OVINE CELL MEDIATED IMMUNITY TO

CHLAMYDIA PSITTACI.

Michael Campbell McCafferty

Thesis presented to the University of Edinburgh for the degree of Doctor of Philosophy in the Faculty of Medicine.

ABSTRACT

Enzootic Abortion of Ewes (EAE) is an economically important disease of ewes, caused by the gram negative, intracellular bacterium, *Chlamydia psittaci*. The disease results in lamb loss from abortion and the perinatal death of weak lambs. Vaccines have controlled EAE for more than thirty years, however in the last decade vaccine efficacy has been poor.

The primary aim of this project was to examine the cell mediated immune responses to *C.psittaci* in sheep and to identify potentially immunoprotective antigens for future vaccine studies, by their ability to stimulate both T-cell proliferation and cytokine production.

Preliminary studies determined the parameters of an antigen driven, ovine lymphocyte transformation assay for *C.psittaci*, employing whole chlamydial elementary bodies (EB) as antigen. It was shown that peripheral blood mononuclear cells (PBMC) from post abortion animals would proliferate in response to chlamydial EB, although lymphocytes from naive ewes also proliferated to a lesser degree. Further studies in mice showed this latter response could be caused by a cross reaction with harmless, enteric bacteria.

The development of proliferative responses of the PBMC to both EB and mitogens was also measured during gestation. Infection at this time led to the development of lasting T-cell responses and a transient suppression of the response to the T-cell mitogen, Con A. In addition, both mitogen and antigen specific responses were disrupted in the immediate pre-parturition period. These responses returned to control levels soon after lambing.

The T-cell proliferative response was further characterised by probing chlamydial EB which had been separated by SDS page electrophoresis and blotted onto nitrocellulose. Individual antigens were then added to cultures of PBMC and T-cell lines generated from post abortion animals. Four antigens were identified with approximate weights of 70, 50, 38 and 30kDa which stimulated proliferation. The ability of individual chlamydial proteins to stimulate cytokine production in these cultures was also tested. The four antigens above also stimulated the production of γ-IFN in the PBMC and T-cell lines from all sheep tested.

Finally, the importance of γ-IFN in a chlamydial infection was investigated in an *in vivo* mouse model, where neutralising γ-IFN with a monoclonal antibody resulted in an increase in the severity of infection in both thymic and athymic mice, when compared with control animals. Increased numbers of viable chlamydiae were isolated from tissues and increased pathological changes and serum interferon levels were demonstrated.

The results presented in this thesis provide evidence for the involvement of cell mediated responses in ovine immunity to *C.psittaci*. Both T-cell proliferation and γ-IFN production can be measured, although how the responses interact with B-cells and antibody has yet to be elucidated.

DECLARATION.

I declare that this thesis has been composed by myself and that the work contained within it, except on occassions which are clearly stated, was performed by myself.

DEDICATION.

This thesis is dedicated to the memory of my father

Michael Campbell McCafferty

February 11th 1941-June 12th 1976

ACKNOWLEDGEMENTS.

I wish to thank my supervisors at the Moredun Research Institute, Dr David Buxton and Dr David Haig, for their help and advice throughout the darkest hours of this project. I am also indebted to Dr Sarah Howie, from the Immunobiology unit of the University of Edinburgh Medical School, for acting as my university supervisor during the course of my studies.

I would also like to thank Kate Thomson, Stephen Maley and Ian Anderson for their excellent technical assistance and also Dr Gareth Jones, Dr Alan Herring and Dr James Dooley for hours of useful discussion and advice.

My thanks go to Dr John Spence and Ms Audrey Craik for their help and advice pertaining to computer facilities and to Dr David Henderson and the staff of the clinical department for their care of animals, large and small, during this project.

Many thanks must also go to the staff of the Moredun Research Institute and to members of the now defunct Pathology department, especially Keith, Nin, Annie, Jackie, Ann, Andy and Wendy for their support, given unfailingly after long Friday nights in the "library".

I would especially like to thank my mum and my step father, whose continual encouragement and support throughout my studies have made all this possible. Finally, my love and thanks go to Anna, who probably still can't believe its all over, for putting up with the long hours, the piles of references and the making of endless cups of tea.

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Chapter 1:

INTRODUCTION.

THE ORGANISM.

Members of the genus *Chlamydia* are unique Gram-negative bacteria. They are obligate, intracellular pathogens which lack the ability to synthesise high energy compounds such as adenosine tri-phosphate and guanosine tri-phosphate, leading Moulder (1974) to coin the phrase "energy parasites". Chlamydial disease has almost as many forms as hosts. Chlamydia infect arthropods, molluscs, over one hundred and thirty species of birds and several species of mammal (Ward, 1983). Avian strains can cause acute respiratory infection in man (Macfarlane and Macrae, 1983) as well as birds and the mammalian strains are known to cause pneumonia, conjunctivitis, polyarthritis, synovitis, enteritis, seminal vesiculitis, sporadic encephalomyelitis and abortion (Storz and Krauss, 1985). Indeed, 20% of ovine abortion reported annually in the U.K. is due to *C.psittaci* (Aitken, 1986a).

Taxonomy

It is perhaps ironic that the Chlamydiae, from the Greek word *chlamys* meaning a cloak, have been so named, since from the earliest days much about them has been "cloaked" in taxonomic confusion. In 1893, Nocard, isolated what he thought was the agent of psittacosis from the wings of psittacine birds he had been studying (Bedson, 1958). He named this organism *Bacillus psittacosis* and although the agent described was probably *Salmonella typhimurium* the name persisted until 1930. Meanwhile, in 1907, Halberstaedter and von Prowazek isolated what they thought was a protozoan from conjuctival scrapings, and called this

parasite *chlamydazoon*. Later the chlamydiae were thought to be viruses, since they could not be grown by normal plating techniques. This theory continued until 1966 when Moulder showed that the chlamydiae were in fact intracellular bacteria with no relationship to viruses.

It was not until 1930 that at least five groups isolated the true agent of psittacosis (Bedson, Western and Simpson, 1930; Krumwiede, McGrath, and Oldenbusch, 1930; Lillie, 1930; Coles, 1930; Levinthal,1930), the latter three leading to inclusion bodies being known as Lillie, Coles, Levinthal bodies or LCL bodies. At this time Lillie proposed the name *Rickettsia psittacosis* and the link with the rickettsiales was formed. This link is perhaps not surprising since the rickettsiales are all Gram-negative obligate intracellular, parasites (Moulder, Hatch, Kuo, Schachter and Storz, 1984).

However, there are differences between the two orders (Moulder et al,1984) including the chlamydial development cycle with alternate cell types, as well as their lack of structural muramic acid and glutamate oxidation. Therefore in 1971, Storz and Page, suggested that they should be classified in a separate order to be called Chlamydiales. However, during the interim period from 1930, many taxonomic names were proposed and the proliferation of names used in scientific literature during the early 1960's, including

Bedsonia, Miyagawenalla, Neorickettsia, as well as psittacosis and ornithosis agents, prompted Page (1966) to suggest the unification of all the members of the group in one family, Chlamydiacae with one genus, Chlamydia .

The first division of the genus Chlamydia was based on chemical and morphological characteristics (Gordon and Quan, 1965). It was found that all the members of the genus could be split into two smaller groups. Group A chlamydiae, produced compact cytoplasmic inclusions which contained glycogen. This group was made up from the agents associated with trachoma, inclusion conjunctivitis and other primary human infections. Group B chlamydiae, developed diffuse inclusions with no glycogen present and comprised all the other chlamydiae from avian and mammalian hosts. The two distinct groups were confirmed when it was reported that the group A chlamydiae were inhibited by Sodium sulfadiazine and group B were not (Lin and Moulder, 1966). It was then suggested that these findings be formalised (Page, 1968) with the recognition of two species within the genus to be called *Chlamydia trachomatis* and *Chlamydia psittaci* respectively. Recently a third species has been postulated (Grayston, Kuo, Wang, and Altman, 1986; Grayston, Kuo, Campbell and Wang, 1989), consisting of the TWAR strains of *C.psittaci*. These are respiratory pathogens which unlike the other members of the species, seem to be restricted to human infections. They also differ on the basis of DNA analysis, serology and elementary body ultrastucture.

C.trachomatis has been further subdivided using a microimmunofluorescence technique (Wang and Grayston, 1971), which revealed the presence of fifteen serotypes of human origin denoted LGV 1-3 and Trachoma A-K, and also a single murine strain which causes pneumonitis. However, *C.psittaci*, perhaps because of its diverse host range is a more heterogeneous species with nine mammalian immunotypes (Perez-Martinez and Storz, 1985), which correlate with the 8 biotypes previously described (Spears and Storz, 1979) and up to seven avian strains (Toyofuku, Takashima, Arikawa and Hashimoto, 1986). The important mammalian immunotypes in sheep are Immunotype 1 associated with ovine abortion; Immunotype 3 associated with polyarthritis and Immunotype 9 associated with inapparent intestinal infection. In all cases, however, the target cells of the chlamydiae are epithelial cells (Kuo, 1986).

Life cycle.

The chlamydiae have a unique life cycle (Bedson, 1933 ;1936; Bedson and Bland, 1932; 1934), which involves two specialised, functionally distinct forms. The first of these is the infectious Elementary Body (EB). The EB is the extracellular stage of the chlamydial life cycle and is a rigid structure, resistant to osmotic lysis. It is approximately 300nm in diameter and has equivalent amounts of DNA and RNA. Unlike other Gram-negative organisms, the chlamydial cell wall does not possess peptidoglycan and thus the structural rigidity of the EB is thought to be due to

the formation of disulphide bonds which cross link cysteine rich outer membrane proteins, which appear to be produced when the infectious EB is formed (Hatch, Allan, and Pearce, 1984; Newhall and Jones, 1983).

The exact mode of entry of the EB into the host cell is unknown, however in 1978 Byrne and Moulder demonstrated that invasion of target cells and subsequent survival was possible by parasite induced phagocytosis. This phenomenon was inhibited by heating, suggesting a thermo-labile surface structure was involved. It was also shown that this induction of uptake was restricted to EB, since fewer reticulate bodies (RB) are phagocytosed in comparitive studies (Brownridge and Moulder, 1979). More recently a surface membrane protein has been implicated in the attachment of chlamydia to the host cell (Hackstadt, 1986; Wenman and Meusar, 1986). The mode of entry of the chlamydiae has been reviewed recently (Wyrick, Choong, Davis, Knight, Royal, Maslow and Bagnell, 1989), and parasite directed phagocytosis, microfilament dependant and independant phagocytosis and pinocytosis via coated and non-coated pits have all been suggested. The reasons for the many possible modes of entry that have been reported may be due to the different target cells used, the varied visualisation techniques employed or the use of infectivity enhancing techniques such as centrifugation.

Once inside the cell, the chlamydiae survive by inhibiting phagosome-lysosome fusion (Friis, 1972), perhaps in the same manner as other intracellular pathogens such as *Toxoplasma gondii* (Jones and Hirsch, 1977) and *Mycobacterium tuberculosis* (Lowrie,

1983). *Mycobacterium leprae*, on the other hand, escapes from the phagosome into the cytoplasm before fusion (Sibley, Franzblau and Krahenbuhl, 1987). The exact mechanisms by which these effects are achieved have yet to be defined. Chlamydial intracellular RBs, however, are incapable of inhibiting phagosome-lysosome fusion (Brownridge and Moulder, 1979) since, when compared with EBs, more are destroyed upon uptake by macrophages. Phagosome-lysosome fusion inhibition is thus restricted to and is triggered by EBs (Eissenberg and Wyrick,1981). When yeast and EBs are concomitantly phagocytosed by macrophages fusion occurs only with yeast laden vacuoles, showing that chlamydial EBs cause specific and not general suppression of fusion (Eissenberg and Wyrick, 1981). Later it was shown that purified EB envelopes alone could inhibit fusion and in this case the ability to inhibit fusion was not lost on heating the envelopes (Eissenberg, Wyrick,Davis and Rumpp, 1983). However, large numbers of EBs, rather than causing higher rates of infection, actually cause reduced rates due to a phenomenon known as immediate cytotoxicity (Moulder, Hatch, Byrne and Kellog, 1976). This is thought to be caused by membrane lesions resulting from the parasite-induced phagocytosis (Friis, 1972). Therefore, larger numbers of EBs give rise to more ingestion, causing more lesions, leading to irreparable membrane damage and cell death, before the invading chlamydiae can replicate.

With survival assured the EBs remain inside a distinct vesicle and transform into the metabolically active, reproductive form of the organism, the RB. RBs can be up to 1600nm in diameter and due to the protein synthesis necessary for metabolism and reproduction,

contain three times as much RNA as DNA (Tamura and Manire, 1967). They divide by binary fission within the confines of the endosome, which increases in size to form a prominent inclusion. Metabolically the chlamydiae are limited (Hatch, 1988), fragments of various pathways are present, but they cannot generate ATP (Moulder, 1962), and therefore possess an ATP uptake system (Hatch, Al-Hossainy and Silverman, 1982). ATPase activity appears to be intrinsic and is regulated by the cysteine rich outer membrane proteins (CRP) in EB (Peeling, Peeling and Brunham, 1989). However, the chlamydiae are dependant on the host cell for basic metabolites and other factors which enable it to synthesise protein, DNA and RNA. The RBs, after division, mature and condense into infectious EBs within the inclusion which then ruptures, causing cell lysis and freeing the mature EBs into the surrounding environment to begin the cycle again.

Persistence vs Latency?

The disease states caused by *C.psittaci* in ruminants fall into two groups; chronic disease such as conjunctivitis and arthritis, due to immunotype 2 and intestinal infection due to immunotype 9, and the second form, acute single episode diseases such as enzootic abortion of ewes (EAE), caused by immunotype 1. In both cases there is a persistence or latency of infection, both *in vitro* (Rodolakis, Bernard, Souriau, Layadi and Buzoni-Gatel, 1989), where avirulent strains induced completely inapparent persistent infections, and *in vivo* where following infection in ewes, *C.psittaci* can persist until the following lambing season when the animals become pregnant, at which time a proportion will abort (McEwan,

Littlejohn and Foggie, 1951). *C.psittaci* can also persist in goats as it has been isolated from them after abortion, when they are considered to be immune (Brown, Amos, Lavin, Girjes, Tims and Woolcock, 1988). Further evidence of this comes from recent work in sheep which showed that independant of the time of infection, pathological changes did not occur in the placenta until after day 90 (Buxton, Barlow, Finlayson, Anderson and Mackellar, 1990). However, it has still to be demonstrated whether these phenomena are due to a low level persistant infection existing in equilibrium with the immune system, or are simply due to a latent form of *C.psittaci*. A persistent infection in EAE suggests that EBs are constantly released, but are held in check until the conditions around the placenta are correct, after day 90; whereas a latent infection would suggest a switching off of the growth cycle, possibly an as yet undetected developmental form, which restarts when triggered by an unknown factor, perhaps hormonal, after day 90.

Support for a latent form of *C.psittaci* came in 1980 when a cryptic form of the parasite which did not stain with Giemsa was suggested to explain an *in vitro* demonstration of a persistant infection (Moulder, Levy and Schulman, 1980). Evidence for chronic persistence comes from *in vitro* studies where addition of lymphokines prevented the growth of *C.psittaci* in culture until the lymphokine was removed from the culture and normal growth restarted unaffected (Byrne and Faubion, 1982). Further *in vivo* studies demonstrated the reactivation of an apparently resolved infection when the host was immunocompromised (Yang, Kuo and Chen, 1983).

This situation is analogous with that encountered in *Toxoplasma gondii* infections, where in the immunocompetent host *T.gondii* form tissue cysts containing slow growing bradyzoites (Dubey and Beattie, 1988), which are hidden from the immune system, within host cells (Sims, Hay and Talbot, 1988; Sims, Hay and Talbot, 1989). These tissue cysts rupture periodically and release large numbers of infective organisms which are subsequently dealt with by a competent immune system (Conley and Jenkins, 1981; Ferguson, Hutchison and Petterson, 1989). However, in the immunocompromised host, such as AIDS patients, the release of the infective organisms can lead to recrudescence and can result in meningoencephalitis (Luft, Conley and Remington, 1983).

Morphology and ultrastructure.

While inclusions are easily seen, single EBs are only just visible by light microscopy, their size approaching the limit of resolution. The EBs of *C.psittaci* and *C.trachomatis* have a coccoid shape, whereas the EBs of the TWAR strains, the proposed species *C.pneumoniae*, are pear shaped (Chi, Kuo and Grayston, 1987). All EBs contain a single electron dense nucleoid core surrounded by cytoplasm composed of ribosomes and amorphous material.

The higher magnification possible with electron microscopy shows in greater detail the surface ultrastructure of the EB. The smooth outer membrane is punctuated by a series of projections, in a single area (Matsumoto, 1982a), which are arranged hexagonally with a centre to centre spacing of 35-50nm (Matsumoto and Higashi, 1975). Individual projections protrude from a hole in the middle of

a 30nm wide "flower" or "rosette" (Matsumoto, 1982b). However, these projections break away easily from the surface of the EB and negative staining has shown them to be 45nm in length and 6nm in diameter. Each appears to be a tapering, hollow structure (Matsumoto, 1988). The rosettes are made up from nine individual subunits which are 3-5nm in size. These are arranged around a central hole of 10-12nm from which the projection emerges (Matsumoto, 1973).

Although the RB are larger and more flexible they possess similar projections to those of the EB. These projections, originating in the cytoplasm, extend through the cell wall via the rosette and enter the host cell cytoplasm, passing through the inclusion membrane. This further strengthens the hypothesis that they are involved in transmembrane molecular transport (Matsumoto, 1981; Matsumoto, 1982c).

Outer membrane preparations, prepared from disrupted EBs show that there is a further, internal, hexagonal arrangement of fine particles, 10nm in diameter (Matsumoto and Manire, 1970). These structures repeat every 16.7nm (Matsumoto, 1979). The outer surface of the outer membrane also has a collection of fine particles 5-6nm wide and 8nm from centre to centre. These structures are thought to play a major role in the rigidity and structural integrity of the outermost surface of the EB cell wall (Matsumoto, 1979; 1982a; 1988).

ANTIGENS OF THE CHLAMYDIAE.

The chlamydiae have small genomes, of approximately 1000Kbp, with the capacity to encode an estimated 400-600 proteins (Stephens, 1988). This is similar to prokaryotes which live in or on eukaryotic cells , such as the Rickettsia with 1650Kbp (Moulder, 1988), but is much smaller than typical, bacterial genomes, such as *Escherichia coli* which has approximately 4000Kbp.

Of the 400-600 proteins synthesised by the chlamydiae many have been shown to be shared by *C.trachomatis* and *C.psittaci* and recent cross reactivity studies have shown that much of this shared antigenicity extends to the putative *C.pneumoniae* strains (Campbell, Kuo and Grayston, 1990). The most important of these will be discussed in greater detail below and include the major outer membrane protein (MOMP), at approximately 40Kd (Hatch, Vance and Al-Hossainy, 1981); CRP, at 60Kd and 12Kd (Newhall, Batteiger and Jones, 1982); a protein thought to be a heat shock product at 57Kd (Morrison, Lyng and Caldwell, 1989); three putative adhesins at 38Kd, 30Kd and 18Kd (Hackstadt, 1986; Wenman and Meusar, 1986) and the genus specific glycoprotein lipopolysaccharide (LPS) (Bedson, 1936; Nurminen, Leinonen, Saikku and Makela, 1983).

Major Outer Membrane Protein (MOMP).

This protein, so called because it comprises 60% of the protein mass in the outer membrane (Caldwell, Kromhout and Schachter, 1981; Salari and Ward, 1981), is the most well characterised of the

chlamydial proteins. It is a surface exposed acidic protein (Batteiger, Newhall and Jones, 1985) containing nine cysteine residues per polypeptide chain (Hatch *et al*, 1984; Stephens, Mullenbach, Sanchez-Pescador and Agabian, 1986; Stephens, Sanchez-Pescador, Wagar, Inouye and Urdea, 1987). These cysteine residues are important in the formation of disulphide linked oligomers in the outer membrane, which are necessary for the structural integrity of the organism (Newhall and Jones, 1983; Hatch *et al*, 1984). The cross linkages are also thought to be important for the porin-like activity of MOMP (Bavoil, Ohlin and Schachter, 1984) and as described above the developmental cycle of the chlamydiae (Newhall and Jones, 1983; Bavoil *et al*, 1984; Hatch *et al*, 1984).

Although antigenically cross reactive, MOMPs from different species and strains exhibit slightly different molecular weights (Salari and Ward, 1981; Hatch *et al*, 1981; Campbell *et al* 1990). The variations in weight are localised in four variable domains present in all MOMP genes where the amino acid sequence has been deduced thus far (Stephens *et al* 1986, 1987; Zhang, Morrison Caldwell and Baehr, 1989a). These variable domains, not surprisingly, are also the location of the antigenic sites of MOMP, including genus, species, sub-species and serotype specific epitopes (Caldwell *et al*, 1981; Caldwell and Schachter, 1982; Newhall, Terho, Wilde, Batteiger and Jones, 1986). Structural studies of MOMPs from various serovars and biotypes of *C.trachomatis* and *C.psittaci*, using peptide mapping, suggest that MOMP represents a serological mosaic of antigenic determinants

located on both the conserved and variable domains (Caldwell and Judd, 1982; Ma, Chen and Kuo, 1987). Molecular analysis, aided by the cloning of the MOMP gene (Allan, Cunningham and Lovett, 1984), has confirmed these findings by looking at the MOMP sequence and locating specific epitopes in the variable regions (Baehr, Zhang, Joseph, Su, Nano, Everett and Caldwell, 1988; Conlan, Clarke and Ward, 1988), and recent sequencing of the MOMP gene of the ovine abortion strain of *C.psittaci* S26/3, should increase knowledge of its role in the ovine immune response (Herring, Tan, Baxter, Inglis and Dunbar, 1989).

Several antibody studies, described in more detail below (Caldwell and Perry, 1982; Peeling, Maclean and Brunham, 1984), have shown that MOMP has a role in infectivity and this is supported by the finding that trypsin cleavage of surface exposed, specific variable domains of *C.trachomatis* serovar B MOMP also inhibit infectivity (Su, Zhang, Barrera, Watkins and Caldwell, 1988). However, despite this evidence involving MOMP in infectivity and the fact that it is the single most predominant surface protein, the efficacy of MOMP based vaccines has only been partially demonstrated in *C.trachomatis* infections (Taylor, Whittum-Hudson, Schachter, Caldwell and Prendergast, 1988), although in EAE, where vaccines have been shown to be effective in the past (McEwan *et al*, 1951), stronger evidence exists for the potential success of a *C.psittaci* ovine abortion strain MOMP enriched vaccine (Tan, Herring, Anderson, and Jones, 1990).

Cysteine rich outer membrane proteins (CRP).

There are two further proteins of the chlamydiae which contain a large proportion of cysteine residues and like MOMP are to be found on the surface of the EB, although unlike MOMP are not found on the RB. The CRP have molecular weights of approximately 12Kd and 60Kd and are found in both *C.psittaci* and *C.trachomatis* (Newhall and Jones, 1983; Hatch *et al*, 1984). Again there are slight differences among strains, *C.trachomatis* trachoma biovars have a singlet protein at 60Kd, whereas the lymphogranuloma venereum biovars have a doublet (Batteiger *et al*, 1985), as do *C.psittaci* strains (Hatch *et al*, 1984). The CRP are extensively cross linked by disulphide bonds to form macromolecular subunits with each other and with MOMP over the surface of the chlamydial EB and this confers the structural rigidity and osmotic stability so characteristic of the EB which has been described above (Newhall and Jones, 1983; Bavoil *et al*, 1984; Hatch *et al*, 1984).

The 60Kd CRP is highly antigenic and possesses genus and species specific epitopes (Newhall *et al*, 1982; Newhall and Basinski, 1986). N terminus sequencing (Newhall and Basinski, 1986) and recently, cloning and sequencing of the gene for the 60Kd protein of *C.trachomatis*, has been performed (Clarke and Lambden, 1988; Clarke, Ward and Lambden, 1988) and the results reveal the presence of 24 cysteine residues. DNA hybridisation studies of a *C.trachomatis* 60Kd CRP gene probe with *C.psittaci* DNA has indicated the presence of genus conserved regions within the same gene (Clarke *et al*, 1988). Further CRP gene DNA sequence analysis has

shown a high level of homology between trachoma and lymphogranuloma venereum strains (Watson, Lambden, Ward, Clarke, 1989). The low molecular weight CRP of 12Kd also possesses antigenic determinants, which are both biovar and species specific epitopes (Zhang, Watkins, Stewart and Caldwell, 1987a).

Adhesins.

Specific binding of EBs with host receptor molecules have been suggested as the method of endocytic uptake of the chlamydiae (Byrne, 1976; Ward and Murray, 1984). If this is the case then the receptor molecules would be expected to be cell wall components (Levy and Moulder, 1982) and proteinaceous (Byrne, 1976). A 38Kd, heat labile, surface protein of *C.trachomatis* was recently described (Joseph and Bose, 1991), which inhibited the binding of EBs to HeLa cells and may be the protein responsible for the findings of Byrne and Moulder (1978). Two putative receptors, also termed "adhesins" have been reported by Wenman and Meusar (1986) using a ligand blotting technique. Two proteins in *C.trachomatis*, a 30-31Kd doublet and a 18Kd single protein, were shown to bind radio-iodinated host cell membranes. The second of these was recently shown to be a DNA binding protein, homologous to eukaryotic histone H1 (Wagar and Stephens, 1988; Tao, Kaul and Wenman, 1991; Hackstadt, Baehr and Ying, 1991), and its ability to bind host cell membranes was probably due to its highly basic charge, pI 10.71 (Tao *et al*, 1991). Antibody to these adhesins raised in rabbits inhibit chlamydia-host cell association. Simultaneously, similar proteins were detected in both *C.trachomatis* and *C.psittaci*, although in the latter case there

was no adhesin doublet at 30-31Kd (Hackstadt, 1986). However a 30Kd adhesin has been identified in a meningopneumonitis strain of *C.psittaci* (Wenman, Kaul, and Meusar, 1986), and recently two putative adhesins, identified by the ligand binding technique, have been described for ovine abortion strains of *C.psittaci* (Tan, 1987).

57Kd protein.

This protein is found in both *C.psittaci* guinea pig inclusion conjunctivitis strains, as well as all fifteen serovars of *C.trachomatis* (Watkins, Hadlow, Moos and Caldwell, 1986). It is associated with both EBs and RBs, but does not appear to be immunoaccessible as determined by indirect immunofluoresence and dot immunoblot analysis of whole EBs and is therefore unlikely to be surface exposed. The 57Kd protein has been implicated in ocular hypersensitivity (Morrison, *et al,* 1989), and may be a heat shock protein.

Lipopolysaccharide (LPS).

The LPS of the chlamydiae is a glycolipid first described by Bedson (1936) and shown to be similar to the LPS of enteric bacteria in 1983 (Nurminen *et al*, 1983) and described chemically in 1985 (Nurminen, Rietschel and Brade, 1985). LPS is present in both EBs and RBs and is closely associated on the surface with MOMP (Birkelund, Lundemose and Christiansen, 1988). It forms the basis of the serodiagnostic complement fixation test for chlamydial infection (Stamp, Watt and Cockburn, 1952; Schachter and Caldwell, 1980). The molecular weight of LPS is approximately 3-4Kd. The

immunodominant group is a 2 keto-deoxy sugar (Dhir, Hakomori, Kenny and Grayston, 1972), however D glucosamine, long chain fatty acids and phosphate groups are also present in the molecule. In addition, the LPS of *C.psittaci* strains contains D galactosamine, not found in *C.trachomatis*, but it is not known if this is a species specific characteristic.

Structurally, chlamydial LPS is divided into a lipid A moeity, comprising the amino acids and the fatty acids, and an oligosaccharide core constructed from the keto deoxy sugars. The lipid A is inserted in the outer membrane while the oligosaccharides are exposed on the exterior. Chlamydial LPS lacks other sugars associated with bacterial LPS and appears similar to and cross reacts with the deeply truncated forms of LPS, such as the Salmonella Re mutants (Nurminen, Wahlstrom, Kleemola, Leinonen, Saikku and Makela, 1983). Antigenically, LPS has genus specific epitopes common to all Chlamydiae and epitopes which cross react with enterobacterial LPS (Caldwell and Hitchcock, 1984). Chlamydia specific epitopes are associated with a unique a2-8 linked keto deoxy disaccharide, found in the core of the LPS molecule (Brade, Brade and Kosma, 1988) and which has recently been synthesised (Kosma, Schulz and Brade, 1988). The cross reactive epitopes have also been characterised, and described as a terminal a-pyranoside keto deoxy residue and a 2-4 linked keto deoxy disaccharide. A third epitope of the cross reactive type is thought to reside in the lipid A portion of the LPS molecule and is unmasked only after acid hydrolysis (Brade, Nurminen, Makela and Brade, 1985). This epitope cross reacts with enterobacterial lipid A.

THE DISEASE.

Of all the chlamydia induced diseases of ruminants, the most economically damaging is EAE, the commonest cause of infectious abortion in sheep in Great Britain (Aitken, 1986a). Lamb losses from EAE may cost the British agricultural industry as much as £10 million per annum (I.D.Aitken, personal communication). Abortions occur in 5-10% of ewes in endemically infected flocks, but abortion rates may be as high as 30% in flocks infected with *C.psittaci* for the first time (Stamp, McEwan, Watt and Nisbett, 1950). While ewes of all ages may abort (Linklater and Dyson, 1979), the incidence is usually higher among young animals (Stamp *et al*, 1950). However in both cases recovery is usually uneventful.

Enzootic Abortion of Ewes (EAE).

The disease was first described in 1936 (Greig, 1936) although shepherds had recognised it as a problem for many years before this. It is characterised by gross inflammation and necrosis of the placenta, leading to the birth of weak or dead lambs, possibly resulting from poor placental transfer of nutrients. Indeed, the disease was first thought to be due to environmental factors and dietary deficiency (Greig, 1936). However, the causative agent of EAE was identified as psittacosis lymphogranuloma agent in 1950 (Stamp *et al*, 1950) and a series of studies culminated in the development of an inactivated, whole organism vaccine (McEwan *et*

al 1951; McEwan, Dow and Anderson, 1955; Littlejohn, Foggie and McEwan, 1952; McEwan and Foggie, 1954;1956). This vaccine was widely used and controlled the incidence of EAE without completely eliminating it (Foggie, 1973).

Subsequently, however, the incidence of EAE has increased again. Previously unaffected flocks have become infected, while the abortion rate in properly vaccinated flocks has risen to 7%, not significantly lower than in unvaccinated flocks (Linklater and Dyson, 1979). The loss of efficacy of the vaccine was thought to be due to either the emergence of novel, more virulent strains of *C.psittaci*, which the vaccine strain (A22) no longer mimicked or a loss of immunogenicity of the vaccine strain caused by its prolonged laboratory passage (Linklater and Dyson, 1979; Aitken, Anderson and Robinson, 1985). A second abortion strain of *C.psittaci*, S26/3, was therefore added to the vaccine.

Despite the introduction of this bivalent vaccine, throughout the '80s the number of cases of ovine abortion attributable to *C.psittaci* was greater than 40% of all diagnosed samples (Veterinary Investigation Diagnostic Analysis) and chlamydial abortion remains a problem in much of Europe, particularly in those countries with a high sheep population such as Great Britain, France, Greece and Germany (Aitken, 1986b). Chlamydial abortion is also a serious problem in America (Shewan, 1980), however, although there have been reports of it occurring in Australia, it is not considered a problem, due to various environmental factors and differences in sheep husbandry (Seaman, 1985).

Clinical signs.

The usual indicator of EAE is the discovery of dead lambs 2-3 weeks before lambing (Aitken, 1991). Earlier symptoms, including an occasional vulval discharge, are seldom noticed or recorded under normal farming conditions. Initial diagnosis can be made from the gross pathology of the foetal membranes. Necrotic placentas with oedematous thickening of the inter-cotyledonary regions are typical of chlamydial abortions. However, despite the obvious placental damage, aborted foetuses are well formed with little degenerative change. After abortion ewes may have a vulval discharge for 7-10 days, but after this dries up there are seldom any further clinical signs and most ewes recover with no side effects. On occasion metritis may be caused by the retention of a placenta and this can lead to a loss of condition and even the death of some ewes. Confirmation of diagnosis is by isolation of *C.psittaci* in eggs or by tissue culture or more conveniently by the demonstration of EBs in smears from infected tissue (modified Ziehl-Neelson stain- Stamp *et al*, 1950).

Pathogenesis

In 1950, Stamp and his co-workers were the first to isolate the then called psittacosis-lymphogranuloma agent (now known to be *C.psittaci*) from the foetal membranes of experimental animals suffering from enzootic abortion of ewes (EAE) (Stamp *et al*, 1950). They also described the appearance of a typical infected placenta,

with the irregular thickening of the chorion due to oedema and the severe inflammatory immune response. Necrosis of the cotyledons associated with the presence of large numbers of infectious chlamydia was also noted.

Understanding of the pathology involved in these gross changes was furnished by Studdert (1968) and Novilla and Jensen (1970). Briefly, from day 60 of gestation in normal pregnancy, haematomata form in the arcades of the placentome. These haematomata are formed from maternal blood from the septal capilliaries and may provide a route whereby the chlamydial EBs in the dams circulation can infect the foetal membranes from the maternal blood supply. However, although the haematomata form around day 60 of gestation, it is not until after day 90 that the first signs of chlamydial infection can be detected (Buxton *et al,* 1990), irrespective of the time of infection. In field infections clinical signs also appear to be independant of the time of infection and are similar regardless of when the sheep become infected (Buxton *et al,* 1990). This phenomenon is unlike the situation in toxoplasmosis in sheep when the time of infection has a direct bearing on the severity and eventual outcome of the disease (Blewett and Watson, 1983).

In the early stages, *C.psittaci* replicates in the foetal trophoblast cells adjacent to the haematomata. After continuing cycles of infection and replication the trophoblast cells are so badly damaged that cell debris, infectious EBs and inflammatory exudate containing mainly neutrophils, builds up at the maternal/foetal interphase. Infection then progresses deeper into

the placentome and laterally into the chorionic membranes. Later in the advanced stages of infection this can lead to extensive necrosis of the cotyledons and the chorionic membranes with an accumulation of exudate in the placentome and on the chorion. Occasionally necrosis and infiltration of neutrophils and macrophages into the maternal septa can be detected (Novilla and Jensen, 1970) and this may lead to metritis.

In the experimental situation when infection results in the death of the foetus before day 120 of gestation, the foetal membranes are usually retained within the uterus for some time before expulsion, causing severe autolysis which has prevented worthwhile analysis from being carried out. After 120 days there is noticeable foetal splenic and lymph node enlargement and fluid is found in the pleural and peritoneal cavities. However, macroscopically little foetal pathology is seen, but the microscopic lesions which are found in foetal tissues, most commonly in the liver, mark sites of focal inflammation with accumulation of reticulo-endothelial cells suggesting that the innate foetal immune system is responding to the infection.

From the wide distribution of these lesions it would appear that entry to the foetus is through the chorionic blood vessels and from there into the foetal circulation via the liver. Despite this, foetal sera tested for anti-chlamydial antibody by the complement fixation test have proved negative, although anti-chlamydial antibody has been demonstrated by an immunoperoxidase method employed by Buxton and colleagues (Buxton *et al*,1990).

Transmission.

The natural route of infection of *C.psittaci* in EAE has yet to be identified. However, it is possible that lambs become infected perinatally (McEwan *et al,* 1951), although infection can also occur transplacentally. What is known, however, is that infection and abortion can take place within a single lambing season (Blewett, Gisemba, Miller, Johnson and Clarkson, 1982), presumably from contact with infected material, highlighting the need to immediately remove animals which have aborted from animals still to lamb. However this finding does not rule out the possibility of transplacental infection also occurring. In both cases it is thought that *C.psittaci* enters a period of latency until the following lambing season when pregnancy triggers the infection of the placenta (McEwan *et al.*, 1951; Wilsmore, Parsons and Dawson, 1984).

Experimentally, typical EAE lesions can be reproduced by parenteral inoculation, either sub-cutaneously or intra-venously (Studdert and McKercher, 1968) and also by oral dosing, where there is some evidence to suggest that the portal of entry is tonsillar tissue (Jones and Anderson, 1988). Again, this finding supports the view that *C.psittaci* infection occurs by the ingestion of infected material or by inhalation of aerosols (Aitken, 1986b; 1991). This may also account for the observation that EAE outbreaks are rare in the highlands where extensive sheep farming is the common practice, but are economically crippling in the lowlands where the farming is more intensive.

Another possible route is venereal transmission, but although it has been shown that sero-conversion occurs after artificial insemination with infected semen, intravaginal infection or natural service, it is unlikely that this method of transmission contributes to the epidemiology of EAE (Appleyard, Aitken and Anderson, 1985). Finally there is little evidence of animal vectors being involved, unlike *T.gondii* for example, and although there is one report of tick borne transmission to cattle (McKercher, Wada, Ault and Theis, 1980) this has not been repeated and is not thought to constitute a major mode of transmission.

Treatment and Prevention.

C.psittaci is sensitive to many antibiotics such as tetracycline, oxytetracycline, tylosine and erythromycin. However, blanket treatment can only moderate the severity and incidence of abortion. Treating all ewes throughout gestation is expensive and so antibiotics are a retrospective treatment, dependant on rapid diagnosis, which does not eliminate infection or reverse placental damage. Therefore chemotherapy would not appear to be a practical means of control in all flocks (Foggie, 1973; Aitken, 1986b), however long acting tetracycline can be used to protect expensive breeding stock in flocks known to be at risk. The first dose (20mg/Kg) being given at or around 100 days, when the infection will have taken hold, and repeated if necessary at 14 day intervals.

Good farming practices can prevent or limit infection. Animals sero-negative for *C.psittaci* should be bought from flocks with no history of EAE, although even this measure will not guarantee infection-free animals. The problem lies in the inability of the complement fixation test and ELISA methods to distinguish naive and susceptible animals from those vaccinated and protected, ewes infected and about to abort, and convalescent and protected sheep. Therefore vigilance is necessary in detecting an outbreak of EAE within a flock, since aborting animals may infect those which have still to lamb (Blewett *et al*, 1982).

All infected material, the foetus, the placenta and contaminated bedding and food, should be buried or destroyed and where appropriate, lambing pens should be disinfected and if possible not re-used. Affected ewes should be isolated for at least 7-10 days, until the vaginal discharge dries up. After lambing, if the number of affected ewes is small, it is advisable to cull them to diminish the risk of transmission. However, if infection was widespread the animals should be retained since they will be immune to further infection and their lambing potential will not be affected as ewes seldom abort twice.

The difficulty in distinguishing naive and susceptible sheep from infected sheep, vaccinated sheep and convalescent sheep highlights the need for an effective, extensive programme of immunoprophylaxis by vaccination and at present this is the major weapon in the control of EAE. Vaccination has been used against

EAE since the 1950s and controlled the disease to the point where it was no longer considered a problem. The vaccine contained a single EAE isolate, grown in egg yolk sacs and inactivated with formalin. In the past decade reduction in vaccine efficacy (Linklater and Dyson, 1979) has led to many attempts to improve the quality of the current vaccine, including the addition of a second strain to the commercial vaccine.

A live vaccine was also demonstrated in 1973 (Yilmaz and Mitscherlich, 1973). This tested the ability of the "P" strain of *C.psittaci* to act as an attenuated vaccine and the authors claimed that it induced protection without stimulating the complement fixing antibodies normally detected after vaccination. Another vaccine employed an abortion strain of *C.psittaci*, AB7 treated with nitrosguanidine to form a temperature sensitive mutant (Rodolakis and Bernard, 1984). Again this vaccine has been reported to be effective in reducing the incidence of EAE. However, to date neither of these vaccines have been commercially produced and tested. More recently a purified EB vaccine (Anderson, Tan, Jones and Herring, 1990) and a MOMP enriched vaccine (Tan, Herring, Anderson and Jones, 1990) have also been tested and proven successful.

Zoonosis.

As well as being of veterinary and economic importance, *C.psittaci* is also an important zoonotic pathogen of humans. Avian strains have long been known to cause respiratory disease when transferred to humans (Schachter, 1986), but there have also been

reports of respiratory illness among laboratory staff (Barwell, 1955) and vaccine production workers (Baker and Cooper, 1983), caused by ovine abortion strains of *C.psittaci*. There is also a further, more serious risk from ovine abortion strains of *C.psittaci* and that is their potential to cause abortion in infected women (Buxton, 1986). The link between *C.psittaci* and human abortion has only recently been confirmed (Johnson, Matheson, Williams, Laing, Jandial, Davidson-Lamb, Halliday, Hobson, Wong, Hadley, Moffat and Postlethwaite, 1985), but has been suspected for some time (Roberts, Grist and Giroud, 1967).

Clinical symptoms include fever, headache, nausea and malaise, although photophobia, rigors and vomiting can be presented by individual patients (Buxton, 1986). Abortion occurs within a week of these symptoms and patients exhibit disseminated intravascular coagulation, which may be caused by a bacterial endotoxin such as LPS (Lester and Roth, 1977). Patients may also develop renal and hepatic dysfunction before recovery (Buxton, 1986). The increased danger for the mother in the human disease is thought to be due to the direct contact between the maternal circulation and the placenta which does not occur in sheep. Histological examination of the placenta shows inflammation of the mesoderm and, as in the ovine disease, there is damage to the trophoblast cells where chlamydial inclusions can be demonstrated (Wong, Gray, Buxton, Finlayson and Johnson, 1985). Therefore as with the sheep placenta, *C.psittaci* has a predilection for the human placenta and pregnant women are advised to avoid working with sheep, particularly during the lambing period.

IMMUNE RESPONSES TO CHLAMYDIAE.

The immune responses to the Chlamydiae were reviewed recently (McCafferty, 1990) and the main points have been highlighted and updated below.

Humoral Immune Responses.

Of the 400-600 proteins encoded by the chlamydial genome (Stephens, 1988) more than 100 can be resolved by SDS-PAGE (Salari and Ward, 1981) or by two dimensional electrophoresis (Batteiger *et al*, 1985). However, only a few of these are immunogenic. In a human system, 14 protein bands from *C.trachomatis* were recognised, using antisera from infected patients (Newhall *et al*, 1982). Rabbit immune sera detected eight proteins of *C.pneumoniae* (Campbell *et al*, 1990), guinea pig sera reacted to a similar number of bands from a guinea pig inclusion conjunctivitis strain of *C.psittaci* (Batteiger and Rank, 1987) and in preliminary immunoblotting studies in sheep, three prominent antigens from *C.psittaci* ovine abortion strains were demonstrated (McClenaghan, Herring, Aitken, and Honeycombe, 1986). More recently 12-14 immunoreactive bands were recognised by convalescent ovine lymph fluid (Huang, Tan, Buxton, Anderson and Herring, 1990) and nine were identified by sera from sheep, which had been vaccinated and challenged with *C.psittaci* (Anderson *et al*, 1990).

Specific IgM and IgG antibodies are induced by these immunoreactive antigens following infection (Page, Patterson, Reopke and Glaser, 1967). Originally, Isa and co-workers (Isa,

Linscott and Jawetz, 1968) claimed that only IgG was produced in monkeys, even in a primary infection. However this was later shown not to be the case and the earlier lack of IgM activity was due to either inapparent previous infections or cross-reacting antigens (Isa, 1973). The ability of the immunofluorescence test, used to detect IgM, in primates has also been questioned (Juchau, Linscott, Schachter and Jawetz, 1972), since high affinity IgG may affect the ability of low affinity IgM to bind to chlamydial antigen.

In several antibody studies epitopes present on MOMP have been shown to be important in chlamydial infectivity. The neutralising ability of anti MOMP IgG has been demonstrated, when surface cross linking of the EB interfered with the development cycle of the organism after internalisation (Caldwell and Perry, 1982), lending weight to the hypothesis that the reduction of disulphide bonds and general "loosening" of the rigid EB structure is important in the chlamydial life cycle (Newhall and Jones, 1983; Hatch *et al*, 1984; Newhall, 1987). This effect has also been demonstrated using an anti MOMP species specific monoclonal antibody, which was effective against more than one serovar of *C.trachomatis* (Peeling *et al*, 1984). Zhang and his co-workers have also obtained similar results, demonstrating neutralisation (Zhang, Stewart, Joseph, Taylor and Caldwell, 1987b; Zhang, Stewart and Caldwell, 1989b).

Neutralisation studies with monoclonal antibodies against other chlamydial proteins recognised by immunoblot techniques have not been as successful. While the CRP role in the structure and

function of the chlamydiae is pivotal and they contain biovar and species specific epitopes, studies indicate that the epitopes are not immunoaccessible (Zhang *et al*, 1987a) and neutralisation tests have not been done. There have also been no successful attempts at neutralisation of infectivity with monoclonal antibody reacting with LPS. The adhesins, with a putative role in host cell attachment, would be candidates for neutralising antibody studies and indeed polyclonal rabbit serum raised against these proteins was capable of inhibiting attachment *in vitro* (Wenman and Meusar, 1986). These results take on further significance in light of studies by Russell and Alexander (1988) which demonstrated prophylaxis in cutaneous leishmaniasis using two antigens known to be involved in the attachment of another intracellular parasite, *Leishmania mexicana*, to its host cell, the macrophage.

Following infection in ewes, CF antibody is produced against epitopes found on chlamydial LPS. Titres rise to a peak at about fourteen days after abortion and remain high for several weeks (Stamp *et al*, 1952). Neutralising antibody appears later, and titres remain higher for longer (McEwen and Foggie, 1954), but neither CF nor neutralising antibody titres correlate with immunity (Storz and Krauss, 1985).

Recent work has concentrated on which subclass of IgG is produced in the humoral response. This interest was stimulated when it was shown by ELISA (Schnorr, Perez-Martinez and Storz, 1987), that IgG2 was the dominant subclass in bovine chlamydial infections. However, similar work with sheep and goat antibodies

showed that IgG1 is dominant (Krauss, Semler, Schmeer and Somner, 1985; Schmeer, Krauss, Apel, Adami, Muller and Schneider, 1987). The significance of this is unknown at present but work on how antigens stimulate different subclasses of antibody may prove useful, particularly in light of the fact that gamma-interferon enhances murine IgG2 production and decreases IgG1 production *in vitro* (O'Gara, Umland, De France and Christiansen, 1988).

However laboratory animal studies dealing with passive transfer of antibodies have produced conflicting results. Passively transferred serum antibody in non-immune guinea pigs resulted in titres higher than those associated with natural immunity to the guinea pig inclusion conjunctivitis strain used (Watson, Mull, McDonald, Thompson and Bear, 1973). However on subsequent challenge the disease was neither prevented nor slowed. Buzoni-Gatel and co-workers (Buzoni-Gatel, Rodolakis and Plommet, 1987) found that immune sera transferred to mice infected with *C.psittaci* led to eradication of the organism from liver and spleen within 6 days, at which stage the liver and spleen of control mice were still infected. Recently polyclonal and monoclonal antibodies have been shown to protect mice from abortion caused by infection with *C.psittaci* ovine abortion strains (Buzoni-Gatel, Bernard, Anderson and Rodolakis, 1990), but to date the monoclonal antibodies used have not been characterised and their chlamydial protein specificity is not known. In sheep however, passive

transfer of hyperimmune sera has failed to protect ewes from subsequent abortion, after experimental infection with ovine abortion strains of *C.psittaci* (G.E.Jones, personnal communication).

Whether these conflicting reports are due to the different host animals used is uncertain, but they and other research showing that B cell deficient mice can resolve chlamydial genital infection and remain immune (Ramsey, Soderberg and Rank, 1988) and that guinea pigs treated with anti thymocyte serum to remove their cell mediated immune mechanisms cannot resolve infection (Rank, Soderberg, Sanders and Batteiger, 1989), suggest that cell mediated immune mechanisms may play an important role in chlamydial immunity.

Cell-mediated immunity.

Cell-mediated immunity (CMI) is a function of specific T-cell cytotoxicity and delayed type hypersensitivity (DTH) reactions, and of natural immune mechanisms including natural killer cell activity and phagocytosis by cytokine-activated macrophages.

Cytotoxicity attributed to both natural and T-cell mediated mechanisms has been demonstrated in spleen cells from mice infected with *C.psittaci* Cal 10 strain (Lammert, 1982). In comparison, cytotoxicity could not be found in lymphocyte preparations taken from the spleen, lymph nodes or peritoneal cavities of mice infected with the LGV strains of *C.trachomatis* (Pavia and Schachter, 1983). Further work with human lymphocytes also failed to

demonstrate cytotoxicity against the LGV strains of *C.trachomatis* (Qvigstad and Hirschberg, 1984). The role of T-cell cytotoxicity is therefore unresolved, although the reported differences may be due to the different species used. It remains to be elucidated whether these mechanisms play a role in protective immunity to ovine *C.psittaci* infection.

That T-cell mediated immunity to *C.psittaci* occurs in sheep can be demonstrated by DTH tests (Wilsmore, Abduljahl, Parsons and Dawson, 1984b; Dawson, Zaghloul and Wilsmore, 1986). After abortion, ewes develop a positive DTH reaction; however, if primary infection occurs outwith pregnancy no DTH response is generated. In the latter case it is possible that the organism may persist at too low a level to stimulate the DTH response, but may cause abortion of the following pregnancy. Immunity is then generated, presumably due to the higher levels of replication within the placenta and substantial challenge of the ewe with antigen as a result of parturition (Storz, 1971). This may explain the earlier findings that following abortion ewes display immunity (Stamp et al, 1950). The generation of CMI may prevent further chlamydaemias and thereby stop subsequent placental infection even though ewes may still harbour a low level infection.

Cytokine-activated phagocytosis by macrophages may occur as a result of DTH responses. In 1983 Murray and colleagues (Murray, Byrne, Rothermel and Cartelli, 1983) showed that oxygen-independent activity of macrophages against intracellular parasites, including *C.psittaci*, was enhanced by a cytokine, originally termed

macrophage activating factor (MAF) but later shown to be interferon gamma (IFN-γ). The same year IFN was shown to be also responsible for oxygen dependent activation of macrophages against intracellular parasites (Nathan, Murray, Weibe and Rubin, 1983). However, as early as 1975 Borges and Johnson had demonstrated that products in the supernatant of activated T-cells could effectively reduce the intracellular replication of *T.gondii*. Cytokine-activated macrophages have been associated with restriction of chlamydial replication, possibly leading to persistent infections (Moulder et al, 1980). Huebner and Byrne (1988) found that while activated macrophages did not clear infection, they were necessary if *Chlamydia* infected mice were to survive, indicating that their action was bacteriostatic. This *in vivo* effect of IFN-γ had been described previously in a series of *in vitro* experiments (Byrne and Faubion, 1982). Recently, Byrne and colleagues (Byrne, Schobert, Williams and Kruegar, 1989) have also demonstrated a cytotoxic effect on *C.psittaci* infected cells mediated by IFN-γ.

At the time that IFN-γ was identified as a macrophage activator it was also implicated as the factor in crude cytokine preparations which induced inhibition of *C.psittaci* replication in macrophages (Rothermel, Rubin and Murray, 1983; Byrne and Krueger, 1983). It was shown that anti-IFN-γ antibodies could remove this inhibition and precipitate a recrudescence of infection. Recently, catabolism of essential amino acids induced by IFN-γ was identified as the

mechanism for this inhibition (Byrne, Lehman and Landry, 1986). This is not the first time, however, that parasite and host competition for essential amino acids has been cited as a mechanism for inhibition of replication due to bacteriostasis (Hatch, 1975).

Other phagocytes involved in the response to chlamydial infection are polymorphonuclear cells or neutrophils (Register, Davis, Wyrick, Shafer and Spitznagel, 1987). These cells are attracted to sites of inflammation by the release of arachidonic acid metabolites from activated macrophages. However this does not seem to be the case with other intracellular parasites such as *T.gondii* (Locksley, Fankhauser and Henderson, 1985) which appear to affect the host cell's arachidonic acid metabolism altering the concentrations of leucotrienes produced, thereby reducing the inflammatory response to the infection and lowering the numbers of neutrophils at the site of infection.

Granules from neutrophils, of patients with chronic granulocytic leukaemia, were fractionated in order to examine which compounds were important in the response to Chlamydia spp. The heavier fractions, which contained lysozyme, reduced *C.trachomatis* infectivity, whereas the lighter fractions, mol wt<13Kd, had a detrimental effect on *C.psittaci*. Granules eluted in the lower molecular weight fractions contain cationic proteins, but the important individual ones are unknown at present.

Further *in vitro* techniques also show that CMI has a role to play. These include specific antigen-induced migration inhibition of peritoneal exudate cells from guinea pigs infected with *C.psittaci* (Seynk, Kerian, Stites, Schanzlin, Ostler, Hanna, Keshishyan and Jawetz, 1981) and the proliferation of lymphocytes from lymph nodes of sheep (Russo and Giauffret, 1978), and also from guinea pigs with *C.psittaci* antigens (Seynk *et al*, 1981), and in human T-cell clones using *C.trachomatis* antigens (Qvigstad and Hirschberg, 1984). In each case migration inhibition and lymphocyte proliferation were demonstrated, indicating that T-cells had been exposed to and had recognised chlamydial antigen. Lammert and Wyrick (1982), using a similar mouse lymphocyte proliferation assay, found that responses to the T cell mitogens concanavalin A and phytohaemagglutinin were reduced 1-2 weeks after infection and that proliferation in response to chlamydial antigen was suppressed. Chlamydiae-induced proliferation occurred only 4 weeks after infection, by which time mitogen responses had returned to normal.

Treatment of guinea pigs with compounds known to be selectively immunosuppressive has shown that CMI is not the only important immune response to *C.psittaci*. When cyclophosphamide is administered at levels which deplete humoral responses, but have no effect on CMI, there is no resolution of disease (Modabber, Bear and Cerny, 1976). There is therefore cooperation between the humoral and the cell-mediated systems, although the exact mechanism by which this occurs is not yet known (Buzoni-Gatel *et*

al, 1987; Rank et al, 1989). Two possibilities are suggested; antibody-dependent cell cytotoxicity and opsonisation. Wyrick and colleagues (Wyrick, Brownridge and Ivens, 1978) demonstrated that opsonised EB were taken up and destroyed in macrophages, presumably because they could no longer prevent phagosome-lysosome fusion, while it has also been shown that although immune sera can transfer immunity the effect is increased by T-cell transfer (Buzoni-Gatel, 1985).

Immunomodulation by C.psittaci.

Of importance in any review on immunity is the consideration of modulation of the host immune response following invasion by the pathogen. With relevance to *C.psittaci* infection this may occur by pathogen-induced cytokine release, hormonal alterations and direct suppression of the immune system.

One such example, the restrictive effects of IFN-γ, has been discussed above. However IFN-γ also has profound effects upon the immune system, particularly leading to the expression of Class II major histocompatibility complex (MHC class II) antigens on a variety of cell types. It is the presence of processed peptides from foreign antigens in association with MHC class II molecules on cell surfaces that is recognised by T-cells and thus initiates specific immune responses. In mice infected with *C.psittaci* increased expression of MHC II antigens has been described on macrophages (Paulnock, Huebnar, Guagliardi, Leitzke, Albrecht and Byrne, 1986). This may well play a role in the generation of immune responses at sites of infection.

C.psittaci may also have a direct modulatory effect on the immune system by inducing immunosuppression through infection of macrophages, which are important in the presentation of antigen to the T-cell and also important phagocytic cells. Thus, while not killing the macrophages, chlamydial infection may alter their ability to perform their normal functions and interfere with the immune system on many different levels. It has also been shown that antigen from *C.psittaci* can directly inhibit lymphocyte proliferation *in vitro* (Lammert, 1982). This is similar to the immunosuppressive substance recently shown to be released by *Mycobacterium leprae* (Liew, 1988).

In addition, hormones have been shown to enhance *C.trachomatis* infections in many animal models (Kuo, 1988) and in EAE it seems reasonable to suggest that hormones may stimulate the infection of the placenta which only occurs after 90 days into gestation (Buxton et al, 1990). Alteration in the levels of many hormones affect many functions of the immune system and immunosuppression associated with pregnancy is a well recognised phenomenon (Tomasi, 1983).

AIMS:

It is clear, therefore, that little is known about the cellular mechanisms which may be important in chlamydial immunity. In addition our understanding of ovine immunity to *C. psittaci* is even more limited. This thesis is designed to further our knowledge of ovine cell mediated immunity to *C. psittaci* by investigating the development of proliferative responses to EB after an initial infection during gestation. In addition, T-cell lines will be generated in order that the individual proteins from the EB which stimulate this proliferation in vaccinated and post-abortion animals can be examined. The importance of γ-IFN in the cell mediated response to *C. psittaci* will also be investigated both *in vitro* and *in vivo*. T cell lines will be used to determine which individual proteins from *C. psittaci* stimulate the production of γ-IFN *in vitro*. Finally, an *in vivo* model will be used to investigate the role of endogenous γ-IFN in the resolution of a chlamydial infection.

Chapter 2:

MATERIALS AND METHODS.

Media:

BHK-21 growth medium: A 10X solution of BHK-21 medium (Gibco Ltd, Paisley, Scotland) was used to prepare the growth medium. It was prepared by adding 20 mls of 10x BHK-21 medium to 20 mls of newborn calf serum (Gibco Ltd, Scotland), 20 mls of Tryptose, Phosphate Broth, 4 mls of sodium bicarbonate, 0.4 ml streptomycin and 1 ml Mycostatin. Finally this was made up to 200 mls with distilled water.

BHK-21 maintenance medium was prepared as above except that the newborn calf serum concentration was reduced to 2%.

BHK-21 treatment medium was prepared as the maintenance medium above except that 20 ml of the IDU solution was added before making up the final volume to 200 ml.

BHK-21 infection medium was prepared as the maintenance medium above except that Chlamydial Transport Medium containing a live inoculum of *C. psittaci* was added before the final volume was made to 200 ml.

Chlamydial Transport Medium was prepared by adding 74.6g of Sucrose, 0.512g of Potassium di-hydrogen orthophosphate, 1.237g of di sodium hydrogen orthophosphate and 0.721g of L-glutamic acid to 1000 ml of distilled water. In addition, 100 ml of foetal calf serum (Gibco Ltd, Paisley), 30 ml of mycostatin and 10 ml of Streptomycin were added before the medium was filter sterilised and dispensed in 4 ml amounts and stored at $4^{o}C$.

Sucrose Phosphate Glutamate was prepared by adding 75g of Sucrose, 0.52g of Potassium di hydrogen orthophosphate, 1.22g of Di-sodium hydrogen orthophosphate and 0.72g of glutamic acid to 1000 mls of distilled water. After membrane filtration this solution was dispensed in 20 ml amounts and stored at $4^{o}C$.

T Cell Lines and Proliferation Assays

All T cell lines were generated by using tissue culture grown elementary bodies of *C. psittaci* at a final concentration of 10 ug/ml (see page 56). In addition, while a minimum of two cell lines were raised from each animal, all graphs in Chapters 5 and 6 are from single T cell lines from single animals which are representative both of others in their group and the data discussed. All proliferation assays discussed in the thesis were after five days of culture and used ^{3}H thymidine with a specific activity of 2 Ci/mmol.

Preparation of Uninfected BHK-21 and ST-6 cells (see page 89)

Uninfected cells were prepared by harvesting monolayers using glass beads to disrupt the cells. After centrifugation (7 mins, 450xg), pellets were weighed and cell suspensions were prepared at 0.001%, 0.01%, 0.1% and 1% weight/volume.

Data Handling

The results of proliferation assays discussed in this thesis are presented as Stimulation Indices (SI) where

$$SI = \frac{\text{counts/minute (exp)}}{\text{counts/minute (control)}}$$

Mean Stimulation Indices ± Standard error (as calculated by minitab) are presneted in table of porliferative response where animals are grouped. In these tables the symbol '-' refers to an SI ≤ 1. In tables of γ-IFN production in Chapter 6 this symbol refers to production of γ-IFN equal to or below background.

All statistical analysis was carried out using a students t-test on logarithmically transformed counts per minute.

The following table is presented to clarify the calculation of SI used in this thesis. It corresponds to the results from conventially raised mice in table 3.10 on page 94 of the thesis.

DATA IN COUNTS PER MINUTE

	MEDIUM CONTROL	1ug/ml antigen	5	10	20
	251	872	595	2525	2817
MOUSE 1	138	910	463	902	2731
	211	411	922	1048	1652
	243	495	1099	367	1327
MOUSE 2	226	242	867	851	1701
	161	158	244	1007	1872
	131	274	399	1741	1667
MOUSE 3	145	220	537	632	1231
	176	150	494	717	1502
	141	142	260	1405	757
MOUSE 4	172	193	182	702	1342
	122	93	297	675	901
MEAN±SE	176±45	346±81	529±84	1047±170	1625±181
SI±SE	1	2±.5	3±.5	6±1	9±1

ANIMALS.

a) Sheep.

Three Dorset ewe lambs, originally born by caesarean section and kept in gnotobiotic conditions in a positive pressure isolator, for 8wk before being transferred to a clean loose box, were used as a source of non-infected control sheep for use in lymphocyte transformation assays and western blot analysis.

Scottish Blackface ewes from the breeding flocks of the Moredun Research Institute were used in all peripheral blood and T cell line studies. They were divided into three groups;

(i) 8 sero-negative ewes were challenged with a mixture of the S26/3 and A22 abortion strains of *C.psittaci* and subsequently aborted. Four were used 6 months after abortion and four 18 months after abortion.

(ii) 3 non pregnant ewes were given one injection of a commercial vaccine and lymphocytes were used in assays after 2-4 weeks.

(iii) 9 sero-negative pregnant ewes were left unchallenged and were allowed to lamb normally.

b) Mice.

Inbred Balb-C and minimally inbred Porton mice were supplied from the breeding colony maintained at the Moredun Research Institute.

Both athymic nude (Nu/Nu) and their thymic hairy (Nu/+) litter mates with an MF1 background were supplied by Harlan-Olac Ltd

(Oxford, England.) and maintained under sterile conditions in positive pressure isolators in the gnotobiotic unit at the Moredun Research Institute. The mice were fed on irradiated food and given distilled water and sterile litter.

BACTERIA.

a) *Chlamydia psittaci.*

The **S26/3** isolate was derived from an outbreak of abortion in a fully vaccinated flock in 1979. It was suggested that due to antigenic drift the organism had managed to evade the immune response generated by the vaccine and so it was included in the new commercial vaccine.

b) *Escherichia coli.*

The X215A isolate was recovered from a sheep rectal swab at the Moredun Research Institute, in 1990. Since the sheep was clinically normal and the isolate was negative for the adhesin K99, it was assumed to be non-pathogenic.

c) *Salmonella typhimurium.*

The SW15/A strain, an avirulent Aro A mutant with a smooth LPS mutation, was constructed at the Moredun Research Institute by Dr J.Oliver.

GROWTH OF C.PSITTACI.

The growing of *C.psittaci*, in eggs and in tissue culture (TC), is described below. The TC method is that developed by Anderson

(1984). Egg grown inoculum was used to infect all experimental animals while *in vitro* assays incorporated TC grown *C.psittaci* to lessen the possibility of inducing cellular proliferation due to contaminating egg derived proteins.

a) The growth of C.psittaci in hens' eggs.

(i) Inoculation of eggs.

Six day old embryonated hens' eggs were obtained from a specific pathogen free flock, raised on an antibiotic free diet at the Poultry Research Centre, Roslin, Midlothian, Scotland. The eggs were incubated overnight and candled to determine viability. At this time the air space was marked, the eggs were numbered and the outer shell was disinfected by washing in absolute alcohol. A 21G 1.5in needle was used to punch a hole into the air space and 0.2ml of the inoculum was injected into the yolk sac cavity. The punched hole was again disinfected and sealed. The eggs were then placed on their sides in a humidified incubator at 37^{o}C.

(ii) Harvesting the yolk sacs.

Infected eggs were candled daily and any which died up to four days later were discarded. Embryos which died up to twelve days after inoculation were disinfected with absolute alcohol and the outer shell was removed from the air space. Sterile instruments were used to cut away the shell membrane and the chorioallantoic membrane. The contents of the egg were emptied into a petri dish and the surrounding membranes removed. A small piece of the yolk sac membrane was cut and placed on a precleaned slide where it was smeared using a scalpel. The smear was air dried, heat fixed and

stained using a Modified Ziehl Neelsen method. Smears were examined microscopically using the x100 oil immersion objective and only those yolk sacs which produced smears containing several hundred elementary bodies per field of vision were harvested. Harvesting was completed by cutting the membrane from the embryo and allowing excess yolk to drain away before transferring the yolk sac to a numbered bijou before storing at -70°C.

(iii) Preparation of inoculum.

Infected yolk sacs were pooled in a plastic universal bottle and the weight was determined before transferring into a mortar. The yolk sacs were then ground with a pestle and sterile sand and resuspended with an equal volume of sucrose phosphate glutamate to give a 1:2 dilution of the original yolk sac material. This was partially purified using centrifugation at 200g for 10min which caused the suspension to become separated into three layers. The middle layer was collected and stored in 1ml amounts at -70°C.

(iv) Modified Ziehl Neelsen staining.

Air dried membrane smears were heat fixed and stained in a 10% solution of strong carbol fuchsin (BDH Chemicals Ltd, Poole, Dorset) for 15min. The smears were then washed in tap water and decolourised with 1% acetic acid. They were then washed again in tap water and counterstained with 0.8% malachite green (BDH Chemicals Ltd, Dorset) until the smear appeared blue-green, when it was given a final wash in tap water and blotted dry. Smears were then examined with a light microscope, using the x100 oil immersion objective lens.

b) The growth of C.psittaci in tissue culture.

(i) Seeding of flasks and TRAC bottles.

The Baby Hamster Kidney Cells (BHK-21) used in this study were a mycoplasma free heteroploid cell line obtained from the Wellcome Research Laboratories and were used at pass levels of between 25 and 35. Both the $80cm^2$ falcon flasks (Flow laboratories, Rickmansworth, England.) and the TRAC bottles (Bibby Sterilin Ltd, Stone, Staffordshire, England) and coverslips were seeded at a concentration of $1.5x10^5$ BHK-21 cells/ml in growth medium and were allowed to grow to confluence for 24hr at 37^oC. Flasks were seeded with 20ml of cells and coverslips with 2ml.

(ii) Treatment of BHK-21 cell monolayers.

Flasks and coverslips were placed in a sterile air hood and the growth medium removed and replaced by an equal volume of treatment medium containing 10% 5-iodo-2-deoxyuridine (IDU). IDU, an analogue of thymidine, is incorporated into the DNA/RNA of BHK cells during replication, causing faulty protein synthesis and an increased susceptibility to chlamydial infection. After the medium was replaced the flasks and coverslips were returned to the incubator for 3 days at 37^oC.

(iii) Infection of the monolayers with *C.psittaci*.

The treatment medium was decanted from the flasks and TRAC bottles and 20ml of fresh maintenance medium, containing viable chlamydial elementary bodies, was added. The flasks were then

centrifuged at 2,500g at 4°C for 30min on a Coolspin. to ensure good contact of the *Chlamydia* with the cell monolayer and so optimise chlamydial infection of the cells. The flasks were then placed in an incubator set at 37°C, for a further three days.

(iv) Harvesting of *C.psittaci* from cell monolayers.

The maintenance medium was again removed from the flasks under aseptic conditions and 4ml of chlamydial transport medium was added to each flask along with sterile glass beads. The flasks were then shaken so that the cells were both removed from the bottom of the flask and disrupted to free the *chlamydia*. The chlamydial transport medium was then aspirated off using a pipette and stored in a sterile bijou, at -70°C until required.

(v) Titration of *C.psittaci* for inoculation.

The relative strength of each aliquot of inoculum was calculated by adding serial dilutions of each to TRAC bottles and coverslips in triplicate as described above. The coverslips were then treated with Giemsa's stain (BDH Chemicals Ltd, Dorset.) and the number of inclusions present on the monolayer at the lower dilutions counted using a light microscope. Typically inclusions were counted at dilutions of between 10^{-4} and 10^{-9}, since above this the monolayer was too badly damaged and inclusions too numerous to allow accurate counting and below this there were too few inclusions to count. Results were recorded as the number of inclusion forming units/ml of inoculum.

(vi) Giemsa staining of *C.psittaci* infected monolayers.

As well as being used to calculate the relative strength of each aliquot of inoculum, TRAC bottles and coverslips were also used to monitor the percentage of infected cells. They were infected with 2ml of the infection medium at the same time as the flasks and were stained prior to harvesting of the flasks. The coverslips were removed from the TRAC bottles and were placed on a rack in methanol for 5min in order to fix the monolayer. The rack was then placed in 2.5% Giemsa stain for 20min. The coverslips were then washed in water to remove excess stain and dehydrated in the following series of acetone and xylene solutions, for 10sec. (acetone, 2:1 acetone/xylene, 1:1 acetone/xylene, 1:2 acetone/xylene, xylene). The coverslips were then mounted on glass slides with DPX (BDH Ltd) and examined at a magnification of 400 under a light microscope. Chlamydial inclusions stained dark purple in the blue cytoplasm of the BHK cells.

PURIFICATION OF CHLAMYDIAL ELEMENTARY BODIES.

Both egg grown and tissue culture grown *C.psittaci* were purified in the same way, using the method described by McClenaghan and colleagues in 1984.

(i) Removal of gross cell debris.

When the elementary bodies were to be purified from the BHK cell monolayer the harvested suspension was further disrupted by

sonication for 1min on ice, decanted into 50ml tubes and centrifuged for 5min at 1,000g at 4°C. This low speed spin removed the largest of the cell debris while leaving the elementary bodies free in the supernatant.

(ii) Purification of *C.psittaci* over 30% urografin cushion.

Urografin (Schering Health Care Ltd, West Sussex, U.K.) is the trade name of a mixture of sodium diatrizoate and meglumine diatrizoate and is used to prepare cell density gradients of varying concentrations. The urografin was supplied at a density of 370mg/ml and was diluted with 20mM Tris/50mM KCl buffer (pH 7.4) until the desired concentration was obtained. For the first stage of elementary body purification this was 30%. This was then poured into the bottom of a polypropylene tube suitable for use with the SW28 rotor of an L8 Beckman ultracentrifuge (Beckman Instruments Ltd, High Wycombe, England.). These tubes held 36ml and so 25ml of supernatant was carefully layered on top of the 10ml cushion. Each tube was then balanced to within 0.01g with Tris/KCl buffer. The SW28 rotor and swing out buckets were removed from the cold room and the tubes were placed inside. The rotor was placed on the ultracentrifuge and the supernatants were centrifuged at 53,000g for 45min at 4°C. The supernatants were discarded and the pellets were resuspended in 1ml of sucrose phosphate glutamate and stored at -70°C until they were required for purification through a 30-60% density gradient.

(iii) Purification of *C.psittaci* through a 30-60% density gradient.

The gradients were prepared with a Gibson minipulse 2 gradient maker. Firstly, 10ml of each of a 30% and a 60% solution of urografin were prepared, in 2 sterile universals. A tube was run from the 30% solution into the 60% solution via the pump and the former was almost allowed to enter the latter before the run was started. Two tubes were then run from the 60% solution to individual test tubes, suitable for use in the SW40 rotor of a Beckman ultracentrifuge (Beckman Instruments Ltd), into which the gradients were poured at a steady pump rate of 300 units on the minipulse dial. The semipurified elementary bodies were removed from the freezer and thawed on a waterbath at 37^{o}C and carefully layered onto the gradients. The tubes were again balanced to within 0.01g and placed into the buckets of the SW40 rotor and centrifuged for 120min at 53,000g at 4^{o}C. After centrifugation two distinct bands were seen, the upper layer a mixture of elementary and reticulate bodies and the lower solely elementary bodies. For the purposes of this thesis both bands were collected and washed 3 times in Tris/KCl buffer, using the SW28 buckets and rotor at a relative centrifugal force of 53,000g for 45min at 4^{o}C. The pellets were resuspended and then frozen at -70^{o}C after the protein concentration had been calculated.

PREPARATION OF SOLID PHASE ANTIGEN.

Antigen that had been transferred to a solid support, usually nitrocellulose (Bio-Rad laboratories Ltd, Watford, England), was required to enable a more sensitive investigation of the

proliferative responses to individual proteins from *C.psittaci*. Polyacrylamide gels were run, using the Laemmli method (1970), to separate the chlamydial proteins before they were blotted onto nitrocellulose by the method of Towbin and his colleagues (1979) and prepared for proliferation assays by the method of Young and Lamb (1986) adapted by Abou-Zeid and his co-workers (1987).

(i) Sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Preparing the gel.

Chlamydial proteins were separated using the Bio-Rad mini-gel system (Bio-Rad Laboratories, England). Glass plates were cleaned by wiping with alcohol to precipitate any contaminating proteins and assembled according to the manufacturers instructions. Various concentrations of polyacrylamide gels were prepared, depending on which proteins were required to be resolved. The resolving gel solution was then transferred to the gel mould, formed by the assembled glass plates, until the upper layer was approximately 1cm from the top of the smaller glass plate. Water saturated butanol was then layered on top to prevent an air meniscus forming. The gel was left to set for 30-45min. The water saturated butanol was then poured off and the stacking gel was layered on top and a perspex comb for moulding the desired wells for the samples and the standards was inserted. Once set the comb was removed and the prepared gel transferred to the running tank. Electrophoresis running buffer was added to the upper and lower reservoirs and trapped air bubbles were removed by agitating the buffer with a pipette.

Running the gel.

Samples and standards were diluted 1:2 with sample buffer and boiled for 5min in a capped tube. Different concentrations were added, again dependant on the eventual use of the gel (200µg were added for T-Cell blotting; 20µg were added for western blotting). 20µl of standards (Dalton mark VII L, Biorad) were added to the outside wells. A constant voltage of 100V was applied across the gel and electrophoretic separation was carried out until the dye front was approaching the bottom of the gel. The power was then switched off and the gel was removed from the glass plates for staining or blotting.

Coomassie Brilliant Blue staining of separated proteins.

The resolved proteins were fixed in the gel for 2-3hr using the fixative solution. After fixation the gel was placed in Coomassie Brilliant Blue (BDH Chemicals Ltd, Poole, Dorset, England) for 1hr before being destained with the destain solution until the required degree of visualisation was achieved. The destaining process was more efficient with frequent changes of destain solution and gentle agitation.

(ii) Immunoblot transfer of proteins to nitrocellulose.

This transfer was performed using a Bio-Rad electrophoresis unit. A plastic grid was placed on the bench and a sponge soaked in transfer buffer was placed on top. Next a square of blotting paper cut to the size of the gel was soaked in transfer buffer and placed on the sponge. The gel was then removed from the plates and

placed on the filter paper before being covered with a sheet of Bio-Rad 0.45µm nitrocellulose membrane also soaked in transfer buffer. Care was taken at this point to ensure that there were no bubbles breaking the contact of the nitrocellulose with the gel. A further sheet of blotting paper was added before a second sponge and plastic grid completed the "sandwich" effect. The "sandwich" was then placed into the transfer unit and covered with transfer buffer. The proteins were transferred overnight at 30V or for at least 3hr at 40V.

WESTERN BLOTTING.

The nitrocellulose containing the transferred proteins was stained with ponceau red (Sigma Chemical Company, Poole, Dorset.) for 30sec at room temperature before the dye was poured off and the nitrocellulose was washed in running tap water until the red stained protein bands appeared. The position of the standards were marked as were the limits of the sample. The standard strip was removed and the nitrocellulose was washed in PBS with 0.05% TWEEN 20 and 10% horse serum for 1hr at 37^{o}C to remove the remaining ponceau red and to lower background colouring by acting as a blocking agent. The nitrocellulose was cut into 4mm strips with a scalpel blade and placed in individual sections of a blotting tray (Bio-Rad Laboratories Ltd, England). Test sera were diluted to 1:40 and positive serum, a pool of sera from recently aborted ewes, was added at a dilution of 1:1000. Each serum was added to the strips in a 2ml amount and the strips were incubated for 1hr at 37^{o}C. After 3 washes in PBS with 0.05% TWEEN 20, 2ml of horse radish peroxidase (HRP) conjugated to a donkey anti sheep

immunoglobulin antibody (Scottish Antibody Production Unit) was added to each strip which were then incubated for 1hr at 37°C. After a further 3 washes the strips were placed in a dish and covered with 3,3 diaminobenzidine (DAB) (Sigma) substrate until the colour was sufficient to visualise the reactive proteins. The strips were then washed in water to prevent any further enzyme substrate reaction, placed between two sheets of blotting paper and allowed to dry.

PREPARATION OF NITROCELLULOSE BOUND ANTIGENS FOR PROLIFERATION ASSAYS.

(i) Visualising transferred proteins.

Transferred proteins were visualised by staining with Ponceau Red, which is not permanent and can be removed by washing. The nitrocellulose membrane was submerged in the stain for 1min and was then washed in running water until the bands became visible. At this stage the major bands were marked or cut completely from the nitrocellulose. The standards were also marked to allow measurement of distance travelled and the calculation of Rf values, a measure of the distance travelled by each protein divided by it's molecular weight, at a later date.

(ii) Dissolving the nitrocellulose.

The nitrocellulose was then placed on a large sheet of filter paper, to support the membrane when it was to be cut. A scalpel blade and a rule were thoroughly cleaned in alcohol and used to measure and cut the remaining nitrocellulose membrane into 2mm

strips. Each strip was placed in a numbered glass bijou for identification and 0.5ml of dimethyl sulfoxide (DMSO) (BDH Chemicals Ltd) was added. The bijoux were then left at room temperature for 1hr to ensure all the nitrocellulose had dissolved.

(iii) Resolubilising the nitrocellulose.

After 1hr, 1ml of sterile 0.05M bicarbonate buffer at pH 9.6, also used as a coating buffer for ELISA plates to aid protein binding, was added dropwise to the bijoux during vigourous mixing on a vortex mixer. Time and care was taken when adding the buffer, especially with the first few drops, since large clumps of nitrocellulose will resolubilise if it is added too quickly. The nitrocellulose suspension was then left for 1hr at room temperature and then transferred to sterile 1.5ml eppendorfs. DMSO was removed from the nitrocellulose, since it is toxic to cells, by washing 3 times, in Hanks Balanced Salt Solution (HBSS) containing 5% foetal calf serum (Sigma) 2% penicillin/streptomycin and 8% bicarbonate, on a microcentaur at 11,000g for 7min. The nitrocellulose was then resuspended in 1ml of Iscoves Modified Dulbecco's Medium containing 5% foetal calf serum, 2% penicillin/streptomycin, 8% bicarbonate and 2-mercapto ethanol (IMDM) and frozen at -20^{o}C until it was required for use in a lymphocyte transformation assay.

PREPARATION OF LYMPHOCYTES FOR IN VITRO ASSAYS.

Lymphocytes were prepared for *in vitro* assays in the three ways described below depending on the donor animal. T-cell lines were

also developed from post abortion sheep by the method of Kurnick and his colleagues (1979) for human T-cells and Louis and colleagues (1982) for murine cells and adapted for sheep by Haig and his co-workers (1990).

a) Peripheral blood lymphocytes.

Blood was collected in 20ml evacuated tubes (Becton Dickinson UK Ltd, Oxford, England.) containing 10 units/ml of preservative free heparin (Evans Ltd, Dunstable, England.). After thorough mixing the blood was placed in plastic universal bottles and centrifuged in an MSE Centaur at 450g for 30min, after which the "buffy coat" was collected from the interface of the red blood cells and the plasma. It was diluted 1:2 with autologous plasma to a volume of 4ml and passed over 5ml of lymphoprep (Nycomed UK Ltd, Birmingham, England.) in a sterile 10ml centrifuge tube at 650g for 40 min. Cells at the interface were collected and washed 3 times at 450g for 7min in HBSS. The cells were then resuspended in IMDM and counted, by taking 10µl of cell suspension and adding it to 10µl of 0.1% nigrosin and counting in an improved Neubauer (Weber Scientific International Ltd, Teddington, Middlesex) counting chamber. The cells were then adjusted to $2x10^6$/ml in IMDM.

b) Spleen cells.

Mice were killed and their spleens removed with sterile instruments under aseptic conditions. The spleens were placed in sterile petri dishes (Nunc Gibco Ltd, Paisley, Scotland.) and the cells were flushed from them using HBSS in two 5ml syringes with 26G3/8in hypodermic needles. The collected cells were then transferred to

10ml tubes and centrifuged at 450g for 7min to pellet them. The cells were resuspended and passed over lymphoprep, as previously described, to remove contaminating red blood cells and washed 3 times in HBSS. The cells were then resuspended at $2x10^6$cells/ml in IMDM and used in proliferation assays as described below.

c) Mesenteric lymph node cells.

Rats were killed with CO_2 gas and their mesenteric lymph nodes were removed aseptically with sterile instruments and placed in sterile HBSS. The nodes were transferred to sterile petri dishes where the fat was removed before the nodes were cut into small pieces and placed in a stomacher bag. After stomaching for 10sec to break up the nodes and free the cells, the contents of the bag were filtered into a 10ml centrifuge tube, to remove the gross cell debris. The cells were pelleted as before, washed 3 times in HBSS and resuspended to $2X10^6$cells/ml in IMDM and used in assays described below.

d) T-cell lines.

(i) Generation of T-cell lines.

PBL were prepared as described previously for specific antigen driven proliferation assays. They were then adjusted to $2x10^6$cells/ml in IMDM. A supraoptimal concentration of antigen (10µg/ml) was added and the cells were incubated in 2ml volumes in 24 well plates (Costar Northumbria Biologicals Ltd, Northumberland, England.) in a humid incubator for 3-5 days. After this time the cells were centrifuged at 450g for 7min and resuspended in 3ml of HBSS. The resuspended cells were passed over 5ml of lymphoprep in

a 10ml sterile centrifuge tube at 650g for 30min to remove dead cells. Viable blast cells were seen at the interface and were collected. These were then washed 3 times in HBSS at 450g for 7min and resuspended in IMDM. The blasts were then counted as before in an equal volume of 0.1% nigrosin and resuspended at a concentration of $2x10^5$cells/ml in IMDM containing 15 units/ml of human recombinant interleukin 2 (IL-2) (Biogen Ltd, Switzerland.) and incubated for 3-5 days. Fresh IL-2 was added after 3-4 days. After this time, washing over lymphoprep was possible if the amount of cell debris required it and the cells were rested in IMDM without IL-2 for 48hr. The donor animal was then bled again in order to produce an autologous source of antigen presenting cells with which to feed the T-cell line. PBL were prepared as before from the collected blood and were irradiated with 4,000 rads from a caesium source, a dose calculated to allow antigen processing by the cells, but to prevent further cell proliferation. After washing the irradiated cells were resuspended to a concentration of $2x10^6$/ml. Dead cells were removed from the viable blast cells as before by passing over lymphoprep and after washing 3 times, the blasts were readjusted to $2x10^5$ cells/ml. 1ml of blast cells was then added to 1ml of irradiated antigen presenting cells and the total of 2ml added to each well of a 24 well plate and incubated for a week with a supraoptimal dose of antigen. Again IL-2 was added to expand the blasts for 3-7 days with fresh IL-2 being added after 3-4 days. This cycle was repeated 3-4 times to generate a homogeneous T-cell line. Larger quantities of cells were produced when necessary by expanding the blasts for longer in IL-2 and splitting back to $2x10^5$/ml when required.

(ii) Phenotypic analysis of T-cell lines.

When T-cell lines had been produced, it was necessary to phenotype the cells in order to show that the T-cells present were in fact $CD4^+$ helper cells. This analysis was carried out by flow cytometry using the fluorescence activated cell scanner (FACscan, Becton Dickinson, Oxford, England.).

Labelling.

After 3 cycles of antigen stimulation and IL-2 expansion, and before the cells were used in a proliferation assay $5x10^5$ cells were added to each of 5 tubes and washed 3 times in Earles Balanced Salt Solution (EBSS). The first stage antibodies were mouse monoclonal antibodies raised against sheep leucocyte markers (supplied by Dr M.R.Brandon, University of Melbourne.). Following washing, the cells were pelleted and resuspended in 50µl of tissue culture supernatant containing one of the monoclonal antibodies or a control antibody. The tubes were then incubated for 40min at 4^oC with occasional agitation. Unbound antibody was removed by washing 3 times in EBSS. The second stage antibody, a rabbit anti mouse immunoglobulin conjugated to fluorscein isothiocyanate (FITC) (Dakopatts, Glostrup, Denmark), was then added as 50µl of a 1:50 dilution. The tubes were again incubated for 40min at 4^oC with occasional agitation. Unbound antibody was removed by further washing in EBSS and the cells were resuspended in 0.5ml of 2% paraformaldehyde in EBSS for fixing.

Analysis.

The Becton Dickinson FACscan utilises an argon ion laser beam (15mA, 488nm), through which the cells pass. Scattered light caused by the passage of the labelled cells is then detected and converted by the "consort 30" software package (Becton Dickinson) In this way labelled cells were detected and counted with the result given as a percentage of labelled cells in the counted population.

(iii) Freezing T-cell Lines.

A stock of prepared T-cell lines were frozen after expansion in IL-2 when sufficient numbers of blast cells allowed. Cells were pelleted at $4^{o}C$ in a refrigerated centrifuge and resuspended to $2x10^{7}$cells/ml in IMDM containing 40% FCS and 10% fresh DMSO. Working on ice, 1ml aliquots were added to cryotubes which were immediately sealed and subjected to controlled freezing ($-1^{o}C$/min). Cells were then frozen under liquid nitrogen for storage.

(iv) Thawing of T-cell Lines.

Cryotubes containing 1ml of frozen blast cells at a concentration of $2x10^{7}$/ml were thawed rapidly in $37^{o}C$ waterbath. Then, as the last ice crystals were melting, the cells were transferred to a sterile centrifuge tube. IMDM containing 50% FCS was added dropwise slowly to give a final total of 3ml and the cells centrifuged at 450g for 7min. The cells were then resuspended to 3ml in IMDM containing 30% FCS and centrifuged again before finally resuspending them in IMDM containing 5% FCS. The viable blasts were counted and adjusted to $2x10^{5}$cells/ml as

described before. These were incubated in a 5% CO_2 incubator with irradiated antigen presenting cells prepared as described, a supra optimal dose of antigen and IL-2 at 5U/ml. After one week the cell line was continued as before beginning with expansion in IL-2.

LYMPHOCYTE PROLIFERATION ASSAYS.

These assays were used to determine the proliferative response of lymphocytes taken from experimental animals to specific antigen in the form of whole chlamydial EBs and non-specific mitogens of T-cells and B-cells. The solid phase assays were based on the technique of Lamb and Young (1986) as adapted by Abou-zeid and his colleagues (1987).

a) Soluble antigen specific lymphocyte proliferation assays.

Peripheral blood lymphocytes prepared as described were added to the wells of a 96 well microtitre plate (Nunc, Gibco Ltd, Paisley, Scotland.) in 0.1ml amounts and incubated with 0.1ml of varying concentrations of chlamydial antigen preparations in triplicate wells. Also added were different concentrations of the B cell mitogen, bacterial lipopolysaccharide (LPS) from *Salmonella minnesota* Re (Sigma Chemical Company Ltd) and a T cell mitogen, Concanavalin A (Con A) again at varying concentrations (Calbiochem Ltd, La Jolla, California.) all in triplicate wells. A medium control was incorporated by adding 0.1ml of IMDM again to triplicate wells. Plates were incubated for 3-5 days in a humid incubator and were pulsed for the final 18hr with tritiated thymidine (^3H) (Amersham International PLC, Amersham, England.) which was added at a concentration of 0.5μC/well. The plates were

then harvested using the Skatron system (Skatron Ltd, Suffolk, U.K.) and the filter mats were dried before the discs were removed and placed in scintillation vials. 1ml of scintillant was added to each vial which was then capped. The collected vials were read on a beta-scintillation counter (Canberra Packard) using 1min counts and the mean counts from triplicate wells were calculated.

b) Solid phase antigen proliferation assays.

Peripheral blood lymphocytes were incubated in triplicate wells of a 96 well microtitre plate by adding 100µl of a $2x10^6$/ml solution as previously described. Medium, B-cell and T-cell controls were added to the blood cells (as previously described) and further controls, nitrocellulose only and nitrocellulose blotted with BSA (Sigma Chemical Company Ltd, Poole, England.), were added. 50µl of resolubilised nitrocellulose bound antigen fractions were added to triplicate wells along with 50µl of medium. The plates were then incubated and harvested as before.

COMPLEMENT FIXATION TEST.

The complement fixation test (CFT) used was that routinely employed for the serological diagnosis of chlamydial infection in sheep. The CFT is based on the complement fixing properties of heat stable antibodies to LPS, which are produced early in a chlamydial infection, and was developed at the Moredun Research Institute (Stamp *et al*, 1951). Briefly, the test relies on the ability of the anti-LPS antibody binding the chlamydial antigen and fixing complement. Therefore if there is no antibody present no complement is fixed and the red blood cells are lysed. If antibody

is present the complement is fixed and no lysis occurs. The test was carried out in round bottomed, 96 well microtitre plates. Serum samples were heat inactivated at 56°C for 1hr and were then diluted 1:8 in complement fixation buffer (CFT buffer). 25µl of CFT buffer was added to all rows of the plate except the top row A, then, 25µl of each heat inactivated sample was added to the top two rows of a column and the final well of the column. After mixing, 251 from row B was transferred to row C and again thoroughly mixed. This process was repeated to dilute each test sample from 1:8 in row A to 1:512 in row G. Standard sera pooled from positive and negative ewes were also added to columns and treated in the same way as the test sera. Batches of chlamydial antigen, grown in yolk sacs, were standardised before being added in 25µl volumes to all wells, except the bottom row of each column. This row was used as a control for the serum to ensure that it was not fixing complement in a non-specific way. Guinea pig complement (Wellcome research laboratories, England) was diluted in CFT buffer, to a standard dilution pre-titrated, when required. It was kept on ice and 25µl was added to all wells on the plate. A complement control was added to the final column on the plate. This involved a serial dilution of complement from row A to row H in the same way as the test serum was diluted, to ensure that the standard dilution of complement used was correct and did not cause non specific lysis of the cells. CFT buffer was added to each well in this column to replace the serum and the antigen. The plates were then stored overnight at 4°C. The next day the plates were incubated for 30min at 37°C while sensitised red blood cells were prepared. Briefly, sheep red blood cells, stored at 4°C in

Alseviers solution, were diluted to 4% v/v and mixed 1:2 with haemolytic serum. The cells were placed in a water bath and mixed frequently to ensure maximum sensitisation over a 30 min period. When sensitised, 25µl of the red blood cell mixture was added to each well of the plate, which was then shaken to thoroughly mix the complement, antigen and serum with the blood cells. The plate was then placed in an incubator for 15min at 37°C, after which time it was shaken again and replaced in the incubator for a further 15min. When finally removed from the incubator the plate was shaken once more and the contents were left to settle on the work bench for 2hr at room temperature. After first checking the results of the incorporated controls the plates were ready to be read and the titres of the test samples calculated. A full pellet with no lysis was scored as a 4 for that dilution, and no pellet with full lysis was scored as a 0. Anything in between was scored a 2.

CYTOKINE DETECTION ASSAYS.

a) Interferon assays.

These assays are based on the anti-viral effects of the interferons (IFN) in protecting normal fibroblasts from infection. The target cells selected depended on the species of interferon that was being investigated. The virus used was Semliki Forest Virus (SFV), which has a wide host range and also has the advantage of a rapid replication rate allowing the assay to be read after 48hr.

(i) Cells.

Assays for murine interferon were carried out using the murine L929 fibroblast cell line, as target cells. Ovine interferons were investigated using ST-6 cells, originally cultured from an ovine adenocarcinoma of the small intestine, as target cells.

(ii) Assay protocol.

The target cells were plated out in flat bottomed, 96well microtitre plates (Costar, Northumbria Biologicals Ltd, Northumberland, England.). Cell densities differed between assays, $5x10^3$cells/well in 100µl of growth medium (IMDM supplemented with 5% serum) for ST-6 cells and $2.5x10^3$cells/well in 100µl of medium for L929 cells. The lower density used in the latter case was necessary, because it was found that if the L929 cells were allowed to become confluent they were unaffected by the addition of the virus and showed no cytopathic effect (CPE). After 24hr, in a humid incubator, the test samples were serially diluted in serumless medium, so that any interferon present would be titrated and a final titre of interferon units/ml calculated. These samples were added to duplicate, triplicate or quadruplicate wells of the 96 well plate in 100µl volumes. Following a further 24hr incubation, the medium was removed from the plate and replaced with 200µl of medium supplemented with 2% FCS and containing SFV (supplied by Dr A.G.Morris, University of Warwick). The SFV had been titrated on ST-6 cells and stored at -70^{o}C as a stock solution of 10^5 tissue culture infectious doses (TCID) and was used in the assay at 10^2 $TCID_{50}$. The plate was replaced in the incubator for

48hr when the degree of CPE was read, using an inverted microscope. Titres were given as the inverse of the lowest dilution which reduced the CPE by 50%. A positive control was incorporated by the addition of various titrated dilutions of recombinant human α-interferon (supplied by Dr M.Scott, Wellcome Laboratories, Beckenham, Kent) to the cells. This interferon was known to be protective to the ST-6 cells to a level of 1U/ml in ovine assays. Recombinant murine γ-interferon, produced in the supernatant of transfected Chinese hamster ovary cells (supplied by Dr A.G.Morris, University of Warwick.) was used for the same purpose in murine assays. Both controls also served to standardise results between assays performed at different times.

b) γ-Interferon neutralisation assays.

In order to determine whether the interferon present in the test sample was in fact γ-IFN or was α/β IFN it was necessary to first of all incubate the test samples for 3hr at room temperature (RT) with a monoclonal antibody known to neutralise the activity of γ-IFN, after which the assay was carried out as before. A rat IgG monoclonal antibody, was used to neutralise murine γ-IFN and a murine monoclonal IFN 9 (supplied by Dr P.R.Wood, Parkville, Australia), originally raised against bovine γ-IFN but which was shown to cross react with and neutralise ovine γ-IFN, was used in ovine assays. A random monoclonal antibody was added as a control and the difference between the titres of the respective samples was taken as being due to γ-IFN.

ASCITES PRODUCTION.

High titre murine γ-IFN neutralising antibody was produced in the form of ascitic fluid from the cell line R46A2 (supplied by Dr A.Mowatt, University of Glasgow.). Athymic nude mice were kept in aseptic conditions in a positive pressure isolator and were primed with an intraperitoneal injection of pristane (Sigma Chemical Company Ltd), an immunosuppressant. 7 days later this was followed by an injection of $5x10^6$cells intraperitoneally to each mouse. As tumours developed the ascitic fluid produced was drained, using a 23G 3/8in hypodermic needle, collected in 10ml centrifuge tubes and clarified by centrifugation at 450g for 15min. This removed gross cell debris. The titre of the pooled ascitic fluid was calculated against a known level of γ-IFN activity, aliquoted and stored at -20^{o}C.

MICROSCOPIC EXAMINATION OF TISSUES.

Small blocks of tissue were cut and were fixed for 2-4hr in cold modified bouin (Finlayson, Buxton, Anderson and Donald, 1985), before being processed by the St Marie method at 4^{o}C. Briefly, this involved leaving the tissue in 70% ethanol overnight before dehydrating through alcohols. The tissue was then rinsed in a 2:1 alcohol/xylene solution and then a 1:2 alcohol/xylene solution for 10min and finally washed 3 times in xylene for 20min each time before embedding in paraffin wax. Sections 4μm thick were cut and placed on poly-L-lysine coated slides and dried overnight at 37^{o}C and stained with haemotoxylin and eosin (Stevens, 1977).

IMMUNOPEROXIDASE STAINING.

The immunoperoxidase method described below was a direct staining method which employed the IgG fraction of pooled ovine hyperimmune antisera against the A22 abortion strain, directly conjugated to the marker enzyme HRP (Finlayson *et al*, 1985). Tissues were fixed and processed using the St Marie method described above. Sections 4µm thick were cut and dewaxed in xylene, rinsed in alcohol and endogenous peroxidase blocked with 1% H_2O_2/methanol for 30min. The slides were rinsed twice, in PBS containing 2% egg albumin for 10min to reduce non-specific binding of the antibody. The conjugated antiserum was diluted 1:10 with a solution of 1% BSA in PBS, was layered onto the section and left in a humidity chamber at RT for 90min. Sections were then rinsed in PBS/2% egg albumin, washed twice in Tris/HCl buffer (pH 7.6), and substrate, 4µg of DAB in 10ml Tris/HCL, added for 10min at RT before being dehydrated through alcohol and mounted in xylene with DPX.

IN SITU HYBRIDISATION.

This method utilises the ability of DNA and RNA to bind to complementary strands of DNA and RNA. In the method used in this thesis an RNA probe was used to detect strands of chlamydial RNA. For *in situ* hybridisation tissues were fixed in 4% paraformaldehyde rather than cold bouin as previously stated.

(i) Preparation of slides.

The slides were prepared by the method of Tourtellotte and his co-workers (Tourtellotte, Verity, Schmid, Martinez and Shapshak, 1987). Briefly, slides were washed in 1N HCL for 20min and then soaked in tap water for 1-2hr, rinsed in absolute alcohol and wiped dry. When dry, the slides were placed in a solution of Denhardts in 0.15% Standard Saline Citrate (SSC), allowed to incubate overnight at RT and fixed in a solution of 3:1 ethanol/acetic acid for 20min again at RT. The slides were then acetylated by exposing them to a solution of triethanolamine (pH 8.0) and acetic anhydride, and agitating gently for 10min. After dehydrating through alcohols the slides incubated overnight at 70^{o}C in a 1% (v/v) solution of the organosilane γ-aminopropyltriethoxysilane, which covalently bonds the section to the slide without affecting the specificity or sensitivity of the hybridisation. The next day they were washed in tap water and baked overnight at 100^{o}C. At this stage the slides could be stored in dust free boxes for up to 6 months at RT. When required the slides were activated by first rinsing in a 10% gluteraldehyde in PBS solution (pH 7.0) for 30min at RT. Then the slides were stabilised in a 0.1% sodium periodate solution for 15min and rinsed in PBS. Activated slides could be stored at RT for 2 weeks.

(ii) Preparation of coverslips.

Coverslips were first cleaned in 1M HCL for 30min and rinsed in

distilled water. After washing in 95% alcohol for 30min the coverslips were dried on gauze and immersed in sigmacote (Sigma Chemical Company) and again dried on gauze. They were then baked for 2hr at 180°C in a covered glass dish.

(iii) Preparation of sections.

Sections 6µm thick were cut from tissue blocks, processed by the St Marie method described above, mounted on the prepared slides using poly-L-lysine and dried overnight at 37°C. The sections were dewaxed in xylene which in turn was removed by washing in alcohol and the sections were air dried. They were then washed in graded alcohols, rinsed 3 times in PBS and placed in a 2x SSC solution for 10min.

(iv) Staining.

The prepared sections were incubated in pre-hybridisation buffer for 2hr at RT. In order to save reagents sections were not immersed in the buffer instead 300µl of buffer was layered on each section. Similarly, only 30µl of hybridisation buffer containing the probe conjugated to digoxigenin was added to to the sections, which were mounted with the prepared coverslips and then incubated overnight at 42°C. The sections were then washed in 2x SSC for 1hr and then in 1xSSC for 1hr. At this stage RNase was added to remove any unhybridised, conjugated probe in order to lessen the background staining. This was carried out for 30min at 37°C. The RNase was removed when the sections were rinsed in 0.5xSSC for 30min at RT. The sections were stained by placing them in a 100mM Tris/HCL 150mM sodium chloride buffer (pH 7.5) (buffer 1) for 1min and then

immersing them in buffer 1 containing 2% normal sheep serum and 0.3% Triton X-100 for 30min. The detecting antibody was an anti-digoxigenin antibody conjugated to alkaline phosphatase diluted 1:500 in buffer 1, containing 1% normal sheep serum and 0.3% Triton X-100 and 100µl was layered onto the sections which were incubated overnight at 4°C. The following day the sections were placed in buffer 1 containing 1.5% (W/V) levamisole for 10min after which the buffer was replaced with a 100mM Tris/HCL 100mM sodium chloride 50mM magnesium chloride solution (buffer 2) containing 1.5% (W/V) levamisole for 10min. The colour was developed with a phosphatase substrate made in buffer 2 and 500µl were layered on each section for 2-4hr. Colour development was stopped with a solution of 10mM Tris/HCL 1mM EDTA pH 8.0. Finally the sections were counterstained in 0.25% light green in 70% alcohol, dehydrated and mounted in DPX.

MEDIA AND SOLUTIONS.

These media and solutions were used in all the methods described above unless otherwise stated.

a) Growth of C.psittaci.

Tryptose, phosphate broth (TPB) was prepared by dissolving 29.5g of powdered medium (Difco Laboratories Ltd, Surrey, England.) in 500ml of distilled water and making the resultant solution up to 1000ml. The solution was dispensed in 20ml amounts and autoclaved.

Sodium bicarbonate solution was prepared by dissolving 80g of sodium bicarbonate (BDH Ltd) in 995ml of distilled water and adding 5ml of 0.4% phenol red as an indicator. This solution was sterilised by membrane filtration.

5-iodo-2-deoxyuridine (IDU) powder (Sigma) was dissolved in 200ml of BHK-21 maintenance medium to give a final concentration of 800µg/ml and then filter sterilised.

Streptomycin. One vial of streptomycin sulphate (Evans medical Ltd, Greenford, England.) was dissolved in 10ml of HBSS, giving a final concentration of 100,000µg/ml.

Gentimycin was supplied by Nicholas Laboratories (Slough, England.) in the form of gentimycin sulphate in 1g vials, which were then dissolved in 100ml of distilled water to give a concentration of 10,000µg/ml.

Mycostatin was obtained from E.R.Squibb and sons (Hounslow, England.). The contents of a 1g vial was resuspended in 100ml of distilled water, to give a final concentration of 5000 units/ml.

BHK-21 maintenance medium was prepared as described appendix 1 except that the newborn calf serum concentration was reduced to 2%.

BHK-21 treatment medium prepared as for the maintenance medium above except that 20ml of the IDU solution was added before making up the final volume to 200ml.

BHK-21 infection medium was prepared as for the maintenance medium except that CTM containing live inoculum was added before the final volume was made.

b) Purification of chlamydial elementary bodies.

20mM Tris 50mM KCl. 1.21g of Tris(hydroxymethyl) amino methane (BDH Ltd) and 7.26g of KCl (BDH Ltd) was dissolved in 400ml of distilled water and the pH was then adjusted to 7.4 with concentrated HCl. The volume was then made up to 500ml.

Urografin. A 30% urografin cushion was prepared by adding 8.76g of urografin (Schering) to 14g of Tris/KCl to give a final volume of 20ml. A solution of 60% urografin for the density gradient was prepared by adding 17.52g of urografin to 8g of Tris/KCl buffer to produce 20ml.

c) Preparation of solid phase antigen.

Acrylamide. A solution of 30% acrylamide was prepared by dissolving 29.2g of acrylamide (Bio-Rad laboratories, Watford, England.) and 0.8g of N'N' methylene bis acrylamide (Bio-Rad laboratories) in 80ml of distilled water. When fully dissolved the solution was adjusted to 100ml.

As acrylamide is a potent neurotoxin great care was taken when handling it. Gloves were worn at all times.

Tris buffer. A 1.0M solution of Tris was prepared by dissolving 12.1g of Tris in 80ml of distilled water. The pH was adjusted to 6.8 with concentrated HCl and the final volume adjusted to 100ml with distilled water. A 1.5M Tris buffer was prepared by adding 18.2g of Tris to distilled water as before. After dissolving the pH was adjusted to 8.8 with concentrated HCl and the volume was corrected to 100ml.

Sample buffer. 20g of glycerol (BDH Ltd), 4g of sodium dodecyl sulphate (SDS) (BDH Ltd) and 2mg of bromophenol blue (BDH Ltd) were added to 25ml of 1M Tris pH 6.8 and 5ml of mercaptoethanol (BDH Ltd). The pH was adjusted with concentrated HCl and distilled water was added to give a final volume of 100ml.

Running buffer was prepared in 5litre volumes by adding 5g of SDS and 72g of Glycine (BDH Ltd) to 5litre of distilled water. The pH of this solution was approximately 8.2.

Immunoblot Transfer Buffer. Proteins were transferred from the gel to the 0.45µm nitrocellulose membrane (Bio-Rad laboratories) using an immunoblot transfer buffer prepared by dissolving 2.9g of Tris and 14.5g of glycine in 200ml of methanol before making the final volume up to 1000ml by adding distilled water.

d) Complement Fixation Test (CFT).

CFT buffer was obtained from Oxoid in tablet form. A tablet was dissolved in 1000ml of PBS.

Sheep red blood cells were collected in a 3.7% sodium citrate solution to prevent clotting. They were washed in PBS and stored in Alseviers solution.

Complement was obtained freeze dried from the Wellcome Foundation, Beckenham, Kent England. Each vial was reconstituted in distilled water before being stored immediately at -70^{o}C.

Haemolysin in the form of rabbit haemolytic serum for sheep red blood cells was also obtained from the Wellcome Foundation. It was stored at 4^{o}C.

e) Microscopic examination of tissues.

Scott's tap water substitute was made up from 1litre of distilled water 3.5g of sodium bicarbonate and 20g of magnesium sulphate.

Mayer's haemotoxylin was 1g of haemotoxylin, 0.2g of sodium iodate and 50g of potassium alum which were dissolved overnight in 1litre of distilled water. The next day 50g of chloral hydrate and 1g of citric acid was added and the solution was boiled for 5min and allowed to cool before being filtered.

Eosin was prepared by dissolving 4g of eosin yellowish in 20ml of alcohol and making up to 400ml with distilled water.

f) In situ hybridisation.

50x Denhardts is a solution of 1% Ficoll, 1% polyvinylpyrrolidine, 1% BSA in distilled water.

The acetylation solution was 350ml of a 0.1% triethanolamine (18.6g/l) in distilled water (pH 8.0) and 0.88ml (0.25% v/v) of acetic anhydride.

Pre-hybridisation buffer was prepared in 2.5ml amounts to save reagents. It was composed of

Deionised formamide	1.25ml
SSC (20x)	0.5ml
Denhardts (50x)	0.05ml
Herring sperm DNA (10mg/ml)	0.125ml
Yeast tRNA (10mg/ml)	0.0625ml
Dextran sulphate (50% W/V)	0.5ml

The hybridisation buffer differed from the pre-hybridisation buffer in that 1µg of probe was diluted in 10µl of 10mM Tris/1mM EDTA/ 600mM Sodium chloride. This solution was diluted 1:200 in the pre-hybridisation buffer.

RNase was added as a 1µg/ml solution in 1x SSC with 500mM Sodium chloride and 10mM Tris (pH 7.5).

Buffer 1 was prepared by dissolving 12.1g of Tris and 8.76g of sodium chloride in 1000ml of distilled water (pH 7.5).

Buffer 2 was again 12.1g of Tris with 5.8g sodium chloride and 10.16g of magnesium chloride dissolved in 1000ml of distilled water.

The buffer 3 used to stop the colour development was a solution of 1.21g of Tris and 67mg of EDTA dissolved in 200ml of distilled water (pH 8.0).

The alkaline phosphatase substrate was prepared by adding 45µl of NBT solution (Boerhinger Mannheim), 35µl of ^{x}-phosphatase (Boerhinger, Mannheim) and 33.2µl of levamisole (7.5% w/v) to 10ml of buffer 2.

Chapter 3a :

THE PROLIFERATIVE RESPONSES OF OVINE PERIPHERAL BLOOD MONONUCLEAR CELLS TO C.PSITTACI ELEMENTARY BODIES: PRELIMINARY STUDIES.

Introduction.

After chlamydial abortion sheep are considered to be solidly immune and do not abort again (Stamp *et al*, 1950). Cell mediated mechanisms have been implicated in immunity to *C.psittaci* (Ramsey *et al*, 1988; Rank *et al*, 1989). In other studies delayed type hypersensitivity reactions (DTH) have been used as a measure of cell mediated immune (CMI) responses to *C.psittaci* in sheep (Wilsmore *et al*, 1984; Wilsmore *et al*, 1986; Dawson *et al*, 1986). Another method of assessing CMI is to measure antigen stimulated proliferation of lymphocytes in previously infected animals. In order to achieve the aims of this thesis by investigating the ovine cell mediated response to *C.psittaci*, it was first of all necessary to look for specific proliferation in response to the extracellular EB of *C.psittaci* in the peripheral blood mononuclear cells (PBMC) of animals which had been infected and which subsequently aborted. As well as measuring CMI by lymphocyte proliferation to *C.psittaci* antigen , a second aim of the study outlined below was to determine the optimum conditions for the *in vitro* proliferation assay which was to be used and to investigate the concentrations of mitogens and antigen which would give optimum responses in the assay. This should allow a measurement of the degree of reactivity to chlamydial antigen within a population of infected sheep.

Experimental procedure.

PBMC were collected in vacutainers containing preservative-free heparin from 6 blackface ewes. Group 1 contained 3 ewes which had been infected with *C.psittaci* and had aborted in the previous

lambing season and group 2 contained 3 ewes, sero-negative for *C.psittaci* by CFT, which had been obtained from a farm known to have been free from chlamydial abortion for the previous three years (see table 3.1). The PBMC were used in an *in vitro* proliferation assay and were stimulated with various doses of Con A, LPS and a purified preparation of EBs grown in BHK-21 cells. Concentrations ranged from 0.5μg/ml-10μg/ml for Con A, 1μg/ml-100μg/ml for LPS and 1μg/ml-100μg/ml for the EBs.

Table 3.1: Experimental design for the determination of the optimum conditions for *in vitro* proliferation assays.

Group	(n)	Sero-converted	Aborted	PBMC
1	3	+	+	+
2	3	-	-	+

Results.

All concentrations of antigen and mitogen quoted in the results section are the final concentrations in the wells of the 96 well plates.

PBMC responses to LPS.

There was no significant difference in the level of response the cells from the animals, in both the uninfected and the post abortion groups gave, when stimulated by LPS, in the dose range tested (see table 3.2, figure 3.1). The peak response was given by

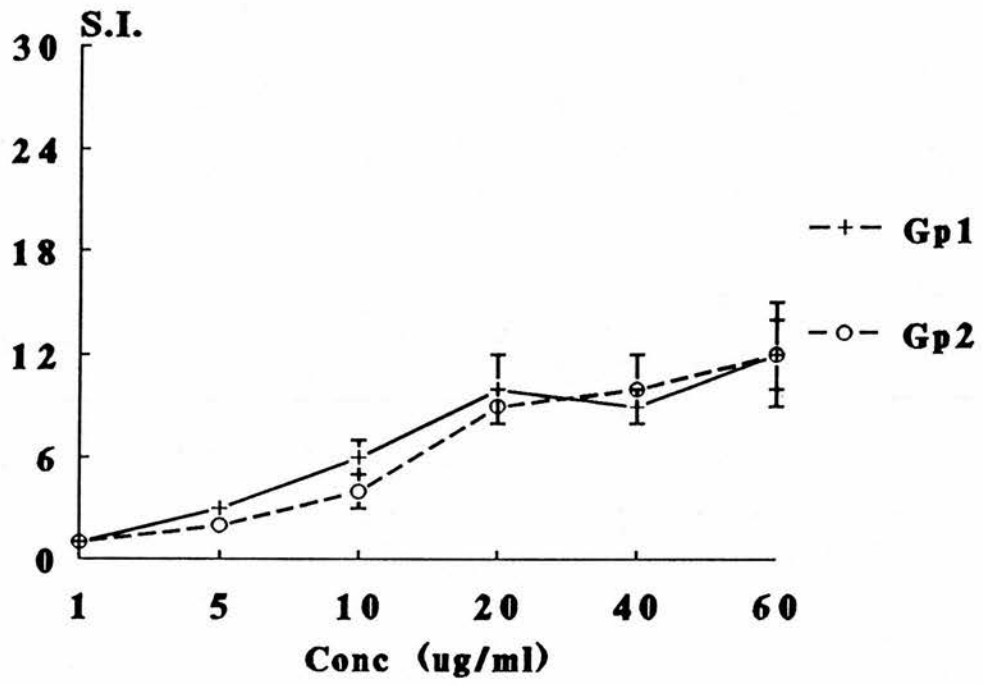
a final concentration of 100μg/ml, which was the highest concentration tested. For the purpose of this thesis the optimum concentration was considered to be 20μg/ml, which gave SIs of 10 and 9 in groups 1 and 2 respectively.

Table 3.2: Proliferative response to LPS of the peripheral blood mononuclear cells from group 1 post abortion ewes and group 2 uninfected ewes. Results expressed as SI$\pm$se. Background counts per minute (cpm) ranged between 683$\pm$132 and 1239$\pm$150.

	Response to LPS (μg/ml)					
Group	1	5	10	20	40	60
1	1$\pm$0	3$\pm$.5	6$\pm$1[a]	10$\pm$2[a]	9$\pm$1[a]	12$\pm$2[a]
2	-	2$\pm$.5	4$\pm$1[a]	9$\pm$1[a]	10$\pm$2[a]	12$\pm$3[a]

[a] $P<0.05$ when compared with medium controls.

Fig 3.1: Proliferative response to LPS of the peripheral blood mononuclear cells from group 1 post abortion ewes (+-+) and group 2 uninfected ewes (o-o).



PBMC responses to Con A.

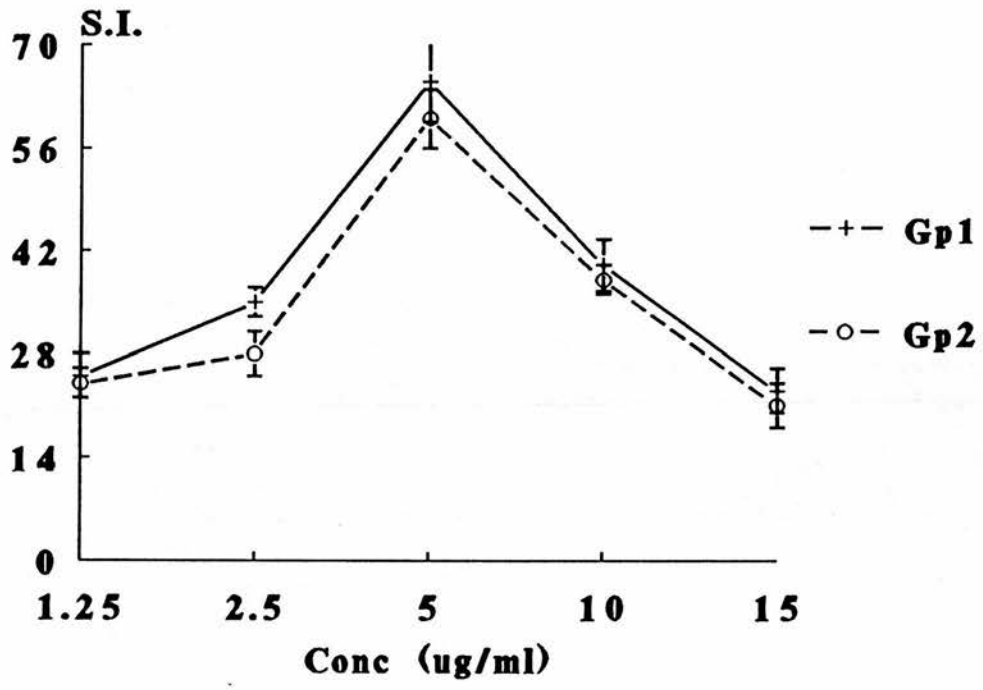
The PBMC from both groups 1 and 2 were stimulated by Con A in the dose range that was tested (see table 3.3, figure 3.2). The response peaked at a final concentration in the well of 5μg/ml in both groups and the magnitude of the proliferation was the same in both groups at all concentrations tested. The peak response gave a stimulation index (SI) of 60 in group 1 and 65 in group 2 and for the purposes of this thesis this was considered the optimum dose.

Table 3.3: Proliferative response to Con A of peripheral blood mononuclear cells from group 1 post abortion ewes and group 2 uninfected ewes. Results expressed as Stimulation Index (SI) $\pm$ standard error (se).

	Response to Con A (μg/ml)				
Group	1.25	2.5	5	10	15
1	25 ± 3^{a}	35 ± 2^{a}	65 ± 5.5^{a}	40 ± 3.5^{a}	23 ± 3^{a}
2	24 ± 2^{a}	38 ± 3^{a}	60 ± 4^{a}	38 ± 2^{a}	21 ± 3^{a}

[a] $P<0.05$ when compared with medium controls.

Fig 3.2: Proliferative response to Con A of peripheral blood mononuclear cells from group 1 post abortion ewes (+-+) and group 2 uninfected ewes (o-o).



PBMC response to EBs.

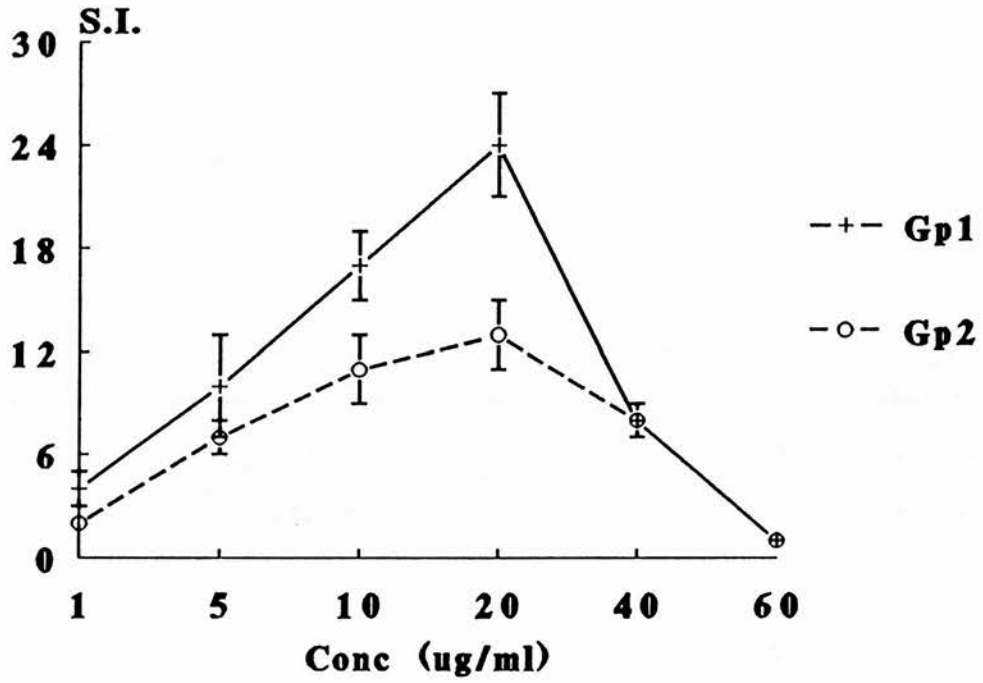
At the lower concentrations of purified EBs (<40μg/ml), lymphocytes from both groups of animals gave responses which were significantly above background ($P<0.05$) (see table 3.4, figure 3.3). A concentration of 20μg/ml stimulated the peak response, with cells from group 1 giving a SI of 24 and cells from group 2 giving a lower, but still significant SI of 13. At concentrations greater than this level there was little or no response in either group.

Table 3.4: Proliferative response to chlamydial elementary bodies of the peripheral blood mononuclear cells from group 1 post abortion ewes (+-+) and group 2 uninfected ewes (o-o).

	Response to chlamydial EBs (μg/ml)					
Group	1	5	10	20	40	60
1	4±1	10±2[a]	17±2[a]	24±3[a]	8±1.5[a]	-
2	2±.5	7±2[a]	11±2[a]	13±2[a]	8±1[a]	-

[a] $P<0.05$ when compared with medium controls.

Fig 3.3: Proliferative response to chlamydial elementary bodies of the peripheral blood mononuclear cells of group 1 (+-+) post abortion ewes and the group 2 uninfected ewes (o-o).



Discussion.

The stimulation of the PBMC by LPS and Con A mitogens was similar in both uninfected and post abortion ewes. The optimum concentration for each mitogen was the same for each group of sheep and the level of the response at this dose did not differ between the groups.

The PBMC from post abortion animals gave a strong proliferative response to EBs, whereas the cells from uninfected animals were less reactive. The PBMC used in the assay were collected approximately 6 months after the animals had aborted, showing that the cell mediated proliferative responses induced by infection with *C.psittaci* are long lasting. Whether this effect is due to a memory T-cell population or is brought about by the immune system constantly being boosted by a low level persistant infection, or a *Chlamydia* infected environment is not clear. There is no recorded evidence for a persistant infection continuing in sheep after abortion, but this may be due to the difficulty of isolating *C.psittaci* at this stage, rather than being because persistance does not occur. It is known that *Chlamydia* can lie dormant from one lambing season to the next (Stamp *et al*, 1950) and that it is seldom isolated from sheep even days after infection (Huang *et al*, 1990). However, reisolation has been achieved after abortion in goats (Brown *et al*, 1989).

The PBMC from the control sheep also mounted a proliferative response to the EB antigen preparation, which although lower than post abortion PBMC responses was still significantly above medium

control background levels. It is unclear whether this is due to a mitogenic effect of chlamydial LPS, known to be contained in the EB preparation, or whether it is the result of a cross reaction, since *C.psittaci* is known to cross react serologically with other enteric bacteria such as *Salmonella* (Nurminen *et al*, 1984). Reactions to inoculum components are unlikely to have been a problem, since the inoculum used for *in vivo* challenge was grown in egg yolk sacs and the purified EBs used in the *in vitro* proliferation assay were tissue culture grown.

Further study is required to examine this question of cross reaction in greater detail and also to determine why there was a virtual absence of proliferation in response to EBs at concentrations above 20μg/ml. One possibility is that it was due to a toxic component in the preparation, since the antigen preparation also contains BHK-21 cell debris and possibly traces of the gradient medium, Urografin.

Chapter 3b:

THE PROLIFERATIVE RESPONSES OF OVINE PERIPHERAL BLOOD MONONUCLEAR CELLS TO C.PSITTACI ELEMENTARY BODIES: FURTHER STUDIES.

Introduction.

In the preceding experiments, two apparent problems arose with the preparation of EBs used as a specific stimulus for the *in vitro* lymphocyte transformation assays employed in this work. The first was the reduced responsiveness of the cells stimulated by the higher concentrations of EBs and the second was the unforseen proliferation of naive cells from uninfected sheep which, although it was at a lower level than that of the primed cells, was significantly above the background response. In the first instance it was decided to investigate whether the antigen preparation was toxic for living cells. Secondly, an investigation was undertaken to determine whether the EB preparation used was inducing a non-specific proliferation in naive cells, by acting as a mitogen, or if the response seen was due to a cross reaction between the EBs and other antigens, probably bacterial, that the animals had been exposed to.

Experimental Procedure.

Experiment 1:Is there a toxic component in the EB preparation ?

PBMC from the post abortion animals and the uninfected animals used previously were collected and set up in duplicate, *in vitro* lymphocyte transformation assays. The cells were stimulated with various concentrations of the individual components of the purified EB preparation grown in BHK-21 cells, including uninfected BHK-21 cells and Urografin, and a control EB preparation grown in ST-6 cells, a sheep fibroblast cell line (Norval *et al*, 1981). One of

the duplicate assays was treated as a transformation assay and was harvested and read as normal. The cells from the other assay were removed at day 5 and were counted (see table 3.5). Nigrosine was used to assess cell viability.

Table 3.5: Design of experiment to determine the toxic factor present in the chlamydial elementary body (EBs) preparation.

PBMC tested against	PBMC tested for	
	Cell viability %	Proliferation
BHK-21/EB	+	+
ST-6 /EB	+	+
BHK-21 only	+	+
ST-6 only	+	+
Urografin	+	+

Experiment 2a: Is the EB preparation mitogenic or is there cross reaction with other bacteria ?

In order to determine whether or not *C.psittaci* has non-specific mitogenic properties for PBMC, 8 Wistar rats and 8 Balb-C mice were used (see table 3.6). Group 1 contained 4 rats and 4 mice which were raised conventionally while group 2 contained 4 rats and 4 mice raised in a positive pressure isolator and were given distilled water and irradiated food and bedding. Neither group recognised chlamydial antigen as determined by CFT. At 8 weeks old

the mice and the rats were killed and the spleens of the mice and the mesenteric lymph nodes of the rats were aseptically removed. Single cell preparations of these tissues were set up in an *in vitro* proliferation assay and stimulated as before with Con A, LPS and EBs from the S26/3 strain of *C.psittaci*.

Table 3.6: Design for experiment to determine whether the EB preparation is a mitogen or whether there is cross reactivity with other enteric bacteria.

Group	Species	(n)	Housed	Infection	Cells used
1a	Mouse	4	Conventional[a]	Uninfected[c]	Spleen
1b	Rat	4	"	"	Lymph node
2a	Mouse	4	Isolator[b]	"	Spleen
2b	Rat	4	"	"	Lymph node
3	Mouse	4	"	*C.psittaci*[d]	Spleen
4	"	4	"	*S.typhimurium*[e]	"
5	"	4	"	*E.coli*[f]	"

[a] conventional small animal cages.
[b] cages placed in positive pressure isolator.
[c] *C.psittaci* given i.p.
[d] *S.typhimurium* given i.p.
[e] *E.coli* given i.p.

Experiment 3: What are the possible sources of a cross reaction ?

Possible sources of the cross reaction were investigated using 12 Balb-C mice. Group 3 contained 4 mice, which were raised in a positive pressure isolator in the same way as group 1 above, but

these mice were infected with 10^5 inclusion forming units of *C.psittaci* at 4 weeks old. Group 4 again contained 4 mice raised in an isolater, but were infected with 10^7 avirulent *S.typhimurium*. A further 4 mice, group 5, were also raised in an isolator, but were infected at 4 weeks with 10^7 avirulent *E.coli* (see table 3.6). Again, after four weeks the mice were killed, their spleens were removed aseptically and single cell suspensions were prepared and used to set up a proliferation assay. As well as stimulation with Con A and LPS the cells were also given a homologous challenge with the bacteria they had been infected with and a heterologous challenge with the other two bacteria, all at concentrations of 1, 5, 10 and 20μg/ml.

Results.

Toxicity.

At higher concentrations of EB (40μg/ml), whether grown in BHK-21 cells or in ST-6 cells, proliferative responses were significantly diminished ($p<0.05$) when compared to those of lower doses. However, proliferation was significantly higher ($P<0.05$) and the percentage viability of the stimulated lymphocytes was greater in ST-6 grown EB preparations, than in BHK-21 grown EBs, when used at this concentration (see table 3.7). There was no significant difference between the preparations at any other concentration tested ($P>0.05$). The proliferation induced in response to the non-EB, cellular components of the preparations and to Urografin was negligible with a SI of 1 and this was not significantly above background ($P>0.05$).

The percentage viability of the cells cultured with the higher concentrations of both ST-6 and BHK-21 EB preparations and the cells stimulated by preparations of uninfected BHK-21 cells and ST-6 cells could not be calculated, since there were few live cells present and most fields under the microscope contained a large amount of cell debris (see table 3.8). The cell debris was present in the 1:10 and the 1:100 dilution of the uninfected BHK-21 cells, however in the ST-6 cells it was only present in the 1:10 dilution and a viability of 30% was recorded at the 1:100 dilution. The viability of cells cultured with Urografin, LPS and Con A remained constant for all dilutions and was between 75% and 85%. These findings are summarised in table 3.9.

Table 3.7: Proliferative response of peripheral blood mononuclear cells (PBMC) when stimulated by individual components of the EB preparation (SI). Background cpm ranged between 203±21 and 301±25.

Component	Concentration				
	5µg/ml	10	20	40	60
BHK-21+EB	3±1	7±1[a]	11±2[a]	4±.5[a]	-
ST-6 +EB	4±1[a]	8±1[a]	12±1[a]	8±1[a]	-
	0.001%(w/v)		0.01%	0.1%	1%
BHK-21 only	-		-	-	-
ST-6 only	-		-	-	-
	0.001%(v/v)		0.01%	0.1%	1%
Urografin	-		-	-	-

[a] $P<0.05$ when compared with medium control cpm.

Table 3.8: Cell viability (%) of PBMC when stimulated by individual components of the EB preparation.

Component	Concentration				
	5μg/ml	10	20	40	60
BHK-21+EB	70	65	40	cd	cd
ST-6 +EB	75	70	55	30	cd
		0.001%(w/v)	0.01%	0.1%	1%
BHK-21 only		55	40	cd	cd
ST-6 only		75	50	30	cd
		0.001%(v/v)	0.01%	0.1%	1%
Urografin		70	75	70	70

cd: cell debris

Table 3.9: Summary of the toxicity and the proliferation induced by the individual components of the EB preparation.

Component	Toxicity	Proliferation
BHK-21/EB	+	+
ST-6 /EB	+	+
BHK-21 only	+	-
ST-6 only	+	-
Urografin	-	-

Mitogenicity vs high dose suppression.

The cells of the mice and rats of group 1, which had been raised conventionally, all responded to Con A and LPS ($P<0.05$) with SI of 78 and 30 respectively. Mouse spleen cells also responded to EBs with a SI of 6, as did the rat lymph node cells with a SI of 9 ($p<0.05$) when compared with medium controls. However, the cells from the mice and rats in group 2, which were raised under aseptic conditions in a positive pressure isolator, while responding to Con A and LPS ($P<0.05$) did not respond to the EB preparation (see tables 3.10 and 3.11, figures 3.4 and 3.5). The responses of the cells in this group treated with the mitogens Con A and LPS were similar to those of their conventionally raised litter mates in group 1 ($P>0.05$). The response to the EB preparation in both mice and rats in group 2 did not rise above a SI of 1.

Table 3.10: Proliferative response to EBs of the mesenteric lymph node cells of rats raised conventionally and raised in isolators expressed as stimulation index (SI)±se

Housed	Conc(μg/ml)			
	1	5	10	20
Conventional	2±.5	3±.5	6±1[a]	9±1[a]
Isolator	-	-	-	-

[a] $P<0.05$ when compared with medium control cpm.

Table 3.11: Proliferative response to EBs of the spleen cells of mice raised conventionally and raised in isolators expressed as SI±se

Housed	Conc(μg/ml)			
	1	5	10	20
Conventional	2±.5	3±1	4±1[a]	7±1[a]
Isolator	-	-	-	-

[a] $P<0.05$ when compared with medium control cpm.

Fig 3.4 : Proliferative response to EBs of the mesenteric lymph node cells of rats raised conventionally (+-+) and raised in isolators (o-o).

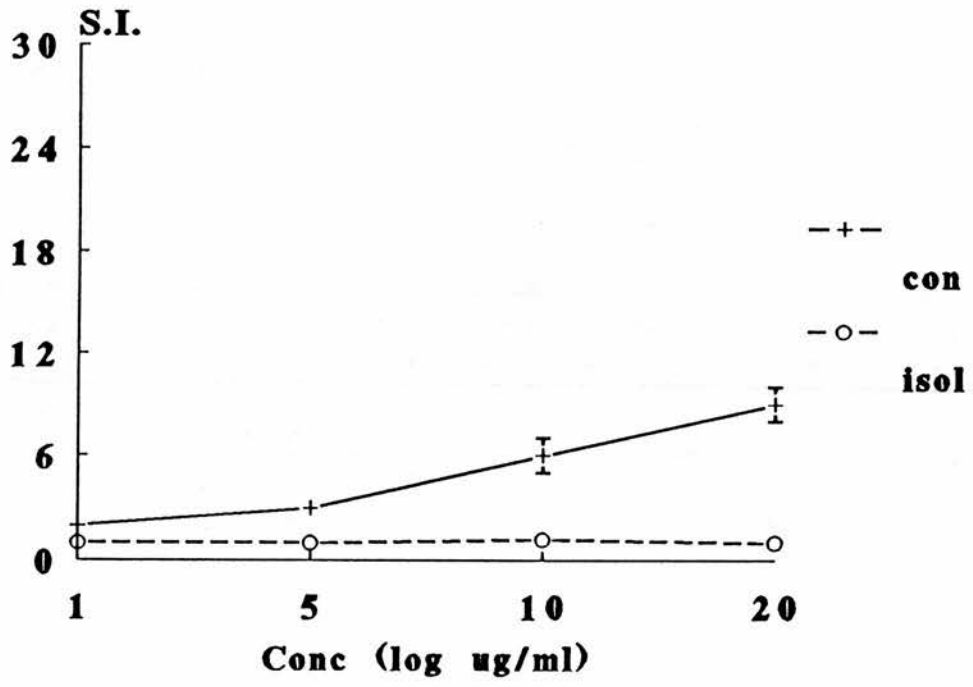
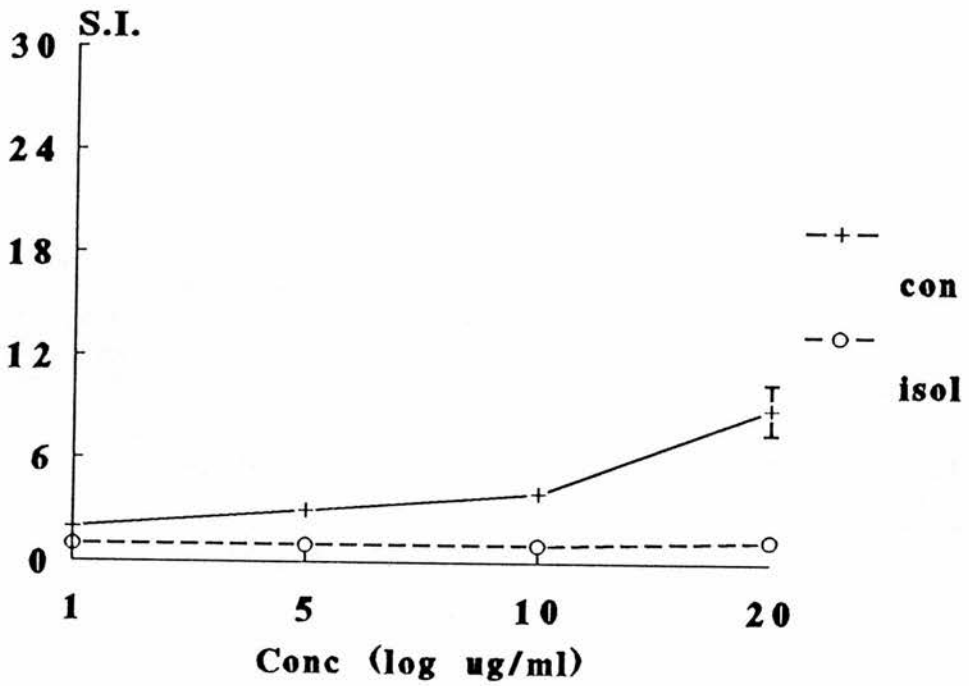


Fig 3.5 : Proliferative response to EBs of the spleen cells of mice raised conventionally (+-+) and raised in isolators (o-o).



In the third experiment the spleen cells from the mice in all three groups gave a proliferative response when given a homologous challenge *in vitro*. The peak response to *C.psittaci* in the chlamydia infected group 3 was a SI of 27 ($P<0.05$) (see table 3.12, figure 3.6), in group 4, the Salmonella infected mice gave a SI of 19 ($P<0.05$) in response to *S.typhimurium* (see table 3.13, figure 3.7) and for group 5 the peak response to *E.coli* was a SI of 16 ($P<0.05$) (see table 3.14, figure 3.8). In addition, each group also gave significant, but lesser responses, to heterologous stimulation with the other two bacteria ($P<0.05$).

Table 3.12: Proliferative response to EBs, *S.typhimurium* and *E.coli* of the spleen cells of mice infected with *C.psittaci*. Background cpm ranged between 261±32 and 345±37.

	Concentration (μg/ml)			
	1	5	10	20
C.psittaci	20±1.5[a]	26±2[a]	27±1.5[a]	15±1[a]
S.typhimurium	7±1[a]	9±2[a]	10±2[a]	6±1.5[a]
E.coli	3±1	3±1	5±1.5[a]	7±1[a]

[a] $P<0.05$ when compared with medium control cpm.

Table 3.13: Proliferative response to EBs, *S.typhimurium* and *E.coli* of the spleen cells of mice infected with *S.typhimurium*.

	Concentration (μg/ml)			
	1	5	10	20
C.psittaci	2±.2	3±.2	7±1[a]	3±.5
S.typhimurium	11±2[a]	12±1.5[a]	18±1.5[a]	19±1[a]
E.coli	5±1[a]	7±1[a]	9±1[a]	11±3[a]

[a] P<0.05 when compared with medium control cpm.

Table 3.14: Proliferative response to EBs, *S.typhimurium* and *E.coli* of the spleen cells of mice infected with *E.coli*.

	Concentration (μg/ml)			
	1	5	10	20
C.psittaci	3±.5	3±.2	5±.5[a]	7±1[a]
S.typhimurium	4±1[a]	4±1[a]	8±1.5[a]	12±2[a]
E.coli	3±.5	4±.5[a]	9±2[a]	16±1.5[a]

[a] P<0.05 when compared with medium control cpm

Fig 3.6 : Proliferative response to EBs (+-+), *S.typhimurium* (o-o) and *E.coli* (□-□), of the spleen cells of mice infected with *C.psittaci*.

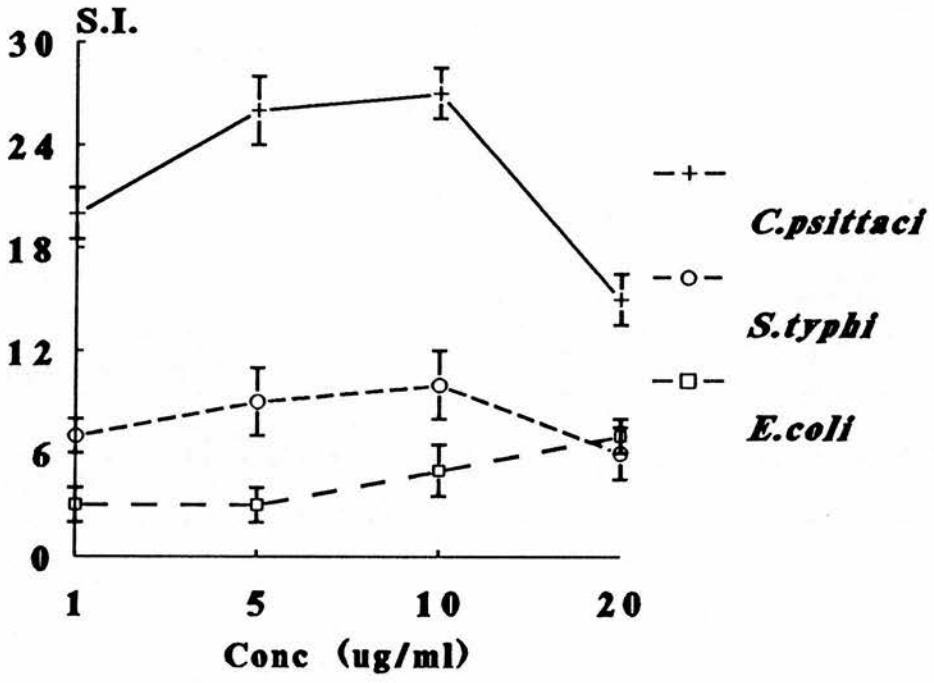


Fig 3.7 : Proliferative response to EBs (+-+), *S.typhimurium* (o-o) and *E.coli* (□-□), of the spleen cells of mice infected with *S.typhimurium*.

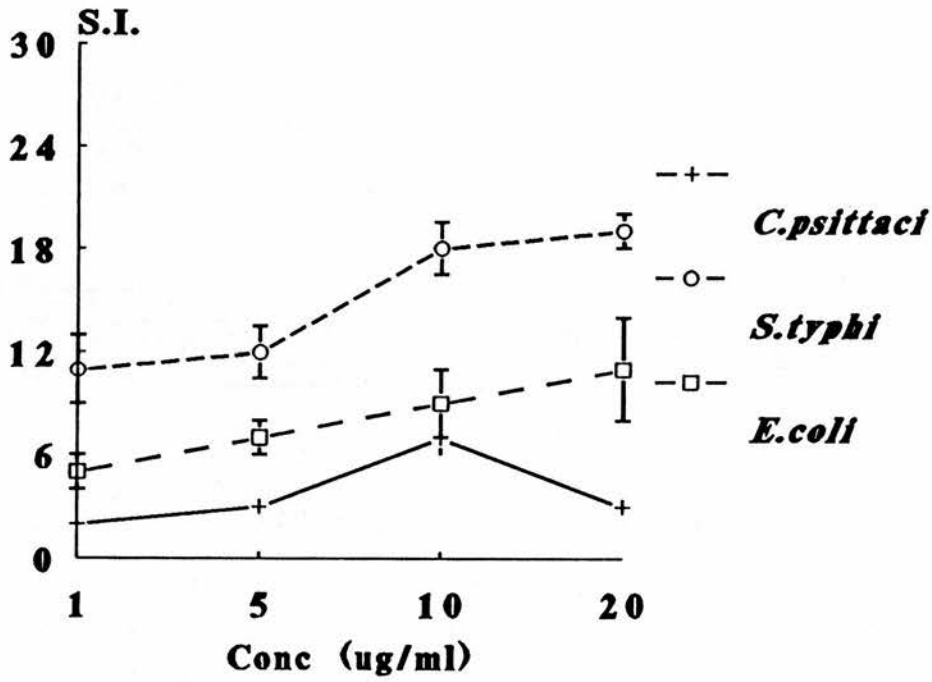
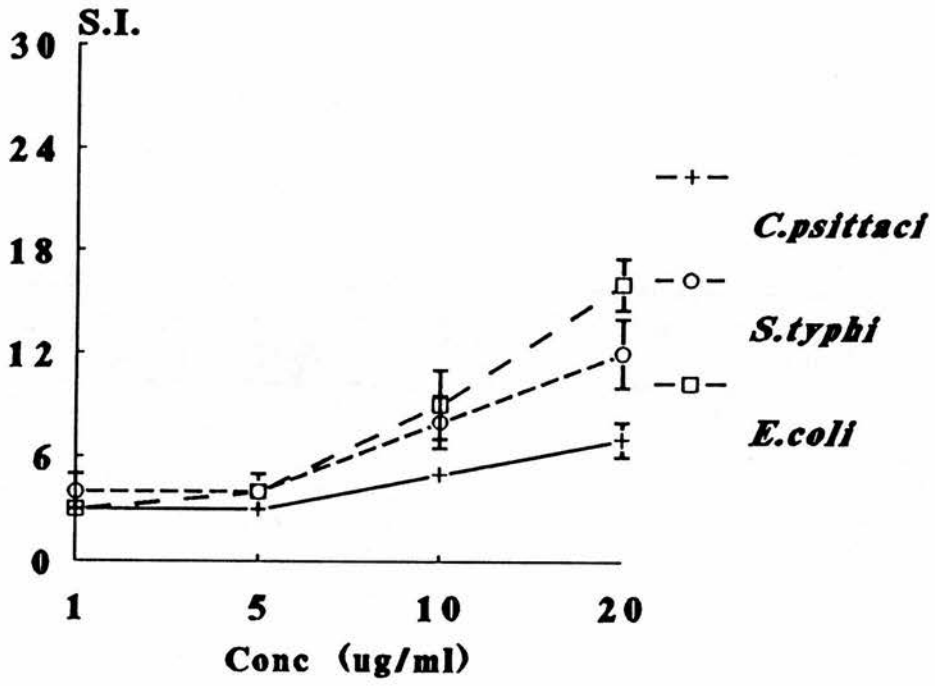


Fig 3.8 : Proliferative response to EBs (+-+), *S.typhimurium* (o-o) and *E.coli* (□-□), of the spleen cells of mice infected with *E.coli*.



Discussion.

Toxicity.

It is clear from the cell counts presented above that the decrease in the proliferative response at higher concentrations of EB preparations is the result of toxicity. Toxicity would appear to be present in both the EBs grown in ST-6 cells and BHK-21 cells, although it is not known if the toxicity is due to the same component. The toxin(s) which is present in the preparation is also present in uninfected BHK-21 cells and ST-6 cells and may be released from the cells when they are disrupted during purification to release the EBs. In any purified preparation of EBs there is a degree of host cell contamination which is carried over due to the apparent "stickiness" of the EBs themselves. This makes further purification difficult although an extra washing stage in the purification process does appear to alleviate the problem without removing it. Urografin was shown to be neither toxic for living cells nor mitogenic.

Mitogenicity vs cross reaction.

It was decided to use mice and rats for the study to examine the question of mitogenicity in EBs for two reasons. Firstly, one can achieve greater control of the environmental conditions in which they are raised than is possible with sheep. Also as the mitogens LPS and Con A act by cross linking sugar residues on lymphocyte receptors and are therefore cross species reactive, the mitogenic effect could still be detected. The initial experiment, involving the mice and rats from group 1 and group 2 showed conclusively that the response to *C.psittaci* in uninfected animals is a cross

reaction with other factors derived from the environment rather than a mitogenic effect. The cells from the isolator reared animals, and therefore derived from a protected environment, displayed normal mitogenic responses when compared with the cells from conventionally reared animals. However they did not mount a proliferative response to the EB preparation used to stimulate the cells. Conventionally raised animals, which would normally be exposed to a very much greater range of antigens in the form of bacteria and other organisms in non-sterile food, water and bedding, did mount a significant response to the EB preparation.

The most obvious candidates for this cross reaction are commensal enterobacteria, since they readily become established when animals are raised conventionally. Two of the most common were therefore included in the second experiment. As *C.psittaci* is known to cross react serologically with the LPS of *Salmonella* (Nurminen *et al*, 1984), *S.typhimurium* was used in the study along with an *E.coli*, isolated from the faeces of a sheep at the Moredun Research Institute. It is not suggested that the strains selected were those strains that caused the proliferative response in the uninfected sheep, mice and rats, they were merely intended to be representative of normal gut flora and thus examine the possibility that enterobacteria might be the cause of the cross reaction.

Infection with any of the three bacteria used would seem to cause a marked cross reaction with the others and would explain the responses seen in cells from conventionally reared, uninfected animals. Stimulation by cross reacting antigens may even be

responsible for maintaining long term memory lymphocyte populations against *Chlamydia, in vivo* (Beverly, 1990). However, a homogeneous challenge results in a higher SI than a heterogeneous challenge and this again is seen when sero-negative ewes are compared with infected animals. These experiments show that care should be taken to ensure that uninfected control animals used in any experiment are of a known background and are free of *C.psittaci*, since the cross reactions may lead to ambiguous results.

Conclusions.

The evidence indicates that sheep can become infected in one lambing season, carry the infection for a year, abort and are then immune to further chlamydial challenge (Stamp *et al*, 1950). However, it is as yet unknown when these mechanisms develop. Antibody responses to *C.psittaci* do not develop until just before abortion, as measured by the CFT (Stamp *et al*, 1952) and western blot analysis (Tan, 1989), and it may be that proliferative mechanisms do not occur until this time. A knowledge of this could go some way to explaining the mechanism behind the ability of *C.psittaci* to cause latent infection and subsequent immunity. However, further study must be undertaken with great care, as it has been shown that purified preparations of EB can be toxic and will also cross react with other enteric bacteria in uninfected animals.

Chapter 4:

THE DEVELOPMENT OF PROLIFERATIVE RESPONSES OF OVINE PERIPHERAL BLOOD MONONUCLEAR CELLS TO C.PSITTACI DURING PREGNANCY.

Introduction.

In the previous chapter proliferative responses were demonstrated in the PBMC of post abortion animals. In other published studies, *in vitro* proliferation of ovine PBMC to chlamydial EBs has also been demonstrated in infected, pregnant sheep 42 and 50 days after infection (Dawson *et al*, 1986). The following study sought to examine the development of these proliferative responses in the PBMC of infected animals through the later stages of gestation and up until lambing/abortion, employing an *in vitro* lymphocyte transformation assay. The effects of both pregnancy and infection on the lymphocyte proliferative responses, as defined by their reactivity to B and T cell mitogens, were also measured. In addition cytospin smears of the peripheral blood preparations were examined for *C.psittaci* organisms by an immunoperoxidase method, in an attempt to determine whether the chlamydaemia described by Storz (1971) occurred late in gestation allowing *C.psittaci* to travel from the site of latency to the placenta via the peripheral blood system.

Experimental Procedure.

Twelve ewes (5-6 years old) were obtained from a farm known to have been free from EAE for at least three years. Oestrus was synchronised and the ewes were tupped. After 8 weeks the sheep were examined with an ultrasound scanner to confirm that they were pregnant. The animals were then split into two groups of 6 (see table 4.1).

Table 4.1: Experimental design monitoring the development of proliferative responses of peripheral blood mononuclear cell (PBMC) to *C.psittaci* in pregnant sheep.

Group	(n)	Infected day 90	PBMC sampled	Aborted
1	6	+	+	4/6
2	6	-	+	0/6

Group 1 were infected with *C.psittaci* on day 90 of gestation. Each animal was given a 1ml suspension of the S26/3 strain, cultured in egg yolk sacs and stored in liquid nitrogen until required. The material was diluted in PBS to give a dose of $10^{5.5}ELD_{50}$/ml and was injected subcutaneously over the left shoulder. Separate needles and syringes were used for each animal. Group 2 were retained as unchallenged control ewes and were housed separately in a clean, high security animal house. All animals were screened for antibody to *T.gondii* and were found to be negative. After abortion/lambing the extent of any macroscopic chlamydial lesions in each placenta was estimated visually and recorded as a percentage of the total area of the placental sample. Vaginal swabs and samples of placenta were collected for culture. The length of gestation from tupping to lambing/abortion was also noted as was the birth weight of live lambs. Any lamb which did not survive 48hr was deemed to be non-viable and the loss was attributed to *C.psittaci*, for the purpose of this experiment.

Before infection of group 1 PBMC were collected from all 12 animals and this was repeated thereafter every 14 days, one group being bled one week and the other group being bled the following week, until after abortion/lambing. Further samples were collected 4 weeks after abortion/lambing. The blood was collected in evacuated test tubes containing preservative-free heparin. Using an *in vitro* lymphocyte transformation assay the proliferative response of each ewe was measured against the B cell mitogen LPS, the T cell mitogen Con A and a purified preparation of chlamydial EBs grown in cultured BHK cells. Cytospin smears of the peripheral blood were also prepared on each occasion and were tested for the presence of *C.psittaci* by immunoperoxidase and *in situ* hybridisation.

Results.

Clinical Differences.

A summary of the clinical differences described below is presented in table 4.2.

Table 4.2: Summary of the clinical differences between infected sheep (Gp 1) and uninfected sheep (Gp 2).

Group	Length of Gestation (d)	Viable Lambs	Average Weight (kg)	Weight viable lamb/ewe (kg)	Infected placenta
1	134±5[a]	3/8	3.9±0.5	1.95	5/5
2	143±3[a]	8/8	4.3±1.2	5.7	0/6

[a] $P<0.05$ when groups are compared.

I) Length of Gestation.

All lambs/foetuses were recovered from both groups and placentas were collected for isolation studies from eleven of the twelve animals. Animals in group 1 which had been infected at day 90 had a significantly shorter gestation period, 134±5 days as compared with 146±3 days for the uninfected animals in group 2 ($p<0.05$).

II) Lamb Weight.

Two of the 6 infected ewes in group 1 produced live lambs whereas in group 2 all 6 animals produced live lambs. In group 1, only 3 of the 8 lambs produced, were born live (37.5%) with a mean weight of 3.9±0.5kg. All 8 lambs in group 2, were born live and they had a mean birthweight of 4.3±1.2kg. The latter lambs although born with a greater birthweight were not significantly heavier. However, when the weight of viable lamb produced per ewe was calculated for each group the uninfected animals in group 2, produced 5.7kg of lamb per ewe and the infected animals produced only 1.95kg.

III) Isolation of *C.psittaci* from placental tissue.

Placentas were recovered from 5 of the 6 infected ewes and all had macroscopically visible areas of typical chlamydial lesions. The areas affected ranged from an estimated 5% of one placenta, up to 90% of the placenta in another case. *C.psittaci* was isolated from all 5 placentas and from the vaginal swabs taken from all 6

animals in group 1. In group 2 no macroscopic signs of chlamydial infection were found on any of the placentas and all attempts at culture from the placental tissue and the vaginal swabs proved negative.

Lymphocyte Responses.

I) LPS.

The responses of the PBMC to the B cell mitogen LPS, from both groups remained similar throughout the duration of the experiment (see table 4.3, figure 4.1). Both groups showed increased activity from a mean stimulation index (SI) of 2 at day 90 to one of 10 just before parturition, after which levels dropped again to a SI of 2 where they remained. Although the responses between the groups did not differ significantly, there were significant increases in the magnitude of the mean response in both groups between day 90 and day 132 just before parturition ($p<0.05$). Similarly the drop in response in both groups between day 132 and parturition was also significant ($p<0.05$).

Table 4.3: Proliferative response of ovine PBMC to 20μg/ml LPS, during gestation, at lambing and 30 days after lambing of infected and uninfected sheep. Results are expressed as SI$\pm$se. Background cpm ranged between 1,172$\pm$131 and 1,537$\pm$162.

	day gestation					day post lambing	
Group	90	104	118	132	lambing	14	30
1	2$\pm$.5	4$\pm$.4[a]	4$\pm$1[a]	10$\pm$1[ab]	3$\pm$.4[ac]	2$\pm$.3	3$\pm$.2[a]
2	2$\pm$.5	nd	6$\pm$1[a]	8$\pm$1[ab]	3$\pm$.5[ac]	3$\pm$.5[a]	3$\pm$.7[a]

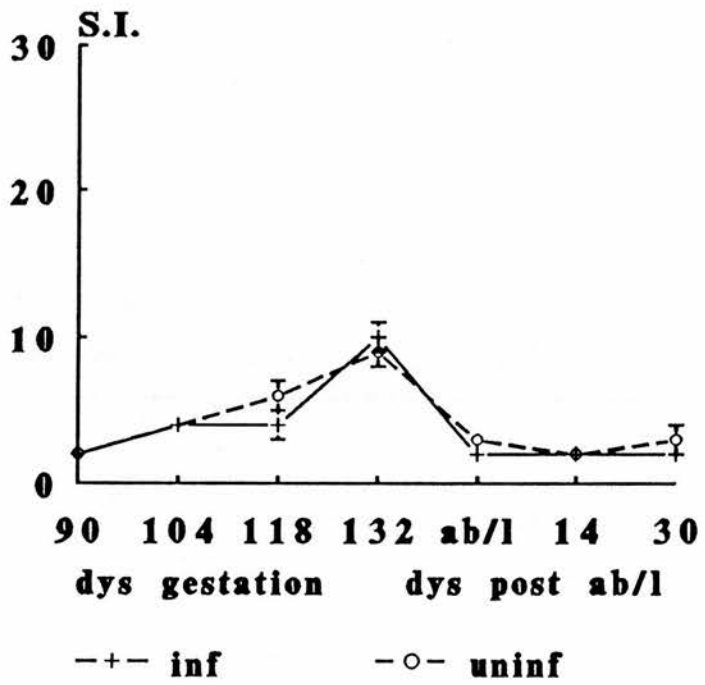
[a] P<0.05 compared with medium control cpm

[b] P<0.05 compared with pre-infection cpm

[c] P<0.05 compared with previous bleed cpm

nd: not done

Figure 4.1: Proliferative response of ovine PBMC to 20μg/ml LPS, during gestation, at lambing and 30 days after lambing of infected (+-+) and uninfected (o-o) sheep.



II) Con A.

Prior to infection of group 1 animals the PBMC from both groups of animals responded with similar magnitude to the T cell mitogen Con A (see table 4.4, figure 4.2). Thereafter and until parturition the mean response in group 1 was significantly suppressed, decreasing from a SI of 155 pre-infection, to one of 70 at parturition ($p<0.001$). However, within 14 days of parturition it rose to the pre-infection level. Group 2 responses on the other hand showed a slight increase from a mean SI of 180 at day 90 to one of 220 in the cells collected just before parturition at day 132. At this point they were significantly higher than those in group 1 ($p<0.05$). However, the response to Con A in these animals also became markedly suppressed around parturition, dropping from a SI of 220 to a SI of 120 at parturition ($p<0.05$). At this time they were again slightly higher, but not significantly different from the responses of the infected animals ($p>0.05$). The level of response in these uninfected animals also returned to normal soon after parturition, in a similar manner to the infected ewes.

Table 4.4: Proliferative response of ovine PBMC to 2.5μg/ml Con A, during gestation, at lambing and 30 days after lambing of infected and uninfected sheep.

	day gestation					day post lambing	
Group	90	104	118	132	lambing	14	30
1	155±25[a]	102±20[ab]	82±20[abd]	70±15[abd]	71±10[ab]	75±15[abd]	163±34[a]
2	180±30[a]	nd	213±30[ad]	225±40[ad]	110±45[abc]	152±50[ad]	167±35[a]

[a] P<0.05 when compared with medium control cpm

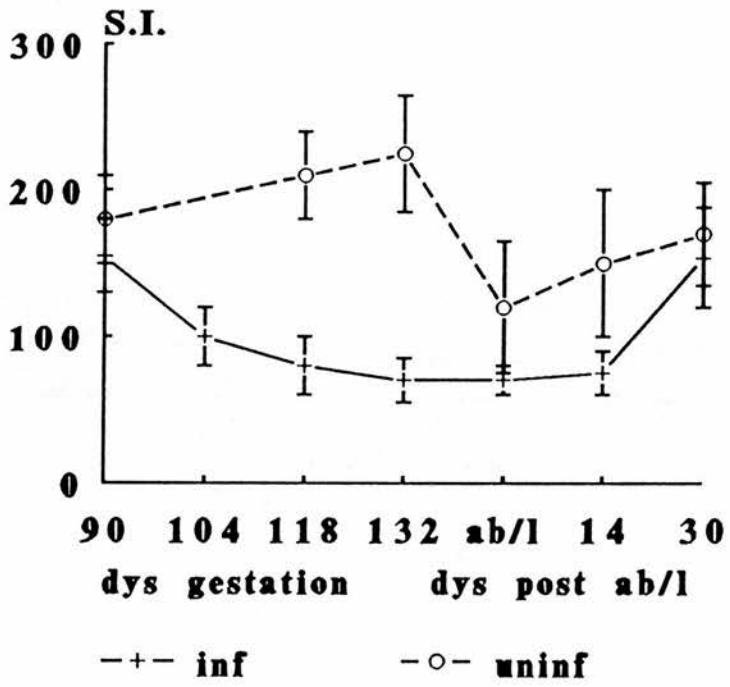
[b] P<0.05 when compared with pre-infection cpm

[c] P<0.05 when compared with previous bleed cpm

[d] P<0.05 when groups are compared

nd: not done

Figure 4.2: Proliferative response of ovine PBMC to 2.5μg/ml Con A, during gestation, at lambing and 30 days after lambing of infected (+-+) and uninfected (o-o) sheep.



III) Specific Antigen.

Proliferative responses to crude EB preparations were again similar and of small magnitude in both groups before infection at day 90 (see table 4.5, figure 4.3). However, within 14 days the responses in group 1 had risen from a mean SI of 3 to 43 ($p<0.05$). The group response remained at this heightened level until day 132, a few days before parturition. During the same period of time the responses in group 2 also rose slightly and significantly ($P<0.05$), from a mean SI of 3 to one of 10. At every comparable bleed from day 104 the mean response in group 1 was higher than in group 2 ($p<0.05$). As with the mitogens the responses to specific antigen dropped in the days prior to lambing/abortion. In this case in group 1 the mean SI fell from 39 to one of 11 between days 132 and parturition ($p<0.05$). After this it rose again to between 13 and 20 where it remained for the duration of the experiment. Group 2 levels dropped to a mean SI of between 3 and 6 where they remained for the remainder of the experiment.

Table 4.5: Proliferative response of ovine PBMC to 20μg/ml EBs, during gestation, at lambing and 30 days after lambing of infected and uninfected sheep.

	day gestation					day post lambing	
Group	90	104	118	132	lambing	14	30
1	2±.5	42±8[ab]	37±9[abd]	39±11[abd]	11+2[abcd]	16+3[abd]	13±2[abd]
2	2±1	nd	7±3[abd]	11±4[ab]	11±3[ab]	3±.5[ac]	4±1[a]

[a] P<0.05 when compared with medium control cpm

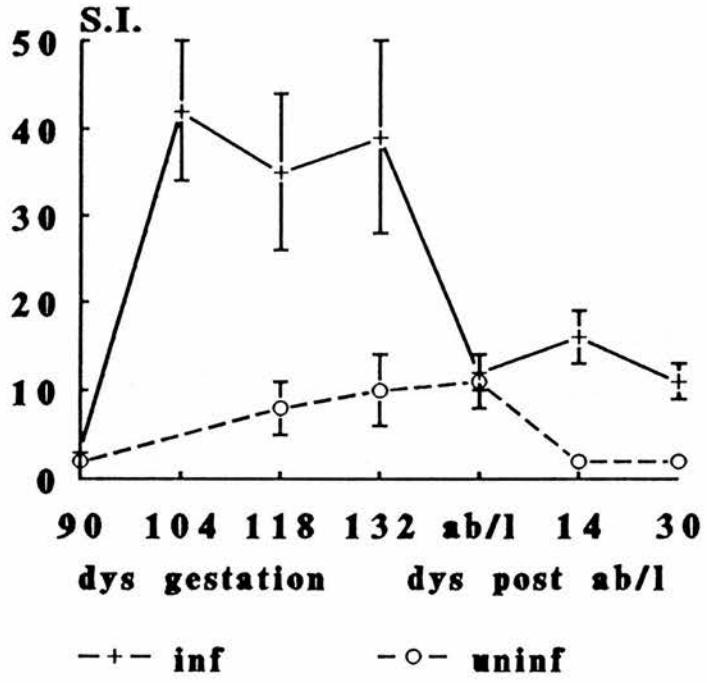
[b] P<0.05 when compared with pre-infection cpm

[c] P<0.05 when compared with previous bleed cpm

[d] P<0.05 when groups are compared

nd: not done

Figure 4.3: Proliferative response of ovine PBMC to 20μg/ml EBs, during gestation, at lambing and 30 days after lambing of infected (+-+) and uninfected (o-o) sheep.



Detection of *C.psittaci* in the peripheral blood.

No *C.psittaci* could be detected in the cytospin preparations taken from the peripheral blood of any ewe, in either group at any of the time points sampled.

Discussion.

Clinical Differences.

Infection of group 1 animals at day 90 of gestation, resulted in demonstrable clinical differences between this group and the uninfected group with increased lamb mortality, decreased length of gestation and reduced birth weight of live lambs. The lamb mortality and the reduction in the length of gestation and of birthweight were consistent with that seen regularly in field infections (Aitken, 1991). These clinical signs almost certainly arise from the placental damage incurred. The resulting loss in placental function leads to the death of the foetus or the early birth of weakly lambs (Aitken, 1991). Hormonal changes measured in infected animals (Leaver, Howie, Aitken, Appleyard, Anderson, Jones, Hay, Williams and Buxton, 1989; Leaver, Howie, Appleyard, Aitken and Hay, 1987) may be due to this placental damage and may be the cause of early births and abortions. While the placentas from the two ewes in group 1 which produced live lambs had fewer, smaller areas of necrotic tissue than the others in the group this is not always the case in experimental chlamydiosis (G.E.Jones, personal communication). It is perhaps not surprising that there is a correlation between the

length of pregnancy and lamb survival (G.E.Jones, personal communication), and this was the case in group 1, since the two ewes which produced live offspring had the longest gestation periods.

In the field, where an initial infection will result in up to 30% of animals aborting (Stamp *et al*, 1950) it is not clear why some animals abort and others do not, when presumably all are susceptible to infection and abortion.

Lymphocyte responses.

The similarity in the responses of the PBMC of both groups of animals to the B-cell mitogen LPS throughout this study seems to suggest that a chlamydial infection has no effect on the non-specific proliferative responses of ovine B-cells. However, the significant rise in response in both groups measured between day 90 and day 132 suggests that there is possibly some pregnancy associated factor which increases the responsiveness of lymphocytes to this mitogen, since a similar effect was recorded in the response of uninfected animals to the T-cell mitogen Con A. These results do not necessarily mean that there is a heightened response to specific antigens and the experiment, as designed, does not answer questions about immunosuppression associated with pregnancy (Tomasi, 1983), since the responses are those of PBMC and therefore say nothing about local immune responses around the developing foetus.

Although there appears to be little difference in the response of the two groups to LPS there is a considerable difference in the response of the infected sheep to the T-cell mitogen Con A when compared with uninfected animals. In the infected group a marked suppression was identified, from 14 days after infection. Once again the reason for this is not clear, but it has been seen before in mice where infection with the *C.psittaci* Cal 10 strain, decreased the response of spleen cells to both T and B-cell mitogens (Lammert and Wyrick, 1982). However, the finding in sheep is further complicated by the fact that the antigen specific lymphocyte response to EBs of *C.psittaci* at the time of the mitogen suppression does not appear to be affected, since it remains high until the immediate pre-parturient period. These results show that any postulated immunosuppression associated with pregnancy does not affect the peripheral immune system's ability to mount a strong proliferative response to specific antigen *in vitro*. Despite this strong proliferative response there has been little antibody shown to be produced until after abortion when titres rise to reach a maximum after 14 days (Stamp *et al*, 1952) as measured by the CFT and Western blot analysis (Tan, 1990). This is further complicated when considered beside the finding that the B-cell response to LPS increases from day 90 until before parturition. Thus it would seem that proliferation is intact, but that specific antibody responses are not apparent. Although not measured in this study ovine DTH responses to *C.psittaci* have been demonstrated in pregnant animals (Dawson *et al*, 1986) and previously the development of DTH had been reported as coinciding with the induction of suppression of

humoral responses in mice (Basten, 1981) and this may possibly explain why there is no specific antibody detected. Both pregnancy associated immunosuppression (Tomasi, 1984) and the intracellular nature of *C.psittaci* (Hahn and Kaufman, 1981) may also affect the ability to mount an antibody response. However, in the case of *T.gondii* infection of pregnant sheep neither of these prevent a strong antibody response (McColgan, Buxton and Blewett, 1988).

A common factor in both of the groups was the sudden drop, of the PBMC responses to the purified EBs and both mitogens in the few days prior to lambing/abortion. A reduced responsiveness to T-cell mitogens has been described in human pregnancy (Tomoda, Fuma, Miwa, Saiki and Ishizuka, 1976), bovine pregnancy (Wells, Burrells and Martin, 1977) and ovine pregnancy (Burrells, Wells and Sutherland, 1978). In the latter study, the response to phytohaemagglutinin (PHA) and pokeweed mitogen (PWM) were measured. In both cases, as with Con A, there was a decrease in response in the immediate pre-parturient period which remained for some days after lambing. The actual time that the suppression began differed between the two mitogens used in the study and both differed from the time of suppression of the Con A stimulated cells. This time lapse may be the result of the different modes of action of the three mitogens, but the overall effect is undoubtedly associated with pregnancy and the onset of parturition. Similar immunosuppressive effects have been reported in patients with multiple trauma injury and are associated with prostaglandin E_2 release, tissue damage and bacterial endotoxins (Green and Faist, 1988), all of which are associated with chlamydial abortion. In

addition, prostaglandin E_2 release is thought to play a role in the onset of labour (Bleasdale and Johnston, 1984) and may be the reason for the immunosuppression in uninfected animals. Defects in the immune system brought about by these factors include diminished DTH responses (Hansbrough, Zapata-Sirvent, Peterson, Wang, Bender, Claman and Boswick, 1984), reduced T dependant antibody responses (Wood, O'Mahoney, Rodrick, Eaton, Demling and Mannick, 1986) and decreased proliferative responses to mitogens (Leguit and Zeijlemaker, Schellekens and Eijsvoogel. 1973. Meines, 1973), all of which are factors in the immune response of *C.psittaci* infected, pregnant ewes.

Detection of *C.psittaci* in the peripheral blood.

There are many reasons which could explain the failure to detect *C.psittaci* in the peripheral blood. The first and most obvious is that the organism may not be there. However, the possibility should be considered that the immunoperoxidase method used was not sensitive enough to highlight trace amounts of EBs. The method also requires *C.psittaci* to have infected the PBMC, since any free EBs would have been washed away during the preparation of the blood.

Conclusions.

It is clear from the evidence presented above that pregnancy alters the PBMC response to mitogens and to chlamydial antigens *in vitro* immediately before parturition. What effect this has on the outcome of a chlamydial infection is uncertain, because when this phenomenon is apparent infection of the placenta is already

underway. Mitogen responses of PBMC as measured by Con A are also suppressed by chlamydial infection, but the mechanisms involved are unknown since they do not prevent proliferation in response to to the EBs themselves.

The effect infection with *C.psittaci* has on the development of PBMC responses in pregnant sheep has been detailed. Also since the responses are long lasting in animals following abortion, there is strong evidence for their role in protective immunity, since sheep seldom abort again.

Chapter 5:

PROLIFERATIVE RESPONSES OF OVINE PERIPHERAL BLOOD MONONUCLEAR CELLS AND CD4+ T-CELLS TO ELEMENTARY BODY PROTEINS.

Introduction.

In previous chapters, it has been shown that a proliferative response to EBs can be demonstrated *in vitro* in lymphocytes from infected pregnant animals during gestation and that this response can still be measured at least 6 months after abortion. What is as yet unresolved, however, is which chlamydial antigens are responsible for stimulating this proliferative response? The chlamydial genome encodes between 400-600 potentially antigenic proteins (Stephens, 1988), of which more than 100 can be visualised by SDS-PAGE eletrophoresis (Salari and Ward, 1981). Of these proteins, 12-14 bands from the EAE strain S26/3, are recognised by ovine convalescent sera and lymph fluid (Huang *et al*, 1990) and 9 bands, from the same strain, are recognised by ovine post vaccine challenge sera (Anderson *et al*, 1990). This study was designed to identify which chlamydial proteins are T-cell reactive using antigen specific T-cell lines developed from PBMC and the method of Young and Lamb (1986) which involves the separation of chlamydial EBs by SDS-PAGE. The separated proteins are then blotted onto nitrocellulose and individual proteins are cut from the nitrocellulose and dissolved in dimethylsulphoxide (DMSO). The dissolved nitrocellulose is then resolubilised as a fine particle suspension which allows individual antigens to be tested in a proliferation assay. In addition it was hoped that any differences there may be between the responses of both vaccinated

and post-abortion animals would also be identified and may help to explain the finding that animals require to be revaccinated every 3 years (Aitken, 1991), while post-abortion animals do not abort again (Aitken, 1991).

The T-cell lines were also tested for proliferation against recombinant MOMP and two synthetic peptides (supplied by Dr A.J.Herring, Moredun Research Institute), based on the MOMP sequence obtained from the S26/3 EAE strain of *C.psittaci* (Herring *et al*, 1989). MOMP was chosen as a possible recombinant protein vaccine, because of its importance in terms of both mass (Caldwell *et al*, 1981) and function (Newhall and Jones, 1983) and, because of the success of a MOMP enriched test vaccine (Tan *et al*, 1991).

Experimental procedure.

The sheep used in the experiment were placed in 5 groups (see table 5.1). Group 1 consisted of three sero-negative blackface ewes from the previous lambing season. Group 2 contained three Dorset ewes, which had been raised under gnotobiotic conditions for 8 weeks before being transferred to and maintained in a clean loose box. The 3 blackface ewes in group 3 were given 1 dose of the commercially available vaccine (Coopers Animal Health Ltd, Breakspear Road, Middlesex, England) as per manufacturers instructions. Four blackface ewes which had aborted in the previous lambing season (6 months earlier) and another 4 which had aborted in the season before (18 months earlier) were placed in

group 4a and 4b respectively. Group 5, consisted of 2 blackface ewes which were injected intra-muscularly with keyhole limpet haemocyanin (KLH) and which were used to produce negative control T-cell lines.

PBMC were collected from the animals and were stimulated with whole EB as well as individual EB protein fractions in lymphocyte transformation assays. The latter were separated on 10% or 12% SDS-PAGE gels, blotted onto nitrocellulose and resolubilised as a fine particle suspension. Con A and LPS were used as control mitogens.

T-cell lines were generated from the PBMC of sheep which responded strongly in the above PBMC proliferation assays. The lines were phenotyped by flow cytometry and were retested in an *in vitro* assay, using the same stimuli as described above. In addition, the T-cell lines were tested against a recombinant form of MOMP and also 2 synthetic peptides, one from variable domain 2 and one from variable domain 4. Monoclonal antibodies against MHC class 1 and MHC class 2 molecules were also added to determine which molecule was used in antigen presentation to the T-cell lines. All assays included the T-cell line raised against KLH as a control.

Finally, samples of the medium were removed from the wells of the 96 well plates, before the addition of the tritiated thymidine, and stored at -20^{o}C until they could be tested for cytokine activity.

Table 5.1: Experimental groups used to determine the ovine peripheral blood mononuclear cells (PBMC) and T-cell line response to biochemically fractionated chlamydial proteins.

Group	(n)	Treatment	PBMC	T-cell lines
1	3	-	+	-
2	3	-	+	-
3	3	vaccinated[a]	+	+/-
4a	4	*C.psittaci* (6mths)[b]	+	+
4b	4	*C.psittaci* (18mths)[c]	+	+
5	2	KLH[d]	+	+

[a] sheep given commercially available vaccine as per instructions

[b] sheep had aborted 6 months previously

[c] sheep had aborted 18 months previously

[d] sheep had been immunised with KLH to provide control T-cell lines

*PBMC was collected at 21 days post vaccination in Gp 3 and 21 days post injection in Gp 5.

Results.

Proliferative response of ovine PBMC to whole chlamydial EBs.

The PBMC from the seronegative ewes in group 1, the uninfected Dorset lambs in group 2 and the KLH infected ewes in group 5, showed similar proliferative responses to whole chlamydial EBs (see table 5.2 and figure 5.1). The peak response gave an SI of between 8 and 10 in all groups. This was significantly higher than background measured by unstimulated cells in medium only. Only 1 animal from group 3 developed a strong proliferative response to

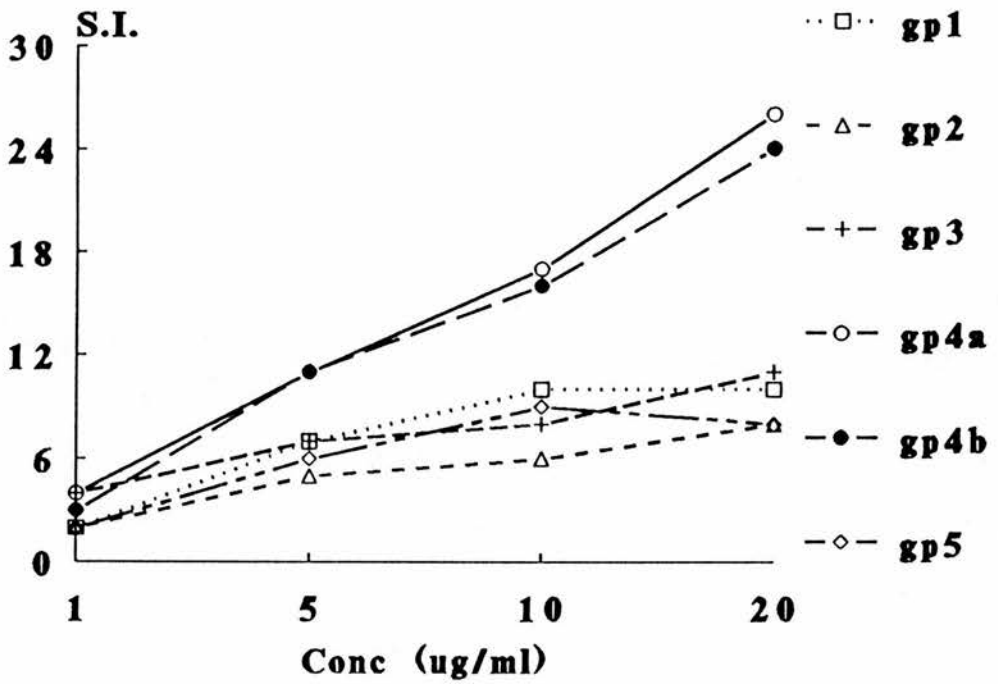
whole EBs and gave an SI of 17, the other two animals gave responses similar to those in groups 1, 2 and 5 (SIs of 7 and 10 respectively). All responses were again significantly above background ($P<0.05$). Finally, the PBMC collected from the sheep in groups 4a and 4b all proliferated strongly in response to stimulation by EBs ($p<0.05$). There was no significant difference between groups 4a and 4b ($P>0.05$) and maximum responses gave SIs of of 26 and 24 respectively.

Table 5.2: The proliferative responses (expressed as a stimulation index (SI)) of ovine PBMC to different concentrations of chlamydial elementary bodies (EBs).

Group	EB (μg/ml)			
	1	5	10	20
1	2±.2	7±1[a]	10±1.5[a]	10±1.1[a]
2	2±.2	5±.7[a]	6±1[a]	8±1[a]
3	4±.5[a]	7±.5[a]	8±1.3[a]	11±2.9[a]
4a	4±.5[a]	11±1.1[a]	17±2[a]	26±2.3[a]
4b	3±.1	11±1[a]	16±2[a]	24±1.9[a]
5	2±.1	6±1[a]	9±1[a]	8±1[a]

[a] $P<0.05$ when compared with medium control cpm.

Figure 5.1: The proliferative response to whole EBs, of ovine PBMC from the uninfected sheep of group 1 (□-□) and group 2 (△-△), vaccinated sheep in group 3 (+-+), infected sheep in group 4a (o-o) and group 4b (o-o) and group 5 (◇-◇) KLH infected sheep.



Proliferative response of ovine CD4+ T-cell lines to whole chlamydial EB.

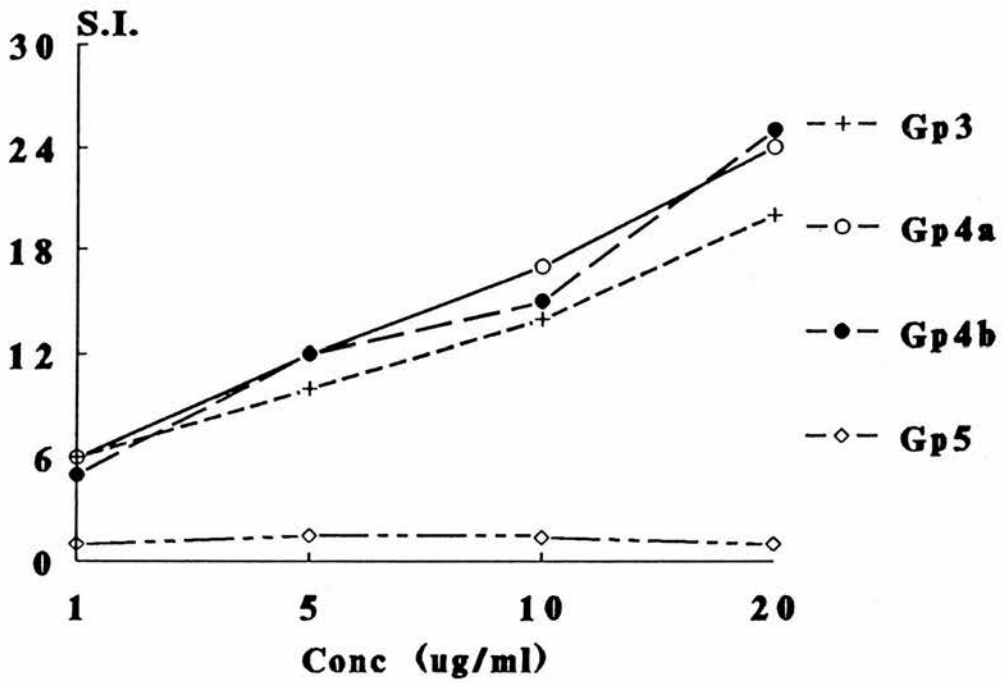
All T-cell lines generated for these experiments were > 90% CD4+ when phenotyped by flow cytometry. There was no significant difference in the proliferative response of the group 4a and 4b T-cell lines when compared either with each other or with the proliferative response of the PBMC of these groups ($P>0.05$). Maximum responses were given on stimulation with 20µg/ml of EBs and resulted in SIs of 24 and 27 respectively (see table 5.3 and figure 5.2). There was a significant rise, however, in the proliferative response of the T-cell lines raised from group 3 animals when compared with the PBMC response of the same group ($P<0.05$). The maximum response was given by 20µg/ml and resulted in an SI of 20. There was no proliferative response to chlamydial EB by T-cell lines generated from group 5 ewes, which had been raised against KLH.

Table 5.3: The proliferative responses (expressed as a stimulation index (SI)) of ovine CD4+ T-cell lines to different concentrations of chlamydial elementary bodies (EB).

Group	EB (μg/ml)			
	1	5	10	20
3	6±1[a]	10±1.1[a]	14±1[a]	20±2[a]
4a	6±1[a]	11±0.8[a]	17±1.1[a]	24±1.9[a]
4b	5±.7[a]	12±0.7[a]	15±1.1[a]	25±1.7[a]
5	-	-	-	-

[a] $P<0.05$ when compared with medium control cpm.

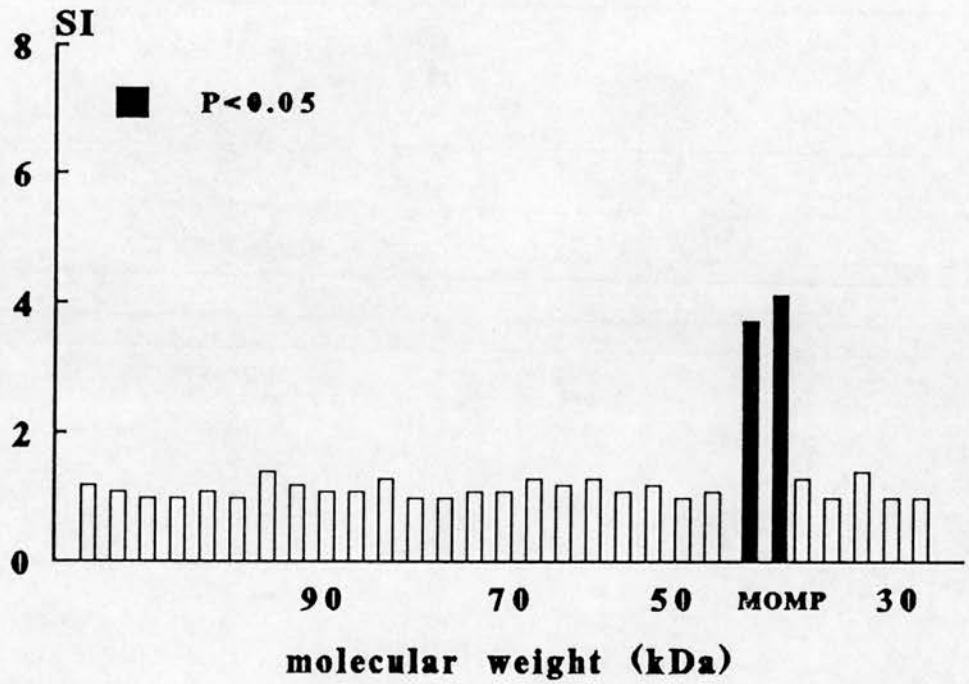
Figure 5.2: The proliferative response to whole EBs, of ovine CD4+ T-cell lines from the vaccinated sheep in group 3 (+-+), infected sheep in group 4a (o-o) and group 4b (o-o) and group 5 (◇-◇) KLH infected sheep.



Proliferative response of ovine PBMC and T-cell lines to biochemically fractionated chlamydial proteins.

There was no significant proliferation in response to any fractionated chlamydial protein in the PBMC of any sheep from groups 1, 2 or 5 ($p>0.05$). T-cell lines generated from group 5 also gave no significant proliferative response when stimulated by the fractionated proteins ($p>0.05$). The PBMC from group 3 sheep only gave significant proliferation, compared with unstimulated background controls, when stimulated by a protein of approximately 38Kd ($P<0.05$) (see figure 5.3). However, the T-cell lines generated from the PBMC of the sheep in this group gave a much wider range of response (see figure 5.4). Significant proliferative responses to fractionated proteins of approximate weights of 30, 38, 50 and 70Kd were consistently obtained ($P<0.05$). On occasion significant responses to proteins of 90 and 45Kd were also seen ($P<0.05$). PBMC from all the animals in groups 4a and 4b responded to many fractionated proteins with approximate weights 30, 38, 50 and 70Kd ($p<0.05$) (see figure 5.5). Other antigens also stimulated significant proliferation in the PBMC of some animals, but not in others ($p<0.05$). These had approximate weights of 18, 45 and 90Kd. T-cell lines generated from the PBMC of these animals gave similar patterns of response when stimulated by fractionated proteins as did the PBMC donor animal (see figure 5.6). The T-cell line proliferative response to individual proteins was higher than the PBMC response, but was not significantly so ($P<0.05$).

Figure 5.3: The proliferative response of group 3 peripheral blood mononuclear cells to nitrocellulose bound chlamydial proteins. Nitrocellulose background control (cpm±se) 623±97.



*MOMP appears on the x-axis merely to emphasise the approximate molecular weight of this protein. It is unknown if protein(s) stimulating proliferation in this area is MOMP.

Figure 5.4: The proliferative response of group 3 CD4+ T-cell lines to nitrocellulose bound chlamydial proteins. Nitrocellulose background control (cpm±se) 821±102.

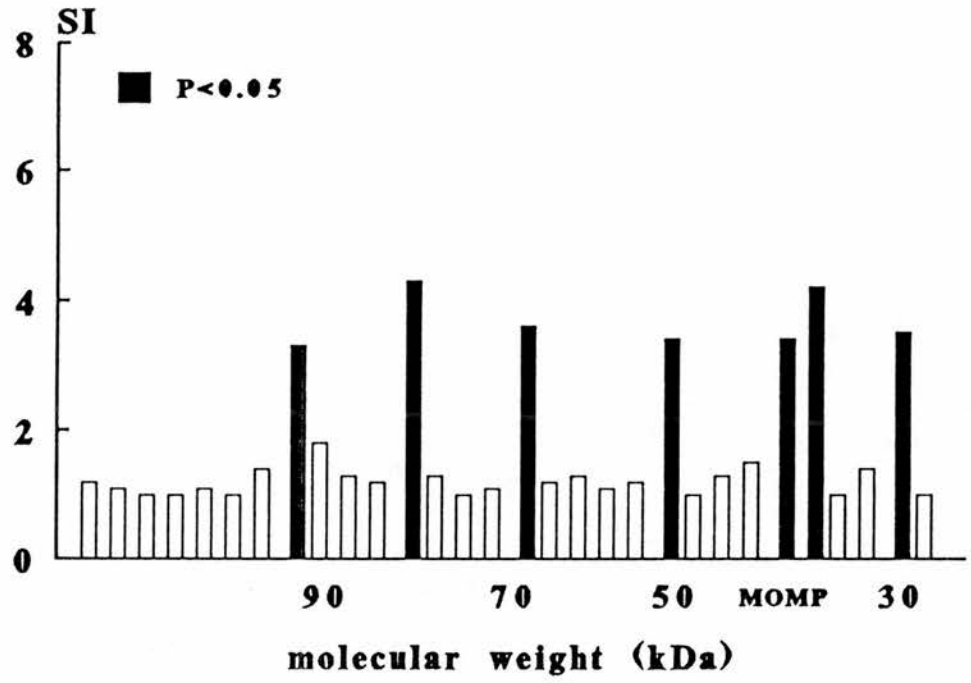


Figure 5.5: The proliferative response of group 4 peripheral blood mononuclear cells to nitrocellulose bound chlamydial proteins. Nitrocellulose background control (cpm±se) 1157±137.

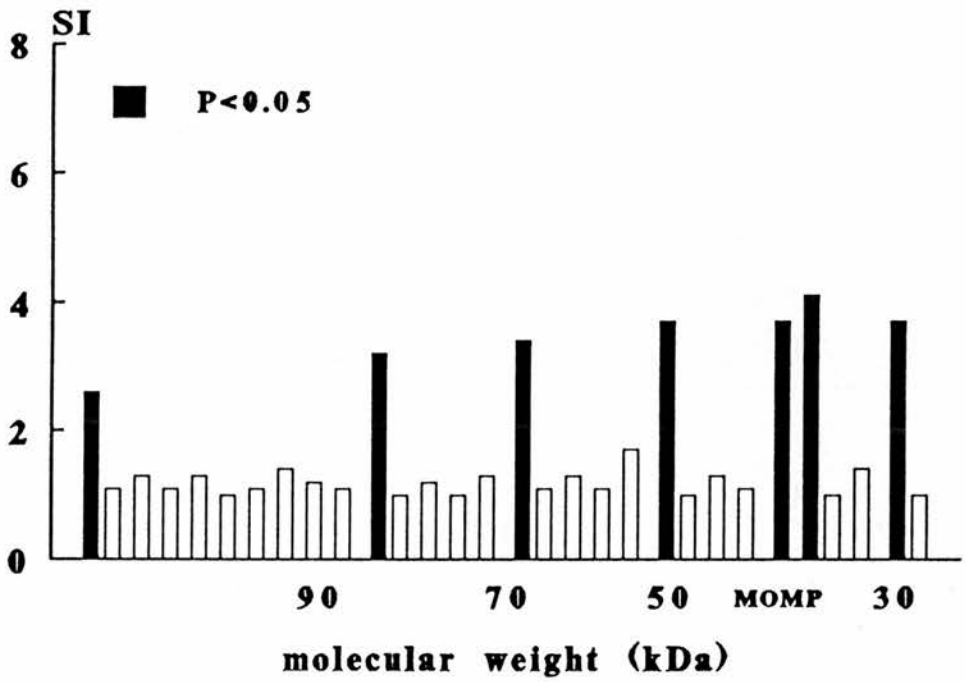
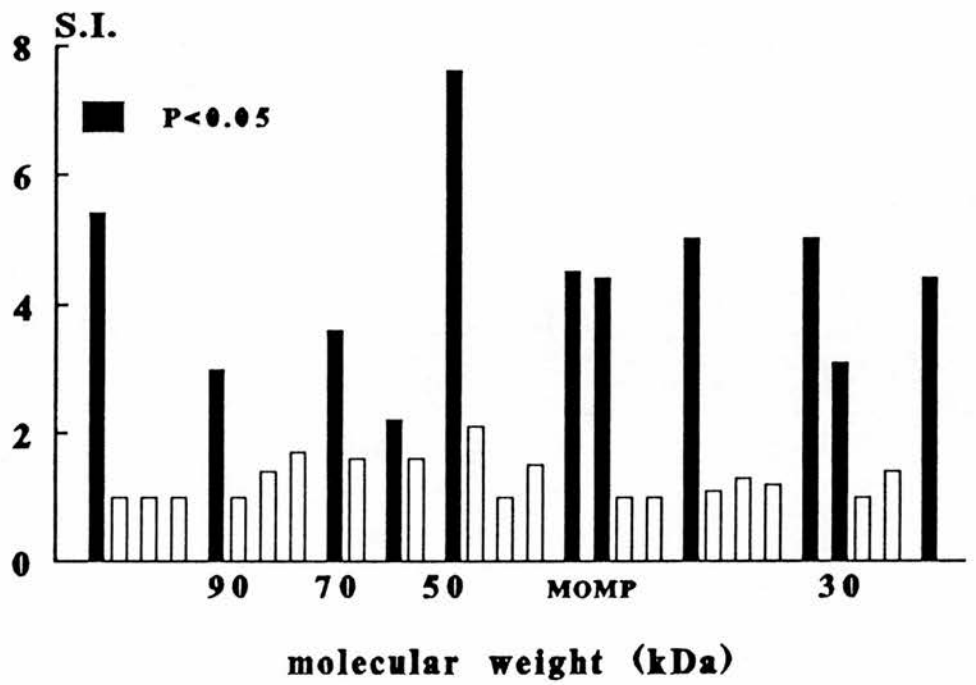


Figure 5.6: The proliferative response of group 4 CD4+ T-cell lines to nitrocellulose bound chlamydial proteins. Nitrocellulose background control (cpm±se) 699±92.



PBMC and T-cell proliferative responses of group 5 sheep to KLH.

PBMC from the group 5 KLH infected ewes responded to whole EBs as described above (table 5.2), they also gave significant proliferative responses when stimulated KLH. The maximum response was given by stimulation with 20μg/ml and resulting in a SI of 16 ($p<0.05$). KLH specific T-cell lines were developed as negative controls and did not respond to whole EBs, LPS or any individual chlamydial protein. Also they did not proliferate in response either to recombinant MOMP or to the synthetic peptides. The lines did, however, respond strongly to both KLH giving a SI of 24 ($p<0.05$) (see table 5.4, figure 5.7) and the T-cell mitogen Con A giving a SI of 67 ($p<0.05$).

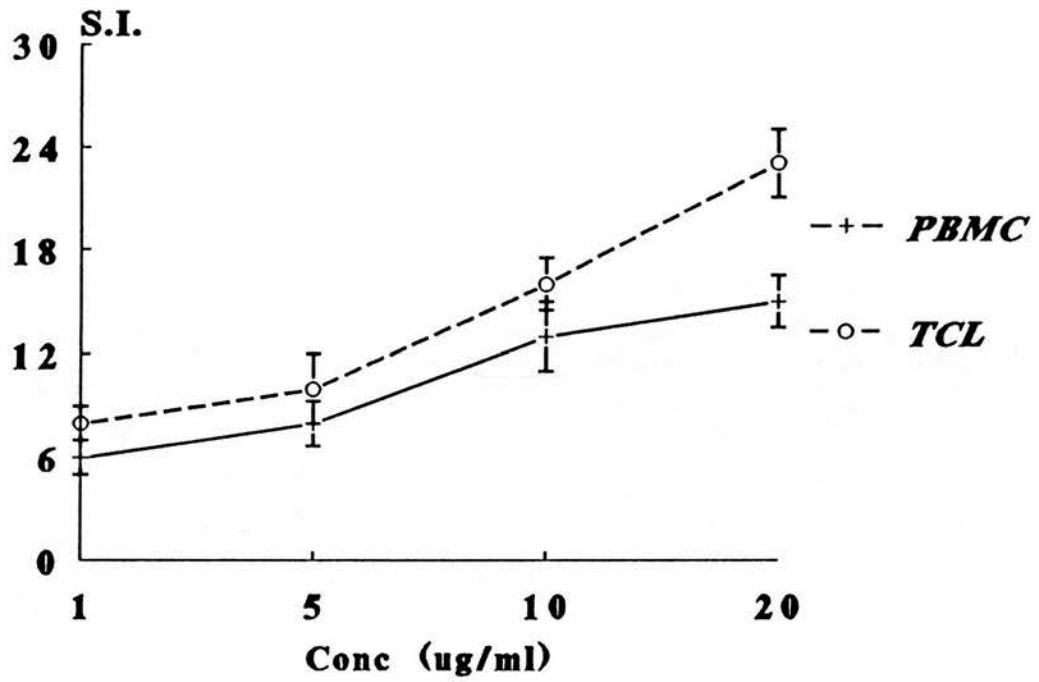
Table 5.4: The proliferative response, expressed as stimulation index (SI)$\pm$se, of group 5 PBMC and CD4+ T-cell lines to Keyhole Limpet Haemocyanin (KLH). Background cpm ranged between 575$\pm$21 and 1,009$\pm$123.

Cell type	KLH (μg/ml)			
	1	5	10	20
PBMC	6 ± 1^{a}	8 ± 1.3^{a}	13 ± 2^{a}	16 ± 1.5^{a}
CD4+ T-cells	6 ± 1^{a}	10 ± 1.7^{a}	16 ± 1.5^{a}	24 ± 2^{ab}

[a] $P<0.05$ when compared with medium control cpm

[b] $P<0.05$ when groups are compared.

Figure 5.7: The proliferative response to KLH of the PBMC (+-+) and CD4+ T-cell lines (o-o) from the group 5 KLH immunised sheep.



Proliferative responses of group 4 T-cell lines to recombinant MOMP and to two synthetic peptides from variable domain 2 and 4.

T-cell lines generated from the PBMC of sheep in group 4, were stimulated by recombinant MOMP (rMOMP) and two synthetic peptides VD2 and VD4 (see table 5.5 and figure 5.8 and 5.9). The T-cells gave significant proliferative responses and a SI of 7±1.5 when stimulated by 2.5μg/ml of rMOMP ($p<0.05$). The maximum proliferative response to the peptides were given with stimulation by 1μg/ml and gave SIs of 15 for VD2 and 18 for VD4 ($P<0.05$).

Table 5.5: Proliferative response of group 4a/4b CD4+ T-cell lines to recombinant MOMP and to two synthetic peptides from variable domain 2 (VD2) and variable domain 4 (VD4) of MOMP. Background cpm ranged between 941±87 and 1,442±121.

	Concentration (μg/ml)			
	.6	1.2	2.5	5
rMOMP	4±0.8[a]	5±1.3[a]	7±1.5[a]	4±1[a]
	.25	.5	1	2
VD2	11±1.7[a]	15±1.5[a]	15±2[a]	7±0.7[a]
VD4	10±1.4[a]	15±1.7[a]	18±2.3[a]	8±1.6[a]

[a] $P<0.05$ when compared with medium control cpm.

Figure 5.8: The proliferative response of the group 4a/4b CD4+ T-cell lines, to rMOMP (+-+).

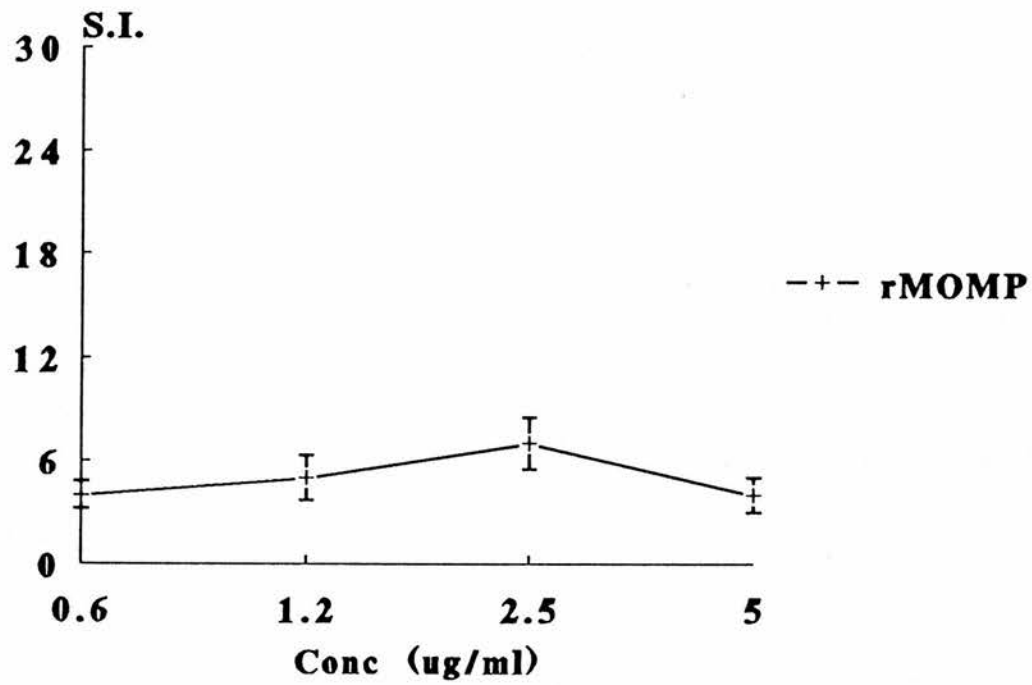
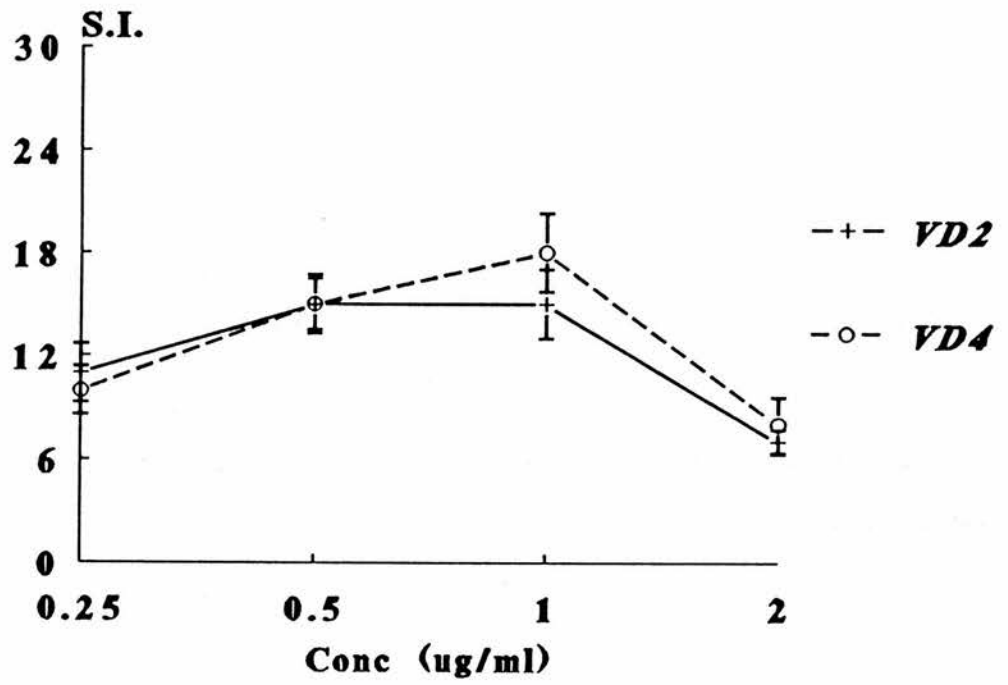


Figure 5.9: The proliferative response of the group 4a/4b CD4+ T-cell lines, to the synthetic peptides of rMOMP from VD2 (+-+) and VD4 (o-o).



Inhibition of proliferative responses of a group 4 sheep (2171) by monoclonal antibody against MHC class II molecules.

A T-cell line, generated from the PBMC of a single sheep in group 4, was stimulated with 20µg/ml of EBs, 2.5µg/ml of rMOMP and 1µg/ml of each of the synthetic peptides. The proliferation of the T-cells in response to the various stimulations was significantly inhibited by the addition of a monoclonal antibody against MHC class II molecules ($p<0.05$), but was unaffected by the addition of a monoclonal antibody against MHC class I molecules ($p<0.05$). The response to EBs was lowered from an SI of 18 to one of 8 (see table 5.6 and figures 5.10, 5.11, 5.12 and 5.13). The decrease in response to rMOMP was from an SI of 7 to one of 2 and the proliferative response to the peptides VD2 and VD4 dropped from 16 to 6 and from 17 to 7 respectively.

Table 5.6: Inhibition of antigen driven proliferation of group 4a sheep 2171, T-cell line in response to 20μg/ml EB, 1μg/ml VD2 and VD4 and 2.5μg/ml of rMOMP, by monoclonal antibody directed against MHC class I and class II. Background cpm ranged between 1,081±111 and 1,371±211.

Antigen	antibody	Concentration of antibody (%v/v)			
		0.5	1.0	2.5	5.0
EBs	MHC class I	18±1	19±1.7	20±1.5	20±1.8
EBs	MHC class II	16±2	10±1.5[a]	9±1[a]	8±1.5[a]
rMOMP	MHC class I	6±1	7±1.2	6±1.3	6±1.1
rMOMP	MHC class II	5±1.5	3±1.2[a]	2±0.8[a]	2±0.6[a]
VD2	MHC class I	15±1.5	14±1.7	15±2	15±1.5
VD2	MHC class II	15±1.3	10±1.8[a]	8±1.3[a]	6±1.5[a]
VD4	MHC class I	17±1.7	17±1.5	16±1	17±1.2
VD4	MHC class II	16±1	12±1.2[a]	10±1.5[a]	7±1[a]

[a] $P<0.05$ when compared with MHC class I control.

Figure 5.10: The diminished proliferative response of the T-cell line derived from group 4a sheep, 2171, to 20µg/ml of chlamydial EB, when blocked by MHC class I and II monoclonal antibody. Medium control background counts were 1121±101.

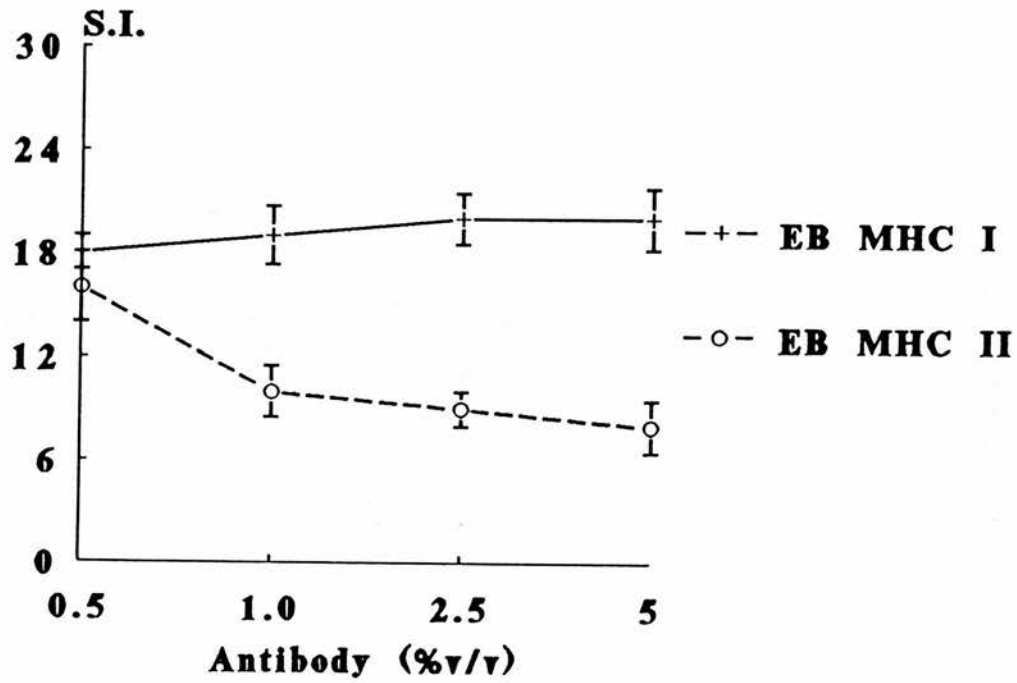


Figure 5.11: The diminished proliferative response of the T-cell line derived from group 4a sheep, 2171, to 2.5µg/ml of rMOMP, when blocked by MHC class I and II monoclonal antibody. Medium control background counts were 1121±101.

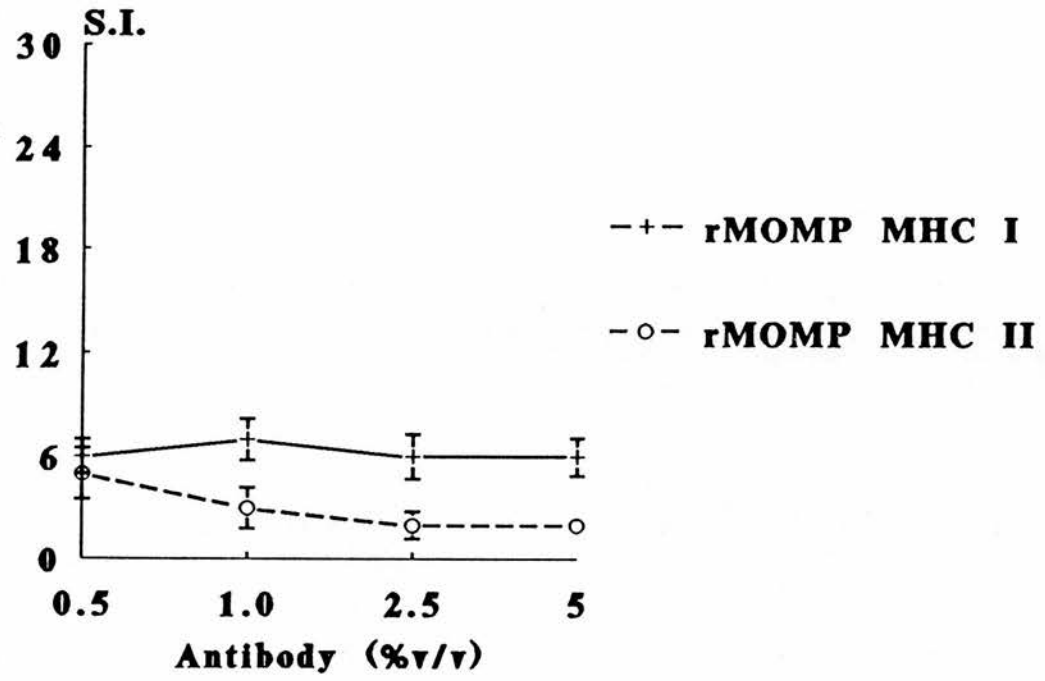


Figure 5.12: The diminished proliferative response of the T-cell line derived from group 4a sheep, 2171, to 1.2µg/ml of synthetic peptide VD2, blocked by MHC class I and II monoclonal antibody. Medium control background counts were 1121±101.

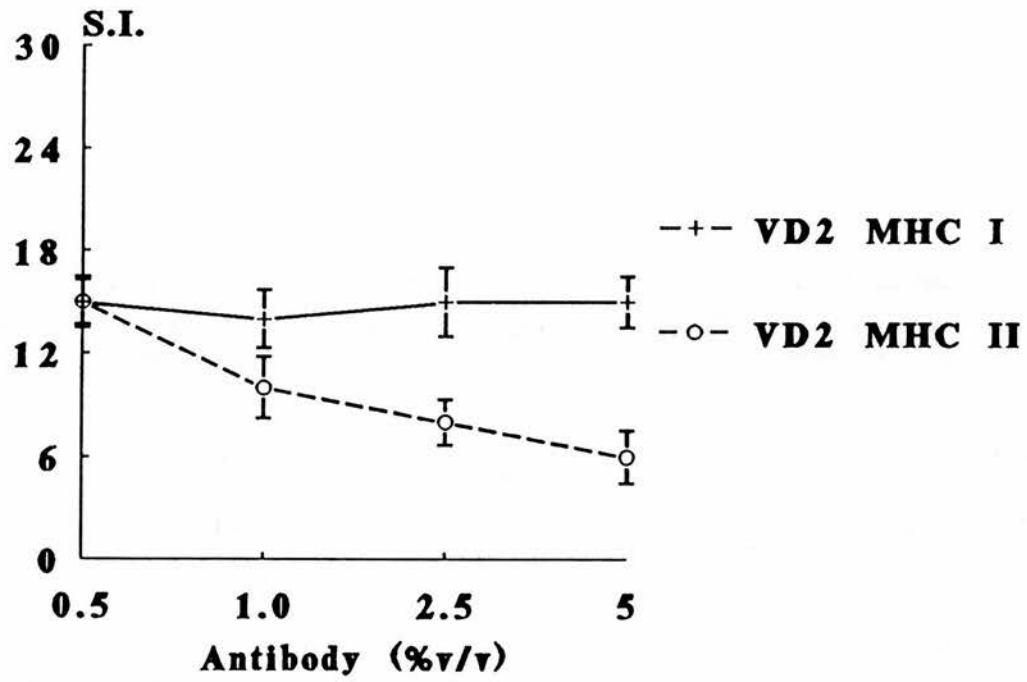
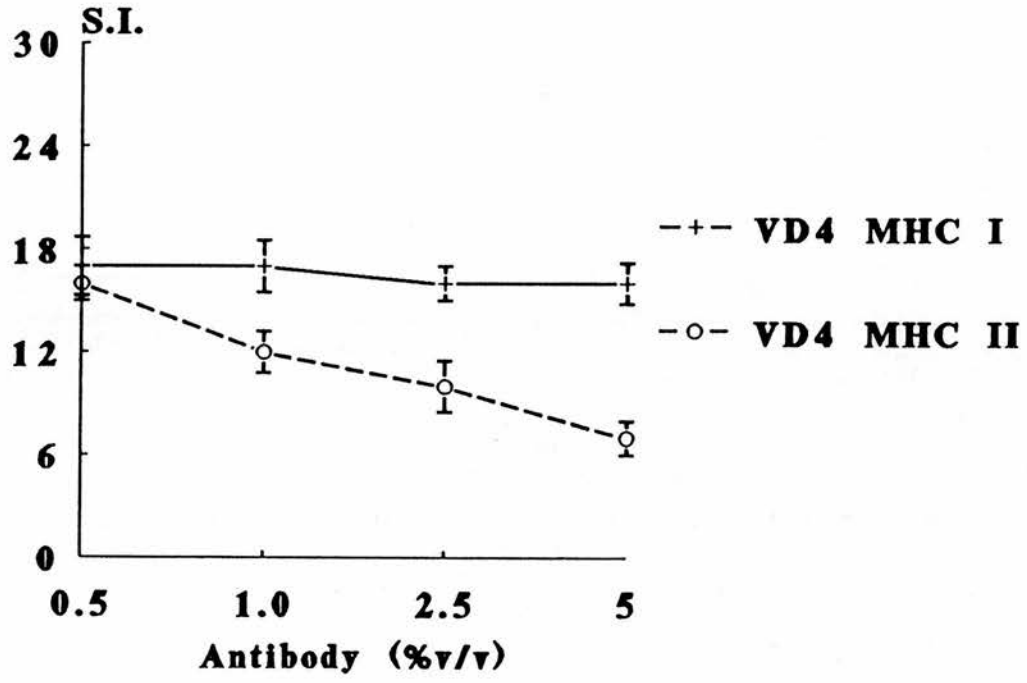


Figure 5.13: The diminished proliferative response of the T-cell line derived from group 4a sheep, 2171, to 1.2µg/ml of synthetic peptide VD4, blocked by MHC class I and II monoclonal antibody. Medium control background counts were 1121±101.



Discussion.

Some current vaccine strategies are based on the use of molecular biological techniques to produce recombinant proteins representing protective antigens, which, it is hoped will confer protective immunity against specific infectious agents encountered in the field. (Arnon, 1984). While simple techniques, such as western blot analysis of serum samples will identify antibody reactive proteins, with intracellular parasites such as *Chlamydia*, cell mediated immunity and T helper cell (CD4+) reactive proteins are thought to be more important (Hahn and Kaufman, 1981) and therefore measurement of T-cell reactivity may be more productive.

Western blot analysis of chlamydial proteins from EAE strains has shown a number of bands that are recognised by ovine convalescent and post vaccine sera (Huang *et al*, 1990; Anderson *et al*, 1990). However, before immunoblotting the proteins are separated on SDS-PAGE gels. This process involves boiling and reducing the protein to aid separation, but this leads to denaturation of the protein and antibodies, many of which are directed against discontinuous, conformational epitopes, will not generally react with them once they have been denatured (Berzofsky, 1980). Therefore some immune recognition may be lost.

However, unlike antibodies T-cells do not generally recognise discontinuous epitopes, (Gell and Benacerraf 1959), but instead react to non native, processed proteins in peptide form, presented by an antigen presenting cell via MHC restriction molecules

(Ishizaka, Okudaira and King, 1975). Denaturation of the protein should not therefore affect the ability of such T-cells to recognise and respond to a protein. Therefore, western blot analysis of T-cell reactive proteins using the method of Young and Lamb (1986) was used in this study to identify T-cell reactive proteins which in the future could be selected as candidate antigens for future vaccine studies.

The technique, while being a rapid method of screening proteins, does have some disadvantages, since the unequal transfer of different proteins to nitrocellulose, which limits the efficiency of a Western blot, may also affect this assay (Young and Lamb, 1986). There are also the possibilities that some epitopes may be destroyed or that the decreased immunogenicity of the nitrocellulose bound antigen may mean that this technique is unsuitable for weakly antigenic proteins (Young and Lamb, 1986).

One method used in this study to overcome the problem of the cell type specificity of the PBMC responses and to focus on CD4+ T-cell responses was the generation of CD4+ T-cell lines. The polyclonal CD4+ cell lines raised against EBs have a greater specificity, since only T-cells reactive to the selecting antigen are generated (Riedlinger, Grencis and Wakelin, 1986) and, since an increased number of cells in the line should be responsive, sensitivity should also be increased (Chiller, Defrietas, Chesnut, Grey and Skidmore, 1982). This phenomenon was demonstrated in the

different results obtained with the PBMC of the vaccinated ewe and the T cell line generated from the PBMC. The PBMC only responded to one protein, whereas the T cell line exhibited a much wider range of antigen response.

The reason why PBMC from animals in group 3, responded only to a protein with a molecular weight similar to that reported for MOMP is unclear. It may be simply that MOMP is the most abundant protein in the vaccine preparation and other proteins do not stimulate an *in vivo* response to the same degree as a natural infection because the antigenic load in the vaccine is lower than that encountered in a natural infection at the time of abortion. Alternatively, it may be that the other proteins have been altered by the vaccine production process, which includes inactivation in formaldehyde for 14 days (McEwan *et al*, 1955) rendering them unrecognisable by the T-cells. However, since the T-cell lines recognised some of the other proteins the first explanation is the more likely. Further evidence for this theory, comes from the fact that the other two ewes in this group did not develop strong proliferative responses to EBs and T-cell lines could not be generated from the PBMC from these animals. Again this may be due to altered immunogenicity or immunoaccessibility or simply due to the antigenic load being too small. However, since one animal in the group developed apparently normal immune responses antigenic alteration is improbable. This does not mean that the commercial vaccine offered no protection, since infection of the animal would result in a secondary boost to the immune system and may confer

protection. Differences between the antigens recognised by this group and those seen by PBMC from groups 4a and 4b are again probably due to the difference in the dose of antigen each received.

Uninfected animals from group 1 and 2, did not show a significant response to any fractionated protein. On many occasions in individual animals, however, there were responses to certain fractions, including that closest to the buffer front, which would have contained both the low molecular weight proteins and chlamydial LPS. Another reactive fraction was the first one, which contained unresolved large proteins and non-reduced EBs which had not entered the gel. Most sero-negative animals also gave small responses to a protein of approximately 55Kd which could be the putative heat shock protein described by Morrison *et al,* 1989. All of these could be the source of the cross-reaction described in previous chapters.

In groups 4a and 4b the antigens which stimulated proliferative responses varied between animals, but there was little or no variation between those which gave a response at the PBMC level and those which responded at the T-cell level for any given animal. The differences between individual sheep was expected as there are also differences seen at the antibody level (Anderson *et al,* 1990; Tan, 1989). In all animals there were 4 antigens which consistently gave significant proliferative responses ($P<0.05$) indicating that these may be immunodominant. These were of approximate molecular weights 30Kd, 38Kd (MOMP), 50Kd, and 70Kd.

However, there were other proteins which stimulated T-cell proliferation in some animals and not others within the group. It is unclear whether these antigens will also be important in stimulating a protective immune response. Of the 4 putative immunodominant antigens two have weights which are similar to two well characterised chlamydial proteins, namely MOMP (Caldwell *et al*, 1981) and the 30Kd adhesin (Hackstadt, 1984). Further study is necessary, however, to determine whether or not these are actually the two proteins stimulating the proliferation.

In an attempt to clarify whether T-cells recognise MOMP, the T-cell lines raised from the post abortion animals in group 4a and 4b were also tested against recombinant MOMP and two synthetic peptides derived from MOMP and based on the MOMP sequence obtained from the S26/3 EAE strain of *C.psittaci* (Herring *et al*, 1989). In a small group of 5, all animals tested responded both to recombinant MOMP and to the peptides. In each case the proliferation in response to the antigen was reduced by addition of antibodies against MHC class II molecules, further emphasising that the T cell lines were functional CD4+ T-cells. The KLH specific T cell line was used as a negative control and did not respond to either the recombinant protein or to the peptides. MOMP was chosen as a possible recombinant protein vaccine, because of its importance in terms of both mass (Caldwell *et al*, 1981) and function (Newhall and Jones, 1983) and, because of the success of a MOMP enriched test vaccine (Tan *et al'* 1990). Thus while supporting the use of a recombinant MOMP vaccine by demonstrating

post abortion immune recognition, further large scale studies are required to determine whether a single protein vaccine will be successful in generating the immune response necessary for protection.

Chapter 6:

PRODUCTION OF GAMMA INTERFERON BY OVINE PERIPHERAL BLOOD MONONUCLEAR CELLS AND CD4+ T-CELL LINES IN RESPONSE TO STIMUL ATION BY CHLAMYDIAL ELEMENTARY BODIES AND BIOCHEMICALLY FRACTIONATED CHLAMYDIAL PROTEINS.

Introduction.

In the previous chapter ovine PBMC and CD4+ T-cell proliferation in response to individual antigens of *C.psittaci* was demonstrated. In this study cytokine release by activated lymphocytes will be investigated as a further indicator of ovine cell mediated immune responses to *C.psittaci*. Cytokines are the coordinators of the immune and the inflammatory responses (Arai, Lee, Miyajima, Miyatake, Arai and Yokata, 1990) and as such are necessary for the development of protective immune responses, since cell to cell interactions, such as T-cell help for B-cell antibody production, require intercellular messengers (Arai *et al*, 1990). Specifically this study will investigate gamma interferon (γ-IFN) production by activated PBMC and T-cells. γ-IFN was originally identified as an anti-viral agent (Wheelock, 1965), but its role in enhancing the killing of intracellular bacteria has also been established (Nathan *et al*, 1983; Murray, 1988).

The importance of γ-IFN in chlamydial immunity has been demonstrated *in vitro* and *in vivo*. γ-IFN activates macrophages (Nathan *et al*, 1983; Arai *et al*, 1990) and activated macrophages have been associated with the restriction of chlamydial replication *in vitro* (Moulder *et al*, 1980). Huebnar and Byrne (1988) also found that activated macrophages, while not clearing infection, were necessary if mice were to survive an *in vivo* infection. A

similar effect has been described previously *in vitro* (Byrne and Faubion, 1982). γ-IFN mediated cytotoxicity has also been demonstrated against *C.psittaci* infected fibroblasts *in vitro* (Byrne *et al*, 1989).

The interferons have been shown to be distinct from each other and have been well characterised in many species. In mouse and man they have been classified by their biological and physiochemical characteristics (Stewart, 1980). In sheep similar differences exist and a biologically active molecule was identified as γ-IFN on the basis of its acid labile, antiviral activity (Entrican, Haig and Norval, 1989). Recent cloning of the cDNA of this molecule (McInnes, Logan, Redmond, Entrican and Baird, 1990) has revealed a 96% homology with the published bovine γ-IFN sequence at both the nucleic acid level and in its predicted amino acid structure (Cerretti, McKereghan, Larsen, Cosman, Gillis and Baker, 1986).

The following study was designed to examine the production of γ-IFN by ovine PBMC and CD4+ T-cell lines, when stimulated by biochemically fractionated chlamydial proteins, in order to determine which chlamydial antigens stimulate both T-cell proliferation and γ-IFN production in an *in vitro* assay. The presence of γ-IFN in the samples tested was detected by a bioassay and also by a sandwich ELISA. The latter was developed as a diagnostic assay for detecting tuberculosis in cattle (Rothel,

Jones, Corner, Cox and Wood, 1990) and employs monoclonal antibodies raised against bovine γ-IFN (Wood, Rothel, McWaters and Jones, 1990), but which cross react with ovine γ-IFN (Rothel *et al*, 1990).

Experimental procedure.

Samples of conditioned media were collected from the PBMC and the T-cell lines stimulated by fractionated antigens in experiments carried out in the previous chapter. The samples were removed from the wells of the 96 well plates in 100μl volumes and triplicate wells were pooled, prior to the addition of tritiated thymidine to the 96 well plates. The samples were then stored at -20^{o}C until they were tested for interferon activity.

Activity was measured in an interferon bioassay and positive samples were further characterised in a γ-IFN neutralising bioassay (see materials and methods). The presence of γ-IFN was also measured directly by a sandwich ELISA method (Commonwealth Serum Labs, Parkville, Australia). Briefly this involved the addition of conditioned medium to plates coated with an anti-γ-IFN monoclonal antibody. A second, horse radish peroxidase conjugated, antibody was then added and a coloured substrate was added to visualise the bound γ-IFN. Conditioned medium from stimulated PBMC was added in 50μl volumes and 25μl volumes were added from T-cell line conditioned medium. Concentrations of γ-IFN were assessed and calibrated using recombinant bovine γ-IFN (Ciba-Geigy, Saint Aubin, Switzerland.)) as a positive control. All groups, sizes, and treatments were the same as those described in the previous chapter (see table 6.1).

Table 6.1: Experimental groups used to determine the ovine peripheral blood mononuclear cell (PBMC) and T-cell line γ-IFN production in response to biochemically fractionated chlamydial proteins.

Group	(n)	Treatment	PBMC	T-cell lines
1	3	-	+	-
2	3	-	+	-
3	3	vaccinated[a]	+	+/-
4a	4	*C.psittaci* (6mth)[b]	+	+
4b	4	*C.psittaci* (18mth)[c]	+	+
5	2	KLH[d]	+	+

[a] sheep were vaccinated with commercial vaccine as per instructions

[b] sheep had aborted 6 months previously

[c] sheep had aborted 18 months previously

[d] sheep were immunised with KLH to provide control T-cell lines.

Results.

PBMC production of γ-IFN after stimulation by EBs, LPS and Con A.

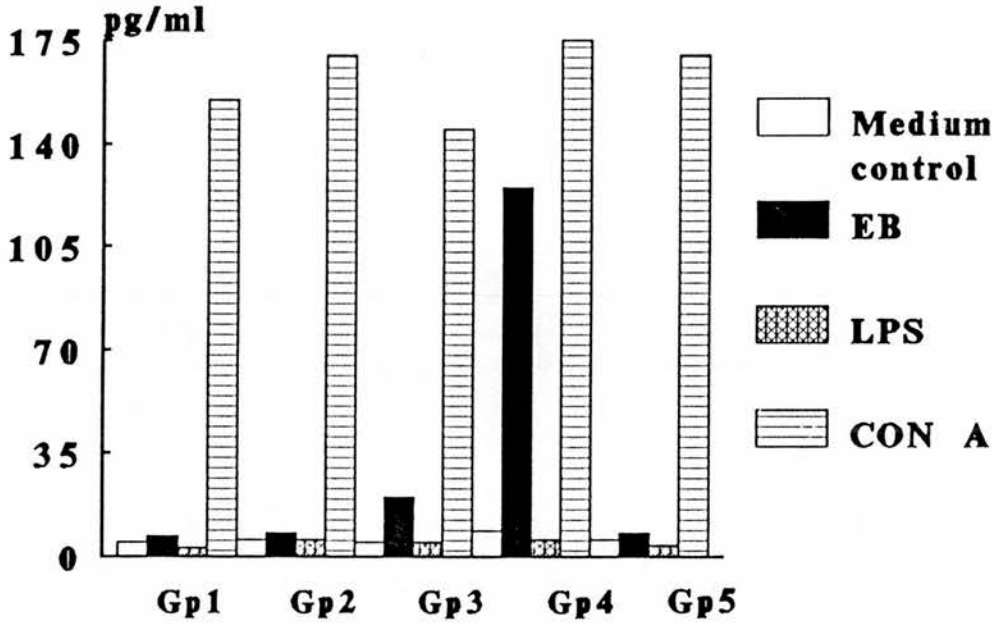
Unstimulated background controls of the PBMC of all sheep tested gave results below the sensitivity of the ELISA (<40pg/ml) and titres of 32U/ml by bioassay. The PBMC of group 1, group 2 and group 5 animals which had not been infected with *C.psittaci*, did not produce γ-IFN detected by ELISA or bioassay, when stimulated by whole EBs or LPS (see table 6.2 and figure 6.1). However, they did produce γ-IFN when stimulated by Con A. Concentrations

measured by ELISA were between 140 and 175pg/ml of IFN and gave titres of between 128U/ml and 256 in the bioassay. The PBMC from the vaccinated animals in group 3 did produce increased levels of Y-IFN when stimulated by EBs. Concentrations were the equivalent of 40pg/ml by ELISA and gave titres of 64U/ml in the bioassay. There was no response to LPS, but Con A responses were similar to the previous groups. Finally, the cells from the group 4a/4b animals, which will be considered as a single group (see previous chapter), produced higher levels of Y-IFN than group 3 animals in response to EBs. Optical densities measured by ELISA were ten times above medium controls and this corresponded to 125pg/ml and gave titres of 256U/ml. LPS and Con A responses were as those described for other groups.

Table 6.2: Production of Y-IFN (pg/ml±se and U/ml) by ovine PBMC stimulated with EB (20°g/ml), LPS (20°g/ml) and Con A (5°g/ml).

	Response to					
	EB		LPS		Con A	
Gp	pg/ml	U/ml	pg	U/ml	pg	U/ml
1	-	-	-	-	155±20	128
2	-	-	-	-	170±18	256
3	40± 3	64	-	-	145±32	128
4a/b	125±22	128	-	-	175±12	256
5	-	-	-	-	170±24	128

Figure 6.1: Production of γ-IFN by ovine PBMC stimulated with 20μg/ml of EBs, 20μg/ml of LPS and 2.5μg/ml of Con A, in the five experimental groups.



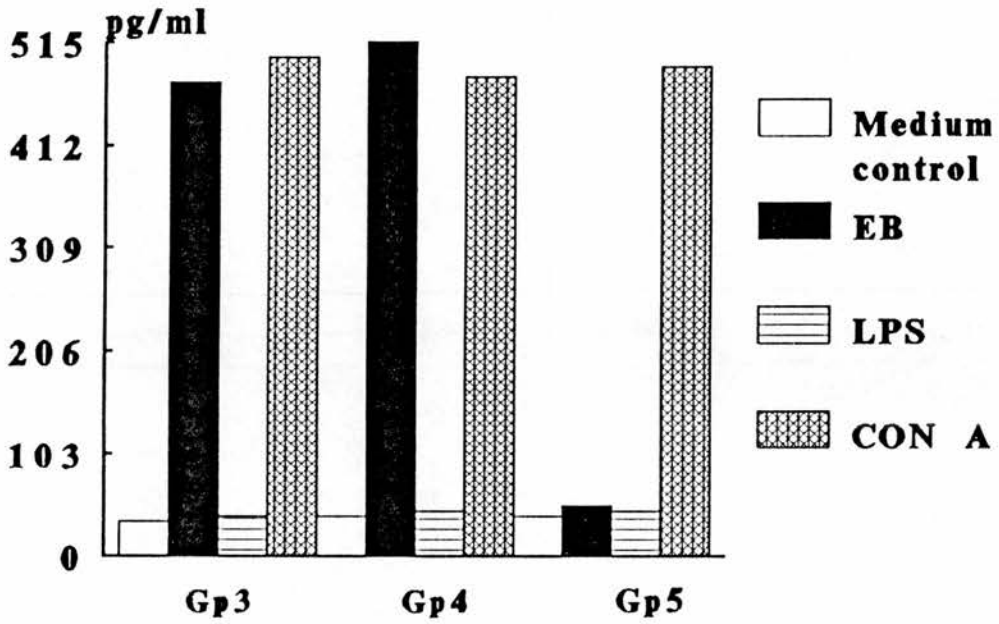
T-cell line production of γ-IFN after stimulation by EBs, LPS and Con A.

The level of production of γ-IFN was increased in all T-cell lines when compared to PBMC results as measured by the concentration present in the unstimulated controls. Unstimulated T-cells produced background concentrations of γ-IFN of between 40 and 45 pg/ml as compared with the negative results given by control PBMC (see above). No T-cell line produced γ-IFN in response to LPS. The T-cell line generated from the best responding animal in group 3 (see previous chapter) produced γ-IFN when stimulated by EBs and Con A (see table 6.3 and figure 6.2). EB stimulation resulted in concentrations of 475±45 pg/ml and Con A stimulated cells produced 500±42 pg/ml of γ-IFN as measured by ELISA, bioassay titres were 512U/ml. Group 4a/4b responses gave similar rises in OD as the PBMC in the group and were ten times greater than background. This gave a concentration of 515±60pg/ml and a titre of 512U/ml in response to EBs and Con A produced 480±55 pg/ml, but had a titre of 1024. Group 5 T-cell lines did not respond to EBs, but gave high readings in response to Con A similar to the other T-cell lines described above.

Table 6.3: Production of Y-IFN (pg/ml±se and U/ml) by ovine T-cell lines stimulated with EB (20°g/ml), LPS (20°g/ml) and Con A (5°g/ml).

	Response to					
	EB		LPS		Con A	
Gp	pg/ml	U/ml	pg	U/ml	pg	U/ml
3	475±45	512	-	-	500±42	512
4a/b	515±60	512	-	-	480±55	1024
5	-	-	-	-	490±40	512

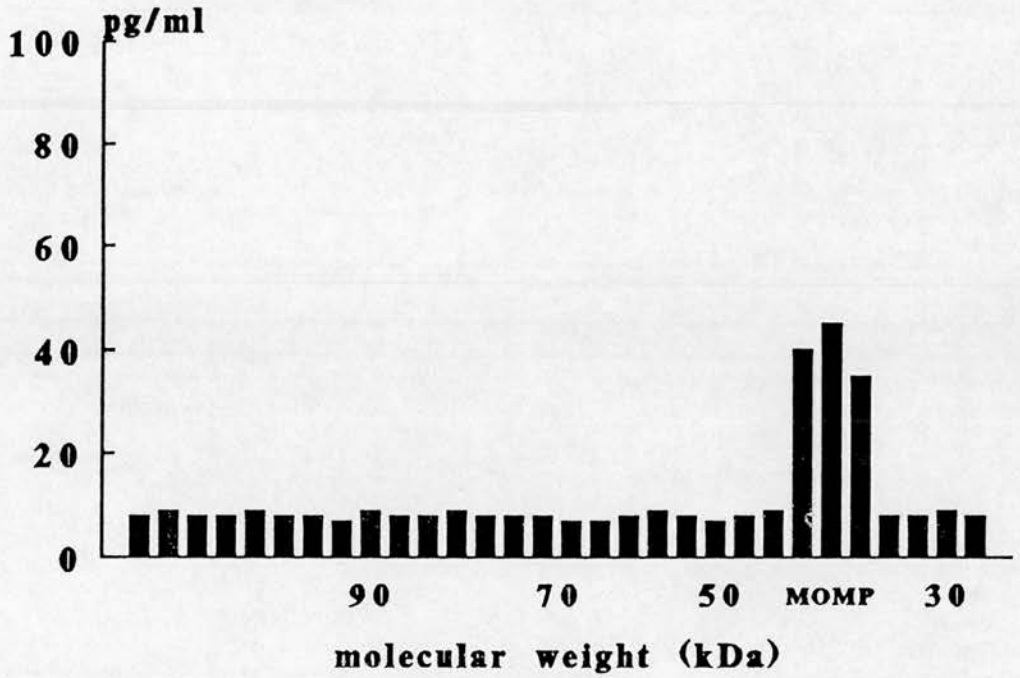
Figure 6.2: Production of γ-IFN by ovine CD4+ T-cell lines stimulated by 20μg/ml of EBs, 20μg/ml LPS and 2.5μg/ml of Con A.



PBMC and T-cell line response to biochemically fractionated chlamydial protein.

There was no response to any fractionated protein from either the PBMC or T-cell lines generated from groups 1, 2 or 5. In group 3 PBMC from the 2 animals with a low proliferative response only produced γ-IFN when stimulated by a protein of approximately 38Kd (see figure 6.3). The third animal gave a wider range of response to antigens of approximately 70, 50, 38 and 30Kd (see figure 6.4). T-cell lines from this animal produced more γ-IFN than the PBMC and were also stimulated by proteins of 90 and 60Kd (see figure 6.5). Group 4a/4b PBMC and T-cell lines produced γ-IFN in response to the same bands (see figure 6.6 and 6.7). All animals responded to proteins of 70, 50, 38 and 30Kd and some responded to bands of 90. 60 and 18Kd. Again concentrations of γ-IFN were greater in the T-cell lines.

Figure 6.3: Production of γ-IFN by the PBMC from the low responding sheep in group 3, stimulated by biochemically fractionated chlamydial proteins.



*Graphs represent ɣ -IFN production from single sheep representative of each group and the data discussed.

Figure 6.4: Production of γ-IFN in the PBMC from the high responding sheep in group 3, stimulated by biochemically fractionated chlamydial proteins.

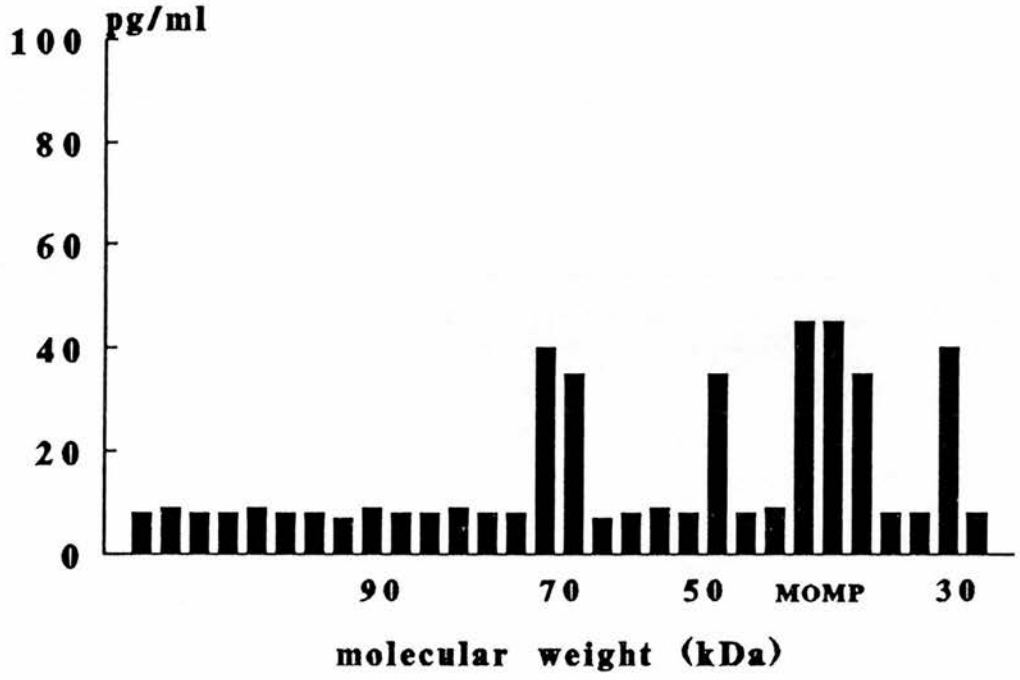


Figure 6.5: Production of γ-IFN by the CD4+ T-cell lines generated from the sheep in group 3, stimulated by biochemically fractionated chlamydial proteins.

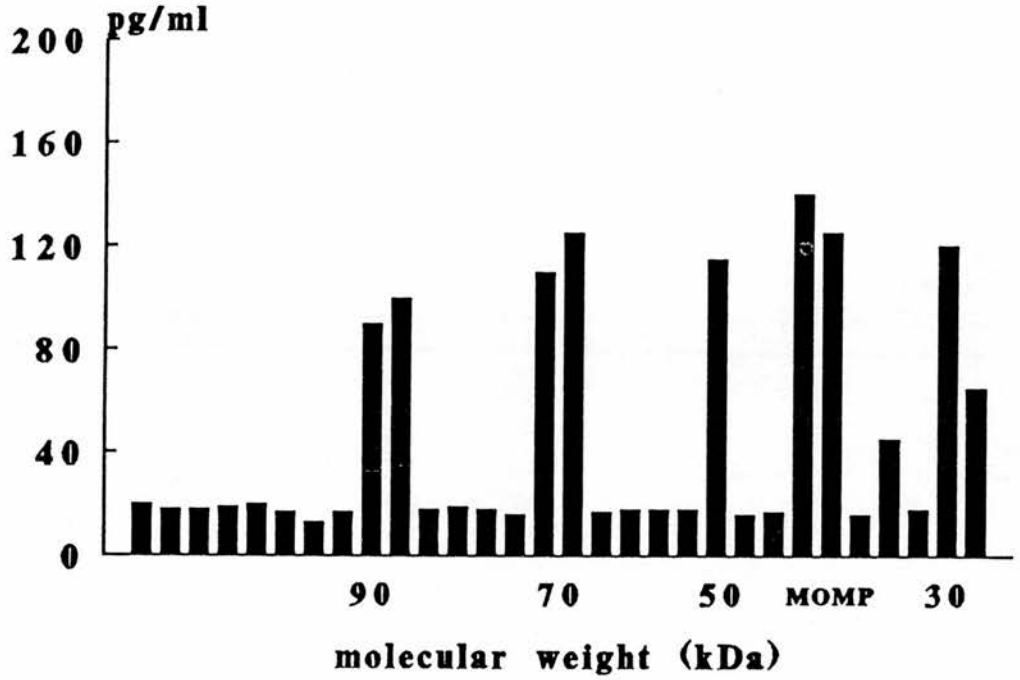


Figure 6.6: Production of γ-IFN by the PBMC from the sheep in group 4, stimulated by biochemically fractionated chlamydial proteins.

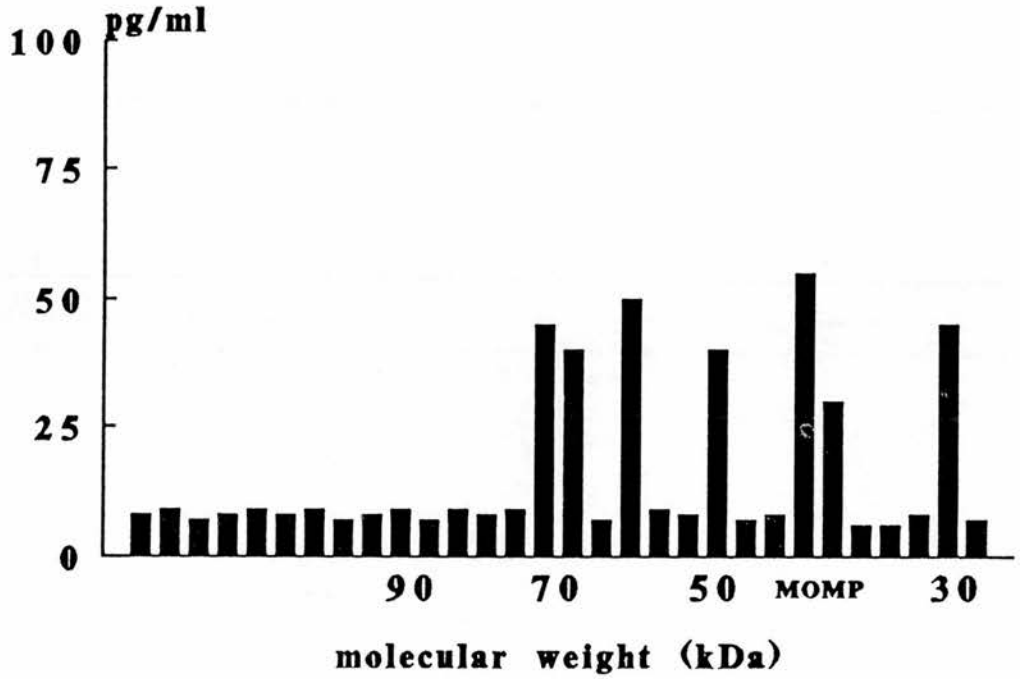
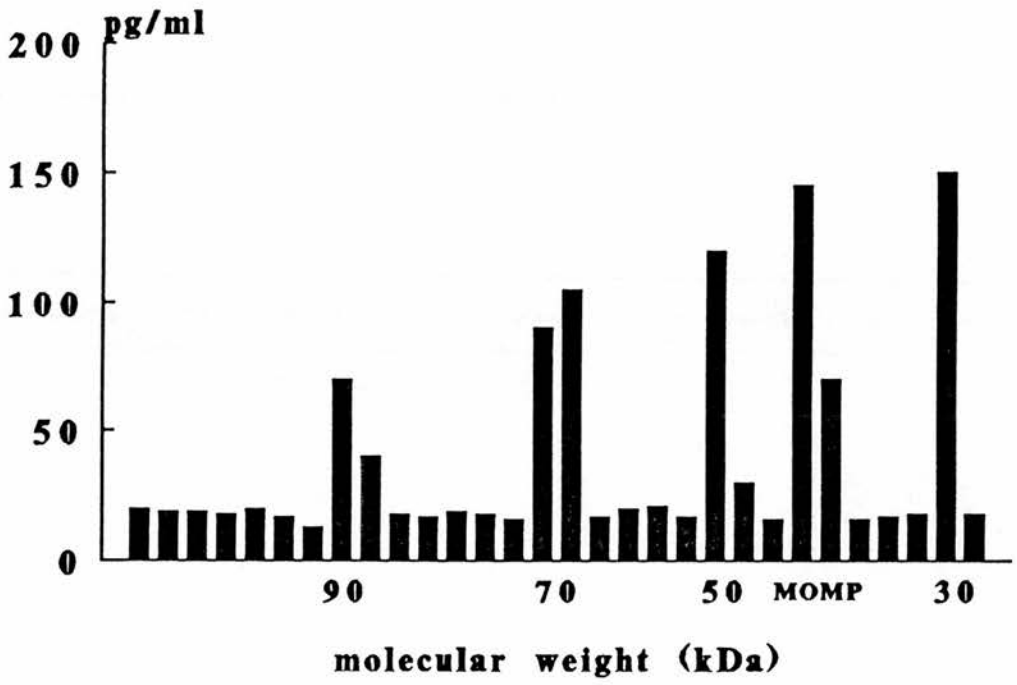


Figure 6.7: Production of γ-IFN by the CD4+ T-cell lines generated from the sheep in group 4, stimulated by biochemically fractionated chlamydial proteins.



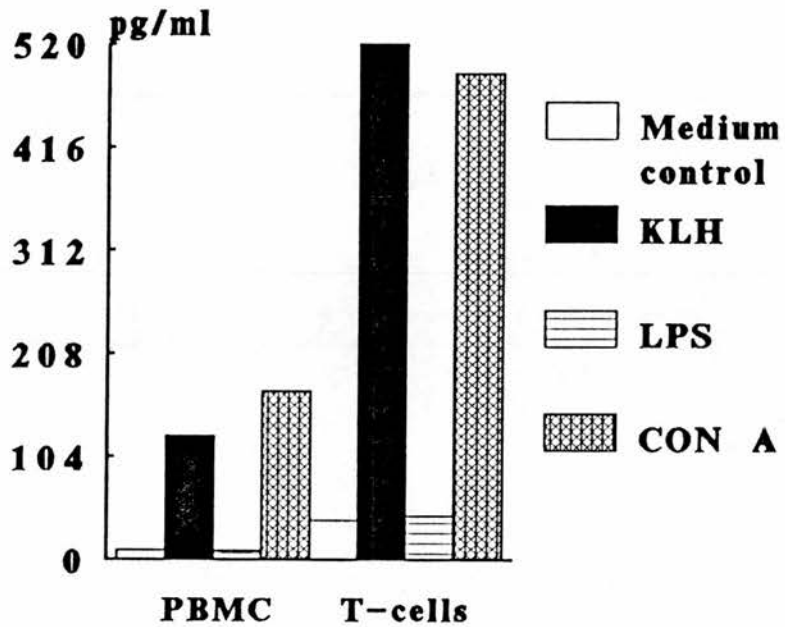
Group 5 PBMC and T-cell line γ-IFN production in response to stimulation by KLH.

The PBMC from the sheep in group 5 produced γ-IFN in response to stimulation by KLH (see table 6.4 and figure 6.8). Levels of γ-IFN detected were 125pg/ml by ELISA and bioassay titres of 256U/ml. T-cell lines generated from these PBMC again produced greater amounts of γ-IFN in their medium controls and cells stimulated with 20°g/ml of KLH produced 520pg/ml and titres of 512U/ml.

Table 6.4: The production of γ-IFN by PBMC and CD4+ T-cell lines from sheep in group 5 in response to 20°g/ml of KLH.

Group 5	pg/ml±se	U/ml
PBMC	125±21	256
T-cells	520±42	512

Figure 6.8: Production of Y-IFN by the PBMC and CD4+ T-cell lines generated from the sheep in group 5, stimulated by 20μg/ml of KLH, 20μg/ml of LPS and 2.5μg/ml of Con A.



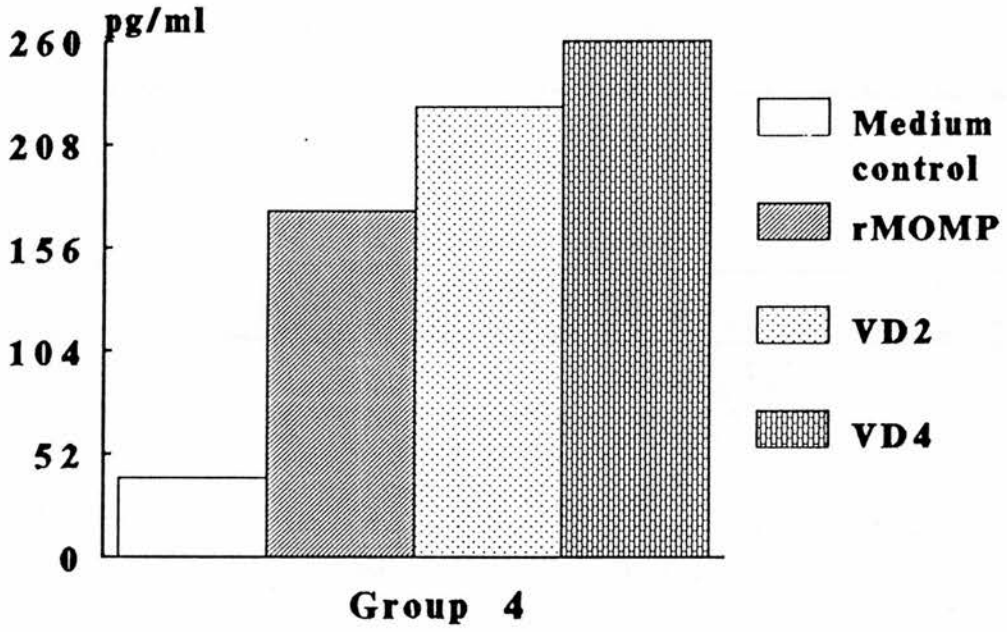
Group 4a/4b T-cell line production of γ-IFN in response to rMOMP and synthetic peptides.

Five T-cell lines from group 4a/4b were stimulated by rMOMP and two synthetic peptides described in the previous chapter. All produced γ-IFN (see table 6.5 and figure 6.9), cells stimulated with rMOMP produced 75pg/ml±8 by ELISA and gave bioassay titres of between 128 and 256U/ml. The cells stimulated by the synthetic peptides gave 227pg/ml±33 and titres of 512U/ml for VD2. Results for VD4 were 260pg/ml±48 and a titre of 256U/ml.

Table 6.5: The production of γ-IFN by T-cell lines, from sheep in group 4a/4b, to 2.5°g/ml rMOMP and 0.5°g/ml of the synthetic peptides VD2 and VD4.

	pg/ml±se	U/ml
rMOMP	175± 8	128-256
VD2	227±33	512
VD4	260±48	256

Figure 6.9: The production of γ-IFN by T-cell lines, from sheep in group 4a/4b, to 2.5µg/ml rMOMP and 0.5µg/ml of the synthetic peptides VD2 and VD4.



Discussion.

The two detection systems used in this series of experiments may not detect similar quantities of γ-IFN. The bioassay will only detect active molecules of γ-IFN which can protect the target cells from viral infection, while the monoclonal antibodies in the ELISA, as well as detecting active molecules may also detect any non-biologically active molecules of γ-IFN which may be present in the conditioned media. Therefore both assays were used to detect γ-IFN.

The major difference between the PBMC and T-cell line production of γ-IFN was the increased concentrations of γ-IFN detected in the conditioned medium taken from the unstimulated control cells of the T-cell lines. This result was not unexpected, since even though PBMC assays have more cells/well the preparations will contain many non-γ-IFN producing cells such as B-cells and monocytes, whereas the T-cell lines contain >90% CD4+ cells (see previous chapter), which are all capable of producing γ-IFN (Watanabe, Taguche, Iwata, Kawadi and Hanaoka, 1983). Increased production will also result from the increased number of cells which will be sensitive to *C.psittaci* and which will produce γ-IFN upon stimulation by EBs. Differences in background levels within groups also correlated with the different proliferative backgrounds in individual sheep seen in the previous chapter. High proliferative backgrounds resulting in high levels of γ-IFN and low proliferative backgrounds producing low levels.

There was very little difference between the responses of the group 4a/4b PBMC and T-cell lines to individual proteins other than the difference in the concentrations of γ-IFN produced, which has already been discussed. This confirms the finding of the previous chapter where similar results were reported in the proliferative response of the post abortion group 4 animals and would suggest that a strong cell mediated immunity existed in the PBMC of these animals. This may explain why animals seldom abort more than once (Stamp *et al*, 1950).

The detection of γ-IFN in the conditioned media of the PBMC and T-cell lines of infected animals stimulated by EBs correlated well with the proliferation reported in the previous chapter. However, animals which were uninfected in groups 1, 2 and 5, and which showed some proliferation in response to EBs did not produce increased detectable levels of γ-IFN and amounts present in EB stimulated cells from these groups were similar to quantities in unstimulated control cells. It is possible that the proliferation described for these groups is due to the stimulation of non-γ-IFN producing cells, such as B-cells. Another intriguing possibility is that the proliferation is due to T-cell subsets similar to those described for mice (Bottomly, 1988). Murine T-helper cells have been subdivided into 2 groups with Th1 cells producing γ-IFN and IL-2 while Th2 cells produce IL-4 (Mossman and Coffman, 1987). Obviously, if a similar differentiation occured in sheep then the proliferation recorded when no γ-IFN was detected could be due to the stimulation of Th2 cells. However, PBMC from the high

responding animal in group 3, while proliferating only in response to a protein of approximately 38Kd, also produced γ-IFN when stimulated by the three other immunodominant antigens with approximate weights of 70, 50 and 30Kd described in the previous chapter. The reason for cells producing γ-IFN when no proliferation is present is less clear, although it has been reported previously for T-cell lines and clones (Hecht, Longo and Matis, 1983). It is possible that high levels of proliferation of non-γ-IFN producing cells within the unstimulated controls may lower the stimulation index of proliferating PBMC to insignificant levels. This would leave the background concentrations of γ-IFN unchanged and the increased amount of γ-IFN produced by proliferating cells would be detected.

These findings would suggest that the production of γ-IFN by PBMC and T-cell lines in response to stimulation by EBs was a more specific test than the measurement of cellular proliferation, when examining the immune status of sheep to *C.psittaci*. This has already been shown to be the case in bovine tuberculosis, where the ELISA method used in this study has been used as a diagnostic test (Wood, La Corner, Rothel, Baldock, Jones, Cousins, McCormick, Francis, Creeper and Tweddle, 1991). It may be that the production of γ-IFN by stimulated PBMC may prove to be a useful diagnostic tool for other intracellular bacteria such as *C.psittaci*. However, a protective role for γ-IFN against ovine abortion strains of *C.psittaci* has yet to be demonstrated.

Chapter 7:

A MOUSE MODEL OF A CHLAMYDIAL INFECTION TO INVESTIGATE THE EFFECT OF ENDOGENOUS GAMMA INTERFERON ON THE RESOLUTION OF DISEASE.

Introduction.

In previous chapters the *in vitro* cell mediated responses of sheep to *C.psittaci* have been investigated and certain antigenic proteins have been identified which stimulate both the proliferation of T-cells and the production of γ-IFN by those same cells. Recently, a large body of evidence has been gained from both *in vitro* and *in vivo* experiments which shows the importance of γ-IFN in the immune response to many intracellular pathogens such as *Trypanosoma cruzi, Toxoplasma gondii, Listeria monocytogenes , Rickettsia conorii* and Vaccinia virus (Borges and Johnson, 1975; Nakane, Numata, Asano, Kohanawa, Chen and Minagawa, 1990; Manor and Sarov, 1990; Dunn and North, 1991; McCabe, Meagher and Mullins, 1990; Karupiah, Blanden and Ramshaw, 1990).

In chlamydial immunity it was reported as early as 1982 that lymphokines restricted the growth of *C.psittaci* in murine macrophages *in vitro* (Byrne and Faubion, 1982) and that the inhibition of growth could be suppressed by adding anti γ-IFN antibody to the culture (Byrne and Kreugar, 1983; Rothermel *et al*, 1983). The ability of γ-IFN to activate the microbicidal activity of host macrophages (Nathan *et al*, 1983) as well as cause suicide-like cell destruction of bacteria-infected cells triggered by bacterial LPS (Byrne *et al*,1988; Dijkmans, Decock, Heremans, Van Damme and Billiau, 1989; Dijkmans, Van Damme, Cornette, Heremans and Billiau, 1990) could well be important in in resistance to *C.psittaci*.

In order to determine the *in vivo* effects of endogenous γ-IFN on the resolution of a live infection of the S26/3 ovine abortion isolate of *C.psittaci* a mouse model of the early stages of infection was constructed. Normal mice and athymic nude mice were employed, together with the *in vivo* use of a monoclonal antibody which neutralised γ-IFN, with the intention of altering the pathogenesis and the severity of the infection. As activated T-cells (Watanabe *et al*, 1983) and natural killer (NK) cells (Welsh, 1984) produce γ-IFN, athymic mice were used to determine the effect of removing T-cells and their products and it was hoped that the antibody treated groups would highlight the differences due to the removal of γ-IFN.

Experimental procedure.

Athymic nude mice (nu/nu) on a MF1 background and their hairy litter mates (nu/+) (Harlan Olac Ltd, Oxford, England) were divided into 9 groups (see table 7.1). Group A contained 10 nu/+ mice infected intra-peritoneally (i.p.) with 10^6 IFU of *C.psittaci* S26/3, grown in egg yolk sacs. Group B also contained 10 nu/+ mice infected with a similar dose of *C.psittaci* but also treated with a murine γ-IFN neutralising monoclonal antibody. The antibody, a rat/mouse hybrid, was a gift from Dr A.Mowatt, Glasgow University. Mice in group B were injected i.p. on day -1, 0, 1 and 3, with 200μl of PBS containing enough monoclonal antibody to neutralise 10^5 units of murine γ-IFN, as measured in a bioassay. Group C and D were each comprised of 12 nu/nu mice and were treated in the same way as groups A and B respectively.

Control groups E and F contained 6 nu/nu and 6 nu/+ mice respectively and were not infected with *C.psittaci*, but were treated with the monoclonal antibody in the same manner as groups B and D. The 6 nu/nu mice in group G were given a control inoculum of 450µl of uninfected egg yolk sac on day 0 and 200µl of a control antibody of rat IgG on day -1, 0, 1 and 3. Groups H and I, containing 6 nu/nu and 4 nu/+ mice respectively were given a control inoculum of 450µl of uninfected egg yolk sac on day 0.

Within the groups, half the mice were killed on day 3 and the other half were killed on day 5 in a chamber with CO_2 gas. After killing, animals were weighed and blood was taken in order that sera could be analysed for γ-IFN activity. The mice were examined macroscopically for visible lesions. Using aseptic precautions, the spleen from each animal was removed and weighed before samples of spleen, liver and lung were taken for attempted isolation of viable *C.psittaci* and for histopathological studies. The latter were immediately fixed in 10 per cent formal saline while samples for isolation were frozen at -70^{o}C in chlamydial transport medium until required.

Tissue sections for histopathology were stained with haemotoxylin and eosin (HE) while serial sections were stained with an immunoperoxidase method to demonstrate chlamydial antigen. Selected tissue sections were treated with an *in situ* hybridisation method for the demonstration of chlamydial RNA.

Table 7.1: The group sizes and treatments used to evaluate the role of endogeneous gamma interferon in the resolution of a live chlamydial infection in mice.

Group	(n)	Type	Treatment	Day killed
A	5	Nu/+[b]	*C.psittaci* only[c]	3
	5			5
B	5	Nu/+	*C.psittaci*+Antibody[d]	3
	5			5
C	6	Nu/Nu[a]	*C.psittaci* only	3
	6			5
D	6	Nu/Nu	*C.psittaci*+Antibody	3
	6			5
E	3	Nu/Nu	Antibody only	3
	3			5
F	3	Nu/+	Antibody only	3
	3			5
G	3	Nu/Nu	Yolk Sac[e]+ Rat IgG[f]	3
	3			5
H	3	Nu/Nu	Uninfected Yolk Sac	3
	3			5
I	2	Nu/+	Uninfected Yolk Sac	3
	2			5

[a] athymic mice on a MF1 background [b] thymic MF1 littermates
[c] mice given 10^6 IFU of *C.psittaci* i.p.
[d] mice given murine Y-IFN neutralising monoclonal antibody
[e] mice given uninfected egg yolk sac as a control innoculum
[f] mice given rat IgG as a control antibody

Results.

The *C.psittaci* infection in mice produced several histological changes in the tissues examined. In the spleen, there was an expansion of the periarteriolar lymphoid sheaths (PALS) associated with an increase in overall spleen weight (Fig 1). Phagocytic

vacuoles were also present in the red pulp and to a small extent the white pulp and there was an increased presence of polymorphonuclear cells (PMN). With the liver macroscopic lesions were found on the surface in some mice. These appeared as small white foci or streaks of necrosis, usually 1-2mm across. They had slightly depressed surfaces and were often bordered by a thin red haemorrhagic zone. Microscopically the hepatocytes and the sinusoidal lining cells within these foci had undergone coagulative necrosis and at the clearly demarcated interphase with adjacent normal tissue there was often a clear zone of haemorrhage within the lesion (Fig 2). There was virtually no inflammatory cell infiltration within these foci nor was any significant amount of antigen demonstrable (Fig 3).

A more consistent microscopic finding in the liver was multiple, small foci of inflammation. The likely development of the lesion was as follows. The foci commenced in the sinusoids (Fig 4). Initially Kupffer cells appeared prominent and antigen was seen in some (Fig 5), as the reticulo-endothelial system was stimulated. The sinusoids became congested both with aggregates of these cells as they proliferated, and by blood borne inflammatory cells. By day 5 the foci were larger and were composed mainly of PMN in the centre of the focus while the periphery was mainly composed of

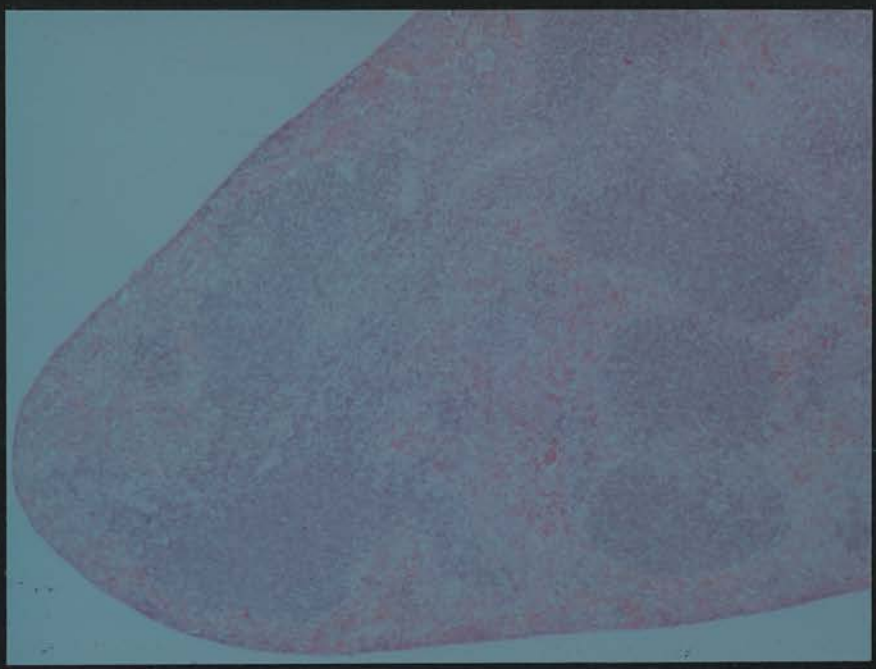
Figure 1A (upper): A section of spleen from an uninfected athymic mouse from group G, on day 3

(HEx16)

Figure 1B (lower): A section of spleen from an infected athymic mouse from group C, on day 3

(HEx16)

Note the clear increase in size of the infected spleen in the lower photograph and also the marked expansion of the periarteriolar lymphoid sheaths.



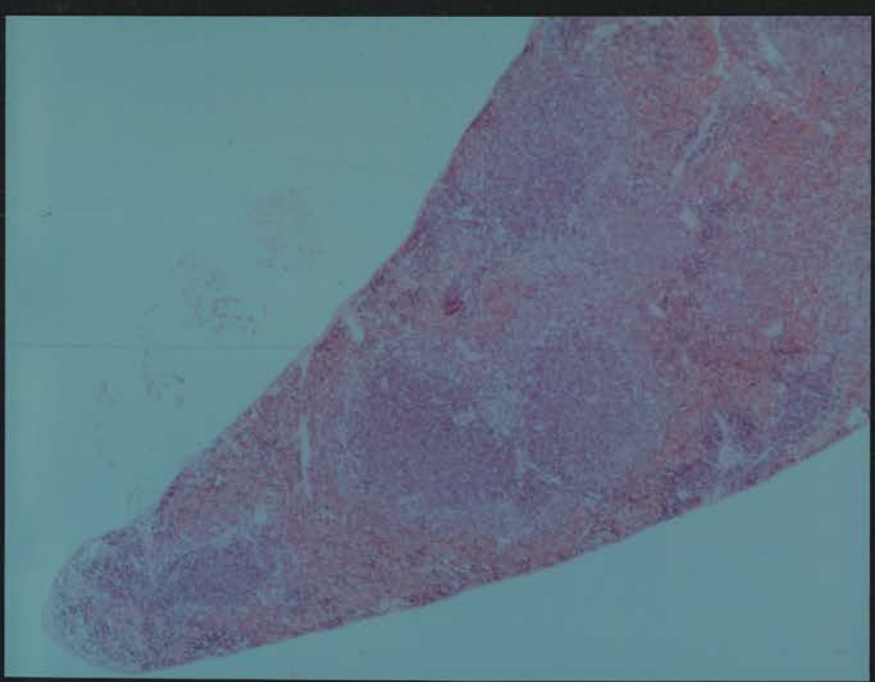
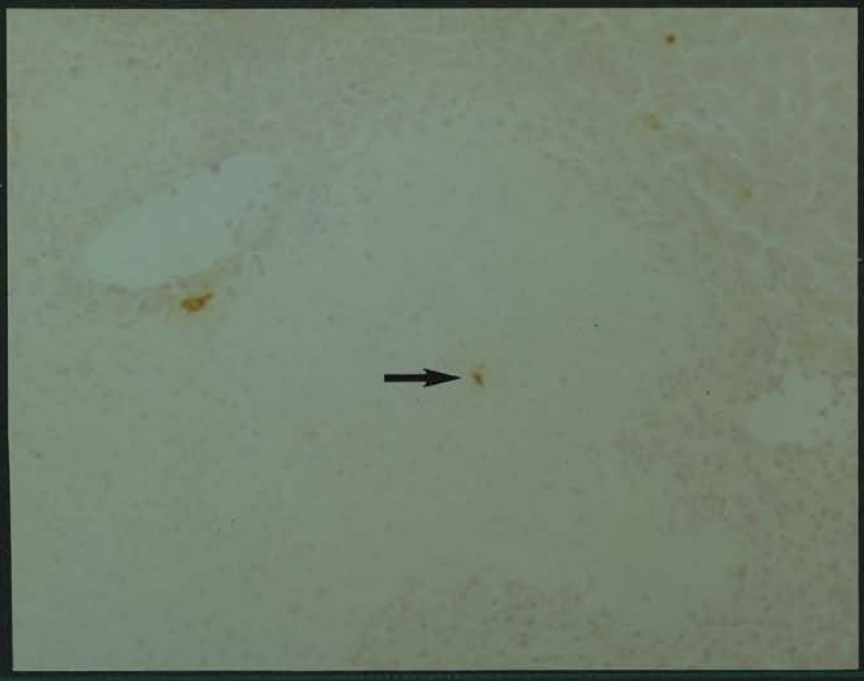


Figure 2: A section of liver from an infected, athymic mouse from group C, on day 3. The photograph depicts a large area of necrosis (N) with a zone of haemorrhage around its periphery (H). There is also a lack of inflammatory cell infiltration within the necrotic area.

(HEx40)

Figure 3: A section of liver from an infected athymic mouse from group D, on day 3 treated with an immunoperoxidase technique to detect the presence of chlamydial antigen. Very little antigen (arrow) can be detected within the necrotic area

(IPx40)



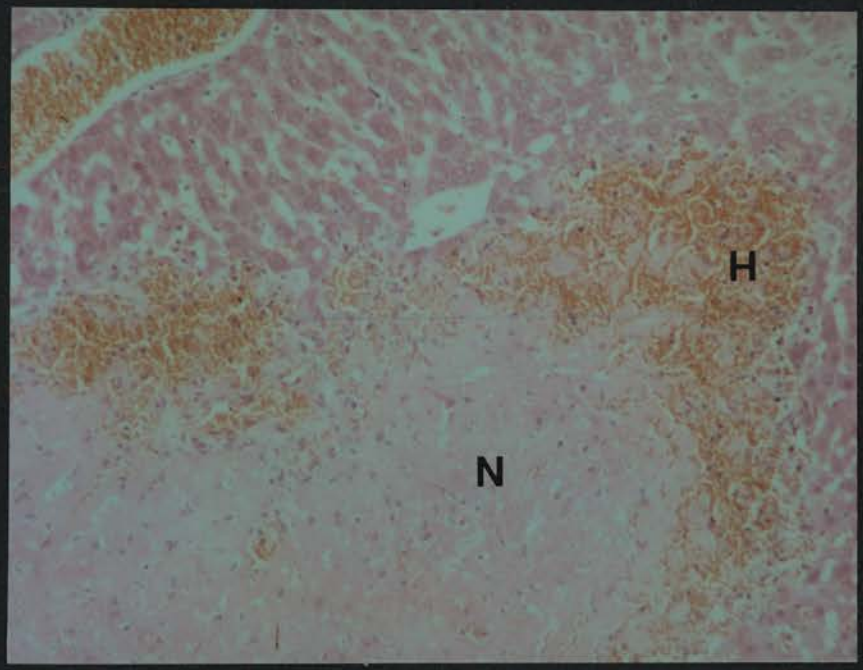
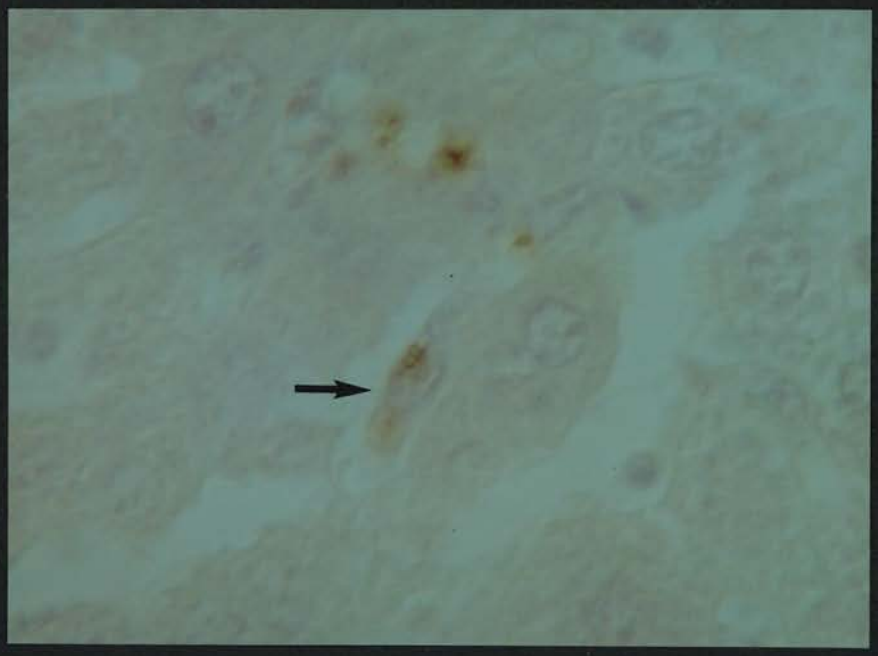

H
N

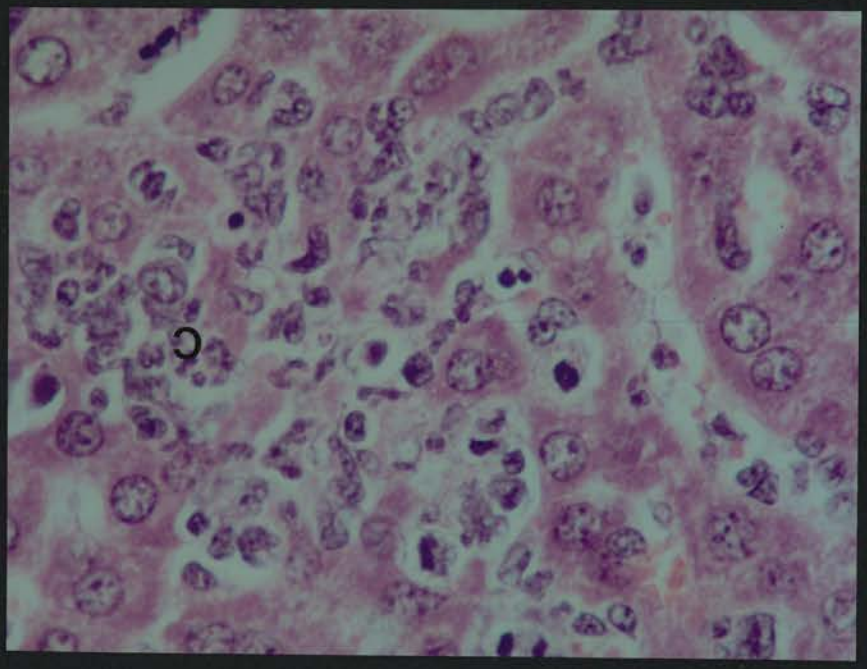
Figure 4: A section of liver from an infected thymic mouse from group B, on day 5. The photograph shows a marked inflammatory cell infiltration (C) within the sinusoids, adjacent to many normal hepatocytes.

(HEx160)

Figure 5: A section of liver from an infected athymic mouse from group C, on day 3 treated with an immunoperoxidase technique to detect the presence of chlamydial antigen. The Kupffer cell (arrow) lining the sinusoid has chlamydial antigen detected in the cytoplasm.

(IPx250)





reticulo-endothelial cells. Surrounding hepatocytes did not appear to be a primary target as only a very few displayed irreversible degenerative changes (Fig 6). These foci of inflammation should not be confused with the focal necrosis described above in which large numbers of hepatocytes become necrotic. Chlamydial antigen was readily demonstrated in many of these foci by the immunoperoxidase method, as was chlamydial RNA by an *in situ* hybridisation method. Chlamydiae were seen to be both extracellular and within inflammatory cells. A cellular thickening of the alveolar septa in the lungs of infected mice was also noted (Fig 7), and in some cases there appeared to be an increase in the number of alveolar macrophages.

The five control groups (E-I) showed no significant abnormalities either macroscopically or on histopathological examination. No chlamydial antigen was detected following staining with the immunoperoxidase method. No chlamydia were isolated from any tissue in these control groups and spleen size remained constant. Therefore, as an aid to clarity the control groups have been omitted from all results tables except for the table of spleen weights.

Spleen.

I) Weights.

Spleen weights are given both as actual weight in grams and also as per cent body weight in table 7.2. There was a significant increase in groups B and D (antibody treated and infected) on day 3 when compared with groups A and C and the control groups E-I

Figure 6: A section of liver from an infected athymic mouse from group C, on day 5. The photograph shows a small focus of inflammation surrounded by normal hepatocytes.

(HEx160)

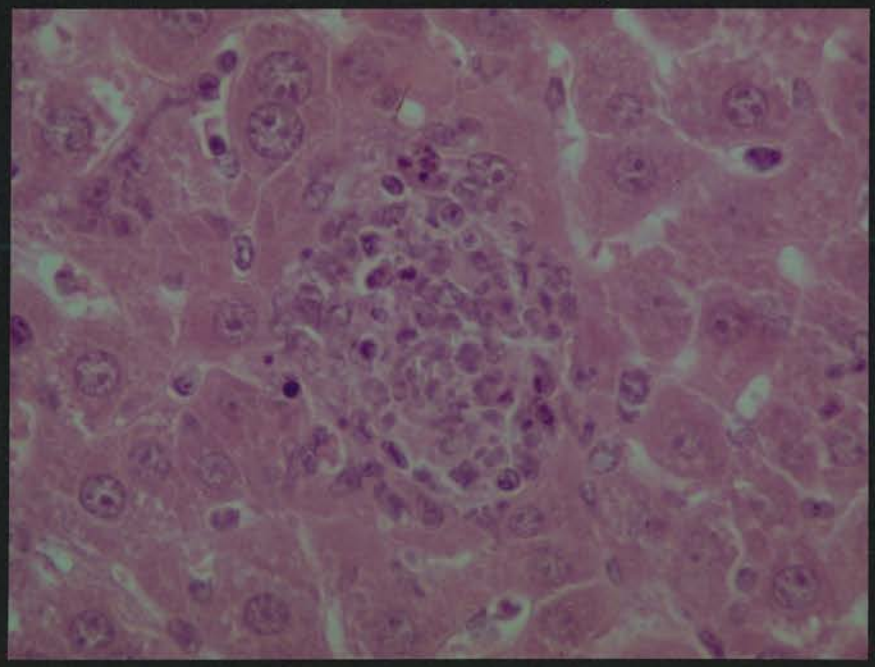


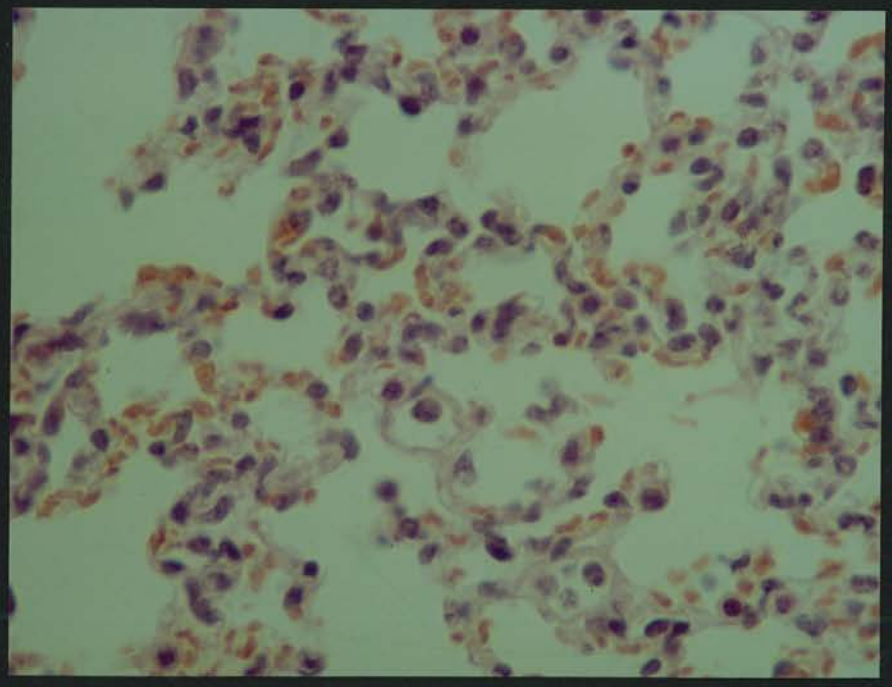
Figure 7A (upper): A section of lung from an uninfected athymic mouse from group G, on day 5.

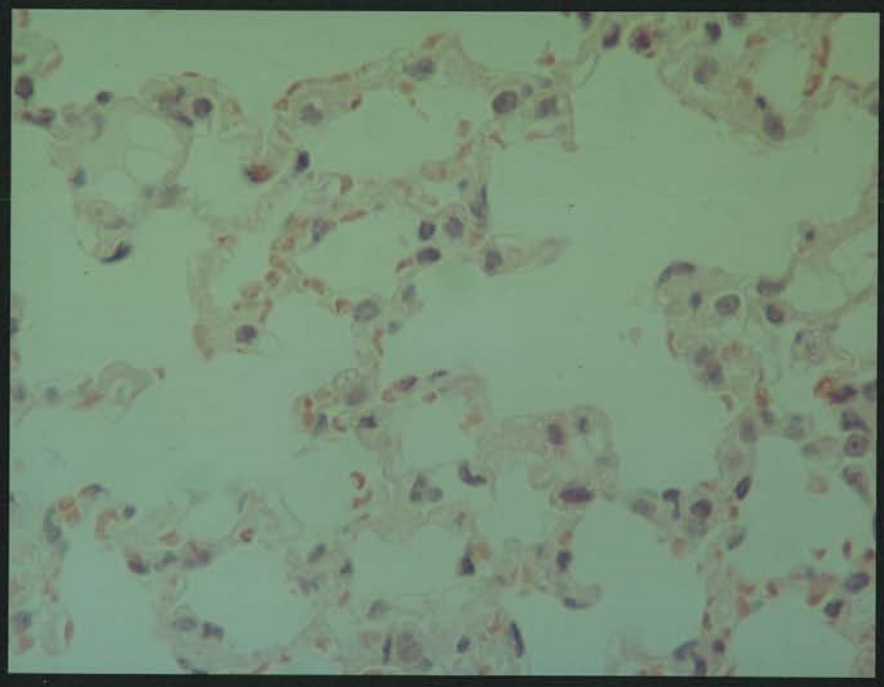
(HEx160)

Figure 7B (lower): A section of lung from an infected athymic mouse from group D, on day 5.

(HEx160)

Note the clear increase in the number of cells in the alveolar septa in photograph 7B.





($p<0.002$ for actual weight and $p<0.01$ for percent body weight). On day 5, groups A to D had all increased in size significantly ($p<0.02$ for both actual weight and percent body weight), whereas the control groups had remained the same ($p>0.05$). In addition, by day 5 the weights of the spleens in groups A to D were significantly greater than the control groups ($p<0.01$ and $p<0.008$, respectively) and those in group B were significantly greater than those in other infected groups ($p<0.01$ and $p<0.003$).

Table 7.2: Mean body weight (g±se), mean weights of spleens (g±se) and the mean weights of spleens expressed as a percentage of the mean body weight of the mice from a given experimental group.

Group	Day killed	Body weight	Weight spleen	% Weight
A	3	29.52±1.36	0.120±0.015	0.41±0.04
	5	31.29±2.81	0.276±0.050[ac]	0.85±0.09[ac]
B	3	30.87±0.60	0.190±0.010[b]	0.62±0.03[b]
	5	28.65±0.80	0.308±0.008[ac]	1.01±0.01[ac]
C	3	22.53±1.35	0.107±0.010	0.46±0.02
	5	24.35±1.20	0.167±0.020[ac]	0.68±0.06[ac]
D	3	25.37±0.80	0.180±0.007[b]	0.71±0.03[b]
	5	23.79±1.60	0.210±0.020[ac]	0.88±0.06[ac]
E	3	26.29±0.80	0.107±0.007	0.40±0.03
	5	25.94±0.70	0.110±0.015	0.43±0.06
F	3	35.34±1.03	0.090±0.005	0.25±0.02
	5	36.16±0.70	0.080±0.000	0.22±0.01
G	3	28.31±1.30	0.090±0.010	0.34±0.03
	5	29.89±2.10	0.087±0.007	0.32±0.02
H	3	26.44±1.06	0.087±0.007	0.30±0.03
	5	26.50±0.50	0.110±0.019	0.38±0.09
I	3	35.56±1.60	0.115±0.005	0.32±0.01
	5	37.94±0.10	0.105±0.005	0.28±0.01

[a] $P<0.05$ when compared with day 3 results of same group

[b] $P<0.05$ when compared with control day 3 results

[c] $P<0.05$ when compared with control day 5 results

II) Histopathology.

a) Prominence of Periarteriolar Lymphoid Sheaths (PALS).

In all infected groups PALS were more prominent at days 3 and 5, than in control groups. There was no recognisable difference within any single group between day 3 and 5, however, in both groups B and D the PALS did appear to be more prominent than the PALS of groups A and C on both day 3 and day 5.

b) Vacuoles and cytolytic debris.

By day 3, in groups A and C (animals which were not treated with antibody), vacuoles containing pyknotic cells and cytolytic debris (Fig 8), were frequently scattered throughout the red pulp and to a much lesser extent, the white pulp of the spleen, but by day 5, they were very few in number. In group D, similar numbers of vacuoles were present on both day 3 and 5, while in group B they were relatively uncommon on both days.

c) Polymorphonuclear cells (PMN).

The degree of PMN infiltration was less on day 5 than on day 3 in groups A and B, although in mice in group B, larger numbers of PMN were observed on both day 3 and 5. In groups C and D, the degree of PMN infiltration was greater on day 5 than day 3, although antibody treated mice did have larger numbers on both days.

d) Antigen.

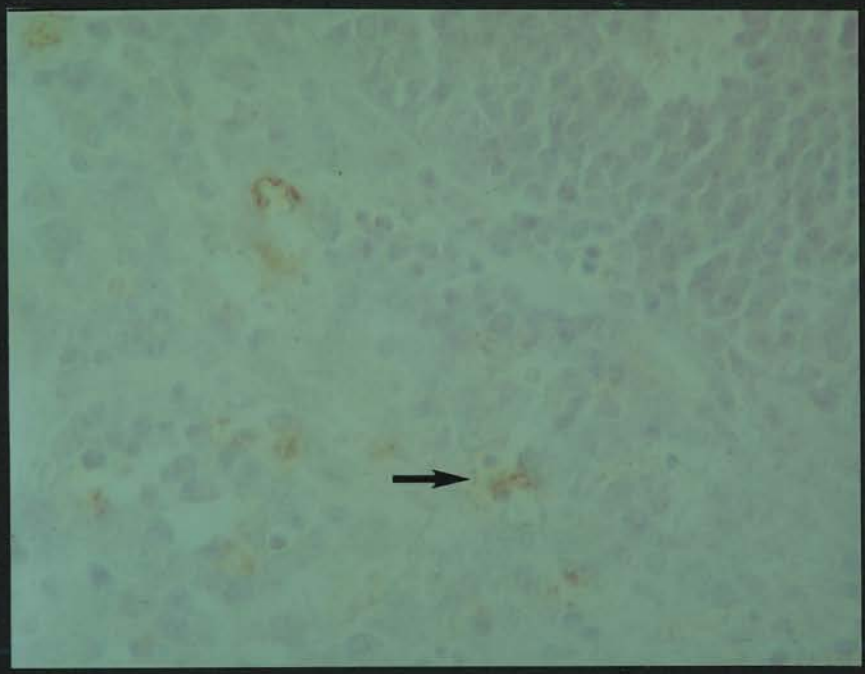
Antigen was detected in all groups of infected mice, on both days. It was mainly associated with the vacuoles (FIG 9) described

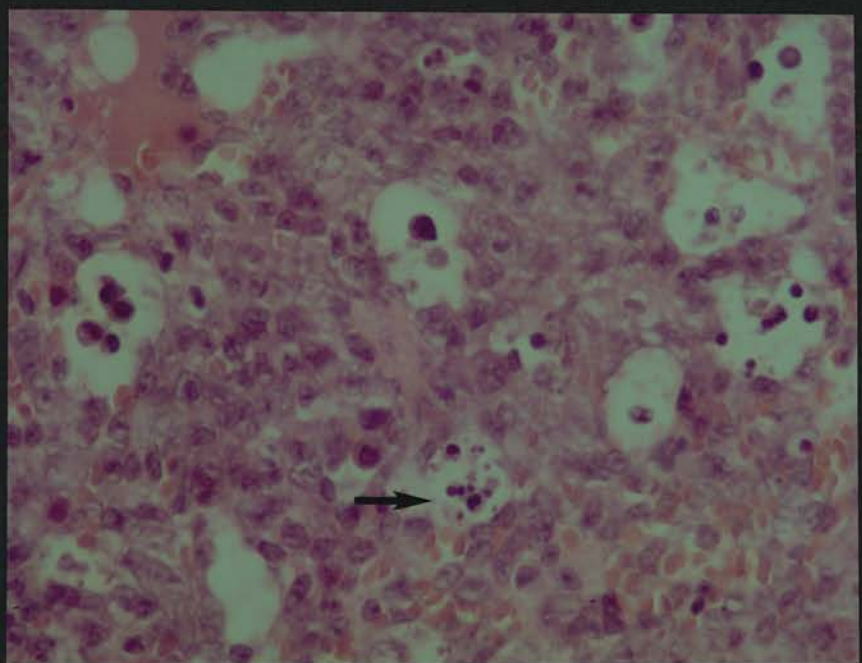
Figure 8: A section of spleen from an infected athymic mouse from group C, on day 3. Within the tissue there are vacuoles (arrow) which contain cytolytic debris and pyknotic cells.

(HEx160)

Figure 9: A section of spleen from an infected athymic mouse from group C, on day 3 treated with an immunoperoxidase technique. The photograph demonstrates the presence of antigen (arrow) within the vacuoles described above.

(IPx160)





above, although not exclusively so, since there was also a lesser amount of antigen detected in the red pulp in the form of EB inclusions. In groups A and B, the amount of antigen detected was similar on day 3 and 5, whereas in the athymic groups C and D the amount of antigen detected in the spleen was greater than that detected in groups A and B. There was also more antigen detected on day 5 when compared with day 3 in groups C and D. More inclusions could also be found in the red pulp of the spleens from groups C and D.

III) Isolation.

The number of viable *C.psittaci* per gram of tissue, was lower in group A than in B, C or D at day 3 (significantly lower than the two antibody treated groups, B and D. $p=<0.01$). On day 5 the numbers of viable chlamydiae isolated in group A were significantly less than on day 3 ($p=<0.002$) and were also significantly less than for any other group at day 5 ($p=<0.03$). In group C the numbers of chlamydiae isolated at day 3, although higher than group A were not significantly so ($p=>0.05$), and there was little change by day 5 ($p=>0.05$). Mice in group D had more viable chlamydiae in their spleens than any other group at day 3 ($p=<0.001$) and day 5 ($p=<0.0005$) and also had significantly more on day 5 than on day 3 ($p=<0.002$).

Table 7.3: Summary of the findings in the spleens of the infected groups A to D.

Group	Day	Prominance of PALS	Vacuoles	PMN	Antigen	Isolation (IFU x 10^5)
A	3	+	++	±	+	5.6±0.8
	5	+	-	±	±	0.1±0.1[a]
B	3	+(+)	+	++	+	9.0±0.6
	5	+(+)	+	±	+	13.0±4.0[a]
C	3	+	++	±	+	8.3±1.1
	5	+	±	+	++	13.0±1.1[a]
D	3	+	++	+	++	19.0±1.6
	5	+(+)	++	++	+++	46.0±2.5[a]

[a] $P<0.05$ when compared with day 3 isolation results of same group

Conclusions.

* *C.psittaci* infection caused an increase in spleen weight and made the PALS more prominent.
* Anti-γ-IFN monoclonal antibody treatment further accentuated this.
* The latter treatment also correlated with a greater detection of viable chlamydiae in the spleen.

* Athymic mice harboured greater numbers of viable chlamydiae than mice with thymuses.
* More antigen was detected in the tissue sections of spleens from athymic mice than from similarly treated mice with thymuses.

Liver.

I) Necrosis.

In group A, at necropsy macroscopic lesions could be seen on the surface of the livers of 2 of 5 mice on day 3, however by day 5, there were no visible signs of necrosis in this group (see table 7.4). In group B, on day 3, 2 of 5 mice had visible hepatic necrosis, and by day 5 all 5 mice were similarly affected. In group C, lesions of necrosis were seen in all 6 mice on day 3 and 2 of 6 on day 5 while in group D it was only seen in 2 animals on day 3, but in all mice on day 5.

Table 7.4: Summary of the presence of macroscopic lesions detected on the liver tissue of the infected mice on day 3 and day 5.

Group	day 3	day 5
A	2/5	0/5
B	2/5	5/5
C	6/6	2/6
D	2/6	6/6

II) Histopathology.

a) Foci of inflammation: frequency and size.

There were no significant differences in the frequency of inflammatory foci in any group A to D on day 3 ($P>0.05$) (see table 7.5) In all groups, however, the frequency was significantly higher on day 5 than on day 3 ($P<0.001$). Also on day 5, there were significantly more foci of inflammation in groups B and D when compared with the day 5 results of groups A and C ($P<0.03$). On day 3, groups A and C had significantly fewer inflammatory cells in each focus when compared with groups B and D ($P<0.05$). Again, in all groups the number of cells in each focus was significantly higher on day 5 than on day 3 ($P<0.001$). On day 5, groups B and D had significantly more cells in each focus than any other group ($P<0.04$).

Table 7.5: Analysis of the inflammation in the livers of mice infected with *C.psittaci*: Frequency and size of inflammatory foci.

Group	Day	Inflammatory foci/ per field	Mean number of cells/ per focus
A	3	0.64 ± 0.16	40.24 ± 5.8
	5	1.84 ± 0.2[a]	112.28 ± 19[a]
B	3	1. ± 0.17	57.8 ± 6
	5	4.24 ± 0.35[ab]	129.5 ± 17[a]
C	3	1. ± 0.16	41.5 ± 4.4
	5	1.43 ± 0.14[a]	88.2 ± 11.9[a]
D	3	1.23 ± 0.19	55.8 ± 5.8
	5	2.70 ± 0.3[a]	206.0 ± 28[ab]

[a] $P<0.05$ when compared with day 3 results in same group

[b] $P<0.05$ when compared with day 5 results of all groups

b) Antigen.

A small amount of antigen was detected in the livers of mice in groups A, B, C and D sampled at day 3 (see table 7.6). On day 5, significantly more antigen was detected in all groups ($P<0.04$), with the largest difference in chlamydiae detected being found in group B. The antigen was generally intracytoplasmic and within the foci of inflammation (Fig 10), particularly those with large infiltrates of PMN. Mice given monoclonal antibody (groups B and D)

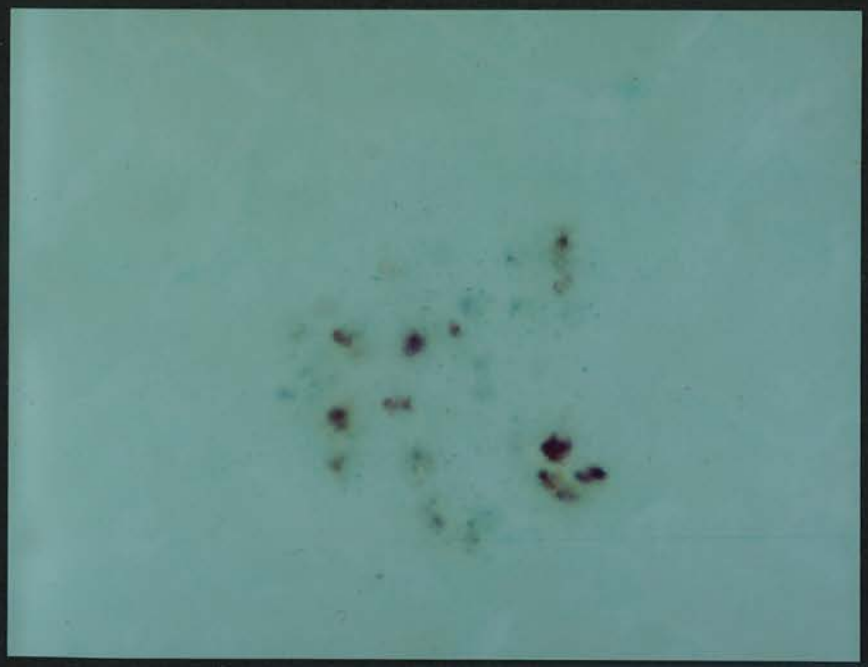
Figure 10A (upper): A section of liver from an infected athymic mouse from group C, on day 5 treated with an immunoperoxidase technique to detect the presence of chlamydial antigen.

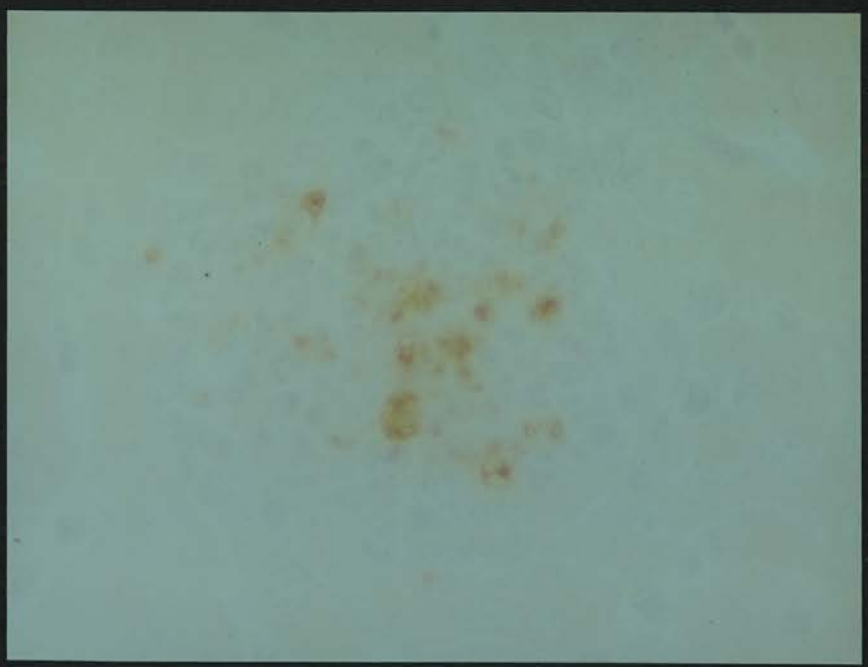
(IPx160)

Figure 10B (lower): A section of liver from an infected athymic mouse from group C, on day 5 treated with an *in situ* hybridisation technique employing chlamydial RNA.

(ISHx160)

In the upper photograph a brown staining reaction product demonstrates the presence of chlamydial antigen within the cytoplasm of inflammatory cells. In the lower photograph the presence of chlamydial RNA is detected within the cytoplasm of cells in an inflammatory focus.





had significantly greater amounts of antigen detected on day 5 when compared with groups A and C ($P<0.04$), as well as containing more chlamydial inclusions (Fig 11), which were not associated with the inflammatory response, than groups A and C, on both days.

Table 7.6: Summary of the presence of chlamydial antigen detected in the inflammatory foci of the liver tissue from infected mice on day 3 and day 5.

	% cells containing antigen	
Group	day 3	day 5
A	3.6±1.0	10.8±2.9[a]
B	7.4±2.1	23.0±2.9[a]
C	4.0±1.3	15.0±4.3[a]
D	8.0±1.6	32.6±6.1[ab]

[a] $P<0.05$ when compared with day3 results

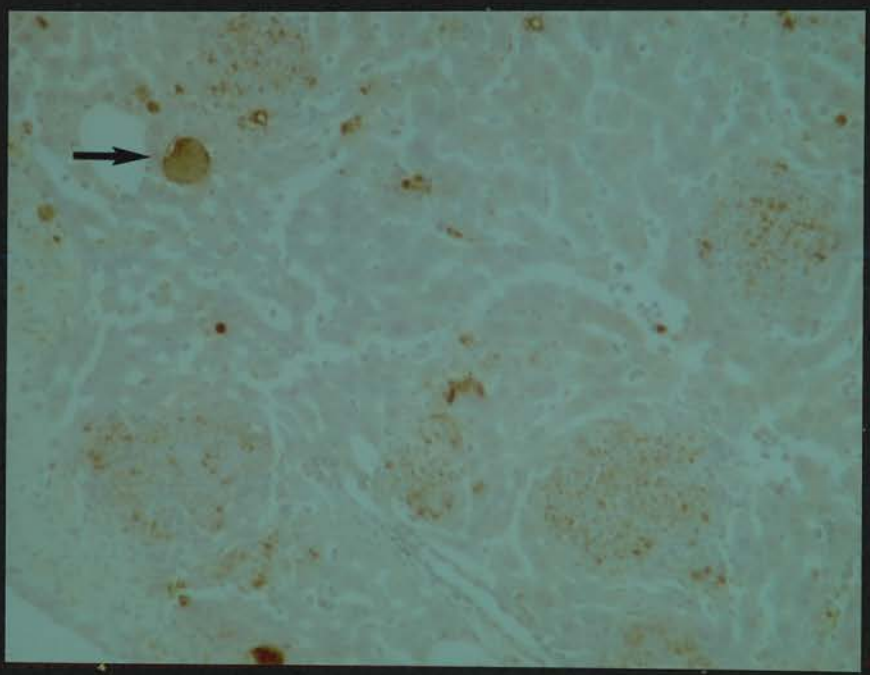
[b] $P<0.05$ when compared with day5 results

III) Isolation.

More viable *C.psittaci* were isolated per gram of hepatic tissue from group C and D, on day 3 and 5, than from mice in group A or B (day 3 $P=<0.005$, day 5 $P=<0.002$) (see table 7.7). At day 3, while athymic mice in group D contained significantly more organisms ($P=<0.009$) than thymic mice in group B, there was no difference between group B and the untreated athymic group C ($P=>0.05$). However, on day 5, significantly more viable chlamydiae

Figure 11: A section of liver from an infected athymic mouse from group D, on day 5 treated with an immunoperoxidase tecnique to detect the presence of chlamydial antigen. The photograph shows large areas of brown staining chlamydial antigen as well as chlamydial inclusions (arrow) common in animals treated with monoclonal antibody.

(IPx40)



were isolated from mice in group B than from mice in group C (P=<0.01). Groups B and D both showed a significant increase between day 3 and day 5 (P=<0.02) while in both groups A and C a significant decrease was detected in the number of viable organisms in hepatic tissue (P=<0.0007).

Table 7.7: Summary of the findings in the livers of groups A to D.

Group	Day	Focal Necrosis	Inflammation Frequency	Inflammation Size	% cells containing Ag	Isolation (IFUx10^3)
A	3	1/5	+	+	+	3.3±0.2
	5	1/5	++	++	++	0.2±0.3[a]
B	3	2/5	+	+	+	7.4±0.2
	5	4/5	+++	+++	+++	32.0±6.0[a]
C	3	6/6	+	+	+	7.3±0.5
	5	2/6	++	++	++	3.7±0.3[a]
D	3	1/6	+	+	+	16.0±2.0
	5	5/6	+++	+++	+++	51.0±8.5[a]

[a] P<0.05 when compared with day 3 results in same group

Conclusions.

* Focal necrosis was more common in athymic mice.
* Anti-Y-IFN monoclonal antibody treatment of both athymic and thymic mice caused more necrosis on day 5.
* The inflammatory response was similar in thymic mice and in athymic mice.

* Antibody treatment of both athymic and thymic mice produced a greater inflammatory response.
* More viable *C.psittaci* were isolated from athymic mice.
* More viable *C.psittaci* were isolated from mice given antibody.
* More antigen was detected in athymic mice when compared with thymic mice.
* Antibody treatment increased the amount of antigen detected in both athymic and thymic mice.

Lung.

I) Histopathology.

a) Hypercellularity.

All groups infected with *C.psittaci* showed an increased cellularity of the alveolar septa when compared with the lungs from animals in the uninfected control groups E to I (see table 7.8). This was in part due to the presence of PMN, particularly in groups C and D. In group C, (athymic mice not treated with antibody) numbers of PMN in the alveolar septa were minimal on day 3, whereas in group D, at this time, (athymic mice given antibody) they were more frequent. The presence of PMN did not appear to be a prominent feature of the increased cellularity of alveolar septa in groups A and B. PMN were detected in the lungs of mice in group B on both days but could only be found on day 5, in group A. Alveolar macrophages were more frequent in all cases on both days.

b) Antigen.

No antigen could be detected in the lung sections from group A or C, on day 3 or 5, by the immunoperoxidase method and little or no chlamydial RNA could be detected by *in situ* hybridisation (see table 7.8). In groups B and D, antigen/RNA was detected by both methods, but only on day 5 (Fig 12).

II) Isolation.

Viable chlamydiae were isolated from all the lungs of groups A, B, C and D on both day 3 and 5 (see table 7.8). In group A the number of chlamydia isolated was less on day 5 than on day 3 ($P=<0.007$), but in all other groups the numbers were increased significantly on day 5 ($P=<0.02$). More chlamydiae were isolated from mice in groups B or D than from mice in groups A or C mice on both days (day 3 $P=<0.0006$; day 5 $P=<0.007$) and more chlamydiae were isolated from athymic mice in groups C and D than from thymic mice in groups A and B, on both days ($P=<0.005$; $P=<0.006$).

Figure 12: A section of lung from an infected athymic mouse from group D, on day 5 treated with an immunoperoxidase technique. Note the presence of chlamydial antigen within the cytoplasm of cells in the lung.

(IPx160)

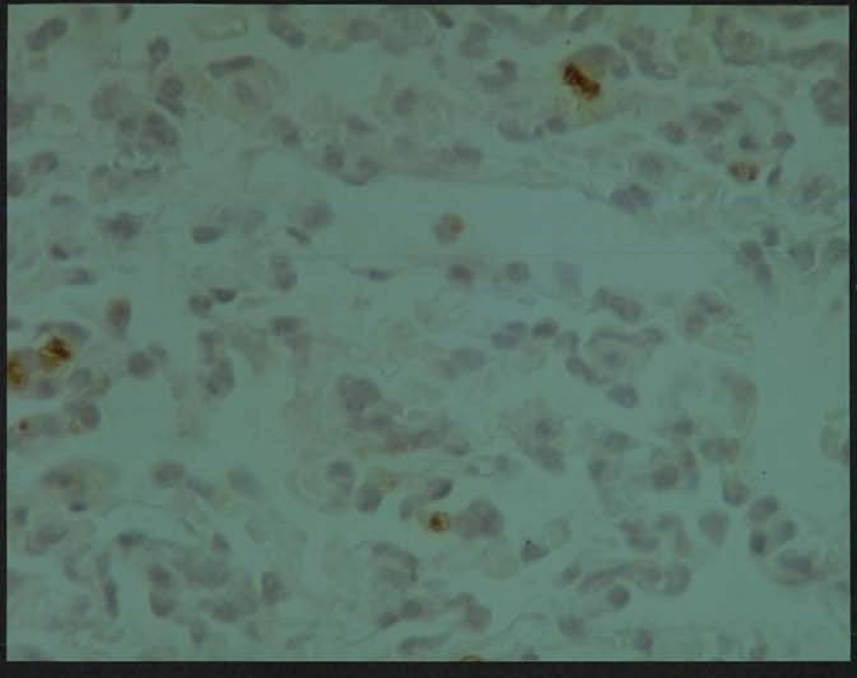


Table 7.8: Summary of the findings in the lung tissue of groups A to D.

Group	Day	Alveolar Hypercell.	Hypercellular cell type mono/PMN	Antigen	Isolation (10^3)
A	3	+	+/-	-	1.1±0.2
	5	+	+/+	-	0.3±0.1[a]
B	3	+	+/-	-	4.2±0.3
	5	+	+/-	+	14.0±2.4[a]
C	3	+	+/-	-	1.6±0.3
	5	+	+/+	-	3.7±0.5[a]
D	3	+	+/+	-	7.8±0.3
	5	+	+/+	+	36.0±5.1[a]

[a] $P<0.05$ when compared with the day 3 results of the same group

Conclusions.

* *C.psittaci* infection stimulated an increase in cellularity of the alveolar septa of all mice.
* Chlamydial antigen was only detected in mice treated with antibody and only on day 5.
* More viable *C.psittaci* were isolated from mice treated with antibody.
* More viable *C.psittaci* were isolated from athymic mice than from thymic mice.

Interferons.

The sera of all mice were tested for the presence of interferon in an anti-viral bioassay. Activity was only detected in mice from groups B and D treated with antibody (see table 7.9). In group B, 5 of 5 mice tested on day 3 were positive with titres ranging from 40 U/ml to 320 U/ml, but only 1 mouse tested on day 5 was positive, with a titre of 80 U/ml. In group D all 6 mice tested on day 3 were positive for interferon activity and 5/6 tested on day 5 were also positive. Titres on day 3 ranged from 160-320U/ml in both cases, but were not significantly higher than those in group B ($p=>0.05$).

All positive sera were tested again for the presence of γ-IFN using a γ-IFN neutralising bioassay. All IFN positive sera proved to be negative for γ-IFN with the exception of one mouse from group D, day 5, which had a titre of 160U/ml.

Conclusion.

* Treatment of mice with anti-γ-IFN monoclonal antibody stimulated an interferon-like activity in the serum.
* This activity was not due to the presence of γ-IFN (with one exception)
* Interferon-like activity was detected in both athymic and thymic mice.

Table 7.9: Summary and titres of interferon containing sera from infected groups A-D

Group	Day	α/β-IFN	U/ml	γ-IFN	U/ml
A	3	0/5	-	-	-
	5	0/5	-	-	-
B	3	5/5	120±25	0/5	-
	5	1/5	80	0/1	-
C	3	0/6	-	-	-
	5	0/6	-	-	-
D	3	6/6	140±16	0/6	-
	5	5/6	60±10	1/5	160

Discussion.

It has been demonstrated in this chapter that infection with *C.psittaci* causes cellular changes in tissues of mice. In the liver, large foci of necrosis may occur while smaller foci of inflammation develop. In the spleen the PALS expand and become prominent and cytolytic vacuoles form, while in the lungs there is a slight cellular thickening of the alveolar septa. In all tissues examined from the infected groups (A to D), while the same basic histopathological changes were seen, treatment with the monoclonal antibody against murine γ-IFN altered the severity and probably the rate of change. In both groups B and D, antibody treatment resulted in the earlier appearance of and the development of greater numbers of inflammatory foci. The numbers of cells in each focus was also

greater when compared with their untreated counterparts. In addition to this the numbers of vacuoles in the spleen, while decreasing with time in untreated mice, were maintained in antibody treated groups. The function of these vacuoles is not clear although it seems likely that they represent sites of phagocytosis, possible effete single macrophages, since they contained cell debris and chlamydial antigen. Therefore in both the liver and the spleen it would appear that in mice not given monoclonal antibody the initial infection was being resolved, while in mice treated with anti-γ-IFN monoclonal antibody infection was not being so readily controlled. This resulted in further tissue damage, allowing the suggestion that γ-IFN has an important role to play in the immune control of chlamydial infection.

Chlamydial infection caused a significant rise in spleen weights when compared with any of the uninfected control groups. In addition, antibody treated mice showed the greatest increase associated with a greater prominence of PALS, suggesting that the treatment stimulated an increase in blastogenesis. This effect could be due to the removal of suppressive effects that γ-IFN might exert on proliferation as it has been identified as a potent suppressor of proliferation *in vitro* (Richard, Forget and Turcotte, 1991). *In vitro* studies have also shown that activated splenic macrophages can suppress the proliferative response of lymphocytes to mitogens (Tomioka, Saito and Yamada, 1990). The results presented above may be an *in vivo* demonstration of these experimental phenomena based on the removal of γ-IFN and a consequent reduction in the number of activated macrophages.

Foci of necrosis, visible with the naked eye, were present in the livers of all infected animals at day 3 and day 5 and γ-IFN, appeared to be associated with its onset and severity , since the apparent neutralisation of the cytokine delayed the appearance of necrotic tissue until day 5 in the majority of mice in groups B and D mice. These foci of coagulative necrosis, which rarely contained inflammatory cells or antigen but were often bordered by a thin, red outer zone of haemorrhage, had the appearance of infarcts. Whether this was a result of increased numbers of chlamydiae in the organs of antibody treated mice causing the release of sufficient cell debris to block hepatic blood vessels and cause necrosis is not clear.

γ-IFN has also been shown to cause cell death in mouse and rat fibroblasts (Dijkmans *et al*, 1989) and this action is enhanced by bacterial LPS (Dijkmans *et al*, Billiau, 1990). Chlamydial LPS has been demonstrated on the surface of infected cells (Richmond and Stirling, 1981) and in small chlamydial infections this cytotoxic reaction may provide a protective immune response, since infected cells would be destroyed, releasing immature, non-infectious RBs. Cell debris from the damaged tissue may also attract phagocytic cells to the site of infection and aid the clearance of the organisms. The lack of detectable antigen within the large areas of necrosis described above, suggests that a direct effect on chlamydia infected cells as described above is an unlikely explanation for the widespread necrosis seen. However, it may be

that tissue damage caused by this mechanism contributes to the pathogenesis of chronic chlamydial infections. It remains a possibility that the necrosis described was caused by a combination of the above factors.

Another major feature of the pathology of the infected animals was the presence of foci of inflammation within the liver. These foci increased both in number and in size, as determined by the number of inflammatory cells they contained on day 5 when compared with day 3. Groups B and D which had been treated with antibody had more frequent and larger foci than their group A and C counterparts. It seems likely that this is linked to the finding that larger numbers of viable chlamydiae were recovered from the hepatic tissue of groups B and D. Tissue damage and cell debris caused by the increased infection would in turn attract more phagocytic cells and create large foci of inflammation. This hypothesis is strengthened by the finding that most of the detectable antigen and chlamydial RNA was associated with these foci. Thus infection of the Kupffer cells within the hepatic sinusoids was in some way related to the formation of these foci.

Two methods of detecting chlamydiae in tissue sections were used in this study. The first was a direct immunoperoxidase method and the second was by *in situ* hybridisation using a specific RNA probe. The two methods were employed initially to determine whether one was more sensitive than the other. Both proved equally sensitive and confirmed the results obtained from the other. Thus it seems

reasonable to conclude that in this experiment chlamydial RNA was always associated with chlamydial antigen, there being no evidence to support the suggestion that the organism could remain undetected in a latent form by not expressing antigen.

The amount of antigen detected in the spleen was greatest in the athymic mice of group C. It also increased between day 3 and day 5, whereas in thymic mice in group A, the amount of antigen detected decreased with time. The administration of antibody to both types of mice led to an increase in the amount of antigen detected at any time. This pattern was repeated in the other tissues examined, sometimes with large differences seen between the different groups and the largest amount of antigen being in mice given antibody. In the lungs in particular, antigen was only detected in groups given antibody and then it was only at day 5 that any could be visualised.

The site of the detected antigen varied between those mice which had been given antibody and those which had not. In the spleen, for example, while all groups had antigen present in cytolytic vacuoles in the red pulp, to where it had presumably been carried by the blood, antibody treated groups also had inclusions present, indicating that chlamydial replication was taking place at these sites. A similar picture was seen in the liver where inclusions were seen in hepatocytes in mice injected with the monoclonal

antibody. Again the inclusions appeared to be growing unhindered and free from inflammatory cell infiltration. These findings would suggest that γ-IFN has a role to play in the clearance of *C.psittaci* from infected tissue.

The sera of the infected mice were tested for the presence of interferon and specifically γ-IFN, but as in previous reports there was no detectable interferon in the sera of infected mice not injected with monoclonal antibody (Williams *et al*, 1988: Zhong *et al*, 1989). Sera from antibody treated mice did contain interferon and in these animals it was of the α/β type. It is not clear why α/β IFN was produced in this instance, but it is known that many intracellular bacteria such as *Listeria monocytogenes* and *Brucella abortus* can induce α/β–IFN after systemic infection of the host (Nakane and Minegawa, 1981; Youngner and Stinebring, 1964). Therefore the production of α/β-IFN may be a further indication of the extent of exacerbation of the pathology caused by injection of the monoclonal antibody. Another factor to consider is that bacterial lipopolysaccharide is known to induce α/β-IFN in murine macrophages (Maehara and Ho, 1977) and it is possible that the lipopolysaccharide from the increased multiplication of *C.psittaci* in antibody treated animals may have induced the production of the α/β-IFN in the macrophages. Only one mouse had any detectable γ-IFN in its serum, but the reason for this is unknown. This mouse also had less detectable antigen and fewer viable chlamydia, in its tissues than other mice in its group.

Conclusions.

It is clear from this study that γ-IFN has a role to play in the early immune response of mice to *C.psittaci*. Infected mice, treated with a γ-IFN neutralising antibody, consistently developed more severe lesions and harboured a greater number of viable chlamydiae in the spleen, liver and lung, when compared with untreated, but infected control mice. However, the role of T-cells at this time is less clear. While there was often a slight exacerbation of infection in athymic mice, the differences were not always significant, an exception to this being the greater numbers of viable chlamydiae isolated from the tissues of the athymic mice. Therefore it would appear that T-cells also have a role to play in the clearance of a chlamydial infection. While in this experiment the exact nature of this has yet to be elucidated, it may be that T-cells become more important in immune control as the infection proceeds beyond the time scale examined in this study, as would appear to be the case in sheep (Chapter 4 and 5).

Chapter 8:

GENERAL DISCUSSION

The main aim of this thesis was to examine the ovine immune response to *C.psittaci*. Work in other species has shown the importance of both humoral and cellular response to this organism (Buzoni-Gatel *et al*, 1987) and studies carried out previously at the Moredun Research Institute have examined and characterised the humoral response of sheep to *C.psittaci* (Tan, 1989). The experiments contained within this thesis were therefore designed to extend our understanding of ovine cell mediated immunity to *C.psittaci*, by examining the development of maternal lymphoproliferative responses during gestation, and determining which antigens of *C.psittaci* provoked both proliferative and cytokine mediated responses after abortion. An attempt was also made to demonstrate the *in vivo* importance of one of these cytokines in the resolution of a chlamydial infection.

As the results of specific experiments were discussed in the relevant chapters, this final chapter will attempt to bring together the main findings of the thesis and discuss them in relation to what is already known about ovine chlamydial infections.

The route of transmission of *C.psittaci* is still poorly understood although recent findings suggest that it is passed on by the ingestion of contaminated placentas and bedding (Aitken, 1990) and the tonsil has been implicated as a possible portal of entry

(Jones and Anderson, 1988). It is clear, however, that once an infection is established *C.psittaci* can lie dormant and not cause abortion until the following year. However, the site of latency is not known.

Studies in mice have shown that after infection the organisms spread to many tissues of the body including the spleen, liver and lungs (Chapter 7). In all the tissues studied the initial response was invariably by cells of the reticulo-endothelial system (Chapter 7). Proliferation of these phagocytic cells was followed by focal infiltration by PMN, possibly attracted by chemotatic factors and cell debris (Chapter 7). PMN are also important in human chlamydial infections (Register *et al.*, 1987) where low molecular weight fractions of PMN granule proteins have been shown to inhibit *C.psittaci* infectivity and fractions containing lysozyme to inhibit *C.trachomatis* infectivity. While the reason for this species difference is unknown, it may be pertinent that *C.trachomatis* has been shown not to grow well in "professional" phagocytes which are lysozyme rich (Register *et al.*, 1987). However, the role of PMN in chlamydial infection remains unclear, since in earlier work it was shown that the presence of PMN in infected mice did not correlate with survival (Heubner and Byrne, 1984).

Research has shown that cytokines are also important during the early stages of infection (Chapter 7). Gamma interferon has both an *in vivo* effect on the severity of the disease (Chapter 7) and in the resolution of infection (Williams, Bonewald, Roodman, Byrne, Magee and Schachter, 1989), possibly by virtue of its synergism

with tumour necrosis factor (TNF-α) (Shemer-Avni, Wallach and Sarov, 1989). The mechanisms by which γ-IFN works are not known. A direct effect, potentiated by bacterial LPS, which causes suicide-like destruction of infected cells has been postulated (Dïjkmans *et al.*, 1990), as has a microbiostatic effect caused by γ-IFN enhanced degradation of the essential amino acid tryptophan (Byrne *et al.*, 1986). This latter effect may prove to be the immunological basis for the persistence or latency which is common in chlamydial infections, particularly EAE (Chapter 1). It is clear that after infection, *C.psittaci* is difficult to isolate from sheep (Huang *et al.*, 1990) but that it will reappear in the placenta of an infected sheep after day 90 (Buxton *et al.*, 1990). It may be that γ-IFN forces the *C.psittaci* to adopt a latent form (Moulder *et al.*, 1980) until a trigger releases the *C.psittaci* from its intracellular site with recrudescence of infection resulting in abortion or premature birth (Aitken, 1990).

The intracellular nature of the chlamydiae mean that cytotoxic cells, important in viral infections, may also be important in containing the infection during the latent phase and in maintaining immunity to *C.psittaci* after abortion. Natural Killer cells (NK cells) mediate natural resistance against tumours, viruses and intracellular parasites (Herberman and Ortaldo, 1981) although the mechanism whereby NK cells recognise foreign antigen and lyse infected cells is not yet fully understood (Surianni, Tagliaferri and Arnti, 1990). The ability of NK cells to produce γ-IFN also has a role to play in host defence against bacterial and viral infections (Dunn and North, 1991) and therefore it is tempting to

suggest that γ-IFN from NK cells and $CD4^+$ cells may help resolve a chlamydial infection (Chapter 6 and 7) and/or maintain it in a latent state (Chapter 1). While the importance of NK cells in chlamydial immunity is unclear, it is known that as well as producing γ-IFN, NK cell activity itself is augmented by this cytokine (Djeu, Heinbaugh, Holden and Herberman, 1979). Ovine γ-IFN is produced in response to *C.psittaci* antigens by peripheral blood mononuclear cells (Chapter 6) and is also important in resolving chlamydial infection (Chapter 7) perhaps by aiding macrophages and NK cells. It is also interesting to note that during gestation NK cells lose their activity. While γ-IFN augments the activity of NK cells estradiol suppresses their activity (Seaman, Blackman, Gindhart, Roubinian, Loeb and Talal, 1978) as does stress (Shavit, Lewis, Terman, Gale and Liebeskung, 1984), both of which feature during pregnancy. If NK cells were important in chlamydial immunity this reduction in activity during gestation could lead to recrudescence of a latent infection and might explain why placental infection only develops after day 90 (Buxton *et al.*, 1990).

As well as non-specific cytotoxicity by NK cells there is also the possibility that specific cytotoxicity by $CD8^+$ T cells, is also important in chlamydial immunity. $CD8^+$ T cells have a receptor which recognises specific foreign antigen, in association with MHC Class I molecules on the surface of infected cells (reviewed in Kaufmann, 1988). It may be that these $CD8^+$ T cells recognise chlamydial peptides expressed on the surface of infected cells and lyse them. This cytotoxicity, both specific and non-specific, may

prove to be an important mechanism for limiting the intracellular cycle of a chlamydial infection, (Chapter 1), because lysis of infected cells would release non-infectious RBs which cannot then infect other cells. The prematurely released RBs would then be open to attack from other components of the immune response such as phagocytes and antibody.

Murine $CD8^+$ cells also release γ-IFN in response to purified protein derivative (PPD) after infection with *Mycobacterium bovis* (Sonnenfield, Mandel and Merigan, 1979) and adoptive transfer of $CD8^+$ cells partially protects mice against malaria, a mechanism which can be reversed by γ-IFN neutralising antibody (Schofield. Schellekens, Nussenweig and Nussenweig, 1987.) Villarquiran, Ferreirra, 1987). These findings may suggest an indirect or a direct role for γ-IFN in the protection afforded by $CD8^+$ T-cells against intracellular bacteria and since γ-IFN is important in chlamydial immunity (Chapter 6 and 7), this may be further evidence of a putative role for $CD8^+$ T-cells in the ovine immune response to *C.psittaci*. However, the importance of cytotoxicity, in the immune response to *C.psittaci* in sheep, remains to be elucidated.

One unanswered question surrounding the period of latency/persistence between initial infection and abortion (Chapter 1) concerns the trigger which signals both the end of the latent phase and the reemergence of infection, with the subsequent colonisation of the placenta. It is possible that the chlamydiae undergo periodic release from the site(s) of latency, but the placenta is not colonised until after day 90, because favourable

conditions do not exist until that time. It is also possible that the chlamydiae are only reactivated after day 90 by a hormonal/chemical signal, which is either absent or at a low concentration before this time, and which triggers their release. Ovine T-cell proliferative responses can be measured following infection at 90 days gestation (Chapter 4). Similar experiments to those conducted in chapter 4, but employing sheep infected before tupping may help answer this question. If during the measurement of T-cell proliferation to *C.psittaci* EBs, from infection to subsequent lambing/abortion, there is little or no response until after day 90, it would suggest that *C.psittaci* is released from the site of latency at this time. If, however, proliferative responses to EBs could be measured throughout gestation, it may suggest there is a periodic release of *C.psittaci* constantly boosting the immune response. These releases of chlamydiae may be limited by the immune system until the organisms colonise the placenta after day 90 when conditions are more favourable. This latter suggestion is strengthened by the unpublished finding that sheep infected with *C.psittaci* show intermittent phases of fever during gestation (D. Buxton, personal communication). It is possible that this fever is caused by the release of *C.psittaci* and a subsequent activation of macrophages and release of the pyrogen IL-1.

After abortion sheep are solidly immune and do not abort again (Chapter 1). Both T-cell proliferative responses (Chapter 5), cytokine release (Chapter 6) and B-cell antibody production (Tan, 1989) can be measured at this time and all three may be involved in

long term immunity. It is hoped that the identification of proteins which caused both proliferation (Chapter 5) and cytokine release (Chapter 6) will prove useful in the future development of an improved vaccine against EAE. Further study will be necessary to determine if individual proteins can be used to produce effective immunity against bacterial infections such as *C.psittaci*. It is encouraging to note that murine immunisation against cutaneous Leshmaniasis has been obtained using defined membrane surface antigens reconstituted into liposomes (Russell and Alexander, 1988). Recombinant chlamydial proteins, such as the MOMP, of the ovine abortion strain S26/3 (Herring *et al.*, 1989), will provide useful tools for this analysis.

While the T-cell lines used in Chapters 5 and 6 were polyclonal, future studies may be further advanced by using T-cell clones which are reactive against single epitopes. These clones could then be used to map epitopes on synthetic peptides based on the known sequences of chlamydial proteins in the same manner as has been used to successfully map epitopes on synthetic peptides from *Plasmodium vivax* (George, Law, Rich and Martin, 1990), *Schistosomiasis mansoni* (Reynolds, Kunkel, Thomas and Higashi, 1990), *Mycobacterium tuberculosis* (Barnes, Mehra, Hirschfield, Fong, Abou-Zeid, Rook, Hunter, Brennan and Modlin, 1989) and Influenza A virus (Gao, Liew and Tite, 1989).

Finally, a major aspect of ovine chlamydiology which requires further study is the question of diagnosis of infection. At present, the most common diagnostic techniques for EAE are based on

the detection of antibody with either the complement fixation test (Stamp *et al.*, 1950) or by Western blot analysis (Tan, 1989), however neither detect high levels of antibody until after abortion (Chapter 1). Cellular techniques can detect differences between infected and naive sheep during gestation (Chapter 4), but the lymphocyte transformation assays described in this thesis (Chapter 3) are currently labour intensive, expensive and require the use of radioisotopes. Therefore, this test is impractical for the screening of large numbers of sheep for the presence of a chlamydial infection. An ELISA for the detection of γ-IFN (Chapter 6) has been developed as a diagnostic test for the detection of *Mycobacterium bovis* (Wood, Corner and Plackett, 1990) and has recently undergone extensive field trials in Australia with documented success (Wood, La Corner, Rothel, Baldock, Jones, Cousins, McCormick, Francis, Creeper and Tweedale, 1991). The kit has recently been approved for use in the diagnosis of bovine tuberculosis by the Australian Animal Health Committee (Wood *et al.*, 1991). As results in Chapter 6 show, using similar techniques to the Australian workers, differences between post abortion ewes and naive sheep could be detected in the limited number of animals tested. To determine whether or not the test was specific enough to differentiate between pathogenic abortion strains of *C.psittaci* and harmless enteric isolates a larger trial would have to be initiated. Success may come from the development of synthetic peptides specific for *C.psittaci* abortion isolates, as it has been shown that even small peptides can stimulate the production of γ-IFN from primed T-cells (Chapter 6). A further question to be answered would be whether there was a correlation

between detection of γ-IFN and protective immunity to abortion which could allow the development of an assay for vaccine efficacy in sheep. Again, however, a large scale trial would be necessary to evaluate the suitability of a γ-IFN ELISA for this purpose.

Therefore, there is still much work to be done before the full role of cell mediated immunity in ovine chlamydial infection has been elucidated. The results presented in this thesis indicate an important role for both $CD4^+$ T-cells and γ-IFN in the maintenance of protective immunity. In addition, immunodominant antigens have been described which may aid future, sub-unit vaccine development. It is hoped that this thesis will provide the groundwork for future work which will further define the immunodominant antigens of *C.psittaci* and the cellular components of the ovine immune response to them, and therefore lead to an even greater understanding of chlamydial immunity in sheep.

Chapter 9:
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APPENDIX:

PAPERS RESULTING FROM THIS THESIS

Veterinary Microbiology, 25 (1990) 87–99
Elsevier Science Publishers B.V., Amsterdam

Immunity to *Chlamydia psittaci* with particular reference to sheep

Michael Campbell McCafferty
Moredun Research Institute, 408 Gilmerton Road, Edinburgh, EH17 7JH, Great Britain

(Accepted 25 January 1990)

ABSTRACT

McCafferty, M.C., 1990. Immunity to *Chlamydia psittaci* with particular reference to sheep *Vet. Microbiol.*, 25: 87–99.

Chlamydia psittaci, a zoonotic bacterium, is the causal agent of enzootic abortion of ewes, an important disease of sheep in many European countries. The major thrust of current chlamydial research is directed towards the human pathogen *Chlamydia trachomatis*. This review attempts to bring together relevant information concerning the host immune response to all members of the genus *Chlamydiae* and show how this has led to an increased understanding of the ovine humoral and cell mediated immune responses to *C. psittaci* while emphasising areas where there is still a lack of knowledge. Specifically the review looks at the common immuno-accessible antigens of the *Chlamydiae* and the antibody responses produced during infection, as well as covering the role of T cells and cytokines in the protective immune response.

INTRODUCTION

This review discusses what is currently known about the pathogenesis of and immune response to chlamydial infections in sheep. Members of genus *Chlamydia* are Gram-negative bacteria. They are obligate, intracellular pathogens which lack the ability to synthesise high energy compounds such as adenosine tri-phosphate, leading Moulder (1974) to coin the phrase "energy parasites". At present two species are recognised *Chlamydia trachomatis* and *C. psittaci*, (Moulder, 1984) although a third species *C. pneumoniae* has been proposed (Grayston et al., 1989).

Chlamydial disease has almost as many forms as hosts. Chlamydia infect arthropods, molluscs, over 130 species of birds and several species of mammels (Ward, 1983). Avian strains can cause acute respiratory infection in man (Macfarlane and Macrae, 1983) as well as birds and the mammalian strains are known to cause pneumonia, conjunctivitis, polyarthritis, synovitis, enteritis, seminal vesiculitis, sporadic encephalomyelitis and abortion (Storz and Krauss, 1985). Indeed, 20% of ovine abortion reported annually

0378-1135/90/$03.50

in Great Britain is due to *C. psittaci* (Aitken, 1986). However, the target cells in all cases are epithelial cells and infiltrating macrophages (Kuo, 1986).

C. trachomatis strains are mainly human pathogens which can be sub-divided into three biovars; the trachoma strains, which cause oculo-genital disease; the lymphogranuloma venereum (LGV) strains, include all other human strains; and the mouse pneumonitis strains. *C. trachomatis* is the most common causative agent of preventable blindness in man, as well as being the cause of the most common sexually transmitted infection in the world (Ward, 1983).

The third species, *C. pneumoniae*, has been proposed to include the TWAR strains of *C. psittaci*. These strains, named after the first isolates discovered TW-183 from Taiwan and AR-39 from Seattle (Grayston et al., 1986), cause acute respiratory infections, but unlike other species of *C. psittaci* appear to be host-specific, specific only to man.

Mammalian strains of *C. psittaci* have been subgrouped into nine immunotypes by Perez-Martinez and Storz (1985), using an indirect microimmunofluorescence technique. These immunotypes show patterns of either disease or host specificity and correlate well with the eight bio-types of *C. psittaci* earlier defined by Spears and Storz (1979). Of particular importance in sheep are immunotype 1, causing abortion; and immunotype 2, causing arthritis and conjunctivitis. Immunotype 9 also has been isolated from sheep faeces but has not been associated with any disease (Perez-Martinez and Storz, 1985). In the case of immunotype 1 strains, target cells are fetal trophoblast cells whereas immunotype 2 has a predilection for synovial and corneal cells.

Chlamydial infections are complicated by the organism's intracellular life cycle first described by Bedson and Bland (1932). This involves two forms, the infectious, extracellular elementary body (EB) and the non infectious, intracellular reticulate body (RB). The EB gains access to the cell where it transforms into an RB and begins to replicate within a cytoplasmic membrane or inclusion vacuole using the hosts' cells energy. The by now large numbers of RBs then revert to the EB form again and burst from the cell ready to infect further healthy cells.

PERSISTENCE OF INFECTION

The disease states caused by *C. psittaci* fall into two groups; chronic disease such as conjunctivitis and arthritis, due to immunotype 2, or single episode diseases such as enzootic abortion of ewes (EAE), caused by immunotype 1. However, in both cases there is a persistence or latency of infection, both in vitro (Rodolakis et al., 1989), where virulence affects the frequency at which persistence occurs, and in vivo. It has been shown that following initial infection in young lambs, *C. psittaci* can persist until the following season when the animals become pregnant at which time a proportion abort (McEwen et

al., 1951). Infection can also persist and *C. psittaci* can be isolated from goats after abortion, when the animals are considered to be immune (Brown et al., 1988). In 1980 Moulder and co-workers suggested a cryptic form of the parasite, which did not stain with Giemsa, to explain the persistence of infection they demonstrated in vitro. These cryptic forms revert to dividing RBs when triggered by a factor as yet unknown allowing the disease cycle to begin again.

However, persistance is still not fully understood but may be partly due to the unique interactions between macrophages and *C. psittaci. C. psittaci* can exist within the macrophage (Kuo, 1988) which is one of the principal antigen presenting cells of the immune system. This may have profound implications in the generation of immune responses during infection. The chlamydiae achieve their survival by inhibiting phagosome-lysosome fusion (Friis, 1972), perhaps in the same manner as other intracellular pathogens such as *Toxoplasma gondii* (Jones and Hirsch, 1972) and *Mycobacterium tuberculosis* (Lowrie, 1983). *Mycobacterium leprae*, on the other hand, escapes from the phagosome into the cytoplasm before fusion (Sibley et al., 1987). The exact mechanisms by which these effects are achieved have yet to be defined, but Byrne and Moulder (1978) showed that uptake of chlamydiae was parasite-induced and that this phagocytosis was inhibited by heating the chlamydiae, suggesting that a heat-labile structure on the surface of the organism was responsible for their uptake. It was also noted that the addition of large numbers of viable chlamydiae did not induce the uptake of heat-inactivated organisms, showing that the unknown component was not a general promoter of phagocytosis.

Intracellular RBs are incapable of inhibiting phagosome-lysosome fusion (Brownridge and Moulder, 1979) and cannot induce uptake by the target cell since, when compared with EBs, fewer RBs are phagocytosed and more are destroyed upon uptake by macrophages. Phagosome–lysosome fusion inhibition is thus restricted to and is triggered by EBs (Eissenberg and Wyrick, 1981). When yeast and EBs are concomitantly phagocytosed by macrophages fusion occurs only with yeast laden vacuoles, showing that chlamydial EBs cause specific and not general suppression of fusion (Eissenberg and Wyrick, 1981). Later it was shown that purified EB envelopes alone could inhibit fusion and in this case the ability to inhibit fusion was not lost on heating the envelopes (Eissenberg et al., 1983). However, large numbers of EBs, rather than causing higher rates of infection, actually cause reduced rates due to a phenomenon known as immediate cytotoxicity (Moulder et al., 1976). This is thought to be caused by membrane lesions resulting from the parasite-induced phagocytosis (Friis, 1972). Therefore, larger numbers of EBs give rise to more ingestion, causing more lesions, leading to irreparable membrane damage and cell death, before the invading chlamydiae can replicate.

ANTIBODY RESPONSES INDUCED BY ANTIGENS OF *C. PSITTACI*

There are many surface antigens on *C. psittaci* which may stimulate a humoral immune response. Sixteen to eighteen bands, separatable by SDS–PAGE electrophoresis, react in immunoblotting experiments using sera from ewes recovering from a chlamydial infection (Tan et al., 1988). Two of the most prominent are a 38–42 kD protein antigen described for two strains of *C. trachomatis* and one of *C. psittaci* (Hatch et al., 1981) and a carbohydrate antigen, first described by Bedson (1936) and later shown to be lipopolysaccharide (LPS) (Nurminen et al., 1983). These two antigens are associated on the surface of chlamydiae (Birkalund et al., 1988).

Due to its prominence on the surface of all strains studied, the 38–42 kD protein antigen has been called the major outer membrane protein (MOMP) (Salari and Ward, 1981). In 1982, Caldwell and Schachter showed that MOMP contained a complex hierarchy of epitopes, including species, subspecies, and serotype specific epitopes. Cloning of the MOMP gene of *C. trachomatis* (Allan et al., 1984), and recent sequencing of the MOMP gene of the *C. psittaci* ovine abortion strain S26/3 (Herring et al., 1989) should increase knowledge of its role in the immune response as should work by Conlan and his co-workers on the primary amino acid sequence of the epitopes (1988).

LPS is genus-specific, being found on all strains of chlamydiae, and forms the basis of the complement fixation test(CF) used in serodiagnosis of disease. It has been shown to cross-react immunologically with the mutant Re determinant of other bacterial LPS (Nurminen et al., 1983). Caldwell and Hitchcock (1984) subsequently demonstrated that there was also a species-specific epitope on chlamydial LPS. The chemical nature of this epitope has been described by Nurminen and colleagues (1985) and recently a trisaccharide related to chlamydial LPS was synthesised by Kosma and co-workers (1988).

Other antigens of importance in chlamydial infection are recognised in immunoblot experiments using sera from convalescent ewes (Tan et al., 1988) and man (Newhall et al., 1982) and include three cysteine-rich proteins of molecular weights 15 kD, 60 kD, 62 kD found on *C. psittaci* (Hatch et al., 1984) and *C. trachomatis* (Batteiger et al., 1985). These are absent from RB's and the lack of disulphide bridges may explain the less rigid structure of the RB cell wall. There are also two adhesins of 31 kD and 18 kD which appear to be eukaryotic cell binding proteins (Hackstadt, 1986). These proteins are not denatured by heating unlike the envelope proteins described by Byrne and Moulder (1978). The 18 kD protein has been cloned in *E. coli* and antibody raised against the expressed product has a neutralising effect on chlamydial infectivity (Kaul et al., 1987). These adhesins take on further significance in light of studies by Russel and Alexander (1988) which demonstrated

prophylaxis in cutaneous leis. maniasis using two antigens known to be involved in the attachment of another intracellular parasite, *Leishmania mexicana,* to its host cell, the macrophage.

Specific IgM and IgG antibodies are induced by these immunoreactive antigens following infection (Page et al., 1967). Originally, Isa et al. (1968) claimed that only IgG was produced in monkeys, even in a primary infection. However this was later shown not to be the case and the earlier lack of IgM activity was due to either inapparent previous infections or cross-reacting antigens (Isa, 1973). The ability of the immunofluorescence test used to detect IgM in primates has also been questioned (Juchau et al., 1972), since high affinity IgG may effect the ability of low affinity IgM to bind to chlamydial antigen.

Following infection in ewes, CF antibody is produced against epitopes found on chlamydial LPS. Titres rise to a peak at about 14 days after abortion and remain high for several weeks (Stamp et al., 1952). Neutralising antibody appears later, and titres remain higher for longer (McEwen and Foggie, 1954), but neither CF nor neutralising antibody titres correlate with immunity (Storz and Krauss, 1985).

Recent work has concentrated on which subclass of IgG is produced in the humoral response. This interest was stimulated when Schmeer and co-workers (1985) showed by ELISA, that IgG2 was the dominant subclass in bovine chlamydial infections. However, similar work with sheep antibodies showed that IgGl is dominant (Krauss et al., 1985). The significance of this is unknown at present but work on how antigens stimulate different subclasses of antibody may prove useful, particularly in light of the fact that gamma-interferon enhances murine IgG2 production and decreases IgGl production in vitro (O'Gara et al., 1988).

However, laboratory animal studies dealing with passive transfer of antibodies have produced conflicting results. Watson and colleagues (1973) passively transferred serum antibody into non-immune guinea pigs which resulted in titres higher than those associated with natural immunity. However, on subsequent challenge the disease was neither prevented nor slowed. Buzoni-Gatel and co-workers (1987) found that immune sera transferred to mice infected with *C. psittaci* led to eradication of the organism. Whether this is due to the different animal models used is uncertain, but both authors agree that cell-mediated immune mechanisms may predominate in resistance to this disease.

CELL-MEDIATED IMMUNITY

Cell-mediated immunity (CMI) is a function of specific T cell cytotoxicity and delayed type hypersensitivity (DTH) reactions, and of natural immune

mechanisms including natural killer cell activity and phagocytosis by cytokine-activated macrophages.

Cytotoxicity attributed to both natural and T cell mediated mechanisms has been demonstrated in spleen cells from mice infected with *C. psittaci* Cal 10 strain (Lammert, 1982). In comparison, cytotoxicity could not be found in lymphocyte preparations taken from the spleen, lymph nodes or peritoneal cavities of mice infected with the LGV strains of *C. trachomatis* (Pavia and Schachter, 1983). Further work with human lymphocytes also failed to demonstrate cytotoxicity against the LGV strains of *C. trachomatis* (Qvigstad and Hirschberg, 1984). The role of T cell cytotoxicity is therefore unresolved, although the reported differences may be due to the different species used. It remains to be elucidated whether these mechanisms play a role in ovine *C. psittaci* infection.

That T cell mediated immunity to *C. psittaci* occurs in sheep can be demonstrated by DTH tests (Wilsmore et al., 1984; Dawson et al., 1986). After abortion, ewes develop a positive DTH reaction, however, if primary infection occurs outwith pregnancy no immunity is generated. In the latter case it is possible that the organism may persist at too low a level to stimulate the DTH response, but may cause abortion of the following pregnancy. Immunity is then generated, presumably due to the higher levels of replication within the placenta and substantial challenge of the ewe with antigen as a result of parturition (Storz, 1971). This explains the earlier findings that after aborting ewes display immunity (Stamp et al., 1950). The generation of CMI may prevent further chlamydaemias and thereby stop subsequent placental infection even though ewes may still harbour a low level infection.

Cytokine-activated phagocytosis by macrophages may occur as a result of DTH responses. In 1983, Murray and colleagues showed that oxygen-independent activity of macrophages against intracellular parasites, including *C. psittaci*, was enhanced by a cytokine and IFN-g was later shown to be the cytokine, originally termed macrophage activating factor (MAF), responsible for oxygen dependent activation of macrophages against intracellular parasites (Nathan et al., 1983). However, as early as 1975 Borges and Johnson had demonstrated that products in the supernatant of activated T cells could effectively reduce the intracellular replication of *T. gondii*. IFN-g is released by activated T cells. Cytokine-activated macrophages have been associated with restriction of chlamydial replication, possibly leading to persistent infections (Moulder et al., 1980). Huebner and Byrne (1988) found that while not clearing infection, activated macrophages were necessary for survival of infected mice indicating that their action was bacteriostatic. This effect of IFN-g had been described previously (Byrne and Faubion, 1982). Recently, Byrne and colleagues (1989) have also demonstrated a cytotoxic effect on *C. psittaci* by infected cells activated by IFN-g.

At the time IFN-g was identified as a macrophage activator it was also im-

plicated as the factor in crude cytokine preparations which induced inhibition of *C. psittaci* replication in macrophages (Rothermel et al., 1983; Byrne and Krueger, 1983). It was shown that anti-IFN-g antibodies could remove this inhibition and precipitate a recrudesence of infection. Recently, catabolism of essential amino acids induced by IFN-g was identified as the mechanism for this inhibition (Byrne et al., 1986). This is not the first time, however, that parasite and host competition for essential amino acids has been cited as a mechanism for inhibition of replication due to bacteriostasis (Hatch, 1975).

Other phagocytes involved in the response to chlamydial infection are polymorphonuclear cells or neutrophils (Register et al., 1987). These cells are attracted to sites of inflammation by the release of arachidonic acid metabolites from activated macrophages. However, this does not seem to be the case with other intracellular parasites such as *T. gondii* (Locksley et al., 1985) which appear to affect the host cell's arachidonic acid metabolism altering the concentrations of leucotrienes produced, thereby reducing the inflammatory response to the infection and lowering the numbers of neutrophils at the site of infection.

Granules from neutrophils, of patients with chronic granulocytic leukaemia, were fractionated in order to examine which compounds were important in the response to *Chlamydia* spp. The heavier fractions, which contained lysozyme, reduced *C. trachomatis* infectivity, whereas the lighter fractions, Mw < 13 kD, had a detrimental effect on *C. psittaci*. Granules eluted in the lower molecular weight fractions contain cationic proteins, but the important individual ones are unknown at present.

Further in vitro techniques also show CMI has a role to play. These include specific antigen-induced migration inhibition of peritoneal exudate cells from guinea pigs infected with *C. psittaci* (Seynk et al., 1981) and the proliferation of lymphocytes from lymph nodes of sheep (Russo and Giauffret, 1978), and from guinea pigs with *C. psittaci* antigens (Seynk et al., 1981), and in human T cell clones using *C. trachomatis* antigens (Qvigstad and Hirschberg, 1984). In each case migration inhibition and lymphocyte proliferation were demonstrated, indicating that T cells have been exposed to and have recognised chlamydial antigen. Lammert and Wyrick (1982), using a similar mouse lymphocyte proliferation assay, found that the response to the T cell mitogens concanavalin A and phytohaemagglutinin was reduced 1–2 weeks after infection and that proliferation in response to chlamydial antigen was suppressed. Chlamydiae-induced proliferation occurred only 4 weeks after infection, by which time mitogen response had returned to normal.

Treatment with compounds known to be selectively immunosuppressive has shown that CMI is not the only important immune response to *C. psittaci*. When cyclophosphamide is administered at levels which deplete humoral responses, but have no effect on CMI, there is no resolution of disease

(Modabber et al., 1976). There is therefore cooperation between the humoral and the cell-mediated systems, although the exact mechanism by which this occurs is not yet known (Buzoni-Gatel et al., 1987; Rank et al., 1989). Two possibilities are suggested; antibody-dependent cell cytotoxicity and opsonisation. Wyrick and colleagues (1978) demonstrated that opsonised EBs were taken up and destroyed in macrophages, presumably because they could no longer prevent phagosome–lysosome fusion, while it has also been shown that although immune sera can transfer immunity the effect is increased by T cell transfer (Buzoni-Gatel et al., 1985).

IMMUNOMODULATION BY *C. PSITTACI*

Of importance in any review on immunity is the consideration of modulation of the host immune response following invasion by the pathogen. With relevance to *C. psittaci* infection this may occur by pathogen-induced cytokine release, hormonal alterations and direct suppression of the immune system.

One such example, the restrictive effects of IFN-g, has been discussed above. However, IFN-g also has profound effects upon the immune system, particularly leading to the expression of class II major histocompatibility complex (MHC class II) antigens on a variety of cell types. It is the presence of processed peptides from foreign antigens in association with MHC class II molecules on cell surfaces that is recognised by T cells and thus initiates specific immune responses. In mice infected with *C. psittaci* increased expression of MHC II antigens has been described on macrophages (Paulnock et al., 1986). This may well play a role in the generation of immune responses at sites of infection.

C. psittaci may also have a direct modulatory effect on the immune system by inducing immunosuppression through infection of macrophages, which are important in the presentation of antigen to the T cell and also important phagocytic cells. Thus, while not killing the macrophages, chlamydial infection may alter their ability to perform their normal functions and interfere with the immune system on many different levels. It has also been shown that antigen from *C. psittaci* can directly inhibit lymphocyte proliferation in vitro (Lammert, 1982). This is similar to the immu osuppressive substance recently shown to be released by *Mycobacterium l rae* (Liew, 1988).

In addition, hormones have been shown to en ıance *C. trachomatis* infections in many animal models (Kuo, 1988) and i EAE it seems reasonable to suggest that hormones may stimulate the infecti n of the placenta which occurs at or about 90 days into gestation (Buxton et al., 1990). Alteration in the levels of many hormones affect many functio s of the immune system and immunosuppression associated with pregnancy is a well recognised phenomenon. (Tomasi, 1983).

CONCLUSION

It is obvious that knowledge of the mechanisms involved in the ovine immune response to *C. psittaci* is scanty. More work is required on the antigens protective in the immune response as well as on the cellular interactions involved in generating a protective immune response. It will also be necessary to elucidate the nature of the balance between the immune system of the host and persistent infection. it is anticipated that current studies of these parameters will provide information to allow rational design of an effective vaccine which may also prevent the establishment of persistent infections in sheep.

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