at 7 days after infection and remained at a constant level from 4 weeks after infection until the end of the experiment (Fig. 6.5). Low levels of rotavirus-specific IgG antibodies were found in serum from 14 days after infection and increased markedly from 42 days after infection (Fig. 6.6). Rotavirus-specific IgG antibodies were observed in nasal secretions from 14 days after infection, with a similar picture as seen in serum (Fig. 6.7). In intestinal scrapings, no rotavirus-specific IgG antibodies were found in the first 10 days after infection, however at 52 days after infection rotavirus-specific IgG antibodies were present in the intestine (Fig. 6.8). The control group had no detectable levels of rotavirus-specific IgG antibodies in serum, nasal secretions, or intestinal scrapings.

Fig. 6.5: Mean numbers of rotavirus-specific IgG secreting cells in blood



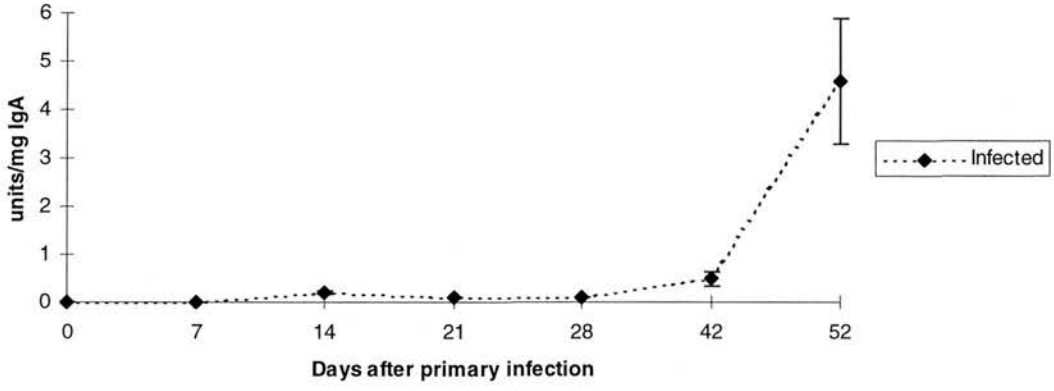
Numbers of rotavirus-specific IgG ASC in blood after rotavirus infection. Results expressed as IgG ASC/10^6 peripheral blood lymphocytes and given as mean with SD.

Fig. 6.6: Mean rotavirus-specific IgG antibody responses in serum



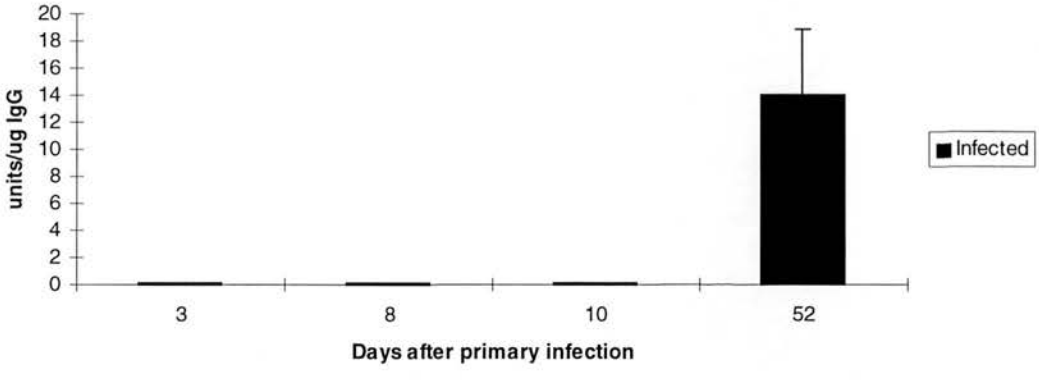
Rotavirus-specific IgG antibody responses in serum after rotavirus infection. Results expressed in units/mg IgG and given as mean with SD.

Fig. 6.7: Mean rotavirus-specific IgG antibody responses in nasal secretions



Rotavirus-specific IgG antibody responses in nasal secretions after rotavirus infection. Results expressed as units/mg IgG and given as mean with SD.

Fig. 6.8: Mean rotavirus-specific IgG antibody responses in intestinal scrapings

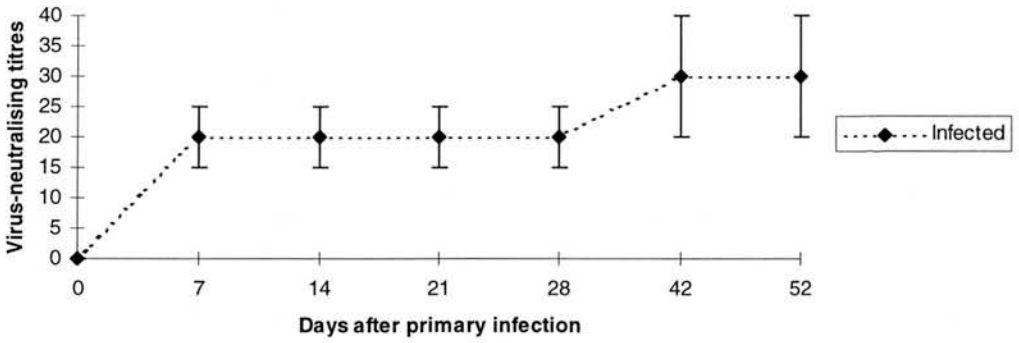


Rotavirus-specific IgG antibody responses in intestinal scrapings after rotavirus infection. Results expressed in units/μg IgG and given as mean with SD.

6.3.3 Neutralising titres against rotavirus

The infected group developed neutralising antibodies against rotavirus K923 in serum from 7 days post-infection while the control group had no detectable neutralising antibodies (Fig. 6.9). Low heterotypic neutralising antibodies (maximum VNT=10) were detected against the bovine rotavirus strain UK in the infected group from 7 days after infection. In both groups, nasal secretions and intestinal scrapings had no detectable neutralising antibodies against rotavirus K923 or rotavirus UK.

Fig. 6.9: Mean neutralising titres in serum against rotavirus K923

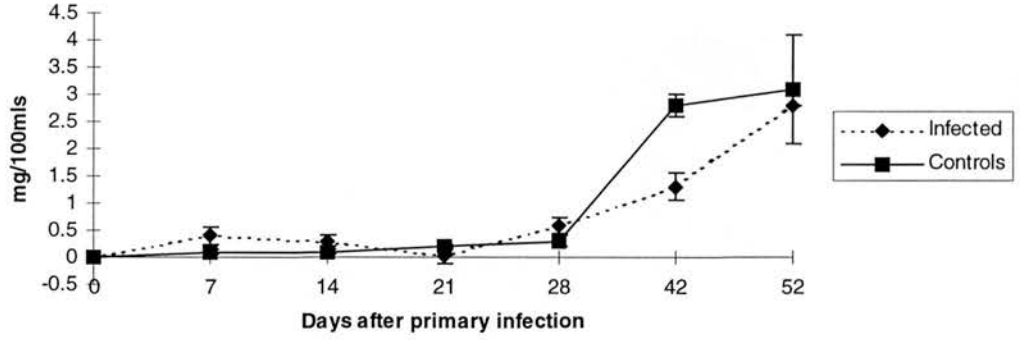


Neutralising titres against K923 after rotavirus infection. Results given as mean with SD.

6.3.4 Total immunoglobulin A and G concentrations

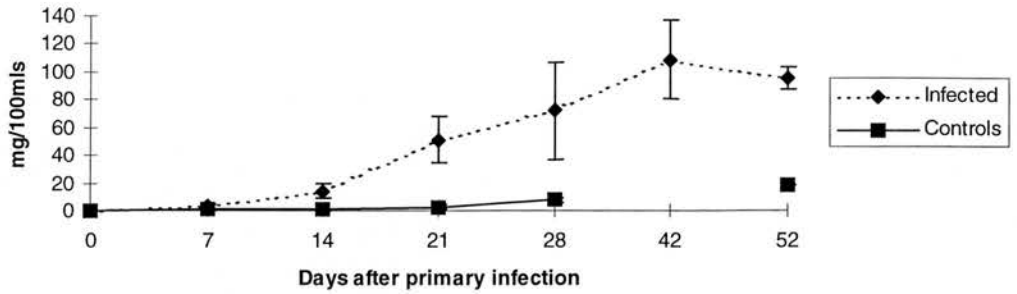
No significant differences were found in total IgA concentrations in serum after rotavirus infection between the infected (0.1 - 2.8 mg/100ml) and control groups (0.1 - 3.1 mg/100ml) (Fig. 6.10). In nasal secretions, the infected group (0.1 - 95 mg/100 ml) had significantly ($p<0.05$) increased total IgA concentrations from 21 days after infection compared to the control group (0.1 - 18 mg/100ml) (Fig. 6.11). No significant differences were found in total IgA concentrations in intestinal scrapings at 3 and 52 days after infection between the infected (0.1 - 3.2 mg/100mls) and control groups (0.1 - 1.4 mg/100mls) (Fig. 6.12).

Fig. 6.10: IgA concentrations in serum



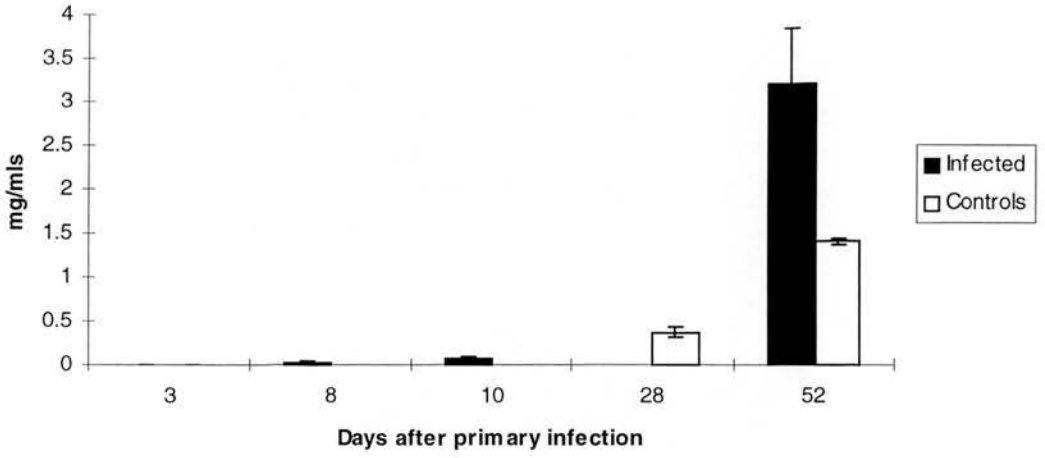
IgA concentrations in serum after rotavirus infection. Results expressed in mg/100ml and given as mean with SD.

Fig. 6.11: IgA concentrations in nasal secretions



IgA concentrations in nasal secretions after rotavirus infection. Results expressed in mg/100 ml and given as mean with SD. (In control group day 42 missing).

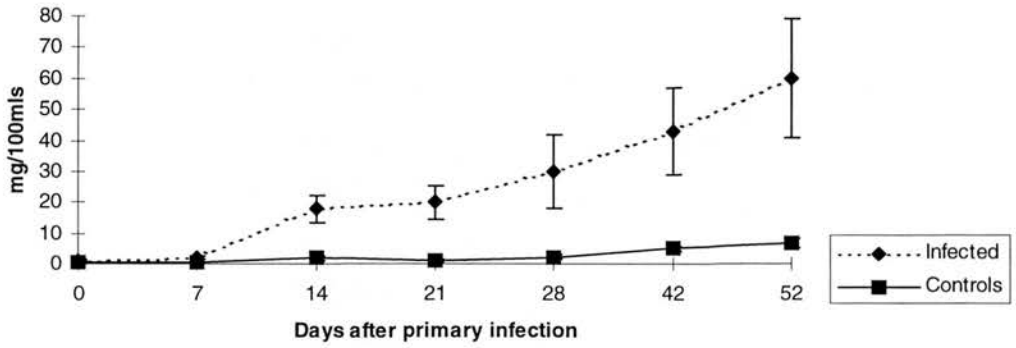
Fig. 6.12: IgA concentrations in intestinal scrapings



IgA concentrations in intestinal scrapings after rotavirus infection. Results expressed in mg/100mls and given as mean with SD.

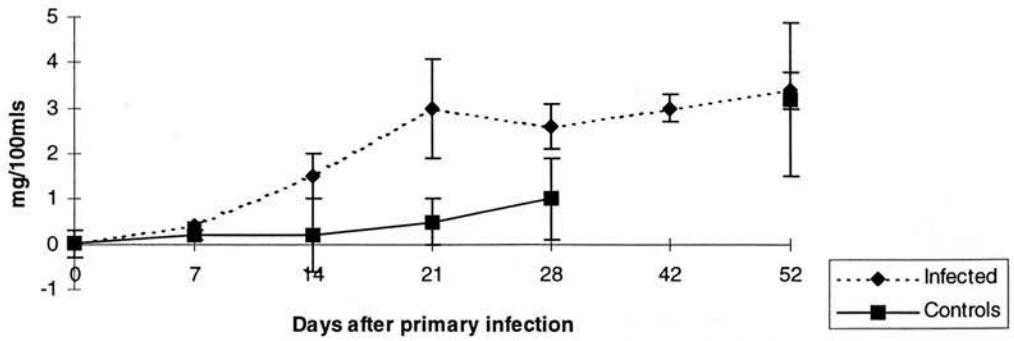
Total IgG concentrations in serum were significantly increased ($p<0.05$) after rotavirus infection in the infected group (1.5 - 60 mg/100ml) compared to the control group (1.4 - 6.7 mg/100ml) (Fig. 6.13). No significant differences were found in IgG concentrations in nasal secretions between the infected group (0.1 - 3.4 mg/100ml) and control group (0.2 - 3.2 mg/100ml) (Fig. 6.14). No significant differences were found in intestinal scrapings at 3 and 52 days after infection between the infected (0.01 - 0.1 mg/100mls) and control groups (0.01 - 0.4 mg/100mls) (Fig. 6.15).

Fig. 6.13: IgG concentrations in serum



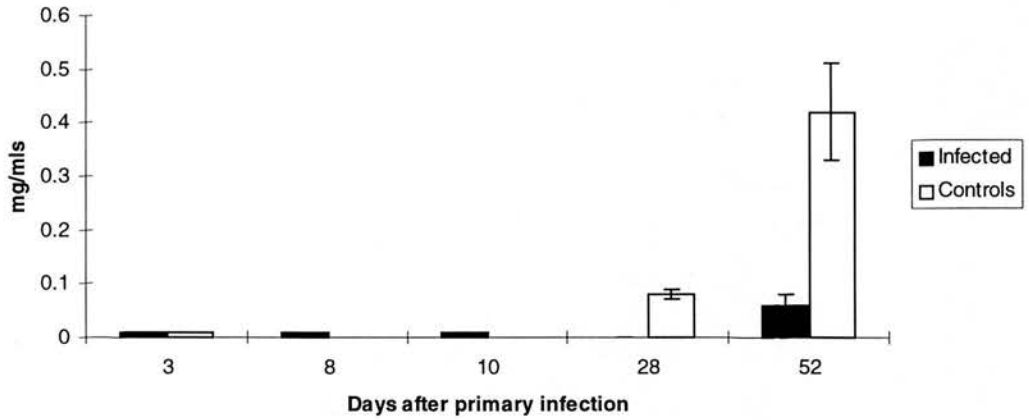
IgG concentrations in serum after rotavirus infection. Results expressed in mg/100ml and given as mean with SD.

Fig. 6.14: IgG concentrations in nasal secretions



IgG concentrations in nasal secretions after rotavirus infection. Results expressed in mg/100ml and given as mean with SD. (In control group day 42 missing).

Fig. 6.15: IgG concentrations in intestinal scrapings



IgG concentrations in intestinal scrapings after rotavirus infection. Results expressed in mg/100mls and given as mean with SD.

6.3.5 Proliferation of lymphocytes in blood and GALT

Peripheral blood lymphocytes from both groups responded to Con A (range infected: 2971±2193 - 10041±8631; controls: 3144±808 - 13933±6955) and PWM (range infected: 16341±11115 - 27982±6911; controls: 11664±1342 - 30694±1604) but not to ovalbumin (data not shown) and rotavirus (Table 6.2). A moderate increase in proliferation against rotavirus was seen in the infected group from 42 days after infection.

Table 6.2: Rotavirus-specific lymphocyte proliferation in blood in the infected and control group.

Day	0	7	14	21	28	42	52
Group							
Infected	113±109	253±160	328±389	86±48	67±27	198±102	220±115
	1.8	1.5	1.0	0.9	0.9	2.2	1.9
Controls	626±1089	297±166	141±133	296±313	67±25	527±298	ND
	1.5	0.8	1.1	1.4	1.1	0.7	

Results given in mean counts per minute ±SD and stimulation index (stimulated wells vs unstimulated wells); ND = not done

Lymphocytes isolated from MLNs and JPPs in both groups responded to Con A (range MLNs: 8063±1081 - 15893±14401; JPPs: 3536±3731 - 23853±447) and PWM (range MLNs: 13440±3818 - 24130±18248; JPPs: 9624±2586 - 31647±3823). In both groups lymphocytes isolated from IPPs responded to Con A (range 64±23 - 454±557) or PWM (236±252 - 1264±1206). In the cultures of IELs and LPLs in both groups no or a low response was observed to Con A (range IELs: 52±9 - 386±275; LPLs: 55±8 - 417±456) and PWM (range IELs: 70±46 - 140±46; LPLs: 56±5 - 354±95).

Lymphocytes isolated from JPPs from the infected group responded significantly to rotavirus at 52 days after infection, while a smaller non-significant increase in proliferation was observed earlier at 10 days after infection (Table 6.3).

Table 6.3: Rotavirus-specific lymphocyte proliferation in GALT in infected and control group.

Group	Days after infection / Tissue	3	8	10	28	52
Infected	MLN	270[1]	191±109	833;1103	ND	566±344
			0.3	1.1		1.5
Control		303;427	ND	ND	130;304	161;138
		0.3			0.9	0.9
Infected	JPP	794;387	314±87	1157;4367	ND	3001±2482
		1.6	1.5	4.1		9.9*
Controls		291;364	ND	ND	124;141	139;80
		1.3			0.8	1.5
Infected	IPP	161;555	94±17	183;346	ND	160±6.0
		1.1	1.3	1.0		1.7
Controls		122;231	ND	ND	118;90	73;52
		1.0			0.8	1.3
Infected	IEL	340;119	123±14	107;317	ND	80±21
		0.9	1.3	0.6		0.9
Controls		155;130	ND	ND	104;112	86;103
		0.7			1.1	1.3
Infected	LPL	194;341	122±6.0	294;289	ND	124±55
		2.3	1.3	0.8		0.8
Controls		167;115	ND	ND	131;101	116;108
		1.0			1.3	1.1

Results given in mean (n>2) counts per minute±SD or for each animal (n=2) and stimulation index; * $p<0.05$; [1] one animal; ND = not done

6.3.6 Phenotypic analysis of lymphocytes in blood and GALT

No significant changes in $CD4^+$ or $\gamma\delta^+$ T cells were observed in blood between the infected and control groups. No clear picture can be given about $CD8^+$ T cells due to missing values. No comparison can be made for $CD45R^+$ cells between both groups, but no change was observed in the infected group with time. A slight increase was seen with time in the expression of light chain in both groups. (Table 6.4).

Table 6.4: Percentage of lymphocyte sub-populations in peripheral blood in the infected and control group.

Marker	Days after infection / Groups	0	7	14	21	28	42	52
CD4	Infected	13 (11)	9.1 (4.3)	9.1 (0.9)	8.8 (1.5)	9.1 (2.1)	ND	9.1 (0.8)
	Controls	24 (12)	14 (5.4)	13 (2.1)	12 (3.0)	21 (9.4)	11.6;6.7	22.2;20.7
CD8	Infected	18 (6.4)	ND	ND	ND	ND	ND	ND
	Controls	16 (2.6)	ND	ND	ND	ND	16.9;10.8	14.6;11.9
γδTcR	Infected	32 (15)	42 (13)	43 (2.2)	42 (6.5)	47 (6.7)	ND	26 (0.9)
	Controls	39 (5.3)	38 (20)	39 (13)	28 (6.6)	42 (16)	49.4;46.7	47.3;44.3
L chain	Infected	20 (3.2)	19 (4.2)	13 (2.5)	7.7 (1.6)	24 (5.4)	ND	29 (4.7)
	Controls	17 (8.1)	16 (6.0)	21 (6.0)	27 (5.9)	35 (7.1)	41.1;40.1	43.7;33.0
CD45R	Infected	19 (14)	23 (8.1)	14 (0.3)	16 (10)	23 (5.3)	ND	ND
	Controls	39 (1.3)	ND	ND	ND	ND	40.5;19.2	32.1;42.2

Results given as mean (n>2) with (SD) or from each animal (n=2); ND = not done

The $CD4^+$ T cells decreased in MLNs and JPPs in both groups followed by recovery. An increase was observed with time in IELs and LPLs in both groups. In IPPs, $CD4^+$ T cells showed a slight increase with time in both groups. No clear picture can be drawn between both groups for the $CD8^+$ T cells. The $\gamma\delta^+$ T cells showed an increase in both groups in MLNs, JPPs, and IPPs with time. In the infected group, these cells decreased with time in IELs with no changes in LPLs. An

increase of $\gamma\delta^+$ T cells was observed in IELs and LPLs in the control group. The expression of light chain increased with time in MLNs and IPPs in both groups while similar levels with time were observed in IELs and LPLs for both groups. No changes were observed in JPPs in the infected group while an increase was seen in the control group. No clear picture is seen in $CD45R^+$ cells in both groups due to missing values (Table 6.5 and 6.6).

Table 6.5: Percentage of lymphocyte sub-populations in GALT in the infected group.

Marker	Days after infection / Tissue	3	8	10	52
CD4	MLN	59.2;62.5	16 (1.0)	14.1;20.7	24 (1.7)
	JPP	24.0;29.7	4.8 (2.3)	4.4;6.0	18 (9.3)
	IPP	2.3;1.5	2.2 (0.8)	3.6;2.7	13 (5.1)
	IEL	7.5;5.8	43 (25)	75.9;62.1	63 (9.8)
	LPL	5[1]	24 (4.0)	42.1;28.4	38 (20)
CD8	MLN	18.3;10.7	ND	ND	ND
	JPP	5.9;3.2	ND	ND	ND
	IPP	9.5;1.0	ND	ND	ND
	IEL	17.5;13.8	ND	ND	ND
	LPL	14[1]	ND	ND	ND
γδTcR	MLN	9.3;9.6	6.2 (2.1)	5.5;3.4	18 (1.2)
	JPP	7.7;6.2	3.1 (1.2)	1.9;1.6	13 (3.3)
	IPP	6.5;3.7	1.7 (1.2)	1.5;2.3	15 (3.5)
	IEL	12.9;25.1	5.4 (1.7)	10.7;9.0	5.9 (1.7)
	LPL	2.9[1]	9.5 (5.6)	7.1;3.7	5.7 (2.7)
Light Ch.	MLN	17.1;16.9	15 (4.2)	11.3;16.5	30 (1.3)
	JPP	36.7;22.0	29 (8.7)	22.0;26.4	35 (8.8)
	IPP	22.1;17.2	36 (5.3)	41.5;31.2	46 (6.1)
	IEL	17.1;15.5	10 (5.6)	3.8;7.7	21 (1.7)
	LPL	35[1]	18 (5.9)	17.7;11.4	23 (2.0)
CD45R	MLN	23.3;46.2	43 (16)	32.7;50.5	ND
	JPP	48.6;50.6	59 (20)	46.1;73.9	ND
	IPP	92.9;88.3	88 (8.8)	86.1;91.9	ND
	IEL	17.7;33.8	31 (6.2)	32.2;46.3	ND
	LPL	31[1]	13 (5.0)	21.1;15.9	ND

Results given as mean (n>2) with (SD) or from each animal (n=2); [1]one animal;
ND = not done

Table 6.6: Percentage of lymphocyte sub-populations in GALT in the control group.

Marker	Days after infection / Tissue	3	28	52
CD4	MLN	54.6;52.4	26.1;19.7	16.5;27.3
	JPP	39.9;44.4	4.0;9.2	4.4;23.7
	IPP	2.3;3.2	1.3;3.7	5.3;7.5
	IEL	9.5;8.0	66.6;57.8	49.3;50.8
	LPL	14.7;15.0	59.8;66.1	31.9;45.2
CD8	MLN	9.8;15.7	ND	11.7;9.6
	JPP	4.6;8.4	ND	1.8;2.3
	IPP	2.3;1.5	ND	1.7;1.0
	IEL	31.6;19.5	ND	12.3;3.6
	LPL	8.0;17.0	ND	4.0;5.8
γδTcR	MLN	11.2;9.0	14.0;19.8	24.4;24.6
	JPP	3.0;5.1	2.8;3.0	6.8;11.6
	IPP	1.8;2.1	4.1;5.7	6.0;8.4
	IEL	6.5;15.4	13.4;16.4	28.8;16.7
	LPL	6.2;16.1	24.5;14.6	26.9;25.3
Light Ch.	MLN	18.4;23.6	43.9;47.0	52.0;39.5
	JPP	25.1;25.5	44.1;36.7	47.5;40.7
	IPP	23.2;31.1	40.0;39.6	32.5;29.9
	IEL	17.7;23.8	21.7;25.1	22.6;18.8
	LPL	20.0;26.2	43.9;24.6	35.4;38.0
CD45R	MLN	35.1;31.3	ND	65.6;57.6
	JPP	51.0;35.3	ND	51.6;50.0
	IPP	90.6;90.0	ND	83.2;68.0
	IEL	29.3;19.5	ND	27.4;69.7
	LPL	23.8;32.6	ND	33.9;36.6

Results given from each animal (n=2); ND = not done

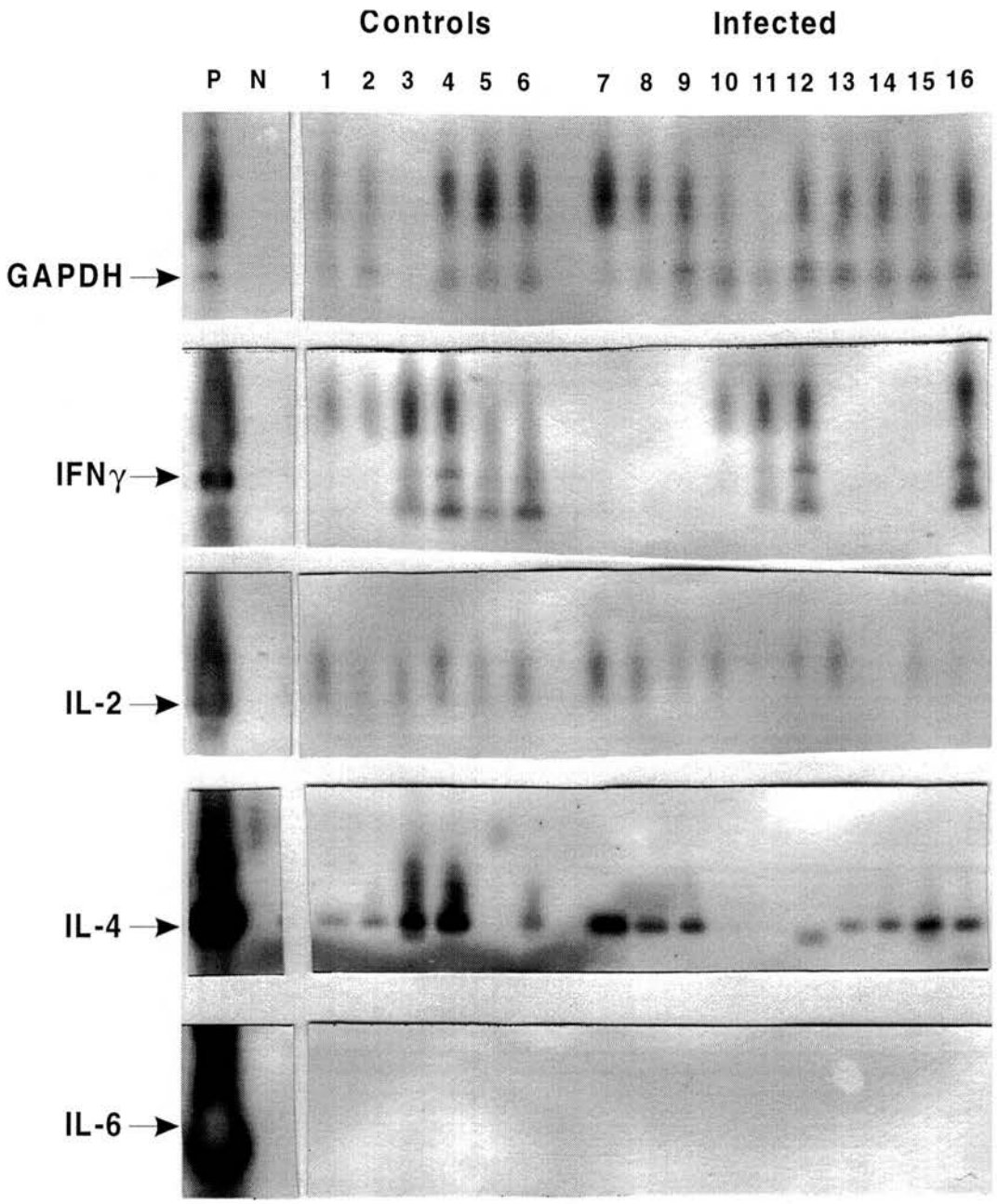
6.3.7 Cytokine expression of lymphocytes in JPPs and MLNs

Lymphocytes isolated from JPPs and MLNs in all animals except two expressed GAPDH indicating a successful RT-PCR.

In JPPs, 2 of 5 infected lambs expressed mRNA for IFNγ 8-10 days after infection while in the control group, mRNA for IFNγ was seen at 28 days in both lambs. Transcripts for IL-2 were found in both groups at all time points, although no clear bands are visible in the groups and the positive control. mRNA for IL-4 was observed in both groups from 3 days after infection. The infected group had an increased level compared to the control group at 3 and 52 days after infection. The control group showed a high level of mRNA for IL-4 at 28 days. No mRNA for IL-6 was observed in either group (Plate 6.1).

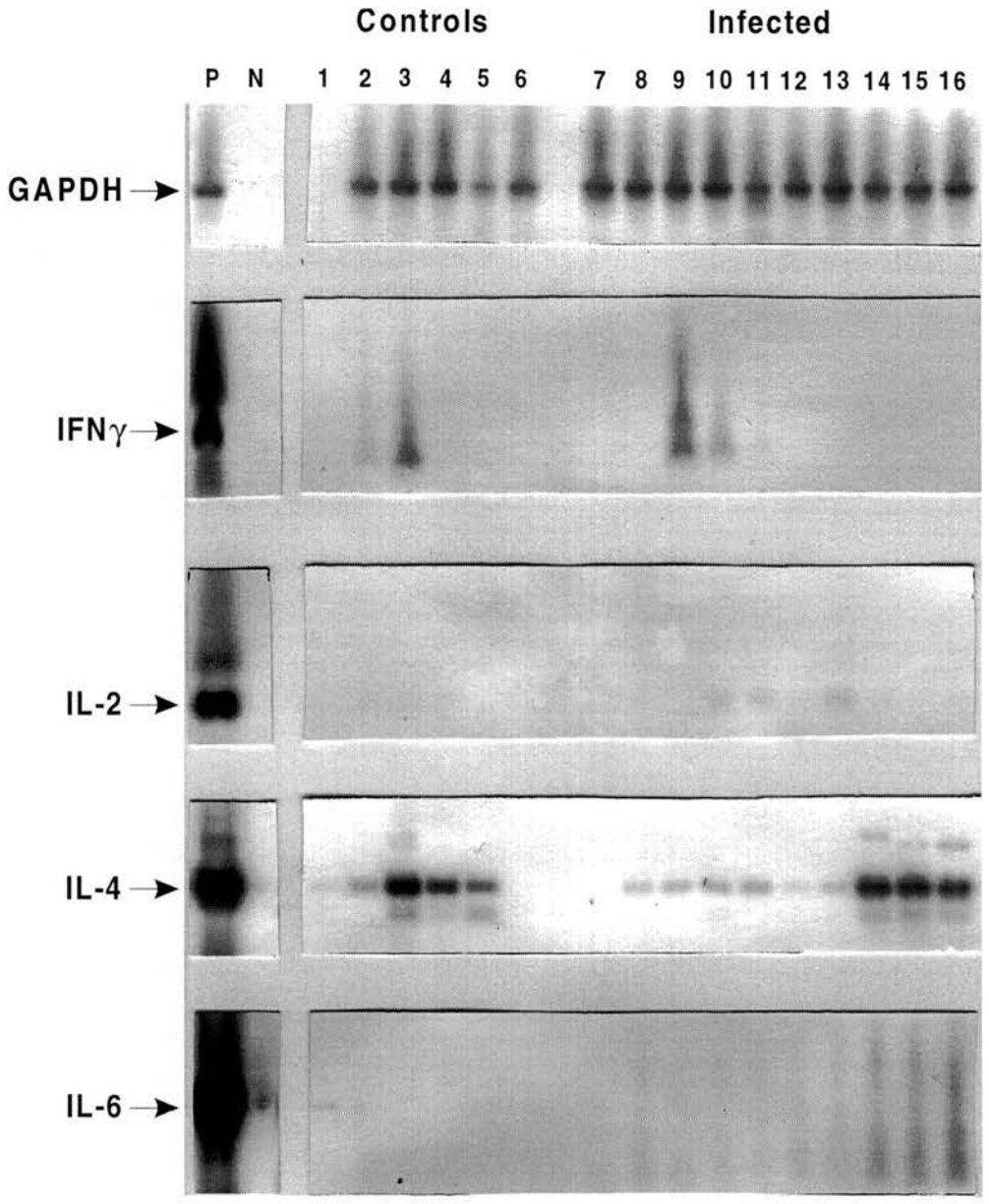
In MLNs, 1 of 3 infected lambs showed mRNA for IFNγ 8 days after infection while in the control group no clear bands were observed, although 1 of 2 lambs had IFNγ transcripts at 28 days. Transcripts for IL-2 were observed in both groups, but at a lower level compared to the expression in JPPs. Transcripts for IL-4 were detected in both groups from 3 days after infection. The control group showed a high level of expression 28 days after infection and declined at 52 days after infection. The infected lambs showed mRNA at 8 and 10 days after infection with high levels of IL-4 transcripts at 52 days after infection. This level of mRNA for IL-4, 52 days after infection was higher in the infected group compared to the control group. No clear picture can drawn for IL-6 as no clear bands are visible, however the band intensity increased in the infected group with time (Plate 6.2).

Plate 6.1: Cytokine expression in JPPs in the control and infected groups



GAPDH and cytokine expression in lymphocytes isolated from JPPs from infected lambs and control lambs. P = positive PCR control; N = negative control; Controls: 1-6 (1-2, 3-4, 5-6 killed at 3, 28, and 52 days respectively); Infected: 7-16 (7-8, 9-11, 12-13, and 14-16 killed at 3, 8, 10, and 52 days respectively).

Plate 6.2: Cytokine expression in MLNs in the control and infected groups



GAPDH and cytokine expression in lymphocytes isolated from JPP from infected lambs and control lambs. P = positive PCR control; N = negative control; Controls: 1-6 (1-2, 3-4, 5-6 killed at 3, 28, and 52 days respectively); Infected: 7-16 (7-8, 9-11, 12-13, and 14-16 killed at 3, 8, 10, and 52 days respectively).

6.4 DISCUSSION

In this study, lambs infected orally at 5-6 days of age with a live lamb passaged ovine rotavirus strain K923 excreted rotavirus within 48 hours of infection. The lambs showed no clinical signs (e.g., diarrhoea) and continued to excrete virus for 6-7 days which was of a similar duration as described in a previous study (Snodgrass *et al,* 1976b). However, in this study one-day-old lambs excreted virus within a period of 24 hours. In similar studies, calves and lambs infected orally with bovine rotavirus strains NCDV and UK respectively started virus excretion within 2-3 and 2 days respectively. They continued to excrete virus for 5-6 and 6-7 days respectively (Saif, 1987; Bruce *et al,* 1995). No virus was detected in the faeces of the non-infected control lambs as expected.

The rotavirus-specific antibody response in serum seen in this study follows the "classical" pattern with the appearance of specific IgA followed by specific IgG. Similar observations have been described in humans and calves infected with rotavirus (Hjelt *et al,* 1985a; 1986; Saif, 1987; Grimwood *et al,* 1988). In humans, circulating rotavirus-specific IgA antibodies were detected at 1-2 weeks with the peak recorded at 30 days after infection and these were detectable till 8 weeks after infection (Grimwood *et al,* 1988; Offit *et al,* 1993). In the human intestine, rotavirus-specific IgA antibodies were seen from 7 days after infection and persisted for 3-4 weeks (Davidson *et al,* 1983; Grimwood *et al,* 1988, Coulson *et al,* 1990). In calves, rotavirus-specific IgA antibodies in serum were seen from 14 days after infection with undetectable levels 5 weeks after infection (Saif, 1987). Faecal rotavirus-specific IgA antibodies were seen from 8 days after infection (Oldham *et al,* 1993). In humans, rotavirus-specific IgG antibodies were detectable from 30 days after infection and continued to rise and were still detectable 6-12 months later. Rotavirus-specific IgG antibodies were only occasionally detected in the intestine and may derive from high serum antibody levels (Grimwood *et al,* 1988). In calves, serum rotavirus-specific IgG antibodies were found from 14-21 days after infection in calves and increased with time (Saif, 1987; Oldham *et al,* 1993).

The high numbers of rotavirus-specific IgG ASC observed in blood did not correlate with high levels of circulating rotavirus-specific IgG antibodies while a relative lower number of rotavirus-specific IgA ASC correlated with relatively higher levels of circulating rotavirus-specific IgA antibodies. This could suggest that the humoral immune response is dominated by a specific IgA response after an intestinal infection.

These results demonstrate that a "classical" humoral immune response in serum and nasal secretions is seen in lambs after infection but that other mechanisms rather than antibodies in the intestinal lumen could be involved in viral clearance. The finding of the common mucosal immune system described in man and various animals is in contrast with the observation made in this study that rotavirus-specific antibodies in nasal secretions preceded rotavirus-specific IgA antibodies in the intestinal lumen. It is possible that these rotavirus-specific IgA antibodies reside in the intestinal cell and were not detectable with the methods used in this study, as described in a previous study in which mice were protected against a rotavirus infection by intracellular intestinal non-neutralising VP6 specific antibodies (Burns *et al,* 1996). The lack of rotavirus-specific IgA antibodies in the intestine could be due to the immaturity of the IgA transport from the mucosal epithelium to the intestinal lumen.

Homotypic neutralising titres in serum were seen from 7 days after infection and remained at a constant level during the course of the study. Low heterotypic neutralising antibodies against a bovine strain in serum were observed from 7 days after infection. No neutralising antibodies were detected in nasal secretions and intestinal scrapings during the course of the study. No neutralising antibodies were detected in the non-infected control group as expected.

Similar observations have been made in rabbits, calves, and lambs infected with rotavirus. A homotypic serum neutralising response was present from 7-9 days after infection in older rabbits while in younger rabbits the presence of neutralising titres was delayed but at 21 days after infection all rabbits had neutralising antibodies. A heterotypic neutralising response was seen in some but not all rabbits after primary infection (Conner *et al,* 1991). In calves and lambs, a homotypic

neutralising response in serum was found from 7 days after infection (Saif, 1987; Bruce *et al,* 1995). In rabbits, intestinal neutralising antibodies were detected from 5-7 days after infection (Conner *et al,* 1991) which is in contrast with our finding of a lack of neutralising antibodies at mucosal surfaces. This suggests that mechanisms other than neutralising antibodies might be involved in virus protection as shown in previous studies. Infection of mice with different rotavirus strains (serotype 3) resulted in protection against subsequent challenge with EDIM, however no neutralising antibodies against EDIM were present in the intestine (Ward *et al,* 1992b). Mice receiving hybridoma cells producing non-neutralising IgA VP6 specific antibodies through transplantation were protected against a rotavirus infection. When directly administered into the lumen they were not effective (Burns *et al,* 1996). These findings of previous studies and the lack of neutralising antibodies found in this study support the hypothesis that *in vivo* intracellular viral inactivation by secretory IgA during transcytosis is a possible mechanism of host defence against rotavirus infection.

Low IgA concentrations were observed in serum, nasal secretions, and intestinal scrapings in both groups from 3 days after infection. No IgA was detected in serum, nasal secretions, and presumptively in intestinal scrapings at the start of the study. In serum, total IgA concentrations increased in both groups with time with a strong increase in the control group from 28 days after infection. In nasal secretions, the infected group had significantly increased IgA concentrations compared to the control group. In the infected group, a peak was observed at 42 days after infection and correlated with the peak observed in rotavirus-specific IgA antibodies. IgA concentrations in intestinal scrapings increased in both groups with time, however a moderate, but insignificant, increased immunoglobulin concentration was seen in the infected group 52 days after infection.

Low IgG concentrations were seen in serum in both groups at the start of the study. In nasal secretions and presumptively in intestinal scrapings no IgG was detected at the start of the experiment, but 3 days after infection low IgG concentrations were observed in both groups. Serum IgG concentrations increased in both groups with time but the infected group had significantly increased

concentrations. In nasal secretions, IgG increased in both groups with time, however the increase in the control group was delayed. IgG in intestinal secretions increased in both groups with time.

The ELISA used in this study to determine the immunoglobulin concentrations is more sensitive than single radial immunodiffusion (SRD) used in a previous study (Smith, 1975; Smith *et al,* 1976). Using SRD, the limits of detection were 1.2 mg/100mls and 2.4mg/100mls for IgG and IgA respectively while the limits of detection for the ELISA were 0.1mg/100mls for both IgA and IgG.

The low immunoglobulin concentrations in serum, nasal secretions, and intestinal scrapings in 6 day old gnotobiotic lambs are in contrast to concentrations found in 10 day old specific pathogen free (SPF) lambs (Smith *et al,* 1976). These SPF lambs had IgG concentrations 15-20×higher in serum than observed in the gnotobiotic lambs. The difference in concentrations could be due to the faster colonisation of bacteria in SPF lambs compared to the limited bacterial flora with a possible slower colonisation gnotobiotic lambs. The colonisation of bacteria might stimulate immunoglobulin concentrations. This low or lack of total antibodies in gnotobiotic lambs after birth confirms that no placental transfer of antibodies takes place unlike humans (McLean and Holmes, 1980). This also suggests that the humoral immune response has to mature after birth.

In a previous study in SPF lambs either infected intranasally with live parainfluenza 3 virus (PI 3) or given no treatment, no serum IgA was detected (Smith *et al,* 1976). Similar increases in serum IgA were observed in both the infected and control gnotobiotic lambs, however the immunoglobulin concentrations observed were lower or close to the detection limit of the method used with the SPF lambs. This could explain why in the SPF lambs no serum IgA was observed. In nasal secretions, SPF and gnotobiotic control lambs had a similar pattern of IgA concentrations. Both infected groups had increased IgA concentrations compared to the controls. The infected SPF lambs had an IgA level that was twice that of the controls while the gnotobiotic lambs had a level 4-5 times that of the controls.

IgG concentrations in serum were higher in both infected groups compared to the control groups. The two infected groups had a similar increase in IgG concentrations in serum, however the increase in IgG in the SPF control lambs was

higher than the one observed in the gnotobiotic control lambs probably due to the faster colonisation of bacteria in the SPF lambs. In nasal secretions, the increase in IgG concentrations was similar for all SPF and gnotobiotic lambs.

These results show that the measurement of immunoglobulin concentrations by ELISA is more sensitive than SRD especially at low concentrations. The status of the animal, gnotobiotic, SPF or conventional, or the presence of bacterial LPS, is an important factor in the maturation of the humoral immune response. The significantly increased IgA and IgG concentrations observed in nasal secretions and serum respectively in the infected group could be due to the increased levels of rotavirus-specific antibodies. The increases in IgA and IgG in the control group are probably due to normal maturation in a gnotobiotic environment.

Peripheral blood lymphocytes from both groups responded to Con A and PWM illustrating functional T and B cells. However, these lymphocytes did not significantly respond to rotavirus antigen, although a moderate increase in proliferation (stimulation index) was observed in the infected group 42 and 52 days after infection. Cells were viable as seen in the responses to ConA and PWM. The non-responsiveness of these PBLs to rotavirus could indicate that the number of precursors to rotavirus is low. In contrast to this study, in gnotobiotic lambs infected with bovine tissue culture lysate rotavirus-primed cells appeared in the blood 7-8 days after infection (Bruce *et al,* 1995).

In the GALT, lymphocytes from MLNs and JPPs all responded to Con A and PWM while a lower response was observed in IPPs. The response to Con A was lower than the response to PWM in IPPs. These results give evidence that functional T and B cells are present in JPPs and MLNs and that IPPs have more functional B cells than T cells as expected as PWM is mainly a B cell stimulant. No response or a reduced response to Con A and PWM compared to MLNs was observed in IELs and LPLs, reflecting similar observations seen in other studies (Mowat *et al,* 1989; Mosley *et al,* 1991). This could suggest poor priming, effective suppressor mechanisms, or activated and fully differentiated T and B cells in IELs and LPLs. The cell viability of IELs and LPLs after culture was not examined. The lack of

response to Con A, PWM, and rotavirus could be due to non-viable cells in these 5-day-old cultures.

Only lymphocytes from JPPs responded to rotavirus antigen in the infected groups. A response was seen at 10 days after infection, which was not significant, but a significant proliferation was seen 52 days after infection. This illustrates the development of specific effector, memory cells, and the presence of professional APCs. No response to rotavirus was seen in the control group. The observation that virus-primed cells were seen in JPPs was similar to a previous study in gnotobiotic lambs infected with a bovine tissue culture lysate (Bruce *et al,* 1995). However, in the previous study, virus-primed cells were seen in JPPs 7 days after infection while in this study a response was observed 10 days after infection. This suggests that the uptake and presentation of rotavirus via M cells seems possible (Landsverk *et al,* 1991) and that it is possible that the uptake and presentation of antigens takes longer when infected with the homologous rotavirus strain. In the previous study, virus primed cells were seen in MLNs and IPPs 7 days after infection this is in contrast with the findings in this study. The lack of virus primed cells in MLNs could be due to the delayed presentation of antigen in JPPs. It is likely that virus primed cells are delayed in the MLNs and appear after 10 days. The observation of a lack of proliferation in MLNs 52 days after infection suggests this is not the case or that virus primed cells by-passed the MLNs and went into the circulation and no memory cells reside within the MLNs. The non-responsiveness of MLNs to rotavirus could also be explained due to a lack of professional APCs in these cultures. Addition of autologous APCs and recombinant IL-2 could stimulate rotavirus antigen presentation to lymphocytes resulting in proliferation to rotavirus (Bruce *et al,* 1994).

The response in IPPs observed in the previous study suggests that rotavirus-specific memory cells have arisen at the site as a result of an unknown mode of uptake and presentation as IPPs lack M cells. No response was seen in IPPs in this study suggesting that IPPs are unlikely to participate in inducing a specific immune response and that IPPs have a key function in B cell differentiation as a primary lymphoid organ and do not participate in specific immune responses.

Few changes were observed in T and B cells in blood between the infected and control group. No changes were seen in $CD4^+$ and $\gamma\delta^+$ T cells with time in both

groups. A slight increase in the expression of light chain on B cells was seen in both groups but this is probably due to a general increase in B cells and antibodies observed in both groups. No clear picture can be drawn from the $CD8^+$ T cells due to missing data but in a previous study in lambs infected with a bovine rotavirus lysate no changes were observed in $CD8^+$ T cells in blood (Bruce *et al,* 1995). Due to missing data, no comparison can be made from the $CD45R^+$ cells between both groups, however no changes were observed in the infected group with time. In the previous study mentioned above, a significant increase in $CD45R^+$ cells was seen 2-3 days after infection. If a similar change has occurred in this study, this change is missed due to the weekly sampling.

In both groups, $CD4^+$ T cells decreased in MLNs and JPPs followed by a recovery while increases were seen in IELs and LPLs with time, this probably due to the maturation of the immune system. Cytotoxic $CD4^+$ T cells in humans have shown to play a protective role against herpes simplex virus (Yasukawa and Zarling, 1984). These cells could be involved in viral clearance. No clear picture can be drawn for the $CD8^+$ T cells as data is missing in both groups. This study did not examine the activity of cytotoxic T lymphocytes so no conclusion can be made about the involvement of $CD8^+$ cytotoxic T cells in viral clearance and protection in this study. These cells have been shown to be important in viral clearance in infected animals (Offit and Dudzik, 1988; London *et al,* 1989; Offit *et al,* 1991). However, these cells are not necessary for a viral clearance as seen in several studies as β_2-microglobulin knockout mice resolve a primary infection. B-cell deficient mice, depleted of $CD8^+$ T cells before infection, gradually resolved a viral infection, suggesting different mechanisms other than antibodies and $CD8^+$ T cells could be involved (Franco and Greenberg, 1995; McNeal and Ward, 1995). The $\gamma\delta^+$ T cells increased in MLNs, JPPs, and IPPs in both groups while in IELs and LPLs these cells showed an increase in the control group with time. In the infected group a decrease was observed in the IELs with no change in LPLs with time. This decrease in the $\gamma\delta^+$ T cells in IELs in the infected group could suggest that an increase in $CD8^+$ T cells occurred in these effector sites. Depletion of $\gamma\delta^+$ T cells in calves had no effect on rotavirus excretion, suggesting that this mechanism is not important in viral clearance but the precise role of $\gamma\delta^+$ T cells is still unclear (Oldham *et al,* 1993). The increased expression of light

chain with time in MLNs in both groups, and in JPPs in the control group correlated with the increased antibody levels found in serum and at mucosal surfaces. No changes were observed in the JPPs in the infected group. Similar levels of expression were found in IELs and LPLs between the groups. No conclusion can be drawn for $CD45R^+$ cells due to missing data but high levels were found in IPPs. The high percentage of light chain and $CD45R^+$ cells found in IELs could be due to contamination by LPLs during the isolation process as B cells are relatively rare (±10%) in IELs (Nagi and Babiuk, 1987).

In this study, no clear picture can be drawn concerning which lymphocyte sub-population is involved in the cellular immune response after a primary rotavirus infection. The decrease observed in the $\gamma\delta^+$ T cells in IELs in the infected groups could suggest a possible increase in $CD8^+$ T cells, however due to the missing values and the lack of information of the cytotoxic activity, no conclusions can be made.

IFNγ transcripts in JPPs were seen in two animals of the infected group 8-10 days after infection which could suggest a Th1-like response with the involvement of $CD8^+$ T cells, however no data is available in the control group at the same time point. In MLNs, one animal of the control group had IFNγ expression 28 days after infection while one animal of the infected group had IFNγ transcripts 8 days after infection. The presence of IFNγ in MLNs in the control group could be involved in the maturation of the immune system. No clear comparison can be made between the groups. IL-2 expression in JPPs was seen in both groups indicating activated T cells. mRNA for IL-4 in JPPs was in greater quantities in the infected group 3 and 52 days after infection compared to the control group suggesting a response directed towards a Th2-like response after rotavirus infection. The increased expression of IL-4 observed in the infected group 3 days after infection is the first indication of an immune response and preceded the rotavirus-specific ASC in serum and rotavirus-specific antibodies in serum and nasal secretions. This high expression of IL-4 3 days after infection could indicate an induction of B cells in the JPPs, for example a switch from IgM^+ to IgA^+ B cells resulting in rotavirus-specific IgA antibodies in the circulation from 7 days after infection. Homing to the intestinal surface probably occurs as rotavirus-specific IgA antibodies were detected in the intestine 52 days

after infection. Strong IL-4 mRNA expression was seen in the control group 28 days after infection which correlates with the strong increase seen in IgA concentrations in serum after 28 days of infection suggesting a maturation in the immune system. In MLNs, the infected group expressed IL-4 mRNA in the first 10 days after infection with a high expression 52 days after infection and this correlated with the rotavirus-specific IgA antibodies observed in the intestine 52 days after infection. This supports the hypothesis of an induction of a Th2-like immune response after rotavirus infection. In the control group, IL-4 mRNA was expressed at all time points but at 3 and 52 days after infection these were lower than observed in the infected group. A similar high expression of IL-4 mRNA was seen at 28 days after infection as in the JPPs. No expression of IL-6 mRNA was observed in JPPs and MLNs in both groups. The data described here indicates that the primary immune response to rotavirus is directed in favour of a Th2-like response as seen in the increased expression of IL-4 in JPPs 3 days after infection rather than a Th1-like immune response.

A similar observation of an immune response in favour of Th2-type activity was seen in rats infected with *Trichinella spiralis.* Although cytokines of both the Th1- and Th2-type were observed in the intestinal lymph in the first 8 days after infection, the Th2-type cytokines were predominant. Worm expulsion was associated with increased levels of IL-4 and IL-5 (Ramaswamy *et al,* 1996). In the present study, viral clearance could not be associated with an increased level of cytokine, although the levels of IL-4 in MLNs increased with time. Mice infected with *Heligmosomoides polygyrus* showed increased expression of Th2-type cytokines with no elevated levels of Th1-type cytokines (Svetic *et al,* 1993).

In the present study, the role of IFNγ in a primary infection with rotavirus is still unclear. The involvement of IFNγ in a primary infection with *Trichinella spiralis* in mice is also unclear due to contrasting results (Pond *et al*, 1989; 1991; Grencis *et al,* 1991).

This study describes the primary immune response to rotavirus in gnotobiotic lambs. After infection, rotavirus-specific IgA ASC and antibodies preceded rotavirus-specific IgG ASC and antibodies in blood and nasal secretions, however no rotavirus-specific antibodies were detected in the intestinal lumen in the first 10 days

after infection while viral clearance occurred within 8-9 days. IgA and IgG concentrations in nasal secretions and serum respectively were significantly increased compared to non-infected controls. Rotavirus-primed lymphocytes were seen only in JPPs from 10 days after infection. No conclusion can be made about the role of cytotoxic T lymphocytes in viral clearance as no data is available about $CD8^+$ T cells and the activity of cytotoxicity was not examined. The expression of cytokines does not indicate a role of cytotoxic T lymphocytes but favours a Th2-like immune response. However, the mechanisms of viral clearance are still unclear. Possible mechanisms could be: 1) intestinal intracellular rotavirus-specific IgA antibodies as specific antibodies are detected in nasal secretions and with the existence of a common mucosal immune system described in man and animals, antibodies are expected in the intestine but they may reside within the cell; 2) natural killer cell activity as NK cells have been reported to be responsible for the regulation of cytomegalovirus infection in mice (Welsh *et al,* 1991); 3) $CD4^+$ T cell cytotoxic activity; 4) an unknown immunological method; or 5) a non-immunological method like depletion of villous epithelial cells susceptible to rotavirus infection (McNeal *et al,* 1995)

CHAPTER 7

INDUCTION OF THE MUCOSAL IMMUNE RESPONSE IN GNOTOBIOTIC LAMBS BY VACCINATION WITH INACTIVATED ROTAVIRUS OR RECOMBINANT ROTAVIRUS ANTIGENS MIXED WITH OR INCORPORATED INTO ISCOMS

7.1 INTRODUCTION

Oral rotavirus vaccines are being developed for children. The most promising vaccines are a quadrivalent vaccine and a multivalent bovine reassortant vaccine Bernstein *et al,* 1995; Clark *et al,* 1996a,b; Kapikian *et al,* 1996). In ruminants, particularly cattle, rotavirus vaccination is based on maternal immunisation aimed at a passive transfer of rotavirus neutralising antibodies via colostrum to the offspring (Snodgrass *et al,* 1980).

Oral vaccination has the advantage that the antigen is delivered at the site of infection and a specific immune response at mucosal surfaces is induced. ISCOMs have successfully been used as a mucosal adjuvant with influenza virus subunits, herpes simplex virus, measles virus fusion protein, feline leukemia virus, and Epstein-Barr virus. Specific antibody responses or cell-mediated responses to the viral components have been successfully induced (Osterhaus *et al,* 1985; Wahren *et al,* 1987; Lövgren, 1988; DeVries *et al,* 1988; Jones *et al,* 1988; Morgan *et al,* 1988; Sundquist *et al,* 1988).

Antigens used can either be incorporated into the ISCOM matrix or mixed with the ISCOM matrix. In 1988, the first successful commercial ISCOM-based vaccine (Iscovac Equi F, Iscotec) was available to induce protection in horses against influenza. The vaccine was given intranasally and based on a mixture of antigens and ISCOM matrix (Sundquist *et al,* 1988).

The aim of this study was to determine if one oral vaccination with inactivated rotavirus mixed with ISCOMs or recombinant VP6 incorporated into ISCOMs could induce immunological priming and protection against a subsequent challenge, and to characterise the different immune mechanisms involved.

The VP6 protein was chosen to be incorporated into ISCOMs as it is the most abundant protein of the virion, it carries antigenic determinants that are common to all group A rotaviruses, and cross-reactivity between VP6 proteins of different rotavirus strains has been shown *in vitro* in parenterally immunised mice with rotavirus (Bruce *et al,* 1994).

7.2 EXPERIMENTAL DESIGN

7.2.1 Animals

Gnotobiotic cross-Suffolk lambs were maintained as described in chapter 2. Lambs were divided into four groups. All lambs were vaccinated orally at 6-7 days of age, challenged 20-21 days later with 1 gram of faeces containing $10^{8.5}$ ffu of live lamb passaged ovine rotavirus strain K923 which was diluted in 5mls of PBS, and killed 8-9 days later.

7.2.2 Vaccines

Group 1 (n=6) were vaccinated with 500μg of ISCOM matrix (Iscotec, Sweden) diluted in 5mls of PBS (PBS/ISC). Group 2 (n=5) received a mixture of 500μg of ISCOM matrix and 1ml of inactivated bovine rotavirus strain UK diluted in PBS to a total volume of 5mls (RV/ISC). Group 3 (n=3) were vaccinated with 1ml of inactivated bovine rotavirus (strain UK) diluted in 4mls of PBS (RV). The rotavirus given to groups 2 and 3 had a titre of $10^{6.8}$ ffu/ml before inactivation with BEI (see 2.2.4). Group 4 (n=3) received 104μg of recombinant VP6 (strain UK) (VP6) (provided by Ms A. Wood and Ms. I. Campbell) incorporated into 519μg of Quil A (kindly done by Ms A. Bellman, Iscotec, Sweden), and diluted in PBS to a total volume of 4mls (VP6/ISC).

7.2.3 Samples

Blood and nasal secretions were taken at regular intervals and faecal swabs were taken daily after vaccination and challenge. Lambs were killed eight or nine days after challenge. At necropsy, MLNs, JPPs, IPPs, small intestine and intestinal scrapings were taken.

Faeces were examined for the presence of viral RNA. Blood, nasal secretions, and intestinal scrapings were tested for rotavirus-specific antibodies and total IgA and IgG levels were measured by ELISA. Rotavirus-specific antibody secreting cells

were determined in PBLs by ELISPOT. Lymphocyte sub-populations and proliferation were determined in PBLs, MLNs, JPPs, IPPs, IELs, and LPLs by FACS-analysis and lymphocyte stimulation tests respectively. The expression of cytokine mRNA (IFNγ, IL-2, IL-4, and IL-6) was measured in MLNs and JPPs by RT-PCR and hybridisation.

7.3 RESULTS

7.3.1 Response after vaccination

7.3.1.1 Rotavirus-specific antibody secreting cells and antibodies

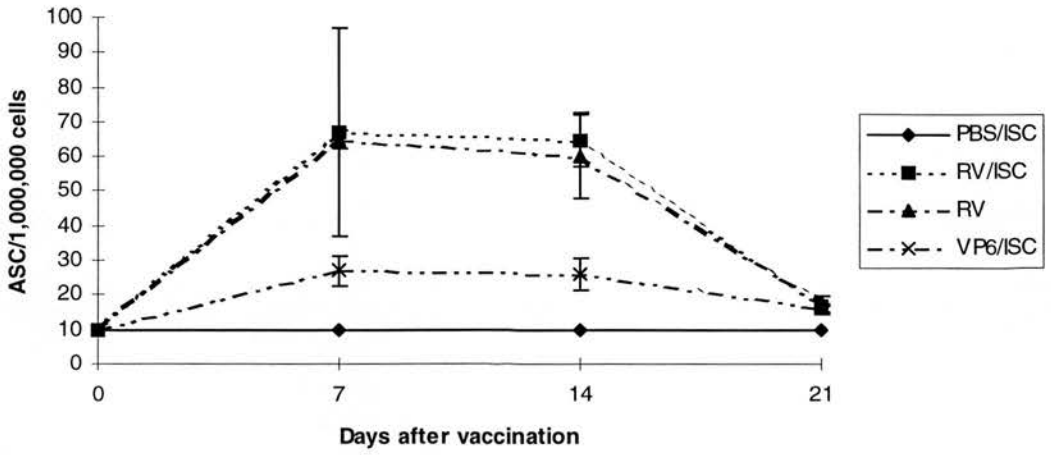
None of the groups had rotavirus-specific IgA ASC or antibodies in blood or nasal secretions after vaccination.

Significantly ($p<0.01$) increased numbers of rotavirus-specific IgG ASC were found in blood in all rotavirus vaccine groups (the RV/ISC, RV, and VP6/ISC groups) from 7 days after vaccination (Fig. 7.1). The numbers of rotavirus-specific IgG ASC were significantly ($p<0.05$) higher in the RV/ISC and RV groups than the VP6/ISC group.

The RV/ISC and RV groups had significantly increased levels of rotavirus-specific IgG antibodies in serum from 11 days after vaccination, but no such IgG antibodies were found in the VP6/ISC group (Fig. 7.2). No rotavirus-specific IgG antibodies were found in nasal secretion in any of the groups after vaccination.

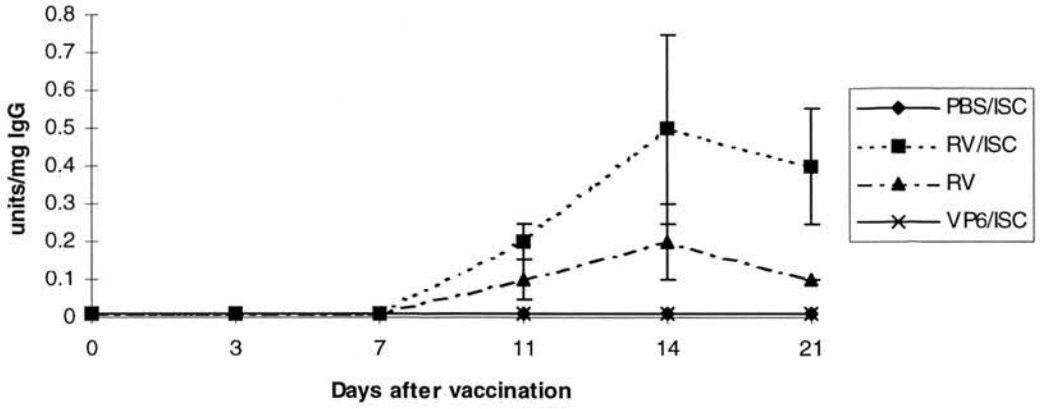
No virus neutralising antibodies were found after vaccination in serum and nasal secretions in any group.

Fig. 7.1: Mean numbers of rotavirus-specific IgG ASC in blood



Numbers of rotavirus-specific IgG ASC in blood after vaccination. Results expressed in ASC/1,000,000 peripheral blood lymphocytes and given as mean with SD.

Fig. 7.2: Mean rotavirus-specific IgG antibody responses in serum



Rotavirus-specific IgG antibody responses in serum after vaccination. Results expressed in units/mg IgG and given as mean with SD.

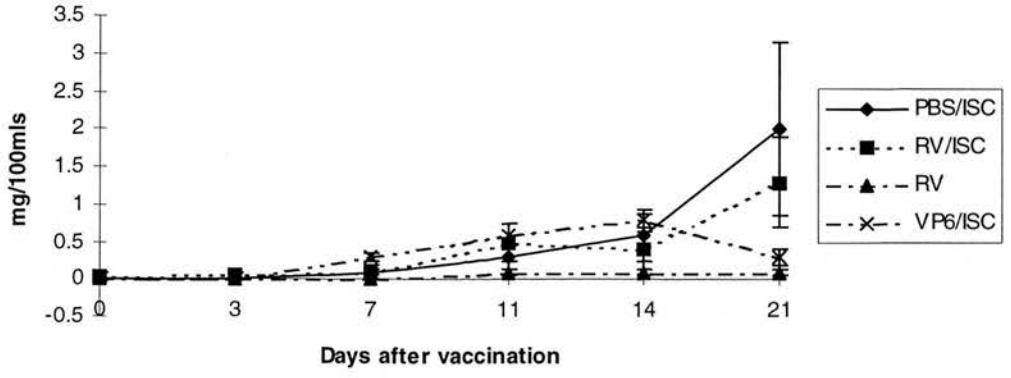
7.3.1.2 Total immunoglobulin A and G concentrations

IgA in serum was not detected in any lambs until 7 days after vaccination. Thereafter all the ISCOM groups showed increases in IgA with no difference between these groups. IgA concentrations were 2.0, 1.3, and 0.3 mg/100mls for the PBS/ISC, RV/ISC, and VP6/ISC groups respectively 21 days after vaccination. The RV group did not show any increase in IgA at any stage and had an IgA concentration of 0.1 mg/100mls 21 days after vaccination (Fig. 7.3a).

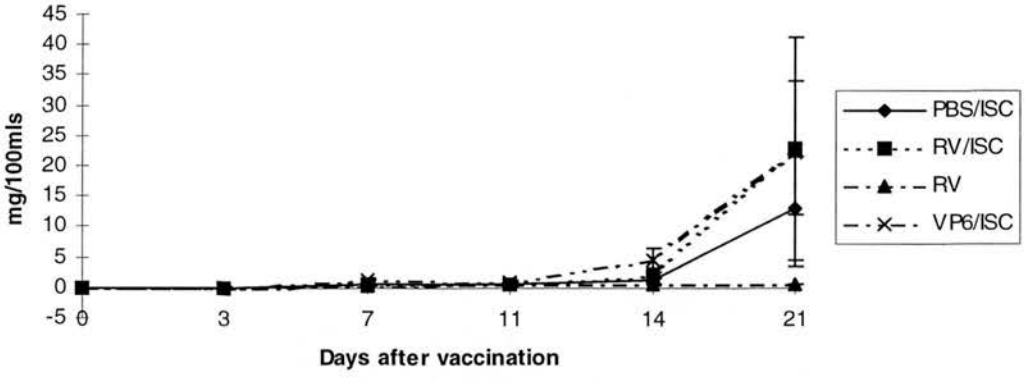
In nasal secretions, IgA was not detected in any lambs until 7 days after vaccination. Thereafter all the ISCOM groups had increased IgA concentrations with no difference between the groups. IgA concentrations were 13.4, 22.9, and 22.4 mg/100mls for the PBS/ISC, RV/ISC, and VP6/ISC groups respectively 21 days after vaccination. The RV group showed no similar increase in IgA concentration after vaccination. An IgA concentration of 0.5 mg/100mls was found 21 days after vaccination (Fig. 7.3b).

Fig. 7.3: IgA concentrations in serum (a) and nasal secretions (b)

a)



b)



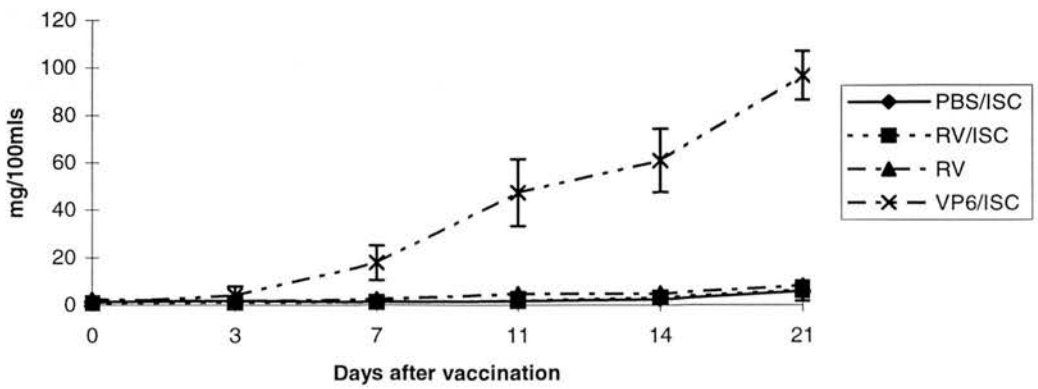
IgA concentrations in serum (a) and nasal secretions (b) after vaccination. Results expressed in mg/100mls and given as mean with SD.

All lambs had low IgG concentrations in serum at the start of the experiment and showed increases in IgG in serum with time with significant ($p<0.01$) increases observed in the VP6/ISC compared to the other groups. The VP6/ISC group had an IgG concentration of 97 mg/100mls 21 days after vaccination while the PBS/ISC, RV/ISC, and RV groups had IgG concentrations of 6.0, 6.3, and 8.2 mg/100mls respectively (Fig. 7.4a).

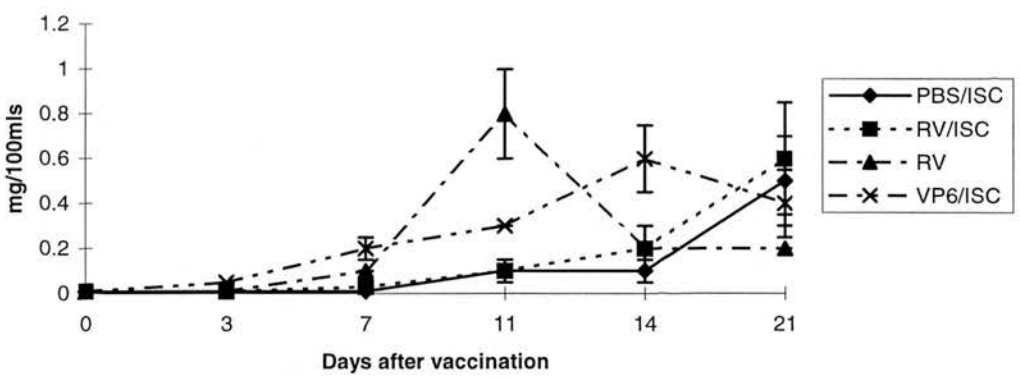
In nasal secretions, IgG was not detectable in the rotavirus vaccine groups until 7 days after vaccination while detectable IgG in the PBS/ISC group was found from 11days after vaccination. No differences in IgG concentrations were observed between the groups from 11 days after vaccination. IgG concentrations were 0.5, 0.6, 0.2, and 0.4 mg/100mls for the PBS/ISC, RV/ISC, RV, and VP6/ISC groups respectively 21 days after vaccination (Fig. 7.4b).

Fig. 7.4: IgG concentrations in serum (a) and nasal secretions (b)

a)



b)



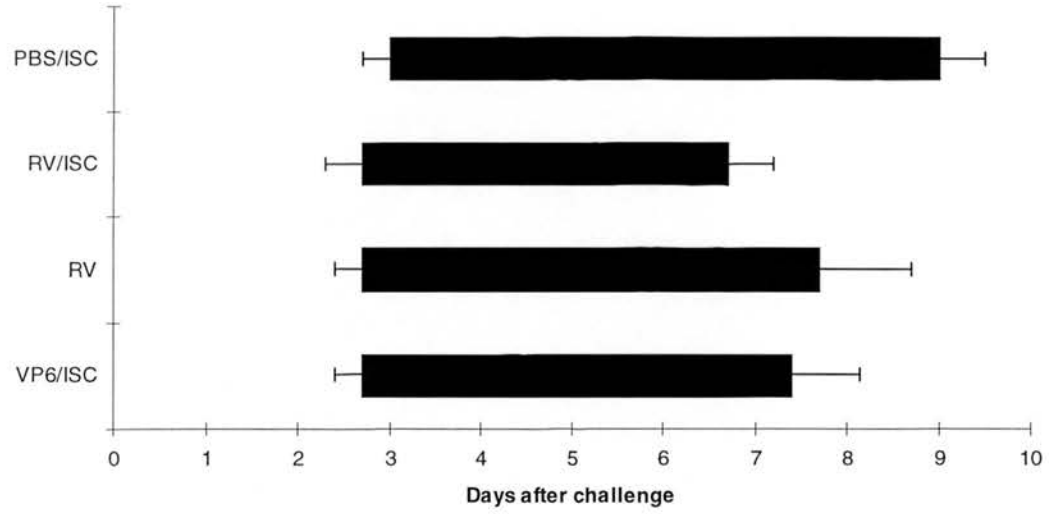
IgG concentrations in serum (a) and nasal secretions (b) after vaccination. Results expressed in mg/100mls and given as mean with SD.

7.3.2 Responses after challenge

7.3.2.1 Viral clearance

No significant difference was observed between the groups in the time to onset of viral excretion. The PBS/ISC group commenced excreting virus 72 hours after challenge while the rotavirus vaccine groups started excreting virus between 48-72 hours after challenge. The number of positive tested days was reduced to 4.0, 5.0, and 4.7 for the RV/ISC, RV, and VP6/ISC groups respectively. However, in the RV/ISC group the number of positive days was significantly ($p<0.05$) reduced than the number of positive days (5.7) found in the PBS/ISC group, although both RV and VP6/ISC groups had p-values of 0.11 and 0.10 respectively. The rotavirus vaccine groups together cleared the virus in a significantly ($p<0.05$) reduced number of days (7.0 days vs 9.0 days in the PBS/ISC group) (Fig. 7.4).

Fig. 7.4: Mean viral excretion in days after challenge

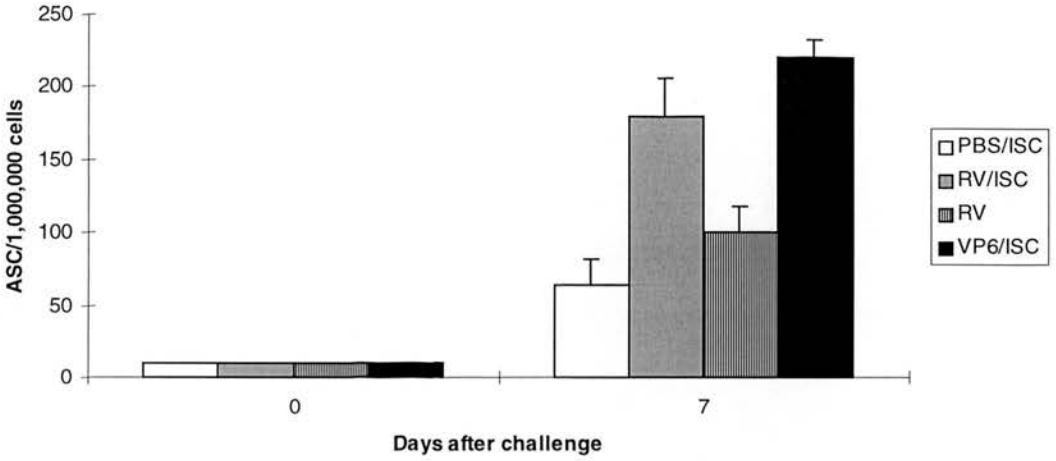


Viral excretion after vaccination and subsequent challenge. Results expressed in days and given as mean with SD

7.3.2.2 Rotavirus-specific antibody secreting cells and antibodies

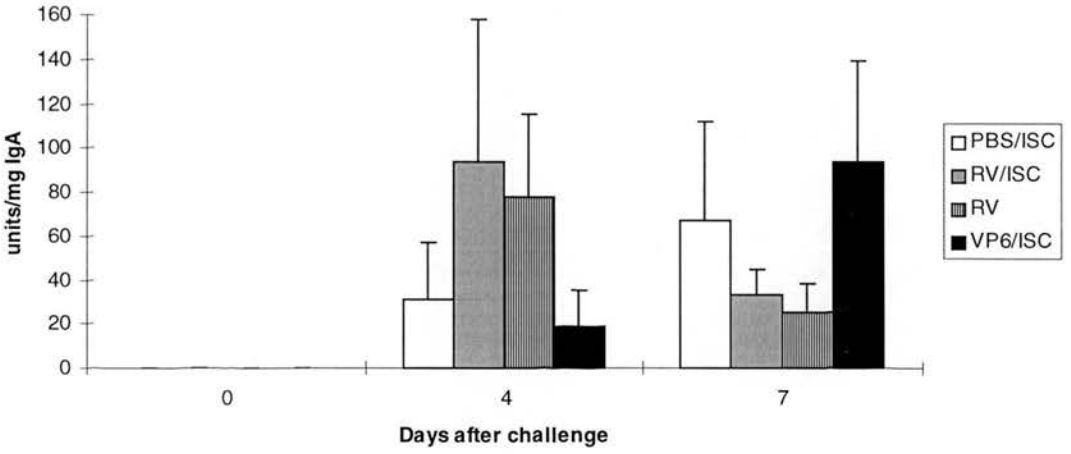
After challenge, rotavirus-specific IgA ASC were observed in all groups. The RV/ISC and VP6/ISC groups had significantly ($p<0.05$) higher numbers of circulating rotavirus-specific IgA ASC than the PBS/ISC group 7 days after challenge (Fig 7.5). After challenge, rotavirus-specific IgA antibodies were detected in serum in all groups, however no significant differences were found between the groups (Fig. 7.6). Rotavirus-specific IgA antibodies were found in nasal secretions in all groups and all rotavirus vaccine groups had significantly ($p<0.05$) higher levels 7 days after challenge than the PBS/ISC group (Fig. 7.7). After challenge, rotavirus-specific IgA antibodies were detected in gut scrapings in all groups but at significantly ($p<0.01$) higher levels in the RV/ISC and RV groups. The levels of rotavirus-specific IgA antibodies in intestinal scrapings were much lower then observed in serum and nasal secretions (Fig. 7.8).

Fig. 7.5: Mean numbers of rotavirus-specific IgA ASC in blood



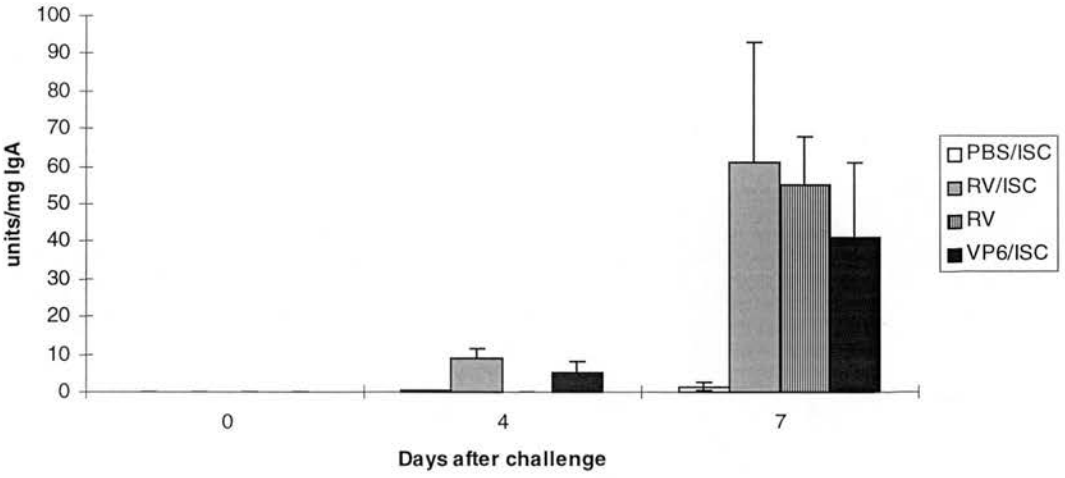
Numbers of rotavirus-specific IgA ASC in blood after challenge. Results expressed in ASC/1,000,000 peripheral blood lymphocytes and given as mean with SD.

Fig. 7.6: Mean rotavirus-specific IgA antibody responses in serum



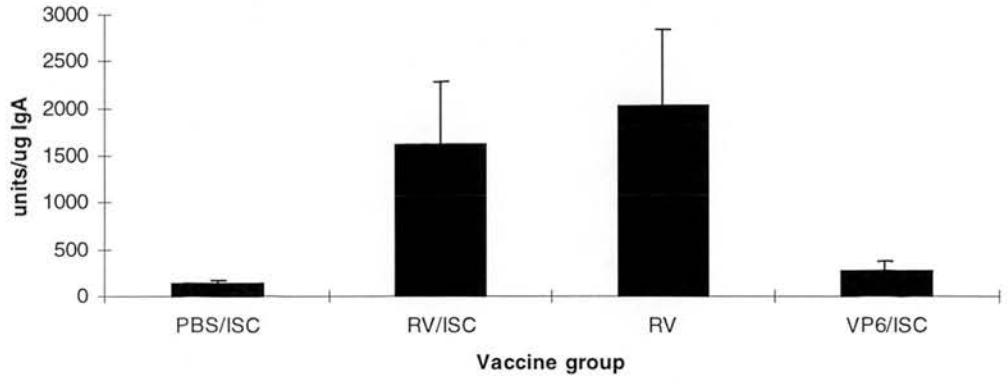
Rotavirus-specific IgA antibody responses in serum after challenge. Results expressed in units/mg IgA and given as mean with SD.

Fig 7.7: Mean rotavirus-specific IgA antibody responses in nasal secretions



Rotavirus-specific IgA antibody responses in nasal secretions after challenge. Results expressed in units/mg IgA and given as mean with SD.

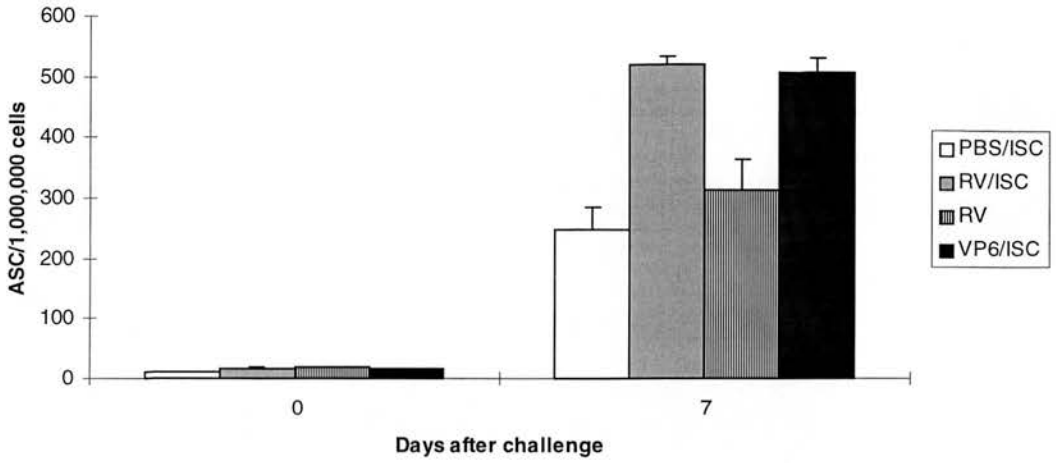
Fig. 7.8: Mean rotavirus-specific IgA antibody responses in gut scrapings



Rotavirus-specific IgA antibody responses in gut scrapings after challenge. Results expressed in units/µg IgA and given as mean with SD. The intestinal scrapings were collected at necropsy 1-2 weeks after challenge.

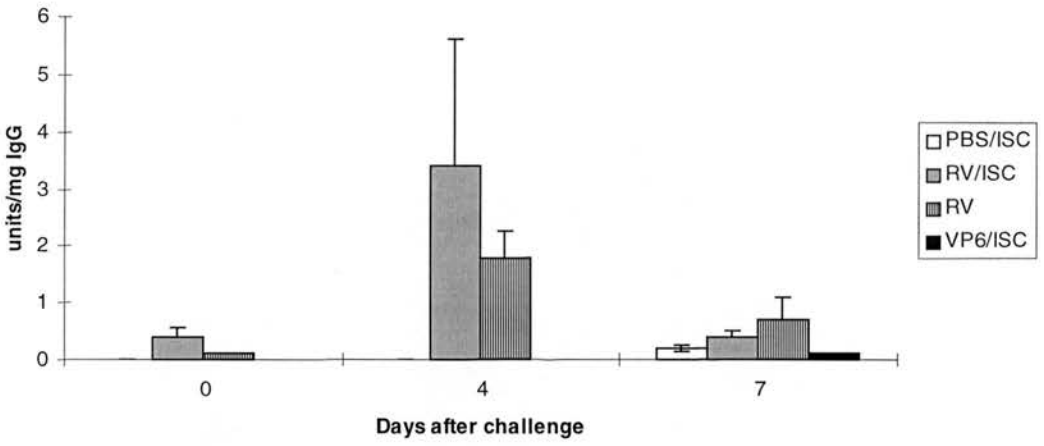
All groups had increased numbers of rotavirus-specific IgG ASC in blood after challenge with significantly ($p<0.05$) increased numbers in the RV/ISC and VP6/ISC groups compared to the PBS/ISC and RV groups 7 days after challenge (Fig. 7.9). Significantly ($p<0.05$) increased rotavirus-specific IgG antibodies were detected in serum in the RV/ISC and RV groups 4 days after challenge (Fig. 7.10). In the RV/ISC and RV groups, these rotavirus-specific IgG antibodies significantly ($p<0.05$) declined afterwards while detectable levels of rotavirus-specific IgG antibodies were observed in the PBS/ISC and VP6/ISC groups 7 days after challenge (Fig. 7.10). In nasal secretions, significantly increased rotavirus-specific IgG antibodies were observed in the RV/ISC and RV groups 4 days after challenge. Detectable levels of rotavirus-specific IgG antibodies were seen in the PBS/ISC and VP6/ISC groups 7 days after challenge (Fig 7.11). Rotavirus-specific IgG antibodies in intestinal scrapings were only seen in the RV/ISC and RV groups with no detectable levels found in the PBS/ISC and VP6/ISC groups (Fig. 7.12).

Fig. 7.9: Mean numbers of rotavirus-specific IgG ASC in blood



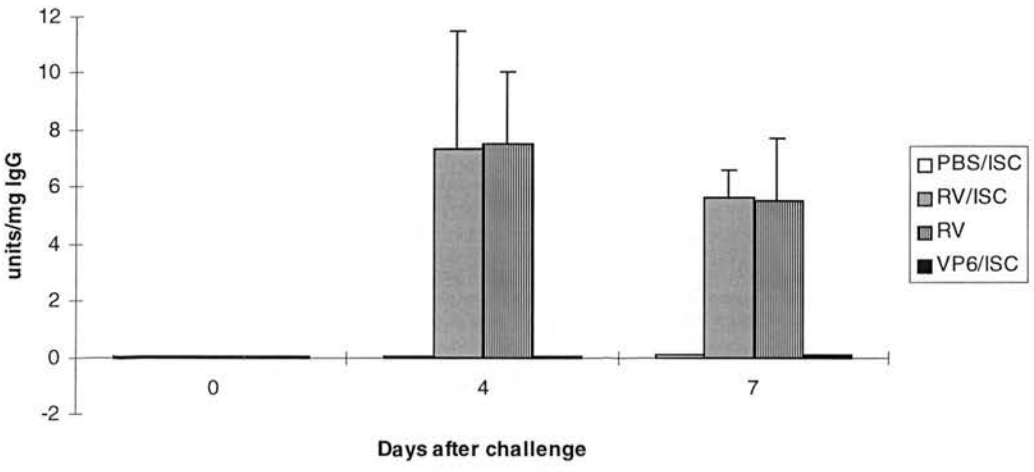
Numbers of rotavirus-specific IgG ASC in blood after challenge. Results expressed in ASC/1,000,000 cells and given as mean with SD.

Fig. 7.10: Mean rotavirus-specific IgG antibody responses in serum



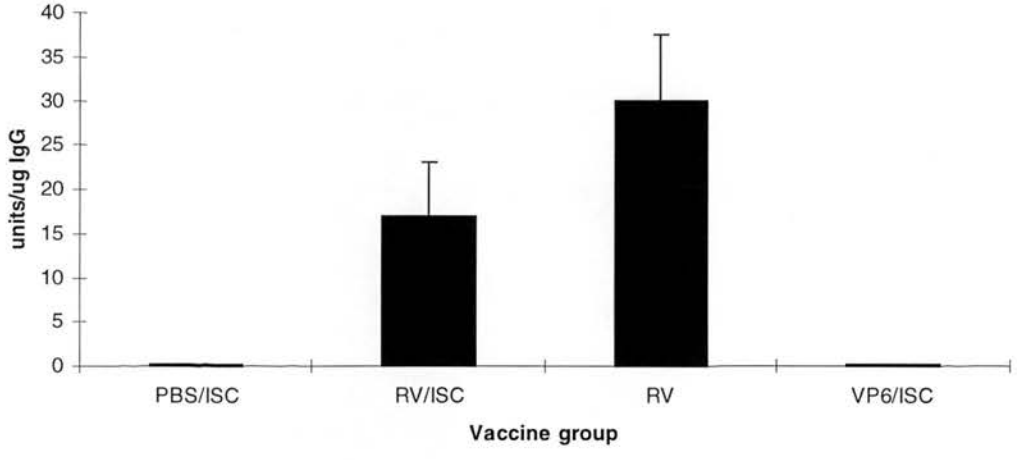
Rotavirus-specific IgG antibody titres in serum after challenge. Results expressed in units/mg IgG and given as mean with SD.

Fig. 7.11: Mean rotavirus-specific IgG antibody responses in nasal secretions



Rotavirus-specific IgG antibody responses in nasal secretions after challenge. Results expressed in units/mg IgG and given as mean with SD.

Fig. 7.12: Mean rotavirus-specific IgG antibody responses in gut scrapings



Rotavirus-specific IgG antibody responses in gut scrapings after challenge. Results expressed in units/µg IgG and given as mean with SD. The intestinal scrapings were collected at necropsy 1-2 weeks after challenge.

7.3.2.3 Neutralising titres against rotavirus

All lambs developed circulating rotavirus neutralising antibodies against K923 7 days after challenge. The PBS/ISC, RV/ISC, RV, and the VP6/ISC groups had mean neutralising titres (range) of 160 (80-160), 80 (20-160), 80 (40-160), and 160 (80-160) respectively, with no significant differences between the groups. A low heterotypic response (VNT=10) was observed in serum in all groups against bovine rotavirus (UK) No neutralising antibodies were detected in nasal secretions and intestinal scrapings.

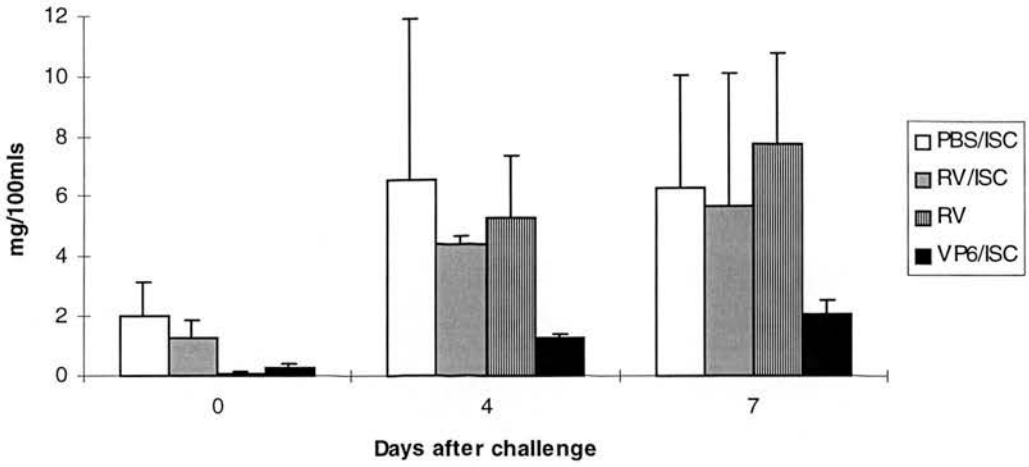
7.3.2.4 Total immunoglobulin A and G concentrations

All groups showed increased IgA concentrations in serum 4 days after challenge. IgA concentrations increased to 6.3, 5.6, 7.8, and 2.1 mg/100mls for the PBS/ISC, RV/ISC, RV, and VP6/ISC groups respectively 7 days after challenge (Fig. 7.13).

IgA was detected in nasal secretions before vaccination in all ISCOM groups. All groups showed increases in IgA by 4 days after challenge, but there was no increase thereafter. IgA concentrations were 32.1, 24.4, 5.9, and 31.2 mg/100mls for the PBS/ISC, RV/ISC, RV, and VP6/ISC groups respectively 7 days after challenge (Fig. 7.14). Individual animal concentrations varied widely in serum and nasal secretions. No significant differences were seen between any groups at 4 and 7 days after challenge, or between day 0 and day 4 and 7 within a group.

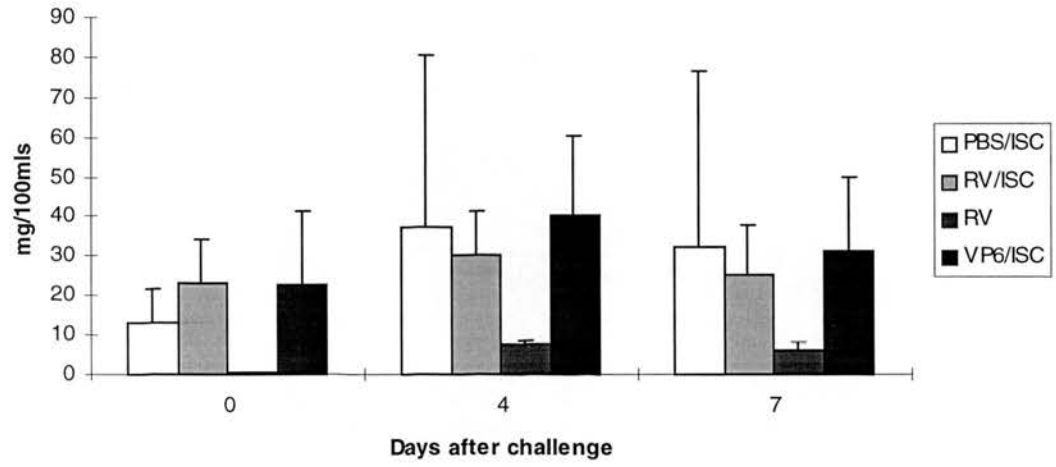
No significant differences in IgA concentrations in intestinal scrapings were observed between the groups (Fig. 7.15).

Fig. 7.13: IgA concentrations in serum



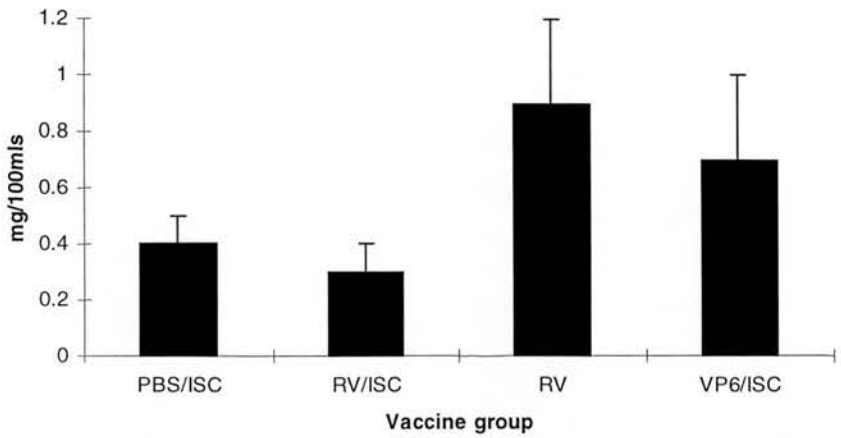
IgA concentrations in serum after challenge. Results expressed in mg/100mls and given as mean with SD.

Fig. 7.14: IgA concentrations in nasal secretions



IgA concentrations in nasal secretions after challenge. Results expressed in mg/100mls and given as mean with SD.

Fig 7.15: IgA concentrations in intestinal scrapings



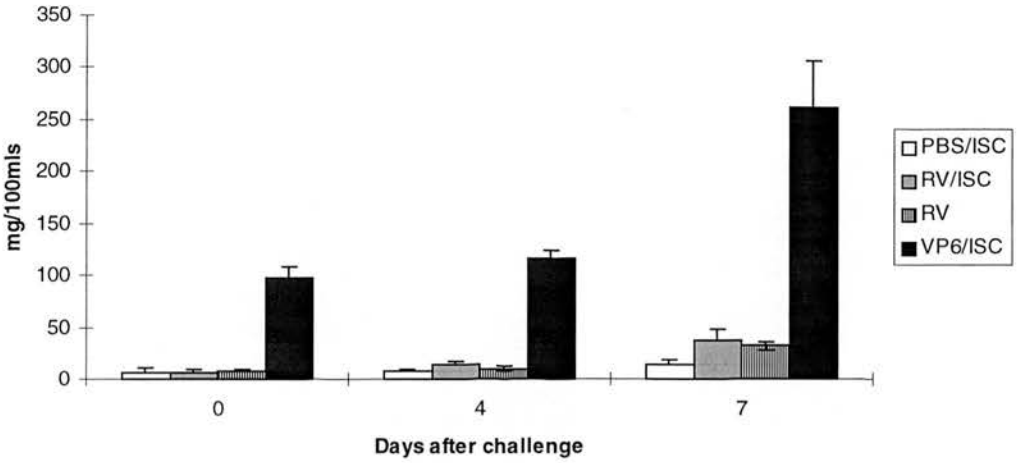
IgA concentrations in intestinal scrapings. Results expressed in mg/100mls and given as mean with SD. The intestinal scrapings were collected at necropsy 1-2 weeks after challenge.

IgG was detected before vaccination in all groups. All groups showed increases in IgG concentrations from 4 to 7 days after challenge. The VP6/ISC group had significantly ($p<0.01$) increased levels in serum compared to the other groups. IgG concentrations were 14.3, 37.3, 32.1, and 261 mg/100mls for the PBS/ISC, RV/ISC, RV, and VP6/ISC groups respectively 7 days after challenge (Fig 7.16).

In nasal secretions, IgG was detected before vaccination in all groups. No clear increases were seen in all groups after challenge. IgG concentrations were 0.7, 1.7, 0.5, and 0.4 mg/100mls for the PBS/ISC, RV/ISC, RV, and VP6/ISC groups respectively 7 days after challenge (Fig. 7.17). Individual animal concentrations varied widely.

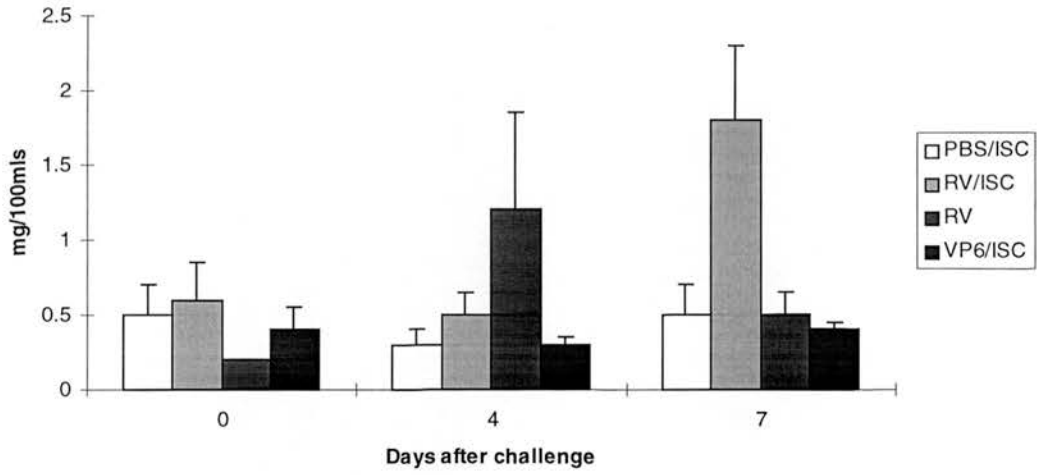
IgG was found in intestinal scrapings in all groups. A higher IgG concentration was seen in the VP6/ISC group, but this was not significant (Fig. 7.18).

Fig. 7.16: IgG concentrations in serum



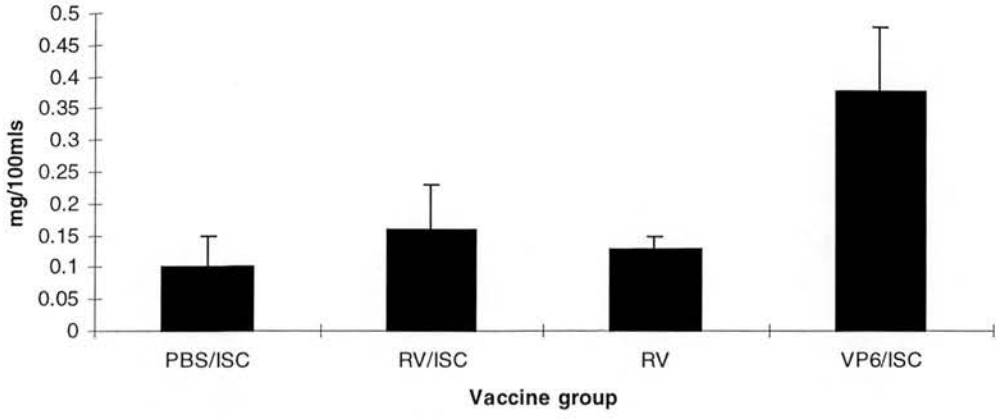
IgG concentrations in serum after challenge. Results expressed in mg/100mls and given as mean with SD.

Fig 7.17: IgG concentrations in nasal secretions



IgG concentrations in nasal secretions after challenge. Results expressed in mg/100mls and given as mean with SD.

Fig 7.18: IgG concentrations in intestinal scrapings



IgG concentrations in intestinal scrapings after challenge. Results expressed in mg/100mls and given as mean with SD. The intestinal scrapings were collected at necropsy 1-2 weeks after challenge.

7.3.2.5 Proliferation of lymphocytes in blood and GALT

Peripheral blood lymphocytes responded to Con A (range PBS/ISC: 1514±1855 - 8183±11353; RV/ISC: 6219±6200 - 11800±8452; RV: 1527±1332 - 10200±13873; VP6/ISC: 5593±5894 - 13266±5037) and PWM (range PBS/ISC: 5463±5925 - 19167±15737; RV/ISC: 8091±5990 - 32739±8014; RV: 7730±2568 - 33942±8119; VP6/ISC: 17685±3517 - 34078±3311) at each time point in each group. No response was seen to ovalbumin in all groups. No response was observed to UK in any group after vaccination, but the VP6/ISC group showed a significant ($p<0.05$) proliferation 1 week after challenge compared to the other groups (Table 7.1).

Table 7.1: Peripheral blood lymphocyte proliferation in response to UK *in vitro*.

Days after vaccination Group	0	7	14	21[1]	28
PBS/ISC	61±41 0.9	37±28 0.6	111±62 0.8	35±14 0.6	81±77 1.0
RV/ISC	123±98 0.9	101±101 1.1	383±477 0.8	40±43 1.0	100±31 0.6
RV	408±558 1.1	242±358 1.1	447±584 0.7	52±38 1.2	37±11 0.9
VP6/ISC	607±749 1.1	164±119 1.4	54±32 1.0	77±53 1.2	605±151 2.7*

Results expressed in mean counts per minute±SD and stimulation index (stimulated wells vs unstimulated wells); [1] = day of challenge; *=$p<0.05$.

GALT lymphocytes were obtained 1-2 weeks after challenge. Lymphocytes isolated from MLNs and JPPs in all responded to Con A (range in MLNs for all groups: 5782±472 - 25339±7839; JPPs: 2459±1841 - 13345±5205) and PWM (range in MLNs for all groups: 10929±1890 - 25339±7839; JPPs: 10251±4873 - 25186±10474). IPP lymphocytes in all groups responded to Con A (range for all groups: 154±95 - 2202±4764) or PWM (range for all groups: 2209±1511 - 4150±8312). IELs and LPLs from the all groups showed no response either to Con A (range in IEL for all groups: 139±20 - 159±113; LPL: 131±51 - 241±157) or PWM (range in IELs for all groups: 63±28 - 128±34; LPLs: 111±31 - 212±134). No response was seen to ovalbumin in all groups irrespective of tissue.

Lymphocytes isolated from JPPs from all groups responded to UK. Due to high animal to animal variations, this was only significant in the RV/ISC and RV groups. An increased proliferation to UK was seen in the lymphocytes isolated from MLNs in the RV/ISC and RV groups, but this was not significant (Table 7.2).

Table 7.2: GALT lymphocyte proliferation in response to UK *in vitro* 1-2 weeks after challenge.

Tissue Group	MLN	JPP	IPP	IEL	LPL
PBS/ISC	97±50 0.4	744±1126 6.4	103±128 1.0	77±34 0.9	110±113 0.6
RV/ISC	497±794 1.7	415±164* 2.0	79±46 0.7	51±19 1.0	66±32 0.6
RV	869±1035 5.0	1433±182* 12	169±106 2.5	86±36 1.5	90±62 0.3
VP6/ISC	120±76 0.7	4121±5154 6.4	86±26 1.7	84±43 1.1	79±14 0.6

Results given in mean counts per minute±SD and stimulation index; * = $p<0.05$.

7.3.2.6 Phenotypic analysis of lymphocytes in blood and GALT

Results are summarised in Table 7.3 and 7.4 with FACS profiles shown in the appendix. The PBS/ISC group had significantly ($p<0.05$) increased $CD4^+$ T cells in blood after challenge compared to pre-vaccination levels while no changes were observed in the percentage of $CD8^+$ T cells in blood. The $\gamma\delta^+$ T cells were significantly increased 1-3 weeks after vaccination and remained elevated after challenge. A significantly ($p<0.05$ and $p<0.01$) increased expression of light chain and $CD45R^+$ cells in blood was observed after challenge. The RV/ISC group showed a similar increase in $CD4^+$ T cells after challenge as observed in the PBS/ISC group. No changes were seen in the percentage $CD8^+$ T cells. The $\gamma\delta^+$ T cells were significantly ($p<0.05$) increased 2 weeks after vaccination but declined 21 days after vaccination. After challenge, an increase was observed in the percentage of these cells. The expression of light chain showed increases after vaccination with a significant ($p<0.05$) increase compared to pre-vaccination level after challenge. The percentage of $CD45R^+$ cells was significantly ($p<0.01$) increased at 21 days after vaccination compared to pre-vaccination levels. After challenge, similar levels were seen between the PBS/ISC and RV/SC groups. The RV group showed a similar pattern in $CD4^+$ T cells as the PBS/ISC and RV/ISC groups. No changes were seen in the percentage of $CD8^+$ T cells compared to pre-vaccination levels. The $\gamma\delta^+$ T cells were significantly increased 2 weeks after vaccination but declined 21 days after vaccination. After challenge, an increase was observed in the percentage of these

cells. The expression of light chain increased after vaccination with a significant ($p<0.05$) increase compared to pre-vaccination levels seen 2 weeks after vaccination and 1 week after challenge. The percentage of $CD45R^+$ T cells increased significantly ($p<0.05$) to similar percentages as seen in the PBS/ISC group after challenge. The VP6/ISC group showed no changes in $CD4^+$ and $CD8^+$ T cells in blood after vaccination or challenge. A significant ($p<0.05$) decrease in $\gamma\delta^+$ T cells was seen 2-3 weeks after vaccination. No changes were found in the expression of light chain. This is in contrast with the increases observed in the PBS/ISC, RV/ISC, and RV groups. The percentage of $CD45R^+$ cells showed no changes after vaccination or challenge compared to pre-vaccination levels. Pre-vaccination levels of $CD45R^+$ cells in the VP6/ISC group were significantly ($p<0.05$) higher compared to the PBS/ISC, RV/ISC, and RV groups.

Table 7.3: Mean percentage of lymphocyte sub-populations in peripheral blood in vaccinated groups.

Marker	Days after vaccination / Groups	0	7	14	21	28
CD4	PBS/ISC	13 (6.0)	14 (10)	23 (12)	16 (3.9)	32 (9.5)**
	RV/ISC	12 (5.9)	14 (8.3)	22 (6.9)	22 (4.4)	34 (6.1)**
	RV	21 (1.9)	25 (3.8)	27 (3.7)	23 (0.6)	33 (5.2)*
	VP6/ISC	36 (4.9)	34 (14)	35 (6.4)	36 (9.0)	37 (9.5)
CD8	PBS/ISC	24;20	30;27	14;14	23;20	24;19
	RV/ISC	17;15	25;25	17;17	12;21	20;28
	RV	27 (2.9)	28 (4.2)	15 (6.3)	14 (4.0)	24 (1.3)
	VP6/ISC	19 (4.9)	16 (6.6)	17 (5.9)	16 (4.1)	18 (3.4)
γδTcR	PBS/ISC	41 (8.8)	67 (10)*	69 (3.6)*	61 (5.9)*	53 (4.0)
	RV/ISC	44 (13)	61 (13)	65 (4.3)*	45 (9.1)	62 (6.2)
	RV	45 (8.8)	51 (2.7)	64 (8.6)*	51 (10)	57 (3.7)
	VP6/ISC	40 (6.2)	24 (15)	26 (5.4)*	24 (7.5)*	23 (11)
L chain	PBS/ISC	19 (5.8)	18 (4.6)	24 (6.3)	26 (5.8)	41(12)*
	RV/ISC	17 (6.4)	18 (5.7)	27 (9.4)	30 (9.6)	43 (7.8)*
	RV	19 (2.0)	21 (2.1)	34 (4.9)*	31 (9.5)	30 (2.6)*
	VP6/ISC	17 (3.5)	15 (5.5)	18 (4.0)	22 (6.1)	22 (6.3)
CD45R	PBS/ISC	22 (12)	19 (11)	17 (5.3)	21 (3.4)	42 (4.1)**
	RV/ISC	21 (12)	23 (9.1)	25 (11)	42 (4.9)**	42 (5.0)**
	RV	27 (3.9)	36 (7.0)	33 (2.7)	26 (6.7)	44 (7.9)*
	VP6/ISC	44 (5.9)	40 (15)	40 (5.3)	46 (0.3)	42 (4.1)

Results given as mean ($n>2$) with (SD) or from each animal ($n\leq2$); * = $p<0.05$; ** = $p<0.01$ (vaccination/challenge levels vs pre-vaccination (baseline) levels within one group)

The percentage of $CD4^+$ T cells was significantly ($p<0.05$) higher in MLNs in both the RV and VP6/ISC groups and in JPPs in the VP6/ISC group compared to the PBS/ISC group. The VP6/ISC group had a significantly ($p<0.01$) lower percentage of $CD4^+$ T cells in IELs and LPLs compared to the PBS/ISC group. Similar percentages were observed in IPPs between the groups. No differences were seen in the percentage of $CD8^+$ T cells in MLNs, JPPs, and IPPs while in IELs and LPLs the VP6/ISC group had a significantly ($p<0.05$) higher percentage compered to the PBS/ISC group. The percentage of $\gamma\delta^+$ T cells was significantly ($p<0.05$) lower in MLNs, JPPs, IPPs, IELs and LPLs in the VP6/ISC group than the PBS/ISC group. The expression of light chain was similar in MLNs, JPPs, and IELs in all groups, however in IPPs and LPLs the VP6/ISC group had a significantly ($p<0.05$) lower percentage compared to the PBS/ISC group. The VP6/ISC group had a significantly ($p<0.05$) higher percentage of $CD45R^+$ cells in MLNs than the PBS/ISC group while a significantly ($p<0.05$) lower percentage was seen in IELs. No differences in the percentage of $CD45R^+$ cells were seen in JPPs, IPPs, and LPLs between the groups (Table 7.4).

Table 7.4: Mean percentage of lymphocyte sub-populations in GALT in vaccinated and challenged groups 1-2 weeks after challenge.

Tissue	Marker Groups	CD4	CD8	γδTcR	L Chain	CD45R
MLN	PBS/ISC	24 (6.6)	33;33	19 (4.6)	32 (12)	42 (13)
	RV/ISC	24 (14)	17;32	15 (6.8)	35 (4.8)	52 (8.6)
	RV	40 (4.5)*	43 (4.6)	21 (8.4)	29 (5.2)	54 (4.3)
	VP6/ISC	48 (8.5)*	37 (11)	4.6 (1.2)*	24 (3.4)	66 (5.2)*
JPP	PBS/ISC	19 (11)	13;15	17 (6.0)	44 (20)	58 (16)
	RV/ISC	23 (8.1)	22;22	14 (7.4)	37 (12)	64 (11)
	RV	20 (4.1)	12 (3.1)	8.4 (4.0)	34 (10)	43 (13)
	VP6/ISC	42 (4.1)*	24 (18)	2.7 (0.6)*	20 (6.5)	62 (12)
IPP	PBS/ISC	16 (11)	8.1;19	14 (5.2)	57 (19)	85 (6.1)
	RV/ISC	13 (6.5)	17;6.2	13 (7.4)	51 (8.9)	81 (9.9)
	RV	23 (5.5)	9.0 (4.1)	18 (6.0)	62 (3.7)	92 (4.0)
	VP6/ISC	10 (11)	7.0 (7.6)	2.6 (0.5)*	28 (5.0)*	85 (13)
IEL	PBS/ISC	46 (20)	30;27	21 (4.6)	26 (13)	42 (9.1)
	RV/ISC	50 (15)	25;22	21 (7.6)	19 (4.6)	45 (4.1)
	RV	54 (32)	30 (2.5)	19 (15)	18 (1.1)	44 (0.9)
	VP6/ISC	4.1 (2.0)**	48 (12)*	7.3 (2.7)*	14 (3.0)	30 (2.7)*
LPL	PBS/ISC	36 (12)	34;34	20 (7.7)	28 (7.0)	32 (7.3)
	RV/ISC	38 (4.8)	25;32	20 (5.4)	28 (5.7)	30 (8.4)
	RV	44 (14)	19 (9.7)	22 (7.2)	24 (2.4)	28 (6.2)
	VP6/ISC	7.6 (4.8)**	46 (7.2)*	9.2 (4.4)*	14 (5.9)*	30 (9.5)

Results given as mean (n>2) with (SD) or from each animal (n≤2); * = $p<0.05$;
** = $p<0.01$ (rotavirus vaccinated groups vs PBS/ISC group)

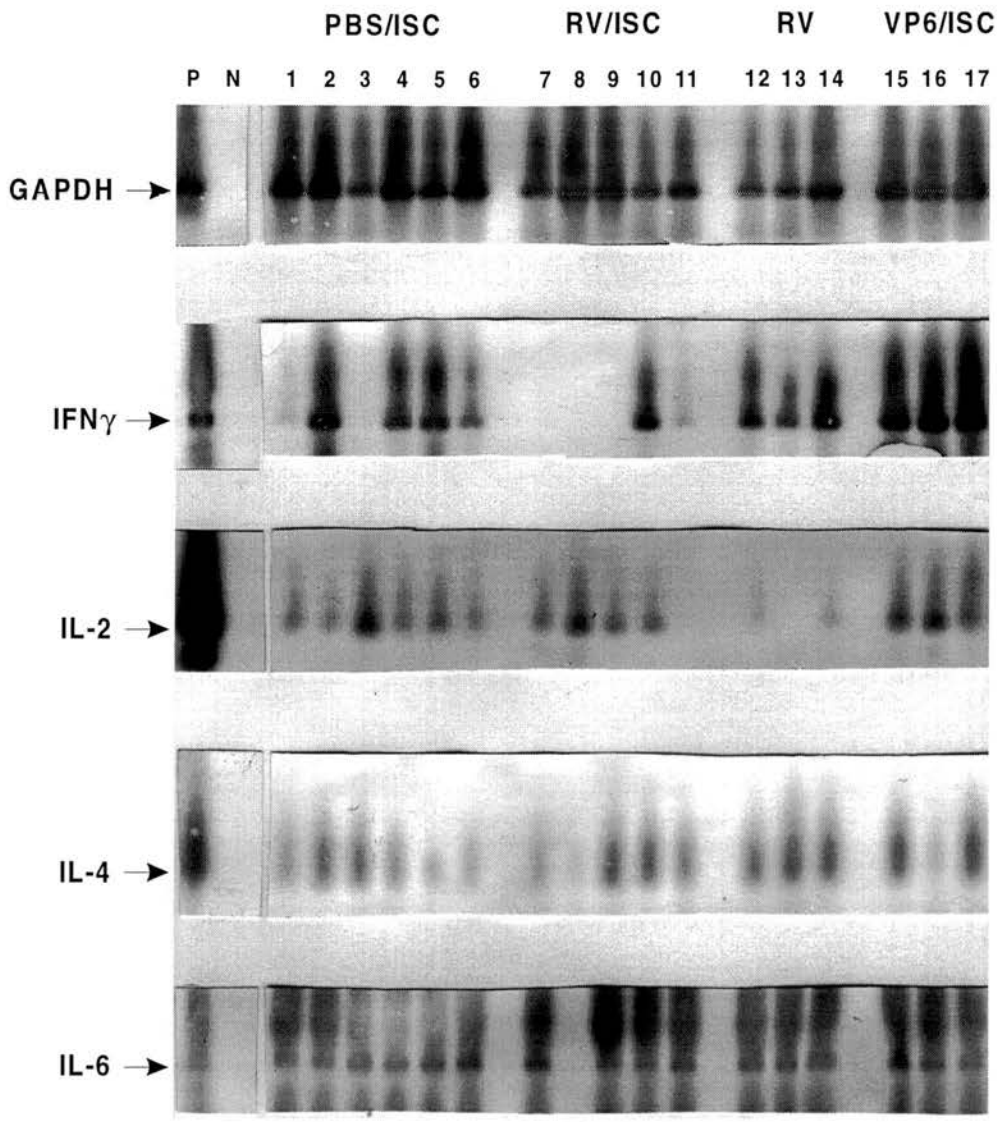
7.3.2.7 Cytokine expression of lymphocytes in JPPs and MLNs

GAPDH expression was seen in all JPPs. In MLNs, GADPH expression was seen in all animals except in animal no. 10. GAPDH expression indicates a valid RT-PCR and if no GAPDH expression was seen, animals were excluded from results.

In JPPs, IFNγ transcripts were seen in 4 of 6 animals from the PBS/ISC group, 1 of 5 animals from the RV/ISC group and in all animals from the RV and VP6/ISC groups. The RV/ISC group had a significant ($p<0.05$) suppression of IFNγ. Transcripts for IL-2 were found in the ISCOM-vaccinated groups while no clear expression was observed in the RV group. IL-4 and IL-6 transcripts were seen in 4 of 5 animals from the RV/ISC group and in all animals from the PBS/ISC, RV, and VP6/ISC groups (Plate 7.1).

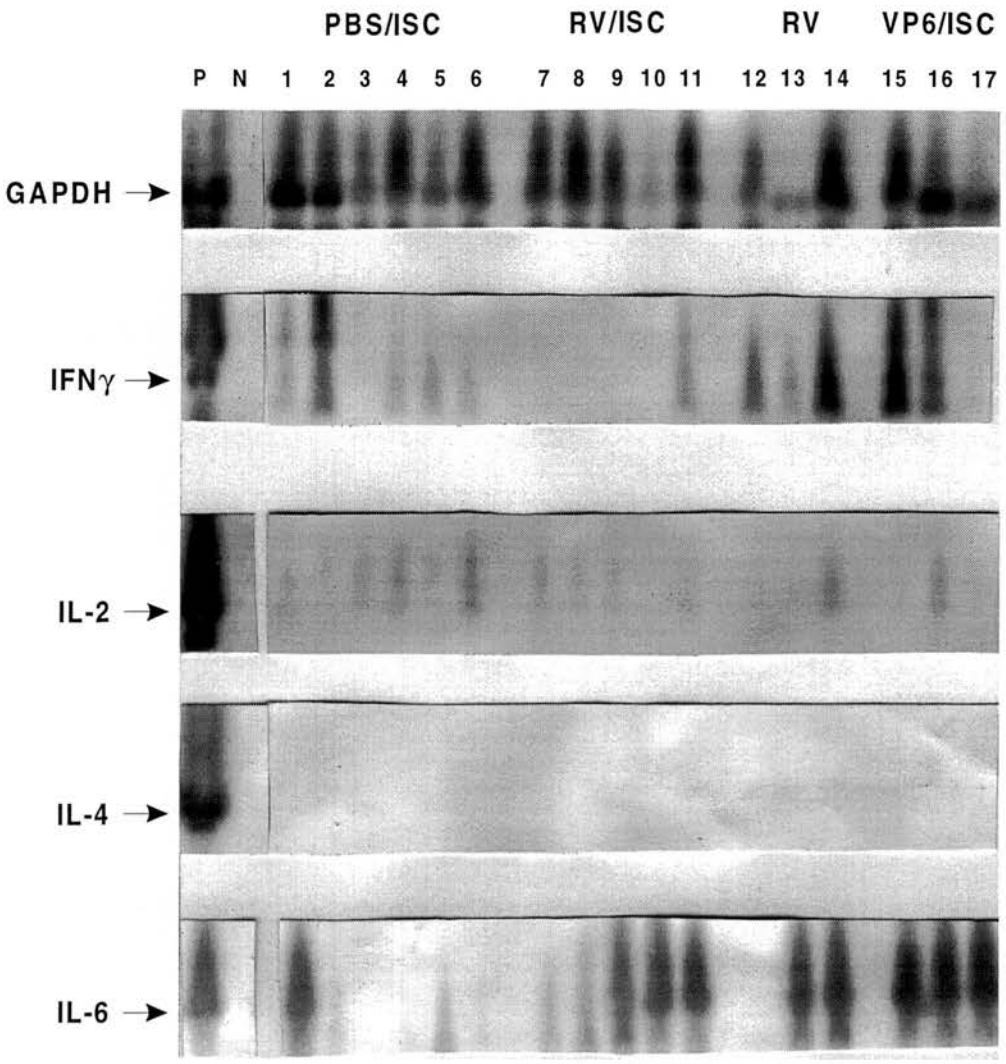
In MLNs, no clear picture can be drawn about the expression of IFNγ as no clear bands are visible in test samples as seen in JPPs. Faint IL-2 transcripts were seen in all groups although no clear bands were visible in both test and positive samples. No transcripts for IL-4 were seen in any group. IL-6 expression was seen in all groups, although the PBS/ISC group had a significant ($p<0.05$) lower expression of IL-6 than the other groups (Plate 7.2).

Plate 7.1: Cytokine expression in JPPs in the vaccinated and challenged groups



GAPDH and cytokine expression in lymphocytes isolated from JPPs from vaccinated and challenged groups. P = positive PCR control; N = negative control; PBS/ISC: 1-6; RV/ISC: 7-11; RV: 12-14; VP6/ISC: 15-17.

Plate 7.2: Cytokine expression in MLNs in the vaccinated and challenged groups



GAPDH and cytokine expression in lymphocytes isolated from MLNs from vaccinated and challenged groups. P = positive PCR control; N = negative control; PBS/ISC: 1-6; RV/ISC: 7-11; RV: 12-14; VP6/ISC: 15-17.

7.4 DISCUSSION

In this study, lambs were vaccinated orally at 6-7 days of age either with a single dose of PBS mixed with ISCOMs, RV mixed with ISCOMs, RV alone or recombinant VP6 incorporated into ISCOMs. The PBS/ISC group showed no specific response. Rotavirus-specific IgG ASC in blood were observed in the RV/ISC, RV, and VP6/ISC groups 7 days after vaccination with declining numbers from 14 days. The RV/ISC and RV groups had three times more rotavirus-specific IgG ASC than the VP6/ISC group. Circulating rotavirus-specific IgG antibodies were detected in the RV/ISC and RV groups from 11 days after vaccination. No rotavirus-specific IgG antibodies were seen in the VP6/ISC group reflecting the lower numbers of IgG ASC. No vaccination regime induced any rotavirus-specific IgA ASC or antibodies. All 3 viral and subunits vaccines primed the immune system as observed in increases in rotavirus-specific IgG ASC and antibodies. Rotavirus-specific IgG ASC preceded rotavirus-specific IgG antibodies in blood indicating that cell trafficking occurred after mucosal priming from the intestine to the circulation compartment.

In previous studies, mice given a single oral dose of 100μg ovalbumin incorporated into ISCOMs showed a specific IgG response, although the levels were generally low. No specific IgA response was observed after one dose, however when given 3 or 6 doses, a specific IgA response was seen. The most critical factor in inducing a specific IgA response was the number and/or frequency of vaccinations rather than the antigen dose. This may reflect the need for repeated stimulation of a relatively small number of specific-precursors to obtain and/or maintain a specific mucosal IgA response (Mowat *et al,* 1991; 1993).

A rotavirus-specific IgG response was seen in serum in mice vaccinated orally with inactivated whole rotavirus incorporated into microspheres. No rotavirus-specific IgA was found in serum and intestinal scrapings after vaccination. No response was seen in mice vaccinated with the same amount of free rotavirus (Khoury *et al,* 1995). This is in contrast with the observation seen in the present study.

The lack of virus-specific IgA after one vaccination was similar to the observation seen in mice vaccinated orally with influenza subunits incorporated into

ISCOMs (Ghazi *et al,* 1995). No specific IgA antibodies in the intestine or serum were seen in mice vaccinated orally with tetanus toxoid and cholera toxin. A similar specific IgG response was seen in serum after one oral dose (Jackson *et al,* 1993).

In a previous study in which horses were vaccinated intranasally with whole inactivated influenza virus incorporated into ISCOMs or virus alone, a significantly higher antibody response was seen in the horses vaccinated with the incorporated antigen (Mumford *et al,* 1994). In contrast to the present study the group given recombinant VP6 incorporated into ISCOMs had a lower antibody response than the given rotavirus mixed with ISCOMs or rotavirus alone. In the present study the major inner core protein (VP6), a non-neutralising antigen, was incorporated and not the whole virus including the neutralising antigens. The presence of neutralising antigens in a vaccine could explain the higher antibody response after vaccination compared to a vaccine composed of non-neutralising antigens.

No IgA was detected in serum and nasal secretions in any group at the start of the study. Low detectable immunoglobulin levels were observed in nasal secretions and serum in all groups from 3 and 7 days after vaccination respectively. IgA concentrations increased in all groups after vaccination. The ISCOM vaccinated groups had higher IgA concentrations in serum and nasal secretions 21 days after vaccination compared to the RV group, but this difference was not significant. IgA concentrations in serum in any vaccinated group were similar to those observed in the infected and control groups (see chapter 6) in the first 21 days after vaccination/infection. In nasal secretions, the ISCOM vaccinated groups had IgA concentrations which were 2-3 times lower as seen in the infected group. The RV group had similar levels than the control group, which were lower than the ISCOM vaccinated and infected groups.

Low detectable IgG concentrations were observed in all groups at the start of the study. No IgG was found in nasal secretions and presumptively in intestinal scrapings at the start of the study but low detectable levels were seen from 7 days after vaccination. In serum, the VP6/ISC groups had significantly increased IgG concentrations compared to the other groups. In nasal secretions, no differences in IgG concentrations were seen between the groups after vaccination. IgG

concentrations found in serum in the PBS/ISC, RV/ISC, and RV vaccinated group were 2-3 times lower than observed in the infected group 21 days after vaccination/infection. The control group had concentrations, which were 2-3 times lower than observed in these three vaccinated groups. The VP6/ISC group had levels 5 times higher than observed in the infected group. A similar observation of significantly increased concentrations was seen in sheep vaccinated orally with ISCOMs and a high antigen dose as observed in chapter 5. In nasal secretions, the infected group had increased IgG compared to the vaccinated and control groups 21 days after vaccination/infection.

After birth, total IgA and IgG increased with time in serum and at mucosal surfaces due to the maturation of the immune system and the bacterial colonisation of the gut as described for the control group in chapter 6.

ISCOMs have a non-specific effect on total IgA concentrations in nasal secretions. Vaccination of the animal, especially when the antigen is incorporated into ISCOMs has an enhancing effect on total IgG concentrations in serum.

Vaccinated lambs were challenged with a live lamb passaged ovine strain K923, 3 weeks after vaccination. All vaccinated lambs commenced excreting virus between 48-72 hours after challenge which was 48 hours later than observed in a study in which one-day-old lambs were infected (Snodgrass *et al,* 1976b). The PBS/ISC, RV/ISC, RV, and VP6/ISC groups excreted virus for an average of 5.7, 4.0, 5.0, and 4.7 days respectively. Lambs given the rotavirus vaccines were partially protected after one single dose as seen in a reduced period of virus excretion compared to the PBS/ISC group. A significant reduction in the virus excretion period was seen only in the RV/ISC group. The small number of animals in the RV and VP6/ISC groups influenced the outcome of the results as seen in the low p-values. It is possible that bigger groups could show a significantly reduced period of virus excretion and that multiple vaccine doses could induce full protection. However, multiple doses of a vaccine comprising antigen (rotavirus) alone could induce oral tolerance (Mowat, 1994).

In a previous study, no protection was seen in mice vaccinated intranasally with a single dose of influenza subunits incorporated into ISCOMs and challenged 3

weeks later with live influenza virus. All mice had detectable live virus in the lungs. No clear picture could be given about the excretion period as mice were killed 3 days after challenge (Ben Ahmeida *et al,* 1993).

Age resistance has been shown in 12 day-old lambs which excreted virus for only 1-2 days following a primary infection (Snodgrass *et al,* 1976b). A similar age resistance was not seen in the present study in the PBS/ISC vaccinated group maybe due to a different breed of lambs or a different antigen dose.

Rotavirus-specific IgA ASC and antibodies were detected in blood and rotavirus-specific IgA antibodies in nasal secretions in all groups after challenge. The RV/ISC and VP6/ISC groups had a significantly increased number of ASC in blood with differences observed in rotavirus-specific IgA antibodies in serum. In nasal secretions, the RV/ISC, RV, VP6/ISC groups had significantly higher levels of rotavirus-specific IgA antibodies. Rotavirus-specific IgA antibodies appeared first in serum followed by nasal secretions. In the intestine, the RV/ISC and RV groups had significantly higher levels of rotavirus-specific IgA antibodies. Virus-specific IgA ASC and antibodies were seen only after infection/challenge with a live virulent virus strain.

Rotavirus-specific IgG ASC were boosted after challenge with significantly higher numbers found in the RV/ISC and VP6/ISC groups. Rotavirus-specific IgG antibodies were seen in all groups but the RV/ISC and RV groups had significantly higher levels at 4 days after challenge. In nasal secretions, all groups had rotavirus-specific IgG antibodies but the RV/ISC and the RV group had significantly higher levels. Rotavirus-specific IgG antibodies in the intestine were only seen in the RV/ISC and RV groups. The RV/ISC and RV groups showed a secondary IgG response as seen in rapidly increased levels at 4 days while the PBS/ISC and VP6/ISC groups had a slower humoral immune response with detectable levels 7 days after challenge.

The high numbers of rotavirus-specific IgG ASC did not correlate with high levels of rotavirus-specific IgG antibodies in blood. However, high numbers of rotavirus-specific IgA ASC correlated with high levels of rotavirus-specific IgA antibodies in blood. This could suggest that an IgA response is dominant after a

mucosal infection with live virus despite mucosal priming after vaccination. A similar observation was reported in chapter 6 in lambs after a primary infection with live rotavirus.

The presence of rotavirus-specific antibodies in the intestine 1-2 weeks after challenge is in contrast to the lack of rotavirus-specific antibodies in lambs infected with rotavirus (see chapter 6). The presence of rotavirus-specific antibodies in the intestine in the rotavirus vaccine groups is probably due to maturation of the immune system.

The significantly increased rotavirus-specific IgA antibodies in the intestine and rotavirus-specific IgG antibodies in serum, nasal secretions, and intestine observed in the RV/ISC and RV groups after challenge is probably due to immunological priming after vaccination. B cells are absolutely necessary for the development of immunity to rotavirus as specific antibodies are essential in viral clearance when reinfected (Franco and Greenberg, 1995; Franco *et al,* 1997a). The significantly increased specific antibody response observed in the RV/ISC and RV groups could suggest a similar finding as these groups are reinfected with rotavirus. Priming also occurred in the VP6/ISC group, although to a lesser degree. The fact that whole rotavirus was used in the RV/ISC and RV groups rather than the VP6 protein could explain this difference in priming.

No rotavirus-neutralising antibodies in serum and nasal secretions were seen in any group after vaccination. After challenge, all groups developed neutralising antibodies against the challenge strain K923 1 week after challenge but no significant differences were found between the groups. Low heterotypic neutralising antibodies were seen against the bovine rotavirus strain UK.

No neutralising antibodies were detected in nasal secretions and gut scrapings 1 week after challenge while rotavirus-specific antibodies were present at mucosal surfaces. This could indicate that the neutralising activity might be effected during transport of these secretory IgA antibodies by epithelial cells or the secretory component of these antibodies could influence the neutralising activity in the lumen.

Mice primed parenterally with the same recombinant VP6 antigen emulsified with Freunds complete adjuvant (CFA) and boosted with rotavirus strain UK

emulsified in IFA, showed significantly increased neutralising antibodies 1-2 weeks after boosting compared to mice primed with PBS in CFA and boosted as above (Bruce *et al,* unpublished data). In the present study, lambs were killed 1-2 weeks after challenge with the last sampling 7 days after challenge. It is possible that we missed this boosted neutralising response in the primed groups due to either the time of killing, a different immune response in sheep compared to mice, or that CFA might be better in inducing serum neutralising antibodies than ISCOMs.

The lack of neutralising antibodies in the intestine indicates that a mechanism other than neutralising antibodies is responsible for viral clearance.

IgA concentrations in serum and nasal secretions increased in all groups after challenge with no significant differences seen between the groups. No significant differences were seen in intestinal scrapings. All vaccinated groups had boosted IgA concentrations in serum 1 week after challenge compared to the infected and control groups at the same point in the experiment (28 days after infection/vaccination) (see chapter 6). In nasal secretions, the RV group had similar concentrations of IgA to the control group while in the PBS/ISC, RV/ISC, and VP6/ISC groups, concentrations were 2-3 times lower as seen in the infected group.

IgG concentrations increased in all groups in serum after challenge but significantly increased IgG concentrations were only seen in the VP6/ISC group. In nasal secretions, no increase in IgG concentrations was observed in all groups after challenge. No differences in IgG concentrations were seen in intestinal scrapings between the groups after challenge. In serum, IgG concentrations in the PBS/ISC, RV/ISC, and RV groups were similar as observed in the infected group, however the VP6/ISC group had a level 7-10 times higher. In nasal secretions, all vaccinated groups had similar concentrations to the infected and control groups. The observation that the significantly higher IgG concentrations in serum in the VP6/ISC group did not correlate with significantly higher IgG concentrations in nasal secretions and intestinal scrapings might suggest that IgG antibodies at mucosal surfaces are produced locally and not by transudation from serum.

Rotavirus-primed lymphocytes were not detected in blood in any group after vaccination, but were seen in the VP6/ISC group 1 week after challenge. Mice fed single doses of 50-100μg ovalbumin in ISCOMs were able to generate systemic T cell immunity (Mowat *et al,* 1991). The lack of response in the PBS/ISC, RV/ISC, and RV groups together with the response seen in the VP6/ISC group could suggest that vaccines comprising a mixture of antigens and ISCOMs are taken up within different cells at different sites within the intestine. In the VP6/ISC group, uptake of antigens and ISCOMs is likely to occur at the same place due to the incorporation of the antigen into the ISCOMs. This could suggest that different mechanisms of antigen presentation and priming are taking place in the VP6/ISC group compared to the PBS/ISC, RV/ISC, and RV groups. Epithelial cells could play an important role in antigen presentation and the priming of T and B lymphocytes when the vaccine is a mixture of antigen and adjuvant. When antigen incorporated vaccines are used, M cells in the JPPs could play an essential role in antigen presentation and priming.

Lymphocytes from MLNs and JPPs from all groups responded to Con A and PWM indicating the presence of functional T and B cells. In IPP, lymphocytes in all groups responded less to Con A than PWM, which was expected, as PWM is mainly a B cell stimulant and IPP consists of mainly B cells. No response to Con A and PWM was observed in IELs and LPLs and this was consistent with the observation reported in chapter 6 in which a similar lack of response was seen to Con A and PWM in IELs and LPLs. The lack of response in the IEL and LPL population to any antigen could be due to the lack of viable cells in these cultures.

Lymphocytes from JPPs from all groups responded to UK. This was only significant in the RV/ISC and RV groups. No significance response was observed in the other two groups due to high animal-to-animal variations within a group. In MLNs, an increased proliferation was seen in the RV/ISC and RV groups, however this was not significant. This together with the finding of increased rotavirus-specific antibodies in these two groups could suggest that a Th2-like immune response was induced after vaccination.

In the PBS/ISC, RV/ISC, and RV groups, the proportion of $CD4^+$ T cells significantly increased in blood after challenge, but no clear picture of the VP6/ISC

group was evident due to a high percentage observed at the start the study. This increase in $CD4^+$ T cells in blood correlates with the big increase in rotavirus-specific antibodies in serum after challenge. No changes were seen in $CD8^+$ T cells in all groups after vaccination or challenge. $\gamma\delta^+$ T cells significantly increased in the PBS/ISC after vaccination and remained elevated after challenge. In the RV/ISC and RV groups, $\gamma\delta^+$ T cells significantly increased after vaccination but a decline was observed in the percentage of $\gamma\delta^+$ T cells 21 days after vaccination. After challenge, the percentage of these cells increased in both groups. This decline in $\gamma\delta^+$ T cells in the RV/ISC and RV groups could be a result of homing. The percentage of $\gamma\delta^+$ T cells were significantly decreased in the VP6/ISC group after vaccination and remained at a similar level after challenge. The precise role of $\gamma\delta^+$ T cells is still unclear but they may be involved with the maturation of the immune system or they could have a cytotoxic role as an increase in these cells was observed in the RV/ISC and RV groups after challenge (Goodman and Lefrancois, 1988). In a previous study, depletion of $\gamma\delta^+$ T cells had no effect on the virus excretion in calves infected with rotavirus suggesting that these cells are not important in viral excretion (Oldham *et al,* 1993). However, in another study in calves infected with rotavirus, $\gamma\delta^+$ T cells were involved in a rotavirus-specific immune response (Parsons *et al,* 1993). The expression of light chain increased in the PBS/ISC, RV/ISC, and RV groups after vaccination with significant increases in all three groups after challenge compared to pre-vaccination levels. This is probably due to a general increase in B cells and antibodies. No increase was seen in the VP6/ISC group and this was in contrast with the significantly increased IgG antibodies in serum observed in this group. The RV/ISC group had significant increases in $CD45R^+$ cells after vaccination whilst in the PBS/ISC and RV groups increases were seen after challenge. No changes were seen in $CD45R^+$ cells in the VP6/ISC group as a high percentage was observed at the start of the experiment.

In this study, the total percentage of the different lymphocyte markers in blood usually exceeded 100%. A possible explanation could be the fact that lymphocytes can express more cell surface markers. It has been shown that naïve ovine $CD4^+$ T cells also express CD45R. This could influence the total percentage of cells, as these $CD4^+$ T cells will be analysed twice. The percentage of these cells in

these lambs will be high at the start of the experiment, as they have not been exposed to antigens. However, after antigen stimulation *in vitro* naïve $CD4^+$ T cells showed a decreased expression of CD45R; a phenotypic change that also occurs when naïve T cells undergo transition to memory cells *in vivo* (MacKay *et al,* 1990). It had been shown in sheep that $\gamma\delta^+$ T cells also can express CD8 however at a lower level than $\alpha\beta^+$ T cells (MacKay *et al,* 1990). When bronchoalveolar lavage fluid lymphocytes obtained from sheep were stimulated *in vitro* with Con A, the co-expression of CD4 and CD8 increased (Begara, 1994). In this study, a clear answer about double-positives in the lambs used in this experiment can not be given as no two-colour immunofluorescence analyses were done. However double-positives have been shown in sheep so they could have had an influence on the total percentage of lymphocytes or technical reasons could have been the cause of total percentages exceeding 100%.

The percentage of $CD4^+$ T cells was significantly higher in MLNs in the RV and VP6/ISC groups and in JPPs in the VP6/ISC group, whilst in IELs and LPLs a significantly lower percentage was observed in the VP6/ISC group than in the PBS/ISC group. No changes were found in IPPs between the groups. The percentage of $CD8^+$ T cells in MLNs, JPPs, and IPPs was similar for all groups. In IELs and LPLs, the VP6/ISC group had a significantly higher percentage than the PBS/ISC group. This observation correlates with the lower percentage of $CD4^+$ T cells observed in IELs and LPLs in this group suggesting a bigger involvement of $CD8^+$ T cells in this group. The VP6/ISC group had significantly lower $\gamma\delta^+$ T cells in MLNs, JPPs, IPPs, IELs, and LPLs than the PBS/ISC group, which correlates with the lower levels seen in blood. No differences were observed in IELs and LPLs between the groups. The expression of light chain was similar in MLNs, JPPs, and IELs for all groups. In IPPs and LPLs, the VP6/ISC group had a significantly lower percentage. The lower expression of light chain in LPLs reflects the lower expression observed in blood in this group. The percentage for $CD45R^+$ cells was similar in all tissues for all groups, although the VP6/ISC group had a significantly higher percentage in MLNs and a significantly lower percentage in IELs.

In this study, the lymphocyte sub-populations in each group could suggest that different mechanisms were involved in viral clearance. The immune response in

the VP6/ISC group is in favour of Th1-like response with the involvement of $CD8^+$ T cells in IELs. In the PBS/ISC, RV/ISC, and RV groups there was no clear indication of a Th1- or Th2-like immune response.

IFNγ transcripts in JPPs were seen in the PBS/ISC, RV, and VP6/ISC groups. In the RV/ISC group, a significant down-regulation of IFNγ was seen, as 4 of 5 samples showed no expression. In MLNs, no distinct bands were visible for IFNγ. This could suggest that in the RV/ISC group viral clearance is not dependent on a Th1-like response. The observation of IFNγ expression in the VP6/ISC group correlates with the increased percentage of $CD8^+$ T cells observed in IELs. The ISCOM groups had mRNA for IL-2 in JPPs whilst faint bands were observed in MLNs. This could suggest that ISCOMs are a stimulant for IL-2 expression. Transcripts for IL-4 were seen JPPs without any clear differences and no expression of IL-4 was seen in MLNs. All groups had IL-6 expression in JPPs. In MLNs, the PBS/ISC group had a significantly lower expression of IL-6 than the other groups that could indicate that rotavirus vaccination induces increased expression of IL-6.

A down-regulation of IFNγ as in the RV/ISC groups was also seen in mice infected with *Shistosoma mansoni*. These mice also showed elevated Th2-type responses (Pearce *et al,* 1991). Mice vaccinated either with inactivated respiratory syncytial virus (RSV) or live RSV showed different cytokine profiles after a subsequent challenge. Vaccination with inactivated RSV resulted in a predominantly Th2- type response while vaccination with live virus gave a predominantly Th1-type response after challenge (Tang *et al,* 1997). In a previous study, feeding mice ovalbumin in ISCOMs primed $CD4^+$ T cell responses in JPPs of both the Th1- and Th2-type (Maloy *et al,* 1995). A similar observation was seen in the present study.

The cytokine expression could suggest that both Th1- and Th2-like immune responses were involved in the PBS/ISC, RV, and VP6/ISC groups while a Th2-like immune response is involved in the RV/ISC groups.

This study describes the effect of vaccination with a single oral dose of PBS/ISC, RV/ISC, RV, and VP6/ISC on the immune response in lambs against a subsequent challenge. Quantitative differences in response between the VP6/ISC

group and other groups are difficult to interpret due to the impossibility of comparing the different vaccine doses. However, qualitative differences are apparent.

After vaccination, priming occurred in the RV/ISC, RV, and VP6/ISC groups resulting in partial protection against a subsequent challenge as seen in a reduced period of viral clearance. Immunological priming was adequate without ISCOMs. Different mechanisms were likely to be induced when different vaccines were used as seen in the tested parameters. No clear picture is available for the PBS/ISC group as evidence for both Th1- and Th2-like responses were seen. The RV/ISC group favours a Th2-like response as a down-regulation of IFNγ and increased rotavirus-specific antibodies were observed. The RV group showed both Th1- and Th2-like responses and no clear conclusion can be made. A Th1-like response was seen in the VP6/ISC group as seen in the expression of IFNγ together with a significantly higher percentage of $CD8^+$ T cells in IELs, although no information is available about the cytotoxic activity of these cells.

The study shows that the VP6 protein is a good inducer of both Th1- and Th2-cells as seen in increased $CD4^+$ T cells in MLNs and JPPs together with the presence of mRNA for IFNγ and IL-4 in lymphocytes isolated from these tissues. Th cell responses against the VP6 protein have also been observed in spleen cells from mice, parenterally inoculated with a bovine rotavirus strain and/or a porcine rotavirus strain (Bruce *et al,* 1994; Baños *et al,* 1997).

Several immune mechanisms could be induced after vaccination with the VP6 protein. A cytotoxic immune response could be involved, and may be related to the increased percentage of $CD8^+$ T cells found in IELs. Mice vaccinated with plasmid DNA encoding the VP6 protein showed virus-specific cytotoxic T cell responses (Chen *et al,* 1997). Mice given hybridomas excreting VP6 specific antibodies were protected against rotavirus infection by blocking virus replication in the intestinal epithelium; these antibodies were not active in the intestinal lumen (Burns *et al,* 1996). The protective mechanism induced in this study could be intracellular non-neutralising rotavirus VP6 specific IgA antibodies as no neutralising antibodies were present at mucosal surfaces. A similar mechanism was also suggested in mice vaccinated with plasmid DNA encoding the VP6 protein

which had significant reductions in viral excretion after a subsequent rotavirus infection (Chen *et al,* 1997).

Vaccination with VP6 was partially protective as a reduced virus excretion ($p=0.10$) was observed. In this study, a single dose was given. It is possible that when multiple doses are given, full protection could be achieved. The small number of animals used in this study could have influenced the results.

The VP6 protein has the potential to be used in a subunit vaccine, as it is the most abundant protein of the virion and it carriers antigenic determinants that are common to all group A rotaviruses. Cross-reactivity between VP6 proteins of different rotavirus strains has been shown *in vitro* in parenterally immunised mice with rotavirus (Bruce *et al,* 1994; Baños *et al,* 1997).

In this study, ISCOMs were used as a mucosal adjuvant although immunological priming was adequate without ISCOMs. A significant reduction in viral excretion that was only observed in the group given whole virus mixed with ISCOMs. However, the groups given the subunit vaccine or inert rotavirus alone showed a reduced virus excretion but the small number in the group influenced the results. The use of subunit vaccines or vaccines composed of inert virus alone should be investigated further.

CHAPTER 8

GENERAL DISCUSSION

Diarrhoeal diseases have a world-wide impact on both human and animal health and are a major cause of morbidity and mortality. Rotavirus is the most important agent of viral gastroenteritis in children and young animals, accounting for approximately 870,000 human deaths annually and substantial economic losses in the agricultural business. Rotavirus infects the gastrointestinal tract, a mucosal surface. Mucosal surfaces are the main entry port of infections and the gut associated lymphoid tissues play an important role in mucosal immunity.

Induction or boosting of mucosal immunity is crucial for protection against these mucosal infections or reinfections and the local immune response can be mediated by different components. The humoral specific effector mechanisms could involve locally produced secretory IgA, or IgG derived from serum or possibly locally produced. The cellular effector mechanisms could involve cytotoxic T lymphocytes, helper T lymphocytes or natural killer cells.

This study concerns parenteral and oral vaccination against rotavirus in adult sheep using different antigen doses and adjuvants, oral vaccination in gnotobiotic lambs to induce immunological priming and protection against a subsequent challenge, and the characterisation of the primary immune response against rotavirus in lambs.

Parenteral vaccines are not very effective at inducing a mucosal immune response in naïve animals. However, in previously exposed humans and animals parenteral vaccination can induce or boost an immune response at mucosal surfaces (Saif and Bohl, 1980; Svennerholm, 1980). Oral vaccines have the advantage that antigen is delivered to the site of infection and may induce specific mucosal immune responses in naïve animals and could boost mucosal immune responses in previously exposed animals.

A detailed characterisation of the mucosal immune response against rotavirus is not available although several mechanisms have been shown to be involved, such as cytotoxic T lymphocytes, T helper lymphocytes, and rotavirus-specific antibodies (Offit and Dudzik, 1988; 1989; Conner *et al,* 1991; Coulson *et al,* 1992; Oldham *et al,* 1993).

A commercial rotavirus dam vaccine for ruminants is available based on rotavirus lysate emulsified with an oil-based adjuvant such as IFA (Wells *et al,* 1978; Snodgrass *et al,* 1980; Castrucci *et al,* 1984; Saif *et al,* 1984; Archambault *et al,* 1988; Möstl and Bürke, 1988; Bellinzoni *et al,* 1989). This vaccination relies on the passive transfer of maternal rotavirus neutralising antibodies via colostrum and milk to the offspring. These maternal antibodies absorbed into the circulation but mainly present in the lumen of the neonatal intestine are an effective mediator of protection. Parenteral vaccination could also be useful in boosting mucosal immune responses in the elderly for protection against reinfection.

A parenteral vaccine based on the commercial rotavirus dam vaccine, a mixture of rotavirus lysate and IFA, was able to stimulate rotavirus-specific secretory IgA responses at mucosal surfaces in adult sheep, although they were short-lived, and systemic rotavirus-specific antibody responses. In humans, specific ASC induced by parenteral vaccination with *Salmonella typhi* Ty21a were mostly directed to the systemic compartment although a part of the specific ASC had mucosal homing attitudes (Kantele *et al,* 1997). A similar observation was observed in this study in which the majority of rotavirus-specific antibodies were associated with the systemic compartment. The response seen in nasal secretions could reflect the response seen at the intestinal surface. Stimulation of IgA antibodies in the intestine and other mucosal surfaces such as the respiratory tract could increase protection against reinfection by a mucosal pathogen.

Parenteral vaccines could be used against endemic enteric infections. Firstly, aimed at a passive transfer of immunity from the adult to the offspring. Secondly aimed at an increased level of specific IgA antibodies at mucosal surfaces to protect against those infections which are clinically important in adults such as bovine coronavirus in cows and *Escherichia coli* in Traveller's diarrhoea in humans. Parenteral vaccines could be a potential alternative to oral vaccines especially in adults.

Parenteral vaccination with different antigen doses could influence the immune response while the use of other adjuvants could be more favourable as IFA has known side effects. At a low rotavirus antigen dose, ISCOMs and IFA were

successful as adjuvants in inducing antibody responses while at a high rotavirus antigen dose, ISCOMs and microspheres induced antibody responses. Increases were seen in rotavirus-specific IgA antibodies in nasal secretions although no differences were observed in rotavirus-specific antibodies in intestinal scrapings. A dose effect was observed in rotavirus-specific IgG antibodies in serum and nasal secretions, however this was not significant. The small numbers in each group may have influenced the outcome of results. Further research with larger groups should be conducted to test if these preparations can stimulate rotavirus-specific IgA antibodies at mucosal surfaces and if rotavirus vaccines can be improved using a higher antigen load.

Oral vaccination of adult sheep using different antigen doses and adjuvants had no significant effect on the immune response although transient increases in rotavirus-specific IgA antibodies were observed in nasal secretions in animals vaccinated either with ISCOMs or microspheres. The possibility exists that the response was short-lived and this was missed in this study due the time of sampling.

Oral vaccination in sheep was not effective with the vaccines used although different types of vaccines and antigen presentations could be more successful. For example incorporating the antigen into the adjuvant could have a more significant effect on the immune response. In this circumstance antigens are more protected against the environment and both antigens and adjuvant are taken up at the same place. This might not be the case when antigens and adjuvants are mixed, with the possibility of different routes of uptake being used.

Protection of the young ruminant against natural rotavirus infection is based on passively derived maternal neutralising antibodies mainly present in the intestinal lumen through the feeding of colostrum and milk. Increased maternal neutralising antibodies in colostrum is achieved by parenterally vaccinating the dam (Snodgrass *et al,* 1980; Castrucci *et al,* 1984; Bellinzoni *et al,* 1989).

Parenteral priming of the young could induce a suppression of the specific humoral immune response when subsequently challenged. This was observed in a previous study in which mice and piglets showed a lack of intestinal specific F4

antibodies upon challenge with live F4$^+$ *Escherichia coli* after parenteral priming with killed *Escherichia coli* F4 bacteria (Bianchi *et al,* 1996).

Oral vaccinations have the advantage that antigens are delivered to the site of infection and induce an active local immunity. Several mucosal adjuvants such as ISCOMs, cholera toxin, and microspheres have been shown to enhance the immune response as antigens given alone are often poorly immunogenic and may provoke oral tolerance when given as multiple doses (Mowat, 1994).

ISCOMs were used as a mucosal adjuvant to induce immunological priming and protective immunity in gnotobiotic lambs against a subsequent challenge with live virulent rotavirus. Rotavirus antigens were given orally either mixed with or incorporated into the ISCOMs. Vaccines comprised either rotavirus lysate mixed with ISCOMs, rotavirus alone, or a recombinant VP6 protein incorporated into ISCOMs and resulted in immunological priming and partial protection against a subsequent challenge. The only significant reduction in viral clearance was seen in the group vaccinated with rotavirus mixed with ISCOMs. Reductions in viral clearance were also seen in the group given rotavirus alone and in the group given a vaccine comprising recombinant VP6 incorporated into ISCOMs. The small numbers in the last two groups influenced the outcome of the results.

Different protective immunological mechanisms were induced when the different vaccines were used. In the non-vaccinated group both Th1- and Th2-like responses were seen. Similar observations were seen in the group given rotavirus alone. Rotavirus mixed with ISCOMs induced a Th2-like response while the group given recombinant VP6 protein incorporated into ISCOMs induced mainly a Th1-like response.

All groups had rotavirus-specific IgA antibodies present in the intestine at necropsy however; these were non-neutralising while neutralising antibodies were seen in circulation. Several hypotheses can be made about the lack of neutralising activity of these intestinal secretory antibodies. The neutralising activity might be affected during transport of these secretory IgA antibodies by epithelial cells or the secretory component of these antibodies could influence the neutralising activity in the lumen.

Lambs were given a single oral dose of vaccine although multiple doses have been reported to induce greater protection. Mice given two doses of an intranasal influenza/ISCOM vaccine were relatively more protected against challenge than mice given one dose (Ghazi *et al,* 1995). It is unclear if multiple doses would induce full protection in lambs and this should be investigated further.

The reduced viral clearance observed when recombinant VP6 was used in the vaccine could be of great importance as all group A rotaviruses share common VP6 epitopes and this could have potential for a future vaccine. Mice parenterally immunised with a bovine or porcine rotavirus strain had Th cells in the spleen which showed responses against the VP6 proteins of other rotavirus strains (Bruce *et al,* 1994; Baños *et al,* 1997). Similar cross-reactivity was seen here as the recombinant VP6 antigen was based on the bovine rotavirus UK strain while the challenge virus was an ovine rotavirus strain K923. Mice vaccinated with plasmid DNA encoding for VP6 showed significant reductions in viral excretion (Chen *et al,* 1997).

To complete this study more groups should be included. Groups given whole rotavirus antigen incorporated into ISCOMs, VP6 given as a mixture with ISCOMs, and groups given multiple doses of the vaccines used with the emphasis on the VP6 vaccines. Although, the potential of inert rotavirus as a vaccine should also be further examined. The observation of neutralising activity in circulation with a lack of neutralising activity in the gut should be further investigated.

Characterisation of the primary immune response in gnotobiotic lambs after a rotavirus infection showed a "classical" primary humoral immune response. Rotavirus-specific IgA antibodies preceded rotavirus-specific IgG antibodies. These antibodies were seen in serum and nasal secretions after infection while rotavirus-specific antibodies were not detected in the gut in the first 10 days after infection. The virus was cleared within 8-9 days. Infection with rotavirus increased IgA and IgG concentrations in nasal secretions and serum respectively. The predicted involvement of cytotoxic T cells was not investigated due to technical reasons. However, the cytokine expression results did not indicate a Th1-like immune response involved in viral clearance but favoured a Th2-like immune response as rotavirus-specific antibodies were present in the intestine after the first 10 days of

infection. Antibodies are essential in protection against reinfection (Franco and Greenberg, 1995; Franco *et al,* 1997a).

The precise mechanisms of viral clearance are still unclear although several mechanisms could be involved. Intestinal intracellular non-neutralising rotavirus-specific IgA antibodies have been suggested by recent studies but the relevance to this case is not known (Burns *et al,* 1996; Chen *et al,* 1997). Further research has to determine the role of these intracellular IgA antibodies. The role of cytotoxic T cells is still unclear as tests are difficult to perform because of a lack of available target cells, but the effectiveness of cytotoxic T cells has been reported in mice (Dharakul, 1990; Offit and Dudzik, 1990). Other cells such as natural killer cells or cytotoxic $CD4^+$ T cells could play a role in viral clearance.

This study tried to characterise the primary immune response in lambs against rotavirus. A few mechanisms are suggested but more research has to be done to determine the importance of each mechanism involved in viral clearance. Viral clearance is very likely to be the result of several specific mechanisms. More animals should be included to determine when rotavirus-specific antibodies appear in the intestinal lumen.

In summary, parenteral vaccination in previously exposed sheep with RV/IFA induced significantly increased systemic immune responses and of more importance significantly boosted rotavirus-specific IgA antibodies at mucosal surfaces.

A clear adjuvant effect was observed in humoral immune response between the adjuvants used at each antigen dose. IFA and ISCOMs were more successful at a low antigen dose then microspheres, while at the high antigen dose ISCOMs and microspheres proved to be more successful. No significant effect was observed on rotavirus-specific IgA antibodies at mucosal surfaces and no significant dose effect was seen on the level of rotavirus-specific antibodies but these could be due to the small numbers in each group.

Oral vaccination in previously-exposed sheep was not successful in increasing rotavirus-specific antibodies either in circulation or at mucosal surfaces,

however a single oral vaccination with inactivated virus in gnotobiotic lambs resulted in partial protection against subsequent challenge. Partial protection was seen in the groups given rotavirus lysate mixed with ISCOMs, rotavirus lysate alone, or recombinant VP6 incorporated into ISCOMs, however this was only significant in the rotavirus/ISCOM group. Different immune mechanisms were induced when different vaccines were used each resulting in partial protection although the small number in each group influenced the outcome of the results. Partial protection was not a result of neutralising antibodies as no neutralising activity was detected in the gut. Vaccination with recombinant VP6 incorporated into ISCOMs could be important in the future for vaccine development as all group A rotaviruses have the same epitopes on the VP6 antigen.

In laboratory animals (e.g., SCID and knockout mice), several immune mechanisms have been identified but the role of each component in the primary immune response is still unclear. The primary immune response to rotavirus in gnotobiotic lambs is more complex as a clear mechanism was not characterised and protection is probably a result of multiple immune mechanisms acting together. Several possible mechanisms are being suggested but the role of each should be further investigated especially in an out-bred population.

REFERENCES

Abraham, R. and P.L. Ogra. 1994. Development of mucosal immune responses to viruses. *Isr. J. Med. Sci.* **30**:323-327.

Abreu-Martin, M.T. and S.R. Targan. 1996. Regulation of immune responses in the intestinal mucosa. *Crit. Rev. Immunol.* **16**:277-309.

Adams, W.R. and L.M. Kraft. 1967. Electron microscopic study of the intestinal epithelium of mice infected with the agent of epizootic diarrhea of infant mice (EDIM) virus. *Am. J. Pathol.* **51**:39-60.

Allan, C.H., Mendrik, D.L., and J. Trier. 1993. Rat intestinal M-cells contain acidic endosomal-lysosomal compartments and express class II major histocompatibility complex determinants. *Gastroenterology* **104**:698-708.

Anderson, E.L., Belshe, R.B., Bartram, J., Crookshanks-Newman, F., Chanock, R.M., and A.Z. Kapikian. 1986. Evaluation of rhesus rotavirus vaccine (MMU 18006) in infants and young children. *J. Infect. Dis.* **153**:823-831.

Andrew, M.E., Coupar, B.E.H., Boyle, D.B., and G.L. Ada. 1987. The roles of influenza virus haemagglutinin and nucleoprotein in protection: analysis using vaccinia virus recombinants. *Scand. J. Immunol.* **25**:21-28.

Archambault, D., Morin, G., and Y. Elazhary. 1988. Influence of immunomodulatory agents on bovine humoral and cellular responses to parenteral inoculation with bovine rotavirus vaccines. *Vet. Microbiol.* **17**:323-334.

Arnaud-Battandier, F., Hague, N.E., Lum, L.G., Elson, C.O., and W. Strober. 1980. Tissue distribution of IgA receptor-bearing cells in mouse and guinea pigs with special reference to the lymphoid population of the gastrointestinal tract. *Cell Immunol.* **55**:106-113.

Ashley, C.R., Caul, E.O., Clarke, S.K.R., Corner, B.D., and S. Dunn. 1982. Rotavirus infections of apes. *Lancet* **2**:477.

Ashwell, J.D. 1988. Are B lymphocytes the principal presenting cells in vivo? *J. Immunol.* **140**:3773-3778.

Babiuk, L., and M. Campos. 1993. Respiratory vaccines for farm animals. In: "Vaccines for veterinary applications". A.R. Peters Ed. Butterworth-Heinemann Ltd, Oxford. p. 83-116.

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

Bass, D.M., MacKow, E.R., and H.B. Greenberg. 1991. Identification and partial characterization of a rhesus rotavirus binding glycoprotein on murine enterocytes. *Virology* **183**:602-610.

Beagley, K.W., and C.O. Elson. 1992. Cells and cytokines in mucosal immunity and inflammation. *Gastroenterology* **21**:347-366.

Beagley, K.W., Eldridge, J.H., Lee, F., Kiyono, H., Everson, M.P., Koopman, W.J., Hirano, T., Kishimoto, T., and J.R. McGhee. 1989. Interleukins and IgA synthesis: human and murine interleukin 6 induce high rate secretion in IgA-committed B cells. *J. Exp. Med.* **169**:2133-2148.

Begara, I.M., 1994. Immunopathological studies in the ovine lung during the course of natural and experimental maedi-visna virus infection. Thesis. University of Edinburgh.

Bellinzoni, R.C., Mattion, C.N., La Torre, J.L., and E.A. Scodeller. 1987. Incidence of rotavirus in beef herds in Argentina. *Res. Vet. Sci.* **42**:257-259.

Bellinzoni, R.C., Blackhall, J., Baro, N., Auza, N., Mattion, N., Casaro, A., La Torre, J.L., and E.A. Scodeller. 1989. Efficacy of an inactivated oil-adjuvanted rotavirus vaccine in the control of calf diarrhoea in beef herds in Argentina. *Vaccine* **7**:263-268.

Ben Ahameida, E.T.S., Gregoriadis, G., Potter, C.W., and R. Jennings. 1993. Immunopotentiation of local and systemic humoral immune responses by ISCOMs, liposomes and FCA: role in protection against influenza A in mice. *Vaccine* **11**:1302-1309.

Bergeland, M.E., McAdaragh, J.P., and I. Stotz. 1977. Rotaviral enteritis in turkey poults. In: "Proceedings of the 26th western poultry disease conference". Univ. California, Davies. p. 129-130.

Berke, G. 1995. Unlocking the secrets of CTL and NK cells. *Immunol. Today* **16**:343-346.

Bern, C. and R.I. Glass. 1994. Impact of diarrheal diseases worldwide. In: "Viral infections of the gastrointestinal tract". A.Z. Kapikian Ed. Marcel Dekker, Inc., New York. p.1-26.

Bernstein, D.I., Glass, R.I., Rodgers, G., Davidson, B.L., and D.A. Sack. 1995. Evaluation of rhesus rotavirus monovalent and tetravalent reassortant vaccines in US children. US Rotavirus Vaccine Efficacy Group. *JAMA* **273**:1191-1196.

Besredka, A. 1919. De la vaccination contre les etats typhoides par la voie buccals. *Ann. Inst. Pasteur* **33**:882-903.

Besser, T.E., Gay, C.C., McGuireT.C., and J.F. Evermann. 1988. Passive immunity to bovine rotavirus infection associated with transfer of serum antibody into the intestinal lumen. *J. Virol.* **62**:2238-2242.

Bianchi, A.T.J., Scholten, J.W., van Zijderveld, A.M., van Zijderveld, F.G., and B.A. Bokhout. 1996. Parenteral vaccination of mice and piglets with $F4^+$ *Escherichia coli* suppresses the enteric anti-F4 response upon oral infection. *Vaccine* **14**:199-206

Biron, C.A., Byron, K.S., and J.L. Sullivan. 1988. Severe herpesvirus infection in an adolescent without natural killer cells. *N. Engl. J. Med.* **320**:1731-1735.

Bishop, R.F. 1993. Development of candidate rotavirus vaccines. *Vaccine* **11**:247-254.

Bishop, R.F., Davidson, G.P., Holmes, I.H., and B.J. Ruck. 1973. Virus particles in the epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis. *Lancet* **1**:149-151.

Bishop, R.F., Lund, J., Cipriani, E., Unicomb, L., and G. Barnes. 1990. Clinical serological and intestinal immune responses to rotavirus infection of humans. In "Medical Virology 9" Maza, M. de la and E.M. Peterson Eds. Plenum Press, New York. p. 85-110.

Bjerke, K. and P. Brandtzaeg. 1988. Lack of relation between expression of HLA-DR and secretory component in follicle-associated epithelium of human Peyer's patches. *Clin. Exp. Immunol.* **71**:502-507.

Blandford, G. and R.B. Heath. 1972. Studies on the immune response and pathogenesis of sendai virus infection of mice. I. The fate of viral antigens. *Immunology* **22**:637-649.

Bohl, E.H. and L.J. Saif. 1975. Passive immunity in transmissible gastroenteritis of swine: immunoglobulin characteristics of antibodies in milk after inoculating virus by different routes. *Infect. Immun.* **11**:23-32.

Bohl, E.H., Gupta, P.K., Olquin, F.M.W., and L.J. Saif. 1972. Antibody responses in serum, colostrum and milk of swine after infection or vaccination with transmissible gastroenteritis virus. *Infect. Immun.* **6**:289-297.

Bohl, E.H., Frederick, G.T., and L.J. Saif. 1975. Passive immunity in transmissible gastroenteritis of swine: intramuscular injection of pregnant swine with a modified live-virus vaccine. *Am. J. Vet. Res.* **36**:267-276.

Bohl, E.H., Kohler, E.M., Saif, L.J., Cross, R.F., Agnes, A.G., and K.W. Theil. 1978. Rotavirus as a cause of diarrhea in pigs. *J. Am. Vet. Med. Assoc.* **172**:458-463.

Bohl, E.H., Theil, K.W., and L.J. Saif. 1984. Isolation and serotyping of porcine rotaviruses and antigenic comparison with other rotaviruses. *J. Clin. Microbiol.* **19**:105-111.

Brandtzaeg, P. and K. Bjerke. 1990. Immunomorphological characteristics of human Peyer's patch. *Digestion* **46** (S):262-273.

Brandtzaeg, P., Halstensen, T.S., Kett, K., Krajci, P., Kvale, D., Rognum, T.O., Scott, H., and L.M. Sollid. 1989. Immunobiology and immunopathology of human gut mucosa: humoral immunity and intraepithelial lymphocytes. *Gastroenterology* **97**:1562-1584.

Bridger, J.C. and G.N. Woode. 1975. Neonatal calf diarrhoea: identification of a reovirus-like (rotavirus) agent in faeces by immunofluorescence and immune electron microscopy. *Brit. Vet. J.* **131**:528-535.

Bromander, A., Holmgren, A., and N. Lycke. 1991. Cholera toxin stimulates IL-1 production and enhances antigen-presentation by macrophages *in vitro*. *J. Immunol.* **146**:2908-2913.

Browning, G.F., Chalmers, R.M., Sale, C.S.H., Fitzgerald, T.A., and D.R. Snodgrass. 1991. Homotypic and heterotypic serum and milk antibody to rotavirus in normal infected and vaccinated horses. *Vet. Microbiol.* **27**:231-244.

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

Bruce, M.G., Campbell, I., van Pinxteren, L., and D.R. Snodgrass. 1995. Intestinal cellular immunity after primary rotavirus infection. *J. Comp. Path.* **113**:155-164.

Bryden, A.S., Thouless, M.E., and T.H. Flewett. 1976. Rotavirus and rabbits. *Vet. Rec.* **99**:323.

Bull, C.G. and C.M. McKee. 1929. Respiratory immunity in rabbits. VII. Resistance to intranasal infection in the absence of demonstrable antibodies. *Am. J. Hyg.* **9**:490-499.

Bull, D.M. and M.A. Bookman. 1979. Isolation and functional characterization of human intestinal mucosal lymphoid cells. *J. Clin. Invest.* **59**:966-9754.

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

Burrows, W. and I. Havens. 1948. Studies on immunity to Asiatic cholera. V. The absorption of immunoglobulin from the bowel and its secretion in the urine and feces of experimental animals and human volunteers. *J. Infect. Dis.* **82**:231-250.

Burrows, W., Elliott, M.E., and I. Havens. 1947. Studies on immunity to Asiatic cholera. IV. The excretion of coproantibody in experimental enteric cholera in the guinea pig. *J. Infect. Dis.* **81**:261-281.

Burrows, W., Deupree, N.G., and D.E. Moore. 1950a. The effect of X-irradiation on experimental enteric cholera in the guinea pig. *J. Infect. Dis.* **87**:158-168.

Burrows, W., Deupree, N.G., and D.E. Moore. 1950b. The effect of X-irradiation on fecal and urinary antibody response. *J. Infect. Dis.* **87**:169-183.

Butcher, E., Rouse, R., Coffman, R., Nottenberg, C., Hardy, R., and I. Weismann. 1982. Surface phenotype of Peyer's patches germinal center cells. Implications in the role of germinal center in B cell differentiation. *J. Immunol.* **129**:2698-2707.

Butcher, E. 1986. The regulation of lymphatic traffic. *Curr. Top. Microbiol. Immunol.* **128**:85-122.

Carlens, O. 1928. Studien über das Lymphatische Gewebe des Darmkanals bei einigen Haustieren, mit besonderer Berücksichtigung der embryonalen Entwicklung, der Mengenverhältnisse und der Altersinvolution dieses Gewebenes im Dünndarm des Rindes. *Z. Anat. Entwicklungsgesch.* **86**:393-493.

Carlson, J.R. and R.L. Owen. 1987. Structure and functional role of Peyer's patches. In: "Immunopathology of the small intestine". M.N. Marsh Ed. John Wiley and Sons Ltd., Chichester. p. 21-40.

Castrucci, G., Frigeri, F., Ferrari, M., Cilli, V., Caleffi, F., Aldrovandi, V., and A. Nigrelli. 1984. The efficacy of colostrum from cows vaccinated with rotavirus in protecting calves to experimentally induced rotavirus infection. *Comp. Immun. Microbiol. Infect. Dis.* **7**:11-18.

Caul, E.O. 1994. Human coronaviruses. In: "Viral infections of the gastrointestinal tract". Kapikian, A.Z. Ed. Marcel Dekker, Inc., New York. p. 603-626.

Cerf-Bensussan, N. and D. Guy-Grand. 1991. Intestinal intraepithelial lymphocytes. *Gastroenterol. Clin. North Am.* **20**:549-576.

Cerretti, D.P., McKereghan, K., Larsen, A., Cosman, D., Gillis, S., and P.E. Baker. 1986a. Cloning, sequence and expression of bovine interferon-gamma. *J. Immunol.* **136**:4561-4564.

Cerretti, D.P., McKereghan, K., Larsen, A., Cantrell, M.A., Anderson, D., Gillis, S., Cosman, D., and P.E. Baker. 1986b. Cloning, sequence and expression of bovine interleukin 2. *Proc. Natl. Acad. Sci. USA* **83**:3223-3227.

Challacombe, S.J., Rahman, D., Jeffery, H., Davies, S.S., and D.T. O'Hagan. 1992. Enhanced secretory IgA and systemic IgG antibody responses after oral immunization with biodegradable microparticles containing antigen. *Immunology* **76**:164-168.

Chen, K.S. and G.V. Quinnan. 1989. Efficacy of inactivated influenza vaccine delivered by oral administration. *Curr. Top. Micribiol. Immunol.* **146**:101-106.

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

Childers, N.K., Bruce, M.G., and J.R. McGhee. 1989. Molecular mechanisms of immunoglobulin A defense. *Ann. Rev. Microbiol.* **43**:503-536.

Chodirker, W.B. and T.B. Tomasi. 1963. Gamma-globulins: quantitative relationships in human serum and nonvascular fluids. *Science* **14**:1080-1081.

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156-159.

Clark, H.F., Furukawa, T., Bell, L.M., Offit, P.A., Perrella, P.A., and S.A. Plotkin. 1986. Immune responses of infants and children to low-passage bovine rotavirus (strain WC3). *Am. J. Dis. Child.* **140**:350-356.

Clark, H.F., Borian, F.E., Modesto, K., and S.A. Plotkin. 1990. Serotype 1 reassortant of bovine rotavirus WC3, strain W179-9, induces a polytypic antibody response in infants. *Vaccine* **8**:327-332.

Clark, H.F., Offit, P.A., Ellis, R.W., Eiden, J.J., Krah, D., Shaw, A.R., Pichichero, M., Treanor, J.J., Borian, F.E., Bell, L.M., and S.A. Plotkin. 1996a. The development of multivalent bovine rotavirus (strain WC3) reassortant vaccine for infants. *J. Infect. Dis.* **174**(S1):S73-80

Clark, H.F., Offit, P.A., Ellis, R.W., Krah, D., Shaw, A.R., Eiden, J.J., Pichichero, M., and J.J. Treanor. 1996b. WC3 reassortant vaccines in children: brief review. *Arch. Virol.* **12** (S):187-198.

Clevers, H., MacHugh, N.D., Bensaid, A., Dunlap, S., Baldwin, C.L., Kaushal, A., Iams, K., Howard, C.J., and W.I. Morrison. 1990. Identification of a bovine surface antigen uniquely expressed on $CD4^{-}CD8^{-}$ T cell receptor γ/δ^{+} T lymphocytes. *Eur. J. Immunol.* **20**:809-817.

Coffin, S.E., Klinek, M., and P.A. Offit. 1995. Induction of virus-specific antibody production by lamina propria lymphocytes following intramuscular inoculation with rotavirus. *J. Infect. Dis.* **172**:874-878.

Coffman, R.L., Lebman, D.A., and B. Schrader. 1989. Transforming growth factor β specially enhances IgA production by lipopolysaccharide-stimulated murine B lymphocytes. *J. Exp. Med.* **170**:1039-1044.

Conner, M.E. and R.W. Darlington. 1980. Rotavirus infection in foals. *Am. J. Vet. Res.* **41**:1699-1703.

Conner, M.E., Gilger, M.A., Estes, M.K., and D.Y. Graham. 1991. Serologic and mucosal immune response to rotavirus infection in a rabbit model. *J. Virol.* **65**:2562-2571.

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

Coulson, B.S., Grimwood, K., Bishop, K., and G.L. Barnes. 1989. Evaluation of end-point titration, single dilution and capture enzyme immunoassays for measurements of antirotaviral IgA and IgM in infantile secretions and serum. *J. Virol. Meth.* **26**:53-66.

Coulson, B.S., Grimwood, K., Masendycz, P.J., Lund, J.S., Mermelstein, N., Bishop, R.F., and G.L. Barnes. 1990. Comparison of rotavirus immunoglobulin A coproconversion with other indices of rotavirus infection in a longitudinal study in childhood. *J. Clin. Microbiol.* **28**:1367-1374.

Coulson, B.S., Grimwood, K., Hudson, I.L., Barnes, G.L., and R.F. Bishop. 1992. Role of coproantibody in clinical protection of children during reinfection with rotavirus. *J. Clin. Microbiol.* **30**:1678-1684.

Craig, S.W. and J.J. Cebra. 1971. Peyer's patches: an enriched source of precursors for IgA-producing immunocytes in the rabbit. *J. Exp. Med.* **134**:188-200.

Cruickshank, R., Duguid, J.P., and R.H.A. Swain. 1965. Medical microbiology. A guide to the laboratory diagnosis and control of infection. E&S Livingstone Ltd, London.

Crouch, C.F. 1985. Vaccination against enteric rota and coronavirus in cattle and pigs: enhancement of lactogenic immunity. *Vaccine* **3**:284-291.

Cubitt, W.D. 1994. Caliciviruses. In: "Viral infections of the gastrointestinal tract". Kapikian, A.Z. Ed. Marcel Dekker, Inc., New York. p. 549-568.

Czerkinsky, C.S.J., Prince, S.M., Michalek, S., Jackson, S., Moldaveanu, Z., Russell, M.W., McGhee, J.R., and J. Mestecky. 1987. IgA antibody producing cells in peripheral blood after antigen ingestion: evidence for a common mucosal immune system in humans. *Proc. Natl. Acad. Sci. USA* **84**:2449-2463.

Davidson, G.P., Hogg, R.J., and C.P. Kirubakaran. 1983. Serum and intestinal immune responses to rotavirus enteritis in children. *Infect. Immun.* **40**:447-452.

Davies, A. 1922. An investigation into the serological properties of dysentery stools. *Lancet* **ii**:1009-1012.

Davies, S.S., Hardy, J., and J. Fara. 1986. Transit of pharmaceutical dosage forms through the small intestine. *Gut* **27**:886-892.

De Diego, M., Rodríguez, F., Alcaraz, C., Gómez, N., Alonsa, C., and J.M. Escribano. 1994. Characterization of the IgA and subclass IgG responses to neutralizing epitopes after infection of pregnant sows with the transmissible gastroenteritis virus or the antigenically related porcine respiratory coronavirus. *J. Gen. Virol.* **75**:2585-2593.

DeMol, P., Zissis, G., Butzler, J.P., Mutwewingabo, A., and F.E. André. 1986. Failure of live attenuated oral rotavirus vaccine. *Lancet* **ii**:108.

De Vries, P., van Binnendijk, R.S., van der Martel, P., Van Wezel., A.L., Voorma, H.O., Sundquist, B., Uytdehaag, F.G.C., and A.D.M.E. Osterhaus. 1988. Measles virus fusion protein presented in an immune-stimulating complex (iscom) induces haemolysis-inhibiting and fusion-inhibiting antibodies, virus-specific T cells and protection in mice. *J. Gen. Virol.* **69**:549-559.

Dertzbaugh, M.T. and C.O. Elson. 1991. Cholera toxin as a mucosal adjuvant. In: "Topics in vaccine adjuvant research". Spriggs, D. and W. Koff Eds. CRC Press, Florida. p. 119-132.

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

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

Dogget, T.A. and R. Curtiss. 1992. Delivery of antigens by recombinant virulent salmonella strains. *Adv. Exp. Med. Biol.* **327**:165-173.

Durkin, H.G., Bazin, H., and B.H. Waksman. 1981. Origin and fate of IgE bearing lymphocytes. I. Peyer's patches as differentiation sites of cells simultaneously bearing IgA and IgE. *J. Exp. Med.* **154**:640-648.

Dwyer, R.M., Powell K., Lyons, E., Donahue, J.M., Roberts, A.W., and M. Osborne. 1988. The aetiology of infectious diarrhoea in thoroughbred foals in central Kentucky. *Equin Vet. J.* **5** (S):59-60.

Ebrahimi, B., Roy, D.J., Bird, P., and D.R. Sargan. 1995. Cloning, sequencing and expression of the ovine interleukin-6 gene. *Cytokine* **7**:232-236.

Edelman, R. 1980. Vaccine adjuvants. *Rev. Infect. Dis.* **2**:370-383.

Eldridge, J., Gilley, R., Staas, J., Moldoveanu, Z., Meulbroek, J., and T. Tice. 1989. Biodegradable microspheres: vaccine delivery system for oral immunization. *Curr. Top. Microbiol. Immunol.* **146**:59-66.

Eldridge, J., Hammond, C., Meulbroek, J., Staas, J., Gilley, R., and T. Tice. 1990. Controlled vaccine release in the gut-associated lymphoid tissues. I. Orally administrated biodegradable microspheres target the Peyer's patches. *J. Contr. Rel.* **11**:205-214.

Entrican, G., McInnes, C.J., Rothel, J.S., and D.M. Haig. 1992. Kinetics of ovine interferon-gamma production: detection of mRNA and characterisation of biological activity. *Vet. Immunol. Immunopath.* **33**:171-178.

Erlandsen, S.L. and D.G. Chase. 1972. Paneth cell function: phagocytosis and intracellular digestion of intestinal microorganisms. I. Hexamita muris. *J. Ultrastruc. Res.* **41**:296-318.

Estes, M.K. and M.K. Cohen. 1989. Rotavirus gene structure and function. *Microbiol. Rev.* **53**:410-449.

Estes, M.K., Palmer, E.L., and J.F. Obijeski. 1983. Rotaviruses: a review. *Curr. Top. Microbiol. Immunol.* **105**:123-184.

Eugster, A.K. and T. Sidwa. 1979. Rotavirus in diarrheic feces of a dog. *VM Sac.* **74**:817-819.

Eugster, A.K., Strother, J., and D.A. Hartfield. 1978. Rotavirus (reovirus-like) infection of neonatal ruminants in a zoo nursery. *J. Wildl. Dis.* **14**:351-354.

Fahey, K.J., Snodgrass, D.R., Campbell, I., Dawson, A. McL., and C. Burrells. 1981. IgG_1 antibody in milk protects lambs against rotavirus diarrhoea. *Vet. Immunol. Immunopath.* **2**:27-33.

Fazekas de St. Groth, S. 1951. Studies in experimental immunology of influenza. IX. The mode of action of pathotopic adjuvants. *Austr. J. Exp. Biol. Med. Sci.* **29**:339-352.

Ferguson, A. and D.M.V. Parrott. 1972. The effect of antigen deprivation on thymus-dependent and thymus-independent lymphocytes in the small intestine of the mouse. *Clin. Exp. Immunol.* **12**:477-484.

Fernelius, A.L., Ritchie, A.E., Classick, L.G., Norman, J.O., and C.A. Mebus. 1972. Cell culture adaptation and propagation of a reovirus-like agent of calf diarrhea from a field outbreak in Nebraska. *Arch. Ges. Virusforschung* **37**:114-130.

Fijtman, N.L., Barrandeguy, M.E., Cornaglia, E.M., and A.A. Schudel. 1987. Varaitions and persistency of electropherotypes of bovine field isolates. *Arch. Virol.* **96**:275-281.

Fitzgerald, T.A., Munoz, M., Wood, A.R., and D.R. Snodgrass. 1995. Serological and genomic characterisation of group A rotaviruses in lambs. *Arch. Virol.* **140**:1541-1548.

Flewett, T.H. 1982. Clinical features of rotavirus infections. In: "Virus infections of the gastrointestinal tract" Tyrell, D.A.J. and A.Z. Kapikian Eds. Marcel Dekker Inc, New York. p. 125-146.

Flewett, T.H., Bryden, A.S., and H. Davies. 1973. Virus particles in gastroenteritis. *Lancet* **2**:1497.

Flewett, T.H., Bryden, A.S., Davies, H., Woode, G.N., Bridger, J.C., and J.M. Derrick. 1974a. Relationship between virus from acute gastroenteritis of children and newborn calves. *Lancet* **2**:61-63.

Flewett, T.H., Davies, H., Bryden, A.S., and M.J. Robertson. 1974b. Diagnostic electron microscopy of feces. II. Acute gastroenteritis associated with reovirus-like particles. *J. Clin. Pathol.* **27**: 608-614.

Flewett, T.H., Bryden, A.S., and H. Davies. 1975. Virus diarrhea in foals and other animals. *Vet. Rec.* **97**:477.

Fontaine, J., Favre, H., Fargeaud, D., Roulet, C., and M. Dupasquier. 1974. Inactivation du virus aphteux. Influence des agents inactivants sur la conservation du virion. *Rapport de la Réunion du Groupe de Recherches de la Commission Européenne pour le Contrôle de la Fièvre Aphteuse.*

Forrest, B.D. 1992. Indirect measurement of intestinal immune responses to an orally administered attenuated bacterial vaccine. *Infect. Immun.* **60**:2023-2029.

Fossum, C., Bergstrom, M., Lövgren, K., Watson, D.L., and B. Morein. 1990. Effect of iscoms and their adjuvant moiety (matrix) on the initial proliferation and Il-2 responses: comparison of spleen cells from mice inoculated with iscoms and/or matrix. *Cell Immunol.* **129**:414-425.

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

Franco, M.A., Tin, G., and H.B. Greenberg. 1997a. $CD8^+$ T cells can mediate almost complete short-term and partial long-term immunity to rotavirus in mice. *J. Virol.* **71**:4165-4170.

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

Frey, A., Giannasca, K.T., Weltzin, R., Giannasca, P.J., Reggio, H., Lencer, W.I., and M.R. Neutra. 1996. Role of glycocalyx in regulating access of microparticles to apical plasma membranes of intestinal epithelial cells: implications for microbial attachment and oral vaccine targeting. *J. Exp. Med.* **184**:1045-1059.

Fukudome, K., Yoshie, O., and T. Konno. 1989. Comparison of human, simian, and bovine rotaviruses for requirement of sialic acid in hemagglutination and cell adsorption. *Virology* **172**:196-205.

Fynan, E.F., Webster, R.G., Fuller, D.H., Haynes, J.R., Sanroro, J.C., and H.L. Robinson. 1993. DNA vaccines: protective immunization by parenteral, mucosal, and gene-gun inoculations. *Proc. Natl. Acad. Sci. USA* **90**:11478-11482.

Germann, T. and E. Rüde. 1995. Interleukin-12. *Int. Arch. Allergy Immunol.* **108**:103-112.

Ghazi, H.O., Potter, C.W., Smith, T.L., and R. Jennings. 1995. Comparative antibody responses and protection in mice immunised by oral or parenteral routes with influenza virus subunit antigens in aqueous form or incorporated into ISCOMs. *J. Med. Microbiol.* **42**:53-61.

Giannasca, P.J. and M.R. Neutra. 1994. Interactions of microorganisms with intestinal M cells: mucosal invasion and induction of secretory immunity. *Infect. Ag. Dis.* **2**:242-248.

Gibson, P.R., Verhaar, H.J.J., Selby, W.S., and D.P. Jewell. 1984. The mononuclear cells of human mesenteric blood, intestinal mucosa, and mesenteric lymph nodes: compartmentalisation of NK cells. *Clin. Exp. Immunol.* **56**:445-452.

Girard, H.C. Bayramoglu, O., Erol, N., and A. Burgut. 1977. Inactivation of O_1 FMD virus by the binary ethylene imine (BEI). *Bull. Off. Int. Epiz.* **87**:201-217.

Glass, R.I., Lang, D.R., Ivanoff, B.N., and R.W. Compans. 1996. Introduction: rotavirus - from basic research to a vaccine. *J. Infect. Dis.* **174** (S1):S1-2.

Godson, D.L., Campos, M., and L.A. Babiuk. 1992. The role of intraepithelial leukocyte-mediated cytotoxicity in enteric antiviral defense. *Viral Immunol.* **5**:1-13.

Goodman, T. and L. Lefrancois. 1988. Expression of the γδ T-cell receptor on intestinal $CD8^+$ intraepithelial lymphocytes. *Nature* **333**:855-858.

Goodman, T. and L. Lefrancois. 1989. Intraepithelial lymphocytes. Anatomical site, not T cell receptor form, dictates phenotype and function. *J. Exp. Med.* **170**:1569-1581.

Grencis, R.K., Hültner, L., and K.J. Else. 1991. Host protective immunity to *Trichinella spiralis* in mice: activation of Th cells subsets and lymphokine secretion in mice expressing different response phenotypes. *Immunology* **74**:329-332.

Grimwood, K., Lund, J.C.S., Coulson, B.S., Hudson, I.L., Bishop, R.F., and G.L. Barnes. 1988. Comparison of serum and mucosal antibody responses following severe acute rotavirus gastroenteritis in young children. *J. Clin. Microbiol.* **26**:732-738.

Groene, W.S. and R.D. Shaw. 1992. Psoralen preparation of antigenically intact noninfectious rotavirus particles. *J. Virol. Meth.* **38**:93-102.

Haan, A. de, Renegar, K.B., Small Jr., P.A., and J. Wilschut. 1995. Induction of a secretory IgA response in the murine female urogenical tract by immunization of the lungs with liposome-supplemented viral subunit antigen. *Vaccine* **13**:613-616.

Hammond, M. and P.J. Timoney. 1983. An electron microscopic study of the viral flora in cases of canine gastroenteritis. *Cornell. Vet.* **73**:82-97.

Hanlon, P., Hanlon, L., Marsh, V., Byass, P., Shenton, F., Hassan-King, M., Jobe, O., Sillah, H., Hayes, R., M'Boge, B.H., Whittle, H.C., and B.M. Greenberg. 1987. A trial of an attenuated bovine rotavirus vaccine (RIT 4237) in Gambian children. *Lancet* **ii**:1342-1345.

Hannant, D., Mumford, J.A., and D.M. Jessett. 1988. Duration of circulating antibody and immunity following infection with equine influenza virus. *Vet. Rec.* **122**:125-128.

Hein, W.R. and C.R. MacKay. 1991. Prominence of γδ T cells in the ruminant immune system. *Immunol. Today* **12**:30-34.

Hein, W.R., Dudler, L., and C.R. MacKay. 1989. Surface expression of differentiation antigens on lymphocytes in the ileal and jejunum Peyer's patches of lambs. *Immunology* **68**:365-370.

Hendrickson, B.A., Rindisbacher, L., Cothesy, B., Kendall, D., Waltz, D.A., Neutra, M.R., and J.G. Seidman. 1996. Lack of association of secretory component with IgA in J chain-deficient mice. *J. Immunol.* **157**:750-754.

Heremans, J.F., Heremans, M.T., and H.E. Schultze. 1959. Isolation and description of a few properties of the β_{2A}-globulin of human serum. *Clin. Chim. Acta* **4**:96-102.

Herring, A.J., Inglis, N.F., Ojeh, C.K., Snodgrass, D.R., and J.D. Menzies. 1982. Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver-stained polyacrylamide gels. *J. Clin. Microbiol.* **16**:473-477.

Herrmann, J.E., Chen, S.C., Fynan, E.F., Santoro, J.C., Greenberg, H.B., Wang, S., and H.L. Robinson. 1996. Protection against rotavirus infections by DNA vaccination. *J. Infect. Dis.* **174** (S1):S93-97.

Heufler, C., Koch, F., Stanzl, U., Topar, G., Wysicka, M., Trinchieri, G., Enk, A., Steinman, R.M., Romani, N., and G. Schuler. 1996. Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-γ production by T helper 1 cells. *Eur. J. Immunol.* **26**:659-668.

Hjelt, K., Graubelle, P.C., Schiotz, P.O., Andersen, L., and P.A. Krasilnikoff. 1985a. Intestinal and serum immune response to a naturally acquired rotavirus gastroenteritis in children. *J. Ped. Gastr. Nutr.* **4**:60-66.

Hjelt, K., Graubelle, P.C., Nielsen, O.H., Schiotz, and P.A. Krasilnikoff. 1985b. Rotavirus antibodies in the mother and her breast-fed infant. *J. Ped. Gastr. Nutr.* **4**:414-420.

Hjelt, K., Graubelle, P.C., Andersen, L., Howitz, P., and P.A. Krasilnikoff. 1986. Antibody response in serum and intestine in children up to six months after naturally acquired rotavirus gastroenteritis. *J. Ped. Gastr. Nutr.* **5**:74-80.

Hobbs, J.R. and G.W. Hepner. 1968. Deficiency of M-globulin in coeliac disease. *Lancet* **i**:217-220.

Hoglund, S., Dalsgaard, K., Lovgren, K., Sundquist, B., Osterhaus, A., and B. Morein. 1989. ISCOMs and immunostimulation with viral antigens. In: "Subcellular Biochemistry". Harris, J.R.I. Ed. Plenum Publ., New York. p. 39-68.

Holmes, I.H. 1983. Rotaviruses. In: "The Reovirdea". Joklik W.K. Ed. Plenum Press, New York. p. 359-423.

Holmgern, J. 1991. Mucosal immunity and vaccination. *FEMS Microbiol. Immunol.* **89**:1-10.

Holmgern, J., Czerkinsky, C., Lycke, N., and A. Svennerholm. 1992. Mucosal immunity: implications for vaccine development. *Immunobiol.* **184**:157-179.

Holmgern, J., Lycke, N., and C. Czerkinsky. 1993. Cholera toxin and cholera B subunit as oral-mucosal adjuvant and antigen vector systems. *Vaccine* **11**:1179-1184.

Hörnqvist, E., Goldschmidt, T.J., Holmdahl, R., and N. Lycke. 1991. Host defense against cholera toxin is strongly $CD4^+$ T cell dependent. *Infect. Immun.* **59**:3630-3638.

Hoshino, Y. and A.Z. Kapikian. 1994. Rotavirus antigens. In: "Rotaviruses". Ramig, R.F. Ed. Springer-Verlag, Berlin. p. 179-227.

Husband, A.J. and J.L. Gowans. 1978. The origin and antigen-dependent distribution of IgA-containing cells in the intestine. *J. Exp. Med.* **148**:1146-1160.

Inman, L. and J. Cantey. 1983. Specific adherence of *E. coli* (strain RDEC-1) to membranous (M) cells of the Peyer's patches in *E. coli* diarrhea in the rabbit. *J. Clin. Invest.* **71**:1-8.

Jackson, R.J., Fujihashi, K., Xu-Amano, J., Kiyono, H., Elson, C.O., and J.R. McGhee. 1993. Optimizing oral vaccines: induction of systemic and mucosal B-cell and antibody responses to tetanus toxoid by use of cholera toxin as an adjuvant. *Infect. Immun.* **61**:4272-4279.

Jarry, A., Robaszkiewicz, M., Brousse, N., and F. Potet. 1989. Immune cells associated with M cells in the follicle-associated epithelium of Peyer's patches in the rat. *Cell Tissue Res.* **255**:293-298.

Jones, R.C., Hughes, C.S., and R.R. Henry. 1979. Rotavirus infection in commercial laying hens. *Vet. Rec.* **104**:22.

Jones, P.D., Tha Hla, R., Morein, B., and G.L. Ada. 1988. Cellular immune responses in the murine lung to local immunization with influenza A virus glycoproteins in micelles and iscoms. *Scand. J. Immunol.* **27**:645-652.

Kagnoff, M.F. 1996. Mucosal immunology: new frontiers. *Immunol. Today* **17**:57-59.

Kantele, A., Kantele, J.M., Savilahti, E., Westerholm, M., Arvilommi, H., Lazarovits, A., Butcher, E.C., and P.H. Mäkelä. 1997. Homing potentials of circulating lymphocytes in humans depend on the site of activation. Oral, but not parenteral typhoid vaccination induces circulating antibody-secreting cells that all bear homing receptors directing them to the gut. *J. Immunol.* **158**:574-579.

Kapikian, A.Z. 1994. Norwalk and norwalk-like viruses. In: "Viral infections of the gastrointestinal tract". Kapikian, A.Z. Ed. Marcel Dekker Inc. New York. p. 471-518.

Kapikian, A.Z., and R.M. Chanock. 1990. Rotaviruses, In: "Virology". Fields, B.N. and D.M. Knife Eds. Raven Press, New York. p. 1353-1404.

Kapikian, A.Z., Wyatt, R.G., Dolin, R., Thornhill, T.S., Kalica, A.R., and R.M. Chanock. 1972. Visualization by electron microscopy of a 26nm particle associated with acute infectious non-bacterial gastroenteritis. *J. Virol.* **10**:1075-1081.

Kapikian, A.Z., Kim, H.W., Wyatt, R.G., Rodriquez, W.J., Ross, S., Cline, W.L. Parrott, R.H., and R.M. Chanock. 1974. Reovirus-like agent in stools: association with infantile diarrhea and development of serologic tests. *Science* **185**:1049-1053.

Kapikian, A.Z., Flores, J., Hoshino, Y., Midthun, K., Gorziglia, M., Green, K.Y., Chanock, R.M., Potash, L., Sears, S.D., Clements, M.L., Halsey, N.A., Black, R.E., and I. Perez-Schael. 1989. Prospects for development of a rotavirus vaccine against rotavirus diarrhea in infants and young children. *Rev. Infect. Dis.* **11**:S539-S546.

Kapikian, A.Z., Flores, J., Vesikari, T., Ruuske, T., Madore, H.P., Green, Y., Gorziglia, M., Hoshino, Y., Chanock, R.M., Midthun, K., and I. Perez-Schael. 1991. Recent advances in development of a rotavirus vaccine for prevention of severe diarrheal illness of infants and young children. In: "Immunology of milk and the neonate" Mestecky, J., Blair, C., and P.L. Ogra Eds. Plenum Press, New York. p. 255-264.

Kapikian, A.Z., Hoshino, Y., Chanock, R.M., and I. Pérez-Schal. 1996. Efficacy of a quadrivalent rhesus rotavirus-based human rotavirus vaccine aimed at preventing severe rotavirus diarrhea in infants and young children. *J. Infect. Dis.* **174** (S):S65-S72.

Karupiah, G., Coupar, B.E.H., Andrew, M.E., Boyle, D.B., Philips, S.M., Müllbacher, A., Blanden, R.V., and I.A. Ramshaw. 1990. Elevated natural killer cell responses in mice infected with recombinant vaccinia virus encoding murine IL-2. *J. Immunol.* **144**:290-298.

Kawanishi, H., Saltzman, L.E., and W. Strober. 1983a. Mechanisms regulating IgA class-specific immunoglobulin production in murine gut-derived associated lymphoid tissues. I. T cells derived from Peyer's patches that switch sIgM B cells to sIgA B cells in vitro. *J. Exp. Med.* **157**:443-450.

Kawanishi, H., Saltzman, L.E., and W. Strober. 1983b. Mechanisms regulating IgA class-specific immunoglobulin production in murine gut-derived associated lymphoid tissue. II. Terminal differentiation of post switch sIgA-bearing Peyer's patch B cells. *J. Exp. Med.* **158**:649-668.

Kawanishi, H., Ozato, K., and W Strober. 1985. The proliferative response of cloned Peyer's patch switch T cells to syngeneic and allogeneic stimuli. *J. Immunol.* **34**:3586-3591.

Kelsall, B.L. and W. Strober. 1996. Distinct populations of dendritic cells are present in the subepithelial dome and T cell regions of the murine Peye's patch. *J. Exp. Med.* **183**:237-247.

Keren, D.F. 1992. Antigen processing in the mucosal immune system. *Immunology* **4**:217-226.

Kern, S.E., Keren, D.F., and C.L. Pierson. 1987. Bacterial overgrowth and mucosal changes in isolated (Thiry-Vella) ileal loops in rabbits: effects of intraluminal antibodies. *Lab. Invest.* **57**:336-341.

Khoury, C.A., Moser, C.A., Speaker, T.J., and P.A. Offit. 1995. Oral inoculation of mice with low doses of microencapsulated, noninfectious rotavirus induces virus-specific antibodies in gut-associated lymphoid tissue. *J. Infect. Dis.* **172**:870-874.

Kimman, T.G. and F. Westenbrink. 1990. Immunity to human and bovine respiratory syntical virus. *Arch. Virol.* **112**:1-25.

Kimman, T.G., Westenbrink, R., Schreuder, B.E.C., and P.J. Straver. 1987. Local and systemic antibody responses to bovine respiratory syntical virus infection and reinfection in calves with and without maternal antibodies. *J. Clin. Microbiol.* **25**:1097-1106.

Kohbata, S., Yokoyama, H., and E. Yabuuchi. 1986. Cytopathogenic effect of *Salmonella typhi* GIFU 10007 on M cells of murine ileal Peyer's patches in ligated ileal loops: an ultrastructural study. *Microbiol. Immunol.* **30**:1225-1237.

Kraal, G., Weissman, I.L., and E.C. Butcher. 1983. Differences in vivo distribution and homing of T cell subsets to mucosal vs nonmucosal lymphoid organs. *J. Immunol.* **130**:1097-1102.

Kraft, L.M. 1957. Studies on the etiology and transmission of epidemic diarrhea of infant mice. *J. Exp. Med.* **101**:743-755.

Kroese, G.M., Butcher, E.C., Stall., A.M., Lalor, P.A., Adams, S., and L.A. Herzenberg. 1989. Many of the IgA producing plasma cells in murine gut are derived from self-replenishing precursors in the peritoneal cavity. *Int. Immunol.* **141**:713-720.

Kurt-Jones, E.A., Liano, D., Hayglass, K.A., Benacerraf, B., Sy, M., and A.K. Abbas. 1988. The role of antigen-presenting B cells in T cell priming *in vitro*. Studies of B cell deficient mice. *J. Immunol.* **140**:3773-3778.

Kurtz, J.B. 1994. Astroviruses. In: "Viral infections of the gastrointestinal tract". Kapikian, A.Z. Ed. Marcel Dekker, Inc., New York. p. 569-580.

La Bonnardiere, C., Cohen, J., and M. Contrepois. 1981. Interferon activity in rotavirus infection newborn calves. *Ann. Rech. Vet.* **12**:85-91.

Lamm, M.E. 1976. Cellular aspects of Immunoglobulin A. *Adv. Immunol.* **22**:223-290.

Landsverk, T., Halleraker, M., Aleksandersen, M., McClure, S., Hein, W., and L. Nicander. 1991. The intestinal habitat for organized lymphoid tissues in ruminants; comparative aspects of structure, function and development. *Vet. Immunol. Immunopath.* **28**:1-16.

Larsen, H.J. and T. Landsverk. 1986. Distribution of T and B lymphocytes in the jejunum and ileocaecal Peyer's patches of lambs. *Res. Vet. Sci.* **40**:105-111.

Lebman, D.A., Griffin, P.M., and J.J. Cebra. 1987. Relationship between expression of IgA by Peyer's patch cells and functional IgA memory cells. *J. Exp. Med.* **166**:1405-1418.

Lebman, D.A., Nomura, D.Y., Coffman, R.L., and F.D. Lee. 1990. Molecular characterization of germ-line immunoglobulin A transcripts produced during transforming growth factor type β-induced isotype switching. *Proc. Natl. Acad. Sci. USA* **87**:3962-3966.

Lecatsas, G. 1972. Electron microscopic and serological studies on simian virus SA11 and the "related" O agent. *Onderstepoort J. Vet. Res.* **39**:133-138.

Lecce, J.G., King, M.W., and R. Mock. 1976. Reovirus-like agent associated with fatal diarrhea in neonatal pigs. *Infect. Immun.* **14**:816-825.

Lecce, J.G., Cummins, J.M., and A.B. Richards. 1990. Treatment of rotavirus infection in neonate and weaning pigs using natural human interferon alpha. *Mol. Biother.* **2**:211-216.

Lefrancois, L. 1994. Basic aspects of intraepithelial lymphocyte immunobiology. In: "Handbook of mucosal immunology". Ogra, P.L., Mestecky, J., Lamm, M.E., Strober, W., McGhee, J.R., and J. Bienenstock Eds. Academic Press, Inc., San Diego. p. 287-297.

Liang, X., Lamm, M.E., and J.G. Nedrud. 1988. Oral administration of cholera toxin-sendai virus conjugate potentiates gut and respiratory immunity against sendai virus. *J. Immunol.* **141**:1495-1501.

Light, J.S. and H.L. Hodes. 1943. Studies on epidemic diarrhea of the newborn: isolation of a filterable agent causing diarrhea in calves. *Am. J. Public Health* **33**:1451-1454.

Light, J.S. and H.L. Hodes. 1949. Isolation from cases of infantile diarrhea of a filterable agent causing diarrhea in calves. *J. Exp. Med.* **90**:113-135.

Liu, L.M. and G.G. MacPherson. 1994. The role of dendritic cells in the uptake and presentation of oral antigens. In: "In Vivo Immunology". Heinen, H., *et al.* Eds. Plenum Press, New York. p. 81-86.

London, S.D., Cebra, J.J., and D.H. Rubin. 1989. Intraepithelial lymphocytes contain virus-specific, MHC-restricted cytotoxic T cell precursors after gut mucosal immunization with reovirus serotype 1/Lang. *Reg. Immunol.* **2**:98-102.

Lövgren, K. 1988. The serum antibody response ditributed in subclass and isotypes after intranasal and subcutaneous immunisation with influenza virus immunostimulating complexes. *Scand. J. Immunol.* **27**:241-245.

Lövgren, K., Kaberg, H., and B. Morein. 1990. An experimental influenza subunit vaccine (ISCOM): induction of protective immunity to challenge infection in mice after intranasal or subcutaneous administration. *Clin. Exp. Immunol.* **82**:435-439.

Lycke, N. and J. Holmgren. 1986. Strong adjuvant properties of cholera toxin on gut mucosal immune responses to orally presented antigens. *Immunology* **59**:301-308.

Lycke, N., Lindholm, L., and J. Holmgren. 1985. Cholera antibody production *in vitro* by peripheral blood lymphocytes following oral immunization of humans and mice. *Clin. Exp. Immunol.* **62**:39-47.

Lyscom, N. and D.E. Brueton. 1982. Intraepithelial, lamina propria and Peyer's patch lymphocytes of the rat small intestine: isolation and characterisation in terms of immunoglobulin markers and receptors for monoclonal antibodies. *Immunology* **45**:775-783.

MacDonald, T.T. and P.B. Carter. 1979. Requirement for a bacterial flora before mice generate cells capable of mediating the delayed hypersensitivity reaction to sheep red blood cells. *J. Immunol.* **122**:2624-2629.

MacKay, C.R., Hein, W.R., Brown, M.H., and P. Matzinger. 1988. Unusual expression of CD2 in sheep: implications for T cell interactions. *Eur. J. Immunol.* **18**:1681-1688.

MacKay, C.R., Beya, M.F., and P. Matzinger. 1989. Gamma-delta T cells express a unique surface molecule appearing late during thymic development. *Eur. J. Immunol.* **19**:1477-1483.

MacKay, C.R., Marston, W.L., and L. Dudler. 1990. Naive and memory T cells show distinct pathways of lymphocyte recirculation. *J. Exp. Med.* **171**:801-817.

MacKenzie, C.D., Taylor, P.M., and B.A. Askonis. 1989. Rapid recovery of lung histology correlates with clearance of influenza virus by specific $CD8^+$ cytotoxic cells. *Immunology* **67**:375-381.

McDermott, M.R., Lukacher, A.E., Braciale, V.L., Braciale, T.J., and J. Bienenstock. 1987. Characterization and *in vivo* distribution of influenza-virus-specific T-lymphocytes in the murine respiratory tract. *Am. Rev. Respir. Dis.* **135**:245-249.

McGhee, J.R. and H. Kiyono. 1993. New perspectives in vaccine development: mucosal immunity to infections. *Infect. Ag. Dis.* **2**:55-73.

McGhee, J.R., Mestecky, J., Dertzbaugh, M.T., Eldridge, J.H., Hirasawa, M., and H. Kiyono. 1992. The mucosal immune system: from fundamental concepts to vaccine development. *Vaccine* **10**:75-88.

McGuire, T.C. and T.B. Crawford. 1973. Passive immunity in the foal: measurement of immunoglobulin classes and specific antibody. *Am. J. Vet. Res.* **34**:1299-1303.

McLean, B. and I.H. Holmes. 1980. Transfer of antirotaviral antibodies from mothers to their infants. *J. Clin. Microbiol.* **12**:320-325.

McNeal, M.M. and R.L. Ward. 1995. Long-term production of rotavirus antibody and protection against reinfection following a single infection of neonatal mice with murine rotavirus. *Virology* **211**:474-480.

McNeal, M.M., Broome, R.L., and R.L. Ward. 1994. Active immunity against rotavirus infection in mice is correlated with viral replication and titers of serum rotavirus IgA following vaccination. *Virology* **204**:642-650.

McNeal, M.M., Barone, K.S., Rae, M.N., and R.L. Ward. 1995. Effector functions of antibody and $CD8^+$ cells in resolution of rotavirus infection and protection against reinfection in mice. *Virology* **214**:387-397.

McNulty, M.S. Allan, G.M., Thompson, D.J., and J.D. Boyle. 1978. Antibody to rotavirus in dogs and cats. *Vet. Rec.* **102**:534-535.

Madden, D.R., Gorga, J.C., Strominger, J.L., and D.C. Wiley. 1991. The structure of HLA-B27 reveals nonamer self-peptides bound in an extended conformation. *Nature* **353**:321-325.

Maddox, J.F., MacKay, C.R., and M.R. Brandon. 1985. Surface antigens, SBU-T4 and SBU-T8 of sheep lymphocytes subsets by monoclonal antibodies. *Immunology* **55**:739-748.

Malherbe, H.H. and R. Harwin. 1963. The cytopathic effects of vervet monkey viruses. *S. Afr. Med. J.* **37**:401-411.

Malherbe, H.H. and M. Strickland-Cholmley. 1967. Simian virus SA11 and the related O agent. *Arch. Virol.* **22**:235-245.

Maloy, K.J., Donachie, A.M., and A.M. Mowat. 1995. Induction of Th1- and Th2-$CD4^+$ T cell responses by oral or parenteral immunization with ISCOMs. *Eur. J. Immunol.* **25**:2835-2841.

Martines, J., Philips, M., and R.G. Feachem. 1991. Diarrheal diseases. In: "Evolving health priorities in developing countries". Jameson, D.T. and W.H. Mosley Eds. World Bank, Washington, DC. p. 1-49.

Marx, P.A., Compans, R.W., Gettie, A., Staas, J.K., Gilley, R.M., Mulligan, M.J., Yamshchikov, G.V., Chen, D., and J.H. Eldridge. 1993. Protection against vaginal SIV transmission with microencapsulated vaccine. *Science* **260**:1323-1327.

Maxson R.T., Jackson, R.J., and S.D. Smith. 1995. The protective role of enteral IgA supplementation in neonatal gut origin sepsis. *J. Pediatr. Surg.* **30**:231-234.

Mayrhofen, G., Pugh, C.W., and A.N. Barclay. 1983. The distribution, ontogeny and origin in the rat of Ia-positive cells with dendritic morphology and of Ia antigen in epithelia, with special reference to the intestine. *Eur. J. Immunol.* **13**:112-122.

Mazanec, M.B., Nedrud, J.G., Kaetzel, C.S., and M.E. Lamm. 1993. A three-tiered view of the role of IgA in mucosal defense. *Immunol. Today* **14**:430-435.

Mebus, C.A., Underdahl, N.R., Rhodes, M.R., and M. Twiehaus. 1969. Calf diarrhea (scours): reproduced with a virus from a field outbreak. *Bull. Neb. Agric. Exp. Station* **233**:1-16.

Mebus, C.A., Kono, M., Underdahl, N.R., and M.J. Twiehaus. 1971a. Cell culture propagation of neonatal calf diarrhea (scours) virus. *Can. Vet. J.* **12**:69-72.

Mebus, C.A., Stair, E.L., Underdahl, N.R., and M.J. Twiehaus. 1971b. Pathology of neonatal calf diarrhea induced by reo-like virus. *Vet Pathol.* **8**:490-505.

Méndez, E., Arias, C.F., and S. López. 1993. Binding to sialic acids is not an essential step for entry of animal rotaviruses to epithelial cells in culture. *J. Virol.* **76**:5253-5259.

Merchant, A.A., Groene, W.S., Cheng, E.H., and R.D. Shaw. 1991. Murine intestinal antibody response to heterologous rotavirus infection. *J. Clin. Microbiol.* **29**:1693-1701.

Mestecky, J. 1987. The common mucosal immune system and current strategies for induction of immune responses in external secretions. *J. Clin. Immunol.* **7**:265-276.

Mestecky, J. and J.R. McGhee. 1987. Immunoglobulin A (IgA): molecular and cellular interactions involved in IgA biosynthesis and immune responses. *Adv. Immunol.* **40**:153-241.

Mestecky, J., Russell, M.W., Jackson, S., and T.A. Brown. 1986. The human IgA system: a reassessment. *Clin. Immunol. Immunopathol.* **40**:105-114.

Midthun, K. and A.Z. Kapikian. 1996. Rotavirus vaccines: an overview. *Clin. Microbiol. Rev.* **9**:423-434.

Minamoto, N., Oki, K., Tomita, M., Kinjo, T., and Y. Suzuki. 1988. Isolation and characterization of rotavirus from feral pigeon in mammalian cell cultures. *Epidemiol. Infect.* **100**:481-492.

Mochizuli, M. and M. Ata. 1986. Characterization of a canine rotavirus hemagglutinin. *Jpn. J. Vet. Sci.* **48**:1011-1014.

Mojaverian, P., Ferguson, R., Vlasses, P., Rocci, M., Oren, A., Fix, J., Caldwell, L., and C. Gardner. 1985. Estimation of gastric residence time of the Heidelberg capsule in humans: effect on varying food composition. *Gastroenterology* **89**:392-397.

Moldoveanu, Z., Clements, M.L., Prince, S.J., Murphy, B.R., and J. Mestecky. 1995. Human immune responses to influenza virus vaccines administered by systemic or mucosal routes. *Vaccine* **13**:1006-1012.

Morein, B. and L. Åkerblom. 1992. The iscom-an approach to subunit vaccines. In: "Recombinant DNA Vaccines". Isaacson, R.I. Ed. Macel Dekker, Inc., New York. p. 369-386.

Morein, B., Ekstrom, J., and K. Lovgren. 1990. Increased immunogenicity of a non-amphipathic protein (BSA) after inclusion into iscoms. *J. Immunol. Meth.* **128**:177-181.

Morgan, A.J., Finerty, S., Lövgren, K., Scullion, F.T., and B. Morein. 1988. Prevention of Epstein-Barr (EB) virus induced lymphoma in cottontop tamarins by vaccination with the EB virus envelope glycoprotein gp340 incorporated into iscoms. *J. Gen. Virol.* **69**:2093-2096.

Moser, C.A., Speaker, T.J., Berlin, J.A., and P.A. Offit. 1996. Aqueous-based microencapsulation enhances virus-specific humoral immune responses in mice after parenteral inoculation. *Vaccine* **14**:1235-1238.

Mosley, R.L., Whetsell, M., and J.R. Klein. 1991. Proliferative properties of murine intestinal intraepithelial lymphocytes (IEL): IEL expressing TCR$\alpha\beta$ or TCR$\gamma\delta$ are largely unresponsive to proliferative signals mediated via conventional stimulation of the CD3-TCR complex. *Int. Immunol.* **3**:563-569.

Mosmann, T.R. and R.L. Coffman. 1989. Th1- and Th2- cells: different patterns of lymphokine secretions lead to different functional properties. *Ann. Rev. Immunol.* **7**:145-173.

Mosmann, T.R. and K.W. Moore. 1991. The role of IL-10 in crossregulation of Th1- and Th2-responses. *Immunol. Today* **12**:A49-A53.

Mosmann, T.R., Cherwinsky, H., Bond, M.W., Giedlin, M.A., and R.L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* **136**:2348-2357.

Möstl, K. and F. Bürke. 1988. Incidence of diarrhoea and of rotavirus- and coronavirus-shedding in calves, whose dams had been vaccinated with an experimental oil-adjuvanted vaccine containing bovine rotavirus and bovine coronavirus. *J. Vet. Med.* **35**:186-196.

Mowat, A.M. 1987. The cellular basis of gastrointestinal immunity. In: "Immunopathology of the small intestine". M.N. Marsh Ed. John Wiley & Sons, Chichester. p. 41-73.

Mowat, A.M. 1990. Human intraepithelial lymphocytes. *Springer Semin. Immunopath.* **12**:165-190.

Mowat, A.M. 1994. Oral tolerance and regulation of immunity to dietary antigens. In: "Handbook for Mucosal Immunology". Orga, P.L., Mestecky, J., Lamm, M.E., Strober, W., McGhee, J.R., and J. Bienenstock Eds. Academic Press, Florida. p. 185-201.

Mowat, A.M. and A.M. Donachie. 1991. ISCOMS-A novel strategy for mucosal immunizations? *Immunol. Today* **12**:383-385.

Mowat, A.M., McInnes, I.B., and D.M.V. Parrot. 1989. Functional properties of intra-epithelial lymphocytes from mouse small intestine. IV. Investigation of the proliferative capacity of IEL using phorobol ester and calciumionophore. *Immunology* **66**:398-403.

Mowat, A.M., Donachie, A.M., Reid, G., and O. Jarrett. 1991. Immune-stimulating complexes containing Quil A and protein antigen prime class I MHC-restricted T lymphocytes *in vivo* and are immunogenic by the oral route. *Immunology* **72**:317-322.

Mowat, A.M., Maloy, K.J., and A.M. Donachie. 1993. Immune-stimulating complexes as adjuvants for inducing local and systemic immunity after oral immunization with protein antigen. *Immunology* **80**:527-534.

Much, D. and I. Zajac. 1972. Purification and characterization of epizootic diarrhea of infant mice virus. *Infect. Immun.* **6**:1019-1024.

Mukkur, T.K.S., Walker, K.H., Baker, P., and D. Jones. 1995. Systemic and mucosal intestinal antibody response of sheep immunized with aromatic-dependent live or killed *Salmonella typhimurium*. *Comp. Immun. Microbiol. Infect. Dis.* **18**:27-39.

Mumford, J.A., Jessett, D., Dunleavy, U., Wood, J., Hannant, D., Sundquist, B., and R.F. Cook. 1994. Antigenicity and immunogenicity of experimental equine influenza ISCOM vaccines. *Vaccine* **12**:857-863.

Murakami, M. and T. Honjo. 1995. Involvement of B-1 cells in mucosal immunity and autoimmunity. *Immunology Today* **16**:534-539.

Murphy, B.R. 1994. Mucosal immunity to viruses. In: "Handbook for Mucosal Immunology". Orga, P.L., Mestecky, J., Lamm, M.E., Strober, W., McGhee, J.R., and J. Bienenstock Eds. Academic Press, Florida. p. 333-346.

Murphy, B.R. and M.L. Clements. 1989. The systemic and mucosal immune response of humans to influenza A virus. *Curr. Top. Microbiol. Immunol.* **146**:107-116.

Murray, P.D., McKenzie, D.T., Swain, S.L., and M.F. Kagnoff. 1987. Interleukin 5 and interleukin 4 produced by Peyer's patch T cells selectively enhance immunoglobulin A expression. *J. Immunol.* **139**:2669-2674.

Nagi, A.M. and L.A. Babiuk. 1987. Bovine gut-associated lymphoid tissue-Morphologic and functional studies. I. Isolation and characterization of leukocytes from the epithelium and lamina propria of bovine small intestine. *J. Immunol. Methods* **105**:23-37.

Nakaoka, R., Tabata, Y., and Y. Ikada. 1995. Potentially of gelatin microsphere as immunological adjuvant. *Vaccine* **13**:653-661.

Nedrud, J.D., Liang, X., Hague, N., and M.E. Lamm. 1987. Combined oral/nasal immunization protects mice from sendai virus infection. *J. Immunol.* **139**:3484-3492.

Netherwood, T., Wood, J.L.N., Townsend, H.G.G., Mumford, J.A., and N. Chanter. 1996. Foal diarrhea between 1991 and 1994 in the United Kingdom associated with *Clostridium perfringens*, rotavirus, *Strongyloides westeri*, and *Cryptosporidium* spp. *Epidemiol. Infect.* **117**:375-383.

Neutra, M.R. and J.R. Kraehenbuhl. 1992. M cell mediated antigen transport and monoclonal IgA antibodies for mucosal immune protection. *Adv. Med. Biol.* **237**:142-150.

Neutra, M.R., Frey, A., and J.P. Kraehenbuhl. 1996. Epithelial M cells: gateways for mucosal infection and immunization. *Cell* **86**:345-348.

Newby, T.J., Stokes, C.R., and F.J. Bourne. 1982. Immunological activities of milk. *Vet. Immunol. Immunopath.* **3**:67-94.

Offit, P.A. and H.F. Clark. 1985. Protection against rotavirus-induced gastroenteritis in a murine model by passively acquired gastrointestinal but not circulating antibodies. *J. Virol.* **54**:58-64.

Offit, P.A. and K.I. Dudzik. 1988. Rotavirus-specific cytotoxic T lymphocytes cross-react with target cells infected with different rotavirus serotypes. *J. Virol.* **62**:127-131.

Offit, P.A. and K.I. Dudzik. 1989. Rotavirus-specific cytotoxic T lymphocytes appear at the intestinal mucosal surface after rotavirus infection. *J. Virol.* **63**:3507-3512.

Offit, P.A. and K.I. Dudzik. 1990. Rotavirus-specific cytotoxic T lymphocytes passively protect against gastroenteritis in suckling mice. *J. Virol.* **64**:6325-6328.

Offit, P.A., Shaw, R.D., and H.B. Greenberg. 1986. Passive protection against rotavirus-induced diarrhea by monoclonal antibodies to surface proteins VP3 and VP7. *J. Virol.* **58**:700-703.

Offit, P.A., Greenberg, H.B., and K.I. Dudzik. 1989. Rotavirus-specific protein synthesis is not necessary for recognition of infected cells by virus-specific cytotoxic T lymphocytes. *J. Virol.* **63**:3279-3283.

Offit, P.A., Cunningham, S.L., and K.I. Dudzik. 1991. Memory and distribution of virus-specific cytotoxic T lymphocytes (CTLs) and CTL precursors after rotavirus infection. *J. Virol.* **65**:1318-1324.

Offit, P.A., Hoffenberg, E.J., Pia, E.S., Panackal, P.A., and N.L. Hill. 1992. Rotavirus-specific helper T cell responses in newborns, infants, children, and adults. *J. Infect. Dis.* **165**:1107-1111.

Offit, P.A., Hoffenberg, E.J., Santos, N., and V. Gouvea. 1993. Rotavirus-specific humoral and cellular immune responses after primary symptomatic infection. *J. Infect. Dis.* **167**:1436-1440.

Offit, P.A., Coupar, B.E.H., Svoboda, Y.M., Jenkins, R.J., McCrea, M.A., Abraham, A., Hill, N.L., Boyle, D.B., Andrew, M.E., and G.W. Both. 1994. Induction of rotavirus-specific cytotoxic T lymphocytes by vaccinia virus recombinants expressing individual rotavirus genes. *Virology* **198**:10-16.

Ogra, P.L. and D.T. Karzon. 1969. Distribution of poliovirus antibody in serum, nasopharynx and alimentary tract following segmental immunization of lower alimentary tract with poliovaccine. *J. Immunol.* **102**:1423-1430.

Ogra, P.L., Coppola, P.R., MacGillivray, M.H., and J.L. Dzierba. 1974. Mechanism of mucosal immunity to viral infections in γA immunoglobulin-deficiency syndromes. *Proc. Soc. Exp. Biol. Med.*. **145**:811-816.

Ogra, P.L., Chiba, Y., Beutner, K.R., and A. Morag. 1976. Vaccination by non-parenteral routes: characterization of immune response. *Dev. Biol. Stand.* **33**:19-26.

Ogra, P.L., Fishaut, M., and M.R. Gallagher. 1980. Viral vaccination via the mucosal route. *Rev. Infect. Dis.* **2**:352-369.

Ojeh, C.K., Snodgrass, D.R., and A.J. Herring. 1984. Evidence for serotype variation among bovine rotaviruses. *Arch. Virol.* **79**:161-171.

Oldham, G., Bridger, J.C., Howard, C.J., and K.R. Parsons. 1993. *In vivo* role of lymphocyte subpopulations in the control of virus excretion and mucosal antibody responses of cattle infected with rotavirus. *J. Virol.* **67**:5012-5019.

Orange, J.S. and C.A. Biron. 1996. An absolute and restricted requirement for IL-12 in natural killer cell IFN-γ production and antiviral defense. *J. Immunol.* **156**:1138-1142.

Osebold, J.W. 1982. Mechanisms of action by immunologic adjuvants. *J. Am. Vet. Med. Assoc.* **181**:983-987.

Osterhaus, A., Weijer, K., Uytdehaag, F., Jarrett, O., Sundquist, B., and B. Morein. 1985. Induction of protective immune responses in cats by vaccination with feline leukemia virus iscom. *J. Immunol.* **135**:591-596.

Owen, R.L., Pierce, N.F., Apple, R.T., and W.C. Cray Jr. 1986. M cell transport of *Vibrio cholerae* from the intestinal lumen into Peyer's patches: a mechanism for antigen sampling and for microbial transepithelial migration. *J. Infect. Dis.* **153**:1108-1118.

Panja, A. and L. Mayer. 1994. Diversity and function of antigen-presenting cells in mucosal tissues. In: "Handbook for Mucosal Immunology". Orga, P.L., Mestecky, J., Lamm, M.E., Strober, W., McGhee, J.R., and J. Bienenstock Eds. Academic Press, Florida. p. 177-183.

Parrot, D.M.V., Tait, C., MacKenzie, S., and A.M. Mowat. 1982. Analysis of the effector functions of different populations of mucosal lymphocytes. *Ann. NY Acad. Sci.* **409**:307-320.

Parsons, K.R., Hall, G.A., Bridger, J.C., and R.S. Cook. 1993. Number and distribution of T lymphocytes in the small intestine mucosa of calves inoculated with rotavirus. *Vet. Immunol. Immunopath.* **39**:355-364.

Pearce, E.J., Caspar, P., Grzych, J.M., Lewis, F.A., and A. Sher. 1991. Down-regulation of Th1- cytokine production accompanies induction of Th2-responses by a parasitic helminth, *Schistosoma mansoni*. *J. Exp. Med.* **173**:159-167.

Pensaert, M., Callebaut, P., and E. Cox. 1994. Enteric coronaviruses of animals. In: "Viral infections of the gastrointestinal tract". Kapikian, A.Z. Ed. Marcel Dekker, Inc., New York. p. 627-696.

Perez-Schael, I., Blanco, M., Vilar, M., Garcia, D., White, L., Gonzalez, R., Kapikian, A.Z., and J. Flores. 1990. Clinical studies of a quadrivalent rotavirus vaccine in Venezualan infants. *J. Clin. Microbiol.* **28**:553-558.

Philips, A.D. 1981. Small intestinal mucosa in childhood in health and disease. *Scand. J. Gastroenterol.* **16** (S):65-85.

Philips, A.D. 1989. Mechanisms of mucosal injury: human studies. In: "Viruses and the gut", Proceedings of the Ninth BSG.SK&F International Workshop, Farthing, M.J.G. Ed. Swan Press, London. p. 30-40.

Pickering, L.K., Morrow, A.L., Herrera, I., O'Ryan, M., Estes, M.K., Guilliams, S.E., Jackson, L., Carter-Campbell, S., and D.O. Matson. 1995. Effect of maternal rotavirus immunization on milk and serum antibody titers. *J. Infect. Dis.* **172**:723-728.

Pollard, M. and N. Sharon. 1970. Responses of Peyer's patches in germ-free mice to antigenic stimulation. *Infect. Immun.* **2**:96-102.

Pond, L., Wassom, D.L., and C.E. Hayes. 1989. Evidence for differential induction of helper T cell subsets during *Trichinella spiralis* infection. *J. Immunol.* **143**:4232-4237.

Pond, L., Wassom, D.L., and C.E. Hayes. 1991. Influence of resistant and susceptible genotype, IL-1, and lymphoid organ on *Trichinella spiralis*-induced cytokine secretion. *J. Immunol.* **149**:957-965.

Prem, S.P. and Y.S. Lyoo. 1993. Immunogens of rotaviruses. *Vet. Microbiol.* **37**:299-317.

Prince, G.A., Horswood, R.L., and R.M. Chanock. 1985. Quantitative aspects of passive immunity to respiratory syncytial virus infection in infant cotton rats. *J. Virol.* **55**:517-520.

Puck, J.M., Glezen, W.P., Frank, A.L., and H.R. Six. 1980. Protection of infants from infection with influenza A virus by transplacentally acquired antibody. *J. Infect. Dis.* **142**:844-849.

Puri, N.K., MacKay, C.R., and M.R. Brandon. 1985. Sheep lymphocytes antigens (OLA): II. Major histocompatibility complex class II molecules. *Immunology* **55**:725-733.

Rahman, M.M., Yamauchi, M., Hanada, N., Nishikawa, K., and T.Morishima. 1987. Local production of rotavirus-specific IgA in breast tissue and transfer to neonates. *Arch. Dis. Childhood* **62**:401-405.

Ramaswamy, K., Negrao-Correa, D., and R. Bell. 1996. Local intestinal immune responses to infections with *Trichinella spiralis*. *J. Immunol.* **156**:4328-4337.

Ramsay, A.J., Ruby, J., and I.A. Ramshaw. 1993. A case for cytokines as effector molecules in the resolution of virus infection. *Immunol. Today* **14**:155-157.

Reed, D.E., Daley, C.A., and H.J. Shave. 1976. Reovirus-like agent associated with neonatal diarrhea in pronghorn antelope. *J. Wildl. Dis.* **12**:488-491.

Renegar, K.B. and P.A. Small Jr. 1991. Passive transfer of local immunity to influenza virus infection by IgA anitbody. *J. Immunol.* **146**:1972-1978.

Reuman, P.D., Pagani, C.M.A., Ayoub, E.M., and P.A. Small Jr. 1983. Maternal-infant transfer of influenza-specific immunity in the mouse. *J. Immunol.* **130**:932-936.

Reuman, P.D., Ayoub, E.M., and P.A. Small Jr. 1987. Effect of passive maternal antibody on influenza illness in children: a prospective study of influenza A in mother-infant pairs. *Pediatr. Infect. Dis.* **6**:398-403.

Reynolds, J.D. and B. Morris. 1983. The evolution and involution of Peyer's patches in foetal and postnatal sheep. *Eur. J. Immunol.* **13**:627-635.

Reynolds, J.D. and R. Pabst. 1984. The emigration of lymphocytes from the Peyer's patches in sheep. *Eur. J. Immunol.* **14**:7-13.

Rubenstein, A.S. and M.F. Miller. 1982. Comparison of an enzyme immunoassay with electron microscopic procedures for detecting rotavirus. *J. Clin. Microbiol.* **15**:938-944.

Rudzik, R., Clancy, R.L., Perey, D.Y.E., Day, R.P., and J. Bienenstock. 1975. Repopulation with IgA-containing cells of bronchial and intestinal lamina propria after transfer of homologous Peyer's patch and bronchial lymphocytes. *J. Immunol.* **114**:1599-1604.

Ryan, M.J., Ramsay, M., Brown, D., Gay, N.J., Farrington, C.P., and P.G. Wall. 1996. Hospital admissions attributable to rotavirus infections in England and Wales. *J. Infect. Dis.* **174** (S):S12-18.

Saif, L.J. 1987. Development of nasal, fecal and serum isotype-specific antibodies in calves challenged with bovine coronavirus or rotavirus. *Vet. Immunol. Immunopath.* **17**:425-439.

Saif, L.J. 1990. Comparitive aspects of enteric viral infections. In: "Viral diarrheas of man and animals". Saif, L.J. and K.W. Theil Eds. CRC Press, Florida. p. 9-31.

Saif, L.J. and E.H. Bohl. 1979. Passive immunity in transmissible gastroenteritis of swine: immunoglobulin classes of milk antibodies after oral-intranasal inoculation of sows with a live low cell culture-passaged virus. *Am. J. Vet. Res.* **40**:115-117.

Saif, L.J. and E.H. Bohl. 1980. Passive immunity against enteric viral infections. In: Proceedings third international symposium on neonatal diarrhoea. Saskatoon, Canada. p. 83-98.

Saif, L.J. and D.J. Jackwood. 1990. Enteric virus vaccines: theoretical considerations, current status, and future approaches. In: "Viral diarrheas of man and animals". Saif, L.J. and K.W. Theil Eds. CRC Press, Florida. p. 313-329.

Saif, L.J., Bohl, E.H., and P.K. Gupta. 1972. Isolation of porcine immunoglobulins and determination of the immunoglobulin classes of transmissible gastroenteritis viral antibodies. *Infect. Immun.* **6**:600-611.

Saif, L.J., Redman, D.R., Smith, K.L., and K.W. Theil. 1983. Passive immunity to bovine rotavirus in newborn calves fed colostrum supplements from immunized or non-immunized cows. *Infect. Immun.* **41**:1118-1131.

Saif, L.J., Smith, K.L., Landmeier, B.J., Bohl, E.H., Theil, K.W., and D.A. Todhunter. 1984. Immune responses of pregnant cows to bovine rotavirus immunization. *Am. J. Vet. Res.* **45**:49-58.

Saif, L.J., Rosen, I., and A.V. Parwani. 1994. Animal rotaviruses. In: "Viral infections of the gastrointestinal tract". Kapikian, A.Z. Ed. Marcel Dekker, Inc., New York. p. 279-368.

Scott, A.C., Luddington, J., Lucas, M., and F.R. Gilbert. 1978. Rotavirus in goats. *Vet. Rec.* **103**:145.

Selby, W.S., Janossy, G., and D.P. Jewell. 1981. Immunohistological characterisation of intraepithelial lymphocytes of the human gastrointestinal tract. *Gut* **22**:169-176.

Selby, W.S., Janossy, G., Bofill, M., and D.P. Jewell. 1983. Lymphocyte subpopulations in the human small intestine. The findings in normal mucosa and in the mucosa of patients with adult coeliac disease. *Clin. Exp. Immunol.* **52**:219-228.

Seow, H.F. Rothel, J.S., and P.R. Wood. 1993. Cloning and sequencing an ovine interleukin-4-encoding cDNA. *Gene* **124**:291-293.

Shalaby, W.S. 1995. Development of oral vaccines to stimulate mucosal and systemic immunity: barriers and novel strategies. *Clin. Immunol. Immunopath.* **74**:127-134.

Shaw, R.D., Merchant, A.A., Groene, W.S., E.H. Cheng. 1993. Persistance of intestinal antibody response to heterologous rotavirus infection in a murine model beyond 1 year. *J. Clin. Microbiol.* **31**:188-191.

Sicinski, P., Rowinski, J., Warchol, J.B., Jarzcebek, Z., Gut, W., Szcygiel, B., Biellecki, K., and G. Koch. 1990. Poliovirus type 1 enters the human host through intestinal M cells. *Gastroenterology* **98**:56-58.

Siebers, A. and B.B. Finlay. 1996. M cells and the pathogenesis of mucosal and systemic infections. *Immunol. Today* **4**:22-29.

Singh, M., Li, X.M., Wang, H., McGee, J.P., Zamb, T., Koff, W., Wang, C.Y., and D.T. O'Hagan. 1997. Immunogenicity and protection in small-animal models with controlled-release tetanus toxoid microparticles as a single-dose vaccine. *Infect. Immun.* **65**:1716-1721.

Smith, W.D. 1975. The nasal secretions and serum antibody responses of lambs following vaccination and aerosol challenge with parainfluenza 3 virus. *Res. Vet. Sci.* **19**:56-62.

Smith, W.D., Dawson, A.McL., Wells, P.W., and C. Burrells. 1975. Immunoglobulin concentrations in ovine body fluids. *Res. Vet. Sci.* **19**:189-193.

Smith, W.D., Dawson, A.McL., Wells, P.W., and C. Burrells. 1976. Immunoglobulins in the serum and nasal secretions of lambs following vaccination and aerosol challenge with parainfluenza 3 virus. *Res. Vet. Sci.* **21**:341-348.

Snodgrass, D.R., Smith, W., Gray, E.W., and J.A. Herring. 1976a. A rotavirus in lambs with diarrhoea. *Res. Vet. Sci.* **20**:113-114.

Snodgrass, D.R., Herring, J.A., and E.W. Gray. 1976b. Experimental rotavirus infection in lambs. *J. Comp. Pathol.* **86**:637-642.

Snodgrass, D.R., Herring, J.A., Linklater, K.A., and D.A. Dyson. 1977. A survey of rotaviruses in sheep in Scotland. *Vet. Rec.* **100**:341.

Snodgrass, D.R., Angus, K.W., and E.W. Gray. 1979. A rotavirus from kittens. *Vet. Rec.* **104**:222-223.

Snodgrass, D.R., Fahey, K.J., Wells, P.W., Campbell, I., and A. Whitelaw. 1980. Passive immunity in calf rotavirus infections: maternal vaccination increases and prolongs immunoglobulin G1 antibody secretion in milk. *Infect. Immunity* **28**:344-349.

Snodgrass, D.R., Terzolo, H.R., Sherwood, D. Campbell, I., Menzies, J.D., and B.A. Synge. 1986. Aetiology of diarrhoea in young calves. *Vet. Rec.* **119**:31-34.

Snodgrass, D.R., Hoshino, Y., Fitzgerald, T.A., Smith, M., Browning, G.F., and M. Gorziglia. 1992. Identification of four VP4 serological types (P serotypes) of bovine rotavirus using viral reassortants. *J. Gen. Virol.* **73**:2319-2325.

Snodgrass, D.R., Campbell, I., Mwenda, J.M., Chege, G., Suleman, M.A., Morein, B., and C.A. Hart. 1995. Stimulation of rotavirus IgA, IgG and neutralising antibodies in baboon milk by parenteral vaccination. *Vaccine* **13**:408-413.

Solvason, N., Lehuen, A., and J.F. Kearney. 1991. An embryonic source of Ly1 but not convential B cells. *Int. Immunol.* **3**:543-550.

Sonoda, E., Matsumoto, R., Hitoshi, Y., Ishii, T., Sugimoto, M., Araki, S., Tominaga, A., Yamaguchi, N., and K. Takatsu. 1989. Transforming growth factor β induces IgA production and acts additively with interleukin 5 for IgA production. *J. Exp. Med.* **170**:1415-1420.

Spalding, D.M., Koopman, W.J., Eldridge J.H., McGhee, J.R., and R.M. Steinman. 1983. Accessory cells in murine Peyer's patch. I. Identification and enrichment of a functional dendritic cell. *J. Exp. Med.* **157**:1646-1659.

Staats, H.F., Jackson, R.J., Marinaro, M., Takahashi, I., Kiyono, H., and J.R. McGhee. 1994. Mucosal immunity to infection with implications for vaccine development. *Curr. Opin. Immunol.* **6**:572-583.

Steinman, R.M. and M.C. Nussenzweig. 1980. Dendritic cells: features and functions. *Immunol. Rev.* **53**:127-147.

Stevens, S.K., Weisman, I.L., and E.C. Butcher. 1982. Differences in the migration of B and T lymphocytes: organ-selective localisation in vivo and the role of lymphocyte-endothelial cell recognition. *J. Immunol.* **128**:844-851.

Stover, C.K., de la Cruz, V., Bansal, G., Hanson, M., Fuerst, T., Jacobs, W., and B. Bloom. 1992. Use of recombinant BCG as a vaccine delivery vehicle. *Adv. Exp. Med. Biol.* **327**:175-182.

Stover, C.K., Bansal, G., Hanson, M., Burlein, J., Palaszynski, S., Young, J., Koenig, S., Young, D., Sadzeine, A., and A. Barbour. 1993. Protective immunity elicited by recombinant bacille Calmette-Guerin (BCG) expressing outer surface protein A (Osp A) lipoprotein: a candidate Lyme disease vaccine. *J. Exp. Med.* **178**:197-209.

Street, N.E. and T.R. Mossmann. 1991. Functional diversity of T lymphocytes due to secretion of different cytokine patterns. *FASEB J.* **5**:171-177.

Stuker, G., Oshino, L.S., Schmidt, N.J., Holmberg, C.A., Anderson, J.H., Glaser, C.A., and R.V. Herickson. 1979. Virus detection in monkeys with diarrhea: the association of adenoviruses with diarrhea and the possible role of rotaviruses. *Lab. Anim. Sci.* **29**:610-616.

Stuker, G., Oshino, L.S., and N.J. Schmidt. 1980. Antigenic comparisons of two new rotaviruses from rhesus monkeys. *J. Clin. Microbiol.* **11**:202-203.

Sugg, J.Y. and J.M. Neill. 1931. Loss of immune substances from the body. II. Diphtheria antitoxin in human saliva. *J. Immunol.* **20**:463-481.

Sundquist, B.G., Lövgren, K., and B. Morein. 1988. Influenza virus iscoms: antibody response in animals. *Vaccine* **6**:49-52.

Sundquist, B.G., Czifra, G., and L. Stipkovits. 1996. Protective immunity induced in chicken by a single immunization with *Mycoplasma gallisepticum* immunostimulating complexes (ISCOMS). *Vaccine* **14**:892-897.

Sutter, G. and B. Moss. 1992. Nonreplicating vaccinia vector efficiently expresses recombinant genes. *Proc. Natl. Acad. Sci. USA* **89**:10847-10851.

Suzuki, H. and T. Konno. 1975. Reovirus-like particles in jejunal mucosa of a Japanese infant with acute infectious non-bacterila gastroenteritis. *J. Exp. Med.* **115**:199-211.

Svennerholm, A.M., Hanson, L.A., Holmgrem, J., Lindblad, B.S., Nilsson, B., and F. Quereshi. 1980. Different secretory immunoglobulin A antibody responses to cholera vaccination in Swedish and Pakistani women. *Infect. Immun.* **30**:427-430.

Svetic, A., Madden, K.B., Zhou, X. di, Lu, P., Katona, I.M., Finkelman, F.D., Urban Jr., J.F., and W.C. Gause. 1993. A primary intestinal helminth infection rapidly induces a gut-associated elevation of Th2--associated cytokines and IL-13. *J. Immunol.* **150**:3434-3441.

Taguchi, T., McGhee, J.R., Coffman, R.L., Beagley, K.W., Eldridge, J.H., Takatsu, K., and H. Kiyono. 1990. Analysis of Th1- and Th2-cells in murine gut-associated tissues. Frequencies of $CD4^+$ and $CD8^+$ T cells that secrete IFN-γ and IL-5. *J. Immunol.* **145**:68-77.

Tang, Y.W., Neuzil, K.M., Fisher, J.E., Robinson, F.W., Parker, R.A., and B.S. Graham. 1997. Determinants and kinetics of cytokine expression patterns in lungs of vaccinated mice challenged with respiratory syncitial virus. *Vaccine* **15**:597-602.

Taylor, P.M. and B.A. Askonas. 1986. Influenza nucleoprotein-specific cytotoxic T-cell clones are protective *in vivo*. *Immunology* **58**:417-420.

Theil, K.W., Lance, S.E., and C.M. McCloskey. 1996. Rotaviruses associated with neonatal lamb diarrhea in two Wyoming shed-lambing operations. *J. Vet. Diagn. Invest.* **8**:245-248.

Tokunaga, K., Nakamura, Y., Sakata, K., Fujimori, K., Ohkubo, M., Sawada, U., and S. Sakiyama. 1987. Enhanced expression of a glyceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. *Cancer Res.* **47**:5616-5619.

Tomasi, T.B. and J. Bienenstock. 1968. Secretory immunoglobulins. *Adv. Immunology* **9**:1-96.

Tomasi, T.B., Tan, E.M., Solomon, A., and R.A. Prendergast. 1965. Characteristics of an immune system common to certain external secretions. *J. Exp. Med.* **121**:101-124.

Treanor, J.J., Clark, H.F., Pichichero, M., Christy, C., Gouvea, V., Shrager, D., Palazzo, A., and P.A. Offit. 1995. Evaluation of the protective efficacy of a serotype 1 bovine-human rotavirus reassortant vaccine in infants. *Pediatr. Infect. Dis.* **14**:301-317.

Tseng, J. 1982. Expression of immunoglobulin isotypes of lymphoid cells of mouse intestinal lamina propria. *Cell Immunol.* **73**:324-336.

Tzipori, S., Caple, I.W., and R. Butler. 1976. Isolation of a rotavirus from deer. *Vet. Rec.* **99**:398.

Ukae, S., Nakata, S., Adachi, N., Kogawa, K., and S. Chiba. 1994. Efficacy of rhesus rotavirus vaccine (MMU-18006) against gastroenteritis due to serotype 1 rotavirus. *Vaccine* **12**:933-939.

Ulmer, J.B., Donnelly, J.J., Parker, S.E., Rhodes, G.H., Felgner, P.L., Dwarki, V.J., Gromkowski, S.H., Deck, R.R., and C.M. DeWitt. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* **259**:1745-1749.

Umesaki, Y. and H. Setoyama. 1992. Immune responses of mice to orally administrated asialo GM1-specific rabbit IgG in the presence or absence of cholera toxin. *Immunology* **75**:386-388.

Van der Heijden, P.J., Bianchi, A.T., Dol., M., Pals, J.W., Stok, W., and B.A. Bokhout. 1991. Manipulation of intestinal immune responses against ovalbumin by cholera toxin and its B subunit in mice. *Immunology* **72**:89-93.

Van Zaane, D., Ijzerman, J., and P.W. de Leeuw. 1986. Intestinal antibody response after vaccination and infection of calves fed colostrum with or without rotavirus antibody. *Vet. Immunol. Immunopathol.* **11**:45-63.

Vesikari, T. 1993. Clinical trials of live oral rotavirus vaccines: the Finnish experience. *Vaccine* **11**:255-261.

Vesikari, T., Isolauri, E., Delem, A., D'Hondt, E., André, F.E., and G. Zissis. 1983. Immunogenicity and safety of live oral attenuated bovine rotavirus vaccine strain RIT 4237 in adults and young children. *Lancet* **ii**:807-811.

Vesikari, T., Isolauri, E., Delem, A., D'Hondt, E., André, F.E., Beards, G.M., and T.H. Flewett. 1985. Clinical efficacy of the RIT 4237 live attenuated bovine rotavirus vaccine in infants vaccinated before a rotavirus epidemic. *J. Pediatr.* **107**:189-194.

Vesikari, T., Green, K.Y., Flores, J., and A.Z. Kapikian. 1992. Protective efficacy against serotype 1 rotavirus diarrhea by live oral rhesus-human reassortant rotavirus vaccines with human rotavirus VP7 serotype 1 or 2 specificity. *Pediatr. Infect. Dis. J.* **11**:535-542.

Vjady, M., and N. Lycke. 1993. Stimulation of antigen-specific T- and B-cell memory in local as well as systemic lymphoid tissues following oral immunization with cholera toxin adjuvant. *Immunology* **80**:197-203.

Vonderfecht, S.L. and B.I. Osburn. 1982. Immunity to rotavirus in conventional neonatal rotavirus. *J. Clin. Microbiol.* **16**:935-942.

Vordermeier, H.M., Coombes, A.G., Jenkins, P., McGee, J.P., O'Hagan, D.T., Davis, S.S., and M. Singh. 1995. Synthetic delivery system for tuberculosis vaccines: immunological evaluation of the M. tuberculosis 38kDa protein entrapped in biodegradable PLG microparticles. *Vaccine* **13**:1576-1582.

Wadell, G., Allard, A., Johansson, M., Svensson, L., and I. Uhnoo. 1994. Enteric adenoviruses. In: "Viral infections of the gastrointestinal tract". Kapikian, A.Z. Ed. Marcel Dekker, Inc., New York. p. 519-548.

Wahren, B., Nordlund, S., Åkersson, A., Sundquist, B., and B. Morein. 1987. Monocyte and iscom enhancement of cell-mediated response to cytomegalovirus. *Med. Microbiol. Immunol.* **176**:13-17.

Walsh, T.E. and P.R. Cannon. 1936. Studies on acquired immunity in rabbits to intranasal infection with type I pneumococcus. *J. Immunol.* **31**:331-346.

Wannemuehler, M.J., Kiyono, H., Babb, J.L., Michalek, S.M., and J.R. McGhee. 1982. Lipopolysaccharide (LPS) regulation of the immune response: LPS converts germfree mice to sensitivity to oral tolerance induction. *J. Immunol.* **129**:959-965.

Ward, R.L. and D.I Bernstein. 1994. Protection against rotavirus disease after natural rotavirus infection. *J. Infect. Dis.* **169**:900-904.

Ward, R.L., Pax, K.A., Sherwood, J.R., Young, E.C., Schiff, G.M., and D.I. Bernstein. 1992a. Salivary antibody titers in adults challenged with a human rotavirus. *J. Med. Virol.* **36**:222-225.

Ward, R.L., McNeal, M.M., and J.F. Sheridan. 1992b. Evidence that active protection following oral immunization of mice with live rotavirus is not dependent on neutralizing antibody. *Virology* **188**:57-66.

Wassef, J.S., Keren, D.F., and J.L. Mailloux. 1989. Role of M cells in initial antigen uptake and in ulcer formation in the rabbit intestinal loop model of shigellosis. *Infect. Immun.* **7**:858-863.

Webster, R.G., Kawaoka, Y., Taylor, J., Weinberg, R., and E. Paoletti. 1991. Efficacy of nucleoprotein and haemagglutinin antigens expressed in fowlpox virus as vaccine for influenza in chickens. *Vaccine* **9**:303-308.

Webster, R.G., Fynan, E.F., Santoro, J.C., and H. Robinson. 1994. Protection of ferrets against influenza challenge with a DNA vaccine to the haemagglutinin. *Vaccine* **12**:1495-1498.

Weinstein, P.D. and J.J. Cebra. 1991. The preference of switching to IgA expression by Peyer's patch germinal center B cells is likely due to the intrinsic influence of their microenvironment. *J. Immunol.* **147**:4126-4135.

Wells, P.W., Snodgrass, D.R., Herring, J.A., and A.McL. Dawson. 1978. Antibody titres to lamb rotavirus in colostrum and milk of vaccinated ewes. *Vet. Rec.* **103**:46-48.

Welsh, R.M., Brubaker, J.O., Vargas-Cortes, M., and C.L. O'Donnell. 1991. Natural killer (NK) cell response to virus infections in mice with severe combined immunodeficiency. The stimulation of NK cells and the NK cell-dependent control of virus infections occur independently of T and B cell function. *J. Exp. Med.* **173**:1053-1063.

Willoughby, R.E., Yolken, R.H., and R.L. Schnaar. 1990. Rotaviruses specifically bind to the neutral glycosphingolipid asialo-GM1. *J. Virol.* **64**:4830-4835.

Wolf, J.L., Rubin, D.H., Finberg, R., Kauffman, R., Sharpe, A.H., Trier, J.S., and B.N. Fields. 1981. Intestinal M cells: a pathway for entry of reovirus into the host. *Science* **212**:471-472.

Woode G.N., Bridger, J.C., Hall, G.A., and M.J. Dennis. 1974. The isolation of a reovirus-like agent associated with diarrhoea in colostrum-deprived calves in Great-Britain. *Res. Vet. Sci.* **96**:85-88.

World Health Organization. 1973. Mortality due to diarrheal diseases in the world. *WHO Weekly Epidemiol. Rec.* **48**:409-416.

Wu, H.Y. and M.W. Russell. 1994. Comparison of systemic and mucosal priming for mucosal immune responses to a bacterial protein antigen given with or coupled to cholera toxin (CT) B subunit, and effects of pre-existing anti-CT immunity. *Vaccine* **12**:215-222.

Xiang, Z.O., Spitalnik, S., Tran, M., Wunner, W., Cheng, J., and H.C.L. Ertl. 1994. Vaccination with a plasmid vector carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus. *Virology* **199**:132-140.

Yasukawa, M. and J.M. Zarling. 1984. Human cytotoxic T-cell clones directed against herpes simplex virus-infected cells. II. Bifunctional clones with cytotoxic and virus-induced proliferative activities exhibit herpes simplex virus type 1 and 2 specific or type common reactivities. *J. Immunol.* **133**:2736-2742.

Yolken, R., Kinney, J., Wilde, J., Willoughby, and J. Eiden. 1990. Immunoglobulins and other modalities for the prevention and treatment of enteric viral infections. *J. Clin. Immunol.* **10** (S):S80-S87.

Zarozinski, C.C., Fynan, E.F., Selin, L.K., Robinson, H.L., and R.M. Welsh. 1995. Protective CTL-dependent immunity and enhanced immunopathology in mice immunized by particle bombardment with DNA encoding an internal protein. *J. Immunol.* **154**:4010-4017.

Zhou, F., Kraehenbuhl, J.P., and M.R. Neutra. 1995. Mucosal IgA response to rectally administered antigen formulated in IgA-coated liposomes. *Vaccine* **13**:637-644.

APPENDIX

BUFFERS AND REAGENTS

199 M/M: 5mls 10% Yeast extract, 5mls 0.1M glutamine, 5mls amphotericin B, 7mls 8% sodium carbonate, 3.75mls 0.1M sodium hydroxide, 5mls 2.5M hepes, 50mls lactalbumin hydrolysate, 50mls 5% bovine serum albumin, 50mls 199 10× medium with Earle's Salts (Gibco, UK), and made till 500mls with distilled water.

PBS 10×: 800g NaCl, 115g Na_2HPO_4, 20g KCl, and 20g KH_2PO_4 are dissolved in 10 litres of distilled water and diluted 1/10 in distilled water before use.

OPD-SUBSTRATE: 60mg of σ-phenylenediamine dihydrochloride (Sigma, UK) is dissolved 75mls in OPD-buffer (OPD-buffer: 4.64g (w/v) of citric acid and 7.33g (w/v) disodium hydrogen ortophosphate anhydrous is dissolved in 1000mls of distilled water), prior to use 60µl of a 30% (w/w) hydrogen peroxidase (H_2O_2) is added.

AEC-SUBSTRATE: 1 tablet of 3-amino-9-ethylcarbazole (AEC) (Sigma, UK) is dissolved in 2.5mls of N,N-dimethyl-formamide (Sigma, UK) and added to 47.5mls of AEC-buffer (AEC-buffer: 14.8mls of a 0.2M acetic acid solution and 35.2mls of a 0.2M sodium acetate solution was added to 50mls of distilled water). The substrate suspension is filtered twice through two 0.45µm filters. Prior to use 35µl of 30% H_2O_2 was added to suspension.

SDS-EXTRACTION BUFFER: 1%(w/v) sodium dodecyl sulphate (SDS) (BDH, UK) is dissolved in 1×extraction buffer (10×extraction buffer: 11.69g of sodium chloride, 2.42g Tris, and 0.74g EDTA is dissolved in 200mls of distilled water).

RNA-EXTRACTION BUFFER: 5mls of a sterile 250mM sodium citrate solution (pH 7.0) was added to 23.6g guanidine isothiocynate (Sigma, UK) and made up to 47mls of distilled water and mixed well before addition of 2.5mls of a 10% (w/v) sodium lauroyl sarcosine solution (Sigma, UK) and 0.36ml of a 14.2M stock of ß-mercaptoethanol (Sigma, UK).

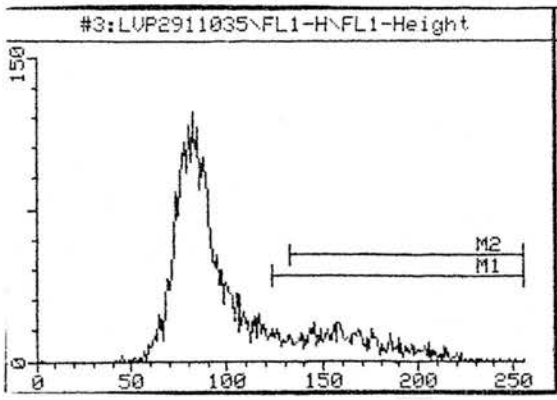
10×MOPS BUFFER: buffer containing 0.2M MOPS [3-(N-morpholino) propanesulfonic acid], 50mM sodium acetate, and 10mM EDTA, this was brought to pH 7.0 and autoclaved.

RNA-LOADING BUFFER: buffer containing 0.75ml of formamide, 0.15ml of 10×MOPS buffer, 0.24ml of 37% (w/v) formaldehyde solution, 0.1ml of glycerol, 0.1ml of a 10% (w/v) bromophenol blue solution, 0.01ml of a 10mg/ml stock solution of ethidium bromide, and 0.1ml of distilled water.

FACS PROFILES

FACS profiles of the different monoclonal antibodies on PBLs.

CD4



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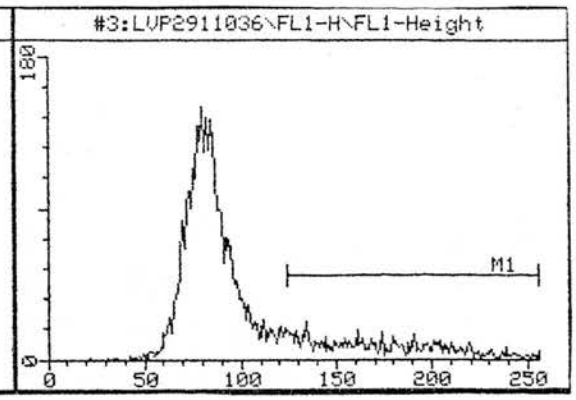

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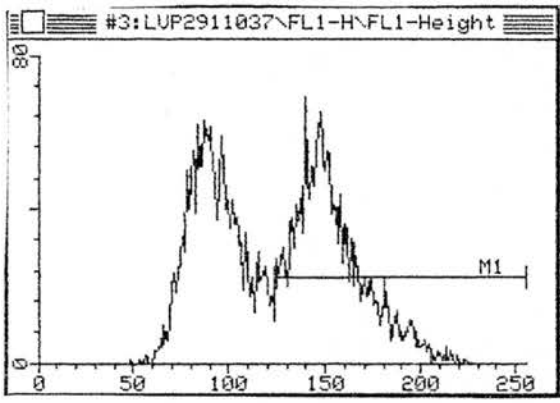

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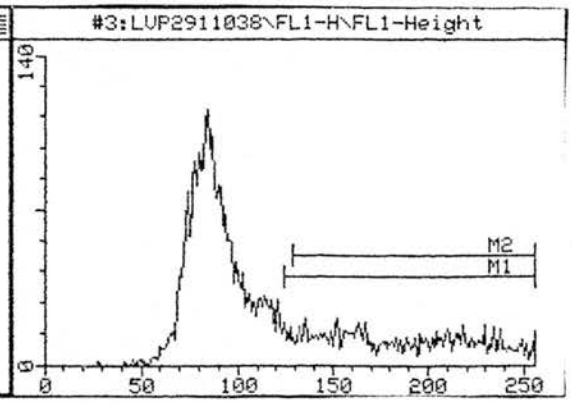

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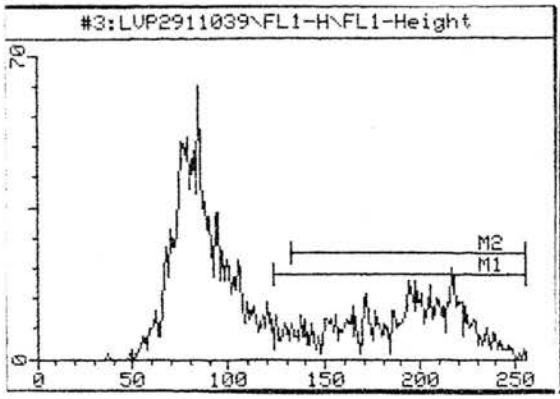

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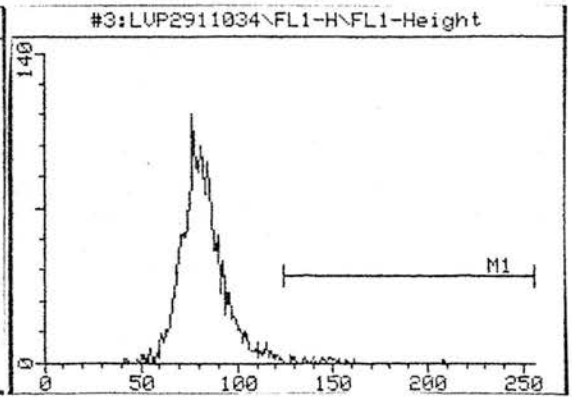

PBLs from lamb W1153 (PBS/ISC group) 1 week after challenge.

FACS PROFILES

FACS profiles of the different monoclonal antibodies on MLNs.

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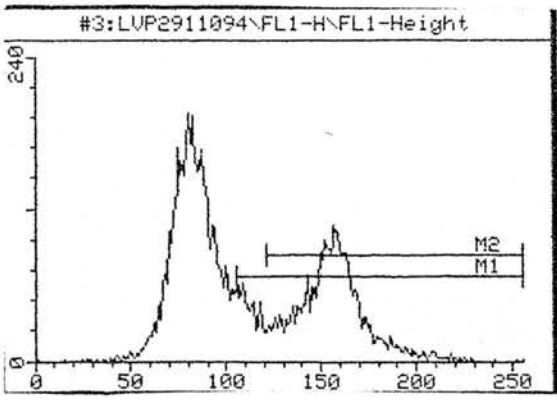

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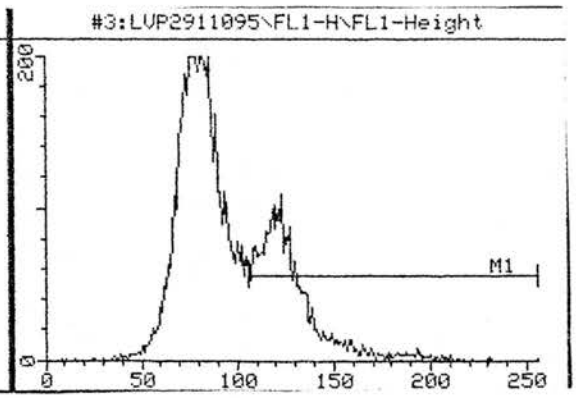

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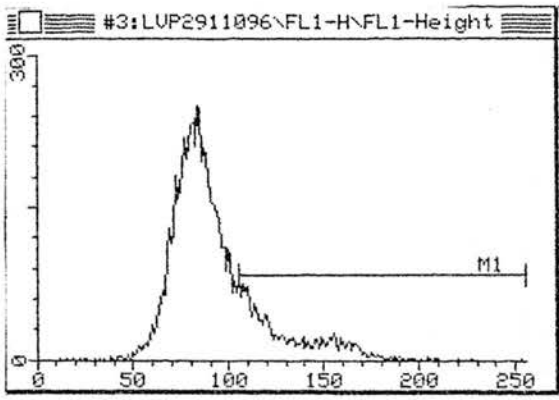

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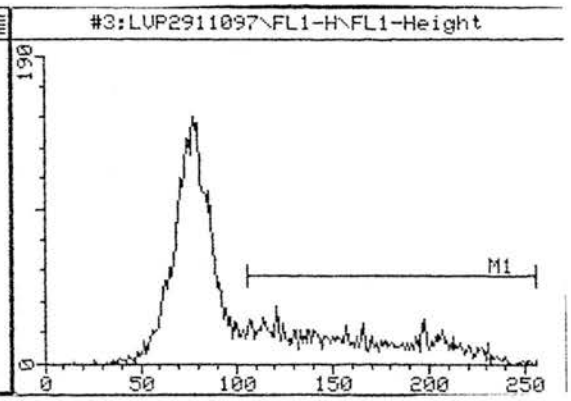

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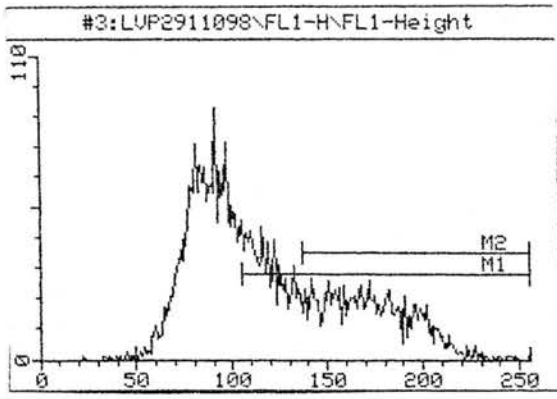

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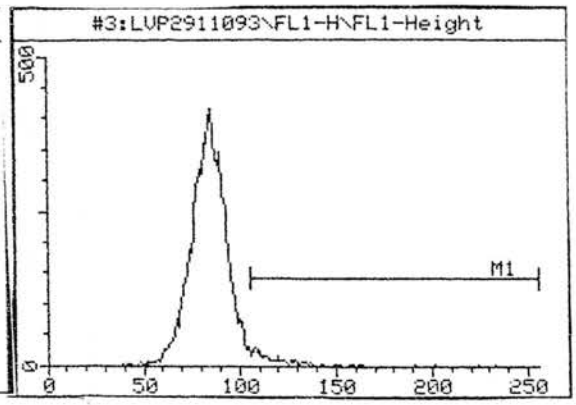

MLNs from lamb W1155 (RV/ISC group) 1-2 weeks after challenge.

FACS PROFILES

FACS profiles of the different monoclonal antibodies on JPPs.

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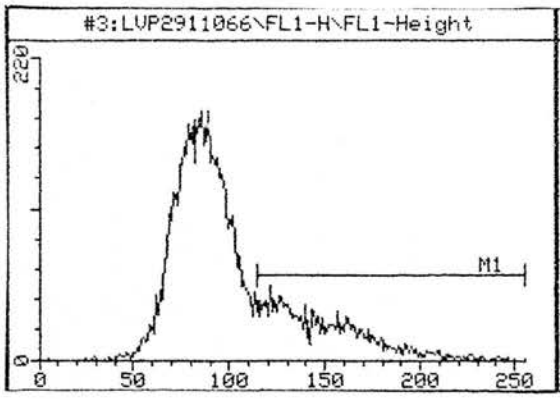

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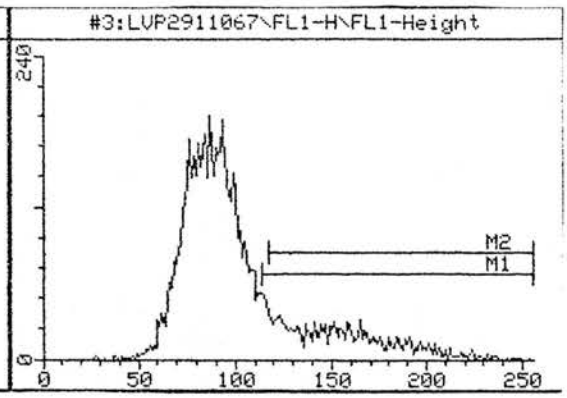

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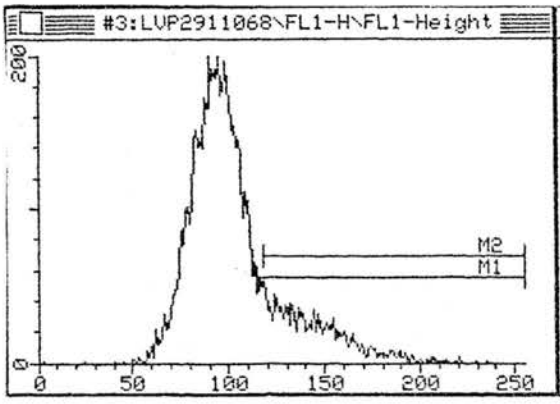

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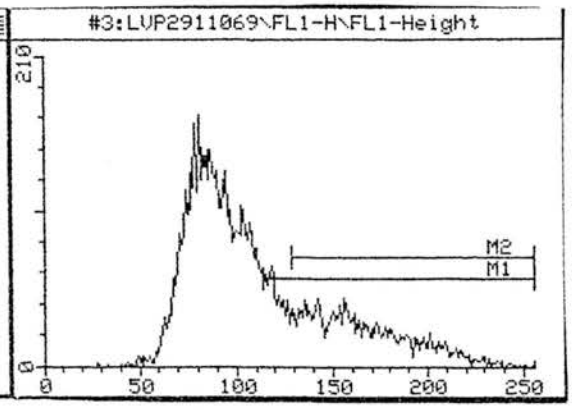

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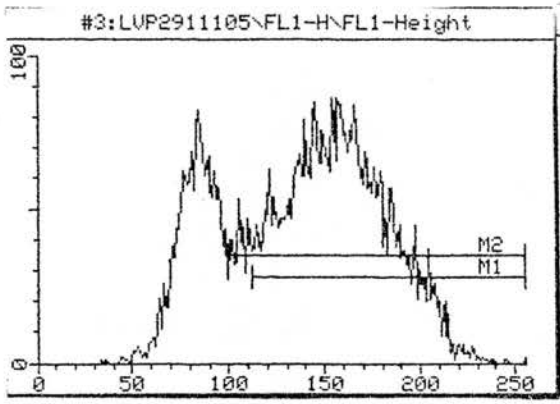

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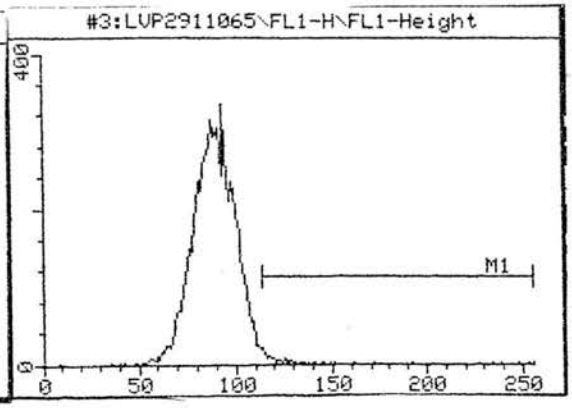
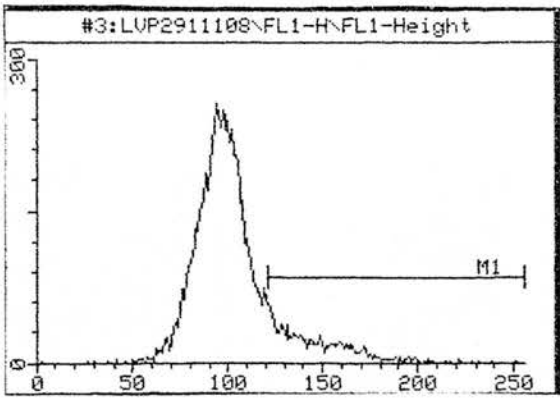


JPPs from lamb W1154 (RV/ISC group) 1-2 weeks after challenge.

FACS PROFILES

FACS profiles of the different monoclonal antibodies on IPPs.

CD4



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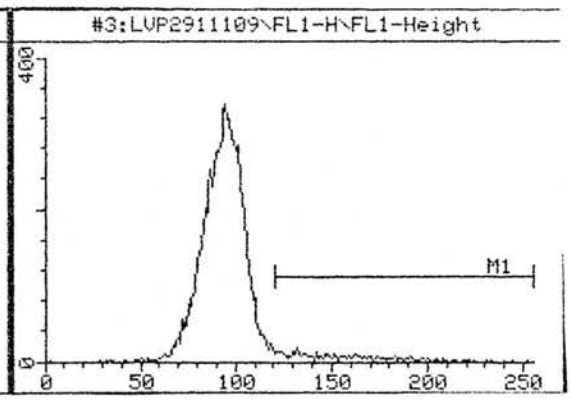

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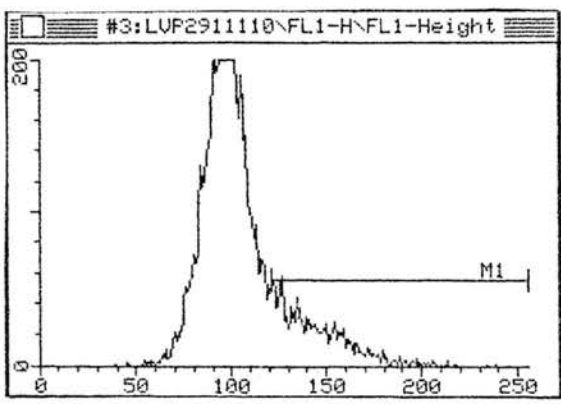

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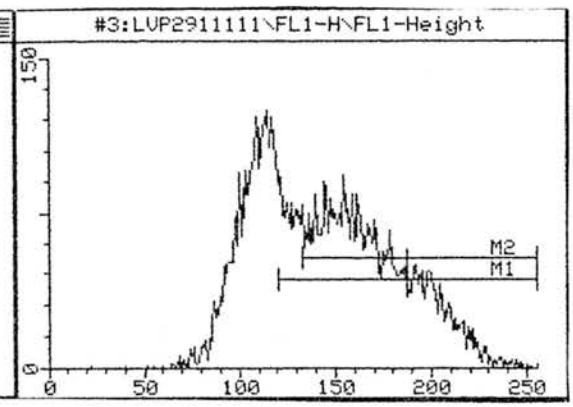

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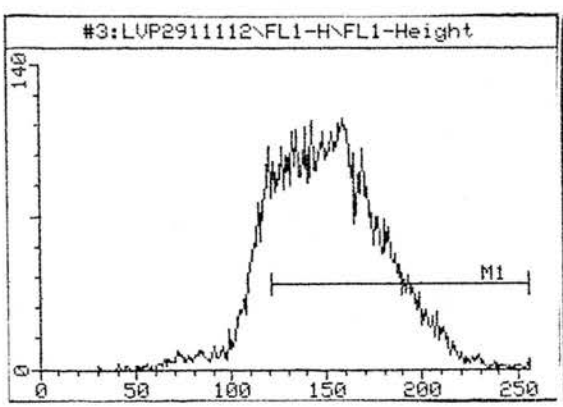

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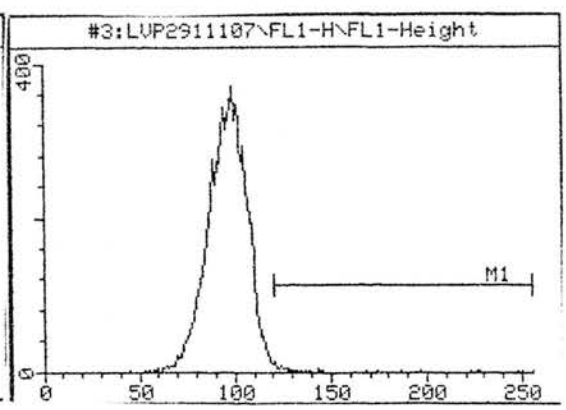

IPPs from lamb W1155 (RV/ISC group) 1-2 weeks after challenge.

FACS PROFILES

FACS profiles of the different monoclonal antibodies on IELs.

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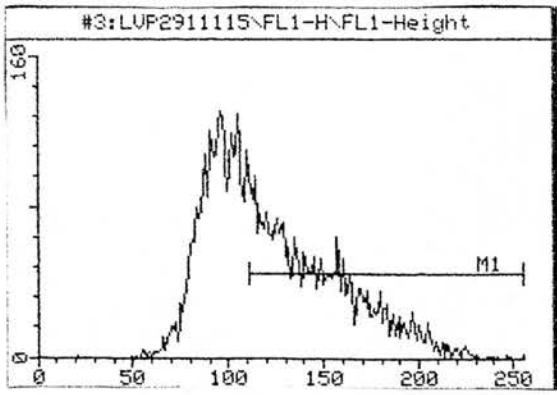

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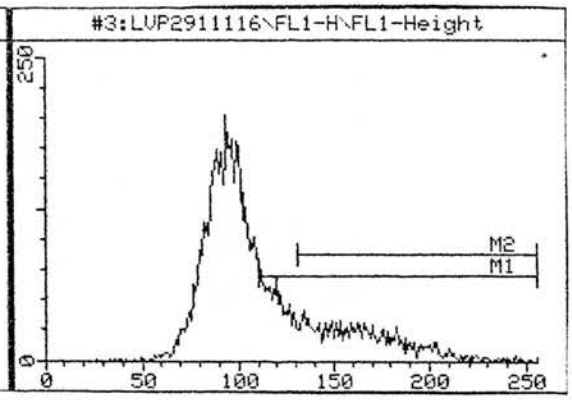

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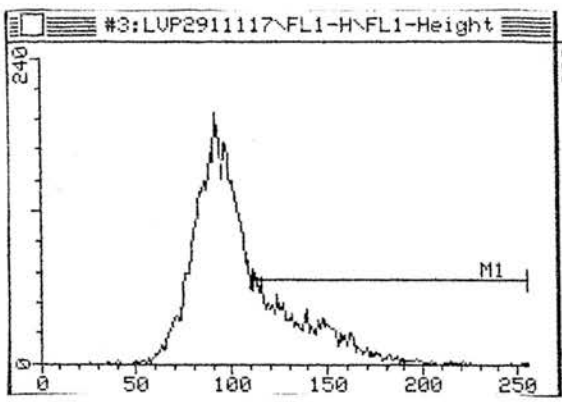

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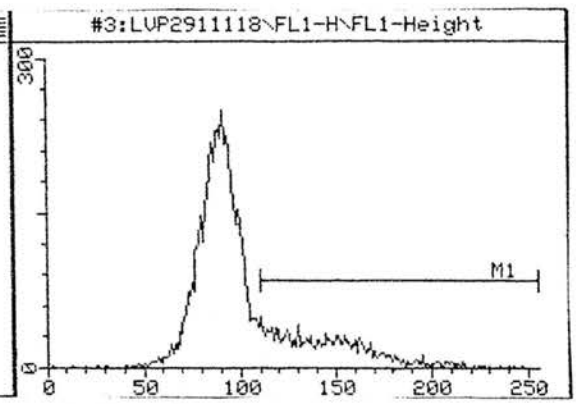

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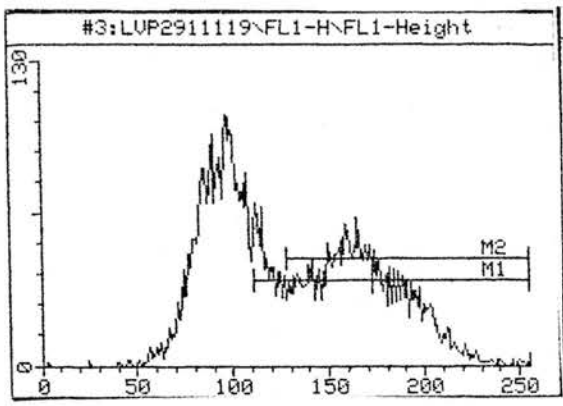

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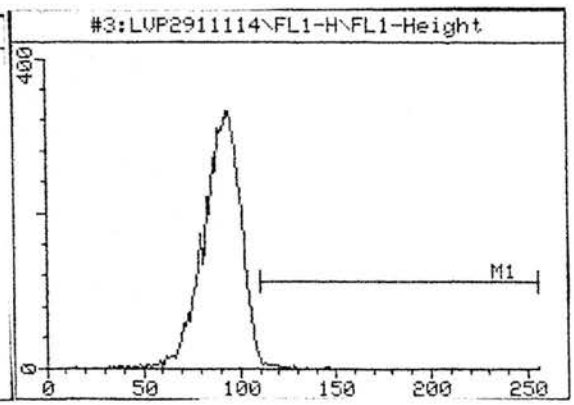
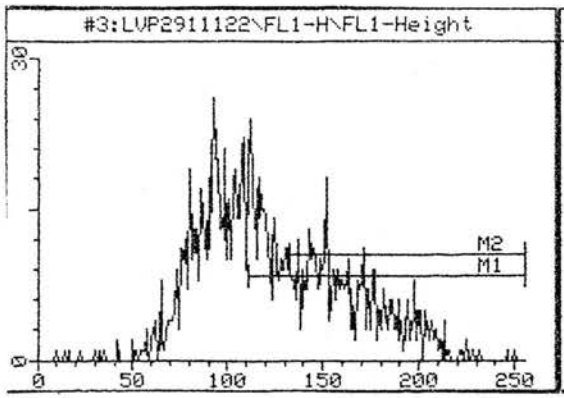


IELs from lamb W1155 (RV/ISC group) 1-2 weeks after challenge.

FACS PROFILES

FACS profiles of the different monoclonal antibodies on LPLs.

CD4



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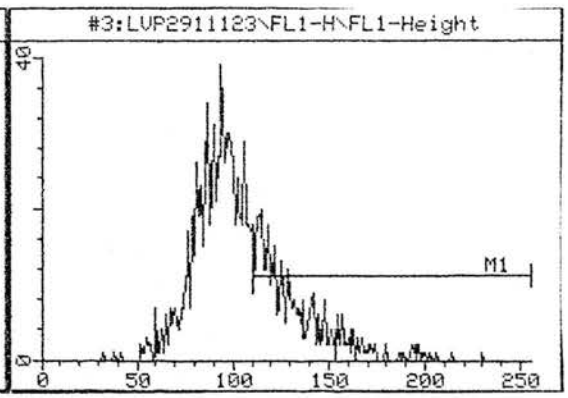

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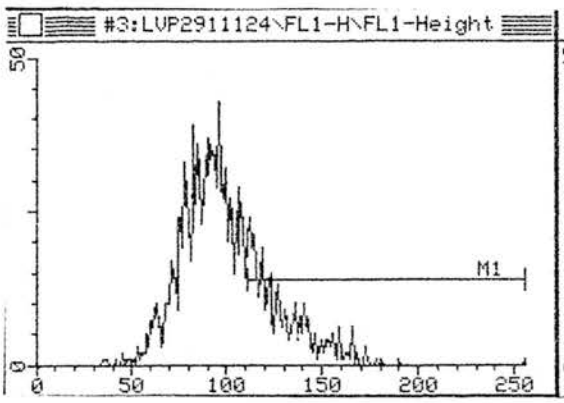

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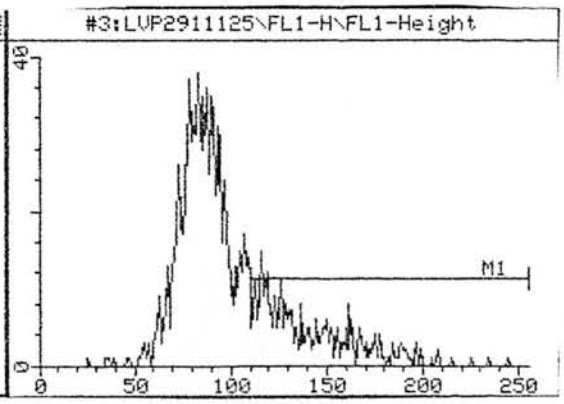

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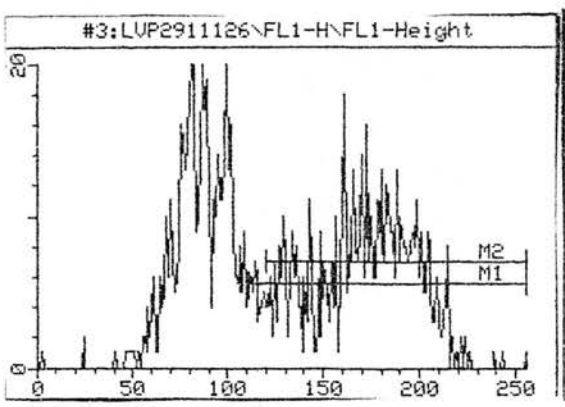

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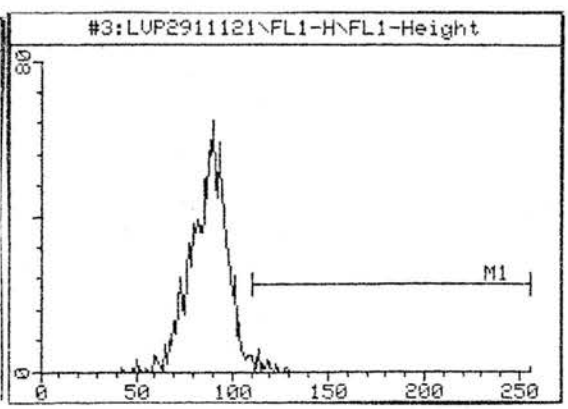

LPLs from lamb W1155 (RV/ISC group) 1-2 weeks after challenge.