The role of T cells in the immune response to *Dictyocaulus viviparus* in calves

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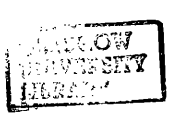

ABSTRACT

The aim of this study was to further characterise the immune responses to infection with normal L3 of *D. viviparus* in calves and, in particular, to investigate the role of T cells in these responses. Infection with *D. viviparus* induces a strong acquired immunity so this parasite provided an ideal model in which to study immune-regulation in the bovine host. Various immunological parameters were measured in calves after single and multiple infections and attempts were made to correlate these parameters with the clearance of infection and the development of resistance.

Both primary infection, and challenge infection, of immune calves resulted in an increase in mRNA for IL-4, but not IL-2, in peripheral blood mononuclear cells and cells collected by bronchoalveolar lavage (BAL). There were significant increases in serum parasite-specific IgG1, IgE and IgA antibodies in serum, both after primary infection and in BAL fluid after challenge. Together, these findings suggest that there was a Th2-type response following primary and challenge infections with *D. viviparus*.

Some of the calves had high IgG2a(A1) levels in serum and BAL fluid and these calves secreted more first stage larvae in their faeces than calves with low IgG2a(A1). This perhaps indicates that these calves were unable to clear the primary infection as efficiently as those with low IgG2a(A1). Compared with the other animals, unstimulated PBMC from one of the calves with high IgG2a(A1) produced very low levels of spontaneous IL-4 but produced higher levels of IL-2 and IFNγ, suggesting a bias in the Th1 direction.

Primary infection with D. viviparus stimulated the proliferation of $\gamma\delta^+$ T cells to parasite antigen, both peripherally and locally. Associated with this was a down-regulation of proliferative responses of $CD4^+$ and $CD8^+$ cells to parasite antigen. This reciprocal relationship implies that activated $\gamma\delta^+$ T cells may have induced factors which down-regulated $CD4^+$ and $CD8^+$ cells. However, after tertiary infection there was proliferation of $CD4^+$ and $CD8^+$ cells but not $\gamma\delta^+$ T cells. These findings suggest that the proposed down-regulatory activity of $\gamma\delta^+$ T cells may have been induced by the presence of parasites.

In conclusion, the results presented demonstrate that a Th2-type response was important in the immune response to *D. viviparus* and that $\gamma\delta^+$ T cells appeared to play a prominent role.

The research reported in this thesis
is my own original work, except where otherwise stated,
and has not been submitted for any other degree.

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1. GENERAL INTRODUCTION

1.1 The bovine lungworm *Dictyocaulus viviparus*

1.1.1 History of *D. viviparus*

Bovine parasitic bronchitis is caused by the trichostrongylid nematode, *Dictyocaulus viviparus*. The disease usually affects calves in their first grazing season and is characterised by increased respiratory rates and coughing. It is a major cause of morbidity and production loss in temperate climates in Europe, Canada, U.S.A., Australia and New Zealand (Urquhart, 1985).

The presence of worms within the main-stem bronchi and trachea of cattle was first reported by Ruysch (1744), but he did not associate these parasites with any specific clinical entity. The link between disease and the presence of parasites was first described by Nicholls (1755), who attributed the disease to small "tape worms". The parasite was originally named *Gordius viviparus* by Bloch (1782) and it underwent several name changes before being assigned to the genus *Dictyocaulus* by Railliet and Henry (1907). In addition to cattle, *D. viviparus* has been demonstrated in the respiratory tract of deer, buffalo and reindeer (Urquhart, 1985).

A knackeryard survey in the UK between August and October 1952 found that parasitic bronchitis was the main cause of death in over 50% of the carcasses examined (Jarrett *et al.*, 1954). In the early 1950s, several studies were performed to increase the understanding of the epidemiology of *D. viviparus* in an attempt to develop methods for its control (Jarrett *et al.*, 1954, 1955a, 1955b, 1957a, 1958; Michel, 1956; Michel and Shand, 1955; Rose, 1960). These studies led to the development of a radiation-attenuated larval vaccine (Jarrett *et al.*, 1959a) which was launched commercially in 1959 and is still widely and successfully used to control parasitic bronchitis throughout Europe (Urquhart, 1985).

1.1.2 Life cycle of *D. viviparus*

D. viviparus has a direct life cycle with an external free-living stage. Infection is initiated by ingestion of infective third stage larvae (L3) from contaminated pasture. Following ingestion, the L3 exsheath in the rumen (Pfeiffer and Supperer, 1980), penetrate the small intestinal wall and begin their migration to the lungs. Details of the parasite's migration have been investigated using experimental infections (Soliman, 1953; Jarrett *et al.*, 1957b; Poynter *et al.*, 1960). These studies concluded that, following penetration of the small intestinal mucosa, larvae migrate via the lymphatics through the mediastinal lymph nodes to the thoracic duct, which empties into the cranial vena cava. The larvae then pass via the right side of the heart into the pulmonary circulation where they emerge through the pulmonary capillaries into the alveoli. Poynter *et al.* (1960) found L3 in the lungs 24 h after a challenge of 200,000 L3. However, Jarrett and Sharp (1963) did not recover larvae from the lungs until five days when smaller doses were administered. The parasites found in the lungs at this time were fourth stage larvae (L4). The authors suggested that the L3 moulted to L4 in the mesenteric lymph nodes and that the previous observation of L3 in the lung was due to the extremely high doses of larvae used. It was concluded that in more natural circumstances, L4's would penetrate the pulmonary capillaries at approximately seven days post infection (pi) (Jarrett and Sharp, 1963). From the alveoli, the larvae migrate up the respiratory tree and by Day 15 pi most parasites have moulted to the fifth stage (L5) (Jarrett *et al.*, 1957b). From Day 17, the parasites grow very rapidly with adult females reaching up to 8 cm, and the males 5 cm, in length (Smythe, 1937).

Adult females produce embryonated eggs which hatch almost immediately to release first stage larvae (L1). The majority of the L1 are coughed up and swallowed to be excreted in the faeces. L1s can be detected in the faeces by 21 to 24 days pi (Jarrett *et al.*, 1957b). Faecal larval counts ranging from 50 to 1000 larvae g^{-1} have been recorded (Urquhart, 1985). Parasite patency lasts between three to six weeks, after which the majority of worms are expelled (Jarrett *et al.*, 1957b). Small numbers of adult parasites may persist for several months in some animals (Jarrett *et al.*, 1955b). This was confirmed by a survey of cattle on farms and in abattoirs which demonstrated that animals could harbour lungworms for up to six months in the absence of reinfection (Cunningham *et al.*, 1956).

It has been proposed that *D. viviparus* may enter a state of arrested development at the early L5 stage (Pfeiffer, 1976). This event is thought to be induced when parasites are exposed to adverse environmental conditions, for example in autumn, prior to uptake (Pfeiffer, 1976), or when parasites are undergoing development in hosts which have some degree of immunity (Jarrett *et al.*, 1955b). Cessation of development at this stage has been observed to last for up to 6 months (Pfeiffer and Supperer, 1980).

L1 passed in faeces develop to infective L3 within three days under optimal conditions of temperature and humidity (Urquhart *et al.*, 1987), but at lower temperatures this may take up to 26 days (Rose, 1956). The free-living stages do not feed, but it is thought that they obtain their nutrition from food granules present within their intestinal cells (Daubney, 1920). The L3 retain the cuticles of one or both of the preceding stages and this provides a degree of protection while they are in the environment (Taylor, 1942). The L3 is the most environmentally resistant of the free-living stages and a proportion are able to survive over the winter to the next grazing season (Jarrett *et al.*, 1954; Duncan *et al.*, 1979). Clinical disease occurs in late summer due to a build up of larvae on the pasture from infections which have established in spring.

1.1.3 Pathogenesis of *D. viviparus*

The first description of the pathology of field cases of *D. viviparus* was by Jarrett *et al.* (1954) and more detailed accounts of the pathogenesis were reported using experimental infections (Jarrett *et al.*, 1957a, 1957b, 1960). A summary of these findings follows.

The period during which larvae enter the host and migrate to the lungs is described as the penetration phase. Generally, there are no clinical signs associated with this phase. During the pre-patent phase of infection, larvae enter the alveoli and migrate up the respiratory tree and this results in coughing, tachypnoea and hyperpnoea, the severity of which depends on the size of the infecting dose and on the individual calf, i.e. size and disease status. When larvae appear in the alveoli, they elicit an acute inflammatory response of polymorphs, macrophages and multinucleate giant cells (Jarrett *et al.*, 1957a, 1957b).

Similar infiltrates are observed in the bronchioles as the larvae make their way up the respiratory tree (Jarrett *et al.*, 1957a, 1957b). These infiltrates can be so severe as to cause blockage of the bronchiolar lumen and collapse of dependent alveoli (Jarrett *et al.*, 1960). Heavily infected animals may die during this pre-patent phase from respiratory failure caused by the associated alveolar collapse, interstitial emphysema and pulmonary oedema (Jarrett *et al.*, 1957a).

By Day 25 pi mature adults in the bronchi and trachea cause bronchitis and tracheitis, with hyperplasia of the bronchiolar epithelium and infiltration of the bronchiolar mucosa with large numbers of eosinophils, some neutrophils and plasma cells (Jarrett *et al.*, 1957a, 1957b). Aspiration of freshly laid eggs and L1 into the bronchioles and alveoli induces infiltrates of neutrophils, macrophages and multinucleate giant cells and dense masses of eosinophils, containing central areas of necrosis (Jarrett *et al.*, 1957a, 1960). This exacerbates the pathology and this severe pneumonia may lead to widespread interstitial emphysema and pulmonary oedema with the production of hyaline membranes on alveolar surfaces (Jarrett *et al.*, 1957a, 1957b). Animals will cough frequently and be tachypnoeic, hyperpnoeic and sometimes dyspnoeic. They may be anorexic, dehydrated and, on occasion, pyrexic (Jarrett *et al.*, 1960). The highest level of mortality is seen during this stage of infection.

Most animals which survive the patent phase expel the adult worms and gradually recover with a steady abatement of clinical signs. However, approximately 25% of severely infected animals may show a sudden exacerbation of clinical signs, which may be fatal (Jarrett *et al.*, 1960). The pathology associated with these cases is a diffuse alveolar epithelialisation involving proliferation of Type II pneumocytes, giving the lung an enlarged and rubbery appearance (Jarrett *et al.*, 1957a, 1957b, 1960). It has been suggested that this is a response to aspiration of dead parasites or their breakdown products (Urquhart *et al.*, 1987).

If exposed to a particularly heavy challenge, immune cattle may show clinical signs, known as re-infection syndrome. This is due to the migration and death of large numbers of larvae in the lungs (Jarrett *et al.*, 1960) and is transient.

1.1.4 Control of *D. viviparus*

The *D. viviparus* irradiated larval vaccine (Huskvac, Intervet UK Ltd., Cambridge, UK) has been in use for almost 40 years and has been highly effective in the control of parasitic bronchitis. Although vaccinated calves exhibit a high level of resistance to infection, the immunity is not absolute and maintenance of this immunity is dependant on boosting by natural field challenge (Urquhart, 1985). Therefore, vaccinated calves may become immune carriers (Cornwell and Berry, 1960) and under severe challenge they may show clinical signs, although this is rare (Jarrett and Sharp, 1963; Urquhart, 1985).

Anthelmintics, such as the avermectins, benzimidazoles and the imidazothiazoles, developed for the control of gastrointestinal nematodes, have also been used to control *D. viviparus* infections. The traditional combination of strategic grazing combined with a single anthelmintic treatment has been less effective for the control of parasitic bronchitis. This is because increases in pasture L3 are unpredictable, occurring any time between June and October (Duncan *et al.*, 1979), and relatively small numbers of parasites are required to induce clinical disease. Other control methods involve strategic treatments with anthelmintics over the grazing season, or the administration of intra-ruminal boluses which release anthelmintic in pulses (Armour *et al.*, 1988) or as sustained release (Kerboeuf *et al.*, 1996; Grimshaw *et al.*, 1996). These anthelmintics are highly efficacious and successive use of such strategies may eventually eliminate pasture contamination, with the consequence that animals may not be exposed to sufficient parasite antigen to stimulate protective immunity. In unvaccinated calves, the development of immunity to *D. viviparus* will be related to the level of challenge experienced during the period of prophylaxis (Eysker *et al.*, 1990b) and to the effectiveness of the prophylactic strategy used (Fisher and Jacobs, 1995). It could be anticipated that whole herds may become susceptible and severe outbreaks may occur. Furthermore, there have been concerns regarding the putative adverse effects of anthelmintics and their residues in animal products and the environment; for example, ivermectin in faeces has been shown to have a detrimental effect on the fauna present (Strong and Wall, 1990).

1.1.5 The immune response to *D. viviparus*

Infection with *D. viviparus* induces a strong acquired immunity, however, the exact mechanisms involved in protection to this parasite remain unclear.

1.1.5.1 The site of the immune response

Work by Michel (1956) lead to the initial assumption that the sites of the immune response were the mesenteric lymph nodes and/or lymphatic vessels. His studies, using the ovine lungworm, *D. filaria,* in a mouse model, found that resistance to infection was lost when the mesenteric lymph nodes were bypassed. Later work has disputed this hypothesis and it is now thought that the most likely location of a protective immune response is the respiratory tract. For example, in calves immunised by either vaccination or by previous infection, a significant number of larvae reach the lungs within 24 h of challenge with 200,000 L3 (Poynter *et al.*, 1960) or seven days after challenge with 1000 L3 (Jarrett and Sharp, 1963). These studies demonstrate that in immune calves most larvae reach the lungs, thus the protective immune response is likely to occur in the respiratory tract. In addition, after subcutaneous injection of L3, where there is no subsequent migration through the mesenteric lymph nodes, calves were protected against re-infection (Wade and Swanson, 1958; Sweitlikowski, 1969). More recently, subcutaneous (Bain and Urquhart, 1988) or intravenous (Canto, 1990) vaccination with irradiated larvae were demonstrated to be as effective as the oral route of administration. Despite these findings, little attention has been paid to the possible effector mechanisms acting in the lungs.

A feature of infection in immune animals, which has not been demonstrated in susceptible animals, is the development of accumulations of inflammatory cells around larvae which have died in the bronchioles (Simpson *et al.*, 1957; Jarrett and Sharp, 1963). One study found lower numbers of these "lymphoid nodules" and higher numbers of parasites in the lungs of calves which died compared with calves that survived an experimental challenge (Pirie *et al.*, 1971). The authors suggested that the nodules arose due to the immunological responses against larvae in the bronchioles (Pirie *et al.*, 1971). However, Poynter *et al.* (1970) found that the presence of nodules did not always correlate with immunity and they stated that the precise relationship between the two was unclear.

1.1.5.2 The role of parasite antigens

Molecules which are excreted/secreted by parasitic nematodes and antigens present on their surface represent the host-parasite interface, so are of particular interest from an immunological point of view.

Excretory/secretory products

Adult excretory/secretory (E/S) material has been shown to produce significant, but variable, levels of protection against challenge with *D. viviparus* in guinea pigs (McKeand *et al.*, 1995). A wide range of functional molecules are released during *in vitro* culture of parasitic nematodes and proteases of the cysteine, serine and metalloprotease classes have been identified in the E/S products of *D. viviparus* (Britton *et al.*, 1992b). These enzymes are thought to have a variety of functions including invasion of the host and parasite feeding. *D. viviparus* L3 and adult E/S products have also been shown to contain superoxide dismutase (SOD) activities (Britton *et al.*, 1994). These anti-oxidants are thought to protect the parasite from oxidant-mediated damage arising from normal cellular catabolism or from the respiratory burst of activated host immune effector cells (Babior *et al.*, 1973). High levels of acetylcholinesterase (AChE) have been detected in the E/S products of *D. viviparus* (McKeand *et al.*, 1994a). It has been hypothesised that AChE may assist parasites in overcoming the host's immune effector mechanisms by breaking down ACh and interfering with the local effects of Ach, including mucous secretion (Hafez, 1977), lysosomal enzyme release (Ignarro and Colombo, 1972), neutrophil chemotaxis (Koyama *et al.*, 1992) and histamine release (Kaliner and Austen, 1975).

The E/S products which have been studied have been derived from *in vitro* cultured parasites. Adult *D. viviparus* die after a few days in culture, and it is unclear whether the products released reflect the physiological secretions of the parasites *in vivo.* However, serum from infected individuals has been shown to immuno-precipitate E/S antigens of *D. viviparus* (Britton *et al.*, 1993a; McKeand *et al.*, 1994a). Such serum has also been shown to specifically inhibit E/S proteinase (Britton *et al.*, 1992b), SOD (Britton *et al.*, 1994) and AChE (McKeand *et al.*, 1994a) activities. Therefore *D. viviparus* E/S products are highly

antigenic. The importance of the E/S products in protective immunity is still unclear and considerable heterogeneity in the recognition of particular E/S antigens has been demonstrated in individual guinea pigs (McKeand *et al.*, 1994b) and calves (Britton *et al.*, 1992a).

1.1.5.3 Surface antigens

Antibody responses to a wide range of cuticular and surface antigens have been demonstrated in parasitic nematodes; these include responses to the cuticular collagens (Pritchard *et al.*, 1988), the insoluble protein matrix of the epicuticle (Betschart *et al.*, 1987) and molecules of the surface coat (Smith *et al.*, 1981; Blaxter *et al.*, 1992). Specific antibody responses have been demonstrated against the surfaces of L3, adult (Canto *et al.*, 1992; Britton *et al.*, 1993b; McKeand *et al.*, 1996), L1 and egg (Britton *et al.*, 1993b) stages of *D. viviparus*. Grencis *et al.* (1986) obtained protection to *Trichinella spiralis* infection after immunisation with antigens which were stripped from the surface of larvae. This study emphasised the importance of surface antigens in the stimulation of protective immunity and, more recently, a 30 kDa surface-associated antigen was identified in adult *Brugia spp.* which is highly conserved across the filarial species (Devaney *et al.*, 1990) and elicits an immune response early in the course of infection (Maizels *et al.*, 1989). However, the nematode surface is not merely an inert extracellular covering, but it is a very dynamic structure (Philipp and Rumjaneck, 1984) and a rapid turnover of surface molecules has been shown in a variety of nematodes including *Toxocara canis* (Smith *et al.*, 1981), *T. spiralis* (Phillip *et al.*, 1980) and *Caenorhabditis elegans* (Politz and Phillip, 1992). This potential to shed surface-exposed antigens may provide the parasite with a means of evading host effector mechanisms. Indeed, McKeand and Kennedy (1995) demonstrated surface shedding of antibody by adult *D. viviparus* following incubation *in vitro* for 24 h at 37^{0}C.

1.1.5.4 Antibody responses to *D. viviparus*

Using passive transfer experiments, Jarrett *et al.* (1955a) showed that antibody was protective against *D. viviparus*. Since this finding, studies on immunity to *D. viviparus* have focused on humoral responses following infection and vaccination. Early studies measured whole IgG in serum using the complement fixation test (CFT) and indirect haemaglutination techniques and these experiments detected whole IgG from Day 30 (Jarrett *et al.*, 1959a) and Day 80 (Cornwell and Michel, 1960) after infection. However, in vaccinated calves, where there were no adult parasites, little or no serum IgG was detected (Cornwell, 1960; Bos *et al.*, 1986). Others also found that calves vaccinated with the 400 Gy-irradiated L3 vaccine had little or no antibody to adult antigen, despite being immune to subsequent challenge (Jarrett *et al.*, 1961; Cornwell, 1960). From these studies it can be concluded that the levels of total parasite-specific antibody do not act as an accurate gauge of the immune status of an animal.

Using CFT, Cornwell (1963) found the same general pattern of response to saline extracts of a range of parasite stages and a more recent study, using whole parasite extracts, found similar responses to L4 and adult stages (Wassal, 1991). It is likely that different parasite stages share common antigens, so extracts of whole parasites will include a number of cross-reactive antigens. In agreement with these observations, sera from vaccinated calves recognised adult E/S antigens, despite the fact that vaccinated calves are not exposed to the adult stage of the parasite (Britton *et al.*, 1993a), suggesting identical or cross-reactive antigens are released from larvae and adults. In contrast, stage-specific antigens have been demonstrated on the surface of *D. viviparus*: vaccinated calves, in which adult parasites do not develop, do not produce antibody specific to the surface of this stage (Britton *et al.*, 1993b; McKeand *et al.*, 1996).

In another study of antibody isotype responses to surface antigens, calves were infected with 2000 larvae on Days 0 and 48 and with 10,000 larvae on Day 76 (McKeand *et al.*, 1996). IgM antibodies to the adult surface increased by Day 48 pi and decreased slightly by Day 76 pi. This decrease was accompanied by a substantial increase in IgG1, which increased again on Day 104 (McKeand *et al.*, 1996). This class switch in antibody isotype between Days 48 to 76 coincided with the time of death of adult parasites in the lungs (Jarrett and Sharp, 1963) and so may have been important in mediating parasite expulsion.

1.1.5.5 The role of eosinophils

As with other helminth infections, *D. viviparus* is associated with blood (Weber and Rubin, 1958; Mackenzie and Michel, 1964) and tissue (Jarrett *et al.*, 1960) eosinophilia. After infection with normal larvae, two peaks of blood eosinophilia occur (Weber and Rubin, 1958; Cornwell, 1962a; Mackenzie and Michel, 1964). It was suggested that the first peak of eosinophilia, which was between nine to 15 days after infection, was stimulated by larvae developing in the lungs and the second peak, which was around 40 days after infection, by the aspiration of eggs and first stage larvae following parasite patency (Cornwell, 1962a). Vaccination with irradiated larvae stimulated a single peak of eosinophilia and a second dose of vaccine gave rise to a similar response (Cornwell, 1962a; Mackenzie and Michel, 1964).

Eosinophils have been shown to adhere to and kill a number of helminth parasites *in vitro*, including *T. spiralis* newborn and infective larvae (Bass and Szedja, 1979), *Wuchereria bancrofti* microfilariae (Higashi and Chowdhury, 1970) and *Schistosoma mansoni* schistosomulae (Capron *et al.*, 1983). Bovine eosinophils have been shown to adhere to *D. viviparus* L3 with an associated reduction in parasite motility (Knapp and Oakley, 1981). A link was suggested between eosinophilia and resistance to *D. viviparus* infection when a correlation was observed between eosinophil levels and survival in an outbreak of parasitic bronchitis (Djafard *et al.*, 1960). However, there is no direct evidence of parasite rejection or killing by eosinophils *in vivo*. In fact, although eosinophils adhere to the surface of *T. canis* L2, and this adherence initiates eosinophil degranulation, these larvae show little damage and even slough off the adherent cells (Fattah *et al.*, 1986).

1.2 T cells in nematode infections

1.2.1 T cells in the bovine

Since the mid-1980's a large number of monoclonal antibodies (Mab) specific for cattle leukocyte cell-surface antigens have been produced, allowing rapid progress to be made in understanding the functions of bovine lymphocyte subsets and the biochemical characterisation of some of the molecules with which the Mabs react. A number of collaborative workshops have been held to help study the various Mabs, and at these workshops the Mabs have been clustered into groups recognising the same antigens. It was found that many of the Mabs recognised antigens equivalent to human differentiation antigens, so these were designated BoCD and given the equivalent CD number to the human cluster. However, various clusters were established for which there was no human equivalent, for which there was insufficient data to firmly establish homology with a human CD antigen, these were assigned workshop cluster (WC) designations.

As in the mouse and man (Kelso *et al.*, 1991), mature peripheral bovine T cells can be divided based on three parameters. The first is the expression of $\alpha\beta$ or $\gamma\delta$ T cell receptors for antigen. The second is the expression of BoCD8 (Ellis *et al.*, 1986) or BoCD4 (Bensaid and Hadam, 1991) accessory molecules, where the reciprocal expression of CD8 and CD4 reflects their function as receptors for Class I or II major histocompatibility complex (MHC) determinants, respectively. Class I molecules generally display peptides derived from intracellular sources, whereas class II molecules display peptides of extracellular origin (Braciale *et al.*, 1987). The third parameter that sub-divides bovine T cells is the expression of activation markers such as BoCD45 (Howard *et al.*, 1991), and a variety of adhesion and homing receptors such as L-selectin (Howard *et al.*, 1992b) and E-selectin (Walcheck *et al.*, 1993).

1.2.1.1 Gamma delta T cells

In humans and mice the majority of T cells express the αβ receptor, however, in ruminants, a large population of cells in the peripheral circulation express the γδ T cell receptor (Hein and Mackay, 1991). During the first one to two weeks after birth, the numbers of $\gamma\delta^+$ cells in peripheral blood expand rapidly so that by three weeks after birth, they constitute 60% of the total T cell population (Wyatt *et al.*, 1994). As the animal ages, the proportion of $\gamma\delta^+$ T cells gradually declines, so that in a mature animal (five to eight years old) $\gamma\delta^+$ T cells comprise five to ten percent of the total T cell population (Clevers *et al.*, 1990).

The majority of ruminant γδ T cells, and no other cell types, express a newly identified member of the scavenger receptor cysteine-rich (SRCR) domain family, designated WC1 (Wijngaard *et al.*, 1992). The tissue distribution of $WC1^+$ T cells is unusual in that they localise at epithelial surfaces and very few are found in the usual areas where T cells are found: few $WC1^+$ T cells are found in the thymus, lymph nodes or spleen (Hein and Mackay, 1991). $\gamma\delta^+$ T cells have been demonstrated in the pseudostratified epithelium of the bovine trachea (Hein and Mackay, 1991) and in bronchoalveolar lavage fluid (BALF) collected from humans (Spinozzi *et al.*, 1996) and rats (McMenamin *et al.*, 1995), so they may play an important role in immune responses in the respiratory tract. There is an additional smaller subpopulation of $\gamma\delta^+$ T cells in the bovine which do not express WC1 (Sopp *et al.*, 1991; Wyatt *et al.*, 1994). The exact tissue distribution of these $WC1^-$ cells is unknown, however, recent studies have found that they make up a significant proportion of mammary gland secretions (Park *et al.*, 1993) and large populations of these cells have been demonstrated in the bovine spleen (Wyatt *et al.*, 1994).

The localisation of $WC1^+$ T cells to epithelial surfaces is thought to reflect their embryonic origins (Hein and Mackay, 1991). In the neonatal ruminant, the thymic medulla contains prominent Hassalls' bodies with dense accumulations of $WC1^+$ T cells (Hein and Mackay, 1991) and it is presumed that $\gamma\delta^+$ T cells originate from these ectodermally-derived cells (Mackay *et al.*, 1989). Moreover, bovine $WC1^+$ T cells avidly bind human E-selectin which is preferentially expressed on venules in the skin, and unlike conventional $\alpha\beta^+$ T cells, prior antigen stimulation and differentiation to a memory lymphocyte are not required for this interaction (Walcheck *et al.*, 1993). The avid binding to this epithelial homing receptor suggests a possible mechanism for the epithelial localisation of $\gamma\delta^+$ T cells.

The function of $\gamma\delta^+$ T cells is still unclear. However, the fact that they are located on epithelial surfaces indicates that they may act as a first line of defence, monitoring pathogens and immune-mediated damage and regulating immune cell activity. Compared with antigen recognition by $\alpha\beta^+$ T cells, recognition by $\gamma\delta^+$ T cells is not always MHC-restricted. Schild *et al.* (1994) showed that $\gamma\delta^+$ T cells bound MHC molecules at a site away from the peptide-binding groove where the $\alpha\beta$ receptors normally bind. In addition, $\gamma\delta^+$ T cell activation did not require antigen processing (Schild *et al.*, 1994). Furthermore, the type of antigen presenting cell (APC) expressing the MHC molecule and the species of origin of the APC does not appear to effect the ability of stimulator cells to activate the $\gamma\delta^+$ T cells (Schild *et al.*, 1994). This implies that antigens can be recognised directly by $\gamma\delta^+$ T cells, allowing greater flexibility than classical $\alpha\beta^+$ T cell type responses. In agreement with this, $\gamma\delta^+$ T cell hybridomas from neonatal mice have been shown to be activated directly using purified protein derivative (PPD) from *Mycobacterium tuberculosis* (O'Brien *et al.*, 1989). A major antigenic component of the PPD of *M. tuberculosis* is a heat shock protein (hsp65), which is homologous to eukaryotic heat shock proteins and recombinant hsp65 also activated the $\gamma\delta$ T cells directly (O'Brien *et al.*, 1989). This suggests that $\gamma\delta^+$ T cells may recognise and be activated by self-antigens. Therefore, $\gamma\delta^+$ T cells may recognise transiently-expressed self-antigens on injured and infected cells, as well as self-antigens on activated leukocytes.

Further evidence for a role for $\gamma\delta^+$ T cells in immune surveillance in a mouse model was provided by Ferrick *et al.* (1995), who demonstrated that $\gamma\delta^+$ T cells were involved in establishing primary immune responses to *Listeria monocytogenes* and *Nippostrongylus brasiliensis*. These authors measured cytokine responses by $\alpha\beta^+$ and $\gamma\delta^+$ T cells and demonstrated that $\gamma\delta$ T cells were the major source of IL-4 (Ferrick *et al.*, 1995). $\gamma\delta^+$ T cell-deficient mice infected with *L. monocytogenes* developed large abscesses compared with normal mice (Mombaerts *et al.*, 1993), which supports a role for $\gamma\delta^+$ T cells in controlling the immune response in this infection. There is some evidence that $\gamma\delta^+$ T cells play a similar role in the bovine. For example, Park *et al.* (1993) demonstrated down-regulation of the proliferative response of $CD4^+$ T lymphocytes to *Staphylococcous aureus* antigens by activated bovine $\gamma\delta^+$ T cells obtained from mammary gland secretions. A mechanism for inhibition of immune responses by $\gamma\delta$ T cells was suggested by Hsieh *et al.* (1996) who showed increased production of IL-10 by splenic $\gamma\delta^+$ T cells in mice infected

with *L. monocytogenes*, which was followed by a decrease in IFNγ and reduced inflammation and tissue damage. While the above studies imply a role for $\gamma\delta^+$ T cells in inhibition of Th1 responses, McMenanin *et al.* (1995) adoptively transferred antigen-specific-IgE tolerance to an aerosol of ovalbumin antigen in rats by injecting $\gamma\delta^+$ T cells intraperitoneally. The $\gamma\delta^+$ T cells were demonstrated to produce high levels of IFNγ in response to ovalbumin antigen and it was postulated that the IFNγ inhibited the Th2 response and IgE production (McMenanin *et al.*, 1995). Therefore, $\gamma\delta^+$ T cells seem to act as local regulators in tissue, both initiating and controlling immune responses to pathogens which induce either Th1 or Th2 responses.

1.2.1.2 Bovine $CD4^+$ and $CD8^+$ cells

The bovine equivalent of CD4 was first described by Baldwin *et al.* (1988). Mabs to this molecule were found to bind to 20 to 30% of PBMC which were also found to be MHC Class II positive. $CD4^+$ T cells activate macrophages and B cells so are important in the control of effector mechanisms to kill both intracellular and extracellular pathogens (Janeway and Travers, 1994). The importance of $BoCD4^+$ cells in immunity to bovine viral diarrhoea virus was demonstrated by Howard *et al.* (1992a) when calves injected with anti-bovine CD4 Mab had prolonged diarrhoea and an increase in virus titre in blood, whereas there was no change in either clinical signs or virus titre in those injected with anti-bovine CD8 or WC1. Moreover, T cell clones which proliferated in response to antigen in cattle infected with bovine leukaemia virus (Gatei *et al.*, 1993) and *Theileria parva* (Baldwin *et al.*, 1992) were found to be $CD4^+$.

As in the mouse and man, naive $BoCD8^+$ cells differentiate into cytotoxic T cells. Cytotoxic cells specific to bovine leukaemia virus (Gatei *et al.*, 1993) and *T. parva* (Teale *et al.*, 1986; Goddeeris *et al.*, 1991) have been found to be $BoCD8^+$. Anti-MHC Class I Mab partially inhibited the cytotoxic function of $BoCD8^+$ cells harvested from *T. parva* infected cattle (Ellis *et al.*, 1986). $CD8^+$ cells have been reported to suppress immune responses in the bovine (Lutje and Black, 1992). For example, Harp *et al.* (1995) found an increase in $BoCD8^+$ cells in the spleens of calves infected with *Cryptosporidium parvum* and this was

associated with greatly reduced proliferative responses for cells cultured with parasite antigen compared with cells incubated in medium alone.

1.2.2 Immune responses to nematodes

Nematodes, unlike microbial or protozoal pathogens, are too large to be phagocytosed by cells of the host's immune and reticulo-endothelial system. Instead, T helper cells direct a whole spectrum of effector mechanisms which may lead to parasite damage or death. These effector mechanisms include antibody-dependent cell-mediated cytotoxicity, mast cell degranulation and complement activation by both the classical and alternative pathways (David, 1990).

Mastocytosis, eosinophilia and increased IgE which are often observed in nematode infections, are also hallmarks of Type I hypersensitivity reactions, but the functional significance of these responses is unclear. There is a large body of evidence to support the view that they are part of the host's defence against parasites, however, other studies have indicated that the allergic response may be an immunopathological or immunoregulatory consequence which may promote parasite survival. Studies in the mouse suggest that mucosal mast cells protect against tissue dwelling nematodes like *T. spiralis* and *Strongyloides ratti* (Grencis *et al.*, 1993; Nawa *et al.*, 1994). However, the lumen dwelling parasite *N. brasiliensis* is apparently expelled by mice without the need for mast cells (Nawa *et al.*, 1994). In fact, the fecundity of *N. brasiliensis* is reduced in mast cell deficient mice, perhaps implying that mast cells improve worm fecundity (Arizono *et al.*, 1993; Newlands *et al.*, 1995). Also, although studies in sheep have shown strong correlations between mastocytosis and levels of sheep mast cell protease (SMCP) with expulsion of *Haemonchus contortus* and *Trichostrongylus vitrinus* infections (Miller *et al.*, 1995; Emery *et al.*, 1993), *H. contortus* was expelled rapidly by hyperimmune sheep when mucosal mast cell numbers were low (Huntley *et al.*, 1992).

IgE-dependent activation of eosinophils leads to the release of granule proteases which appear to be cytotoxic to *S. mansoni* (Gounni *et al.*, 1994). However, these granule proteases have also been shown to be toxic to mammalian cells (Spry *et al.*, 1992) suggesting an immunopathological consequence. A seroepidemiological study of *Schistosoma haematobium* in humans by Hagan *et al.* (1991) demonstrated that parasite-

specific IgE antibodies were beneficial and that reinfection was significantly less likely in those with high levels of IgE. However, infections with helminths also results in disproportionately high levels of total, non-parasite-specific IgE (Jarrett and Bazin, 1974). This excess IgE could saturate IgE receptors on mast cells, basophils, eosinophils and macrophages, preventing specific mediator release. It has been postulated that excess IgE may bind the type A isoform of the low affinity IgE receptor (CD23) on B cells, so reducing synthesis of parasite-specific IgE (Pritchard, 1993). Moreover, eosinophils are thought to be the prime effector cells in the pathophysiology of asthma (Frigas and Gleich, 1986; Seminario and Gleich, 1994).

1.2.3 T helper cell subsets

1.2.3.1 Introduction

Studies in mice have shown that on activation, naive T cells, which produce only IL-2 (Street *et al.*, 1990), give rise to Th0 cells which produce a wide range of cytokines (Firestein *et al.*, 1989; Street *et al.*, 1990). The Th0 cells, in turn, develop into Th1 or Th2 cells, which can be differentiated by the various cytokines they produce (Mosmann and Coffman, 1989). Upon activation, both subsets produce mRNA for interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor (TNF) α and β. Th1 cells induce IL-2, IFNγ and lymphotoxin (LT), while Th2 cells induce IL-4, IL-5, and IL-6 (Mosmann and Moore, 1991). This differential secretion of cytokines suggests that Th1 and Th2 cells may be involved functionally in different immune responses (Street *et al.*, 1990). In the mouse, Th1 responses are important in cell-mediated immunity, largely dominated by IFNγ which acts on macrophages and monocytes to up-regulate processes known to be beneficial to phagocytosis of opsonized micro-organisms (Ceretti *et al.*, 1986b). Th2 cells, in the mouse, are responsible for antibody-mediated immunity, dominated by IL-4 which has a major role in the activation of B cells, in particular in promotion of IgG1 (Vitetta *et al.*, 1985) and IgE (Coffman and Carty, 1986) production. This strict distinction between Th1 and Th2 cells seems to be more absolute in inbred mice following exposure to particular pathogens than in outbred populations exposed to several

pathogens. For example, human Th1 clones can secrete IL-4 (Haanen *et al.*, 1991), while Th2 clones can secrete IL-2 and IFNγ (Yssel *et al.*, 1992). Therefore, human T cell clones are defined on the basis of the ratios of IL-4 and IFNγ that they produce (de Waal Malefyt *et al.*, 1995)

1.2.3.2 Regulation of T helper cell responses

T cells require the presence of IL-4 in order to differentiate into Th2 cells (Finkelman and Urban, 1992). Cells, other than T cells, have been shown to produce IL-4 and these may provide a source of IL-4 for Th2 differentiation. *In vivo* studies have shown that IgE-primed mast cells and basophils produce IL-4 (Bradding *et al.*, 1992; Bradding *et al.*, 1995). Moreover, it has been demonstrated that the bee venom allergen, phospholipase A2, induced IgE-independent mediator release, including IL-4, from rodent mast cells, suggesting that these cells may be a primary source of IL-4 in allergic reactions (Dudler *et al.*, 1995). Another potential primary source of IL-4 is the $\gamma\delta^+$ T cell, as described in *N. brasiliensis* infected mice (Ferrick *et al.*, 1995). Yet another possible source of IL-4 was demonstrated by Yoshimoto and Paul (1994), when they found that mice, injected with anti-CD3 or *Staphylococcal* enterotoxin B, expressed IL-4 mRNA from a population of $CD4^+$ cells bearing the surface marker NK1.1. Therefore, mast cells, basophils, $\gamma\delta^+$ T cells and $CD4^+NK1.1^+$ cells are all potential sources of IL-4 in primary infections which initiate Th2 responses.

IL-13 has many biological activities which are shared with IL-4, including induction of B cell proliferation and IgE synthesis. IL-13 is produced by human $CD4^+$ Th0, Th1 and Th2 clones, $CD8^+$ cells (de Waal Malefyt *et al.*, 1995) and IgE-activated mast cells (Burd *et al.*, 1995). It has been shown that naive human $CD4^+CD45RO^-$ T cells, stimulated using anti-CD3 Mab, produced large amounts of IL-13, IL-5 and IFNγ, but no IL-4, and that these cells induced efficient IgE production by B cells (Brinkmann and Kristofic, 1995). Therefore, these cells may drive IL-13-dependent IgE production by themselves and this IgE could prime mast cells and eosinophils for IL-4 production.

IFNγ and IL-12 are both produced by macrophages and it is likely that intracellular bacteria and viruses induce Th1 type responses through macrophage activation (Romagnani,

1992). Monoclonal antibodies against IFNγ given to mice vaccinated against *S. mansoni* caused a dramatic reduction in Th1-associated immunity (Sher *et al.*, 1990), confirming that IFNγ is important in driving Th1 responses. IFNγ and IL-12 promoted the differentiation of allergen-specific T cells *in vitro* to Th0 or Th1, instead of Th2 clones (Parronchi *et al.*, 1992). Therefore, these cytokines not only upregulate Th1 responses, but they also act to inhibit Th2 responses. In agreement with this, exogenous IL-12 inhibited Th2 responses in the popliteal lymph nodes following footpad injection of *S. mansoni* eggs in mice (Oswald *et al.*, 1994). This suppression was blocked if the mice were simultaneously injected with neutralizing anti-IFNγ monoclonal antibody (Oswald *et al.*, 1994), indicating that IL-12 acted via IFNγ. These findings suggest that IL-12 has enormous potential as an immunomodulator, and indeed, Wynn *et al.*, (1995) showed that administration of IL-12 significantly increased vaccine-induced immunity to *S. mansoni* in the mouse. Moreover, Urban *et al.* (1996) found that exogenous IL-12 prevented *C. parvum* infection in mice through IFNγ-dependent mechanisms.

IL-10 is produced by murine Th2 cells (Sher *et al.*, 1991), $\gamma\delta^+$ T cells (Hseih *et al.*, 1996), B cells and mast cells (Mosmann and Moore, 1991) and was shown to inhibit cytokine production, including IFNγ, by Th1 cells in mice (Fiorentino *et al.*, 1989). This inhibitory role for IL-10 on IFNγ production was confirmed in mice infected with *N. brasiliensis*. These animals produced large quantities of IL-10, and addition of anti-IL-10 Mab increased IFNγ synthesis (Mossman and Moore, 1991). IL-10 was also shown to be responsible for the downregulation of Th1 responses to *S. mansoni* eggs (Sher *et al.*, 1991). Similarly, IL-10 knockout mice developed chronic enterocolitis due to uncontrolled inflammatory Th1 responses to gut flora antigens (Kuhn *et al.*, 1993). However, this is not the situation in all hosts. For example, IL-10 is produced by human (Yssel *et al.*, 1992) and bovine (Brown *et al.*, 1994c) Th0, Th1 and Th2 clones and has been shown to inhibit cytokine production and proliferation of all three Th cell types in these species rather than just Th1 cells (Yssel *et al.*, 1992; Brown *et al.*, 1994c). In these experiments, IL-10 primarily affected antigen presenting cell (APC) function, and did not directly inhibit human (Yssel *et al.*, 1992) and bovine (Brown *et al.*, 1994c) $CD4^+$ T cells.

Therefore, crossregulation of antibody responses and delayed type hypersensitivity reactions may, at least, be partially explained by mutual inhibition of Th1 and Th2 cells.

1.2.3.3 T helper cell responses in rodent nematode infections

Immunity to nematode parasites has been most intensively studied using intestinal parasites in rodents. It has been found that these infections are generally characterised by high levels of IgE, eosinophils and mast cells. IgE has been demonstrated to be regulated by IL-4 (Coffman and Carty, 1986) and IL-13 (de Waal Malefyt *et al.*, 1995), while eosinophils are regulated by GM-CSF, IL-3, and IL-5 (Yamaguchi *et al.*, 1988; Spry *et al.*, 1992). Mast cells are regulated by IL-3, IL-4, IL-9 and IL-10 (Madden *et al.*, 1991). All these cytokines are produced by Th2 cells, demonstrating a correlation between nematode infections and induction of Th2 responses. Moreover, Th2 cell responses have been associated with expulsion of *N. brasiliensis* (Street *et al.*, 1990), *Heligmosomoides polygyrus* (Urban *et al.*, 1991) and *Trichuris muris* (Else and Grencis, 1991). However, the situation appears to be more complicated as a number of factors may affect the specific immune response to infection.

Different parasite antigens may determine the Th cell subset which is activated and exposure to specific parasite antigens, presented during the course of infection, may determine the Th subset which predominates. This hypothesis was proposed by Else *et al.* (1992) when they demonstrated that early larval stages of *T. muris* induced a Th2 response and later stages, a Th1 response. It may be that parasites possess triggers for both Th1 and Th2 responses and that different individuals vary in their sensitivity to these stimuli. For example, Else and Grencis (1991) observed that *T. muris* triggered Th1 responses in a susceptible mouse strain, but Th2 responses in a resistant mouse strain.

The differential induction of Th cell subsets is not a systemic phenomenon and expression of different cytokine profiles in different immunological compartments is a common feature of the immune response. Splenic $CD4^+$ T cells from uninfected mice produced more IL-4 than mucosal lymphoid tissue $CD4^+$ cells, while peripheral lymph node cells secreted the highest levels of IFNγ (Tonkonogy and Swain, 1993). Compartmentalisation was demonstrated in mice infected with *T. spiralis,* when simultaneous Th1 and Th2 responses were demonstrated in the spleen and mesenteric lymph nodes, respectively (Kelly *et al.*, 1991). This compartmentalisation must be considered when analysing immune responses. Moreover, T cells from one compartment are able to

respond to different pathogens with different Th responses. For example, a strong skewing of the cytokine environment towards Th2 in PBMC from humans infected with filarial parasites did not prevent the predominance of Th1 cytokines in response to *M. tuberculosis* (Sartono *et al.*, 1996). Therefore, when analysing immune responses to parasites, the parasite stages, the anatomical location of the parasite and the genetic make-up of the host may all bias the results.

1.2.4 T helper subsets in the bovine

It has been shown that Th1- and Th2- like subsets exist in humans (Romagnani, 1991), but it is still not entirely clear if the same Th subsets exist in cattle and, if so, if they play similar roles. Much of the initial work in cattle has focused on immunity to intracellular protozoal infections, where, in mice, Th1 cytokines have been shown to play an important role (Finkelman and Urban, 1992). When investigating Th cell populations in clones from cattle infected with the intra-erythrocytic protozoan parasite *Babesia bovis*, Brown *et al.* (1993b) found that infection induced heterogeneous lymphocyte subpopulations. They found that the unrestricted Th0 subset predominated and identified one Th1 clone. Others studying cattle infected with *T. parva*, which infects the lymphocytes, found that infection stimulated production of IL-2 receptors (Dobbelaere *et al.*, 1990; Coquerelle *et al.*, 1989) and IFNγ (Entrican *et al.*, 1991), suggesting a Th1 type response.

The only work reported to date on Th cell responses in bovine helminth infections described the isolation of T cell clones from cattle infected with the trematode *Fasciola hepatica*. Most clones were Th0, however, some Th2 clones were identified, expressing IL-4 but little or no IL-2 or IFNγ (Brown *et al.*, 1994b).

All of the above T cell studies in the bovine have relied exclusively on cells cloned from a single time point in infection. As it is well known that the cloning environment has a significant impact on the cell subsets produced (Gajewski *et al.*, 1991), it is not clear whether the *in vitro*-derived clones truly reflect the *in vivo* situation. With this lack of knowledge on Th cell responses to bovine helminth infections, this study was designed to examine T cell responses to *D. viviparus* in calves.

1.3 Objectives

The aim of the work described in this thesis was to study immunity induced by *D. viviparus* at the level of the T cell. With this goal in mind, the following parameters were examined:

- Isotype and IgG subclass levels over the time course of three infections.
- Polyclonal and antigen-specific proliferative responses of PBMC and isolated T cell populations.
- Cytokine responses following infection.

In addition to measuring peripheral responses, local responses in the lung were analysed in BALF and in the lymphocyte populations in the local draining lymph nodes.

2. MATERIALS AND METHODS

2.1 Experimental calves

Three month-old male Friesian calves weighing between 120 to 195 kg were obtained from a helminth-free source. The calves were maintained indoors, fed on hay and a proprietary concentrate feed and offered water *ad libitum*. All calves were examined at least once daily.

2.1.1 Provision of material for assay optimisation

For preliminary experiments, three groups of calves (seven animals in total) were infected orally with *D. viviparus* L3, at 10 L3 kg^{-1}. Blood samples were collected weekly by jugular venepuncture. Faecal samples were collected daily to monitor larval excretion. Tracheo-bronchial lymph nodes (T-B LNs) were collected at post-mortem, 28 to 35 days pi.

2.1.2 Longitudinal experiment

To allow parameters to be measured in immune versus naïve animals, a longitudinal experiment was undertaken. Five calves, Group A, were infected orally with 10 L3 kg^{-1} on Days 0, 65 and 112 pi. They were treated with 7.5 mg kg^{-1} fenbendazole (Panacur, Hoechst UK Ltd, Milton Keynes) on Day 104, to ensure that they were parasite-free before the tertiary, challenge infection. The three remaining calves, Group B, acted as challenge controls and remained uninfected until Day 112 when they were infected orally with 10 L3 kg^{-1}. Group A calves were blood sampled weekly throughout the experiment. Group B calves were blood sampled on three occasions prior to challenge, i.e. on Day 0, Day 29 and Day 111, and weekly thereafter. BALF was collected on Days 0, 19, 26, 75 (19 days after the secondary infection), 86 (21 days after the 2nd infection), 122 (10 days after the tertiary infection) and 133 (21 days after the tertiary infection) from Group A and B calves. Faecal

samples were collected daily from Day 19 after each infection for two weeks or until no L1 were detected in the faeces. Calves were monitored clinically throughout the experiment; rectal temperature and respiratory rate were recorded daily during the pre-patent and patent periods of each infection. Symptomatic therapy was administered when required (see results). All calves were killed on Day 147 (35 days after the tertiary infection). At post-mortem, the numbers of adult parasites in the lungs were counted and the T-B and pre-crural LNs were collected.

2.2 Collection of samples

2.2.1 Blood samples

Blood was collected by jugular venepuncture into vacutainer tubes (Becton Dickinson UK Ltd, Oxford), or into 100 ml bottles containing 25 ml of 35% sodium citrate (BDH).

2.2.2 Bronchoalveolar lavage

BALF was collected using a modification of a method first described by Fogarty *et al.*, (1983). Briefly, plastic tubing (Orme Scientific Ltd, Manchester) of 11 mm diameter was passed via the nose into the trachea. A longer, narrower (6 mm) tube was passed through the first tube and carefully advanced to reach the terminal bronchus, usually in the right caudal lung lobe as this is the first to exit from the trachea. Warm sterile phosphate-buffered saline (PBS) (180 ml) (Appendix 1.1) was carefully infused, then aspirated, recovering an average of 30% of the fluid. The BALF was centrifuged at 700 g for 5 min at room temperature with 100% brake. The supernatant was filtered through a Whatman No 1 filter (BDH), then concentrated using a Centriprep device (Amicon Ltd., Stonehouse) with a molecular weight cut-off of 10 kDa. This process was repeated until the samples were 12% of their original volume. The samples were stored at -20°C.

The cell pellet which remained after initial centrifugation was resusupended in 10 ml of tissue culture medium (TCM) (Appendix 1.7), and filtered through sterile gauze. The

filtrate was washed twice in TCM by centrifuging as described above, the supernatant discarded and the pellet resuspended in 10 ml TCM. After the second wash, 0.5 ml of the cell suspension was retained for routine cytology. The remaining 9.5 ml were centrifuged as before and the pellet resuspended in 100 µl diethyl pyrocarbonate (DEPC)-treated water (Appendix 1.12) and transferred to a Nunc cryopreservation tube (Costar UK Ltd., High Wycombe). The samples were snap-frozen and stored in liquid nitrogen.

2.2.3 Parasitological monitoring

Faecal samples were collected from the rectum and placed in plastic faecal pots. *D. viviparus* L1 were counted using the modified Baermann technique (Henriksen, 1965).

Adult parasites were obtained from the respiratory passages of experimentally infected calves killed between 28 to 35 days post-infection/challenge. Parasites were recovered by perfusion of the lungs via the pulmonary artery following the technique of Inderbitzen and Eckert (1978). Material collected from the trachea was passed through "Maxa" milk filters (Fullwood and Bland Ltd, Ellesmere), from which adult parasites were retrieved, and counted.

2.3 Preparation of parasite material

2.3.1 Infective larvae

Infective L3 of *D. viviparus* were supplied by Intervet, U.K. These were stored in PBS at 4°C. Following storage, live larvae were separated from the dead larvae by centrifugation through a Percoll (Sigma-Aldrich Company, Poole) gradient. Briefly, 5 ml of a larval suspension were carefully layered onto 5 ml of 45% Percoll/PBS and centrifuged at 1000 g for 20 min. This procedure pelleted the live larvae at the bottom of the tube, whilst the dead larvae remained at the Percoll/PBS interface. The pellet of live larvae was washed three times in PBS and resuspended in 10 ml of PBS. The larvae were counted and infective doses prepared.

2.3.2 Parasite antigens

Adult parasites

Adult parasites were obtained from the lungs of infected animals as described (Section 2.2.3). For the preparation of adult homogenate in PBS, 100 parasites were washed three times in PBS at 4°C and resuspended in 1 ml PBS in a glass homogeniser (Jencons Scientific Ltd., Bedfordshire) on ice. For adult homogenate in proteinase inhibitors, the parasites were homogenised in 1 ml of 10 mM Tris buffer containing a cocktail of proteinase inhibitors (Appendix 1.2). The homogenates were centrifuged at 13,000 g for 30 min at 4°C and the supernatants aliquoted and frozen at -70°C.

Third stage larvae

Aliquots of 1 x 10^6 *D. viviparus* L3 were washed three times in PBS at 4°C, and resuspended in 0.5 ml PBS or Tris/proteinase inhibitors. Parasites were sonicated on ice in a MSE Soniprep 150 ultrasonic disintegrator (MSE, Scientific Instruments, Crawley), at an amplitude of 16 μm, for approximately seven cycles of 0.5 min with 1 min rests between cycles. Samples were examined using a light microscope to ensure that the parasite cuticles were adequately disrupted. The supernatant was obtained and stored as described for the adult homogenate.

First stage larvae and eggs

A combination of L1 and eggs were obtained from the cultures of adult parasites. Briefly, adult parasites, collected from the lungs as described above, were immediately placed in PBS at 37°C. The parasites were washed three times in PBS at 37°C and then in three changes of Hank's balanced salt solution (HBSS, Sigma) containing 100 units ml^{-1} penicillin,

100 μg ml^{-1} streptomycin (Gibco BRL, Paisley, Scotland), 125 μg ml^{-1} gentamicin (Gibco BRL) and 25 μg ml^{-1} amphoteracin B (Gibco BRL). This was followed by one wash in nematode culture medium (NCM) (Appendix 1.3). Parasites were cultured at a concentration of two adults ml^{-1} of medium at 37°C with 5% CO_2 for three days in NCM, replacing with fresh medium every 24 h. The media removed was rich in eggs and L1 which were washed three times in PBS at 4°C and resuspended in 0.5 ml PBS or Tris/proteinase inhibitors and sonicated as described for L3.

Excretory / secretory products

The excretory/secretory (ES) products from adult parasites were collected from cultures of adult parasites as described above for L1 and eggs. The media collected after culture was centrifuged to pellet the eggs and L1, then the supernatant was concentrated using a Centriprep device (Amicon Ltd.) with a molecular weight cut-off of 10 kDa. This was repeated until the incubation dye from the culture fluid was no longer visible. The concentrated ES material was stored at -70°C.

2.4 Protein assay

To measure the concentration of protein in the antigen preparations, a Coomassie blue, dye-based, protein assay was used (Bio-Rad Laboratories Ltd., Hemel Hemptstead). This assay was performed according to the manufacturer's instructions.

2.5 SDS-polyacrylamide gel electrophoresis

To compare different antigen preparations, parasite proteins were separated using 7.5 to 12% SDS-PAGE mini-gels, using the method of Laemmli (1970). The separating gel (Appendix 1.4) was poured first and allowed to polymerise before the stacking gel (Appendix 1.4) was poured. After the stacking gel had polymerised, SDS-PAGE running

buffer (Appendix 1.4) was added to the gel tank. To prepare the samples, 10 µl of sample buffer (Appendix 1.4) were mixed with 10 µl of sample, containing 10 µg protein. To cleave disulphide bonds, 5% 2-mercaptoethanol (Sigma) was included in the sample buffer. Samples were heated to 100°C for 5 min to denature the proteins. The gels were run at 20mA per gel for approximately 1 h.

Molecular weights of parasite proteins were estimated by reference to molecular weight markers of either the very low 2.512 to 16.949 kDa, low 14.4 to 97.4 kDa or high 14.4 to 200 kDa range (all Sigma). In order to visualise the polypeptides, gels were incubated for 2h in 0.1% Coomassie Brilliant Blue R-250 (BDH) in 45% methanol, 10% acetic acid in distilled deionised water (ddH_2O). Gels were destained in 20% methanol, 7% acetic acid in ddH_2O until the background was clear (approximately 2 h). Gels were photographed using Polaroid high speed, black and white film, Type 667. Prior to drying, gels were soaked in 10% acetic acid, 1% glycerol in ddH_2O and then dried for 2 to 3 h in an Easy Breeze gel drier (Hoeffer Scientific Instruments, Newcastle-under-Lyme).

2.6 Western Blotting

Western blotting was used to transfer the separated proteins from the gel by applying an electric field perpendicular to the plane of the gel. Proteins were transferred to a nitrocellulose matrix which was then probed with antibodies. After standard SDS-PAGE, gels were equilibrated for 30 min in Tris-glycine transfer buffer (Appendix 1.5). The nitrocellulose transfer membrane (Biotrace NT, Gelman Sciences Ltd, Northampton) was immersed in the buffer for 15 min, then the gel and membrane were carefully loaded into the TE series transfer electrophoresis unit (Hoeffer) between layers of Whatmann 3MM chromatography paper (BDH) and nylon sponges (Hoeffer). The proteins were transferred at 200 mA for 2 h. The membranes were then cut into strips. To verify protein transfer to membranes, strips were stained with either 0.1% amido black (BDH) in 45% methanol, 10% acetic acid for 15 min, then destained in 20% methanol, 7% acetic acid in ddH_2O until the background was clear (approximately 2 h); or with Ponceau-S (Sigma) and rinsed in ddH_2O. To confirm protein transfer, the gels were stained with Coomassie Brilliant Blue, as described (Section 2.5).

The remaining strips of nitrocellulose were incubated in 10% soya milk (Infasoy, Cow & Gate Nutricia Ltd, Trowbridge) in blot wash buffer (BWB) (Appendix 1.5) for 1 h, then washed three times for 5 min in BWB. Next, strips were incubated with the relevant serum samples at an optimal dilution (1:100 to 1:400) for 2 h at room temperature, then washed three times, as before. Strips were then incubated with 1:500 dilution of rabbit anti-bovine alkaline phosphatase conjugate (Sigma) in BWB for 1 h, followed by three washes in BWB. The strips were incubated in the substrate 3-bromo-4-chloro-3-indolyl phosphatase (Sigma) for 5 min and the reaction was stopped by rinsing in ddH_2O.

2.7 Antibody ELISA

An ELISA was used to detect anti-*D. viviparus* antibodies in serum and BALF. Blood samples were stored for 24 h at 4°C, after which sera were separated by centrifugation at 700 g for 20 min, then stored at -20°C. The method of preparation of concentrated BALF samples was described in Section 2.2.2.

Ninety-six-well polystyrene microtitre plates (Immulon 4, Dynatech Laboratories Ltd., Billinghurst) were coated with 100 µl $well^{-1}$ of an optimal dilution of *D. viviparus* antigen (see results), diluted in carbonate buffer (Appendix 1.6), and incubated for 2 h at 37°C or overnight at 4°C. The plates were then washed three times in ELISA buffer (PBS with 0.05% polyoxyethylene-sorbitan monolaurate, Tween 20, Sigma). To reduce non-specific binding, 100 µl ELISA buffer with 5% soya milk (Infasoy) were added to each well and the plates incubated for 1 h at room temperature, then washed as above. Optimal dilutions of control and test serum, or BALF, in ELISA buffer with 2% soya milk, were added to duplicate wells (100 µl $well^{-1}$). The plates were incubated for 30 min at 37°C. After washing, 100 µl of optimally diluted (see results) mouse monoclonal antibody to bovine IgG1, IgG2, IgA (Serotec Ltd., Oxford), IgM (Sigma), IgE (kindly provided by Dr Laurel Gershwin, University of California at Davis, California) or peroxidase-conjugated rabbit anti-bovine IgG (Sigma) were added to each well. The plates were incubated for 30 min at 37°C, then washed as before. Where necessary, 100 µl of peroxidase-conjugated goat anti-mouse IgG (Sigma) were added to each well and the plates incubated for 30 min at 37°C. After washing, 100 µl of 3,3, 5,5- tetramethylbenzidine (TMB) peroxidase

substrate (Dynatech) were added to the wells. Plates were incubated at room temperature for an optimum time, which was assessed during initial titration experiments for each antibody and antigen. Optical densities (OD) were read at 630 nm using an automatic plate reader (Dynatech MR5000).

The optimum dilutions of antigen and antisera were determined for each isotype/subclass, using checkerboard titrations of pooled positive and negative sera and BALF. All serum or BALF samples were assayed for a particular isotype/subclass on the same day. The threshold ODs were calculated from two standard deviations above the mean OD of 20 control sera samples collected from uninfected calves or, alternatively, from the mean of 17 pre-infection BALF samples collected from the eight experimental calves.

2.8 Preparation of cells

2.8.1 Isolation of PBMCs

Blood was collected into 32% sodium citrate solution, then centrifuged at 1000 g for 25 min at room temperature with 80% brake. The buffy coat was collected and diluted 1:4 with TCM (Appendix 1.7) and carefully layered over an equal volume of Histopaque 1083 (Sigma). Centrifuging at 900 g for 35 min with 30% brake resulted in a pellet of red blood cells, a clear band of Histopaque, an opaque layer containing PBMC and a layer of TCM above. The TCM was aspirated to approximately 1 cm above the opaque layer, which was then collected into a fresh tube. This cell-rich suspension was diluted 1:5 in TCM and centrifuged at 1500 g for 5 min at room temperature with 100% brake to wash the cells free of Histopaque. To remove platelets, the PBMC were washed in TCM by centrifuging at 250 g for 10 min with 0% brake. This was repeated three times, after which the numbers of live and dead cells were counted in an improved Neubauer haemocytometer (Weber Scientific International Ltd., Teddington), using Trypan blue (0.4% solution, Sigma) to stain the dead cells.

2.8.2 Isolation of lymphocytes from lymph nodes

The LNs were carefully dissected from the carcass, ensuring the capsules remained intact, and placed in Hank's washing solution (HWS: Appendix 1.8) at 37°C. After 10 min, any remaining fat and connective tissue still adhering to the nodes were removed. The nodes were then washed three times in HWS at 37°C, for 10 min each wash, then transferred to a plastic petri-dish containing 10 ml TCM with 20% heat inactivated foetal calf serum (FCS: Gibco, BRL). The nodes were cut into 5 mm pieces which were sieved gently through a sterile plastic tea strainer using the plunger of a 20 ml syringe. The cell-rich TCM/FCS was then aspirated three to four times to obtain a single cell suspension which was transferred into a 15 ml centrifuge tube and allowed to settle for 5 min. The supernatant was aspirated, leaving the larger debris, and centrifuged at 300 g for 7 min with 100% brake. The supernatant was discarded and the cells resuspended in 10 ml TCM and counted, as above. All manipulations were carried out under sterile conditions.

2.8.3 Cryopreservation

The cell suspensions were centrifuged at 300 g for 5 min and the supernatant discarded. The pellets were resuspended in FCS to half the required volume and an equal volume of cryopreservation medium (Appendix 1.9) was added and the suspension immediately aliquoted into 1 ml Nunc cryopreservation tubes (Costar). These were frozen, wrapped in cotton wool in polystyrene boxes, at -70°C for 24 h, after which they were moved to liquid nitrogen. To defrost cells, cryopreservation tubes were brought to 37°C rapidly by plunging them into a 37°C waterbath. The cells were resuspended in 10 ml of TCM, centrifuged at 1000 g for 5 min and the supernatant discarded.

2.9 Lymphocyte proliferation assays

PBMC or LN-derived lymphocytes were incubated, in triplicate, in 96-well, half-area, flat-bottomed tissue culture plates (Costar) in TCM/10% FCS with either 2%

penicillin/streptomycin (5000 iu penicillin, 5000 iu streptomycin, Gibco BRL) or 10,000 μg ml^{-1} ciproxin (ciprofloxacin hydrochloride, Bayer plc, Basingstoke). In some cases, 10^{-5}M 2-mercaptoethanol (Sigma) was used in the culture medium. The cells were cultured in the presence of various concentrations of Concanavalin A (ConA: Sigma), parasite antigens (see results), or in medium alone and incubated for up to seven days at 37°C, 5% CO_2. The cells were pulsed with 0.5 μCi tritiated thymidine (^3H: Amersham International plc, Little Chalfont) before harvesting. This was performed using an automated harvester (Filtermate 196, Packard). Next, 25 μl of scintillation cocktail (MicroScint-O, Packard) were added to each well and the wells sealed with adhesive sheets (Topseal A, Packard). The amount of ^3H incorporated was measured using a scintillation counter (TopCount microplate scintillation counter, Packard).

2.10 Preparation of supernatants for cytokine analysis

PBMC or LN-derived cells were cultured in 24-well plates (Costar) in 1 ml of TCM/10% FCS as above. Cells were incubated for 24 to 72 h in the presence of 2.5 μg ml^{-1} Con A (Sigma), 1.25 μg ml^{-1} lipopolysacharide (LPS, from *Escherichia coli* 0127:B8, Sigma), 10 μg ml^{-1} parasite antigens, or in medium alone. All samples were analysed in duplicate. The contents of each well were collected into Eppendorf tubes and centrifuged at 13,000 g for 5 min at room temperature. The supernatants were collected and stored at -70°C.

2.11 Cytokine bioassays

2.11.1 Interleukin-1

A murine thymic sarcoma cell line (D10), which proliferates in response to human and murine IL-1, was found to respond to bovine IL-1 (Scott, unpublished). This cell line was kindly provided by Dr. C. Lawrence, Department of Veterinary Pathology, University of Glasgow, and used with permission from Dr. S.J. Hopkins, University of Manchester. These cells were grown in 10 ml bioassay culture medium (BCM: Appendix 1.10)

containing 3 μg ml^{-1} Con A (Sigma), 120 iu ml^{-1} human recombinant IL-2 (kindly provided Dr. C. Lawrence, Department of Veterinary Pathology and obtained from J. Nunberg, Cetus Corp., Emeryville, California, USA) and 1 ng ml^{-1} IL-1 (IL-1β: recDNA, Human type, National Institute for Biological Standards and Control, Potters Bar, Hertfordshire). The cells were incubated at 37°C with 5% CO_2, in 25 cm^3 tissue culture flasks (Costar). The cells were split to 1:10 every three to four days.

For the actual bioassay, the D10 cells were used after four days of culture. They were washed three times in BCM, by centrifuging at 1000 g for 5 min and then resuspended at a concentration of 1 x 10^5 ml^{-1} in TCM containing 120 iu ml^{-1} human recombinant IL-2 and 3 μg ml^{-1} Con A. Next, 100 μl of cell suspension were added to each well. The human recombinant IL-1 (NIBSC) was used as a standard in the bioassay. All samples were analysed in triplicate. Seven, tripling dilutions were made of the IL-1 standard, the first dilution was at a final concentration of 90 pg ml^{-1} IL-1 and the seventh at 0.041 pg ml^{-1} IL-1. The first dilution of the samples under study was 1:40 and eight tripling dilutions were made. The cells were incubated for 68 h at 37°C with 5% CO_2 and pulsed with ^3H, before harvesting 4 h later.

The levels of IL-1 in each sample were calculated using a computer programme specifically designed by the Department of Immunology, University of Manchester, for analysis of bioassay results. This programme uses the parallel line approach, i.e. the curves for the titrated unknown samples and the standards should be parallel if the molecule responsible for the activity in the samples/standards is the same. The parallel portions of these curves are then used to measure the displacement from the standard, which is proportional to the biologically active cytokine content of the samples.

2.11.2 Interleukin-6

To measure bovine IL-6, a murine hybridoma cell line (B9) was used, which was kindly provided by Dr. C. Lawrence, Department of Veterinary Pathology with permission from Dr. L. Aarden, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam. These cells were cultured in BCM (Appendix 1.10) containing 9 units ml^{-1} of crude human IL-6 (Cambio, Cambridge). The cells were split to 1:10 every 48 h.

For the bioassay, human recombinant IL-6 (NIBSC) was used as a standard. The bioassay was performed in the same manner as described above for IL-1. The first dilution of the standard was 270 pg ml^{-1} IL-6 and the seventh 0.123 pg ml^{-1} IL-6. The first dilution of test samples was 1:3 and the last 1:6561.

2.12 Measuring bovine interferon gamma using an ELISA

Levels of IFNγ were measured using a commercially available bovine IFNγ ELISA, kindly provided by Dr. R.E. Slaughter, CSL Ltd., Victoria, Australia. The kit was used according to the manufacturer's instructions with recombinant bovine IFNγ (kindly provided by Dr. R.B. Collins, Institute for Animal Health, Compton) used as a standard.

2.13 Analysis of cell populations by fluorescence activated cell sorting

To analyse the phenotype of cell populations, PBMC, LN-derived cells or separated cell populations were labelled with monoclonal antibodies to cell surface markers for bovine lymphocytes. The markers used were:

CC42: Binds boCD2, a pan T cell marker (Howard and Morrison, 1991).
CC8: Binds boCD4 (Howard and Morrison, 1991)
CC63: Binds boCD8 (MacHugh *et al.*, 1991)
CC15: Binds $WC1^+$ cells (Clevers *et al.*, 1990)
CC21: Binds $WC3^+$ cells, thought to be B cells (Naessens *et al.*, 1990)

Cells were resuspended in PBS/BSA/azide (Appendix 1.11) at 2 x 10^7 ml^{-1}. Aliquots of 50 µl cell suspension were added to 5 ml centrifuge tubes (55.475.001, Sarstedt, Leicester) and 50 µl of the appropriate monoclonal antibody, at 1:1000 dilution, were added. After incubation at 4°C for 20 min in the dark, 4 ml of PBS/BSA/azide were added and the tubes were centrifuged at 1000 g for 5 min. The supernatants were discarded and the pellets

vortexed to resuspend the cells. This washing was repeated twice, then 50 µl of FITC-conjugated anti-mouse IgG (1:40) were added to the resuspended cell pellets. The cells were incubated for 30 min at 4°C in the dark, washed three times as before, then resuspended in 0.5 ml of 1% paraformaldehyde in PBS. The cells were stored in the dark at 4°C and analysed using a FACS scan (Coulter Elite, Coulter Corporation, Miami, Florida) within two weeks.

2.14 Magnetic separation and culture of cells

To investigate which T cell subpopulations would proliferate to Con A or parasite antigen, magnetic cell sorting (MACS) was used to separate PBMC before culture *in vitro*. An appropriate amount of magnetic beads (1 ml beads per 1 x 10^7 cells), coated with goat anti-mouse IgG (H+L) (Biomag, Metachem, Northampton), were shaken vigorously in a 15 ml or 50 ml centrifuge tube to resuspend the magnetic particles. A magnet was applied to the tube for 10 min to pull the magnetic particles to the side of the tube. The supernatant was then carefully aspirated, leaving the magnetic particles, which were resuspended in TCM (Appendix 1.7) /10%FCS. The washing of the beads was repeated twice. After the final wash, the beads were resuspended in TCM/10% FCS (1 ml per 1 x 10^7 cells) and the appropriate monoclonal antibody (Section 2.13) added to a final dilution of 1:1000. The antibody was incubated with the beads at 4°C for 20 min and the beads washed three times, as before. The cells which were to be separated were added, along with a minimum of 10 ml TCM/10% FCS per 1 x 10^7 cells, and incubated for 20 min at 4°C. The beads were separated, using a magnet, for 10 min. The supernatant, which contained the negatively selected cell population, was carefully collected and used for further separations. The magnetic particles, which contained the positively selected cells, were resuspended in TCM/10% FCS and these cells were counted (Section 2.8.1) and used in proliferation assays.

The separated populations were analysed by FACS (Section 2.13) to assess the efficiency of the separation. Lymphocyte proliferation assays were then performed on the positively selected populations, as described (Section 2.9), with or without the inclusion of

autologous (from the same animal) or heterologous (from another animal) APC, see results. PBMC irradiated at 2500 rads using a radioactive caesium source were employed as APC.

2.15 RNA isolation

To isolate RNA from PBMC and LN-derived lymphocytes, the TRIzol reagent (Gibco BRL) was used according to the manufacturer's instructions. The quantity of RNA isolated was assessed by measuring the OD of the RNA at 260 nm. To assess the quality of the RNA, the OD was measured at 260 nm and 280 nm, with a ratio of ≥ 2 indicating a pure sample. The quality of the RNA was also assessed by analysis on a 1.2 % agarose gel (Section 2.16).

2.16 Agarose gels for analysis of RNA samples

RNA was visualised on gels containing 1.2% agarose, 20% 10 x 3-N-morpholinopropanesulfonic acid (MOPS, Sigma), 17% formaldehyde (40% w/v, BDH) in DEPC-treated ddH_2O (Appendix 1.12). The samples were prepared by adding 1 µl of ethidium bromide (10 mg ml^{-1}, Sigma), 1 µl of RNA sample dye (Appendix 1.13) and 5 µl formamide (BDH) to 2 to 5 µg of sample RNA in 5 µl of DEPC ddH_2O. The samples were incubated at 65°C for 5 min, then cooled on ice for 5 min before being loaded onto the gel. MOPS (1x) was used as the running buffer. Gels were run at 30 V for 10 min, then 50 V for 50 min. Gels were viewed on a shortwave UV transilluminator and photographed using Polaroid high speed, black and white film, Type 667.

2.17 Reverse transcription and DNA amplification by the polymerase chain reaction

First strand cDNA synthesis was performed using 1 to 2 µg of total RNA. The RNA was suspended in 8 µl DEPC ddH_2O. To reduce DNA contamination of the RNA samples, 1 µl DNAse (Gibco BRL) with 1 µl DNAse buffer (Gibco BRL) were added, followed by incubation at room temperature for 15 min. The DNAse was inactivated by adding 2 µl EDTA (20 mM, Gibco BRL). Prior to reverse transcription (RT), 2 µl oligo(dT) primer (100 ng ml^{-1}) were added to the RNA samples following denaturation for 10 min at 65°C. Next, the samples were cooled slowly to room temperature to allow annealing of the primer to the RNA template. To this mixture were added, 20 units RNAse inhibitor (RNAsin, Promega), 4 µl of 5 x RT buffer (Promega), 2 µl dNTPs (10 mM, Pharmacia) and 15 units of RT enz (Promega). The samples were incubated at 42°C for 1 h, then 52°C for 30 min. The first strand cDNA was stored in ddH_2O at -20°C.

Amplification by PCR was performed on 2 µl of a 1:10 dilution of the first-strand cDNA. The PCR reaction contained 200 µM of each dNTP (Pharmacia), 10 µl *Taq* DNA polymerase buffer (Cetus), 2.5 units Amplitaq DNA polymerase enzyme (Cetus) in a total volume of 100 µl. The reaction was primed using the appropriate 3' and 5' primers for each bovine cytokine (Appendix 2.1 and 2.2). PCR conditions were: denaturation at 94°C for 5 min, followed by 25 to 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, followed by a final extension of 10 min at 72°C. An aliquot was then separated on a 1.5% agarose gel (Section 2.18) to determine the size range of the amplified product.

2.18 Agarose Gel Electrophoresis

Gels containing 1.5% agarose (w:v) and 0.05% ethidium bromide in 1 x Tris acetate buffer (TAE: Appendix 1.15) were used to separate and analyse DNA molecules. DNA sample dye (Appendix 1.14) was added to the samples at a ratio of 1 part dye to 5 parts sample, and the samples loaded. The gels were run in 1 x TAE at approximately 5 V cm^{-1}. In order to estimate the size of the DNA, 0.5 µg of a molecular weight marker (φX–174RF DNA

Hinc II digest, Pharmacia) were run alongside the samples. Gels were viewed and photographed as described (Section 2.16).

2.19 Confirming the identity of PCR products

To confirm the identity of PCR products, they were digested using an appropriate restriction enzyme (Appendix 2) and the size of the resulting fragments assessed by gel electrophoresis. To purify the PCR product, the samples were separated on 1.5% agarose gels (Section 2.18). The DNA band of interest was excised from the gel using a clean scalpel and the DNA purified using a Qiaex DNA gel extraction kit (Qiagen, Germany) according to the manufacturer's instructions. To elute the DNA from the pellet, the samples were resuspended in 30 µl of the appropriate restriction enzyme buffer (Appendix 2.3). After incubating for 15 min at room temperature, the suspension was centrifuged at 13,000 g for 30 sec and the DNA-containing supernatant transferred to a clean tube. The appropriate restriction enzyme was added and was incubated with the DNA for 2 h at 37°C. To stop the reaction, 0.5 M EDTA (Gibco BRL) was added to a final concentration of 10 mM. To precipitate the digested products, 0.6 volumes of 5 M ammonium acetate and 2 volumes of 100 % ethanol were added. The samples were vortexed and frozen at -70°C for at least 30 min and then centrifuged at 12,000 g for 5 min at 4°C and the supernatant discarded. The pellets were air dried, then dissolved in 15 µl ddH_2O before being analysed on 1.5% agarose gels.

2.20 Hybridisation analysis of nucleic acids

2.20.1 Transfer of nucleic acids to membranes

RNA (Northern blotting) and DNA (Southern blotting) were transferred from ethidium bromide-stained agarose gels to Hybond-N membranes (Amersham), essentially by the method of Southern (1975). Transfer was confirmed by viewing the gels on a shortwave

UV transilluminator. After transfer, the membranes were baked for 2 h in an 80°C oven, then crosslinked using a UV crosslinker (GS Gene Linker, UV Chamber, BioRad) at 50 Joules for 30 sec.

2.20.2 Production of cDNA probes

Bacterial transformation

DNA fragments for bovine IL-2 and human β-actin in Bluescript plasmids (Stratagene, Cambridge) and bovine IL-4 and IL-10 in PCR II plasmids (Invitrogen, 9351 NV Leek, The Netherlands) were kindly provided by Dr. R.B. Collins, Institute for Animal Health, Compton. The plasmids were transformed into DH5α cells (MAX Efficiency DH5a™ Competent Cells, Gibco BRL). A 20 µl aliquot of competent cells was allowed to thaw, slowly, on ice. Once thawed, 10 ng of plasmid, containing the recombinant fragments, were added to the cells which were gently tapped to mix, then left on ice for 30 min. The cells were heat-shocked at 42°C for 45 sec, cooled on ice for 2 min and then 90 µl of SOC medium (Appendix 1.18) were added. The transformed cells were shaken at 37°C for 45 min to allow growth and development of antibiotic resistance. After incubation, the cells were mixed, then 100 µl of the suspension were spread on L-broth agar plates (Appendix 1.19) containing 75 µg ml^{-1} ampicillin (Sigma) and 1 µl ml^{-1} Xgal (20% in dimethylformamide, Sigma). The remaining cell suspension was centrifuged at 13,000 g for 30 sec, then resuspended to 100 µl with SOC medium (Appendix 1.18) and spread on a second agar plate. The plates were incubated at 37°C for a minimum of 12 h. To ensure colonies from individual plasmids were chosen, single white colonies were streaked onto antibiotic-containing plates, as above, and grown overnight at 37°C. A single colony from this plate was used in further experiments.

Small-scale DNA extraction and restriction digestion to identify recombinant DNA

A single colony of transformed cells, containing plasmid DNA, was put into 10 ml of L-broth (Appendix 1.19) with the appropriate antibiotics, as above, then shaken overnight at 37°C. DNA was extracted from a 3 ml aliquot of the overnight culture using a Wizard miniprep kit (Promega), according to the manufacturer's instructions, until transfer of the clear, DNA-containing, supernatant to a clean Eppendorf. Then, 1 ml of 100% ethanol was added, the tube inverted to mix and then incubated at -70°C for 30 min. The sample was centrifuged at 13,000 g at 4°C for 10 min, then the supernatant was discarded and 500 µl of 70% ethanol were added. The Eppendorf was vortexed, then centifuged as before for 5 min. The supernatant was discarded and the pellet allowed to dry, before being resuspended in 30 µl of autoclaved ddH_2O.

The plasmid vectors were digested using the appropriate restriction enzymes under the recommended conditions (see Appendix 2.4), then the products were analysed on 1% agarose gels (Section 2.18).

Larger scale DNA production and quantification

Multiple small scale DNA extractions were performed, as above, and the resuspended pellets were pooled. The DNA-containing plasmid vectors were digested (Section 2.21) on a 1% agarose midi-gel. The digested product was seen as a single band. This band was excised using a clean scalpel, the DNA purified using a Quiaex DNA gel extraction kit (Qiagen) (Section 2.19) and the resulting pellet was resuspended in ddH_2O. To estimate the quantity of DNA, a known volume of sample was run on a 1% agarose mini-gel alongside a known quantity of a low molecular weight DNA mass ladder (Pharmacia). Comparing the sizes of bands allowed an approximation of the quantity of DNA in the sample.

2.20.3 Radiolabelling of cDNA probes

To generate labelled cDNA probes, 25 ng DNA, in 11 µl ddH_2O, were denatured by boiling for 10 min, then put on ice for 5 min. Next, 50 µCi of [α^{32}P]-dCTP (3000 Ci $mmol^{-1}$, Amersham) and 4 µl of "high prime" (Boehringer Mannheim) were added and the sample was incubated at 37°C for 30 min. The labelled products were purified on Sephadex G-50 beads (Nick column, Pharmacia). Typically, incorporations of 10^8 to 10^9 cpm were achieved.

2.20.4 Hybridisation of probes to filter bound nucleic acid.

Detection of immobilised DNA or RNA on filters was achieved by hybridisation to radiolabelled nucleic acids. Filters were pre-incubated for 4 h at 65°C in a hybridisation tube (Hybaid), containing 12.5 ml prehybridisation buffer (Appendix 1.21). The freshly prepared DNA probe (see above) was denatured by boiling for 10 min and then cooled on ice for 5 min. The probe (10^6 cpm per cm^2) was added to the tube and the hybridisation mixture was incubated at 65°C overnight. Filters were rinsed and washed twice, each wash for 15 min at 65°C, in 2 x SSC (Appendix 1.22), 0.5% SDS. This was followed by two washes in 1 x SSC, 0.5% SDS and then two washes in 0.1 x SSC, 0.5% SDS. Filters were rinsed in 2 x SSC, to remove SDS, then sealed in polythene. The filters were placed in cassettes with autoradiography film (Kodak scientific imaging film, X-omat, LS) and placed at -70°C. The film was developed from 30 min to 7 d later, depending on the level of hybridisation.

2.21 Expression of recombinant protein

2.21.1 Bacterial transformation and confirmation of identity of DNA

DNA fragments in pET-15b (Novagen Inc., Madison, Wisconsin), encoding repeat units of a single repeat (DvA-1L) (Referred to as Rpt A in this thesis) and three contiguous repeats

(DvA-1JKL) (Referred to as Rpt C-A in this thesis) from the nematode polyprotein allergen/antigen of *D. viviparus* (Britton *et al.*, 1995) were kindly provided by Professor M.W. Kennedy, Division of Infection and Immunity, University of Glasgow, with permission from Dr. C. Britton, Wellcome Unit of Molecular Parasitology, University of Glasgow. These were transformed into BL21(DE3) competent cells (Novagen). This was performed as described in Section 2.20.2, except that the BL21 cells were grown in agar containing 34 μg ml^{-1} chloramphenicol (Sigma) and 50 μg ml^{-1} carbenicillin (Sigma).

To confirm that the transformed cells contained the correct plasmid DNA, a small scale DNA extraction was performed on an overnight culture of cells, see Section 2.20.2. The plasmid DNA was then digested using *Bam*H1 (Promega) at 37°C for 1 h and the products analysed on a 1% agarose gel (Section 2.18).

2.21.2 Small-scale protein expression

To ensure that a large-scale expression of the recombinant Rpt A and Rpt C-A repeats of DVA-1 was going to be successful, a small-scale expression was carried out. A single colony of transformed BL21(DE3) cells (Novagen), containing plasmid DNA, was put into 10 ml of L-broth (Appendix 1.19), containing 34 μg ml^{-1} chloramphenicol (Sigma) and 50 μg ml^{-1} carbenicillin (Sigma), then shaken overnight at 37°C. The following day, 500 μl of culture, containing cells in the stationary phase of growth, were added to 10 ml of L-broth with antibiotics and these were shaken at 37°C for approximately 40 min, until the cells reached the logarithmic phase of growth. The logarithmic phase was determined when the optical density at 600 nm (OD_{600}) of the cell suspension was between 0.6 to 1.0. At this stage, isopropyl-b-D-thiogalactopyranosidase (IPTG, Sigma) was added to a final concentration of 1 mM. The cells were shaken at 37°C for 3 h. Then, 50 ,100, 250 and 500 μl aliquots of the cell suspension were centrifuged at 13,000 g for 2 min and resuspended in 20 μl of SDS-PAGE sample buffer (Appendix 1.4). The samples were analysed by SDS-PAGE (Section 2.5) and Western blotting (Section 2.6).

2.21.3 Large scale expression and purification of recombinant proteins

Induction

A single colony of transformed BL21(DE3) cells (Novagen), containing plasmid DNA, was added to 100 ml of L-broth (Appendix 1.19) containing antibiotics, as above, and shaken overnight at 37°C. The 100 ml of overnight culture were added to 1 L of L-broth with antibiotics and shaken at 37°C, until the cells reached the logarithmic phase of growth. At that point, IPTG (Sigma) was added and the cells were shaken for a further 3 h (see above).

Cell extract preparation

To pellet the cells, the culture was poured into four 250 ml centrifuge flasks and centrifuged at 10,000 g for 10 min. Each pellet was resuspended in 10 ml of ice-cold binding buffer (1x: His.Bind purification kit, Novagen) and the suspensions were pooled. To shear the DNA, the sample was sonicated on ice, at an amplitude of 18 μm, for bursts of about 1 min, with 1 min rests, until the sample cleared. The lysed cells were then centrifuged at 12,000 g for 15 min at 4°C. The supernatant, containing the soluble protein, was filtered through a 0.45 μm syringe filter (Nunc) and stored overnight at 4°C.

Affinity purification

The pET vector carries a His-Tag sequence which is expressed at the N-terminal end of the target protein. The His-Tag sequence binds to divalent cations, so can be immobilised on a Nickel resin. The filtered cell extract containing the target protein was purified using a His.Bind purification kit (Novagen), according to the manufacturer's instructions. All procedures were carried out at 4°C. Briefly, 10 ml columns were filled with His-Bind resin, which was washed, charged with nickel sulphate, then rinsed in binding buffer. Next, 2 ml aliquots of the cell extract were loaded on the column and allowed to drip slowly, until a total of 10 ml had been loaded per column. The flow-through was collected and reloaded in

2 ml aliquots, as before. The column was washed and the bound protein eluted and collected in 1 ml fractions. SDS-PAGE (Section 2.5) was used to determine which fractions contained the recombinant protein. To ensure that all of the recombinant protein had bound to the column, the flow-through was also analysed on the gel.

Thrombin cleavage

The fractions of eluate containing the recombinant protein were pooled and dialysed against 5 L thrombin cleavage buffer (Appendix 1.23) for 24 h at 4°C. The OD_{280} of the dialysed sample was measured and the amount of protein calculated using the equations:

$$Y \times 1280 + W \times 5690 = E$$

$$mwt/E \times OD_{280} = mgml^{-1}$$

where: Y = tyrosine and W = tryptophan, in the sequence.

Keeping 50 µl of sample as an uncut control, 1 unit of thrombin was added per mg of protein and the sample incubated overnight at room temperature. To check the protein had been cut by the thrombin, 5 µg of cut and uncut protein were analysed by SDS-PAGE (Section 2.5). The cut sample was dialysed overnight against 5 L of PBS at 4°C, the OD_{280} measured and the protein content calculated, as above.

3. CLINICAL FINDINGS

3.1 Introduction

Infection with *D. viviparus* results in a strong acquired immunity. Several studies have been performed to identify the level of parasite exposure required to stimulate protective immunity. Early experimental infections with *D. viviparus* used very high numbers of infective larvae and calves which received 50,000 L3 invariably died (Jarrett *et al.*, 1957b). Similarly, calves receiving 5000 L3 showed severe clinical signs, with respiratory rates greater than 100 min^{-1}, adventitious sounds in the lungs, inappetance and weight loss (Jarrett *et al.*, 1957b). Calves infected with 1000 to 2000 L3 retained a good appetite and had respiratory rates which were always below 70 min^{-1} (Jarrett *et al.*, 1957b). Poynter *et al.* (1970) found that complete protection was afforded when calves were infected twice at monthly intervals with 1000 L3, but diminished protection was obtained when 500 L3 were administered. Similarly, Cornwell and Jones (1970a) found that 3-month-old calves, infected twice at four week intervals with 1000 L3, showed clinical signs after initial, but not after subsequent challenge infections. Therefore, 1000 L3 given twice, at monthly intervals, is the lowest infective dose which has been proven to give protection against subsequent challenge without the development of severe clinical signs.

In a more recent study, calves were exposed to "trickle" infections of between two to 100 L3 twice weekly for 3 weeks (ie. a total 12 to 600 L3). Two waves of patency resulted, each lasting approximately four weeks and the number of L1 in faeces was higher during the second wave (Eysker *et al.*, 1993). These data demonstrated that the level of immunity was insufficient after the primary infection to protect against a subsequent infection. Studies in grazing animals have confirmed these findings. For example, the level of immunity after one season was higher in non-treated calves than in oxfendazole treated calves, the latter group presumably having lower parasite exposure (Eskyer *et al.*, 1990a). Other authors have demonstrated that the development of immunity after initial low level infections was insufficient to protect calves against subsequent challenge when turned out to pasture the following year (Eysker and van Miltenburg, 1988). Thus it is essential that an adequate infective dose is given to induce protective immunity.

As the aim of the experiments described in this thesis was to analyse responses during the development of immunity, an experiment was designed in which *D. viviparus* infections were monitored over time. Based on the data summarised above, three-month-old calves were infected with 10 L3 kg^{-1} on one (Group B) or three (Group A) occasions. These calves weighed between 120 to 195 kg at the start of the experiment, therefore they received between 1200 and 2550 L3 per infection. Larvae used at each infection were from the same batch and were used within 48 h of arrival. The larvae were examined microscopically to assess their viability, which was based on the presence of an abundant supply of food granules in the body, coiling of the L3 and wriggling on heating. The infections were administered as described in Chapter 2.

Calves were monitored clinically and the levels of L1 in faeces were enumerated throughout the experiment. The measurement of these parameters enabled a rough assessment of the degree of immunity attained after each infection. To achieve an accurate estimate of changes in clinical status, details were recorded at the same time each day, approximately one hour after being fed and before any handling. Regular clinical examination ensured that symptomatic therapy could be administered to calves to alleviate clinical signs.

Infection with *D. viviparus* induces severe airway pathology with infiltration of large numbers of mast cells (H. Miller, personal communication), eosinophils, neutrophils, macrophages and multinucleate giant cells (Jarrett *et al.*, 1960a). In an attempt to follow the changes in local cellular populations during the course of infections, BAL was performed on the experimental calves pre-infection and two or three times after each infection. The technique used for BAL was first described by Fogarty *et al.* (1983) as a new method for examining pulmonary responses without the need for local or general anaesthesia. Calves were lavaged at weekly intervals over four months with no adverse effects, however, repeated lavage within a short time interval (ie. four lavages, within six hours) was shown to result in alterations of cell composition in the lavage sample (Dalgleish, 1989). In the experiments described here, calves were lavaged with an interval of at least seven days, so the procedure itself was unlikely to have any detrimental effect.

Experimental design

The experimental protocol is explained in Materials and Methods (Section 2.1.2).

3.2 Results

3.2.1 Clinical findings

The results of the clinical findings are summarised below. Detailed results of all the parameters measured are tabulated in Appendix 3.

3.2.1.1 Rectal temperatures

There were slight, but not statistically significant, elevations in rectal temperature in some of the calves during the pre-patent and patent periods of the primary infection. Temperatures remained within normal ranges (101.5 to 103°C) throughout the secondary and tertiary infections (Appendix 3). Calf 166, which died on Day 19 after the second infection, was pyrexic (103-104°F) for the three days before it died. This occurred in spite of intensive therapy (Section 3.2.1.5).

3.2.1.2 Respiratory rates

All the calves had elevated respiratory rates starting at Day 8 to 12 after the primary infection. This corresponds to the pre-patent period during which larvae migrate through pulmonary capillaries into alveoli and into the bronchioles and bronchi (Jarrett and Sharp, 1963). Respiratory rates continued to rise from Day 21 pi and remained elevated throughout the patent phase, when adult worms were in the bronchi (see parasitological data). Respiratory rates declined thereafter. There was a similar, though less prolonged, response after the secondary infection. After the tertiary infection respiratory rates remained within

normal limits. The pattern of responses for all calves in Group A were similar (Appendix 3); Figure 3.1 illustrates graphically the respiratory rates for one of the calves (Calf 165).

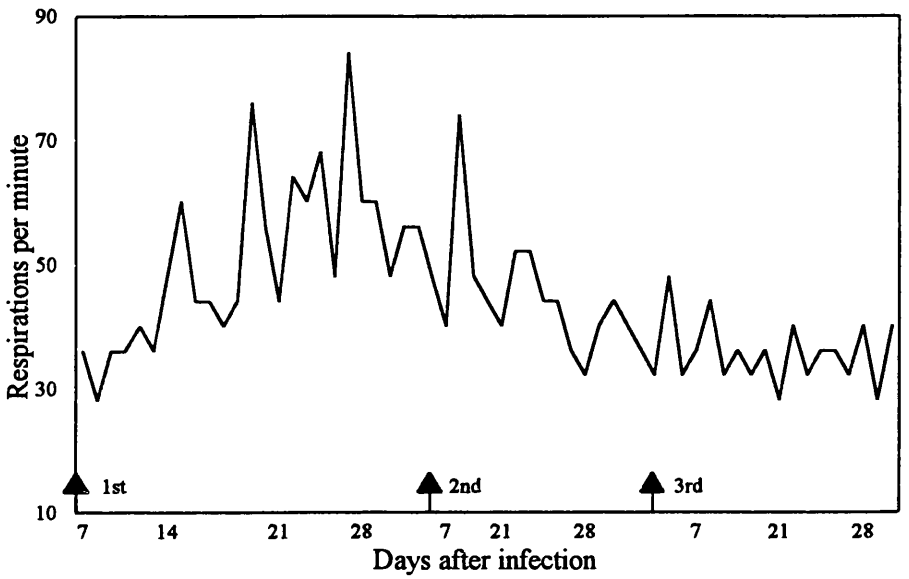

Figure 3.1 Respiratory rates of Calf 165 over three infections with *D. viviparus*.

Respiratory rates were monitored throughout the course of the experiment. This graph shows the respiratory rates for Calf 165 (Group A). The arrows indicate when the infections were administered.

3.2.1.3 Weight gain

The Group B calves, which remained uninfected until the final challenge on Day 112, showed significantly ($p < 0.05$) higher live weight gains from Day 0 to Day 70 (mean 66 kg) than the Group A calves (mean 32 kg). Due to technical difficulties, calves were not weighed after Day 70.

3.2.1.4 Parasitological monitoring

All Group A calves had L1 in their faecal samples from Day 24 after the primary infection (Table 3.1). Larvae were excreted from Day 1-21 to Day 2-14 and reached peak levels (6 to 57 L1 g^{-1} of faeces) at a median time of 34 days pi. Group A calves continued to excrete

larvae for a mean of 68 days after the primary infection. Only one of the calves (Calf 165) excreted larvae after the secondary infection, at very low levels (0.1 L1 g^{-1}), from Day 33 to 35. There was no larval excretion after the tertiary infection in these calves. Parasites were not recovered from the lungs of Group A calves on Day 35 after the tertiary infection.

The three challenge control calves (Group B) had peak larval counts of 28, 11 and 71 L1 g^{-1} in their faeces and 81, 74 and 47 adult parasites were recovered from their lungs at post-mortem (Table 3.1).

Group	Calf No.	Peak L1 g^{-1} in faeces	Range (days)	Adults in lungs
A	165	3.7	21-70	0
	166	57*	24-84	18
	172	0+		0
	174	19	24-49	0
	175	8	24-49	0
B	169	28	23-	81
	170	11	23-	74
	176	71	23-	47

Table 3.1 Parasitological data

L1 in faecal samples were enumerated using the modified Baermann technique. Range indicates the time period over which L1 were detected in faeces. Adults in the lungs: denotes the numbers of parasites recovered from the lungs at post-mortem, on Day 35 after challenge infection. * Calf 166 died on Day 88. + Calf 172 was treated with an anthelmintic on Day 1-18.

3.2.1.5 Therapeutics

Seven out of the eight calves received symptomatic therapy after the primary infection. The actual timing of treatments is detailed in Appendix 3. The drugs used were the non-steroidal anti-inflammatory flunixin (2.2 mg kg^{-1}, Finadyne, Schering-Plough Animal Health, Welwyn Garden City) and the broad spectrum antibiotic enrofloxacin (2.5 mg kg^{-1}, Baytril, Bayer plc,

Bury St. Edmonds). Only one calf, Calf 166 (see below), required treatment during subsequent infections.

One of the calves in Group A, Calf 172, did not improve in response to symptomatic therapy administered from Day 13 to Day 18 pi. The condition of this calf worsened and a salvage treatment of the larvicidal anthelmintic, levamisole, (7.5 mg kg^{-1}, Levacide, Norbrook Labs Ltd., Kidderminster) was given on Day 18. The clinical condition of the calf improved dramatically over the next 24 h. This calf did not excrete L1 in faeces after the primary, secondary or tertiary infections. After the secondary infection, it had an elevated respiratory rate and coughed occasionally. After the tertiary infection, the respiratory rate remained within normal limits.

Calf 166, which had the least severe clinical signs following primary infection, continued to excrete higher numbers of larvae for a longer period than the other calves, and was excreting 20 L1 g^{-1} on Day 84 pi (Appendix 3). This calf was coughing on Day 14 after the secondary infection and, by Day 17, had an elevated temperature (104°F) and respiratory rate (84 min^{-1}). On Days 17, 18 and 19 after the secondary infection, it was treated with flunixin and enrofloxacin, at the dose rates indicated above. The clinical condition of the calf improved slightly on Day 2-18, but worsened again on Day 2-19, so much so that it deteriorated rapidly and died. This calf was excreting 20 L1 g^{-1} of faeces when it died. From the L1 data (Table 3.1) it can be seen that this calf was still harbouring a patent infection when the secondary infection was administered. Moreover, at post-mortem, 18 adult *D. viviparus* were recovered from the trachea and main-stem bronchi (Table 3.1).

3.2.2 Leukocyte populations in BALF

BALF was collected from calves pre-infection, on Days 19 and 26 after primary infection, and on Days 10 and 20 after secondary and tertiary infection. The numbers of leukocytes are presented as percentages rather than actual numbers as the total volume of BALF and therefore the numbers of cells recovered, was very variable from one day to the next (i.e. 8 to 100% recovery). The percentages of cells present gave a more accurate indication of the fluctuations in cellular phenotype over the course of the infections.

On a few occasions it was not possible to pass the plastic tubing into the trachea, as the calf would swallow the tube. After a few attempts, the other nostril was used and, if that was unsuccessful, the calf was left unsampled. Too much intervention would cause trauma and bleeding.

3.2.2.1 Changes in leukocyte populations after infection with *D. viviparus*

The percentage changes found in different leukocyte populations following *D. viviparus* infection are summarised in Table 3.2 and shown graphically in Figure 3.2. The cells recovered pre-infection were predominately pulmonary alveolar macrophages (mean 87%) (Table 3.2). The levels of lymphocytes varied from 0 to 16.3% (mean 9.3%) and neutrophils, 0 to 7.5% (mean 3.0%) and, in approximately half of the samples, a few eosinophils were recovered (mean 0.44%).

The most dramatic increase in cell type observed after infection was in eosinophils. By Day 10 after primary infection (Day 1-10), there was a significant ($p \leq 0.01$) increase in the percentages of eosinophils present, rising from 0.4 to 8%. This increase in eosinophils was reflected in total numbers of cells harvested, the maximum total numbers of eosinophils recovered from infected calves being 10.3×10^5, as opposed to 1.7×10^5 in uninfected calves (i.e. a six fold increase).

By Day 1-19 there was another substantial increase in the percentage of eosinophils, to a mean of 46% ($p \leq 0.01$) compared with 8% on Day 1-10. Again eosinophils rose significantly between Day 1-19 and Day 1-21, to a mean of 76.5% ($p \leq 0.05$). The levels fell thereafter, to a mean of 37.3% by Day 1-26 ($p \leq 0.1$).

There was also a small, though not statistically significant, increase in lymphocytes from Day 0 to Day 1-10 (9.28% rising to 13.1%). After Day 1-10, the mean percentages of lymphocytes remained low. The percentages of neutrophils rose significantly from Day 1-19 to a mean of 6.3% compared with 3.0% in uninfected calves ($p \leq 0.01$), and these continued to rise until Day 1-26 when there was a mean of 12.6% ($p \leq 0.1$). The absolute numbers of neutrophils increased; the maximum recovered from uninfected calves was 1.2×10^6 cells,

whereas the mean number on Day 1-26 was 3.6 x 10^6 cells (ie. a three fold increase). These increases in mononuclear cells were reflected by relative decreases in the percentages of macrophages, which were the dominant cell type pre-infection.

The cellular changes after the secondary infection differed from those observed after the primary infection. The first sample, taken on Day 2-10, showed that the mean percentage of eosinophils was 11.1%. These levels remained fairly static with a mean of 12% on Day 2-21. The percentage of neutrophils increased after the secondary infection with a mean of 35% on Day 2-10 and 40% on Day 2-21. There was a small , but not significant, increase in lymphocytes, with means of approximately 14% on Day 2-10 and 2-21, compared with 9.3% in uninfected calves ($p > 0.1$).

After the tertiary infection, the pattern was different again. There was a significant ($p \leq 0.01$) increase in the percentage of lymphocytes with a mean of 17.6% on Day 3-10. These remained elevated, with a mean of 22.9% on Day 3-21. This increase was reflected in the total numbers of lymphocytes collected, which increased from a mean of 1.0 x 10^6 pre-infection to 2.65 x 10^6 on Day 3-10 and 3.13 x 10^6 on Day 3-21. Neutrophil levels remained high with mean percentages of 27.6% on Day 3-10 and 18.5% on Day 3-21. The mean percentage of eosinophils was 15.8% by Day 3-10 and this decreased to 7% by Day 3-21.

The percentages of alveolar macrophages decreased in infected calves, however, this did not appear to be due to a decrease in actual numbers of macrophages, but rather reflected increases in the other lymphocyte populations i.e. a relative decrease.

Day	No.	PMN	Lymph	Mono	Eosin	Macro
		Mean(std)	Mean(std)	Mean(std)	Mean(std)	Mean(std)
0	12	2.9(2.4)	9.28(5.8)	0	0.4(0.4)	87.2(6.6)
1-10	3	2.1(0.4)	13.1(3.3)	0	*8.0(7.7)	76.6(10.9)
1-19	5	*6.3(3.7)	5.20(4.3)	0	*46.0(18.2)	42.5(17.8)
1-21	2	*11.8(0.8)	2.5(1.5)	0	*76.5(9.5)	9.2(8.8)
1-26	3	*12.6(6.7)	8.8(3.3)	17.0(29.4)	*37.3(19.6)	41.0(21.9)
2-10	4	*35.0(29.2)	14.1(8.0)	0	*11.1(4.1)	22.0(23.5)
2-21	2	*40.0(34.4)	14.2(2.8)	0	*12.0(1.6)	33.4(30.0)
3-10	4	*27.6(16.9)	*17.6(8.4)	0	*15.7(10.3)	39.0(28.1)
3-21	4	*18.5(15.9)	*22.9(16.8)	0	*7.0(4.5)	51.5(32.5)

Table 3.2 Leukocyte populations in BALF after primary, secondary and tertiary infections with *D. viviparus*.

BALF was collected from Group A and Group B calves, pre-infection and pi. A 5% aliquot of the BALF cells was cytospun and the pellet stained with May-Grunwald stain. The numbers of each different cell subset were counted and expressed as a percentage of the total cell population.

Day Day pi that BAL performed after: 1- primary, 2- secondary and 3- tertiary infections.
No. Number of samples included in the mean
PMN Neutrophils
Lymph Lymphocytes
Mono Monocytes
Eosin Eosinophils
Macro Macrophages
Mean Mean percentage of each cell population present
std Standard deviation
* Indicates that the means were significantly different from those present in uninfected calves i.e (Day 0). This was calculated using a two-tailed t test and $p \leq 0.1$ was considered significant.

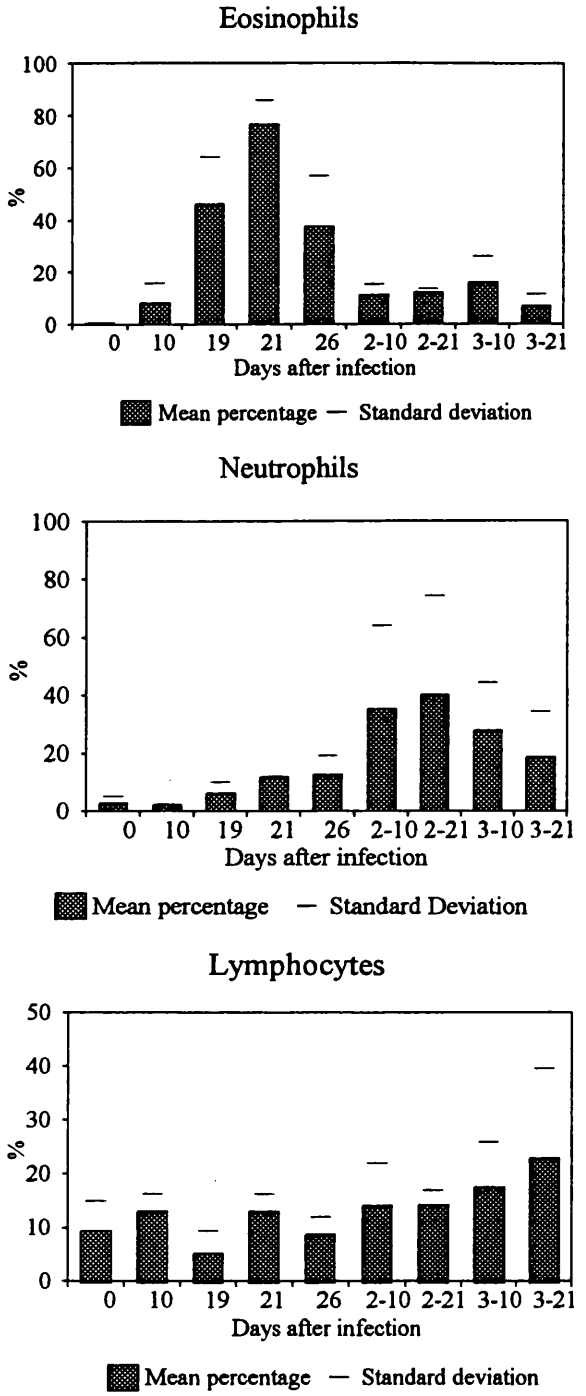

Figure 3.2 Leukocyte populations in BALF after infection with *D. viviparus*.

BALF was collected from Group A and Group B calves, pre-infection and pi. A 5% aliquot of the cells collected was cytospun and the pellet stained with May-Grunwald stain. The numbers of each different cell subset were counted, and expressed as a percentage of the total cell population. The days pi are indicated with 2-, indicating days after the secondary infection and 3- indicating the days after the tertiary infection.

3.3 Discussion

The Group A calves in this study were infected with *D. viviparus* L3 on Day 0 and Day 63, then challenged on Day 112, when, based on previous studies (Canto, 1990), it was assumed that they would be immune. The Group B calves, which acted as challenge controls, remained parasite naive until infection on Day 112. The measurement of clinical parameters, including rectal temperature, respiratory rate, calf weight and, in addition, the enumeration of faecal L1, allowed an indirect assessment of the degree of immunity attained after each infection.

The clinical signs reported after infection with *D. viviparus* L3 depended on the magnitude of the infecting dose, as discussed in the Introduction to this chapter. In this study, respiratory rates for all calves after the primary infection increased by Day 8 to 12, and either remained elevated, or decreased over the next seven days. Further increases were observed around Day 21. These changes were similar to those reported by Cornwell and Jones (1970a) who infected calves with 1000 *D. viviparus* L3. In contrast, Jarrett *et al.* (1955a) observed progressive increases in respiratory rates over the second week, however, the calves in that study received 4000 L3 (Jarrett *et al.*, 1960b). In the present study, the respiratory rates increased after the secondary infection, despite the absence of L1 in the faeces of most calves. This suggested that the larvae reached the lungs, but did not develop to patency. After the tertiary infection, the respiratory rates remained within normal limits, indicating that the parasites did not migrate through the lungs and that the calves were fully immune. Therefore, respiratory rates appeared to provide a sensitive indicator of immune status.

There were small fluctuations in the rectal temperatures of the calves, with slight increases associated with increases in respiratory rates. Apart from Calf 166, none of the increases were significant. In previous experimental studies in vaccinated calves, in calves infected with L3 and in field studies, there has been little reference to fluctuations in body temperature. Anecdotal mention is made in several review papers, associating infection with a rise in rectal temperature. For example, Duncan and Urquhart (1982) reported that temperatures may be elevated (up to 104°F). They considered that elevations in temperature were usually due to damage by *D. viviparus* and not to secondary bacterial infection. In

contrast, Eysker (1994) suggested that pyrexia may occur only if there is an associated secondary bacterial infection. It may be that in mild infections, as in this study, the degree of lung damage is low and unlikely to produce pyrexia, while heavy infections may result in severe lung damage and pyrexia.

The parasitological data combined with the respiratory rates gave an indication of the degree of immunity after primary and secondary infection. Only one calf excreted L1 after the secondary infection and this was at a very low level (0.1 L1 g^{-1}). None of the Group A calves excreted larvae after the tertiary infection. Poynter *et al.* (1970) demonstrated complete protection, with no L1 in faeces and no adults in the lungs, in calves immunised twice with 1000 L3 given 29 days apart and challenged with L3 two weeks later. These data correlate well with the findings observed here.

One of the calves, Calf 172, showed no clinical improvement in response to symptomatic therapy from Day 1-13 to Day 1-18 and required salvage treatment with larvicidal anthelmintic on Day 1-18. This calf did not excrete L1 after the primary, secondary or tertiary infection. It seemed to have developed immunity to *D. viviparus*, despite the absence of an obvious patent infection. Calf 166 had mild clinical signs after primary infection, but required symptomatic therapy from Day 2-17 onwards. The condition of this animal worsened on Day 2-19 leading to death within 24 h. It is unclear why this calf deteriorated so rapidly and why it succumbed to a relatively low level of infection. This considerable individual variation to infection has also been reported by many other workers including (Poynter *et al.* 1970; Simpson *et al.* 1957; Jarrett *et al.* 1957a). In an attempt to eliminate variation due to different infective doses and calf sizes, the calves in this study were weighed and given infective doses per kilogram. There was no correlation between calf weight and clinical signs. None of the calves appeared to have any intercurrent disease which may have increased susceptibility to lung worm. It is more likely that the variations in clinical signs and parasite burdens were due to individual calf susceptibility. It has been demonstrated that there is considerable heterogeneity in the antibody repertoire to *D. viviparus* E/S products of in calves (Britton *et al.*, 1992a) and outbred guinea pigs (McKeand *et al.* 1994b).

The changes in the local cell population during the course of the infections were investigated by enumerating the various leukocyte populations. The baseline levels of the different cell phenotypes for the calves in this study were similar to those found by Fogarty *et al.* (1983), who reported > 94% macrophages in BALF from clinically normal calves.

Similarly, Dalgleish (1989) found that macrophages were the most abundant cell type in samples from uninfected calves. A predominance of macrophages has been demonstrated in the BALF of clinically normal individuals of many other species, including humans (Hunninghake *et al.*, 1979), rats (Egwang *et al.*, 1984), mice and dogs (Cohen and Baktra, 1980).

The most dramatic change in leukocyte populations in BALF after infection with *D. viviparus* was an increase in the percentage of eosinophils, from < 1% in uninfected calves to a mean of 76.5% on Day 21 after primary infection. Similarly, the relative numbers of eosinophils in BALF showed dramatic increases after both primary and secondary infections with *N. brasiliensis* (Egwang *et al.*, 1984) and *T. canis* (Buijs *et al.*, 1994) infections; in both these studies, eosinophils increased approximately 18 fold. In *T. canis* infected mice, significant increases ($p \leq 0.05$) in the numbers of eosinophils and lymphocytes in BALF were observed by Day 14 pi and remained elevated at Day 28 pi (Buijs *et al.* 1994). In rats infected with *N. brasiliensis*, a biphasic increase in total cells, mainly consisting of eosinophils and neutrophils, was demonstrated, with the first peaks on Days four to six pi and the second on Day 16 pi (Egwang *et al.*, 1984). In the present study, the eosinophil levels increased by Day 10 pi and continued to increase until Day 21 and, as with the *T. canis* study (Buijs *et al.*, 1994), no biphasic response was demonstrated. However, in the *N. brasiliensis* study, samples were collected more frequently than in the present study or in that of Buijs *et al.* (1994), so a biphasic response may have gone unnoticed. Indeed, a biphasic peripheral eosinophilia has been demonstrated after *D. viviparus* infection (Weber and Rubin, 1958). The first peak was between nine and 15 days pi, coinciding with larval development in the lungs, and the second peak at approximately Day 40 pi, following parasite patency (Cornwell, 1962a). However, it is unknown if this peripheral biphasic response is reflected locally in the lungs.

Previous studies have demonstrated that helminth parasites release factors which attract eosinophils: the E/S products of the L3 of *Ostertagia ostertagi* were found to be chemotactic for bovine eosinophils (Klesius *et al.*, 1985), and cercarial homogenates or transformation fluid from *S. mansoni* induced a local accumulation of eosinophils and neutrophils when injected into guinea pig skin. In that study, the leukocyte accumulation was correlated with proteolytic activity present in the cercarial homogenates (Teixeira *et al.*, 1993). *D. viviparus* has been demonstrated to release serine-, cysteine- and metallo-

proteinases (Britton *et al.*, 1992b), so these may be involved in eosinophil chemotaxis. The rapid tailing off of the eosinophil response in the present study may be explained by an eosinophil attractant only being released by pre-adult stages of the parasite, or alternatively, host factors, such as antibodies, may inhibit the eosinophil chemotaxis. The second peak of eosinophil numbers in *N. brasiliensis* infection coincided with the peak of rat mast cell protease, an indicator of mucosal mast cell activation (Woodbury *et al.*, 1984). Activated mast cells release mediators such as histamine, protaglandin D2, eosinophil chemotactic factor of anaphylaxis (ECF-A) and monohydroxyecosatetraenoic acids (HETEs) which modulate eosinophil migration (Dessein and David, 1982; Gordon *et al.*, 1990). Mast cells are abundant in *D. viviparus* infected lungs (H. Miller, personal communication) and so may be involved in eosinophil recruitment.

Eosinophils, and particularly their granules, play an important role in the inflammatory response (Spry *et al.*, 1992) and their relevance in airway inflammation has been highlighted by their role in the pathophysiology of asthma (reviewed by Seminario and Gleich, 1994). Eosinophils have been shown to adhere to and kill a number of helminth parasites including *T. spiralis* (Bass and Szedja, 1979) and *S. mansoni* (Capron *et al.*, 1983) and, indeed, bovine eosinophils were shown to adhere to the L3 of *D. viviparus* (Knapp and Oakley, 1981). The present findings of a profound local eosinophilia imply that eosinophils may play an important role in *D. viviparus* infections.

A significant increase in neutrophils was demonstrated in the BALF of *D. viviparus* infected calves on Day 19 after infection. Parasite patency is reached around Day 21 after infection with *D. viviparus,* so there was perhaps a correlation between the presence of neutrophilia and the development of patency. An increase in neutrophils in the lavage fluid of calves has previously been demonstrated after bacterial infection: inhalation exposure to *Pasteurella haemolytica* and *Salmonella typhimurium* (Walker *et al*, 1985; Dalgleish, 1989) induced increases in BALF neutrophil numbers. Neutrophils are the most active cell type mediating antibody-dependent cell-mediated cytotoxicity (ADCC) in cattle (Wardley *et al.*, 1976) and, although there is no evidence that bovine neutrophils can kill helminths, they have been shown to kill the protozoan *Trichomonas fetus* (Aydintug *et al.*, 1993). Neutrophils have been shown to be involved in defence against helminths in other hosts. For example; human neutrophils, together with eosinophils, killed *T. spiralis* newborn larvae via myeloperoxidase (Venturriello *et al.*, 1993). Furthermore, the myeloperoxidase was reported

to be more active than eosinophil peroxidase at killing *T. spiralis* newborn larvae (Buys *et al.*, 1984).

Parasites were shown to produce chemotactic factors for neutrophils; for example, cercarial homogenates and transformation fluid from *S. mansoni* induced a local accumulation of neutrophils (Teixeira *et al.*, 1993). Furthermore, a neutrophil chemotactic factor has been cloned from adult *Dirofilaria immitis* (Owhashi *et al.*, 1993). In some parasite species the chemotactic ability appears to be stage-specific, e.g. newborn larave of *T. spiralis* showed the strongest chemotactic potential for neutrophils, whereas adult worms showed a weaker attraction (Shupe and Stewart, 1991). The release of a putative neutrophil attractant by adult stages could explain the later increase in neutrophils after a primary *D. viviparus* infection, but it does not explain the high percentage of neutrophils demonstrated in BALF after the secondary and tertiary infections, when patency was not reached. An alternative explanation may be that the parasite induces host cells to produce a neutrophil attractant. In support of this theory, various factors which are increased in inflammatory responses, such as endotoxins (Koyama *et al.*, 1991), ACh (Koyama *et al.*, 1992) and tachykinins (Vonessen *et al.*, 1992), have been shown to stimulate the release of neutrophil attractants from bronchial epithelial cells. Similarly, TNFα was shown to regulate the infiltration of neutrophils and, later, eosinophils into the airway of mice after inhalation of *S. mansoni* egg antigen (Lukacs *et al.*, 1995).

The levels of eosinophils and neutrophils remained elevated after the tertiary infection. The major difference in cell phenotypes after the tertiary infection, compared with the first two infections, was the significant ($p \leq 0.01$) increase in the percentages of lymphocytes by Day 3-10. This increase in lymphocytes may be associated with the more rapid termination of infection, suggesting they may be important in the protective response in immune animals. The following chapters will attempt to define the role of T lymphocytes in the immune response to *D. viviparus* infection in calves.

4. ANTIBODY RESPONSES TO *D. VIVIPARUS*

4.1 Introduction

The potential importance of antibodies in protective immunity to *D. viviparus* was first established by Jarrett *et al.* (1955a) when they demonstrated that an intraperitoneal injection of sera from naturally infected calves reduced mean worm burdens of naïve recipients by 95%, compared with challenge controls. More recently, in a similar study by Canto (1990), a mean reduction in worm burdens of 78% was achieved. Passive transfer of immunity against *D. viviparus* has also been carried out successfully using serum from L3 infected guinea pigs (Canto, 1990; McKeand *et al.*, 1995) and serum from guinea pigs immunised with adult E/S products (McKeand *et al.*, 1995). However, serum from calves, immunised by intravenous injections of 1000 Gy irradiated L3, did not confer protection to naïve animals (Canto, 1990). A similar lack of protection was obtained in passive transfer experiments in guinea pigs using serum from animals given 400 Gy and 1000 Gy irradiated L3 (Canto, 1990). Other studies have shown that, in calves vaccinated using 400 Gy irradiated L3 which were strongly immune to challenge, little or no serum IgG was detected to either adult or L4 *D. viviparus* (Bos *et al.*, 1986; Wassall, 1991). These findings suggest that antibody may play a role in immunity during natural infection, but not following vaccination. However, the precise role of antibody in protective immunity to *D. viviparus* remains unclear.

McKeand *et al.* (1996) described a class switch from a predominance of IgM antibodies early after infection with *D. viviparus* to predominately IgG1 and IgG2 antibodies on Day 76, a time point which coincided with the death of adult parasites in the lungs (Jarrett and Sharp, 1963). Different antibody isotypes/subclasses have different effector functions; for example, in mice infected with *T. muris*, different parasite-specific IgG subclasses are induced in resistant strains compared to susceptible strains (Else *et al.*, 1990). Similarly, sheep resistant to *H. contortus* have significantly higher parasite-specific serum IgG1 levels than susceptible sheep (Gill *et al.*, 1993a). As the different IgG subclasses are controlled by different cytokines (see Introduction), examination of subclass responses can shed some light on the underlying T helper cell response. Consequently, a

more detailed time-course study of parasite-specific antibody isotype/subclass responses may help unravel the mechanisms of the immune response.

Five distinct bovine immunoglobulins have been identified; IgM, IgA, IgG1, IgG2 and IgE. Apart from IgG, these Igs are similar to their counterparts in non-ruminants. The two IgG subclasses have pronounced biochemical and physio-chemical differences between one another. IgG1 is an important secretory immunoglobulin in ruminants and is known to be selectively transported from serum into the mammary gland (Newby and Bourne, 1977). Furthermore, high levels of IgG1 were demonstrated in respiratory secretions (Curtain *et al.*, 1971; Watson and Lascelles, 1973). As is the case in other mammals, IgA is also an important secretory immunoglobulin in the bovine. In the respiratory tract, however, IgG1 appears to be quantitatively more important in the lower lungs and IgA in the upper respiratory tract and bronchi (Butler , 1983).

The most likely site for the protective immune response against *D. viviparus* is the lung (reviewed in Chapter 1). It was therefore important to examine specifically the immune responses in the respiratory tract, as these may not be reflected in the peripheral circulation (Befus *et al*, 1986). For example, in calves infected with bovine coronavirus, Heckert *et al.* (1991) found a predominance of IgA in BALF, over that in serum. This compartmentalisation of antibody responses has also been demonstrated in the alimentary tract in *C. parvum* infected lambs, where faecal IgA was found to predominate over serum IgA (Hill *et al.*, 1990).

In an attempt to address the possible role of local antibody in protective immunity in parasitic bronchitis, a longitudinal study was performed in which both local and peripheral antibody responses were measured by ELISA. Calves were exposed to single or multiple infections and subclass and isotype-specific responses were measured in the serum and BALF over the course of the infections.

4.2 Results

4.2.1 Measurement of antigen-specific IgG, IgG1, IgG2, IgM and IgA

4.2.1.1 Specificity of monoclonal antibodies used in the ELISA

Outline

To confirm the specificity of the monoclonal antibodies that were used in the ELISA, purified bovine immunoglobulins (Igs) were obtained from Dr. K. Kenny, Key Laboratories. Ninety six-well plates were coated with purified bovine IgA, IgM, IgG1 and IgG2, at concentrations recommended by Dr. K. Kenny (personal communication), and incubated for 2 h at 37°C. After washing, 100 µl of 5% soya milk were added to each well to reduce non-specific binding and the plates were incubated at room temperature for 1 h. After washing, 100 µl of mouse anti-bovine IgA (1:500, Serotec), IgM (1:1000, Sigma), IgG1 (1:160), IgG2a(A1) (1:160, both Serotec) or total IgG2 (1:25, Mab 8H6, Dr. K. Kenny) were added to each well. The plates were incubated at 37°C for 30 min and washed. Next, 100 µl of peroxidase-conjugated goat anti-mouse IgG (whole molecule) were added to each well and the plates incubated at 37°C for 30 min. After final washing, 100 µl of TMB peroxidase substrate were added to the wells and the ODs measured (as described in Section 2.7).

Results

The results are presented in Table 4.1. Each Mab bound only to the correct isotype /subclass and not to any of the others. Therefore, the Mabs used for the subsequent ELISAs were specific.

Igs \ Mab	IgA	IgM	IgG1	IgG2a(A1)	IgG2(total)
IgA	+	-	-	-	-
IgM	-	+	-	-	-
IgG1	-	-	+	-	-
IgG2	-	-	-	+	+

Table 4.1 Specificity of Mabs used in the ELISA

ELISA plates were coated with purified bovine Igs and Mabs specific to bovine antibodies were added. A peroxidase-conjugated anti-mouse antibody was used to detect bound Mab. + : indicates a positive result and - : indicates a negative result.

4.2.1.2 Serum antibody responses to *D. viviparus* adult homogenate

Outline

The optimum dilutions of antigen and antisera were determined for each isotype/subclass using checkerboard titrations of pooled positive and negative sera. The dilutions used in the assays were:

a) Anti-IgG assay: antigen 2.5 μg ml^{-1}, serum 1:400, peroxidase-conjugated anti-bovine IgG 1:10,000.

b) Anti-IgM assay: antigen 2.5 μg ml^{-1}, serum 1:400, anti-bovine IgM 1:1000, peroxidase-conjugated anti-mouse IgG 1:50,000.

c) Anti-IgA assay: antigen 1.25 μg ml^{-1}, serum 1:50, anti-bovine IgA 1:500, peroxidase-conjugated anti-mouse IgG 1:25,000.

d) Anti-IgG1 assay: antigen 2.5 μg ml^{-1}, serum 1:400, anti-bovine IgG1 1:100, peroxidase-conjugated anti-mouse IgG 1:25,000.

e) Anti-IgG2 assay: antigen 1.25 μg ml^{-1}, serum 1:50, anti-bovine IgG2 1:100, peroxidase-conjugated anti-mouse IgG 1:25,000.

Purified immunoglobulins were not available for standards in the ELISAs. Thus, it was not possible to measure the exact concentration of each immunoglobulin measured nor was it possible to compare the amounts of the different isotypes measured. However, changes in each isotype over the time-course of the experiment were investigated for each animal.

Results

The data in Figure 4.1 to Figure 4.3 demonstrate the serum antibody responses to an homogenate of adult *D. viviparus* for Group A and Group B animals over the course of the three infections.

Increases in total IgG were detected from Day 21 pi in serum samples (Figure 4.1 and Figure 4.3). In Group A calves, total IgG peaked between Days 35 to 49 and then plateaued, rising again 14 days after the secondary infection to peak between Days 19 to 39. After this, the levels of IgG declined slowly and did not increase following the tertiary infection. There were high levels of IgM in the sera collected pre-infection from Calves 169 (Figure 4.3) and 172 (Figure 4.1). These high levels of non-specific IgM may be due to the presence of antigens in the parasites which are cross-reactive with antigens to which the calves have previously been exposed i.e. food items or gut commensal organisms. In all the calves the levels IgM increased from Day 21 pi, peaking between Days 21 to 35 and decreasing and remaining low thereafter. Serum IgA levels initially increased from Day 7 to 28 pi, then decreased gradually after Day 35. From Day 14 after the secondary infection, IgA again increased and then decreased by Day 2-26. Calf 174 had a second increase after the secondary infection, from Day 2-33. There was a short-lived increase in specific IgA from Day 7 after the tertiary infection in two calves (174 and 175), declining by Day 14.

The IgG1 subclass response followed a similar pattern to that of the total IgG in some of the calves. In two calves (174 and 175), IgG1 levels increased rapidly from Day 21 pi, then gradually decreased after Day 49 (Figure 4.2). IgG1 levels rose again after the secondary infection, with a smaller peak than after the primary infection. For the other calves, the levels of IgG1 rose more gradually from Day 21 pi and continued to increase after the secondary infection. In all the calves, levels of IgG1 gradually declined from around Day 2-33, with no increases after the tertiary infection. In contrast to the other isotype/subclass responses, the IgG2a(A1) levels varied more substantially between calves

(Figure 4.2). For example, in Group A, Calves 166 and 174 had high IgG2a (A1) levels whilst IgG2a (A1) levels remained around threshold levels in Calves 165, 172, and 175. For Calf 166 and 174, levels of IgG2a(A1) rose from Day 21 and 28 pi, respectively. There was a further increase from Day 14 after the secondary infection, which peaked on Day 2-26. This dichotomy in IgG2a(A1) responses showed a degree of correlation with faecal L1 counts. For example, Calves 166 and 174, whose IgG2a(A1) levels were high, had L1 counts of 57 and 19 L1 g^{-1}, respectively (Table 3.1). Whereas, the three calves whose IgG2a(A1) levels remained low throughout the three infections had lower L1 counts, with peaks of 0, 2.4 and 3.7 L1 g^{-1} (Table 3.1). For the Group B calves, Calves 169 and 176 had higher IgG2a(A1) levels than Calf 170. Calf 169 excreted 28, Calf 176 excreted 71 L1 g^{-1} whilst Calf 170 excreted 11 L1 g^{-1} (Table 3.1). The association between IgG2a(A1) levels and L1 output is illustrated in Figure 4.4.

Attempts were made to correlate the level of each isotype/subclass with the various clinical parameters measured in individual animals. No correlations were observed between antibody isotype/subclass and temperature or respiratory rate, however, there was correlation between L1 output and IgG2a(A1) levels: animals with elevated IgG2a(A1) levels had higher L1 output than those with low IgG2a(A1). Pearson's correlation was performed between peak L1 count and peak IgG2a(A1) level for the eight calves in the experiment. A correlation of 0.505 was found and this is considered to be a moderate level of correlation.

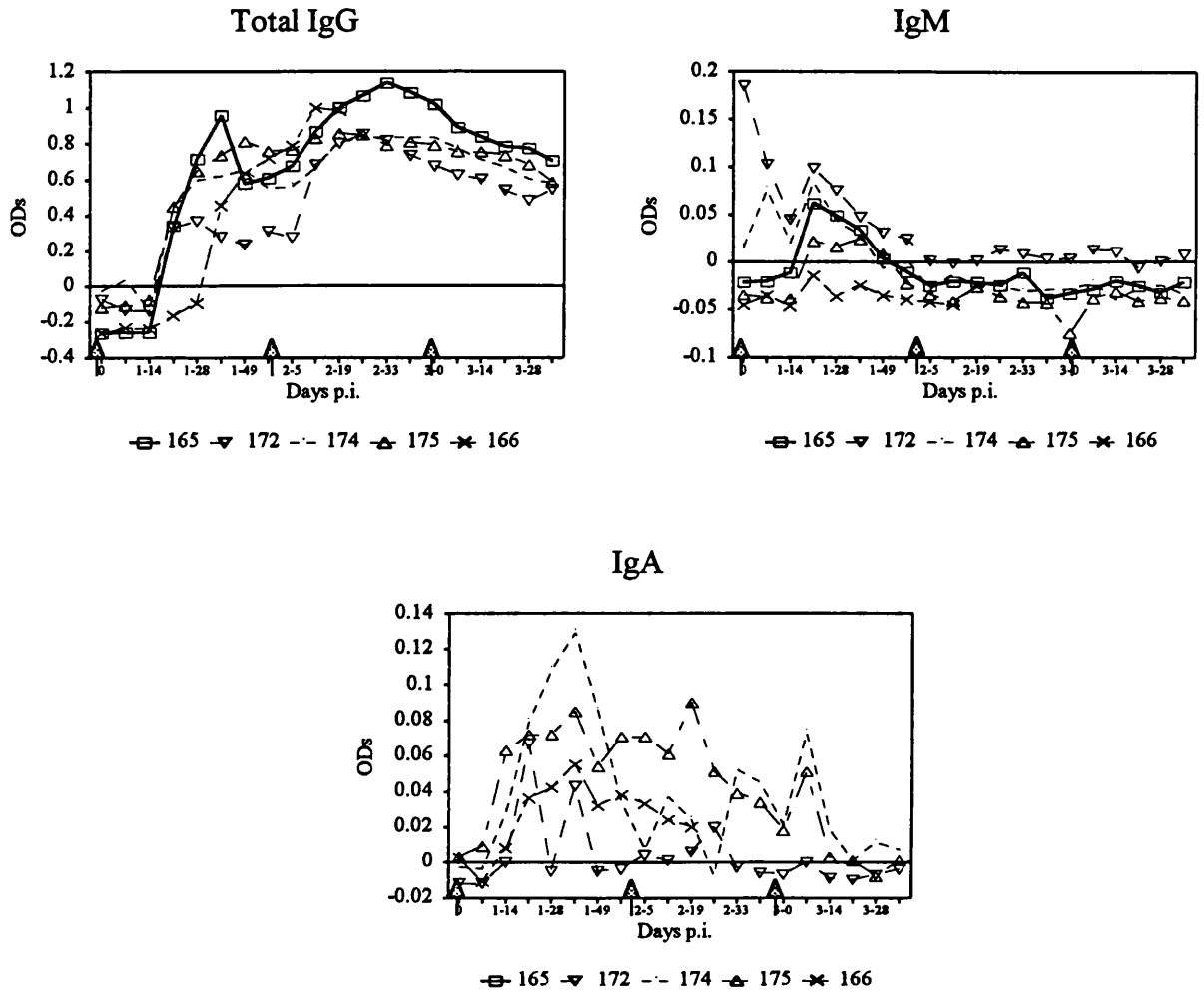

Figure 4.1 ODs for adult homogenate-specific IgG, IgM and IgA in Group A calves
Weekly serum samples from Group A calves were analysed for levels of antigen-specific IgG, IgM and IgA by ELISA. The results show mean ODs for duplicate samples minus the threshold OD. The threshold was two standard deviations above the mean OD of 20 sera samples from uninfected calves. The arrows indicate the days of infection. 1- , 2-, 3-: indicate the days after the primary, secondary and tertiary infections, respectively. See text for ELISA conditions.

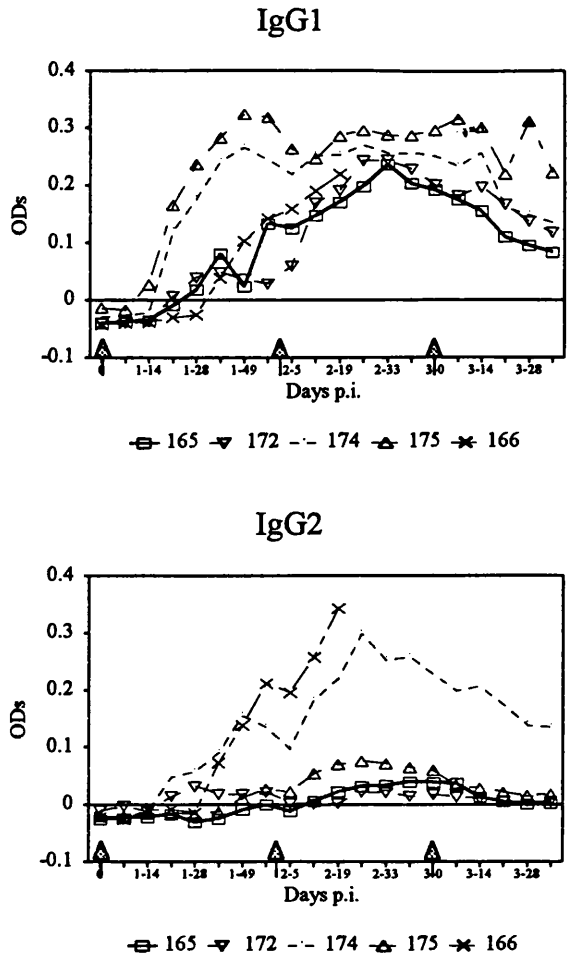

Figure 4.2 ODs for adult homogenate-specific IgG1 and IgG2a(A1) in Group A calves

Weekly serum samples from Group A calves were analysed for levels of antigen-specific IgG1 and IgG2a(A1) by ELISA. The results show mean ODs for duplicate samples minus the threshold OD. The threshold was two standard deviations above the mean OD of 20 sera samples from uninfected calves. The arrows indicate the days of infection. 1- , 2-, 3-: indicate the days after the primary, secondary and tertiary infections, respectively. See text for ELISA conditions.

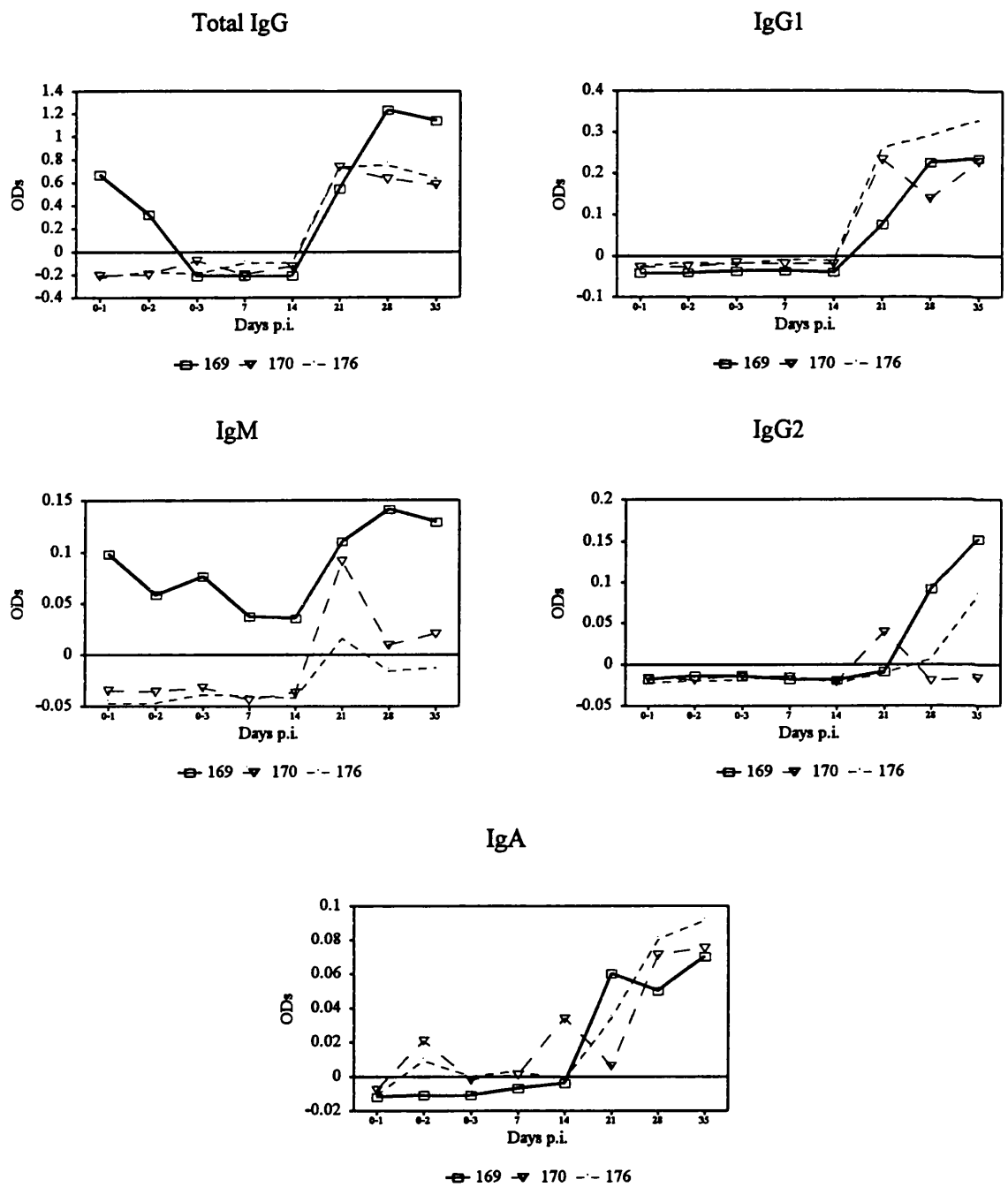

Figure 4.3 ODs for adult homogenate-specific antibodies in Group B calves

Weekly serum samples from Group B calves were analysed for levels of antigen-specific antibodies, by ELISA. The results show mean ODs for duplicate samples minus the threshold OD. The threshold was two standard deviations above the mean OD of 20 sera samples from uninfected calves. O-1, 0-2, 0-3 indicate three pre-infection samples. See text for ELISA conditions.

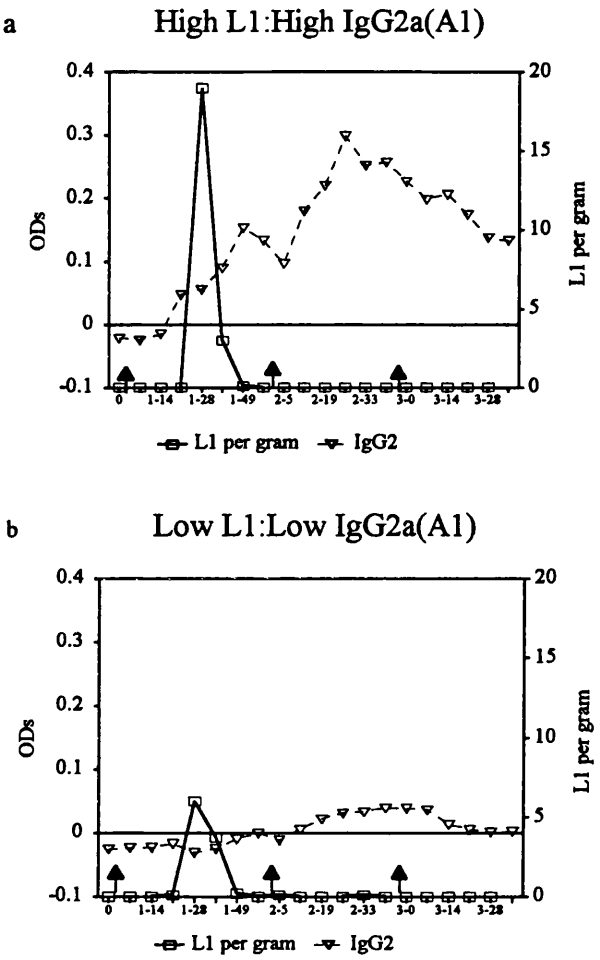

Figure 4.4 IgG2a(A1) antibody levels in calves excreting low or high numbers of L1

Weekly serum samples from Calf 174 (a) and Calf 165 (b) were analysed for levels of IgG2a(A1) antibody to adult homogenate, by ELISA. The results show mean ODs for duplicate samples minus the threshold OD. The threshold was two standard deviations above the mean OD of 20 sera samples from uninfected calves. The numbers of L1 in faeces were measured using the modified Baermann technique. The arrows indicate the days of infection. 1- , 2-, 3-: indicate the days after the primary, secondary and tertiary infections, respectively. See text for ELISA conditions and Baermanisation methods.

4.2.1.3 Antibody responses in BALF

Outline

The optimum dilutions of antigen and antisera were determined for each isotype/subclass using checkerboard titrations of pooled positive and negative BALF. The dilutions used in the assays were:

a) Anti-IgG assay: antigen 1.5 μg ml^{-1}, BALF 1:200, peroxidase-conjugated anti-bovine IgG 1:10,000.
b) Anti-IgM assay: antigen 1.5 μg ml^{-1}, BALF 1:10, anti-bovine IgM 1:1000, peroxidase-conjugated anti-mouse IgG 1:50,000.
c) Anti-IgA assay: antigen 1.5 μg ml^{-1}, BALF 1:20, anti-bovine IgA 1:500, peroxidase-conjugated anti-mouse IgG 1:25,000.
d) Anti-IgG1 assay: antigen 1.5 μg ml^{-1}, BALF 1:10, anti-bovine IgG1 1:160, peroxidase-conjugated anti-mouse IgG 1:25,000.
e) Anti-IgG2 assay: antigen 1.5 μg ml^{-1}, BALF undiluted, anti-bovine IgG2 1:160, peroxidase-conjugated anti-mouse IgG 1:25,000.

Results

The antibody profiles in BALF are illustrated in Figure 4.5. Each graph shows the mean results for Group A compared with Group B calves for a particular isotype/subclass. Complete time courses for all calves for each particular isotype/subclass were analysed in a single experiment on the same day. For each sampling time, the mean ODs for the Group A calves were compared with the means for the Group B calves using a two-tailed t test. Significant differences are indicated in the graphs.

Total IgG levels fluctuated over the course of the experiment, increasing by Day 19 pi and decreasing to pre-infection levels by Day 26. Following the secondary infection, there was an increase in IgG from Day 10. On Day 10 after the tertiary infection there was a substantial increase ($p \leq 0.1$) in BALF IgG in all the calves in Group A which then

decreased again by Day 21 pi. Local IgM responses remained low in all calves, with very small increases after the primary and secondary infections and a further increase after the tertiary infection. IgA levels in BALF increased after each infection, with the most pronounced increase ($p \leq 0.01$) after the tertiary infection. The IgG1 subclass responses reflected the total IgG response in all the calves, with increases on Day 10 after the secondary infection ($p \leq 0.1$) and Day 10 after the tertiary infection ($p \leq 0.05$).

The IgG2a(A1) responses in BALF varied substantially between individual calves, so these results are not presented as means of the data. This variation was related to peak larval counts in the faeces. IgG2a(A1) responses after the primary and secondary infections were low in all calves, however, on Day 10 after the tertiary infection, the IgG2a(A1) response in BALF from Calf 174 which had a peak of 19 L1 g^{-1}, was significantly higher than the levels in Calf 165 excreting only 3.7 L1 g^{-1} (ODs of 0.366 and O.067 respectively) (Figure 4.6).

There were no increases observed in the levels of antibody in BALF for Group B calves until Day 10 (IgA) or Day 20 (IgG, IgM and IgG1) (Figure 4.5). The levels of IgG1 and IgM antibodies were higher than the levels observed in Group A animals after the primary infection. Group B calves had higher levels of larval excretion (Table 3.1) so it may be that a larger lung worm burden accounted for the higher IgG1 and IgM responses in the BALF of these animals.

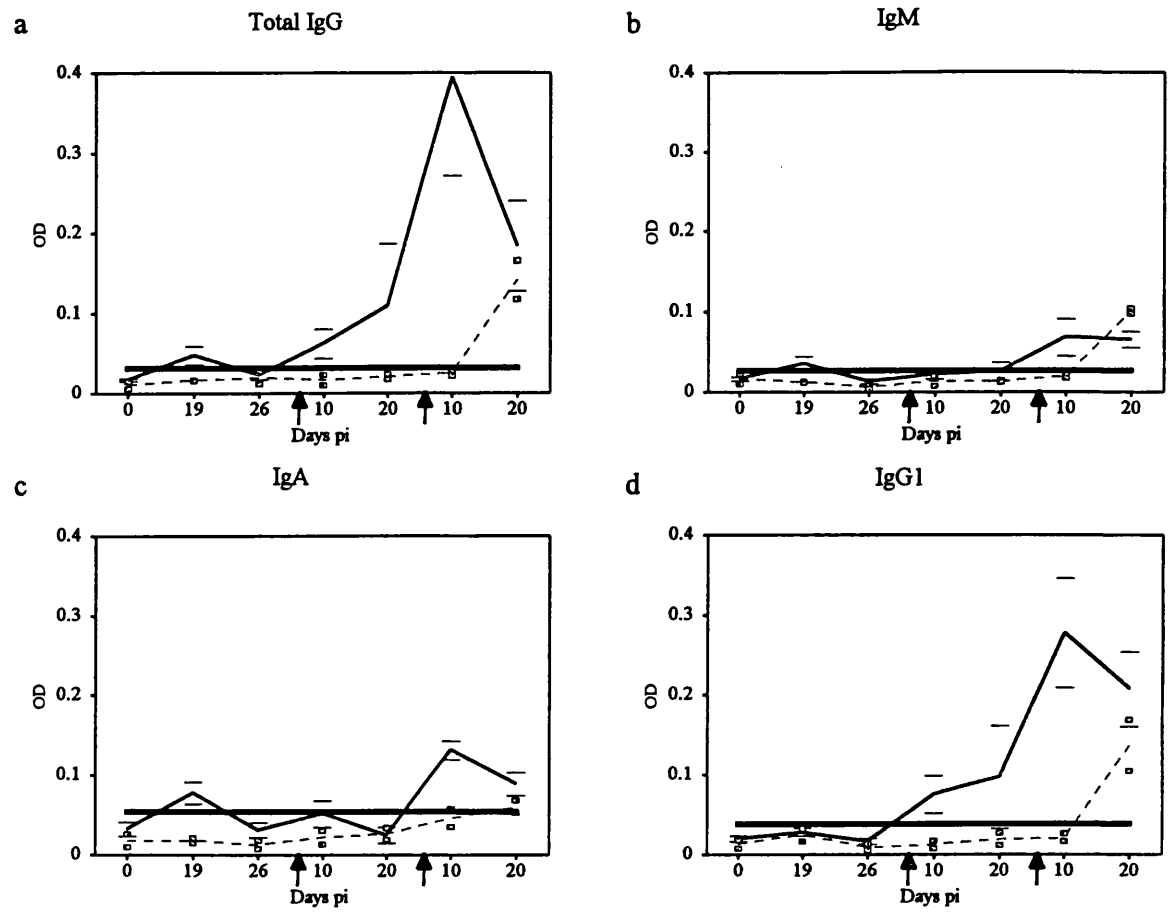

Figure 4.5 Mean ODs for Group A and Group B calves for adult homogenate-specific antibodies in BALF

BALF was collected pre-infection, on Days 19 and 26 after the primary infection and Days 10 and 20 after the secondary and tertiary infections. The arrows indicate the days that infections were administered. Antibody levels were measured by ELISA on BALF samples which had been concentrated to approximately 12% of original volume. Results are presented as mean ODs for Group A (___) compared with Group B (.....) calves. The horizontal lines indicate threshold which was calculated from the mean plus two SDs of ODs of BALF samples from 17 uninfected calves. * : indicates that the differences between Group A and Group B were statistically significant, using a t test. The number following indicates the level of significance.

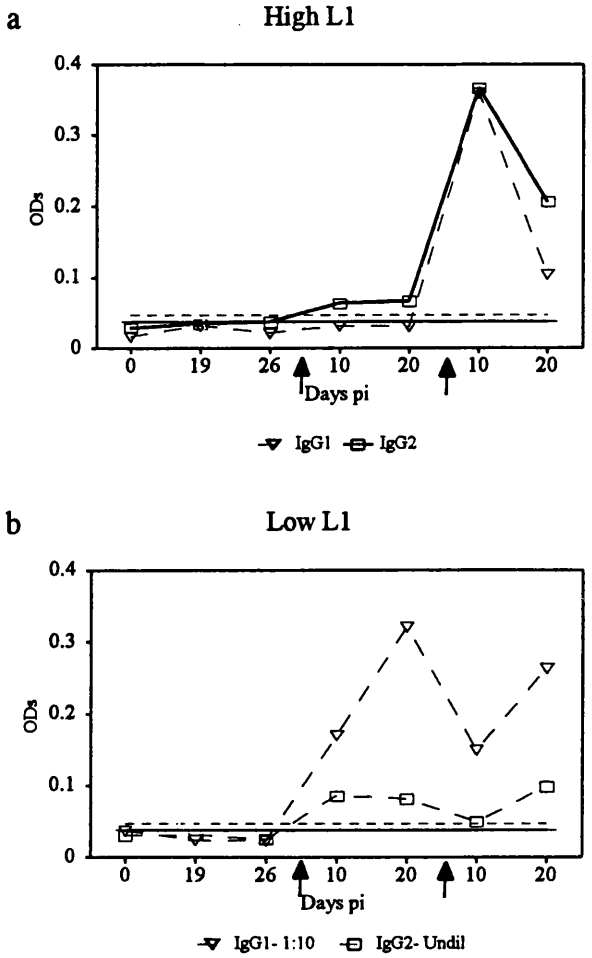

Figure 4.6 IgG2a(A1) in BALF of calf excreting high or low numbers of L1.

BALF was collected pre-infection, on Days 19 and 26 after the primary infection and Days 10 and 20 after the secondary and tertiary infections. Antibody levels were measured by ELISA on BALF samples which had been concentrated to approximately 12% of original volume. Results are presented as ODs. The horizontal lines indicate threshold ODs for IgG1 (___) and IgG2a(A1) (.....). The thresholds were calculated from the mean plus two SDs of ODs of BALF samples from 17 uninfected calves. a) IgG1 and IgG2a(A1) in a calf with a peak L1 excretion of 19 L1 g^{-1}. b) IgG1 and IgG2a(A1) in a calf with a peak L1 excretion of 3.7 L1 g^{-1}. The arrows indicate the days that infections were given.

4.2.1.4 Measuring total parasite-specific IgG2

Outline

The majority of both polyclonal and monoclonal anti-bovine IgG2 reagents are allotype-biased, with more than 80% of reagents specifically detecting IgG2a(A1), rather than IgG2a(A2) (Butler *et al.*, 1994). The allotype bias of the anti-IgG2 reagent used in these studies was kindly tested by Dr. K. Kenny, Key Laboratories, and was shown to be IgG2a(A1)-biased. Dr. Kenny kindly provided a non-biased Mab (Mab 8H6) and this was used to measure total IgG2 levels in the Group A calves.

Results

The results, illustrated in Figure 4.7 show the levels of total IgG2 in Group A calves. IgG2 increased from Day 28 pi and continued to rise after the secondary infection to peak around Day 33 in all calves except Calf 165. The levels of total IgG2 in serum from Calf 165 increased until Day 3-0. There were no increases in total IgG2 after the tertiary infection in any calf. Contrary to the findings for the IgG2a(A1) allotype responses, the pattern of responses for total IgG2 were very similar amongst the calves.

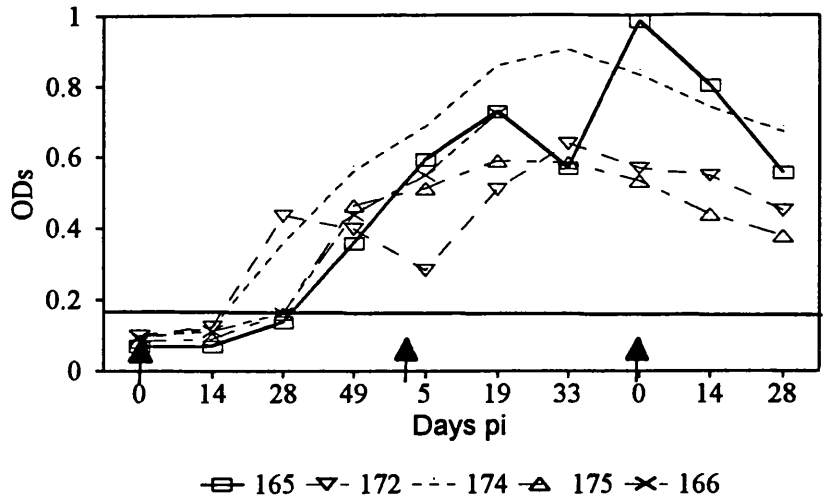

Figure 4.7 Total IgG2 levels in serum from Group A calves.

Levels of total IgG2a were measured in serum samples collected fortnightly from Group A calves. The arrows indicate the days when the infections were given. ELISA plates were coated with adult antigen @ 2.5 μg ml^{-1} . Serum was @ 1:100, Mab 8H6 @ 1:20, anti-mouse peroxidase conjugate @ 1:25,000. The results are expressed in ODs. The horizontal line indicates the threshold which was calculated from the mean plus three SDs of the ODs of 10 uninfected control calves.

4.2.1.5 Antibody responses to different life cycle stages of *D. viviparus*

To establish whether particular antibody isotypes/subclasses preferentially recognised different life cycle stages of *D. viviparus*, levels of the various isotypes/subclasses were analysed to L3 and egg/L1 stages. The optimum dilutions for antigen, serum/BALF and conjugates were determined using checkerboard titrations. The conditions used are summarised in Appendix 3. Figure 4.8 shows the mean serum antibody levels for the Group A calves to adult, L3 and egg/L1 antigens and Figure 4.9, the mean BALF antibody levels. Essentially, the kinetics of antibody responses in both serum and BALF samples to the L3 and the egg/L1 antigens were very similar to those observed against the adult antigen preparation. The OD readings obtained for L3-specific IgM in BALF were similar to those for L3-specific IgG. In contrast, much lower levels of IgM were obtained for adult- and Egg/L1 antigens, compared with IgG.

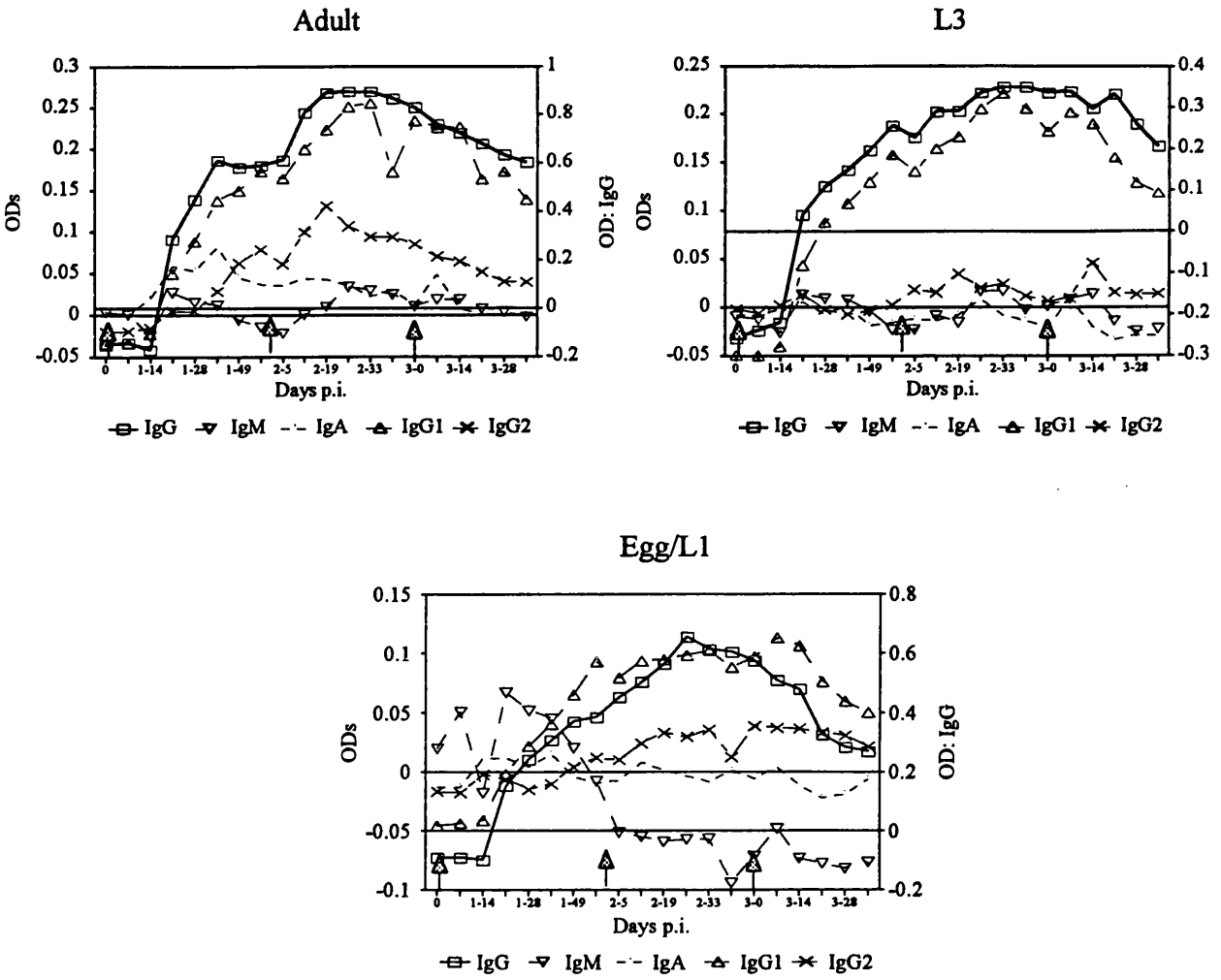

Figure 4.8 Serum antibody responses to different life cycle stages

Levels of serum antibodies to an adult, L3 and L1/egg homogenate were measured in Group A calves over three infections. The results show mean ODs for duplicate samples minus the threshold OD. The threshold was two standard deviations above the mean OD of 20 sera samples from uninfected calves. The graphs show the mean ODs for five Group A calves, minus the threshold OD. The arrows indicate the days the infections were given. 1-, 2-,3- indicate the days after the primary, secondary and tertiary infections respectively.

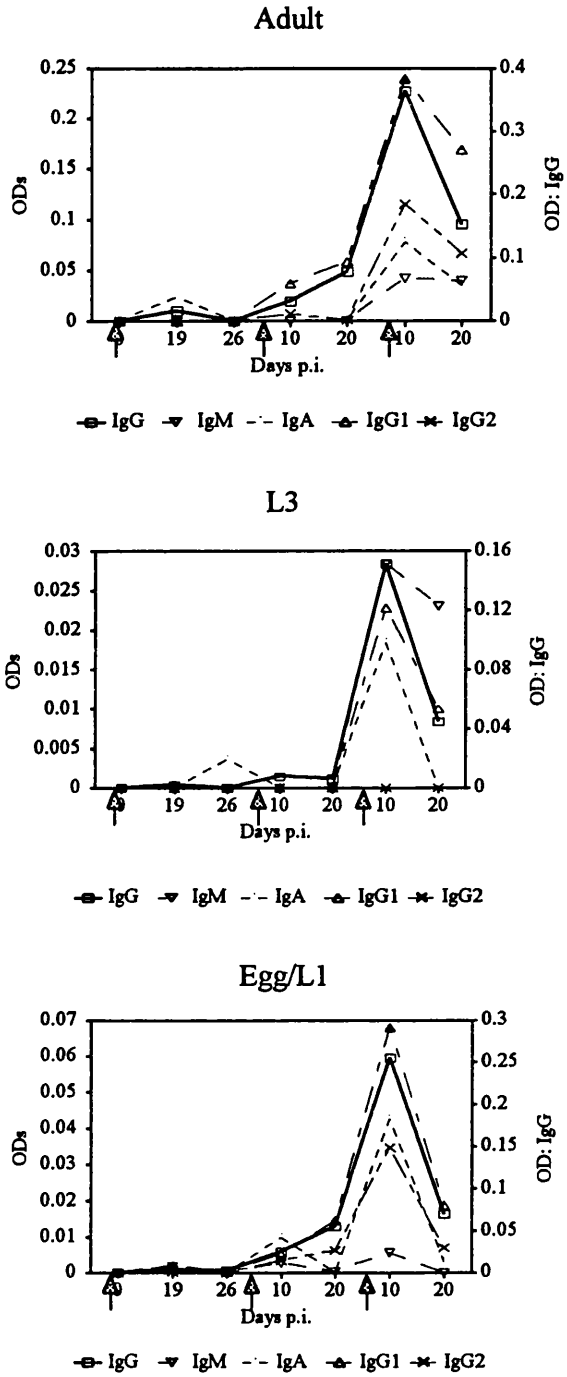

Figure 4.9 BALF antibody responses to different life cycle stages

Levels of BALF antibodies to an adult, L3 and L1/egg homogenate in Group A calves over three infections. The results are expressed as ODs, and the graphs show the mean ODs for five Group A calves. The threshold ODs calculated as from the mean plus two SDs of ODs of BALF samples from 17 uninfected calves were: IgG, 0.031; IgM, 0.026; IgA, 0.054; IgG1, 0.038; IgG2, 0.047. The arrows indicate the days the infections were given.

4.2.2 IgE antibody responses

There are no commercially available monoclonal or polyclonal antibodies to bovine IgE. Recently, two reagents have been made available and these were used to estimate levels of parasite-specific IgE in the serum and BALF samples. A Mab to an enriched and affinity-purified globulin fraction of high titre IgE-containing sera was kindly provided by Dr. L. Gershwin, University of California (Thatcher and Gershwin, 1988). A polyclonal rabbit antisera against a recombinant protein, corresponding to nucleotides 1111 to 1575 of ovine IgE, was kindly provided by Dr. H.D.F.H. Schallig, University of Utrecht. This antisera is raised against approximately two thirds of the Cε3 and Cε4 chains (Engwerda *et al.*, 1992).

In preliminary experiments, it was observed that the anti-IgE Mab bound to *D. viviparus* adult homogenate in a non-specific manner. Similar results were previously reported with *O. ostertagi* adult homogenate, but not with an homogenate prepared from the L3 of *O. ostertagi* (L. Gershwin, personal communication). Therefore, the reactivity of the Mab with antigen derived from other life cycle stages of *D. viviparus* was examined. The Mab bound non-specifically to L1/egg and adult E/S antigens, but not to the L3 antigens (data not shown). Consequently, L3 antigens were used in all further ELISAs to measure parasite-specific IgE.

4.2.2.1 Competitive binding by non-IgE isotypes

Competitive binding between bovine IgE and other isotypes has been observed in calves infected with bovine respiratory syncytial virus (BRSV) (Stewart and Gershwin, 1989). Protein G (Peng *et al.*, 1994) and saturated ammonium sulphate (SAS) (Haba and Nisonoff, 1985) can be used to preferentially precipitate non-IgE antibody isotypes. In this study, a comparison was made between the ability of Protein G and SAS to preferentially bind parasite-specific IgG antibodies in serum from the *D. viviparus* infected calves. Then, IgE levels were compared in untreated serum and in IgG depleted serum and BALF samples. Increasing concentrations (20 to 640 μg ml^{-1}) of Protein G or 27.5% SAS were incubated with serum (diluted 1:20) or BALF (undiluted) and rotated for 50 min at room temperature. The Protein G samples were then centrifuged at 2000 g for 5 min and the SAS samples at

13,000 g for 10 min, both at room temperature, and the supernatants used as samples in ELISAs. These reagents successfully depleted IgG but had no effect on parasite-specific IgE levels (data not shown). In all future experiments to measure IgE, untreated serum and BALF were used.

4.2.2.2 Levels of parasite-specific IgE measured in serum and BALF using a monoclonal antibody to bovine IgE.

Outline

Using checkerboard titration, the following optimal dilutions were chosen for the anti-IgE ELISA using a Mab to bovine IgE:

a) Anti-IgE assay in serum: L3 homogenate at a concentration of 10 $\mu g\ ml^{-1}$, serum at 1:80 dilution, mouse anti-bovine IgE at 1:750 and peroxidase-conjugated goat anti-mouse IgG at 1:40,000.

b) Anti-IgE assay in BALF: L3 homogenate at 2.5 $\mu g\ ml^{-1}$, BALF at 1:5, mouse anti-bovine IgE at 1:750 and peroxidase-conjugated goat anti-mouse IgG at 1:20,000.

Results

Significant increases in parasite-specific IgE were not detected in the serum of calves after primary, secondary or tertiary infection with *D. viviparus* (Figure 4.10 a and b). However, there were increases in parasite-specific IgE in BALF samples (Figure 4.10 c and d). There were large increases in BALF IgE for Calf 165 on Day 20 after the secondary and tertiary infections, these increases corresponded to increases in other antibody isotypes in this calf (data not shown). There was a significant increase in BALF IgE for Calf 175 on Day 10 after the tertiary infection, this also corresponded to increases in the other antibody isotypes in this calf (data not shown). There were increases in BALF IgE after primary infection in the three Group B calves.

In the mouse, both IgG1 and IgE are regulated by IL-4 (Kopf *et al.*, 1993), and so it was of interest to compare the kinetics of these isotype responses. The patterns of the IgE and IgG1 responses in BALF were similar for all the calves (Figure 4.11).

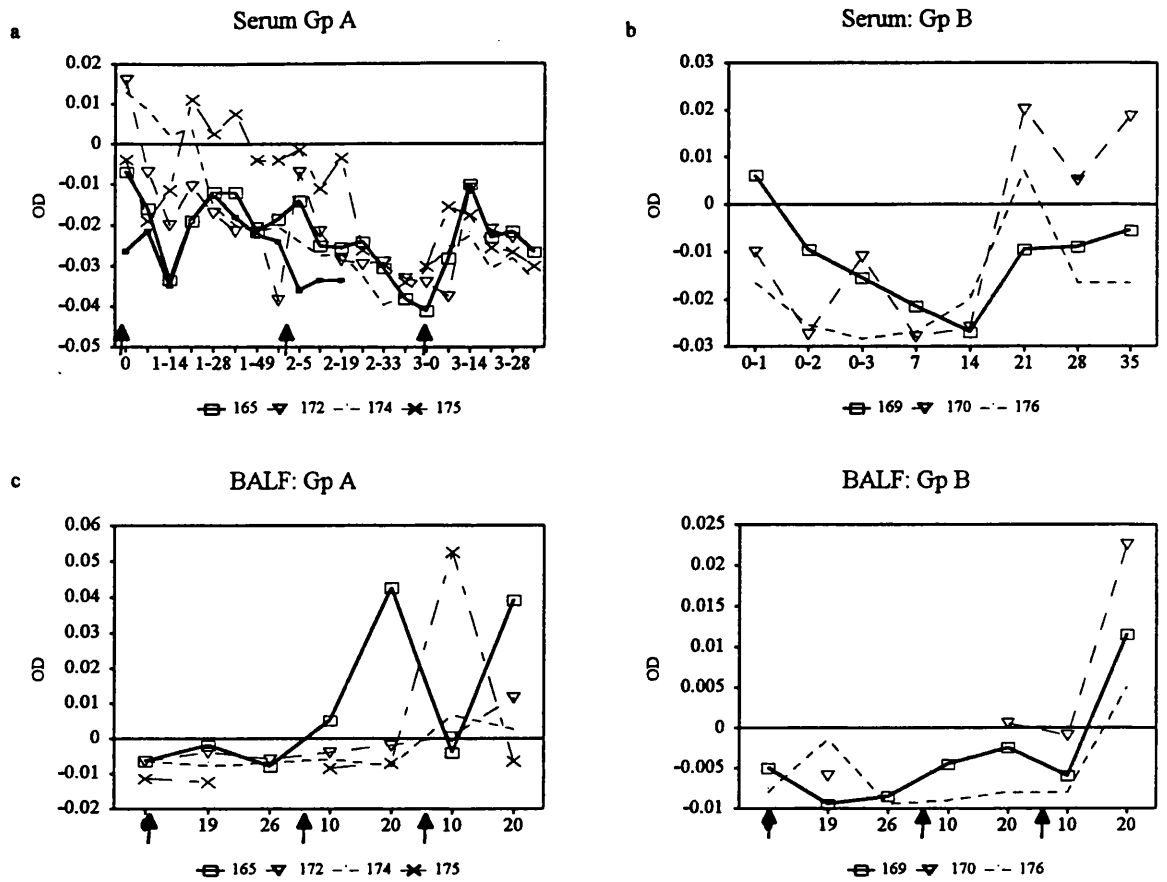

Figure 4.10 IgE in serum and BALF, measured using a Mab to bovine IgE

Serum was collected pre-infection and weekly after infection. BALF was collected pre-infection, on Days 19 and 26 after the primary infection and on Days 10 and 20 after the secondary and tertiary infections. Levels of L3-specific IgE were measured using an ELISA (see text for conditions). Arrows indicate the days that infections were given. Results shown are mean ODs of duplicates minus the threshold, calculated from the mean plus two SDs of 20 control sera or 17 pre-infection BALF samples.

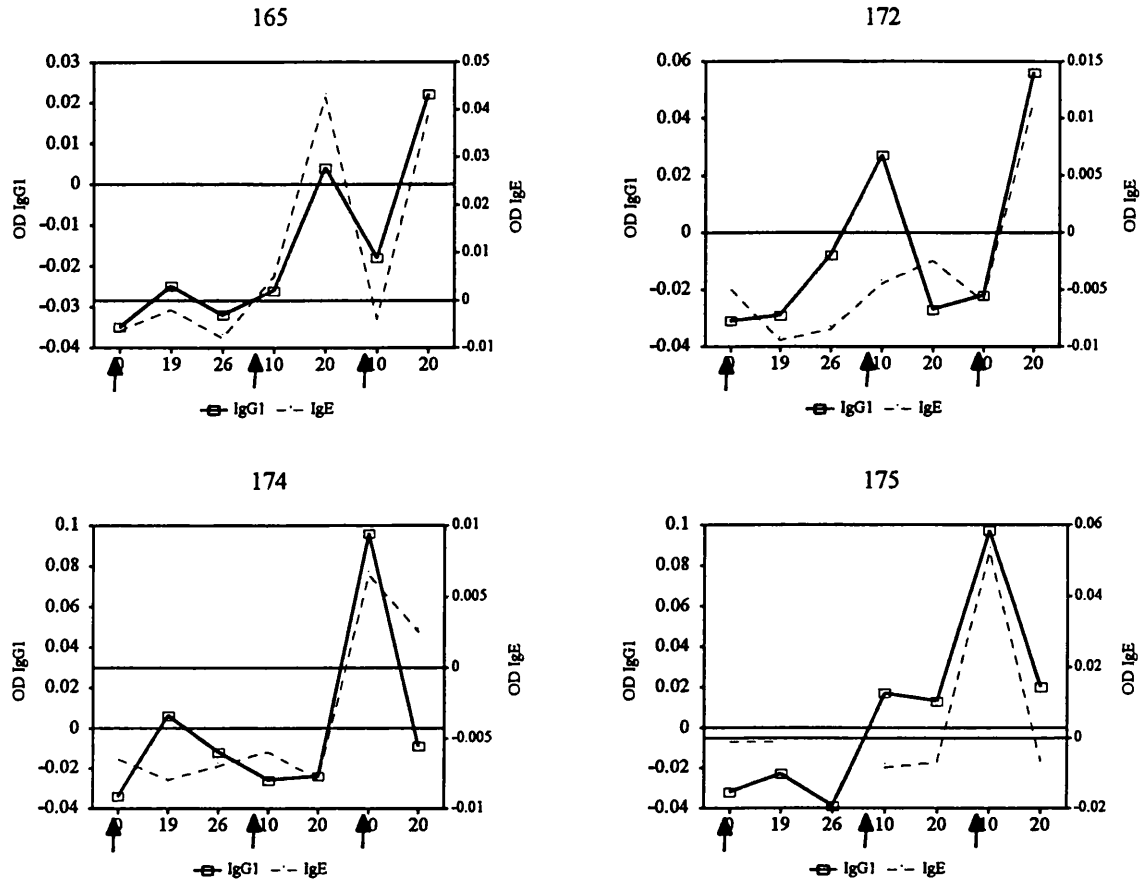

Figure 4.11 Comparison of the kinetics of IgG1 and IgE responses in BALF

BALF was collected pre-infection, on Days 19 and 26 after the primary infection and on Days 10 and 20 after the secondary and tertiary infections. Levels of L3-specific IgE and IgG1 were measured using an ELISA (see text for conditions). Arrows indicate the days that infections were given. Results shown are mean ODs of duplicates minus the threshold, calculated from the mean plus two SDs of 17 pre-infection BALF samples. The lines indicate when the OD for IgE (right axis) was 0.

4.2.2.3 Levels of parasite-specific IgE measured in serum using a rabbit polyclonal antibody to a recombinant protein from ovine IgE.

Levels of parasite-specific IgE were measured in the serum of Group A calves using the polyclonal antibody to ovine IgE. IgE was not measured in BALF using this reagent as the batch of polyclonal antibody ran out and two further batches were unsuitable for ELISA. After checkerboard titrations, the optimal dilutions chosen for ELISA were: *D. viviparus* L3 antigen at 2.5 $\mu g\ ml^{-1}$, serum at 1:40 dilution, rabbit anti-ovine IgE at 1:250 and alkaline phosphate-conjugated anti-rabbit IgG at 1:30,000.

L3-specific IgE was detected in the serum of four out of five Group A calves using this reagent. Increases above threshold were detected from Day 35 after primary to Day 7 after secondary infection (Day 2-7). IgE levels peaked at Days 2-26 to 2-33 and remained high until Day 3-14 (Figure 4.12). There were no increases in parasite-specific IgE above threshold values in serum from Calf 165, whereas, L3-specific IgE was detected in the BALF from this calf using the Mab reagent (Figure 4.9). The pattern of IgE and IgG1 responses in serum were similar for all calves (Figure 4.13) although IgE antibodies increased later than IgG1 and this increase was more gradual.

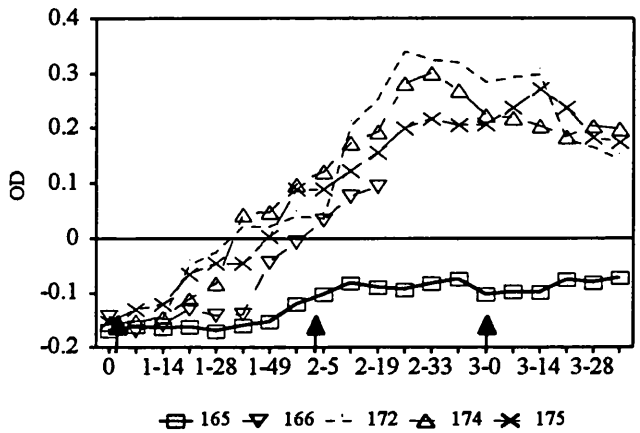

Figure 4.12 ***D. viviparus*-specific IgE in serum, measured using the polyclonal antibody**

Serum was collected pre-infection, and weekly after infection. Levels of L3-specific IgE were measured in an ELISA using a rabbit anti-ovine IgE (see text for conditions). Arrows indicate the days that infections were given. Results shown are mean ODs of duplicates minus the threshold, calculated from the mean plus two SDs of 20 control sera samples. The lines indicate when the OD for IgE was 0.

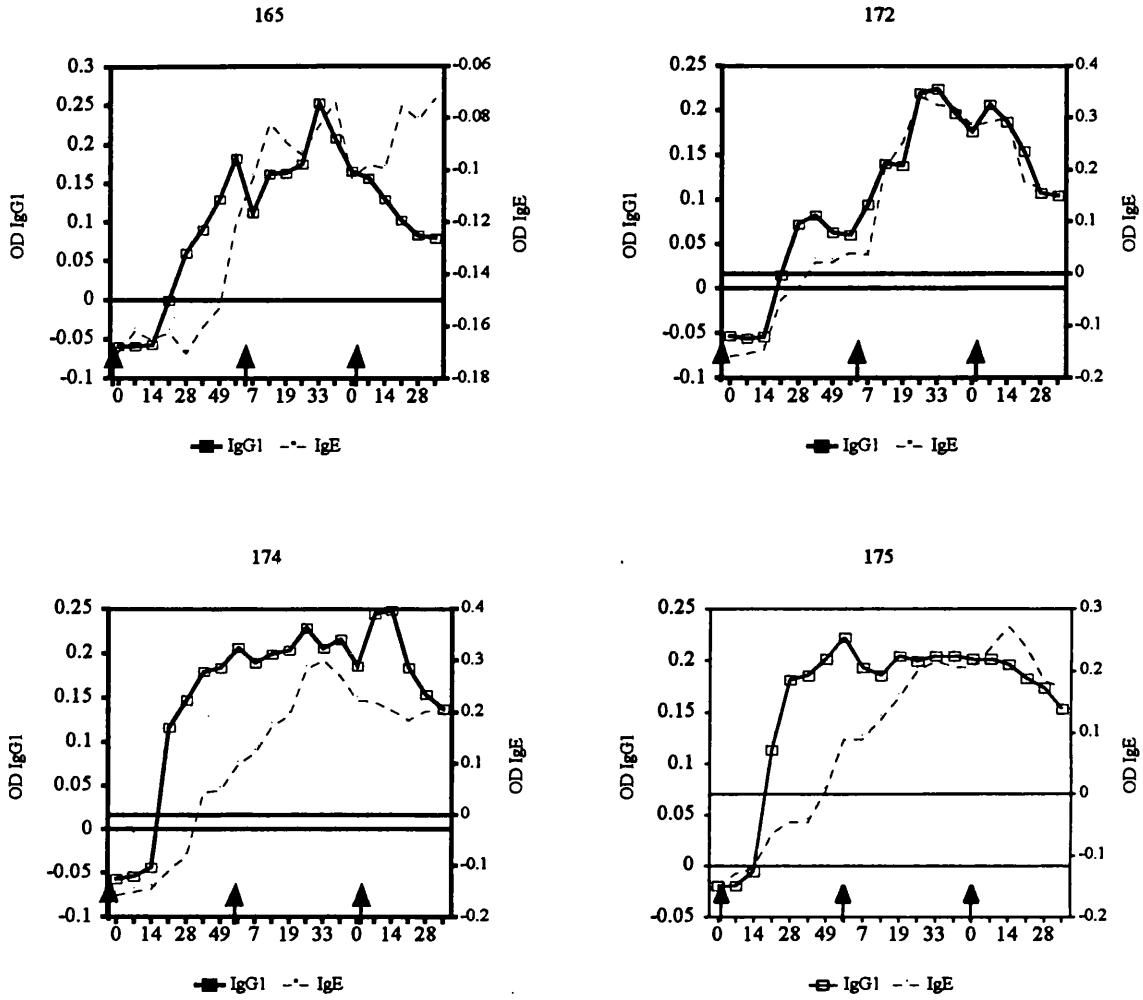

Figure 4.13 Comparison of the kinetics of IgG1 and IgE responses in serum

Serum samples were collected pre-infection, and weekly after infection. Levels of L3-specific IgE were measured using an ELISA (see text for conditions). Arrows indicate the days that infections were given. Results shown are mean ODs of duplicates minus the threshold, calculated from the mean plus two SDs of 20 control sera samples.

4.2.2.4 Attempts to measure total IgE

Various attempts were made to measure total non-parasite-specific IgE, using the Mab, polyclonal Ab or combinations of the two in double sandwich ELISAs. It was not possible to measure total IgE using any of these methods.

4.3 Discussion

Initial studies investigating antigen-specific antibody responses to *D. viviparus* used complement fixation and indirect haemagluttination techniques. These early studies detected increased levels of antigen-specific whole IgG in serum from Day 30 (Jarrett *et al.*, 1959b) or Day 80 (Cornwell and Michel, 1960) pi. More recently, the ELISA has allowed detection of whole IgG antibodies from Day 21 pi (Bos *et al.*, 1986), reflecting the increased sensitivity of ELISA over the earlier techniques. In this study, the kinetics of the IgG antibody response after a primary infection were similar to the previous study using the ELISA (Bos *et al.*, 1986). For example, IgG antibody was detected from Day 21 pi, with peak levels detected around Day 35, followed by a gradual decline. A secondary antibody response was observed in the present study when the calves were reinfected 63 days after the primary infection. Similar results were obtained in some previous studies (Jarrett *et al.*, 1959b), while in other studies, secondary responses were not seen upon re-exposure (Cornwell and Michel, 1960). This discrepancy probably relates to the timing of the secondary infection.

In the study presented here, there was no increase in antibody levels in any of the calves after tertiary infection, even though this infection was administered when the levels of antibody were lower than, or similar to, those observed at secondary infection. The fact that there were no increases in IgG in immune animals suggests that specific IgG responses may be associated with the presence of adult parasites in the lung. This observation is substantiated by the study of Jarrett *et al.* (1957a). In that experiment, calves were infected three times at six monthly intervals and increased IgG was detected after each infection. However, some of these calves continued to excrete larvae after the secondary and tertiary infections, indicating the presence of adult parasites in the lungs. Further evidence that

adult parasites are required to stimulate high levels of parasite-specific IgG, comes from studies in calves vaccinated with 400 Gy irradiated L3. Adult parasites did not develop in these calves and they had low levels of *D. viviparus*-specific IgG (Cornwell, 1962b, Bos *et al.*, 1986).

The early peak in IgM observed here after primary infection, followed by a rapid decline, probably reflects an initial expression of IgM on B cells which switches, after specific activation, to the expression of other isotypes (Snapper and Mond, 1993). An early peak in IgM antibodies to the adult and the L3 surface has been observed in previous studies (Canto *et al.*, 1992; McKeand *et al.*, 1996).

Serum IgA levels increased at very early time points (Day 7 pi in some calves) compared with the other isotypes. IgA is an important immunoglobulin at mucosal sites (Husband, 1987) and the rapid rise in this isotype may be a consequence of the migratory route of the larvae through the intestinal tract. The levels of serum IgA increased after all three infections, whereas there was no increase in serum IgG after the tertiary infection. As discussed above, the major inducer of IgG production appears to be the adult parasites in the lung. One possible explanation for increases in serum IgA after the tertiary infection is that larval antigens are more potent inducers of IgA. Similarly, increases in L3 E/S-specific, but not adult E/S-specific, serum IgA were demonstrated in sheep after infection with *H. contortus* (Schallig *et al.*, 1994).

The kinetics of the local antibody responses, measured in BALF, were distinct from the serum antibody responses. The most dramatic increases in total IgG in BALF were seen after the tertiary infection whereas, in the serum, total IgG gradually decreased after the tertiary infection. In pigs immunised with *Actinobacillus pleuropneumoniae,* local antibodies in BALF were elevated in the absence of a rise in serum antibodies (Hensel *et al.*, 1994). Similarly, there was a predominance of IgA in BALF, over that of serum, in calves infected with bovine coronavirus (Heckert *et al.*, 1991) and BRSV (Kinman *et al.*, 1987). By the tertiary infection, there were no increases in the respiratory rates of the calves (see Chapter 3), suggesting that larvae did not penetrate the lungs in large numbers. Therefore, the parasites may have been killed immediately upon reaching the lungs. Certainly, the large increase in antibody levels in the lungs suggested that the local antibody response was involved in protective immunity. However, immune mechanisms at the various mucosal sites interact through the common mucosal immune system. For example, oral immunisation of pigs with *A. pleuropneumoniae* increased the levels of IgA and IgG in

BALF (Delventhal *et al.*, 1992) and oral immunisation protected against the respiratory pathogen *Pseudomonas aeruginosa* in rats (Cripps *et al.*, 1994). Antigen-specific T cells have also been shown to migrate from immunised gut to lung (reviewed by Dunkley *et al.*, 1995). Therefore, it is likely that there are increases in antibody levels and parasite-specific T cells at all mucosal sites and it would be worthwhile, in future studies, to look at levels of intestinal or faecal antibodies in challenged calves. With this in mind, the tertiary infection may have been terminated in the gut or lower in the lungs.

There was a trend for higher IgG2a(A1) production in serum and BALF in calves excreting higher numbers of L1 in their faeces. However, using a non-biased monoclonal antibody there were no differences demonstrated in total IgG2 levels between individual calves. Therefore, this study has shown a correlation between *D. viviparus* L1 excretion and levels of IgG2a(A1), rather than total IgG2. The A1 and A2 allotypes of IgG2a are structurally different, especially near the hinge region, and they may also have functional differences (Kacskovics *et al.*, 1995). Previous studies with other infectious agents have indicated that the structural differences in the A1 and A2 allotypes may reflect functional differences. For example, *Brucella*-resistant and -sensitive herds were reported to differ in IgG2 allotype expression (Estes *et al.*, 1990), while *Haemophilus somnus* was preferentially recognised by IgG2a of the A2 allotype (Yarnall *et al.*, 1988). A correlation between IgG2a(A1) levels and larval excretion may be relevant, as *in-vitro* studies, using bovine B cells, have shown that IFNγ stimulated production of IgG2, and that IL-2 augmented this response (Estes *et al.*, 1994). Since previous studies have shown that the majority of both polyclonal and monoclonal anti-IgG2 reagents were IgG2a(A1) biased (Butler *et al.*, 1994), the IgG2 measured in the study by Estes *et al.* (1994) was most likely to be IgG2a(A1). This is similar to findings in mice where IFNγ induces IgG2a (Snapper and Paul, 1987). In rodents, IFNγ and IL-2 have been shown to be important in Th1 subset responses (Mosmann and Coffman, 1989). Moreover, T cell clones with similar cytokine profiles to murine Th1 and Th2 subsets have been isolated from cattle infected with *B. bovis* (Brown *et al.*, 1993b) and *F. hepatica* (Brown *et al.*, 1994b), respectively. However, it is unclear if these *in vitro* derived clones truly reflect the *in vivo* situation and whether Th1- and Th2-like subsets exist in cattle.

When responses to other parasite stages were examined (egg/L1, L3), there were no differences in the kinetics of the antibody responses for any of the isotypes/subclasses

examined. It is likely that different parasite stages share common antigens, so extracts of whole parasites will include a number of cross-reactive antigens. Studies using immunofluorescence found that antigens on the surface of various stages of *D. viviparus* were stage-specific (Cánto *et al.*, 1992; Britton *et al.*, 1993b; McKeand *et al.*, 1996) and it may be worthwhile to examine isotype-specific responses to surface antigens over a time course.

Competition between IgE and other antibody isotypes has been demonstrated in previous studies (Stewart and Gershwin, 1989; Ramaswamy and Befus, 1993). However, in this study, there were no changes in the levels of parasite-specific IgE measured in serum or BALF samples after depletion of IgG. Using a Mab against serum-derived bovine IgE, no significant increases in parasite-specific IgE were detected in the serum of calves infected with *D. viviparus*. In contrast, using a polyclonal antibody to a recombinant sheep protein corresponding to a portion of the Cε3 and Cε4 chains, increases in parasite-specific IgE were detected. It is unclear why this paradox exists. The Mab has been successfully used to detect IgE in cattle in other studies, i.e. in bovine respiratory syncytial virus infections (Stewart and Gershwin, 1989) and in light, but not moderate, infections of *O. ostertagi* (Thatcher *et al.*, 1989; Baker and Gershwin, 1993). It may be that the Mab detected only certain epitopes on IgE, which were not available for binding in the serum of infected calves. For example, the low affinity IgE receptor expressed on B cells, eosinophils and monocytes (FceR11) gives rise to soluble fragments by proteolysis which retain binding specificity for IgE (Sutton and Gould, 1993). One possibility is that the Mab recognises either free or FceR11-complexed IgE. Alternatively, if the Mab binds to the Fab region of the IgE, it may compete with the parasite antigen for binding. If one Fab chain was pre-bound to antigen and the other chain to antigen on the ELISA plate, the Mab would be unable to bind to the IgE.

While the Mab was unable to detect IgE in serum, it could detect IgE in BALF. It is likely that the levels of parasite-specific IgE were much higher in BALF than in serum and perhaps there was not enough IgE in serum to be detected using the Mab. Alternatively, the structure of IgE in BALF and serum may differ. Halliwell *et al.* (1993) demonstrated increased antigen-specific IgE in BALF of horses in the absence of increases in serum IgE. Large numbers of mast cells have been reported in the lungs of calves after a primary infection with *D. viviparus* (H.R.P. Miller, unpublished observation). A local increase in

IgE was also found in rats after infection with *N. brasiliensis* (Ramaswamy and Befus, 1993) and this infection is also characterised by pulmonary mastocytosis (Wells, 1971). Mast cells are activated when the high-affinity IgE receptor (FcεR1) is bound by IgE and specific antigen (Gordon *et al*, 1990) and once activated they secrete a wide range of inflammatory mediators. Eosinophils also bind IgE, via FceR1 (Gounni *et al.*, 1994), and large numbers of eosinophils are also found in the lungs of calves infected with *D. viviparus* (Jarrett *et al.*, 1960) and in BALF (Section 3.2.2).

Whatever the mechanism, the Mab was unable to detect *D. viviparus*-specific IgE in serum in this study. A Mab against recombinant Cε3 and Cε4 chains of sheep IgE, has recently been produced by Dr. H.D.F.H. Schallig, University of Utrecht. This has been used successfully in immunohistochemical analysis of IgE secreting cells in the ovine gastrointestinal tract (J. Huntley, personal communication). Two Mabs have been made by immunising mice with purified ovine mast cells as a source of IgE (Shaw *et al.*, submitted). It would interesting to use these new Mab to try to measure IgE in bovine serum.

The kinetics of both the local and the serum IgE and IgG1 antibody responses were very similar. In the mouse, both IgE and IgG1 are regulated by the Th2 cytokine IL-4 (Kopf *et al.*, 1993). In the bovine, it is not known what controls IgG1 and IgE and whether they are likely to be induced by the same cytokine. It was therefore interesting to note that these isotypes had similar kinetics. In BALF, but not in serum, calves with low IgE levels tended to have higher IgG2a(A1) levels. This may have been a coincidental finding, the statistical significance of which could not be assessed, as the numbers of observations were too few. However, in view of the fact that a positive correlation was demonstrated between IgG2 levels and L1 excretion, these findings may not be coincidental, if bovine IgG2 is regulated by Th1- like cells and IgE by Th2- like cells.

In conclusion, in fully immune animals there were significant increases in parasite-specific IgG1, IgE and IgA in BALF, suggesting that local antibody-mediated immune responses may play a protective role. A correlation was found between elevated IgG2a(A1) in serum and BALF and higher L1 secretion. Moreover, there was a negative correlation between IgE and IgG2a(A1) levels in BALF. These findings suggest that calves with a tendency to produce higher levels of IgG2a(A1), and lower levels of local IgE, were less able to clear infection. Whether or not protective immunity to *D. viviparus* is associated with a Th2 cytokine profile will be discussed in Chapter 6.

These separated populations were cultured *in vitro* in the presence of APC, with or without Con A or parasite antigen. The cells were separated using magnetic activated cell sorting (MACS), a method which is rapid and can result in highly purified populations of cells with yields of >90% purity (Semple *et al.*, 1993). Moreover, MACS separation did not cause any significant activation of cells (Semple *et al.*, 1993) and subsequent cell culture showed preservation of function of both positively and negatively selected cells (Stanciu *et al.*, 1996).

Together, the experiments detailed in this Chapter should provide some insight as to the role of T cell subpopulations in immunity to *D. viviparus*.

Results

5.2 T cell Proliferative responses

5.2.1 Optimisation of proliferation assays

Proliferation assays were optimised initially using material collected from *D. viviparus* infected calves which were not those in Groups A and B discussed earlier. These other calves were the same age, breed and sex and were maintained in an identical manner to those in Groups A and B. Blood samples were collected pre-infection and weekly pi. Tracheo-bronchial LNs were collected at post-mortem, on Day 35 pi. The PBMC and LN-derived lymphocytes were isolated and cryopreserved (Section 2.8).

Proliferative responses were assessed in lymphocyte proliferation assays (Section 2.9). Proliferation was measured by the amount of ^{3}H thymidine incorporated into DNA, as counts per minute (cpm). The results were expressed as a stimulation index (SI), calculated by dividing the cpm for cells cultured with mitogen/antigen, divided by the cpm for cells cultured in medium alone. Expressing the proliferative responses as SI allows for variations in background between samples.

5.2.1.1 Con A

Con A was used as a polyclonal stimulant. Concentrations of Con A, from 40 to 0.5 μg ml^{-1}, were used to stimulate PBMC and LN-derived cells from infected and uninfected calves . The concentration of Con A which produced optimal proliferation was 2.5 μg ml^{-1}, with a reduced response at higher and lower concentrations (Figure 5.1). The optimum time for culture of the cells with Con A, before pulsing with ^{3}H thymidine, was 48 h for the PBMC and 72 h for LN-derived cells (not shown).

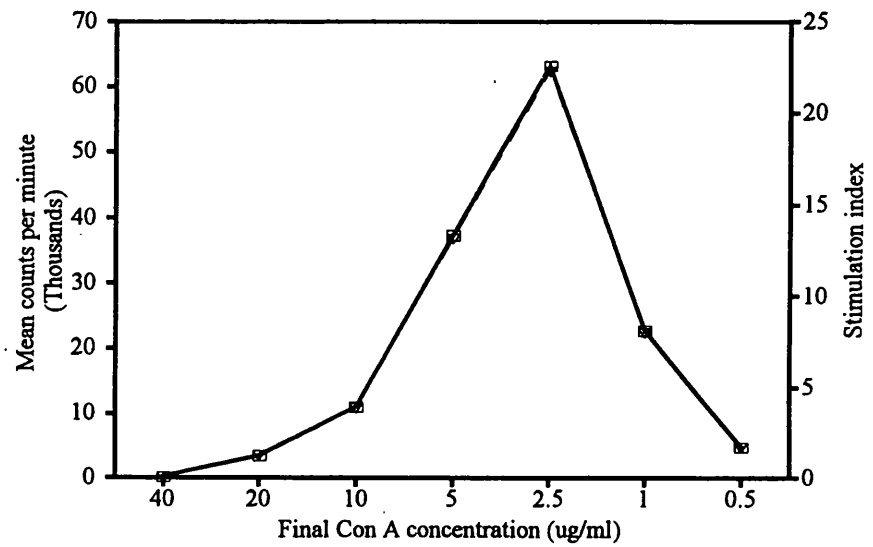

Figure 5.1 Optimisation of Con A concentration for proliferation assays

PBMC isolated from an uninfected calf were cultured in triplicate with or without Con A for 48 h, before pulsing with ^{3}H thymidine. The SI was calculated by dividing the mean cpm for cells cultured with Con A, by the mean for cells cultured in medium alone.

5.2.1.2 Antigen preparation

Investigation of non-specific proliferation

a) Elimination of low molecular weight mitogens

The first titration experiments were carried out using an homogenate of adult *D. viviparus* in PBS (Section 2.3.2). These experiments demonstrated extremely high levels of non-specific proliferation to this antigen preparation, with SI >100 demonstrated for both PBMC and LN-derived cells from uninfected calves. In an attempt to minimise this non-specific response, the parasite antigen was prepared using an alternative protocol recommended by other workers in the field (R. Grencis, personal communication). Briefly, the adult homogenate was ultracentrifuged at 100,000 x g for 30 min at 4°C; the ultracentrifugation step should result in the sedimentation of low molecular weight insoluble material. Antigen preparations, prepared with and without ultracentrifugation, were compared by SDS-polyacrylamide electrophoresis (PAGE). It was observed, however, that there was more variation in banding patterns between different batches of antigen, than between a preparation before and after ultracentrifugation (data not shown). Preliminary results showed that there were no significant differences between proliferative responses to antigen preparations before and after ultracentrifugation (data not shown).

To ensure that the antigen preparation did not contain low molecular weight mitogens which may non-specifically stimulate the cells, the ultracentrifuged antigen preparation was pooled and dialysed using tubing with a molecular weight cut-off of ~12,000 kDa. The preparation was dialysed for 16 h, at 4°C, in three changes each of 1 L PBS (Appendix 1.1) and then filtered through a 0.2 µm syringe filter (Nalgene). The quantity of protein was measured as described in Section 2.4. Following SDS-PAGE, no obvious differences were observed between preparations before and after dialysis. Moreover, there was still significant non-specific proliferation when the dialysed antigen was tested in assays (data not shown).

b) Comparing antigens from different life cycle stages

In an attempt to determine if the non-specific response was specific to the adult stage, experiments were carried out using *D. viviparus* antigens derived from a variety of life cycle

stages: ultracentrifuged homogenates of L3, L1/egg, adult and adult E/S products were tested. The responses were assessed using PBMC from infected and uninfected calves. PBMC were also used from a gnotobiotic calf, which had been born into, and maintained in, a pathogen-free environment at the Institute for Animal Health, Compton, UK. Without exception, these antigens preparations induced non-specific proliferation in cells from uninfected calves and the gnotobiotic calf (see Table 5.1 for an example of some of the results). Adult parasites were more readily available, so the adult homogenate was used in further experiments.

Mitogen	Infected	Uninfected	Gnoto
Con A	92.3	86.8	96.9
Adult	12.4	5.9	54.6
L3	14.4	10.0	17.8
L1/egg	17.4	8.2	23.2
E/S	26.0	45	34.5

Table 5.1 PBMC responses to antigens from different life cycle stages of *D. viviparus*

PBMC from an infected, uninfected and gnotobiotic calf were cultured, in triplicate, with or without 2.5 μg ml^{-1} Con A for 48 h, or 10 μg ml^{-1} of ultracentrifuged homogenates of adult, L3, L1/egg antigens or adult E/S antigens, for 72 h. The results are expressed as SI.

c) Investigation of whether antigen preparations contained endotoxins

To ensure that the dialysed and ultracentrifuged homogenate of adult *D. viviparus* (DvDUH) did not contain bacterial endotoxins, which may have acted non-specifically to stimulate cells (Staugas *et al.*, 1992), the parasite antigen was heated to 100°C for 10 min. This heating inhibited all proliferation (Table 5.2 shows examples of some results), suggesting that the molecules involved were proteins rather than lipopolysachharides (LPS) (Denkers *et al.*, 1994). In additional experiments, polymixin B, which binds to LPS and inhibits its activity (Morrison and Jacobs, 1976) was included in the culture medium. The polymixin B did not inhibit the non-specific proliferative responses (Table 5.2), confirming that this activity was not due to LPS (Staugas *et al.*, 1992; Denkers *et al.*, 1994).

Mitogen	Infected	Uninfected	Gnoto
Con A	51.0	47.0	67.0
DvDUH	71.0	35.0	48.0
Heat treated	0.9	0.4	0.1
with polymixin	65.0	78.0	35.0

Table 5.2 Investigation of endotoxin activity in DvDUH

PBMC from an infected, uninfected and gnotobiotic calf were cultured, in triplicate, with or without 2.5 μg ml^{-1} Con A for 48 h, or 10 μg ml^{-1} of DvDUH for 72 h. Heat treated: the DvDUH was heated to 100°C for 10 min. With Polymixin: Polymixin B was included in culture medium @ 5 μg ml^{-1}. The results are expressed as SI.

d) Examining the possibility that *D. viviparus* contains superantigen-like activity

A characteristic of classical superantigens, such as staphylococcal enterotoxin B, is that they do not require to be processed for efficient antigen presentation (Herman *et al.*, 1991). To determine whether the parasite homogenate behaved in a superantigen-like manner, the requirement of the antigen for processing was assessed. The ability of paraformaldehyde fixed PBMCs to act as APCs, was investigated. T cells were isolated from an infected calf and from the gnotobiotic calf, by passing PBMC through a nylon wool column. Briefly, a 10 ml syringe was filled to the 7 ml mark with scrubbed nylon wool and this was sterilised by autoclaving. The column was rinsed with 30 ml TCM/30% FCS, then incubated at 37°C for 90 min. A further 10 ml of medium was washed through, then 2 x 10^8 cells were added in a 2 ml volume, drop-wise, to the column. One ml of medium was added to wash the cells into the column, which was then incubated at 37°C for 60 min, to allow binding of adherent cell populations. After incubation, the non-adherent T cells were washed through using 20 ml medium.

Isolated T cells from an infected calf and the gnotobiotic calf were cultured with irradiated PBMC (2500 RAD, using a radioactive caesium source), or with PBMC which had been incubated in 1% paraformaldehyde in PBS for 10 min. Table 5.3 shows the results obtained, expressed as SI. Compared with the PBMC, the separated T cells proliferated better to Con A, without the need for APC. In fact the SI were higher without APC. Cells

from both the infected and the gnotobiotic calf proliferated in response to DvDUH in the presence of irradiated, but not paraformaldehyde-fixed APC. Therefore, the proliferation stimulated by DvDUH requires antigen processing.

	Gnotobiotic calf		Infected calf	
	Con A	**Antigen**	**Con A**	**Antigen**
PBMC	48.6	103.5	25.2	11.8
T cells only	66.3	1.2	106.3	2.2
Irrad APC/T cell 2:1	26.5	4.6	40.3	2.8
Irrad APC/T cell 4:1	28.4	18.1	43.0	3.0
Fixed APC only	1.1	1.0	0.8	1.3
Fixed APC/T cell 2:1	55.0	1.2	100.5	1.4
Fixed APC/T cell 4:1	63.2	1.4	100.6	1.2

Table 5.3 The requirement of APC for antigen processing

SI for PBMC and T cells after culture with Con A or DvDUH, in the presence or absence of irradiated or paraformaldehyde-fixed APC. T cells were isolated by passing PBMC, from a gnotobiotic calf and from a calf infected with *D. viviparus* over a nylon wool column. As APC, PBMC were irradiated to 2500 RAD, using a radioactive caesium source, or fixed, by incubating with paraformaldehyde. PBMC or T cells (2×10^6 ml^{-1}) were cultured, in triplicate, with 2.5 μg ml^{-1} Con A for 48 h, or 10 μg ml^{-1} DvDUH for 72 h.

e) Optimisation of antigen concentration

A very large quantity of ultracentrifuged and dialysed adult antigen was prepared, aliquoted and frozen at -70°C. These aliquots were used for all future experiments. The optimum concentration for this antigen preparation was 10 μg ml^{-1} (Figure 5.2) and the optimum time for proliferation was 5 d for LN-derived cells and 3 d for PBMC (not shown). Beyond these time points, the cells had proliferated optimally and begun to die.

As cell numbers would be limiting, half-area microtitre plates (maximum volume 100 µl) were assessed. Cells were stimulated with Con A @ 2.5 µg ml^{-1}. As is illustrated in Figure 5.3, similar results were found using standard and half-area plates.

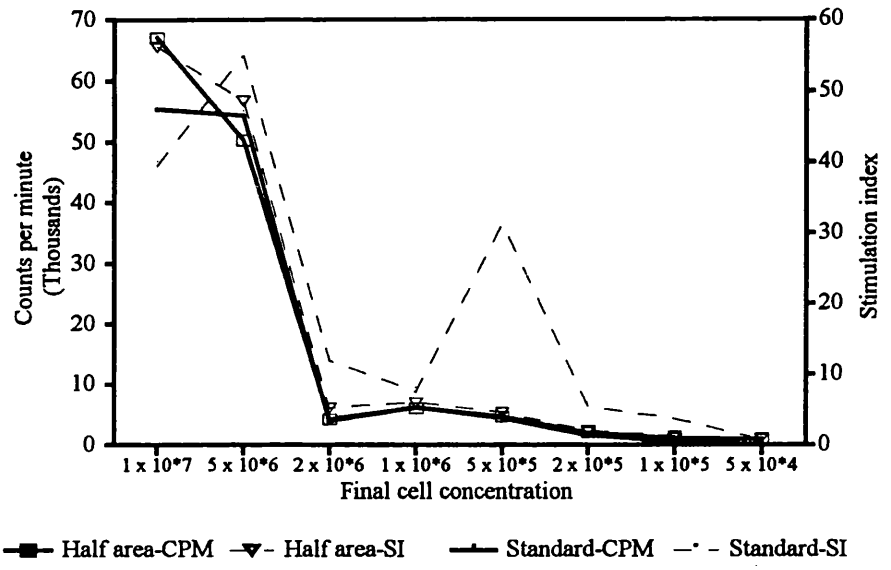

Figure 5.3 Comparing standard and half-area microtitre plates

Comparison of half-area microtitre plates (maximum volume, 100 µl) with normal microtitre plates (maximum volume, 200 µl). PBMC were cultured, in triplicate, with or without Con A @ 2.5 µl ml^{-1} for 48 h.

5.2.1.4 Responses after freezing cells

In time course studies, assays on cells taken at different time points from the same calf were analysed on the same day to avoid between-day bias. This meant that cells had to be cryopreserved in liquid nitrogen. After the cells were defrosted, the numbers of live and dead cells were counted, as described in Section 2.8.1. The percentage of dead PBMC was generally < 10%, however, the percentage of dead LN-derived cells was often much higher

(up to 50%). Hence, before further analyses of LN-derived cells, dead cells were depleted. To deplete the dead cells, separation was performed using Histopaque 1083, as described in Section 2.8.1. The dead cells pelleted to the bottom of the centrifuge tube, while the live cells banded at the interface between the Histopaque and TCM. After separation, there were consistently < 10% dead cells in the LN preparations.

It was important to ascertain whether or not similar proliferative responses were obtained using fresh versus cryopreserved cells. To compare the responses in cells before and after cryopreservation, cells were stimulated with Con A when fresh and again after cryopreservation for 1 month. In the experiment shown (Figure 5.4), it should be noted that the proliferation of the fresh cells was lower than would be expected, with a maximum SI of 23. This experiment clearly shows that cryopreserved cells proliferated well in response to Con A.

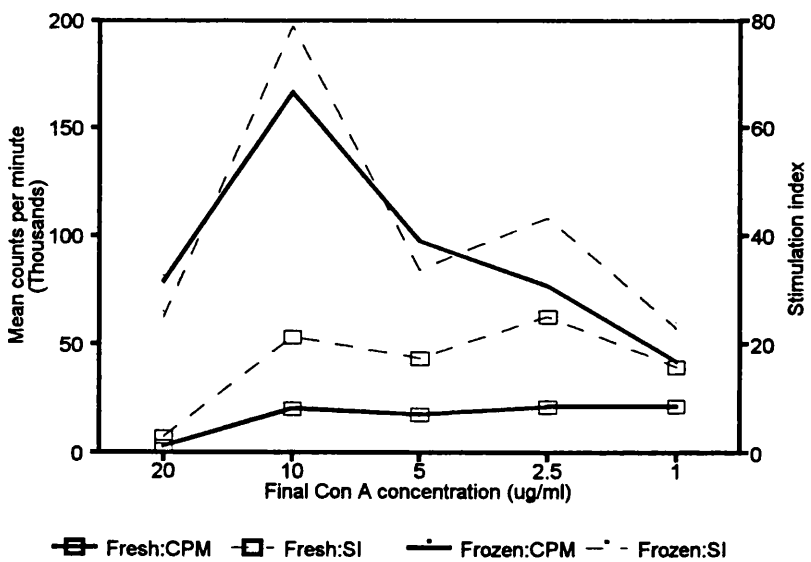

Figure 5.4 Comparison of proliferative responses before and after cryopreservation

PBMC were used fresh or after cryopreservation in liquid nitrogen for one month. PBMC were cultured, in triplicate, with or without 2.5 μg ml^{-1} Con A for 48 h before pulsing with ^{3}H thymidine.

5.2.1.5 Examination of the effect of adding fungizone to cell cultures

From the earlier experiments, it had been observed that there was occasionally fungal contamination of cell cultures. Fungizone is routinely included in culture medium in some laboratories. An experiment was carried out to compare proliferative responses to Con A of three PBMC samples and three LN-derived cell samples in the presence or absence of fungizone in the culture medium. The cells cultured with fungizone had significantly lower SI than those cultured without fungizone (not shown) ($p > 0.02$, using the t-test for matched pairs). It was therefore decided not to include fungizone in future experiments.

5.2.1.6 Examination of the effect of adding 2-mercaptoethanol to culture medium

Other authors have included the reducing agent 2-mercaptoethanol (2-ME) in culture medium in order to enhance cell proliferation (Noelle and Lawrence, 1980; Fiscus *et al.*, 1982). Experiments were performed to compare the SI of PBMC and LN-derived lymphocytes when cultured with or without 2-ME. For all cell populations, under all culture conditions, the proliferation of cells was higher when 2-ME was added to the medium (not shown). When SI were examined, no significant differences were observed in PBMC cultured with or without 2-ME. However, when SI of the LN-derived cells were compared, those cultured with 2-ME had significantly higher responses ($T=6$, $p \leq 0.02$, Wilcoxin's test for matched pairs). Therefore, 2-ME was included in assays where LN-derived cells were used, but not in assays containing PBMC.

5.2.1.7 Optimisation of FCS concentration in culture medium

PBMC and LN-derived cells stimulated with 2.5 $\mu g\ ml^{-1}$ Con A and 10 $\mu g\ ml^{-1}$ DvDUH, were cultured in TCM containing final concentrations of 1.25%, 2.5%, 5% and 10% FCS. The proliferation of stimulated cells increased as the concentration of FCS increased, as did

gnotobiotic calf usually proliferated non-specifically in response to parasite antigen. The experiments to investigate the source of this non-specific proliferation were described above.

The lack of response to DvDUH in PBMC from Calf 165 (Figure 5.5) at Day 0, most probably reflected the low level of viability of the cells, as demonstrated by the lack of response to Con A. If one ignores the other time points for which low viability was suggested by low Con A responsiveness (Days 3-0, 3-14), it can be seen that PBMC from Calf 165 proliferated to antigen throughout the course of the primary, secondary and tertiary infections. The results obtained in the second time course analysis for Calf 165 were roughly similar, with PBMC proliferating at all time points to DvDUH. The absolute level of proliferation varied throughout the two experiments. The proliferation of PBMC cultured in medium alone fluctuated throughout the time course of the experiment, as seen most clearly in the second experiment. This may suggest that some of the cells within the PBMC population were activated *in vivo* and continued to proliferate when cultured *in vitro,* in the absence of stimulation. In the second experiment, the actual proliferative responses to Con A remained fairly stable, apart from a very low response on Day 0. However, the SI for Con A fluctuated more, reflecting the variation in responses for cells cultured in medium alone.

The proliferative responses to Con A of PBMC from Calf 172 (Figure 5.6) fluctuated markedly over the three infections in the first experiment, whereas they remained more stable in the second experiment. These fluctuations in responses probably reflected between-day variation in the success of cryopreservation and/or thawing, rather than a down-regulation of polyclonal responses. The PBMC responses to DvDUH followed a very similar pattern to the responses to Con A. In the second time course experiment for Calf 172, there was only a single time point (Day 3-21) at which the thawed cells appeared to be non-viable. In this experiment, the PBMC showed a very high level of proliferation to parasite antigen, even on Day 0. This result illustrates the problem, discussed previously, of high levels of antigen-specific proliferation in the cells from uninfected animals.

As for Calf 172, the PBMC responses to Con A for Calf 174 (Figure 5.7) fluctuated more in the first experiment than in the second experiment, but on both occasions the responses on Days 3-0 and 3-21 were low. Again the antigen-stimulated responses mimicked the responses to Con A. There was a very high level of proliferation to DvDUH on Day 0 in the second experiment.

The proliferative responses of the PBMC from Calf 175 (Figure 5.8) were low to DvDUH in the first experiment, especially after the primary infection. In the second experiment, apart from on Day 0, the PBMC responded well to Con A at all time points. The responses for cells cultured in medium alone were very variable, as for Calf 165, which made the SI very variable. Contrary to the findings for the other calves, PBMC responses to parasite antigen did not mimic responses to Con A. In the first experiment, the SI for responses to DvDUH on Day O was relatively high, however, the cpm were quite low; this apparently high response is a reflection of the very low cpm for PBMC cultured in medium alone, compared with higher counts for other time points.

The responses for PBMC from Calf 166, the calf which died on Day 2-19, were analysed once only (Figure 5.9). On Day 0 and Day 2-19, there was no proliferation to Con A or parasite antigen, suggesting that the cells were non-viable. At all other time points there were good proliferative responses to both Con A and parasite antigen, with these responses following a similar pattern to each other.

In the first experiment for the Group B calves (Figure 5.10 to Figure 5.12) responses were assessed at three time points pre-infection (0-1: Day 0, 0-2: Day 29 and 0-3: Day 111 = Day 3-0, i.e. the day that the tertiary infection was administered). In the second experiment, responses were only assessed once pre-infection (Day 3-0) (Figure 5.10 to Figure 5.12). The high SI in the three pre-infection samples again illustrated the problems observed with non-specific proliferation to DvDUH. There were differences in the responses from cells collected on three occasions pre-infection from individual calves, highlighting the variability seen in proliferation assays and the difficulties of analysing the results. The proliferative responses to parasite antigen followed a very similar pattern to the responses to Con A in both experiments.

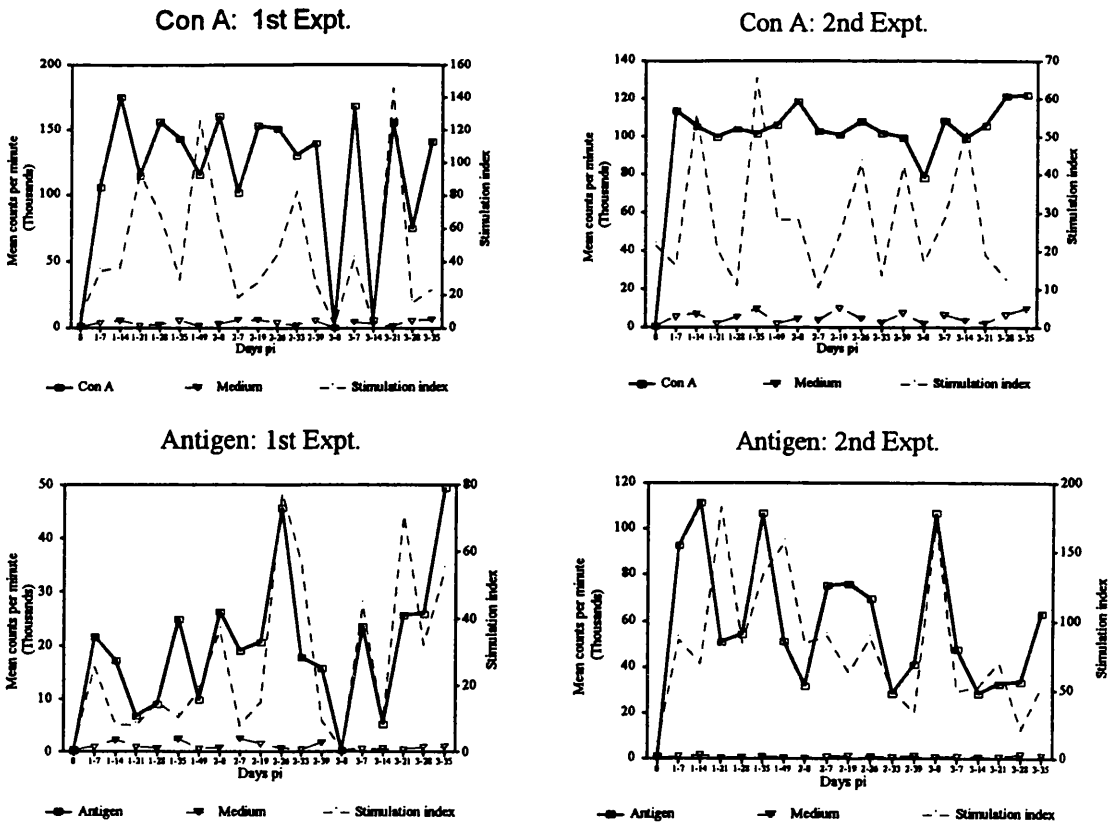

Figure 5.5 Proliferative responses of PBMC from Calf 165

PBMC were cultured, in triplicate, with or without 2.5 μg ml^{-1} Con A for 48 h and 10 μg ml^{-1} DvDUH for 72 h, before pulsing with ^{3}H thymidine. Con A: counts per minute (cpm) (left axis) for cells cultured with Con A. Antigen: cpm for cells cultured with DvDUH. Medium: cpm for cells cultured in medium only. SI: calculated by dividing the mean cpm for cells cultured with antigen, by the mean for cells cultured in medium only. Day pi: 1-, after primary, 2-, after secondary and 3-, after tertiary infections.

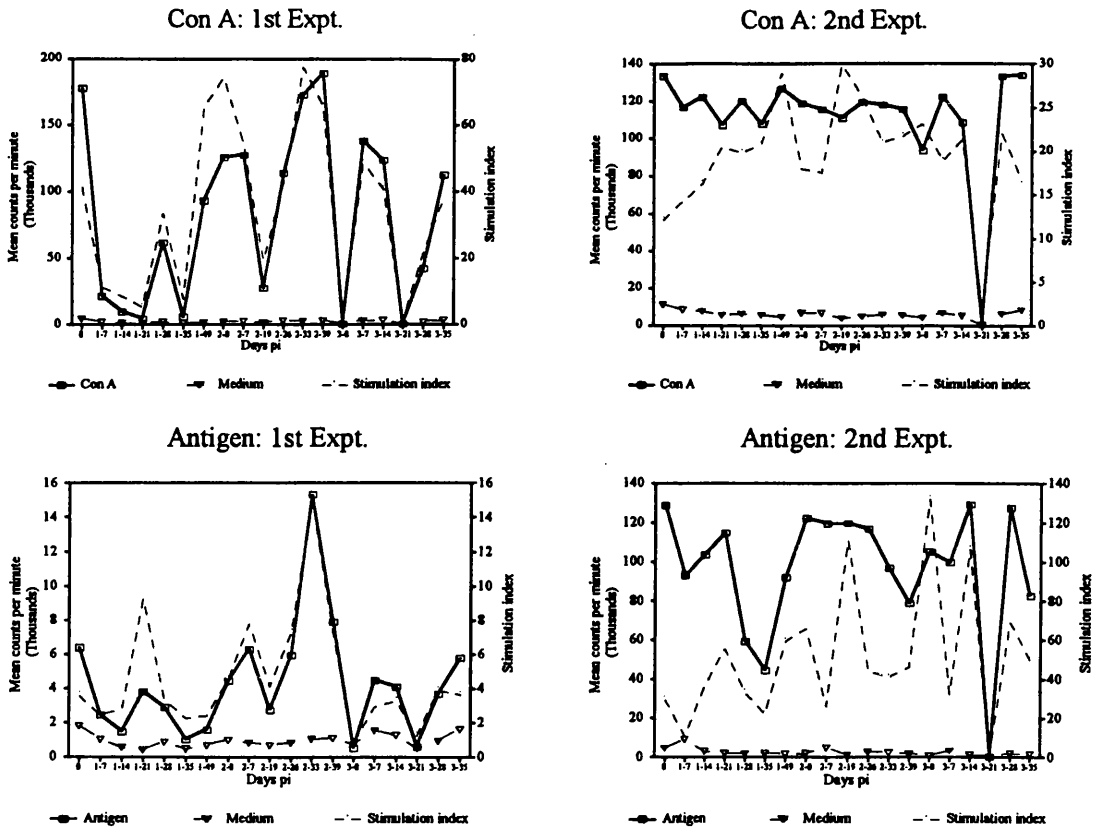

Figure 5.6 Proliferative responses of PBMC from Calf 172

For conditions, see caption for Figure 5.5.

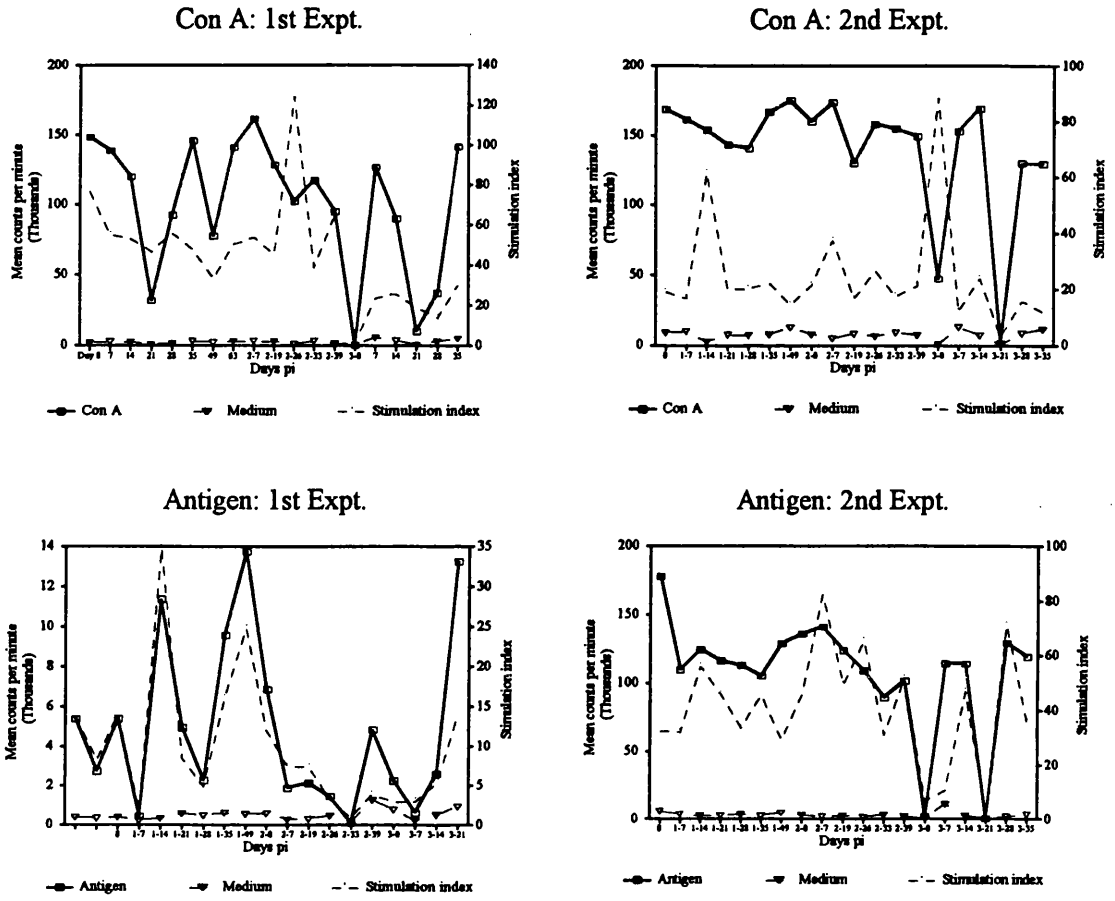

Figure 5.7 Proliferative responses of PBMC from Calf 174

For conditions, see caption for Figure 5.5.

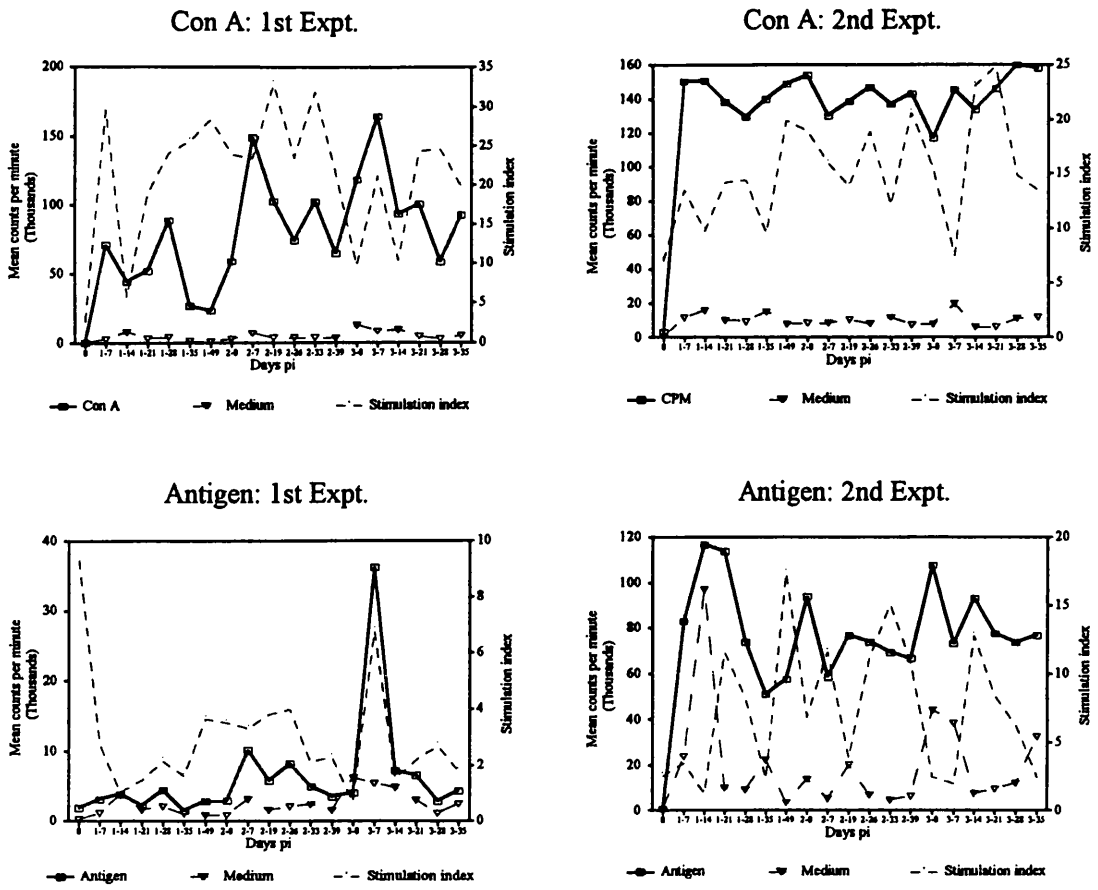

Figure 5.8 Proliferative responses of PBMC from Calf 175

For conditions, see caption for Figure 5.5.

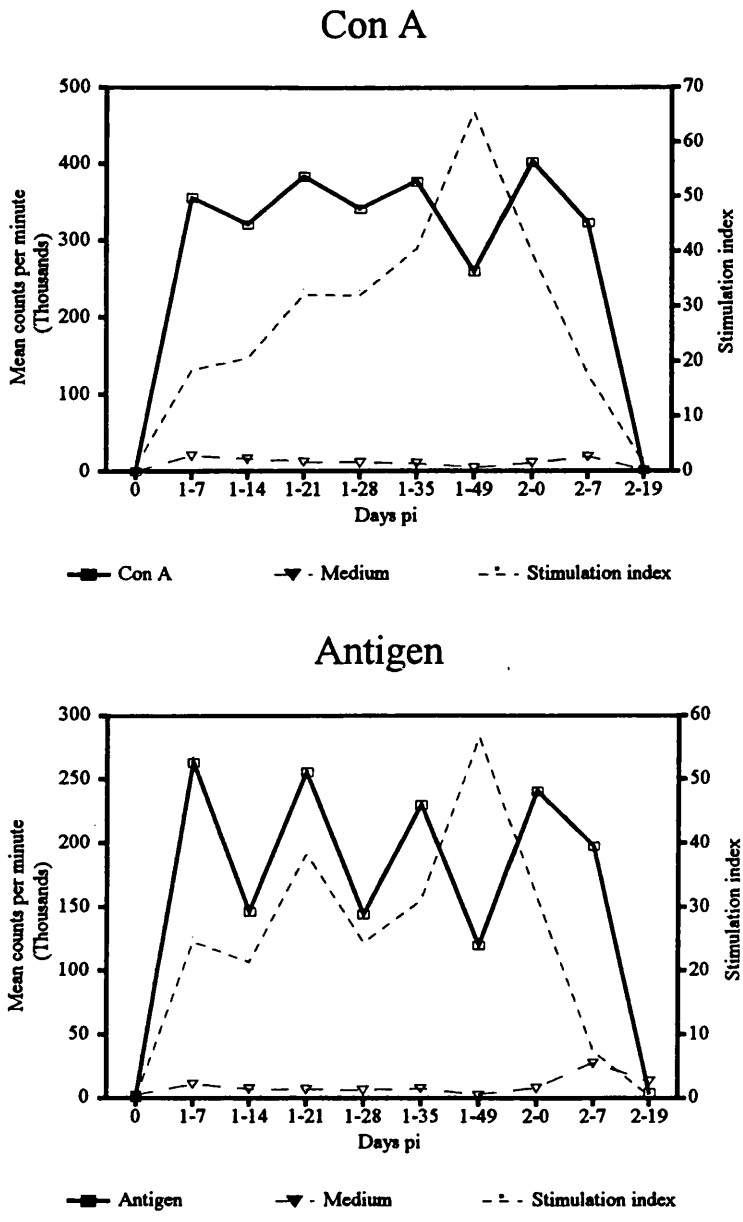

Figure 5.9 Proliferative responses of PBMC from Calf 166

For conditions, see caption for Figure 5.5. This calf died on Day 2-19.

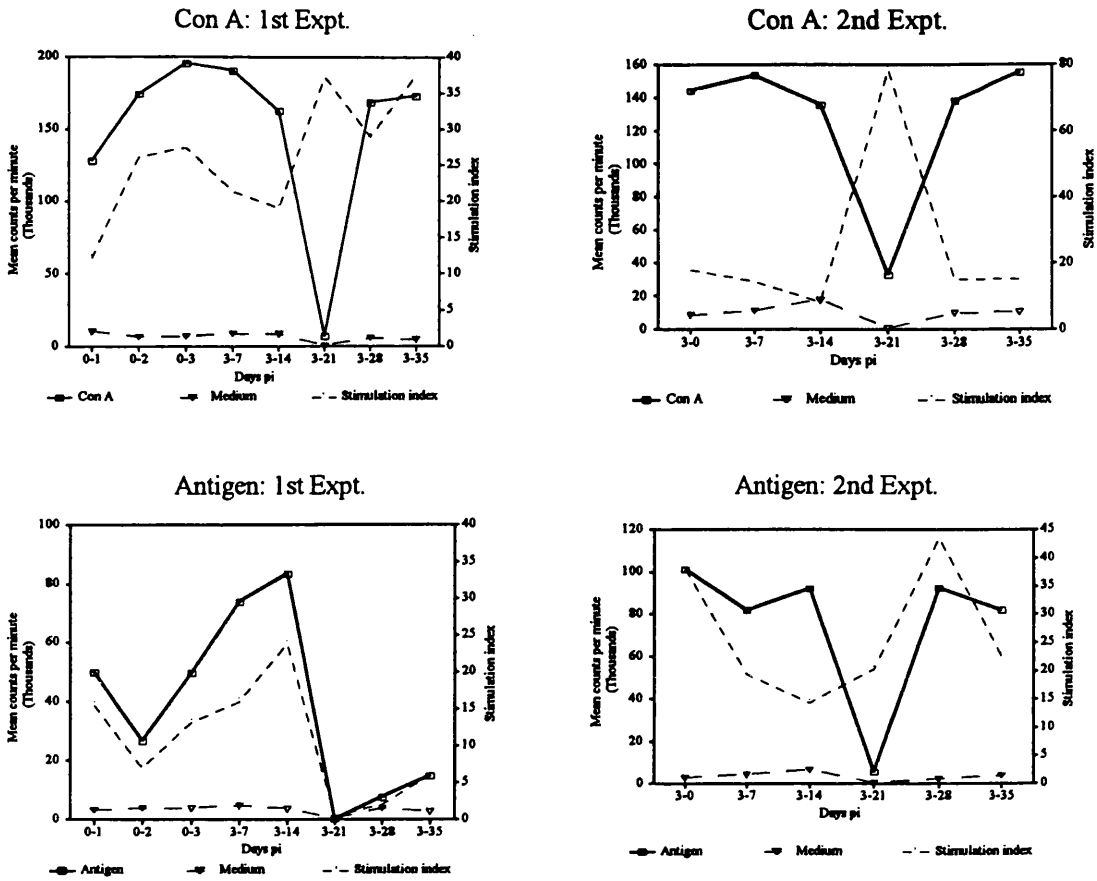

Figure 5.10 Proliferative responses of PBMC from Calf 169

PBMC were cultured, in triplicate, with or without 2.5 μg ml^{-1} Con A for 48 h and 10 μg ml^{-1} DvDUH for 72 h, before pulsing with ^{3}H thymidine. Con A: counts per minute (cpm) (left axis) for cells cultured with Con A. Antigen: cpm for cells cultured with DvDUH. Medium: cpm for cells cultured in medium only. SI: calculated by dividing the mean cpm for cells cultured with antigen, by the mean for cells cultured in medium only. 0-: pre-infection samples. 3-: days after tertiary infection.

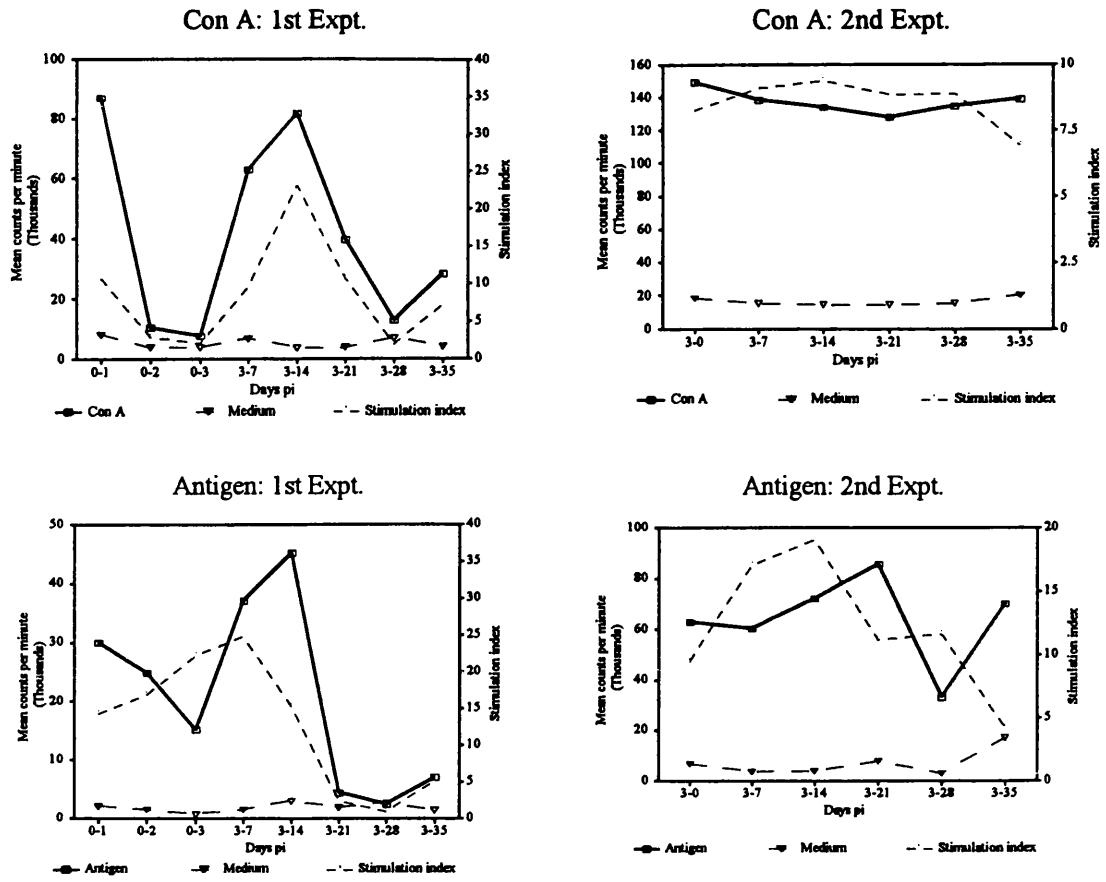

Figure 5.12 Proliferative responses of PBMC from Calf 176

For conditions, see caption for Figure 5.10.

5.2.2.2 Summary

Unfortunately, there was a high degree of variability in these experiments, firstly in responses to Con A and, secondly, with non-specific proliferation to DvDUH in uninfected animals. Responses to the polyclonal T cell mitogen Con A, which evaluates lymphocyte function, varied between samples collected on different days from the same calf. The variations in responsiveness pre-infection are demonstrated in the results for the three Group B calves for cells collected on three different occasions (Figure 5.10 to Figure 5.12). There was no correlation between proliferative responses to Con A and percentages of dead cells, which were generally <10%. However, the percentages of dead cells were assessed by trypan blue exclusion and this may not have been sufficiently sensitive to detect all dead cells. When responses to Con A were poor, it was likely that the cells were non-viable.

Also, the responses to the ultracentrifuged adult homogenate of *D. viviparus* were extremely variable. There were non-specific responses to this antigen in uninfected calves and the gnotobiotic calf. The high level of proliferation of PBMC from uninfected calves to DvDUH effectively ruled out meaningful measurement of antigen-specific responses pi. By and large, the responses to this antigen mimicked the responses to Con A and correlations were assessed between these responses for 96 PBMC samples where the cells were viable. A moderate correlation was demonstrated between responses to Con A and antigen (corr = 0.457). There was no correlation between responses for cells cultured in medium alone and Con A responses (corr = 0.199) or between cells cultured in medium alone and antigen responses (corr = 0.049).

5.2.3 Responses to recombinant antigens

In an attempt to identify an antigen preparation that would induce antigen-specific responses without any non-specific activity, responses to recombinant *D. viviparus* antigens were investigated. These were subunits of a large polypeptide, DvA-1 (Genbank™ Accession No. U2568), found in the E/S products of *D. viviparus* parasites (Britton *et al.*, 1995). DvA-1 is encoded by a gene with significant homology to the *Ascaris* allergen gene ABA-1 (Spence *et al.*, 1993). These genes, and related nematode polyprotein

allergens/antigens comprise a head-to-tail array encoding multiple units of 15 kDa polypeptides (Poole *et al.*, 1992; Tweedie *et al.*, 1993; McReynolds *et al.*, 1993). Two different recombinant antigens were used: Rpt-A, which was a single repeat of the 14 kDa subunit and Rpt C-A, a 42 kDa antigen consisting of three repeat units.

5.2.3.1 Preparation of recombinant antigens

A small quantity of cDNA encoding Rpt A and Rpt C-A, in the pET-15b expression vector, was kindly provided by Professor M.W. Kennedy, Division of Infection and Immunity, University of Glasgow, with permission from Dr. C. Britton, Wellcome Unit of Molecular Parasitology, University of Glasgow. The plasmid was transformed into BL21 (DE3) competent cells (Section 2.22) and DNA was extracted from 3 ml of an overnight culture of the transformed cells, using a Wizard mini-prep kit (Section 2.22). The plasmid vectors were digested using BamH1 (Promega) at 37°C for 1 h. Rpt A cut to give a product of 0.4 kb and the Rpt C-A cut to give a band at 1.2 kb.

To ensure that the vectors would express the desired protein, small scale protein expression was carried out, and the products analysed on a 10% SDS-polyacrylamide gel, (Section 2.5). Bands of the correct size were obtained (not shown). Antibodies raised in rabbits to the two recombinant proteins were kindly provided by Dr. C. Britton and a Western blot performed (Section 2.6). Strong binding was evident to proteins of the correct molecular weight, for both Rpt A and Rpt C-A (Figure 5.13). The serum also bound, much less strongly, to other antigens.

Following the successful small scale expression, a large scale expression was performed for both recombinant antigens and the expressed proteins purified through nickel columns (Section 2.23.2). The proteins were eluted from the columns, the fractions containing the expressed protein were pooled and the histidine tag removed by thrombin cleavage (Section 2.23.2).

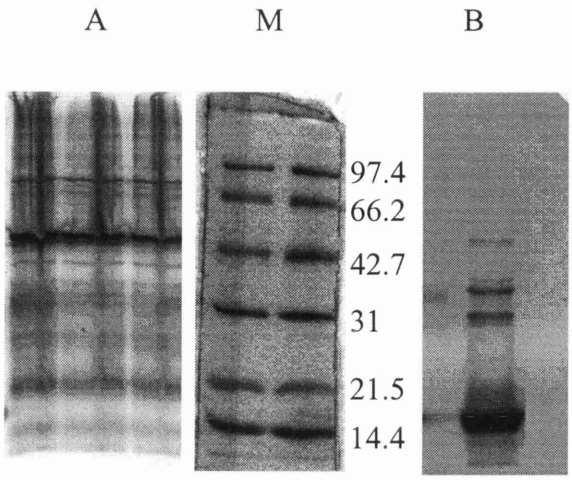

Figure 5.13 Analysis of expression of recombinant proteins by Western blotting
Small scale expression of protein was performed for Rpt C-A and Rpt A. The products were run on 10% SDS-polyacrylamide gels, then transferred to nitrocellulose and incubated with specific antibodies to the recombinant proteins, raised in rabbits. A: Rpt C-A: a product of 42 kDa is recognised. B: Rpt A: a product of 14 kDa is recognised. M: low molecular weight markers, sizes (kDa) are indicated.

Recognition of recombinant antigens by calf sera

The MHC region controls antibody recognition of the ABA-1 allergen in infected mice (Kennedy *et al.,* 1990). Therefore, an ELISA was performed to investigate whether the recombinant antigens were recognised by all the infected calves. Serum samples, collected 35 days after primary infection, were analysed for whole IgG antibody specific for each of the recombinant antigens. Samples from 10 uninfected calves were examined as controls. There were no IgG antibodies detected to Rpt A in any of the calves, as all ODs were below the threshold value (not shown). The ODs for IgG antibody to Rpt C-A for the eight experimental animals are shown in Table 5.4. The threshold OD was 0.099. Six out the eight calves had ODs which were significantly above this threshold. Calf 172, had a very low positive reading and Calf 170 was negative.

Group	Calf No	OD-1	OD-2	Mean OD
A	165	0.271	0.302	0.286
	166	0.210	0.181	0.196
	172	0.106	0.109	0.108
	174	0.196	0.199	0.198
	175	0.202	0.211	0.206
B	169	0.242	0.237	0.240
	170	0.099	0.096	0.098
	176	0.298	0.315	0.306

Table 5.4 Levels of Rpt-A-specific antibodies in infected calves

Levels of specific IgG antibodies to Rpt C-A in serum collected 35 days after primary infection for Group A and Group B calves, were measured by ELISA. The conditions for the ELISA were; antigen @ 5 μg ml^{-1}, serum @ 1:400, anti-bovine IgG @ 1:10,000. OD-1, OD-2: Optical densities in duplicate wells. The threshold OD (0.099) was calculated as the mean plus three SD of the ODs measured in serum from 10 control calves.

A Western blot was performed to confirm the recognition of Rpt C-A by antibodies from infected calves. On the freshly developed blot, it was clear that six out of eight calves recognised a band around 42 kDa. The two calves (Calves 170 and 172) that did not recognise the Rpt C-A antigen in ELISA did not recognise the antigen in the Western blot (Figure 5.14). Many of the calves also recognised another larger band at approximately 60 kDa.

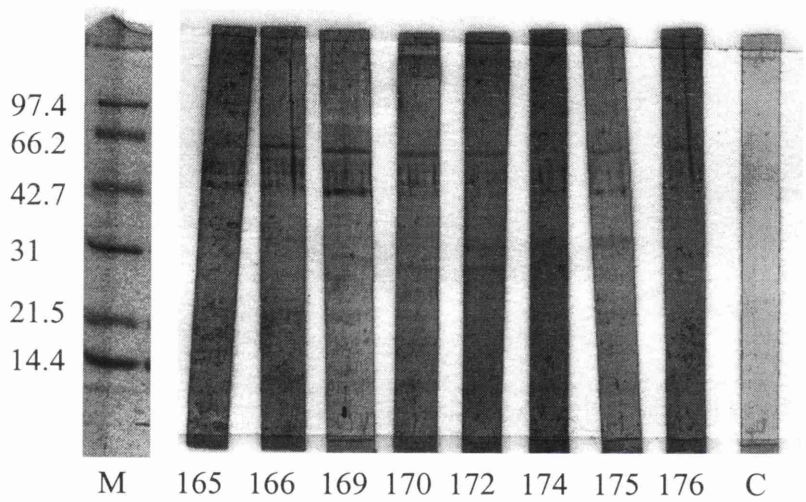

Figure 5.14 Recognition of Rpt C-A by sera from infected calves

Seventy μg of Rpt C-A were run in a continuous well on an SDS-polyacrylamide gel, then transferred to nitrocellulose and incubated in strips with sera from the experimental calves. The calf numbers are indicated. M: low molecular weight protein marker, sizes (kDa) are indicated. C: no serum control.

5.2.3.3 Proliferative responses to recombinant antigen

There was no recognition of Rpt-A by any of the calves in the ELISA, so this protein was not used in the proliferation assays. The triple subunit protein, Rpt C-A was used at a concentration of 10 $\mu g\ ml^{-1}$, the optimal concentration chosen after preliminary titration experiments (not shown).

In attempt to find a suitable control antigen to ensure that the 6-HIS tail on the recombinant protein did not induce non-specific responses, another recombinant protein was sought which also had a 6-HIS tail and to which cattle should not have been exposed. The human immunodeficiency virus NEF protein was kindly provided by Dr. M. Harris, Department of Veterinary Pathology. This recombinant antigen is a structural component of primate immunodeficiency viruses (HIV) and causes proliferation of PBMC from HIV patients. Unfortunately, the NEF antigen induced proliferation of PBMC from the *D. viviparus* infected calves with SI which were higher than those for Rpt C-A, so this was not a suitable control antigen. It was not possible to find another potential control antigen.

Proliferative responses to Rpt C-A were measured for PBMC from calves from Group A and Group B, measured over the time course of the experiment. To calculate the threshold SI, proliferative responses were assessed in 10 PBMC samples from uninfected calves and from the gnotobiotic calf. The threshold, calculated as the mean SI plus three SD for these control cells, was 1.98.

The SI for PBMC cultured with Rpt C-A from the gnotobiotic calf were always < 1.98 (not shown). PBMC from Group A calves (Figure 5.15) proliferated in response to Rpt C-A, with a maximum SI of 7. The kinetics of the response varied between calves and there were no obvious increases in responses as calves became more immune. Proliferative responses for cells cultured in medium alone or in the presence of Rpt C-A were strongly correlated (corr = 0.95). Proliferation of cells cultured in medium alone may indicate activation of the cells *in vivo* which continue to proliferate, in the absence of antigen, when cultured *in vitro*. The fact that the responses for cells cultured with medium alone were highly correlated to responses for cells cultured with Rpt C-A, may indicate that an antigen similar to Rpt C-A stimulated the cells *in vivo*. The kinetics of these responses were different to those observed with Con A and adult homogenate which were similar to one another, as described above. No correlations were demonstrated between responses to Rpt

C-A and Con A (corr = 0.238), nor Rpt C-A and adult antigen (corr = 0.053). The responses for Calf 172 were similar to those for the other calves despite the very low levels of antibody specific to Rpt C-A detected by ELISA.

The results for the Group B calves are shown in Figure 5.16. An antigen-specific response was seen only for Calf 169, on Day 3-14. All other SI were below the threshold SI of 1.98. As for Group A calves, the proliferative responses for cells cultured in medium alone followed a similar pattern to those cultured with Rpt C-A. This pattern of response was again quite different to the responses observed to Con A and adult homogenate, which mimicked one another (see Figure 5.10 to Figure 5.12, Experiment 2).

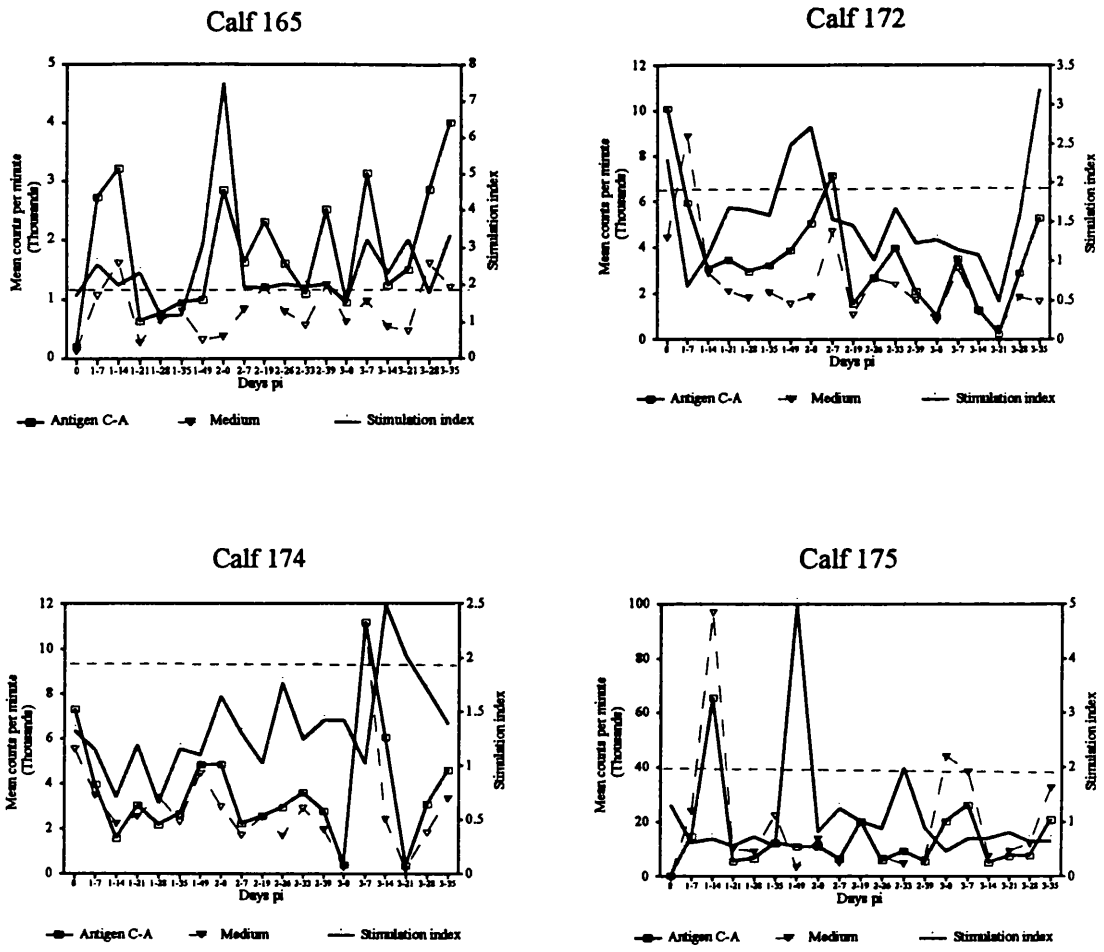

Figure 5.15 Proliferative responses of PBMC from Group A calves to Rpt C-A

PBMC were cultured, in triplicate, with or without 10 μg ml^{-1} Rpt C-A, for 72 h. Antigen: cpm (left axis) for cells cultured with Rpt C-A. Medium: cpm for cells cultured in medium only. Day pi: 1- , after primary, 2-, after secondary and 3- after tertiary infections. The dotted line indicates the threshold SI (1.98) which was the mean plus three SD of 11 control samples.

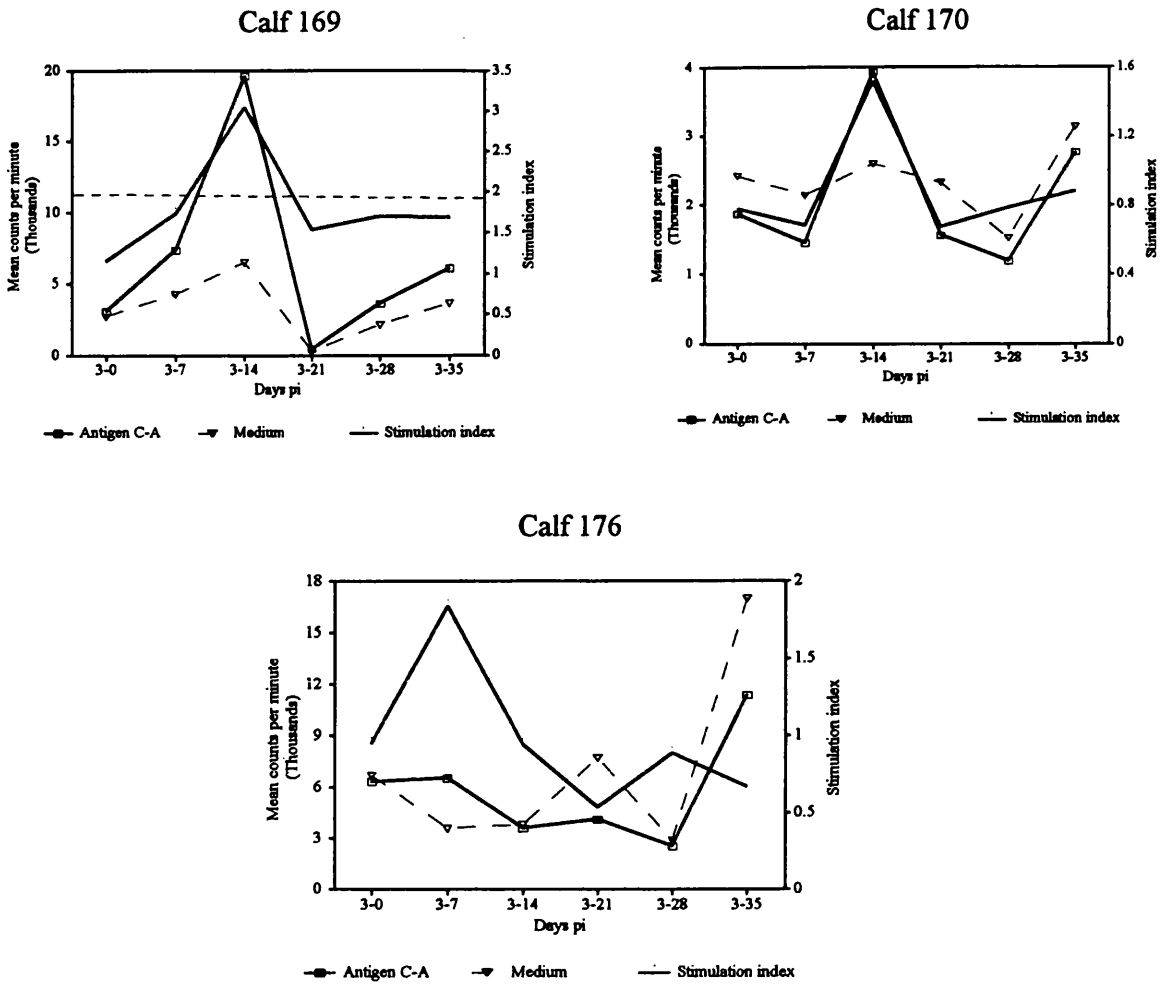

Figure 5.16 Proliferative responses of PBMC from Calf 166 and Group B calves to Rpt C-A

PBMC were cultured, in triplicate, with or without 10 μg ml^{-1} Rpt C-A, for 72 h. Antigen: cpm (left axis) for cells cultured with Rpt C-A. Medium: cpm for cells cultured in medium only. 0-: pre-infection samples. 3-: days after tertiary infection. The dotted line for Calf 169 indicates the threshold SI (1.98) which was the mean plus three SD of 11 control samples. All SIs for Calves 170 and 176 were below this threshold.

5.2.4 Proliferation of LN-derived cells

LNs were collected from all the experimental calves 35 days after the tertiary, challenge infection. The left, right, cranial and middle tracheo-bronchial (T/B) LNs were taken. These all drain different areas of the lungs, but there is considerable overlap in the drainage areas. The pre-crural LN was also collected, as a control node. This node is located just below the skin in the flank and does not drain the lung. The left T/B LN from a gnotobiotic calf, kindly provided by Mrs. E. McInnes, Institute for Animal Health, Compton, was used as an additional control. The lymphocytes were isolated from the LNs and cryopreserved as described previously (Section 2.8.3).

5.2.4.1 Proliferative responses of tracheo-bronchial LN-derived cells to Con A and an adult homogenate of *D. viviparus*

All the T/B LN-derived cells (Figure 5.17), including those from the gnotobiotic calf (not shown), proliferated to Con A and to parasite antigen. There were no significant differences in responses of the different T/B nodes to Con A or parasite antigen (Figure 5.17). The antigen-specific responses of the cranial T/B node were lower than the responses for the other T/B LNs, however, the difference was not statistically significant.

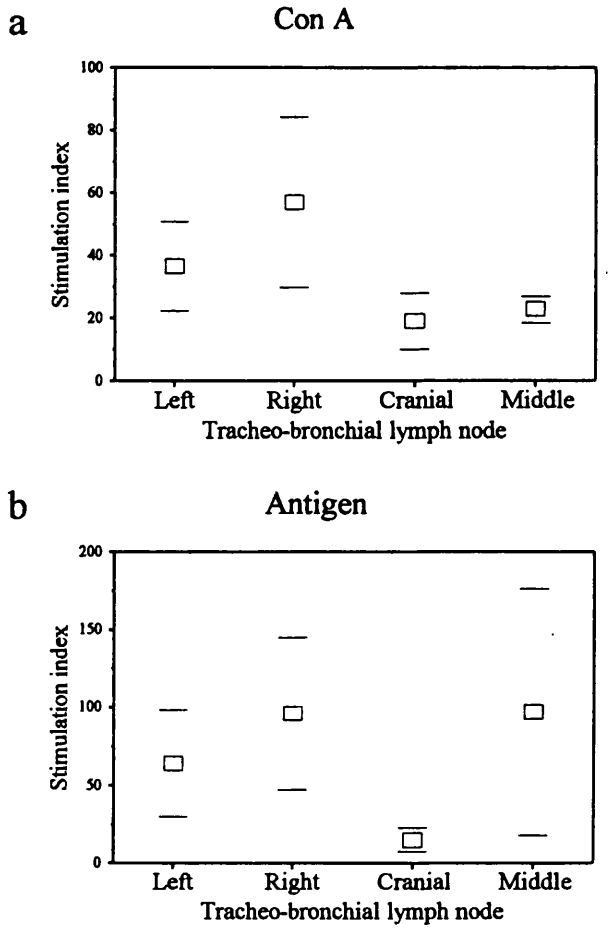

Figure 5.17 Comparison of proliferative responses of different tracheo-bronchial LN-derived cells.

LN-derived cells were cultured, in triplicate, with (a) Con A (2.5 μg ml^{-1}) for 72 h or (b) DvDUH (10 μg ml^{-1}) for 5 d, before pulsing with ^{3}H thymidine. The squares indicate the mean SI for the four infected calves and lines either side denote ± SD.

The left T/B LN was chosen for further experiments as this was the largest of the T/B LNs, so adequate cell numbers could always be assured. Responses to Con A and DvDUH were analysed in left T/B LN from Group A and Group B and from the gnotobiotic calf. This was repeated on four occasions and the results are shown in Table 5.5 and Table 5.6. Despite the fact that the cells used were identical aliquots, prepared at the same time, there was much between-day variation in SI for cells stimulated with Con A and with DvDUH. Even the pattern of responses were different; as some cells which had high SI in one

experiment, had low SI in another experiment. This variability complicated analysis of the results.

Generally, the SI for cells cultured with Con A were lower in Experiments 3 and 4 compared with Experiments 1 and 2. Part of the reason that the SI were lower than expected in some of the samples was due to the proliferation of cells cultured in medium alone. This was best illustrated by Experiment 3 in which Con A-stimulated cells from several of the animals incorporated >100,000 cpm, although the SI were in the range of 20 to 40. In contrast, the SI obtained with antigen-stimulated cells were very high and reflect the low cpm incorporated for wells where cells were cultured in medium alone. The responses to DvDUH in Experiments 3 and 4 were very high and, on most occasions, the SI were higher than the SI to Con A. Con A is a mitogen which stimulates all T cells, while the percentage of antigen-reactive cells should be lower, so antigen-specific SI are usually less than Con A. This provided further evidence that DvDUH may be behaving as a mitogen or superantigen.

The pattern of responses to Con A and DvDUH were very similar and a moderate correlation (corr = 0.448) was demonstrated between these responses. There were no significant differences in the response to Con A or DvDUH for left T/B LN-derived cells from Group A calves, collected on Day 35 after tertiary infection, compared with Group B calves, collected on Day 35 after primary infection ($p > 0.1$).

		C-1		C-2		C-3		C-4	
	Calf	cpm	SI	cpm	SI	cpm	SI	cpm	SI
Gp A	165	57,935	41.3	114,495	57.4	108,987	23.0	45,201	24.4
	166					30,018	35.3	5,556	7.6
	172	2,651	19.5	102,417	162.0	107,466	26.6	44,444	20.7
	174	25,099	28.7	63,347	67.3	83,323	22.0	36,102	15.9
	175			9,034	44.2	1,270	3.0	19,880	17.4
Gp B	169	19,444	40.0	106,258	111.4	105,748	35.0	39,135	36.6
	170	70,062	111.1	106,208	183.1	130,299	40.1	66,606	41.6
	176	22,513	34.8	8,585	34.4	122,340	46.2	39,320	23.7
GN	GN	118,322	32.6	92,777	3.5	144,141	39.0	112,838	11.0

Table 5.5 Proliferative responses to Con A

Left T/B LN-derived cells were cultured @ 2.5 x 10^6 ml^{-1}, in triplicate, with 2.5 μg ml^{-1} Con A for 72 h, before pulsing with ^3H thymidine. C-: columns show results for cells cultured with Con A for 1st, 2nd,3rd and 4th experiments. Cpm: counts per minute. SI: stimulation index. GN: gnotobiotic calf.

		A-1		A-2		A-3		A-4	
	Calf	cpm	SI	cpm	SI	cpm	SI	cpm	SI
Gp A	165	23,605	33.6	45,569	19.21	106,263	98.1	93,440	174
	166					38,390	64.8	16,719	35.6
	172	36,136	80.9	14,980	17.0	151,902	159	98,107	145
	174	1,548	5.2	9,574	6.3	63,364	82.8	46,009	47.7
	175	7,414	29.5	4,313	8.6			66,075	177
Gp B	169	7,281	19.0	14,938	14.6	113,983	103	124,121	151
	170	33,835	81.7	17,576	32.2	124,877	91.5	90,817	118
	176	33.997	109.3	14,053	12.48	144,925	79.2	110,271	169
GN	GN	96,879	34.7	102,060	4.15	122,278	13.4	58,704	0.93

Table 5.6 Proliferative responses to DvDUH

Left T/B LN-derived cells were cultured @ 2.5 x 10^6 ml^{-1}, in triplicate, with 10 μg ml^{-1} DvDUH for 5 d, before pulsing with ^3H thymidine. A-: columns show results for cells cultured with antigen for 1st, 2nd,3rd and 4th experiments. Cpm: counts per minute. SI: stimulation index. GN: gnotobiotic calf.

5.2.4.2 Comparison of proliferative responses of pre-crural and T/B LNs

Responses to Con A and DvDUH were compared between left T/B and pre-crural LNs. As the pre-crural LNs do not drain the site of infection, it might be anticipated that the T cell population at this site would be different to that present in the pulmonary LNs. This experiment was performed on two occasions. The proliferative responses to Con A were similar for the left T/B and the pre-crural LNs (Table 5.7). Moreover, there were no statistically significant differences between the responses in the left T/B and pre-crural LNs to parasite antigen. The proliferative responses to parasite antigen and Con A for cells from the pre-crural LNs were not correlated (corr = 0.212), whereas there was a strong correlation (corr = 0.753) between responses to Con A and DvDUH for cells from the left T/B LNs (Table 5.7). This compares with a moderate correlation (corr = 0.448) which was demonstrated over the four experiments, summarised in Table 5.6. There were no

statistically significant differences in responses of the pre-crural LNs from Group A compared with Group B calves to Con A or DvDUH.

			Con A		DvDUH	
Expt	**Group**	**Calf No.**	**Left**	**Pre**	**Left**	**Pre**
1	**A**	165	24.4	38.9	174	117.1
		166	7.6	30.0	35.6	119.5
		172	20.7	19.7	145	64.2
		174	15.9	20.8	47.7	51.3
		175	17.4	28.0	177	45.8
	B	169	36.6	22.0	151	5.68
		170	41.6	50.9	118	136.4
		176	23.7	27.8	169	5.3
2	**A**	165	23.0	16.2	98.1	24.8
		172	26.6	15.8	159	19.6
		174	22.0	67.7	82.8	126.1
		175	D	24.5	D	56.5
	B	169	35.0	16.2	102.8	125.4
		170	40.1	51.6	91.5	129.8
		176	46.2	23.5	79.2	23.5

Table 5.7 Comparison of proliferative responses of left T/B and pre-crural LNs.

LN-derived cells were cultured @ 2.5 x 10^6 ml^{-1} with 2.5 μg ml^{-1} Con A for 72 h, or 10 μg ml^{-1} DvDUH for 5 d, before pulsing with ^3H thymidine. The results are expressed as SI. The cells collected from the left T-B LN from Calf 175 in the second experiment were dead, so results are not shown (D).

5.2.4.3 Proliferative responses of LN cells to Rpt C-A

Proliferative responses to Rpt C-A were examined in LN-derived cells from the local left T/B LN and the peripheral pre-crural LN (Table 5.8). Cells collected from the left T/B LN from the gnotobiotic calf were also analysed. This experiment was performed on two occasions. The cells from the gnotobiotic calf did not proliferate in response to Rpt C-A

(not shown). The cells from the left T/B LN did not proliferate, or had very low responses (maximum SI = 3), in the presence of Rpt C-A. However, the cells collected from the pre-crural LN from some calves proliferated more vigorously to Rpt C-A (SI = 2 to 18) (Table 5.8). There was a significant difference between the SI for the pre-crural and left T/B LNs (T=8, p = 0.002, Wilcoxin's test for matched pairs). Proliferative responses to Rpt C-A and proliferative responses for cells cultured in medium alone for all the LN-derived cells were strongly correlated (corr = 0.888), as had been observed previously for PBMC (described above). There were no significant differences in the proliferative responses of local or peripheral LN-derived cells collected from Group A or Group B calves to Rpt C-A.

			Left T/B			Pre-crural		
Expt.	**Group**	**Calf No.**	**Rpt C-A**	**Medium**	**SI**	**Rpt C-A**	**Medium**	**SI**
1	**A**	165	791	538	1.47	1,178	795	1.48
		166	500	470	1.06	22,559	1,186	19.02
		172	765	675	1.13	3,605	1,774	2.03
		174	861	964	0.89	2,687	1,665	1.61
		175	586	374	1.57	3,475	2,183	1.59
	B	169	1,037	821	1.26	2,394	754	3.18
		170	1,106	769	1.44	1,798	805	2.23
		176	890	652	1.37	84,493	22,768	3.71
2	**A**	165	1,685	1,083	1.56	8,557	4,168	2.05
		172	2,338	955	2.45	17,143	6,705	2.56
		174	1,223	766	1.60	1,224	776	1.58
		175	106	236	0.45	3,373	2,153	1.57
	B	169	1,997	1,109	1.80	13,862	767	18.07
		170	3,240	1,365	2.37	1,480	812	1.82
		176	5,536	1,829	3.03	63,441	6,615	9.59

Table 5.8 Proliferative responses of LN-derived cells to Rpt C-A

LN-derived cells were cultured, in triplicate, @ 2.5 x 10^6 ml^{-1} with 10 μg ml^{-1} Rpt C-A or in medium alone, for 5 d, before pulsing with ^3H thymidine. SI: stimulation index.

5.3 Changes in leukocyte populations in response to infection

In order to analyse changes in the phenotype of PBMC and LN-derived cells over the course of the infections, the cells were labelled with monoclonal antibodies to surface markers for bovine lymphocytes as described in Section 2.13. Labelling with each of the markers was performed in duplicate. To act as a control, each cell sample was also incubated with the FITC-conjugated anti-mouse antibody. Routinely, for each sample, 20,000 cells were analysed by FACS. The mean percentages of labelled cells in duplicate samples were calculated, then the mean for duplicate conjugate-only controls was subtracted from this figure. Duplicate samples gave highly repeatable results.

5.3.1 Changes in leukocyte populations after a primary infection

PBMC were collected weekly after a primary infection from four calves infected with *D. viviparus* L3. These were not the same animals as those described in the longitudinal experiment. These calves were three month-old, male Friesians and were maintained in a similar manner to the other groups. The cells were analysed by FACS and the mean percentages of $CD4^+$, $CD8^+$, and $\gamma\delta^+$ T cells for the four calves are shown in Figure 5.18.

The $\gamma\delta^+$ T cells increased significantly on Day 1-7 ($p \leq 0.01$), compared with pre-infection levels, peaked on Day 1-14 and returned to pre-infection levels by Day 1-28. There was a later increase in $CD4^+$ cells, seen on Day 1-21 ($p > 0.05$), and these continued to increase on Days 1-28 and 1-35. There were no significant changes in the percentages of $CD8^+$ or B cells (not shown) ($p > 0.1$) throughout the time course. However, a trend was demonstrated for an increase in $CD8^+$ cells on Days 1-28 and 1-35.

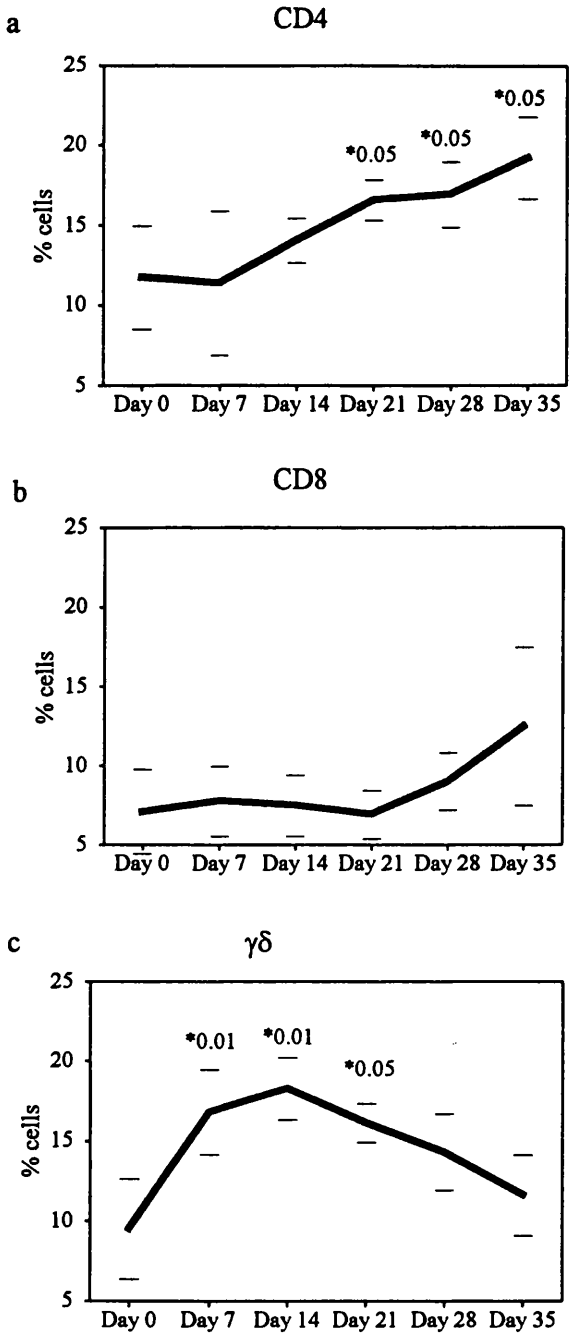

Figure 5.18 T cell populations over a primary infection with *D. viviparus*

Mean percentages of $CD4^+$ (a), $CD8^+$ (b), and $\gamma\delta^+$ T cells (c) in PBMC collected weekly from four calves after a single *D. viviparus* infection. The cells were labelled with Mab for cell surface markers for each cell type, then analysed by FACS. Error bars show standard error of the mean (SEM). * Indicates that the mean was significantly different from the mean on Day 0, using the t-test. The numbers after the * indicate the level of significance, where $p < 0.1$ was considered to be significant.

5.3.2 Changes in T cell populations over three infections

5.3.2.1 PBMC

PBMC samples, collected on Days 0, 1-21, 1-49, 2-19, 3-14 and 3-28, were analysed. These analyses were performed on three calves from Group A. The results are shown in Figure 5.19. The most significant change in cell populations was an increase in $\gamma\delta^+$ T cells as the calves became immune (Figure 5.19c). Compared with pre-infection levels, there was a significant increase in $\gamma\delta^+$ T cells by Day 3-14 and Day 3-28 ($p \leq 0.02$). The percentage of $CD4^+$ cells decreased significantly compared with pre-infection levels ($p \leq 0.1$) on Days 3-14 and 3-28 (Figure 5.19a), while the percentage of $CD8^+$ cells increased on Days 1-21 and 2-19 (Figure 5.19b).

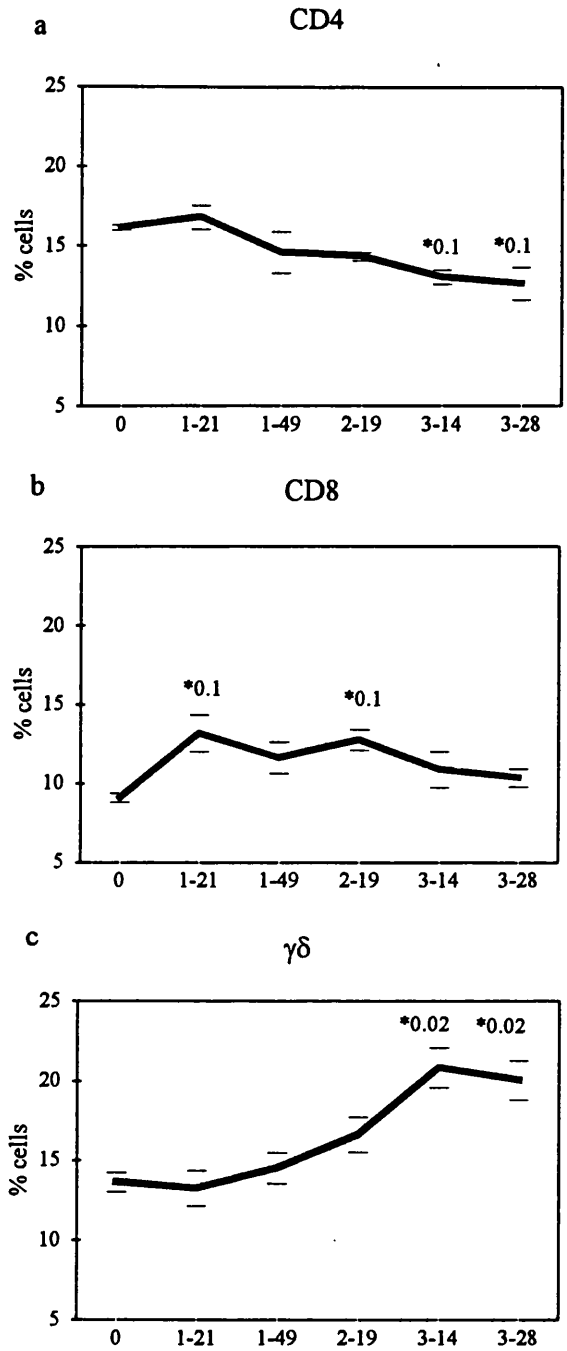

Figure 5.19 T cell populations over three infections

Mean percentages of $CD4^+$ (a), $CD8^+$ (b), and $\gamma\delta^+$ T cells (c) in PBMC collected weekly from three calves over three infections with *D. viviparus*. The cells were labelled with Mab for cell surface markers for each cell type, then analysed by FACS. Error bars show standard error of the mean (SEM). * Indicates that the mean was significantly different from the mean on Day 0, using the t-test. The numbers after the * indicate the level of significance, where $p < 0.1$ was considered to be significant. 1-, 2-, 3- indicates days after primary, secondary or tertiary infections, respectively.

5.3.2.2 LN-derived cells

LNs were collected from calves 35 days after the tertiary infection in Group A calves and 35 days after the primary infection for Group B calves. As controls, a pre-crural LN was collected from infected calves. The T/B LNs from infected calves were much larger than those from uninfected calves, while the left T/B LN from the gnotobiotic calf was very small. The pre-crural LNs were of normal size. The results are expressed as numbers of each cell population as a percentage of the total cells examined.

Comparison of populations from different LNs

For each calf, using the t-test, no significant differences were demonstrated between the percentages of different T cell populations in the left, right, cranial or middle T/B LNs after a primary infection with *D. viviparus* (not shown). There were significant differences in percentages of different T cell populations isolated from the pre-crural LNs (n = 2), collected 35 days after a primary infection, compared with cell populations isolated from T/B LNs (n = 12, Table 5.9). The T/B LNs from infected calves contained a higher percentage of B cells ($p \leq 0.01$), but a lower percentage of T cells (CD2, $p \leq 0.05$), with significantly lower $CD4^+$ ($p \leq 0.05$), $CD8^+$ ($p \leq 0.01$) and $\gamma\delta^+$ T cells ($p \leq 0.01$) than the pre-crural LNs. The ratio of CD4:CD8 cells was higher in the T/B LNs compared with the pre-crural LNs (Table 5.9). The cell population profiles obtained in the left T/B node of the gnotobiotic calf were similar to those collected from the pre-crural LNs but different to those from the T/B nodes from infected calves (Table 5.9). The sum of the percentages of $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T cells was greater than the total number of T cells (CD2). This is most likely due to the presence of cells which express for example, both BoCD4 and BoCD8 or WC1.

Cell Pop.	T/B n=12	Pre-crural n=2	Gnotobiotic n=1
BoCD2$^+$(Pan T cell)	20.7 (6.2)	41.2(2.1)* p≤.05	57.6
BoCD4$^+$	15.4(5.5)	23.4(4.2)* p≤0.05	33.3
BoCD8$^+$	4.6(2.4)	12.1(6.4)* p≤0.01	22.4
WC1$^+$(γδ T cell)	1.2(0.7)	14.25(8.3)* p≤0.01	4.2
WC3$^+$(B cell)	58.2(8.9)	35.8(15.7)* p≤0.01	20.2
CD4:CD8	3.3	1.9	1.5

Table 5.9 Leukocyte populations in LN-derived cells

Mean percentages of different leukocyte populations in T/B and pre-crural LNs collected from infected calves and the left T/B node from a gnotobiotic calf. n: number of LNs analysed. The standard deviations are shown in brackets. *: indicates that the differences between the pre-crural and T/B nodes were significantly different, using a two-tailed t test, the level of significance is shown. CD4:CD8: the ratio of $CD4^+$ to $CD8^+$ cells.

Comparison of LN populations after a primary or tertiary infection

The left T/B LNs collected from Group A and Group B calves were analysed by FACS. The percentage of B cells was significantly higher in LNs from Group B compared with Group A calves ($p \leq 0.1$) (Table 5.10). The percentage of $\gamma\delta^+$ T cells was also significantly higher in Group B compared with Group A calves ($p \leq 0.1$). There were no significant differences in percentages of $CD4^+$ and $CD8^+$ cells nor in the ratios of CD4:CD8 cells.

Cell Pop.	Group A n=4	Group B n=2
BoCD2$^+$(Pan T cell)	43.5(8.9)	36.2(13.3)
BoCD4$^+$	24.1(2.8)	25.0(3.0)
BoCD8$^+$	13.0(5.0)	12.5(5.5)
WC1$^+$(γδ T cell)	2.1(1.6)	5.0(2.0)* p≤ 0.1
WC3$^+$(B cell)	49.7(5.6)	60.5(7.5)* p≤ 0.1
CD4:CD8	1.8	1.9

Table 5.10 LN populations after a primary or tertiary infection

Mean percentages of different leukocyte populations in left T/B LNs collected from Group A compared with Group B calves. n: number of LNs analysed. The standard deviations are shown in brackets. *: indicates that the differences between left T/B LNs collected from Group A and Group B were significantly different, using a two-tailed t test. The level of significance is shown. CD4:CD8 : the ratio of $CD4^+$ to $CD8^+$ cells.

5.4 Changes in leukocyte populations in proliferation assays

To investigate which leukocyte populations were responding in the lymphocyte proliferation assays (Section 5.2.3), cells were labelled with bovine cell surface markers before and after stimulation with Con A or antigen. The PBMC were defrosted and counted (Section 2.8). The cell population in an aliquot of approximately 1.5 x 10^7 cells was analysed immediately by FACS. The remaining cells were cultured at a final concentration of 2.5 x 10^6 ml^{-1} in 5 ml of TCM/5% FCS in 25 ml culture flasks. The cells were incubated with or without Con A @ 2.5 μg ml^{-1} or the dialysed ultracentrifuged homogenate of adult *D. viviparus* (DvDUH) @ 10 μg ml^{-1}. Cells cultured with Con A were analysed 48 h later and those cultured with parasite antigen, 96 h later.

The percentages of dead cells, assessed by staining with Trypan blue (see Section 2.8.1), were observed to increase dramatically after culture. To assess the numbers of dead cells more accurately, cells were stained with the fluorescent stain propidium iodide (5 μg ml^{-1}). The FACS analyser was used to enumerate the dead cells.

5.4.1 Changes in PBMC after proliferation *in vitro*

The variations in PBMC populations after *in vitro* culture with Con A or DvDUH were analysed for one calf. The cells used were collected on Days 1-49, 2-19, 3-14 and 3-28. Figure 5.20 shows the mean percentage of cells pre-stimulation and following stimulation with Con A or DvDUH for Calf 175. At all time points, the percentage of $CD4^+$ T cells decreased after stimulation with Con A or antigen, compared with unstimulated cells (Figure 5.21a). The percentage of $CD8^+$ cells increased slightly after stimulation with Con A on Days 1-49, 2-26 and 3-28 and after stimulation with antigen on Days 1-49, 2-26 and 3-14 (Figure 5.21b). However, there were large increases in the percentages of $\gamma\delta^+$ T cells after stimulation with Con A at all time points and after stimulation with parasite antigen on Days 1-49, 2-26 and 3-28 (Figure 5.21c).

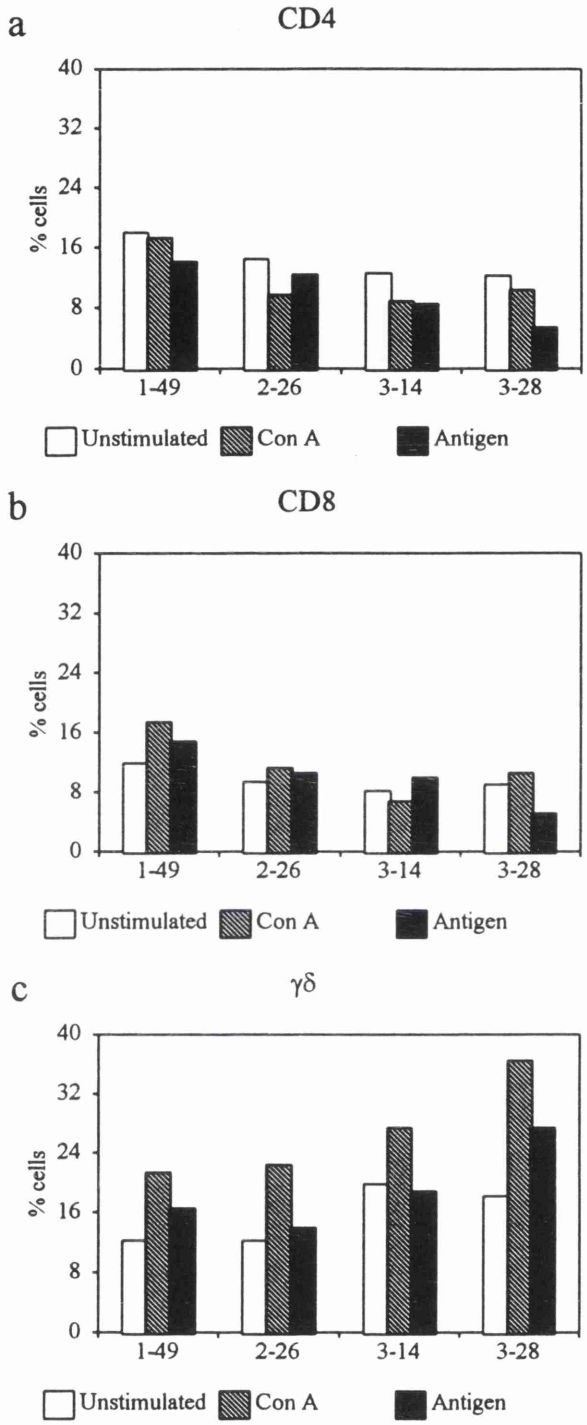

Figure 5.20 Leukocyte populations after stimulation *in vitro*

Percentages of $CD4^+$(a), $CD8^+$(b), and $\gamma\delta^+$ T cells (c) in PBMC from one calf, before and after stimulation with Con A or DvDUH. The cells were labelled with Mabs for cell surface markers for each cell type, then analysed by FACS. 1-, 2-, 3- indicates days after primary, secondary or tertiary infections, respectively. Unstim: before stimulation. Con A: after stimulation with Con A. Antigen: after stimulation with antigen.

5.4.2 Changes in LN-derived cells after proliferation *in vitro*

To investigate which of the cell populations in the LNs were proliferating in response to Con A or parasite antigen, left T/B LN-derived cells from Group A and Group B calves were analysed before and after stimulation *in vitro*. The results for Group A calves are shown in Figure 5.21 and those for Group B calves in Figure 5.22. In Group A calves, the $CD4^+$ and $CD8^+$ populations showed little change after stimulation with Con A or antigen. However, there was a significant increase in $\gamma\delta^+$ T cells after stimulation with both Con A ($p \leq 0.01$) and parasite antigen ($p \leq 0.1$). Moreover, the percentage of $\gamma\delta^+$ T cells increased significantly following culture in medium alone ($p \leq 0.05$). In contrast, there was a significant decrease in B cells after culture under all conditions.

For the Group B calves (Figure 5.22) the percentages of $CD4^+$ cells increased significantly after culture with Con A ($p \leq 0.05$), but not after culture with antigen. As noted with Group A calves, the percentage of B cells decreased significantly following culture with Con A or antigen. There were no other significant changes in the percentages of particular cell populations after culture under any of these conditions.

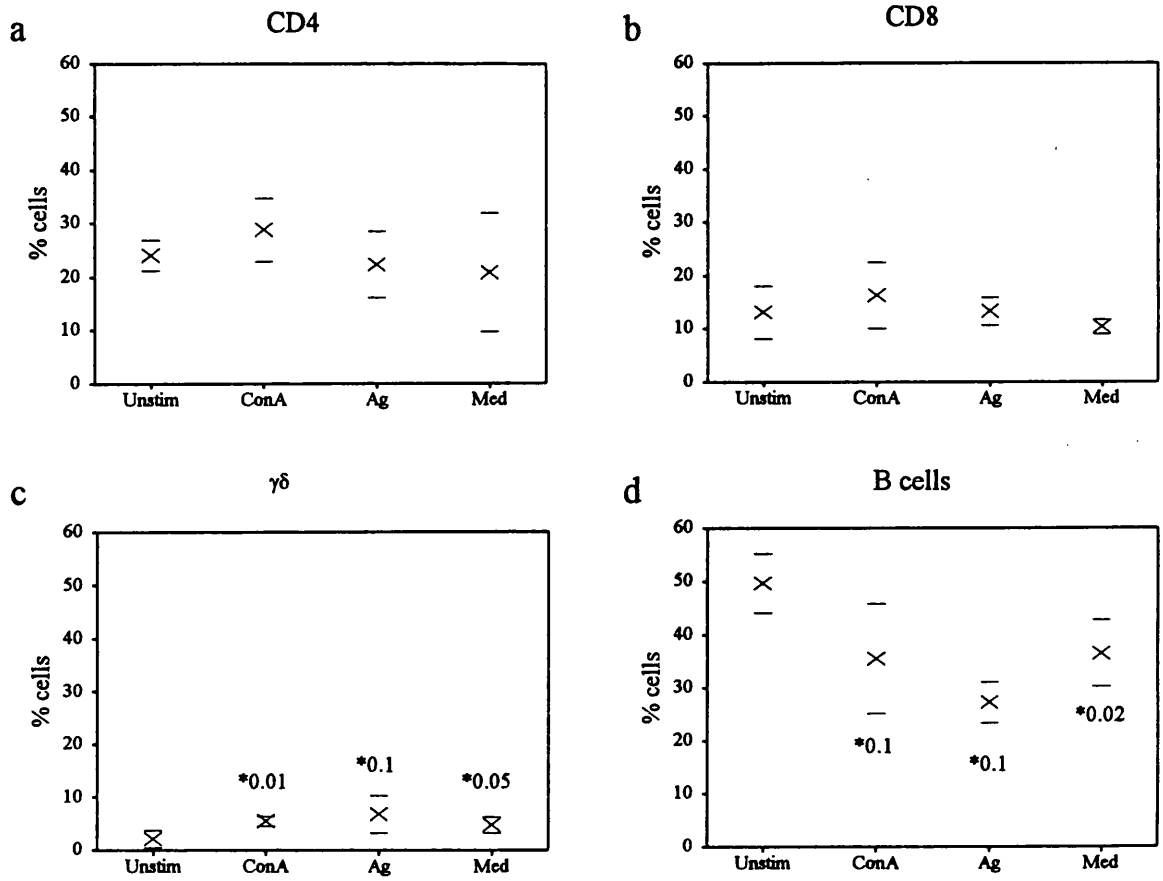

Figure 5.21 Changes in LN-derived cells from Group A after *in vitro* stimulation

Mean percentages of $CD4^+$(a), $CD8^+$(b), $\gamma\delta^+$(c) and B (d) cells in left T/B LN-derived cells from four Group A calves, before and after stimulation with Con A, parasite antigen or culture in medium alone. The cells were labelled with Mabs for cell surface markers for each cell type, then analysed by FACS. Unstim: before stimulation. Con A, Ag, Med: after stimulation with Con A, parasite antigen or culture in medium alone. Error bars show SEM. * Indicates that the mean was significantly different to the mean for unstimulated cells, using the t-test. The numbers after the * indicate the level of significance, where $p \leq 0.1$ was considered to be significant.

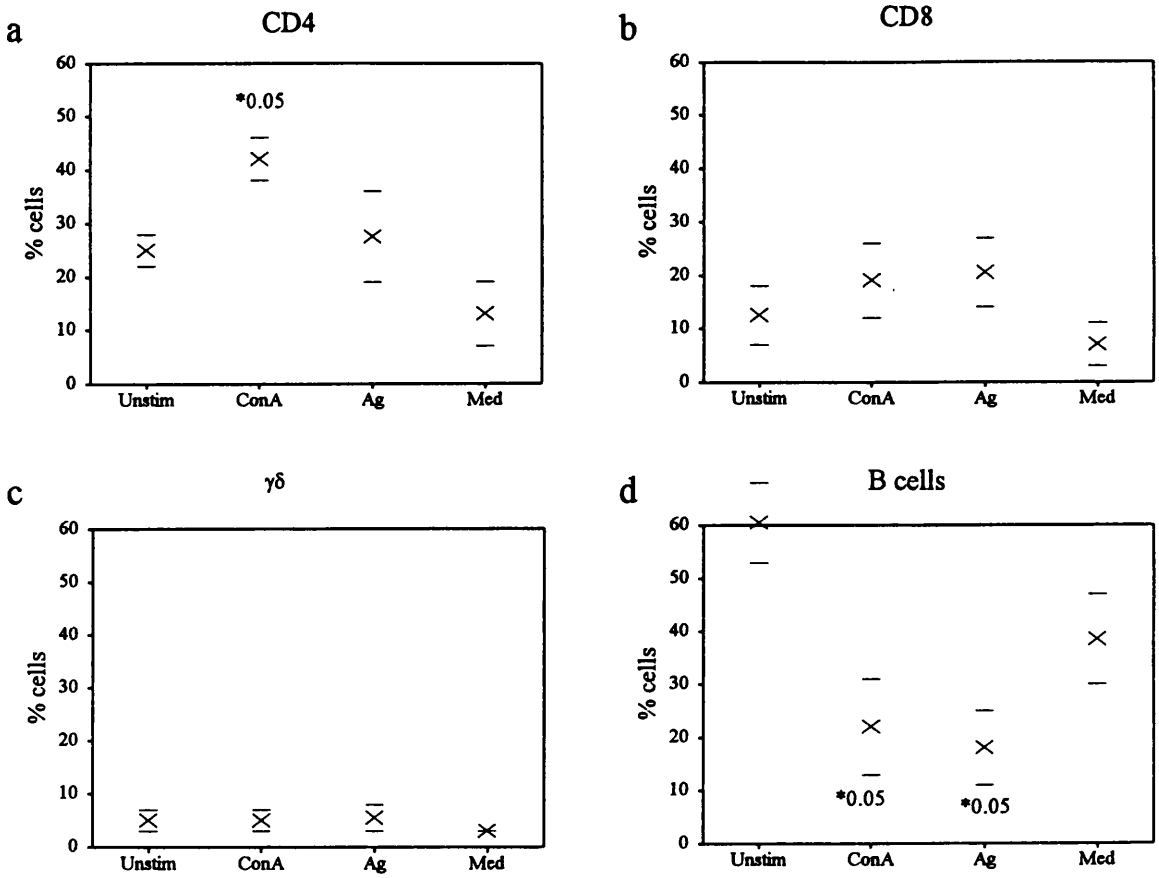

Figure 5.22 Changes in LN-derived cells from Group B after *in vitro* stimulation

Mean percentages of $CD4^+$(a), $CD8^+$(b), $\gamma\delta^+$(c) and B (d) cells in left T/B LN-derived cells from two Group B calves, before and after stimulation with Con A, parasite antigen, or culture in medium alone. The cells were labelled with Mabs for cell surface markers for each cell type, then analysed by FACS. Unstim: before stimulation. Con A, Ag, Med: after stimulation with Con A, parasite antigen or culture in medium alone. Error bars show SEM. *: indicates that the mean was significantly different to the mean for unstimulated cells, using the t-test. The numbers after the * indicate the level of significance, where $p \leq 0.1$ was considered to be significant.

5.5 Proliferation of isolated T cell populations

In the previous series of experiments, which examined changes in cell populations after culture *in vitro*, it appeared that the culture conditions may have favoured the proliferation of $\gamma\delta^+$ T cells. To avoid this potential source of bias, T cell populations were separated prior to culture *in vitro* and the proliferative responses of the separated populations assessed. PBMC were separated using magnetic beads (Section 2.14). The beads were labelled with Mab CC15 to isolate WC1+ cells, CC8 to isolate $BoCD4^+$ cells and CC63 to isolate $BoCD8^+$ cells. The separated cells were then cultured in the presence of APC with Con A, *D. viviparus* adult homogenate or in medium alone.

To optimise conditions for culture of the separated populations, initial titration experiments were performed using PBMC from infected calves (not shown). The optimal concentrations were chosen on the basis of SI. These conditions are shown below. It should be noted that the optimal cell concentrations chosen for $CD4^+$ and $CD8^+$ cells were much lower than for $\gamma\delta^+$ T cells.

Culture medium: TCM/5% FCS with 10^{-5} 2-ME
10,000 μg ml^{-1} Ciprofloxacin

Con A: 2.5 μg ml^{-1} (48 h culture)

Antigen: 10 μg ml^{-1} (5 d culture)

Cell concentrations:

- Whole PBMC: 2.5×10^6 ml^{-1}
- $\gamma\delta^+$ T cells: 2.5×10^6 ml^{-1}.
- $CD4^+$ cells: 5×10^6 ml^{-1}
- $CD8^+$ cells: 5×10^5 ml^{-1}

APC to T cell ratio: 2:1

5.5.1 Antigen presenting cells

Optimising dose of irradiation for preparation of APC

PBMC, irradiated using a radioactive caesium source, were used as APC. To determine the optimum dose of radiation that would inhibit proliferation of the PBMC, but would allow maximal APC function, the PBMC were irradiated at increasing doses of radiation from 500 to 6000 RADS, then cultured in the presence of Con A. The amount of radiation which completely inhibited proliferation to Con A was 2500 RADS (not shown), so this dose was used when preparing APC in subsequent experiments.

Autologous versus heterologous APC

These experiments were designed to investigate whether the cell populations, separated from whole PBMC, would proliferate more efficiently in the presence of Con A and DvDUH with or without autologous (from the same calf) or heterologous (from a different calf) APC. The results of an experiment in one calf are shown in Table 5.11.

The separated populations proliferated in response to Con A, with or without APCs. When comparing SI of the different populations incubated with autologous or heterologous APC, the most noticeable differences were observed with $CD4^+$ cells. This population had higher SI when incubated with heterologous APC compared with autologous APC. This series of experiments were repeated on a number of occasions with cells from different calves, with comparable results.

There were no significant differences in proliferative responses of $\gamma\delta^+$ T cells to Con A ($T = 36$, $n = 8$, $p > 0.1$, Wilcoxin's test for matched pairs) or parasite antigen ($T = 10$, $n = 8$, $p > 0.1$) when autologous or heterologous APC were used. The $CD4^+$ cells had a significantly higher response to Con A when cultured with heterologous APC ($T = 12$, $n = 12$, $p \leq 0.05$), but no differences in responses to antigen ($p > 0.1$). The $CD8^+$ cells had a significantly higher response to parasite antigen when cultured with autologous APC ($T = 2$, $n = 6$, $p \leq 0.1$), but no significant differences in response to Con A. There were no significant differences in responses to antigen for $CD4^+$ and $\gamma\delta^+$ T cells using autologous or

heterologous APC. The proliferation of cells cultured in medium alone, with autologous or heterologous APC, was always low, suggesting that there was no mixed leukocyte response when heterologous APC were used. Heterologous APCs would be much more plentiful so these were used in subsequent experiments.

	No APC		Aut APC		Het APC	
	Con A	Antigen	Con A	Antigen	Con A	Antigen
WC1+ (γδ)	42.0	0.9	62.4	7.5	55.4	6.2
BoCD4$^+$ (CD4)	40.6	0.7	17.9	2.2	67.8	3.6
BoCD8$^+$ (CD8)	40.2	0.8	35.2	6.7	38.4	4.0

Table 5.11 Autologous versus heterologous APC

PBMC from a *D. viviparus* infected calf were separated by MACS, then cultured in triplicate with 2.5 μg ml^{-1} Con A for 2 d or 10 μg ml^{-1} DvDUH for 5 d, in the presence of autologous or heterologous APC.

5.5.2 Proliferative responses of isolated T cell populations

PBMC collected on Days 0, 1-35, 2-35 and 3-35 from four calves, were separated into CD4$^+$, CD8$^+$ and γδ$^+$ T cells (Section 2.14). Aliquots of unseparated PBMC and separated populations (with APC) were cultured in the presence of Con A, DvDUH or in medium alone. APC were cultured on their own in order to assess their activity. Cells for all time points for each individual calf were analysed on the same day, while cells from different calves were analysed on different days. Therefore, within calf, but not between calf comparisons could be made. To assess the efficiency of cell separations, FACS analysis was carried out on the remaining cells. The remaining population (i.e. PBMC after separation) always contained < 5% of CD4$^+$, CD8$^+$ or γδ$^+$ T cells and usually contained < 1%, indicating that the separation was efficient. The subclasses present in the positively selected populations could not be differentiated, because the beads from the magnetic separation, which were still present, were labelled with goat anti-mouse antibody. This would have

interfered with the FACS analysis which utilised an anti-mouse FITC conjugate. Therefore, the separated populations could only be described as enriched, and not pure, populations.

The SI for separated populations are shown in Figure 5.23 to Figure 5.25. As in earlier experiments, unseparated PBMC responded well to parasite antigen at all time points (not shown). The responses for separated cells cultured in medium alone were always very low (<100 cpm, not shown). For each calf, the responses to Con A and parasite antigen followed similar kinetics at most time points. For each cell population, at each time point, the cells from three out of the four calves behaved similarly. In all cases, it was a different calf that behaved aberrantly. The following summary of results describes the findings for three out of four calves for each time point.

The responses of $\gamma\delta^+$ T cells to Con A and antigen tended to be low pre-infection, then increased on Day 35. The responses remained high after the secondary infection, but decreased after the tertiary infection (Figure 5.23). Low responses to Con A on Days 0 and 3-35 implied that these cells were non-viable. However, $CD4^+$ and $CD8^+$ cells, isolated from the same aliquot of PBMC, proliferated well, indicating that the PBMC collected on that occasion were in fact viable. An alternative explanation is that $\gamma\delta^+$ T cells may normally be less responsive to Con A than $CD4^+$ and $CD8^+$ cells and it is only when they are activated that they respond more vigorously.

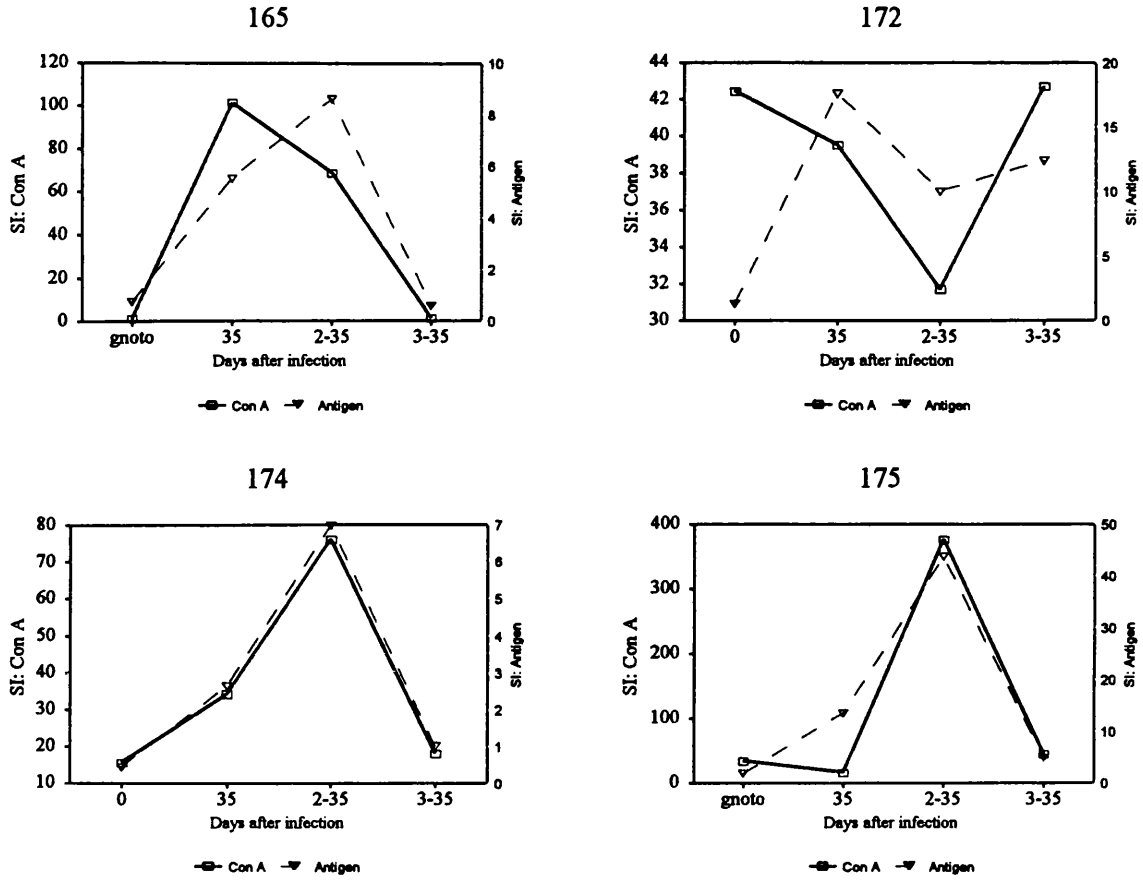

Figure 5.23 SI for MACS separated $\gamma\delta^+$ T cells after culture with Con A or DvDUH

Cells were separated using magnetic beads labelled with Mab CC 15, then cultured *in vitro* with heterologous APC with 2.5 μg ml^{-1} Con A for 48 h or 10 μg ml^{-1} DvDUH for 5 d. Results are expressed as SI. 0: pre-infection. 35: Day 35 after the primary infection. 2-35 and 3-35: Day 35 after the secondary and tertiary infections, respectively. When no pre-infection samples were available, cells from a gnotobiotic (gnoto) calf were used.

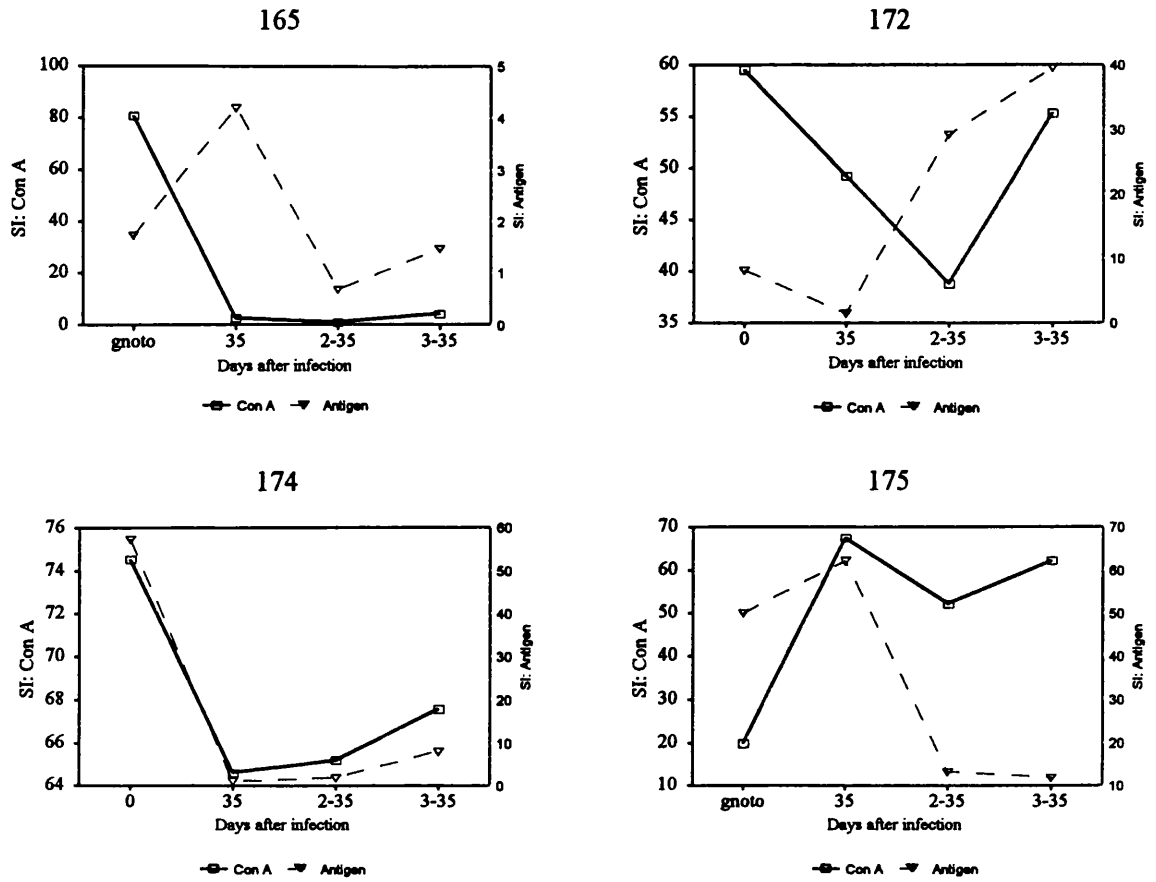

Figure 5.24 SI for MACS separated CD4$^+$ cells after culture with Con A or DvDUH
Cells were separated using magnetic beads labelled with Mab CC8, then cultured *in vitro* with heterologous APC with 2.5 μg ml^{-1} Con A for 48 h or 10 μg ml^{-1} DvDUH for 5 d. Results are expressed as SI. 0: pre-infection. 35: Day 35 after the primary infection. 2-35 and 3-35: Day 35 after the secondary and tertiary infections, respectively. When no pre-infection samples were available, cells from a gnotobiotic (gnoto) calf were used.

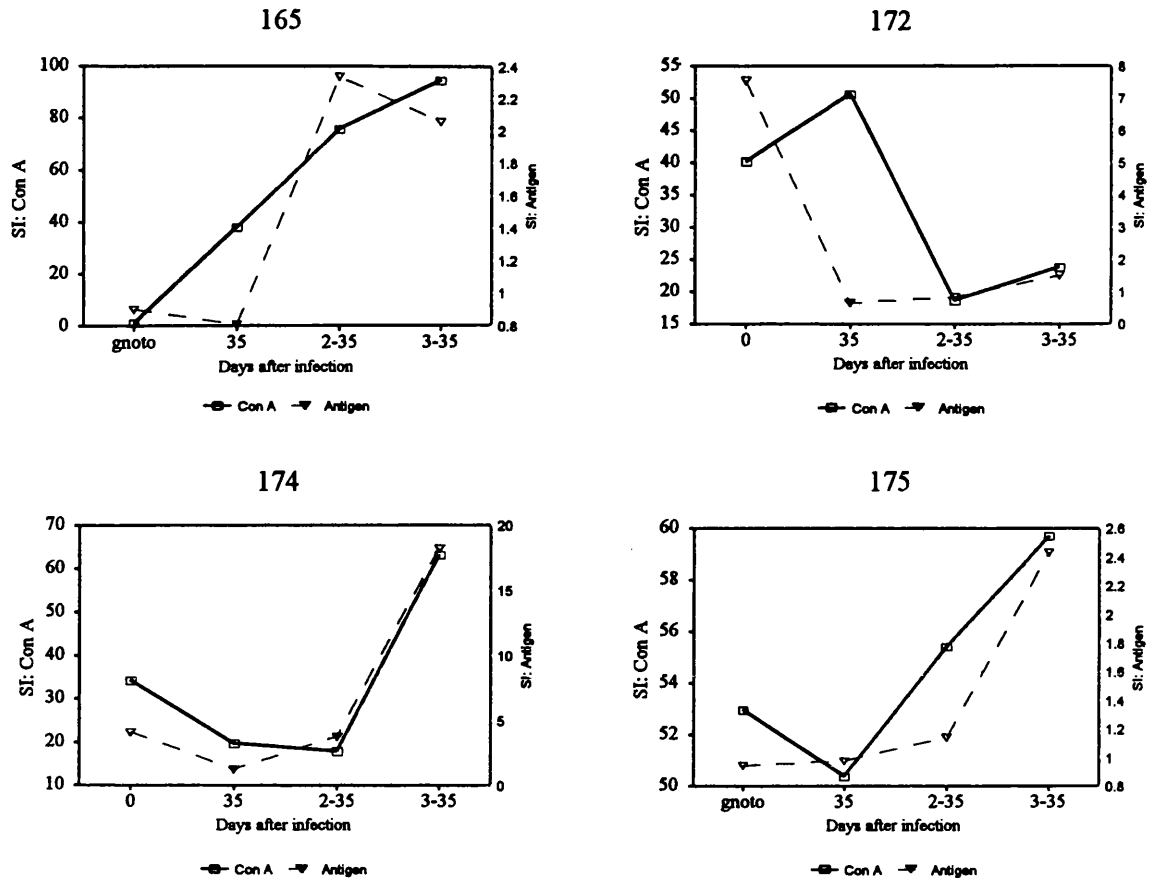

Figure 5.25 SI for MACS separated CD8$^+$ cells after culture with Con A or DvDUH
Cells were separated using magnetic beads labelled with Mab CC63, then cultured *in vitro* with heterologous APC with 2.5 μg ml^{-1} Con A for 48 h or 10 μg ml^{-1} DvDUH for 5 d. Results are presented as SI. 0: pre-infection. 35: Day 35 after the primary infection. 2-35 and 3-35: Day 35 after the secondary and tertiary infections, respectively. When no pre-infection samples were available, cells from a gnotobiotic (gnoto) calf were used.

sheep infected with *T. colubriformis* between 1 and 2 weeks pi, which increased again over weeks 2 to 7 pi.

The addition of 2-ME to culture medium has been shown to partially restore mitogen-induced responses of lymphocyte populations. It is unclear how 2-ME acts, but it is thought to alter an early event in Con A responsiveness, as it was demonstrated to shift maximum responses to one day earlier (Noelle and Lawrence, 1980). Other studies have shown that 2-ME can act as a T cell mitogen in FCS-containing cultures (Lemke and Opitz, 1976) and as a weak B cell mitogen (Lemke and Opitz, 1976; Noelle and Lawrence, 1980). Therefore, the increased proliferation of LN cells observed here may have been due to this mitogenic effect.

The results obtained with PBMC and LN-derived cells stimulated with DvDUH were difficult to interpret. Although cells from infected animals responded well to stimulation with DvDUH, cells from uninfected animals and the gnotobiotic calf also responded. In this study, there was no indication that antigen-specific proliferative responses were suppressed, as has been previously demonstrated in *O. ostertagi* infection in the bovine host (Klesius *et al.*, 1984). Similarly, Gasbarre (1986), demonstrated a decrease in numbers of PBMC which proliferated in response to antigen in *O. ostertagi*-infected compared with naïve calves, by limiting dilution assays.

Because of the high background proliferation in uninfected animals, it was not possible to demonstrate consistent differences pi in responses between the single (Group B) and multiple (Group A) infected calves. Previous studies in uninfected ruminants have produced similar findings. For example, lymphocyte reactivity was demonstrated in naïve lambs in response to *H. contortus* L3 (Torgerson and Lloyd, 1992; Gill, 1994) and adult (Gill, 1994) antigens, while Chauvin *et al.* (1995) demonstrated that E/S products from *F. hepatica* had a mitogenic effect on PBMC from uninfected sheep. Cross *et al.* (1986) also demonstrated non-specific responses to *O. ostertagi* L3 antigen.

Proliferation assays are known to suffer from considerable variability and great differences, both within and between cells from individual animals tested on the same, or different days, have been reported, rendering interpretation difficult (Fiscus *et al.*, 1982). A major source of physiological variation is the genotype of individual animals and this may have been an important factor in this study, where outbred animals were used. Other sources of physiological variation include stress, age, hormonal changes, disease, exercise and circadian rhythm. Technical sources of variation are also numerous and include

of superantigens requires the presence of MHC Class II molecules on APCs, but the MHC allele of the presenting cell does not have to match that of the T cell (Woodland and Blackman, 1993). In the study presented here, separated $CD4^+$ and $CD8^+$ cells proliferated non-specifically to parasite antigen and there were no significant differences in this proliferative response when autologous or heterologous cells were used as APC, providing more evidence for superantigen-like activity in the *D. viviparus* homogenate.

Unlike conventional protein antigens, *S. aureus* (Herman *et al.*, 1991) and *T. gondii* (Denkers *et al.*, 1994) superantigens do not require processing prior to their presentation by MHC Class II molecules. However, not all superantigens behave this way; for example, the bacterial superantigens, P*seudomonas* exotoxin A and Streptococcal M protein, required metabolically active APCs for full stimulation of target cells (Legaard *et al.*, 1991; Majumdar *et al.*, 1993). In the study presented here, separated T cells did not proliferate when paraformaldehyde-fixed PBMC were used as APC, suggesting that antigen processing was required. Similarly, Robinson *et al.*, (1994) found that successful presentation of the *H. polygyrus* 'superantigen' required the presence of metabolically-active MHC Class I positive accessory cells (Robinson *et al.*, 1995) and fixing of accessory cells with paraformaldehyde abrogated the response (Robinson *et al.*, 1994). Therefore, in many ways the *D. viviparus* and *H. polygyrus* nematode antigens appear to act in a similar manner to superantigens.

In the present study, there was a moderate correlation between responses to adult homogenate and responses to Con A in PBMC, with a strong correlation between responses in LN-derived cells. A β-galactosidase-binding lectin has recently been identified in the cuticle of adult *C. elegans* (Arrata *et al.*, 1996). Lectins bind specific carbohydrate moieties on cell membranes and some, including Con A, PHA, and PWM stimulate proliferation of bovine lymphocytes (Pearson *et al.*, 1979). It would be interesting to determine whether lectin-like molecules are present in parasitic nematodes, and if so, whether they can act as non-specific mitogens.

As was observed with the PBMC, it was not possible to demonstrate consistent differences in DvDUH-stimulated proliferative responses between LN-derived cells from Group A and Group B calves. Furthermore, when proliferative responses to DvDUH were compared between LN cells derived from a LN draining the site of infection (T/B LN) and a peripheral LN (pre-crural), no obvious differences were observed. The proliferative

responses of the T/B LN cells to Con A and parasite antigen were strongly correlated, perhaps providing additional evidence that the parasite antigen was acting in a similar manner to a mitogen.

In an attempt to identify an antigen that would not produce activity in cells from naïve animals, two recombinant *D. viviparus* antigens were examined, Rpt A and Rpt C-A. None of the calves had serum IgG antibodies to Rpt A, however, significant levels of serum IgG antibodies to Rpt C-A were demonstrated by ELISA and Western blotting in six of the eight calves pi. These antigens were not recognised by naïve animals. Kennedy *et al.* (1990) reported that the MHC region controlled antibody recognition of ABA-1 in infected mice, so the situation may be similar for DVA-1 in calves. In a previous study, Britton *et al.* (1995) detected antibody using Western blotting to Rpt C-A in pooled serum from three calves after three experimental infections of 5,000 *D. viviparus* L3. The lack of recognition of Rpt-A, which is one of the subunits of Rpt C-A, suggests that in isolation this antigen may not be highly immunogenic.

PBMC and T/B LN cells from the gnotobiotic calf did not proliferate in response to Rpt C-A. Therefore this antigen did not behave like a superantigen. However, when responses to Rpt C-A were examined in PBMC from infected animals, the antigen-specific responses were very low compared with the responses for those stimulated by DvDUH. This was not an unexpected finding as this protein was only a minor proportion of the whole range of proteins present in the DvDUH preparation.

There was a strong correlation between proliferative responses to Rpt C-A and proliferative responses of cells cultured in medium alone, for both PBMC and LN-derived cells. Proliferation of cells cultured in medium alone implies *in vivo* activation. A similar proliferation of PBMC cultured in medium alone was demonstrated in sheep infected with *T. colubriformis* and these responses peaked three weeks pi (Pernthaner *et al.*, 1995).

There were no significant differences in proliferative responses to Rpt C-A in PBMC from challenged immune calves compared with PBMC from calves after a primary infection. Thus the role of Rpt C-A in protective T cell responses in immune calves is unclear.

Perhaps the most interesting point to arise from the experiments with Rpt C-A, was the dichotomy in proliferative responses between the draining T/B LN and the pre-crural LN. A statistically significant difference between these responses was demonstrated ($p \leq 0.002$). However, in response to stimulation with whole *D. viviparus* homogenate, there

were no significant differences in proliferative responses between the T/B and the pre-crural LNs. There are a number of possible explanations for these data. Firstly, there may be inhibition of responses in the local LN. Differential T cell responsiveness at different anatomical sites has been demonstrated previously (De Marez *et al.*, 1995). In that study, good proliferative responses were observed to L3 antigen of *O. ostertagi* in abomasal LN cells of challenged immune calves, whereas PBMC and mediastinal LN-derived cells responded poorly. Although a suppression of local responses was not observed with the whole homogenate, it is important to note that the results obtained with a complex mixture of antigens, such as the homogenate, represent a cumulative total of all responses, some of which may be stimulatory, while others are suppressive. Furthermore, within the homogenate, certain antigens may be immunodominant, although these are not necessarily the antigens seen by the immune system during the course of infection. A local down-regulation of responses may have been parasite or host driven. The parasite travels via the circulation to the lungs, so if it releases factors which act to inhibit T cell responses, these may act in the gut, the mesenteric LNs, the lymph vessels travelling to the thoracic duct, the circulation, the lungs and/or local draining LNs. Certainly, the highest SI to Rpt C-A were seen in the pre-crural LNs and the responses in these peripheral nodes were higher than those seen with PBMC.

When analysing changes in leukocyte populations in response to infection, the results were expressed as changes in percentages of PBMC, rather than actual numbers of cells. It is important to note that although there may have been relative decreases in one cell population compared with another, the actual numbers of both populations may have increased *in vivo*, but one population may have increased much more than the other. This was illustrated by the findings of Gasbarre (1994) who found that although the abomasal LNs of calves infected with *O. ostertagi* greatly increased size with large increases in all lymphocyte populations, the percentage of $CD4^+$ cells decreased. He suggested that the actual numbers probably increased, but at a lower rate than other (non-identified) cell types.

The $\gamma\delta^+$ T cells increased early after primary infection (Days 7 through 21), whereas the $CD4^+$ and $CD8^+$ cells remained at similar ratios, but increased later, on Days 28 and 35 pi. An early increase in percentages of $T19^+$ ($\gamma\delta^+$) T cells on Day 7 pi, which thereafter declined, was demonstrated in sheep infected with *T. axei*, while the $CD4^+$ and $CD8^+$ cells gradually increased over the first four weeks pi (Pernthaner *et al.*, 1996). In this study, the

percentages of $\gamma\delta^+$ T cells were significantly higher after the tertiary infection than after the primary infection, whereas the percentages of $CD4^+$ cells were significantly lower. As the calves were fully immune by the tertiary infection, this implies that $\gamma\delta^+$ T cells may have a role in protective immunity to *D. viviparus*.

The T cell populations in local LNs from infected calves were quite different from those from the gnotobiotic calf, which had relatively more B cells and less T cells. The percentages of different cells collected from the pre-crural LNs of infected calves were similar to those found in the gnotobiotic calf. Therefore changes in LN cell populations observed in the *D. viviparus* infected calves, were in the local drainage LNs only. The percentages of both $CD4^+$ and $CD8^+$ cell populations decreased in infected compared with uninfected calves, but the ratio of CD4:CD8 T cells was higher in infected calves. A similar finding was reported in *F. hepatica* infected sheep when LNs increased in size and had increased CD4:CD8 ratios compared with uninfected sheep (Meussen *et al.*, 1995).

To further define which cells were responding in the *in vitro* proliferation assays, leukocyte populations were analysed by FACS pre- and post-culture with Con A, antigen, or medium alone. The only cell population which proliferated after culture of PBMC or LN-derived cells with parasite antigen was the $\gamma\delta^+$ T cell population. Coupled with the results from the phenotypic analyses of PBMC, it seems likely that the $\gamma\delta^+$ T cell population expanded in response to infection with *D. viviparus*. Moreover, $\gamma\delta^+$ T cells in cultures of LN-derived cells from Group A calves also proliferated after culture in medium alone, suggesting that they had been primed *in vivo*.

Since the culture conditions may have favoured the growth of $\gamma\delta^+$ T cells and the proliferating $\gamma\delta^+$ T cells may have produced factors which inhibited other cell populations, the PBMC were separated prior to culture *in vitro*. Separated $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T cells all proliferated in response to Con A without the need for APC. An earlier study using FACS-separated sheep PBMC demonstrated that $CD4^+$ cells responded to Con A without APC, but that $CD8^+$ and $\gamma\delta^+$ T cells required APC to proliferate (McClure and Hein, 1989). The proliferation observed in the present study, in the purified T cell populations, may be due to small numbers of contaminating APCs in the separated populations. To properly define the requirement of T cells for APC, more stringent separation would be required. This could be achieved by separation by MACS followed by separation using FACS. Alternatively, a different MACS technique is available, where isotype-specific labelled

magnetic beads are used. This allows the positively separated population to be analysed for the presence of contaminating cells belonging to other populations.

None of the cell populations responded to parasite antigen in the absence of APC. This was similar to findings by Torgerson and Lloyd (1993), who demonstrated that T cell enriched cultures of PBMC from *H. contortus* infected sheep responded to Con A, but not parasite antigen, in the absence of APC.

There were no significant differences in proliferative responses of $\gamma\delta^+$ T cells and $CD4^+$ cells to parasite antigen when autologous or heterologous APC were used, suggesting that the cells proliferated to parasite antigen in a non-MHC-restricted manner. $\gamma\delta^+$ T cells bind MHC molecules at a site away from the peptide binding groove where $\alpha\beta$ receptors normally bind, and antigen recognition is not always MHC restricted (Schild *et al.*, 1994). For $CD4^+$ cells, non-MHC restricted proliferation could occur if the antigen was acting in a similar manner to a superantigen or lectin, as discussed above.

$CD4^+$ and $CD8^+$ cells, but not $\gamma\delta^+$ T cells, from uninfected calves proliferated in response to parasite antigen. Superantigens may stimulate $CD4^+$ or $CD8^+$ cells (Herman *et al.*, 1991) and lectins may stimulate a variety of T cell subsets (Pearson *et al.*, 1979). Therefore, the parasite antigen may contain superantigen and/or lectin-like constituents which stimulate $CD4^+$ and $CD8^+$ T cells, but not $\gamma\delta^+$ T cells, to proliferate.

After the primary and secondary infections, the $\gamma\delta^+$ T cells proliferated most vigorously, suggesting that they played a role in the immune response at these time points. $\gamma\delta^+$ T cells have been demonstrated to be important in protozoan infections of cattle. For example, a *B. bovis* antigen stimulated $\gamma\delta^+$ T cells to proliferate (Tetzlaff *et al.*, 1992), while Flynn and Sileghem (1994) found preferential activation of $\gamma\delta^+$ T cells after *T. congolense* infection in trypano-tolerant cattle.

In this study, after the primary and secondary infections the proliferative responses of separated $CD4^+$ and $CD8^+$ were lower than pre-infection. However, after the tertiary infection, there was a decrease in the $\gamma\delta^+$ T cell response and an increase in the $CD4^+$ and $CD8^+$ responses. After the primary and secondary infections, the $\gamma\delta^+$ T cells may act *in vivo* to inhibit the ability of the $CD4^+$ and $CD8^+$ cells to respond to antigen. Activated bovine $\gamma\delta^+$ T cells obtained from mammary gland secretions have been observed to down-regulate the proliferative responses of $CD4^+$ cells to *S. aureus* antigens (Park *et al.*, 1993). A possible mechanism for this inhibition may be via IL-10, which is known to be produced by murine

$\gamma\delta^+$ T cells (Hsieh *et al.*, 1996). IL-10 acts to inhibit Th0, Th1 and Th2 cell responses in cattle (Brown *et al.*, 1994c). $\gamma\delta^+$ T cells were recently shown to play a role in regulation of the immune response to *T. colubriformis* infection in sheep when depletion of $\gamma\delta^+$ T cells during primary infection enhanced protective immunity (McClure *et al.*, 1995). A similar enhancement of immunity was seen after depletion of IFNγ (McClure *et al.*, 1995). These results demonstrated that $\gamma\delta^+$ T cells retarded the potential development of immunity against the parasite, perhaps via the induction of IFNγ. In *D. viviparus* infection and in the experiments of McClure *et al.* (1995), the parasite may induce the $\gamma\delta^+$ T cell response in an attempt to evade the immune response. Alternatively, the $\gamma\delta^+$ T cell response may be host driven, in response to parasite antigen or to transiently-expressed self-antigens on injured cells or activated leukocytes. $\gamma\delta^+$ T cells are thought to act as local tissue regulators, both initiating and controlling immune responses (reviewed in Chapter 1).

Future experiments to try to identify which cells are important in effector mechanisms in protection could include specifically depleting lymphocyte subpopulations *in vivo* using monoclonal antibodies specific for bovine T cell subsets. Using this technique, Howard *et al.* (1992a) demonstrated that $BoCD4^+$ cells played a major role in controlling a primary infection with a non-cytopathic strain of bovine viral diarrhoea virus. In mice, passive transfer of cell populations between inbred mice has also allowed investigation of which subsets are important for protective immunity. Such experiments are not possible in outbred domestic animals, but similar questions may eventually be answered by using progeny derived from embryo splitting and cloning techniques.

6. CYTOKINE ANALYSES

6.1 Introduction

In the mouse and in man, infection with helminth parasites has been shown to be associated with increases in the cytokines which are produced by Th2 cells (Finkelman and Urban, 1992) (reviewed in Chapter 1). T cell clones have been isolated from cattle infected with *F. hepatica* and, although most clones were found to express a wide range of cytokines and were thus classified as Th0 cells, some clones expressed IL-4 and little or no IL-2 or IFNγ (Brown *et al.*, 1994b). It is still unclear whether Th1 and Th2 subsets exist in cattle. The above study and other, similar studies in cattle infected with *B. bovis* (Brown and Logan, 1992; Brown *et al.*, 1993a, 1993b), employed cloned cells and it is known that the cloning environment can influence the type of cell subsets present. There have been no previous studies which have investigated cytokine secretion profiles *in vivo* in helminth-infected cattle. The aim of this part of the study was to investigate cytokine secretion profiles following infection with *D. viviparus.*

IL-1 is synthesised by many cell types, in particular monocytes and macrophages, and acts as a mediator of many biological activities, including numerous immunoregulatory functions (reviewed by Oppenheim *et al.*, 1986). Two related gene products, IL-1α and IL-1β, exist and these have the same biological activities and bind to the same receptor (Dower *et al.*, 1986; Kilian *et al.*, 1986). IL-1β is secreted by bovine monocytes (Werling *et al.*, 1995) and, using RT-PCR, mRNA for bovine IL-1α and IL-1β has been detected in $CD4^+$ cells (Covert and Splitter, 1995). *In vitro* studies demonstrated that recombinant bovine (rBo) IL-1β acted as a modulator of IL-2-induced immunoglobulin secretion, but did not induce proliferation of bovine B cells (Collins and Oldham, 1995). Administration of rBoIL-1β to cattle, activated lymphocytes and primed them for subsequent stimulation by T cell and B cell mitogens (Stabel *et al.*, 1993). Human and murine IL-1 are thought to enhance transcription of a number of factors released from activated T cells (Mizel, 1987). High affinity receptors for IL-1 are present on murine Th2, but not Th1, lymphocytes (Greenbaum *et al.*, 1988). Moreover, the activation of Th2 cells by IL-4 was demonstrated

to be dependent on the presence of IL-1 (Greenbaum *et al.*, 1988) and a bovine IL-1-dependent, IL-4-producing $CD4^+$ T cell clone has been isolated (Stevens *et al.*, 1992).

IL-6 is also a pro-inflammatory cytokine which has many biological activities. It is primarily produced by macrophages and also by T cells and B cells (Titus *et al.*, 1991). IL-6 induces the growth and differentiation of T cells and B cells and IL-1 and IL-6 act synergistically in their induction of T cell activation (reviewed by Titus *et al.*, 1991). Bovine IL-6 has been cloned and sequenced (Droogmans *et al.*, 1992) and shown to be expressed by Con A stimulated $CD4^+$ cells (Covert and Splitter, 1995).

IL-2 is produced by T cells within hours of stimulation by an antigen or mitogen (Smith, 1988) and plays a central role in the regulation of growth, development and activity of cells in the immune systems of mouse and man (Smith, 1988). Murine Th1 and Th2 cells respond to IL-2 (Greenbaum *et al.*, 1988). A growth factor, later identified as IL-2, was isolated from Con A-stimulated bovine PBMC by Brown and Grab (1985). This cytokine supported proliferation of Con A-derived T cell blasts, and, continuous growth of T cell clones, for over 6 months. Other studies showed that long term cultures of bovine cell lines were IL-2 dependent (Miller-Egde and Splitter, 1984). Bovine IL-2 has been cloned, sequenced and expressed (Cerretti *et al.*, 1986a). rBoIL-2 induced proliferation of activated B cells (Collins and Oldham, 1993, 1995) and induced secretion of all classes of immunoglobulins (Collins and Oldham, 1995).

Interleukin-4 is produced by T cells, mast cells and basophils (Gollob and Coffman, 1994). It is produced by Th0 and Th2 cells, but not Th1 cells (Mosmann and Coffman, 1989). T cells require the presence of IL-4 in order to differentiate into Th2 cells (Finkelman and Urban, 1992). For example, in *N. brasilienses* infection, the levels of IL-5, IL-9 and IL-10 were significantly reduced in IL-4 knock-out mice, compared with normal mice (Kopf *et al.*, 1993). IL-4 has been shown to act as a T cell growth factor in mice (Grabstein *et al*, 1987) and humans (Spits *et al.*, 1987). It has a major role in the activation of B cells, promoting proliferation and differentiation. In particular, murine IL-4 has been demonstrated to promote production of IgG1(Vitetta *et al.*, 1985) and IgE (Coffman and Carty, 1986). Together with IL-3, it is also a growth factor for mast cells (Mosmann *et al.*, 1986). Bovine IL-4 has been cloned (Heussler *et al.*, 1992) and was shown to regulate both T and B cell responses (Estes *et al.*, 1995). In the presence of costimulators, IL-4 increased production of IgM, IgG1 and IgE from bovine B cells (Estes *et al.*, 1995).

IFNγ is produced by T cells in response to stimulation with antigen or mitogen and, amongst other functions, has potent anti-viral and anti-proliferative effects (Entrican *et al.*, 1989). It acts on macrophages and monocytes to up-regulate processes known to be beneficial to phagocytosis of opsonized micro-organisms (Entrican *et al.*, 1989). It also acts on T cells to stimulate IL-2 receptor expression, moreover, IL-2-bound receptors upregulate IFNγ secretion by T cells (Gajewski and Fitch, 1988). However, IFNγ selectively inhibits the growth of Th2 cells (Gajewski and Fitch, 1988). Bovine IFNγ has been sequenced and the amino acid homology is 63% with human, and 47% with murine, IFNγ (Cerretti *et al.*, 1986b). It has been demonstrated that T cells were the major source of IFNγ in the bovine. Removal of these cell types from Con A- or PHA-stimulated PBMC abolished IFNγ production (Ishikawa *et al.*, 1994). RboIFNγ can either enhance or suppress production of certain immunoglobulins by B cells. For example, rboIFNγ promoted IgG2 production by activated B cells, but suppressed IgM secretion (Estes *et al.*, 1994).

TNFα is secreted by macrophages and is important in non-antibody-dependent cytotoxicity (Titus *et al.*, 1991). It is an important chemotractant and activator of neutrophils and eosinophils (Billingham, 1987). Bovine TNFα has been cloned and expressed (Cludts *et al.*, 1993). In *P. haemolytica*-infected calves, increased levels of TNFα were detected in the serum (Pace *et al.*, 1993; Horadagoda *et al.*, 1994) and increased mRNA for TNFα was detected in alveolar macrophages (Yoo *et al.*, 1995a, 1995b).

IL-5 is produced by murine Th2 cells (Mosmann and Coffman, 1989), has been shown to enhance IgA synthesis by B cells (Murray *et al.*, 1987) and, together with IL-4, is involved in regulation of IgE production (Takenaka *et al.*, 1993). IL-5 is essential for eosinophilopoiesis (Sher *et al.*, 1990; Takenaka *et al.*, 1993).

Murine IL-10 is produced by Th2 cells (Sher *et al.*, 1991), $\gamma\delta^+$ T cells (Hseih *et al.* 1996), B cells and mast cells (Mossman and Moore, 1991). It inhibits cytokine (IFNγ and IL-2) synthesis by Th1 cells and $CD8^+$ lymphocytes (Sher *et al.*, 1991). Bovine IL-10 has been cloned and expressed (Hash *et al.*, 1994). However, bovine IL-10 mRNA was found to be expressed by Th0, Th1 and Th2 clones and rBoIL-2 inhibited proliferation of Th0, Th1 and Th2 subsets (Brown *et al.*, 1994c) (reviewed in Chapter 1). IL-10 inhibits proliferation of bovine T cells via an effect on APC (Brown *et al.*, 1994c). IL-10 has also

been shown to promote switch-recombination from the IgM to the IgG isotype in human B cells (Malisan *et al.*, 1996).

The aim of this investigation was to determine which cytokines were important in the regulation of the immune response to *D. viviparus*. The levels of cytokines in supernatants from *in vitro* culture, and the expression of cytokine mRNA, were measured in PBMC, LN cells and cells collected by BAL. Cytokines were measured in cell supernatants using bioassays, which measure the levels of biologically active molecules. Cytokine bioassays can be based on various biological affects such as cell proliferation, cytotoxicity, colony formation, or induction of secretion of other cytokine/non-cytokine molecules. These assays are rarely entirely specific for a particular cytokine and can often respond to a number of cytokines and other molecules. Moreover, inhibitors present in biological samples may result in underestimation of cytokine content. Although analysis of mRNA expression provides a measure of gene transcription, it is unknown whether all the protein that is produced is biologically active. However, Degen *et al.*, (1983) found that total translatable mRNA and poly(A) sequences for actin accumulated in parallel with total protein synthesis and they suggested that elevated protein synthesis in activated bovine lymphocytes was regulated by mRNA levels. It was hoped that these two approaches to cytokine analysis would provide some indication as to which cytokines were involved in the immune response to *D. viviparus*.

6.2 Bioassays

6.2.1 Interleukin-1

A murine thymic sarcoma cell line (D10) was used (Section 2.11.1) which has been used previously to measure bovine IL-1 (Winstanley and Eckersall, 1992; Yoo *et al.*, 1995a). Experiments performed to investigate the optimum time for cell culture to enable the detection of maximum levels of IL-1 in supernatants indicated that the highest quantities of IL-1 were detected 24 h after culture of PBMC with LPS, Con A or parasite antigen

(DvDUH) (not shown). The highest quantity of IL-1 was detected when cells were stimulated with LPS, so LPS was used as a positive control in subsequent assays.

Cells were stimulated with doubling dilutions of LPS, from 10 μg ml^{-1} to 0.625 μg ml^{-1}, to determine the concentration which induced the maximum IL-1 secretion. The highest quantities of IL-1 were detected using a concentration of 1.25 μg ml^{-1} of IL-1 (not shown), therefore this concentration was used in future experiments.

6.2.1.1 IL-1 in supernatants from PBMC

Levels of IL-1 were measured in supernatants from PBMC collected fortnightly from four Group A calves, over the three infections with *D. viviparus*. The supernatants used were from cells whose viability was indicated by their ability to proliferate to Con A. The results are shown in (Figure 6.1). IL-1 was detected in supernatants collected from all the calves. There were no consistent increases in IL-1 secretion after infection and the levels fluctuated at different time points, with no obvious pattern.

In Calves 165, 172 and 175, there was less IL-1 detected in supernatants from cells cultured in medium alone, compared with cells cultured with LPS or antigen. However, the PBMC collected after the primary infection from Calf 174, on Days 1-14 and 1-63, secreted more IL-1 when cultured in medium alone, compared to culture with LPS or DvDUH. Unstimulated PBMC from Calf 174 generally secreted more IL-1 than did PBMC from the other calves. The levels of IL-1 secreted by unstimulated PBMC from Calf 174 were highest after the primary infection, moderately high after the secondary infection, but much lower after the tertiary infection.

The patterns of IL-1 secretion from antigen-stimulated cells were very similar to those for cells cultured in medium alone for all the calves, with a strong correlation demonstrated for Calves 165 and 172 and a moderate correlation for Calf 175 (Appendix 4.1). However, there was no correlation for Calf 174. The levels of IL-1 secreted by antigen-stimulated and LPS-stimulated cells were fairly similar, with moderate correlations demonstrated for Calves 165, 172 and 175.

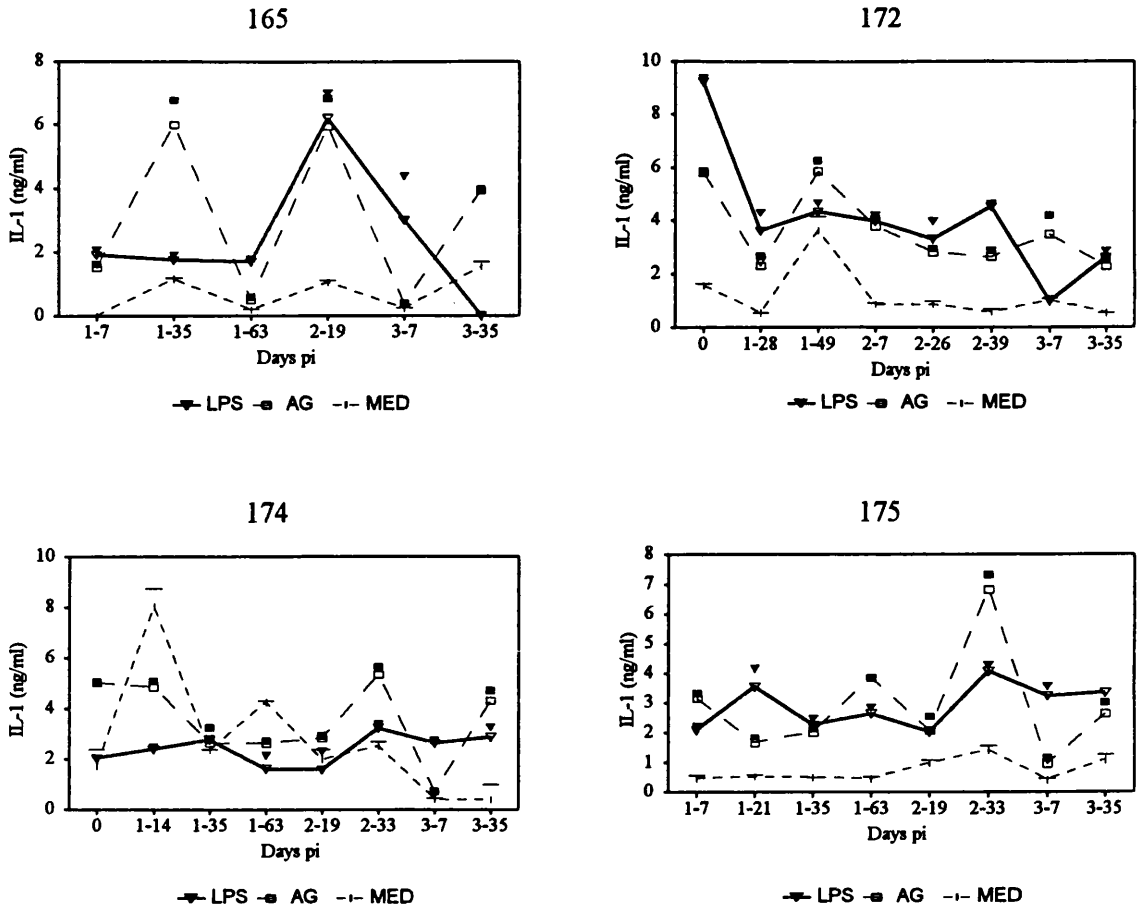

Figure 6.1 IL-1 detected in supernatants from PBMC

PBMC collected fortnightly from four Group A calves (165, 172, 174 and 175) were cultured *in vitro* with 1.25 μg ml^{-1} LPS, 10 μg ml^{-1} DvDUH or in medium alone for 24 h. Culture supernatants were assayed, in triplicate, for IL-1, using rhuIL-1 as a standard. The days pi on which the PBMC were collected are shown, with 1-, 2- and 3- indicating the days after the primary, secondary or tertiary infections, respectively. The standard deviations for the triplicate samples are indicated by markers above each line.

6.2.1.2 IL-1 detected in supernatants from LN-derived cells

Left T/B LN cells from all the calves in Group A and Group B were cultured *in vitro* with 1.25 μg ml^{-1} LPS, 10 μg ml^{-1} DvDUH or in medium alone for 24 h and the supernatants analysed for IL-1. Very low levels of IL-1 were detected in these supernatants, with

picogram (pg), rather than ng, quantities measured (Table 6.1). There were strong correlations (corr = 0.82 to 0.992) between IL-1 detected from left T/B LN cells from each calf when cultured with LPS or parasite antigen. Due to dissimilar variances (measured using the F-test), the levels of IL-1 secreted by LN cells from calves in Groups A and B could not be compared using the t test. However, there appeared to be higher levels of IL-1 secreted by LN cells from Group B calves (Table 6.1).

Group	Calf No.	LPS	Antigen	Medium
A	165	15.1 (±1.8)	15.2 (±5.0)	0
	172	13.7 (±1.8)	13.9 (±4.4)	0
	174	5.6 (±0.4)	3.7 (±0.2)	0
	175	8.7 (±0.1)	5.3 (±0.7)	24.3(±0.9)
B	169	116.4 (±35.2)	19.4 (±3.0)	1.0 (±0.2)
	170	47.3 (±16.0)	13.0 (±1.5)	0.3 (±0.2)
	176	698.1 (±144.0)	286.4 (±86.2)	42.1 (±13.9)

Table 6.1 IL-1 levels in LN-derived supernatants (pg ml^{-1})

Left T/B LN cells were cultured with 1.25 μg ml^{-1} LPS, 10 μg ml^{-1} DvDUH or in medium alone, for 24 h. The levels of IL-1 detected in the supernatants collected from the cell cultures are shown in pg ml^{-1}. 0 = IL-1 not detected.

6.2.2 Interleukin-6

A murine hybridoma cell line (B9) was used to measure bovine IL-6 (Section 2.11.2). Preliminary experiments were carried out using PBMC from an uninfected calf to optimise the conditions for detecting IL-6. Most IL-6 was detected following culture with LPS. However, high levels of IL-6 were detected when B9 cells were cultured with LPS and no culture supernatant (not shown). IL-6 was not detected in the controls where B9 cells were

cultured with Con A or DvDUH only. Therefore, LPS was not suitable as a positive control, so Con A was used to stimulate the production of IL-6 in subsequent assays. An optimum time of 24 h was chosen for future assays.

6.2.2.1 IL-6 in supernatants from PBMC

IL-6 was measured in supernatants from PBMC collected fortnightly from Calves 165, 172 and 174. IL-6 was not detected in supernatants from PBMC from Calves 172 and 174 cultured with DvDUH or in medium alone (not shown). The levels of IL-6 from cells from Calf 165, stimulated with DvDUH/medium alone, were either very low (maximum 144 pg) or were below the sensitivity of the assay (Figure 6.2). Variable quantities of IL-6 were detected in supernatants from PBMC cultured with Con A, with no obvious pattern to secretion (Figure 6.2).

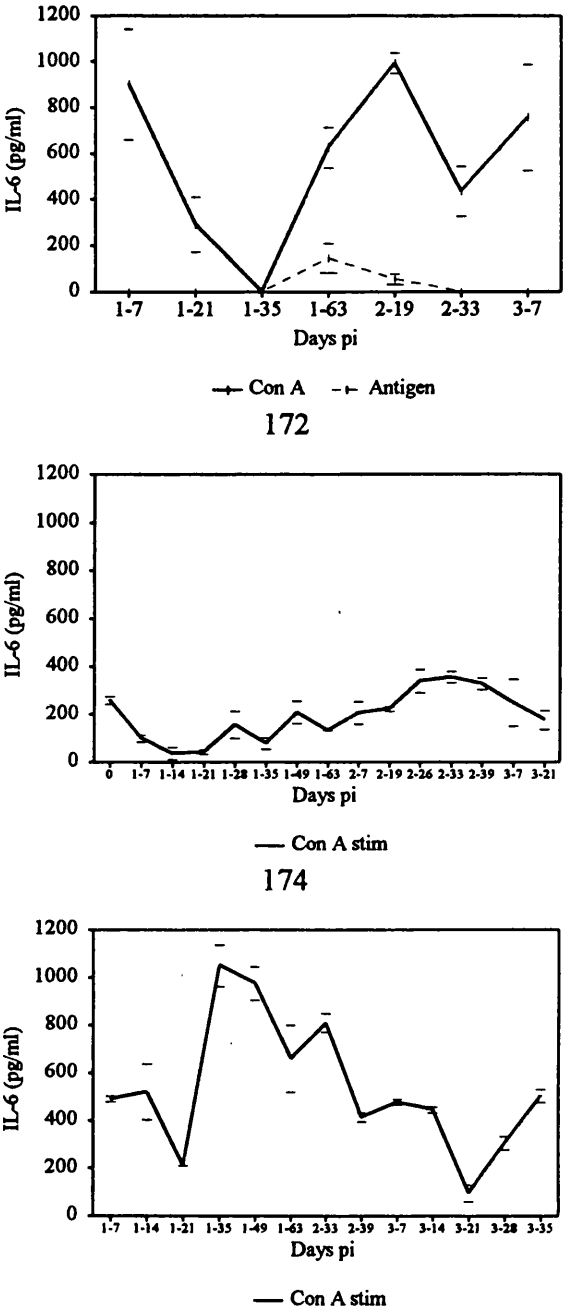

Figure 6.2 IL-6 detected in supernatants from PBMC

PBMC from Calves 165, 172 and 174 (Group A) were cultured *in vitro* with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium alone, for 24 h. Culture supernatants were assayed, in triplicate, for IL-6, using rhuIL-6 as a standard. The days pi when the PBMC were collected are shown, with 1-, 2- and 3- indicating the days after the primary, secondary or tertiary infections, respectively. The SD for the triplicate samples are indicated by error bars.

6.2.2.2 IL-6 in supernatants from LN-derived cells

Left T/B LN cells from all the calves in Group A and Group B were cultured *in vitro* with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium alone, for 24 h. The supernatants were collected and analysed for IL-6, as described above. IL-6 was not detected in any of these culture supernatants (not shown).

6.2.3 Interleukin-2

The levels of bovine IL-2 were measured, in supernatants from cells cultured *in vitro*, by Dr. R.B. Collins, Institute for Animal Health, Compton. This assay used a bovine IL-2 responsive cell line (bovine $CD8^+$ cells), which responds to bovine, porcine and human IL-2. As before, the optimal conditions for IL-2 detection were titrated in preliminary experiments (not shown). The levels of IL-2 secreted by cells cultured with Con A, or in medium alone, decreased each day. However, the levels for cells cultured with DvDUH increased each day. Supernatants collected after 48 h culture were used for subsequent bioassays.

6.2.3.1 IL-2 in supernatants from PBMC

Levels of IL-2 were measured in supernatants from PBMC collected fortnightly from Group A and Group B calves. rboIL-2 was used as a standard in the bioassays. The results are shown in Figure 6.3 and Figure 6.4. Significant quantities of IL-2 were produced by cells from each calf in response to Con A and DvDUH, although the responses were variable and there were no consistent increases after infection. There was little, if any, IL-2 detected in supernatants from cells cultured in medium alone, except for Calf 174, when the levels were higher (maximum 16.2 units ml^{-1}). The levels of IL-2 in supernatants from cells cultured with Con A or DvDUH were very similar, with moderate correlations demonstrated for six of the eight calves (corr = 0.54 to 0.986). IL-2 levels did not correlate with SI, IL-1 or IL-6 levels.

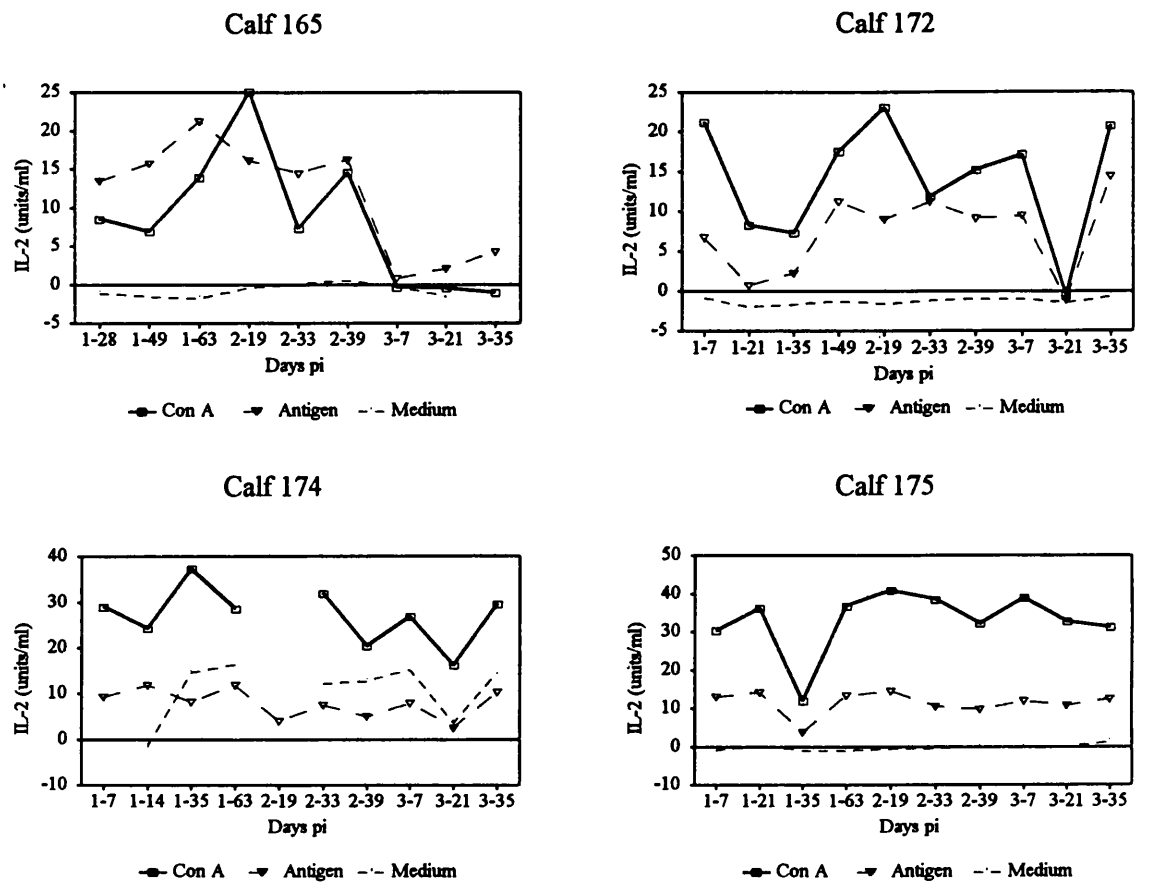

Figure 6.3 IL-2 detected in supernatants from PBMC

PBMC from Calves 165, 172, 174 and 175 (Group A) were cultured *in vitro* with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium alone, for 48 h. Supernatants were assayed in triplicate for IL-2, using a bio-assay. The days pi when the PBMC were collected are shown, with 1-, 2-, 3- indicating the days after the primary, secondary and tertiary infections, respectively.

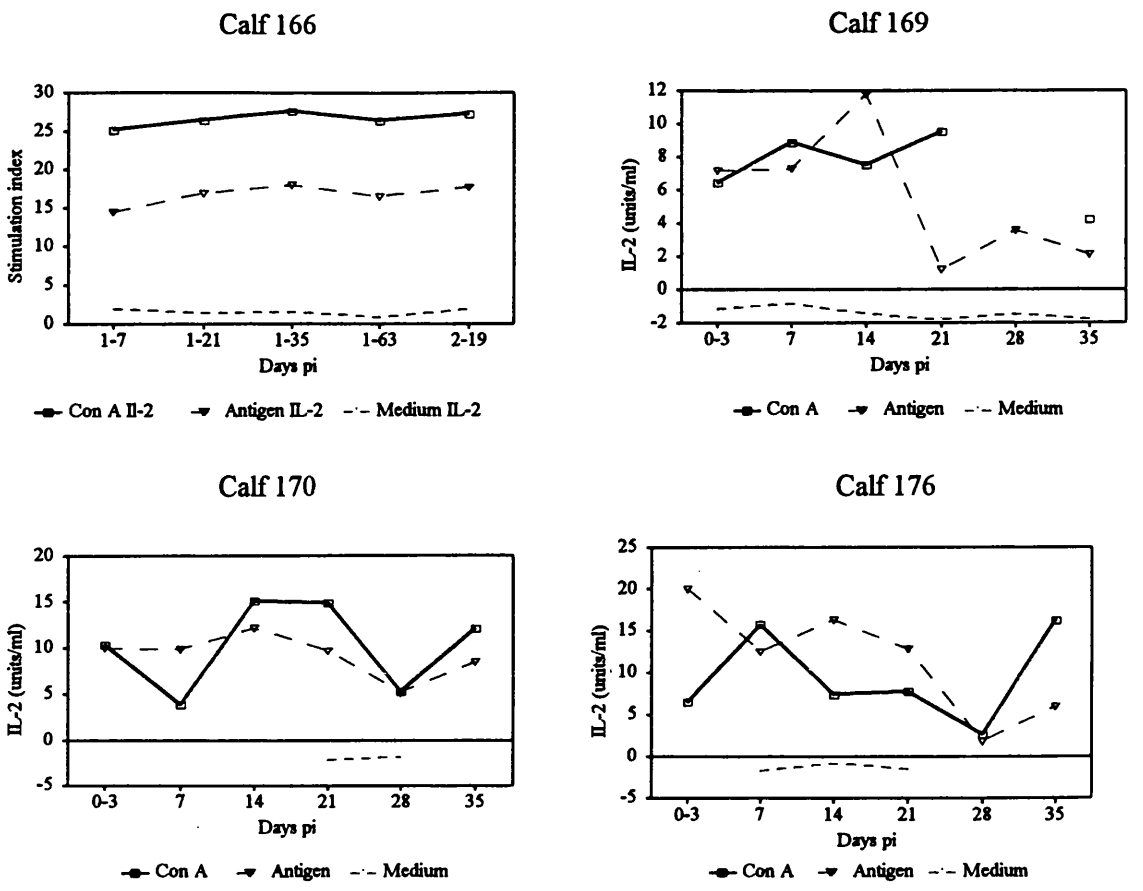

Figure 6.4 IL-2 detected in supernatants from PBMC

PBMC from calves 166 (Group A), 169, 170 and 176 (Group B) were cultured *in vitro* with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium alone, for 48 h. Supernatants were assayed in triplicate for IL-2, using a bio-assay. For Calf 166, the days pi when the PBMC were collected are shown, with 1-, 2-, 3- indicating the days after the primary, secondary and tertiary infections, respectively. For the Group B calves 0-3 indicates the day that the tertiary infection was given.

6.2.3.2 IL-2 in supernatants from LN-derived cells

Left T/B and pre-crural LN cells from all the calves in Group A and Group B were cultured *in vitro* with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium alone, for 48 h. Left T/B LN cells from the gnotobiotic calf were also analysed. The supernatants were collected and analysed for IL-2, as above. The results are shown in Figure 6.5. Very little IL-2 was produced by left T/B LN cells from infected calves in response to DvDUH, whereas cells from the pre-crural LN produced significantly higher quantities of IL-2 in response to antigen ($p < 0.01$). IL-2 is a major growth factor for T cells and, interestingly, the cells from the pre-crural LN proliferated more in response to Rpt C-A than did the T/B LN cells (Section 5.2.4.3). The levels of IL-2 in the supernatants from Con A and antigen-stimulated cells from the gnotobiotic calf were almost equal (Con A: 14.8, Antigen: 15.1, units ml^{-1}). There were no differences between levels of IL-2 in supernatants from cells stimulated with Con A from the T/B compared with the pre-crural LNs ($p > 0.1$).

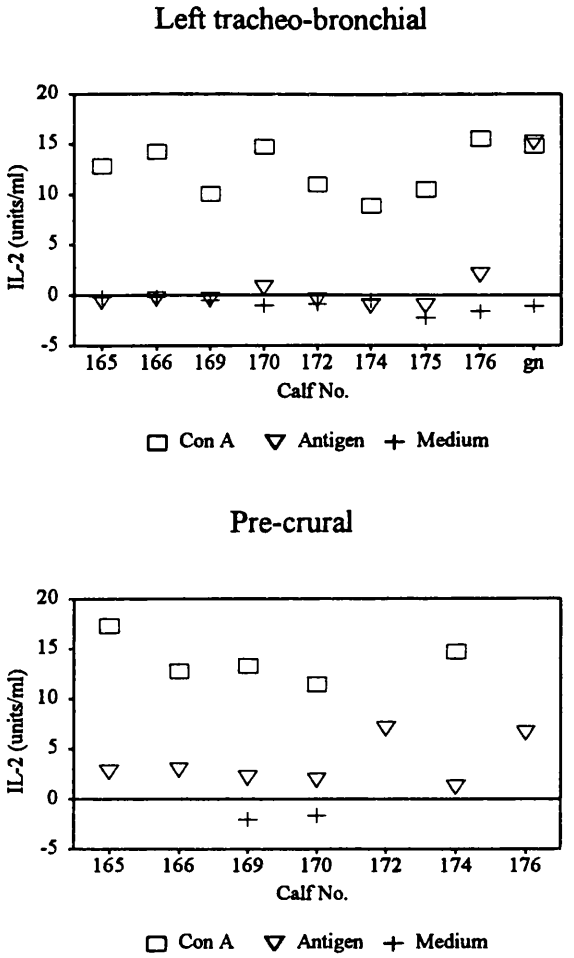

Figure 6.5 IL-2 in LN supernatants

Left T/B and pre-crural LN cells were cultured *in vitro* with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium alone, for 48 h. The supernatants were collected and analysed for IL-2 in a bio-assay, using rboIL-2 as a standard.

6.2.3.3 IL-2 in supernatants from MACS separated cells

To further define the role of different T cell subpopulations in the immune response to *D. viviparus,* the levels of IL-2 were measured in supernatants from separated $CD4^+$, $CD8^+$ and $WC1^+$ (γδ) cells, cultured *in vitro.* PBMC collected pre-infection and on Day 35 after a primary, secondary or tertiary infection were separated using magnetic beads (Section 2.14), then cultured in the presence of heterologous APC, using conditions identical to those described in Section 5.5.2. The cells were cultured in 24-well plates and incubated

for 48 h, when the cytokine supernatants were collected (as described in Section 2.10). The secretion of IL-2 was measured in $\gamma\delta^+$, $CD4^+$ and $CD8^+$ cells from Calves 165, 172, 174 and 175. The pre-infection PBMC from Calves 165 and 175 were accidentally thawed during cryopreservation, so were unsuitable for culture.

The results are illustrated in Figure 6.6 to Figure 6.8. For all three cell populations, IL-2 was only detected in supernatants from cells cultured with Con A. There was no antigen-specific IL-2. Significantly higher levels of IL-2 were secreted from $\gamma\delta^+$ T cells compared with $CD4^+$ cells ($p < 0.02$) and $CD8^+$ cells ($p < 0.02$) (Wilcoxin's test for matched pairs), while levels of IL-2 secreted by $CD4^+$ and $CD8^+$ cells were not significantly different. Cells from Calf 174 produced lower levels of IL-2 than the other calves, despite the fact that cells from this animal proliferated in response to Con A with similar SI to those from the other animals (Section 5.5).

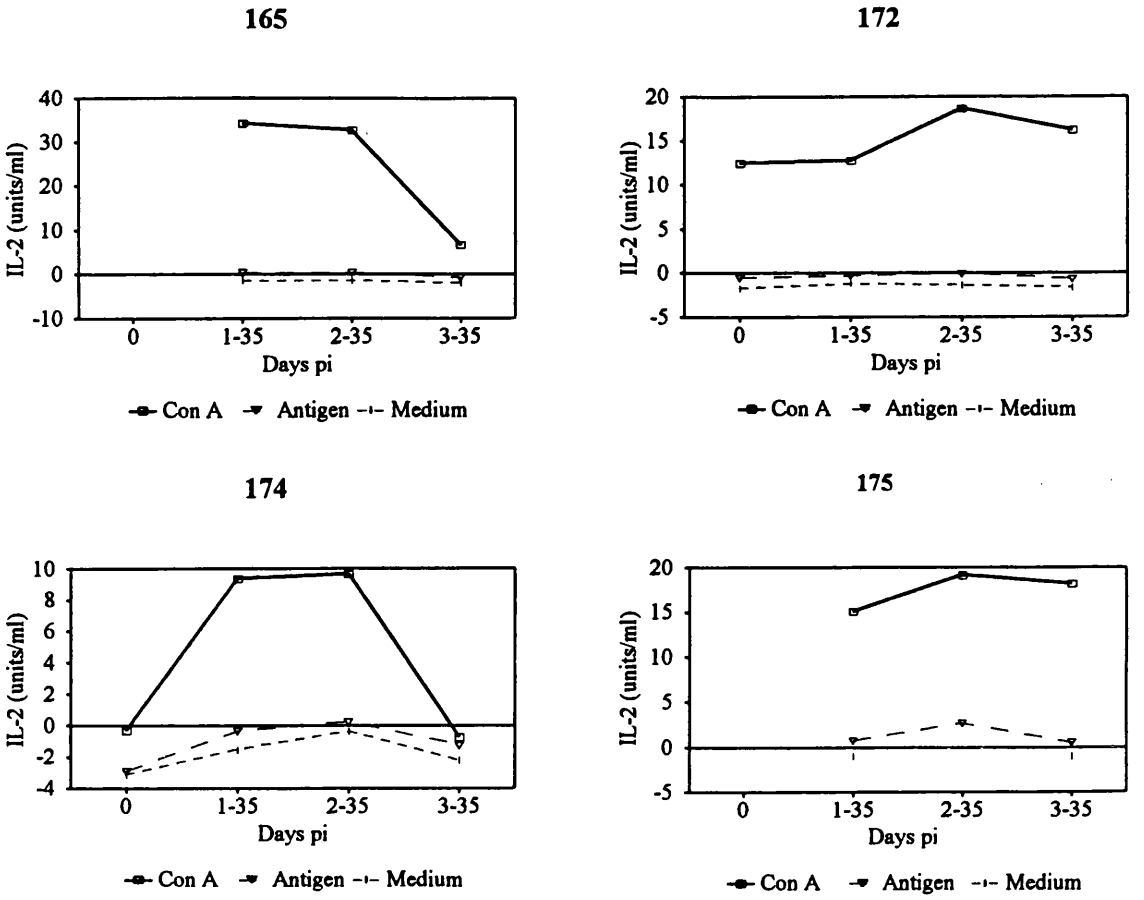

Figure 6.6 IL-2 in supernatants from $\gamma\delta^+$ T cells

PBMC, collected pre-infection (0) and on Day 35 after a primary (1-35), secondary (2-35) and tertiary (3-35) infection, were separated using magnetic beads. The separated $\gamma\delta^+$ T cells were cultured at a concentration of 2.5×10^6 ml^{-1} with heterologous APC, at a ratio of 2 per 1 $\gamma\delta^+$ T cell. The cells were cultured with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium alone, in 24-well plates. Supernatants were collected after 48 h culture and analysed for IL-2. The results are expressed as units of IL-2 per ml of supernatant.

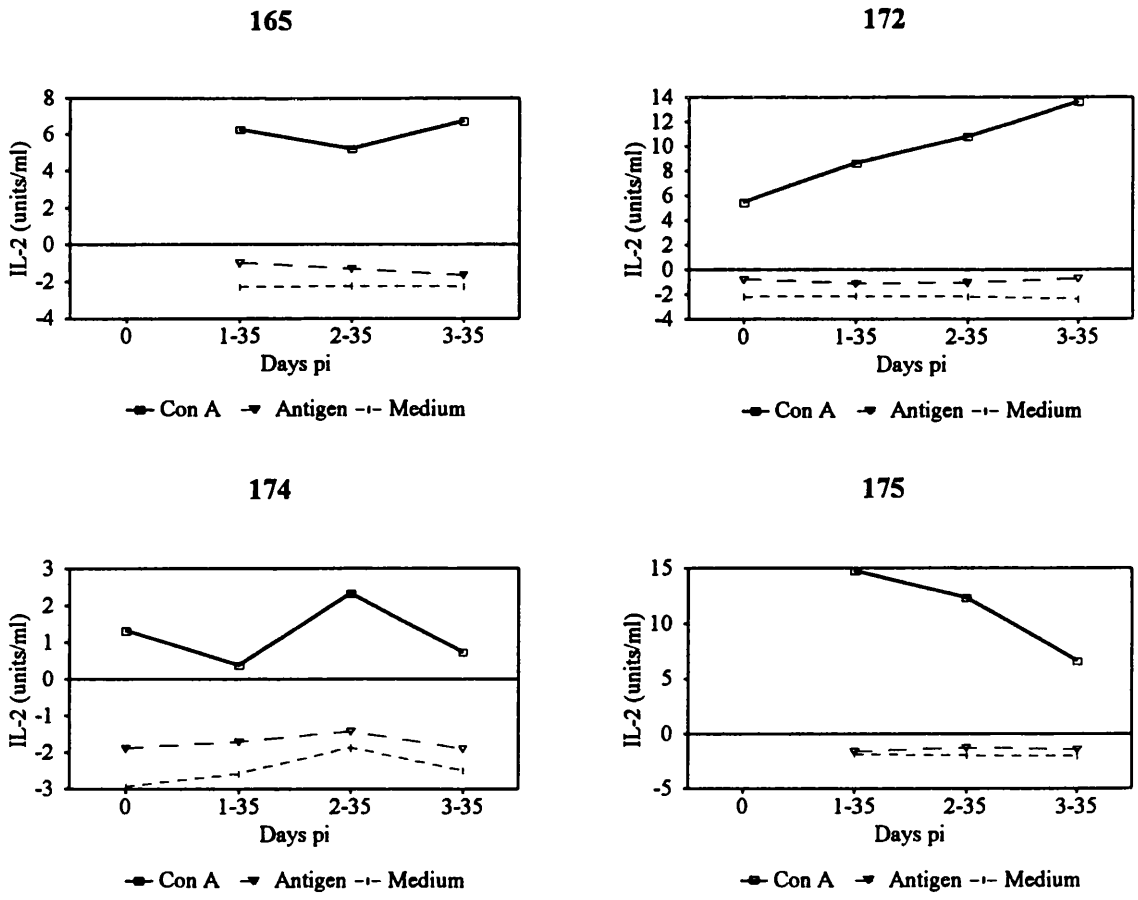

Figure 6.7 IL-2 in supernatants from $CD4^+$ cells

PBMC, collected pre-infection (0) and on Day 35 after a primary (1-35), secondary (2-35) and tertiary (3-35) infection, were separated using magnetic beads. The separated $CD4^+$ cells were cultured at a concentration of 5×10^5 ml^{-1} with heterologous APC, at a ratio of 2 per 1 $CD4^+$ T cell. The cells were cultured with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium alone, in 24-well plates. Supernatants were collected after 48 h culture and analysed for IL-2. The results are expressed as units of IL-2 per ml of supernatant.

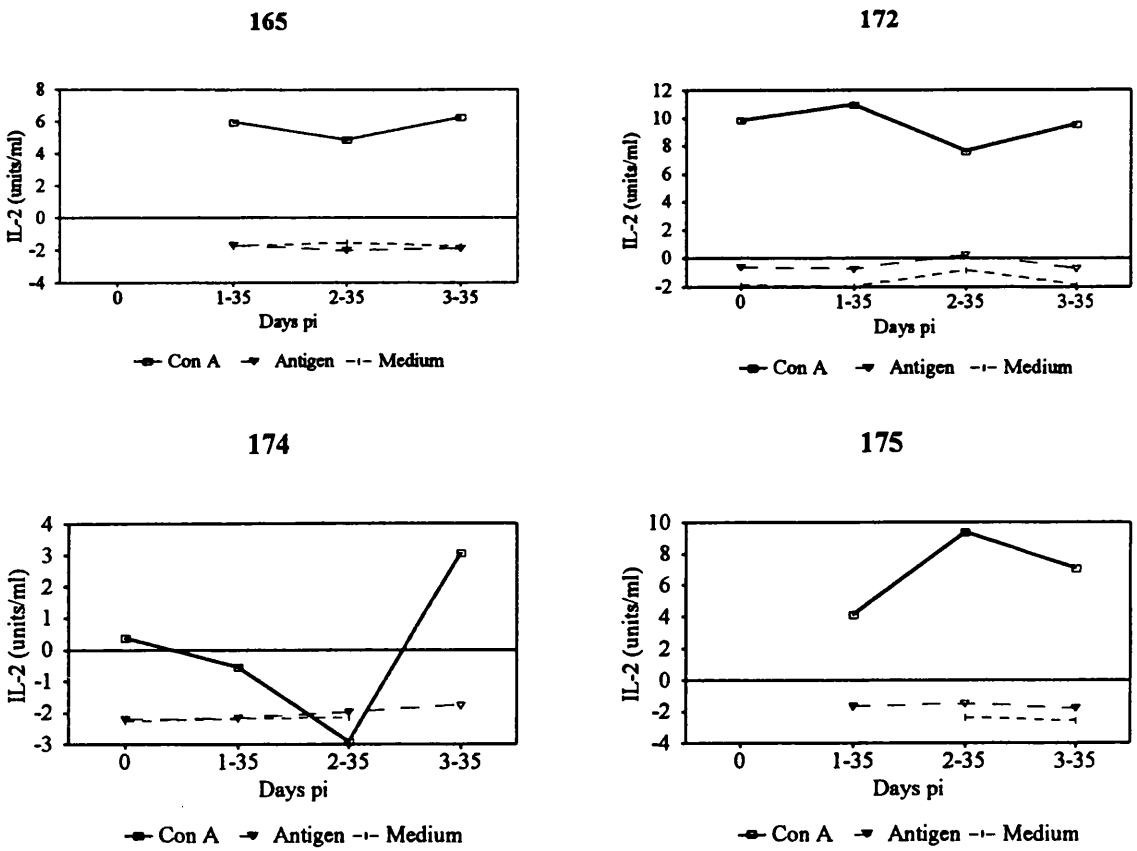

Figure 6.8 IL-2 in supernatants from $CD8^+$ cells

PBMC, collected pre-infection (0) and on Day 35 after a primary (1-35), secondary (2-35) and tertiary (3-35) infection, were separated using magnetic beads. The separated $CD8^+$ cells were cultured at a concentration of 5×10^5 ml^{-1} with heterologous APC, at a ratio of 2 per 1 $CD8^+$ T cell. The cells were cultured with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium alone, in 24-well plates. Supernatants were collected after 48 h culture and analysed for IL-2. The results are expressed as units of IL-2 per ml of supernatant.

6.2.4 Interleukin-4

The levels of bovine IL-4 were measured by Dr. R.B. Collins, Institute for Animal Health, Compton, using a B cell proliferation assay. B cells were isolated from bovine PBMC by MACS separation and were used immediately in the bio-assay. B cells also respond to IL-2, so a rabbit polyclonal antibody to bovine IL-2 (Institute for Animal Health, Compton) was included in the assays to block IL-2 activity. Preliminary experiments showed that levels of IL-4 secreted by cells cultured in medium alone increased each day (not shown), as has been found in samples in other studies (R.B. Collins, personal communication). Significant quantities of IL-4 were detected in supernatants from cells stimulated with Con A and DvDUH by 24 h (not shown), so supernatants taken at this time point were used for future bioassays.

6.2.4.1 IL-4 in supernatants from PBMC

IL-4 was detected in supernatants from PBMC collected fortnightly from all Group A and Group B calves after stimulation with Con A or DvDUH. The results are shown in Figure 6.9 and Figure 6.10. In five out of eight calves (166, 169, 170, 174 and 175), IL-4 levels were higher in supernatants from stimulated cells (Con A/DvDUH) than in those from cells cultured in medium alone. However, for three calves (165, 172 and 176) the levels of IL-4 produced following stimulation (Con A/DvDUH) were similar to those in medium alone.

The levels of IL-4 detected in supernatants from Con A- and antigen-stimulated cells were correlated for five of the eight calves (Appendix 4.2). The levels in supernatants from Con A-stimulated cells and unstimulated cells were correlated in six of the eight calves (Appendix 6.2).

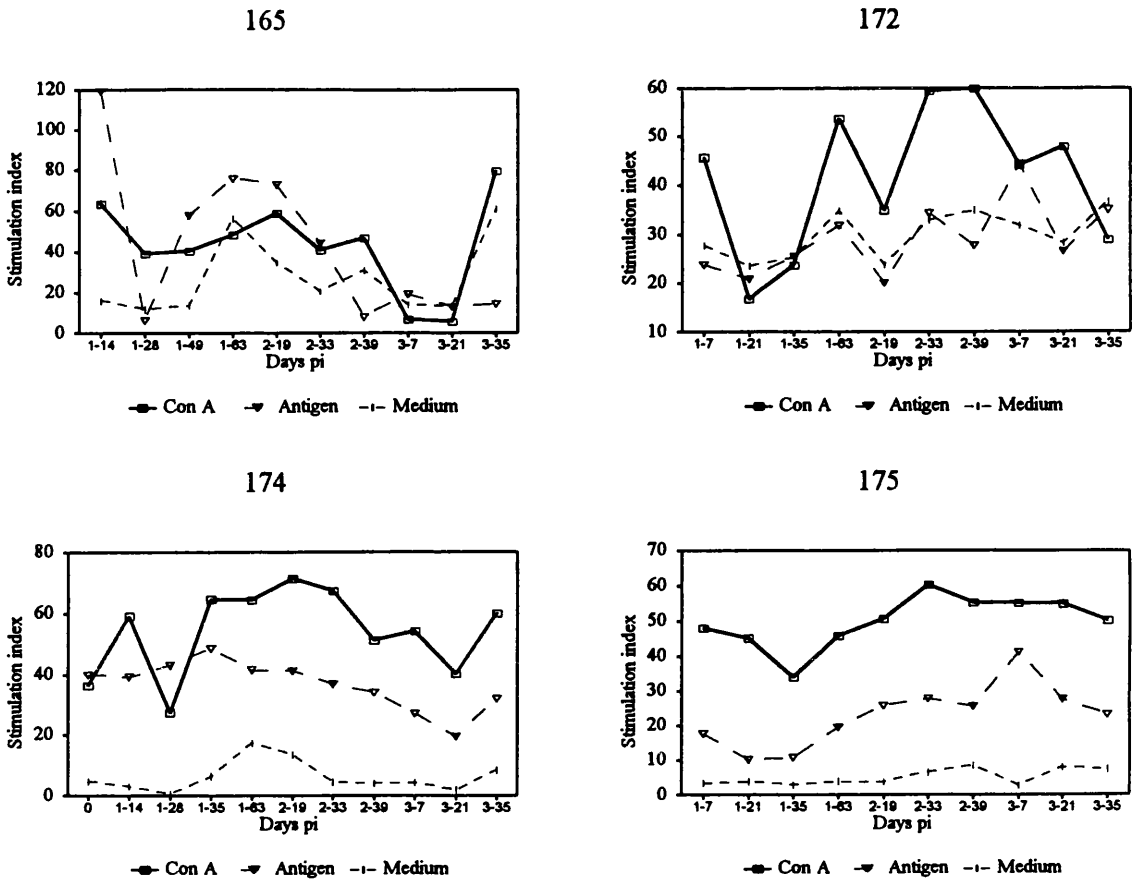

Figure 6.9 IL-4 detected in supernatants from PBMC

PBMC from Calves 165, 172, 174 and 175 (Group A) were cultured *in vitro* with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium alone, for 24 h. Supernatants were assayed in triplicate for IL-4. The results are expressed as a SI. The days pi when the PBMC were collected are shown, with 1-, 2-, 3- indicating the days after the primary, secondary and tertiary infections, respectively.

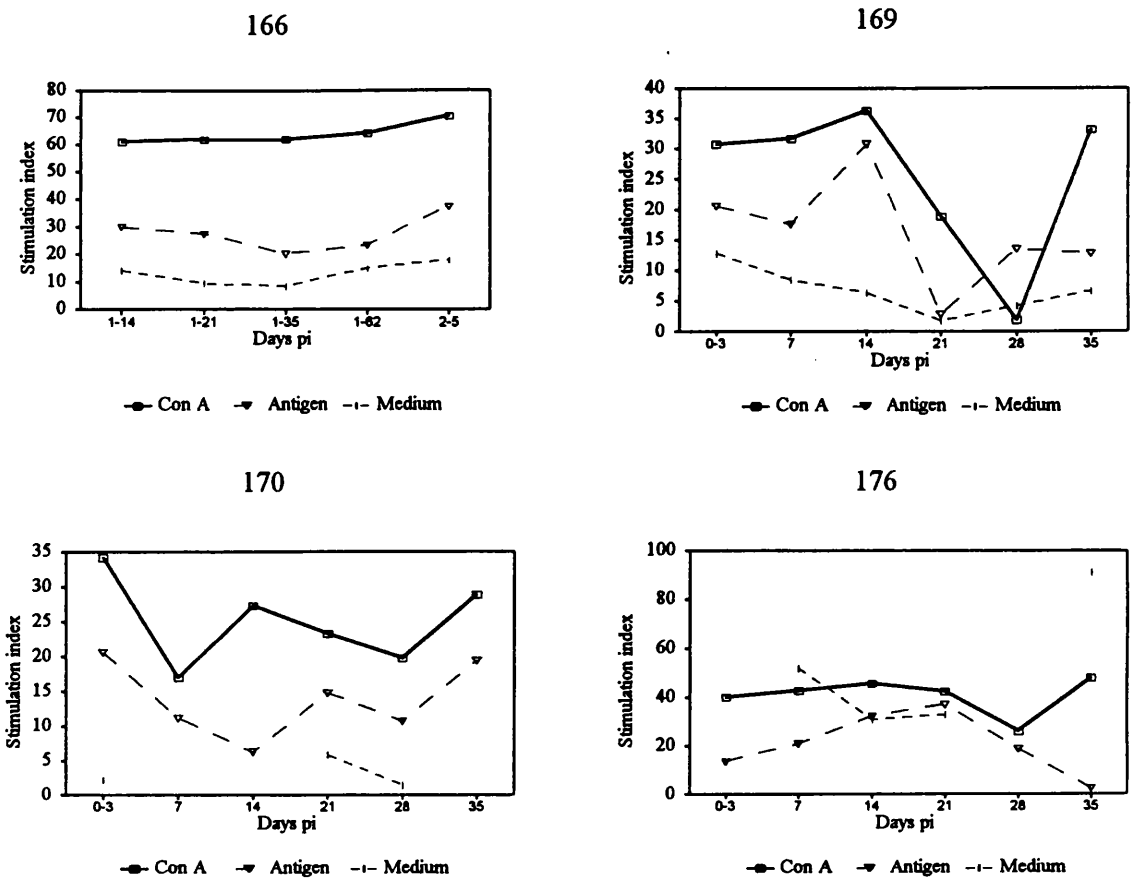

Figure 6.10 IL-4 detected in supernatants from PBMC

PBMC from Calves 166 (Group A), 169, 170 and 176 (Group B) were cultured *in vitro* with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium alone, for 24 h. Supernatants were assayed in triplicate for IL-4. The results are expressed as a SI. For Calf 166, the days pi when the PBMC were collected are shown, with 1-, 2-, 3- indicating the days after the primary, secondary and tertiary infections, respectively. For the Group B calves, 0-3 indicated the day that the tertiary infection was given.

6.2.4.2 IL-4 in supernatants from LN-derived cells

Left T/B LN-derived cells from all the calves in Group A and Group B were cultured *in vitro* with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium alone, for 24 h. The supernatants were collected and analysed for IL-4, as above. The results are shown in Figure 6.11. Significant quantities of IL-4 were detected in the LN supernatants from cells cultured with Con A, antigen and in medium alone, but the levels between these groups were not significantly different ($p > 0.1$). Similarly, the quantities of IL-4 detected in supernatants from cells collected after a primary (Group B, Calves 169,170 and 176) or tertiary (Group A, Calves 165, 172, 174 and 175) infection were not significantly different.

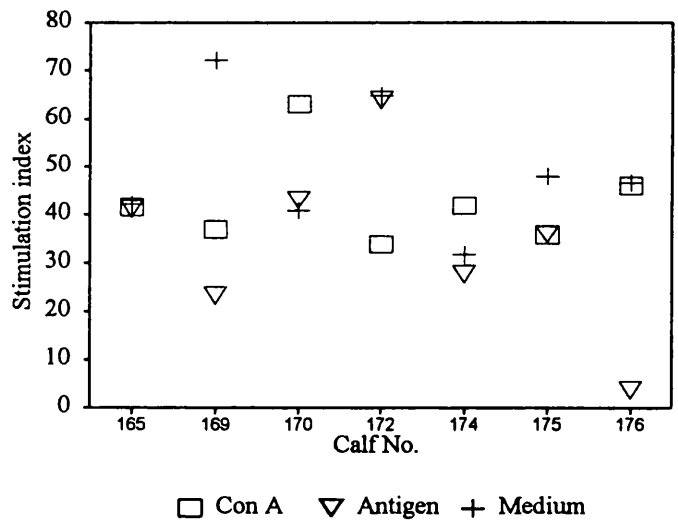

Figure 6.11 IL-4 in LN supernatants

Left T/B LN cells were cultured *in vitro* with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium alone, for 24 h. The supernatants were collected and analysed for IL-4 in a bio-assay. The results are expressed as SI.

6.2.4.3 IL-4 in supernatants from MACS separated cells

The levels of IL-4 were measured in supernatants from separated $CD4^+$, $CD8^+$ and $WC1^+$ cells cultured *in vitro*. PBMC were separated using magnetic beads (Section 2.14), then cultured in the presence of heterologous APC, using conditions identical to those described in Section 5.5.2. The cells were cultured in 24-well plates and incubated for 48 h, when the cytokine supernatants were collected (as described in Section 2.10). The secretion of IL-4 was measured in $\gamma\delta^+$, $CD4^+$ and $CD8^+$ cells from Calves 165, 172, 174 and 175. As mentioned previously, the pre-infection cells from Calves 165 and 175 were unsuitable for culture.

The results are shown in Figure 6.12 to Figure 6.14. Generally, the highest levels of IL-4 were detected in Con A supernatants in all calves for all cell types (mean SI = 11.4 ± 13.3), the exception being the $\gamma\delta^+$ T cells from Calf 174. There was no antigen-specific IL-4 production from $CD4^+$, $CD8^+$ or $\gamma\delta^+$ T cells. The levels of IL-4 produced in response to antigen were low (mean SI = 1.9 ± 1.8) and equivalent to values for cells grown in medium alone (mean SI = 1.9 ± 2.0). Again, Calf 174 provided an exception, where the highest level of IL-4 was detected in cells grown in medium alone. For the $\gamma\delta^+$ T cells, Con A-stimulated IL-4 production was greatest after the primary and secondary infections, and decreased after the tertiary infection (Figure 6.12). For $CD4^+$ and $CD8^+$ cells, the pattern of IL-4 secretion over time was different for each calf. However, for Calves 174 and 175, the kinetics of IL-4 secretion by their $CD4^+$ and $CD8^+$ were similar (Calf 174, corr = 0.98) (Calf 175, corr = 0.93).

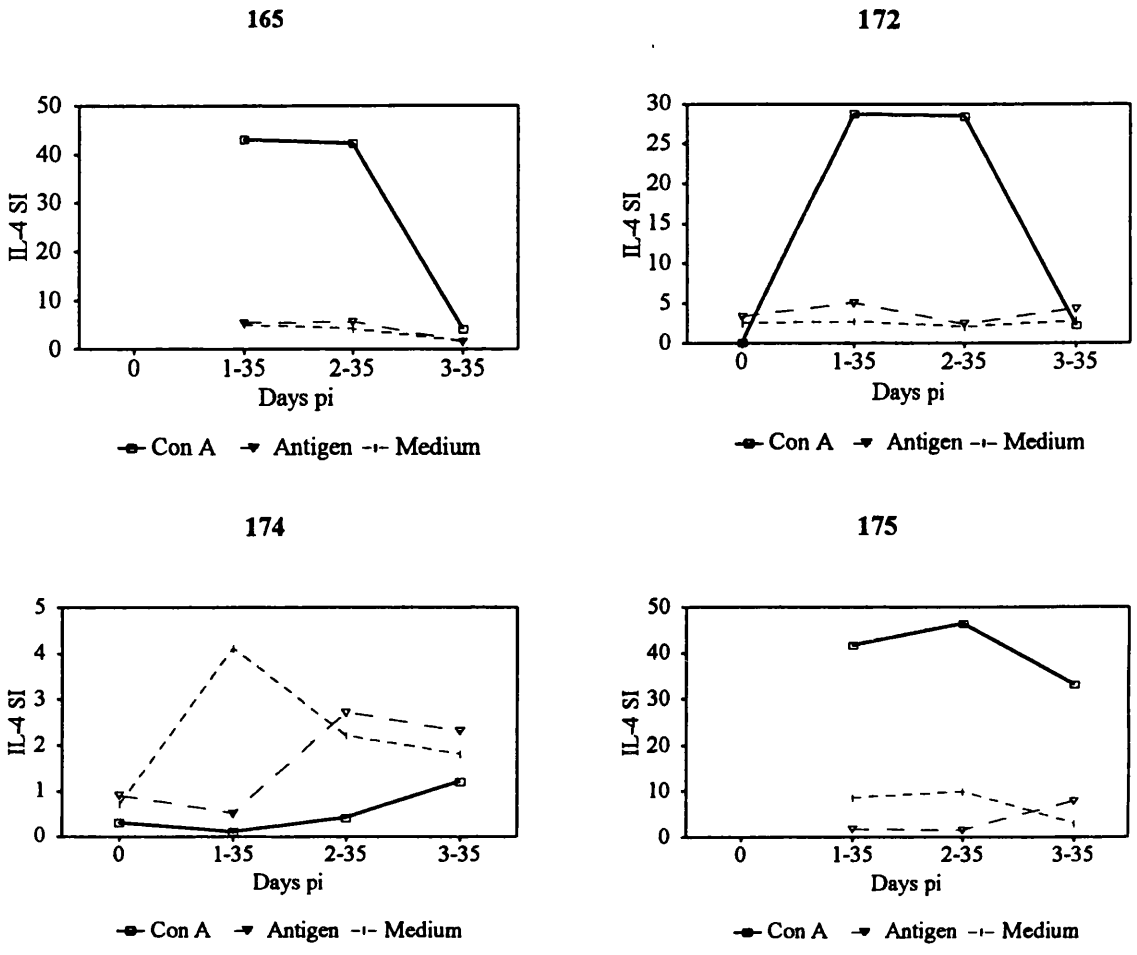

Figure 6.12 IL-4 in supernatants from $\gamma\delta^+$ T cells

PBMC, collected pre-infection (0) and on Day 35 after a primary (1-35), secondary (2-35) and tertiary (3-35) infection, were separated using magnetic beads. The separated $\gamma\delta^+$ T cells were cultured at a concentration of 2.5 x 10^6 ml^{-1} with heterologous APC at a ratio of 2 per 1 $\gamma\delta^+$ T cell. The cells were cultured with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium alone, in 24-well plates. Supernatants were collected after 48 h culture and analysed for IL-4. The results are expressed as SI.

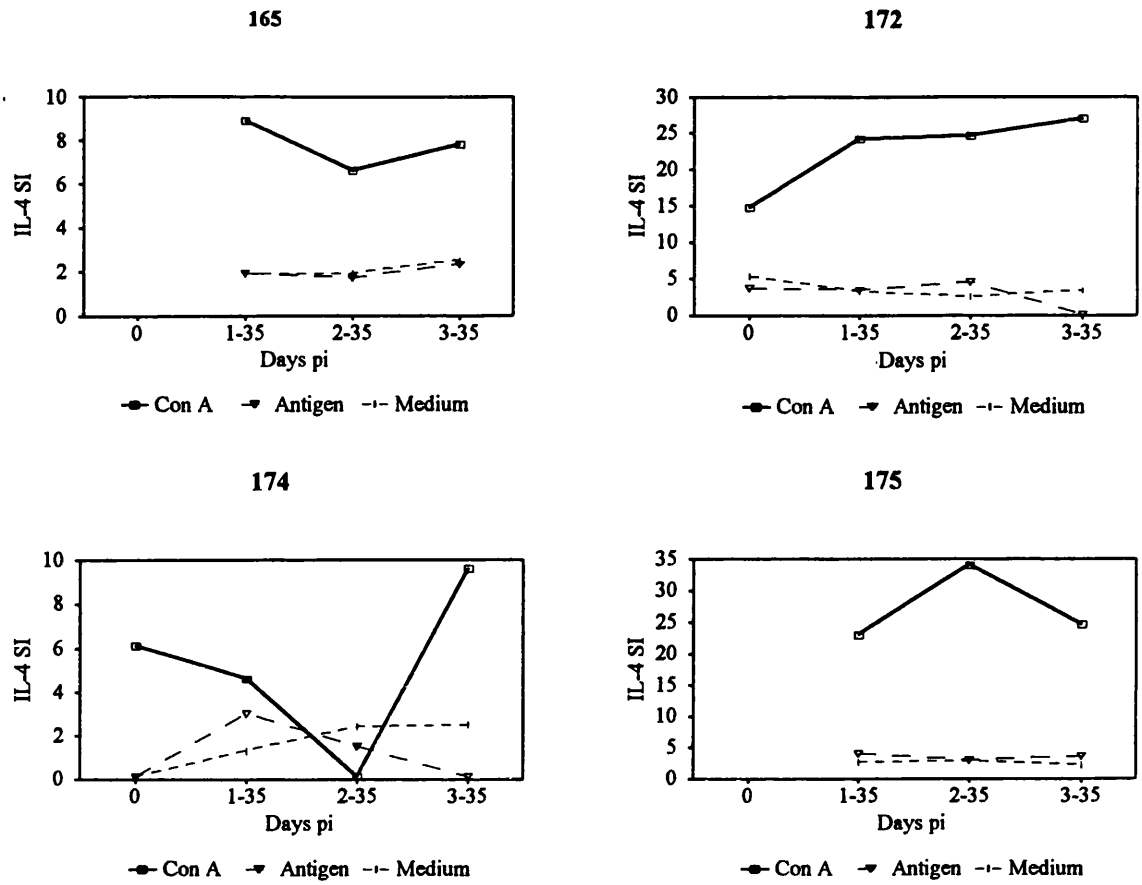

Figure 6.13 IL-4 in supernatants from $CD4^+$ cells

PBMC, collected pre-infection (0) and on Day 35 after a primary (1-35), secondary (2-35) and tertiary (3-35) infection, were separated using magnetic beads. The separated $CD4^+$ cells were cultured at a concentration of 5 x 10^5 ml^{-1} with heterologous APC at a ratio of 2 per 1 $CD4^+$ cell. The cells were cultured with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium alone, in 24-well plates. Supernatants were collected after 48 h culture and analysed for IL-4. The results are expressed as SI.

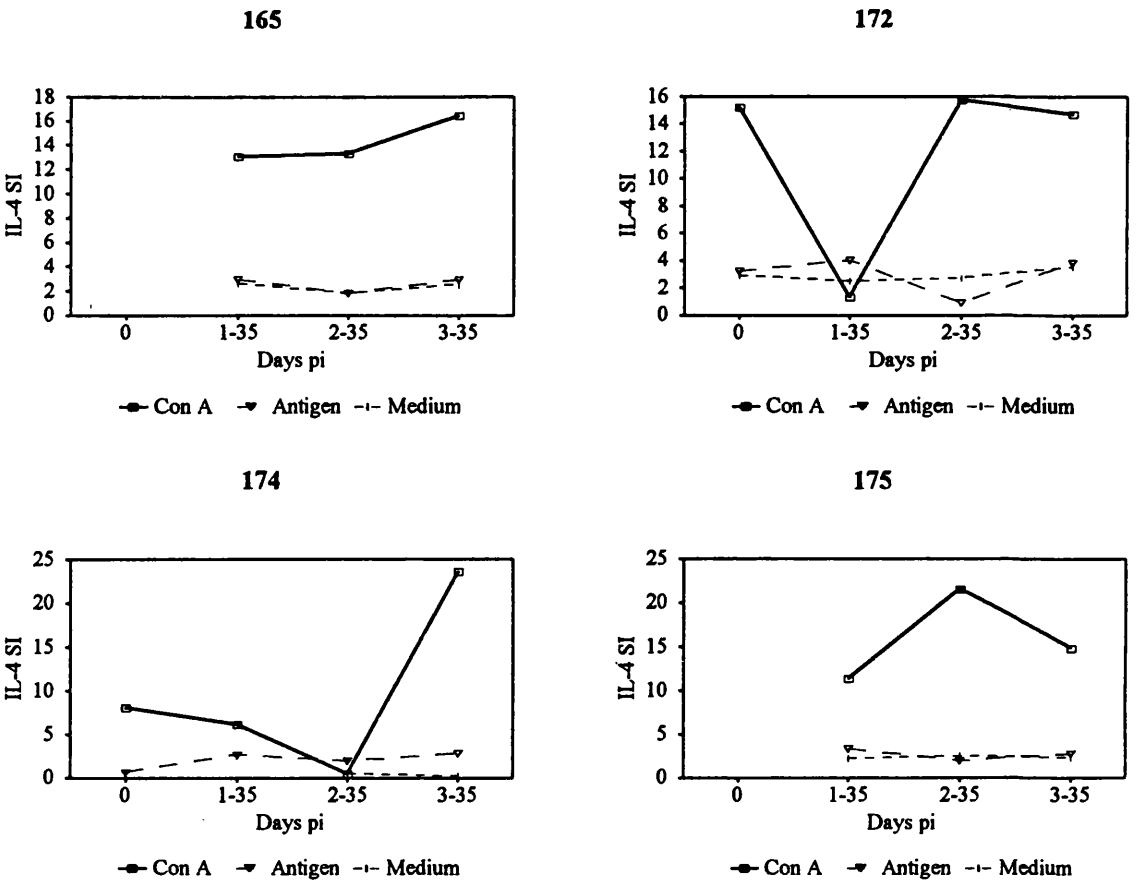

Figure 6.14 IL-4 in supernatants from CD8$^+$ cells

PBMC, collected pre-infection (0) and on Day 35 after a primary (1-35), secondary (2-35) and tertiary (3-35) infection, were separated using magnetic beads. The separated CD8$^+$ cells were cultured at a concentration of 5 x 10^5 ml^{-1} with heterologous APC at a ratio of 2 per 1 CD8$^+$ cell. The cells were cultured with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium alone, in 24-well plates. Supernatants were collected after 48 h culture and analysed for IL-4. The results are expressed as SI.

6.2.5 Interferon-γ

The availability of rboIFNγ has allowed the development of an ELISA for detection of bovine IFNγ (Rothel *et al.*, 1990). This sandwich ELISA detects IFNγ from cattle, sheep, goat and buffalo (Rothel *et al.*, 1990). Levels of IFNγ were measured using a commercially available IFNγ test kit kindly provided by Dr. R.E. Slaughter, CSL Ltd., Victoria, Australia. This ELISA-based kit was used according to the manufacturer's instructions. Initial experiments were performed to optimise the conditions for IFNγ production. Con A was shown to be the most potent inducer of IFNγ in sheep spleen cells and cloned $CD4^+$ T cells and LPS did not induce IFNγ production from these cells (Entrican *et al.*, 1989). Therefore Con A was chosen to stimulate maximum IFNγ production in this study. The levels of IFNγ were measured in duplicate, by ELISA, using positive and negative controls provided with the kit. TCM/5% FCS alone was added to 16 wells, as blanks, and the threshold was calculated as the mean OD for blank wells, plus 3 x SD. The highest quantity of IFNγ was detected in supernatants from cells cultured with Con A and the lowest in supernatants from cells cultured in medium alone (not shown). Levels of IFNγ increased with time of culture (not shown), so 72 h supernatants were chosen for subsequent analyses.

6.2.5.1 IFNγ detected in supernatants from PBMC

Levels of IFNγ were measured in supernatants from PBMC collected weekly from all the calves in Group A and Group B. RboIFNγ was kindly provided by Dr. R.B. Collins, Institute for Animal Health, Compton, and this was used as a standard so that actual quantities of IFNγ could be calculated. The results for Calves 165, 172, 174 and 175 (Group A) are illustrated in Figure 6.15 and the results for Calves 166 (Group A), 169, 170 and 176 (Group B) are illustrated in Figure 6.16. IFNγ was detected in all supernatants. The kinetics of IFNγ secretion varied between calves and there were no consistent increases

at any particular time point pi. In general, the quantities of IFNγ in supernatants from cells cultured in medium alone were lower than in supernatants from cells cultured with Con A or DvDUH, except for Calves 174 and 175. The actual levels of IFNγ, in supernatants from cells cultured with Con A or antigen, were very similar for five calves (Calves 165, 166, 174, 169 and 170) (Figure 6.15 and Figure 6.16) and moderate correlations were demonstrated Appendix 4.3. The levels of IFNγ secreted by PBMC were not correlated with SI, IL-1, IL-6, IL-2 or IL-4 levels.

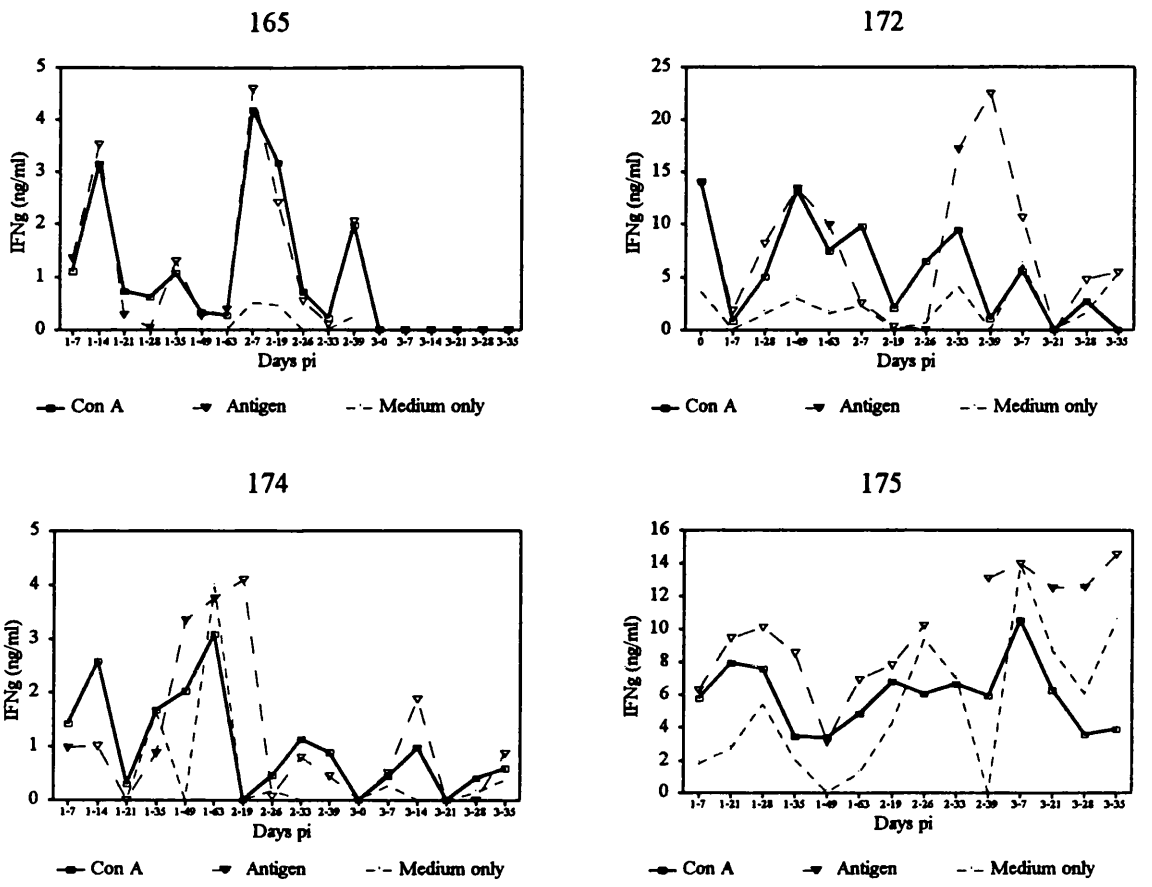

Figure 6.15 IFNγ levels in supernatants from PBMC

PBMC from Calves 165, 172, 174 and 175 (Group A) were cultured *in vitro* with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium only, for 72 h. Culture supernatants were assayed in duplicate for IFNγ, using rboIFNγ as a standard. The results are expressed as ng of IFNγ per ml of supernatant. The days pi which the PBMC were collected are shown, with 1-, 2- and 3- indicating the days after the primary, secondary or tertiary infections, respectively.

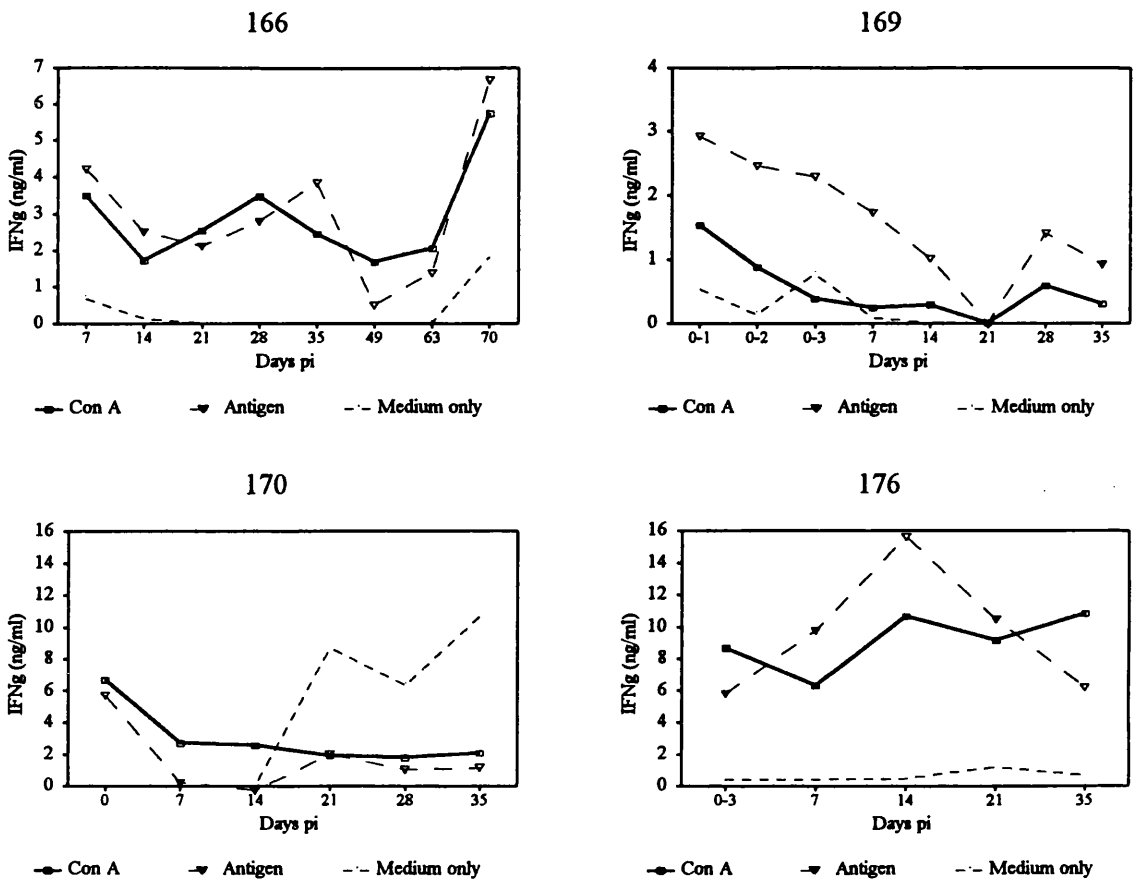

Figure 6.16 IFNγ levels in supernatants from PBMC

PBMC from Calves 166 (Group A), 169, 170 and 176 (Group B) were cultured *in vitro* with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium only, for 72 h. Culture supernatants were assayed in duplicate for IFNγ, using bovine IFNγ as a standard. The results are expressed as ng of IFNγ per ml of supernatant. For Calf 166, the days pi when the PBMC were collected are shown, with 1-, 2-, 3- indicating the days after the primary, secondary and tertiary infections, respectively. For the Group B calves, 0-3 indicated the day that the tertiary infection was given.

6.2.5.2 IFNγ in supernatants from LN-derived cells

Left T/B LN cells from all the calves in Group A and Group B were cultured *in vitro* with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium alone, for 72 h. The supernatants were collected and analysed for IFNγ, using the ELISA kit. IFNγ was not detected in any of these culture supernatants (not shown).

6.2.5.3 IFNγ in supernatants from MACS separated cells

The levels of IFNγ were measured in supernatants from separated $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T cells cultured *in vitro* from Calves 165, 172, 174 and 175. The pre-infection PBMC from Calves 165 and 175 were unsuitable for culture, as described above. PBMC were separated using magnetic beads and cultured as described previously.

The results are illustrated in Figure 6.17 to Figure 6.19. For all three populations, by far the highest quantity of IFNγ was detected in supernatants from cells cultured with Con A, with much lower quantities detected in supernatants from cells cultured with antigen and minimal amounts detected in supernatants from cells cultured in medium alone. In most cases, the pattern of secretion from antigen-stimulated cells followed that for Con A-stimulated cells. The responses in Con A-stimulated cells will be described in more detail. The highest quantity of IFNγ was detected in supernatants from separated $\gamma\delta^+$ T cells (maximum 30 ng ml^{-1}), while $CD4^+$ and $CD8^+$ cells produced much less (maximum 6 and 1.3 ng ml^{-1} respectively). In the two pre-infection samples available (Calves 172 and 174), low levels of IFNγ were detected in supernatants from $\gamma\delta^+$ T cells. The highest levels of IFNγ were detected after the primary and secondary infections for all four calves and levels decreased after the tertiary infection.

In general, separated $CD4^+$ cells secreted very low levels of IFNγ and in many cases no IFNγ could be detected (e.g. Calf 165). Similarly, secretion of IFNγ by $CD8^+$ cells was low.

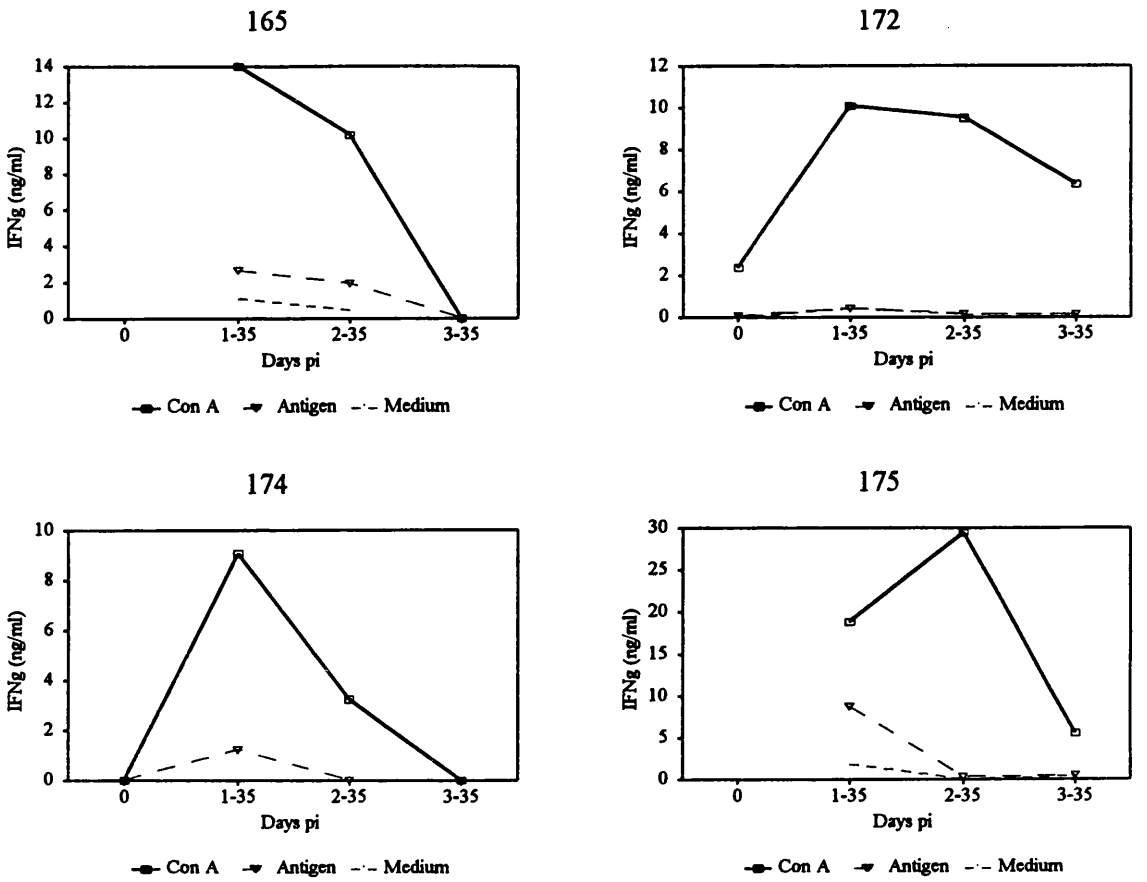

Figure 6.17 IFNγ in supernatants from γδ$^+$ T cells

PBMC, collected pre-infection (0) and on Day 35 after a primary (1-35), secondary (2-35) and tertiary (3-35) infection, were separated using magnetic beads. The separated γδ$^+$ T cells were cultured at a concentration of 2.5 x 10^6 ml^{-1} with heterologous APC at a ratio of 2 per 1 γδ$^+$ T cell. The cells were cultured with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium alone, in 24-well plates. Culture supernatants were assayed in duplicate for IFNγ, using bovine IFNγ as a standard. The results are expressed as ng of IFNγ per ml of supernatant.

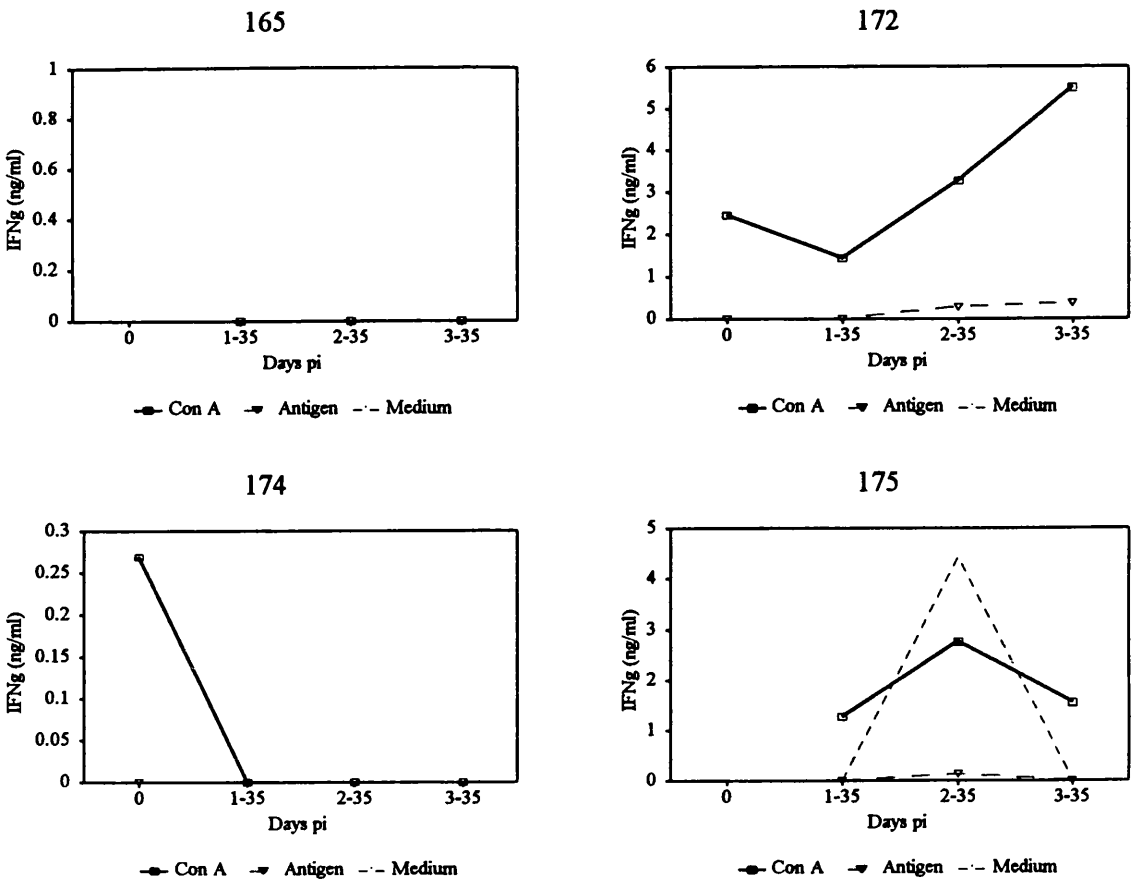

Figure 6.18 IFNγ in supernatants from CD4$^+$ cells

PBMC, collected pre-infection (0) and on Day 35 after a primary (1-35), secondary (2-35) and tertiary (3-35) infection, were separated using magnetic beads. The separated CD4$^+$ cells were cultured at a concentration of 5 x 10^5 ml^{-1} with heterologous APC at a ratio of 2 per 1 CD4$^+$ cell. The cells were cultured with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium alone, in 24-well plates. Culture supernatants were assayed in duplicate for IFNγ, using bovine IFNγ as a standard. The results are expressed as ng of IFNγ per ml of supernatant.

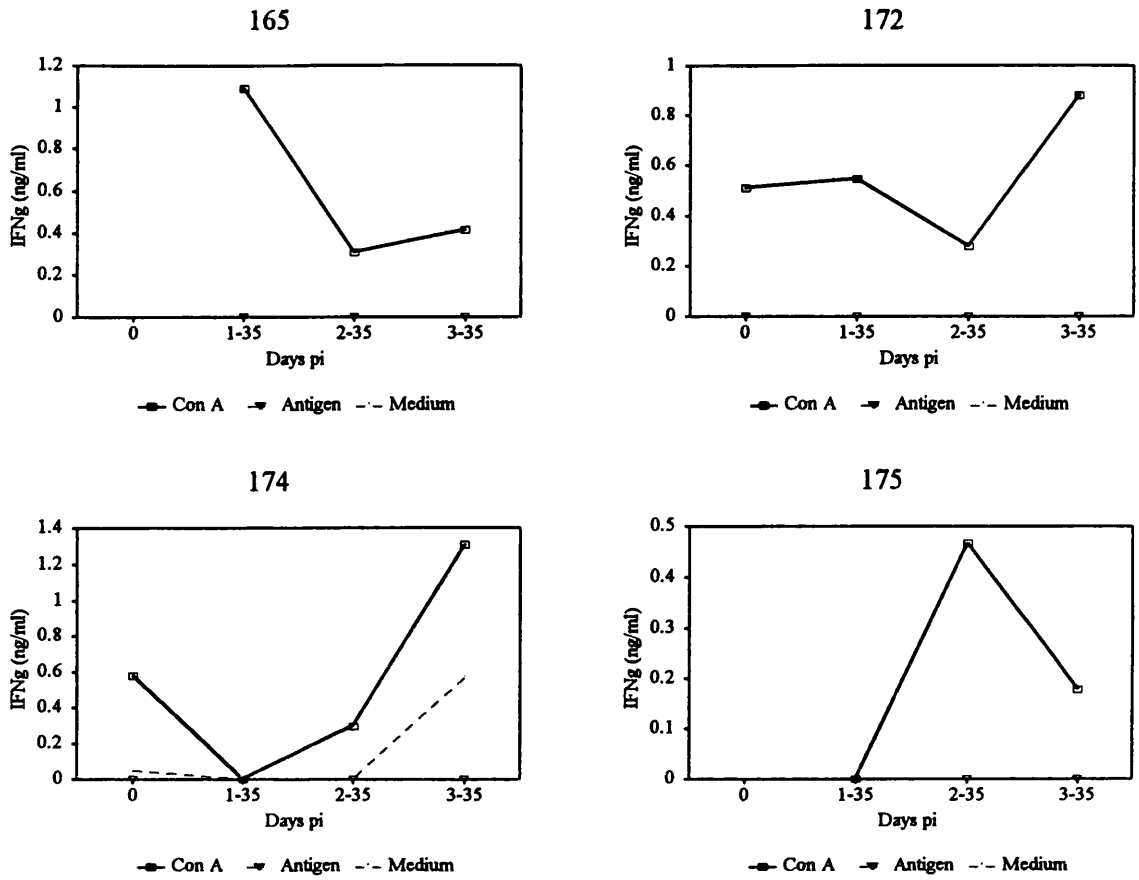

Figure 6.19 IFNγ in supernatants from CD8$^+$ cells

PBMC, collected pre-infection (0) and on Day 35 after a primary (1-35), secondary (2-35) and tertiary (3-35) infection, were separated using magnetic beads. The separated CD8$^+$ cells were cultured at a concentration of 5 x 10^5 ml^{-1} with heterologous APC at a ratio of 2 per 1 CD8$^+$ cell. The cells were cultured with 2.5 μg ml^{-1} Con A, 10 μg ml^{-1} DvDUH or in medium alone, in 24-well plates. Culture supernatants were assayed in duplicate for IFNγ, using bovine IFNγ as a standard. The results are expressed as ng of IFNγ per ml of supernatant.

6.2.5.4 Comparison between cytokine secretion and proliferative responses for MACS separated cells

To determine if the separated cell populations produced increased amounts of cytokines when they proliferated in response to Con A or DvDUH, the cytokine secretion profiles and proliferation assays results were compared. The responses for the whole PBMC populations were also compared. The results are illustrated in Figure 6.20 to Figure 6.23. Generally, the highest quantities of cytokines were secreted by the whole PBMC populations and the next highest by the separated $\gamma\delta^+$ T cells. However, higher levels of IFNγ were secreted by $\gamma\delta^+$ T cells from Calves 165 and 175, stimulated with Con A, compared with the whole PBMC population. Also, higher levels of IL-2 were secreted by Con A stimulated $\gamma\delta^+$ T cells from Calf 165. When γδ T cells proliferated, they tended to secrete increased quantities of all the cytokines. For Calves 165 and 175, strong correlations were demonstrated between SI (based on proliferation assay) and IFNγ, IL-2 and IL-4 (corr = 0.73 to 0.999). However, $\gamma\delta^+$ T cells from Calf 174 did not secrete IL-4 (Figure 6.22), and $\gamma\delta^+$ T cells from Calf 175 secreted very low levels of IL-4 (Figure 6.23). The quantities of cytokines secreted by $CD4^+$ and $CD8^+$ cells remained low, despite good proliferative responses and there were no correlations between SI and cytokine secretion in these cell populations.

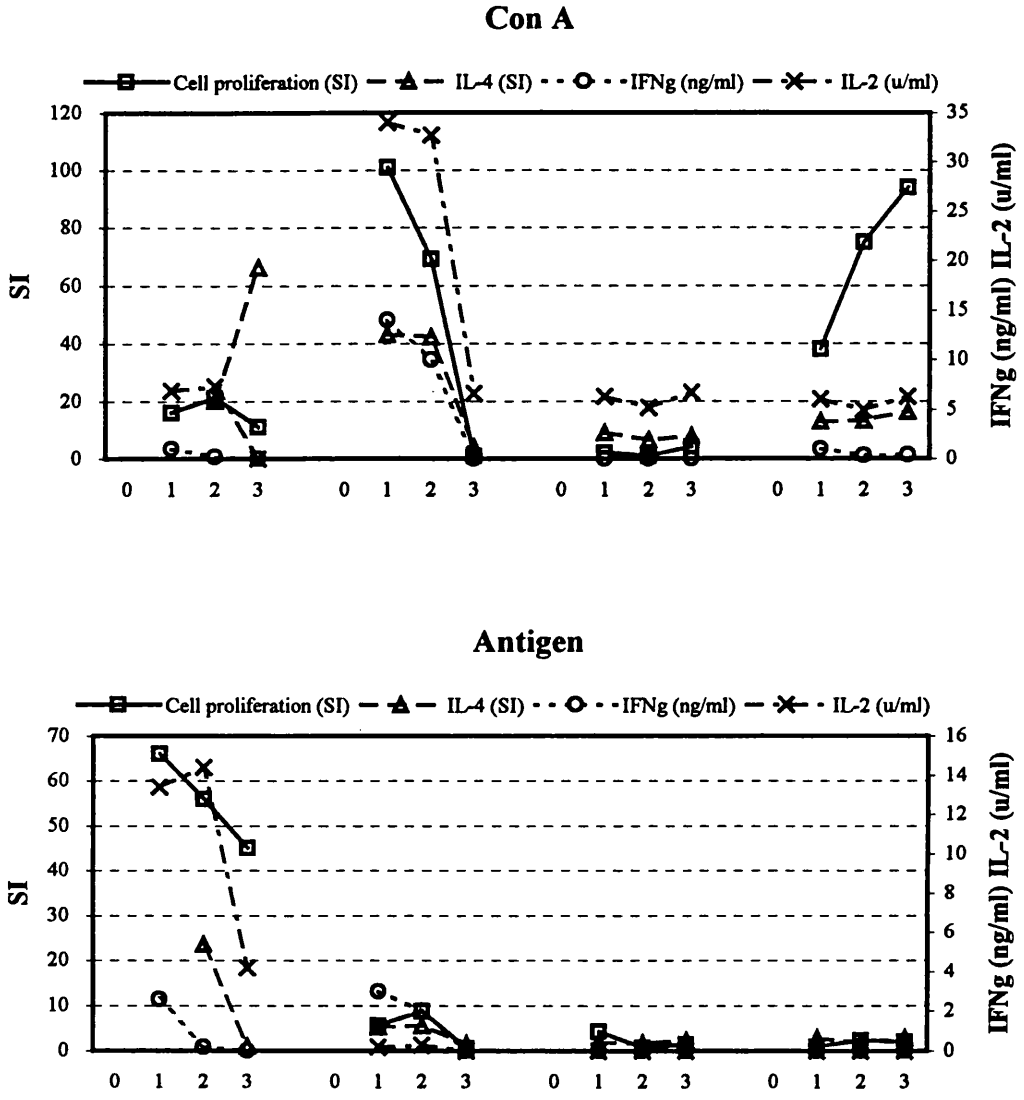

Figure 6.20 Comparison between cytokine secretion and SI for PBMC from Calf 165

PBMC, collected pre-infection (0) and on Day 35 after a primary (1), secondary (2) and tertiary (3) infection, were separated using magnetic beads. The separated cells were cultured with Con A for 3 d, or antigen for 6 d, when proliferation was assessed. Culture supernatants collected after 24, 48 or 72 h were analysed for IL-4, IL-2, and IFNγ, respectively.

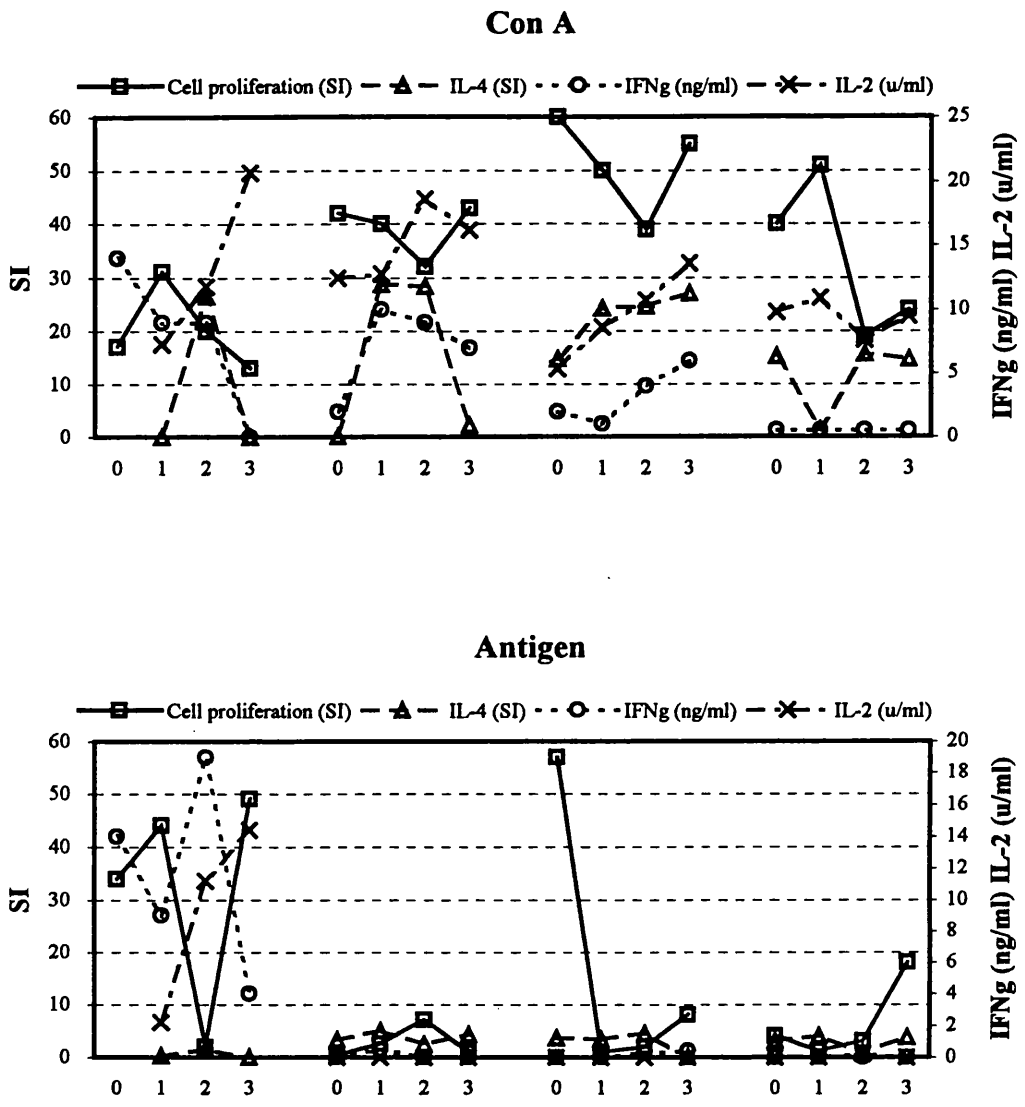

Figure 6.21 Comparison between cytokine secretion and SI for PBMC from Calf 172

For conditions, see Figure 6.20.

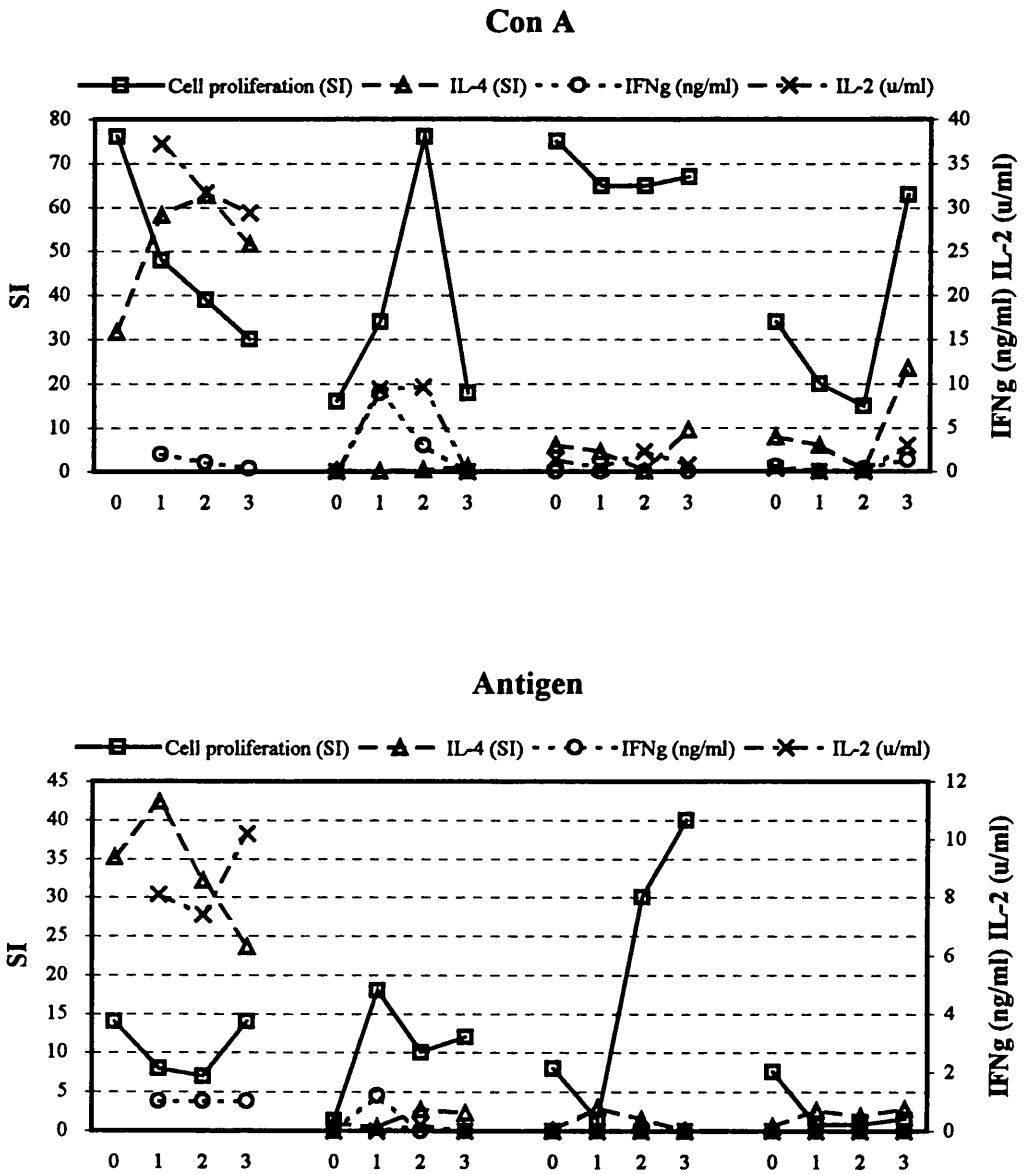

Figure 6.22 Comparison between cytokine secretion and SI for PBMC from Calf 174

For conditions, see Figure 6.20.

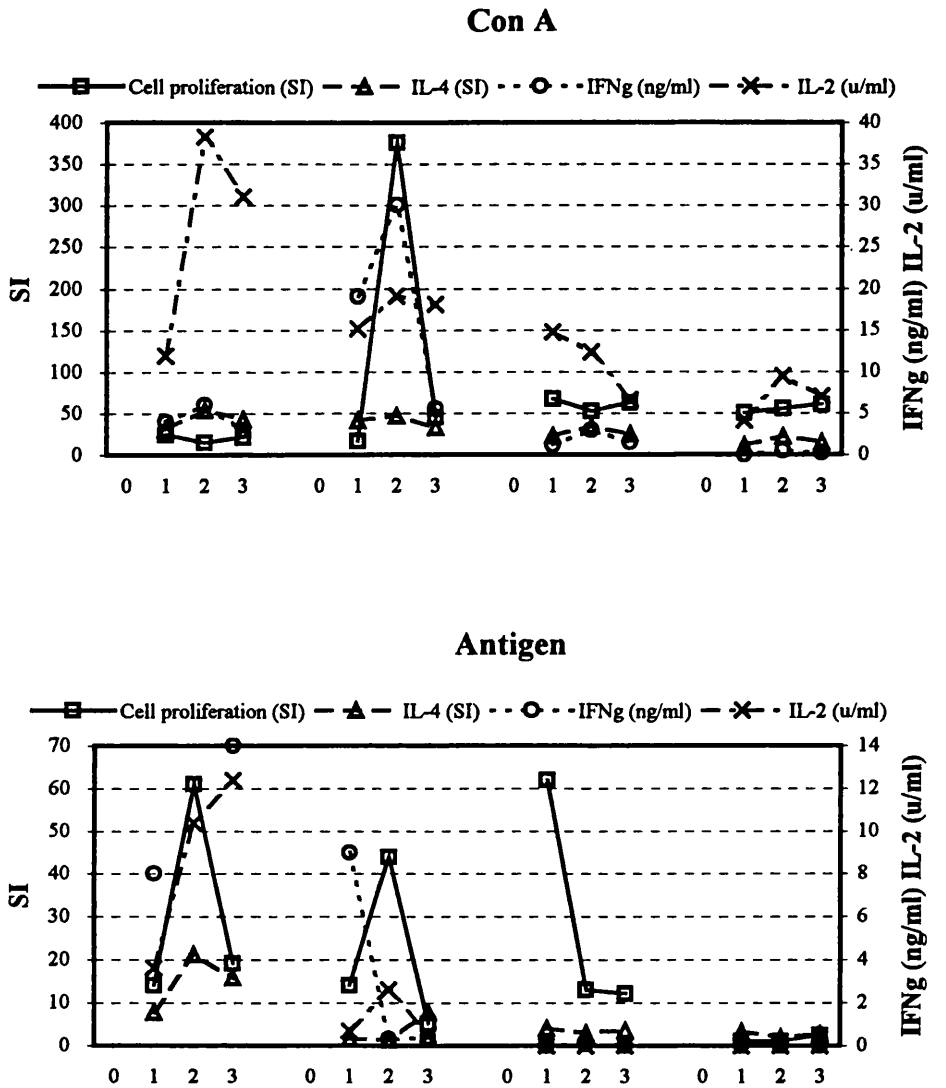

Figure 6.23 Comparison between cytokine secretion and SI for PBMC from Calf 175

For conditions, see Figure 6.20.

6.2.6 Tumour necrosis factor

In an attempt to measure bovine TNF, a murine fibroblast cell line L-929 was used to measure TNF activity in supernatants from LN-derived cells cultured with 2.5 μg ml^{-1} Con A, 1.25 μg ml^{-1} LPS, 10 μg ml^{-1} DvDUH or in medium alone. The cells were cultured for 24 or 48 h before the supernatants were collected. This assay was carried out by Dr. C. Lawrence, in the Department of Veterinary Pathology, University of Glasgow. The assay had been used routinely and successfully to measure TNF activity in supernatants from feline PBMC and LN cells (Lawrence *et al.*, 1995). No TNF activity was detected in any of the supernatants, even when the assays were repeated on supernatants from LN-derived cells from other calves. It was decided that this cell line was not responsive to bovine TNF.

6.3 Measuring mRNA for bovine cytokines

6.3.1 Northern blot analysis

In an attempt to identify differences in mRNA expression for bovine cytokines, Northern blot analysis was performed. RNA was isolated from PBMC (Section 2.15) collected pre-infection and on Day 35 after a primary, secondary or tertiary infection from Calves 165, 172, 174 and 175 (Group A). The RNA was transferred from the gel to nylon membranes and, after drying, was cross-linked to the membrane (Section 2.20.1). Specific cDNA probes were prepared for bovine IL-2, IL-4 and IL-10 (Section 2.20.2). The probes were radiolabelled (Section 2.20.3), then hybridised to the RNA overnight at 65°C (Section 2.20.4). After washing, the filters were placed in cassettes with autoradiograph film and stored at -70°C.

The cDNA probes for IL-2 and IL-4 did not hybridise to RNA from any of the calves. However, the IL-10 probe hybridised to a transcript of the expected size for bovine

IL-10 (740 bp) in PBMC from all the calves. RNA for IL-10 was detected at all time points for Calf 174 (Figure 6.24). There was very little IL-10 detected pre-infection, the highest level of hybridisation was after the primary infection, with less after the secondary and tertiary infections. The probe hybridised to RNA from the other three calves, but the degree of hybridisation was less than for Calf 174. Since the degree of hybridisation was so low, these results are nor shown. For Calf 165, IL-10 transcript was detected after the primary and secondary infections. For Calf 172, IL-10 transcript was detected after the secondary infection. For Calf 175, IL-10 transcript was detected after the secondary and tertiary infections. Only small amounts of IL-10 transcript were detected on all the autoradiographs and any differences in signal that were detected were also small and may have been due, at least in part, to unequal loading of RNA on the gel.

Despite relatively large quantities of RNA being used for the Northern analyses, no IL-2 or IL-4 and very little IL-10 were detected, therefore this technique did not appear to be sensitive enough to detected mRNA for bovine cytokines.

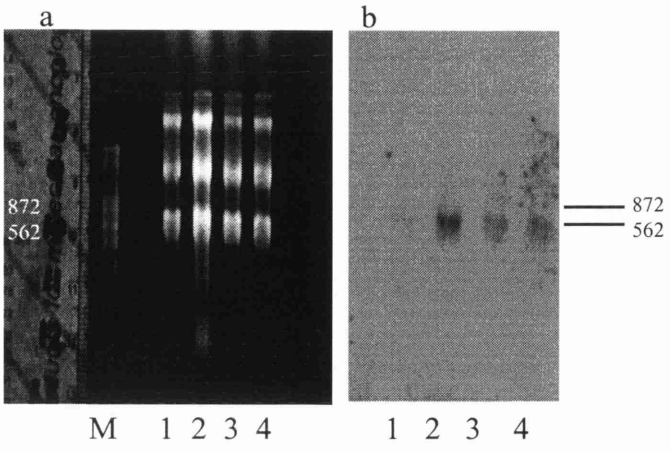

Figure 6.24 Northern blot to detect bovine IL-10

RNA (15 μg), isolated from PBMC collected 1) pre-infection and on Day 35 after the 2) primary, 3) secondary and 4) tertiary infections from Calf 174 was separated on an agarose gel (a). M: RNA markers (Promega, G315). The RNA was transferred to a nylon membrane and probed with a cDNA for bovine IL-10 (b). The lines indicate marker sizes (bp) above and below the product. The product detected was of the expected size (740 bp).

6.3.2 The use of RT-PCR to detect mRNA for bovine cytokines

6.3.2.1 Controls for reverse transcription

RT-PCR was used to investigate the expression of mRNA for various bovine cytokines. RNA was isolated from PBMC or LN cells (Section 2.15) which had been incubated overnight with 2.5 μg ml^{-1} Con A. The RNA samples were analysed on 1.2% agarose mini-gels to assess their quality and quantity (Section 2.16). To reduce contamination with genomic DNA, the RNA was incubated with DNAse (Gibco BRL) prior to RT-PCR (Section 2.17). Reverse transcription was performed on DNAse treated and untreated (control) samples. PCR was performed, using primers for bovine β actin, on RNA and reverse transcribed cDNA which had, and had not, been treated with DNAse. The bovine β actin gene was used as a constitutively expressed control. This set of controls was performed each time RNA samples were reverse transcribed. An aliquot of the PCR products was separated on a 1.5% agarose gel (Section 2.18) to determine the size and range of the amplified products.

Figure 6.25 shows an example of the results when there was DNA contaminating the RNA. Lane 2 shows a band when RNA was used in the PCR, indicating DNA contamination. When the RNA was DNAse treated before undergoing PCR, there was no band (lane 6), indicating that the DNAse treatment successfully removed DNA from the RNA sample. Contamination with DNA was confirmed when RT-PCR was performed without DNAse treatment (lane 2), there was a large band of the expected size, with a smear above (lane 3). DNAse treatment, to reduce contaminating DNA, followed by RT-PCR gave clear bands of the expected size (270bp) (lanes 4 and5).

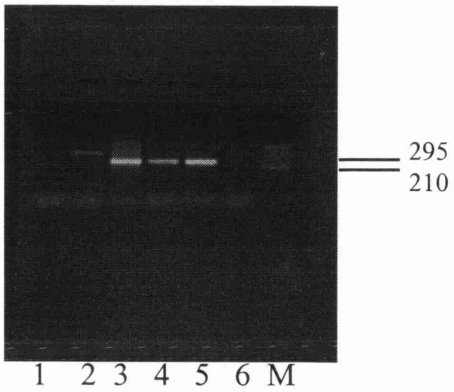

Figure 6.25 Controls for reverse transcription

RNA was isolated from Con A-stimulated PBMC from Calf 174, collected on Day 49 pi. An aliquot of RNA was DNAse treated, then reverse transcribed and amplified, using primers for β actin. As controls; RNA was amplified directly, or after DNAse treatment; RNA was reverse transcribed, but not DNAse treated. M: φX-174RF DNA *Hinc* II digest markers, the lines indicate the marker sizes (bp) on either side of the product. The expected product of 270 bp was obtained.

Lane 1: β actin primers only, no cDNA or RNA.

Lane 2: 2 μl RNA.

Lane 3: 2 μl cDNA, not DNAse treated.

Lane 4: 2 μl 1:10 cDNA, DNAse treated.

Lane 5: 2 μl cDNA, DNAse treated.

Lane 6: 2 μl RNA, DNAse treated .

6.3.2.2 Detection of mRNA in PBMC from infected calves

RNA was isolated from Con A-stimulated PBMC from an infected calf and RT-PCR performed using primers for bovine β actin, IFNγ and IL-4 as described in Section 2.17. The conditions for the PCR were:

Denaturation	94°C	5 min	
Step 1	94°C	1 min	} 30 cycles
Step 2	55°C	1 min	
Step 3	72°C	3 min	
Extension	72°C	10 min	

Hold @ 4°C.

The results are shown in Figure 6.26. Products of the correct size were observed using all the primers. However, with IFNγ and IL-4, there were extra bands, above those of the expected size. This PCR was repeated on several occasions using PBMC from different calves. The results were always similar (not shown).

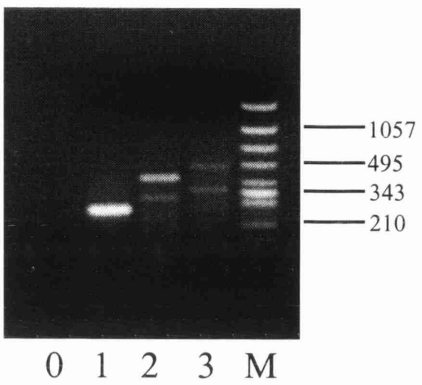

Figure 6.26 mRNA for IFNγ and IL-4 in PBMC from an infected calf

Photograph of an ethidium bromide-stained agarose gel of PCR products. RNA, isolated from PBMC from an infected calf, was reverse transcribed and amplified using primers for: Lane 0: primer only control, Lane 1: bovine β actin(270 bp), Lane 2: IFNγ (316 bp), Lane 3: IL-4 (349 bp) and Lane 4: φX-174RF DNA *Hinc* II digest markers. The lines indicate some of the marker sizes (bp).

6.3.2.3 Restriction digestion to confirm the identity of PCR products

To confirm the identity of the PCR products, the appropriate sized bands were excised from the agarose gel and the DNA purified using a Qiaex DNA gel extraction kit (Section 2.19). The DNA was incubated with the appropriate restriction enzyme (Appendix 2.2) and the products separated on a 1.5% agarose gel. Figure 6.27 shows an example of purified IL-4 products. The PCR product did not fully digest, but partial digestion yielded products of approximately the correct size (207 and 141 bp).

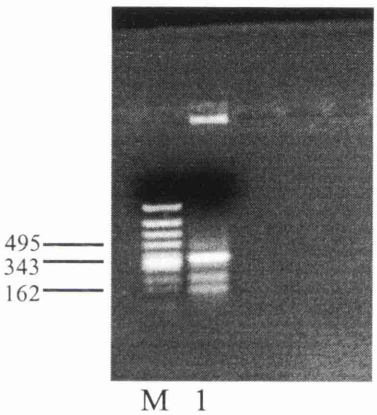

Figure 6.27 Restriction digest of PCR product purified following amplification using IL-4 primers

PCR products from RT-PCR, using IL-4 primers, from PBMC from an infected calf. The lower band (349 bp) was excised, the DNA was purified and digested using PstI @ 37°C for 1 h. The DNA was then precipitated and resuspended in a small volume and run on a 1.5% agarose gel. This ethidium bromide-stained gel shows the products of digestion. M: φX-174RF DNA *Hinc* II digest markers. Lane 1: uncut IL-4 (349 bp) and digest products (207 and 141 bp).

6.3.2.4 Modification of PCR conditions in an attempt to obtain a single product

In an attempt to obtain a single product after PCR, various PCR parameters were tested which included:

- Using a hot start, i.e. all reagents were added to the PCR reaction, except the Amplitaq. The mixture was heated to 95°C for 3 min, then the Amplitaq was added and PCR cycles started, without the initial denaturation step.
- Varying the number of cycles from 25 to 40.
- Varying the annealing temperature; 54, 55, 56 and 58°C.
- Varying the quantity of cDNA used, from 0.25 to 150 ng.
- Varying the concentration of dNTPs used, from 100 to 800 μM of each dNTP.
- Varying the quantity of primers used, from 0.132 to 1.32 μg of each primer.
- Varying the concentration of magnesium in the reaction, from 1.5 to 4 μM.

None of these had the desired effect of eliminating the spurious band. Next, it was decided to take 10 μl of the PCR product and use this in a second PCR reaction, using identical conditions to those for the first reaction. This yielded a large quantity of product which produced a single band on an agarose gel.

6.3.2.5 mRNA for bovine cytokines in PBMC and LN-derived cells from infected and uninfected calves.

RNA was isolated from PBMC and LN cells collected pre-infection and on Day 35 after primary infection. In an attempt to up-regulate expression of cytokine mRNA, the cells were stimulated with Con A for 12 h. RNA was isolated and RT-PCR was performed using primers for bovine IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, TNFα and IFNγ, using the conditions described above. Bovine β actin was used as a standard to compare the quantity of mRNA in each reaction. In all samples, including those from uninfected calves, a PCR product of the expected size was obtained, using all the primers (Figure 6.28 to Figure

6.30). The β actin signal was similar for all samples, suggesting that the amount of input RNA was similar. Although this was not a quantitative measure, there were no obvious differences in the amount of PCR product obtained in the different samples for TNFα, IL-1α, IL-1β (Figure 6.28), IL-10 (Figure 6.29) and IFNγ (Figure 6.30). When expression of bovine IL-6 was measured, there was less IL-6 expressed by PBMC from an uninfected compared with an infected calf (Figure 6.29). There appeared to be less IL-2 expressed by PBMC, from both infected and uninfected calves, compared with LN cells (Figure 6.30). There also appeared to be less IL-4 expressed by PBMC and LN cells from an uninfected compared to an infected calf (Figure 6.30).

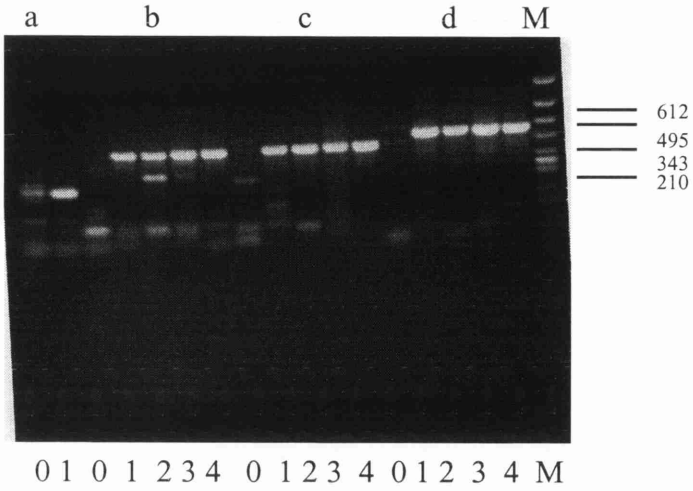

Figure 6.28 PCR products obtained using primers for IL-1α , IL-1β and TNFα

RNA was isolated from PBMC and left T/B LNs from an infected and an uninfected calf. RT-PCR was performed using primers for bovine a) βactin (271bp), b) TNFα (466bp), c) IL-1α (480bp) and d) IL-1β (558 bp). 0: primers only, 1: LN cells from an infected calf, 2: LN cells from an uninfected calf, 3: PBMC from an uninfected calf, 4: PBMC from an infected calf. M: φX-174RF DNA *Hinc* II digest markers. The lines indicate some of the marker sizes (bp).

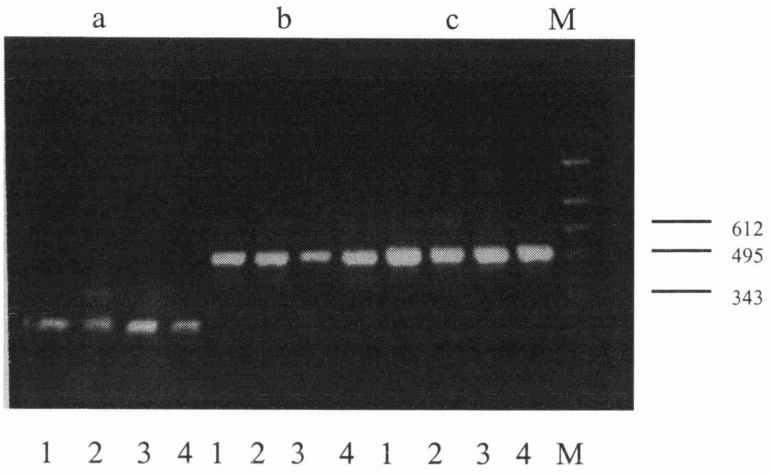

Figure 6.29 PCR products obtained using primers for IL-6 and IL-10

RNA was isolated from PBMC and left T/B LNs from an infected and an uninfected calf. RT-PCR was performed using primers for bovine a) βactin (270 bp), b) IL-6 (505 bp) and c) IL-10 (517 bp). 1: LN cells from an infected calf, 2: LN cells from an uninfected calf, 3: PBMC from an uninfected calf, 4: PBMC from an infected calf. M: φX-174RF DNA *Hinc* II digest markers. The lines indicate some of the marker sizes (bp).

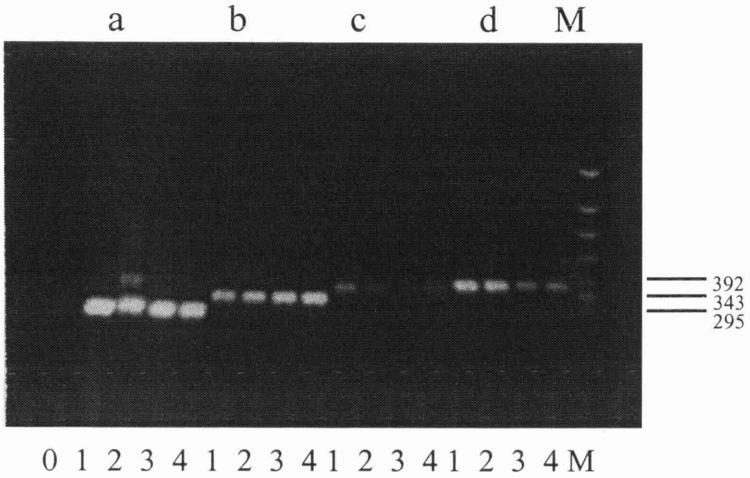

Figure 6.30 PCR products obtained using primers for IL-2, IL-4 and IFNγ

RNA was isolated from PBMC and left T/B LNs from an infected and an uninfected calf. RT-PCR was performed using primers for bovine a) βactin (270 bp), b) IFNγ (316 bp), c) IL-4 (349 bp) and d) IL-2 (373 bp) 0: No cDNA, actin primers. 1: LN cells from an infected calf, 2: LN cells from an uninfected calf, 3: PBMC from an uninfected calf, 4: PBMC from an infected calf. M: φX-174RF DNA *Hinc* II digest markers. The lines indicate some of the marker sizes (bp).

6.3.2.6 Cytokine transcripts using RNA from unstimulated cells

It was decided to investigate whether a more quantitative result could be obtained using cells which had not been stimulated previously with Con A. RNA was isolated from PBMC from an uninfected calf and from PBMC collected on Day 28 after a primary infection. RT-PCR was performed using primers for β actin, IL-2, IL-4, IL-6, and IFNγ. No differences were apparent in expression of any of the cytokines between uninfected and infected PBMC (Figure 6.31).

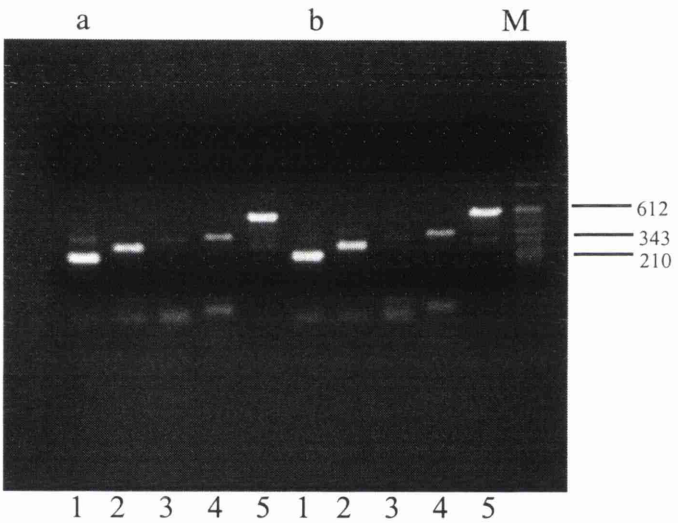

Figure 6.31 Cytokine transcripts in unstimulated PBMC from an infected and uninfected calf

RNA, isolated from unstimulated PBMC from an a) infected and b) uninfected calf, was reverse transcribed, then amplified, using primers for bovine 1) β actin (270 bp) , 2) IFNγ (316 bp) 3) IL-4 (349 bp), 4) IL-2 (373 bp) and 5) IL-6 (505bp). M: φX-174RF DNA *Hinc* II digest markers. The lines indicate some of the marker sizes (bp).

6.3.3 RT-PCR and Southern analysis.

The initial RT-PCR technique required two sets of PCR cycles to obtain a product which could be seen on an ethidium bromide-stained gel: however, blotting the PCR product and probing with radiolabelled DNA provides an additional amplification step and is a more sensitive indicator of the presence of DNA.

6.3.3.1 Optimisation of PCR conditions

In an attempt to quantify the PCR product, PCR conditions were optimised so that doubling dilutions of input cDNA resulted in a corresponding decrease in the signal observed on blots. A large quantity of RNA was isolated from PBMC from an infected calf (Calf 166, Day 2-19) and this was reverse transcribed. The PCR conditions were:

2 µl 1/10 cDNA (approximately 20 ng)
5 µl *Taq* DNA polymerase buffer
1 µl 20 µM primer 1
1 µl 20 µM primer 2
100 µM each dNTP
ddH_2O to 45 µl.

Denaturation	94°C	5 min	
Then added 1.25 units Amplitaq DNA polymerase enzyme made up to 5µl with dd H_2O.			
Step 1	94°C	1 min	} 15 to 30 cycles
Step 2	55°C	1 min	
Step 3	72°C	1 min	
Extension	72°C	10 min	
Hold @ 4°C.			

Using primers for bovine β actin, IL-2 and IL-4, PCR was performed using doubling dilutions of cDNA, from 1:10 to1:640. For β actin, this was repeated using 15, 20 or 25 PCR cycles and for IL-2 and IL-4, using 20, 25 and 30 PCR cycles. The products were run on agarose gels and Southern blotting was performed. Products of the correct size were obtained for all PCR amplifications (Figure 6.32). It should be noted that there was some evidence of contamination in the primer only lane for IL-2, however, this was not a consistent finding. From these results it was concluded that the optimum number of cycles required for β actin was 20 and for IL-2 and IL-4 was 30.

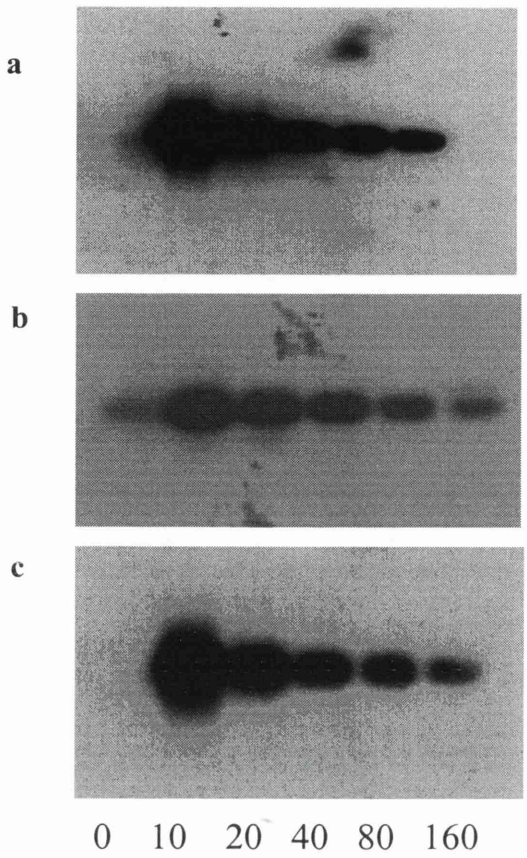

Figure 6.32 Optimising conditions for RT-PCR and Southern analysis

A semi-quantitative RT-PCR was performed on RNA isolated from PBMC from Calf 166. Doubling dilutions of cDNA from 1:10 to 1:160 were used. The products were probed with a cDNA probe for a) human βactin, b) bovine IL-2 and c) bovine IL-4. These results were obtained using a) 20 , b) and c) 30 PCR cycles. 0: no cDNA, 10, 20 etc : dilutions of cDNA @ 1:10, 1:20, etc.

6.3.3.2 Expression of mRNA for bovine IL-2 and IL-4 in PBMC by Southern analysis

To compare mRNA expression for bovine IL-2 and IL-4 in PBMC before and after infection with *D. viviparus*, RNA was isolated from PBMC from Calves 165, 172, 174 and 175 collected pre-infection and Day 35 after primary, secondary and tertiary infection. RT-PCR was performed, using primers for β actin as a standard. The expression of β actin was fairly consistent at all time points (Figure 6.33). When compared with relative intensity of the corresponding band of β actin, there were no obvious differences in expression of IL-2 at different time points for each calf (Figure 6.34).

For Calves 165 and 172 there was no expression of IL-4 mRNA pre-infection (lane 0, Figure 6.35). IL-4 was detected after the primary infection, with additional increases after the secondary and the tertiary infections for Calf 172. For Calf 165, the levels of IL-4 after the primary and secondary infections were similar and there was increased expression after the tertiary infection. The expression of IL-4 mRNA in PBMC from Calves 174 and 175 was more variable (Figure 6.36). There was no (Calf 174), or very low (Calf 175) expression of IL-4 pre-infection. For both calves, IL-4 increased after the primary infection, but decreased after the secondary infection. IL-4 was not detected after the tertiary infection for Calf 175, whereas IL-4 and IL-2 were detected. There was no tertiary infection time point for Calf 174.

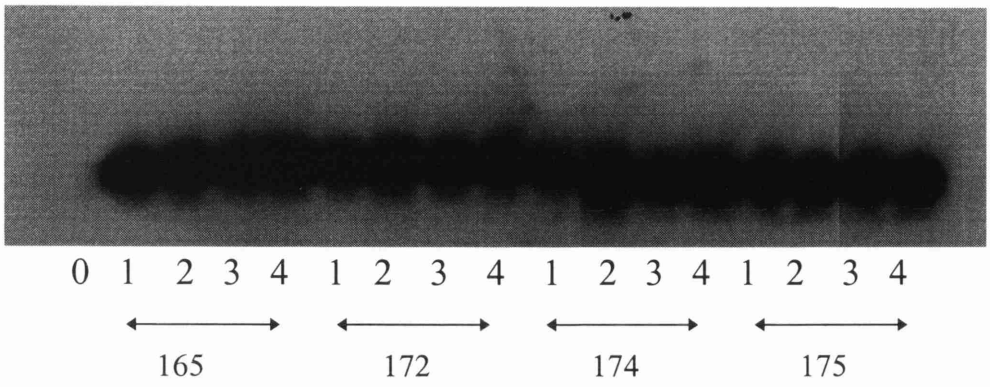

Figure 6.33 βactin mRNA expression in PBMC

RNA was isolated from PBMC from Calves 165, 172, 174, and 175, collected pre-infection (1) and 35 days after the primary (2), secondary (3) and tertiary (3) infections. A semi-quantitative RT-PCR was performed and the products were probed with a cDNA probe for human βactin. 0: primer only control.

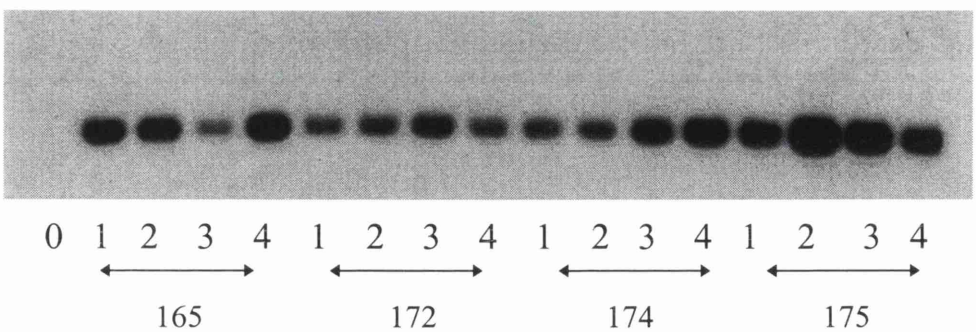

Figure 6.34 IL-2 mRNA expression in PBMC

RNA was isolated from PBMC from Calves 165, 172, 174, and 175, collected pre-infection (1) and 35 days after the primary (2), secondary (3) and tertiary (4) infections. A semi-quantitative RT-PCR was performed and the products were probed with a cDNA probe for bovine IL-2. 0: primer only control.

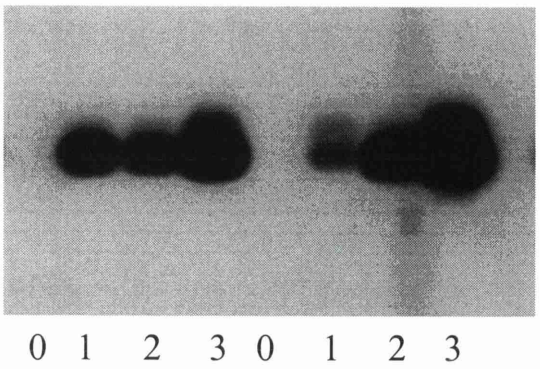

Figure 6.35 IL-4 mRNA expression in PBMC

RNA isolated from PBMC from Calves 165 a) and 172 b) collected pre-infection (0) and 35 days after the primary (1), secondary (2) and tertiary (3) infections. A semi-quantitative RT-PCR was performed and the products were probed with a cDNA probe for bovine IL-4.

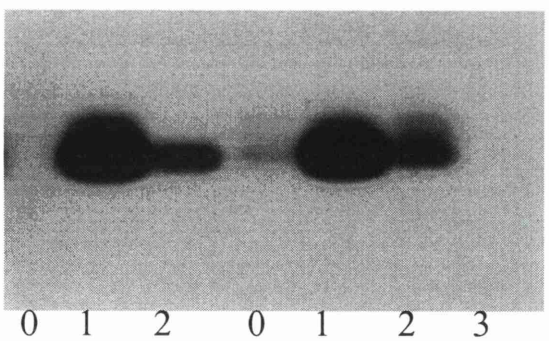

Figure 6.36 IL-4 mRNA expression in PBMC

RNA isolated from PBMC from Calves 174 a) and 175 b) collected pre-infection (0) and 35 days after the primary (1), secondary (2) and tertiary (3) infections. A semi-quantitative RT-PCR was performed and the products were probed with a cDNA probe for bovine IL-4.

6.3.3.3 Expression of mRNA for bovine IL-2 and IL-4 in cells collected by BAL

RNA was isolated from a mixed population of BALF cells and RT-PCR was performed, using primers and probes for β actin, IL-2 and IL-4. The cells used were collected from uninfected calves, on Day 21 after the primary infection (1-10, 1-21) and on Day 10 and 21 after the tertiary infection. The expression of β actin was fairly consistent at all time points (Figure 6.37), with slight increases in expression from samples collected after the tertiary infection. IL-2 mRNA was not detected in cells from BAL collected from two calves pre-infection. Although transcripts were detected in cells collected from calves after infection, when compared with β actin expression, there were no obvious differences in expression of IL-2 at different time points (Figure 6.38). IL-4 mRNA was not detected pre-infection (not shown), but was expressed in cells collected from six calves after the primary infection and three calves after the tertiary infection (Figure 6.39). The highest levels of IL-4 were detected on Day 3-10 for all three calves analysed for that time point; by Day 3-21, levels had decreased, despite the fact that β actin levels were high in these samples.

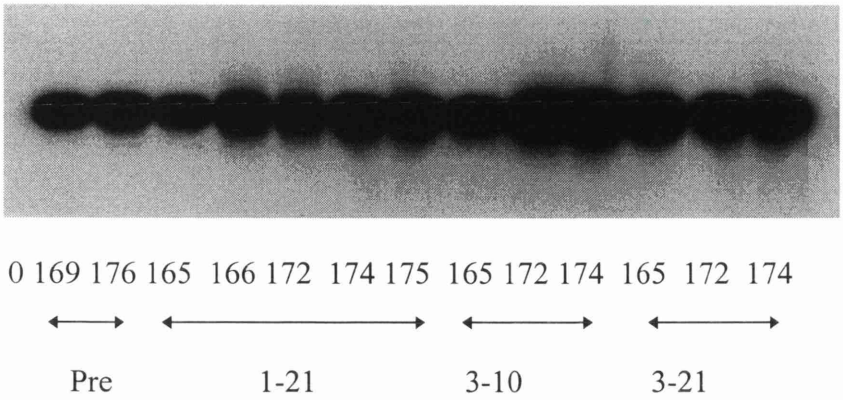

Figure 6.37 βactin mRNA expression in BALF cells

RNA was isolated from BALF cells collected from Calves 169 and 176 pre-infection (pre), Calves 165, 166, 172, 174 and 175 on Day 21 after the primary (1) infection and Calves 165, 172 and 174 on Day 10 (2) and Day 21 (3) after the tertiary infection. A semi-quantitative RT-PCR was performed and the products were probed with a cDNA probe for human βactin. 0: no cDNA control.

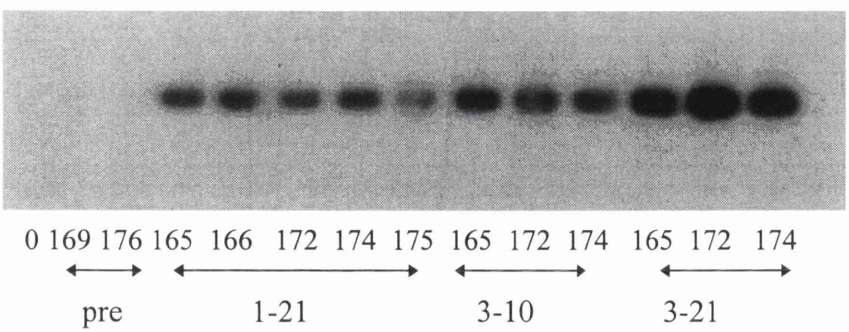

Figure 6.38 IL-2 mRNA expression in BALF cells

RNA was isolated from BALF cells collected from Calves 169 and 176 pre-infection (pre), Calves 165, 166, 172, 174, and 175 on day 21 after the primary (1) infection and Calves 165, 172 and 174 on Day 10 (2) and Day 21 (3) after the tertiary infection. A semi-quantitative RT-PCR was performed and the products were probed with a cDNA probe for bovine IL-2. 0: no cDNA control.

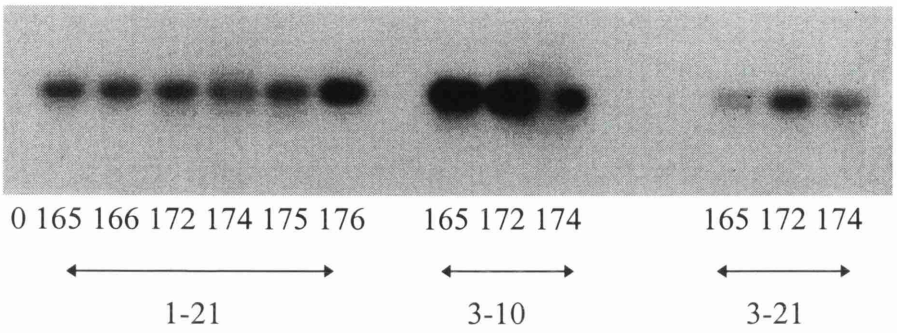

Figure 6.39 IL-4 mRNA expression in BALF cells

RNA was isolated from BALF cells from Calves 165, 166, 172, 174, 175 and 176 collected on Day 21 after the primary (1) infection and for Calves 165, 172 and 174 after the secondary (2) and tertiary (3) infections. A semi-quantitative RT-PCR was performed and the products were probed with a cDNA probe for bovine IL-4. 0: no cDNA control.

6.3.4 Detection of mRNA for bovine IL-5

Through a collaboration with Dr. H-F. Seow, CSIRO Division of Animal Health, Victoria, Australia, the bovine IL-5 gene was recently cloned and sequenced by Dr. R.B. Collins, Institute for Animal Health and, based on this sequence, primers were designed for use in PCR reactions (Appendix 2.4). RNA was isolated from cells collected by BAL at a time point when eosinophils constituted 25% of the cell population, i.e. when it was likely that there would be expression of mRNA for IL-5. RT-PCR was performed using the newly designed primers for bovine IL-5. Using one round of PCR cycles (35 cycles), no product was identified on an ethidium bromide-stained agarose gel (not shown). When the product from the first set of cycles was used in a second set of PCR cycles (30 cycles), a faint band was seen of the expected size (327 bp) (lane 3, Figure 6.40). However, there was also a band detected around 400 bp, which may have been a contaminant. The RT-PCR was repeated, using PBMC from an infected calf and using LN cells from an infected calf, but no products were detected convincingly.

Despite the fact that the BAL samples contained a high percentage of eosinophils, only very small quantities of mRNA for IL-5 detected were in these cells. IL-5 was not detected in PBMC or LN cells from infected calves (not shown). It was beyond the scope of this study to prepare DNA probes for bovine IL-5. RT-PCR and Southern blotting may have provided a more sensitive indicator of mRNA for IL-5.

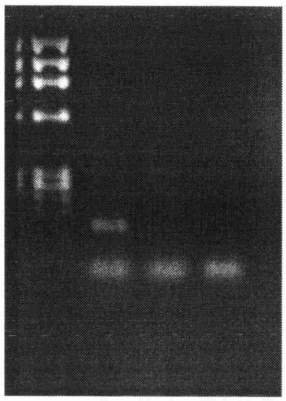

Figure 6.40 Detection of mRNA for IL-5 using RT-PCR

RNA was isolated from BALF cells collected from Calf 166 on Day 2-19. The RNA was reverse transcribed and amplified using primers for (1) bovine actin (270 bp), (2) and (3) IL-5 (327 bp). M: φX-174RF DNA *Hinc* II digest markers.

1: 2 μl 1:10 cDNA, β actin primers.

2: 2 μl 1:10 cDNA, IL-5 primers.

3: 2 μl cDNA, IL-5 primers.

6.4 Discussion

It was anticipated that measurement of cytokine profiles during the course of infection with *D. viviparus* may have provided some indication of a Th cell bias. To this end, a variety of cytokines were measured by bio-assay/ELISA and the more sensitive technique of RT-PCR was employed to detect cytokine transcripts.

IL-2 was detected in supernatants from PBMC following stimulation with parasite antigen or Con A. However, there was no consistent pattern to IL-2 secretion between individual calves and no obvious increases after infection with *D. viviparus* compared with preinfection levels. The pattern of IL-2 secretion in response to antigen-stimulation generally followed the pattern in response to Con A-stimulation and moderate to strong correlations were demonstrated between these responses. Despite the fact that IL-2 is the major growth factor for T cells, no correlations were observed between IL-2 levels and proliferative capacity.

The observation that very little antigen-specific IL-2 was produced by the draining T/B LNs, while cells from the pre-crural LNs produced significantly higher amounts ($p < 0.01$) was extremely interesting. Moreover, T/B LN cells from the gnotobiotic calf secreted very high levels of antigen-specific IL-2. A possible explanation is that the parasite may release factors which specifically inhibit IL-2. For example, taeniastatin an E/S product of *Taenia taeniaeformis* inhibited IL-1, IL-2 and IL-2-receptor expression in murine cells (Leid *et al.*, 1986). It was also noted that no IFNγ was detected in the T/B LNs and that these cells had significantly lower proliferative responses to Rpt C-A compared with the pre-crural LNs (Chapter 5). Another possible explanation may be that the major source of cytokines were the $\gamma\delta^+$ T cells. The percentages of $\gamma\delta^+$T cells in the T/B LNs of infected calves were significantly lower than those in the pre-crural LNs (Section 5.3.2.2).

When IL-2 expression was examined by RT-PCR, levels of transcript were similar between infected and uninfected PBMC and this was substantiated by similar findings using the more sensitive RT-PCR and Southern analysis. Hutchinson *et al.*, (1994) also detected mRNA for IL-2 in control cattle in Con A-stimulated PBMC, using RT-PCR. In this study, T/B LN cells appeared to express more IL-2 than did the PBMC both pre-infection and pi. This contrasts with the findings from the analysis of IL-2 protein and suggests that, if there

was indeed down-regulation of IL-2 secretion in the draining T/B LNs, that it may have been at a post-transcriptional level. It would be interesting to perform a more detailed study of mRNA expression by local and peripheral LN-derived cells in uninfected and *D. viviparus*-infected calves.

Neither IL-2 nor IL-4 mRNA were detected in BALF cells collected pre-infection. The cell population in BALF changed after infection (Chapter 3), in particular there was a dramatic decrease in the percentage of macrophages. The high percentage of macrophages (> 80%) in pre-infection BALF samples could have diluted any message for IL-2 or IL-4 from other cell types. It is possible that the levels of expression of mRNA for IL-2 and IL-4 were related to the percentages of different cell types present in the BALF sample. However, no correlations between cell types and cytokine expression were demonstrated (results not shown).

High levels of IFNγ were secreted by antigen-stimulated cells. This was similar to results obtained in supernatants from Con A-stimulated cells and moderate to strong correlations were demonstrated between these parameters. IFNγ was not detected in supernatants from T-B LN cells. Suppression of local IFNγ secretion was previously demonstrated in antigen-stimulated mediastinal LN lymph cells in rats infected with *N. brasiliensis* (Uchikawa *et al.*, 1994). These findings again suggest that parasite factors may inhibit immune responses in the local draining LN, as discussed above for IL-2.

Perhaps the most compelling data came from the measurement of IL-4 levels. After infection, PBMC from some calves (165 and 172) produced high levels of spontaneous IL-4, while others (174 and 175) had lower levels of spontaneous IL-4. This finding contrasts markedly with the low levels of IL-1, IL-2, IL-6 and IFNγ in unstimulated cells from most animals (apart from IL-2 for Calf 174, and IFNγ for Calves 170, 174 and 175). RT-PCR analysis of IL-4 mRNA expression in PBMC tended to confirm the IL-4 secretion profiles. For Calves 165 and 172, no mRNA for IL-4 was detected pre-infection, but transcripts were detected after infection, with increases after the secondary and tertiary infections. For Calves 174 and 175, IL-4 transcripts were detected after the primary infection, which decreased after the secondary and tertiary infections. These obvious changes in IL-4 mRNA expression after infection are very exciting and suggest that infection with *D. viviparus* may induce a Th2-type cytokine response.

In contrast to the findings with IL-2 and IFNγ, IL-4 was detected in supernatants from T/B LN cells and the levels of IL-4 were similar whether the cells were cultured with Con A, antigen or in medium alone. The LN cells proliferated to parasite antigen but did not produce IL-2 or IFNγ, but they did produce IL-4. This suggests that there may be a local Th2-type response and the proliferation that was detected in response to DvDUH may have reflected an expansion of Th2 cells. It would be interesting to further investigate this hypothesis by determining whether a neutralising antibody to bovine IL-4 (when this becomes available) could inhibit this proliferation. For example, a polarised antigen-specific Th2 proliferative response to *Brugia pahangi,* in mice, was blocked by anti-IL-4 antibody (Osborne *et al.,* 1996). In this study, further measurement of the local IL-4 responses via RT-PCR and Southern analysis of BALF cells confirmed a local increase in IL-4 following infection.

Attempts were made to identify the cellular source of the various cytokines by analysis of MACS-separated T cell populations. In response to Con A, $\gamma\delta^+$, $CD4^+$, and $CD8^+$ T cells separated from PBMC secreted IL-2. The highest levels of IL-2 were secreted by Con A-stimulated $\gamma\delta^+$ T cells and there was a positive correlation between the SI for $\gamma\delta^+$ T cells in proliferation assays and IL-2 secretion. This contrasts with the findings of Brown *et al.* (1994a) who found that supernatants from Con A-stimulated $\gamma\delta^+$ T cell clones from cattle infected with *B. bovis* secreted neither IL-2 nor IL-4. Moreover, Northern analysis did not detected expression of mRNA for IL-2 in Con A-stimulated $\gamma\delta^+$ T cells (Brown *et al.*, 1994a). However, studies in mice have demonstrated IL-2 secretion by activated $\gamma\delta^+$ T cells when stimulated *in vitro* (Cron *et al.,* 1989).

Antigen-specific IL-2 was detected only at very low levels in supernatants from separated $\gamma\delta^+$ T cells populations from two calves (174 and 175). No IL-2 was detected in any of the other separated populations, even when there were good proliferative responses. The IL-2 detected in supernatants from antigen-stimulated whole PBMC populations may have come from another source other than T cells. Alternatively, factors produced by other cells may have been involved in its production.

Again, the major source of IFNγ was $\gamma\delta^+$ T cells, and in fact, there were higher levels of IFNγ detected in supernatants from MACS separated $\gamma\delta^+$ T cells than in supernatants from whole PBMC populations for Calves 165 and 175, and similar levels for Calf 172. This suggests that $\gamma\delta^+$ T cells are potent IFNγ producers. The lower levels of

IFNγ in PBMC populations may indicate that factors released by other cells in the whole population inhibit IFNγ production by $\gamma\delta^+$ T cells. Brown *et al.* (1994a) found that $\gamma\delta^+$ T cell clones from cattle infected with *B. bovis* secreted only IFNγ, though mRNA for TNFα was also detected in some clones. The findings of this study that bovine $\gamma\delta^+$ T cells were an important source of IFNγ confirm those of Brown *et al.* (1993b, 1994a). Very low levels of IFNγ were secreted by separated $CD4^+$ and $CD8^+$ cells, whereas, Ishikawa *et al.* (1994) demonstrated that both $CD4^+$and $CD8^+$ T cells, from uninfected cattle, produced IFNγ. Moreover, IFNγ was secreted by $CD4^+$ T cell clones from cattle infected with *B. bovis* (Brown *et al.*, 1993b). However, neither of these authors compared the quantities of IFNγ secreted by $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T cells.

In response to Con A, IL-4 was produced by $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T cells, with the highest levels in $\gamma\delta^+$ T cells for three out of the four calves. To date, only Th1-type cytokine production has been demonstrated by bovine $\gamma\delta^+$ T cells (Brown *et al.*, 1994a). However, that study investigated cells cloned from cattle infected with *B. bovis* and there have been no similar studies which have investigated cells from cattle infected with nematodes where it would be expected that a Th2-type response would be more dominant. Ferrick *et al.* (1995) demonstrated *in vivo* production of IL-4 by peritoneal and splenic $\gamma\delta^+$ T cells in mice infected with *N. brasiliensis*. Moreover, mRNA for another Th2 cytokine, IL-5, was demonstrated in $\gamma\delta^+$ T cells in the gastrointestinal tract of sheep infected with *T. colubriformis* (Bao *et al.*, 1996). Therefore, this study provides the first evidence of IL-4 production by ruminant $\gamma\delta^+$ T cells.

The analyses of IL-1, IL-5, IL-6, IL-10 and TNFα were not as exhaustive as those for IL-2, IL-4 and IFNγ. Significant quantities of IL-1 were secreted by PBMC after stimulation with LPS or parasite antigen at all time points, including pre-infection. These results were substantiated by the RT-PCR studies, in which IL-1 transcripts were detected in Con A-stimulated PBMC from infected and uninfected calves. IL-1 transcripts have been detected in uninfected calves in other studies (Ito and Kodoma, 1994). The levels of IL-1 secreted from T/B LN cells were much lower than those from PBMC. As discussed previously, it would be interesting to determine whether this finding related to an ability of the parasite to down-regulate specific cytokines. Alternatively, the cells which secrete IL-1 may have been less numerous in the LNs than in PBMC population. The ubiquitous and multifunctional nature of IL-1 complicates the interpretation of these results. The isolation

of a bovine IL-1 dependent IL-4-producing $CD4^+$ T cell clone (Stevens *et al.*, 1992) suggests that further investigation of the role of IL-1 in cattle may provide novel information. It may be more rewarding to look at IL-1 production, or its action, in individual cell populations.

A pronounced local eosinophilia (Chapter 3) and increase in IgE (Chapter 4) were demonstrated in calves after infection with *D. viviparus*, therefore it would have been useful to correlate these findings with IL-5 levels. However, there are no bioassays for bovine IL-5 and until very recently this cytokine had not been cloned from the bovine. Although the sequence was made available and primers designed and used for PCR, there was only a very faint product detected in a BALF cell population, which contained a high percentage of eosinophils. IL-5 was not detected in PBMC or LN cells. mRNA for IL-5 was detected in cells collected by BAL from humans with atopic asthma (Robinson *et al.*, 1992, 1993). Furthermore, IL-5 was detected in supernatants from mediastinal LN cells from mice infected with *T. muris* (Else *et al.*, 1992). Further studies are required to ascertain whether IL-5 is induced in response to infection with *D. viviparus*.

LPS was demonstrated to be a potent inducer of mRNA for IL-6 in bovine monocytes (Heussler *et al.*, 1992), so should have been a good candidate to stimulate bovine cells to produce high levels of IL-6. Unfortunately, LPS, on its own, induced proliferation of the murine hybridoma cell line (B9). Therefore Con A was used instead of LPS. Although there are no previous reports of B9 cells responding to bovine IL-6, these cells have been used successfully on many occasions to measure IL-6 from other species including rats (Osawa *et al.*, 1995), man (Hashim *et al.*, 1995), cats (Lawrence *et al.*, 1995) and chickens (Rath *et al.*, 1995). However, B9 cells may respond to other cytokines including human IL-11 (Lu *et al.*, 1994; Schwabe *et al.*, 1996) and human IL-13 (Labitlebouteiller *et al.*, 1995). Moreover, some cytokines act to inhibit the proliferation of IL-6-dependent B9 cells, including human TNFα, human TGFβ and murine IFNs (Schwabe *et al.*, 1996).

IL-6 was detected in supernatants from Con A-stimulated bovine PBMC, but at much lower levels than IL-1, with pg rather than ng quantities being detected. IL-6 was detected in supernatants from antigen-stimulated PBMC from only one calf, and this was at very low levels. There was no increase in secretion of IL-6 by cells from infected compared with uninfected calves and no IL-6 was detected in supernatants from LN cells. These low

levels of IL-6 may indicate that the assay used was not sufficiently sensitive; alternatively, IL-6 activity may have been inhibited by other cytokines present, for example IFNγ (Schwabe *et al.*, 1996). There are no previous reports of the B9 cell line being used to measure bovine IL-6 and given the nature of the results obtained here, no conclusions could be drawn regarding the levels of biologically active IL-6 secreted by these bovine cells.

Using RT-PCR, mRNA for bovine IL-6 was detected in PBMC and LN cells from both uninfected and infected calves. In Con A-stimulated cells, more mRNA was expressed in PBMC from an infected compared to an uninfected calf, but there were no differences detected in unstimulated cells. Using similar methods, Covert and Splitter (1995) also detected mRNA for bovine IL-6 in Con A-stimulated cells from uninfected cattle. Similar to IL-1, IL-6 is a ubiquitous and multifunctional cytokine and more specific studies would be required to examine the role of this cytokine in the bovine. Human and murine IL-6 are thought to be involved in the regulation of IgA synthesis (Beagley *et al.*, 1989; Hiroi *et al.*, 1995; Ichinose *et al.*, 1996), therefore, it would be interesting to investigate this role for IL-6 in the bovine, using the *D. viviparus* model, as high levels of IgA were detected in BALF (Chapter 4).

Using RT-PCR, no differences were detected in the expression of mRNA for TNFα in Con A-stimulated PBMC or LN cells from infected and uninfected calves. Further studies would be required to determine the role of TNFα in the immune response to *D. viviparus*.

In the absence of bioassays for the detection of bovine IL-10, RT-PCR was performed using specific primers. No obvious differences were detected in the expression of mRNA for IL-10 in PBMC and LN cells from infected and uninfected calves. Northern analysis also detected the presence of IL-10 transcripts in unstimulated PBMC from both uninfected and infected calves. These mRNA levels appeared to increase after infection. A recent paper described $\gamma\delta^+$ T cells as a major source of IL-10 in the spleen cells of mice infected with *L. monocytogenes* (Hsieh *et al.*, 1996). It may be that in the study described here, $\gamma\delta^+$ T cells, which were elevated after infection and proliferated in a parasite-specific manner, were the major source of the large amounts of mRNA for IL-10 that were detected by Northern analysis. Neither IL-2 nor IL-4 transcripts were detected by this method, perhaps indicating that there were higher levels of mRNA for IL-10.

In conclusion, following infection with *D. viviparus* there was upregulation of mRNA for IL-4 but not IL-2, demonstrated using RT-PCR and Southern blotting, in PBMC and BALF cells. This suggests a Th2-type response, both peripherally and locally. There was a local down-regulation of IL-2, IFNγ and IL-1 secretion in the T/B LNs and this correlated to a down-regulation of proliferative responses to the recombinant antigen (Chapter 5). Finally, there appeared to be a population of $\gamma\delta^+$ T cells in infected calves which secreted IL-2, IL-4 and IFNγ and the secretion of these cytokines was correlated to the proliferative activity of the cells.

7. CONCLUDING DISCUSSION

A current paradigm in immunology is the concept of Th1 and Th2 subsets producing cross-regulatory cytokines and giving rise to different types of immune response. To date, there is insufficient data from bovine studies to determine whether Th1 and Th2 subsets exist *in vivo*. However, the present study provides preliminary evidence of a Th2-like response following infection with *D. viviparus*.

In this study, RT-PCR and Southern analysis of PBMC demonstrated an up-regulation of IL-4 expression following infection with *D. viviparus*. Moreover, transcripts of bovine IL-10 were detected by Northern analysis of PBMC collected after infection. Evidence for a local Th2-type response was also provided, when increases in IL-4 transcripts were demonstrated in BALF cells, using RT-PCR. Significant increases in local IgG1, IgE and IgA were demonstrated in BALF, the most pronounced increases observed after the tertiary infection, when the animals were fully immune. These antibody isotypes have been shown to be regulated by Th2 cytokines: IL-4 up-regulates production of IgG1, *in vitro*, in mice (Vitteta *et al.*, 1985) and cattle (Estes *et al.*, 1995); IL-10 was found to induce switch-recombination from IgM to IgG in human B cells (Malisan *et al.*, 1996); IgE is regulated by IL-4 (Coffman and Carty, 1986) and IL-13 (de Waal Malefyt *et al.*, 1995); IL-4, IL-5 and IL-6 have all been implicated as playing roles in enhancing IgA synthesis (Beagley *et al*, 1989; Hiroi *et al.*, 1995; Ichinose *et al.*, 1996). Interestingly, the highest levels of IL-4 expression in BALF cells were detected on Day 10 after the tertiary infection. This coincided with the peak levels of IgG1, IgE and IgA in BALF. This suggests that IL-4 may be important for class-switching in cattle, as described in mice (Finkelman and Urban, 1992).

There were significant increases in serum IgG1 and IgE after the primary and secondary infections, providing further evidence for a Th2-type response. However, the levels of these Igs did not increase after the tertiary infection, when the animals were immune. The fact that passive transfer experiments using serum from *D. viviparus* L3-infected calves (Canto, 1990) and guinea pigs (Canto, 1990; McKeand *et al.*, 1995) were successful, indicates that serum antibody must play some role in the protective immune response. However, the results from this study and from studies in vaccinated calves where

little or no serum IgG was detected (Bos *et al.*, 1986; Wassal, 1991), suggest that serum antibodies may not be essential for protective immunity.

The pronounced serum antibody responses demonstrated after the primary and secondary infections suggest a role for IgG1 in limiting these infections. However, no correlations were demonstrated between serum IgG1 levels and L1 excretion. These findings are similar to those in mice infected with *H. polygyrus.* Higher IgG1 responses were demonstrated in strains of mice in which infection was cleared rapidly, compared with responses in strains in which the infection became chronic (Wahid and Behnke, 1993), suggesting a role for IgG1 in parasite clearance. However, no correlations were demonstrated between the intensity of the IgG1 response within mouse strains and the loss of worms by particular individuals (Wahid and Behnke, 1993). It was concluded that IgG1, *per se*, did not play a role in clearance of primary *H. polygyrus* infections in mice. The results of this study therefore suggest that local antibodies in BALF may play an important role in protective immunity to *D. viviparus,* but the precise role of serum antibodies remains unclear.

Evidence was provided of Th1-type cytokine responses in some calves, although these animals also secreted Th2-type cytokines. It is likely that outbred animals such as cattle, exposed to a wide array of pathogens, will secrete both Th1 and Th2 cytokines (Haanen *et al.*, 1991; Yssel *et al.*, 1992). In this respect, cattle may be similar to humans, where in response to particular pathogens, T cells secrete a higher ratio of Th1 or Th2 cytokines (de Waal Malefyt *et al.*, 1995). This contrasts with the situation in inbred mice, where T cell subset responses are much more polarised (Mosmann and Coffman, 1989).

Of interest was the observation that calves with higher IgG2a(A1) secreted more larvae than calves with lower IgG2a(A1). Perhaps this indicates that these calves were unable to clear the primary infection as efficiently as those with low IgG2a(A1). IFNγ is an important cytokine for the induction and differentiation of Th1-type cells *in vivo* in mice (Mosmann and Coffman, 1989) and has been shown to stimulate the secretion of IgG2a(A1) from bovine B cells *in vitro* (Estes *et al.*, 1994). Preliminary evidence that high IgG2a(A1) may be associated with Th1-type responses in cattle *in vivo* was provided by the analysis of cytokine secretion profiles here. One of the Group A calves (Calf 174) had high IgG2a(A1) levels and high L1 excretion. Compared with the other Group A animals, unstimulated PBMC from this calf produced very low levels of spontaneous IL-4, but

produced higher levels of IL-2 and IFNγ, suggesting a bias in the Th1 direction. For Calf 174, the pattern of high IL-2 and low IL-4 levels in supernatants from unstimulated PBMC persisted throughout the three infections. In contrast, the levels of IFNγ in this calf were elevated after the primary infection, but were very low by Day 19 after the secondary infection. Interestingly, the pattern of IgG2a(A1) response was very similar, with events occurring slightly later. The peak IgG2a(A1) was on Day 2-19 and thereafter levels declined. This preliminary evidence (albeit for a single animal) suggests that IFNγ may drive the IgG2a(A1) response *in vivo* in the bovine, which would agree with the *in vitro* findings of Estes *et al.* (1994).

The results of proliferation assays were fraught with inconsistencies. A high degree of variability is common in these assays, particularly when using outbred animals (Fiscus *et al.*, 1982). The non-specific proliferation of cells from uninfected calves to whole adult parasite homogenate complicated the interpretation of results. However, a variety of experiments were carried out to further define the nature of this non-specific response and these yielded a number of interesting findings. The cellular sources of the non-specific proliferation appeared to be $CD4^+$ and $CD8^+$ cells and not $\gamma\delta^+$ T cells. The $CD4^+$ and $CD8^+$ cells proliferated to the whole adult homogenate in the presence of autologous or heterologous APC, implying that the antigen was not being presented in a conventional manner. Proliferation required the presence of metabolically active APC, demonstrated by a lack of response using paraformaldehyde-fixed APC. This type of non-specific proliferation is similar to that reported for an *H. polygyrus* antigen, which was considered to contain a superantigen (Robinson *et al.*, 1994). Classically, superantigens, such as *Staphylococcal* enterotoxin B1, do not require to be processed for efficient antigen presentation (Herman *et al.*, 1991), however the superantigens *Pseudomonas* exotoxin A and Streptococcal M protein did require metabolically active APCs (Legaard *et al.*, 1991; Majumdar *et al.*, 1993). It is therefore possible that the *D. viviparus* and *H. polygyrus* adult homogenates contain superantigens.

A recombinant protein (Rpt C-A), excreted in the E/S products of *D. viviparus* (Britton *et al.*, 1995), induced antigen-specific responses in PBMC and LN-derived cells from infected calves. The actual proliferative responses to the protein were very low compared with the responses observed with the whole adult homogenate. However, this was not unexpected as the recombinant protein represents a minor proportion of the range

of proteins in the whole adult homogenate. A correlation was demonstrated between proliferative responses to Rpt C-A and the proliferation of PBMC cultured in medium alone. This may imply that the cells had been activated *in vivo* by Rpt C-A, or a similar antigen. Similar *in vivo* activation of PBMC has been observed in sheep infected with *T. axei* (Pernthaner *et al.*, 1995).

When analysing proliferative responses in the LNs, cells from the peripheral pre-crural LNs had significantly higher responses to Rpt C-A compared with those from the local T/B LNs. This suggests that there may have been a local inhibition of proliferative responses. Cytokine analyses demonstrated local down-regulation in secretion of IL-2, IFNγ and IL-1, but not IL-4, in the T/B LNs. *D. viviparus* may produce factors which down-regulate proliferative responses, as has been demonstrated for an E/S product of *T. taeniaeformis* (Leid *et al.*, 1986). Alternatively, the responses may have been inhibited, indirectly, by secretion of cross-regulatory cytokines which were induced by the parasite. mRNA for IL-10 was detected using Northern analysis whereas mRNA for IL-2 and IL-4 were not, implying that there were higher levels of mRNA for IL-10 following infection with *D. viviparus*. During *N. brasiliensis* infections, mice synthesise greatly increased quantities of IL-10 (T.R. Mosmann, unpublished, cited by Mosmann and Moore, 1991). IL-10 is an important immunoregulator (Moore *et al.*, 1993) and was demonstrated to inhibit cytokine production and proliferation of bovine Th0, Th1 and Th2 clones (Brown *et al.*, 1994c), so may have been involved in down-regulation of responses in this system. It would be interesting to investigate this hypothesis by measuring mRNA for IL-10 in the pre-crural and T/B LNs of infected calves.

The ruminant immune system contains a very large number of $\gamma\delta^+$ T cells (Hein and Mackay, 1991), so it would not be surprising to find that they played an important role in bovine immune responses. There were significant increases in the percentages of $\gamma\delta^+$ T cells in the PBMC and T/B LN cell populations after infection with *D. viviparus*. Moreover, after stimulation of whole PBMC populations *in vitro*, the $\gamma\delta^+$ T cells, but not the $CD4^+$ and $CD8^+$ cells, proliferated. Separated $\gamma\delta^+$ T cells populations proliferated in a specific manner to parasite antigen, but this was mainly after the primary and secondary infections, with low responses after the tertiary infection. In contrast, the $CD4^+$ and $CD8^+$ cells which proliferated most vigorously were those from uninfected calves and those collected after the tertiary infection. Therefore, $CD4^+$ and $CD8^+$ cell responses were diminished after the

primary and secondary infections and this was associated with an increased proliferation of the $\gamma\delta^+$ T cells. This reciprocal relationship implies that activated $\gamma\delta^+$ T cells may have induced factors which down-regulated $CD4^+$ and $CD8^+$ cells. A possible mechanism may be via IL-10, which was shown to be produced by murine $\gamma\delta^+$ T cells (Hseih *et al.*, 1996). A role for $\gamma\delta^+$ T cells in immune surveillance was discussed in Chapter 1 and recent evidence has implied that they act as local tissue regulators. Immune responses to pathogens which induced either Th1- (Mombaerts *et al.*, 1993; Park *et al.*, 1993) or Th2-type (McMenamin *et al.*, 1995) responses were controlled by $\gamma\delta^+$ T cells. Hence, the $\gamma\delta^+$ T cell response may be parasite-driven to evade the host's immune response, or host-driven to avoid excessive immunopathology. The lack of $\gamma\delta^+$ T cell responses after the tertiary infection ties in with their role as immune regulators. After the primary and secondary infections, the $\gamma\delta^+$ T cells may have been activated by parasite factors to which the calves were not exposed after the tertiary infection. Alternatively, after the primary and secondary infections, the $\gamma\delta^+$ T cells may have been activated by self-antigens (O'Brien *et al.*, 1989), expressed as a result of local tissue damage caused directly and indirectly by the parasite.

The high degree of immunity induced by *D. viviparus* made it an ideal model in which to study immune responses in cattle. The results presented demonstrate that a Th2-type response was important in the immune response to this parasite and that $\gamma\delta^+$ T cells appeared to play a prominent role. These exciting findings suggest great potential for this parasite model in future studies of immune responses in ruminants. For example, it would be interesting to further investigate of the role of $\gamma\delta^+$ T cells in the regulation of the immune response to *D. viviparus*. This could include investigating whether $\gamma\delta^+$ T cells produce IL-10 and whether addition of $\gamma\delta^+$ T cells, from infected and uninfected calves, to $CD4^+$ and $CD8^+$ cells inhibited proliferative responses to parasite antigen. Another avenue which could be explored would be to use this model to look for the source of IL-4 for initiation of Th2 responses. Mast cells (Bradding *et al.*, 1995) and $\gamma\delta^+$ T cells (Ferrick *et al.*, 1995) are potential sources and these were both up-regulated in calves after infection with *D. viviparus*. Future *in vitro* and *in vivo* studies could investigate whether *D. viviparus* antigens induce release of IL-4 by mast cells or $\gamma\delta^+$ T cells. Moreover, further characterisation of the antigens that are important in up-regulation of IL-4 may help unravel the mechanisms involved and provide ideas for new vaccines.

APPENDICES

1: RECIPES

1.1 Phosphate buffered saline

Stock Buffer

60 g	di-sodium hydrogen orthophosphate (Na_2HPO_4: BDH)
13.6 g	sodium dihydrogen orthophosphate (NaH_2PO_4: BDH)
8.5 g	sodium chloride (NaCl: BDH)

1 x PBS

80 ml stock made up to 2 litres (L) with 0.9% saline. pH adjusted to 7.2.
(0.9% saline: 18 g NaCl made up to 2 L with ddH_2O)

1.2 Tris / proteinase inhibitors

10 mM	Tris base (Boehringer Mannheim, Lewes, East Sussex) / HCl pH 8.3 containing:
2 mM	ethylenediaminetetra acetic acid (EDTA: Sigma)
1 mM	phenylmethylsulponylfluoride in isopropanol (Pms-F: Sigma)
5 µM	pepstatin in methanol (Sigma)
2 mM	1,10 phenanthroline in ethanol (Sigma)
5 µM	leupeptin (Sigma)
5 µM	antipain (Sigma)
25 µg/ml	N-*p*-tosyl-L-lysine chloromethyl ketone (TLCK: Sigma)
50 µg/ml	N-tosyl-L-phenyl alanine chloromethyl ketone (TPCK: Sigma)

1.3 Nematode Culture Medium

RPMI 1640 medium (Dutch modification without L-glutamine) (Gibco BRL) supplemented with:

2.4 µg/ml	L-glutamine (Gibco BRL)
100 IU/ml	penicillin (Gibco BRL)
100 µg/ml	streptomycin (Gibco BRL)
5 µg/ml	amphoteracin B (Gibco BRL)
0.25 µg/ml	gentamicin (Gibco BRL)
1 mg/ml	D-glucose (BDH)
0.4 µg/ml	glycil-L-histidyl-L-lysine (Sigma)
0.5 ng/ml	glutathione (Sigma)
0.01 mM/ml	sodium pyruvate (Sigma)
0.01 mg/ml	sodium bicarbonate (Sigma)

1.4 SDS-Page Reagents

Tris Buffers:

1.5M Tris

18.171 g Tris base (BDH) made up to 100 ml with ddH_2O.
pH adjusted to 8.8 with 1 M HCl. Stored at 4°C.

0.5M Tris

6.057 g Tris base (BDH) made up to 100 ml with ddH_2O.
pH adjusted to 6.8 with 1 M HCl. Stored at 4°C.

Separating Gel:

acrylamide	Ultrapure protogel (30% acrylamide, 0.8% bisacrylamide stock solution: Flowgen, Sittingbourne, England)
SDS	sodium dodecyl sulfate (Sigma)
$(NH_4)_2S_2O_8$	Ammonium peroxodisulphate (BDH)
TEMED	Tetramethylethylenediamine (Sigma)

For 10 mls

	10%	**12%**	**15%**
ddH_20	4 ml	3.3	2.3
30% acrylamide	3.3 ml	4	5
1.5 M Tris (pH 8.8)	2.5 ml	2.5	2.5
10% SDS	0.1 ml	0.1	0.1
10% $(NH_4)_2S_2O_8$	0.1 ml	0.1	0.1
TEMED	0.004 ml	0.004	0.004

Stacking Gel:

ddH_20	6.1 ml
30% acrylamide	1.3 ml
0.5 M Tris (pH 6.8)	2.5 ml
10% SDS	0.1 ml
10% $(NH_4)_2S_2O_8$	0.05 ml
TEMED	0.01 ml

Sample Buffer

5 g	SDS (Sigma)
5 ml	1 M Tris, pH 7.5
2 ml	100 mM Pms-F (Sigma)
1 ml	100 mM EDTA (Sigma)
10 ml	glycerol (Sigma)
2 ml	0.2% (w/v) bromophenol blue (BDH)

Added to ddH_20 and made up to 100 ml. Just before use, added 5% 14.3 M 2-mercaptoethanol (Sigma). For "native" gel: omitted SDS, PMS-F and EDTA.

Running Buffer

3.3 g	Tris base (BDH)
14.42 g	Glycine (BDH)
1 g	SDS (Sigma)

Tris base and glycine were dissolved in 700 ml ddH_2O. SDS was added, then the solution was made up to 1 L with dd H_2O. The pH was adjusted to 8.3 with HCl.

1.5 Western blotting

Tris-glycine transfer buffer (pH 8.2)

3 g	Tris base (BDH)
14.4 g	Glycine (BDH)
1 g	SDS (Sigma)
200 ml	methanol

Made up to 1 L with ddH_2O. Adjusted to pH 8.2 with HCl.

Blot wash buffer (pH 7.2)

1.21 g	Tris base (BDH)
8.75 g	NaCl (BDH)
0.5 ml	polyoxyethylene-sorbitan monolaurate (Tween 20, Sigma)

Made up to 1 L with ddH_2O. Adjusted to pH 7.2 with HCl.

1.6 Carbonate buffer (pH 9.6)

1.5 g	sodium carbonate anhydrous (Na_2CO_3, BDH)
2.93 g	sodium hydrogen carbonate ($NaHCO_3$, BDH)
0.2 g	sodium azide (NaN_3, Sigma)

Made up to 500 ml with ddH_2O and adjusted pH to 9.6, stored at 4°C.

1.7 Tissue Culture Medium

RPMI	Dutch modification (without L-Glutamine)(Gibco BRL) with:
2.5%	L-glutamine 200 mM (Gibco BRL)
0.5%	Hepes buffer 1 M (Sigma)

1.8 Hank's washing solution

0.35 g	sodium bicarbonate (BDH)
9.8 g	Hanks' balanced salts (Sigma)
50 ml	pen/strep 5000 iu penicillin and 5000 iu streptomycin (Gibco BRL)
50 ml	amphoteracin B (Gibco BRL)
6.25 ml	gentamicin 50 mg/ml (Sigma)

Made up to 1 L with autoclaved ddH_2O.

1.9 Cryopreservation medium

RPMI	Dutch modification (without L-Glutamine)(Gibco BRL) with:
2.5 ml	5000 iu penicillin and 5000 iu streptomycin (Gibco BRL)
20%	Heat inactivated FCS (Gibco, BRL)
20%	DMSO (BDH)

1.10 Bio-assay culture medium

89 ml	RPMI Dutch modification (without L-Glutamine)(Gibco BRL)
1 ml	L-glutamine 200 mM (Gibco BRL)
100 µl	2 mercapto-ethanol 2 x 10^{-5} M (Sigma)
100 µl	gentamicin 50 mg/ml (Sigma)
10 ml	Heat inactivated foetal calf serum (Gibco BRL)

1.11 PBS/BSA/azide

5 g	bovine serum albumin (BSA, Boehringer Mannheim)
0.5 g	sodium azide (NaN_3, Sigma)

Added to 500 ml PBS.

1.12 DEPC treated water

Added 0.1% diethyl pyrocarbonate (DEPC, Sigma) to ddH_2O. Allowed to stand for 2 h at room temperature to inhibit RNAses. Autoclaved to remove traces of DEPC.

1.13 RNA sample dye

50%	glycerol (Sigma)
1 mM	EDTA (Sigma)
0.25%	bromophenol blue (BDH)
0.25%	xylene cyanol FF (Sigma)

Made in DEPC ddH_2O. Stored at 4°C.

1.14 DNA sample dye

30%	glycerol (Sigma)
0.25%	bromophenol blue (BDH)
0.25%	xylene cyanol FF (Sigma)

Made in ddH_2O. Stored at 4°C.

1.15 Tris-acetate buffer (TAE) 50x

242 g	Tris base (BDH)
57.1 ml	glacial acetic acid (BDH)
100 ml	0.5 M EDTA (Sigma)

Made up to 1 L in ddH_2O.

1.16 SOB medium

2 g	tryptone (Difco, West Molesey, Surrey)
0.5 g	yeast extract (Difco)
1 ml	1 M NaCl (BDH)
0.25 ml	1 M potassium chloride (KCl, BDH)

Made up to 100 ml in ddH_2O. Shaken until solutes dissolved, then autoclaved.

1.17 2 M magnesium stock solution

1 M	magnesium chloride ($MgCl_2$, BDH)
1 M	magnesium sulphate ($MgSO_4$, BDH)

Sterilised through a 0.2 μm syringe filter (Nalgene).

1.18 SOC medium

10 ml	SOB medium (Appendix 1.16)
100 µl	2 M D-glucose (BDH)
100 µl	2 M Mg stock solution (Appendix 1.17).

Sterilise through a 0.2 µm syringe filter (Nalgene).

1.19 L-broth

10 g	NaCl (BDH)
10 g	tryptone (Difco)
5 g	yeast extract (Difco)
15 g	bacto-agar (Difco): For agar plates]

Made up to 1 L with ddH_2O, then autoclaved.

1.20 Denhart's solution (50x)

5 g	Ficoll (Pharmacia)
5 g	polyvinylpyrrolidine (Sigma)
5 g	BSA (Fraction V, Sigma)

Made up to 500 ml with ddH_2O, then stored at -20°C.

1.21 Prehybridisation buffer

12.5 ml	20 x SSC (Appendix 1.21)
5 ml	50 x Denhart's solution (Appendix 1.22)
2.5 ml	10% (w/v) SDS (Sigma)
100 µl	salmon sperm DNA (Sigma)

Made up to 50 ml with sterile water. Heated to 65°C. Denatured approximately 200 µl of salmon sperm DNA by boiling for 10 min and cooling on ice for 5 min. Added 100 µl of the denatured salmon sperm to the warm buffer and used immediately.

1.22 SSC (20 x)

175.32 g	NaCl (BDH)
88.23 g	sodium citrate (Sigma)

Made up to 1 L with ddH_2O and stirred well.

1.23 Thrombin cleavage buffer

24.22 g	Tris base (BDH)
87.66 g	NaCl (BDH)
3.67 g	calcium chloride ($CaCl_2$, BDH)

Made up to 1 L with ddH_2O. This was 10 x buffer. For 1x buffer; diluted 1:10 with ddH_2O.

Cytokine	Access. No. (Ref)	Sequence: 5'to 3'	Size of insert
bobactin 3'	u02295	GAGAAGCTGTGCTACGTCGC	270 bp
5'	(1.)	CCAGACAGCACTGTGTTGGC	
boIL-2 3'	m12791	GAGGCACTTAGTGATCAAGTC	373 bp
5'	(2.)	TGCTGCTGGATTTACAGTTGC	
boIL-4 3'	m77120	TCAGCGTACTTGTGCTCGTC	349 bp
5'	(3.)	GTCTCACCTACCAGCTGAATC	
boIL-5 3'	none	TCAGCTTTCCATGCTCCACTC	327 bp
5'		ATGAATAGACTGGTGGCAGAG	
boIL-6 3'	x57317	CAGCTACTTCATCCGAATAGC	505 bp
5'	(4.)	AGGCAGACTACTTCTGACCAC	
boIL-10 3'	u00799	CGTTGTCATGTAGGATTCTATG	517 bp
5'	(5.)	ACAGCTCAGCACTGCTCTGTT	
boIL-1a 3'	m37210	GTAGAGTGCACAGTCAAGGCT	480 bp
5'	(6.)	TACAGCTTCCAGAGTAACGTG	
boIL-1b 3'	m35589	AGTGAAGTTCAGGCTGCAAGCT	558 bp
5'	(7.)	TGACGCACCCGTTCAGTCAA	
boTNFa 3'	z14137	ATGATCCCAAAGTAGACCTGCC	466 bp
5'	(8.)	ACTCAGGTCATCTTCTCAAGCC	
boINFg 3'	m29867	GGTGACAGGTCATTCATCAC	316 bp
5'	(9.)	GCAAGTAGCCCAGATGTAGC	

Appendix 2.1 Details of primers used for bovine cytokines

Refs:

1. Davey and Wildeman, 1995
2. Cerretti *et al.*, 1986a
3. Heussler *et al.*, 1992
4. Droogmans *et al.*, 1992
5. Hash *et al.*, 1994
6. Maliszewski *et al.*, 1988
7. Leong *et al.*, 1988
8. Cludts *et al.*, 1993
9. Cerretti *et al.*, 1986b

1 GGCCAGTGCA AGCTTGCATG CCTGCAGGTC GACTCTAGAG GATCCCCCGC

51 GGATCCGCCA ATGCTGTAGA AAGTACCATG AATAGACTGG TGGCAGAGAC

101 CTTGACACTG CTCTCCACGC ATCAAACTCT GCTGATAGGT GATGGGAACT

151 TGATGATTCC TACTCCTCAG CATACAAATC ACCAACTATG CATTGAAGAA

201 GTCTTTCAGG GAATAGACAC ATTGAAGAAT CAAACTGCAC AAGGGGATGC

251 TGTGAAAAAA ATATTCCGAA ACTTGTCTTT AATAAAAGAA TACATAGACC

301 TCCAAAAAAG GAAGTGTGGA GGAGAAAGAT GGAGAGTGAA ACAATTCCTC

351 GACTACCTGC AAGTTTTCCT TGGTGTGATA AACACAGAGT GGAGCATGGA

401 AAGCTGAGTC GACGCGGGGT ACCGAGCTCG AA

Appendix 2.2 Sequence for bovine IL-5 (R. Collins, Compton)

The regions chosen for PCR primers are underlined.

Cytokine	Accession Number	PCR product size (bp)	Restriction Enzyme	Product sizes (bp)
IL-1α	m37210	480	*Hae*III	423,58
IL-1β	m35589	558	*Pst*I	300,247,14
IL-2	m12791	373	*Hinc*II	276,98
IL-4	m77120	349	*Pst*I	207,141
IL-6	x57317	505	*Ecor*II	250,90
IL-10	u00799	517	*Hinc*II	326,193
TNFα	z14137	466	*Bsa*I	307,161
IFNγ	m29867	316	*Ecor*II	141,177

Appendix 2.3 Restriction enzymes used to confirm the identity of bovine cytokine PCR products.

cDNA	Access. No.	Length	Vector	Insert site	Restriction enz.	Temp
boIL-2	M12791	544bp (78-622)	pBluescript	*Eco*RV	*Hind*III, Pst1	37°C
Human β-actin	X00351	640bp (528-1168)	pBluescript	*Hind*III, *Eco*R1	*Hind*III, Pst 1	37°C
boIL-4	M77120	348bp (70-418)	PCRII		*Bst*XI	55°C
boIL-10	U00799	739bp (1-740)	PCRII		*Eco*R1	37°C

Appendix 2.4 Cytokine cDNAs

This table shows details of the cytokine cDNAs which were used to make probes for Northern and Southern blotting. Also included are details of the vectors in which the cDNAs were expressed and the restriction enzymes and conditions used to digest the vector to release the cDNA.

Samples tested	Antigen	Isotype/ subclass	Antigen conc.	Serum/BALF dilution	Mab dilution	Conjugate dilution
Serum	**L3**	IgG	0.6 μg ml^{-1}	1:400	n/a	1:10,000
		IgM	0.6 μg ml^{-1}	1:200	1:1000	1:50,000
		IgA	0.6 μg ml^{-1}	1:50	1:500	1:25,000
		IgG1	0.6 μg ml^{-1}	1:200	1:160	1:25,000
		IgG2a(A1)	0.6 μg ml^{-1}	1:200	1:320	1:25,000
	Egg/L1	IgG	0.6 μg ml^{-1}	1:400	n/a	1:10,000
		IgM	0.6 μg ml^{-1}	1:100	1:1000	1:25,000
		IgA	0.6 μg ml^{-1}	1:25	1:500	1:25,000
		IgG1	0.6 μg ml^{-1}	1:100	1:160	1:25,000
		IgG2a(A1)	0.6 μg ml^{-1}	1:50	1:160	1:25,000
BALF	**L3**	IgG	1.25 μg ml^{-1}	1:100	n/a	1:10,000
		IgM	1.25 μg ml^{-1}	1:20	1:1000	1:25,000
		IgA	0.3 μg ml^{-1}	1:20	1:500	1:25,000
		IgG1	0.3 μg ml^{-1}	1:20	1:160	1:25,000
		IgG2a(A1)	0.3 μg ml^{-1}	1:10	1:160	1:25,000
	Egg/L1	IgG	1.25 μg ml^{-1}	1:50	n/a	1:10,000
		IgM	1.25 μg ml^{-1}	1:25	1:1000	1:25,000
		IgA	1.25 μg ml^{-1}	1:25	1:500	1:25,000
		IgG1	1.25 μg ml^{-1}	1:25	1:160	1:25,000
		IgG2a(A1)	1.25 μg ml^{-1}	undil.	1:160	1:25,000

Appendix 3 Optimal dilutions of antigen and antisera for each antibody isotype for L3 and Egg/L1 measurements.

Checkerboard titrations of antigen, sera/BALF and antisera allowed optimum dilutions to be determined so that the highest ratios for background:noise were obtained. The table shows the optimum dilutions used when measuring serum and BALF antibody levels to L3 and egg/L1 antigens. For IgG, a peroxidase-conjugated anti-bovine IgG was used. For the other isotypes/subclasses an unconjugated anti-bovine Mab was used, followed by an anti-mouse peroxidase-conjugate.

	165	172	174	175
LPS v Ag	0.48 M	0.62 M	0.07	0.40 M
LPS vs Med	0.46 M	0.25	0.07	0.47 M
Ag vs Med	0.82 S	0.83 S	0.02	0.64 M

Appendix 4.1 Correlation coefficients between IL-1 levels.

IL-1 levels were measured in supernatants from PBMC collected fortnightly from calves 165, 172, 174 and 175, cultured with LPS, Con A or antigen. Pearson's correlation coefficients were calculated for samples collected at each time point, cultured under different conditions. The strength of the correlations are indicated. S: strong 0.70 to 0.89. M: moderate 0.40 to 0.69.

Group	Calf	Con A v Ag	Ag v Med	Con A v Med
A	165	0.40 M	0.31	0.43 M
	166	0.70 S	0.39	0.39
	172	0.36	0.71 S	0.59 M
	174	0.30	0.31	0.64 M
	175	0.77 S	0.21	0.56 M
B	169	0.52 M	0.56 M	0.53 M
	170	0.59 M	0.07	-0.12
	176	-0.03	-0.98 S	0.68 M

Appendix 4.2 Correlation coefficients between IL-4 levels.

IL-4 levels were measured in supernatants from PBMC collected weekly from the calves and cultured with LPS, Con A or antigen. Pearson's correlation coefficients were calculated for samples collected at each time point, cultured under different conditions. The strength of the correlations are indicated. S: strong 0.70 to 0.89. M: moderate 0.40 to 0.69.

		Con A v Ag	Con A v Med	Ag v Med
Group A	**165**	0.976S	0.810M	0.719M
	166	0.887M	0.895M	0.859M
	172	0.367	0.382	0.324
	174	0.472M	0.635M	0.430M
	175	0.309	0.457M	0.690M
Group B	**169**	0.813M	0.456M	0.696M
	170	0.852M	-0.567M	-0.138
	176	0.164	0.207	0.027

Appendix 4.3 Correlation coefficients between IFNγ levels

IFNγ levels were measured in supernatants from PBMC, collected weekly from Group A and Group B calves, cultured with Con A, antigen or in medium alone. Pearson's correlation coefficients were calculated for samples collected at each time point, cultured under different conditions. The strength of the correlations are indicated. S: strong 0.70 to 0.89. M: moderate 0.40 to 0.69.

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