IDENTIFICATION OF *COWDRIA RUMINANTIUM* PROTEINS THAT INDUCE SPECIFIC CELLULAR IMMUNE RESPONSES

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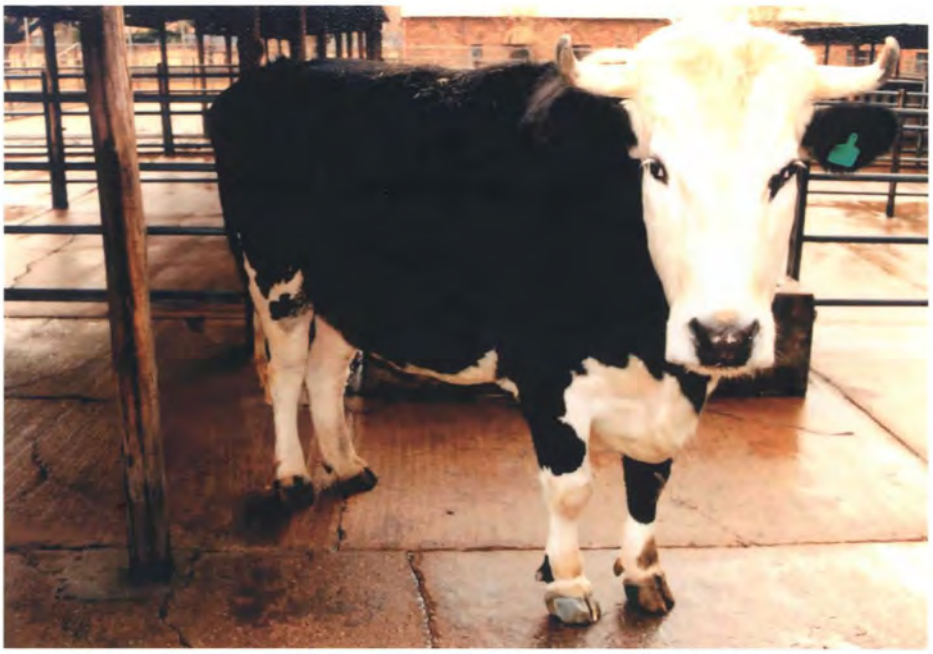

This thesis is dedicated to B9191.

ABSTRACT

Cowdria ruminantium (*Cowdria*) is an obligate intracellular pathogen that causes heartwater in ruminants. Cellular immunity and the type I cytokine IFN-γ have been implicated in protective immunity to heartwater. The aim of this thesis was to identify proteins of the Welgevonden isolate of *Cowdria* that induce lymphocyte proliferation and IFN-γ production. Differential centrifugation was found to be the simplest and most efficient method of *Cowdria* purification. *Cowdria* organisms were fractionated into their constituent proteins of between 11 and 168 kDa by continuous flow electrophoresis. The resulting fractions were tested for their ability to stimulate lymphocyte proliferation *in vitro*. In an attempt to simulate the natural infective process, peripheral blood mononuclear cells (PBMC) were obtained from two cattle rendered immune by infection and treatment and assayed in proliferation assays with the proteins fractions. In a parallel study, four cattle were immunised with inactivated *Cowdria* to determine whether their lymphocytes responded similarly. *Cowdria*-specific proliferation was detected for only a brief period after immunisation by infection with live organisms. This response was only detected again two to three years later. In contrast, PBMC from animals immunised with inactivated organisms were continuously responsive for at least three years. Only *Cowdria* proteins with molecular masses of 11, 12, 14 to 17 and 19 to 23 kDa induced proliferative responses in PBMC obtained from all six animals. Cell surface phenotypic analysis of *Cowdria* specific T-cell lines indicated that $CD4^+$ lymphocytes were enriched over time with a concomitant increase in antigen-specific proliferation and IFN-γ production. Proteins of molecular masses 13 to 18 kDa induced $CD4^+$ lymphocyte

proliferation and IFN-γ production by T-cell lines from all the animals tested. Antibodies raised in a chicken and in rabbits to these low molecular weight proteins had low titres and specificity. Two-dimensional electrophoresis indicated that proteins within a single molecular weight range comprised several components with different pIs, revealing the complexity of the *Cowdria* proteome. This complicates the search for potentially protective antigens. Nevertheless, since they cause proliferation and IFN-γ production by lymphocytes from immunised cattle, these low molecular weight proteins merit further investigation as potential vaccine antigens.

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ABBREVIATIONS

Abs	absorbance
Ag	antigen
APC	antigen presenting cell
B	bovine
BA	bovine aorta
bp	base pairs
BSA	bovine serum albumin
BSV	bovine saphenic vein
CAPS	cyclohexylamine propane sulphonic acid
CD	cluster of differentiation
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulphonate
Ci	curie
CNBr	cyanogen bromide
ConA	Concanavalin A
Cpm	counts per minute
CTL	cytotoxic T lymphocytes
DC	differential centrifugation
2DE	2-dimensional electrophoresis
DEAE	diethylaminoethyl
dH_2O	distilled water
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
DNA	deoxyribonucleic acid
E_5	calf endothelial cell line
EC	endothelial cell
ECF	East Coast fever
ECL	enhanced chemiluminescence

EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
FCA	Freund's complete adjuvant
FCS	foetal calf serum
FIA	Freund's incomplete adjuvant
Fig	figure
FITC	fluorescein isothiocyanate
G	gauge
g	gravitational force
h	hour
HBSS	Hanks' balanced salt solution
HEPES	2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethane sulphonic acid
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
IAC	immunoaffinity chromatography
IEF	isoelectric focusing
IFA	indirect immunofluoresence assay
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.m.	intramuscular
IPI	interferon-gamma production index
IU	international unit
i.v.	intravenous
kDa	kilodalton
mAb	monoclonal antibody
MAP1	major antigenic protein 1
MB	macro bead
mg	milligram
MHC	major histocompatibility complex

min	minute
ml	millilitre
MPO	myeloperoxidase
MSP	major surface protein
nd	not done
NK	natural killer
neg	negative
NK	natural killer
no	number
OVI	Onderstepoort Veterinary Institute
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGC	Percoll density gradient centrifugation
pI	isoelectric point
pos	positive
PSIAC	positive selection immunoadsorbent chromatography
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
R	ribosomal
s.c.	subcutaneous
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SI	stimulation index
TEMED	tetramethylenediamine
Tc	T cytotoxic
TCGF	T-cell growth factor
Th	T helper
TNF	tumor necrosis factor
Tris	tris(hydroxymethyl)aminomethane

Tween	polyoxyethylene sorbitan monolaurate
μg	microgram
μl	microlitre

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Communications resulting from this study

Publications:

1. Brayton, K.A., J. Fehrsen, E.P. de Villiers, M. van Kleef, and B.A Allsopp. 1997. Construction and initial analysis of a representative λZAPII expression library of the intracellular rickettsia *Cowdria ruminantium*: cloning of *map*1 and three other *Cowdria* genes. Veterinary Parasitology. 72:185-199.

2. Van Kleef, M., N.J. Gunter, H. Macmillan, B.A. Allsopp, V. Shkap, and W.C. Brown. 2000. Identification of *Cowdria ruminantium* antigens that stimulate proliferation of lymphocytes from cattle immunized by infection and treatment or with inactivated organisms. Infection and Immunity. 68:603-614.

3. Van Kleef, M., H. Macmillan, N. J. Gunter, E. Zweygarth, B. A. Allsopp, V. Shkap, D. H. Du Plessis, and W. C. Brown. 2002. Low molecular weight proteins of *Cowdria ruminantium* (Welgevonden isolate) induce bovine $CD4^+$-enriched T-cells to proliferate and produce Interferon-γ. Veterinary Microbiology. 85:259-273.

Conference presentation:

1. Van Kleef, M., N.J. Gunter, H. Macmillan, B.A. Allsopp, V. Shkap and W.C. Brown. Identification of *Cowdria ruminantium* antigens that stimulate proliferation and interferon-gamma production by lymphocytes from cattle. BioY2K combined millennium meeting, Rhodes University, Grahamstown, S.A. 23-28 January 2000.

Conference posters:

1. Van Kleef, M., N.J. Gunter, B.A. Allsopp, D.H. du Plessis, and W.C. Brown. Identification of *Cowdria ruminantium* proteins that induce lymphocyte proliferation in an immune animal. Society for Tropical Veterinary Medicine Conference, 4th biennial meeting, Montpellier, France. 5-9 May 1997.

2. Du Plessis, D.H., M. van Kleef, J. Fehrsen, N.J. Gunter and H. Macmillan. Identification of protective and diagnostic epitopes of *Cowdria ruminantium*. Vaccines and Immunotherapeutics in the 3rd Millenium Conference. 7th biannual meeting, Lorne, Australia. 19-22 March 2000.

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Cowdria ruminantium (*Cowdria*) is an obligate intracellular pathogen that causes heartwater in ruminants. The disease is controlled primarily through immunisation by infection with virulent blood and subsequent treatment with antibiotics to prevent a serious course of the disease. This is a cumbersome procedure that has numerous disadvantages including its unsuitability for use in countries where potential vectors are present, but which do not harbour the disease. There is thus a real need for an improved vaccine, especially in terms of increased efficacy and improved safety. Subunit vaccines are potentially safe, cheap, heat stable, easy to administer and can induce a broad immune response with lifelong memory (Liljeqvist & Ståhl, 1999). Several findings suggest that cellular immunity plays an important role in protection against heartwater (du Plessis, 1982; du Plessis *et al.*, 1991 & 1992a; Martinez, 1997; Mwangi *et al.*, 1998a, b & c; Totté *et al.*, 1997 & 1998). Accordingly an important objective in many vaccination strategies is the activation of lymphocytes with particular effector functions. In order to develop a subunit vaccine it will therefore be necessary to first identify antigens that are involved in inducing protective cellular immunity. The search for such antigens is the subject of this thesis.

1.2. LITERATURE REVIEW

1.2.1. THE DISEASE – heartwater

1.2.1.1. Economic impact

Improvement of livestock productivity in the developing world is severely constrained by tick-borne parasites and the diseases they transmit (Musoke *et al.*, 1997). Among the most important are *Theileria parva* (East Coast fever), *T. annulata* (bovine tropical theileriosis), *Babesia bigemina* and *B. bovis* (babesiosis), *Anaplasma marginale* (anaplasmosis) and *Cowdria ruminantium* (heartwater). All constitute a major impediment to the introduction of more productive cattle breeds in endemic areas and cause high morbidity and mortality. While certainly significant, the precise economic impact of heartwater on livestock production cannot be determined. The reasons for this are that the disease is seldom reported and diagnosis is only rarely confirmed. Moreover, the use of acaricides, resistance of certain animal breeds and the existence of enzootic stability can mask the incidence of the disease. At least 17.5 million animals are nevertheless considered to be at risk. This figure includes 8.7 million animals in the small scale farming sector which represents food security for rural people, as well as 8.8 million in the commercial sector. Heartwater therefore represents a significant economic burden (livestock census performed by the Directorate of Animal Health, Pretoria, 1996). It is estimated that in South Africa 40 to 50% of all livestock deaths within endemic areas are caused by heartwater (FAO, 1996). Consequently it is a major stumbling block preventing movement of stock from heartwater free to heartwater enzootic areas (Provost & Bezuidenhout, 1987). A safe

and effective method of control would therefore have a tremendous positive economic and social impact on rural and peri-urban communities. It could also benefit the environment by allowing farmers to introduce high-grade animals with improved productivity, thereby reducing the need for large herds.

1.2.1.2. Heartwater

Cowdria was first identified as the causative agent of heartwater by Edmund Cowdry, an American parasitologist working at Onderstepoort, (Cowdry, 1925). This disease is non-contagious and transmitted by ticks of the genus *Amblyomma* to wild and domestic ruminants. It occurs in sub-Saharan Africa and the Caribbean (Provost & Bezuidenhout, 1987). It is also a serious threat to livestock on the American mainland since the disease is apparently capable of spreading from Guadeloupe to surrounding islands by infected ticks that are carried by migrating birds. In this way it may eventually reach the American mainland where potential vectors are present, but where the disease is currently absent (Barré *et al.*, 1987). This is an additional reason for the interest in developing a subunit vaccine as the current vaccine consists of virulent organisms in ovine blood and can therefore not be used in the event of an American outbreak.

Losses from heartwater due to mortality usually occur when susceptible animals are moved to, or are raised in endemic areas, when tick control methods fail and when tick vectors spread. The disease is characterised by the development of petechiae on the conjunctiva of the eye, high fever, severe nervous symptoms, hydrothorax and hydropericardium. Within the different species of animals the incubation period of heartwater varies considerably, ranging between 9-29 days.

The route of infection, virulence of the stock and the amount of infective material administered all influence the incubation period. The morbidity and mortality rates are largely influenced by the species, breed and age of the animal, the virulence of the *Cowdria* isolate, immunisation and tick control programs, specific chemotherapy and the season (van De Pypekamp & Prozesky, 1987). The pathology differs between breeds of ruminants and *Cowdria* isolates, but it is thought to be the result of increased capillary permeability leading to transudation and oedema (Clark, 1962). Very little direct lysis and few cytopathic changes are observed in infected endothelial cells of moribund animals. Cytopathic effects are, however, observed in endothelial cells that contain no organisms. No correlation is observed between the concentration of the organism and the extent of lesions in particularly the lungs of infected animals (Pienaar, 1970). Treatment, once neurological signs have developed, becomes more difficult as not enough is known about the pathophysiology to make any supportive treatment really effective (van Amstel & Oberem, 1987).

1.2.1.3. Diagnosis

Diagnosis of heartwater is often difficult in the live animal because early clinical signs of the disease are not decisively characteristic. A further complicating factor is that the course of the disease is usually too fast for early treatment. Considering the epidemiology, symptoms and lesions at autopsy, one can usually only make a tentative diagnosis. A definite diagnosis depends upon the microscopic demonstration of the causative organism in the vascular endothelial cells of the brain. In the field, the most reliable diagnostic method remains the examination of brain smears from the first animal that has died followed by monitoring the daily rectal temperatures

of all potentially infected animals (Camus & Barré, 1987). Treatment of heartwater during the early febrile stages presents few problems and recovery can usually be expected when either sulphonamides or tetracyclines are used.

1.2.1.4. Hosts

Domestic cattle, sheep, goats and certain game species are naturally susceptible to heartwater. These animals remain carriers for long periods (av. 246 days) after recovery (Andrew & Norval, 1989). In addition, many wildlife species can become infected. *Cowdria* antigen has been detected by blood or tick transmission studies or by polymerase chain reaction (PCR) assays in African buffalo, African elephant, blesbuck, eland, giraffe, impala, Kafue lechwe, sitatunga, springbuck, tsessebe, waterbuck and wildebeest. Serological evidence of antibodies to *Cowdria* exists in both black and white rhinoceros species. This, however, has yet to be confirmed through isolation of the organism (Deem, 1998). The eland, giraffe, kudu and blue wildebeest can act as experimental reservoirs of *Cowdria*. These wild ruminants are natural hosts for the tick vector and occur commonly within heartwater endemic areas of Africa. They are, therefore, likely to be important in the epidemiology of the disease as natural reservoirs of *Cowdria* infection (Peter *et al.*, 1998). It is clear, therefore, that considerable risks are associated with the translocation of wild ruminants from heartwater-endemic areas to heartwater-free areas with large populations of susceptible ruminants in addition to the tick species that are capable of transmitting the disease.

1.2.1.5. Vectors

The only vectors known to be capable of transmitting *Cowdria* are 13 species of ticks in the genus *Amblyomma*, the two most important of which are *A. variegatum* and *A. hebraeum* (Fig 1.1. Walker & Olwage, 1987). *Amblyomma* is a three-host tick. Birds as well as small, medium and large mammals are hosts to the immature stages (larvae and nymphs) while adult ticks prefer larger mammals. Ticks acquire the pathogen while feeding either on animals with the disease, or on carrier animals. Transmission of the organism occurs trans-stadially from larva to nymph, from nymph to adult or from larva through nymph to adult (Bezuidenhout, 1987; Horak *et al.*, 1987). *Cowdria* can be maintained by one generation of ticks infected as larvae, for more than three years (Neitz, 1968). *A. variegatum* was introduced into Guadeloupe in the early 1800s and is now established on 15 islands in the Caribbean. The presence of *Cowdria* on three Caribbean islands, in conjunction with the widespread distribution of *A. variegatum*, presents a disease threat to the American mainland (Barré *et al.*, 1987).

1.2.1.6. Control

Disease control is based on attempting to eliminate the tick vectors using acaricides. Intensive dipping is expensive and only effective under certain conditions. A major problem is that this approach can result in tick populations developing acaricide resistance (Musoke *et al.*, 1997). Protective immunity against heartwater can be induced in ruminants by infection with ovine blood containing virulent organisms and subsequent treatment with antibiotics to prevent a serious course of the disease. Although this has become the standard practice in Southern Africa it has major limitations: (1) the possibility of unwanted transmission of other pathogens; (2)

unsuitability for use in countries (e.g. America) where potential vectors are present, but which do not harbour the disease; (3) a cold chain is required which is generally unreliable and impractical in developing countries; (4) intravenous administration is required; (5) loss of vaccinated animals due to virulence of the vaccine and (5) protection is not assured against all isolates (van der Merwe, 1987). A thorough understanding of the disease, the causative agent and the mechanisms of protective immunity to heartwater thus all need to be taken into account in the development of an improved vaccine.

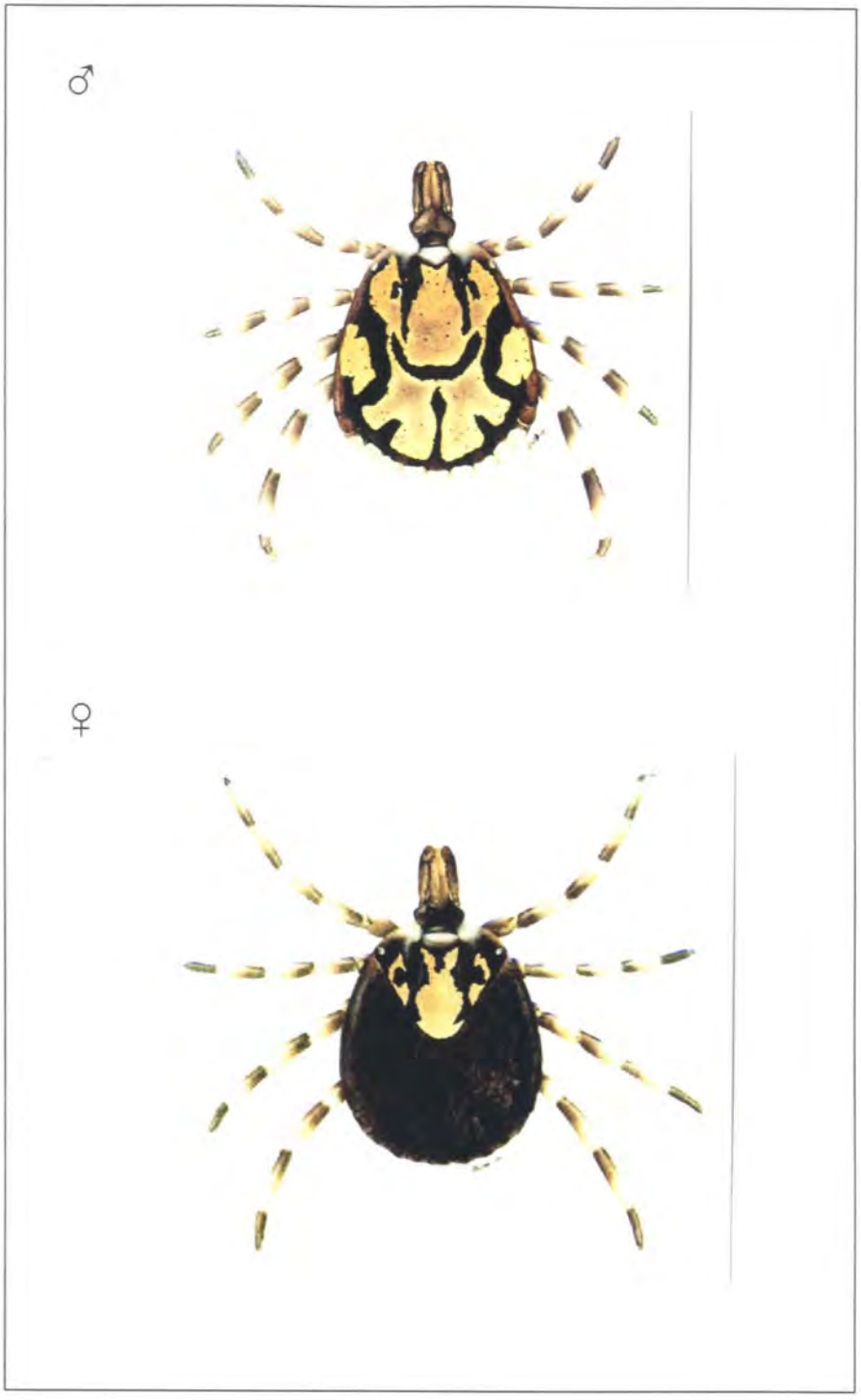

FIG. 1.1. *Amblyomma hebraeum* male and female ticks (Walker & Olewage, 1987).

1.2.2. THE ORGANISM - *Cowdria ruminantium*

1.2.2.1. Classification

Cowdria is the only species in the genus *Cowdria* and is currently classified as a member of the *Ehrlichieae* tribe in the family *Rickettsiaceae*, order *Rickettsiales* (Ristic & Huxoll, 1984). Rickettsiae are bacterial parasites of eukaryotic cells and are transmitted by arthropods (Williams and Vodkin, 1987). *Cowdria*, a typical Rickettsiae, is pleomorphic in shape and varies in size between 0.49 and 2.7 μm in diameter. It develops within membrane-bound vacuoles in the cytoplasm of endothelial cells where multiplication occurs mainly by binary fission and multiple budding and endosporulation (Pienaar, 1970). However, recent genetic analysis of 16S rRNA genes, groESL and surface protein genes lead to the proposal that the genus *Ehrlichia* be changed to include *Ehrlichia (Cowdria) ruminantium* comb. nov. (Dumler *et al.*, 2001).

1.2.2.2. Developmental cycle

***In vitro*:** Electron microscopic studies in endothelial cell cultures indicated that *Cowdria* has a *Chlamydia*-like development cycle. Large colonies of reticulate bodies result after division by binary fission within the intracytoplasmic vacuoles. These reticulate bodies consist of a fine network of filaments interspersed with numerous ribosomes. After three to four days in cell culture, they develop into smaller intermediate bodies characterised by an electron-dense core. These condense further into electron-dense bodies shortly before disruption of the host cells, releasing them into the culture medium. A new infectious cycle is initiated when the released

elementary bodies invade other endothelial cells. Each infectious cycle lasts between five and six days (Jongejan *et al.*, 1991). This correlates with the putative developmental cycle observed *in vivo* where the cycle in reticulo-endothelial cells is complete within three to four days (du Plessis, 1975). Figure 1.2. shows vacuole bound *Cowdria* colonies within the cytoplasm of *in vitro* cultured endothelial cells.

In the vertebrate host: Organisms initially replicate in the reticulo-endothelial cells in lymph nodes. They are then released into the efferent lymph stream and eventually into the blood where endothelial cells are parasitised. The organisms, which are in contact with endothelial cell membranes, are taken up through phagocytosis and enclosed in a vacuole. The vacuole membrane is therefore probably derived from host cell membranes. *Cowdria* then exists within the membrane bound vacuole in the cytoplasm of endothelial cells, developing from a single granule to a large group. This eventually causes the cell to rupture and disseminate the organisms into the blood, thus repeating the cycle (du Plessis, 1970; Prozesky & du Plessis, 1987).

In the tick vector: *Cowdria* initially appears to develop in the midgut epithelial cells. Subsequent stages of the organism may invade and develop in salivary gland acini cells. This suggests that it may be transferred to the vertebrate host via the salivary glands rather than by gut regurgitation (Prozesky & du Plessis, 1987; Bezuidenhout, 1987).

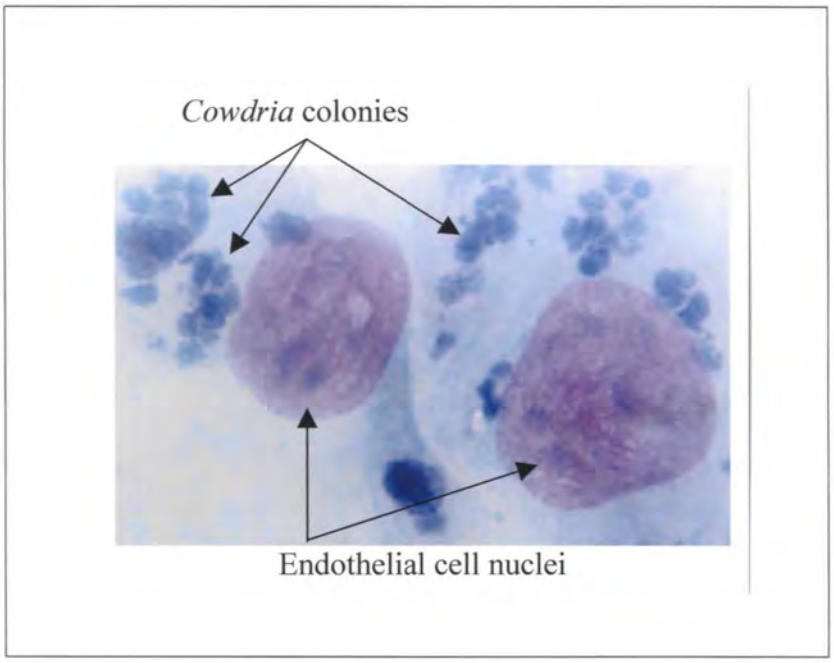

FIG. 1.2. *Cowdria ruminantium* in cultured bovine endothelial cells.

1.2.2.3. Antigenic and molecular characterisation.

Antigenic and molecular characterisation of *Cowdria* may help to provide solutions to the current problems experienced with diagnosis and vaccine development. Fifty-six isolates of *Cowdria* have reportedly been made from 19 different countries and of these, 15 originate in South Africa (de Villiers, 2001). Random amplified polymorphic DNA assays can differentiate between isolates and may be used for typing *Cowdria* (Perez *et al.*, 1997). The best characterised proteins of *Cowdria* are the 32 kDa (major antigenic protein 1, MAP1; Jongejan & Thielemans, 1989; Rossouw *et al.*, 1990), 21 kDa (MAP2; Mahan *et al.*, 1994a) proteins as well as the GroEL and GroES heat shock proteins (Lally *et al.*, 1995), the genes for which have been cloned (van Vliet *et al.*, 1994; Brayton *et al.*, 1997; Mahan *et al.*, 1994a; Lally *et al.*, 1995). Although these proteins are all conserved among isolates, genetic and antigenic diversity have been observed in the case of MAP1 (Reddy *et al.*, 1996; Perez *et al.*, 1998). This protein also varies in size (31 to 33 kDa) between the different isolates (Barbet *et al.*, 1994). Furthermore, comparisons of the 16S ribosomal RNA gene sequence (Allsopp *et al.*, 1999) indicated the existence of genetic diversity and serological analysis detected antigenic differences between isolates (Rossouw *et al.*, 1990; Jongejan *et al.*, 1988; Perez *et al.*, 1998). Therefore besides the presence of conserved proteins, there is genetic and antigenic diversity between isolates from geographically different areas. Whether there is a correlation between these two types of diversity remains to be determined.

Most serodiagnostic methods such as the indirect fluorescent antibody test (du Plessis *et al.*, 1987), immunoblotting (Mahan *et al.*, 1993) and enzyme-linked immunosorbent assays (Jongejan *et al.*, 1993) developed so far for the diagnosis of *Cowdria* are essentially nonspecific due mainly to cross-reactions with *Ehrlichia* species. Not surprisingly, therefore, the amino acid sequences

of the GroES and GroEL proteins of *Cowdria* are similar to those of the analogous proteins of *E. chaffeensis* (Lally *et al.*, 1995). Furthermore the MAP1 protein sequence is similar to the 30 kDa proteins of *E. canis* (Ohashi *et al.*, 1998a), the N-terminal of *E. chaffeensis* P28 protein and all the members of the *E. chaffeensis* outer membrane protein 1 family (Ohashi *et al.*, 1998b). Furthermore, the MAP2 protein is recognised by *E. canis* antiserum and protein sequence similarities were identified from *E. canis* and *E. chaffeensis* (Bowie *et al.*, 1999). MAP-1 antibodies cross-react with a 27 kDa protein of *E. canis* (Mahan *et al.*, 1993) and antibodies to *Ehrlichia* species react with the MAP1 protein (Jongejan *et al.*, 1993). An antigenic region of MAP1, designated MAP1b has nevertheless been identified as being specific to *Cowdria* and is used as an antigen in an indirect ELISA. This assay can detect antibodies in serum from domestic ruminants (van Vliet *et al.*, 1995; Mboloi *et al.*, 1999). However it cannot be used as an indicator of *Cowdria* exposure in cattle from areas of endemic heartwater due to undetectable antibody levels within a period of weeks after infection (Semu *et al.*, 2001).

Developing *Cowdria* specific DNA probes has also been difficult due to cross-hybridisation with *Ehrlichia* species. The gene of the 28 kDa immunodominant outer membrane protein of *E. chaffeensis* has been shown to be similar to the *Cowdria map*1 gene and the *omp-1* multigene family of *E. chaffeensis* (McBride *et al.*, 1999). Indeed, it has been proposed that the *map*1 gene is a member of a multigene family containing both conserved and variable genes (Sulsona *et al.*, 1999). However, a genomic DNA fragment termed pCS20 identified as specific for *Cowdria* was shown to be conserved between different isolates. Its biological function is still unknown (Waghela *et al.*, 1991). A PCR assay based on primers derived from the pCS20 sequence was developed and shown to be more sensitive and specific than a pCS20 DNA probe (Peter *et al.*,

1995). This PCR is currently used in epidemiological studies.

The small-subunit ribosomal RNA (16S rRNA) sequence has been used to determine the phylogenetic relationship of *Cowdria* to other Rickettsiales. *Cowdria* is phylogenetically closely related to *Ehrlichia* genogroup III species such as *E. canis* and *E. chaffeensis* and distantly related to the rickettsial species *Anaplasma marginale* in the *Ehrlichia* genogroup III complex (van Vliet *et al.*, 1992; Allsopp *et al.*, 1997). A comparative study indicated that the pCS20 probe is the most sensitive probe to use for *Cowdria* genotype identification and it shows no cross-hybridisation with *Ehrlichia* spp. On the other hand, the 16S probe was best for gathering phylogenetic information (Allsopp *et al.*, 1999).

Thus while cross-reactions with *Ehrlichia* species hamper diagnosis, divergences between isolates further complicate the search for an improved vaccine against heartwater. In order to place the strategies described later in this thesis into perspective, various aspects of cell-mediated immunity are briefly summarised below. A discussion of the specific immune responses to *Cowdria* and related pathogens follows this.

1.2.3. CELLULAR IMMUNE RESPONSES TO INTRACELLULAR PATHOGENS

Healthy vertebrates protect themselves against pathogens by means of many different mechanisms comprising both innate and specific immunity. Innate immunity cannot distinguish one pathogen from another and functions in much the same way against most infectious agents. It provides early defence and can in turn induce specific immune responses. A specific immune response can distinguish between different pathogens and is stimulated by exposure to such infectious agents. It is a complex mechanism that increases in magnitude and defence capabilities with each successive exposure to a particular pathogen. Not all pathogens grow in extracellular spaces where they are accessible to antibodies. All viruses and some bacterial pathogens and parasites enter cells where they are safe from antibody attack. To eliminate these invaders, a different system of recognition and response exists, namely the cell-mediated immune response. Cell-mediated immunity is a localised reaction to intracellular pathogens mediated by lymphocytes and phagocytes rather than antibodies. It is not possible however to consider cell-mediated and antibody-mediated responses as entirely separate. Cells are involved in the initiation of antibody responses and antibody acts as an essential link in some cell-mediated responses. Moreover no cell-mediated response is likely to occur in the total absence of antibody (Abbas *et al.*, 1997; Janeway & Travers, 1994; Schirmbeck *et al.*, 1993.).

1.2.3.1. The distribution of lymphoid tissues in the body

Lymphocytes arise from stem cells in bone marrow, and differentiate in the central lymphoid organs; B cells in bone marrow and T-cells in the thymus. They migrate from these tissues through

the blood stream to the peripheral lymphoid tissues - the lymph nodes, spleen, and gut associated lymphoid tissues such as tonsils, Peyer's patches, and the appendix. These peripheral lymphoid tissues are the sites of lymphocyte activation by antigen. Lymphocytes that circulate in the blood stream enter the peripheral lymphoid organs, and are eventually carried by lymph to the thoracic duct where they re-enter the blood stream (Janeway & Travers, 1994).

Adaptive/cellular immune responses are not initiated at the site where a pathogen first establishes a focus of infection. Pathogens infecting peripheral sites will be trapped in the lymph nodes directly downstream of the site of infection. Those pathogens that enter the blood will be trapped in the spleen and pathogens infecting mucosal surfaces will accumulate in the Peyer's patches or tonsils. All of these lymphoid organs contain professional antigen presenting cells (APC; macrophages, dendritic cells and B cells) specialised for capturing antigen and activating T-cells. Naïve lymphocytes circulate continuously from the blood stream to the lymphoid organs and back to the blood making contact with many APC every day. Naïve T-cells that recognise their specific antigen on the surface of an APC cease to migrate and differentiate into effector cells. This step takes several days and at the end of this period, the armed effector T-cells leave the lymphoid organ and re-enter the blood stream so that they can migrate to sites of infection. The number of cells that can bind and respond to a given antigen is initially very small (Abbas *et al.*, 1997; Janeway & Travers, 1994). Therefore, to generate sufficient specific effector lymphocytes to fight infection and protect the host, an activated lymphocyte must first proliferate before differentiating into effector cells (clonal expansion; Burnet, 1959).

1.2.3.2. Clonal selection

Each naïve lymphocyte present in the body has a single type of receptor that is specific for a particular epitope and can therefore only recognise one specific antigen. Lymphocytes with receptors to self-antigens are eliminated early in development, assuring tolerance of self. When the receptor on a mature lymphocyte interacts with its antigen, that cell is activated to become a blast cell (lymphoblast) and starts to divide/proliferate. This gives rise to a clone of identical progeny, all of whose receptors bind the same antigen. Antigen specificity is thus maintained as the lymphocytes first proliferate before they differentiate into effector cells. The differentiated effector cells will thus bear receptors of identical specificity to those of the parental cell. The immune response will cease once these effector cells eliminate all antigen. Not all cells become effector cells but remain as memory lymphocytes, which allow the host to respond more rapidly and intensely to a later infection/challenge with the same antigen (Burnet, 1959).

1.2.3.3. Classes of lymphocytes and their effector functions

The cells of humoral immunity are B-lymphocytes, which express antibodies on their surface that can recognise antigen and develop into antibody-secreting cells. B cells recognise regions on proteins, nucleic acids, polysaccharides, lipids and small chemicals. T lymphocytes are the cells of cellular immunity. The T lymphocytes respond to a variety of pathogens through the use of either $\alpha\beta$ or $\gamma\delta$ T-cell receptor heterodimers on their surfaces. The $\alpha\beta$ T lymphocytes (helper and cytotoxic T lymphocytes) recognise linear fragments of protein antigen, defined by amino acid sequence, bound to major histocompatibility complex (MHC) molecules. Upon activation helper T lymphocytes (Th cells) secrete a number of cytokines that stimulate or regulate activation, growth

and differentiation of lymphocytes and inflammatory cells. In this way they also provide an amplification mechanism. The Th cells do not directly attack foreign antigen or pathogen, but create conditions that allow other cells to do so efficiently. Cytotoxic T lymphocytes (CTL) similarly recognise foreign antigen on the target cells. They release perforin 1, which creates holes in the target cell or induces programmed cell death (apoptosis). The mechanism whereby cytotoxic mediators are released allows CTLs to kill single infected cells in tissue without creating wide spread tissue damage. They also produce cytokines but to a lesser extent. The γδ T-cells directly recognise proteins and non-peptide antigens and thus mediate cellular immune functions without antigen processing. The functions of γδ T-cells are similar to that of αβ T-cells i.e. they secrete various cytokines and express cytolytic functions. They also play a role in maintenance of host cell integrity and homeostasis. Their preferential localisation in epithelial layers was taken as evidence for their surveillance functions at these important sites of microbial entry. They also play a role in antimicrobial immunity (Abbas *et al.*, 1997; Janeway & Travers, 1994; Kaufmann, 1996).

1.2.3.4. Professional antigen presenting cells

Professional antigen presenting cells (APC) must be capable of presenting peptide fragments of antigen on MHC molecules and of delivering a co-stimulatory signal. There are 2 classes of MHC: MHC class I is expressed on all nucleated cells of the body while MHC class II is expressed by antigen presenting cells (Roitt, 1991; Kuby, 1994; Weenink & Gautam, 1997). In order for a T cell to recognise and respond to the antigen, an APC must express MHC molecules that the T cell recognises as self. This is known as MHC restriction (Zinkernagel & Doherty, 1997). Interaction between the Th cells or CTL's and the APC or target cells involves multiple T-cell surface proteins

that recognise different ligands on the APC or target cells. In addition to the T-cell receptor, Th cells and CTL's contain co-receptors called CD4 and CD8 respectively that interact with non-polymorphic regions of the MHC class II and class I respectively. These co-receptors facilitate the interactions of T-cells with APC or target cells. B cells, macrophages and dendritic cells are the main cell types involved in the initial presentation of exogenous antigens to naïve T-cells. These cells vary in their means of antigen uptake, MHC expression, co-stimulator expression, the antigen they present, and their locations in the body (Abbas *et al.*, 1997; Janeway & Travers, 1994).

An important consideration when searching for vaccine antigens is that T-cells with different functions recognise peptides produced in two distinct intracellular compartments within the APC. The mode of antigen processing and presentation determines which T-cell subset is activated in an immune response. Two alternate pathways have been defined: MHC class II restricted presentation of an extracellular protein antigen and MHC class I restricted presentation of an endogenously synthesised protein (Brodsky & Guagliardi, 1991).

MHC class II restricted presentation

Extracellular protein is internalised by endocytosis into a phagosome by an APC and processed in the resulting endosome to generate peptides. Nascent Class II MHC molecules are associated with an invariant chain that blocks peptide binding during the initial stages of class II assembly and export, and contributes to the endosomal targeting of these molecules. Vesicles containing either peptides or MHC class II molecules/invariant chains fuse. The invariant chain dissociates and the peptides bind to MHC class II molecules. The peptide-MHC complex is then expressed on the cell surface and presented to a $CD4^+$ T-cell. Thus extracellular antigens preferentially stimulate $CD4^+$

Th cells (Weenink & Gautam, 1997).

MHC class I restricted presentation

When a parasite infects a cell, its protein is degraded in the cytosol into peptides and transported to the endoplasmic reticulum. Here the foreign peptides bind to MHC class I molecules that are not associated with an invariant chain and are therefore free to bind to peptides produced within the cell. The peptide-MHC complexes are then transported through the Golgi into exocytic vesicles that fuse with the plasma membrane. These complexes are expressed on the cell membrane and presented to $CD8^+$ T-cells. In contrast to the MHC class II pathway, synthesis of antigenic proteins within a cell thus preferentially stimulates $CD8^+$ CTL's (Townsend and Bodmer, 1989; Ojcius *et al.*, 1996).

The division of the antigenic world into class I versus class II presentation systems correlates fairly well with the division into endogenously synthesized antigens versus exogenously introduced soluble proteins. There is however evidence to suggest that class I restricted T-cells can be induced against exogenously introduced, nonreplicating antigens of which a few examples are given here. Immunisation with a soluble T-Ag fragment of SV40 without adjuvant (Schirmbeck *et al.*, 1993), purified HIV-1 envelope protein in immunostimulating complexes (ISCOMs) (Takahashi *et al.*, 1990), viral proteins in the presence of cellular debris (Bachmann *et al.*, 1994), soluble recombinant HIV protein entrapped in biodegradable microparticles (Moore *et al.*, 1995), injection of virus-like particles without adjuvant (Schirmbeck *et al.*, 1996) and synthetic peptides entrapped in microparticles (Nixon *et al.*, 1996) have all led to induction of CTL responses. There therefore seems to be no absolute requirement for a protein to be

endogenously synthesized in the APC in order to induce class I restricted CTL. Rather the form of the endocytosed antigen and its subcellular localisation may determine class I versus class II presentation. The mechanism of the alternate pathways remains unclear. Peptides may be generated by vacuolar processing, as this appears to occur without cytosolic antigen delivery. Transit of peptides through the cytosol to the endoplasmic reticulum also appears not to be necessary. Peptides generated by vacuolar processing may bind to MHC-I molecules that have already exited the endoplasmic reticulum/Golgi. They could therefore bind MHC-I molecules directly within vacuolar compartments or to MHC-I molecules on the surface after peptide regurgitation from intracellular compartments (Harding & Song, 1994). This alternative pathway provides a mechanism whereby non-viable antigen preparations can conceivably be used to effectively induce CD8+ T-cell responses when such responses are necessary for protective immunity.

1.2.3.5. Cytokines

Cytokines mediate communication within the immune system and between immune and other cell types (Gallagher *et al.*, 1993). They are small soluble proteins produced by one cell that alter the behaviour or properties of another. Cytokines that are produced by T-cells are called interleukin (IL-) followed by a number. An individual cytokine is able to stimulate the production of many others, generating a network that orchestrates the host's immune response to infection. Cytokines are important mediators of the protective and pathological immune responses (Kuby, 1994). A critical role for cytokines in the pathogenesis and control of a number of protozoan diseases has now been established (McKeever *et al.*, 1997).

$CD4^+$ T-cells exert most of their helper functions through secreted cytokines, which either act on the cells that produced them or modulate the responses of other cells. To generate an appropriate immune response, antigen stimulated T-cells must differentiate to a particular phenotype of cytokine production (Caruso *et al.*, 1998). Three different subsets of $CD4^+$ cells have been described on the basis of the cytokines that they produce: (1) Th1 subset which produces IL-2, interferon-γ (IFN-γ) and tumor necrosis factor-β (TNF-β); (2) Th2 subset which produces IL-4, IL-5, IL-6, IL-10 and IL-13 and (3) Th0 subset which produces cytokines characteristic of both Th1 and Th2 subsets (Mosmann *et al.*, 1986; Mosmann & Coffman, 1989; Street & Mosmann, 1991; Cox & Liew, 1992; Abbas *et al.*, 1996; Mosmann & Sad, 1996). Cells producing large amounts of transforming growth factor β (TGF-β) have been termed Th3 (Chen *et al.*, 1994). The Th1 cytokines, of which IFN-γ is the principal effector cytokine, mediate macrophage activation and delayed-type hypersensitivity reactions while the Th type 2 cytokines act as growth and/or differentiation factors for B cells (Romagnani, 1996). IL-2 and IL-4 are the autocrine growth factors of Th1 and Th2 subsets respectively (Mosmann & Coffman, 1989). Cytokines secreted by one subset can block the production or activity of the cytokines secreted by the other subset and thus exhibits cross-regulation. For example, IFN-γ secreted by Th1 cells inhibits proliferation of the Th2 subset, and IL-10 secreted by Th2 cells downregulates the secretion of IFN-γ and IL-2 by the Th1 subset (Fig. 1.3.; Kuby, 1994). Pathogens often appear to stimulate only one Th subset, different subsets at different stages of infection or both simultaneously, but at different sites (Scott & Kaufmann, 1991). Distinct cytokine-secreting subsets of $CD8^+$ T-cells have also been identified: Tc1 cells secrete IL-2 and IFN-γ and Tc2 cells secrete IL-4, IL-5 and IL-10 (Mosmann & Sad, 1996). IFN-γ is secreted by CTL and Th1 cells at about the same level whereas most other cytokines are secreted at lower levels by CTL and

Th1 cells (Street & Mosmann, 1991). Similarly Th1 γδ cells produce IL-2 and IFN-γ and Th2 γδ cells produce IL-4, IL-5, IL-6 and IL-10 (Hayday, 2000).

There is no doubt that the Th1/Th2 paradigm defined for murine and human Th cell clones is an oversimplification of a more complex immunoregulatory network. Many infections are cleared by a combination of inflammatory and antibody responses. As more sensitive assays for cytokine detection have been developed, more heterogeneity among Th cell clones has been discovered. There is evidence to suggest that a T-cell response to a given pathogen or antigen is heterogeneous, even though a predominant type 1 or type 2 response usually occurs. Expression of IL-2, IL-4, IFN-γ, and IL-10 in cattle is differentially regulated. Furthermore IL-10 is not a Th2 cytokine in cattle as reported in mice, but as with human T-cells, IL-10 is expressed by all subtypes of Th cells (Brown *et al.*, 1998b).

Production of IFN-γ is important for optimal defence against intracellular pathogens especially those living in mononuclear phagocytes (Billiau *et al.*, 1998). Of particular relevance to this thesis is the fact that IFN-γ has been identified as playing an important role in protection against almost all of the tick-borne diseases including heartwater (Brown *et al.*, 1996 & 1999; Preston *et al.*, 1992; Kodama *et al.*, 1987; Totté *et al.*, 1996 & 1999a). Accordingly, work on anti-tick borne disease vaccines is targeted at vaccines that stimulate IFN-γ producing $CD4^+$ T-cells.

1.2.3.5.1. Interferons

Interferons (IFNs) are amongst the most important cytokines involved in modulating the functions of macrophages (Gessani & Belardelli, 1998). During viral infections macrophages are among the first cells in any organ to be exposed to the intruders. They are generally considered to be the major producers of IFN soon after infection. Most IFN's belong to one of three classes: α, β or γ. Two additional classes have also been discovered: ω and τ. IFN-α, β, ω and τ are classified as type I IFNs as they have more in common structurally and functionally. IFN-γ stands by itself as a type II molecule. Both type I and II are able to interfere with viral replication in cells and modulate activities of the immune system. Essentially all cell types in the body can produce IFN type I, but only T lymphocytes, natural killer (NK) cells and macrophages (all members of the immune system) release IFN-γ. They do so not when they are infected, but when they are alerted to the presence of viruses, bacteria or parasites in other cells, or to the emergence of cancer (Johnson *et al.*, 1994).

Type II IFN: IFN-γ is a 45-kDa homodimeric glycosylated protein produced only under pathologic circumstances (trauma, infection, cancer and autoimmunity) by particular activated lymphocyte populations ($CD4^+$, $CD8^+$), NK cells and macrophages (Billiau *et al.*, 1998). It has immunomodulatory effects on a variety of immune cells, exerting its multiple biological activities by controlling the expression of many genes. This leads to macrophage activation and T-cell differentiation towards a Th-1 type immune response. Production of IFN-γ by either T or NK cells requires co-operation of accessory cells, mostly mononuclear phagocytes, which also need to be in some state of activation (Gessani & Belardelli, 1998). IFN-γ production is up regulated by IL-12 and down-regulated by IL-10. IFN-γ can therefore be considered as a virtual

immunologic switch (Johnson *et al*, 1994).

A few of the most important functions of IFN-γ are listed briefly: 1) enhancement of the expression of antigen presentation to T-cells, thus amplifying the recognition phase of the immune response; 2) promotion of differentiation of T lymphocytes into the Th 1 subset; 3) activation of macrophages and neutrophils up-regulating their respiratory burst; 4) stimulation of the cytolytic activity of NK cells; 5) activation of vascular endothelial cells promoting $CD4^+$ T lymphocyte adhesion and facilitation of lymphocyte extravasation; 6) co-responsibility for the adhesion of lymphocytes to endothelial cells in postcapillary veins; and 7) regulation of chemokine secretion by endothelial cells. The net effect of these varied activities of IFN-γ is to promote macrophage inflammatory reactions (Abbas *et al.*, 1997; Janeway & Travers, 1994).

The mechanisms of immunity to diseases and the identity of antigens recognised by protective immune responses can indicate which antigens are likely to be effective in subunit vaccines and also highlight the type of antigen delivery system that will be required for a vaccine to induce a protective immune response. Understanding how immune responses are regulated in ruminants is critical for devising strategies to direct an immune response toward a desired effector function required for either preventing infection or eliminating disease. The following section illustrates that knowledge of the protective immune responses to parasites can be utilized in the search for protective antigens.

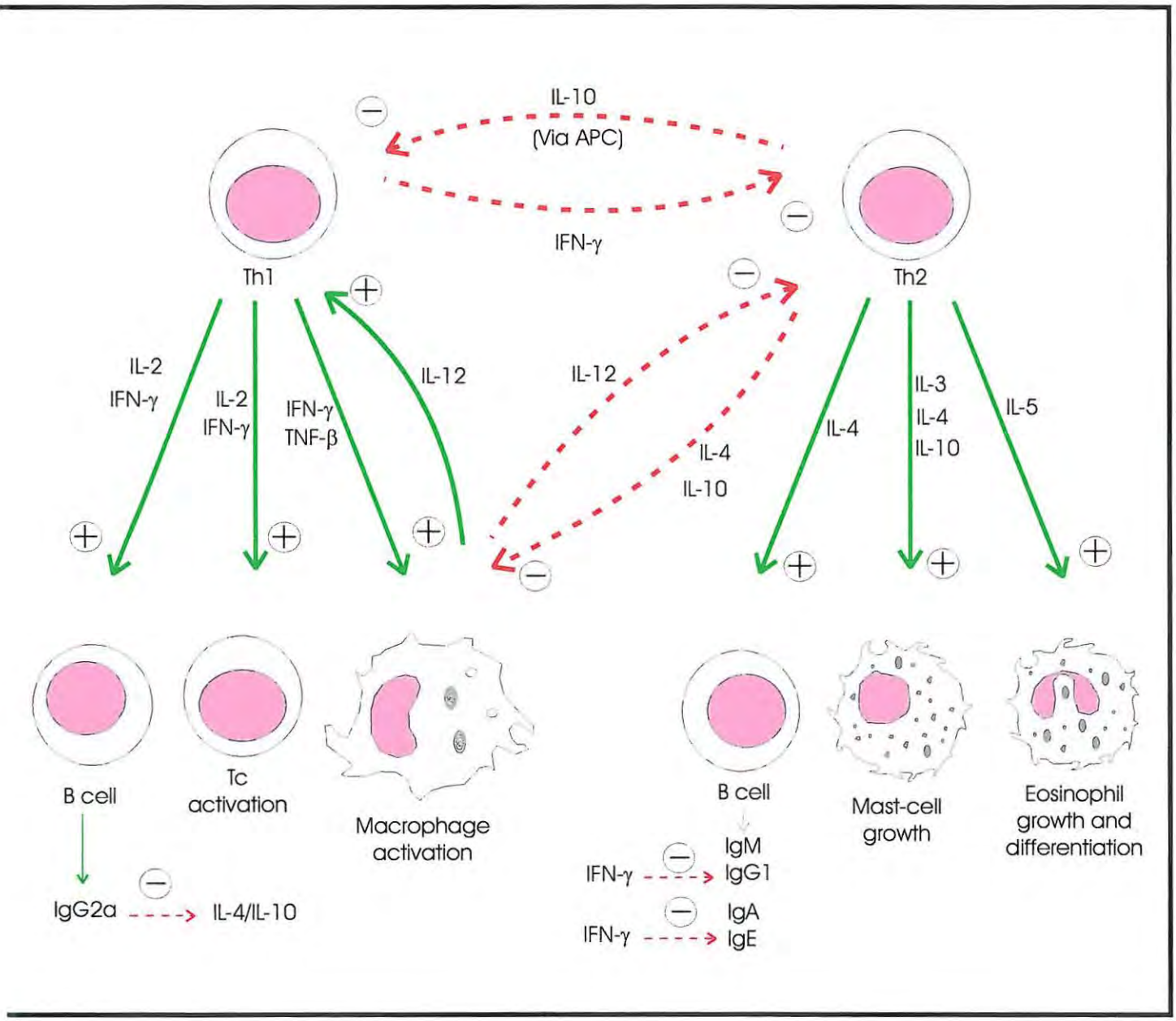

FIG. 1.3. Cytokines secreted from Th1 and Th2 lymphcyte subsets and their cross-regulation. Solid arrows indicate stimulatory effects and dashed arrows indicate inhibitory effects (adapted from Kuby, 1994).

1.2.4. ANTIGENICALLY DEFINED VACCINES

One of the most effective and most versatile means to combat infectious diseases is vaccination (Mäkelä, 2000). In 1798, Edward Jenner was able to prevent smallpox by inoculation with pus from cowpox sores containing the virus *Vaccinia* (Jenner, 1798). It took nearly 100 years before the next vaccines were developed by Pasteur: killed attenuated *Pasteurella multocida* for animals in 1880 (Pasteur, 1880), anthrax in 1881 (Pasteur *et al.*, 1881) and a rabies vaccine in 1885 (Pasteur, 1885). These were followed by bacterial cell vaccines for typhoid fever (Salmon & Smith, 1886), cholera (Kolle, 1896), and the plague (Haffkine, 1887). After these initial successes, and notwithstanding advances in our knowledge, the rate of new vaccine development in the following century was modest by comparison.

The two basic types of current vaccines, i.e. presenting the microbe in an attenuated but live form or as an inactivated, killed vaccine, were already among the first vaccines introduced in the 1800's. A third type of vaccine introduced against diphtheria and tetanus in the 1920's is a purified bacterial component. These purified single component vaccines are attractive as the immune response is maximally directed to the molecule relevant for protection and additional components that could cause adverse reactions are avoided. Microbes (such as *Cowdria*) that cannot be grown, or can only be grown with difficulty *in vitro*, pose a special problem to vaccine development. A real breakthrough was the genetic technology that allows for microbial genes to be transferred and expressed as recombinant products in easily grown organisms, such as *Escherichia coli, Bacillus subtilis* and yeast. However, selection of an appropriate host is important if the recombinant product requires post-translational modifications (Mäkelä, 2000;

Liu, 1998). Recombinant DNA technology has also made it possible to use safe viral (Boyle, 1994) or bacterial vectors (Hodgson, 1994) for the expression of protective antigens from dangerous pathogens i.e. recombinant vector vaccines. The molecular biological revolution, which started in the early 1950s, produced the first recombinant vaccine for human use, the hepatitis B vaccine in the 1980s (McAleer *et al.*, 1984). In 1990 the use of naked DNA encoding immunogenic proteins as a vaccine was reported (Wolff *et al.*, 1990).

Most vaccines are designed as a prophylatic measure, i.e. to stimulate the immune response so that on subsequent exposure to the particular infectious agent, infection is either prevented or limited so that clinical disease does not occur. A complicating factor is that whole proteins from pathogens may contain epitopes that inhibit protective immune responses. Some protein sequences may even mask the recognition of cryptic determinants that would be elicited by peptide immunisation. These negative effects may be avoided if T-cell epitopes that specifically stimulate protective immune responses are identified. The conventional approach to vaccine design is the identification of dominant antigenic epitopes associated with protective immune responses and the construction of synthetic or recombinant epitopes in a form suitable for immunisation (Cox & Liew, 1993).

T-cell epitopes can be identified by measuring T-cell proliferation in the presence of overlapping peptides covering the whole protein (Fujii *et al.*, 1997; Burton *et al.*, 1999). Peptides that induce specific CTL responses can be identified by e.g. pulsing dendritic cells with a panel of peptides and assaying for CTL responses (Jin *et al.*, 2000). These methods require the synthesis of a great number of peptides. For example, to test a 20 mer peptide with 10 amino acid overlaps spanning

the length of a given protein one would need to construct (x/10)-1 peptides, where x is the length of the protein in amino acid residues. Amino acid substitutions within epitopes has led to the identification of MHC binding and TCR contact residues (Burton *et al.*, 1999). Although this method has been used successfully to predict T-cell epitopes, with the assurance that no potential epitope will be missed, the cost and labour required often makes this strategy impractical (Sercarz *et al.*, 1993). Synthetic peptide libraries generated by solid-phase synthesis strategies have been developed into another powerful tool for identifying of biologically active substances. Although synthetic peptide libraries do not yield natural epitopes, they may lead to the identification of the natural sequences from databases. However protein databases are incomplete and genomic sequencing is therefore important for the successful application of this method of epitope identification (Hiemstra *et al.*, 2000).

Another approach that is sometimes successful is the use of epitope prediction algorithms. T-cell epitopes are predicted from protein sequences of known cytotoxic and helper T-cell epitopes where analysis has revealed similarities within their primary sequences. This method reduces the number of peptides to be examined as only those that meet the requirements of the algorithm are sequenced. Such prediction templates have been successfully used to define eight helper and three cytotoxic epitopes in four different proteins (Rothbard and Taylor, 1988). The specificity of interaction of the T-cell receptor with the antigenic fragment is greater than that of the presenting cell. T-cell antigenic sites are frequently amphipathic structures i.e. one portion of the molecule is hydrophobic and the other hydrophilic. The polar portion of the molecule may be recognised by the T-cell receptor whereas the apolar side may interact with the presenting cell (Berzofsky *et al.*, 1987). An amphipathic helix model algorithm (AMPHI algorithm) was

developed in which antigenic sites are postulated to be helices with one face predominantly polar and the opposite face predominantly apolar. Using this algorithm, 18 of the 23 known immunodominant helper T-cell antigenic sites located on 12 proteins have been identified (Margalit *et al.*, 1987). In 1996, the EpiMer algorithm was developed which searches a protein's primary amino acid sequence for known MHC-binding motifs. It identifies regions of the sequence that contains clusters of binding motifs, which may permit a peptide derived from that sequence to bind to genetically distinct MHC molecules. This algorithm does not predict all possible epitopes within a given protein antigen but may predict those epitopes able to bind to multiple MHC alleles. It was used to predict peptides of four HIV proteins that have potential to bind to multiple MHC alleles (Roberts *et al.*, 1996). A similar algorithm, the EpiMatrix, is a non-anchor-based computer algorithm that predicts MHC-binding regions of proteins by searching for regions that score well in a previously defined MHC-binding peptide matrix. The advantage of matrices is that peptides can be given a score that represents the potential of each amino acid in the sequence to promote or inhibit binding. This technique was used to identify the top 2% of peptides from the viral sequence with the highest potential to bind to HLA-B7. Ten out of the 55 peptides synthesised were shown to be binders (Jin *et al.*, 2000). Another study found that a statistically significant tendency of T immunogenic peptides is to contain clusters of rare tetrapeptides. This result was used to locate potential T epitopes in the HIV *gag* protein that were recognised by a HLA-A2 restricted human cytotoxic T cell line (Claverie *et al.*, 1988). The methods developed by Rothbard and Taylor (1988), Margalit *et al.* (1987) and Claverie *et al.* (1988) were used to predict sequences of the ROP2 protein of *Toxoplasma gondii* containing potential T-cell epitopes. An epitope was identified (predicted by all three methods) that is recognised by a high percentage of the immune population that further strengthened its potential

as a vaccine candidate (Saavedra *et al.*, 1996).

While the abovementioned approaches have been successful in the design of vaccines against viral infections, they have been less so in attempts to combat protozoan infections, mainly because of the complexity of the responses against eukaryotic cells. Furthermore, the organisms are antigenically complex and have life cycles in which the various stages frequently differ antigenically from one another. The parasites themselves often have the ability to manipulate the immune response for their own survival, evading the consequences of immune attack. In addition, the protective proteins must first be identified and their amino acid sequence determined before these techniques can be applied.

In parasitic infections, one of the two immunological pathways (Th1 & Th2) is protective and the other counter-protective. However, it should be possible, by systematically investigating both T-cell subsets and cytokine profiles, to obtain a very good idea about which components of the host reaction to infection are essential and protective. As particular epitopes drive the immune system towards either Th1 or Th2 responses, vaccines can be designed to induce either response. The approaches used to identify antigens for subunit vaccine development are guided by the type of immune responses that are likely to mediate protection (Musoke *et al.*, 1997) as is illustrated in the examples discussed below.

1.2.4.1. Malaria

The malarial parasite has a complex life cycle that presents several targets for attack, each of which is susceptible to different types of host immune response (Jones & Hoffman, 1994). Several epitopes have already been characterised and development of multivalent vaccines is currently a promising area of research (Kima *et al.*, 1992). Immunisation with radiation-attenuated sporozoites protects animals and humans against malaria. Antibodies and cytotoxic T lymphocytes against the circumsporozoite protein are thought to be important in mediating this immunity. However, immunisation with subunit vaccines based on the circumsporozoite protein has not given the complete protection found after immunisation with attenuated sporozoites. Thus other antigens may contribute to the protective immunity observed. Mice immunised with P815 mouse tumor cells transfected with a mixture of sporozoite surface protein 2 and circumsporozoite genes were completely protected against malaria (Khusmith *et al.*, 1991). Immunisation with recombinant yeast retrotransposon (Ty) and the modified Ankara vaccinia virus containing a single *Plasmodium berghei* class I-binding epitope provided 95% sterile protection against malaria in mice (Plebanski *et al.*, 1998). In another study protection was elicited in malaria-naïve monkeys by vaccination with the C terminus of the 19 kDa protein of the major merozoite surface protein (MSP-1) fused to tetanus toxoid universal T-cell epitopes P30 and P2 (Egan *et al.*, 2000). These studies emphasise the importance of delivery systems and multi-antigen combinations if protective immunity against malaria is to be achieved.

1.2.4.2. Bacteria and viruses

The development of protective immunity against many intracellular bacterial pathogens has proven problematic, as induction of protective immunity requires recovery from sublethal infection with viable forms of the bacteria. Such infection results in the *in vivo* activation of specific cell-mediated immune responses and both $CD4^+$ and $CD8^+$ T lymphocytes may function during the induction of this protective immunity. The vaccination of experimental animals with plasmid DNA containing genes encoding unique proteins has proven effective in generating both humoral and cellular antigen specific immune responses (Cornell *et al.*, 1999). Such immunisation has also led to protective immunity in several animal models of viral disease: influenza (Ulmer *et al.*, 1993); HIV (Boyer *et al.*, 1997) and rabies (Xiang *et al.*, 1994). Historically, experimental infection of mice with *Listeria monocytogenes* has evolved as the prototypic model for characterising protective immunity to intracellular bacterial pathogens. Genetic immunisation with a specifically designed plasmid DNA construct was shown to mimic the antigen specific immune CTL response observed following sublethal infection with this pathogen and to provide protective immunity (Cornell *et al.*, 1999). These results demonstrate that this experimental approach may therefore be applied to preventing diseases caused by intracellular bacterial pathogens.

1.2.4.3. Babesiosis

Babesia bovis is an intraerythrocytic protozoan parasite causing a virulent form of babesiosis in cattle. Vaccination against babesiosis is achieved using organisms of reduced virulence. Anti-*Babesia* vaccines are targeted at epitopes that stimulate IFN-γ producing $CD4^+$ T-cells as all

Babesia specific $CD4^+$ T-cells that have been examined to date produce IFN-γ (Brown *et al.*, 1996). Identification of the antigens involved has focused on the surface of the merozoite and infected erythrocytes. Several different strategies have been used to identify potentially protective *B. bovis* proteins (reviewed in Brown & Palmer, 1999). One strategy involved testing biochemically fractionated merozoite antigens systematically in immunisation and challenge experiments. In another approach, proteins were fractionated by continuous flow electrophoresis and selected by their ability to stimulate IFN-γ secreting $CD4^+$ T-cells isolated from immune cattle. Fractions with apparent molecular masses of 20-23, 40, 51-52 and 58-60 kDa were identified (Brown *et al.*, 1995). Several of these fractions stimulated proliferation of T-cell lines from each of the four immune cattle, suggesting that the fractions are not likely to be restricted to a single MHC class II allele (Brown *et al.*, 1998a). A third approach involved proteins selected on the basis of the function required for parasite survival. This involved defining merozoite surface proteins and apical complex proteins implicated in erythrocyte invasion, which would be subject to neutralising antibodies. The rhoptry associated protein-1 (RAP-1) antigen was one of a few identified by all three approaches (reviewed in Brown & Palmer, 1999). This protein meets certain criteria likely to be essential in development of a subunit vaccine against *Babesia*. For instance it contains T- and B-cell epitopes conserved among strains and which are not subject to antigenic variation. They can thus be recognised by a diverse repertoire of MHC class II molecules. Vaccination with this protein resulted in partial protection of cattle (Brown *et al.*, 1998a). Vaccine development against babesial parasites still remains a challenge, however, and the search for protective proteins and their epitopes continues.

1.2.4.4. Theileriosis

Theileria annulata is an intracellular parasite of bovine macrophages. It transforms the host cell into a rapidly expanding population of metastatic cells. An effective vaccine based on schizont-infected cells is available (Musoke *et al.*, 1997). Both innate and adaptive responses contribute to recovery from infection and resistance to challenge, while the cytokines produced by infected and uninfected cells influence the outcome of infection. Nothing is known about the stimuli that activate innate immune responses, the antigens that stimulate adaptive immune responses to *T. annulata* or how the host's immune system recognises parasite antigens. It is speculated that IFN-γ and IFN-α may play an important role in innate immunity, and resistance may largely be due to the speed with which innate immune responses develop. Due to the complexity of the *T. annulata* life cycle, there are three potential targets for a subunit vaccine: the sporozoite (to reduce the infective dose), the schizont (to control proliferation of infected mononuclear cells and pathology) and the merozoite/piroplasm (to reduce infection of erythrocytes and anaemia, and to limit transmission). The types of antigens to be included in a subunit vaccine should therefore include the following: 1) Sporozoite antigens that will initiate $CD4^+$ T-cell responses. These will generate parasiticidal cytokines, such as TNF-α and IFN-γ, and/or activate macrophages to produce nitric oxide; 2) Schizont antigens that will stimulate $CD4^+$ T-cells to produce IL-2 and IFN-γ. The IL-2 will enhance T-cell proliferation and the IFN-γ will activate macrophages to produce parasiticidal factors such as TNF-α and nitric oxide and IL-12 to activate NK cells to produce parasiticidal IFN-γ; 3) Antigens stimulating specific responses such as cytotoxic T-cells. These cells will eliminate schizont-infected cells and generate parasiticidal by-products like IFN-γ, TNF-α and nitric oxide; and 4) Invariant molecular structures recognised by the innate immune responses. These may include lipid and carbohydrate antigens as well as protein antigens

(reviewed in Preston *et al.*, 1999). Trials with two recombinant antigens, SPAG-1 (from the sporozoite surface) and TAMS (from the merozoite surface) induced partial protection against sporozoite or merozoite challenge, respectively. Efficacy depended on the delivery system used (Reviewed in Boulter & Hall, 1999). Further studies are under way to identify additional antigens and to improve the efficacy of vaccine delivery.

T. parva infects and transforms bovine lymphocytes, causing a severe lymphoproliferative disease called East Coast fever (ECF). The current method of immunisation against ECF is infection with cryopreserved sporozoites and simultaneous treatment with long acting oxytetracycline (Musoke *et al.*, 1997). MHC-class I-restricted parasite-specific cytotoxic T lymphocytes are believed to be the major protective immune effector mechanism deployed by cattle against *T. parva* (reviewed in McKeever *et al.*, 1999). There is evidence that $CD4^+$ T-cell help is required for both the induction and recall of this response. Sporozoites are naturally poor immunogens but antibodies raised against the sporozoite surface antigen p67 neutralise infection *in vitro* and can protect against needle delivered challenge with the parasite (Musoke *et al.*, 1997). A recombinant fusion p67 protein formulated in saponin was used to immunise cattle. Six out of nine cattle were immune to challenge. Current efforts are directed towards increasing the degree of protection by improving the immunisation scheme, and identification of schizont antigens recognised by cytotoxic T lymphocytes, which may provide more long lasting immunity. Candidate vaccine antigens currently under evaluation are the sporozoite molecule (SPAG1) which induces sporozoite neutralising antibodies and two merozoite antigens namely the 30 kDa and Mag 1 (McKeever *et al.*, 1999).

1.2.4.5. Rickettsia

Rickettsiae (of which *Cowdria* is a member) are small obligate intracellular coccobacillary bacteria that reside free in the cytosol of host cells (Winkler, 1990). Immunity to members of the genus Rickettsia (*Rickettsia tsutsugamushi, R. typhi, R. conorii, R. akari, R. prowazekii*) is thought to be mediated primarily by cellular mechanisms. This has been suggested on the basis of well established *in vitro* parameters of cell mediated immunity including lymphocyte proliferation in response to specific antigenic stimulation (Jerrells *et al.*, 1986; Osterman, 1985). In particular, antigen specific helper T-cells (producing IFN-γ) were indicated to play an important role in protection against rickettsial infection (Kodama *et al.*, 1987). This cytokine has been shown to inhibit the growth of intracellular rickettsiae (Wisseman & Waddell, 1983; Turco & Winkler, 1983). In addition the principal effector of rickettsial killing has been shown to be the macrophage (Osterman, 1985). A 47 kDa (Hickman *et al.*, 1993) and 56 kDa protein (Seong *et al.*, 1997) of *R. tsutsugamishi* and a 198 kDa protein of *R. conorii* have been identified as candidates for vaccine development against scrub typhus and boutonneuse fever respectively.

1.2.4.6. Anaplasmosis

Anaplasma is an intraerythrocytic rickettsia within genogroup II of the ehrlichial complex. It causes babesiosis in cattle. Several methods are available for immunisation against anaplasmosis. These include live virulent or less virulent, attenuated or killed organisms. An undesirable feature of these methods is that inoculation is invariably followed by infection which can be severe in some cases. In anaplasmosis, *Anaplasma marginale* clearance is effected by antibody against surface B-cell epitopes (called major surface proteins, MSP) in combination with $CD4^+$ T-cell

mediated macrophage activation that enhances opsonisation and microbial killing (Palmer *et al.*, 1999; Brown *et al.*, 1998 c & d). $CD4^+$ T-cells expressing IFN-γ play a very important role by providing help to B cells for production of opsonising IgG2 antibodies. They also affect macrophages by increasing receptor expression, phagocytosis, phagolysosomal fusion and release of rickettsiacidal nitric oxide (Brown *et al.*, 1999). Immunisation with purified MSP has been shown to prevent *A. marginale* rickettsemia upon challenge. Protection correlated with specific antibodies to MSP epitopes (Brown *et al.*, 1998c). However, rapid generation of MSP antigenic variants limits the effectiveness of this vaccine. Identifying invariant conserved MSP B-cell epitopes and linked T-cell epitopes that induce sufficient immunity to limit morbidity and mortality rather than prevention of infection is the current goal of *Anaplasma* vaccine research.

In each of the above diseases, all recombinant antigens evaluated so far fall short of the requirement to induce immunity comparable to that induced by the current live/attenuated vaccines. The failure of recombinant antigens to induce solid protection, in spite of promising results with native proteins, may result from the inability of the expression systems currently employed to make secondary modifications to the expressed products that are important for immune recognition. Moreover, additional antigens may be essential for the induction of appropriate responses. It is clear from the above examples that antigens capable of inducing the production of Th1 cytokines including IFN-α and IFN-γ have potential in the development of a recombinant vaccine. Nevertheless much research is still warranted. The following section discusses what is known about the immune responses to *Cowdria*. This knowledge will ultimately help in development of an improved heartwater vaccine.

1.2.5. IMMUNE RESPONSES TO *Cowdria ruminantium*

The earliest work done in order to try and unravel the immune response to *Cowdria* indicated that antiserum from heartwater immune cattle was not able to passively transfer protective immunity to naïve animals (Alexander, 1931; du Plessis, 1970). This finding does not, however, exclude the participation of antibodies in opsonisation, complement-mediated killing and antibody-dependent cell mediated cytotoxicity in combination with a cellular immune response directed towards heartwater. As *Cowdria* is largely an intracellular parasite, both $CD8^+$ cytotoxic T-cells and $CD4^+$ helper T-cells may be important in the development of protective immunity. It has been shown that cattle immunised against heartwater by infection and treatment generate lymphocyte responses characterised by a mixture of $CD4^+$, $CD8^+$ and γδ T-cells and expression of IFN-γ, TNF-α, TNF-β and IL-2. Proliferation was observed *in vitro* when peripheral blood mononuclear cells (PBMC) were exposed to either autologous infected endothelial cells or infected monocytes, but not to elementary bodies of the organism. The endothelial cells required pre-treatment with T-cell growth factors to induce class II MHC expression prior to infection and their subsequent use as stimulators of PBMC (Mwangi *et al.*, 1998a). $CD4^+$ T-cell responses specific for the MAP1 and γδ T-cell responses specific for the MAP2 recombinant antigens of *Cowdria* are also induced by this method of immunisation. Both proteins induced a T helper type 1 immune response (Mwangi *et al.*, 1998c).

Several studies have demonstrated that animals can be protectively immunised with either culture-attenuated (Jongejan, 1991) or inactivated organisms (Martinez *et al.*, 1994; Mahan *et al.*, 1995), the latter especially suggesting that the development of a subunit vaccine for *Cowdria*

may indeed be feasible. Vaccination with culture-attenuated organisms has disadvantages in that cross-protection against different isolates is not complete and it would therefore be necessary to attenuate several strains to cover the entire antigenic repertoire. A further complication is that only certain strains can be attenuated by *in vitro* passage which obviously limits the use of this method of vaccination (Jongejan, 1991). On the other hand immunisation with inactivated *Cowdria* in an adjuvant could potentially allow many strains to be included in a vaccine. PBMC from animals made resistant to challenge by vaccination with inactivated organisms contain *Cowdria*-specific, MHC class II-restricted, IFN-γ-producing, $CD4^+$ T lymphocytes (Totté *et al.*, 1997). $CD4^+$ T-cell lines generated using *Cowdria* lysates responded strongly to whole lysates but not to recombinant MAP1 or MAP2 proteins (Totté *et al.*, 1998). When these cell lines were stimulated with soluble *Cowdria* proteins purified by fast performance liquid chromatography, a single peak which included proteins between 20 and 32 kDa induced proliferation (Totté *et al.*, 1999a). On the other hand, γδ-T-cell lines responded specifically to recombinant MAP1 and MAP2 (Totté *et al.*, 1998). Flow cytometric analysis of PBMC showed no significant change in the immune cell population after vaccination and boosting with inactivated organisms. Nevertheless significant changes occurred after challenge, including an initial progressive depletion of $CD4^+$, $CD8^+$ and γδ T-cell subsets and a rise in numbers and activation of monocytes. This was followed by an increase in $CD8^+$ T lymphocytes (Martinez, 1997). This is in accordance with previous findings suggesting that $CD8^+$ T-cells play a major role in immunity to heartwater (du Plessis, 1982; du Plessis *et al.*, 1991 & 1992a).

Cattle immune to lethal challenge produced IFN-α, whereas animals that died did not produce this cytokine (Totté *et al.*, 1994a). However, IFN-α alone could not completely inhibit the *in*

vitro growth of *Cowdria* suggesting that other factors may be required for complete inhibition. The type 2 cytokine IFN-γ has been shown to be a very potent inhibitor of *Cowdria* growth in endothelial cells. However, the mechanisms whereby IFN-γ inhibits the growth of *Cowdria* still remains to be determined (Totté, *et al.*, 1999a) but there are at least four pathways through which it could play a role in protection. It might directly affect endothelial cells, making them in some way unsuitable for Cowdria growth. The effect appears to depend on the dose since low doses inhibited growth without inducing nitric oxide or cytotoxicity (Totté *et al.*, 1996). On the other hand, high doses also inhibited growth, but increased nitric oxide production and induced apoptosis of infected and non-infected endothelial cells (Mutunga *et al.*, 1998). Another important role of IFN-γ is the upregulation of MHC class I and II expression on various cell types (Coutinho *et al.*, 1991). This effect on monocytes will lead to enhanced presentation of *Cowdria* antigens to the immune system. IFN-γ also leads to increased phagocytosis by monocytes and release of reactive oxygen intermediates, nitric oxide and lysosomal enzymes such as myeloperoxidase (MPO). Recombinant MPO confers protection against heartwater in a murine model, possibly through a direct toxic effect on extracellular organisms (Tournay *et al.*, 1996). *Cowdria* has also been shown to elicit *de novo* synthesis of IL-1β, IL-6 and IL-8 mRNA in bovine brain endothelial cells *in vitro*. This effect was potentiated by INF-γ (Bourdoulous *et al.*, 1995). IL-1 and IL-6 can act as co-stimulatory signals for T- and B-cell activation (Weaver & Unanue, 1990). It can be speculated that by secreting these cytokines, brain endothelial cells may contribute to protective immune responses. Consequently, antigens capable of inducing the production of these cytokines by immunocompetent cells should be investigated if a subunit vaccine against heartwater is to be developed.

To date, stocks of *Cowdria* from geographically widely separated areas have shown either complete, partial or no cross-immunity *inter se* or against the reference (vaccine) Ball3 isolate, when tested in goats (Jongejan *et al.*, 1988), sheep (du Plessis *et al*, 1989) or mice (Stewart, 1989). Antigenic differences between the isolates complicate immunisation against the disease and may explain some of the disappointing results that have been reported using the current vaccine (Uilenberg, 1983). Immunoblotting of *Cowdria* proteins with sheep or bovine anti-serum did not reveal identical or unique antigenic properties, which could explain the differences in pathogenicity, and cross-immunity observed amongst the various isolates (Rossouw *et al.*, 1990). This may be an indication that the proteins involved are polymorphic. Polymorphic proteins ranging between 23 to 31 kDa, that fall into the size range that induces proliferation of *Cowdria* specific, IFN-γ-producing $CD4^+$ T-cell lines derived from immune cattle (Totté *et al.*, 1999b), were in fact identified by immunoblotting (Perez *et al.*, 1998).

The discovery of murinotropic isolates of *Cowdria* has provided a suitable model for immunological studies at reasonable cost (du Plessis & Kümm, 1971; Mackenzie & MacHardy, 1987). Investigations in a mouse model confirmed results obtained in bovines which showed that both $CD4^+$ and $CD8^+$ lymphocytes (du Plessis *et al.*, 1991 & 1992a; Byrom *et al.*, 2000a) and the Th type 1 cytokine IFN-γ (Byrom *et al.*, 2000b) are important in protective immunity. A naked DNA vaccine expressing the MAP1 gene of *Cowdria* has been shown to be able to protect between 23-88% of immunised mice against a lethal challenge (Nyika *et al.*, 1998). However, immunisation with denatured MAP1 excised from SDS-PAGE gels and emulsified in complete Freund's adjuvant did not protect goats and sheep despite high antibody titres being present before challenge (van Kleef *et al.*, 1993). These findings suggest that a murine model may not

be suitable in all cases for evaluating putative protective proteins or recombinant vaccines against heartwater.

Knowledge of the effector mechanisms which result in immunity to a particular disease, is without a doubt a great advantage during the development of a new vaccine (Watson *et al.*, 1994). It allows a logical approach to the selection of protective antigens, choice of adjuvant and vaccination strategies. Vaccines of the future should therefore be designed to take into account the cytokine profiles produced during immunity. It may well be that protective vaccines that induce sterile immunity are unattainable and that preventing disease rather than infection would be adequate. During the last two decades, developments in recombinant DNA technology have created new opportunities for vaccine production both through genetic manipulation of pathogens and by enabling defined antigenic subunits that induce protective immune responses to be identified (Morrison *et al.*, 1999). The application of this new technology so far may have been somewhat slow to yield vaccine products. This is due in part to the fact that until recently there was limited knowledge of the immunology of many of the target diseases and of how antigens are processed and recognised by the immune system. Recent advances in immunology coupled to further developments in the application of DNA technology can nonetheless be expected to provide a firm foundation for the rational development of new vaccines.

A rational subunit vaccine development strategy for *Cowdria* should thus be aimed at inducing a Type 1 immune response together with cross-immunity. For this to be attained, the proteins or other constituents of the pathogen that actually interact with the immune system and cause the appropriate responses need to be identified. Once this is achieved, their encoding genes will need

to be located and incorporated into an appropriate vaccine delivery system. Finally, the prototype vaccine must, of course, be effective under realistic field conditions.

1.3. RATIONALE

As indicated in the preceding sections, it is clear that cellular immunity plays a major role in protection against intracellular pathogens such as *Cowdria*. The anti-parasitic activities of parasite-activated $CD4^+$ T-cells and macrophages are effected primarily through the production of the inflammatory cytokines TNF-α, TNF-β and IFN-γ. These cytokines activate neutrophils and macrophages, resulting in enhanced phagocytosis (Kumaritalake *et al.*, 1991; Langhorne *et al.*, 1989) and production of reactive nitrogen and oxygen intermediates which are toxic for intracellular parasites (Langhorne *et al.*, 1989; Liew & Cox, 1991; Rockett *et al.*, 1991). Thus from the current state of knowledge it is clear that any rational attempt to develop a recombinant vaccine for *Cowdria* should focus on components of the parasite that can potentially induce the appropriate cellular immune responses. Therefore, because protective immunity to heartwater has been shown to be mediated by a Th1 type cellular immune response (section 1.2.5.), with particular involvement of IFN-γ, this thesis will focus on identification of proteins that induce such responses.

Owing to the size (ranging between 1546 kb and 1692 kb) and complexity of the *Cowdria* genome (de Villiers *et al.*, 2000a & b), it is likely to be impractical to empirically evaluate each

new gene in a variety of immunological contexts for its ability to express a protective antigen. Therefore, for the development of a subunit vaccine it is important to identify *Cowdria* proteins that stimulate T lymphocytes and in turn, relate these to protective responses. **Chapter 2** of this thesis investigates strategies for the purification of *Cowdria* for use in cellular immunological assays. **Chapter 3** sets the stage for ensuing immunological studies by immunising cattle with *Cowdria* as a source of primed lymphocytes. To simulate the natural infective process the PBMC used were obtained from animals rendered immune by infection and treatment. In a parallel study four cattle were immunised with inactivated *Cowdria* to determine whether their lymphocytes also respond similarly. These lymphocytes and the *Cowdria* organisms purified as in Chapter 2 were used to optimise the proliferation assay. **Chapter 4** describes how *Cowdria* proteins were fractionated by continuous flow electrophoresis and the fractions tested for their ability to stimulate lymphocyte proliferation *in vitro*. In **Chapter 5**, a preliminary study is described investigating the development of tools for the identification of genes that encode the proteins identified in Chapter 4. In **Chapter 6**, the response induced by low molecular weight proteins of *Cowdria* was characterised by determining the phenotype of the responding lymphocyte population and ascertaining whether IFN-γ was produced. In conclusion, **Chapter 7** summarises the main results of the thesis and discusses the possibilities for future research.

CHAPTER 2

PURIFICATION OF *COWDRIA*

2.1. INTRODUCTION

Having a source of pure *Cowdria* is an important factor in determining the nature of immunity to heartwater and for identifying the proteins that may play a role in protective immunity. The aim of this part of the study was therefore to prepare antigen suitable for use in screening lymphocytes from cattle for their cellular immune responses. The Welgevonden isolate was chosen because the pathological patterns caused by this isolate resemble those caused by the Ball3 isolate presently used in the live vaccine (Prozesky & du Plessis, 1985). Additionally, unlike the Ball3 isolate, the Welgevonden isolate is pathogenic to mice, permitting viability tests to be determined in these animals. It also elicits total immunity against more isolates than any other (du Plessis *et al.*, 1989). For the experiments conducted in this thesis, *Cowdria* organisms were multiplied by *in vitro* cell culture in bovine endothelial cells. The organism was first cultured *in vitro* in endothelial cells in 1985 (Bezuidenhout *et al.*, 1985). This was considered a major breakthrough for research in heartwater and was probably the starting point of all modern *Cowdria* research. Culture conditions have, however, been immensely improved over the years, resulting in significantly higher yields (Zweygarth *et al.*, 1997). A prerequisite for all protein and DNA research is that preparations should be as free as possible from contaminating host material. Because *Cowdria* grows intracellularly, it is extremely difficult to obtain organisms free of such material. During the infectious cycle of *Cowdria*, the organism ruptures the host cell and is released into the culture medium whereafter it can re-infect other cells. This means that *Cowdria*

cannot be harvested directly in a pure form. Two methods have been investigated to purify the organism namely, isopycnic Percoll density gradient centrifugation (Neitz *et al.*, 1987) and wheat germ lectin chromatography (Vermeulen *et al.*, 1987). Both these methods succeeded in only partially purifying the organism. Because no really efficient and reliable purification method was available, three different purification procedures were investigated as ways of preparing *Cowdria* antigen. These were differential centrifugation, a combination of differential centrifugation and discontinuous zonal Percoll density-gradient centrifugation, and finally immunoaffinity chromatography.

Differential centrifugation was previously examined as an initial step in purifying *Cowdria* from cell culture debris (Rossouw *et al.*, 1990). Electron micrograph studies showed that the organisms sedimented at ≤ 30,000g together with the cellular debris. In addition it is well known that nuclei sediment at 1000g together with large cellular debris (Bohinski, 1983). Because the DNA may interfere with ensuing purification steps and investigations, it is important also to remove contaminating DNA. Accordingly, in order to minimise *Cowdria* losses, the first centrifugation step was carried out at 1000g followed by one at 30,000g (section 2.2.3.). A similar procedure was used to prepare inactivated *Cowdria* organisms for immunisation of cattle and sheep and for immunological studies using the Gardel isolate (Martinez *et al.*, 1994; Totté *et al.*, 1997).

Cattle have been successfully immunised by preparing inactivated organisms obtained by a combination of differential centrifugation and discontinuous Percoll density centrifugation (Mahan *et al.*, 1995). In addition this method has been reported to yield sufficiently pure

organisms for DNA extraction with < 10% bovine DNA contamination (de Villiers *et al.*, 1998). This approach (section 2.2.4.) was therefore also investigated for antigen preparation.

Isolating a particular cell population (in this case *Cowdria*) according to its cell surface properties provides an alternative to methods which exploit physical differences between cell types. Affinity chromatography allows cells to be selectively retained on an immobilised ligand (antibody) which interacts specifically with a cell surface component (antigen). Cells may subsequently be recovered by desorption with various buffers. Immunoaffinity purification techniques offer simplicity, specificity, rapid single step isolation and minimal contamination by non-specific proteins or cellular debris. Antibodies are eminently suitable for this purpose. Due to its high degree of specificity, immunoaffinity chromatography was also investigated. The so-called major antigenic protein of *Cowdria* (MAP1) was chosen as the target antigen using an anti-MAP1 IgG as the corresponding antibody. The immune MAP1 anti-serum was previously shown to have high titres and specificity towards *Cowdria* in immunoblotting (van Kleef *et al.*, 1993). When used in positive selection immunoaffinity chromatography, it was expected to provide the necessary specificity. Anti-MAP1 IgG was therefore used to prepare an immunoaffinity matrix using either CNBr-activated Sepharose 6MB (macrobeads) or Triazine activated agarose 4XL. This chapter describes the different purification procedures, and their apparent effectiveness as methods of purifying *Cowdria*.

2.2. MATERIALS AND METHODS

2.2.1. *IN VITRO* CULTIVATION

The Welgevonden isolate of *Cowdria* was cultured either in bovine saphenic vein (BSV) endothelial cells (prepared from B9191) or a calf endothelial cell line (E_5), as described by Zweygarth and co-workers (1997). The cultures were kindly provided by Dr E. Zweygarth and Ms. A Josemans of the Parasitology Division, OVI.

2.2.2. CRUDE CELL CULTURE PREPARATIONS

Crude preparations of the Welgevonden isolate-infected or non-infected cell cultures were prepared as detailed previously (Rossouw *et al.*, 1990). Non-infected or infected (harvested when maximally infected with organisms) BSV cells were centrifuged for 30 min at 10,000g. The resulting pellet was suspended in phosphate buffered saline (PBS: 0,14 M sodium chloride, 1 mM potassium phosphate, 8 mM sodium phosphate and 3 mM potassium chloride, pH 7.4). The preparations were stored at -20°C. These crude cell culture preparations were used for SDS-PAGE.

2.2.3. DIFFERENTIAL CENTRIFUGATION

Differential centrifugation was done with either infected E_5 or BSV cell cultures by a modification of the procedure described by Totté *et al.* (1997). The cultures were centrifuged at 1000g for 10 min. The resultant supernatants were centrifuged at 30,000g for 30 min. The pellets were resuspended in PBS containing sodium benzyl penicillin (0.12 mg/ml) and streptomycin

sulphate (0.198 mg/ml). The suspensions were subjected to five freeze-thaw cycles performed with liquid nitrogen, to inactivate the organism, and stored at -20°C. The infected E_5 cell culture preparations were used for immunisation of cattle and the infected BSV cell culture preparations were used as antigen in proliferation assays (Chapter 3).

2.2.4. PERCOLL DENSITY GRADIENT CENTRIFUGATION

Percoll density gradient centrifugation (PDGC; Mahan *et al.*, 1995) was performed with *Cowdria*-infected BSV cell cultures. The infected cultures were centrifuged at 1500g for 30 min and the resulting supernatant at 30,000g for 30 min. The organisms were suspended in 1 ml PBS and loaded onto a step gradient of 0-10-20-30-40% Percoll (1.13 ± 0.0005 g/ml; Pharmacia) prepared in PBS. The gradient was centrifuged at 400g for 30 min and the *Cowdria* organisms harvested from the 0% layer. They were washed twice in PBS at 30,000g for 30 min. The resultant pellet was resuspended in PBS and stored at -20°C. These preparations were used as antigen in proliferation assays (Chapter 3).

2.2.5. IMMUNOAFFINITY CHROMATOGRAPHY

Immunoaffinity chromatography was performed as described previously (Brayton *et al.*, 1997) with purified goat anti-MAP1 IgG (van Kleef *et al.*, 1993) coupled to either CNBr-activated Sepharose 6MB (Pharmacia) or Triazine activated agarose 4XL (ACL).

2.2.5.1. Production of polyclonal goat anti-MAP1 serum

A goat was immunised with the MAP1 protein of *Cowdria* as described by van Kleef *et al.* (1993). Preparative SDS-PAGE was performed with crude *Cowdria*-infected preparations (section 2.2.7.). An amount of 1.9 mg protein was loaded per 1.5 x 120 x 160 mm gel, corresponding to approximately114 µg of the 31 kDa protein. After preparative SDS-PAGE the gel was stained with 0.3 M copper chloride (section 2.2.7.1.). The 31 kDa (MAP1) protein bands were excised from four gels, fragmented and stored at –70°C until required for immunisation. Protein from a single gel was used per immunisation. The goat was immunised at four weekly intervals firstly with antigen emulsified in Freund's complete adjuvant (FCA) then Freund's incomplete adjuvant (FIA), and followed by two immunisations without adjuvant. The goat was bled two weeks after each immunisation. The antiserum specificity was evaluated and titres determined by immunoblotting (section 2.2.8.2.) using crude infected and non-infected culture preparations (section 2.2.2.) as antigen in the electro-blots (section 2.2.8.).

2.2.5.2. Purification of anti-MAP1 IgG by DEAE Affi-Gel® Blue chromatography

Purification of IgG by DEAE Affi-Gel® Blue (Bio-Rad) was performed at 10°C according to the manufacturers instructions. Goat anti-MAP1 serum (obtained on day 98, two weeks after the fourth immunisation) was dialysed against 0.03 M sodium chloride, 0.02 M Tris and 0.02% sodium azide, pH 8.0 (Buffer A). A column (15 x 650 mm) of DEAE Affi-Gel® Blue (100 ml bed volume) was prepared. The dialysed immune serum sample was applied to the column at 1 ml antiserum per 4 ml gel bed volume. The IgG was eluted with three bed volumes of Buffer A at a flow rate of 1 ml/min. Fractions with approximately the same volume as that of the immune serum applied were collected and the absorbancy recorded at 280 nm. Fractions equivalent to

eight times the volume of antiserum applied to the column were combined beginning with the first tube of the unbound protein peak. The absolute amount of IgG obtained was determined by converting the absorption values into concentration values: Absorbance at 280 nm = 1.0 (1 cm pathlength) = 0.75 mg IgG/ml (Robyt & White, 1987). The gel was regenerated with two bed volumes of 2 M sodium chloride, 0.2 M Tris and 0.02% sodium azide, pH 8.0 followed by three bed volumes of buffer A.

2.2.5.3. Coupling of anti-MAP1 IgG to CNBr-activated Sepharose 6MB

Coupling of purified goat anti-MAP1 IgG to CNBr-activated Sepharose 6MB was done at room temperature as described by the manufacturer (Pharmacia). A concentration of 16 mg IgG per 1 g gel at a gel:buffer ratio of 1:2 is recommended. The pooled fraction of DEAE Affi-Gel® Blue purified goat anti-MAP1 IgG was concentrated with polyethylene glycol and dialysed against 0.2 M sodium bicarbonate and 0.5 M sodium chloride, pH 8.5 (coupling buffer). The freeze-dried CNBr-activated Sepharose 6MB was swollen for 15 min at room temperature in 1 mM hydrochloric acid. The swollen gel was washed on a sintered glass filter (G3) with 5 ml coupling buffer per gram of dry gel and immediately transferred to the concentrated and dialysed anti-MAP1 IgG. This suspension was mixed for 2 h, whereafter the buffer was replaced with 1 M ethanolamine and mixed for a further 2 h. Thereafter the gel was washed 10 times with 50 ml of alternatively 500 ml 0.1 M acetate and 0.5 M sodium chloride, pH 4.0 and 500 ml coupling buffer. This matrix was used for immunoaffinity chromatography purification of *Cowdria* (section 2.2.5.5.).

2.2.5.4. Coupling of anti-MAP1 IgG to Triazine activated agarose 4XL

Coupling of purified goat anti-MAP1 IgG to Triazine activated agarose 4XL was done as described by the manufacturer (Affinity Chromatography Ltd.). To this end, the freeze-dried Triazine activated agarose 4XL was swollen for 5 min in an excess of distilled water and then washed with 20 volumes of distilled water on a sintered funnel. The gel was washed with a further seven volumes of 50 mM sodium acetate, pH 5.0 containing 0.5 M sodium chloride (coupling buffer) and allowed to drain under gravity. The gel was then weighed out into a container suitable for rotary tumbling. A solution of 10 mg/ml IgG was prepared in 50 mM sodium acetate, pH 5.0 and sufficient solid sodium chloride was added to bring the solution to 0.5 M sodium chloride. The volume of the solution just exceeded that of the gel. An equal volume of swollen gel and IgG solution was mixed by rotary tumbling at 4°C for 2 h. The gel was washed with ten volumes of coupling buffer and resuspended in an excess solution of 2 M imidazole, pH 5.0. The gel was mixed for 2 h at room temperature. It was next washed with five volumes of water and five volumes of 20% (v/v) ethanol containing 0.02% (w/v) sodium azide and stored at 4°C in the same solution. This matrix was used for immunoaffinity chromatography purification of *Cowdria* (section 2.2.5.5.).

2.2.5.5. Immunoaffinity chromatography

Immunoaffinity chromatography was performed as described previously (Brayton *et al.*, 1997). Anti-MAP1 IgG coupled Sepharose 6MB or Triazine activated agarose 4XL was packed into a column (15 x 200 mm) and equilibrated at a flow speed of 2 ml/min with ten bed volumes of PBS containing 0.02% sodium azide, pH 7.4 (equilibration buffer) at room temperature. A crude infected cell culture preparation (1.5 mg/1 g gel) was applied to the column at a flow rate of 0.2

ml/min in a volume not exceeding the void volume. After incubating for 30 min the unbound material was washed off with two bed volumes of PBS (equilibration buffer). The flow was then reversed and a volume of 3 M potassium thiocyanate, 50 mM Tris and 0.02% sodium azide, pH 9.0 (desorption buffer) not exceeding the void volume was passed through the column at a flow rate of 2 ml/min. After an incubation period of 60 min the bound material was eluted with two bed volumes of desorption buffer at a flow rate of 0.2 ml/min. A volume of 2 ml fractions were collected and the absorbance recorded at 280 nm. Fractions were pooled into an eluate (unbound material) and retentate (bound material). The pooled fractions were centrifuged at 30,000g for 30 min. Samples of the pellets were prepared for Bio-Rad protein assay (section 2.2.6.), immunoblotting (section 2.2.8.) and electron microscopy (section 2.2.9.).

Various elution buffers were investigated for the desorption of *Cowdria* organisms from anti-MAP1 coupled Triazine-activated agarose 4XL. The gel was evenly divided into 6 aliquots (i.e. 3 ml aliquots). Immunoaffinity chromatography was performed as described above with different buffers as indicated in Table 2.1.

Table 2.1. Desorption buffers used in immunoaffinity chromatography of *Cowdria* organisms on anti-MAP1 coupled Triazine activated agarose 4XL.

Desorption condition	Equilibration/wash buffer	Elution/desorption buffer
Chaotropic	PBS	Potassium thiocyanate; 50 mM Tris, pH 9.0
Low pH	0.01 M phosphate, pH 6.8	1) 100 mM glycine pH 2.5; 2) 1/20 volume 1 M phosphate, pH 8.0
High pH	0.01 M phosphate, pH 8.0	1) 100 mM phosphate acid, pH 12.5; 2) 1/20 volume 1 M phosphate, pH 6.8
High salt	10 mM phosphate, pH 7.2	3.5 M magnesium chloride, 10 mM phosphate, pH 7.2
Organic solvent	PBS	10% Dioxane in PBS
Denaturing agent	PBS	2 M guanidine hydrochloride

All buffers contained: 0.154 M sodium chloride, 0.02% sodium azide.

2.2.6. Protein determination

Protein concentrations were determined by the Bio-Rad protein micro assay (Bio-Rad; Hercules, California) using bovine serum albumin as a standard.

2.2.7. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SDS-PAGE was performed with crude infected and non-infected cell culture preparations (section 2.2.2.) by modifications of the method described by Laemmli (1970). The stacking gel contained 4% acrylamide, 0.1% bisacrylamide, 0.1% sodium dodecyl sulphate (SDS) in 0.1 M Tris buffer (pH 6.8). The separating gel contained 12% acrylamide, 0.3% bisacrylamide, 0.1% SDS in 0.4 M Tris buffer (pH 8.8). Gels were polymerised by adding ammonium persulphate and tetremethylenediamine at final concentrations of 0.05% and 0.1% respectively in the stacking gel and 0.05% each in the separating gel. Large gels of 1.5 x 120 x 160 mm were prepared. The

samples (40 μg/well – 15 well comb or 1.9 mg protein/well – preparative comb) were dissolved in 0.06 M Tris buffer (pH 6.8), 16% glycerol, 2 % SDS, 2.5% dithiothreitol (DTT) and 0.001% bromophenol blue by heating at 100°C for 10 min. Electrophoresis was performed in an electrode buffer containing 0.02 M Tris, 0.1 M glycine and 0.06% SDS, pH 8.3. The gels were electrophoresed for 2 h at a constant current of 45 mA whilst the mobile front was in the stacking gel. Once the front entered the separating gel, electrophoresis was performed at a constant current of 65 mA for 3 h.

2.2.7.1. Copper chloride stain

After SDS-PAGE the gels were briefly rinsed in several changes of distilled water and soaked for 5 min with 0.3 M copper chloride stain (Lee *et al.*, 1987). They were then rinsed in several changes of distilled water in which they were stored.

2.2.8. ELECTROBLOTTING ONTO PVDF MEMBRANES

Electrophoretic transfer of SDS-PAGE separated proteins was achieved by a modification of the procedure described by Moos *et al.* (1988). Proteins were transferred to a polyvinylidene (PVDF) membrane (Millipore) by electrophoresis in 10 mM cyclohexylamine propane sulphonic acid (CAPS) buffer, pH 9.0 at 0.25 A for 1 h 45 min. The blotted sheet was air-dried and stored at – 20°C until further use.

2.2.8.1. Staining of proteins on PVDF membranes

Proteins were stained by soaking the membrane for 10 min with 0.25% Coomassie brilliant blue, 5% methanol, 1% acetic acid and 5% distilled water. The membrane was destained with 5% methanol, 1% acetic acid and 5% distilled water for 10 min and rinsed in distilled water for a further 10 min, after which it was left to dry. It was stored at -20°C.

2.2.8.2. Immunostaining of PVDF membranes

Cowdria antigens were identified by immunoblotting techniques (Millipore) with polyclonal antiserum as described previously (Rossouw *et al.*, 1990). Dried, electro-blotted (section 2.2.8.), PVDF membranes were wet with methanol and rinsed briefly in distilled water. When goat antiserum was used in immunoblotting, the membranes were blocked with 5% Elite milk powder in 20 mM Tris and 0.9 M sodium chloride, pH 7.4. The membranes were then incubated in sera diluted 1:20 (unless otherwise stated), with a solution containing 1 % Elite milk powder, 0.05% Tween 20, 20 mM Tris and 0.9 M sodium chloride, pH 7.4 (incubation buffer), with gentle agitation for 90 min at room temperature. The membranes were washed three times for 5min with 0.1% Elite Milk powder in 20 mM Tris and 0.9 M sodium chloride, pH 7.4 (wash buffer). This was followed by gentle agitation of the membrane for 90 min at room temperature in peroxidase-conjugated IgG, which was diluted 1:500 with incubation buffer. After washing three times for 5 min with wash buffer the proteins were detected by immersing the membrane into the developing solution. Developing solution: a) 20 ml of 2.8 mM 4-chloro-1-naphtol in ice cold ethanol. b) 100 ml of 0.015% hydrogen peroxide in 20 mM Tris, pH 7.4. Mix solutions a) and b) and incubate with membrane for 20 min. After termination of color development by rinsing in distilled water, the membrane was air dried and stored at –20°C.

2.2.9. ELECTRON MICROSCOPY

Samples were kindly prepared for electron microscopic studies by the staff of the Electron Microscopy Division at the OVI, Onderstepoort. Samples to be examined were centrifuged at 10,000g for 30 min at 4°C. The pellets were fixed with 2.5% glutaraldehyde in 0.1 M Millonig's phosphate buffer pH 7.4 for 24 h at 4°C (all subsequent procedures were performed at room temperature unless otherwise stated). This step was followed by two rinses of 10 min each with Millonig's phosphate buffer. Post fixing was carried out in 1% osmium tetroxide in Millonig's phosphate buffer for 45 min and rinsed twice with 0.1 M Millonig's phosphate buffer for 10 min each. Dehydration was achieved by the stepwise addition of 50%, 70%, 90%, 95% and 3 x 100% ethanol for 15 min each. A solution of 50:50 (propylene oxide:ethanol) was then added and incubated for 10 min followed by two incubations in 100% propylene oxide for 10 min each. The samples were then incubated stepwise by adding resin:propylene oxide (30:70, 50:50, 70:30 and 2 x 100% resin) for 15 min each. The samples were left in capsules to polymerise for 24 h at 65°C. Ultra-thin sections of 0.05 μm were cut with a Reichert-Jung Ultracut microtome. These sections were stained for 30 min in aqueous solutions of 5% uranyl acetate and for 30 min in lead citrate. The sections were then rinsed with 0.02% sodium hydroxide followed by double distilled water and left to dry. The grids were examined with a Joel JEM 1200 EX electron microscope.

2.3. RESULTS

2.3.1. PURIFICATION OF *COWDRIA* ORGANISMS

2.3.1.1. Differential centrifugation

The total protein yields, as determined by Bio-Rad protein assay, that were obtained from nine differential centrifugation purifications varied from between 2 x 10^{-4} and 1 x 10^{-2} mg/cm^2 culture flask. This was not due to variability in the purification procedure, but rather due to variations in yields of the organism after *in vitro* culture. The resulting pellets were investigated for their suitability as an antigen in proliferation assays (Chapter 3).

2.3.1.2. Percoll density gradient centrifugation

A total of 100 μg protein was obtained after Percoll density gradient centrifugation. This preparation was also tested in proliferation assays (Chapter 3).

2.3.1.3. Immunoaffinity chromatography

Anti-MAP1 serum with a titre of 1:125,000 in immunoblots was subjected to DEAE Affi-Gel® Blue chromatography for anti-MAP1 IgG purification (section 2.2.5.2.). A representative chromatogram obtained when 15 ml of antiserum was passed through a 100 ml bed volume of DEAE Affi-Gel® Blue is shown in Fig. 2.1. A total of five purifications were done with a yield of 110 mg of IgG per purification. This purified anti-MAP1 IgG was pooled and coupled to

CNBr-activated Sepharose 6MB (section 2.2.5.3.) with a coupling efficiency was 95%. Immunoblotting with the MAP1 IgG, before and after coupling, on infected and uninfected culture antigen indicated that it was specific for MAP1 (31 kDa) and other *Cowdria* proteins, with no cross-reactions with any of the host cell proteins (Fig. 2.2.). Immunoaffinity chromatography (section 2.2.5.5.) was performed using crude *Cowdria* preparations (section 2.2.2.). A representative chromatogram is shown in Fig. 2.3. The capacity of the anti-MAP1 coupled Sepharose 6MB varied between runs and with different batches of Sepharose. For example, 1 mg purified *Cowdria* was obtained per 0.8 to 40 g dry gel. This is shown by the differences in the two chromatography runs illustrated in Fig 2.3 where the amount of bound material (retentate) varied vastly between the two chromatograms. Light microscopic examinations of the eluate and desorbed retentate showed that not all the organisms that passed through the column were retained on the matrix. This occurred even when less (half; 3 mg) material was loaded onto the gel column. This resulted in fewer organisms binding and concomitantly lower yields. Electron micrographs of the purified organisms show that the harsh desorption conditions of 3 M potassium thiocyanate pH 9.0 did not visibly damage the organisms (Fig. 2.4.). There were however, some organisms present with irregular and ruptured membranes, perhaps resulting from the desorption conditions.

In an attempt to optimise protein yields, an alternative matrix, namely Triazine activated Agarose 4XL together with various desorption eluants (see Table 2.1.) was investigated. The eluants included a chaotropic agent, low and high pH, high salt/low pH, an organic solvent and denaturing agent. Purified anti-MAP1 IgG was coupled to Triazine-activated Agarose 4XL (section 2.2.5.4.) with a coupling efficiency of 86%. Immunoaffinity chromatography (section

2.2.5.5.) was performed using crude *Cowdria*. A similar elution pattern was found as seen in Fig 2.3. (soiid red line). Elution conditions of high pH resulted in the best protein yields, which was followed closely by chaotropic elution conditions (Table 2.2). When the Triazine-activated Agarose 4XL and the chaotropic potassium thiocyanate desorption buffer was used yields of 1 mg protein/0.1 g gel were obtained. The use of this alternative matrix therefore did not lead to improved protein yields.

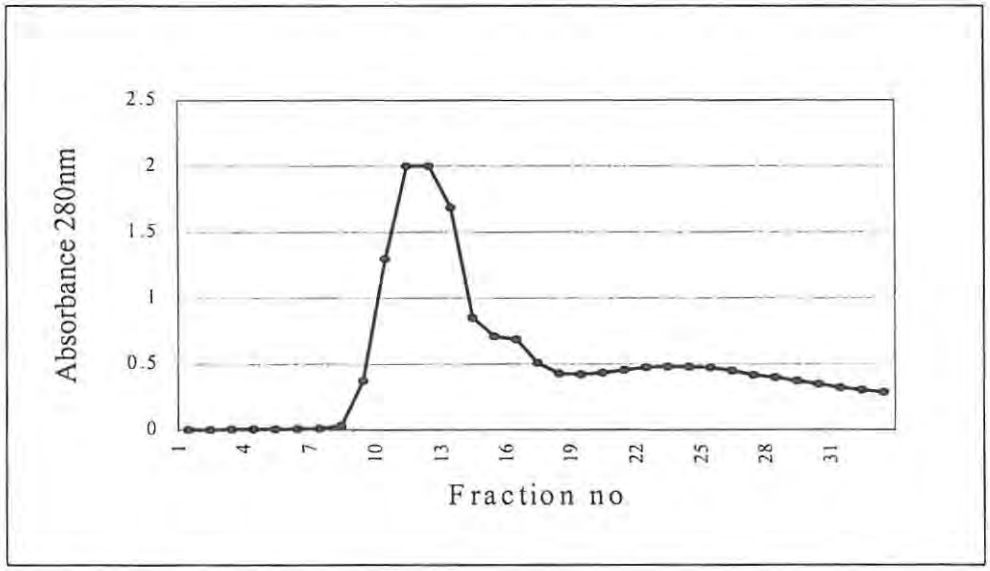

FIG 2.1. DEAE Affi-Gel® Blue chromatography of a goat anti-MAP1 serum. The void volume contained anti-MAP1 IgG. A volume of 15 ml dialysed antiserum was loaded onto a 100 ml bed volume of DEAE Affi-Gel® Blue matrix and eluted with 0.02 M Tris; 0.028 M sodium chloride, pH 8.0.

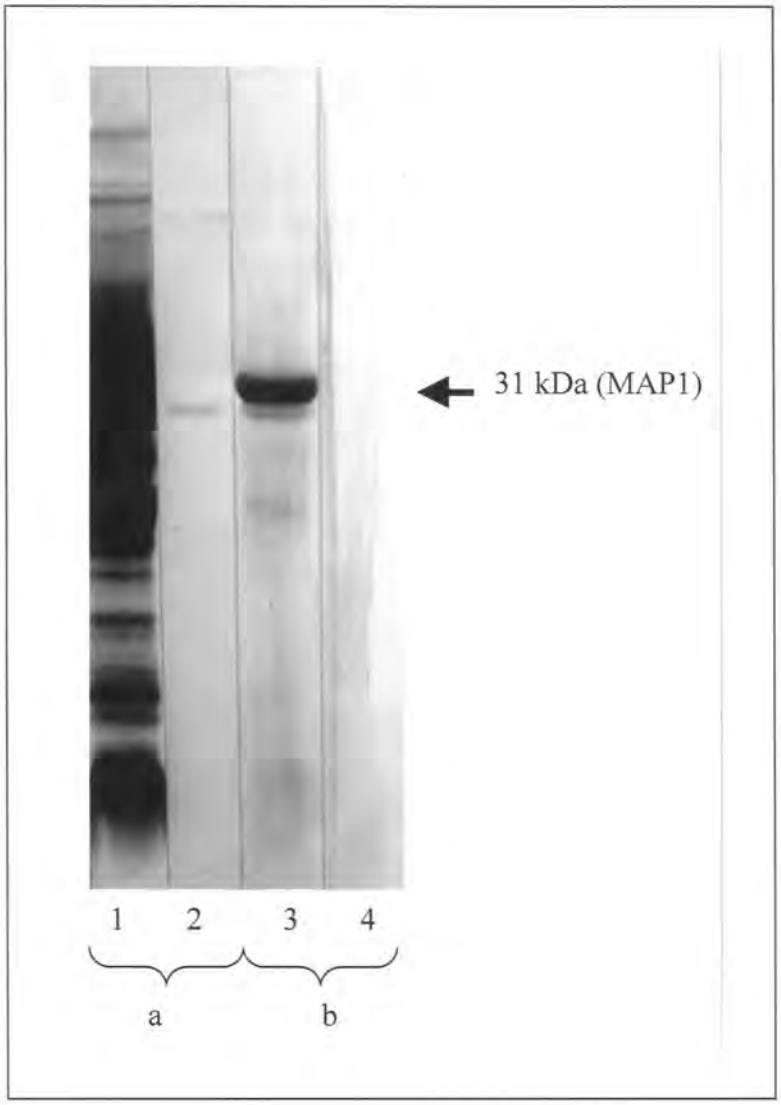

FIG. 2.2. Immunoblot of crude *Cowdria* infected and uninfected preparations probed with DEAE purified MAP1 IgG. The western blot was probed with purified MAP1 IgG before (a, 1/10 dilution) and after (b, undiluted) coupling to CNBr-activated Sepharose 6MB. Lanes 1 and 3: infected cell culture and Lanes 2 and 4: uninfected cell culture.

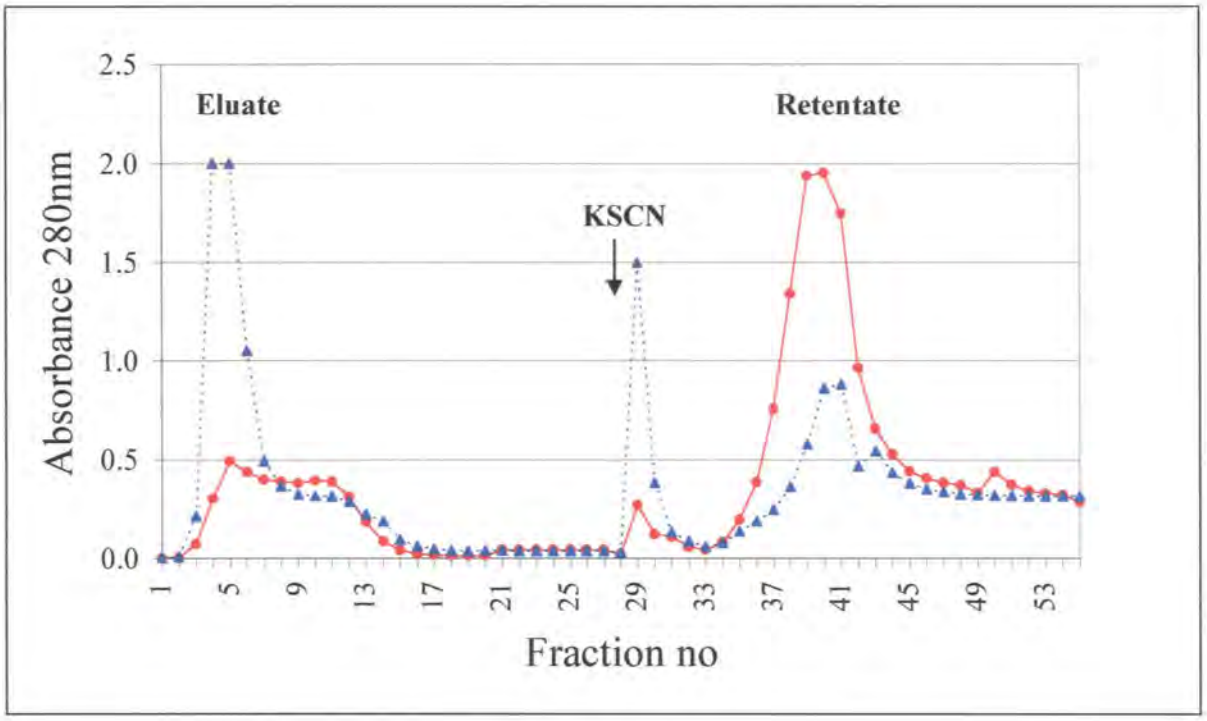

FIG 2.3. Chromatograms showing extreme variability in two immunoaffinity chromatography elution profiles of *Cowdria* preparations. Organisms were purified from a crude cell culture preparation, infected with the Welgevonden isolate, by immunoafinity chromatography with anti-MAP1 coupled to CNBr-activated Sepharaose 6MB. The solid red line represents a profile obtained after a high yield. Dotted blue line represents a profile obtained after low yield. Fractions 3-17 = eluate; fractions 32-46 = desorbed retentate. The arrow indicates the application of desorption buffer (3 M potassium thiocyanate (KSCN); 50 mM Tris; pH 9.0).

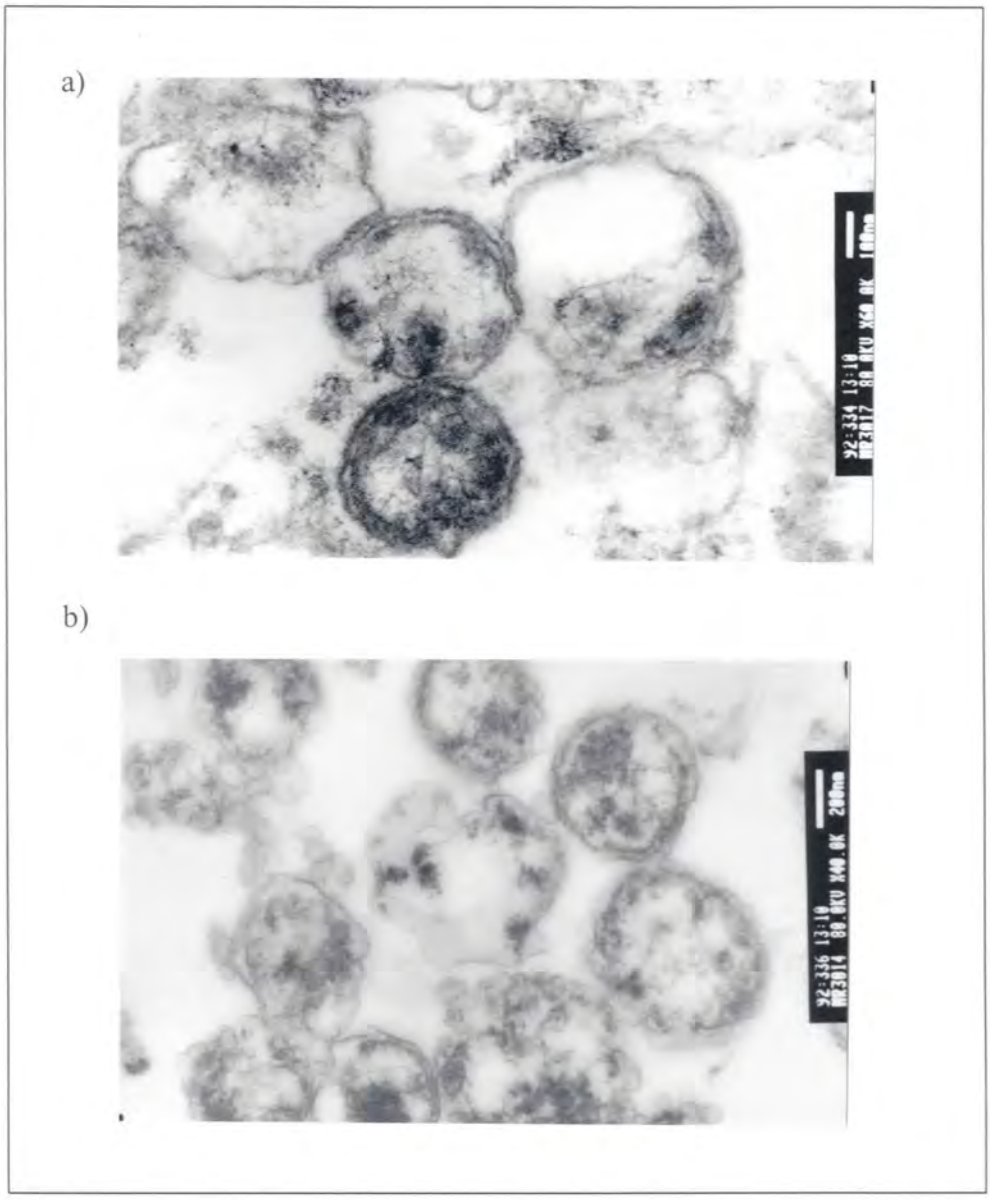

FIG 2.4. Electron micrographs of *Cowdria* organisms a) before and b) after immunoaffinity chromatography. *Cowdria* was purified by immunoadsorbent chromatography on an anti-MAP1 IgG coupled CNBr-activated Sepharose 6MB matrix. The bound organisms were desorbed with 3 M potassium thiocyanate, pH 9.0.

TABLE 2.2. Comparison of protein yields obtained after immunoaffinity chromatography using anti-MAP1 IgG coupled to Triazine-activated Agarose 4XL and various desorption buffers.

Desorption by	Desorption buffer	Protein yield μg
high pH	100 mM phosphate pH 12.5	220
chaotropic agent	3 M potassium thiocyanate; 50 mM Tris, pH 9.0	203
denaturing agent	2M guanidine hydrochloride	138
low pH	100 mM glycine pH 2.5	7
organic solvent	10% Dioxane	#
high salt/low pH	3.5 M magnesium chloride; 10 mM phosphate pH 7.2	#

buffer interference with protein determination

2.4. DISCUSSION

When comparing the three methods evaluated for *Cowdria* purification, the quickest and most efficient method was differential centrifugation, followed by Percoll density centrifugation and lastly despite its theoretical advantages, immunoaffinity chromatography. During immunoaffinity chromatography it was hoped that *Cowdria* organisms could be retained on the column by a goat anti-MAP1 IgG coupled to the matrix via surface-expressed MAP1 proteins. The contaminating endothelial cell debris was eluted with PBS and the organisms released using a chaotropic agent. Discouragingly low yields were obtained, however, and not all the *Cowdria* organisms that passed through the column bound to the matrix. This appeared to be irrespective of sample load. This finding may indicate that the MAP1 protein was either not expressed, or was not exposed on the surface of all the organisms. Alternatively the protein may not be present during all the developmental stages of the organism. It is also possible that it is not a true surface protein, but

rather gets exposed as a result of membrane damage. Electron microscopy of the organisms after immunoadsorbent chromatography showed both ruptured and intact membranes. In an effort to try and improve the yields, an alternative matrix Triazine-activated Agarose 4XL and various desorption buffers were examined. A 7.7% increase in yield was obtained using a high pH eluant instead the chaotropic eluant and the Agarose 4XL matrix. However, the Agarose 4XL matrix resulted in lower yields than the Sepharose 6MB matrix using the chaotropic eluant.

Purification of *Cowdria* still remains somewhat of a challenge to every researcher working with the organism. For instance, immunoaffinity-purified *Cowdria* (prepared by the author of this thesis) was used for the construction and analysis of a λZAPII expression library containing approximately 10^4 *Cowdria* genome equivalents (Brayton *et al.*, 1997). The DNA isolated in this manner only contained 3-5% bovine DNA which was a substantial improvement over previous methods (Ambrosio *et al.*, 1987). Organisms purified in this manner were also used for genome studies by pulsed field gel electrophoresis. The DNA appeared to be degraded, however, and was therefore unsuitable for this application. Discontinuous Percoll density gradient centrifugation resulted in DNA of a high quality and yield, but with an unacceptable bovine DNA content of 10%. Discontinuous Percoll density gradient centrifugation used in combination with magnetic cell separation resulted in only <1% bovine DNA contamination (de Villiers *et al.*, 1998). Immunoaffinity purified organisms (prepared by the author of this thesis) were also a source of DNA for the construction of a cosmid library for molecular genetic studies. Seven clones were isolated representing 10% of the *Cowdria* genome (Brayton *et al.*, 1999). In Chapter 3 the results of the purification methods described in this chapter are compared from an immunological point of view.

CHAPTER 3

PROLIFERATIVE RESPONSES OF PBMC TO *COWDRIA* PREPARATIONS

3.1. INTRODUCTION

T-cell mediated immunity is technically far more difficult to measure than humoral immunity because T-cells do not make a secreted antigen-binding product. There is no simple binding assay for such responses and *in vitro* T-cell assays require considerable time and expertise. Cell-mediated immunity is determined by measuring lymphocyte proliferation. This response is due to the interaction of antigen-responsive T lymphocytes with the specific immunising antigen *in vitro* (Osterman, 1985). When T-cells meet their specific antigen they are stimulated to undergo division. This response is usually accompanied by a morphological change to a blast cell. The degree of lymphocyte stimulation may be assayed either by determining the percentage of blast cells in the culture or by measuring the amount of a radioactive DNA analogue incorporated into newly synthesised DNA. However, T-cell proliferation only tells one that cells able to recognise that antigen have been previously activated. It does not reveal what effector functions they mediate. Consequently, the effector phase of T-cell responses must be assessed by assays that detect cytolytic activity or the secretion of cytokines by the responding cells. The isolation and continuous growth *in vitro* of T lymphocytes can thus be used to extend our knowledge of cellular immune responses induced by parasitic infections (Gasbarre & Urban, 1982). They can also be adapted to the study of both the inductive and effector phases of T-cell responses. While

knowledge of the bovine immune system is still lagging behind the mouse and the human, investigations over the past 10 years have immensely increased the knowledge of the bovine immune system. This has led to the development of several useful reagents. These can now be applied to study the mechanisms of immunity to cattle diseases and the identity of antigens recognised by protective immune responses.

This chapter describes how proliferation of lymphocytes from experimental cattle was used to assess their biological responses to various mitogens and to *Cowdria*. Peripheral blood mononuclear cells (PBMC) from the blood of cattle is the most accessible source of lymphocytes and antigen presenting cells for *in vitro* proliferation assays. In addition PBMC, in contrast to T-cell lines or clones, represent the most heterogeneous T-lymphocyte repertoire. They are therefore useful as initial probes in immunological studies. Immunodominant proteins of a parasite that induces antigen specific lymphocyte proliferation in a number of animals can be recognised by this method (Zhu *et al.*, 1993). Developing vaccine alternatives based on the strategy of selecting antigens that stimulate a strong lymphocyte response may help to resolve the current vaccine dilemma. The experimental cattle used in the studies described in this thesis were immunised with ovine blood stabilate infected with a virulent Welgevonden isolate followed by treatment with an antibiotic to prevent a serious course of the disease. This method of immunisation simulates natural infection as *Cowdria* is allowed to develop, but only the early stages of the disease are established. In addition, infection and treatment is the current method of vaccinating ruminants against heartwater (van der Merwe, 1987). Ruminants immunised with inactivated organisms are also protected against heartwater (Martinez *et al.*, 1994; Mahan *et al.*, 1995). Inclusion of cattle, immunised by this method, in investigations described within this

thesis can indicate whether their lymphocytes respond similarly to those obtained from naturally infected immune animals. Primed lymphocytes from experimental cattle immunised by both approaches together with various purified *Cowdria* preparations (prepared as in Chapter 2) were used to optimise the lymphocyte proliferation assay. This assay was then utilised to monitor the cellular immune response after immunisation.

3.2. MATERIALS AND METHODS

3.2.1. EXPERIMENTAL CATTLE

3.2.1.1. History of experimental cattle

Seven outbred cattle were used in the immunological studies described within this thesis. Their details are given in Table 3.1. They were raised and kept in a heartwater free area. During the studies the cattle were stabled under tick free conditions. The Animal Ethics Committee, OVI, approved all experiments involving the cattle.

TABLE 3.1. Details of experimental cattle.

Animal no.	Species	Breed	Sex	Origin	Birth date
9191	Bovine	Fresian x Hereford	ox	Blood vaccine breeding programme, OVI	09/10/1992
1359	Bovine	Nguni x Bonsmara	ox	Kaalplaas, OVI	14/11/1995
809	Bovine	Nguni x Bonsmara	ox	Kaalplaas, OVI	16/10/1995
821	Bovine	Nguni x Bonsmara	ox	Kaalplaas, OVI	23/10/1995
1351	Bovine	Nguni x Bonsmara	ox	Kaalplaas, OVI	02/11/1995
775	Bovine	Nguni x Bonsmara	ox	Kaalplaas, OVI	27/09/1995
816	Bovine	Nguni x Bonsmara	ox	Kaalplaas, OVI	18/10/1995

3.2.1.2. Serological screening of experimental cattle

Cattle were screened by indirect immuno-fluorescence (IFA) for the presence of antibodies to *Cowdria*, *Babesia* and *Theileria* and by competition ELISA for *Anaplasma*. Staff of the Parasitology Department at the OVI, Onderstepoort, kindly performed these assays.

3.2.1.3. Immunisation by infection and treatment

Cattle were immunised by infection and treatment as described by van der Merwe (1987). Animals B9191 (11 months old) and B1359 (15 months old) were inoculated intravenously (i.v.) with 5 ml sheep blood stabilate infected with the Welgevonden isolate. The animals temperatures were monitored daily. They were treated on the third day of a rising febrile reaction by intramuscular (i.m.) injection with long-acting oxytetracycline (Liquamycin LA, Pfizer) at a dosage rate of 20 mg/kg body weight. The animals were challenged with the same batch of homologous stabilate at the following intervals: B9191 at one month and three years; and B1359 at six months and eight months after immunisation. The animals temperatures were monitored daily after challenge. A body temperature of 1°C above the average daily temperature was considered to be a febrile reaction.

3.2.1.3.1. Determination of infectivity of blood stabilate

As a positive control to verify the viability and virulence of the stabilate, mice were inoculated intravenously (i.v.) with 0.2 ml per mouse of the stabilate on the same day that the cattle were immunised (Prozesky & du Plessis, 1985). Post mortem examinations were done, by staff of the Pathology Division at OVI, on the mice that succumbed to determine the cause of death. The

presence of *Cowdria* in the mice was confirmed by immunohistochemical identification of the organism in formalin-fixed tissue sections (section 3.2.1.4.2.).

3.2.1.4. Immunisation with inactivated *Cowdria*

Cattle were immunised with inactivated organisms as described by Martinez *et al.*, (1997). Animals B809 (26 months old), B821 (26 months old), B1351 (31 months old) and B775 (32 months old) were injected subcutaneously (s.c.) with 50 μg inactivated *Cowdria* protein (prepared as in section 2.2.3.) in Montanide ISA50 adjuvant (SEPPIC) not exceeding a total volume of 1 ml. One animal (B816; 32 months old) received only PBS in adjuvant as control. The animals were injected twice at an interval between 14 and 16 weeks.

3.2.1.4.1. Determination of infectivity of the inactivated *Cowdria*-infected cell culture stabilate

In order to confirm that the preparation contained inactivated *Cowdria*, mice were inoculated intravenously with 0.2 ml per mouse of the same preparation (Prozesky & du Plessis, 1985). The mice were monitored for 30 days post inoculation. The absence of *Cowdria* organisms in the organs of surviving mice that were subsequently sacrificed was confirmed by immunohistochemical staining of the organism in formalin-fixed tissue sections (section 3.2.1.4.2.).

3.2.1.4.2. Immunohistochemical identification of *Cowdria* in formalin-fixed tissue sections

Immunohistochemical identification of *Cowdria* in formalin-fixed tissue sections was kindly performed by staff of the Department of Pathology, Faculty of Veterinary Science, University

of Pretoria (Jardine *et al.*, 1995). The brains of mice were fixed in 10% buffered formalin for at least 24 h. Tissue blocks were embedded in paraffin wax at a temperature not exceeding 60°C and subsequently processed according to standard histological practices. Sections were affixed to poly-L-lysine-coated glass slides and dried overnight in an incubator at 60°C. The tissue sections were dewaxed in xylene for 5 min then, rehydrated through graded alcohols and washed in distilled water for 3 min. Endogenous peroxidases were quenched by immersing the sections in 3% hydrogen peroxide in methanol for 5 min, after which the sections were rinsed twice for 5 min in distilled water. Sections were subjected to an antigen-retrieval protocol to expose sequestered antigens where the sections were placed in distilled water and boiled in a microwave on full power for two consecutive 5 min periods. Sections were cooled for 15 min, rinsed twice in distilled water and then rinsed in PBS containing 0.1% bovine serum albumin. Non-specific antibody binding was blocked with 10% normal rabbit serum (Labotec) for 20 min. The sections were blotted and the slides covered in a 1:500 dilution of primary goat anti-*Cowdria* MAP1 antibody (prepared as described in section 2.2.5.1.) for 60 min at room temperature. Following two further 5 min washes in PBS the sections were incubated for 30 min at room temperature with a 1:500 dilution of biotinylated rabbit anti-goat secondary antibody (Dako A/S). Two further washes in PBS were followed by covering the sections with an avidin-biotin-complex (Dako A/S) for 30 min according to the manufacturers recommendations. The sections were again rinsed in PBS and treated for 8 min with the perioxidase substrate diaminobenzamine tetrahydrochloride (BDH). Slides were finally rinsed in distilled water, counterstained with Mayer's haemotoxylin, dehydrated, mounted and examined with standard light microscope.

3.2.2. FICOLL PURIFICATION OF PBMC

PBMC were purified from whole blood by a modification of the method described by Coligan *et al.* (1991). A volume of 60 ml blood was collected into a syringe containing 2 ml 0.5 M ethylenediaminetetraacetic acid (EDTA). The blood was diluted 1:2 with Hanks' Balanced salt solution (HBSS; without magnesium, calcium and phenol red) containing 0.001 M EDTA. This mixture was either over- or underlayed with 15 ml Ficoll (Histopaque 1077-1; Sigma or Lympho separation medium; ICN Biomedicals) and centrifuged at room temperature for 30 min at 900g. The PBMC/Ficoll interface was collected and diluted three times with Alsever's solution. It was then centrifuged at 600g for 10 min. The resulting pellet was resuspended in Alsever's solution and recentrifuged at 400g for 10 min. This wash step was repeated until the supernatant was clear. After the last wash, the pellet was resuspended in complete RPMI-1640 medium (RPMI-1640 medium containing 25 mM N-[2-hydroxyethyl]piperazine-N'-[4-butanesulfonic acid], 2 mM L-glutamine, 10% fetal calf serum, $5x10^{-5}$ mercapto-ethanol, 0.1 mg/ml sodium benzylpenicillin and 0.2 mg/ml streptomycinsulphate). Cells were stained with 0.1% Trypan Blue and counted using a haemocytometer.

3.2.3. LYMPHOCYTE PROLIFERATION ASSAY

Proliferation assays were carried out in duplicate or triplicate wells of half-area flat bottom 96 well plates (Costar) at 37°C in a humidified atmosphere containing 5% CO_2 for five days (three days with concanavalin A) as described by Brown *et al.* (1995). Each well (total volume 100 µl) contained complete RPMI-1640 medium, responder cells added at a final concentration of 4 x 10^6 PBMC per ml and various indicated concentrations of infected or non-infected BSV cell

preparations (0.03 - 10 μg antigen/ml). Positive controls were either 2 U concanavalin A/100 μl (three day assay) or 6 U IL-2/100 μl (five day assay). Proliferation was determined by measuring the incorporation of 0.5 μCi of [methyl-^{3}H]thymidine added during the final 18 h of the assay. The cells were harvested, and the radioactivity was determined in a scintillation counter. Results are presented as a stimulation index (SI) ± standard deviation (SD), where SI = mean counts per minute (cpm) of test sample/mean cpm of unstimulated control. The unstimulated control was PBMC in medium. Unless otherwise stated a SI ≥ 2 was considered to be an indication of antigen-specific proliferation.

3.3. RESULTS

3.3.1. SEROLOGICAL SCREENING OF EXPERIMENTAL CATTLE

All experimental cattle were screened for the presence of antibodies to tick-borne parasites before immunisation. Animal B9191 was seronegative to *Cowdria* as determined by an IFA assay. Animals B1359; B809; B821; B1351 and B775 were seronegative to *Cowdria, B. bovis* and *T. mutans* as determined by IFA assays and *Anaplasma* as determined by competition inhibition ELISA.

3.3.2. IMMUNISATION OF EXPERIMENTAL CATTLE FOR THE PREPARATION OF PBMC

3.3.2.1. Determination of the virulence of the blood stabilate

The symptoms before death, the time interval before death (± 12 days) and the post mortem findings all indicated that the mice were infected with *Cowdria*. In addition, the presence of *Cowdria* in tissue sections of the mice was confirmed histopathologically (section 3.2.1.4.2.). These findings indicated that the stabilate was indeed virulent.

3.3.2.2. Monitoring of cattle immunised by infection and treatment

Two cattle (designated B9191 & B1359) were immunised by infection with virulent Welgevonden isolate-infected ovine blood stabilate. The virulence of the stabilate was confirmed as described in section 3.3.2.1. Rectal temperatures were taken daily and both were treated with oxytetracycline on the third day of a febrile reaction in order to prevent development of the disease beyond which point treatment is ineffective (Fig. 3.1.). Following immunisation, both cattle were found to be immune to challenge, with the same virulent blood stabilate, as determined by the lack of a febrile response (Fig. 3.1.) and overt heartwater symptoms.

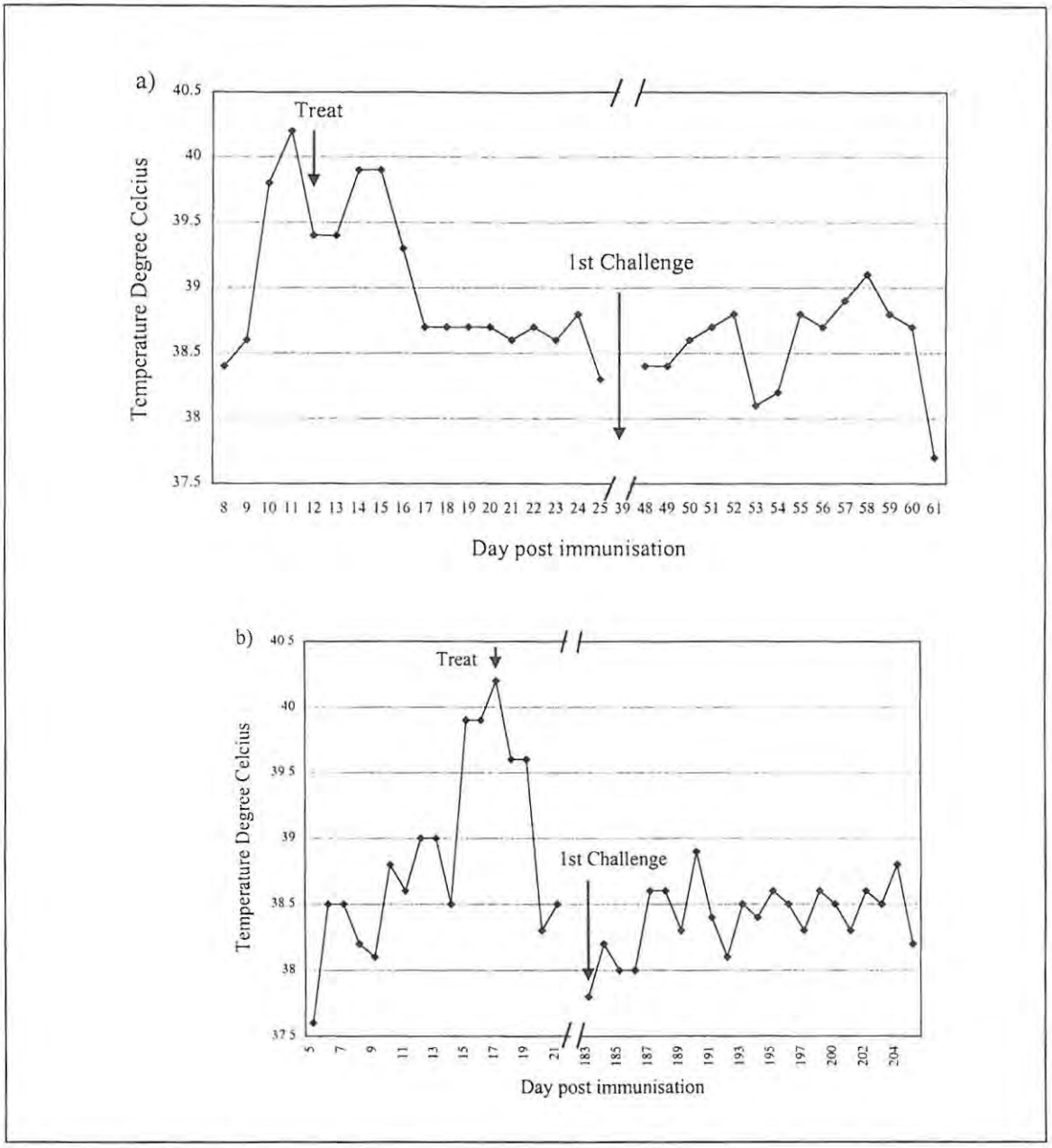

FIG. 3.1. Daily rectal temperatures of two cattle (a) B9191 & (b) B1359 immunised by infection and treatment. Both cattle were immunised on day one by i.v. injection of 5-10 ml of Welgevonden isolate-infected sheep blood stabilate. They were treated with liquamycin on the third day of a febrile reaction ("Treat"). They were challenged by i.v. injection of 5-10 ml of Welgevonden isolate-infected sheep blood stabilate ("Challenge").

3.3.2.3. Determination of infectivity of the inactivated *Cowdria*-infected cell culture stabilate

The mice were monitored for 30 days for disease symptoms during which time none developed. When the mice were sacrificed, no *Cowdria* organisms were found in tissue sections (section 3.2.1.4.2.). This indicated that the *Cowdria*-infected cell culture stabilate was indeed inactive.

3.3.2.4. Monitoring of cattle immunised with inactivated *Cowdria*

The four cattle (designated B809; B821; B1351 & B775) that were immunised with the inactivated stabilate in ISA50 adjuvant and one animal (B816) that was immunised with adjuvant alone, did not develop a febrile reaction or any disease symptoms after immunisation.

3.3.3. PROLIFERATIVE RESPONSES

Because of the simplicity of preparation and the high yields obtained, differentially centrifuged organisms were used in preliminary screening of proliferative responses of lymphocytes from B9191. In these initial assays, PBMC obtained from B9191, were assayed weekly with a range of different antigen concentrations. Once a proliferative response was observed, the assay was optimised as described in sections 3.3.3.3. and 3.3.3.4. The optimised assay conditions were then used in further studies.

3.3.3.1. Proliferative responses of PBMC during the course of immunising cattle by infection and treatment

The cattle were monitored for *Cowdria*-specific lymphocyte proliferative responses during the course of immunisation to establish when the optimal response occurred. Responses obtained during the course of immunisation by infection and treatment are illustrated in Fig. 3.2. Day-to-day variation was observed with PBMC obtained from each of the different animals. To address this variation, unstimulated PBMC controls were assayed on the same assay plates on the same day and used to determine the SI (section 3.2.3.). PBMC tested prior to immunisation or challenge did not respond to *Cowdria* antigens when tested at a range of protein concentrations varying from 0.03 - 10 μg/ml. In contrast, PBMC from both immune animals responded specifically to *Cowdria* antigens after challenge. After challenging B9191 for the second time, *Cowdria* specific lymphocyte proliferation peaked four weeks after challenge (mean cpm 31,764 ± 8702; 164 weeks after immunisation) but was not measurable two weeks later (Fig. 3.2.a). Lymphocytes obtained at this time point of peak proliferation were used to optimise the lymphocyte proliferation assay (sections 3.3.3.3. & 3.3.3.4.). These experiments established optimum assay conditions. By comparison, proliferation assays with PBMC from B1359 indicated an antigen-specific response one week after the first challenge (mean cpm 52,099 ± 3899; 28 weeks after immunisation) and three weeks after the second challenge (mean cpm 48,626 ± 4992; 41 weeks after immunisation). No response was measurable a week later (Fig. 3.2.b). When PBMC from these same cattle were assayed again one to two years later, they showed antigen-specific proliferation with SI ≥ 5.5. This response was measurable for up to three years later. The further duration of the response was not determined.

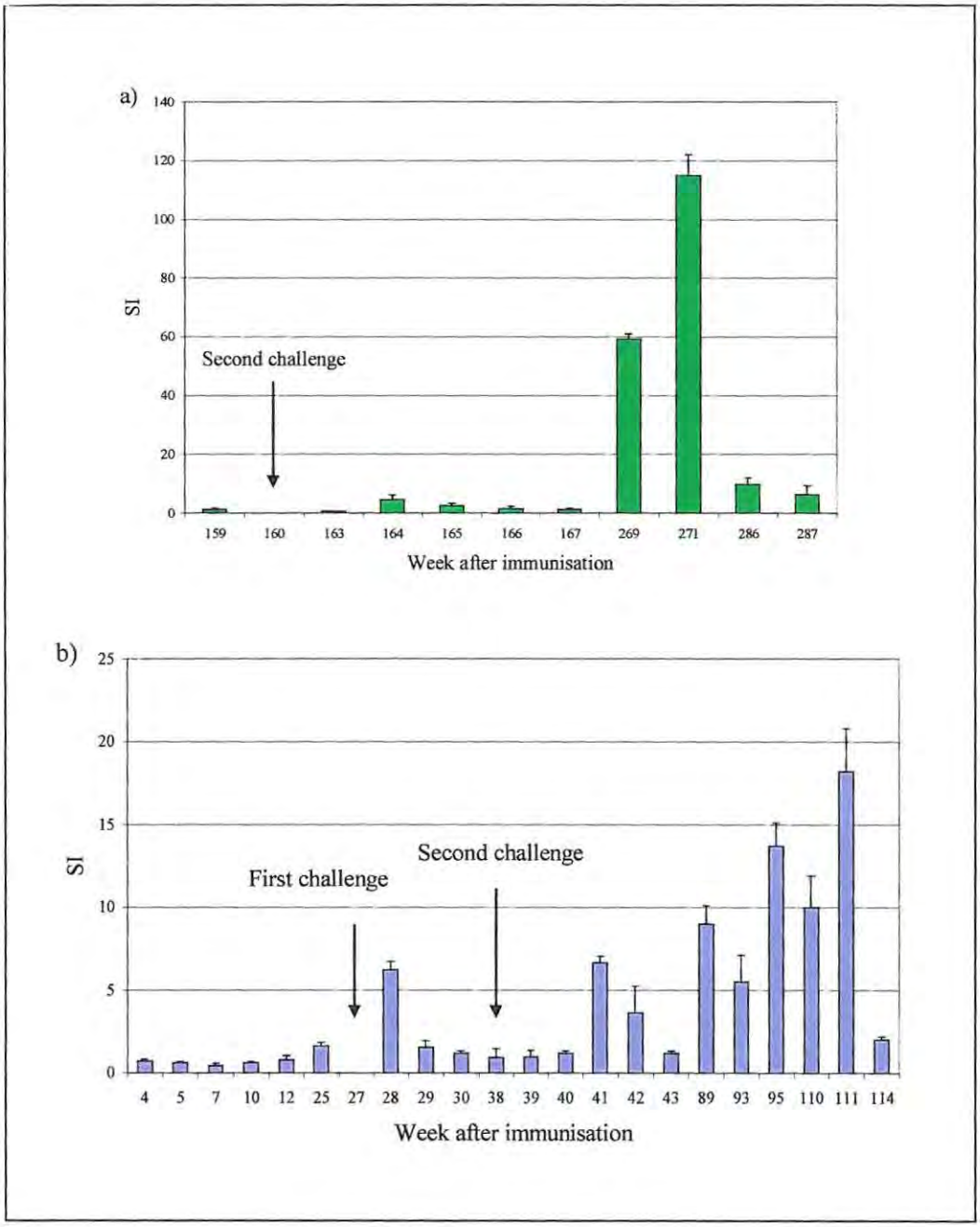

FIG. 3.2. Proliferative responses of PBMC from cattle B9191 (a) and B1359 (b) at various intervals after the animals were immunised against heartwater by infection and treatment. The PBMC were cultured for six days with differentially centrifuged preparations of *Cowdria*-infected BSV cells at a concentration of 1 μg/ml in either duplicate or triplicate wells. The mean cpm for the PBMC controls were: 2065 ± 2194 (B9191) and 3943 ± 3090 (B1359). The arrows (→) indicate the time of challenge. Results are presented as SI ± SD.

3.3.3.2. Proliferative responses of PBMC during the course of immunising cattle with inactivated organisms

Lymphocytes from cattle immunised using inactivated organisms were included to establish whether they responded similarly to lymphocytes from 'naturally' infected cattle. In addition, proliferation assays were conducted during the course of immunisation to determine when the proliferative responses were maximal. In contrast to what was observed when cattle are immunised by infection and treatment, lymphocytes from the cattle immunised with inactivated organisms (B809, B821, B1351 and B775) proliferated specifically in response to *Cowdria* antigen throughout the duration of the investigations (Fig. 3.3.). PBMC collected from the control animal (B816) did not proliferate in response to *Cowdria* antigen either before or after immunisation with adjuvant alone (mean SI < 2; Fig. 3.3.). PBMC from cattle immunised with inactivated organisms could thus be used at any time to detect lymphocyte responses.

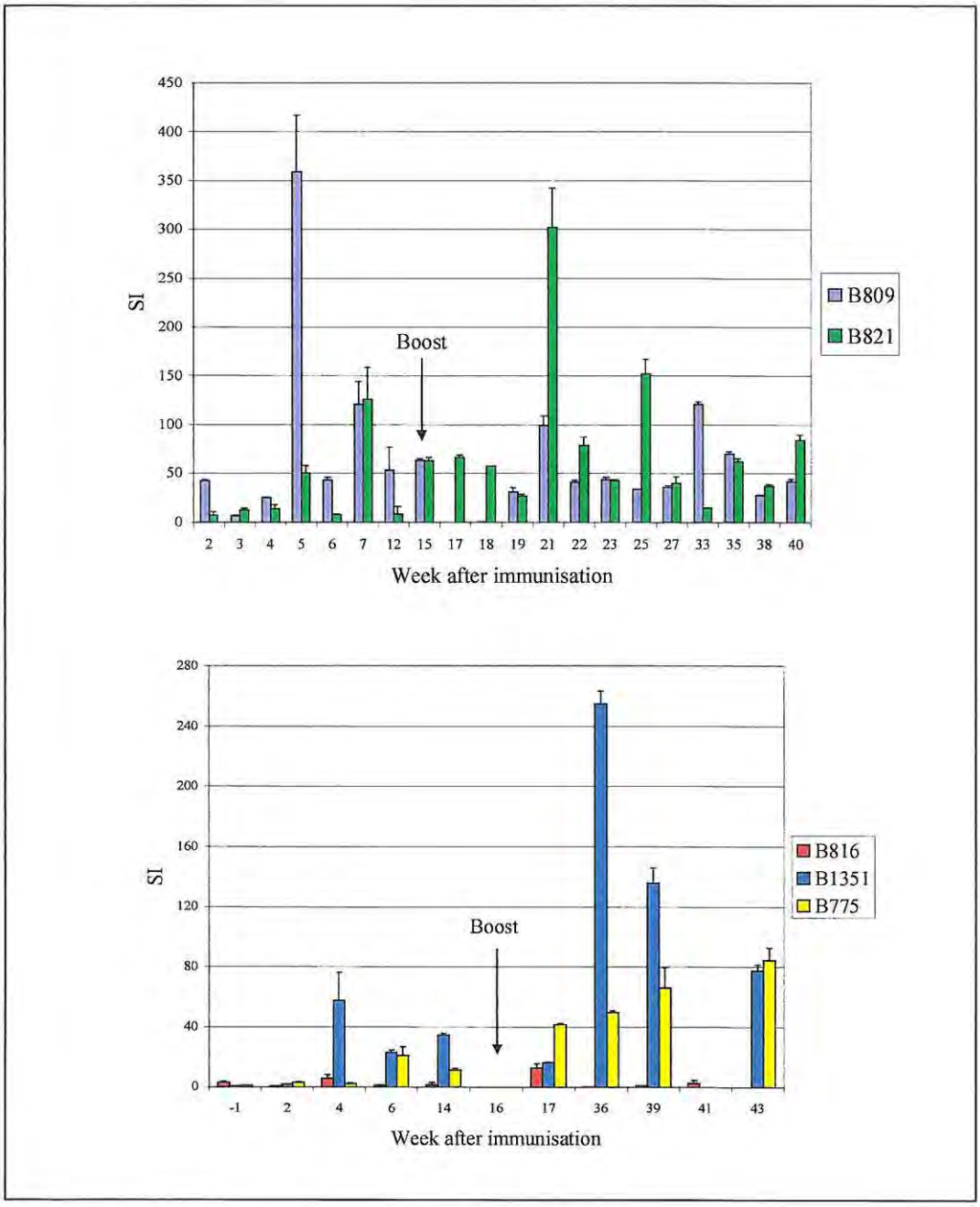

FIG. 3.3. Proliferative responses of PBMC from cattle B809; B821; B816; B1351 and B775 at various intervals after immunising with inactivated *Cowdria.* The PBMC were cultured for six days with differentially centrifuged preparations of *Cowdria*-infected BSV cells at a concentration of 1 μg/ml in either duplicate or triplicate wells. The mean cpm for the PBMC controls were: 15,707 ± 18,666 (B816); 4120 ± 6930 (B809); 2529 ± 2710 (B821); 8948 ± 11,612 (B1351) and 6724 ± 6063 (B775). The arrows (→) indicate the time of boosting. Results are presented as SI ± SD.

3.3.3.3. Proliferation elicited by different *Cowdria* preparations

Cowdria organisms prepared by each of the three different methods described in Chapter 2 were tested for their ability to elicit proliferative responses in PBMC obtained initially from B9191, an animal that had been immunised by infection and treatment (164 weeks post-immunisation). To compare their efficacy, the antigen preparations were assayed in duplicate wells on the same day (section 3.2.3.). The highest lymphocyte proliferative response was obtained with antigen prepared by immunoadsorbent chromatography followed closely by organisms prepared by differential centrifugation and finally, those from Percoll density gradients (B9191a, Fig. 3.4.). Since low yields were obtained by immunoaffinity chromatography, and differential centrifugation offered simplicity and higher yields, the latter was used in subsequent optimisation assays and for the examination of the proliferative responses of immunised cattle. Once these responses had been determined with one individual, the three different methods of *Cowdria* preparation were once again tested to verify their ability to elicit proliferative responses in PBMC from all the experimental cattle. The PBMC were obtained from a non-immune animal, two cattle immunised by infection and treatment (B9191 & B1359) and three immunised with inactivated organisms (B821, B775 & B1351). With the exception of PBMC obtained from B775, the highest lymphocyte proliferative response was obtained with antigen prepared by differential centrifugation (Fig. 3.4.). Because it is extremely difficult to determine the amount of endothelial cell contaminants within *Cowdria* infected preparations and to determine the level of non-specific proliferation, appropriate controls were included in the assays. When differentially centrifuged non-infected BSV cell preparations (prepared from B9191) were used as the antigen in proliferation assays with PBMC obtained from immunised animals, proliferation was consistently significantly lower than that obtained with the infected preparations (Fig. 3.4.).

This indicated that the latter response was *Cowdria*-specific. Differentially centrifuged non-infected BSV cell preparations gave negligible proliferation in assays with PBMC obtained from nine naïve cattle (SI ≤ 1.7; results not shown). These results showed that non-infected BSV cell antigen did not induce alloreactive responses in PBMC from unrelated immunised or non-immune cattle. Owing to its relative simplicity in comparison to immunoadsorbent chromatography, together with the higher proliferative responses induced, differential centrifugation was therefore subsequently routinely used as the method of choice to purify *Cowdria* for use in the immunological assays.

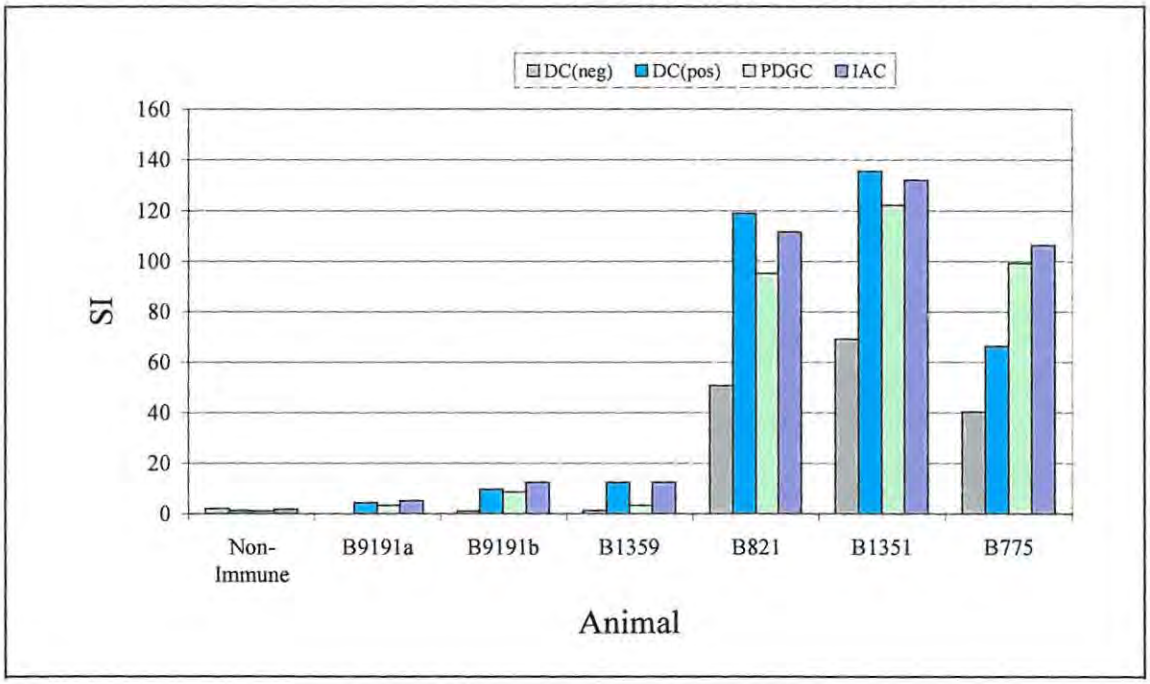

FIG 3.4. Proliferative responses of PBMC from a non-immune animal, two immune animals immunised by infection and treatment (B9191 & B1359) and three animals immunised with inactivated organisms (B821, B1351 & B775) to various *Cowdria* preparations. Differentially centrifuged non-infected BSV cell preparations (DC(neg)) and *Cowdria*-infected BSV cells prepared by differential centrifugation (DC(pos)), Percoll density gradient centrifugation (PDGC) and immunoaffinity chromatography (IAC) were used as antigen in proliferation assays. B9191a = PBMCs were collected 164 weeks post-immunisation, B9191b = PBMCs were collected three years post-immunisation.

3.3.3.4. Optimisation of the lymphocyte proliferation assay

To establish optimal conditions for the lymphocyte proliferation assays, various concentrations of differentially centrifuged antigen were tested. The maximal response was obtained with 1.25 μg/ml antigen when measured with PBMC obtained 28 days post second challenge and with 0.6 μg/ml with PBMC obtained 35 days post second challenge (Fig. 3.5.). In addition, the possible toxic/inhibitory effect of the antigen concentration on the lymphocytes was investigated in the presence of the mitogen Con A and growth factor IL-2. Con A and IL-2 were used as positive controls in the three or five day assays respectively. Antigen concentrations of ≥ 2.5 μg/ml resulted in a 50% inhibition of Con A induced responses (Table 3.2.). Antigen concentrations of ≥ 1.25 μg/ml resulted in a 75% inhibition of IL-2 induced responses (Table 3.2.). Antigen concentrations of between 0.6 and 1.25 μg/ml elicited maximal antigen-specific proliferation as indicated by a higher SI and therefore an average of 1 μg/ml was used in all the ensuing assays.

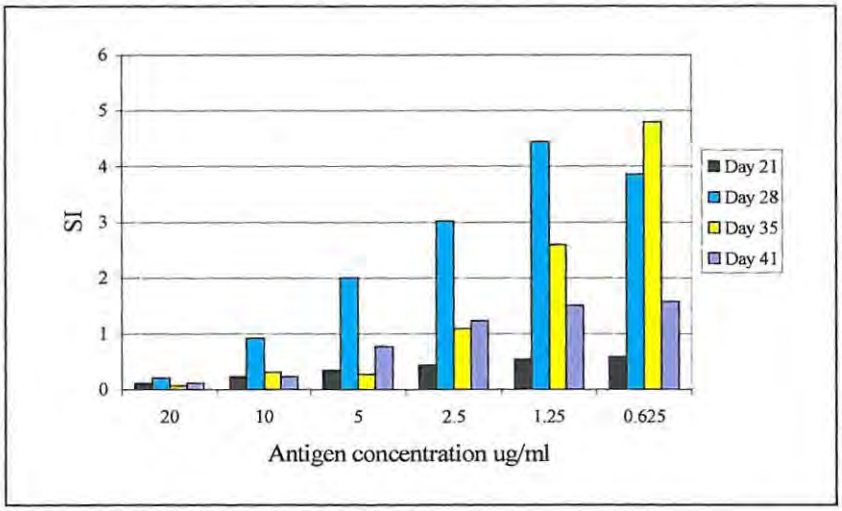

FIG 3.5. Proliferative responses of PBMC to various concentrations of purified *Cowdria* organisms. The PBMC were collected from B9191 at various intervals (21 – 41 days) after the second challenge. The cells were cultured for six days with differentially centrifuged preparations of *Cowdria*-infected BSV cells at concentrations varying between 0.6 and 20 μg/ml in duplicate wells. The mean cpm for the PBMC controls was: 4022 ± 2192.

TABLE 3.2. Proliferative responses of PBMC to various concentrations of purified *Cowdria* organisms in the presence of Con A or IL-2. The PBMC were cultured for four days (Con A) or six days (IL-2) with differentially centrifuged preparations of *Cowdria*-infected BSV cells at concentrations between 20 and 0.6 μg/ml in duplicate wells. The mean cpm for the PBMC controls were: 12,682 ± 1373 (Con A assay) and 7156 ± 749 (IL-2 assay).

Antigen concentration μg/ml	SI					
	Antigen	Antigen + Con A	Con A	Antigen	Antigen + IL-2	IL-2
20	0.2	4.0		0.2	2.7	
10	0.5	1.9		0.9	1.6	
5	1.4	6.1		2.0	2.4	
2.5	1.9	5.9		3.0	4.4	
1.25	1.7	9.7		4.4	3.5	
0.625	1.9	10.9		3.9	12.3	
Control			10.9			11.3

3.4. DISCUSSION

The duration of protective immunity in cattle immunised by infection with *Cowdria* and treatment with antibiotic has been reported to be at least two to three years (du Plessis & Bezuidenhout, 1979; du Plessis *et al.*, 1992b). Indeed animal B9191 was found to be immune to challenge three years after immunisation by this regimen, thus confirming these observations. Proliferation assays after immunisation by infection and treatment detected no *Cowdria*-specific proliferation *in vitro* after a single treatment. A proliferative response was observed, however, between one and four weeks after challenging the cattle. This was followed by a period when no response was detectable. It only reappeared between one and three years after the first immunisation. The long intervals during which no responses were detected hampered progress in evaluating the cellular immune responses of these cattle. This finding highlights the importance of regular monitoring of cattle immunised by infection and treatment for the appearance of reactive lymphocytes. Only once a proliferative response was observed, could the assay be optimised. In contrast, a proliferative response was observed a week after the cattle were immunised with inactivated *Cowdria*, remaining detectable at all times for up to three years afterwards.

The proliferation assay results presented here were obtained from studies undertaken with PBMC. As the animals B9191 and B1359 were immunised with live *Cowdria*, both replicating and circulating organisms should thus be present in these animals. In addition, the organism has been shown to occur in various cell types including macrophages, monocytes, Küpffer cells, reticulum cells of the lymph nodes, fibroblasts and connective tissues as well as cells of the

spleen, brain, pancreas and the heart (Cowdry 1925; du Plessis 1970 & 1975; Ilemobade & Blotkamp, 1978). Their immune responses may therefore have been taking place locally (e.g. in the lymph nodes, spleen etc.) during the periods when no detectable proliferative responses were obtained with circulating peripheral lymphocytes. On the other hand, animals that had been immunised with inactivated *Cowdria* always had responsive circulating lymphocytes present in their blood, irrespective of when they were assayed (Totté *et al.*, 1997), thus confirming the findings of this thesis. Gale and co-workers (1996) also observed a highly variable PBMC proliferative response in cattle immune to a related pathogen *A. marginale.* At certain times after infection, PBMC did not respond in proliferation assays. Sensitised T-cells, however, were readily detected in their spleens at all time points tested. The authors noted that a positive PBMC proliferative response may be indicative of carrier status/immunity against *A. marginale*, and that a negative response is not necessarily indicative of carrier-free status/susceptibility (Gale *et al.*, 1996). Similarly, following vaccination with live *B. bovis* parasites, lymphocyte responses were low and lasted for approximately two weeks. The responsive lymphocytes disappeared from circulation but reactive cells could still be found in the spleen or lymph nodes. In contrast, immunisation with non-living antigen resulted in higher proliferative responses lasting for six months, but this did not correlate with protection (Timms & Stewart 1984). The restricted time period when proliferation was observed when cattle were immunised with live *Cowdria*, as well as the higher proliferation obtained with inactivated organisms are thus not phenomena unique to heartwater.

The early disappearance of a proliferative response by peripheral lymphocytes may be explained by the circulatory pathways of lymphocytes. These cells continually circulate from the blood into

the peripheral lymphoid tissues and return to the blood via the lymphatic ducts. The peripheral T-cell population consists of naïve, effector and memory T-cells. They have different migration routes directed by different receptors present on the lymphocytes that interact with tissue specific adhesion molecules. Naïve lymphocytes circulate through the lymph nodes where they encounter antigen and are activated. On the other hand, memory and effector lymphocytes migrate to sites of infection and inflammation. Memory cells are generally found in tissues where they are most likely to re-encounter an infection (Kuby, 1994; Janeway & Travers, 1994). Many immune responses may therefore initially remain localised at the site of infection. PBMC may not provide information on potential responses. Spleen or lymph node cells may better represent ongoing responses. In the case of immunisation with inactivated organisms in adjuvant, this may be seen as a depot of antigen rather than an infection site. Therefore, specific memory lymphocytes remain in circulation as seen in the results presented here. The timing of the assay is also important as the Th1 and Th2 responses may predominate at different stages of an infection (Mosman & Sad, 1996). Taking the above into consideration, a study of the responses in other immune compartments such as the lymph nodes, the spleen or at the sites of infection may give a more defined picture of the immune response to *Cowdria* during these periods. Furthermore, antigen-specific proliferation was later detected in PBMC from the cattle used in these studies. This may be explained by the return into circulation of responsive lymphocytes. Mwangi and co-workers (1998a) similarly failed to detect a proliferative response before challenge with PBMC collected from animals immunised by infection and treatment. Proliferation was only detected when autologous endothelial cells were pre-treated with T-cell growth factors prior to infection with live organisms, fixed and then used as stimulators. Their results suggest that antigen processing and presentation by infected endothelial cells or monocytes may be essential for the

induction of specific T-cell responses. This may provide an alternative explanation for the periods during which no proliferation was observed.

Differential centrifugation was found to be the purification method of choice as antigen obtained in this manner induced the highest, most specific proliferative responses. Similar results were obtained with the Gardel isolate (Totté *et al.*, 1997). The low proliferative responses to BSV cell antigen of PBMC from cattle immunised by infection and treatment indicates that any endothelial cell contaminants that may be present after differential centrifugation did not result in any non-specific background proliferation. In contrast, elevated proliferation values were obtained with PBMC from cattle that had been immunised with inactivated organisms and assayed with differentially centrifuged non-infected BSV cell preparations. This may have been due to an adjuvant effect (Emery *et al.*, 1990) or the presence of residual endothelial cell debris in the differentially centrifuged preparations that were used for immunising these cattle. However, BSV cell antigen did not induce alloreactive responses in PBMC from the cattle and was therefore able to be used as a source of *Cowdria* for the ensuing immunological studies.

To summarise, cattle immunised by infection and treatment or with inactivated organisms could be used to supply reactive PBMC. In addition, conditions for performing the lymphocyte proliferation assays were optimised. These studies provided an important foundation for further investigations aimed at identifying immunodominant *Cowdria* T-cell antigens.

CHAPTER 4

FRACTIONATION OF *COWDRIA* INTO ITS CONSTITUENT PROTEINS AND ANALYSIS OF PROLIFERATIVE RESPONSES INDUCED IN PBMC

4.1. INTRODUCTION

The previous chapter described how lymphocytes from cattle immunised either by infection and treatment or with inactivated organisms proliferate *in vitro* in response to whole *Cowdria*. With vaccine development in mind, it is important to identify proteins that are likely to be involved in protective immunity. One possible source of such proteins could be from an expression library. A representative λZAPII expression library of the Welgevonden isolate of *Cowdria* has been constructed. It resulted in molecular clones of *map*1 and three other *Cowdria* genes being obtained (Brayton *et al.*, 1997). This library should therefore be able to provide a large number of *Cowdria* fusion proteins. However, when cloning DNA of bacterial origin, the cloned promotors may sometimes be active in the host cell leading to expression of foreign proteins that can be toxic to the host. This was indeed found to be a problem with the λZAPII library. It was therefore unsuitable as a ready source of recombinant proteins for use in proliferation assays. Another problem is that screening expression libraries, whether by immune serum or in proliferation assays, is highly labour intensive. For example, *Salmonella* genomic DNA fragments were subcloned into *E. coli* by using the λgt11 expression vector. The resulting library

was first screened by immunoblot methods with specific polyclonal antisera. In an examination of more than 5000 cloned *Salmonella* genomic segments, not one of the immunoblot-positive clones stimulated antigen-specific proliferation of sensitised lymphocytes. When the expressed antigens were each tested for their capacity to stimulate lymphocyte proliferation, only five of the 2000 recombinant phages tested stimulated an antigen-specific proliferative response (Warren *et al.*, 1990). There are, however, reports that PBMC cannot be used to screen recombinant DNA libraries for T-cell reactivity because *E. coli* components stimulate PBMC, thereby masking the specific response. Because T-cells do not recognise antigens directly, non-specific binding cannot be adsorbed out. The problem could be overcome by establishing T-cell clones which lead to reduced background proliferation. Screening 1750 pools of recombinant antigens with a mixture of eight T-cell clones identified two recombinant phage clones that stimulate T-cell proliferation (Mustafa *et al.*, 1998).

Antigen recognition by T-cells is minimally dependent on the native conformation of the antigen (Benjamin *et al.*, 1984; Unanue, 1984). This makes it practical to obtain proteins for lymphocyte proliferation assays by electrophoretic methods, even if the protein gets denatured. For example, protective malarial parasite proteins ranging between 25 to 40 kDa have been identified by vaccination trials with fractions obtained by denaturing continuous flow electrophoresis (CFE) of *P. chaudi adami* schizont proteins (Kima *et al.*, 1992). Similarly, Brown and co-workers have successfully identified several antigens of *B. bovis* merozoites (also obtained by means of CFE) that stimulated proliferation of T-cell lines and clones (Brown *et al.*, 1995; Brown & Palmer 1999; Stich *et al.*, 1999). Since T-cells are required for protective immunity in malaria (Grun & Weidanz, 1981; Brake *et al.*, 1988) and babesiosis (Brown *et al.*, 1991) these findings suggest

that potentially protective *Cowdria* T-cell antigens could similarly be identified using CFE fractionated proteins. The advantages include high resolution of proteins with as little as 2% difference in apparent molecular size as well as the ability to fractionate cell membrane-associated and other proteins that are insoluble in their native state. Analysis is thus not limited to soluble proteins and the optimisation of protein solubilisation with various nonionic or zwitterionic detergents, which is required for isoelectric focusing, is eliminated.

In an attempt to identify which of the multitude of proteins comprising *Cowdria* were responsible for the T-cell responses described in the previous chapter, BSV cell cultures infected with the Welgevonden isolate of *Cowdria* were fractionated by continuous flow electrophoresis (CFE) under denaturing and reducing conditions. Non-infected BSV cell cultures were also fractionated to serve as a negative control. Essentially 100% of the SDS can be removed by acetone precipitation (Hager & Burgess, 1980). CFE fractions may therefore be used in subsequent proliferation assays without adverse effects caused by the detergent. Each fraction was tested for its ability to stimulate lymphocyte proliferation *in vitro*. As was described in the previous chapter, the PBMC used in the proliferation assays were obtained from cattle which were either immunised by infection and treatment, or with inactivated organisms.

4.2. MATERIALS AND METHODS

4.2.1. FRACTIONATION BY CONTINUOUS FLOW ELECTROPHORESIS

CFE of crude *Cowdria*-infected or non-infected BSV cell preparations was performed using a Prep-Cell Apparatus (Bio-Rad) essentially as described by Brown and co-workers (1995) with the following modifications. The separating gel contained either 7% or 15% acrylamide, 0.3% bisacrylamide, in 0.4 M Tris buffer (pH 8.8) and was polymerised by the addition of 0.25% ammonium persulphate and 0.025% tetremethylenediamine (final concentration). The stacking gel contained 4% acrylamide, 0.1% bisacrylamide, in 0.1 M Tris buffer (pH 6.8) and was polymerised by the addition of 0.5% ammonium persulphate and 0.1% tetremethylenediamine (final concentration). A separating gel of 8 cm was cast and the volume of stacking gel was two and a half times that of the sample. Approximately 10 mg of protein was solubilised in SDS-PAGE sample buffer (section 2.2.7.), boiled and electrophoresed under reducing conditions on 28-mm (internal diameter) cylindrical gels consisting of either 7% or 15% acrylamide. Fractions of 2.5 ml were eluted and collected over the course of 15 hr (7% acrylamide gel; 180 fractions) or 7.5 hr (15% acrylamide gels; 175 fractions). The fractions were stored at -70°C. They were precipitated by adding 8 times the volume of ice-cold acetone, incubated at -20°C for 16 h and centrifuged at 10,000g for 10 min. The acetone was aspirated, the precipitates resuspended in 70% cold ethanol and centrifuged at 10,000g for 30 min. After ethanol removal the pellets were air dried, suspended in PBS containing antibiotics and stored at -70°C. All the fractions (pooled at six fractions per sample to give a final concentration of 5 % (v/v) per fraction per well) were tested in proliferation assays (section 3.2.3.) with PBMC from all the experimental cattle. The

one tailed Student *t* test was used to determine the levels of significance of SIs between the non-infected and infected BSV cell CFE fractions.

4.2.2. SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

A volume of 20 µl of every tenth precipitated fraction was analysed by SDS-PAGE as described in section 2.2.7.). A vertical mini-gel system (EC-120) with acrylamide gels of either 7% for the 7% Prep-Cell fractions or 12% for the 15% Prep-Cell fractions were used.

4.2.2.1. Silver staining

Protein bands were detected by silver staining, according to the manufacturers recommendations, using one of three silver stain kits: Silver stain kit (SIGMA), Gel Code (Pierce) or Silver Snap (Pierce).

4.3. RESULTS

4.3.1. FRACTIONATION OF *COWDRIA*-INFECTED AND NON-INFECTED BSV CELL CULTURES INTO CONSTITUENT PROTEINS BY CONTINUOUS FLOW ELECTROPHORESIS

In order not to exclude any *Cowdria* proteins that may be associated with the host cell, fractionation by CFE was carried out with crude preparations. Cell culture proteins between 50-168 kDa were optimally fractionated on a 7% gel and between 11-38 kDa on a 15% gel (Fig.

4.1.). In a similar control experiment, non-infected BSV cell culture proteins between 50-123 kDa were fractionated on a 7% gel and between 11-34 kDa on a 15% gel (results not shown). There were artefactual bands at approximately 66 kDa on the analytical SDS-PAGE gels which were used to determine the molecular weights of the fractions (Fig. 4.1.). Similar bands were also observed by Brown and co-workers (1995) and may be due to a chemical reaction by the electrophoresis reagents (Beis & Lazou, 1990). Other protein bands, for example at fractions 5; 15; 25; 35; 65 and 75 on gel a) and fraction 150 on gel b) may be due to proteolytic degradation. A total of 700 fractions were collected and prepared for lymphocyte proliferation assays (section 3.2.3.) by acetone precipitation (section 4.2.1.). Due to the large number of fractions to be assayed and the relatively small quantities of protein within each fraction, it was not practical to standardise them with regard to concentration. Therefore, a randomly chosen CFE fraction (with molecular weight of approximately 100 kDa) was tested in proliferation assays at final concentrations of 3-24% (v/v) in the presence or absence of ConA or IL-2. This was done to establish optimum working concentrations and to determine whether inhibitory or toxic substances may be present in the fractions after CFE and/or precipitation. Concanavalin A- or IL-2-induced proliferative responses were not inhibited at any of the concentrations tested (Fig. 4.2. & Fig. 4.3.). Based on these findings together with previous results obtained by other workers (Brown *et al.*, 1995), a final concentration of 5% (v/v) was selected for testing with *Cowdria* immune PBMC.

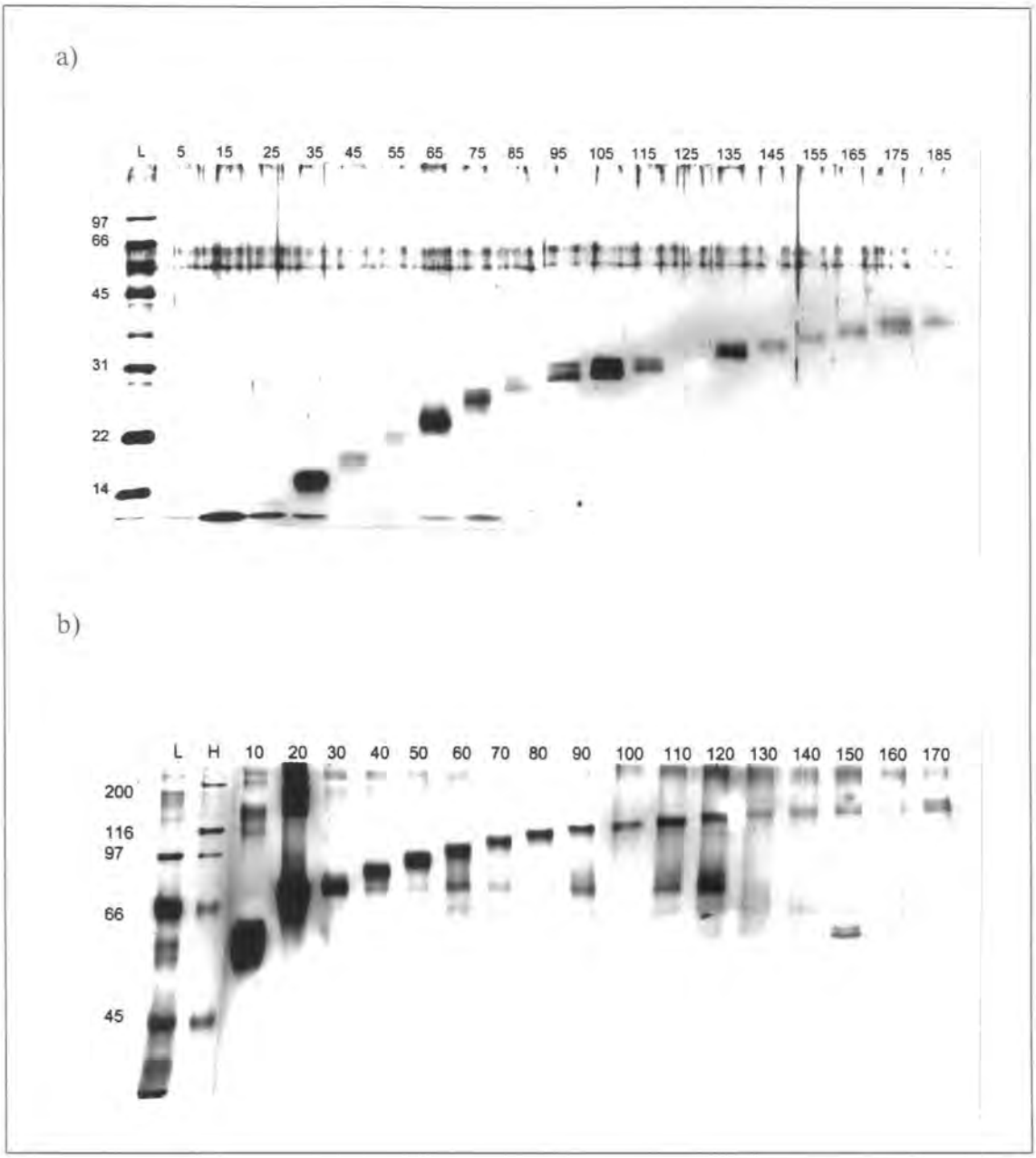

FIG. 4.1. SDS-PAGE analysis of *Cowdria*-infected BSV cell preparations fractionated by CFE. Crude *Cowdria*-infected BSV cell preparations were fractionated by CFE on either 15% or 7% acrylamide gels. The fractions were precipitated with acetone and resuspended in 500 µl PBS. A volume of 20 µl of every 10th precipitated fraction was subjected to analytical SDS-PAGE. A 12% acrylamide was used to analyse the 15% CFE fractions (a) and a 7% gel to analyse the 7% CFE fractions (b). Proteins were detected by silver staining. The relative mobilities of the low molecular weight standards (lane L) and high molecular weight standards (lane H) are indicated on the left of each panel in kilodaltons.

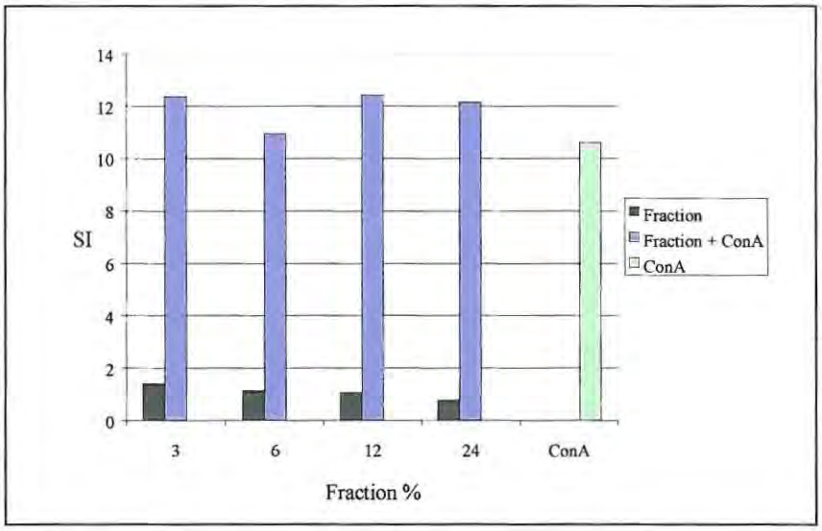

FIG. 4.2. Proliferative responses of PBMC to various percentages of a 100 kDa CFE *Cowdria* fraction in the presence of Con A. The PBMC were cultured for four days with 3, 6, 12, and 24% (v/v) of a CFE fraction in the presence of 2 U Con A/well. The mean cpm for the PBMC controls were: 12,682 ± 1373.

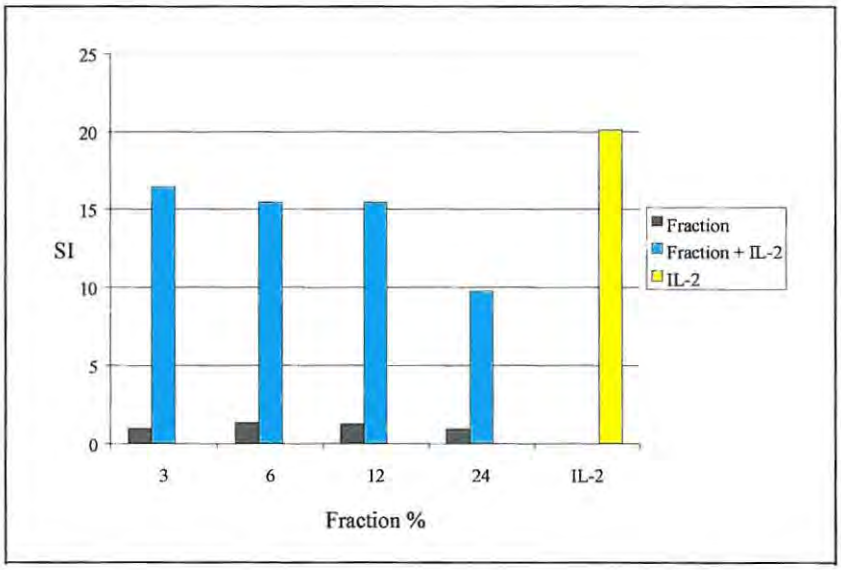

FIG. 4.3. Proliferative responses of PBMC to various percentages of a 100 kDa CFE *Cowdria* fraction in the presence of IL-2. The PBMC were cultured for six days with 3, 6, 12, and 24% (v/v) of a CFE fraction in the presence of 6 U IL-2/well. The mean cpm for the PBMC controls were: 2821 ± 124.

4.3.2. PROLIFERATIVE RESPONSES INDUCED BY *COWDRIA* PROTEINS FRACTIONATED BY CONTINUOUS FLOW ELECTROPHORESIS

The degree of proliferation (ie. SI; section 3.2.3.) of PBMC that was induced by pools of CFE fractions which had been prepared either from infected or non-infected control cultures was determined for each experimental animal (B9191, B1359, B809, B821, B1352, B775 and B816 given below). It was impractical to screen each fraction individually, therefore, they were pooled at six per sample before being assayed in triplicate for their ability to induce lymphocyte proliferation. A mean SI baseline (SI_{neg}) for CFE fractions prepared from non-infected BSV cultures was calculated for each animal. The one-tailed Student *t* test was used to determine whether there was a statistically significant difference between the SI obtained from each pool of CFE fractions prepared from infected BSV cell cultures and the SI_{neg}.

4.3.2.1. Proliferative responses of PBMC from cattle immunised by infection and treatment to CFE fractions

Animal B9191: Fractions from *Cowdria*-infected and non-infected BSV cells were assayed with PBMC collected from animal B9191 at week 291 after immunisation. The SI_{neg} was determined to be 1 ± 0.1 and 2 ± 0.1 for the 7% and 15% polyacrylamide CFE fractions respectively. An examination of the fractions derived from the CFE using a 7% polyacrylamide gel, revealed that those inducing *Cowdria* specific proliferation were localised to the first 24 fractions i.e. those with estimated molecular weight of ≤ 74 kDa. In addition, a further four discrete pools with higher molecular weights induced a lower level of proliferation (50 to 67; 81 to 111; 115 to 140 and 151 to 162 kDa; Fig. 4.4.a & Table 4.1.). A similar preparation was fractionated on a 15%

polyacrylamide gel to resolve the low molecular weight proteins. The stimulatory fractions ranged from 11 to 38 kDa (Fig. 4.4.b & Table 4.2.). Due to the relatively high SI_{neg} of 2 obtained for the 15% CFE gel, a SI ≥ 4 was considered as antigen specific for this preparation. Ten groups of fractions had SI ≥ 4. The molecular weights in these groups ranged from 11 to 24 and 26 to 38 kDa (Fig. 4.4.b & Table 4.2).

Animal B1359: Fractions from *Cowdria*-infected BSV cells were assayed with cryopreserved PBMC collected 41 weeks after immunising B1359. In a similar way to those of B9191, lymphocytes from this animal responded specifically to *Cowdria* fractions with relatively low molecular weights, ie. in the range of 11 to 23 and 26 to 27 kDa (Fig. 4.5.b & Table 4.2.). In addition, a pool of high molecular weight proteins of approximately 85 to 90 kDa also induced lymphocyte proliferation (Fig. 4.5.a & Table 4.1.). In the case of this animal's PBMC, the SI_{neg} was determined to be 0.4 ± 0.1 and 0.7 ± 0.2 for the 7% and 15% CFE fractions respectively. The low SI_{neg} values obtained indicated that no alloreactive responses were induced by the BSV cells present in CFE fractions. A comparison of the results obtained with B9191 showed that proteins in the molecular weight range of 11 to 23, and 26 to 27 kDa were recognised by lymphocytes from both immune cattle.

4.3.2.2. Proliferative responses of PBMC from cattle immunised with inactivated organisms to CFE fractions

Animals B809; B821; B775; B1351 and B816: Fractions from *Cowdria*-infected BSV cells were assayed in lymphocyte proliferation assays with PBMC collected one week before, and between 18 and 25 weeks after immunisation commenced. As controls, fractions from non-infected BSV

cells were assayed with PBMC collected between 41 and 57 weeks. The proliferative responses of PBMC from B809 induced by the different CFE fractions are represented in Fig. 4.6. to illustrate the type of responses obtained. The responses of PBMC from animals B821; B1351; B775 and B816 induced by CFE fractions of *Cowdria*-infected BSV cells are summarised in Table 4.1. and Table 4.2. No antigen specific proliferation was detected with PBMC from animals before immunisation with inactivated organisms, with a mean SI ≤ 2 being obtained (B809: Fig. 4.6.; results of remaining cattle not shown). A $SI_{neg} < 1.0$ was observed for all cattle, except for the control animal, when PBMC were tested with 7% and 15% CFE fractions from non-infected endothelial cells (B809: Fig. 4.6.; results of remaining cattle not shown). The low SI_{neg} values obtained indicated that no alloreactive responses were induced by the presence of BSV proteins in the CFE fractions. PBMC from the experimental animal B809 that was immunised with inactivated organisms responded specifically and significantly to fractions containing proteins of 11 to 31; 60 to 80; 85 to 134 and 145 to 148 kDa (Fig. 4.6. & Table 4.1. & Table 4.2.). PBMC from cattle B821, B1352 and B775 responded in a similar manner to *Cowdria* fractions (Table 4.1. & Table 4.2). The SI_{neg} for the control animal B816 was determined to be 3.6 ± 0.9 and 4 ± 0.5 for the 7% and 15% CFE fractions respectively. The high SI_{neg} of 4 obtained was taken into consideration and a SI ≥ 8 was considered as antigen-specific for this particular animal. The high SI values obtained for this control animal (immunised with adjuvant alone) may have been as a result of an unknown adjuvant effect. However, six groups of fractions which induced significant proliferation of PBMC from animal B816 were identified. They had the following approximate molecular weights: 13; 18; 26; 27; 29 and 30 kDa (Table 4.2.).

Fractions that induced PBMC to proliferate were compared between animals immunised by infection and treatment and those immunised with inactivated *Cowdria*. Proteins with molecular weights of approximately 11; 12; 14 to 17 and 19 to 23 kDa were found to induce lymphocytes to proliferate in both sets of immunised animals (see Table 4.2).

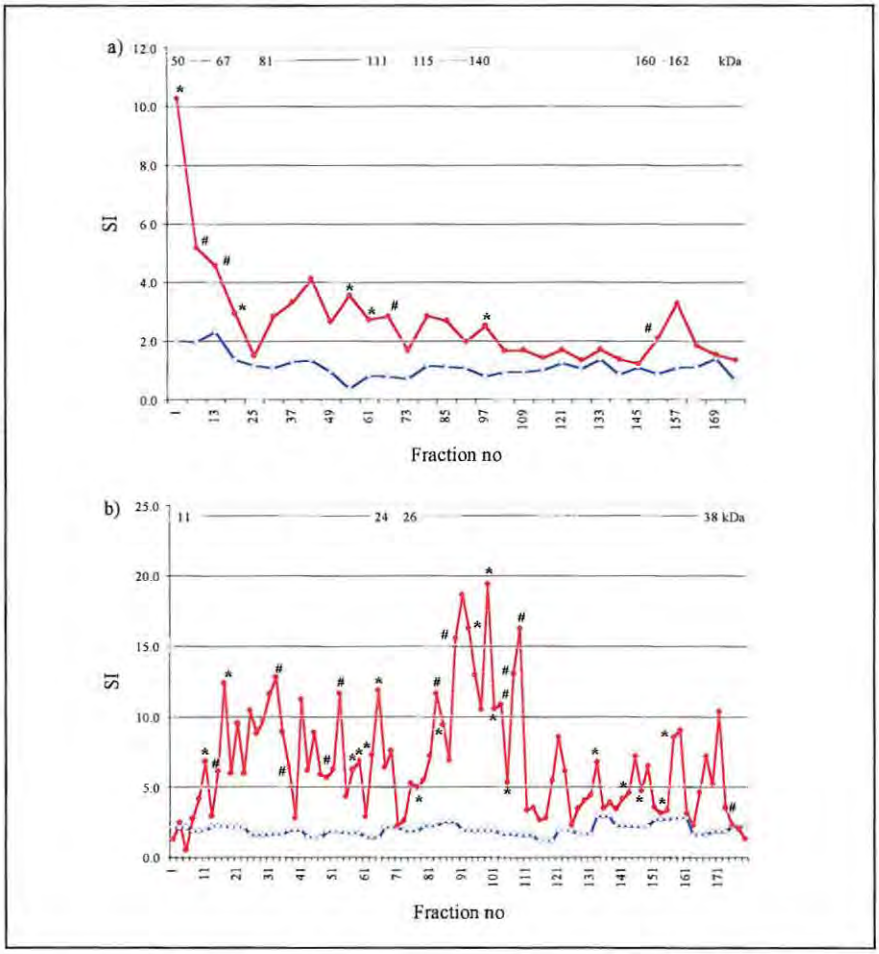

FIG. 4.4. Proliferative responses of PBMC obtained from animal B9191 when exposed to *Cowdria*-infected and non-infected BSV cell preparations fractionated by CFE. *Cowdria*-infected or non-infected BSV cells were electrophoresed on either 7% (a) or 15% (b) acrylamide gels. Proteins were eluted from the gels, precipitated with acetone and resuspended in PBS. Open circles ○: Fractions from control non-infected BSV cell preparations were pooled (six per sample) and assayed in triplicate wells for stimulation of PBMC collected 291 weeks after immunisation. Closed circles ●: Fractions from infected BSV cell preparations were pooled (two per sample) and assayed in triplicate wells for stimulation of PBMC collected 291 weeks after immunisation. The mean cpm for the PBMC controls were 1378 ± 361. The results are presented as SI. (* = $P \leq 0.05$; # = $P \leq 0.1$). The approximate molecular weight (kDa) of the fractions inducing *Cowdria*-specific proliferation are shown above the charts.

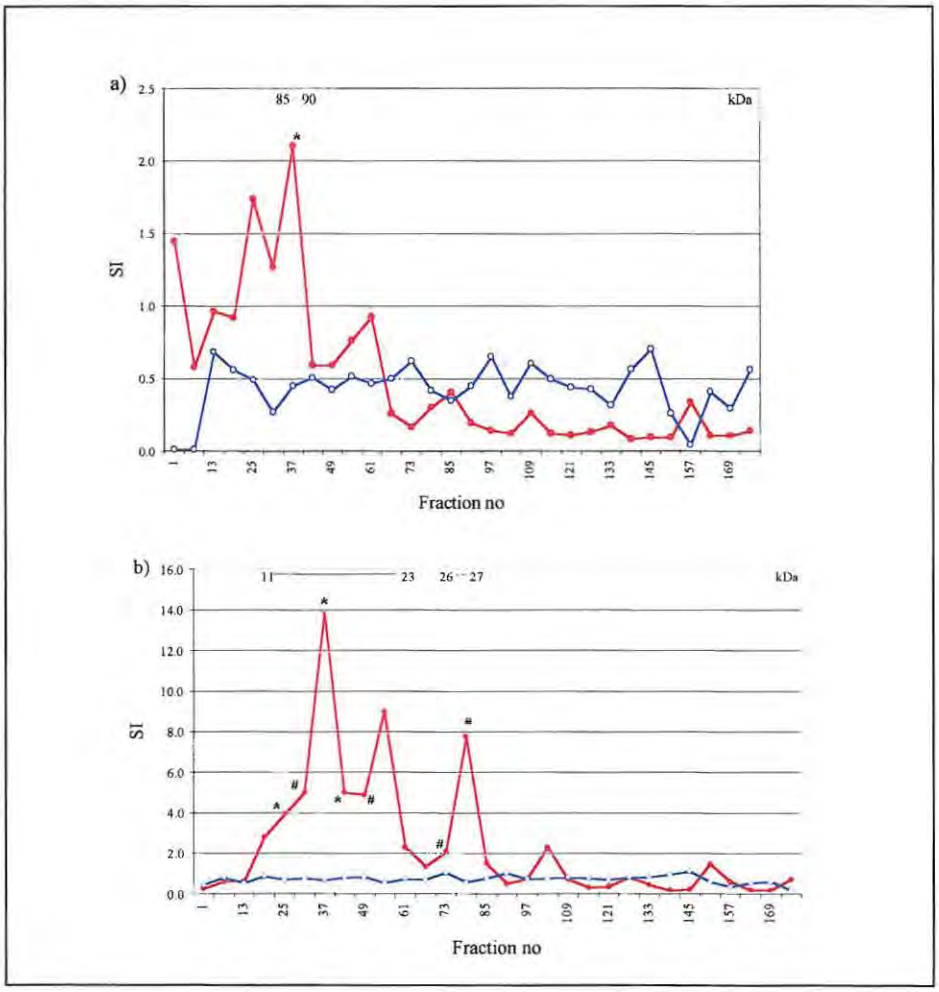

FIG. 4.5. Proliferative responses of PBMC obtained from experimental animal B1359 when exposed to *Cowdria*-infected and non-infected BSV cell preparations fractionated by CFE. *Cowdria*-infected and non-infected BSV cell preparations were electrophoresed on either 7% (a) or 15% (b) acrylamide gels. Proteins were eluted from the gels, precipitated with acetone and resuspended in PBS. Open circles ○: Fractions from control non-infected BSV cell preparations were pooled (six per sample) and assayed in triplicate wells for stimulation of PBMC collected 111 weeks after immunisation. Closed circles ●: Fractions from *Cowdria*-infected BSV cell preparations were pooled (six per sample) and assayed in triplicate wells for stimulation of PBMC collected 41 weeks after immunisation. The mean cpm for the PBMC controls were 2434 ± 2134. The results are presented as SI. (* = $P \leq 0.05$; # = $P \leq 0.1$). The approximate molecular weights (kDa) of the fractions inducing *Cowdria*-specific proliferation are shown above the charts.

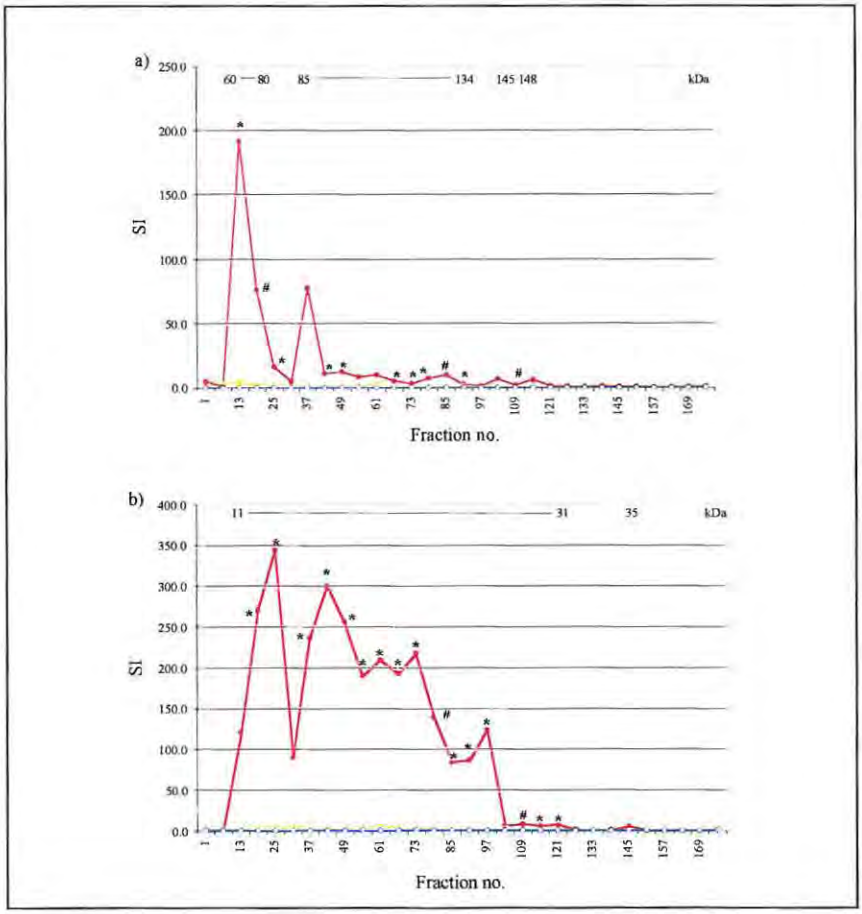

FIG. 4.6. Proliferative responses of PBMC obtained from experimental animal B809 when exposed to *Cowdria*-infected and non-infected BSV cell preparations fractionated by CFE. *Cowdria*-infected or non-infected BSV cell preparations were electrophoresed on either 7% (a) or 15% (b) acrylamide gels. Proteins were eluted from the gels, precipitated with acetone and resuspended in PBS. Closed squares ■: Fractions from infected BSV cell preparations were pooled (six per sample) and assayed in triplicate wells for stimulation of PBMC collected one week before immunisation (non-immune animal). Open circles ○: Fractions from control non-infected BSV cell preparations were pooled (six per sample) and assayed in triplicate wells for stimulation of PBMC collected 57 weeks after commencing immunisation. Closed circles ●: Fractions from infected BSV cell preparations were pooled (six per sample) and assayed in triplicate wells for stimulation of PBMC collected 18 weeks after commencing immunisation. The mean cpm for the PBMC controls were 6505 ± 11,797. The results are presented as SI. (* = $P \leq 0.05$; # = $P \leq 0.1$). The approximate molecular weights (kDa) of the fractions inducing *Cowdria*-specific proliferation are shown above the charts.

TABLE 4.1. Summary of the proliferative responses of PBMC obtained from two cattle immunised by infection and treatment (B9191 & B1359), four cattle immunised with inactivated *Cowdria* (B809; B821; B1351 & B775) and one control ox immunised with adjuvant only (B816) when exposed to *Cowdria*-infected BSV cell preparations fractionated by CFE on a 7% poyacrylamide gel.

Fraction no[a]	Stimulation Index[b] Animal No:							kDa
	B9191	B1359	B809	B821	B1351	B775	B816	
1	**10***	1	**5**	2	**31**	**87**	0	
7	**5#**	1	1	1	**17**	**54**	1	<55
13	**5#**	1	**192***	6	**18**	**53**	1	60
19	**3***	1	**76#**	3	**9**	**26**	5	67
25	2	2	**16***	1	**8**	**8**	4	75
31	**3**	1	**5**	1	**12**	**18**	3	81
37	**3**	**2***	**78**	1	**9**	**19**	3	85
43	**4**	1	**11***	2	**7**	**11**	4	91
49	**3**	1	**12***	1	**7**	**13**	4	97
55	**4***	1	**8**	1	**9**	**21**	4	102
61	**3***	1	**10**	1	**8**	**17**	3	106
67	**3#**	0	**5***	1	**5**	**5**	2	109
73	2	0	**3***	1	**5**	**3**	2	112
79	**3**	0	**7***	1	**9**	**12**	2	115
85	**3**	0	**10#**	1	**10**	**21**	3	120
91	2	0	**3***	1	**8**	**5**	1	126
97	**3***	0	2	1	**8**	**12**	2	135
103	2	0	**7**	1	**7**	4	2	141
109	2	0	**2#**	1	**7**	6	4	145
115	1	0	**6**	1	**7**	5	4	149
121	2	0	2	1	**6**	1	2	153
127	1	0	1	1	**5**	2	2	157
133	2	0	1	1	**5**	4	3	158
139	1	0	2	1	**6**	4	2	158
145	1	0	1	1	**7**	**5**	4	158
151	**2#**	0	1	1	**11**	**14**	2	160
157	**3**	0	1	1	**11**	**15**	2	164
163	2	0	1	1	**12**	**16**	1	166
169	2	0	1	1	**8**	**6**	2	167
175	1	0	1	1	**12**	**20**	1	167

[a]Fractions from *Cowdria*-infected BSV cells were pooled six per sample and assayed in triplicate wells for stimulation of PBMC

[b]Boldface numbers: SI greater than twice background proliferation.

*$P \leq 0.05$; #$P \leq 0.1$

TABLE 4.2. Summary of the proliferative responses of PBMC obtained from two cattle immunised by infection and treatment (B9191 & B1359), four cattle immunised with inactivated *Cowdria* (B809; B821; B1351 & B775) and one control ox immunised with adjuvant only (B816) when exposed to *Cowdria*-infected BSV cell preparations fractionated by CFE on a 15% poyacrylamide gel.

Fraction no[a]	Stimulation Index[b] Animal No:							kDa
	B9191	B1359	B809	B821	B1351	B775	B816	
1	1	0	0	1	0	**3***	0	11
7	**5#**	1	0	1	**2**	**31***	0	11
13	**7***	1	**120**	**31#**	**11***	**50***	0	11
19	**7***	**3**	**270***	**196***	**11***	**42***	4	11-12
25	**10#**	**4***	**344***	**129***	**13***	**50***	**10#**	13
31	**11***	**5#**	**90**	**41**	**17***	**45***	3	14-16
37	**7***	**14***	**237***	**154***	**14***	**52***	7	16-17
43	**7**	**5***	**300***	**64***	**8***	**46***	**10***	18
49	**8**	**5#**	**256***	**56***	**8***	**44***	6	19-21
55	**6***	**9**	**190***	**26#**	**14***	**42***	7	22-23
61	**7***	**2**	**209***	**47**	**12***	**52***	3	23
67	**5**	1	**192***	**4***	**9***	**39***	7	24-25
73	**4**	**2#**	**217***	**3***	**7***	**33***	**8***	26
79	**8**	**8#**	**139#**	**3**	**14***	**34***	**11***	27
85	**11***	**2**	**84***	**3**	**13***	**34***	**10**	28
91	**16***	1	**87***	1	**7***	**38***	6	29
97	**13***	1	**123***	2	**7***	**24***	**11***	29
103	**10***	**2**	**6#**	1	**12***	**22***	**10***	29-30
109	**8#**	1	**8**	1	**12***	**33***	7	30
115	4#	0	**6***	1	**8***	**23***	6	31
121	**6**	0	**7***	1	**8***	**26***	6	31
127	4	1	1	1	**12***	**24***	7	32-33
133	**5#**	0	1	1	**13***	**36***	6	33-34
139	**4***	0	1	1	**10***	**24***	7	34
145	**6***	0	**5**	2	**10***	**31***	7	35
151	3	1	0	1	**12***	**30***	7	35-36
157	**7#**	1	0	1	**15***	**37***	5	36-37
163	**5#**	0	0	1	**12***	**28***	7	37-38
169	**6**	0	0	1	**11***	**24***	3	38
175	2	1	0	1	1	1	5	38

[a]Fractions from *Cowdria*-infected BSV cells were pooled six per sample and assayed in triplicate wells for stimulation of PBMC.

[b]Boldface numbers: SI greater than twice background proliferation.

The blue shaded area indicates fractions that induce lymphocyte proliferation in both sets of animals.

*$P \leq 0.05$; #$P \leq 0.1$

4.4. DISCUSSION

Continuous flow electrophoresis was successfully used to separate *Cowdria* proteins into discrete molecular weight fractions. A 7% polyacrylamide gel resolved proteins between 50 and 168 kDa (190 fractions) and a 15% polyacrylamide gel those of between 11 and 38 kDa (190 fractions). Each fraction contained only one or two molecular weight components. This high resolution afforded by CFE therefore allowed a relatively fine discrimination of the immunostimulatory proteins despite their having to be pooled for practical reasons. Seven outbred cattle were used in this study of which two were immunised by infection and treatment, four with inactivated organisms in adjuvant and one with adjuvant alone. Although two different methods of immunisation were used, PBMC from all the cattle proliferated in response to a common group of proteins with molecular weights of 11; 12; 14 to 17; 19 to 23 kDa. This is an important observation as lymphocytes from animals expressing different MHC molecules usually recognise different epitopes from the same pathogen. In some cases these may be on different proteins. Whether the cattle used in this study express different MHC molecules requires investigation, but a single antigen-based vaccine is not likely to be effective as it may not be recognised by all individuals (Morrison *et al.*, 1999). Accordingly, it is important to identify antigens that bind permissively to a number of MHC molecules because they can potentially overcome the problem of genetic restriction in an outbred population (Agrewala *et al.*, 1998). The use of multiple antigen vaccines could also overcome this restriction (Liljeqvist & Ståhl, 1999). Furthermore, not only common proteins of an isolate recognised by memory T-cells from genetically different cattle should be studied, but immunodominant epitopes conserved between isolates also need to be characterised. In *A. marginale* T-cell clones derived from cattle immunised with outer

membranes of a Florida strain, demonstrated conservation of certain Th cell epitopes among genetically distinct strains (Brown *et al.*, 1998b). Short-term T-cell lines obtained from cattle with different MHC class II haplotypes and immunised with native MSP2, were used in conjunction with synthetic peptides to identify multiple universal MSP2 epitopes that induce immunodominant memory responses (Brown *et al.*, 2001).

The low background proliferation observed with non-infected BSV cell preparations fractionated by CFE once again confirmed that there were no alloreactive responses to BSV cellular antigens. Furthermore, the cattle immunised with inactivated organisms in adjuvant also showed higher SIs than PBMC from cattle immunised by infection and treatment. It has been shown previously that oil-based adjuvant can induce rapid but short termed cellular responses in the absence of antigen (Emery *et al.*, 1990). The high background detected for the control animal B816 (Table 4.2) may therefore be due to an adjuvant effect.

The MAP1 protein of *Cowdria* has been implicated in protection in an immunisation trial in mice (Nyika *et al.*, et al, 1998). The size of MAP1 varies between different isolates (Barbet *et al.*, 1994). Probing western blots of crude BSV cell culture infected with the Welgevonden isolate, with a polyclonal anti-MAP1 serum indicated that it was approximately 31 kDa (section 2.3.1.3.; van Kleef *et al.*, 1993). CFE fractions in the region of 31 kDa induced PBMC from one out of two cattle immunised by infection and treatment to proliferate. Cells from three out of four cattle immunised with inactivated organisms also responded similarly. The different responses to fractions in the 31 kDa range by PBMC from the different cattle therefore do not appear to be a result of differences in vaccination strategies. It may however, be due to MHC restriction, a

possibility that remains to be determined.

In previous work reported by Totté and co-workers (1999b), IFN-γ producing $CD4^+$ T-cell lines were stimulated with soluble proteins of between 22 and 32 kDa, of the Gardel isolate, that had been purified by fast performance liquid chromatography. Thus it appears that proteins of between 11 and 23 kDa of the Welgevonden isolate and proteins between 22 and 32 kDa of the Gardel isolate induce similar cellular immune responses *in vitro*. This means that only proteins with molecular weights of 22 and 23 kDa fall within the range identified for the Gardel isolate. There can be full-, non- or partial cross-protection between isolates (Jongejan *et al.*, 1988; du Plessis *et al.*, 1989; Stewart, 1989). Consequently these proteins require further characterisation to determine their degree of conservation between the two different isolates. Furthermore, whether they can induce protective responses in outbred animals also needs to be established.

In summary, results presented in this chapter indicate that, as with *P. chabaudi adami* and *B. bovis*, fractionating *Cowdria* organisms by CFE provides a way of identifying potential vaccine antigens. The use of sensitised primary polyclonal lymphocytes permits rapid and simple screening of fractions for the proteins that stimulate specific immune responses. In this way *Cowdria* proteins with the potential to play a role in protection were identified. These proteins need to be further characterised to determine which lymphocyte populations they activate and to identify the cytokines produced upon activation. Experiments addressing these questions are described in Chapter 6. The following chapter describes a preliminary study aimed at obtaining reagents and developing methods that could help to identify the genes encoding the low molecular weight immunoreactive antigens of *Cowdria*.

CHAPTER 5

TOWARDS TOOLS FOR IDENTIFYING THE GENES ENCODING THE IMMUNOREACTIVE *COWDRIA* PROTEINS

5.1. INTRODUCTION

The previous chapter described the identification of *Cowdria* proteins in the molecular weight regions of between 11 and 23 kDa that induce lymphocyte proliferation. Because these proteins induce recall lymphocyte responses they are obvious candidates for further examination aimed at vaccine development. Improving the current heartwater vaccine may be achieved through the development of a suitable subunit vaccine and delivery system or perhaps by using naked DNA as a vaccine. It is, therefore, of paramount importance to identify the genes, which encode the proteins of interest. This may be achieved regardless of whether the low molecular weight proteins represent breakdown products, because the proteins themselves are used to develop the respective tools for gene identification. One novel way of doing this is to use an antiserum to screen phage libraries displaying peptides derived from *Cowdria* (Fehrsen & du Plessis, 1999). This approach could potentially lead to the direct identification of the genes encoding the proteins of which the peptide forms a part, since in phage display, the peptide is physically coupled to its encoding DNA (Smith, 1985). Immunoaffinity selection is achieved by screening a collection of millions of different peptides displayed on the surface of a population of filamentous phages with specific antibodies (Parmley & Smith, 1988). The binding phages are recovered and their attached peptides identified by sequencing the phage DNA. If the genome

sequence is known, epitopes can be identified by directly matching affinity-selected peptides with the DNA sequence of the targeted protein. If the genome sequence is unknown a simple sequence comparison will, however, not be possible. Five different peptide sequences have already been selected from a MAP1-deficient fragmented genome phage display library. Four of the peptides had regions similar to MAP1. However, none could be located to a *Cowdria* open reading frame indicating that cross-reactive epitope mimics had been obtained (Fehrsen & du Plessis, 1999). Not withstanding, once the *Cowdria* genome sequence is at hand, this phage display library can be screened with antiserum specific for the proteins identified in the previous chapter. A direct comparison of the DNA sequence of the antibody-binding peptides with the genomic sequences should allow their encoding genes to be identified. Goats, chickens and rabbits can all be investigated for antiserum production to the low molecular weight proteins of *Cowdria*. Anti-MAP1 serum with high titre (1:125000) has previously been successfully prepared in a goat (van Kleef *et al.*, 1993). Laying hens are usually very good producers of specific antibodies. After immunising a chicken the specific antibodies are transported to the egg yolk from which they can readily be purified (Polson *et al.*, 1985). A laying hen can produce more than 20 g of yolk antibodies (IgY) per year. Animal care expenses are lower for chickens compared to rabbits and goats (Schade *et al.*, 1992; Carlander *et al.*, 2000).

In a more traditional approach the partial amino acid sequence of the proteins could be determined and nucleic acid probes developed to screen genomic libraries. For this, the actual proteins that induce lymphocytes to proliferate must first be purified to homogeneity. This is often easier said than done. Several different steps are generally required, each designed to eliminate a greater portion of unwanted material from the sample of the previous step. Since

there is no universally applicable procedure, each step must be determined solely by trial and error. Minor components, or even relatively abundant ones, cannot be distinguished if their molecular weight is the same as that of one of the major components (Ames & Nikaido, 1976). Two-dimensional electrophoresis offers high resolution and sensitivity for the separation of proteins from a complex biological source since each dimension separates proteins according to independent parameters (O'Farrel, 1975). Proteins are separated according to isoelectric point by isoelectric focusing in the first dimension and according to molecular weight by SDS-PAGE in the second dimension. Two-dimensional electrophoresis may therefore be used to determine whether proteins are homogenous when separated by SDS-PAGE.

With the above mentioned possibilities in mind, this chapter describes some preliminary studies aimed at developing suitable tools to identify the encoding genes of the low molecular weight proteins of *Cowdria* that induce cellular immune responses *in vitro*.

5.2. MATERIALS AND METHODS

5.2.1. PREPARATION OF ANTIGEN FOR IMMUNISATION

Unless otherwise stated, the proteins used for immunisation were excised from SDS-PAGE gels (section 2.2.5.1.). In some experiments, continuous flow electrophoresis fractions (section 4.2.1.) containing the proteins of interest were used.

5.2.2. IMMUNISATION SCHEDULE FOR PRODUCING ANTISERUM IN GOATS

Goats were immunised by a modification of the procedure described by van Kleef *et al.* (1993). Two goats were immunised s.c. and i.m. with either the 11 or 15 kDa protein of *Cowdria* as indicated in Table 5.1. Montanide ISA50 was used as adjuvant. One goat was immunised with protein bands corresponding 11 kDa (excised from copper chloride stained SDS-PAGE gels; section 2.2.5.1.) and boosted with a 11 kDa CFE fraction (section 4.2.1.). A second goat was immunised (s.c. and i.m.) and boosted with a 13-16 kDa CFE fraction. The goats were bled two weeks after booster injection.

Table 5.1. Immunisation schedule for producing antiserum in goats.

kDa	Week	Immunised with	kDa
10	0	protein excised from two gels and mixed with ISA50	11
	4	200 µl fraction no 4	11
15	0	400 µl fraction no 28	13
	4	200 µl fraction no 31	14-16

5.2.3. IMMUNISATION SCHEDULE FOR PRODUCING ANTIBODIES IN A CHICKEN

A chicken was immunised i.m.with the 15 kDa protein of *Cowdria* as indicated in Table 5.2. using the protein band excised from a copper chloride stained SDS-PAGE gel (section 2.2.7.1.). ISA50 was used as adjuvant. A precipitated protein fraction of 15 kDa obtained by CFE (section 4.2.1.) was administered without adjuvant as a final boost. The IgY was harvested from the chicken egg yolks (section 5.2.6.).

Table 5.2. Immunisation schedule for producing antibodies in a chicken.

Week	Immunised with:
0	protein excised from ½ gel and mixed with ISA50
3	protein excised from ¼ gel and mixed with ISA50
4	protein excised from ¼ gel and mixed with ISA50
11	1 ml precipitated CFE fraction no 21-29

5.2.4. IMMUNISATION SCHEDULE FOR PRODUCING ANTISERUM IN RABBITS

Five rabbits were immunised s.c. and i.m. (as indicated in Table 5.3) with one of the following *Cowdria* protein regions: ≤ 13; 14 to 16; 17 to 19; 18 to 22 and 23 to 28 kDa, and essentially as described (van Kleef *et al.*, 1993). The proteins were obtained by excising the respective protein bands from copper chloride stained SDS-PAGE gels (section 2.2.7.1.). The homogenised gels were mixed with Freund's complete adjuvant for the first immunisation, followed by Freund's incomplete adjuvant for the second immunisation. The third and fourth boosters were administered with only homogenised gel and no adjuvant. The rabbits were bled two weeks after each immunisation.

Table 5.3. Immunisation schedule for producing antiserum in rabbits.

Week	Immunised with
0	protein excised from one gel and mixed with FCA
4	protein excised from one gel and mixed with FIA
8	protein excised from one gel only
12	protein excised from one gel only
17	protein excised from one gel only

5.2.5. PREPARATION OF SERUM FROM BLOOD

Serum was prepared from blood as described previously (Rossouw *et al.*, 1990). Blood collected from immunised animals was left to clot for 2 h at room temperature and then overnight at 4°C. The coagulated blood was centrifuged at 300g for 10 min at room temperature. The serum was siphoned off and stored at -20°C.

5.2.6. PREPARATION OF IGY FROM EGG YOLK

The IgY antibodies were isolated from egg yolk as described (Polson *et al.*, 1985). A volume of χ ml yolk was added to (4 x χ) ml 0.1 M phosphate buffer pH 7.6. To this (0.175 x χ) g polyethylenglycol was added and centrifuged at 5000g for 20 min. The supernatant (volume ψ ml) was passed through a funnel containing a cotton wool plug. To this filtrate (0.085 x ψ) g PEG was added and incubated for 10 min. The solution was centrifuged at 5000g for 25 min and the precipitate was dissolved in (2.5 x χ) ml of buffer. To this (0.3 x χ) g PEG was added, incubated for 10 min and centrifuged at 5000g for 25 min. The pellet was dissolved in 1/4χ ml buffer, 0.1% sodium azide added and stored at 4°C.

5.2.7. PROBING ANTISERUM ON ELECTROBLOTTED *COWDRIA* PROTEINS

SDS-PAGE was performed with crude *Cowdria* infected and with uninfected cell cultures (section 2.2.7.) and western blotted onto PVDF membranes (section 2.2.8.). The goat and rabbit antisera and chicken IgY were used to probe the blots which were developed by an enhanced

chemiluminescent substrate (Super Signal® West Pico chemiluminescent substrate, Pierce) and horse radish peroxidase-labeled anti-immunoglobulins of matching species.

5.2.8. TWO-DIMENSIONAL ELECTROPHORESIS

Two-dimensional electrophoresis was performed by a modification of the method described by O'Farrel (1975).

5.2.8.1. First dimension: Isoelectric focusing

Differentially centrifuged Welgevonden infected and non-infected cell culture preparations were dissolved in a 1:1 (v/v) ratio in sample buffer. Various sample buffers and preparation conditions were investigated for their ability to optimally solubilise the protein samples. Samples were prepared by one of the following protocols: **Protocol 1:** antigen was centrifuged at 30,000g and a CHAPS solution (10 mM CHAPS, 9.5 M urea and 2.5% DTT) added to the pellet. After incubating for 10 min at 37°C Triton solution (4% Triton X-100, 9.5 M Urea, 2.5% DTT and 4% ampholines) was added and incubated for a further 10 min at 37°C. **Protocol 2:** CHAPS solution was added to the antigen and incubated for 10 min at 37°C. A Triton solution was then added and incubated for a further 10 min at 37°C. **Protocol 3:** antigen was centrifuged at 30,000g. Thereafter IEF buffer A (8 M urea; 2% CHAPS; 2% ampholines; 0.3% DTT and Bromophenol blue) was added to the pellet and incubated for 10 min at 37°C. **Protocol 4:** antigen was centrifuged at 30,000g and IEF buffer A added to the pellet. **Protocol 5:** IEF buffer A was added to the antigen and incubated for 10 min at 37°C. **Protocol 6:** IEF buffer A was added to the antigen. After following a specified protocol, the samples were centrifuged at 24,000g for 10 min

and the resulting supernatant fluid loaded onto the IEF gel. Standard proteins (IEF mix pH 3.6-9.3, isoelectric focusing marker, SIGMA) were also run on the gel. Isoelectric focusing was performed using an Ampholine PAG plate, pH 3.5-9.5 (Pharmacia Biotech) with 1 M sodium hydroxide at the cathode and 1 M phosphoric acid at the anode. Focussing was at 1500 V, 50 mA, 30 W for 1.5 h (according to the manufacturers recommendations, Pharmacia Biotech). The gel was either stained with Coomassie Brilliant Blue (section 5.2.8.1.1.) or prepared for and subjected to SDS-PAGE as described in section 5.2.8.2.

5.2.8.1.1. Staining of IEF gels

The gel was fixed for 60 min in 20% (w/v) trichloroacetic acid and washed 4 x 15 min in a solution containing 25% ethanol and 8% acetic acid in distilled water. The gel was then stained for 15 min with 0.25% Coomassie Brilliant Blue, 5% methanol, 1% acetic acid in distilled water and destained with 5% methanol, 1% acetic acid in distilled water. The gel images were captured with a Lumi Imager® (Roche) when sufficiently destained. In some cases the IEF gels were stained with Silver Snap® (Pierce) after Coomassie Brilliant Blue staining and destaining.

5.2.8.2. Second dimension: SDS-PAGE

The IEF gel was equilibrated in SDS-PAGE sample buffer for 30 min. A 12% polyacrylamide separating gel with a 4% stacking gel was prepared (section 2.2.7.). The top of the stacking gel was covered with 1 ml molten 1% (w/v) agarose. The equilibrated first dimension gel was immediately positioned on top of the molten agarose and imbedded with more agarose. A low molecular weight standard sample was loaded into a precast well. Electrophoresis was performed

as described in section 2.2.7. Protein bands were detected by staining the second dimension SDS-PAGE gel with Gelcode® colour silver stain (Pierce).

5.3. RESULTS

5.3.1. PRODUCTION OF ANTIBODIES TO *COWDRIA* PROTEINS

Goats, chickens and rabbits were used in attempts to prepare antisera specific for the *Cowdria* proteins that induce lymphocyte proliferation, identified in Chapter 4.

5.3.1.1. Antiserum production in goats

Immunoblotting indicated that no antigen-specific antibodies were contained in the antiserum obtained from the two goats that were immunised with either the 10 or 15 kDa protein of *Cowdria*. This was observed at dilutions of 1/20 and development by ECL. Due to the relative simplicity of preparing IgY antibodies in chickens, it was then decided to immunise a chicken.

5.3.1.2. Antibody production in a chicken

The chicken immunised with the 15 kDa protein resulted in IgY that was "specific" in immunoblots for the 12.5; 14 and 16 kDa proteins of *Cowdria* (Fig. 5.1. & Table 5.4). These proteins could only be detected with ECL at an IgY dilution of 1/20. This antibody preparation cross-reacted with MAP1 (31 kDa) and bovine serum albumin (Fig. 5.1.). Due to the low titres

and apparent poor specificity of these antibodies, antiserum preparation in rabbits was considered as yet another alternative.

5.3.1.3. Antiserum production in rabbits

Four rabbits were immunised with *Cowdria* proteins ranging between 23 to 29; 18 to 22; 17 to 19 and ≤ 13 kDa respectively. Only two rabbits produced antiserum recognising *Cowdria* specific proteins between 18 and 24 kDa (Fig. 5.2; Table 5.4.). Once again, these proteins could only be detected with ECL at antiserum dilutions of 1/20. In addition to this, the rabbit antiserum contained antibodies that apparently cross-reacted with MAP1 and other proteins.

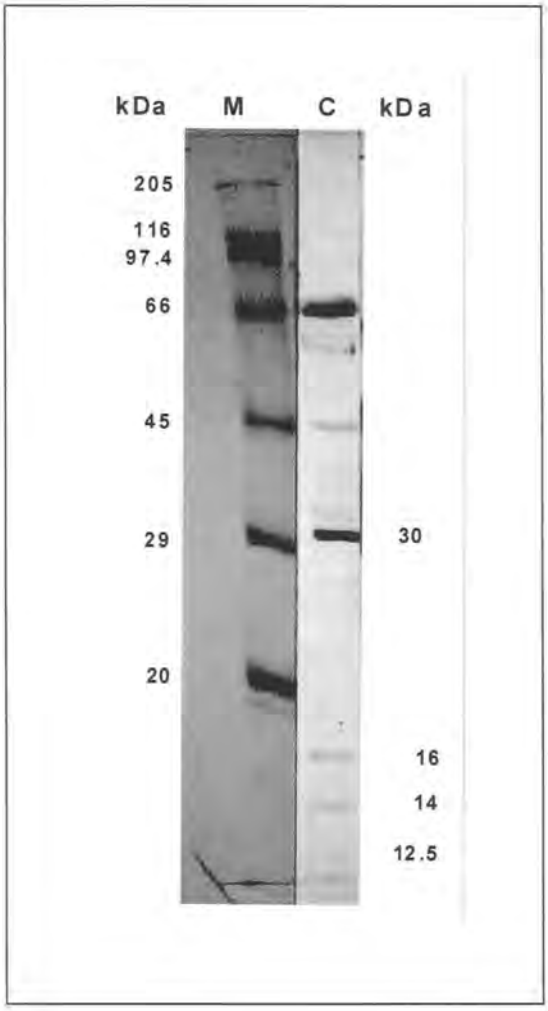

FIG. 5.1. Western blot analysis of *Cowdria*-infected BSV cells probed with chicken IgY raised against the 15 kDa protein of *Cowdria*. *Cowdria*-infected BSV cells were subjected to SDS-PAGE, blotted onto a PVDF membrane and probed with chicken IgY at a dilution of 1:20 (Lane C). The blot was developed by enhanced chemiluminescence and the image captured by a Lumi Imager (Roche). Lane M = molecular weight marker proteins stained with Coomassie Brilliant Blue stain.

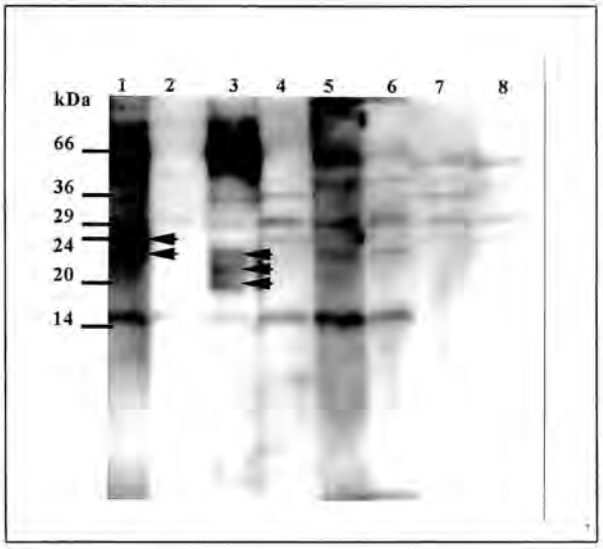

FIG. 5.2. Western blot analysis of *Cowdria*-infected BSV cells probed with rabbit IgG raised against various proteins of *Cowdria*. *Cowdria*-infected BSV cells were subjected to SDS-PAGE, blotted onto a PVDF membrane and probed with rabbit antiserum. Lanes 2; 4; 6 and 8 were probed with prebleed serum from each respective rabbit. Antisera used to probe the blots were raised against *Cowdria* proteins of: Lane 1, 23 to 28 kDa; Lane 3, 18 to 22 kDa; Lane 5, 17 to 18 kDa and Lane 7, ≤ 13 kDa. The arrows indicate antibody binding to *Cowdria* proteins in the molecular weight range of the proteins against which they were raised.

TABLE 5.4. Summary of the results obtained after immunising goats, a chicken and rabbits with various *Cowdria* proteins.

ANIMAL	IMMUNISED WITH kDa	RESULT[#] kDa
Goat A	11	X
Goat B	13-16	X
Chicken A	15	12; 14; 16
Rabbit A	≤13	X
Rabbit B	17-19	X
Rabbit C	18-22	18; 20; 22
Rabbit D	23-29	22; 24

[#]proteins identified corresponding to the molecular weight range of the immunogen.
X no *Cowdria* specific antibodies were obtained

5.3.2. TWO-DIMENSIONAL ELECTROPHORESIS

5.3.2.1. First dimension: Isoelectric focusing

Methods of sample preparation that will minimise protein loss due to insolubility and produce good separation in the IEF gel (first dimension) are important for optimum resolution in the SDS-PAGE second dimension. Optimisation of IEF conditions therefore included comparisons of sample preparation approaches and varying the position of sample loading. Different sample buffers and preparation conditions were tested for their ability to solubilise *Cowdria* proteins as detailed in section 5.2.8.1. Protocol 6 and Buffer A (8 M urea; 2% CHAPS; 2% ampholines pH 3.0 – 10.0; 0.3% DTT and Bromophenol blue) was chosen as the IEF sample buffer for use in subsequent experiments leading to relatively sharp protein bands with little distortion and streaking (Fig 5.3. lane 6). However it must be noted that probably not all the proteins were solubilised as there was a small pellet present after centrifugation regardless of the method used.

It was also observed that the proteins tended to precipitate at the point of application (Fig. 5.3.). To minimise this, various positions of sample application were investigated. The best point of application was determined to be 1 cm from the cathode i.e. at pH 8.6 (Fig 5.4. lane 8). This resulted in adequate protein focusing throughout the gel. Sample application at any other point on the gel led to protein precipitation at the point of application with no migration towards the cathode.

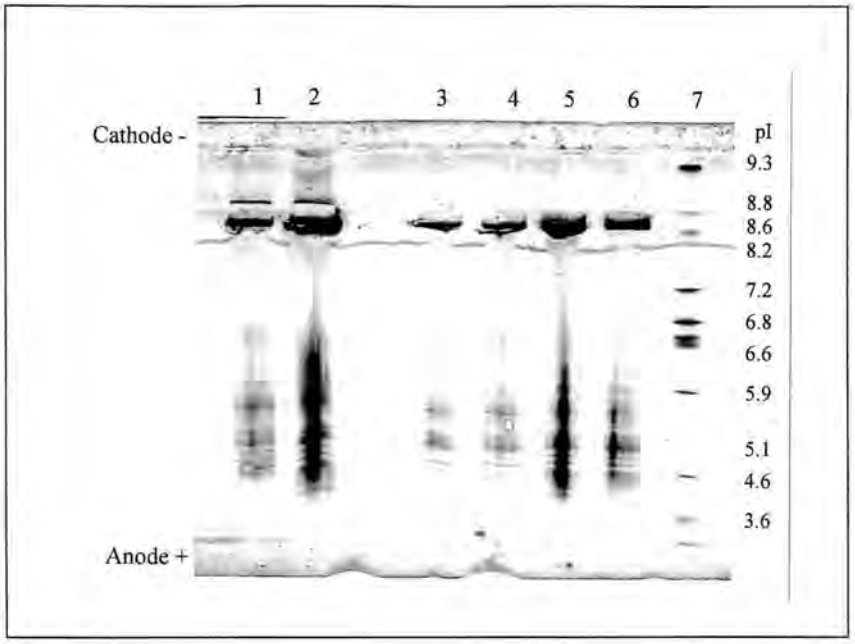

FIG. 5.3. Coomassie Brilliant Blue stained IEF gel showing the efficacy of various sample buffers and preparation conditions in solubilising *Cowdria* proteins for isoelectric focusing. Lane 1: Protocol 1. Lane 2: Protocol 2. Lane 3: Protocol 3. Lane 4: Protocol 4. Lane 5: Protocol 5. Lane 6: Protocol 6. Details of the protocols used are supplied in section 5.2.8.1. After following a specified protocol, the samples were centrifuged at 24,000g for 10 min and the resulting supernatant fluid loaded 1 cm from the cathode buffer strip. The gel (Ampholine PAGplate precast polyacrylamide gel pH range 3.5 to 9.5) was run at constant volts (1500 V; 13 mA; 7 W) for 1 h 30 min.

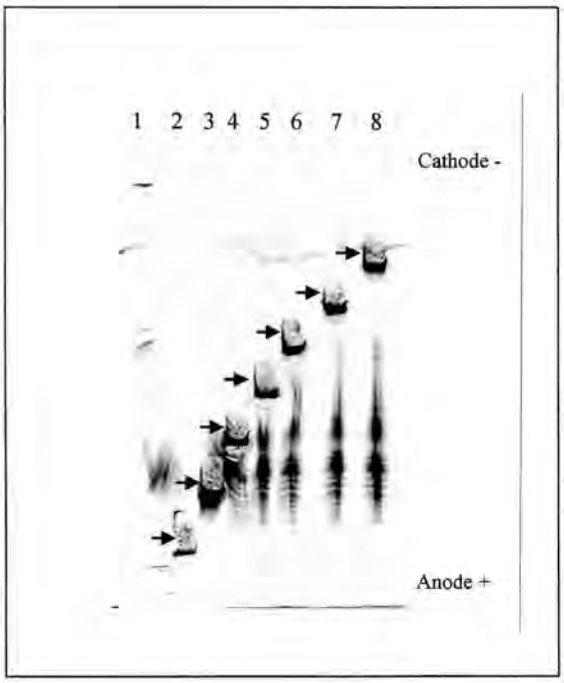

FIG. 5.4. Coomassie Brilliant Blue stained IEF gel showing the effect of loading samples at different positions. Samples were prepared by using protocol 6 (section 5.2.8.1.) Thereafter they were centrifuged at 24,000g. The supernatants were loaded at various indicated positions (➡). The gel (Ampholine PAGplate precast polyacrylamide gel pH range 3.5 to 9.5) was run at constant volts (1500 V; 13 mA; 7 W) for 1 h 30 min.

5.3.2.2. Second dimension: SDS-PAGE

Results obtained after two-dimensional electrophoresis of differentially centrifuged organisms showed that there was more than one protein within a molecular weight region with different pI values (Fig. 5.5. & Table 5.5.). For example, nine proteins separated within the 18 kDa region with estimated pI values of 4.2, 5.7, 5.9, 6.1, 6.2, 6.6, 8.0, 8.3, and 8.5 (Fig. 5.5. & Table 5.5.). Similar results were obtained in the immuno-stimulatory area of between 14 and 23 kDa (Fig. 5.5. & Table 5.5.).

TABLE 5.5. Estimated molecular weights and corresponding pI's of *Cowdria* proteins, separated by two-dimensional electrophoresis, in the molecular weight range of 14 to 23 kDa.

kDa	pI								
14	**4.0**	**8.0**	**8.2**	**8.3**					
15	**5.7**	**7.0**	**7.7**	**8.0**	**8.2**	**8.3**	**8.9**		
16	**4.0**	**6.0**	**6.6**	**7.1**	**7.7**	**8.0**	**8.3**	**8.9**	**9.0**
17	**4.2**	**6.6**	**7.2**	**7.6**	**8.0**	**8.3**			
18	**4.2**	**5.7**	**5.9**	**6.1**	**6.2**	**6.6**	**8.0**	**8.3**	**8.5**
19	**8.3**	**8.5**	**8.8**						
20	**4.0**	**5.7**	**6.8**	**7.6**	**7.9**				
21	**3.7**	**5.7**	**6.0**	**8.7**					
22	**4.0**	**4.4**	**5.5**	**6.2**	**6.5**	**6.8**	**7.1**	**7.2**	**8.1**
23	**3.8**	**5.9**	**8.8**	**9.2**					

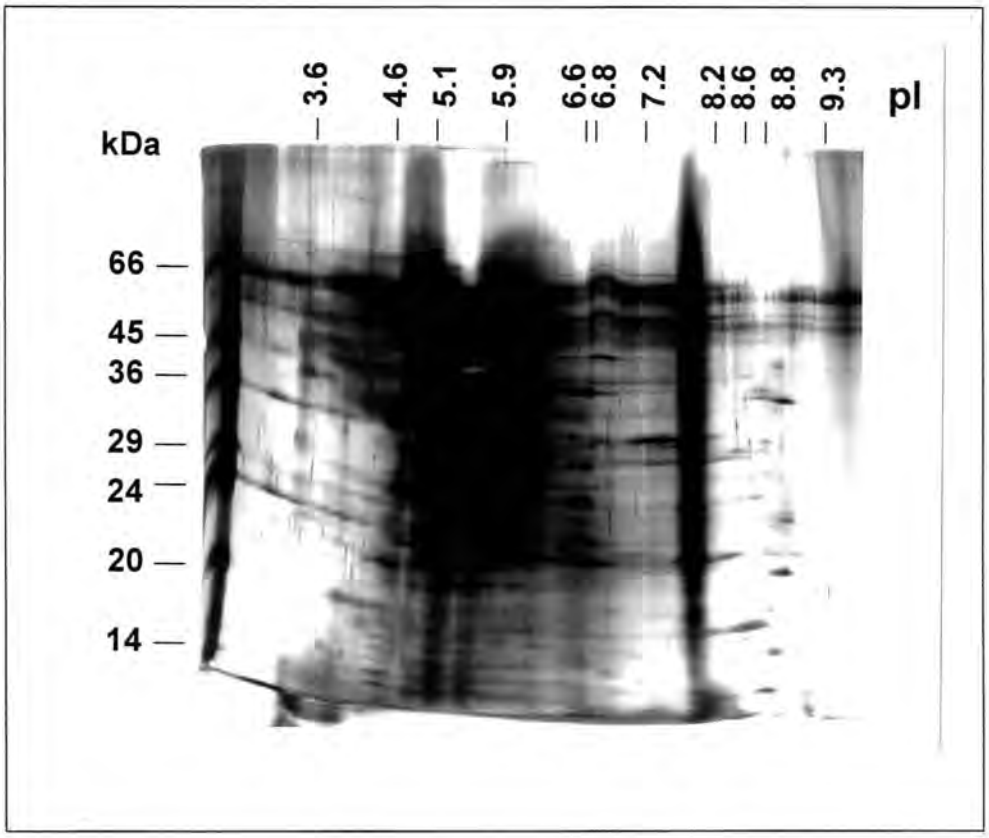

FIG. 5.5. Two-dimensional electrophoretic analysis of a differentially centrifuged *Cowdria*-infected BSV cell preparation. First-dimension electrophoresis was performed with Ampholine PAGplate precast polyacrylamide gel pH range 3.5 to 9.5 run at constant volts (1500 V; 13 mA; 7 W) for 1 h 30 min. The second-dimension was performed with a 12% SDS polyacrylamide gel run on a BIO-RAD vertical electrophoresis unit. Proteins in the second-dimension gel were stained by silver staining.

5.4. DISCUSSION

The inability to produce an antiserum in goats and the low titres and specificity obtained in rabbits and a chicken may be due to sub-optimum protein concentration being administered. Other factors may include the fact that the proteins were denatured, the choice of adjuvant, and/or a low intrinsic immunogenicity. Immunogenicity is the ability to induce a humoral and/or cell-mediated immune response. It is determined by foreignness, molecular weight, chemical composition and complexity of a protein (all four levels of protein organisation – primary, secondary, tertiary and quartenary – contribute). Large insoluble macromolecules generally are more immunogenic than small soluble ones because they are more readily phagocytosed and processed. Alternatively these low molecular weight proteins may simply be poor B-cell immunogens but good T-cell immunogens as evident by the fact that they induce good PBMC proliferative responses *in vitro*. In addition to the above-mentioned factors, the genetic constitution of an animal influences the type, as well as the degree of the response. Genetic control of immune responsiveness is confined to genes within the MHC, genes encoding B-cell and T-cell receptors and genes encoding various proteins involved in immune regulatory mechanisms. Genetic variability in all of these affects the immunogenicity of a given macromolecule in different animals. For each experimental immunogen there will be some combination of optimal dosage, choice of adjuvant and route of administration that will induce a peak immune response in a given animal (Kuby, 1994).

Since only small amounts of the antigen were available, the nowadays unpopular Freund's adjuvant was used. The depot action of the adjuvant protects the antigen and allows its slow

release, thereby ensuring a persistent antibody response (Harlow & Lane, 1988). It has been reported that when acid-treated, naked bacteria are used as immune carriers for protein antigens less antigen was needed for immunising (Bellstedt *et al.*, 1987). This technique should be investigated for antiserum preparation against the low molecular weight proteins of *Cowdria*. Repeated injections can increase antibody levels in antiserum, but a point is eventually reached where no further increase is observed (Eisen & Siskind, 1964). Repeat injections were necessary before a response was detected in immunoblots as shown in this Chapter. It is known that regular boosting also leads to the maturation of the immune response, yielding high-affinity binders (Harlow & Lane, 1988). Antibodies of high affinity, however, are usually capable of displaying a wider range of cross-reactions than antibodies of low affinity (Eisen & Siskind, 1964). When the chicken was immunised with the 15 kDa protein, the resulting IgY recognised proteins of 12, 14, & 16 kDa. It also apparently cross-reacted with proteins corresponding to MAP1 and one with a molecular weight of 66 kDa. Similar cross-reacting antibodies were obtained when rabbits were immunised with proteins of 18 to 29 kDa. This may indicate that high affinity antibodies were generated and this needs to be determined. A physical limitation of immunising with gel fragments is the difficulty in precisely excising the protein band from a complex mixture due to the elastic nature of the preparative polyacrylamide gels. The excised gel may contain not only the band of interest but other protein species as well. In a previous study it was observed that when rabbits were immunised twice with a 27 kDa *Cowdria* protein, the resulting antibodies only recognised the MAP1 protein. The 27 kDa protein was only recognised after a third immunisation. It was also observed that antiserum raised to MAP1 cross-reacted with the 27 kDa and other proteins of between approximately 6 and 94 kDa (van Kleef *et al.*, 1993). Some *Cowdria* proteins may therefore share common epitopes while some bands could perhaps

represent protein degradation products. These factors could be responsible for the observed cross-reactions with other proteins.

Using phage display, it is now possible to obtain antibodies that bind to virtually any chosen target without the use of laboratory animals or hybridomas in a system that completely by-passes the immune system. An advantage of this strategy is that it can be used to obtain novel antibodies that would be impossible to obtain using conventional methods (Palmer *et al.*, 1997). The generation of high-affinity monoclonal antibodies has traditionally involved the production of hybridomas from spleen cells of immunised animals. The process is time-consuming, inefficient and only a small number of antibodies are produced against a few dominant immunogenic epitopes (Liu & Marks, 2000). Antibody display by filamentous bacteriophages is accomplished by fusing the coding sequence of the antibody variable (V) regions to the amino terminus of the phage minor coat protein pIII. Expression of the fusion product and its subsequent incorporation into the mature phage coat results in the antibody being presented on the phage surface, while its genetic material resides within the particle. This linkage between antibody genotype and phenotype allows antigen specific phage antibodies to be enriched using immobilised or labeled antigen. Phages that display a relevant antibody will be retained on a surface coated with antigen, while non-adherent phages are washed away. Bound phages can be recovered from the surface, re-infected into bacteria and re-grown for further enrichment and binding analysis (Winter *et al.*, 1994; Hoogenboom, 1997). The selection of phage antibodies with a range of binding activities and specificities has been successfully achieved from various libraries (Palmer *et al.*, 1997; van Wyngaardt & du Plessis 1998). This technique, therefore, offers a powerful means of generating

antibodies without immunisation and merits investigation for obtaining antibodies to the low molecular weight proteins of *Cowdria*.

Irrespective of the difficulties experienced in raising antibodies to the low molecular weight proteins, a rabbit antiserum was successfully used to screen a *Cowdria* peptide phage display library. Four peptide sequences were identified of between 9 and 29 amino acids in length (Fehrsen & du Plessis, personal communication). These sequences can be compared with that of the *Cowdria* genome sequence once it becomes available, thus providing an indication of which genes are likely to code for these relevant proteins.

An alternative approach for identifying the genes encoding the low molecular weight proteins involves obtaining their partial amino acid sequences. This would enable DNA probes to be designed, which could then be used either to screen genomic libraries or be compared with the *Cowdria* genome thereby allowing the corresponding genes to be identified. For this to be practical, the homogeneity of the proteins needs to be determined. To this end, no technology matches the resolving power of two-dimensional electrophoresis. In the two-dimensional electrophoresis studies a wide pH range of between pH 3 and 9 was used in the first dimension. A 12% polyacrylamide gel provided the second dimension. Initial optimisation entailed testing different solubilisation buffers and various positions for sample loading. SDS successfully solubilises membrane proteins, but CHAPS was used instead because it has no net charge (zwitterionic). A solubilisation buffer containing 8 M urea, 2% CHAPS, 2% ampholines and 0.3% DTT resulted in the sharpest protein bands with minimal distortion and little streaking. The remaining streaking that was seen could have been as a result of low solubility of some proteins

within the gel and/or the presence of nucleic acids. However, the sample used for 2DE was prepared by differential centrifugation, which should have removed any contaminating host cell DNA. Reagents such as DTT are charged and thus migrate out of the pH gradient during IEF. This results in a loss of solubility for some proteins, especially those that are prone to interaction by disulphide bonding. Replacing the thiol-containing DTT with a non-charged reducing agent such as tributyl phosphine may greatly enhance protein solubility during IEF and can be further investigated (Herbert *et al.*, 1997). The position of sample loading was also observed to play an important role in the solubilisation and migration of the *Cowdria* proteins. The best point of sample application on the IEF gel was determined to be 1 cm from the cathode. When the sample was loaded at any other point, the proteins precipitated and none migrated towards the cathode.

These preliminary experiments with 2DE provided an idea of the complexity of the *Cowdria* proteome. Most importantly, protein fractions of one apparent molecular weight actually consisted of more than one component. Various factors can lead to the two-dimensional electrophoresis image being misinterpreted. These could include alternative stable conformations (conformers), sequence polymorphisms, various combinations of different subunits, various states of phosphorylation, methylation or acetylation; protein damage, or differences in oxidation states of the proteins. If the secondary structure of a protein is partially removed then the protein has no unique isoelectric point resulting in smear stripes and poorly resolved bands (Jungblut & Thiede, 1997). Thus, there is considerable room for technical improvements in two-dimensional electrophoresis. For example in the case of *Cowdria*, a more detailed analysis can be done using narrow pH gradients which can provide higher resolution. In order to obtain proteins sufficiently homogeneous for sequencing, the CFE fractions will need to be further resolved using a second

technique such as IEF. A preparative IEF system such as the Rotofor® (Bio-Rad) can be used to fractionate complex protein samples in free solution (Evans *et al.*, 1989; Bier, 1998). Such an approach is therefore a useful adjunct to CFE. These methods therefore provide a means towards further characterising the proteins which have been identified as being involved in cellular immune responses towards *Cowdria*. This will in turn greatly facilitate the identification of their encoding genes.

The following Chapter describes how *Cowdria*-specific T-cell lines were established, characterised and utilised to further narrow down the range of immunostimulatory *Cowdria* proteins.

CHAPTER 6

ESTABLISHING AND CHARACTERISING *COWDRIA*-SPECIFIC T-CELL LINES

6.1. INTRODUCTION

Several findings have implicated cellular immunity in protection against heartwater (du Plessis, 1982; du Plessis *et al.*, 1991 & 1992a; Martinez, 1997; Totté *et al.*, 1997 & 1998; Mwangi *et al.*, 1998 a, b & c; Byrom *et al.*, 2000a & b). Totté and co-workers (1994a) observed significant levels of circulating IFN-α in cattle rendered immune to heartwater challenge. Recombinant IFN-α was, however, only able to retard, but not block, *Cowdria* growth within endothelial cells *in vitro*. It was therefore suggested that additional mechanisms are necessary to control infection (Totté *et al.*, 1994a). A possible candidate was IFN-γ. This cytokine has been shown to be a potent inhibitor of *Cowdria* growth in endothelial cells (Totté, *et al.*, 1993 & 1996; Mutunga *et al.*, 1998). Similar results were obtained with Concanavalin A-stimulated bovine lymphocyte supernatants (Mahan *et al.*, 1994b). This inhibitory activity was abolished by an anti-IFN-γ mAb but not by an anti-TNF-α mAb (Mahan *et al.*, 1996). Furthermore, injecting mice with IFN-γ, but not TNF-α protected them against *Cowdria* (Totté, *et al.*, 1994b). IFN-γ upregulates MHC class I and II expression on various cells (Coutinho *et al.*, 1991) and may thus increase the presentation of *Cowdria* antigens to the immune system. The surface expression of both MHC class I and II has been shown to be strongly inhibited in a dose dependent manner when endothelial cells are infected with *Cowdria* (Totté *et al.*, 1996; Vachiéry *et al.*, 1998). IFN-γ also

leads to enhanced phagocytosis and the release of reactive oxygen intermediates, nitric oxide and lysosomal enzymes such as myeloperoxidase (MPO) by monocytes. Indeed, recombinant MPO has been shown to protect mice against heartwater infection (Tournay *et al.*, 1996). While its involvement, therefore, seems to be beyond dispute, the mechanism whereby IFN-γ inhibits the growth of *Cowdria* still remains to be elucidated (Totté *et al.*, 1999a).

Of particular relevance to the development a new heartwater vaccine is the fact that IFN-γ has been implicated in protection against several other parasitic diseases of ruminants (Kodama *et al.*, 1987; Preston *et al.*, 1992; Brown *et al.*, 1996 & 1999; Totté *et al.*, 1996 & 1999a). Accordingly, work on vaccines for these diseases has been targeted at stimulating IFN-γ producing $CD4^+$ T-cells. For example, several *B. bovis* proteins that stimulated IFN-γ secreting $CD4^+$ T-cells from immune cattle have been identified (Brown *et al.*, 1995). In another study, the MSP2 of *A. marginale* has been shown to induce protective immune responses in cattle that were characterised by a T helper lymphocyte response together with IFN-γ production (Brown *et al.*, 1998c). MSP2 epitopes that induced T helper responses were subsequently identified and are thus potential targets for vaccine development (Brown *et al.*, 2001). With the ultimate aim of developing a subunit vaccine for heartwater, it is important to focus on components of the parasite that can potentially elicit the appropriate cellular immune responses. Therefore, antigens that induce strong cell-mediated immune responses characterised by type 1 $CD4^+$ and $CD8^+$ T-cell responses are those most likely to be useful as vaccine antigens. Evidence presented in Chapter 4 has shown that a number of low molecular weight proteins of *Cowdria*, i.e. those that range from 11 to 23 kDa, induced proliferation of PBMC obtained from cattle immunised either by infection and treatment or alternatively, with inactivated organisms.

As opposed to the multitude of cells in a preparation of PBMC, T-cell lines or clones have many advantages when the aim is to characterise an immune response. For example, the use of T-cell clones is necessary to characterise the cytokine profile induced by a particular pathogen or antigen, particularly since $CD4^+$, $CD8^+$ and γδ T-cell subsets produce similar cytokine profiles (section 1.2.3.5.). T-cell cloning can help to identify which lymphocyte phenotype is stimulated and to establish which cytokines are produced. It was described in Chapter 4 how the use of sensitised primary PBMC permitted rapid and simple screening for the proteins that stimulated specific immune responses to *Cowdria*. Such preparations can be expected to contain only a limited number of lymphocytes specific for the target antigen. Establishment of short term T-cell *lines* selects and enriches for antigen-specific lymphocytes. This can be done by discontinuous (cycles of stimulation and intervening rest), or continuous stimulation (no rest cycle), the latter allowing for more rapid generation (Coligan *et al.*, 1991). A particular antigen-stimulated *clone* on the other hand, expresses only a selected, minor set of effector molecules from the large repertoire. Cloning of T-cells from cell lines can be done in the presence of T-cell growth factor (TCGF), either by limiting dilution or in soft agar. *In vitro* selection based on factors other than antigen specificity, such as the ability to grow well in culture, may occur. This can result in the mortality of whole subpopulations of T-cells and certain antigen specificities being lost (Sredni *et al.*, 1981). Thus only a limited set of effector functions is expressed in a particular T-lymphocyte clone while most effector molecules are usually not produced. T-cell lines are therefore often more informative since they do not exhibit the restricted and selective responses of clones (Brown *et al.*, 2001).

This chapter describes the establishment of *Cowdria*-specific T-cell lines and analyses the phenotype of the lymphocytes stimulated by *Cowdria* antigens. Results presented also confirms the importance of low molecular weight fractions in vaccine development by demonstrating that they induce IFN-γ production by antigen-primed lymphocytes.

6.2. MATERIALS AND METHODS

6.2.1. GENERATION AND MAINTENANCE OF *COWDRIA*-SPECIFIC T-CELL LINES

T-cell lines specific for *Cowdria* were established from PBMC of the cattle numbered B9191, B1359, B809 and B821 by a modification of a procedure described by Brown & Logan (1992), but with some modifications. Four million PBMC were cultured per well in a volume of 1.5 ml complete RPMI in 24-well plates (Costar) together with 1 μg/ml *Cowdria* antigen. After seven days the cells were subcultured to a density of 1 x 10^6 lymphocytes per ml and restimulated with 4 x 10^6 irradiated (3000 rad) autologous PBMC per ml as a source of APC (rest cycle). After seven days the cells were subcultured to a density of 1 x 10^6 lymphocytes per ml and restimulated with 1 μg/ml *Cowdria* antigen together with 4 x 10^6 irradiated autologous PBMC per ml as feeder cells (stimulation cycle). The T-cell lines were maintained by alternating the rest and restimulation cycles. The T-cells were Ficoll purified (section 3.2.2.) and lymphocyte proliferation assays (2.5 x 10^5 T-cells per ml and 2 x 10^6 irradiated PBMC per ml; section 3.2.3.) were performed after a rest cycle and flow cytometry analysis (section 6.2.2.) performed after each cycle.

6.2.2. CELL SURFACE PHENOTYPIC ANALYSIS BY FLOW CYTOMETRY

The surface phenotype composition of the T-cell lines was determined by indirect immunofluorescence staining using specific mAb as described (Lalor *et al.*, 1986). The cells to be phenotyped were centrifuged at 300g for 10 min at 10°C. The pellet was resuspended with complete RPMI and plated at 2 x 10^5 cells/50 µl (maximum of 1 x 10^6) in 96 well v-bottom plates. The appropriate antibody was diluted 1:66 in complete RPMI containing 0.2% NaN_3 (flow cytometry medium) and 50 µl added to each well and incubated for 30 min on ice. The mAbs used were specific for bovine CD2 (clone BAQ95), CD3 (clone MM1A), B cell surface marker B-B2 (clone BAQ44A), CD4 (clone GC50A1), CD8α (clone CACT80C) and γδ T-cell receptor-N24γ (clone GB21A). These Mab were obtained from Dr. William C. Davis (Washington State University, Pullman, WA, USA). A volume of 100 µl flow cytometry medium was added to each well and centrifuged at 300g for 3 min at 10°C. The supernatant liquid was removed and the cells resuspended on a mixer. The cells were washed three times by adding 200 µl flow cytometry medium to each well and centrifuged at 300g for 3 min at 10°C. After the cells were resuspended 50 µl of secondary antibody, viz. fluorescein isothiocyanate (FITC) goat anti-mouse polyvalent antiserum (IgA, IgG and IgM; final dilution 1:250; SIGMA), was added and incubated on ice for 30 min in the dark. Thereafter the cells were washed three times as described above. After the cells were resuspended, 200 µl of 2% formaldehyde in PBS (flow cytometry fixer) was added and the plates placed at 4°C in the dark for 30 min. The samples were transferred to a flow cytometry tube containing 200 µl flow cytometry fixer. The samples were analysed by flow cytometry using a Coulter XL-4 flow cytometer. The percentage of positive cells was determined

by subtracting the percentage of cells stained with the second reagent only from the percentage of cells stained with a given Mab and the second reagent as described (Overton, 1988).

6.2.3. Complement-mediated lysis of γδ T-cells

Complement-mediated lysis of γδ T-cells was performed on the T-cell lines after the fourth week in culture by a modification of the procedure described by Lalor *et al.* (1986). Ficoll purified T-cells were resuspended with diluted mAb specific for bovine γδ T-cells (clones GB21A and GB54A; 1:1000 dilution) and incubated for 30 min at 4°C. The cells were washed twice by centrifugation at 300g for 10 min. Sterile rabbit serum (SIGMA) was added at a dilution of 1:5 and incubated at 37°C for 45 min. The resultant cells were Ficoll purified (section 3.2.2.) to obtain viable cells. Samples were analysed by flow cytometry (section 6.2.2.) and in proliferation assays (section 3.2.3.).

6.2.4. Bovine IFN-γ ELISA

To determine whether crude antigen and CFE fractions induce IFN-γ production, supernatants were collected on day five of the proliferation assay i.e. before labeling with tritium. The levels of bovine IFN-γ in these supernatants (diluted 1:5) were determined using the BOVIGAM™ ELISA (CSL, Ltd., Australia). Results of IFN-γ ELISA are presented as IFN-γ production index (IPI) where IPI = mean absorbance at 450 nm (Abs_{450nm}) of test sample/mean Abs_{450nm} of unstimulated control (PBMC or T-cell line with APC). Indices greater than twice unstimulated control were considered positive.

6.2.5. GENERATION OF *COWDRIA*-SPECIFIC T-CELL CLONES

T-cell lines were cloned by limiting dilution. Ficoll purified T-cells were cloned in 96 well round bottom plates at 0.3; 1; 3; or 10 T-cells per well by a modification of the method described by Coligan *et al.*, (1991). After Ficoll purification (section 3.2.2.), the cells were counted and their concentration adjusted to 1 x 10^6 cells/ml. They were then diluted as indicated in Table 6.1. A volume of 100 µl feeding mix (complete RPMI, 5 x 10^4 irradiated autologous PBMC, 2 µg/ml *Cowdria* antigen and 20% TCGF) was added to each well in three 96 well plates. A volume of 100 µl of each dilution was added to the wells as follows: Plate 1: two rows @ 10 cells/well (A-B); two rows @ 3 cells/well (C-D) and four rows @ 1 cell/well (E-H). Plate 2: four rows @ 1 cell/well (A-D) and four rows @ 0.3 cells/well (E-H). Plate 3: seven rows @ 0.3 cells/well (A-G) and one row of irradiated PBMC alone (H). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cells were fed at intervals of either one or two weeks by removing 50% of the medium and replacing it with fresh feeding mix. After approximately two to four weeks, the contents of one 96 well (showing growth) was transferred up successively to a 48 well (0.75 ml/well) and 24 well (1.5 ml/well) tissue culture plates in the presence of feeding mix. The cells were finally maintained in 24 well plates. Those in the remaining wells were fed with feeding mix until ready for transfer.

TABLE 6.1. Dilution of T-cells for cloning by limiting dilution.

Dilution no	Dilution factor	Cells/ml	Volume required ml	Cells/100 µl	Cells/well final
1		$1x10^6$	5		
2	2x	$5x10^5$	10		
3	10x	$5x10^4$	10		
4	10x	$5x10^3$	10		
5	10x	500	10		
6	5x	100	**10**	**10**	**10**
7	3x	33.3	**9**	**3.3**	**3.3**
8	3x	11.1	**18**	**1.1**	**1.1**
9	3x	3.3	**15**	**0.33**	**0.33**

6.2.5.1. Preparation of T-cell growth factor

T-cell growth factor was prepared from PBMC as described by Brown & Garb (1985) with the following modifications. PBMC from several non-immune cattle were screened for ability to produce adequate T-cell growth factor. The cattle were reared in tick free conditions at the blood vaccine production facilities at OVI. Two million PBMC were cultured per ml complete medium (section 3.2.2.) in culture flasks (Costar) with 2 µg/ml Con A for 48 h. The culture medium was harvested and centrifuged for 10 min at 400g. The supernatants were divided into tubes containing 0.1 M α-methyl mannoside and stored at –20°C. Prior to use the tubes were thawed to 37°C and filter sterilised. Samples were assayed in proliferation assays to determine T-cell growth factor efficacy. The T-cell growth factor was used for generating T-cell clones by limiting dilution (Section 6.2.5.).

6.3. RESULTS

6.3.1 ESTABLISHING *COWDRIA*-SPECIFIC T-CELL LINES

6.3.1.1. Phenotypic analysis and proliferation assays of *Cowdria*-specific T-cell lines

Initial attempts at establishing T-cell lines by weekly stimulation with *Cowdria* antigen failed (results not shown). By using a strategy of alternatively stimulating cells for seven days with antigen and resting them for a further seven days (section 6.2.1.), T-cell lines could, however, be repeatedly and successfully established. These lines could be maintained in culture for up to six weeks (Table 6.2.) after which they suddenly lost their specificity (results not shown). Weekly phenotypic analyses (section 6.2.2.) were performed to determine which T-cell subpopulations were present over time. After the first stimulation cycle of seven days, the cells from animal B809 (immunised with inactivated organisms) contained 5.6% B cells, 13.1% $CD8^+$, 31.2% γδ and 31.5% $CD4^+$ T-cells (Table 6.2.). The fact that these percentages do not add up to 100% may be due to the large gate sizes that were used. A large gate (100% lymphocyte inclusivity) may also include monocytes, granulocytes and red blood cells (Givan, 1994). Therefore, resulting in a decrease in the % of T cell subpopulations as seen here. When these lymphocytes were tested in a proliferation assay, a SI of 2 was obtained (Table 6.2.). By alternatively stimulating T-cell lines with antigen and resting without antigen, the culture was enriched by 43% for $CD4^+$ T-cells and depleted by 27% for γδ T-cells. This led to greater antigen specificity as can be seen by the greater SI of 11 and 20 obtained at four and six weeks respectively (Table 6.2.). The cell lines showed consistently lower background proliferation after exposure to non-infected endothelial cell antigen as indicated by a SI of 1. Similar results were

obtained for the other cell lines from animal B821 immunised with inactivated organisms, as well as animals B1359 and B9191 immunised by infection and treatment (Tables 6.2 & 6.3.). In contrast, PBMC assayed at the same time showed a higher non-specific proliferation than did the T-cell lines in response to non-infected endothelial cell antigen (Table 6.4.). The T-cell lines were thus conclusively more antigen-specific than the PBMC.

6.3.1.2. Complement mediated lysis of γδ T-cells

Because the cell lines contained a mixture of $CD4^+$ and γδ T lymphocytes, an attempt was made to remove the γδ T-cells by complement-mediated cell lysis (section 6.2.3.) using a mAb specific for the γδ T-cell receptor. Following lysis, the percentage of $CD4^-$ T-cells increased in all lines, reaching levels of 85-88% in three of the lines (Table 6.3.). The percentage of γδ T-cells was reduced in all but one of the cell lines (Table 6.3.). Nevertheless, when the percentage of γδ T-cells was reduced to 3-5% of the total number of lymphocytes (i.e. in cell lines B821 and B9191 after culture for four weeks), the SI in response to *Cowdria*-infected endothelial cell antigens increased, attaining values of 108 and 240 respectively. Thus, the strong proliferative responses shown by cell lines consisting almost entirely of $CD4^+$ T-cells demonstrates that the responder cells in the population were likely to be predominantly of this phenotype. This does not rule out the possibility that some γδ T-cells could have contributed to the proliferative response observed. It is, however, unlikely that their contribution could have exceeded 3-5% of the total response.

6.3.1.3. Proliferative response of T-cell lines to CFE fractions of *Cowdria*

Cowdria antigens were fractionated by CFE (section 4.2.1.) to determine the size of the proteins

which were immunostimulatory to the $CD4^+$ enriched T-cells. The T-cell lines derived from animal B9191 responded specifically by proliferating in response to fractions containing proteins of 11 to 25, 27, 32, 33 and <55 kDa (Tables 6.5. and 6.6.). The T-cell lines derived from animals B1359, B890 and B821 responded in the same way to the protein fractions as can be seen in Tables 6.5. and 6.6. Proteins of between 97 and 168 kDa did not induce $CD4^+$ enriched lymphocytes from any of the four cattle to proliferate (data not shown). When the protein fractions that induced the $CD4^+$-enriched cell lines to proliferate were compared between the animals immunised either by infection and treatment or with inactivated organisms, those with molecular weights of approximately 11 to 21 kDa were observed to be common to both immunisation groups (Table 6.5.).

6.3.1.4. IFN-γ production by T-cell lines in response to *Cowdria* CFE fractions

IFN-γ is a cytokine that is associated with a type I immune response. To ascertain whether the CFE fractions also induce IFN-γ production, supernatants were collected from proliferation assays performed with PBMC and T-cell lines that had been exposed to CFE fractions and crude antigen (section 6.2.4.). Partially purified *Cowdria* organisms induced PBMC to produce IFN-γ (Table 6.4.). PBMC that were stimulated with non-infected endothelial cell antigens produced low levels of IFN-γ. In contrast the T-cell lines were, once again, higher responders than PBMC, producing more IFN-γ in response to *Cowdria* antigen than to non-infected endothelial cell antigen (Table 6.4.). CFE fractions obtained from non-infected BSV cells did not induce either significant proliferation or IFN-γ production by PBMC (Fig. 6.1.b).

The $CD4^+$ enriched T-cells derived from animal B9191 responded specifically by proliferating

and producing IFN-γ after stimulation with *Cowdria* antigen fractions containing proteins of 11 and 13 to 23 kDa. The cells from animals B1359, B809 and B821 responded similarly to the low molecular weight proteins (Table 6.5.). The production of IFN-γ correlated significantly with proliferation for animals B809 ($r^2 = 0.88$) and B821 ($r^2 = 0.96$) which were immunised with inactivated organisms. However, the correlation between IFN-γ production and proliferation was less significant for animals B9191 ($r^2 = 0.12$) and B1359 ($r^2 = 0.18$) which were immunised by infection and treatment. This was evident from the observation that $CD4^+$ enriched T-cells derived from animal B9191 responded specifically by producing IFN-γ without detectable proliferation to antigen fractions containing proteins of 26, 28, 29, 30, 31 and 33 to 38 kDa (Table 6.5.). This was also observed with animal B1359 and to a lesser degree animal B821 (Table 6.5.). To summarise, the continuous flow electrophoresis fractions of molecular weights of 13 to 18 kDa induced $CD4^+$ lymphocyte proliferation and IFN-γ production by T-cell lines from all the animals immunised either by infection and treatment or with inactivated organisms (Table 6.5.).

TABLE 6.2. Proliferative responses and phenotype analyses of *Cowdria*-stimulated T-cell lines.

Animal no.	Week[a]	Cycle completed	% lymphocytes in T-cell line[b]				SI[c]	
			B-cells	$CD8^+$	γδ	$CD4^+$	Non-infected EC	Infected EC
B809	1	Stimulate	5.6	13.1	31.2	31.5	nd	2
	2	Rest	3	5.3	18.7	35.9	1	4
	3	Stimulate	1.2	2.7	8.7	50.2	nd	nd
	4	Rest	1.2	3.8	8	69.2	1	11
	5	Stimulate	1	8.4	7.1	57.8	nd	nd
	6	Rest	0.6	3.3	3.8	74.2	2	20
B821	1	Stimulate	4.4	8.5	29.2	24.5	nd	2
	2	Rest	3	5.5	15.8	57.3	1	20
	3	Stimulate	0	2.1	13.5	59.2	nd	nd
	4	Rest	0	1.1	8.6	70.9	1	59
	5	Stimulate	4	2.2	4.9	52.6	nd	nd
	6	Rest	16.2	3.9	12.2	58.5	1	180

[a]The cell lines were initiated from PBMC at the beginning of week 1.
[b] Flow cytometry analyses were performed at the end of each cycle.
[c] Proliferation assays were performed at the end of each cycle.
Non-infected endothelial cell (EC) – 1 μg/ml non-infected EC preparation.
Infected EC – 1 μg/ml *Cowdria* infected EC preparation.
nd – not done.

TABLE 6.3. Proliferative responses and phenotype analyses of T-cell lines.

Animal no.	Week[a]	Cycle completed	% lymphocytes[b]		SI[c]	
			γδ	$CD4^+$	Non-infected EC	Infected EC
B809	1	Stimulate	0.2	30.6	nd	nd
	2	Rest	0.9	50.9	1	2
	3	Stimulate	1.5	55.8	nd	nd
	4	Rest	4.7	55.4	2	24
	4*		8.4	85.5	1	37
B821	1	Stimulate	28.2	45	nd	nd
	2	Rest	12.2	66.3	1	11
	3	Stimulate	8.4	67.1	nd	nd
	4	Rest	7.4	35.7	1	62
	4*		2.6	85.5	1	108
B1359	1	Stimulate	28.8	34.4	nd	nd
	2	Rest	22.1	35.6	3	51
	3	Stimulate	19.2	48	nd	nd
	4	Rest	15.2	47.9	1	30
	4*		19	56.3	1	151
B9191	1	Stimulate	43.8	26.2	nd	nd
	2	Rest	28.1	49.6	1	38
	3	Stimulate	14.4	75.7	nd	nd
	4	Rest	13.3	64.5	2	143
	4*		5.1	88	2	240

[a]The cell lines were initiated from PBMC at the beginning of week 1.
[b] Flow cytometry analyses were performed at the end of each cycle.
[c] Proliferation assays were performed at the end of each cycle.
Non-infected endithelial cell (EC) – 1 μg/ml non-infected EC preparation.
Infected EC – 1 μg/ml *Cowdria* infected EC preparation.
* - after complement-mediated γδ T-cell lysis.
nd – not done.

TABLE 6.4. Proliferation and IFN-γ production by PBMC and T-cell lines after stimulation with *Cowdria*-infected and non-infected BSV cell preparations.

Animal no.	Sample	SI		IPI	
		Non-infected EC	Infected EC	Non-infected EC	Infected EC
B821	PBMC	24	73	2	5
	T-cell line	1	73	1	14
B809	PBMC	1	2	3	7
	PBMC	6	12	2	4
	T-cell line	2	15	1	2
B1359	PBMC	1	2	1	2
	T-cell line	1	10	1	2
	PBMC	4	14	3	4
	T-cell line[a]	1	30	1	2
	T-cell line[b]	1	151	1	3
B9191	PBMC	nd	nd	1	3
	PBMC	nd	nd	7	6
	T-cell line	1	18	3	7
	PBMC	6	128	1	3
	T-cell line[a]	2	143	1	16
	T-cell line[b]	2	240	0.3	6

[a] Week 4, before complement mediated γδ T-cell lysis.
[b] Week 4, after complement mediated γδ T-cell lysis.
Non-infected endothelial cell (EC) - 1 μg/ml non-infected EC preparation.
Infected EC - 1 μg/ml *Cowdria* infected EC preparation.
nd – not done.

TABLE 6.5. Proliferative responses and IFN-γ production by $CD4^+$ enriched T-cell lines exposed to *Cowdria*-infected BSV cell preparations fractionated by continuous flow electrophoresis through a 15% polyacrylamide gel.

Fraction no.	kDa	Animal no.							
		B9191		B1359		B809		B821	
		SI	IPI	SI	IPI	SI	IPI	SI	IPI
1	≤11	1	1	1	1	**4**	1	1	1
7	≤11	1	**4**	1	1	**13**	**5**	**5**	**4**
13	11	**8**	**2**	1	**2**	**32**	**6**	**67**	**12**
19	11-12	**10**	1	**4**	**3**	**41**	**8**	**61**	**11**
25	13	**14**	**2**	**3**	**3**	**53**	**11**	**66**	**10**
31	14-16	**9**	**4**	**2**	**2**	**45**	**11**	**116**	**14**
37	16-17	**7**	**2**	**4**	**3**	**33**	**10**	**107**	**15**
43	18	**9**	**3**	**3**	**2**	**25**	**5**	**16**	**5**
49	19-21	**4**	**5**	**3**	1	**24**	**4**	**7**	**2**
55	22-23	**4**	**4**	1	1	**24**	**5**	**4**	**2**
61	23	**6**	**2**	1	1	**7**	1	**4**	**3**
67	24-25	**2**	1	1	**2**	**13**	1	**3**	**2**
73	26	1	**2**	1	1	**17**	0.3	1	1
79	27	**2**	1	1	**2**	**9**	0.3	1	1
85	28	1	**2**	1	1	**2**	1	**3**	**2**
91	29	1	**2**	1	1	**4**	1	1	1
97	29	1	**2**	1	1	**2**	1	1	1
103	29-30	1	1	1	**2**	**3**	1	1	1
109	30	1	**2**	1	1	1	1	1	**3**
115	31	1	**2**	1	1	0.2	1	1	**3**
121	31	1	**2**	1	**2**	0.2	**2**	1	**3**
127	32-33	**2**	1	1	1	1	1	1	**2**
133	33-34	1	**3**	1	**2**	0.2	1	1	1
139	34	1	**2**	1	1	0.2	1	1	1
145	35	1	1	1	**2**	0.4	1	1	1
151	35-36	1	**2**	1	**2**	0.2	1	**4**	1
157	36-37	1	**2**	1	1	0.4	1	1	1
163	37-38	1	**2**	1	1	0.2	1	1	1
169	38	1	1	1	**5**	1	1	1	1
175	38	1	1	1	1	1	1	1	1

Boldface numbers: SI and IPI ≥ 2 times unstimulated control.
Blue area indicates the molecular weights that induce proliferation and IFN-γ production by T-cell lines from all cattle.

TABLE 6.6. Proliferative responses of $CD4^+$ enriched T-cell lines to *Cowdria* proteins fractionated by continuous flow electrophoresis through a 7% polyacrylamide gel.

Fraction no.	kDa	Proliferation (SI) of animal no:			
		B9191	B1359	B809	B821
1	<55	**7**	1	**36**	**85**
7	55-59	1	1	**2**	**2**
13	60-66	1	1	**8**	**2**
19	67-74	1	1	**4**	1
25	75-80	1	1	**2**	1
31	81-84	1	1	1	1
37	85-90	1	1	**2**	1
43	91-96	1	1	**2**	1

Boldface numbers: SI $\geq$ 2 times unstimulated control.

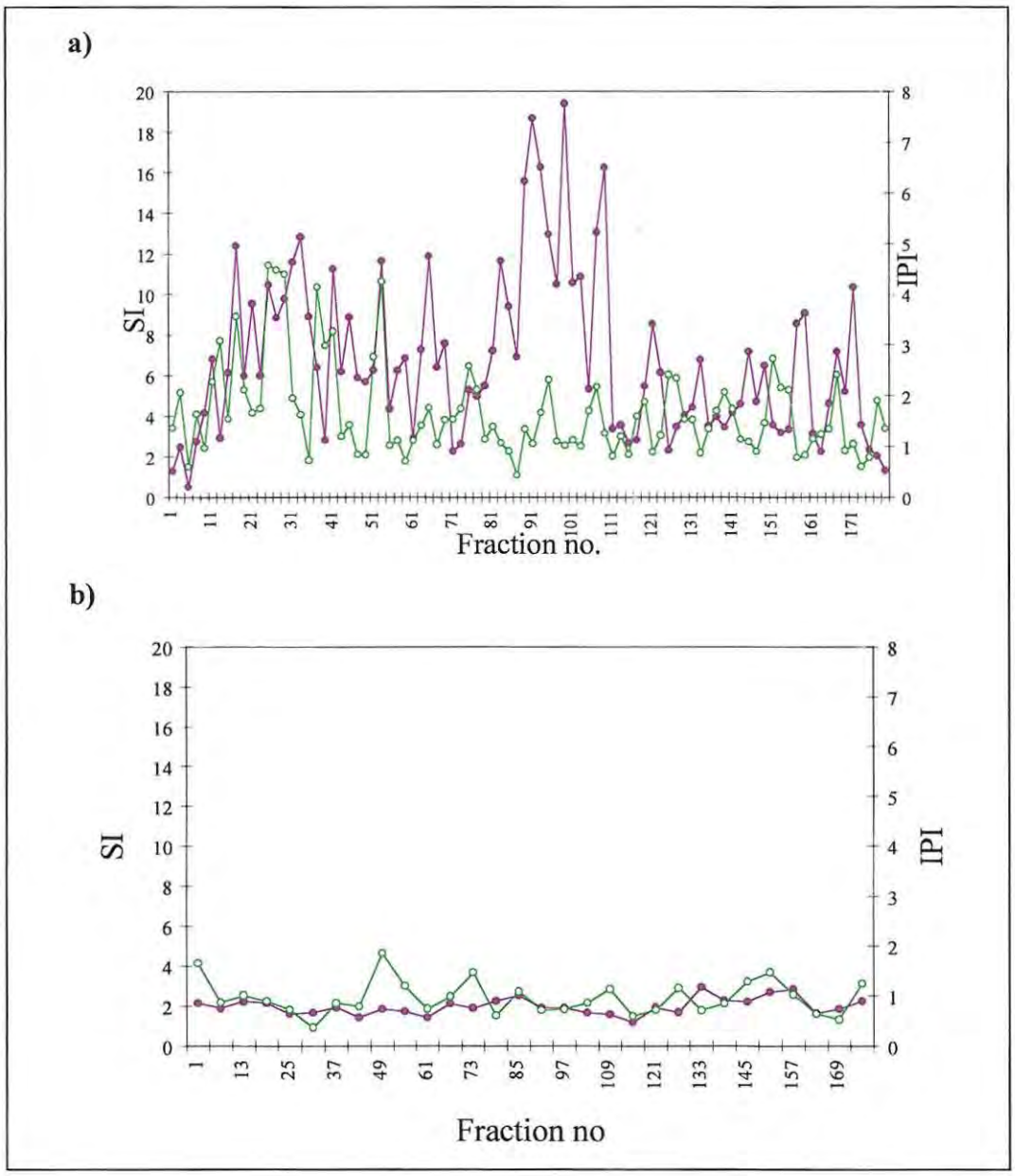

Fig. 6.1. Proliferative responses and IFN-γ production of PBMC from B9191 after stimulation with *Cowdria* infected (a) and non-infected (b) BSV cell preparations fractionated by CFE. *Cowdria*-infected and non-infected BSV cell preparations were electrophoresed on 15% polyacrylamide gels. Proteins were eluted from the gels and collected as 2.5 ml fractions, precipitated with acetone and resuspended in PBS. Fractions were pooled either six (a) or two (b) per sample and assayed in triplicate wells for PBMC proliferation (closed circle ●) and IFN-γ production (open circle ○). Supernatants were collected on day 5 of the proliferation assays for determination of IFN-γ production.

6.3.2. T-CELL CLONES

To characterise in detail the $CD4^+$ T cell response to *Cowdria,* attempts were made at cloning reactive lymphocytes. Numerous attempts at cloning enriched $CD4^+$ T-cell lines (after complement mediated lysis of γδ T-cells of the T-cell lines after the fourth week in culture; section 6.2.3.) by limiting dilution (section 6.2.5.) were however, unsuccessful. Cloning T-cells from PBMC cultured for seven days led to γδ T–cell clones that did not proliferate in response to *Cowdria* antigen. Efforts to clone *Cowdria*-specific T-cells were then abandoned.

6.4. DISCUSSION

It was described in Chapter 4 how proteins of 11, 12, 14 to 17 and 19 to 23 kDa specifically induced proliferation of PBMC from cattle immunised either by infection and treatment, or with inactivated organisms (van Kleef *et al.*, 2000). The nature of the responding cell population was, however, not determined. By generating T-cell lines or clones that on exposure to *Cowdria* antigens enrich for antigen-specific lymphocytes it was hoped to determine the phenotype of the responding lymphocytes and to establish which cytokines they produced.

One week after initiation of the T-cell lines, $CD4^+$ lymphocytes were the predominant cells in the cultures. A progressive enrichment of $CD4^+$ T-cells was observed during the following weeks. This led to greater specificity in their proliferative response towards *Cowdria* antigen, with a concomitant lower background proliferation. This was observed for all the T-cell lines

established, whether from cattle immunised by infection and treatment or with inactivated organisms. Enrichment of $CD4^+$ T-cells over time was similarly observed with *B. bovis* where a rest/stimulation cycle was also used (Brown and Logan, 1992). Phenotypic analysis of lymphocytes recovered from cultures six days after stimulation with *Babesia* merozoite antigen had fewer γδ T-cells than the starting PBMC population (Brown *et al.*, 1994). Helper T cells are central to both humoral and cellular immunity and $CD4^+$ T-cells act through the production of IFN-γ as both helper cells for immunoglobulin secretion and as effector cells to activate macrophages. Identifying immunogens that induce these cells to respond and produce IFN-γ in the majority of animals in an outbred population is thus likely to be important in vaccine development.

Studies described in this chapter indicate that *Cowdria* proteins of 11 to 21 kDa induced $CD4^+$-enriched lymphocytes to proliferate, but only proteins of from 13 to 18 and one of 31 kDa induced lymphocytes from all four cattle tested to produce IFN-γ. Proteins between 11 to 12 and 19 to 23 kDa induced lymphocytes from three of the four cattle to produce IFN-γ. Thus, although the two pairs of cattle were immunised differently, their lymphocytes responded similarly to the fractions. However, the highest proliferation values were obtained with the T-cell lines from those that had been immunised with inactivated organisms. It was shown (Chapter 4) that no alloreactive responses were induced by fractions from non-infected BSV cells when they were assayed with PBMC from unrelated cattle (van Kleef *et al.*, 2000). Therefore it is unlikely that the responses observed resulted from residual BSV antigens.

Interestingly, the fractions in the MAP1 molecular weight region (i.e. 31 kDa) induced $CD4^+$ T-cell lines from all four cattle to produce IFN-γ in the absence of detectable proliferation. Fractions within this region did however induce proliferation of PBMC from four out of the six cattle tested (see Chapter 4). Because the lymphocytes were obtained from cattle immunised either by infection and treatment, or with inactivated organisms, this is unlikely to be simply a result of the immunisation method. This observation may indicate that these fractions stimulate macrophages to produce IFN-γ-inducing cytokines, such as IL-12 and IL-18 (Probst *et al.*, 1997; Shoda *et al.*, 1999), a possibility that needs further investigation (see below).

In the case of the Gardel isolate of *Cowdria*, specific MHC class II-restricted IFN-γ-producing $CD4^+$ T lymphocytes were detected in cattle made resistant to challenge by vaccinating with inactivated organisms (Totté *et al.*, 1997). $CD4^+$ T-cell lines generated from these cattle using *Cowdria* preparations responded strongly to whole preparations, but not to recombinant MAP1 protein (Totté *et al.*, 1998). In contrast, Mwangi and co-workers observed that when cattle were immunised by infection and treatment with the Plumtree and Mbizi isolates, $CD4^+$ Th type-1 responses specific for the recombinant MAP1 were induced (Mwangi *et al.*, 1998 b & c). Similarly when mice were immunised by infection and treatment with the Crystal Springs isolate, the recombinant MAP1 protein induced splenocytes to proliferate and produce IFN-γ (Byrom *et al.*, 2000b). These conflicting results may be due to different immunisation strategies or to the different isolates used in the investigations. The inconsistent induction of proliferation and IFN-γ production by MAP1 may also indicate that additional, and as yet unidentified proteins that can induce these responses were present in these fractions.

Polymorphic immunodominant antigens ranging from 23 to 29 kDa have been identified using goat antiserum raised against the Gardel isolate and cross-adsorbed with the Senegal isolate (and vice versa) to probe western blots. As the two isolates differ genetically and do not cross-protect, these antigens were identified as having potential for use in vaccine development (Perez *et al.*, 1998). In this study with the Welgevonden isolate, however, proteins in this range were poor inducers of proliferation and IFN-γ production. These results highlight the need to take potential differences between isolates into account in the search for universal protective antigens of *Cowdria*.

Attempts to clone *Cowdria*-specific lymphocytes for further cytokine characterisation failed. Only non-responding clones with the γδ T-cell phenotype were obtained. It has been reported that the cloning efficiency of γδ T-cells is low. However, in cases where they have been cloned from parasite-stimulated T cells lines they do not proliferate specifically in response to parasite antigen as was also observed in this study (Tetzlaff *et al.*, 1992). In cattle one year of age and younger γδ T-cell subsets comprise between 15 to 26% of the PBMC population. This percentage declines to less than 10% of PBMC in animals three to five years of age (Clevers *et al.*, 1990). Functional studies of ruminant γδ T-cells are limited and inconclusive but bovine γδ T-cells are known to localise on certain epithelial surfaces where they presumably mount a first line of defence against pathogens. The γδ T-cells are also selectively recruited to sites of active inflammation and the antigens recognised are usually heat shock or stress proteins (Hein & Macay, 1991). Brown and co-workers were the first to demonstrate expression of NK-like activity and inflammatory cytokines IFN-γ and TNF-α by bovine γδ T-cells expressing the WC1 determinant (Brown *et al.*, 1994). However, it remains to be determined whether γδ T-cells play an important role in

protective immunity against heartwater.

The results of the experiments with the T-cell lines confirm the findings made with PBMC (Chapter 4) namely that low molecular weight proteins of *Cowdria* are likely to be involved in the protective immune response of cattle to heartwater. These findings are extended by the demonstration that proteins of from 13 to 18 kDa in the case of the Welgevonden isolate induce specific $CD4^+$ T-lymphocyte proliferation and the production of IFN-γ. Since IFN-γ is likely to play an important role in protective immunity, proteins that were identified as being able to induce both the production of this cytokine, as well as elicit lymphocyte proliferation merit being investigated as potential vaccine antigens.

CHAPTER 7

CONCLUDING DISCUSSION

The current live vaccine for heartwater has numerous disadvantages. A suitable recombinant vaccine could conceivably overcome many of these. Accordingly, the main aim of the investigation described in this thesis was to identify those proteins of the Welgevonden isolate of *Cowdria* that are likely to be involved in protective immunity. The Welgevonden isolate was chosen since it induces cross-protective immunity against more isolates than any other and is also highly virulent (du Plessis *et al.*, 1989). Cellular immune responses, in particular those associated with IFN-γ producing $CD4^+$ lymphocytes, are known to play an important role in protection against heartwater (du Plessis, 1982; du Plessis *et al.*, 1991 & 1992a; Martinez 1997; Totté *et al.*, 1997 & 1998; Mwangi *et al.*, 1998a, b & c; Byrom *et al.*, 2000a & b). $CD4^+$ lymphocytes play a pivotal role in protective immunity. They secrete cytokines that stimulate or regulate activation, growth and differentiation of both B- and T-lymphocytes and inflammatory cells thereby enabling these cells to function optimally in the host's defence against pathogens. The Th1 subset is responsible for cell-mediated immune responses to intracellular pathogens. Of particular relevance to protection against *Cowdria* is IFN-γ, a potent inhibitor of *Cowdria* growth in endothelial cells (Totté *et al.*, 1993 & 1996; Mutunga *et al.*, 1998). Proteins that induce Th1 associated responses are therefore likely to be in some way involved in a protective response. Consequently, their ability to induce lymphocyte proliferation together with a concomitant production of IFN-γ is a valid criterion when searching for potentially protective proteins of *Cowdria*.

Differential centrifugation, Percoll density gradient centrifugation and immunoaffinity chromatography were three methods that were investigated to purify *Cowdria* for use in the immunological assays (Chapter 2). Despite the high specificity offered by the immunoaffinity purification method and the comparable SIs obtained in proliferation assays, the yields were unacceptably low. Surprisingly, although differential centrifugation is a relatively crude purification technique, organisms prepared in this way resulted in the highest SIs in most proliferation assays. It was therefore used to purify *Cowdria* for use in all assays in which PBMC from immunised cattle were screened for responsive lymphocytes.

A total of seven outbred cattle were used as a source of primed lymphocytes. Of particular significance to vaccine development is that cattle are a natural host of heartwater. While not an easy option from a practical point of view, this approach eliminated the inherent disadvantages of using a heterologous experimental system such as the mouse. Two cattle were immunised by infection and treatment, four with inactivated organisms and as a control, one with adjuvant alone. An unexpectedly erratic and limited period when lymphocyte proliferation could be detected in cattle immunised by infection and treatment initially hampered progress (Chapter 3). In contrast, lymphocyte proliferation could be detected within a week, and subsequently for at least two years in cattle immunised with inactivated organisms. Regular monitoring for the possible reappearance of responsive circulating lymphocytes was therefore necessary. This was important because these lymphocytes were initially crucial for optimising the proliferative assay and ultimately the identification of candidate vaccine antigens. Fortunately, responsive circulating lymphocytes reappeared between one to three years after immunisation. Because these cattle were infected with live organisms, one possible reason for the absence of circulating

reactive lymphocytes may be that they were recruited to sites of infection, as discussed in Chapter 3.

Continuous flow electrophoresis was used to fractionate preparations of *Cowdria* into its constituent proteins in the molecular weight ranges of between 11 and 168 kDa (Chapter 4). This allowed a relatively accurate identification of the proteins that induce cellular immune responses. In this way proteins of 11, 12, 14 to 17, 19 to 23 kDa were initially identified on the basis of inducing proliferation of PBMC obtained from two cattle immunised by infection and treatment. They also induced proliferation of PBMC from four cattle immunised with inactivated organisms (Chapter 4). Further screening of fractions with enriched $CD4^+$ T-cell lines, established from cattle either immunised by infection and treatment or with inactivated organisms, further narrowed down the range of immunologically important proteins. Proteins of between 13 and 18 kDa were shown to induce $CD4^+$ lymphocytes to proliferate and produce to IFN-γ (Chapter 6). Thus, although two disparate methods of immunisation were used, the circulating lymphocytes responded similarly to the same set of proteins. Epitopes from these proteins were thus able to prime lymphocytes *in vivo* and the responses could be recalled *in vitro*. This finding accentuates the potential importance of these proteins for inclusion into a recombinant vaccine. In another study, Totté and co-workers (1999b) purified soluble proteins of the Gardel isolate of between 22 and 32 kDa using high performance liquid chromatography. They similarly induced $CD4^+$ lymphocytes, from cattle immunised with inactivated organisms, to proliferate and produce IFN-γ (Totté *et al.*, 1999b). Therefore, evidence is mounting that low molecular weight proteins can induce the type of cellular immune responses known to play a role in protective immunity to heartwater. It remains to be determined, however, whether they can be exploited to induce actual

protective immune responses *in vivo*.

For recombinant vaccine development, the next important step would be to identify the genes encoding these proteins. As mentioned in Chapter 5 this can potentially be achieved either by screening phage display or other expression libraries with specific antiserum or by screening genomic libraries with probes developed from specific partial amino acid sequences. Antisera were raised to these low molecular weight proteins (Chapter 5) and used to screen a *Cowdria* phage display library. Four peptide sequences were identified (Fehrsen & du Plessis, personal communication) and will be compared with the *Cowdria* genome sequence once it becomes available. The genomes of the Welgevonden and Gardel isolate are being sequenced. Having these sequences should greatly facilitate the task of identifying the cognate genes.

Two-dimensional electrophoresis patterns or protein expression profiles can describe the protein composition (proteome) of e.g. a *Cowdria* isolate. In Chapter 5, a two-dimensional electrophoresis pattern of the Welgevonden isolate indicated that any one of the molecular weight regions shown to induce proliferation apparently contained several components, each with differing pIs. In order to obtain the partial amino acid sequence of a protective protein within a molecular weight region, each fraction therefore needs to be further examined using a second method. Isoelectric focusing offers high resolution and can be adapted for preparative applications. A useful apparatus for this purpose is the Rotofor® (Bio-Rad) which fractionates complex protein samples in free solution (Evans *et al.*, 1989; Bier, 1998). Accordingly, it may be used as an adjunct to CFE for further fractionation. The resulting isoelectric focusing fractions can then be similarly assayed for their ability to induce $CD4^+$ lymphocytes to proliferate and to

produce IFN-γ. The partial amino acid sequence of these proteins may then be determined and their encoding genes identified by comparison with the *Cowdria* genome sequence when it becomes available. This may be possible irrespective of whether the amino acid sequence was obtained from the intact protein or from a breakdown product represented within the low molecular weight region. Alternatively, protein and nucleotide sequence databases can be searched for homologues. This may lead to a putative gene sequence. A short sequence of ten amino acids is usually sufficient to identify protein homologues, but peptide mass mapping in combination with peptide sequences can be more effective (Pandey & Mann, 2000). Protein sequencing by mass spectrometry has largely replaced the classical Edman degradation because it is more sensitive, can deal with protein mixtures and offers much higher throughput (Yates, 2000). Accordingly, the *Cowdria* proteins identified in this study can now be characterised by this means to determine their accurate protein mass, obtain peptide mass maps and sequences. Databases can then be searched directly with the data generated for protein homologue identification and thus determine the possible encoding gene sequence (Yates, 2000).

Different cross-protection patterns (Jongejan *et al.*, 1988 & 1991; du Plessis *et al.*, 1989 & 1990), the antigenic diversity (Rossouw *et al.*, 1990; Jongejan *et al.*, 1988) and their genetic diversity (Perez *et al.*, 1997) between isolates of *Cowdria* indicate that different proteins may be required from each isolate in order to obtain universal protection. There is consequently a need to determine whether there is a correlation between genetic diversity, antigenic diversity and cross-immunity. Understanding these differences may help to identify factors responsible for the varying degrees of cross-protection, immunological cross-reactivity and virulence attributed to the multitude of isolates. A more complete understanding of the *Cowdria* proteome as well as

its genome will thus benefit vaccine design. For instance, comparing the two-dimensional electrophoresis patterns of different isolates that *do not* cross-protect can reveal proteins that are unique or common to the different isolates. Such studies can perhaps help to find antigens that protect against all the isolates. New technological developments such as using different fluorescent dyes to separately label two protein samples for visualising on a single two-dimensional gel can provide simple image analysis with improved accuracy (Orange *et al.*, 2000). It is also important to investigate the degree of change of an identified protein during *Cowdria*'s infectious cycle and after passage through the tick and ruminant host. This will indicate whether the protein remains conserved. It is important in vaccine design to look for highly conserved antigens that are not subject to variation (Brown *et al.*, 2001). Although genomics and proteomics are a powerful combination, immunological analyses of identified proteins will still have to be undertaken to evaluate the potential vaccine candidates. Ultimately, both rational and empirical approaches are needed in vaccine research (van Regenmortel, 2000).

Lymphocytes do not recognise the entire protein molecule. They only recognise discrete sites called epitopes presented in combination with MHC. Furthermore, studies with *Mycobacterium tuberculosis* indicated that two peptides from the same protein can differentially induce either a Th1 or Th2 response (Agrewala *et al.*, 1998). This may pose problems if a protective response favours one subset while the other causes adverse effects. Thus it may be beneficial to identify the minimal epitopes of *Cowdria* proteins recognised by T-cells. Core epitopes can be determined by progressively truncating the N- and C- terminal residues and assaying the resulting peptides in T-cell proliferation assays. This is cost and labor intensive (Burton *et al.*, 1999). Alternatively, epitope prediction algorithms can rapidly identify T cell epitopes, as

discussed in Chapter 1 (Margalit *et al.*, 1987; Rothbard & Taylor 1988; Claverie *et al.*, 1988; Roberts *et al.*, 1996). However, it must be borne in mind that the flanking residues of the core epitopes may play a role in modulating their immunogenicity (Moudgil *et al.*, 1998). Furthermore, these epitopes need to be characterised for recognition by different MHC molecules and whether they confer better protection or not than the whole protein.

A successful recombinant *Cowdria* vaccine should induce immunity that is comparable to, or if possible better than that induced by the live vaccine in terms of the degree of protection and duration of immunity. An advantage of a recombinant vaccine is that the immune stimulus can be maximally directed to the molecules relevant for protection while additional components that could cause adverse or other unwanted reactions are avoided. Studies with *P. yoelii* illustrated that not only is the appropriate vaccine delivery system crucial, but of equal importance is the incorporation of multi antigens (Khusmith *et al.*, 1991). The appropriate antigen delivery system must thus be chosen and exploited to generate optimum protective responses. The choice is influenced by the nature of the desired response, which in the case of heartwater appears to be Th1 cell mediated. The infrastructure and financial capacity of developing countries, where heartwater is prevalent, must also be considered (Musoke *et al.*, 1997). Recombinant vaccines have many advantages for those countries because they can easily be produced on a commercial scale, are stable, reproducible and cheap (Wright *et al.*, 1992). There are at least three different ways that recombinant DNA technology can be exploited for vaccine development. These are recombinant antigen vaccines, live antigen delivery vectors or DNA vaccines (Ada, 1997; Melnick, 1989). The disadvantage of a recombinant antigen vaccine is the requirement of production and purification of large quantities of antigen thus making them expensive (Musoke

et al., 1997). Furthermore, expressed peptides need to be combined with an appropriate carrier or adjuvant to improve their immunogenicity and to stimulate the desired immune response (Liljeqvist & Ståhl, 1999). In contrast to this, the vaccinia virus live antigen delivery vector is easy to manipulate, has a wide host range, possesses about 100 different promotors, and there is room for inclusion of DNA coding for at least 10 different antigens (Ada, 1997; Melnick, 1989). It also offers the potential to incorporate cytokines or other co-stimulatory molecules that have the capacity to bias the T-cell response to a Th1 or Th2 cytokine profile (Morrison *et al.*, 1999). Protein plus DNA encoding IL-12 has been shown to produce immunity that lasts much longer. IL-12 is a co-stimulatory factor able to enhance the production of IFN-γ, stimulate lymphocyte proliferation and prevent apoptosis in Th1 cells. It plays a role in maintaining Th1 cells after vaccination (Gurunathan et al., 1998). All these aspects will probably need to be taken into account in the development of a *Cowdria* recombinant vaccine.

From the multitude of possible proteins of the Welgevonden isolate of *Cowdria*, six molecular weight regions ranging from 13 to 18 kDa were identified as being able to induce specific $CD4^+$ lymphocyte proliferation and the production of IFN-γ. Since such cellular immune responses play an important role in protective immunity, these proteins therefore merit being further investigated. Genomic, proteomic and vaccine studies can now be focussed on proteins in these molecular weight ranges.

APPENDIX A

BUFFERS & SOLUTIONS

Alsever's solution

114 M dextrose

27 mM sodium citrate.$2H_2O$

71 mM NaCl

Adjust to pH 6.1 with 1 M citric acid and filter sterilise.

Complete RPMI medium

RPMI-1640 medium containing:

25 mM 25 mM N-[2-hydroxyethyl]piperazine-N'-[4-butanesulfonic acid]

2 mM L-glutamine

10% fetal calf serum

$5x10^{-5}$ mercapto-ethanol

0.1 mg/ml sodium benzylpenicillin

0.2 mg/ml streptomycinsulphate

Hanks' balanced salt solution

5.4 mM KCl

0.3 mM Na_2HPO_4

0.4 mM KH_2PO_4

4.2 mM $NaHCO_3$

0.6 mM $MgSO_4$

137 mM NaCl

5.6 mM D-glucose

Add H_2O to 1 liter and adjust to pH 7.4

Millonig's Phosphate buffer, pH 7.4

0.1 M NaH_2PO_4

0.1 M $NaHPO_4$

4% sucrose

2.5 mM $CaCl_2$

Phosphate-buffered saline, pH 7.4

1.9 mM NaH_2PO_4

8.1 mM $NaHPO_4$

154 mM NaCl

SDS-PAGE sample buffer

0.06 M Tris buffer (pH 6.8)

16% glycerol, 2 % SDS

2.5% dithiothreitol (DTT)

0.001% bromophenol blue

REFERENCES

Abbas, A.K., K.M. Murphy, and A. Sher. **1996**. Functional diversity of helper T lymphocytes. Nature. 383:787-793.

Abbas, A.K., A.H. Lichtman, and J.S. Pober. **1997**. Cellular and molecular immunology. Third edition. W.B Saunders Company.

Ada, G. **1997**. Overview of vaccines. Molecular Biotechnology. 8:123-134.

Agrewala, J.N., and R.J. Wilkinson. **1998**. Differential regulation of Th1 and Th2 cells by p91-110 and p21-40 peptides of the 16-kD α-crystallin antigen of *Mycobacterium tuberculosis*. Clinical and Experimental Immunology. 114:392-397.

Alexander, R.A. **1931**. Heartwater. – The present state of our knowledge of the disease. Report of the Directorate of Veteterinary Services and Animal Industries Onderstepoort. 17:89-150.

Allsopp, M.T.E.P., E.S. Visser, J.L. du Plessis, S.W. Vogel, and B.A. Allsopp. **1997**. Different organisms associated with heartwater as shown by analysis of 16S ribosomal RNA gene sequences. Veterinary Parasitology. 71:283-300.

Allsopp, M.T.E.P., C.M. Hattingh, S.W. Vogel, and B.A. Allsopp. **1999**. Evaluation of 16S, *map*1 and pCS20 probes for detection of *Cowdria* and *Ehrlichia* species. Epidemiology and Infection. 122:323-328.

Ambrosio, R.E., J.L. du Plessis, and J.D. Bezuidenhout. **1987**. The construction of genomic libraries of *Cowdria ruminantium* in an expression vector gt11. Onderstepoort Journal of Veterinary Research. 54:255-256.

Ames, G.F., and K. Nikaido. **1976**. Two-dimensional gel electrophoresis of membrane proteins. Biochemistry. 15:616-623.

Andrew, H.R., and R.A.I. Norval. **1989**. The carrier status of sheep, cattle and African buffalo recovered from heartwater. Veterinary Parasitology. 34:261-266.

Bachmann, M.E., T.M. Kündig, G. Freer, Y. Li, C.Y. Kang, D.H. Bishop, H. Hengartner, and R.M. Zinkernagel. **1994**. Induction of protective cytotoxic T cells with viral proteins. European Journal of Immunology. 24:2228-2236.

Barbet, A.F., S.M. Semu, N. Chigagure, P.J. Kelly, F. Jongejan, and S.M. Mahan. **1994**. Size variation of the major immunodominant protein of *Cowdria ruminantium*. Clinical and Diagnostic Laboratory Immunology. 1:744-746.

Barré, N., G. Uilenberg, P.C. Morel, and E. Camus. **1987**. Danger of introducing heartwater onto the American mainland: Potential role of indigenous and exotic *Amblyomma* ticks. Onderstepoort Journal of Veterinary Research. 54:405-417.

Beis, A., and A. Lazou. **1990**. Removal of artifactual bands associated with the presence of 2-mercaptoethanol in two-dimensional polyacrylamide gel electrophoresis. Analytical biochemistry. 190:57-59.

Bellstedt, D.U., P.A. Human, G.F. Rowland, and K.J. van der Merwe. **1987**. Acid-treated, naked bacteria as immune carriers for protein antigens. Journal of Immunological Methods. 16:249-255.

Benjamin, D.C., J.A. Berzofsky, I.J. East, F.R.N. Gurd, C. Hannum, S.J. Leach, E. Margoliash, J.G. Michael, A. Miller, E.M. Prager, M. Reichlin, E.E. Sercarz, S.J. Smith-Gill, P.E. Todd, and A.C. Wilson. **1984**. The antigenic structure of proteins: a reappraisal. Annual Review of Immunology. 2:67-101.

Berzofsky, J.A., K.B. Cease, J.L. Cornette, J.L. Spouge, H. Margalit, I.J. Berkower, M.F. Good, L.H. Miller, and C. Delisi. **1987**. Protein antigenic structures recognised by T cells: Potential applications to vaccine design. Immunological Reviews. 98:9-52.

Bezuidenhout, J.D. **1987**. Natural transmission of heartwater. Onderstepoort Journal of Veterinary Research. 54:349-351.

Bezuidenhout, J.D., C.L. Patterson, and B.J.H. Barnard. **1985**. *In vitro* cultivation of *Cowdria ruminantium*. Onderstepoort Journal of Veterinary Research. 52:113-120.

Bier, M. **1998**. Recycling isoelectric focusing and isotachophoresis. Electrophoresis. 19:1067-1063.

Billiau, A., H. Heremans, K. Vermeire, and P. Matthys. **1998**. Immunomodulatory properties of interferon-γ. Annals of the New York Academy of Sciences. 856:22-32.

Bohinski, R. C. **1983**. Modern concepts in biochemistry. Fourth edition. Allyn and Bacon, INC.

Boulter, N., and F.R. Hall. **1999**. Immunity and vaccine development in bovine theilerioses. Advances in Parasitology. 44:41-97.

Bourdoulous, S., A. Bensaid, D. Martinez, C. Sheikboudou, I. Trap, A.D. Strosberg, and P. O. Couraud. **1995**. Infection of bovine brain microvessel endothelial cells with *Cowdria ruminantium* elicits IL-1β, -6 and –8 mRNA production and expression of an unusual MHC class II Dqα transcript. Journal of Immunology. 20:613-622.

Bowie, M.V., G.R. Reddy, S.M. Semu, S.M. Mahan, and A.F. Barbet. **1999**. Potential value of major antigenic protein 2 for serological diagnosis of heartwater and related Ehrlichial infections. Clinical and Diagnostic Laboratory Immunology. 6:209-215.

Boyer, J.D., K.E. Ugen, B. Wang, M. Agadjanyan, L. Gilbert, M.L. Bagarazzi, M. Chattergoon, P. Frost, A. Javadian, W.V. Williams, Y. Refaeli, R.B. Ciccarelli, D. McCallus, L. Coney, and D.B. Weiner. **1997**. Protection of chimpanzees from high-dose heterologous HIV-1 challenge by DNA vaccination. Nature Medicine. 3:526-32.

Boyle, D.B. **1994**. Viral vaccines for animal disease control. In: Wood, P.R., P. Willadsen, J.E. Vercoe, R.M. Hoskinson, and D. Demeyer (Eds.). Vaccines in Agriculture: Immunological approaches to animal health and production. CSIRO, Australia. p187-192.

Brake, D.A., C.A. Long, and W.P. Weidanz. **1988**. Adoptive protection against *Plasmodium chabaudi adami* malaria in athymic nude mice by a cloned T cell line. The Journal of Immunology. 140:1989-1993.

Brayton, K.A., J. Fehrsen, E.P. de Villiers, M. van Kleef, and B.A Allsopp. **1997**. Construction and initial analysis of a representative λZAPII expression library of the intracellular rickettsia *Cowdria ruminantium*: cloning of map1 and three other *Cowdria* genes. Veterinary Parasitology. 72:185-199.

Brayton, K.A., E.P. de Villiers, J. Fehrsen, C. Nxomani, N.E. Collins, and B.A Allsopp. **1999**. *Cowdria ruminantium* DNA is unstable in a Super Cos1 library. Onderstepoort Journal of Veterinary Research. 66:111-117.

Brodsky, F.M., and L.E. Guagliardi. **1991**. The cell biology of antigen processing and presentation. Annual Review of Immunology. 9:707-744.

Brown, W.C., and D.J. Garb. **1985**. Biological and biochemical characterization of bovine interleukin 2. Studies with cloned bovine T cells. Journal of Immunology. 133:3184-3190.

Brown, W.C., and K.S. Logan. **1992**. *Babesia bovis*: Bovine helper T cell lines reactive with soluble and membrane antigens of merozoites. Experimental Parasitology. 74:188-199.

Brown, W.C., and G.H. Palmer. **1999**. Designing blood stage-vaccines against *Babesia bovis* and *B. bigemina*. Parasitology Today. 15:275-281.

Brown, W.C., K.S. Logan, G.G. Wagner, and C.L. Tetzlaff. **1991**. Cell-mediated immune responses to *Babesia bovis* merozoite antigens in cattle following infection with tick-derived or cultured parasites. Infection and Immunity. 59:2418-2426.

Brown, W.C., W.C. Davis, S.H. Choi, D.E.A. Dobbelaere, and G.A. Splitter. **1994**. Functional and phenotypic characterisation of $WC1^+$ γ/δ T cells isolated from *Babesia bovis*-stimulated T cell lines. Cellular Immunology. 153:9-27.

Brown, W.C., K.S. Logan, S. Zhao, D.K. Bergman, and A.C. Rice-Ficht. **1995**. Identification of *Babesia* bovis merozoite antigens separated by continuous-flow electrophoresis that stimulate proliferation of helper T-cell clones derived from *B. bovis* immune cattle. Infection and Immunity. 63:3106-3116.

Brown, W. C., T. F. McElwain, B. J. Ruef, C. E. Suarez, V. Shkap, C. G. Chitko-Mckown, W. Tuo, A. C. Rice-Ficht, and G. H. Palmer. **1996**. *Babesia bovis* rhoptry-associated protein 1 is immunodominant for T helper cells of immune cattle and contains T-cell epitopes conserved among geographically distant *B. bovis* strains. Infection and Immunity. 64:3341-3350.

Brown, W.C., T.F. McElwain, I. Hötzel, B.J. Ruef, A.C. Rice-Ficht, R.W. Stich, C.E. Saurez, D.M. Estes, and G.H. Palmer. **1998a**. Immunodominant T-cell antigens and epitopes of *Babesia bovis* and *Babesia bigemina*. Annals of Tropical Medicine & Parasitology. 92:473-482.

Brown, W.C., A.C. Rice-Ficht, and D.M. Estes. **1998b**. Bovine type 1 and type 2 responses. Veterinary Immunology and Immunopathology. 63:45-55.

Brown, W.C., V. Shkap, D. Zhu, T.C. McGuire, W. Tuo, T.F. McElwain, and G.H. Palmer. **1998c**. $CD4^+$ T lymphocyte and immunoglobulin G2 responses in calves immunized with *Anaplasma marginale* outer membranes and protected against homologous challenge. Infection and Immunity. 66:5406-5413.

Brown, W.C., D. Zhu, V. Shkap, T.C. McGuire, E.F. Blouin, K.M. Kocan, and G.H. Palmer. **1998d**. The repertoire of *Anaplasma marginale* antigens recognised by $CD4^+$ lymphocyte clones from protectively immunized cattle is diverse and includes major surface protein 2 (MSP-2) and MSP-3. Infection and Immunity. 66:5414-5422.

Brown, W.C., T.F. McElwain, G.H. Palmer, S.E. Chantler, and D.M. Estes. **1999**. Bovine $CD4^+$ T lymphocyte clones specific for rhoptery associated protein-1 (RAP-1) of *Babesia bigemina* provide cognate help for IgG1 and IgG2 synthesis. Infection and Immunity. 67:155-164.

Brown, W.C., T.C. McGuire, D. Zhu, H.A. Lewin, J. Sosnow, and G.H. Palmer. **2001**. Highly conserved regions of the immunodominant major surface protein 2 (MSP2) of the genogroup II Ehrlichial pathogen *Anaplasma marginale* are rich in naturally derived $CD4^+$ T lymphocyte epitopes that elicit strong recall responses. Journal of Immunology. 166:1114-1124.

Burnet, F.M. **1959**. The clonal selection theory of acquired immunity. Cambridge University Press. London.

Burton, M.D., B. Blaher, C. Suphioglu, R.E. O'Hehir, F.R. Carbone, and J.M. Rolland. **1999**. T-cell receptor contact and MHC binding residues of a major rye grass pollen allergen T-cell epitope. Allergy and Clinical Immunology. 103:255-261.

Byrom, B., A.F. Barbet, M. Obwolo, and S.M. Mahan. **2000a**. $CD8^+$ T cell knockout mice are less susceptible to *Cowdria ruminantium* infection than athymic, $CD4^+$ T cell knockout, and normal C57Bl/6 mice. Veterinary Parasitology. 93:159-172.

Byrom, B., M. Obwolo, A.F. Barbet, and S.M. Mahan. **2000b**. A polarized Th1 type immune response to *Cowdria ruminantium* infection is detected in immune DBA/2 mice. Journal of Parasitology. 86:983-992.

Camus, E., and N. Barré. **1987**. Diagnosis of heartwater in the live animal: experiences with goats in Guadeloupe. Onderstepoort Journal of Veterinary Research. 54:291-294.

Carlander, D., H. Kollberg, P. Wejåker, and A. Larsson. **2000**. Peroral immunotherapy with yolk antibodies for the prevention and treatment of enteric infections. Immunologic Research. 21:1-6.

Caruso A., S. Licenziati, D. Morelli, S. Fiorentini, D. Ricotta, Fabio, Malacarne, L. Sfondrini, and A. Balsari. **1998**. Segregation of type 1 cytokine production in human peripheral blood lymphocytes: phenotypic differences between IFN-γ and IL-2 producing cells in the $CD8^+$ T cell subset. European Journal of Immunology. 28:3630-3638.

Chen, Y., V.K. Kuchroo, J. Inobe, D.A. Hafler, and H.L. Weiner. **1994**. Regulatory T cell clones induced by oral tolerance: Suppression of autoimmune enchepalomyelitis. Science. 265:1237-1240.

Clark, R. **1962**. The pathological physiology of heartwater (*Cowdria ruminantium*). Onderstepoort Journal of Veterinary Research. 29:25-33.

Claverie, J-M., P. Kourilsky, P. Langlade-Demoyen, A. Chalufour-Prochnicka, G. Dadaglio, F. Tekaia, F. Plata, and L. Bougueleret. **1988**. T-immunogenic peptides are constructed of rare sequence patterns. Use in the identification of T epitopes in the human immunodeficiency virus *gag* protein. European Journal of Immunology. 18:1547-1553.

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

Coligan, J.E., A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober. **1991**. Current protocols in immunology. John Wiley and Sons.

Cornell, K.A., H.G.A. Bouwer, D.J. Hinrichs, and R.A. Barry. **1999**. Genetic immunization of mice against *Listeria monocytogenes* using plasmid DNA encoding listeriolysin O. Journal of Immunology.163:322-329.

Coutinhou, G.C., O. Durieu-Trautmann, A.D. Strosberg, and P.O. Couraud. **1991**. Catecholamines stimulate the IFN-γ-induced class II MHC expression on bovine brain capillary endothelial cells. Journal of Immunology. 147:2525-2531.

Cowdry, E. V. **1925**. Studies on the aetiology of heartwater. I. Observation of a rickettsia, *Rickettsia ruminantium* (n.sp.) In the tissues of infected animals. Journal of Experimental Medicine. 42:231-252.

Cox, F.E.G., and F.Y. Liew, **1992**. T-cell subsets and cytokines in parasitic infections. Immunology Today. 13: 445-448.

Cox, F.E.G., and F.Y. Liew. **1993**. T-cell subsets and cytokine profiles in the design of anti-protozoal vaccines. p93-100. In: McKeever, D.J. (Ed.). Novel immunization strategies against protozoan parasites. Proceedings of a workshop held at ILRAD, Nairobi, Kenya. ILRAD, Kenya.

Deem, S.L. **1998**. A review of heartwater and the threat of introduction *of Cowdria ruminantium* and *Amblyomma* spp. Ticks to the American mainland. Journal of Zoo and Wildlife Medicine. 29:109-113.

De Villiers, E.P. **2001**.Genome analysis of *Cowdria ruminantium*. Ph.D. thesis. University of Utrecht, Utrecht, Netherlands.

De Villiers, E.P., K.A. Brayton, E. Zwyegarth, and B.A. Allsopp. **1998**. Purification of *Cowdria ruminantium* organisms for use in genome analysis by pulse-field electrophoresis. Annals of the New York Academy of Sciences. 849:313-320.

De Villiers, E.P., K.A. Brayton, E. Zwyegarth, and B.A. Allsopp. **2000a**. Genome size and genetic map of *Cowdria ruminantium*. Microbiology. 146:2627-2634.

De Villiers, E.P., K.A. Brayton, E. Zwyegarth, and B.A. Allsopp. **2000b**. Macrorestriction fragment profiles reveal genetic variation of *Cowdria ruminantium* isolates. Journal of Clinical Microbiology. 38:1967-1970.

Du Plessis, J.L. **1970**. Pathogenesis of heartwater: I. *Cowdria ruminantium* in the lymph nodes of domestic ruminants. Onderstepoort Journal of Veterinary Research. 37:89-96.

Du Plessis, J.L. **1975**. Electron microscopy of *Cowdria ruminantium* infected reticulo endothelial cells of the mammalian host. Onderstepoort Journal of Veterinary Research. 42:1-14.

Du Plessis, J.L. **1982**. Mice infected with a *Cowdria ruminantium*-like agent as a model in the study of heartwater. D.V.Sc. Thesis, University of Pretoria. South Africa.

Du Plessis, J.L., and J.D. Bezuidenhout. **1979**. Investigation on the natural and acquired resistance of cattle to artificial infection with *Cowdria ruminantium*. Journal of the South African Veterinary Association. 50:334-338.

Du Plessis, J.L., and N.L. Kumm. **1971**. The passage of *Cowdria ruminantium* in mice. Journal of the South African Veterinary Medical Association. 42:217-221.

Du Plessis, J.L., E. Camu, P.T. Oberem, and L. Malan. **1987**. Heartwater serology: some problems with the interpretation of results. Onderstepoort Journal of Veterinary Research. 54:327-329.

Du Plessis, J.L., L. van Gas, J.A. Olivier, and J.D. Bezuidenhout. **1989**. The heterogenicity of *Cowdria ruminantium* stocks: Cross-immunity and serology in sheep and pathogenicity to mice. Onderstepoort Journal of Veterinary Research. 56:195-202.

Du Plessis, J.L., F.T. Potgieter, and L. van Gas. **1990**. An attempt to improve the immunization of sheep against heatwater by using different combinations of 3 stocks of *Cowdria ruminantium*. Onderstepoort Journal of Veterinary Research. 57:205-208.

Du Plessis, J.L., P. Berche, and L. van Gas. **1991**. T cell-mediated immunity to *Cowdria ruminantium* in mice: The protective role of Lyt-2^+ T cells. Onderstepoort Journal of Veterinary Research. 58:171-179.

Du Plessis, J.L., C. Gray, and M.F. van Strijp. **1992a**. Flow cytometric analysis of T cell response in mice infected with *Cowdria ruminantium*. Onderstepoort Journal of Veterinary Research. 59:337-338.

Du Plessis, J.L., P.J. Loock, and C.J.F. Ludemann. **1992b**. Adult *Amblyomma hebraeum* burdens and heartwater endemic stability in cattle. Onderstepoort Journal of Veterinary Research. 59:75-89.

Dumler, J.S., A.F. Barbet, C.P. Bekker, G.A. Dasch, G.H. Palmer, S.C. Ray, Y. Rikihisa, and F.R. Rurangirwa. **2001**. Reorgainsation of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int Journal Syst Evol Microbiol. 51:145-165.

Egan, A.F., M.J. Blackman, and D.C. Kaslow. **2000**. Vaccine efficacy of recombinant *plasmodium falciparum* merozoite surface protein 1 in malaria-naïve, -exposed-, and/or – rechallenged *aotus vociferans* monkeys. Infection and Immunity. 68:1418-1427.

Eisen, H.N., and G.W. Siskind. **1964**. Variations in affinities of antibodies during the immune response. Biochemistry. 3:996-1008.

Emery, D.L., J.S. Rothel, and P.R. Wood. **1990**. Influence of antigens and adjuvants on the production of gamma-interferon and antibody by ovine lymphocytes. Immunology and Cell Biology. 68:127-136.

Evans, C.H., A.C. Willson, and B.A. Gelléri. **1989**. Preparative isoelectric focusing in ampholine electrofocusing columns versus immobiline polyacrylamide gel for the purification of biologically active leukoregulin. Analytical Biochemistry. 177:358-363.

Fehrsen, J., and D.H. Du Plessis. **1999**. Cross-reactive epitope mimics in a fragmented-genome phage display library derived from the rickettsia, *Cowdria ruminantium*. Immunotechnology. 4:175-184.

Fujii, S., K. Ono, A. Takeuchi, T. Aki, S. Shigeta, O. Suzuki, T. Jyo, and U. Yamashita. **1997**. Identification of T-cell epitope sequences on an important mite antigen. Clinical and Experimental Allergy. 27:1086-1094.

Gale, K.R., M.G. Gartside, C.M. Dimmock, H. Zakrzewski, and G. Leatch. **1996**. Peripheral blood lymphocyte proliferative responses in cattle infected with or vaccinated against *Anaplasma marginale*. Parasitology Research. 82:551-562.

Gallagher, R.B., R. Brines, and D. Girdlestone. **1993**. Why immunopharmacology? Immunology Today. 14:241.

Gasbarre, L.C., and J.F. Jr. Urban. **1982**. Assessment of T lymphocyte responses induced by parasite antigens. Veterinary Parasitology. 10:119-129.

Gessani, S., and F. Belardelli. **1998**. IFN-γ Expression in macrophages and its possible biological significance. Cytokine & Growth Factor Reviews. 9:117-123.

Givan, A.L. **1992**. Flow cytometry first principles. Wiley-liss.

Grun, J.L., and W.P. Weidanz. **1981**. Immunity to *Plasmodium chabaudi adami* in the B-cell-deficient mouse. Nature. 290:143-145.

Gurunathan, S., C. Prussin, D.L. Sacks, and R.A. Seder. **1998**. Vaccine requirements for sustained cellular immunity to an intracellular parasitic infection. Nature Medicine. 4:1409-1415.

Haffkine, W.M. **1897**. Remarks on the plague prophylactic fluid. British Medical Journal. 1:1461.

Hager, D.A., and R.R. Burgess. **1980**. Elution of proteins from sodium dodecyl sulphate-polyacrylamide gels, removal of sodium dodecyl sulphate, and renaturation of enzymatic activity: results with sigma subunit of *Escherichia coli* RNA polymerase, wheat germ DNA topoisomerase and other enzymes. Analytical Biochemistry. 109:76-86.

Harding, C.V., and R. Song. **1994**. Phagocytic processing of exogenous particulate antigens by macrophages for presentation by class I MHC molecules. The Journal of Immunology. 153:4925-4933.

Harlow, E., and H.D. Lane. **1988**. Antibodies: A laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y., U.S.A.

Hayday, A.C. 2000. γδ Cells: A right time and a right place for a conserved third way of protection. Annual Review of Immunology. 18:975-1026.

Hein, W.R., and C.R. Mackay. **1991**. Prominence of γδ T cells in the ruminant immune system. Immunology Today. 12:30-34.

Herbert, B.R., M.P. Molloy, A.A. Gooley, B.J. Walsh, W.G. Bryson, and K.L. Williams. **1998**. Improved protein solubility in two-dimensional electrophoresis using tributyl phosphine as reducing agent. Electrophoresis. 19:845-851.

Hickman, C.J., C.K. Stover, S.W. Joseph, and E.V. Oaks. **1993**. Murine T-cell response to native and recombinant protein antigens of *Rickettsia tsutsugamishi*. Infection and Immunity. 61:1674-1681.

Hiemstra, H.S., J.W. Drijfhout, and B.O. Roep. **2000**. Antigen arrays in T cell immunology. Current Oponion in Immunology. 12:80-84.

Hodgson, A.L.M. **1994**. Bacterial vaccine vectors. In: Wood, P.R., P. Willadsen, J.E. Vercoe, R.M. Hoskinson, and D. Demeyer (Eds.). Vaccines in Agriculture: immunological approaches to animal health and production. CSIRO, Australia.

Hoogenboom, H.R. **1997**. Designing and optimizing library selection strategies for generating high-affinity antibodies. Trends in Biotechnology. 15:62-70.

Horak, I.G., K.M. DE F. MacIvor, T.N. Petney, and V. de Vos. **1987**. Some avian and mammalian hosts of *Amblyomma hebraeum* and *Amblyomma marmoreum* (Acari: Ixodidae). Onderstepoort Journal Veterinary Research. 54:397-403.

Ilemobade, A.A., and Blotkamp, C. **1978**. Heartwater in Nigeria. II. The isolation of *Cowdria ruminantium* from live and dead animals and the importance of routes of inoculation. Tropical Animal Health Production. 10:39-44.

Janeway, C.A., and P. Travers. **1994**. Immunobiology. The immune system in health and disease. Blackwell Scientific Publications. Oxford.

Jardine, J.E., S.W. Vogel, M. van Kleef, and J.J. van der Lugt. **1995**. Immunohistochemical identification of *Cowdria ruminantium* in formalin-fixed tissue sections from mice, sheep, cattle and goats. Onderstepoort Journal of Veterinary Research. 62:277-280.

Jenner, E. **1798**. An inquiry into the causes and effects of the Variolae vaccine. Low, London.

Jerrells, T.R., D.L. Jarboe, and C.S. Eisemann. **1986**. Cross-reactive lymphocyte responses and protective immunity against other spotted fever group Rickettsiae in mice immunized with *Rickettsia conorii*. Infection and Immunity. 51:832-837.

Jin, X., C.G.P. Poberts, D.F. Nixon, J.T. Safrit, L.Q. Zhang, Y.X. Huang, N. Bhardwaj, B. Jesdale, A.S. DeGroot, and R.A. Koup. **2000**. Identification of subdominant cytotoxic T lymphocyte epitopes encoded by autologous HIV Type 1 sequences, using dendritic cell stimulation and computer-driven algorithm. Aids Research and Human Retroviruses. 16:67-76.

Johnson, H.M., F.W. Bazer, B.E. Szente, and M.A. Jarpe. **1994**. How interferons fight disease. Scientific American. 270:68-75.

Jones, T.R., and S.L. Hoffman. **1994**. Malaria vaccine development. Clinical Microbiology Reviews. 7:303-310.

Jongejan, F. **1991**. Protective immunity to heartwater (*Cowdria ruminantium* infection) is acquired after vaccination with *in vitro*-attenuated rickettsiae. Infection and Immunity. 59:729-731.

Jongejan, F., and M.J.C. Thielemans. **1989**. Identification of an immunodominant antigenically conserved 32-kilodalton protein from *Cowdria ruminantium*. Infection and Immunity. 57:3243-3246.

Jongejan, F., G. Uilenberg, and F.F.J. Franssen. **1988**. Antigenic differences between stocks of *Cowdria ruminantium*. Research in Veterinary Science. 44:186-189.

Jongejan, F., T.A. Zandbergen, P.A van de Wiel, M. de Groot, and G. Uilenberg. **1991**. The tick-borne rickettsia *Cowdria ruminantium* has a chlamydia-like developmental cycle. Onderstepoort Journal of Veterinary Research. 58:227-237.

Jongejan, F., N. de Vries, H. Nieuwenhuijs, L. Wassink, and A. van Vliet. **1993**. The major antigenic protein of *Cowdria ruminantium* is conserved within the genus *Ehrlichia*. Revue d'Élevage et de Médecine vétérinaire des Pays tropicaux. 46:145-152.

Jungbult, P., and B. Thiede. **1997**. Protein identification from 2-DE gels by MALDI mass spectrometry. Mass Spectrometry Reviews. 16:154-162.

Kaufmann, S.H.E. **1996.** γ/δ and other unconventional T lymphocytes: What do they see and what do they do? Proceedings of the National Academy of Sciences of the United States of America. 93:2272-2279.

Kodama, K., S. Kawamura, M. Yasukawa, and Y. Kobayashi. **1987**. Establishment and characterization of a T-cell line specific for *Rickettsia tsutsugamushi*. Infection and Immunity. 55:2490-2495.

Khusmith, S., Y. Charoenvit, S. Kumar, M. Sedegah, R. L. Beaudoin, and S. L. Hoffman. **1991**. Protection against malaria by vaccination with sporozoite surface protein 2 plus CS protein. Science. 252:715-718.

Kima, P.E., I.K. Srivastava, and C.A. Long. **1992**. Proteins with molecular masses of 25 to 40 kilodaltons elicit optimal protective responses against *Plasmodium chabaudi adami* infection. Infection and Immunity. 60:5065-5070.

Kolle, W. **1896**. Zur aktiven Immunizierung des Menschen gegen Cholera. Centralblatt für Bacteriologie, Parasitenkunde u. Infektionskrankheiten. 19:97-104.

Kuby, J. **1994**. Immunology. Second edition. W. H. Freeman and Company. New York.

Kumaratilake, L.M., A. Ferrante, and C. Rzepczyk. **1991**. The role of T lymphocytes in immunity to *Plasmodium falciparum*. Enhancement of neutrophil-mediated parasite killing by lymphotoxin in IFN-γ: comparisons with tumor necrosis factor. Journal of Immunology. 146:762-767.

Laemmli, U.K. **1970**. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature. 227:680-685.

Lally, N.C., S. Nicoll, E.A. Paxton, C.M. Cary, and K.J. Sumption. **1995**. The *Cowdria ruminantium* groE operon. Microbiology. 141:2091-2100.

Lalor, P.A., B.M. Morrison, R.M. Goodeeris, R.M. Jack, and S.J. Black. **1986**. Monoclonal antibodies identify phenotypically and functionally distinct cell types in the bovine lymphoid system. Veterinary Immunology and Immunopathology. 13:121-140.

Langhorne, J., S.J. Meding, K. Eichmann, and S.S. Gilliard. **1989**. The response of $CD4^+$ T cells to *Plasmodium chabaudi chabaudi.* Immunological Reviews. 112:71-94.

Lee, C., A. Levin, and A. Branton. **1987**. Copper staining: A five minute protein stain for sodium docecyl sulphate-polyacrylamide gels. Analytical Biochemistry. 166:308-312.

Liew, F.Y., and F.E.G. Cox. **1991**. Nonspecific defence mechanism: the role of nitric oxide. Parasitology Today. 7:A17-A21.

Liljeqvist, S., and S. Ståhl. **1999**. Production of recombinant subunit vaccines: protein immunogens, live delivery systems and nucleic acid vaccines. Journal of Biotechnology. 73:1-33.

Liu, M.A. **1998**. Vaccine developments. Nature Medicine Vaccine Supplement. 4:515-519.

Liu, B., and J.D. Marks. **2000**. Applying phage antibodies to proteomics: Selecting singly chain Fv antibodies to antigens blotted on nitrocellulose. Analytical Biochemistry. 286:119-128.

MacKenzie, P.K.I., and N. McHardy. **1987**. *Cowdria ruminantium* infection in the mouse: A review. Onderstepoort Journal of Veterinary Research. 54:267-269.

Mahan, S.M., N. Tebele, D. Mukwedeya, S. Semu, C.B. Nyathi, L.A. Wassink, P.J. Kelly, T. Peter and A.F. Barbet. **1993**. An immunoblotting diagnostic assay for heartwater based on the immunodominant 32-kilodalton protein of *Cowdria ruminantium* detects false positives in field sera. Journal of Clinical Microbiology. 31:2729-2737.

Mahan, S.M., T.C. McGuire, S.M. Semu, M.V. Bowi, F. Jongejan, F.R. Rurangirwa, and A. F. Barbet. **1994a**. Molecular cloning of a gene encoding the immunogenic 21 kDa protein of *Cowdria ruminantium*. Microbiology. 140:2135-2142.

Mahan, S. M., G. E. Smith, and B. Byrom. **1994b**. Concanavalin A-stimulated bovine T-cell supernatants inhibit growth of *Cowdria ruminantium* in bovine endothelial cells *in vitro*. Infection and Immunity. 62:747-750.

Mahan, S.M., H.R. Andrew, N. Tebele, M.J. Burridge, and A.F. Barbet. **1995**. Immunization of sheep against heartwater with inactivated *Cowdria ruminantium*. Research in Veterinary Science. 58:46-49.

Mahan, S. M., G. E. Sileghem, G. E. Smith, and B. Byrom. **1996**. Neutralisation of bovine concanavalin A T cell supernatant-mediated anti-*Cowdria* activity with antibodies specific to interferon gamma but not to tumor necrosis factor. Parasite Immunology. 18:317-324.

Mäkelä, P.H. **2000**. Vaccines coming of age after 200 years. Federation of European Microbiological Societies Microbiology Reviews. 24:9-20.

Margalit, H., J.L. Spouge, J.L. Cornette, K.B. Cease, C. Delisi, and J.A. Berzofsky. **1997**. Prediction of immunodominant helper T cell antigenic sites from the primary sequence. Journal of Immunology. 138:2213-2229.

Martinez, D. **1997**. Analysis of the immune response of ruminants to *Cowdria ruminantium* infection. Development of an inactivated vaccine. Ph.D. thesis. University of Utrecht, Utrecht, Netherlands.

Martinez, D., J.C. Maillard, S. Coisne, C. Sheikboudou, and A. Bensaid. **1994**. Protection of goats against heartwater acquired by immunization with inactivated elementary bodies of *Cowdria ruminantium*. Veterinary Immunology and Immunopathology. 41:153-163.

Mboloi, M.M., C.P.J. Bekker, C. Kruitwagen, M. Greiner, and F. Jongejan. **1999**. Validation of the indirect MAP-1 enzyme-linked immunosorbent assay for diagnosis of experimental *Cowdria ruminantium* infection in small ruminants. Clinical and Diagnostic Laboratory Immunology. 6:66-72.

McAleer, W.J., E.B. Buynak, R.Z. Maigetter, D.E. Wampler, W.J. Miller, and M.R. Hillerman. **1984**. Human hepatitis B vaccine from recombinant yeast. Nature. 307:178.

McBride, J.W., X-J. Yu, and D.H. Walker. **1999**. Molecular cloning of the gene for a conserved major immunoreactive 28-kilodalton protein of *Ehrlichia canis*: a potential serodiagnostic antigen. Clinical and Diagnostic Laboratory Immunology. 6:392-399.

McKeever, D.J., J. K. Nyanjui, and K. T. Ballingall. **1997**. *In vitro* infection with *Theileria parva* is associated with IL-10 expression in all bovine lymphocyte lineages. Parasite Immunology. 19:319-324.

McKeever, D.J., E.L.N. Taracha, W.I. Morrison, A.J. Musoke, and S.P. Morzaria. **1999**. Protective immune mechanisms against *Theileria parva*: Evolution of vaccine development strategies. Parasitology Today. 15:263-267.

Melnick, J.L. **1989**. Viral vaccines: achievements and challenges. Acta Virol. 33:482-493.

Moore, A., P. McGuirk, S. Adams, W.C. Jones, J.P. McGee, D.T. O'Hagan, and K.H.G. Mills. **1995**. Immunization with a soluble recombinant HIV protein entrapped in biodegradable microparticles induces HIV-specific CD8+ cytotoxic T lymphocytes and CD4+ Th1 cells. Vaccine. 13:1741-1749.

Moos, M., N.Y. Nguyen, and T. Liu. **1988**. Reproducible high yield sequencing of proteins electrophoretically separated and transferred to an inert support. Journal of Biochemical Chemistry. 263:6005-6008.

Morrison, W.I., G. Taylor, R.M. Gaddum, and S.A. Ellis. **1999**. Contribution of advances in immunology to vaccine development. Advances in Veterinary Medicine. 41:181-195.

Mosmann, T.R., and R.L. Coffman. **1989**. Th1 and Th2 cells: different patterns of lymphokine secretion leads to different functional properties. Annual Reviews of Immunology. 7:145-173.

Mosmann, T.R., and S. Sad. **1996**. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunology Today. 17:138-146.

Mosmann, T.R., H. Cherwinski, M.W. Bond, M.A. Giedlin, and R.L. Coffman. **1986**. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. Journal of Immunology. 136:2348-2357.

Musoke, A.J., D. McKeever, and V. Nene. **1997**. Subunit vaccines for the control of tick-borne diseases: implications for the future. Parasitologia. 39:131-137.

Mustafa, A.S., K.E.A. Lundin, and F. Oftung. **1998**. Isolation of recombinant phage clones expressing mycobacterial T cell antigens by screening a recombinant DNA library with human $CD4^+$ Th1 clones. FEMS Immunology and Medical Microbiology. 22:205-216.

Mutunga, M., P. Preston, and K. Sumption. **1998**. Nitric oxide is produced by *Cowdria ruminantium*-infected bovine pulmonary endothelial cells *in vitro* and is stimulated by gamma interferon. Infection and Immunity. 66:2115-2121.

Moudgil, K.D., E.E. Sercarz, and I.S. Grewal. **1998**. Modulation of the immunogenicity of antigenic determinants by their flanking residues. Immunology Today. 19:217-220.

Mwangi, D.M., S.M. Mahan, J.K. Nyanjui, E.L.N. Taracha, and D.J. McKeever. **1998a**. Immunization of cattle by infection with *Cowdria ruminantium* elicits T lymphocytes that recognize autologous infected endothelial cells and monocytes. Infection and Immunity. 66:1855-1860.

Mwangi, D.M., D.J. McKeever, and S.M. Mahan. **1998b**. Cellular immune responses of cattle to Cowdria ruminantium. p309-315. In: Brown, F., and L. R. Haaheim (Eds.). Modulation of the immune response to vaccine antigens. Developments in Biological Standardization. Basel, Karger.

Mwangi, D.M., D.J. McKeever, J.K. Nyanjui, A.F. Barbet, and S.M. Mahan. **1998c**. Major antigenic proteins 1 and 2 of *Cowdria ruminantium* are the targets for T-lymphocyte responses of immune cattle. Annuals of the New York Academy of Science. 849:372-374.

Neitz, W.O. **1968**. Heartwater. Bu. Off. Int. Epiz. 70:329-336.

Neitz, A.W.H., N.M.J. Vermeulen, and G.J. Viljoen. **1987**. Purification of *Cowdria ruminantium* by density gradient centrifugation. Onderstepoort Journal of Veterinary Research. 54:223-231.

Nixon, D.F., C. Hioe, P. Chen, Z. Bian, P. Kuebler, M-L. Li, H. Qui, X-M. Li, M. Singh, J. Richardson, P. McGee, T. Zamb, W. Koff, C.Y. Wang, and D. O'Hagan. **1996**. Synthetic peptides entrapped in microparticles can elicit cytotoxic T cell activity. Vaccine. 14:1523-1530.

Nyika, A., S.M. Mahan, M.J. Burridge, T.C. McGuire, F. Rurangirwa, and A.F. Barbet. **1998**. A DNA vaccine protects mice against the rickettsial agent *Cowdria ruminantium*. Parasite Immunology. 20:111-119.

O'Farrel, P.H. **1975**. High resolution two-dimensional electrophoresis of proteins. The Journal of Biological Chemistry. 250:4007-4021.

Ohashi, N., A. Unver, N. Zhi and Y. Rikihisa. **1998a**. Cloning and characterization of multigenes encoding the immunodominant 30-kilodalton major outer membrane proteins of *Ehrlichia canis* and application of the recombinant protein for serodiagnosis. Journal of Clinical Microbiology. 36:2671-2680.

Ohashi, N., N. Zhi, Y. Zhang and Y. Rikihisa. **1998b**. Immunodominant major outer membrane proteins of *Ehrlichia chaffeensis* are encoded by a polymorphic multigene family. Infection and Immunity. 66:132-139.

Ojcius, M.D., G. Gachelin, and A. Dautry-Varsat. **1996**. Presentation of antigens derived from microorganisms residing in host-cell vacuoles. Trends in Microbiology. 4:53-59.

Orange, P., E. Hawkins, C. Story, and S. Fowler. **2000**. Fluorescence 2-D difference gel electrophoreis. Innovations Forum. Issue 5.

Osterman, J.V. **1985**. Rickettsiae and hosts. Acta virol. 29:166-173.

Overton, W.R. **1988**. Modified histogram subtraction technique for analysis of flow cytometry data. Cytometry. 9:619-626.

Palmer, D.B., A.J.T. George, and M.A. Ritter. **1997**. Selection of antibodies to cell surface determinants on mouse thymic epithelial cells using phage display library. Immunology. 91:473-478.

Palmer, G.H., F.R. Rurangirwa, K.M. Kocan, and W.C. Brown. **1999**. Molecular basis for vaccine development against the Ehrlichial pathogen *Anaplasma marginale*. Parasitology Today. 15:281-286.

Pandey, A., and M. Mann. **2000**. Proteomics to study genes and genomes. Nature. 405:837-846.

Parmley, S.F., and G.P. Smith. **1988**. Antibody-selectable filamentous fd vectors: affinity purification of target genes. Gene. 73:305-318.

Pasteur, L. **1880**. De l'attention du virus du cholera des poules. Competes Rendus Des Séances de L'Académie des Sciences. Paris 91:673-680.

Pasteur, L. **1885**. Méthode pour prévenir la range aprés morsure. Competes Rendus Des Séances de L'Académie des Sciences. Paris. 101:765-772.

Pasteur, L., C.-E. Chamberland, and R. Roux. **1881**. Sur la vaccination charbonneuse. Competes Rendus Des Séances de L'Académie des Sciences. Paris 92:1378-1383.

Perez, J.M., D. Martinez, A. Debus, C. Sheikboudou, and A. Bensaid. **1997**. Detection of genomic polymorphisms among isolates of the intracellular bacterium *Cowdria ruminantium* by random amplified polymorphic DNA and southern blotting. FEMS Microbiology Letters. 154:73-79.

Perez, J.M., D. Martinez, C. Sheikboudou, F. Jongejan, and A. Bensaid. **1998**. Characterization of variable immunodominant antigens of *Cowdria ruminantium* by ELISA and immunoblots. Parasite Immunology. 20:613-622.

Peter, T.F., S.L. Deem, A.F. Barbet, R.A.I. Norval, B.H. Simbi, P.J. Kelly and S.M. Mahan. **1995**. Development and evaluation of PCR assay for detection of low levels of *Cowdria ruminantium* infection in *Amblyomma* ticks not detected by DNA probe. Journal of Clinical Microbiology. 33:166-172.

Peter, T. F., E. C. Anderson, M. J. Burridge, S. M. Mahan. **1998**. Demonstration of a carrier state for *Cowdria ruminantium* in wild ruminants from Africa. Journal of Wildlife Diseases, 34:567-575.

Pienaar, J.G. **1970**. Electron microscopy of *Cowdria (Rickettsia) ruminantium* (Cowdry, 1926) in the endothelial cells of the vertebrate host. Onderstepoort Journal of Veterinary Research. 37:67-78.

Plebanski, M., S.C. Gilbert, J. Schneider, C.M. Hannan, G. Layton, T. Blanchard, M. Becker, G. Smith, G. Butcher, R.E. Sinden, and A.V.S. Hill. **1998**. Protection from *Plasmodium berghei* infection by priming and boosting T cells to a single class I-restricted epitope with recombinant carriers suitable for human use. European Journal of Immunology. 28:4345-4355.

Polson, A., T. Coetzer, J. Kruger, E. Von Maltzahn, and K.J. van der Merwe. **1985**. Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. Immunological Investigations. 14:323-327.

Preston, P.M., C.G.D. Brown, and W. Richardson. **1992**. Cytokines inhibit the development of trophozoite-infected cells of *Theileria annulata* and *Theileria parva* but enhance the proliferation of macroschizont-infected cell lines. Parasite Immunology. 14:125-141.

Preston, P.M., F.R. Hall, E.J. Glass, J.D.M. Cambell, M.A. Darghouth, J.S. Ahmed, B.R. Shiels, R.L. Spooner, F. Jongejan, and C.G.D. Brown. **1999**. Innate and adaptive immune responses co-operate to protect cattle against *Theileria annulata*. Parasitology Today. 15:268-274.

Probst, P., Y.A. Skeiky, M. Steeves, A. Gervassi, K.H. Grabstein, and S.G. Reed. **1997**. A leishmania protein that modulates interleukin (IL-)-12, IL-10, and tumor necrosis factor-alpha production and expression of B7-1 in human monocyte-derived antigen-presenting cells. European Journal of Immunology. 27:2634-2642.

Provost, A., and J.D. Bezuidenhout. **1987**. The historical background and global importance of heartwater. Onderstepoort Journal of Veterinary Research. 54:165-169.

Prozesky, L., and J.L. du Plessis. **1985**. The pathology of heartwater. I. A study of mice infected with the Welgevonden strain of *Cowdria ruminantium*. Onderstepoort Journal of Veterinary Research. 52:71-79.

Reddy, G.R., C.R. Sulsona, R.H. Harrison, S.M. Mahan, M.J. Burridge, A.F. Barbet. **1996**. Sequence heterogeneity of the major antigenic protein 1 genes from *Cowdria ruminantium* isolates from geographical areas. Clinical and Diagnostic Laboratory Immunology. 3:417-422.

Ristic, M., and D.L. Huxoll. **1984**. Tribe II. Ehrlicheae Philip 1957. 948^{AL}. In: N.R. Krieg, and J.G. Holt. (Eds). Berrey's Manual of Systematic Bacteriology, vol. 1. Williams and Wilkins, Baltimore, Maryland.

Roberts, C.G.P., G.E. Meister, B.M. Jesdale, J. Lieberman, J.A. Berzofsky and A.S. de Groot. **1996**. Prediction of HIV peptide epitopes by a novel algorithm. AIDS Research and Human Retroviruses. 12:593-610.

Robyt, J.F., and B.J. White. **1987**. Biochemical techniques theory and practive. Brooks/Cole Publishing Company.

Rockett, K.A., M.M. Awburn, W.B. Cowden and I.A. Clark. **1991**. Killing of *Plasmodium falciparum in vitro* by nitric oxide derivatives. Infection and Immunity. 59:3280-3283.

Roitt, I.M. **1991**. Essential Immunology. Seventh edition. Blackwell Scientific Publications

Romagnani, S. **1996**. Understanding the fole of Th1/Th2 cells in infection. Trends in Microbiology. 4:470-473.

Rossouw, M., A.W.H. Neitz, D.T. de Waal, J.L. du Plessis, L. van Gas, and S. Brett. **1990**. Identification of the antigenic proteins of *Cowdria ruminantium*. Onderstepoort Journal of Veterinary Research. 57:215-221.

Rothbard, J.B., and W.R. Taylor. **1988**. A sequence pattern common to T cell epitopes. The European Molecular Biology Organization Journal. 7:93-100.

Saavedra, R., M.A. Becerril, C. Dubeaux, R. Lippens, M-J. de Vos, P. Herion, and A. Bollen. **1996**. Epitopes recognized by human T lymphocytes in the ROP2 protein antigen of *Toxoplasma gondii*. Infection and Immunity. 64:3858-3862.

Salmon, D.E., and T. Smith. **1886**. On a new method of producing immunity from contagious diseases. American Veterinary Review. 10:63-69.

Schade, R., C. Staak, C. Hendriksen, M. Erhard, H. Hugl, G. Koch, A. Larsson, W. Pollmann, M. van Regenmortel, E. Rijke, H. Spielmann, H. Steinbusch, and D. Straughan. **1999**. The production of avian (egg yolk) antibodies – IgY. The report and recommendations of European Center for the Validation of Alternative Methods workshop 21.

Schirmbeck, R., J. Zerrahn, A. Kuhröber, W. Deppert, and J. Reimann. **1993**. Immunization of mice with the N-terminal (1-272) fragment of simian virus 40 large T antigen (without adjuvants) specifically primes cytotoxic T lymphocytes. European Journal of Immunology. 23:1528-1534.

Schirmbeck, R., W. Böhm and J. Reimann. **1996**. Virus-like particles induce MHC class I-restricted T-cell responses. Intervirology. 39:111-119.

Scott, P, and S.H.E. Kaufmann. **1991**. The fole of T-cell subsets and cytokines in the regulation of infection. Immunology Today. 12:346-348.

Semu, S.M., T.F. Peter, D. Mukwedeya, A.F. Barbet, F. Jongejan, and S.M. Mahan. **2001**. Antibody responses to MAP1B and other *Cowdria ruminantium* antigens are down regulated in cattle challenged with tick-transmitted heartwater. Clinical Diagnostic Laboratory Immunology. 8:388-396.

Seong, S.Y., M.S. Huh, W.J. Jang, S.G. Park, J.G. Kim, S.G. Woo, M.S. Choi, I.S. Kim, and W.H. Chang. **1997**. Induction of homologous immune response to *Rickettsia tsutsugamushi* Boryong with a partial 56-kilodalton recombinant antigen fused with the maltose-binding protein MBP-Bor56. Infection and Immunity. 65:1541-1545.

Sercarz, E.E., P.V. Lehmann, A. Ametani, G. Benichou, A. Miller, and K. Moudgil. **1993**. Dominance and crypticity of T cell antigenic determinants. Annual Review of Immunology. 11:729-766.

Shoda, L.K., D.S. Zarlenga, A. Hirano, and W.C. Brown. **1999**. Cloning of a cDNA encoding bovine interleukin-18 and analysis of IL-18 expression in macrophages and its IFN-γ-inducing activity. Journal of Interferon and Cytokine Research. 19:1169-1177.

Smith, G.P. **1985**. Filamentous fusion phage: Novel expression vectors that display cloned antigens on the virion surface. Science. 228:1315-1317.

Sredni, B., H.Y. Tse, C. Chen, and R.H. Schwartz. **1981**. Antigen-specific clones of proliferating T lymphocytes. Journal of Immunology. 126:341-347.

Stewart, C.G. **1989**. *Cowdria ruminantium* stocks: how significant are they? Zimbabwe Veterinary Journal. 20:149-154.

Stich, R. W., A. C. Rice-Ficht, and W. C. Brown. **1999**. *Babesia bovis*: common protein fractions recognized by oligoclonal *B. bovis*-specific $CD4^+$ T cell lines from genetically diverse cattle. Experimental Parasitology. 91:40-51.

Street, N. E., and T. R. Mosmann. **1991**. Functional diversity of T lymphocytes due to secretion of different cytokine patterns. The Federation of American Societies for Experimental Biology Journal. 5:171-177.

Sulsona, C.R., S.M. Mahan, and A.F. Barbet. **1999**. The *map1* gene of *Cowdria ruminantium* is a member of a multigene family containing both conserved and variable genes. Biochemical and Biophysical Research Communications. 257:300-305.

Takahashi, H., T. Takeshita, B. Morein, S. Putney, R.N. Germain, and J.A. Berzofsky. **1990**. Induction of CD8+ cytotoxic T cells by immunization with purified HIV-1 envelope protein in ISCOMs. Nature. 344:873-875.

Tetzlaff, C.L., A.C. Rice-Ficht, V.M. Woods, and W.C. Brown. **1992**. Induction of proliferative responses of T cells from *Babesia bovis*-immune cattle with a recombinant 77-Kilodalton merozoite protein (Bb-1). Infection and Immunity. 60:644-652.

Timms, P., and N.P. Stewart. **1984**. Immune responses of cattle following vaccination with living and non-living *Babesia bovis* antigens. Veterinary Parasitology. 16:243-251.

Totté, P., D. Blankaert, P. Zilimwabagabo, and J. Wérenne. **1993**. Inhibition of *Cowdria ruminantium* yield by interferons alpha and gamma in endothelial cells. Revue d'Élevevage et de Médecine vétérinaire des Pays tropicaux. 46:189-194.

Totté, P., F. Jongejan, A. L. de Gee, and J. Werenne. **1994a**. Production of interferon alpha in *Cowdria ruminantium* infected cattle and its effect on infected endothelial cell cultures. Infection and Immunity. 62:2600-2604.

Totté, P., N. Vachiery, H. Heremans, A. Billiau, and J. Wérenne. **1994b**. Protection against *Cowdria ruminantium* infection in mice with gamma interferon produced in animal cells. In: Kobayashi, T., Y. Kitagawa, and K. Okumura (eds). Animal Cell Technology: basic and applied aspects. Kluwer Academic Publication. 6:595-599.

Totté, P., N. Vachiery, D. Martinez, I. Trap, K.T. Ballingall, N.D. MacHugh, A. Bensaid, and J. Wérenne. **1996**. Recombinant bovine interferon gamma inhibits the growth of *Cowdria ruminantium* but fails to induce MHC class II following infection of endothelial cells. Veterinary Immunology and Immunopathology. 53:61-67.

Totté, P., D. McKeever, D. Martinez, and A. Bensaid. **1997**. Analysis of T-cell responses in cattle immunized against heartwater by vaccination with killed elementary bodies of *Cowdria ruminantium*. Infection and Immunity. 65:236-241.

Totté, P., D. McKeever, F. Jongejan, A. Barbet, S. Mahan, D. Mwangi, and A. Bensaid. **1998**. Analysis of cellular responses to native and recombinant proteins of *Cowdria*. Annals of the New York Academy of Sciences. 849:155-160.

Totté, P., A. Bensaid, S. M. Mahan, D. Martinez, and D. J. McKeever. **1999a**. Immune responses to *Cowdria ruminantium* infections. Parasitology Today. 15:286-290.

Totté P., J. Nyanjui, A. Bensaid, and D. McKeever. **1999b**. Bovine CD4+ T-cell lines reactive with soluble and membrane antigens of *Cowdria ruminantium*. Veterinary Immunology and Immunopathology. 70: 269-276.

Tournay, C., P. J. Courtoy, L. Marodi, P. Totté, J. Werene, A. Jacquet, L. Garcia-Quintana, A. Bollen, and N. Moguilevsky. **1996**. Uptake of recombinant myeloperoxidase, free or fused Fcγ by macrophages enhances killing activity toward micro-organisms. DNA and Cell Biology. 15:617-624.

Townsend, A., and H. Bodmer. **1989**. Antigen recognition by class I restricted T lymphocytes. Annual Review of Immunology. 7:601-624.

Turco, J., and H.H. Winkler. **1983**. Inhibition of the growth of *Rickettsia prowazekii* in cultured fibroblasts by lymphokines. Journal of Experimental Medicine. 157:974-986.

Uilenberg, G. **1983**. Heartwater (*Cowdria ruminantium* infection): current status. Advances in Veterinary Science and Comparative Medicine. 27:427-480.

Ulmer, J.B., J.J. Donnelly, S.E. Parker, G.H. Rhodes, P.L. Felgner, V.J. Dwarki, S.H. Gromkowski, R.R. Deck, C.M. De Witt, A. Friedman, L.A Hawe, K.R. Leander, D. Martinez, H.C. Perry, J.W. Shiver, D.L. Montgomery, and M.A. Liu. **1993**. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science. 259:1745-1749.

Unanue, E.R. **1984**. Antigen presenting function of the macrophage. Annual Review of Immunology. 2:395-428.

Vachiéry, N. I. Trap, D. Martinez, and A. Bensaid. **1998**. Inhibition of MHC class I and class II cell surface expression on bovine endothelial cells upon infection with *Cowdria ruminantium*. Veterinary Immunology and Immunopathology. 61:37-48.

Van Amstel, S.R., and P.T. Oberem. **1987**. The treatment of heartwater. Onderstepoort Journal of Veterinary Research. 54:475-479.

Van de Pypekamp, H.E., and L. Prozesky. **1987**. Heartwater: An overview of the clinical signs, susceptibility and differential diagnosis of the disease in domestic ruminants. Onderstepoort Journal of Veterinary Research. 54:263-266.

Van der Merwe, L. **1987**. The infection and treatment method of vaccination against heartwater. Onderstepoort Journal of Veterinary Research. 54:489-491.

Van Kleef, M., A.W.H. Neitz, and D.T. De Waal. **1993**. Isolation and characterization of antigenic proteins of *Cowdria ruminantium*. Revue d'Élevevage et de Médecine vétérinaire des Pays tropicaux. 46:157-164.

Van Kleef, M., N.J. Gunter, H. Macmillan, B.A. Allsopp, V. Shkap, and W.C. Brown. **2000**. Identification of *Cowdria ruminantium* antigens that stimulate proliferation of lymphocytes from cattle immunized by infection and treatment or with inactivated organisms. Infection and Immunity. 68:603-614.

Van Regenmortel, M.H.V. **2000**. Molecular design versus empirical discovery in peptide-based vaccines. Coming to terms with fuzzy recognition sites and illdefined structure-function relationships in immunology. Vaccine. 18:216-221.

Van Vliet, A.H.M., F. Jongejan, and B.A.M. van der Zeijst. **1992**. Phylogenetic position of *Cowdria ruminantium* based on analysis of amplified 16S rDNA sequences. International Journal of Systematic Bacteriology. 42:494-498.

Van Vliet, A.H.M., F. Jongejan, M. van Kleef, and B.A.M. van der Zeijst. **1994**. Molecular cloning, sequence analysis, and expression of the gene encoding the immunodominant 32-kilodalton protein of *Cowdria ruminantium*. Infection and Immunity. 62:1451-1456.

Van Vliet, A.H.M., B.A.M. van der Zeijst, E. Cammus, S.M. Mahan, D. Martinez, and F. Jongejan. **1995**. Use of a specific immunogenic region on the *Cowdria ruminantium* MAP1 protein in a serological assay. Journal of Clinical Microbiology. 33:2405-2410.

Van Wyngaardt, W, and D.H. du Plessis. **1998**. Selection of an scFv phage antibody that recognises bluetongue virus from a large synthetic library and its use in ELISAs to detect viral antigen and antibodies. Onderstepoort Journal of Veterinary Research. 65:125-131.

Vermeulen, N.M.J., A.W.H. Neitz, and G.J. Viljoen. **1987**. Purification of *Cowdria ruminantium* by lectin cellular affinity chromatography. Onderstepoort Journal of Veterinary Research. 54:233-246.

Waghela, S.D., F.R. Rurangirwa, S.M. Mahan, C.E. Yunker, T.B. Crawford, A.F. Barbet, M.J. Burridge, and T.C. McGuire. **1991**. A cloned DNA probe identifies *Cowdria ruminantium* in *Amblyomma variegatum* ticks. Journal of Clinical Microbiology. 29:2571-2577.

Walker, J.B., and A. Olwage. **1987**. The tick vectors of *Cowdria ruminantium* (Ixodoidae, Ixodidae, genus *Amblyomma*) and their distribution. Onderstepoort Journal of Veterinary Research. 54:353-379.

Warren, R. L., D. Lu, D. R. Sizemore, L. S. Baron, and D. J. Kopecko. **1990**. Method for identifying microbial antigens that stimulate specific lymphocyte responses: application to *Salmonella*. Proceedings of the National Academy of Sciences of the United States of America. 87:9823-9827.

Watson, D.L., I.G. Colditz, and H.S. Gill. **1994**. Immunological effector mechanisms in ruminants. In: Wood, P.R., P. Willadsen, J.E. Vercoe, R.M. Hoskinson, and D. Demeyer (Eds.). p21-36. Vaccines in Agriculture: Immunological applications to animal health and production.

Weenink, S.M., and A.M. Gautam. **1997**. Antigen presentation by MHC class II molecules. Immunology and Cell Biology. 75:69-81.

Weaver, C.T., and Unanue, E.R. **1990**. The costimulatory function of antigen-presenting cells. Immunology Today. 11:49-55.

Williams, J.C., and M.H. Vodkin. **1987**. Metabolism and genetics of Chlamydias and Rickettsias. Onderstepoort Journal of Veterinary Research. 54:221-221.

Winkler, H.H. **1990**. Rickettsia species (as organisms). Annual Review of Microbiology. 44:131-153.

Winter, G., A.D. Griffiths, R.E. Hawkins, and H.R. Hoogenboom. **1994**. Making antibodies by phage display technology. Annual Review of Immunology. 12:433-455.

Wisseman C.L. Jr, and A. Waddell. **1983**. Interferon-like factors from antigen- and mitogen-stimulated human leukocytes with antirickettsial and cytolytic actions on *Rickettsia prowazekii*: infected human endothelial cells, fibroblasts, and macrophages. Journal of Experimental Medicine. 157:1780-1793.

Wolff, J.A., R.W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, and P.L. Felgner. **1990**. Direct gene transfer into mouse muscle *in vivo*. Science. 247:1465-1468.

Wright, I.G., R. Casu, M.A. Commins, B.P. Dalrymple, K.R. Gale, B.V. Goodger, P.W. Riddles, D.J. Waltisbuhl, I. Abetz, D.A. Berrie, Y. Bowles, C. Dimmock, T. Hayes, H. Kalnins, G. Leatch, R. McCrae, P.E. Montague, I.T. Nisbet, F. Parrodi, J.M. Peters, P.C. Scheiwe, W. Smith, K. Rode-Bramanis, and M.A. White. **1992**. The development of a recombinant *Babesia* vaccine. Veterinary Parasitology. 44:3-13.

Xiang, Z.Q., S. Spitalnik, M. Tran, W.H. Wunner, J. Cheng, and H.C. Ertl. **1994**. Vaccination with a plasmid vector carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus. Virology. 199:132-140.

Yates, J.R. **2000**. Mass spectrometry from genomics to proteomics. TIG. 16:5-8.

Zhu, Y., S.C. Oliveira, and G.A. Splitter. **1993**. Isolation of *Brucella abortus ssb* and *uvrA* genes from a genomic library by use of lymphocytes as probes. Infection and Immunity. 61:5339-5344.

Zinkernagel, R.M., and P.C. Doherty. **1997**. The discovery of MHC restriction. Immunology Today. 18:14-17.

Zweygarth, E., S.W. Vogel, A.I. Josemans, and E. Horn. **1997**. *In vitro* isolation and cultivation of *Cowdria ruminantium* under serum-free culture conditions. Research in Veterinary Science. 63:161-164.