STUDIES ON CYTOKINES AND NITRIC OXIDE IN RELATION TO *COWDRIA RUMINANTIUM* INFECTION

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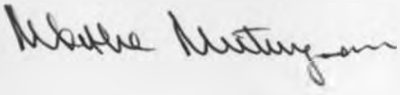
The study presented in this thesis is entirely the product of my own work except where specifically stated in the text and in the acknowledgements.



Mbithe Mutunga

To the memory of my father, Mzee Paul Mulinge, and to my mother, Mama Alice Mulinge, for starting me this way;
And to Danson, for his undivided support during some difficult times, and to Linda and Tito, a constant joy and reminder of how precious life is.

SUMMARY

This thesis describes studies on the pathogenesis of heartwater by investigating the effects of cytokines and nitric oxide (NO) in experimental *Cowdria ruminantium* infections in *in vitro* and *in vivo* models. The latter were carried out in sheep and mice.

In sheep infections, there was increased levels of antioxidation enzymes, glutathione peroxidase and superoxide dismutase with the highest levels coinciding with the period of the clinical disease. *C. ruminantium* was also found to induce increased levels of nitrite, indicative of NO, in plasma where the nitrite levels were found to have an earlier and higher increase in sheep with prior exposure to *C. ruminantium* antigens as compared to naive sheep infections. Mean interferon gamma (IFN–γ) levels in plasma were found to be increased after infection, just prior to or at time of the febrile reaction, with higher levels being recorded in the sheep with prior exposure to *C. ruminantium* antigens than in the naive animals. There was a gradual increase in mean interleukin 8 (IL-8) and tumor necrosis factor alpha (TNF-α) levels after infection and the highest levels coincided with the febrile reaction. Higher levels of IL-8 were recorded in the primary infections than in the sheep with prior exposure to *C. ruminantium* antigens.

To determine the source of the NO detected in *in vivo* studies, *in vitro* studies of *C. ruminantium* infections of bovine pulmonary endothelial cells (BPEC) were conducted and NO was found to be induced by *C. ruminantium* infections. The NO production was found to be enhanced by bovine recombinant IFN–γ and was inhibited by nitric oxide inducible synthase inhibitor, LNMMA. Induction of apoptotic cell death was seen in IFN- γ treated cells with the infected cells showing higher apoptotic cell

death than the uninfected cells. Treatment of elementary bodies with NO was found to reduce both infectivity and viability of *C. ruminantium*. This indicates that NO is released during endothelial infection and has an effect upon infectivity and viability of the organism.

The importance of interferons in the control of *C. ruminantium* infections was further investigated in an *in vivo* model using mice deficient in IFN- γ and IFN-a/b receptors. Infection of these mice resulted in a more severe disease characterised by a shorter incubation period, higher morbidity and mortality rates in relation to time, with more pronounced pathology characterised by increased amount of oedema fluid, infiltration of inflammatory cells and higher lung and brain infection rate as compared to control mice. Electron micrographs from infected mice 7 days post-infection indicated that organisms had developed to later developmental stages in the mice deficient in IFN- γ receptors, where as earlier developmental forms were seen in controls, with mixed stages being seen in the IFN-α/β receptor deficient mice.

Studies then looked at the time course of cytokine production in relation to *C. ruminantium* infection in mice. Infection of mice with *C. ruminantium* induced transient productions of IFN–γ and the biologically active 70 kDa heterodimeric form of IL-12, (IL-12p70) and a corresponding initial reduction in IL-4 and IL-10 levels. IL-12p70 levels were highest on days 2-4 while IFN–γ levels were highest on day 6, just prior to disease onset followed by a dramatic reduction which also coincided with the clinical onset on day 8. Levels of IL-4 and IL-10 were reduced immediately after *C. ruminantium* infection with the lowest levels coinciding with the highest IFN–γ levels on day 6 post-infection. This study showed induction of a transient Th 1 immune

response which was not sustained, possibly resulting in the fatal outcome of infection of mice.

In contrast, inoculations of mice with murine recombinant interleukin 12 (MrIL-12) were found to have immunomodulatory effects on *C. ruminantium* infections in mice and to protect a significant number of mice. The highest protection was afforded by mice inoculated with MrIL-12 from beginning of infection with booster doses every 2 days; 20% of these did not show any clinical signs and were immune to challenge infection with an overall recovery rate of 45% as compared to the control. There was reduction of mortality and morbidity rates in mice inoculated with MrIL-12 before or at time of infection but giving IL-12 after disease onset was found not to offer any protection to the animals against *C. ruminantium* infection. The increased resistance or reduced severity of infection coincided with sustained increase in IFN- γ, nitrite levels and in IgG2a immunoglobulins and a reduction in IL-4 and IL-10.

This study showed that the course taken by *C. ruminantium* infection in both *in vivo* and *in vitro* models is dependent on the cytokine and antibody milieu in plasma and possibly tissues prior to or during infection. Involvement of NO in *C. ruminantium* infections was suggested in this study. Infection in mice was characterised by a transient high IFN-γ response at about day 6 which was not sustained. It is likely that this response controls *C. ruminantium* to some extent because mice deficient in IFN-γ receptors died earlier in the disease with higher infection levels as compared to the control. Sustained release of IFN-γ occurred in mice treated with MrIL-12 and provided protection in a significant proportion. The

failure to sustain IFN-γ in mice may be related to a lack of sustainable IL-12 production.

The importance of a Th 1 type immune response was, therefore, underlined and the possible roles for IL-12 and IFN- γ with involvement of nitric oxide in the killing of the infectious agent suggested. The study showed for the first time the possible roles of nitric oxide in the killing immune response and pathology of the disease and the importance of availability of IL-12 early in infection in the development of protective immune response in mice.

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LIST OF ABBREVIATIONS

µl = microlitres

ml = millilitres

µM = micromolar

g = gram

µg = microgram

ng = nanogram

pg = picogram

C = degrees celsius

DNA = deoxyribonucleic acid

RNA = riboxynucleic acid

BSA = bovine serum albumin

BPEC = bovine pulmonary endothelial cells

OVC = ovine vascular emdotheila cells

EBs = elementary bodies

s.c. = subcutaneous

HEPES = N-[2-hydroxyethyl] piperazine-N-[2-ethanesulfonic acid]

PBS = phosphate buffered saline

SNAP = S-Nitroso - N - acetyl - DL- penicillamine

L-NMMA = N-monomethyl -L arginine

NO = nitric oxide

NO_2^- = nitrite

SD = standard deviation

h = hour

min = minute

X = times

CO_2 = carbon dioxide

IFAT = indirect immunofluorescent antibody test

Fig. = figure

IFN-γ = interferon gamma

BorIFN-γ = bovine recombinant interferon gamma

IFN-α = interferon alpha

IFN-β = interferon beta

TNF = tumor necrosis factor

OvrTNF-α = ovine recombinant tumour necrosis factor alpha

IL-1 =interleukin 1

IL-2 = interleukin 2

IL-4 = interleukin 4

IL-6 = interleukin 6

IL-8 = interleukin 8

IL-10 = interleukin 10

IL-12 = interleukin 12

MurIL-12 = murine recombinant interleukin 12

ELISA = enzyme linked immunosorbent assay

IgG = immunoglobulin

kDa = kilodalton

MHC = major histocompatibility complex

PCR = polymerase chain reaction

OD = optical density

No. = number

WHO = World Health Organisation

BCG = Bacille Calmette Guerin

FITC = fluorescein isothiocynate

EM = electron microscopy

LPS = lipopolysacharide

GPX = glutathione peroxidase

SOD = superoxide dismutase

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INTRODUCTION

Heartwater is an infectious, often fatal, non-contagious, tick-borne disease of wild and domestic ruminants. The disease is caused by a rickettsial organism, *Cowdria ruminantium* (Cowdry, 1925) and transmitted by ticks of the genus *Amblyomma* (Walker and Olwage, 1987). The distribution of the disease follows that of the vector tick (Camus and Barre, 1982). Heartwater has been diagnosed in Sub-Saharan Africa (Provost and Bezuidenhout, 1987) and the surrounding islands (Uilenberg, 1983). Although heartwater is considered to be primarily an African disease, it has also been diagnosed in the West Indies and it seems possible that the disease may spread to the American continent (Camus and Barre, 1987).

Until the sixties, most work on heartwater was done in South Africa, but this has changed in recent years with the increase in awareness of the importance of the disease, due to the increase in importation of exotic breeds of livestock (Camus *et al.*, 1996). The disease has been described as second in importance only to East Coast Fever and trypanosomiasis in the African continent (Uilenberg, 1983), hence, it is especially of significant economic importance in areas where improved breeds and managemental practices are being tried; it is a major hindrance to the development of improved livestock production systems (Ilemobade, 1976; Uilenberg, 1981).

Both domestic and wild ruminants are susceptible to heartwater but only the former manifest clinical disease in field cases while the latter act more as reservoirs and are of epidemiological importance (Camus *et al.*, 1996). The disease occurs in different forms ranging, from peracute to innapparent forms, and is characterised by a febrile reaction followed by disorders in the nervous, respiratory and/or

gastrointestinal systems (Alexander, 1931; Neitz, 1968). Nervous symptoms are the most distinct signs of the disease although not always present (Neitz, 1968).

The pathogenesis of the disease remains unclear and many hypotheses have been put forward concerning this; it has been suggested to be centred in factors which cause increased capillary permeability, and allow seepage of plasma proteins resulting in transudation through the serous membranes, and cardiac insufficiency (Clark, 1962; Owen *et al.*, 1973; Duplessis, 1975a; 1975b; Prozesky and Du Plessis, 1984), but the underlying causes are not known. Although vascular lesions were suggested to be due to the presence of *C. ruminantium* in the vascular wall (Pienaar *et al.*, 1966), later studies showed that there was no direct link between the presence of the infectious agent and cytopathic changes (Pienaar, 1970; Du Plessis, 1985b). Other studies have suggested that the increase in permeability may be due to mediators produced as a result of the disease. Some ascribed the vascular defect to a toxin (Pienaar *et al.*, 1966; Pienaar, 1970; Ilemobade, 1976) but such a toxin was not identified; others suggested inconclusively that this was due to release of vasoactive substances by mast cells (Du Plessis, 1975a;1975 b).

Cell-mediated immune responses have been reported to be important in protection against *C. ruminantium* infections (Du Plessis, 1982; Du Plessis *et al.*, 1991; 1992; Mahan *et al.*, 1994; 1996; Totte *et al.*, 1993; 1994). *C. ruminantium* infects and multiplies primarily in endothelial cells (Cowdry, 1926) and also phagocytic cells (Logan *et al.*, 1987). *C. ruminantium* parasites are, therefore, in obligatory association with the intracellular compartments of the host cells and the implication of this cell-parasite association may be important in understanding the

host-parasite interactions and, hence, the pathogenesis and immune responses of the disease as a whole. This was the basis of the hypothesis from which these studies were developed: Infection of endothelial cells (and other phagocytic cells) by *Cowdria ruminantium* may result in induction of a number of responses leading to changes in the micro-environment of the endothelium and, hence, a disturbance in the homeostatic function of the cardiovascular system as a whole. This may result in a complexity of activities bringing into play body defence mechanisms involving induction of mediators such as cytokines, reactive nitrogen intermediates and oxygen intermediates, as part of the body defence mechanisms as well as contributors to pathology. The extent to which clinical signs and pathology are a consequence of the infectious agent and to what extent they are as a result of the immune responses are not well known. Host defence mechanisms which fight against uptake and establishment of *C. ruminantium* organisms in the intracellular ecological niches will control infection and increase survival, hence the importance of the right extracellular and intracellular ecological environment at the right time.

The aim of this study was, therefore, to investigate the pathogenesis and immune responses induced by the host-parasite interactions in *C. ruminantium* infections, with special emphasis on cytokines and nitric oxide in relation to pathology and immunity to the disease. This involved:

1. Investigation of the disease kinetics induced in sheep of different susceptibility to *C. ruminantium*, by examining the profiles of selected cytokines (interferon gamma (IFN–γ), tumor necrosis factor alpha (TNF–α) and interleukin 8 (IL-8)), nitric oxide

(NO) and enzymes involved in antioxidation processes (superoxide dismutase (SOD) and glutathione peroxidase (GPX)).

2. Examination of the effects of bovine recombinant interferon gamma (BorIFN–γ) and NO on *C. ruminantium* infection of bovine pulmonary endothelial cells *in vitro*.

3. Investigation of the role played by Type II (IFN–γ) and Type I (IFN-α/β) interferons in the protective and pathological mechanisms of *C. ruminantium* infection *in vivo* using mice deficient in IFN–γ and IFN-α/β receptors.

4. Investigation of Th 1 cell type and Th 2 cell type immune responses including cytokines and NO profiles in mice infected with *C. ruminantium*.

5. Assessment of the immune and pathological responses elicited by inoculations of murine recombinant IL-12 on *C. ruminantium* infection of mice.

CHAPTER 1

LITERATURE REVIEW

1.1. *Cowdria ruminantium*

1.1.1. History and geographical distribution

The first record of what was probably heartwater was according to Neitz (1968) made in South Africa by the voortrekker pioneer Louis Trichardt in 1838. He had noticed a fatal disease "nintas" amongst his sheep which he suspected was caused by massive tick infestations. He described these observations in his dairy on the 17th, of February 1838. About 50 years later a farmer, John Webb, reported to the Cattle and Sheep Disease Commission in Grahamstone (South Africa) on a disease similar to the one described by Trichardt, which had by this time come to be referred to as heartwater. He was of the opinion that the disease was introduced into the Eastern Cape at about the time that William Bowker found a bont tick on a cow which had been imported from Zululand in about 1837. Subsequently, according to Henning (1956), the disease was reported from various parts of South Africa (Provost and Bezuidenhout, 1987).

Heartwater has, since then, been described in different areas under different local names; Kaboa meaning trembles (Fulani), Enguruti (Masai), Daji (Hausa), Khader, Magak (Sudanese), Heartwater, blacklung or Dronkgalsiekte (Afrikaans) (Camus *et al.*, 1996). The disease is also widely distributed in Eastern Africa. Prior to 1925, only the signs of the disease were recognised and it was not until 1930 that Daubney (1930) diagnosed the disease first in Kenya by demonstrating the presence of *C. ruminantium* organisms in brain and kidney sections of infected sheep. He also stated that *Amblyomma habraem*, the vector of heartwater in South Africa was not present in Kenya and showed that the disease was transmitted by the

so called "tropical bont tick", *A. variegatum*, the most common member of the genus in Kenya.

The disease was originally found only in the African continent but has been diagnosed in the Caribbean and there is danger of the disease being introduced in the American continent (Camus and Barre, 1982). There is however no answer as to whether the disease is indigenous to the African continent and the fact that the *Amblyomma* species of ticks occur in the Asian and American continent further confuses the issue (Camus *et al.*, 1996). A list of countries were the disease has been diagnosed including the year and author was compiled by Provost and Bezuidenhout (1987) based on summary of articles, according to Camus and Barre (1982).

1.1.2. Aetiological agent

1.1.2.1. Classification

Heartwater is caused by a rickettsial organism, *Cowdria ruminantium*, measuring about 0.2-0.5u (Cowdry, 1925) which is found in the cytoplasm of vascular endothelial cells of the mammalian host. In the Bergy's Manual of Systemic Bacteriology, *C. ruminantium* is classified as being in the family Rickettsiaceae, tribe of Erhlichiae (Ristic and Huxsoll, 1984). *Cowdria* and many of the *Erhlichia* were originally named rickettsia but were renamed later in honour of the German bacteriologist Paul Ehrlich and the American bacteriologist E. V. Cowdry (Moshkovski, 1945; 1947). A taxonomic position near to that of *Erhlichia* was found to be logical after serological cross reactions were shown to exist between *Cowdria* and *Ehrlichia* and DNA sequencing found the two genera to be closely related (Van

Vliet *et al.*, 1992). The close relationship between *Cowdria* and *Erhlichia* was further supported by the finding that *Cowdria* also multiplies within granulocytes, the same host cells for several *Ehrlichia* species (Logan *et al.*, 1987). A close relationship has also been reported between *Cowdria* and *Chlamydia* (Pienaar, 1970; Uilenberg, 1983; Scott ,1987).

1.1.2.2. Morphology and staining characteristics

C. ruminantium has been described in sections stained in Giemsa stain as a coccal, gram-negative organism staining lilac to purple blue (Cowdry, 1925; 1926). Cowdry described the morphology of the organism in infected endothelial cells of experimentally induced cases of heartwater and also in intestinal epithelial cells of the tick *Amblyomma habreum*. His description of the morphology was based on its appearance in paraffin-embedded sections and smears made from the alimentary tract of the invertebrate host. The organism was described as uniform, coccus-shaped bodies of 0.2-0.5u in diameter which occur in dense masses varying from a few individual cells to several hundred in the cytoplasm of infected cells (Cowdry, 1925; 1926). These findings were further confirmed by other researchers (Bezuidenhout, 1984; Kocan *et al.*, 1987). Several researchers have described the pleiomorphic forms of *Cowdria ruminantium* (Jackson and Neitz, 1932; Sahu, Dardiri and Wool, 1983; Stewart and Howell, 1981). Donatien and Lestoguard (1937) observed initial bodies which were described as homogenous masses in the cytoplasm, stained purple with Giemsa stain and measured between 1-2μ and up to 5μ. Jackson (1931) and Jackson and Neitz (1932) described the variations of the clumps or colonies in size and shape; they described the different forms as small (coccoid), medium and large forms. The

large forms were described as having irregular shapes varying from coccoid, ring to horse shoe shapes. There are many other stains which have been used including Loefflers methylene blue, methyl-green pyronine, fuchsin (Prozesky, 1987a), Mallory (Yunker *et al.* 1987), acridine orange and Diff-quik^{R} (Camus and Barre 1988).

The morphology of the organism in the vertebrate host is similar to that in the vector (Cowdry, 1926). Apart from the coccoid forms described by Cowdry (1925 and 1926) and initial bodies described by Donatien and Lestoquard (1937), bulky dark blue forms have been described measuring 2-4μ (Camus *et al* 1996). Electron microscopy enabled the precise morphology to be determined and showed that the colonies are situated inside membrane-lined vacuoles in the cytoplasm of the endothelial cells and that each organism is surrounded by a two unit membrane (Pienaar, 1968; 1970; Du Plessis, 1975a; Stewart and Howell, 1981) .

1.1.2.3. *In vitro* cultivation

Until 1985, *in vitro* cultivation of *C. ruminantium* was a long-story of frustrations due to lack of suitable culture system. In 1985 Bezuidenhout *et al.* managed to propagate *C. ruminantium* in irradiated endothelial cell. It was also demonstrated that cultures can be initiated without the need to retard cell growth (Bezuidenhout, 1987; Byrom and Yunker, 1990). Since 1985, several stocks of *C. ruminantium* (Ball 3, Welgevoden, Senegal, Gardel, Crystal springs, Highway, Palmriver, Mbizi, Lemco, Kiswani) have been successfully used to infect bovine or ovine endothelial cells (Camus *et al.*, 1996). *C. ruminantium* has also been grown in neutrophils *in vitro* (Logan *et al.*, 1987a).

1.1.2.4. Hosts and transmission

Heartwater is a tick-borne disease of both wild and domestic ruminants, although only domestic ruminants (sheep, cattle and goats) suffer the clinical disease, wild ruminants act more as reservoirs (Camus *et al.*, 1996). Heartwater is transmitted by the three host ticks of the genus *Amblyomma* (Bezuidenhout, 1987). *Amblyomma variegatum* has been described as the most important vector and is wide spread in savannah areas of tropical Africa and surrounding islands (Daubney 1930; Walker and Olwage 1987) while *Amblyomma hebraeum* is the most important vector in southern Africa (Lounsbury, 1900, cited by Camus *et al.*, 1996). Other species which have been reported to transmit the disease include *Amblyomma gemma* (Lewis, 1949), *Amblyomma pomposum* (Neitz, 1947), *Amblyomma lepidum* (Karrar, 1966), *Amblyomma thollon* (Mackenzie and Norval 1980) and *Amblyomma sparsum* (Norval and Mackenzie 1981). Transmission is transtadial, although transovarial transmission has also been implicated (Bezuidenhout and Jacobsz, 1986).

1.1.3. The Disease

1.1.3.1. Clinical manifestations

The incubation period and the course of the disease are influenced by the species of animal affected, the virulence of the isolate, the amount of the infectious agent administered, the route of infection, the age and the immune status of the animal (Alexander 1931; Neitz, 1968; Uilenberg 1983). Goats are the most susceptible experimental animals (Camus *et al.*, 1996). In natural infections the incubation period is a day or two longer than in experimental infections (Donatien and Lestoquard,

1937) but is variable. The disease is manifested in different forms in ruminants, ranging from peracute, acute, subacute to mild forms (Camus *et al.*, 1996). In the peracute form, sudden death of animals without any clinical signs is common (Aklahu, 1980). The acute form is the most common form of the disease, with a course of disease ranging from 2-6 days (Camus *et al.*, 1996). It is characterised by a rapid temperature increase of up to 41°C, with other clinical signs occurring 1-2 days after the onset of fever; other clinical signs include respiratory distress and nervous signs, but the latter may not always be present (Alexander, 1931; Clark, 1962; Owen *et al.*, 1973). The subacute form of the disease is similar to the acute form but the clinical signs are less prominent (Camus *et al.*, 1996). The mild form of the disease, also called heartwater fever, has a transient fever followed by natural recovery and this form is considered to be of epidemiological value due to its carrier status (Camus *et al.*, 1996). The mild and subacute forms of the disease have been said to be more frequent among indigenous cattle of all ages, partially immune animals and young calves of all breeds (Uilenberg, 1981).

1.1.3.2. Pathogenesis

Steck (1928), Alexander (1931), Henning (1956), Clark (1962) and Prozesky and Duplessis (1985c) are some of the workers who have described increased capillary permeability leading to lung oedema as the most prominent lesion and as the cause of death. However, the underlying cause of this lung oedema and hydrothorax remains unknown. Gross brain lesions are rare and, even when present, not pathognomonic. Brain oedema, hyperemia and haemorrhages are some of the findings

which have been reported in cattle, sheep and goats (Pienaar *et al.* 1966); the relationship between these and the pathogenesis of the nervous lesions observed in heartwater cases is not clear. Swelling of kidneys, splenomegaly, oedematous lymph nodes and haemorrhages in various surfaces have also been reported (Prozesky and Du Plessis, 1985b).

1.1.3.3. Pathology

The gross lesions are similar in all species and have been well described by Steck (1928), Currason and Delpy (1928), Alexander (1931), Henning, (1956) and Pienaar *et al.*, (1966), Uilenberg, (1971) and Prozesky, (1987c). The main lesions are those associated with increased vascular permeability. These include hydrothorax, lung oedema, hydroperitoneum, haemorrhages on the pericardium and intestines and congested lung. Enlarged spleen was described as common by some authors (Alexander 1931) and as rare by others (Uilenberg, 1971a); these differences were postulated to be strain linked (Camus *et al.*, 1996). Oedema and congestion in the brain have also been described (Pienaar *et al.*, 1966). Lesions in mice have been said to be similar to those described in the ruminants described above and are mainly characterised by effusions in body cavities and splenomegaly (Prozesky and Du Plessis, 1985b).

Microscopic lesions were described by Cowdry (1926), Steck (1928) and Prozesky and Du Plessis, (1985b). The lesions described in the brain of infected animals include necrotic foci and microcavitation in the cerebella cortex, oedema and accumulation of cells in the perivascular spaces and presence of the infectious agent in

the cytoplasm of endothelial cells, although this was found to be rare in mice as compared to ruminants (Prozesky and Du Plessis, 1985b). Cytopathic changes in lung alveolar endothelial cells were described as mild with swelling of mitochondria, dilatation of the endoplasmic reticulum and presence of the infectious agent in the lung endothelial cells (Prozesky and Du Plessis, 1985b).

1.1.3.4. Clinical pathology

Disappearance of eosinophils before onset of the febrile reaction is documented (Clark, 1962; Owen *et al.*, 1973; Ilemobade, 1976; Van Amstel *et al.*, 1988; Mutunga, 1992). Darkening of the plasma in infected animals was reported by Clark, (1962) and was attributed to bilirubin (Van Amstel *et al.*, 1987). Some authors reported the occurrence of leukopaenia (Ilemobade, 1976; Abdel Rahim and Shomein, 1978; Van Amstel *et al.*, 1987) while leukocytosis was reported by Van Amstel *et al.*, (1994). Anaemia has also been reported to occur after onset of fever (Van Amstel *et al.*, 1988; 1994). An increase in glucose and blood urea nitrogen were reported (Mutunga, 1992) and Ilemobade (1976) found increased levels of pyruvate and lactate levels in sera of *C. ruminantium* infected animals.

1.1.4 Diagnosis

Heartwater is often misdiagnosed even by the most experienced veterinarians, because gross lesions are not pathognomonic to the disease. A combination of proper history taking, clinical observations, macroscopic and microscopic pathological findings and serological tests have been used. Currently DNA probes have been developed and are being tried for detection of *C. ruminantium* (Waghela *et al.*, 1991; Yunker *et al.*, 1993.

Detection of *C. ruminantium* in capillary endothelial cell smears has been for a long time the only method of confirming the disease (Purchase , 1945). Treatment of heartwater infected animals has been reported to alter the amount of infectious material (Prozesky, 1987b). Some animals treated of the disease, although not fully recovered, were reported to have fewer macroscopic changes and no organisms were detectable in the brain (Prozesky, 1987a). Identification of *C. ruminantium* in the brain smears 48-60 hours after an animal has been treated is often difficult. The small forms of *C. ruminantium* organisms are more severely affected than the larger forms (Prozesky, 1987a). Confirmation of *C. ruminantium* infection in mice differs to some extent from those in ruminants infected with *C. ruminantium*, as they are not easily found in brain endothelial cells (Prozesky and Du Plessis 1985a). Hyrothorax, hydropericardium, oedema of the lungs and splenomegaly are found in the majority of mice inoculated with *C. ruminantium* isolates infective for mice (Du Plessis, 1975a; Prozesky and Du Plessis, 1985a). The highest concentration of organisms in mice has been seen in the lungs (Prozesky and Du Plessis, 1985b). It is not clear why organisms are detected in very low concentrations in the brains of mice as compared to the brains of ruminants (Prozesky, 1987b; Du Plessis, 1985a). Morphologically there are no differences seen between different isolates (Prozesky, 1987a; Du Plessis, 1985a).

1.1.5 Control of heartwater: treatment and immunisation

Sulphonamides and antibiotics have been used successfully for the treatment of heartwater. Tetracyclines constitute the drug of choice in the treatment of heartwater, with the long acting formulations of oxytetracyclines being preferred (Camus *et al.*, 1996). Prophylactic methods include quarantine, effective tick control

and immunisation methods. Immunisation using infective material (blood or ground-up tick homogenates) followed by treatment after fever onset has been used for a long time (Bezuidenhout, 1989). This is currently the method of immunisation adopted in Southern Africa, using a moderately pathogenic strain of *C. ruminantium* (the Ball 3 isolate) but protection is not fully achieved (Du Plessis *et al.*, 1989). This immunisation principle of infection and treatment has been applied using other stocks of *C. ruminantium*, and, in later trials, infectious agent grown in tissue culture and from the results, it has been concluded that some stocks are difficult to control using others due to lack of cross-protection (Du Plessis *et al.*, 1989; Jongejan *et al.*, 1988). Use of attenuated vaccines has also been attempted; vaccinations using the Senegalese stock, attenuated by passaging in bovine endothelial cells in culture, was reported, but this stock is not cross-protective with other isolates due to differences in its antigenic profile (Jongejan *et al.*, 1993c). Lately, more success has been reported in vaccinations using inactivated *C. ruminantium* organisms; Martinez *et al.*, 1994, reported some success in goats vaccinated with inactivated *C. ruminantium* elementary bodies (EBs) of the Gardel stock. Use of proteins which are common to most isolates, like the 32 kDa, (MAP1) protein, has been attempted (Van Kleef *et al.*, 1993), but this method of immunisation has the antigenic diversity of some of *C. ruminantium* isolates as its major drawback (Camus *et al.*, 1996).

1.1.6. Immune response to *C. ruminantium* infection

Indigenous breeds may acquire a strong innate resistance to *Cowdria* through natural selection (Spreull, 1922; Alexander, 1931; Du Plessis *et al.*, 1983). Variation in the susceptibility to *C. ruminantium* in cattle, sheep and goats has also been

recorded (Du Plessis and Malan, 1987a; Uilenberg, 1971; Spreull, 1922). Differences in the resistance of cattle to *C. ruminantium* infection have been documented. There is general agreement that local breeds developed in endemic areas are more resistant than exotic breeds and it was suggested that they acquire this strong innate resistance through long natural selection (Matheron *et al.*, 1987). However, there have been conflicting reports on the resistance of different breeds to *C. ruminantium* infection (Du Plessis and Malan , 1987a; 1987b), with some authors reporting that *Bos indicus* are more resistant than *Bos taurus* breeds (Van der Merwe, 1979), while others reported that the innate resistance found in African breeds had nothing to do with the breed influence (Uilenberg, 1983).

Some breeds of sheep and goats, like the Persian breed, exhibit strong natural resistance to *C. ruminantium* (Lounsbury, 1904 cited by Camus *et al.*,1996) while the Angora goat is considered the most susceptible domestic ruminant to heartwater followed by the Boer goat, the Merino and the Merino crossbreeds (Spreull, 1922). Younger animals have also been shown to be more resistant to *C. ruminantium* than older animals, and this resistance was suggested to be independent of the immune status of the dam (Neitz and Alexander, 1941). Lambs younger than 8 days and kids up to 6 weeks of age have been found to be resistant to heartwater with calves being resistant for longer periods (3 weeks to 6 months) (Neitz and Alexander, 1941). It was suggested that 78 % of flocks continually exposed to heartwater were immune to heartwater disease (Neitz and Alexander, 1941).

Animals which recover from *C. ruminantium* infection have been shown to be resistant to the disease (Alexander, 1931; Neitz, 1939). Vaccination has been

attempted over the years using materials ranging from bile (Dixon, 1898) to the current vaccination attempts using recombinant antigens (Van Kleef *et al.*, 1993). Theiler (1906) produced a polyvalent serum by combining serum, from infected cattle and sheep, and reported that 20 ml of the vaccine protected goats and sheep against challenge with 5 ml of infected blood injected subcutaneous but not against intravenous challenge. Antibodies were shown not to be protective in *C. ruminantium* infections (Alexander, 1931; Du Plessis, 1970; 1982), but immune serum was reported to inhibit infectivity of the infectious agent in the presence of complement and mouse peritoneal macrophages (Du Plessis *et al.*, 1991). Byrom *et al.*, (1993) showed that hyperimune serum from DBA/2 and Balb'c mice as well as bovine sera had a strong neutralising effect on the infection of endothelial cells *in vitro* but that there was no protection of these mice against intravenous challenge of *C. ruminantium*, and that addition of complement had no beneficial effect.

Cell-mediated immunity has been documented as important in protection against *C. ruminantium* infection (Du Plessis, 1982; Du Plessis *et al.*, 1991; 1992; Mahan *et al.*, 1994; 1996; Totte *et al.*, 1993; 1994). Du Plessis (1982) was able to show that transfer of immune spleen cells protected naive mice against challenge with the Kumm isolate of *C. ruminantium* given 30 and 60 days later. This immunity was reported to be largely mediated by $CD8^+$ T lymphocytes, as immune spleen cells depleted of Lyt-2+ T cells ($CD8^+$ cells) were unable to confer resistance to recipient mice challenged with *C. ruminantium*, whereas the depletion of $L_3T_4^+$ cells ($CD4^+$ cells) had no effect on this protection (Du Plessis *et al.*, 1991).

Infection of bovine brain endothelial cells was shown to induce expression of IL-1B, IL-6 and IL-8 mRNAs and that this effect was potentiated by IFN–γ (Bourdoulous *et al.*, 1995).

1.2. Mechanisms of immunity and pathological damage

Cell mediated immunity is documented as important in the control of *C. ruminantium* infections (Du Plessis, 1982; Du Plessis *et al.*, 1991; 1992a; Mahan *et al.*, 1994; 1996; Totte *et al.*, 1993; 1994). Cytokines, including interferons, tumor necrosis factor and interleukins, and other related mediators (secondary mediators) such as reactive oxygen and nitrogen radicals play important roles in immunological, pathological and physiological processes in humans and animals. This section reviewes some of the literature relevant to this study as concerns immune responses to intracellular infections including cell mediated immune responses, cytokines and secondary mediators in pathological and immunological mechanisms. These included: macrophages and T cells in immune responses; interferons, tumor necrosis factor, interleukins including interleukin 12, interleukin 8, interleukin 4 and interleukin 10; reactive nitrogen intermediates with special emphasis on nitric oxide, and reactive oxygen intermediates and antioxidants.

1.2.1. Immune responses to intracellular infections

1.2.1.1. Cellular responses: macrophages and T cells

Macrophages play a key role in the control of diseases as they are involved in innate resistance phenomena, initiation, and effector mechanisms of specific host response (Bienhoff *et al.*, 1992). Much research has gone into this cellular immunity

mechanism with the early studies being done in tuberculosis where it has been found that the immune response in this disease is cell mediated without humoral antibody responses (Green *et al.*, 1993). Macrophages play a central role as antigen-presenting cells and their activation depends on T cell-derived products such as IFN–γ for killing of intracellular pathogens (Bancroft *et al.*, 1987). IFN–γ has been described as one of the primary T cell products which has macrophage activating properties (Bancroft *et al.*, 1987; Nathan and Sporn, 1991). IFN–γ increases MHC Class I and Class II expression and stimulates the cells to become more active in killing parasites (Pontzer and Russel, 1989; Rhodes *et al.*, 1986).

Although the major macrophage activating cytokine is IFN–γ (Nathan *et al*, 1983) other cytokines have also been found to activate macrophages e.g. TNF-α, IL-4 (Myers and Murtaugh, 1995). However, TNF-α does not seem to play a clear role in antiviral immunity in animals (Myers and Murtaugh, 1995). In parasitic infections IFN–γ produced by either $CD4^+$ T cells (DeMaeyer and DeMaeyer, 1988) and $CD8^+$ cytotoxic T cells (Frasca *et al.*, 1985) appear to play the major role in macrophage activation.

Some products secreted by macrophages have been implicated in microbicidal activity, including cytokines e.g. TNF, reactive oxygen and nitrogen metabolites and enzymes (Richards *et al.*, 1995). Some of these mediators are released by resting unstimulated cells while others like nitric oxide and superoxide anion are released by activated cells upon stimulation of the macrophages and related cells (Elsasser *et al.*, 1995). These molecules may act as cytotoxic effector molecules depending on the susceptibility of the organism in question (Lancaster, 1992).

The most common diseases encountered in domestic animals and, indeed, mammals as a whole, are respiratory diseases, in particular those caused by infectious agents and to a lesser extent allergic and chemically induced lung diseases (Bielefeldt-Ohmann, 1995). The respiratory system is a target of micro-organisms, dust, gases. It has been suggested recently that the respiratory system in particular the lung may play a major role in immune responses and regulation (Bielefeldt-Ohmann, 1995), due to the lung being situated at the interface between the external and internal environments and containing all the cellular constituents of a lymphoid tissue (Holt *et.al.*, 1990). The cascade of events begins with induction of interferon by epithelial cells (IFN-β)and by resident macrophages (IFN-α) (Bielefeldt-Ohmann *et al.*, 1991 and 1995). This is followed by production of IL-6 and IL-8 initially by resident macrophages (Hennet *et al.*, 1992 and Vacheron *et al.*, 1991) and then by bronchial epithelial cells (Becker *et al.*, 1991), pulmonary type II epithelial cells (Standford *et al.*, 1990), fibroblasts (Van Damme *et al.*, 1989), endothelial cells and by macrophages (Vacheron *et al.*, 1991). It has been suggested that TNF-α and IL-1 are the main stimuli to the macrophages for production of IL-6 and IL-8 as well as the pathogens themselves (Van Damme *et al.*, 1989). Although TNF-α may have a cytotoxic effect on EC, the indications are that the main mechanisms for microvascular injury and increased permeability are indirectly mediated via PMN-dependent effects (Bielefeldt-Ohmann, 1995).

The interaction of resting T cells with antigen and cytokines results in their activation, proliferation and differentiation (Hamblin, 1993). The clonal expansion of T cells is dependent on the presence of antigen presenting cells (APCs) which, for

CD4+ T cells, are mononuclear phagocytes or dendiritic cells expressing Class II MHC and for CD8+ T cells are any cells expressing Class I MHC (Hamblin, 1993). The types of cytokine released by different subpopulations of CD4+ T cells are thought to influence different facets of the immune response (Pace *et al.*, 1983; Tripp and Unanue, 1996). According to Mosmann and Coffman (1989), there are two subpopulations of $CD4^+$ T cells which secrete different cytokines; Th 1 cells secrete IFN–γ and IL-2 while Th 2 cells secrete IL-4, IL-6 IL-5 and IL-10. The activities of the cytokines synthesised suggest that the two types of T cells have different functions; Th 1 cells secrete cytokines associated with cellular immunity, where as Th 2 cells secrete cytokines associated with B cell activation, proliferation and differentiation and the two types of cells seem to regulate each other (Hamblin, 1993; Trinchieri, 1993). IFN–γ secreted by Th 1 cells inhibits cytokine production by Th 2 cells and IL-10 secreted by Th 2 cells inhibits cytokine production by Th 1 cells. In high doses TNF-α leads to hemodynamic, pulmonary, metabolic and pathological consequences indistiquishable from endotoxemia and septic shock. It further induces vascular permeability leading to oedema and injury to endothelial cells and appears to play a key role in hypotension and altered metabolism. TNF-α has been incriminated in lung injury in sheep (Redl *et al.*, al; 1990), cattle (Kenison *et al*; 1987) and goats (Van Miert *et al*; 1992). The clonal expansion of CD4+ and CD8+ T cells is critically dependent on the cytokine milieu present, e.g., IL-12, IL-2 (Trinchieri, 1993).

The following are reviews of activities of the various cytokines relevant to the experiments carried out in the studies described in this thesis.

1.2.1.2. Interferons in health and disease

The interferons are divided into two families: Type I (a/b) and Type II (g) interferons and they have also been classified according to the producer cells as leukocyte (a), fibroblast (b) or immune (g) interferons (Pestka, 1983; 1986) but this classification has now changed to type I and II interferons.

1.2.1.2.1. Type I Interferons (IFN-α and IFN-β)

Type I (a /b) were grouped together due to extensive sequence homologies in their amino acid sequences and the observation that they bind to a common cell-surface receptor (Mariano *et al.*, 1992). Type I interferons act on target cells exerting a variety of effects including anti-viral, antiproliferative and immunomodulatory activities. All Type I interferons compete for binding to a single cell-surface receptor complex which initiates the signalling mechanisms for the cellular effects observed. The cellular response to the binding of interferon to the cell induces activation of specific DNA-binding proteins that complex with the promoters of interferon stimulated genes (ISG) at the interferon stimulated response element (ISRE) resulting in the induction of several gene products 2-5A synthatase, MHC class I surface antigens, P 68 kinase and the Mx family of proteins as well as several other proteins whose function is not yet known (Seng and Lengyel, 1992).

IFN-α/β receptors are generally found on all cells poorly responsive to interferon. Cells showing no binding at all of one of the family are relatively rare or deliberate constructs. The IFN-α R chain is implicated in the binding and activities of all IFN- α/β species (Uze *et al.*, 1992).

Following the binding of IFN to its receptor the immediate early response is the activation of transcription of a set of genes containing a conserved response element called ISRE (IFN stimulated response element) in their promoter (Kerr and Stark, 1991). ISRE binds the ISGF -3 transcription factor which consists of four proteins (Schindler *et al.*, 1992).

1.2.1.2.2. Type II Interferons (IFN–γ)

IFN–γ is a glycoprotein of monomer molecule weight 20-25 KDa which is secreted from T cells and natural killer (NK) cells. IFN–γ was originally identified by its *in vitro* anti-viral activity nearly 30 years ago (Wheelock, 1965). IFN–γ exhibits many different biological activities *in vitro*, including anti-viral, anti-proliferative, macrophage activation and induction of MHC class I and II molecules; plays a key role in host defence and in inflammatory processes and exhibits a potential in the treatment of infectious diseases and neoplasia. IFN–γ induces expression of many key antigens including Class I and Class II MHC antigens (Basham and Merrigan, 1983; King and Jones 1983), nitric oxide synthase and other cytokines such as IL-1(Farrar and Schreiber, 1993; Corbett *et al*; 1991). The activity of IFN–γ is synergistic with other cytokines; IFN–γ enhances the cytotoxic activity of TNF-α and other antiviral activities of IFN-α or IFN-β. IFN–γ activates macrophages to become tumouricidal and kill intracellular parasites (Pace *et al* 1983) and enhances B cell maturation and immunoglobulin secretion (Vilcek *et al.*, al; 1985, O'Garra *et al.*, 1988). IFN is responsible for inducing non-specific cell mediated mechanisms of host defence through its ability to activate macrophages (Bancroft *et al.*, 1987; Nathan and Sporn, 1991).

The IFN–γ receptor consists of 2 integral membrane polypeptides that are members of the type II cytokine receptor family; an alpha receptor and a beta subunit that participates in signal transduction only. Human and murine IFN–γ receptors bind their respective ligands with high affinity in a strictly species specific manner.

IFN–γ binds to IFN–γ receptor alpha chain expressed at cell surfaces to form a complex which then associates with IFN–γ receptor beta chain. In brief, this process activates enzymatic reactions and interactions with specific sequences resulting in the development of IFN–γ inducible biologic responses (Shuai *et al.*, 1992).

1.2.1.3. Tumour Necrosis Factor

Tumor necrosis factor is a pleiotropic cytokine capable of altering physiological and immunological sequel as well as mediating the patho-physiological responses of several disease conditions (Myers and Murtaugh, 1995). The central role of the endothelium in homeostasis, especially in the control of vascular permeability, involves complex interactions with the blood components (Myers and Murtaugh, 1995) and studies of the interactions between the endothelium and cytokines such as TNF have contributed significantly to the current appreciation of endothelial participation in homeostasis (Matthews *et al.*, 1987;). TNF-α has modulatory effects on permeability properties of the endothelium. TNF binds to high affinity endothelial cell surface receptors (Matthews *et al.*, 1987;).

Protection mediated by TNF includes its antimicrobial and tumoricidal effect. Most cells have TNF receptors and respond to TNF and lymphotoxin. Cells that are resistant to TNF are different from sensitive cells in that they appear to express proteins that protect them from TNF cytotoxicity (Goeddel *et al.*, 1986; Wallach,

1984). TNF has been shown to increase the formation of superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) (Radeke *et al.*, 1990). Anaerobic conditions, the antioxidant butylated hydroxyl toluene (BHT) and other agents, that diminish the formation of oxygen free radicals such as glucocorticoids and dimethly sulfoxide (DMSO), all block TNF cytotoxicity *in vitro* (Radeke *et al.*, 1990)). It has been suggested that TNF kills cells by generating intracellular oxygen radicals (Matthews *et al.*, 1987; Wong and Goeddle, 1988a; Hepburn *et al.*, 1987). *In vivo* TNF protects animals from some diseases and other insults, such as hyperoxia, that appear to be associated with the production of reactive oxygen and nitrogen radicals (White *et al.*, 1989).

TNF has been found to induce production of SOD (the MnSOD but not the CuZnSOD) with the TNF resistant cells producing more SOD than TNF susceptible cells (Wong and Goeddle, 1988b). MnSOD requires very low levels of TNF (0.1 ng/ml) (Wong and Goeddle, 1988b). Glucorcoticoids and glucose have been shown to inhibit MnSOD induction in some cells (Warner and Wisp, 1990; Cornelius *et al.*, 1987).

The liver is important in regulating metabolism and nutrient use. The liver is both a source and target for TNF (Lancaster, 1992). The functions of hepatocytes change dramatically during the disease state possibly due to the localised production of TNF and other cytokines(Lancaster, 1992). Induction of acute-phase response proteins leads to changes in protein metabolism. Lancaster, 1992, suggested that disturbances of liver function during disease are related to Kupfer cell production of cytokines that misdirect hepatocytes to generate increased amounts of NO and self destruction.

1.2.1.4. Interleukin 12

Interleukin 12 (IL-12) is a heterodymeric glycoprotein composed of unrelated subunits of 35 KDa (p35) and 40 kDa (p40) (Kobayashi *et al.*, 1989; Chan *et al.*, 1991; Trinchieri, 1993) and co-expression of both chains is necessary for biological activity of IL-12. IL-12 is produced by monocyte-macrophages, B cells and possibly other accessory cells in response to bacterial products and parasites (Trinchieri, 1993). IL-12 has been identified to be responsible for bridging innate resistance, involving phagocytes and NK cells, and adaptive immunity, involving Th cells, cytotoxic T cells and B cells (Trinchieri, 1993). Activities of IL-12 include induction of IFN–γ production by resting and activated T and NK cells (Kobayashi *et al.*, 1989; Chan *et al.*, 1991) and enhancement of T cell proliferation in combination with other cytokines, such as IL-2 (Kobayashi *et al.*, 1989; Gately *et al.*, 1992; Perusia *et al.*, 1992). In addition, IL-12 has been demonstrated to induce cell mediated immunity through enhancement of NK and T cell cyotolytic activity (Kobayashi *et al.*, 1989; Gately *et al.*, 1992). Cells induced to proliferate by IL-12 include CD4+ and CD8+ T cells (Gately *et al.*, 1992).

1.2.1.5. Interleukin 8

Interleukin 8 (IL-8) belongs to the chemokine superfamily group of cytokines which exhibit between 20 and 45 % similarity in their amino acid sequences and act on various inflammatory cell types (Taub and Oppenheim, 1993; Oppenheim *et al.*, 1991). IL-8 has been reported to induce directional migration of various cell types, including neutrophils and T lymphocytes (Oppenheim *et al.*, 1991). The cytokine plays a key role in the accumulation of leukocytes at site of inflammation, and, in

addition to being a potent chemoattractant, IL-8 also has a wide range of pro-inflammatory effects (Nicola, 1994). A number of human respiratory diseases exhibit increased IL-8 expression at both the protein and RNA levels in lung histological sections and interstitial fluid (Nicola, 1994). In some disease states, increased levels in IL-8 in body fluids correspond to increased neutrophils (Koch *et al.*, 1992). IL-8 are implicated as major participants in acute as well as more prolonged inflammatory responses (Taub and Oppenheim, 1993).

In vitro biological effects of IL-8 include activation of neutrophils including chemotaxis, shape change, degranulation, respiratory burst, increased cytosolic $Ca2^{+}$, increased adhesion to endothelial cells, fibrinogen, and extracellular matrix proteins, increase expression of CD11a, CD11b, CD11c and CD18 and release of lysosomal enzyme (Nicola 1994; Oppenheim *et al*; 1991; Taub and Oppenheim 1993; Schall, 1991; Matsushima *et al.*, 1992). *In vivo* biological effects of IL-8 on target cells include: neutrophil infiltration in human, primates, mouse, rat, rabbit and dog, neutrophilia, angiogenesis, synovial inflammation and plasma leakage (Oppenheim *et al*; 1991; Taub and Oppenheim, 1993).

1.2.1.6. Interleukin 4

Interleukin 4 is a 18-20 kDa glycoprotein produced primarily by activated T lymphocytes (Nicola, 1994). To date, five natural sources of IL-4 have been identified: some subpopulations of $CD4^{+}$ peripheral T cells (Mosmann and Coffman, 1989); a minor subpopulation of T lymphocytes in spleen and thymus (Zlotinik *et al.*,

1992); a subpopulation of $CD3^+$ $CD4^+$ $CD8^-$ thymocytes that briefly continue to secrete IL-4 after they emigrate to the spleen (Bendelac and Schwartz, 1992).

The many immunoregulatory functions of IL-4 include induction of immunoglobulin isotype switching to IgE expression, and induction of the subpopulation of helper T cells, designated as Th 2 cell, which regulate humoral immunity, eosinophilia, and inflammatory macrophage deactivation (Nicola, 1994). It has been demonstrated that administration of anti-IL-4 in some disease conditions diverts the ensuing response away from a Th 2 cell immune response toward a Th 1 cell immune response (Sadick *et al.*, 1990)

1.2.1.7. Interleukin 10

Interleukin 10 (IL-10) is a secreted 17-21 kDa protein produced by some subclasses of T-lymphocytes, primarily Th 2 type cells, B cells and macrophages after activation by antigen or bacterial products (Nicola, 1994). IL-10 acts on macrophages to down regulate class II MHC molecules on the surface of monocytes and macrophages (de Waal Malefyt *et al.*, 1991a). IL-10 inhibits production of reactive oxygen and nitrogen intermediates including nitric oxide, and cytokine synthesis by activated Th 1 cells and natural killer (NK) cells suggesting that it has anti-inflammatory activities (Moore *et al.*, 1993,; Fiorentino *et al.*, 1991). Production of IL-10 is inhibited by IFN–γ. IL-10 can result in a switch in the immune response from a Th 1 type (cell-mediated) to a Th 2 type (antibody-type response) immune responses (Nicola, 1994).

1.2.2. Reactive nitrogen and oxygen intermediates and antioxidants

1.2.2.1. The endothelium and reactive nitrogen intermediates (Nitric Oxide)

In 1980 Furchgtt and Zawadzki demonstrated the phenomenon of endothelium- dependent relaxation (vasodilatation) in vascular tissue and its mediation by a humoral factor which later became known as endothelium-dependent relaxation factor (EDRF). Bioassay studies revealed the ephemeral nature of this mediator which is protected from breakdown by a superoxide dismutase (SOD) indicating that it is inactivated by superoxide anions (O^{-}_{2}) (Furggott, 1984;1988). EDRF was found to be a potent activator of guanylate cyclase, the enzyme catalysing cyclic guanosine-3'5'-monophosphate (GMP) synthesis. EDRF was also found to inhibit platelet aggregation and adhesion. This property together with its vasodilator effect were shown to be mediated by activation of the soluble guanylate cyclase, leading to increases in cyclic guanosine monophosphate (cGMP) within the platelet or smooth muscle (Furchgott, 1984). It was later suggested that EDRF may be nitric oxide (NO) or closely related (Furchgott, 1988). This was confirmed experimentally by comparing the effects of EDRF and NO on vascular smooth muscle and platelets (Palmer *et al.*, 1987).

Organic nitrates have been used for the treatment of hypertension and they are known to activate the soluble guanylate cyclase. These nitrates have also been shown to generate NO *in vitro* by a non-enzymatic reaction and the activation of guanylate cyclase by these compounds was found to be inhibited by haemoglobin which binds to NO (Moncada *et al.*, 1988).

Evidence has shown that activated macrophages form nitrite (NO^-_2) and nitrate (NO^-_3) from L-arginine and suggested that L-arginine is a potential precursor of NO (Hobbs and Ignarro, 1997). Despite extensive research, the precise mechanism by which nitric oxide synthase (NOS) catalyzes the oxidation of L-arginine to NO remain unclear (Hobbs and Ignarro, 1997). The initial reaction may involve N-hydroxylation of guanidium nitrogen to form N-hydroxyl -L-arginine to NO. However subsequent steps in the conversion of N-hydroxyl -L-arginine to NO and L-citrulline remain unclear (Hobbs and Ignarro, 1997).

NO is now regarded as a unique messenger molecule with diverse biological activities including regulation of vascular smooth muscle tone and platelet activity, non-adrenergic and non-cholinergic neurotransmission, cytotoxic and cytostatic activities to cells and as an immune effector molecule in eliminating certain pathogens (Hobbs and Ignarro, 1997). NO has been said not to have a distinct receptor on target cells; however, the target site for NO was suggested to be iron, bound within certain proteins as heme group or iron-sulfur complex (Hobbs and Ignarro 1997). The mechanisms by which NO kills cells or halts replication is not clear but probably involves inhibition of DNA synthesis by inactivation of ribonucleotide reductase and by direct deaminantion of DNA (Kwon et. a., 1991; Wink *et al.* 1991). Also due to its affinity for iron-sulfur-containing proteins, NO may bind and inhibit cis-aconitase and ubiquinone reductase preventing cells from generating ATP (Staimler *et al.*, 1993). The endothelium has the ability to constantly generate a powerful vasodilator that controls vessel wall diameter and consequently plays a decisive role as a determinant of blood flow and blood pressure (Hobbs and Ignarro, 1997). Importantly the host

cells producing NO possess some inherent immunity against its toxic effects and thereby are not destroyed themselves but the reason for their insusceptibility is unknown (Hobbs and Ignarro, 1997).

Nitric oxide and prostacyclin are both cytoprotective, vasodilators and inhibitors of platelet aggregation and adhesion but it is not known if they act synergestically or not (Rees *et al.*, 1989). Facts still not known about NO are the biological significance and mechanism of this mediator and also whether NO in the vessel wall modulates white cell activation or controls smooth muscle proliferation. NO has been said to possibly participate in nitrosative reactions leading to deamination of DNA and mutagenesis (Wink *et al.*, 1991). Reactions of NO with superoxide leads to formation of the highly toxic oxidant peroxynitrite (Beckman *et al.* 1990). Reaction of NO with iron-sulfur centres in proteins may lead to the inhibition of their activity (Hibbs, 1991).

NO exposure has been incriminated as being able to cause severe lung injury (WHO, 1977; Gaston *et al.*, 1994a). Thiols play an important role in disarming NO. Levels of glutathione rise in lung tissue in response to oxidant stress, such as exposure to high levels of NO contained in cigarette smoke and endogenous RS- NO (RS-NO= S-nitrosothiol or thionitrite) levels increase in the lung in response to immune activation and inflammation (Gaston *et al.* 1994a).

In the central nervous system, RS-NO has been found to also exert protection against NO-mediated toxicity. NO reacts with superoxide to produce peroxynitrite (Lipton *et al.* 1993). In contrast, formation of RS-NO provides a mechanism for limiting the reaction of NO with oxygen radical. Reduced oxygen species are highly

toxic to endothelial cells and are believed to play an important role in the vascular related morbidity seen in patients with hypercystenaemia (Staimler *et al.*, 1993).

Under normal physiological circumstances EDRF (NO) interacts with homocysteine to form S- nitroso-homocysteine (a potent vasodilator and antiplatelet agent) (Staimler *et al.*, 1993). S- nitroso proteins are present in plasma of normal subjects (Staimler *et al.*, 1993) and in the exudate of airway lining fluid in some disease states (Gaston *et al.*, 1994). NO group transfer reactions to plasma membrane proteins containing reactive sulfhydryls has been demonstrated (Lipton *et al.* 1993).

NO has been implicated as having a major role in host defence mechanisms against viruses, bacteria and other intracellular parasites (Green *et al.* 1990). Many investigators readily accept the notion that NO is toxic given its reputation as a reactive radical with a short biological half-life. NO is known to react with molecular oxygen to give nitrogen dioxide which is a strong oxidant and a potent toxin in any biological system, but significant nitrogen dioxide occurs only at NO concentrations far in excess of those seen *in vivo* (Green *et al.* 1990).

1.2.2.2. Reactive oxygen intermediates and antioxidants

Aerobic metabolism and the process of generating ATP from glucose and oxygen by cytochrome oxidase leads to reduction of oxygen (O_2) to water (H_2O) (and occasionally to the superoxide radical ($O_2^{\cdot-}$)) (Grisham *et al.*, 1992a; 1992b). Further reduction of $O_2^{\cdot-}$ by one or two additional electrons yields hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH) respectively. Catalase, superoxide dismutase(SOD) and glutathione peroxidase (GPX) complete the reduction of H_2O_2 to H_2O. These enzymes, SOD, GPX and catalase, process the $O_2^{\cdot-}$ and H_2O_2 and prevent them from

interacting to produce OH and singlet oxygen ($.O_2$) through an iron-dependent reaction (Grisham *et al.*, 1992a).

Enhanced generation of superoxide (O_2-.) or reduced levels of SOD have been implicated in a wide range of pathological conditions (Wond and Goeddle, 1988b). Molecular oxygen is readily reduced by one electron to give superoxide anion (Pauling, 1979). Superoxide radical has been referred by biochemists as a highly reactive and destructive radical (Deng *et al*, 1993). At physiological pH it has little potential to oxidise other molecules. Thus, superoxide acts as an oxidant only in a more acidic environment or when the molecule under attack is a good one-electron donor such as reduced Fe(II)-sulfur centres (Deng *et al*, 1993). Superoxide dismutase (SOD) protects against oxidant stress and ischemic injury in many model systems by an unclear mechanism (Flaherty and Weisfeldt, 1988).

1.3. Concluding remarks

Research on heartwater has advanced knowledge in some aspects of the disease such as the aetiology (morphology and classification) and some aspects of epidemiology including transmission (Camus *et al.*, 1996). However, the knowledge of the pathogenesis and immune mechanisms of the disease remain unclear, with contradictory findings being reported and inconclusive implications of involvement of complement and vaso-active substances (Du Plessis and Malan, 1987a). The disease is of major economic importance in Africa and is a major constraint against improvement of livestock industry, with an estimated loss of millions of dollars incurred every year due to the disease, as summarised by Camus *et al.*, 1996. Currently there are no effective methods of controlling the disease, other than the cumbersome and expensive tick-control methods. Development of an effective vaccine to the disease, would, therefore, be of great importance. This can only be achieved with sufficient knowledge of the pathological and immunological mechanisms elicited by the infection (host-parasite interactions). Armed with these facts, this study was designed to examine the responses elicited by *C. ruminantium* infection of endothelial and phagocytic cells, and how these responses influence the final outcome of the disease. The role played by cytokines, nitric oxide and reactive oxygen intermediates in the pathological and immunological mechanisms of *C. ruminantium* infections was investigated in both *in vitro* and *in vivo* models.

CHAPTER 2

MATERIALS AND METHODS

2.1. *In vitro* cultivation of *Cowdria ruminantium* in endothelial cells

2.1.1. *Cowdria ruminantium* isolates

Three isolates of *C. ruminantium* (Cowdry, 1925), Gardel isolated in Guadeloupe (Uilenberg, 1983), Welgevonden isolated in South Africa (Du Plessis, 1982) and Kathiani isolated in Kenya (Ngumi *et al.*, 1997) were used in this study. The Welgevonden and Gardel isolates were propagated in tissue culture in bovine endothelial cells as described in section 2.2.3. and were used in studies carried out in Edinburgh. The Kathiani (Kenyan) isolate of *C. ruminantium* used was a blood stabilate (blood stabilate No. 327B of 25-10-1994) and was stored at -196°C in liquid nitrogen. This was a generous gift from Kenya Agricultural Research Institute (KARI), Veterinary department, Muguga, and was used in studies in Kenya. The stabilate was transported in ice, snap thawed and used to infect sheep within 30 minutes after thawing.

2.1.2. Bovine Endothelial Cell Cultures

Bovine pulmonary endothelial cells (BPEC), bovine aorta endothelial cells (BAEC) and bovine brain endothelial cells (BBEC) were used in these studies. The BBEC were a gift from Dr Martinez, Guadeloupe (Martinez, 1993) while the BPECs and BACs were isolated and cultured from vascular tissues obtained from abattoir-slaughtered cattle using a modification of the method described by Byrom *et al* (1990). In brief, short segments of the pulmonary artery were immersed in phosphate buffered saline containing antibiotics (PBS/A). Collagenase solution at 1 mg/ml in PBS/A was used to detach the cells which were then cultured in Glasgow minimal

(Sigma Chemicals Ltd., UK Chemicals) and incubated at 37°C in 5% CO_2. The medium was changed every 3 days until the cells were confluent and the cells were propagated and stored as already described for bovine endothelial cells. The cells were identified as endothelial cells based on their morphology.

2.1.4. Infection of endothelial cells

The endothelial cell cultures were ready for infection when the monolayer was almost confluent (90%). The medium was discarded and the cells infected with *C. ruminantium* and fresh medium for infected cells added. This medium for infected cells was comprised of the following (all reagents were obtained from Gibco Laboratories, UK); 100ml Glasgow Minimum Essential Medium (GMEM / BHK-21), 10% Tryptose Phosphate Broth, 10% heat inactivated foetal bovine serum (FCS), 0.02 mM L-glutamine per ml, 0.2 mM HEPES buffer per ml (Gibco Laboratories), 100 IU penicillin G and 100 µg streptomycin per 100 ml of medium. The medium from these infected cultures was replaced after 7 days. Level of infection with *C. ruminantium* was scored in cytospin smears ranging from low (denoted as 1+) to moderate (++2) to high level of infection (3+++). When the cells were heavily infected, *C. ruminantium* elementary bodies (EBs) were passaged to fresh cell cultures or the cells were harvested and stored in culture medium containing 10% dimethyl sulfoxide (DMSO) (Sigma Chemicals Ltd., UK) in liquid nitrogen (-196°C).

2.1.5. Assay for viable *C. ruminantium* EBs by a fluorescein diacetate staining technique

The viability of *C. ruminantium* EBs was quantified by a fluorescein diacetate staining method. The principle behind the test is that fluorescein diacetate is taken in

essential medium (GMEM) (Gibco Laboratories, U.K.) supplemented with 20% heat inactivated foetal calf serum, 2 mM/100 ml L-glutamine, 100 IU/ 100 ml penicillin G and 100 µg/ 100 ml streptomycin. The cells were used for the different studies at passages ranging from 7-10. The cells were passaged by detaching with trypsin and versene (Gibco Laboratories) and stored in 10% DMSO under liquid nitrogen (-196°C).

2.1.3. Ovine Vascular cells

An attempt was made to isolate ovine vascular endothelial cells from peripheral blood of sheep by a method described by Albert Bensaid (Personal Communication, 1995). Peripheral blood mononuclear cells (PBMC) were isolated using the Ficoll Hypaque method (Pharmacia, Uppsala, Sweden). Ten millilitres of heparinised blood was centrifuged at 3000g for 20 minutes to separate different cell fractions of blood. The buffy coat, containing the leucocyte fraction was then removed and mixed with 9 mls PBS containing heparin at 10 U/ml. This was layered on Ficoll Hypaque (sodium metrizoate) (Pharmacia Chemicals, UK) and centrifuged at 1000g for 35 minutes. PBMC were obtained from the Ficoll-PBS interface and washed twice with 20 ml PBS containing heparin by centrifuging at 450g for 10 minutes for each wash. The cells were then cultured in RPMI 1640 medium supplemented with 8% foetal calf serum (Gibco Laboratories and Life Technologies, UK) at 10^7 cells /ml and incubated at 37°C in a moist atmosphere with 5 % CO_2 for 2 hours. Unattached cells were removed by washing the flasks twice with warm complete RPMI 1640 medium. The adherent cells were then grown in Glasgow Minimal Essential Medium (GMEM) containing 20% foetal calf serum and 300 µg/ml endothelial cell growth supplement

(Sigma Chemicals Ltd., UK Chemicals) and incubated at 37°C in 5% CO_2. The medium was changed every 3 days until the cells were confluent and the cells were propagated and stored as already described for bovine endothelial cells. The cells were identified as endothelial cells based on their morphology.

2.1.4. Infection of endothelial cells

The endothelial cell cultures were ready for infection when the monolayer was almost confluent (90%). The medium was discarded and the cells infected with *C. ruminantium* and fresh medium for infected cells added. This medium for infected cells was comprised of the following (all reagents were obtained from Gibco Laboratories, UK); 100ml Glasgow Minimum Essential Medium (GMEM / BHK-21), 10% Tryptose Phosphate Broth, 10% heat inactivated foetal bovine serum (FCS), 0.02 mM L-glutamine per ml, 0.2 mM HEPES buffer per ml (Gibco Laboratories), 100 IU penicillin G and 100 µg streptomycin per 100 ml of medium. The medium from these infected cultures was replaced after 7 days. Level of infection with *C. ruminantium* was scored in cytospin smears ranging from low (denoted as 1+) to moderate (++2) to high level of infection (3+++). When the cells were heavily infected, *C. ruminantium* elementary bodies (EBs) were passaged to fresh cell cultures or the cells were harvested and stored in culture medium containing 10% dimethyl sulfoxide (DMSO) (Sigma Chemicals Ltd., UK) in liquid nitrogen (-196°C).

2.1.5. Assay for viable *C. ruminantium* EBs by a fluorescein diacetate staining technique

The viability of *C. ruminantium* EBs was quantified by a fluorescein diacetate staining method. The principle behind the test is that fluorescein diacetate is taken in

by all cells but is hydrolysed only within live cells to produce green fluorescence (Rotman and Papermaster 1966). Fluorescein diacetate (FD) (Sigma Chemicals Ltd., UK) was prepared at 5mg /ml in acetone and stored in a tightly capped container at -20°C. Ninety µl volume of medium from the test plates was mixed with 10µl of diluted fluorescein diacetate (1:50 in PBS) and incubated for 15 minutes at room temperature in the dark. Viable EBs appeared bright green and were counted using a graded eye piece in a fluorescent microscope and the number of viable cells calculated as follows:

Viable cells/ml =(average number of viable cells in large square) $x10^4$/ml x 1/dilution

$$\% \text{ Viable cells} = \frac{\text{number of viable cells}}{\text{number of viable cells + number of dead cells}} \times 100\%$$

2.1.6. Assay for viable endothelial cells:

The endothelial cell monolayer was examined microscopically for evidence of apoptosis in experiments which were aimed at determining the effect of added interferon gamma on endothelial cell infection with *C. ruminantium*. At the end of the experiment (7 days) the medium from the cultures was removed and the cell monolayer on cover slips stained in Diff quik^R (Baxter Diagnostics, Germany) using standard manufacturer's procedure, and acridine orange (Sigma Chemicals Ltd., UK) to identify and quantify viable and non-viable endothelial cells. For the acridine orange staining, a modification of a previously described method was used (Polunovsky *et al.*, 1994). Acridine orange at 3µg / ml was used. A cytospin preparation of the scraped cells was fixed in 70% ethanol for 10 minutes before staining with acridine orange for 3 h in the dark. A drop of buffered glycerol on a coverslip was placed on the stained cells and the morphology examined using fluorescence microscopy.

Apoptotic cells were identified morphologically by condensation of chromatin, nuclear fragmentation, blebbing of the nuclear membrane and presence of membrane-bound bodies. Non viable cells were counted in five fields at a magnification of x 1000 and expressed as a percentage mean ± SD.

2.1.6.2. DNA extraction and analysis for apoptotic cell death in infected cell cultures

Infected endothelial cells were assessed for DNA fragmentation, a characteristic finding in apoptotic cells. Endothelial cells were detached from culture wells using a rubber policeman and washed twice in PBS by centrifuging at 1500g for 10 min, then resuspended in 1ml lysis buffer (25 mM EDTA, 10mM Tris pH 8, 100mM Nacl, 0.05 % SDS and 0.1 mg /ml proteinase K). The cells were then incubated at 50°C for18 h. Samples were then extracted twice with equal volume of phenol/chloroform and centrifuged at 13000 g for 5 minutes. The top layer was then drawn into a fresh tube and an equal volume of phenol/chloroform added for the second time and 0.2 µg/ml of Rnase A added and incubated at 37°C for 1 h. A further incubation at 37°C for 1 h followed addition of 0.25µg/ml of RNase T. DNA was precipitated overnight in 2 volumes of ethanol, 0.5 volumes of ammonium acetate (7.5µM) and centrifuged at 13,000 g for 10 minutes. The pellet was then resuspended in 50 µl of TE buffer (0.01 M Tris pH 8 and 1 mM EDTA) containing 10µg/ml ethidium bromide and electrophoresed at 10V/cm for 2 h in 1.5 % agarose gel. DNA was visualised under UV fluorescence and photographed with Polaroid MP-4 Land Camera (Polaroid Corporation, USA) using type 55 Polaroid film.

2.2. Nitric oxide and *in vitro* studies

2.2.1. Detection of nitric oxide by the Griess Assay.

Nitric oxide (NO) reacts rapidly with water and oxygen to produce nitrite (NO^-_2). Since this compound is both stable and quantifiable, its concentration in culture medium and plasma may be taken as a measure of nitric oxide production. Nitrite levels were measured as described by Migliorini *et al.*, (1991). NO_2^- levels were assayed in culture supernatants using the Griess assay method. One hundred µl of the test sample were mixed with an equal volume of the Griess reagent (0.1% naphthylethylene diamine 2HCL and 1% sulphanilamide in 5% phosphoric acid) and incubated at room temperature for 10 minutes. The OD value was read at 492 nM on a microplate reader. A standard curve was generated by doubling dilutions within a range of 0 and 100 µM of sodium nitrite in culture medium. All reagents, unless otherwise stated, were from Sigma Chemicals Ltd., UK. Samples were tested in triplicate and results expressed as means ± standard deviation (SD). The basal level of NO_2^- in cell-free medium was determined for each assay and deducted from that of the test samples.

2.2.2. Nitric oxide production in bovine pulmonary endothelial cells infected with *C. ruminantium* and stimulated with bovine recombinant interferon gamma (BorIFN-γ), and the NO inhibitor, L-NMMA

Thermanox glass coverslips (Nunc Inc., USA) of about 13 mm in diameter were sterilised in absolute alcohol for 10 minutes then rinsed in sterile PBS before inserting them in 24 well culture plates (Gibco Laboratories Ltd.) using sterile forceps. BPEC were seeded in the plates and incubated at 37°C in 5% CO_2. At confluence, the cells were infected with *C. ruminantium* EBs obtained from the

supernatant of an infected BPEC culture. The supernatant was centrifuged at 1500 g to spin down endothelial cells, then at 15,000 g to pellet the EBs. The EBs were then resuspended in complete medium and this was used as the inoculum to infect the cells. The plates were then incubated at 37°C for 4 hours after which all the medium was removed and fresh medium was added to the cells containing 0, 25, 50, 75 and 100U/ml bovine recombinant interferon -gamma (BorIFN–γ) provided by Ciba-Geigy Ltd, Basle, Switzerland.. The effect of the NO synthase inhibitor N-monomethyl - L arginine (L-NMMA) (Sigma Chemicals Ltd., UK) at a final concentration of 1 mM was also assessed by culturing infected endothelial cells with or without BorIFN-γ. Twelve replicates were used for each treatment. The other wells were left as uninfected control wells.

2.2.3. Nitric oxide production in ovine vascular cells; Effect of BorIFN-γ, ovine recombinant tumor necrosis alpha (OVrTNF-α) and NO inhibitor L-NMMA

Ovine vascular cells were seeded in 2x24 well plates at a concentration of $4x10^6$ cells per ml. At confluence the media on all the wells was removed after assaying for NO (day 0) and the following added in four wells per plate for each ; bovine recombinant interferon gamma (BorIFN–γ) (Ciba Geigy, Basle Switzerland) at 100 U/ml, ovine recombinant TNF-α (OvrTNF-α) (a generous gift from Gary Entrican, Moredun Research Institute) at 100U/ml, 1mM L-NMMA (Sigma Chemicals Ltd., UK), 1mM L-NMMA and 100U/ml BorIFN–γ, 1mM L-NMMA and 100 U/ml TNF-α, and complete medium (non-infected control). Nitrite levels indicative of nitric oxide production were assayed using the Griess assay described in section 2.2.1 at 0, 6, 20,

48 and 72 hours post-stimulation from all the wells and the means for each treatment and the control determined. The tests were carried out in triplicates.

2.2.4. The effect of SNAP-generated NO on *C. ruminantium* infectivity and viability in vitro

Source, establishment and maintenance of endothelial cell lines infected with the Gardel isolate of *C. ruminantium* have been described elsewhere (sections 2.1.2. and 2.1.4). S- Nitroso - acetyl - DL - penicillamine (SNAP), a nitric oxide releasing molecule were obtained from Sigma Chemicals Ltd., UK. Thermanox^{R} coverslips (Nunc. Inc., USA) of about 13 mm diameter were resterilised in 70 % alcohol and rinsed in PBS before being inserted in wells of 24 well culture plates. Endothelial cells from a confluent plate were trypsinised and subcultured in all the wells at the same concentration. At confluence NO concentration was assayed using the Griess assay before discarding the medium and infecting 16 wells with *Cowdria ruminantium* EBs from a 3+++ culture plate. Eight wells were not infected (controls). SNAP solutions at concentrations of 100 µM, 50 µM and 25 µM were prepared and the level of NO generated by each concentration determined by the Griess assay described previously (section 2.2.1.) . The effects on the viability and infectivity of *C. ruminantium* were assessed by pre-treating EBs with SNAP and also by culturing infected cells in the presence of SNAP in the growth medium.

2.2.4. 1. Assay for the effect of NO pre-treatment of *C. ruminantium*.

The effect of NO on *C. ruminantium* was assessed by infecting BPEC with EBs pre-treated with different concentrations of the NO donor molecule S - nitroso - N - acetyl - DL - penicillamine (SNAP) (Affiniti U.K.). *C. ruminantium* EBs were pre-treated for 2 hours at 37°C in 0, 25, 50 and 100 µM SNAP dissolved in culture medium. The NO level

for each concentration of SNAP was determined using the Griess assay method described above. Viability count of the treated EBs was performed prior to infection using fluorescein diacetate staining method as described above. The EBs were then spun down at 15,000 g for 20 minutes and washed in fresh medium before resuspending them in complete medium and infecting confluent cell monolayers in 24 well culture plates containing coverslips prepared as already described. The plates were incubated at 37°C in 5 % CO_2 for 4 h to allow infection of the cells after which the inoculum was removed from all the wells and replaced with fresh medium. To assess the level and rate of infection, the number of fluorescent foci (colonies) per coverslip and the percentage of infected endothelial cells were determined on specified days post-infection by indirect fluorescent antibody test (IFAT) and giemsa staining of endothelial cells on coverslips respectively. Briefly for IFAT, the coverslips were fixed in 20 % acetone for 10 minutes. An anti-*Cowdria* 32 Kda monoclonal antibody (Mab) at 1:200 in PBS was added to the cells on the coverslips and incubated in a humidified chamber at room temperature for 1 h. After 3 X 5 min washes in PBS the cells were incubated with a biotin-labelled anti-mouse antibody at a dilution of 1:250 and incubated for 1 h in room temperature in a humidified chamber followed by another wash. A further 1 h incubation followed addition of streptavidin FITC at 1: 300 dilution. The cells were viewed under fluorescence microscope and the number of fluorescent foci of infection estimated for the total area of the coverslip. Infection rate was assessed in giemsa-stained coverslips and the number of infected cells per field was expressed as a percentage of the cell count. Counts were made on coverslips per treatment and the mean recorded.

2.2.4.2. Assay for the effect of co-culturing NO in *C. ruminantium*-infected cultures.

Effect of incubating infected cultures with different concentrations of SNAP on viability and multiplication were assessed. BPEC were cultured on coverslips as already described and infected at confluence with *C. ruminantium* EBs in medium containing 0. 25, 50 and 100 μM SNAP, which was left in the cultures through out the experiment. Viability of *C. ruminantium* EBs in these cultures was determined on days 1, 2, 4 and 7 post-infection. This was done by taking aliquots from wells for each treatment, which were then centrifuged at low speed (1000 g) for 5 minutes to spin down endothelial cells and viability of EBs in the supernatant determined by the fluorescein diacetate staining method as described above. To estimate the level of infection in cultures at the end of the experiment, cytospin smears were prepared on day 7 post-infection from each of these treatments and stained in giemsa and a mean estimate of the number of organisms per field assessed for each treatment. This was taken as a reflection of the multiplication rate of the infectious agent in these cultures.

2.2.4.1. Effect of NO on *C. ruminantium* EBs pre-treated with different concentrations of SNAP in vitro

The effect of NO on infectivity of *C. ruminantium* was assessed by infecting BPEC with EBs pre-treated with different concentrations of the NO donor molecule S - nitroso - N - acetyl - DL - penicillamine (SNAP) (Affiniti U.K.). *C. ruminantium* EBs were incubated in 0, 25, 50 and 100 μM SNAP solutions for 2 hours at 37°C after determining the NO level for each concentration of SNAP using the Griess assay method described in section 2.2.1. Viability count of the EBs was performed prior to infection using fluorescein diacetate staining method as described in section 2.1.5. The EBs were then spun down at 15,000 g for 20 minutes and washed in fresh

medium before resuspending them in complete medium and infecting confluent cell monolayers on coverslips set up as described above (section 2.2.4.). The plates were incubated at 37°C in 5% CO_2 for 4 h to allow infection of the cells after which the inoculum was removed from all the wells and replaced with fresh medium. (An aliquot of 500 µl of EBs in the different SNAP concentrations were left at 37°C in 5% CO_2 during the whole course of the experiment and viability of the EBs assessed). Infectivity was assessed by an indirect fluorescence antibody test (IFAT). Briefly, the coverslips were mounted on glass slides (Chance Propper LTD., UK) using a glass glue (Lochtite Glass Bond from Lochtite Ltd., UK) and fixed in 20% acetone for 10 minutes. An anti-*Cowdria* 32 Kda monoclonal antibody (Mab) (a gift from Dr Jongejan, Utrecht) at 1:200 in PBS was added to the cells on the coverslips and incubated in a humidified chamber at room temperature for 1 h. After 3 X 5 min washes in PBS the cells were incubated with a biotin-labelled anti-mouse antibody (Sigma Chemicals Ltd., UK) at a dilution of 1:250 in PBS and incubated for 1 h at room temperature in a humidified chamber followed by another wash. A further 1 h incubation followed addition of streptavidin FITC (Sigma Chemicals Ltd., UK) at 1: 300 dilution in PBS. The cells were viewed under fluorescence microscope and the number of fluorescing cells estimated for the total area of the coverslip. Also to assess infectivity of the EBs to the endothelial cells, infection rates were determined for the different treatments. Two coverslips for each treatment were removed aseptically from wells and stained with giemsa and the mean number of infected cells per field determined and expressed as a percentage of the mean total cell count in the same fields. Counts were done in ten fields for each treatment. The two methods

(giemsa and IFAT) were compared as an assessment of infectivity of EBs after NO treatment.

2.2.4.2. Effect of NO on *C. ruminantium* infected cells cultured in growth medium containing different concentration of SNAP

C. ruminantium EBs were added in BPEC cultures maintained in medium containing 0. 25, 50 and 100 μM SNAP. Viability of *C. ruminantium* was assessed on days 0, 1, 2, 4 and 7 post-infection using the fluorescein diacetate method described previously (section 2.1.5.). This was done by taking 90ul aliquots of the supernatant overlying the cells from all the wells and the viability of EBs assessed after spinning down the endothelial cells at 1000g for 5 minutes.

Cytospin smears were also prepared and stained in giemsa and the mean number of colonies per field was determined after counting colonies from ten fields.

2.3. *Cowdria ruminantium* infections *in vivo*

2.3.1. Experimental animals

2.3.1.1. BALB/c mice

These were obtained from B & K Ltd., UK, and they were 8-10 weeks old at the beginning of experiments. The mice were kept in cages in groups of not more than 5 and allowed 4 days to acclimatise before any inoculations were given.

2.3.1.2. Interferon gamma, and interferon alpha/beta knock out mice

Three groups of specially bred strains of mice were obtained from Department of Veterinary Pathology, Faculty of Veterinary Medicine, University of Edinburgh, with prior consent for experimental work from the originator, Dr Huang, Harvard University, USA. The groups used were as follows; 15 IFN-γ receptor knock out

mice (G 129) in which deletion of the α-chain of IFN type II (γ) receptor had occurred (Huang et.al; 1993); 15 IFN-α/β receptor knock out mice (A 129) in which deletion of IFN type I (α and β) receptor signalling sub unit had occurred (Muller *et. al.,,* 1994) and 15 wild type control strain of the same genetic background (WT 129). The mice were kept in double cleaned cages in groups of not more than five and given food (pellets) and water *ad libitum*.

2.3.1.3. Sheep

Studies on *C. ruminantium* infections in sheep were carried out in the Faculty of Veterinary Studies in Kenya and also at the CTVM, Edinburgh. In studies carried out in Kenya, two breeds of sheep, the Dorper and Red Maasai, were used. Six Red Maasai sheep were obtained from a farm in Ngong, about 15 km south of Nairobi, Kenya. The farm had a history of good management practices including regular tick control practice. Six Dorpers were obtained from the Faculty of Vet. Medicine farm, University of Nairobi, Kabete, Kenya. This farm practices very strict tick-control regime and has no history of cases of heartwater. The different breeds were housed separately in stalls in the isolation unit of the department of Clinical Studies and allowed feed of hay, concentrates and water *ad libitum*.

In the studies carried out in Edinburgh, a total of 16 sheep were used. 10 Scottish black face crosses and 6 merino crosses were obtained from Blythe Bank and South Bank Farms respectively, in Peebleshire, Scotland. They were housed in groups of two and supplied with feed and water *ad libitum*. Prior to infection, all the animals were kept under quarantine and until shown not to be excreting *Salmonella*, and dosed with anthelmintics.

2.3.2. Clinical disease monitoring and sampling in mice

The incubation period was determined from daily rectal temperatures in which onset of hypothermia was taken as the start of clinical disease (Du Plessis, 1982). The mean incubation period, mean course of the disease from onset of hypothermia to death or recovery, morbidity and mortality rates for each group were determined and the differences subjected to statistical analysis for determination of any significant differences. Morbidity and severity of clinical disease was based on visual observation of symptoms, posture, gait, appetite, skin elasticity, awareness, shivering / tremors, any behavioural changes and any other abnormal finding. Blood samples were collected for sera from the tail vein and also from the heart in sacrificed mice immediately after death, on designated days defined for each experiment.

2.3.3. Pathological investigations in mice

Comparative gross, histological and ultrastructural pathological studies were carried out between the groups for both the sacrificed and non-sacrificed mice. On post-mortem examination, the main things noted were the amount of oedema fluid which was measured with one ml syringes and presence of any gross lesions in the lungs, liver, spleen and brain. Brain crush smears were prepared, fixed in 10% methanol for 10 minutes and stained in giemsa for 40 minutes and examined for infected endothelial cells under the light microscope. Twenty fields per slide were examined at a magnification of x 1000 for the presence of infected endothelial cells and the mean counts determined and data for each group of mice analysed statistically using the chi-square method.

2.3.3.1. Histopathological procedures of lung and brain sections.

Lung and brain specimens were fixed in 4 % buffered formaldehyde for histopathological studies. They were processed and stained with haematoxylin and eosin (H&E) and also in giemsa stains as per standard procedures, by the histopathology laboratory of the Veterinary Pathology Department of the University of Edinburgh. Pathological changes and infection rates were assessed from these specimens.

2.3.3.2. Transmission electron microscopy procedures of lung and brain sections.

The transmission electron microscopy work was carried out in the Pre-clinical department, Faculty of Veterinary Medicine of the University of Edinburgh. Lung and brain sections of the sacrificed mice were fixed in 3 % glutaraldehyde in 0.1 M sodium cacodylate buffer immediately after removal. The sections were post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer and then dehydrated in graded ethanol (50, 70 and 90%) for 10 minutes each, and then in 100% ethanol three times for 10 minutes each. After embedding in araldite, 60nm sections were cut on a diamond knife using a Reichert OMU4 Ultracut Ultramicrotome, and mounted on 200 mesh copper grids. The sections were then stained in uranyl acetate and lead citrate using a LKB Ultrostainer. The sections were then viewed and photographed using a Philips 400 Transmission electron microscope. The objective was to compare the pathology and infection rate between the different groups using lung and brain sections .

2.3.4. Preparation of spleen homogenates in mice

Spleens were weighed for all the groups including uninfected controls and their weights compared. Spleen tissue was collected immediately after sacrificing mice or during necropsy examination using aseptic techniques and rinsed and stored in sterile PBS at -20°C. Individual spleens were homogenised in 1% (3-(3 cholamidopropyl dimethylammoni)-1-propanesulfonate (CHAPS; Sigma Chemicals Ltd., UK) in a Dounce tissue homogenizer and 10% wt/vol. homogenates were prepared. They were then left in ice for 1 h and the insoluble debris removed by centrifugation at 2000 g for 20 minutes The clear supernatants were stored at -70°C for cytokine and nitrite assays.

2.3.5. Monitoring of infection in sheep

Rectal temperatures were taken daily and blood samples collected every other day. Heparinised and clotted blood was collected every two days until death or recovery; heparinised blood was used for analysing glutathione peroxidase (GPX) and superoxide dismutase (SOD) levels. Plasma was obtained after centrifugation of the heparinised blood at 2000g for 20 minutes and used for assaying of glucose-6-phosphate dehydrogenase (G-6-PDH), TNF-α, IL-8, IFN-γ, nitrite assays and for DNA extraction for the quantification of infection (parasitaemia) using PCR. Serum was separated from the clotted blood after centrifugation at 1000 g for 20 minutes for use in an indirect ELISA antibody assay test. The plasma and sera samples were stored at -20°C before use.

2.4. Serology

2.4.1. Preparation of ELISA antigen from *Cowdria ruminantium* EBs (EBs)

Culture supernatant from a three positive infected cells was harvested and centrifuged at 1000g for 5 mins in MSE Centaur 2 centrifuge to pellet the endothelial cells. The supernatant was then centrifuged to pellet the EBs at 15,000g for 20 minutes at 4°C and the supernatant discarded. The EBs were resuspended in 20 ml of sterile PBS and centrifuged as above. The pellet was then resuspended in 1 ml PBS and transferred to a 1.5 ml eppendorf and centrifuged in a microcentrifuge at 13,000 g for 10 minutes to pellet the cells. This was repeated a second time and the EBs stored in 500 µl PBS at -20 °C or used to prepare the antigen as follows; After pelleting the EBs, the pellet was resuspended in lysis buffer, at 1 µl of buffer for every 1 µl of culture supernatant. The lysis buffer was comprised of 0.5% Np.40 and 0.5% Sodium deoxycholate in TEN (50mM Tris pH 7.4, 150 mM Sodium chloride and 2 mM EDTA). The mixture was incubated at 37°C in a waterbath for 30 minutes and then passed through a 26G needle several times. The mixture was incubated in the water bath for a further 5 minutes and then centrifuged at 16,000 g for 30 minutes at 4°C (Biofuge 17RS). The supernate was then stored in small aliquots as ELISA antigen at -20°C.

2.4.2. Sero diagnosis of *C. ruminantium* infection by an ELISA method

An indirect ELISA method was used. A 96 well flat bottomed Immulon 1 plate (Nunc. Ltd. UK) was coated with an 100 µl ELISA antigen (EL 14) (Section 2.4.1.) at a dilution 1:1600 in 0.1 M carbonate-bicarbonate buffer (Sigma Chemicals Ltd., UK) and incubated overnight at 4°C. After washing four times with PBS containing 0.05% Tween 20 (hereafter to be referred to as PBST) 100 µl of test and control

(positive and negative) sera samples diluted 1:50 in PBST were added to the wells in duplicates and incubated for 1 h at 37°C on a horizontal shaker. After another wash, 100 µl of the conjugate, anti-sheep IgG peroxidase (Sigma Chemicals Ltd., UK, product code No. A 3415) at 1:6000 dilution in PBST, was added to all the wells and incubated at 37°C for 1 h. A final wash was followed by colour development using 100 µl of the chromogen substrate(OPD) (Sigma Chemicals Ltd., UK). One 30 mg OPD tablet was dissolved in 75 ml distilled water before adding 40 µl/100ml of 30% hydrogen peroxide. The colour development was stopped by adding 100 µl per well of 1 M sulphuric acid to all the wells and the optical density read at 492 nm.

2.4.3. Detection of IgG isotype responses in mice infected with *C. ruminantium* by an indirect ELISA protocol

A modification of the method described by Soldan *et al* (1993) was used. Blood was collected for sera from test and control mice and the sera was used in an indirect ELISA for IgG1, IgG2a, IgG2b and IgG3 isotyping. A 96 well flat-bottomed plate (Immulon 1, Dynatech Laboratories) was coated with 50 µl of a *C. ruminantium* soluble antigen (EL. 15) at a dilution of 1:1000 in 1 M carbonate bicarbonate buffer pH 9.6 (Sigma Chemicals Ltd., UK) and incubated overnight at 4 °C. The plate was then washed three times with PBST (washing buffer), as was done for all the other subsequent steps. The plate was then blocked with 100 µl of normal rabbit serum diluted 1:50 in PBST (blocking buffer) for 1 h at room temperature. Fifty microlitres of the test and control sera diluted at 1:50 in blocking buffer were then added to the wells and the plate incubated at 37°C for 1 h. Fifty microlitres of rat anti-mouse monoclonal antibodies with specificity for mouse, IgG1, IgG2a, IgG2b and IgG3,

product code numbers MCA 336, MCA 421, MCA 422, and MCA 42 (Serotech, UK) diluted 1:1000 in blocking buffer were added and incubated at 37°C for 1 h. An anti-rat IgG whole molecule peroxidase conjugate (Sigma Chemicals Ltd., UK, product code NO. A9037) at 1:1000 dilution in blocking buffer was then added and incubated at 37°C for 1 h. Fifty microlitres of tetramethyl benzimidamine (TMB) microwell peroxidase substrate (Kikergaard and Perry Laboratories, USA) was added and the reaction was stopped after 15 minutes with 50 µl 1 M sulphuric acid and the optical density (OD) value read at 450 nm using an ELISA reader (Multiscan plus, version 2.03 Labsystems) after blanking the plate with negative control and the results were expressed as OD values and the ratio of IgG1: IgG2a was especially noted. To minimise plate variations, the same negative control serum was used for all the plates (serum NO. IV a1) and plate values were adjusted to reduce plate to plate variations by computing an adjustment value from the ratio of the control values on each plate.

2.4.4. Detection of IgG isotype responses in sheep

The same principle as for mice IgG isotyping was essentially utilised. Blood was collected for sera from sheep every 2 days for the detection of IgG1 and IgG2 isotypes. A 96 well flat-bottomed plate was coated with 100 µl of *C. ruminantium* soluble antigen (EL 15) at 1:1000 as already described for mice in section 2.4.3. After washing and blocking the plate with 200 µl of 4% normal rabbit serum at 1:50 in blocking buffer as described for mice in section 2.4.3., 100 µl the test and control samples at dilutions of 1:50 in blocking buffer were added and incubated at 37°C for 1 h. After washing the plate, rat anti-mouse monoclonal antibodies for IgG1 and IgG2 (Sheep Hybridoma IgG1 and IgG2) (a gift from Dr Harrison, CTVM), at a

dilution of 1:50 were added and incubated at 37°C for a further 1 h. 100 µl of goat anti-rat IgG whole molecule peroxidase conjugate (Sigma Chemicals Ltd., UK, product code No. A 9037) was then added at1:1000 in blocking buffer and incubated at 37°C for 1 h after which 100 ul of the substrate tetramethyl benzimidamine (TMB) microwell peroxidase substrate (Kirkegaard and Perry Laboratories, USA) was added and the reaction was stopped after 15 minutes with 100 ul of 1 M sulphuric acid and the OD value read at 450 nm. The ratio of IgG1: IgG2a was compared for the different groups of sheep. Known positive and negative control sera (positive control serum number G476; negative control serum number S68) were used in all the tests to minimise plate variations.

2.5. Biochemical tests;

2.5.1. Glutathione peroxidase assay

The Ransel kit for glutathione peroxidase (GPX) assay was obtained from Randox, UK and the test carried out as per manufacturers instructions. The assay principle is that GPX catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH the oxidised glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+ and the decrease in absorbance at 340 nm is measured.

$2GSH + ROOH \xrightarrow{\text{----------}} ROH + GSSG + H_2O$

$GSSG + NADPH + H+ \xrightarrow{\text{--------}} NADP+ + 2GSH$

Heparinised whole blood was diluted 6 fold and the mean of the decrease in absorbance used to calculate the actual GPX concentration in Units per litre using the formula below (Randox Manual, 1995).

U/l of Haemolysate = 8412 x Change in absorbance at 340 nm per minute x dilution .

2.5.2. Superoxide dismutase assay

The Ransel kit for superoxide dismutase assay was obtained from Randox (UK) and the test carried out as per manufacturers instructions. The assay principle takes into account the role of superoxide dismutase to accelerated dismutation of the toxic superoxide radical (O2), which is produced during oxidative energy processes, to hydrogen peroxide and molecular oxygen. The method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2 -(4-iodophenyl) -3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction.

Xanthine $\xrightarrow{XOD}$ Uric acid + O_2

I.N.T. $\xrightarrow{O_2}$ Formazan dye

OR

$O_2 \xrightarrow{SOD} O_2 + H_2O_2$

Heparinised blood was centrifuged at 3000 g for 10 minutes and the plasma aspirated. The erythrocytes were then washed three times with 0.9% NaCl solution and centrifuged at 3000 g for 10 minutes after each wash. The washed and centrifuged erythrocytes were then made up to 2.0 ml by adding cold distilled water and left to stand for 15 minutes at 4°C. The lysate was then diluted 50 fold with 0.01 M phosphate buffer pH 7.0 giving a final dilution factor of 200.

The degree of inhibition was converted to a percentage using this formula:

$$\% \text{ inhibition} = 100 - \frac{\text{change in absorbance of sample/min} \times 100}{}$$

change in absorbance of blank/min

Actual SOD levels were obtained from a standard curve (plotted using the % inhibition obtained with the standards of known concentrations) using the following formula (Randox Manual, 1995):

SOD units/ml of whole blood = SOD units/ml from std curve x dilution factor.

2.5.3. Nitrite assay in plasma.

The Griess assay described in section 2.2.1. was used for detection of nitrite levels in plasma, substituting culture supernatants with plasma.

2.6. PCR to detect *Cowdria ruminantium* infection in Plasma of infected Sheep

A method was developed in an attempt to detect and assess level of parasitaemia in plasma of infected animals as well as enable us to compare the level of infection and severity of disease in Dorpers infected with a virulent Kenyan strain (Kathiani) and Merinos / Scottish blackface infected with a virulent South African strain, Welgevonden. Previous studies have demonstrated the presence of the parasite in plasma using *C. ruminantium* antigen ELISA method (Viljoen *et. al.*,, 1987). In this study amplification of *C. ruminantium* DNA extracted from plasma was undertaken using oligonucleotide primers derived from the 16S rDNA sequences for *Cowdria* (Dame *et. al.*,, 1992 and Van Vliet et.al., 1992) (see section 2.6.2.).

2.6.1. Protocol for DNA extraction from plasma

Two hundred and fifty microlitres of mixed plasma was processed by centrifuging at 15,000 g to pellet *C. ruminantium* EBs. The supernatant was removed and the pellet resuspended in lysis buffer (25 mM EDTA, 10mM Tris (pH 8), 100mM Nacl, 0.05 %

SDS and 0.1 mg /ml proteinase K). The mixture was incubated at 55°C in a water bath for 1 h then boiled for 5 mins. to inactivate the enzyme. The samples were then kept at -20°C before PCR. As the positive control and also to derive a 'calibration curve' for the PCR, EBs from tissue culture were counted by the fluorescein diacetate staining method described in section 2.1.5. and centrifuged at 15,000 g to pellet the EBs. Known concentrations of EBs of 10,000, 5,000, 2,500, 1,600, 800, 400, 200, 100, 50, and 0 EBs / ml were resuspended in plasma obtained from normal sheep and the EBs processed for DNA extraction as described for the samples. Distilled water was used as negative control.

2.6.2. DNA Primers and their sequences and theoretical amplification specificity

TABLE 1

Primer code	Sequence	Theoretical Primer Specificity
	pCS20 derived primer set from 5'-3'	
Forward	**HE primers derived from the 16S rDNA sequences from 5'-3'**	
HE 1(cr)	CAGTTATTTATAGCTTCGGCTATGAG	*C. ruminantium* (Crystal Spring) (Dame et.al; 1992; Van Vliet *et. al.*,, 1992)
HE 1(cow)	CAGTTATTTATAGCTTCGGCTATRAGTATCTG	*C. ruminantium* (Senegal, Crystal Springs), Sumption, unpublished data)
R= an ambiquous base		
Reverse		
HE 2	GTGGCAGACGGGTGAGTAATGC	*E. canis, E. phagocytophila, E. chaffeensis, E. equi, E ewingi, E. risticii, C. ruminantium and A. marginale*
HE 3 (s)	GGTACCGTCATTATCTTCCC	*E. canis, E. phagocytophila, E. chaffeensis, E. equi, E ewingi, E. risticii, C. ruminantium and A. marginale*
HE 3(l)	CTATAGGTACCGTCATTATCTTCCC	*C. ruminantium* (Senegal, Crystal Springs), *E. canis, E. chaffensis, E. ewingii*

2.6.3. Polymerase chain reaction

PCR master mix was prepared comprised of MilliQ water, the primers HE 1 (forward) and HE 2 or 3 (reverse) at a final concentration of 2 µM, nucleotides (dNTPs) at 2 µM, Magnesium chloride at 50uM, TAQ polymerase at 0.5 µM and PCR Buffer containing 10mM Tris HCL, pH 8.3; 50 mM KCL; 0.001% w/v gelatin, adding the enzyme, TAQ polymerase, last. All the preparations were carried out in a laminar flow hood. The hood surface and the all the tubes, racks and pipettes had been sterilised with UV light. The reagents and samples were kept on ice thoughout the procedure. Forty five microlitres of PCR master mix was put into 0.5 ml reaction tubes and each tube was overlayed with 50 µl of mineral oil. Five microlitres of each test sample and control was added through the oil into the PCR master mix.

2.6.2.2. PCR conditions

The sample mixtures were placed in an Omnigene PCR machine (Hybaid) programmed as follows: denaturation at 94°C for 4 minutes, annealing at 55°C for 1 minutes and extension at 72°C for 1 minutes (1 cycle) followed by 37 cycles of 1 minutes at 94°C, 2 minutes at 45°C and 2 minutes at 72°C with a final extension of 10 min at 72°C.

2.6.2.3. Preparation of agarose gel to visualise PCR products

PCR products were analysed by running the samples in a 1 % agarose gel. Briefly, 0.5 g of agarose (standard grade - Sigma Chemicals Ltd., UK) was put into 50 ml loening buffer (1 x TBE =Tris/Boric acid/EDTA) and heated gently to melt the agarose in a microwave and cooled before adding 0.2 µg/ml of ethidium bromide.

Ten µl of the PCR products were aliquoted into microcentrifuge tubes and 1 µl of loading buffer (80% glycerol, 0.02% bromophenol blue, 19.98% Tris:EDTA) was added. Five µl of each sample and 5 µl Kb ladder was loaded into the gel, which was run at 90 V and visualised under UV light and recorded by photography.

2.7. Cytokine studies

2.7.1. Cytokine studies in mice

2.7.1.1. Quantification of mouse interferon gamma by an ELISA method

A commercial kit for mouse IFN-γ assay was obtained from Genzyme (IFN-γ Duo set kit). Preliminary tests were done using different dilutions of the capture antibody and secondary antibody for optimisation of the reaction to determine the optimal dilutions of the reagents. In summary, a 96 well Maxisorb immunoassay plate (Nunc. Ltd., UK) was coated with 100 µl per well of capture antibody (Hamster anti-mouse IFN-γ) diluted in 0.1 M carbonate buffer (coating buffer) at a concentration of 0.75 µg /ml and incubated overnight at 4°C. The plate was then washed 2-3 times with 250 µl of wash buffer (PBST). The plates were then blocked by incubating with 250 µl per well blocking buffer (PBS with 1% fatty-acid free bovine serum albumen (BSA) (Sigma Chemicals Ltd., UK, product code No. A 6003) for 2 h at 37 °C. A hundred microlitres of the samples (spleen homogenates) and standards (recombinant mouse IFN-γ) diluted in 1% 3-(3 cholamidopropyl dimethylammoni)-1-propanesulfonate (CHAPS) (Sigma Chemicals Ltd., UK) were added to respective wells and incubated for 1 h at 37°C in a horizontal shaker. The standards concentrations ranged from 20 to 1620pg/ml. Initially the standards were diluted in both CHAPS and culture medium to find out if CHAPS as a medium for samples

caused any variations from those in culture medium. The plates were then washed 4-5 times with wash buffer and incubated with 100 µl of the second antibody, biotin-labelled goat anti-mouse IFN-γ diluted in PBST containing 1% BSA (PBST/BSA) at a concentration of 1.0 µg/ml and incubated for 1 h 30 minutes at 37°C in a shaker. After 4-5 washes 100 µl of detection reagent (Horseradish-peroxidase (HRP) conjugated streptavidin) at 1:4,000 in PBST/BSA was added and incubated for 30 min at 37°C in a shaker. One hundred microlitres of tetramethyl benzimidamine (TMB) microwell peroxidase substrate (Kirkegaard and Perry Laboratories, USA) was then added and incubated for 10-30 mins. The reaction was stopped with 100 µl per well of 0.5M H_2SO_4 and the plate read at 450nm within 30 minutes. A calibration curve was derived from the OD of the standards and the amount of IFN-γ in each sample calculated in values of pg/ml.

2.7.1.2. Quantification of mouse total Interleukin 12 (IL-12) by an ELISA method

IL-12 is secreted from cells in three identified forms; heterodimeric p70, homodimeric $p40_2$ and monodimeric p40; heterodimeric p70 is responsible for all the known biological activities (Kobayeshi *et al.*, 1989; Trinchieri *et al.*, 1992). The InterTest ™ Total Mouse IL-12 ELISA kit obtained from Genzyme (USA) was used for assaying for total IL-12. A 96 well plate pre-coated with anti-mouse IL-12 was used to capture mouse IL-12 from the standards and test samples. A hundred microlitres of the standards (recombinant mouse IL-12) and test samples diluted 1:2 in the assay diluent, a buffered protein solution containing 0.2% chloroacetamide (provided by the manufacturer) were added to the wells and incubated for 30 minutes at room temperature. The concentration of the standards were 0, 60, 180, 540 and

1620 pg/ml. The wells were washed with wash buffer provided by the manufacturer (a buffered detergent containing 0.2% chloroacetamide) to remove any unbound material. 100 µl per well of an anti-mouse IL-12 biotinylated antibody diluted 1:50 in assay diluent was added and the plate incubated for 30 minutes at room temperature. The wells were washed again and 100 µl of horseradish peroxidase (HRP)-conjugated streptavidin added and incubated for 30 minutes at room temperature. 100 µl of a ready to use chromogen-substrate solution containing hydrogen peroxide, which had been provided in the kit was added and incubated at room temperature for 10 minutes. The reaction was stopped by adding 100 µl of 1 M sulphuric acid and the absorbance was read at 450 nm. A standard curve was obtained by plotting the concentrations of the mouse IL-12 standards versus the absorbance.

2.7.1.3. Quantification of mouse Interleukin 12 p70 (IL-12 p70) by an ELISA method

The capture ELISA test was performed using an Intertest mouse IL-12 p70 kit obtained from Genzyme (USA). The procedure for the assay was similar to that for total IL-12 except for the primary and secondary antibodies, the standards and also the incubation times were longer for the IL-12 p70 than for total IL-12. The primary antibody used in this assay was an anti-mouse IL-12 p35, while the secondary antibody was a biotinylated mouse anti-IL-12 p70 antibody. The test samples and the standards, recombinant mouse IL-12 p70, at doubling concentrations ranging from 25 to 1600, were incubated for 2 h at room temperature in a shaker. After adding the biotin-labelled anti-mouse IL-12 p70, the plate was incubated for 1 h, and then for 15 minutes after adding the horseradish peroxidase-conjugated streptavidin. All

incubations were performed at room temperature and on a horizontal shaker. The ready to use chromogen - hydrogen peroxide substrate, TMB (Kirkegaard and Perry Laboratories, USA) was then added and the plate incubated for 20 minutes before stopping the reaction with 1M sulphuric acid and the absorbance read at 450 nm.

2.7.1.4. Quantification of mouse interleukin 4 (IL-4) by an ELISA method

A commercial kit for mouse IL-4 assay was obtained from Genzyme (IL-4 Duo set kit). All the volumes added in this assay were 100 µl/well. A 96 well Maxisorb immunoassay microtitre plate (Nunc. Ltd., UK) was coated with the capture antibody, a monoclonal rat anti-mouse IL-4 diluted in 0.1M carbonate buffer , pH 9.5 (coating buffer) and incubated overnight at 4°C. The plate was then washed 5x with a wash buffer (PBST) before blocking the plate with PBS containing 4 % BSA (blocking buffer) and incubated for 2 hours at 37°C. The standards, recombinant mouse IL-4, at concentrations of 0, 100, 200, 400, 600, 800 and 1000 pg /ml and test samples were then added to the wells and incubated for 1 hour at 37°C. The standards were diluted in (CHAPS) since this detergent was used in processing of the mouse spleen homogenates. After washing, the second antibody (biotinylated polyclonal goat anti-mouse IL-4) diluted in PBST containing 1% BSA was then added and incubated at 37°C for 1 hour. The detection reagent (horseradish peroxidase (HRP) conjugated streptavidin) was then added at a dilution of 1:300 in PBST containing 1% BSA and incubated at 37°C for 15 minutes. The ready to use chromogen - hydrogen peroxide substrate, TMB (Kirkegaard and Perry Laboratories, USA) was then added and incubated for 10 minutes at room temperature. The reaction was stopped by adding

100µl of 1 M H_2SO_4 and the absorbance was read using an ELISA reader at 450 nm and the values of IL-4 calculated after plotting a calibration curve from the standards.

2.7.1.5. Quantification of interleukin-10 by by an ELISA method

A commercial ELISA kit for mouse IL-10 assay was obtained from Genzyme Ltd. Fifty microlitres of standards and test samples were added into a microtitre wells of a plate which had been supplied by the manufacturer and coated with an anti-mouse IL-10 antibody, and incubated for 2 hours at room temperature on a horizontal shaker. The standards were diluted with an assay diluent containing a protein at a concentration of 0, 30, 60, 180, 540, and 1080 pg /ml. The plate was then washed 3 times with a wash buffer (provided with the kit as a detergent in buffer). Fifty µl of the detection antibody, biotynylated anti mouse IL-10, was then added and incubated for 1 hour at room temperature on a horizontal shaker. Fifty µl per well of horseradish peroxidase conjugated streptavidin was then added and incubated for 30 minutes at room temperature on a horizontal shaker. Fifty µl of the the ready to use substrate (peroxide) TMB (Kirkegaard and Perry Laboratories, USA) was then added and incubated for 15 minutes or until colour developed and the reaction was stopped with 50 µl of 1 M H_2SO_4 and the absorbance read on an ELISA reader at 450 nm. The values of IL-10 in pg/ml were calculated from the standard curve based on the absorbance obtained from the standards.

2.7.2. Cytokine assays in sheep

2.7.2.1. Immunoassay for ovine interferon gamma (IFN-γ) by an ELISA method

IFN-γ was assayed using two bovine sandwich ELISA kits, one from Lifescreen Ltd., UK, and one from CSL Biosciences, Australia. In summary, 100 µl of plasma test and control samples, and positive and negative controls provided by the manufacturers were added into 96 well microtitre plate, coated with anti- bovine IFN-γ. Fifty µl of incubation buffer provided by the manufacturer was added into all the wells and the plate incubated for 1 hour at room temperature on a horizontal shaker set at 700 rpm. The plate was then washed 3 times with washing buffer, provided by the manufacturer, diluted in distilled water. 100 µl of the conjugate (anti- IFN-γ bovine HRP conjugate) was added and incubated for 1 h at room temperature on a horizontal shaker at 700 rpm. After a wash, 100 µl of the substrate -chromogen solution, TMB (Kikergaard and Perry Laboratories, USA) was added and incubated at room temperature for 15 minutes on a horizontal shaker as above. 200 µl of stop solution, provided with the kit, was then added and the absorbance read at 450 nm.. The results were interpreted on the basis of the negative and positive control readings, in which the expected responses in optical density (OD) were between 0.05 and 0.011 OD's for the negative control and 1.5 and 2.1 OD's for the positive control.

2.7.2.2. Immunoassay for Ovine IL- 8 by an ELISA method

This procedure was carried out in Moredun Research Institute using a procedure optimised for ovine IL-8 assay by David Haig and colleagues. A Dynatech 129B, flat-bottomed plate was coated with 100 µl/well of a 5 µg/ml solution of monoclonal antibody 8MG diluted in 0.1 M carbonate buffer, pH 9.6. The plate was incubated

overnight at 4 °C wrapped in cling film. After a 6 x wash with PBST, the plate was blocked with PBST / 3 % BSA for 1 hour at room temperature. All subsequent steps were followed by a 6 x wash. Fifty microlitres per well of plasma test samples at 1:4 dilution in PBST / 3 % BSA and standards were then loaded in duplicate and incubated at room temperature for 1 hour. The standards were prepared in doubling concentrations ranging from 50 to 6,000 pg/ml in PBST / 3 % BSA. 100 µl/well of polyclonal rabbit anti-IL-8 sera was then added at a dilution of 1:2000 in PBST / 3 % BSA and incubated for 1 hour at room temperature. 50 µl /well of the conjugate, goat anti-rabbit diluted 1:1000 in PBS was then added and incubated for 1 hour at room temperature. 50 µl /well of the chromogen (OPD)-substrate (peroxide) was added and incubated until colour had developed but before colour developed in the blank wells. The reaction was stopped with 50µl/well of 2.5 M H_2S0_4 and the absorbance was read using an ELISA reader and the values of IL-8 calculated after plotting a calibration curve from the standards.

2.7.2.3. Immunoassay for Ovine Tumour Necrosis Factor alpha (TNF-α) by a radioimmunoassay method

This assay was carried out in Moredun Research Institute, courtesy of Dr Chris Hodgson and Gordon Moon, and the method used was that reported by Kenison *et al.*, (1990). All antibody dilutions were prepared in assay buffer consisting of 0.15 PBS, pH 7.4, containing bovine serum albumin (BSA, Sigma Chemicals Ltd., UK) and 0.1% Tween 20 (Sigma Chemicals Ltd., UK). The antigen used, recombinant bovine TNF-α (rboTNF-α) was produced, cloned and expressed in E.coli and purified by HPLC and SDS-PAGE (kindly donated by Dr Gary Entrican, Moredun Research Institute, and obtained from CIBA-GEIGY Ltd., Switzerland). First antibody, anti-

bovine TNF-α antiserum, was raised in rabbit 378 at Moredun Research Institute, 1994. An initial subcutaneous injection (2 sites) of 50 ug rboTNF-α (conjugated to itself using glutaraldehyde) with complete Freunds adjuvant was followed by a similar booster injections at 4 and 8 weeks post-immunisation. Incomplete Freunds adjuvant was used for these booster injections. Blood was collected 12 days post-the second boost, allowed to clot and the serum harvested. Dilutions of this polyclonal antiserum were used in the assay. Second antibody was prepared by mixing 2 parts of a 1:40 dilution of donkey anti-rabbit IgG (Scottish Antibody production Unit, Lanarkshire) with 7 parts of a 6% solution of polyethylene glycol 6000 (BDH) in assay buffer. A radiolabelled TNF-α was used a tracer and was prepared by a modification of the iodogen method used by Peel *et al* (1990). Briefly, 10ug of rboTNF-α was mixed with 0.5m Ci Na125 (Amersham International plc) in 95 ul of borate-buffered saline (BBS), pH 8.3. The reaction was carried out in a glass tube coated with 10ug iodogen (tetrachlorodiphenyglycouracil, Pierce Chemicals) and the reaction allowed to proceed , with the occasional agitation, for 20 minutes at room temperature. Unbound iodine was removed using Sephadex G25 gel filtration column (Uni-Quick, Eldan Scientific) pre-equilibrated with 15 ml elution buffer (BBS containing 0.2% bovine serum albumin and 0.5% Tween 20). The specific activity of the tracer produced by this procedure was approximately 24mCi/mg. A dilution which yielded approximately 10,000 cpm/tube was used in the assay. For each run, dilutions of rboTNF-α containing 500, 100, 20, 4, 0.8 and 0.16 ng/ml in fetal bovine serum were included as standards. The assay was performed in 75 x 11mm polystyrene tubes (Sardedt). 100 ul of the standards, controls and test samples in duplicate were mixed with 100 ul of

polyclonal antibody (1:4000 to 1:8000) and 100 ul of assay buffer and incubated at 4oC for 24 h. At the end of this period, 100 ul of radiolabelled rboTNF-α was added to all the tubes, vortexed and incubated for a further 18h at 4oC. The second antibody (400ul) was then added to all the tubes except total count (TC) tubes and incubated for 2 h at 4oC To enhance pellet formation, 100ul of 1% rabbit serum was added to the tubes and the incubation continued for a further 30 minutes. All tubes except TC were then centrifuged at 3838g for 15 minutes, Bechkman GS-15) and the supernatant aspirated with a glass Pasteur pipette. The radioactivity in the pellet was measured in gamma counter (Canberra-Packard Cobra, UK) and the results were calculated with Cobra software.

2.7.3. RT-PCR for mouse cytokines

2.7.3.1. Isolation of mice spleen cells for RNA extraction

Spleens from the sacrificed mice were weighed and 20 mg of spleen tissue disrupted and homogenised by using aseptic techniques in sterile mortar and pestle within the shortest time possible. RNA extraction of spleen tissue was carried out using RNeasy kit (Qiagen) (Rneasy Mini handbook, 1997). This procedure utilises the selective binding properties of a silica-gel membrane, in which a specialised high-salt buffer system allows upto 100ug of RNA longer than 200 bases to bind to the RNeasy silica-gel membrane. The samples were lysed and homogenised in the presence of a highly-denaturing guanidinium isothiocyanate-containing buffer (RTL buffer) which immediately inactivates RNases to ensure isolation of intact RNA. Complete homogenization was carried out as this shears the high molecular weight genomic DNA. In summary, the tissues were disrupted in 350 ul of RTL buffer and the lysate

was then placed in a QIAshredder column sitting in a 2 ml collection tube and centrifuged for 2 mins. at maximum speed to homogenise. The extraction of total RNA was then done in the shortest time possible, as per the Qiagen commercial kit instructions. The extracted RNA was stored in -20oC in RNAse free water after determining the concentration of RNA by measuring the absorbance at 260 nm (A260) in a spectrophotometer. An absorbance of 1 unit at 260 nm corresponds to 40 ug of RNA per ml (A260 = 1 = 40 ug/ml). The RNA was used in RT-PCR for expression of mouse cytokines in spleen.

2.7.3.2. Reverse transcription of mice RNA to cDNA

Samples of 5 µg of RNA were used for reverse transcription to cDNA using the Super Script pre-amplification system (Life Technologies Ltd., UK) with oligo dT as a primer, according to the manufacturers instructions. 1 µl of Oligo dT primer (500 ug/ml) was added into 5 µg of RNA in 12 µl RNase free water and incubated at 70°C for 10 min on an Omnigene PCR machine (Hybaid). The mixture was then chilled on ice. This allowed the RNA to separate and the oligo dT to bind to the poly A tails of the mRNA molecules. Into each sample 8 µl of superscipt mix containing 4 µl synthesis buffer, 2 µl of 0.1M dithiothreitol (DTT/reducing agent), 1 µl of 10 mM nucleotides (dATP,dCTP, dGTP and dTTP) and 200U per µl of Superscript reverse transcriptase were added and left to stand at room temperature for 5 minutes. The samples were then heated at 42°C for 50 min in an Omnigene machine followed by 90°C for 5 minutes to denature the enzyme. They were then chilled and centrifuged at 10000 g for 15 sec. and the cDNA stored at -20°C.

2.7.3.4. Polymerase chain reaction analysis of cDNA in mice

Polymerase chain reaction (PCR) was performed using 2 ul of the cDNA and primers for cytokine sequences were used to amplify the cDNA during a 40 cycle PCR. All the cytokine primers used were a generous gift from Dr Mark Lawson, Medical School, University of Edinburgh. The PCR mix used in each reaction contained 19 ul milliQ water, 1.2 ul of 50 mM MgCL, 2.4 ul dNTP mix (dATP, dCTP, dGTP, dTTP, each at 10 mM), 1 ul of 25 pM positive and negative primer, 2 ul cDNA suspension, 3 ul PCR buffer and 0.4 ul (2 units) Taq polymerase (Thermometric Ltd., UK). A layer of 20 ul of paraffin oil was added to prevent evaporation and the PCR programme was carried out on an Omnigene PCR machine (Hybaid) with the following conditions; 95°C for 3 mins (denaturation step) for 1 cycle, 95°C X 1 min (denaturation step), 55°C X 1 min (primer attachment step), 72°C X 5 min (elongation step) for 30 cycles and 72°C X 5 min (elongation step) for 1 cycle. The samples were then stored at -20°C before electrophoresis at 90 V was carried out in 2 % agarose gel containing 4 µg/ml ethidium bromide and the PCR products visualised under UV light.

2.7.4. RT-PCR for sheep cytokines

2.7.4.1. PBMC isolation from blood of infected sheep for extraction of RNA

Heparinised blood was collected from infected sheep at days 4, 7, 11 and 13 pos-infection and PBMC separated using Ficoll Hypaque method already described in section 2.1.3. The cells were then cultured in RPMI 1640 for 24 hours before scraping the cells and extracting RNA using an RNeasy^{R} kit (Quiagen Ltd., UK) as

described in section 2.7.3.1. The RNA was used in RT-PCR for detection of expression of sheep cytokines in spleen.

2.7.4.2. Reverse transcription of sheep RNA to cDNA

The procedure was as already described for mouse RT-PCR, section 2.7.3.2.

2.7.4.3. Polymerase chain reaction analysis of sheep cDNA

Polymerase chain reaction (PCR) was performed using 2 µl of the cDNA and primers for cytokine sequences were used to amplify the cDNA during a 40 cycle PCR. All the cytokine primers sets used (IL-4, IL-10 and TNF-α) were a generous gift from John Campell, CTVM, University of Edinburgh, and are as listed below.

MRNA:	**Primer direction:**	**product Size (bp):**	**5'-Sequence-3'**
IL-4	Forward	427	GCATTGTTAGCGTCTCCTGGTAAAC
	Reverse		CTTCATAATCGTCTTTAGCCTTTCC
IL-10	Forward	733	GCTCAGCACTACTCTGTT
	Reverse		GTTCACAGAGAAGCTCAGT
TNF-α	Forward	500	CTCAGGTCATCTTCTCAAGCC
	Reverse		CAGGGCGATGATCCCAAAGTAGACC

The PCR mix used in each reaction contained 19 ul milliQue water, 1.2 µl of 50 mM MgCL, 2.4 µl dNTP mix (dATP, dCTP, dGTP, dTTP, each at 10 mM), 1 ul positive and negative primer, 2 µl cDNA suspension, 3 ul PCR buffer and 0.4 µl (2 units) Taq polymerase (Thermometric Ltd.). A layer of 20 µl of paraffin oil was added to prevent evaporation and the PCR programme was carried out on an Omnigene PCR machine

(Hybaid) in the following conditions; 95°C for 3 mins (denaturation step) for 1 cycle, 95°C X 1 min (denaturation step), 55°C X 1 min (primer attachment step), 72°C X min (elongation step) for 30 cycles and 72°C X 5 min (elongation step) for 1 cycle. The samples were then stored at -20°C before electrophoresis at 90 V was carried out and the PCR products visualised under UV light in 2 % agarose gel containing 4 µg/ml ethidium bromide.

CHAPTER 3

STUDIES ON CYTOKINES, NITRIC OXIDE AND FREE RADICAL SCAVENGER LEVELS DURING INFECTION OF SHEEP WITH *COWDRIA RUMINANTIUM*

3.1. Introduction

The clinical manifestations and pathology of *C. ruminantium* infection are mainly associated with severe respiratory distress, cardiac and nervous embarrassment due to increased capillary permeability leading to oedema (Clark, 1962). Whilst increased capillary permeability may explain the marked fall in blood and plasma volume which occur prior to death in heartwater (Clark, 1962), the cause of such alterations remain obscure.

Despite immunisation and specific chemotherapy, heartwater remains a major obstacle against introducing highly-producing animals into Africa with the aim of upgrading or replacing local stock (Uilenberg, 1981; 1983). Variation in the susceptibility to *C. ruminantium* in cattle, sheep and goats has been recorded (Du Plessis *et al.*, 1987, Uilenberg, 1971, and Spreull, 1922). Differences in the resistance of cattle to *C. ruminantium* infection have been documented and there is general agreement that local breeds developed in endemic areas are more resistant than exotic breeds and it was suggested that they acquire this strong innate resistance through long natural selection (Matheron *et al.*, 1987). There have been conflicting reports on resistance of different breeds to *C. ruminantium* infection (Du Plessis *et al.*, 1987). For instance, *Bos indicus* were reported to be more resistant than *Bos taurus* breeds by Van der Merwe (1979), but Uilenberg (1983) reported that the innate resistance found in African breeds had nothing to do with the breed influence, thus two conflicting reports. The reports on the resistance of indigenous sheep and goats is also controversial with indigenous breeds being reported by some authors to possess a higher degree of natural resistance to the disease than exotic breeds (Spreull, 1922; Alexander, 1931; Uilenberg, 1971; Du Plessis *et al.*, 1983) while some authors reported heavy losses among indigenous breeds (Ilemobade, 1976; Karrar, 1960).

Existence of natural selection among goat populations raised in endemic areas has been suggested (Matheron *et al.*, 1987). The resistance rate acquired in such epidemiological conditions was suggested to depend on the intensity of infectious contact experienced by the population (Matheron *et al.*, 1987). All these reports emphasise the obscure nature of the non-specific factors which play important roles in pathogenesis and resistance to *C. ruminantium* infection.

The anti-microbial role of interferons and other cytokines has been reported in several diseases to be mediated through production of reactive nitrogen and / or oxygen radicals (Seguin *et al.*, 1994). Exposure of cells to certain stimuli including pathogens, cytokines, mediators and chemical products results in increased uptake of oxygen leading to production of reactive oxygen radicals and nitric oxide (Dugas *et al.*, 1995). Nitric oxide is a powerful vasodilator which has been found to play a major role in enhancing vascular permeability, and the latter is a key phenomenon in the pathogenesis of *C. ruminantium* infection (Furchgott *et al.*, 1984). Infection of endothelial cells by *Rickettsia rickettsii,* an organism closely related to *C. ruminantium,* was reported to induce increased production of intracellular peroxide as well as a sustained increase in the levels of superoxide dismutase, but a decrease in glutathione peroxidase and glucose-6-phospatase, scavengers of oxidising radicals (Santucci *et al.*, 1994). These reports prompted the studies reported here, to investigate levels of some of the enzymes involved in antioxidation processes and relate these to the levels of some cytokine and nitric oxide levels and how these may influence the degree of resistance to the disease. The cytokines studied included IFN-γ, TNF–α , which are known to be involved in activation of macrophages and endothelial cells, and IL-8, which is known to activate neutrophils leading to their migration and may have a role in lung damage and exudation.

The aim of this study was, therefore, to investigate the relationship between inflammatory cytokines and biochemical markers of free radical production in relation to *C. ruminantium* infection in both indigenous and exotic breeds of sheep. The work included studies on the levels of enzymes involved in scavenging oxidising agents, nitric oxide and cytokines in sheep infections of *C. ruminantium*. This involved infecting sheep considered to be highly susceptible to the disease (Merino and Black face crosses, and Dorper), sheep immunised with a recombinant antigen of *C. ruminantium* and infection of indigenous African breeds (Red Maasai), which are considered to be resistant to the disease. The initial objectives included investigation of the innate resistance found in most African breeds. However, these objectives were reviewed after all the Red Maasai were found to be serologically positive, an indication of prior exposure to *C. ruminantium* antigens, before entering the experiment as the animals were obtained before my arrival to Kenya to carry out the work. As the field trip to Kenya was only for 7 weeks and given financial constraints, it was not possible to obtain other more suitable animals for the work.

3.2. Materials and Methods

3.2.1. Experimental animals;

Six Scottish Black face and Merino crosses were infected with a virulent South African strain of *C. ruminantium*, the Welgevonden isolate, while six Dorpers were infected with a virulent Kenyan strain , the Kathiani isolate, as the Welgevonden isolate could not be imported into Kenya where this experiment was performed. The reactions were compared to those in animals which had prior exposure to *Cowdria* antigens and which were expected to show clinical signs on infection: six Black Face and Merino crosses immunised with a recombinant antigen, the 58kDa GroE/L of *C. ruminantium*

(Kibor, 1997) and challenged with the Welgevonden isolate at a dose of $3x10^5$ EBs; and six Red Maasai sheep which had been exposed naturally to *Cowdria* antigens and challenged with the Kenyan strain (Kathiani). The sheep were individually identified with numbers as shown in the table below (Table 3.1). Although the Red Maasai were raised in a farm which had not reported heartwater, they were all serologically positive with antibodies to *C. ruminantium* prior to infection as compared to the Dorper which were sero-negative, as tested using an indirect ELISA assay, described below. All the animals were about 12-24 months old. The source and maintenance of the animals has already been described in section 2.3.1.1.

Table 3.1.

Welgevonden sheep infections identification numbers		**Kathiani sheep infections identification numbers**	
Primary sheep	Immunised sheep	Dorpers	Red Maasai
67	66	12	1
68	69	15	2
70	71	17	3
73	72	18	4
75	74	19	5
77	76	20	6

3.2.2. Infectious agent

The Welgevonden (South African) isolate of *Cowdria ruminantium* propagated in tissue culture in bovine endothelial cell lines was used to infect the Scottish Black face and Merino crosses using an infectious dose of $3x10^5$ elementary bodies (EBs) as described in section 2.1.1. The viability count to determine the infectious dose was performed by a fluorescein diacetate staining technique as described in section 2.1.5. The Kenyan isolate of *Cowdria ruminantium* (Kathiani; blood stabilate No. 327B of 25-10-1994) was used as a blood stabilate to infect the Dorpers and the Red Maasai as

described in section 2.1.1. The stabilate had previously been shown to be virulent but the strain had not been isolated in tissue culture and therefore the infectious dose could not be determined.

3.2.3. Clinical disease monitoring and sampling

The disease was monitored in the sheep daily and blood samples collected from all sheep every two days, as described in section 2.3.5. Serum was separated and tested by indirect ELISA assay test, and also used for immunoglobulin isotyping, for *C. ruminantium* antibodies; heparinised blood was collected for assays of glutathione peroxidase (GPX) and superoxide dismutase (SOD) levels; plasma was collected for assays of TNF-α, IL-8, IFN-γ, nitrite and for extraction of *C. ruminantium* DNA for the quantification of infection by PCR.

3.2.4. Serology

3.2.4.1. ELISA for antibody assay prior to infection

An indirect ELISA method was used to assay for *C. ruminantium* antibodies in 12 sheep infected with the Kathiani isolate, as described in section 2.4.2. using a *C. ruminantium* soluble antigen (batch No. 14 (EL14)) prepared as described in section 2.4.1. All the Red Maasai had high antibody optical density prior to infection with a mean value of more than 0.900 as compared to the Dorpers which had low levels of less than 0.060 (Figure 3.2), indicating that, although the Red Maasai were chosen because of no history of heartwater, they were found to have high and uniform antibody levels prior to infection.

3.2.4.2. Anti-*Cowdria* immunoglobulin isotypying

Serum anti-*Cowdria* IgG1 and IgG2 antibody levels were assayed by an ELISA method in 12 sheep infected with the Kathiani isolate, as described in section 2.4.4., using a *C. ruminantium* soluble antigen (batch No. 15 (EL 15)) prepared as described in section 2.4.1.

3.2.5. Biochemical tests;

3.2.5.1. Glutathione peroxidase assay

Glutathione peroxidase (GPX) levels were measured in heparinised blood for all the 24 sheep using a commercial kit (Randox) as described in section 2.5.1. The levels were measured prior to infection from day 0 and there after every 2 days post-infection until death or recovery, up to day 30. The assay principle and method were performed as described in section 2.5.1. The levels were compared and contrasted between the Welgevonden infections (Scottish Black Face and Merino crosses) and the Kathiani infections (Red Maasai and Dorpers). The levels were also compared between days for each group.

3.2.5.2. Superoxide dismutase assay

SOD levels were assayed in heparinised blood for all the 24 sheep using a commercial kit (Randox) as described in section 2.5.2. The levels were measured before and after infection, as for GPX and compared between different groups and for different days within each group.

3.2.5.3. Nitrite assay

Nitrite levels were measured in plasma collected every 2 days for all the 24 sheep after infection with *C. ruminantium* using the Griess assay method, as described in section

2.2.1., with the modification that plasma was used in place of culture supernatants. The levels were compared and analysed between the different groups of sheep and different days for each group.

3.2.6. Cytokine assays by Enzyme Linked Immunosorbent Assay (ELISA)

3.2.6.1. Interferon gamma assay

IFN–γ was measured in plasma collected from all the 24 sheep from day 0 prior to infection and every 2 days thereafter using two bovine sandwich ELISA kits, one from CSL Biosciences, Australia, and one from Lifescreen Ltd., UK, as described in section 2.7.2.1. The OD values obtained from positive and negative controls were used as basis for interpretation of results.

3.2.6.2. Interleukin 8 assay

IL-8 was assayed in the sheep plasma samples of all the 24 sheep collected from day 0 and every 2 days there after up to day 30 by an ELISA test. The procedure for ovine IL-8 assay had been optimised by David Haig and colleagues (Moredun Research Institute) and was performed as described in section 2.7.2.2. The IL-8 values were calculated from a calibration curve and analysed and compared between the different sheep breeds and also between different stages of infection.

3.2.6.3. Tumour necrosis factor assay (TNF–α)

A radio-immunoassay method was used to assay the levels of TNF–α in all the 24 sheep using a bovine recombinant TNF–α cloned and expressed in *E. coli* as antigen, rabbit anti-bovine TNF as primary antibody, donkey anti-rabbit sera as secondary

antibody and radio-labelled TNF–α as tracer as described in section 2.7.2.3. For each run, dilutions of rboTNF–α containing 500, 100, 20, 4, 0.8 and 0.16 ng/ml in fetal bovine serum were included as standards. The assay was carried out in Moredun Research Institute, courtesy of Dr Chris Hodgson and Gordon Moon, using the method reported by Kenison *et al.*, (1990). TNF–α levels were assayed in plasma samples collected before and after infection for all the sheep infections. TNF–α levels were calculated using the calibration curve of the standards and the results analysed and compared for different breeds of sheep and also for different days during infection.

3.2.7. PCR to detect and estimate level of C. ruminantium infection in Plasma

A method to assess relationship between level of infection and severity of disease in the Welgevonden and Kathiani infections was attempted using PCR. For positive control, and also to derive a 'calibration curve' for the PCR, EBs from tissue culture were counted by the fluorescein diacetate method and centrifuged at 15,000 g to pellet the EBs. Known concentrations of EBs (10^5, $10^{2.5}$, $10^{1.25}$, 800, 400, 200, 100 50, and 0 EBs / ml) were resuspended in plasma from normal sheep and the EBs processed for DNA extraction as before. DNA extraction from plasma was performed as already described in section 2.6.1. PCR was performed in a total of 57 samples for days 0, 4, 8, 10, 12 and 14 using *C. ruminantium* DNA primers, HE 1 (forward) , HE 2 or HE 3(reverse) whose sequences and theoretical amplification specificity have been described in section 2.6.2. These primers were not analysed using southern blotting during this study but are routinely used in the laboratory. The PCR conditions used and procedure were as described in section 2.6.3. PCR products were analysed by running the samples in a 1 % agarose gel. The results were visualised under UV light and

recorded by photography (see section 2.6.3.2). The results were compared between the animals and a relationship between the PCR and the other results including levels of cytokines and antioxidants compared.

3.2.8. RT-PCR for expression of Cytokines

Forty ml heparinised blood was collected from 2 Merino and Blackface cross sheep identification numbers 222 and 225, infected with the Welgevonden isolate of *C. ruminantium* at days 4, 7, 11 and 13 post-infection (due to the large amount of blood required for enough PBMC, these 2 other sheep had to be used so as not to interfere with the other experiment by overbleeding). PBMC were then separated using Ficoll Hypaque method as described in section 2.1.3. The cells were cultured in RPMI 1640 for 24 hours before scraping the cells and extracting RNA using a Qiagen kit as described in 2.7.3.1. RT-PCR was carried out using the extracts as described in section 2.7.4.2. and the resultant cDNA used in a PCR using cytokine primers (TNF–α , IL-4 and IL-10) as described in section 2.7.4.3.

3.2.10. Data Analysis

The students t-test, Analysis of Variance (ANOVA) or Mann-Whitney test were used for statistical data analysis of the biochemical and cytokine data sets and results expressed at 0.05 level of significance.

3.3. Results

3.3.1. Clinical and pathological manifestations

3.3.2.1. Welgevonden infections

The disease onset was acute in all the sheep, with severe clinical signs being noted 2 days after fever onset. The mean rectal temperatures in the sheep infected with the Welgevonden isolate were noted to be increased to or more than 41°C by day 8 in both groups (Figure 3.1 and Appendx 1.10) but the trends and the temperature reactions were not significantly different between the infections of naive and primary and immunised groups of sheep (t-test; p=0.355). The mean incubation period, course of fever and days to death were not significantly different between the primary and immunised animals (t test; p>0.05 for the three parameters) (Table 3.2). Clinical signs seen in these sheep included laboured breathing with increased respiratory rate, loss of appetite, depression, unsteady gait, recumbency and in some animals nervous signs just prior to death. Hydropericardium was a common pathological feature and routine brain squash smears stained in giemsa confirmed the disease by demonstrating *C. ruminantium* colonies in each of the dead sheep. Differences were noted in the mean infection rates of endothelial cells of the immunised and primary infections, with the immunised showing an infection rate of 30% with a range of 20-45 while the mean infection rate in the primary infections was 62.95% with a range of 43 -75 as calculated by Kibor (1997). Death occurred within 5-6 days after the febrile reaction (Table 3.2).

3.3.2.2. Kathiani Infections

The mean incubation period for animals which succumbed to the disease was 16 and 20 days for the Dorpers and the Red Maasai respectively (Figure 3.2a and Appendices

1.1 and 1.2). The morbidity rate was higher in the Dorpers with 6 out of 6 showing clinical disease as compared to 2 out of 6 in the Red Maasai (Figure 3.2b and Appendices 1.1 and 1.2). The reacting sheep in both groups showed mild signs and a prolonged course of disease of more than 10 days. The clinical picture was characterised initially by a febrile reaction followed by a slight depression. Other clinical signs included reduced appetite, slight tremors, nasal discharge and slight respiratory distress. Four sheep, two from each group which suffered clinical disease were sacrificed on day 22 post-infection otherwise there were no deaths recorded and spontaneous recovery was seen in the other sheep in the groups.

Fig. 3.1.

Mean temperature changes in primary and immunised sheep infected with the Welgevonden isolate of *C. ruminantium*

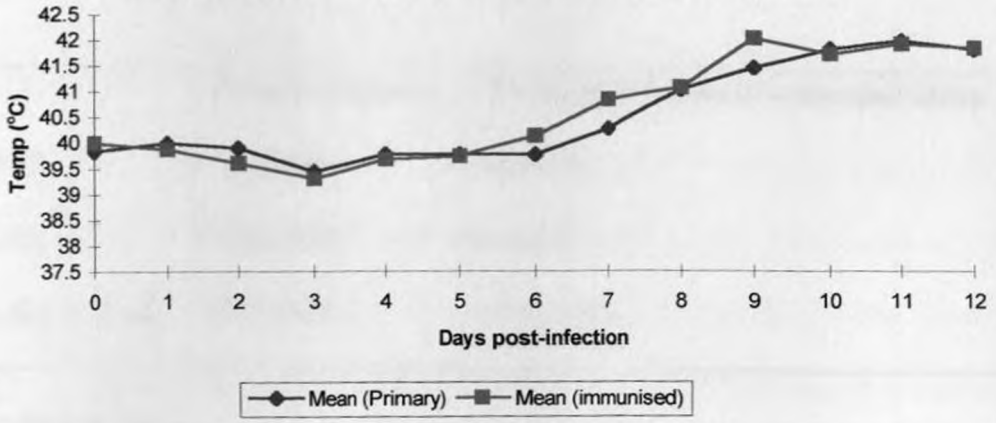
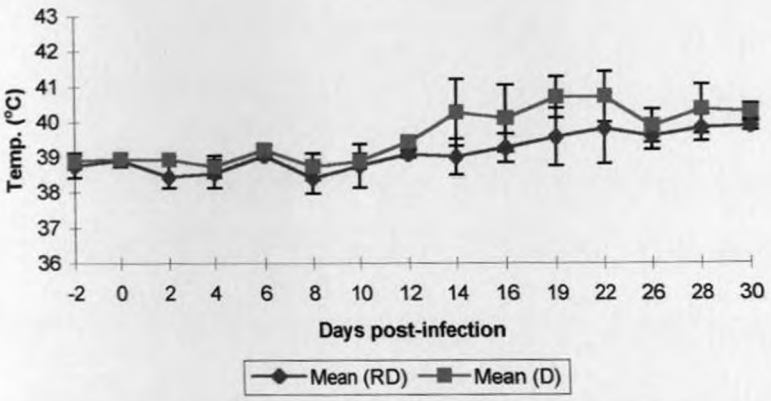


Fig. 3.2.

A. Mean temperature of Red Maasai (RD) and Dorper (D) sheep infected with the Kathiiani isolate of *C. ruminantium*



B. Temperature reactions in individual Red Maasai sheep Nos. 1-6 infected with the Kathiani isolate of *C. ruminantium*. Only sheep No. 3 and 6 had temperature reactions of more than 41°C

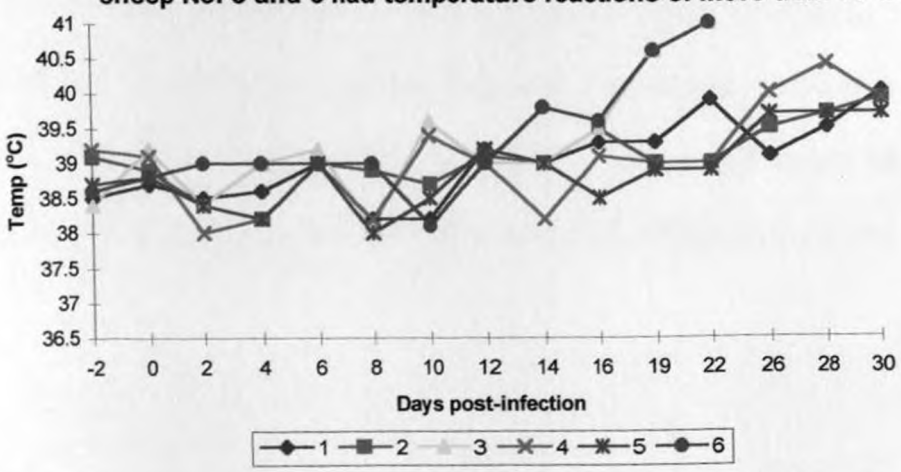


Table 3.2: Clinical data of the Welgevonden infections

	Primary infections	Challenge infections of immunised sheep	
Mean I.P.	7.5+/-0.84	6.83+/-0.	
Mean CF	5.3+/-0.52	6.2+/-1.7	
Mean day of death	11.7+/-0.82	11.7+/- 1.2	

KEY:
IP-Incubation period

CF-Course of Fever

3.3.2. Serology

3.3.2.1. Anti-*C. ruminantium* antibodies

An indirect ELISA test for anti-*Cowdria* antibodies revealed that all the Red Maasai had high optical density (OD) value indicative of antibody levels in serum prior to infection (Figure 3.3a and b and Appendix 1.3.2) with a mean value of more than 0.900 as compared to the Dorpers which had levels with a mean of less than 0.060 as stated above in materials and methods. After infection, all the Red Maasai recorded a decline in the OD value up to days 12/16 after which an increase was noted. A gradual increase in OD value was noted in all the Dorpers with the highest levels being recorded at day 22.

3.3.2.2. IgG1 and IgG 2 immunoglobulin isotypes to *C. ruminantium*

Anti-*Cowdria* immunoglobulin isotypes IgG1 and IgG2 were assayed in the sheep infected with the Kathiani isolate of *C. ruminantium* from day 0 to day 30 post-infection. There were major variations in IgG1 but not IgG2 in individual sheep in the Red Maasai sheep during infection, with those showing a febrile reaction recording higher but transient levels of IgG1 (on day 14) as compared to non-febrile sheep (Figure 3.4a and b and Appendix 1.3.1). Significantly higher mean levels of IgG1 (t test; p=0.002) were recorded in the Red Maasai as compared to the Dorpers but not in the mean IgG2 levels (Figure 3.4c). The Red Maasai had significantly higher mean levels of IgG1 as compared to IgG2 with a ratio of 5:1 (Figure 3.4 c and Appendix 1.3).

Fig. 3.3.

A. Indirect ELISA results in Red Maasai sheep infected with the Kathiiani isolate of *C. ruminantium*. Only sheep No. 3 and 6 showed a temperature reaction of more than 41°C

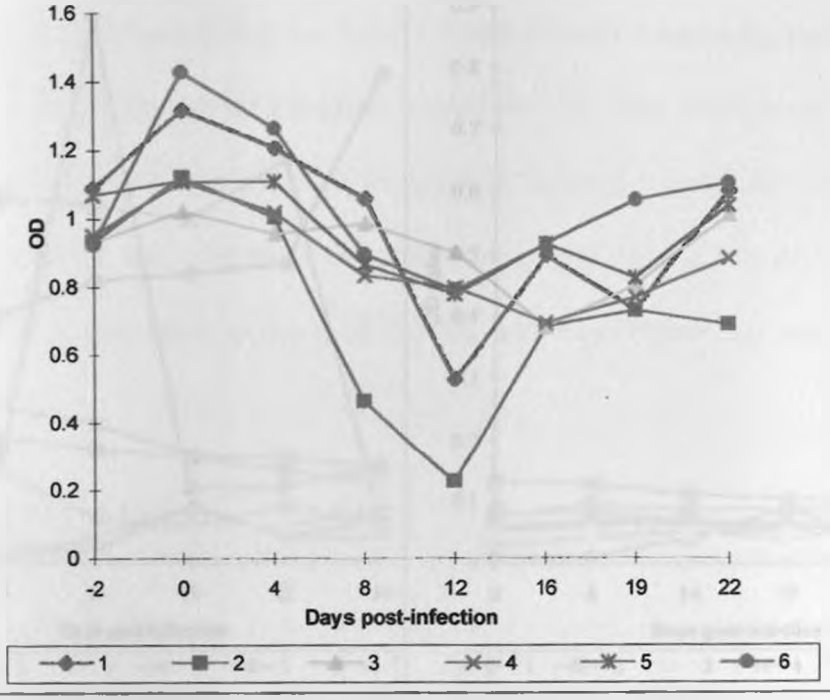


Fig. 3.3.

B. Indirect ELISA results in Dorper sheep infected with the Kathiiani isolate of *C. ruminantium*. Note that all the sheep had temperature reactions of more than 40.5°C Sheep Nos. 12 and 15 had the earliest and most acute reaction to the disease.

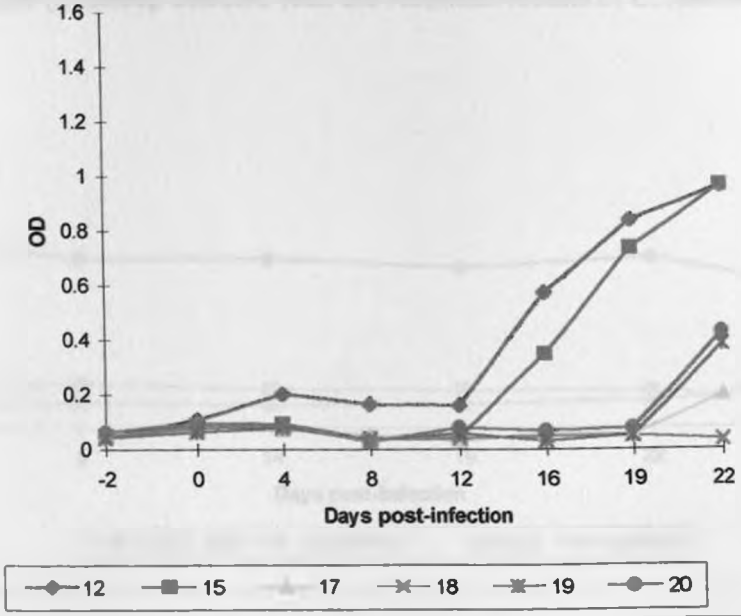
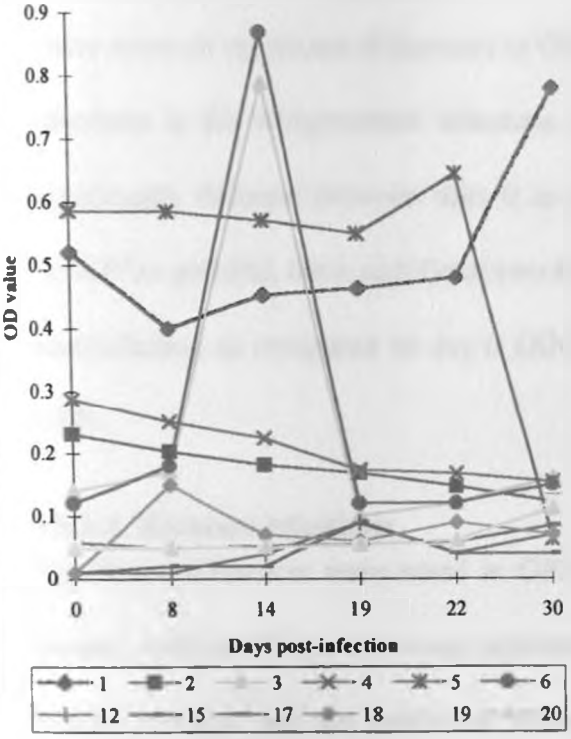


Fig. 3.4.

A. IgG1 levels in Red Maasai sheep Nos. 1-6 and Dorpers sheep Nos. 12, 15, 17, 18, 19, and 20 infected with the Kathiani isolate of *C. ruminantium*



B. IgG2 levels in Red Maasai sheep Nos. 1-6 and Dorpers sheep Nos. 12, 17, 18, 19, and 20 infected with the Kathiiani isolate of *C. ruminantium*

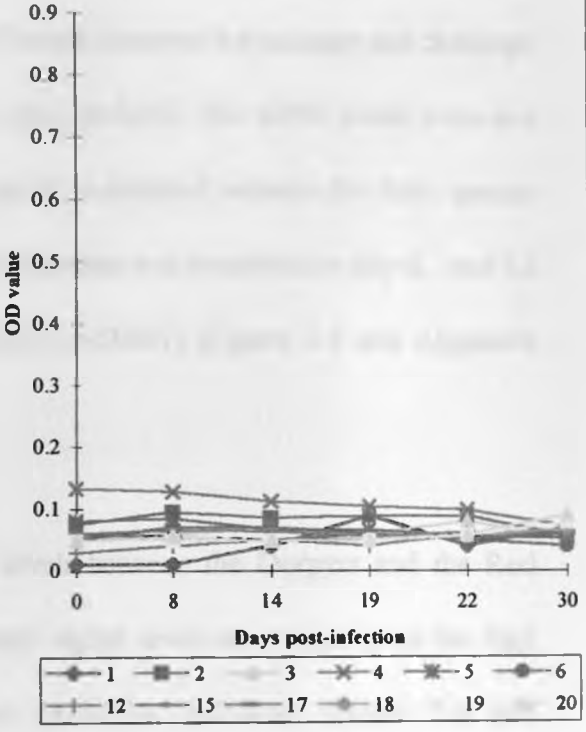
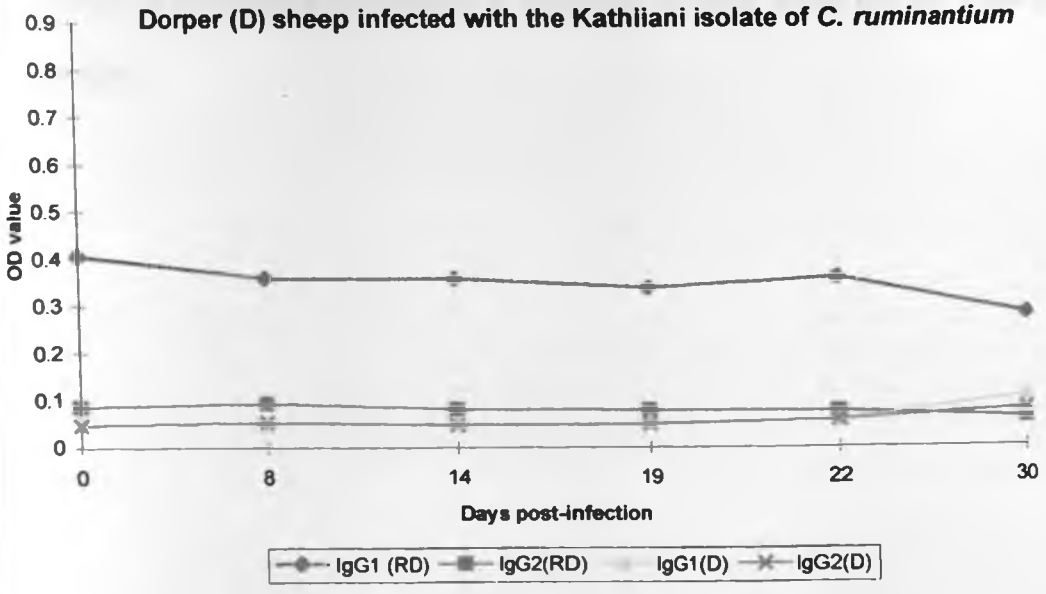


Fig. 3.4.C.

Mean Immunoglobulin levels of IgG1 and IgG2 in Red Maasai (RD) and Dorper (D) sheep infected with the Kathiiani isolate of *C. ruminantium*



3.3.3. Glutathione peroxidase (GPX)

3.3.3.1. Welgevonden infections

There were no significant differences in GPX levels between the primary and challenge infections in the Welgevonden infections (t-test, $p=0.15$). The GPX levels were not significantly different between days 0 to day 6 in infected animals for both groups (ANOVA; $p>0.05$), but a significant two fold increase was recorded on day 8, and 12 post-infection as compared to day 0 (ANOVA; $p<0.001$) (Figure 3.5 and Appendix 1.10).

3.3.3.2. Kathiani infections

Significant differences were noted in GPX levels between the Dorpers and the Red Maasai, with the Dorpers showing significantly higher levels as compared to the Red Maasai through out the course of disease (ANOVA; $p=0.009$) (Figure 3.6 and Appendix 1.5). The levels increased gradually after infection in both breeds, with peak levels coinciding with the febrile reaction and higher levels being recorded in Dorpers and also in the reacting Red Maasai as compared to those which did not show febrile reaction.

Fig. 3.5.

Mean glutathione peroxidase (GPX) levels in primary and immunised sheep infected with the Welgevonden isolate of *C. ruminantium*

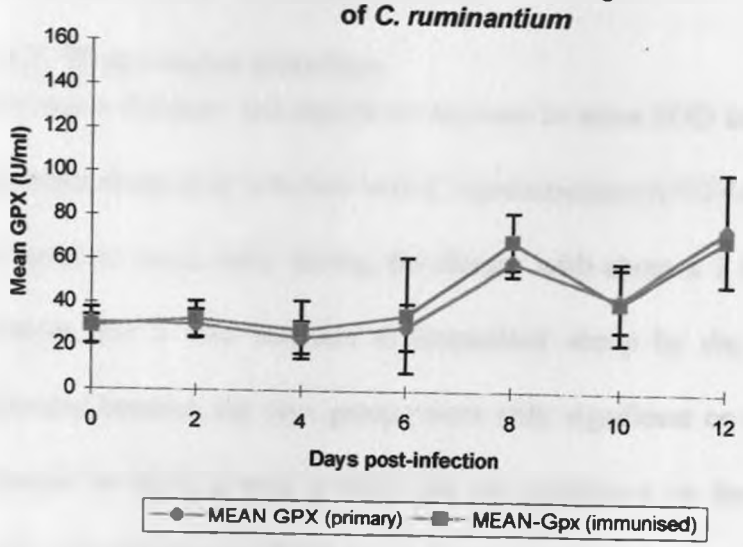
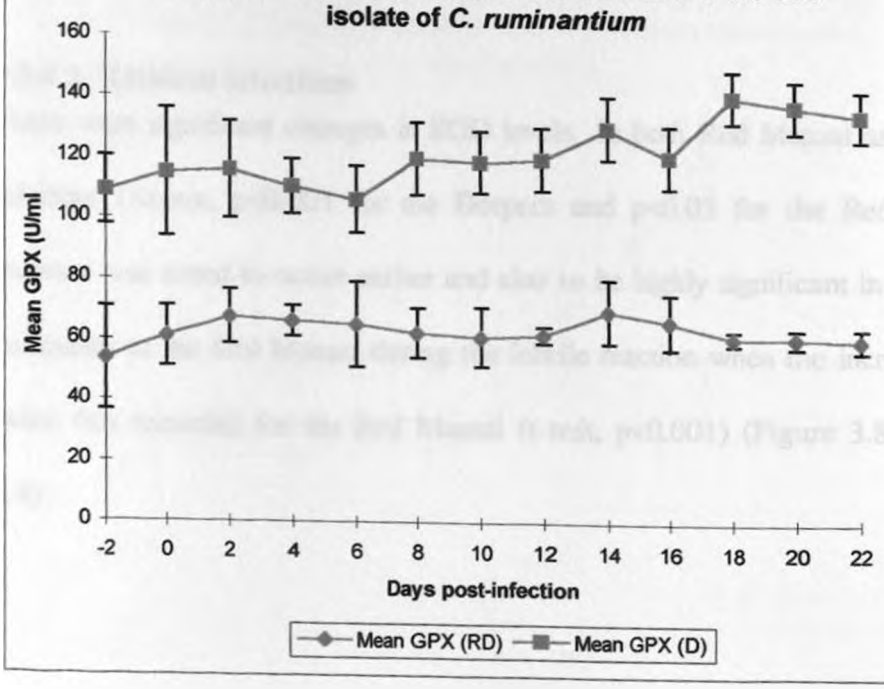


Fig. 3.6.

Mean glutathione peroxidase (GPX) levels in Red Maasai (RD) and Dorper (D) sheep infected with the Kathiani isolate of *C. ruminantium*



3.3.4. Superoxide dismutase (SOD)

3.3.4.1. Welgevonden infections.

There was a dramatic and significant increase in mean SOD levels in both primary and immunised sheep after infection with *C. ruminantium* (ANOVA, p=0.04). The increase was noted to occur early during the disease with about a 3 fold increase in primary infections and 2 fold increase in immunised sheep by day 2 post-infection. The differences between the two groups were only significant on day 2 post-infection as compared to day 0 (t test, p=0.03) but not significant on the other days (ANOVA; p=0.3). The high mean SOD levels were sustained through out the course of the disease in both groups, except on day 10 when there was a slight drop in the immunised group, and the highest mean SOD levels were recorded during the febrile reaction (4-12 days) post-infection (Figure 3.7 and Appendix 1.10).

3.3.4.2. Kathiani infections

There were significant changes in SOD levels in both Red Maasai and Dorper after infection (Anova, $p<0.001$ for the Dorpers and $p<0.05$ for the Red Maasai). The increase was noted to occur earlier and also to be highly significant in the Dorpers as compared to the Red Maasai during the febrile reaction when the increase was about twice that recorded for the Red Maasai (t-test, $p<0.001$) (Figure 3.8 and Appendix 1.4).

FIG. 3.7.

Mean superoxide dismutase (SOD) levels in primary and immunised sheep infected with the Welgevonden isolate of *C. ruminantium*

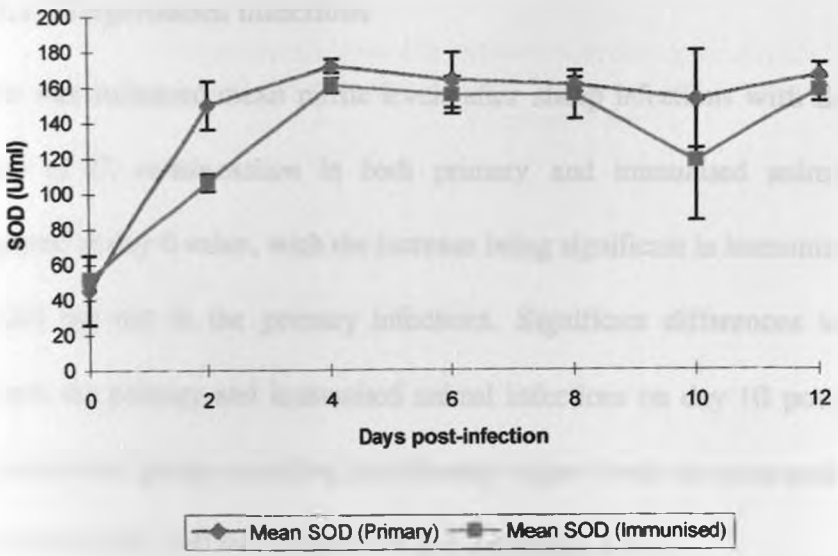
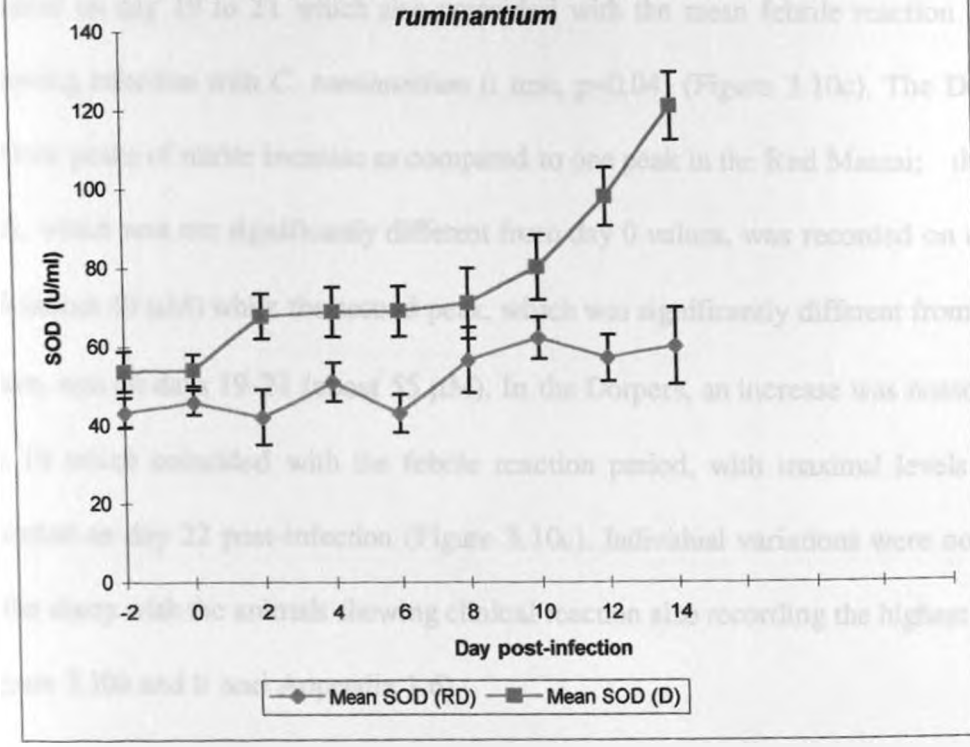


Fig. 3.8.

Superoxide dismutase (SOD) levels in Dorper (D) and Red Maasai (RD) sheep infected with the Kathiani isolate of *C. ruminantium*



3.3.5. Nitrite levels

3.3.5.1. Welgevonden infections

There was increased mean nitrite levels after sheep infections with the Welgevonden isolate of *C. ruminantium* in both primary and immunised animal infections as compared to day 0 value, with the increase being significant in immunised sheep (t-test, p=0.04) but not in the primary infections. Significant differences were also found between the primary and immunised animal infections on day 10 post-infection, with the immunised group recording significantly higher levels as compared to the primary infections (t-test, p=0.04) (Figure 3.9 and Appendix 1.12).

3.3.5.2. Kathiani infections

There were significant increases in mean nitrite levels in both the Red Maasai and the Dorpers on day 19 to 21 which also coincided with the mean febrile reaction period following infection with *C. ruminantium* (t test, p=0.04) (Figure 3.10c). The Dorpers had two peaks of nitrite increase as compared to one peak in the Red Maasai; the first peak, which was not significantly different from day 0 values, was recorded on days 4 to 8 (about 40 μM) while the second peak, which was significantly different from day 0 values, was on days 19-21 (about 55 μM). In the Dorpers, an increase was noted from day 16 which coincided with the febrile reaction period, with maximal levels being recorded on day 22 post-infection (Figure 3.10c). Individual variations were noted in all the sheep with the animals showing clinical reaction also recording the highest levels (Figure 3.10a and b and Appendix 1.6).

Fig. 3.9.

Mean Nitrite levels in Primary and immunised infections of sheep infected with the Welgevonden isolate of *C. ruminanitum*

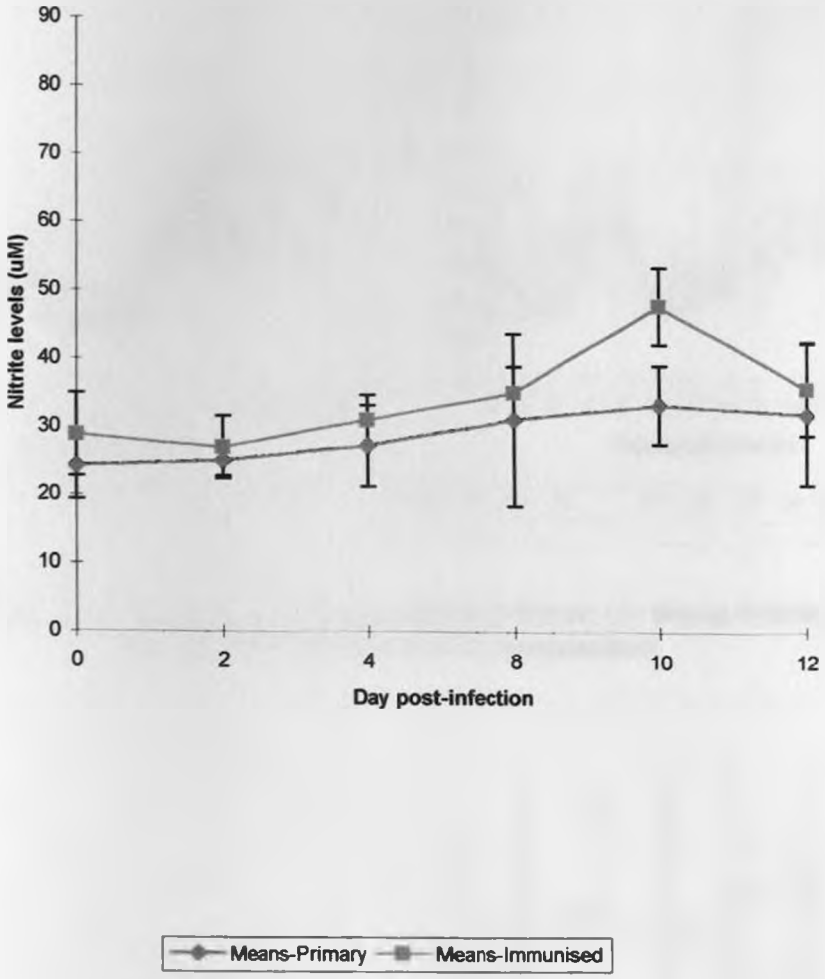
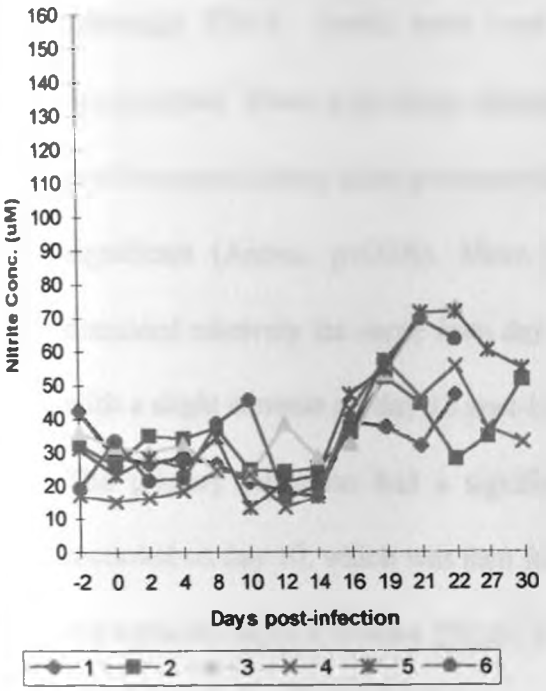
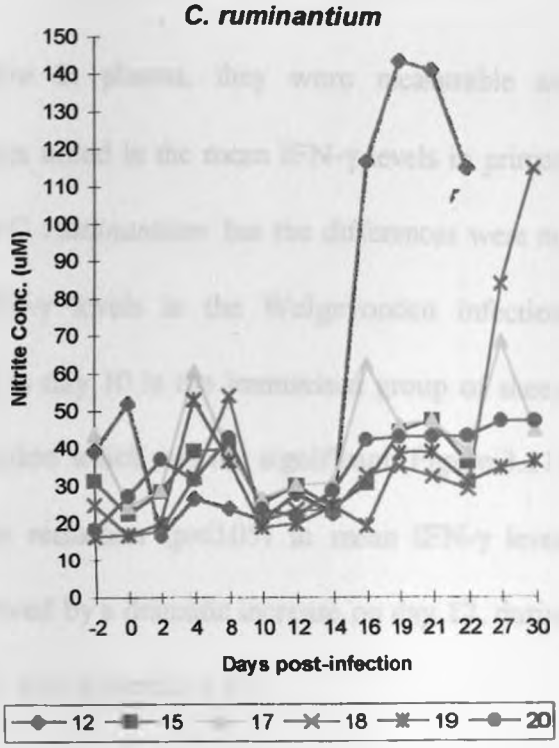


Fig. 3.10.

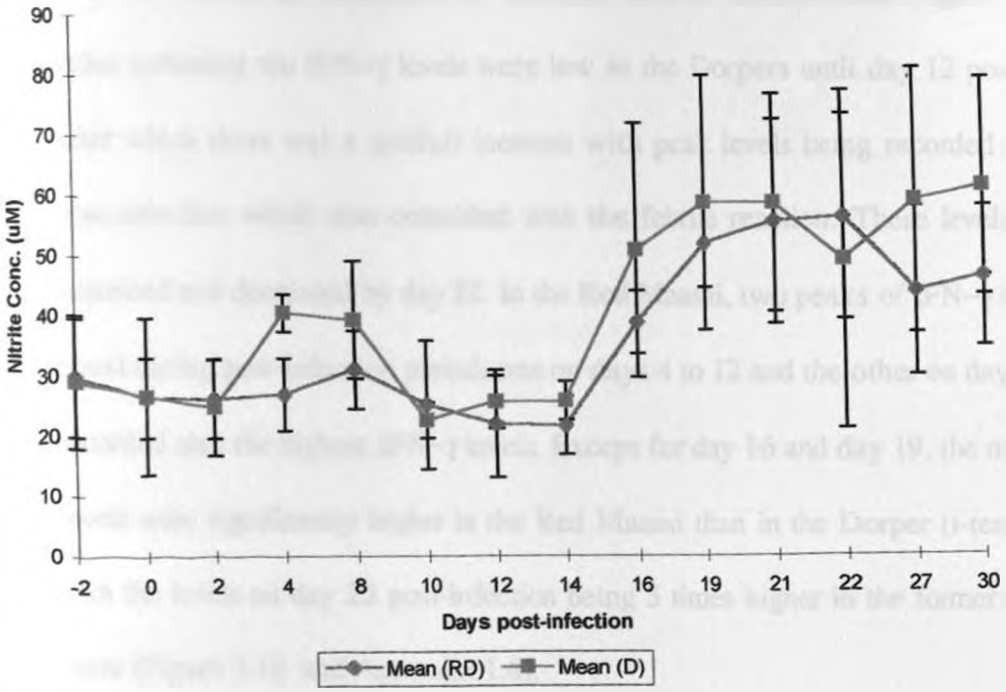
A. Nitrite levels in individual Red Maasai sheep Nos. 1- 6 infected with the Kathiani isolate of *C. ruminantium*



B. Nitrite levels in individual Dorper sheep Nos. 12,15, 17,18, 19 and 20 infected with the Kathiani isolte of *C. ruminantium*



C. Mean nitrite levels in Red Maasai (RD) and Dorper (D) Sheep infected with the Kathiani isolate of *C. ruminantium*



3.3.6. Cytokine assays

3.3.6.1. Interferon gamma (IFN–γ) levels

3.3.6.1.1. Welgevonden infections

Although IFN-γ levels were very low in plasma, they were measurable and reproducible. There were some differences noted in the mean IFN-γ levels in primary and immunised sheep after infection with *C. ruminantium* but the differences were not significant (Anova, p=0.06). Mean IFN–γ levels in the Welgevonden infections remained relatively the same from day 0 to day 10 in the immunised group of sheep, with a slight increase on day 12 post-infection which was not significant (Figure 3.11). The primary infections had a significant reduction ($p<0.05$) in mean IFN-γ levels recorded on day 10, which was then followed by a dramatic increase on day 12, during the terminal stages of disease (Figure 3.11 and Appendix 1.11).

3.3.6.1.2. Kathiani infections

Significant differences (t-test, $p<0.05$) were noted in the mean levels of IFN–γ in the Red Maasai and the Dorpers after infection with *C. ruminantium* (Figure 3.12). OD value reflecting the IFN–γ levels were low in the Dorpers until day 12 post-infection after which there was a gradual increase with peak levels being recorded on day 19 post-infection which also coincided with the febrile reaction. These levels were not sustained and decreased by day 22. In the Red Maasai, two peaks of IFN–γ levels were noted during post-infection period, one on days 4 to 12 and the other on day 22, which recorded also the highest IFN–γ levels. Except for day 16 and day 19, the mean IFN–γ levels were significantly higher in the Red Maasai than in the Dorper (t-test, $p<0.05$), with the levels on day 22 post-infection being 5 times higher in the former than in the latter (Figure 3.12 and Appendix 1.8).

Fig. 3.11.

Mean IFN-γ levels in primary and immunised sheep infected with the Welgevonden isolate of *C. ruminantium*

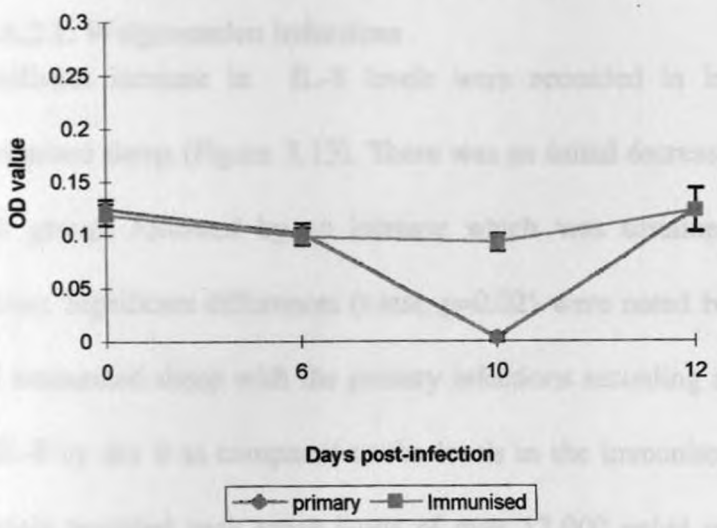
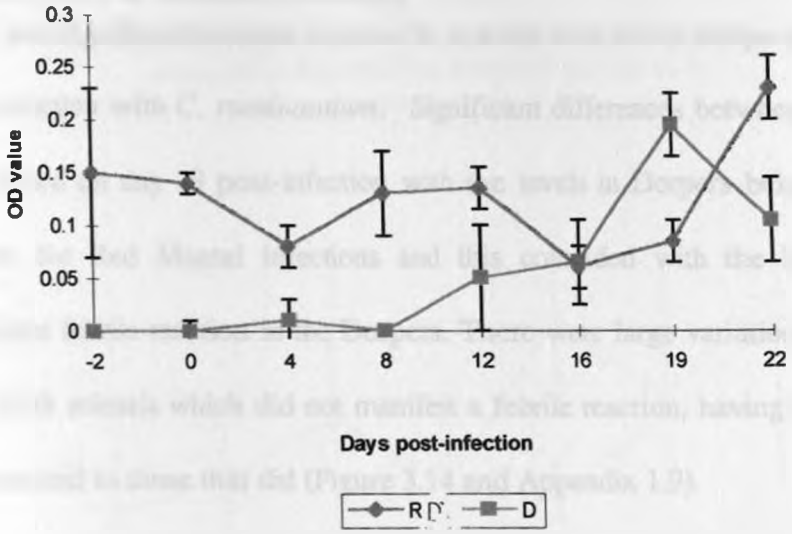


Fig. 3.12.

Mean IFN-γ levels in Red Maasai (RD) and Dorper (D) sheep infected with the Kathiiani isolate of *C. ruminantium*



3.3.6.2. Interleukin 8 (IL-8) levels

3.3.6.2.1. Welgevonden infections

Significant increase in IL-8 levels were recorded in infections of primary and immunised sheep (Figure 3.13). There was an initial decrease recorded on day four in both groups followed by an increase which was sustained through the course of disease. Significant differences (t-test, p=0.02) were noted between infections in naive and immunised sheep with the primary infections recording a dramatic 3 fold increase in IL-8 by day 6 as compared to the levels in the immunised infections. The primary animals recorded peak mean levels of over 17,000 pg/ml on days 8 and 10 , which were about two fold the mean peak level recorded in the immunised group on day 10, of about 9,000 pg/ml (Figure 3.13 and Appendix 1.10).

3.3.6.2.2. IL-8 in Kathiani infections

There was significant increase in mean IL-8 levels in both the Dorpers and Red Maasai after infection with *C. ruminantium*. Significant differences between the two groups were noted on day 19 post-infection with the levels in Dorpers being 3 times higher than in the Red Maasai infections and this coincided with the highest and most consistent febrile reaction in the Dorpers. There were large variations in the levels of IL-8, with animals which did not manifest a febrile reaction, having lower IL-8 levels as compared to those that did (Figure 3.14 and Appendix 1.9).

Fig. 3.13.

Mean IL-8 levels in primary and immunised sheep infected with the Welgevonden isolate of *C. ruminantium*

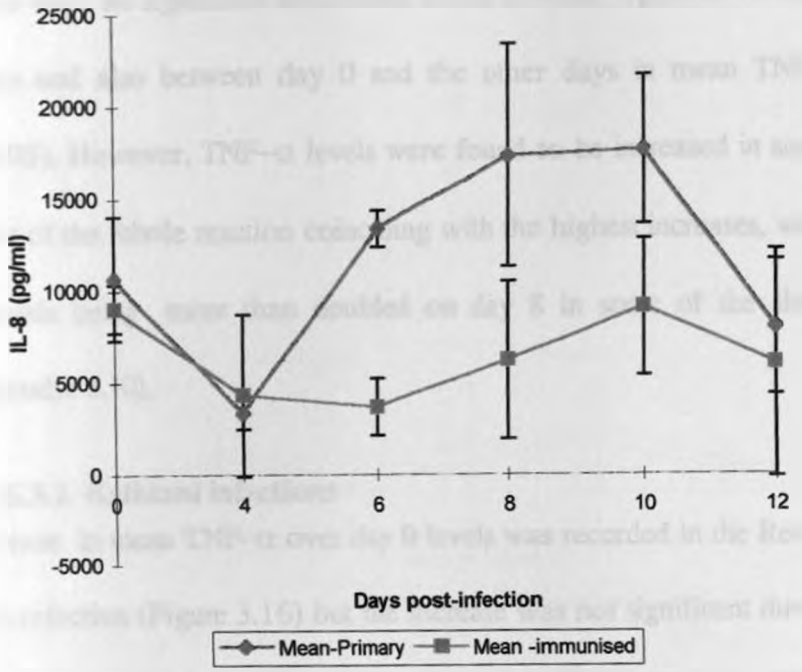
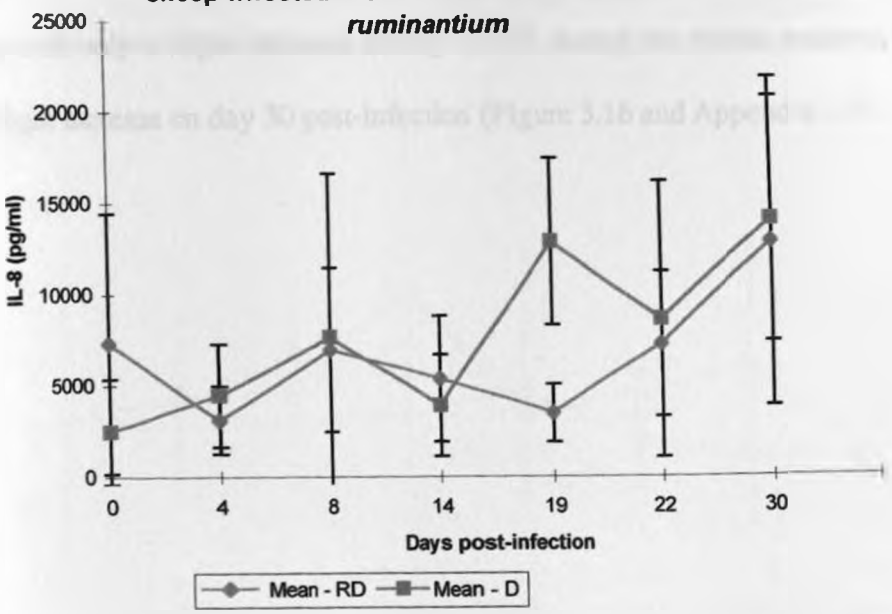


Fig. 3.14.

Mean IL-8 levels in Red Maasai (RD) and Dorper (D) sheep infected with the Kathiani isolate of *C. ruminantium*



3.3.6.3. TNF–α

3.3.6.3.1. Welgevonden infections

There were no significant differences noted between infections of naive and immunised sheep and also between day 0 and the other days in mean TNF–α levels (t test, $p>0.05$). However, TNF–α levels were found to be increased in some sheep, with the onset of the febrile reaction coinciding with the highest increases, with the mean TNF–α levels being more than doubled on day 8 in some of the sheep (Fig 3.15 and Appendix 1.10).

3.3.6.3.2. Kathiani infections

Increase in mean TNF–α over day 0 levels was recorded in the Red Maasai on day 10 post-infection (Figure 3.16) but the increase was not significant due to wide individual variations, with the sheep which showed a febrile reaction showing the highest increase. These levels were not sustained and the mean levels were at the basal level by day 14. There were no significant changes noted following infection of the Dorper sheep, with only a slight decrease on day 19-22, during the febrile reaction, followed by a slight increase on day 30 post-infection (Figure 3.16 and Appendix 1.7).

Fig. 3.15.

Mean TNF-α in sheep infected with the Welgevonden isolate of *C. ruminantium*

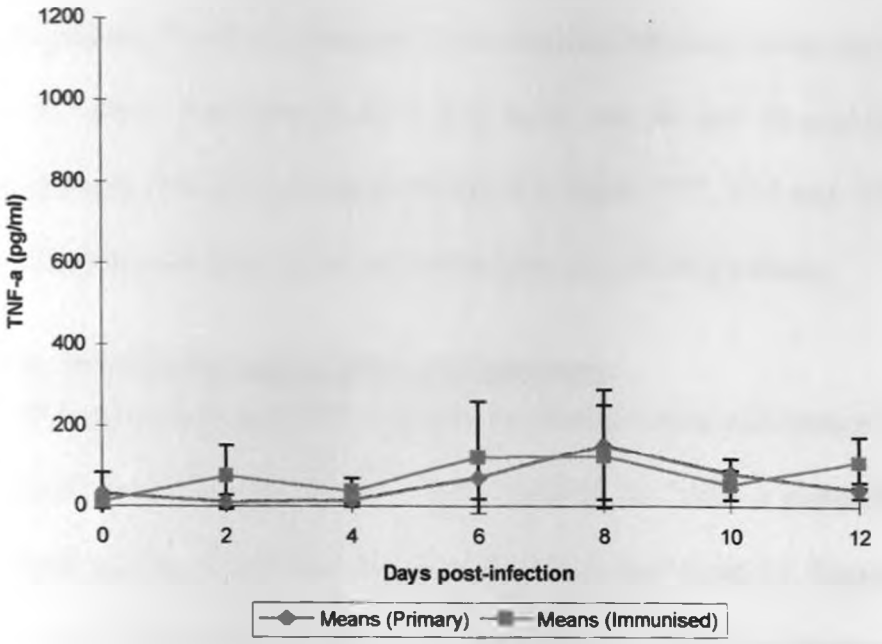
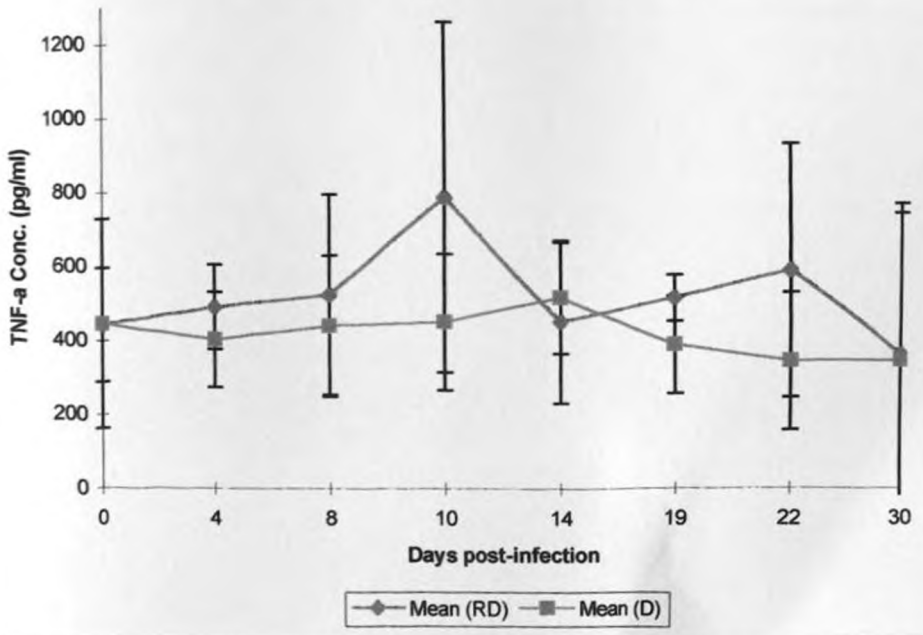


Fig. 3.16.

Mean TNF-a levels in Red Maasai (RD) and Dorper (D) sheep infected with the Welgevonden isolate of *C. ruminantium*



3.3.7. PCR results in Parasitaemia

Positive results were obtained with DNA extracted from Welgevonden infections early in the disease (day 4) as compared to the Kathiani infections where the first noticeable positive result, characterised by a faint band, was on day 10 post-infection in the Dorpers and 14 in the Red Maasai (Table 3.3, Figure 3.17, 3.18 and 3.19). This work was, however, not conclusive and served more as a learning process.

3.3.8. RT-PCR for expression of Cytokines

RT PCR performed using RNA from primary Welgevonden infections of sheep showed increased expression of TNF–α, IL-4 AND IL-10. TNF–α expression was more prominent on day 7 post-infection as compared to days 4 and 11. Expression of IL-10 was found to be more prominent on days 4 and 11 whereas expression of IL-4 was more prominent on day 7 (Table 3.4, Figure 3.19 and 3.20). However, non-specific bands in the gels made it very difficult to the validity of these results and this part of work, as for the one described in section 3.3.7., served more as a learning procedure.

Table 3.3.

Data showing PCR results of DNA extracted from plasma of sheep infected with both the Welgevonden and Kathiani isolates of *C. ruminantium*. Positive results (P) showed amplification of a 388 bp product and these results were visualised in agarose gel. Positive results were noted in the Welgevonden infections from day 4 while in the Kathiani infections, the first positive results were noted on day 10 in the Dorpers and day 14 in the Red Maasai.

	PCR results					
Sheep No	Day 0	Day 4	Day 8	Day 10	Day 12	Day 14
66	-	+	+	+	+	D
67	-	+	+	+	+	D
68	-	+	+	+	+	D
69	-	-	-	+	-	D
70	ND	ND	ND	ND	ND	ND
71	ND	ND	ND	ND	ND	ND
72	ND	ND	ND	ND	ND	ND
73	ND	ND	ND	ND	ND	ND
74	ND	ND	ND	ND	ND	ND
75	ND	ND	ND	ND	ND	ND
1	ND	ND	ND	ND	ND	ND
2	ND	ND	ND	ND	ND	ND
3	-	-	-	-	-	-
4	-	-	-	-	-	-
5	ND	ND	ND	ND	ND	ND
6	-	-	-	-	-	+
12	-	-	-	-	+	+
15	-	-	-	-	-	+
17	-	-	-	+	-	-
18	ND	ND	ND	N	ND	ND
19	ND	ND	ND	ND	ND	ND
20	ND	ND	ND	ND	ND	ND

Key:
- = Negative
+ = Positive
ND = not done
D = Animal had died

Fig. 3.17.
Agarose gel of PCR products of DNA extracted from plasma of sheep infected with the Welgevonden isolate of *C. ruminantium* and compared with sheep infected with the Kathiani isolate of *C. ruminantium*. DNA extracted from known levels of *C. ruminantium* elementary bodies (EBs) at different dilutions was used as positive control and to give an indication of the level of rickettsaemia. The results showed amplification of a 388bp product in the Welgevonden infections at a much earlier stage of the disease (day 8) but not in the Kathiani DNA.

A. Upper gel:
Lanes:
1 - KB ladder
2 - 10,000 EBs
3 - 5,000 EBs
4 - 2,500 EBs
5 - 1000 EBs - cut off point
6 - 500 EBs
7 - 100 EBs
8 - 46 EBs
9 - negative control (distilled water)

Lower gel
Lanes:
1 - KB ladder
2 - Welgevonden infection Day 0 (sheep No. 66)
3 - Welgevonden infection Day 0 (sheep No. 67)
4 - Welgevonden infection Day 0 (sheep No. 68)
5 - Kathiani (Dorper) infection Day 0 (sheep No. 12)
6 - Kathiani (Dorper) infection Day 0 (sheep No. 15)
7 - Kathiani (Dorper) infection Day 0 (sheep No. 17)

B: Lanes:
1. - KB ladder
2. - Positive control
3 - Welgevonden infection Day 8 (sheep No. 66)
4 - Welgevonden infection Day 8 (sheep No. 67)
5 - Welgevonden infection Day 8 (sheep No. 68)
6 - Kathiani (Dorper) infection Day 8 (sheep No. 12)
7 - Kathiani (Dorper) infection Day 8 (sheep No. 15)
8. - negative control(distilled water)

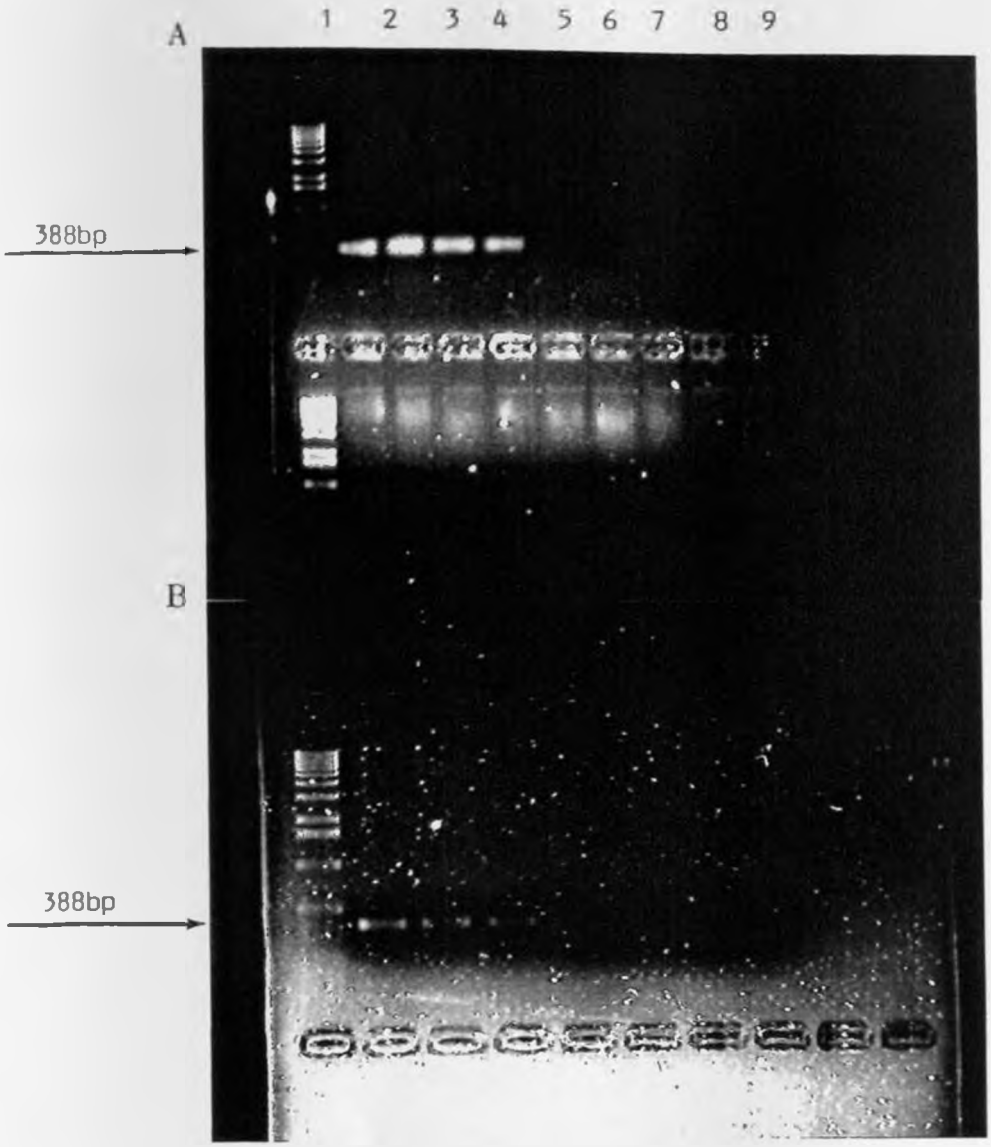

A
1 2 3 4 5 6 7 8 9
388bp
B
388bp

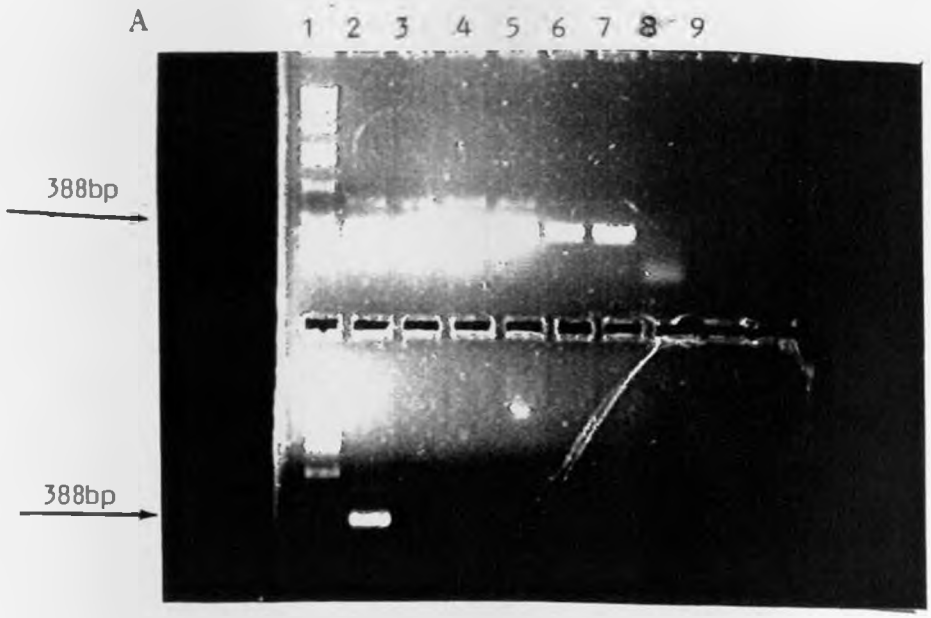
Fig. 3.18.
Agarose gel of PCR products of DNA extracted from plasma of sheep infected with the Welgevonden isolate of *C. ruminantium* and compared with sheep infected with the Kathiani isolate of *C. ruminantium*. DNA extracted from known levels of *C. ruminantium* elementary bodies (EBs) at different dilutions was used as positive control and to give an indication of the level of rickettsaemia. The results showed amplification of a 388bp product in the Welgevonden DNA on days 12 (A) and 4 (B) but not in the Kathiani DNA.

A. Upper gel:
Lanes:
1 - KB ladder
2 - 10,000 EBs
3 - 5,000 EBs
4 - 2,500 EBs
5 - 1000 EBs
6 - 500 EBs
7 - 100 EBs - cut off point
8 - 46 EBs
9 - negative control (distilled water)

Lower gel
Lanes:
1 - KB ladder
2 - Welgevonden infection Day 12 (sheep No. 66)
3 - Welgevonden infection Day 12 (sheep No. 67)
4 - Kathiani (Dorper) infection Day 12 (sheep No. 12)
5 - Kathiani (Dorper) infection Day 12 (sheep No. 15)
6 - Welgevonden infection Day 0 (sheep N0.66)
7 - Kathiani (Dorper) infection Day 0 (sheep No. 12)

B:
1 - KB ladder
2 - negative control (distilled water)
3 -Welgevonden infection Day 4 (sheep No. 66)
4 - Welgevonden infection Day 4 (sheep No. 67)
5 - Welgevonden infection Day 4 (sheep No 68)
6 - Kathiani (Dorper) infection Day 4 (sheep No. 12)
7 - Kathiani (Dorper) Day 4 (sheep No. 15)
8 - Kathiani (Dorper) Day 4 (sheep No. 17)

A



B

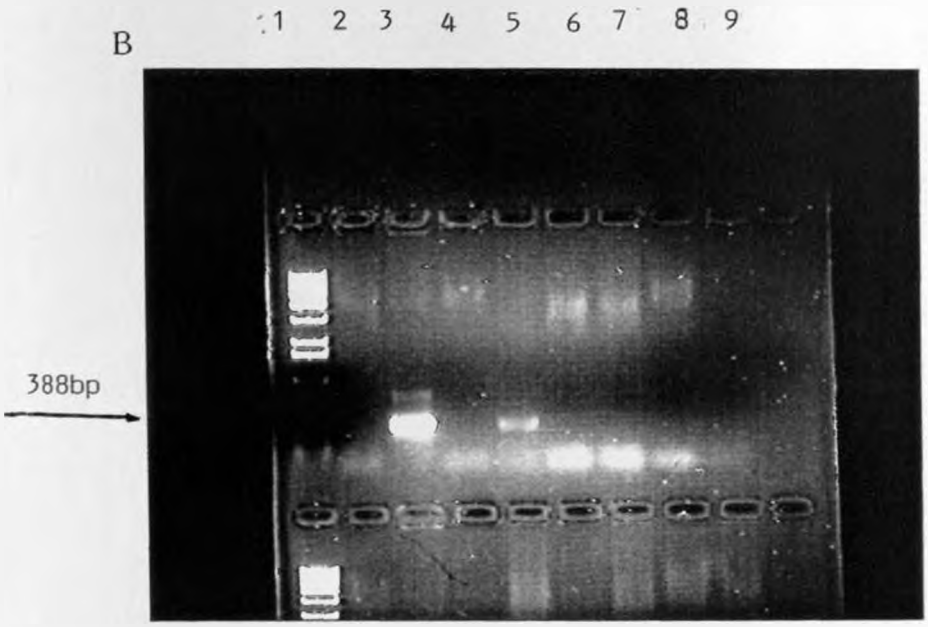
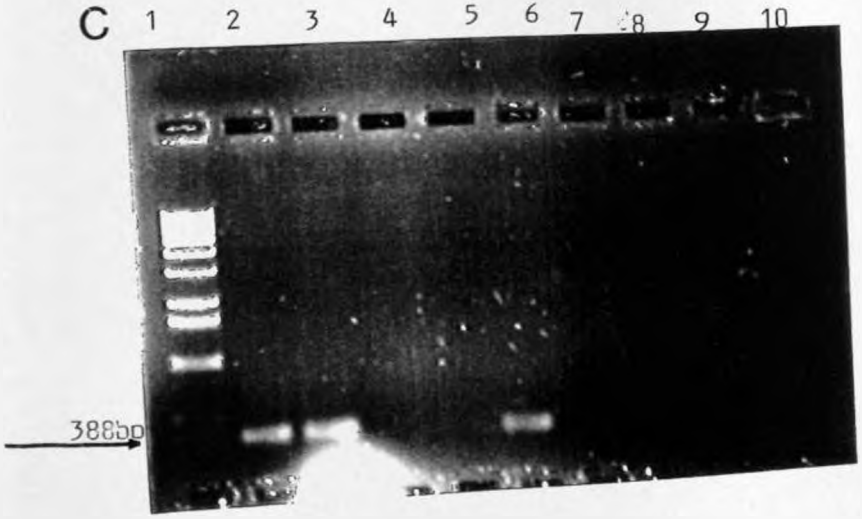


Fig. 3.19 . Agarose gel of PCR products of DNA extracted from plasma of sheep infected with the Welgevonden isolate of *C. ruminantium* and compared with sheep infected with the Kathiani isolate of *C. ruminantium*. DNA extracted from known levels of *C. ruminantium* elementary bodies (EBs) at different dilutions was used as positive control and to give an indication of the level of rickettsaemia. The results showed amplification of a 388bp product in the Welgevonden and Kathiani DNA on days 10 (A), 12 (B) and 14 (C) (Kathiani infections only). There was indication of higher levels of *C. ruminantium* DNA in the Welgevonden infections.

A. Lanes:
1 - KB ladder
2 - 10,000 EBs
3 - 5,000 EBs
4 - 2,500 EBs
5 - 1000 EBs
6 - 500 EBs
7 - 100 EBs - cut off point
8 - 46 EBs
9 - negative control (distilled water)
Lower gel
Lanes:
1 - KB ladder
2 - Welgevonden infection Day 10 (sheep No. 66 - immunised)
3 - Welgevonden infection Day 10 (sheep No. 67 -primary)
4 - Welgevonden infection Day 10 (sheep No. 68- primary)
5 - Welgevonden infection Day 10 (sheep No. 69- immunised)
6 - Kathiani (Dorper) infection Day 10 (sheep No. 12)
7 - Kathiani (Dorper) infection Day 10 (sheep No. 15)
8 - Kathiani (Dorper) infection Day 10 (sheep No. 17)
9 - Kathiani (Dorper) infection Day 10 (sheep No. 18)
B:
Lane:
1 - KB ladder
2 - negative control (distilled water)
3 - Kathiani (Dorper) infection Day 12 (sheep No. 12)
4 - Kathiani (Dorper) infection Day 12 (sheep No. 15)
6 - Welgevonden infection Day 12 (sheep No. 66)
C:
Lane:
1. - Marker
2. - Kathiani (Dorper) infection day 14 (sheep No. 12)
3. - Kathiani (Dorper) infection Day 14 (sheep No. 15)
4. - Kathiani (Dorper) infection Day 14 (sheep No. 18)
5. - Kathiani (Dorper) infection Day 14 (sheep No. 3)
6. - Kathiani (Dorper) infection Day 14(sheep No. 6)
7. - negative control (negative plasma)


388bp


C
1
2
3
4
5
6
7
8
9
10
388bp

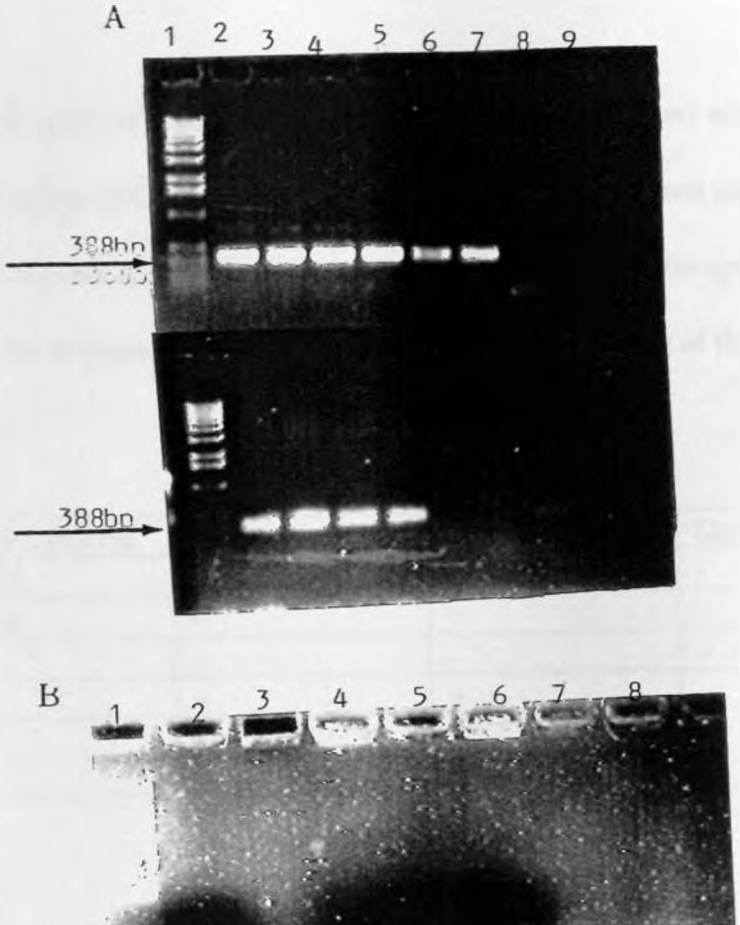

A
1 2 3 4 5 6 7 8 9
388bp
388bp
B
1 2 3 4 5 6 7 8

Table 3.4.
RT-PCR results performed using RNA extracted from sheep infected with the Welgevonden isolate of *C. ruminantium*. The cDNA was amplified using TNF–α-a , IL-4 and IL-10 primers. The results were not conclusive due to non-specific signals and possibly due to degradation of DNA as shown by the smearing of the gel (Fig. 3.20 and 3.21)

Sheep No and Cytokine	Day 4	Day 7	Day 11
222 -TNF–α-a	+	-	-
225 -TNF–α-a	-	+	-
222-IL-4	+	-	+
225-IL-4	+	+	-
222-IL-10	-	-	-
225-IL-10	+	-	-

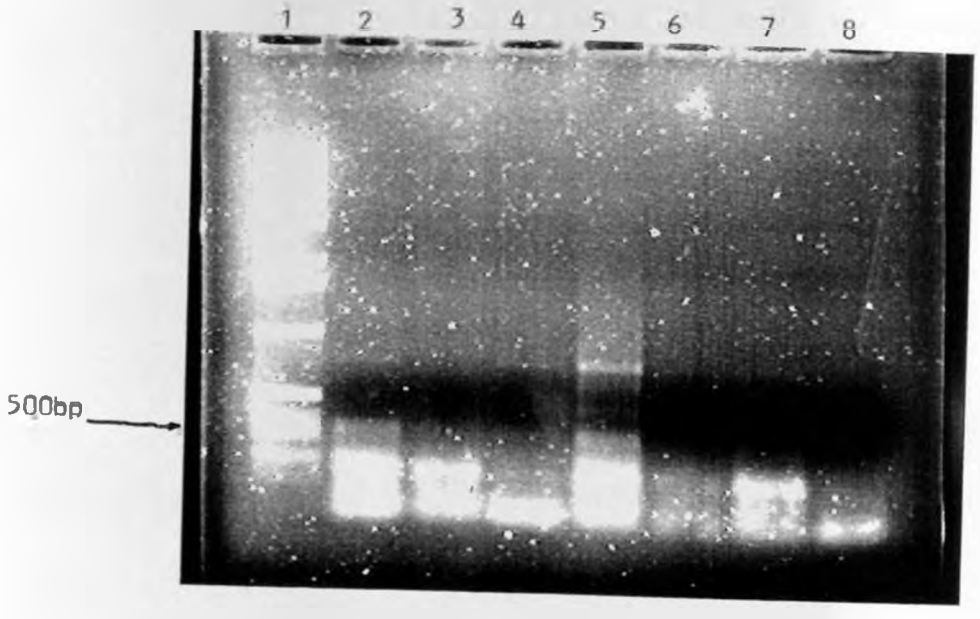
Key:
- = Negative
+ = Positive

Fig. 3.20.

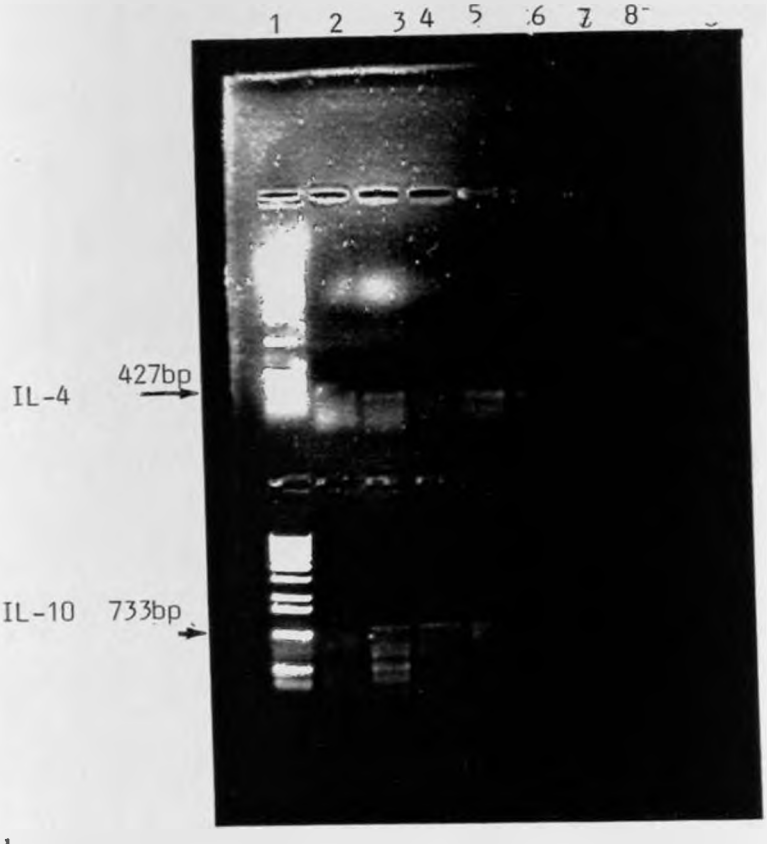
RT-PCR using RNA extracted from sheep infected with *C. ruminantium* on days 4, 7 and 11 post-infection. The cDNA was amplified with TNF-a primers (500 bp). Positive results were obtained on days 4 and 7 but not on day 11.

Lanes:

1. - Marker
2. - Sheep No. 222 Day 4
3. - Sheep No. 225 Day 4
4. - Sheep No. 222 Day 7
5. - Sheep No. 225 Day 7
6. - Sheep No. 222 Day 11
7. - Sheep No. 225 Day 11
8. - negative control



1. **Fig. 3.21**
RT-PCR using RNA extracted from sheep infected with *C. ruminantium* on days 4, 7 and 11 post-infection. The cDNA was amplified with IL-4 (427 bp) and IL-10 (733bp) primers. Positive results were obtained on days 4 and 7 but not on day 11 post-infection for IL-4 and on day 4 and 11 but not 7 post-infection for IL-10. post-infection.



A:
Lanes:
1. - Marker
2. - Sheep No. 222 Day 4
3. - Sheep No. 225 Day 4
4. - Sheep No. 222 Day 7
5. - Sheep No. 222 Day 11
6. - Sheep No. 225 Day 11
7. - negative control

B:
Lanes:
1. - Marker
2. - Sheep No. 222 Day 4
3. - Sheep No. 225 Day 4
4. - Sheep No. 222 Day 7
5. - Sheep No. 225 Day 11
6. - Sheep No. 225 Day 11
7. - negative control

3.4. DISCUSSION

The Welgevonden infections manifested an acute course of the disease characterised by short incubation periods (6-7 days), high temperature reactions, pronounced respiratory distress signs and also short course of disease with a mortality rate of 100%. There were no significant differences between primary infections and those immunised with the recombinant antigen, in terms of incubation period and course of disease (Table 3.2), although the infection rates in the brain endothelial cells were found to be significantly higher in the primary as compared to the immunised sheep (Kibor, 1997). The Kathiani infections in Dorper and Red Maasai showed a significantly longer incubation period ($p<0.05$) with a mean of 16 and 19 days respectively, and longer course of the disease (>10 days). Only 33% of the Red Maasai showed a febrile reaction while the remaining 67 % had no overt clinical signs. All Dorper sheep manifested a mild to acute form of the disease, with the fever as the main clinical sign. There were no mortalities recorded as a result of infection with the Kenyan strain, except four sheep which were sacrificed after fever onset. The short incubation periods and the high mortality rates in the Welgevonden infections may have been due to a dose effect, since $3x10^5$ EBs used in the Welgevonden infections resulted in a 7-8 day incubation period and death by day 12 as compared to the longer incubation periods and mild form of disease in the Kathiani infections where the infectious dose in the blood stabilate was not known.

Glutathione peroxidase (GPX) and superoxide dismutase (SOD) act as antioxidants and are involved in inactivation of reactive oxygen intermediates in the body (Flaherty and Weisfeldt, 1988). The levels of these enzymes were noted to increase significantly ($p<0.05$) after *C. ruminantium* infection. However, there were

differences noted in the trends and time at which each enzyme increased, and these differences could not be explained in this study. SOD was noted to increase early after infection (about day 2 post-infection) in both groups of sheep infected with the Welgevonden isolate of *C. ruminantium*, while the early SOD increase in the Kathiani infections was noted in the Dorpers but not in the Red Maasai. On the other hand, GPX levels remained relatively low early in the disease with increases occurring later (after day 6 post-infection).

One possible explanation for the increase in antioxidising enzymes may be that infection of animals with *C. ruminantium* results in activation of phagocytic cells leading to changes in cell aerobic metabolism. The process of generating ATP from glucose and oxygen by cytochrome oxidase leads to reduction of O_2 to oxidising radicals (reactive oxygen intermediates (ROI)) including superoxide, hydroxyl ions and glutathione peroxide which are harmful to the body (Grisham *et al.*, 1992). A positive feedback machinery may then be stimulated as part of the body defence mechanism leading to release of the relevant antioxidising enzymes, as seen in this study. The release of oxidising radicals, has been reported by several authors to be part of the body defence mechanisms to rid itself of pathogenic organisms and tumour cells, a situation which may also increase the inflammatory response and therefore the pathology (Wong *et al.*, 1988a; Yamzuhin *et al.*, 1989; Mathews *et al.*, 1987). Induction of SOD by TNF–α was reported to be a possible protective mechanism in the body to rid itself of pathogens and tumour cells (Wong *et al.*, 1988b; Yamzuhin *et al.*, 1989). Mathews *et al.* (1987) reported tumour cell killing by TNF–α was inhibited by anaerobic conditions, free-radical scavengers and inhibitors of arachidonic metabolism.

The indirect indication of increase in ROI may also be linked to the high nitrite levels, indicative of NO, noted in all the *C. ruminantium* infected animals. Higher levels of nitrite indicative of NO were recorded in all the sheep after infection with *C. ruminantium*, with the highest levels coinciding with the febrile reaction. It was also of utmost interest to note that animals previously exposed to *C. ruminantium* antigens (the group immunised with a recombinant antigen and the Red Maasai) showed earlier production of nitrite levels as compared to that recorded in primary infections with the maximal levels being attained on day 10 post-infection in the Welgevonden isolate infections and day 19 post-infection in the Kathiani infected sheep. This may implicate nitric oxide as being involved in, or released as a result of the immune responses to *C. ruminantium* infections and this issue is addressed by the *in vitro* studies described in chapter 4.

Mean IFN–γ levels were found to be increased after infection in Dorper sheep and the Red Maasai, with the latter recording IFN-γ levels five times higher than the susceptible sheep, but this increase in the latter was possibly not due to infection as the levels were high even prior to infection. The increased IFN–γ levels were found to correspond to increased nitrite levels indicating a possible link between the two. Anti-microbial effects of IFN–γ through induction of NO have been reported by several workers (Lane *et al.*, 1994; Sequin *et al.*, 1994;. Oswald *et al.*, 1994; Migliorin *et al.*, 1991); for instance, involvement of IFN–γ and CD8+ T cells in the induction of nitric oxide synthase has been shown to be protective against *Plasmodium berghei* infections in mice (Sequin *et al.*, 1994). IFN–γ elevation in infection of immunised sheep as compared to primary infections points to the already documented role of cell-mediated immunity in *C. ruminantium* infection in both sheep and mice (Du Plessis *et al.*, 1992;

Mahan *et al.*, 1996). IFN–γ also activates B cells to increase antibody production and induces MHC class II molecules. The killing of organisms by IFN–γ activated macrophages, an important defence mechanism in cell-mediated immunity, has been said to involve both ROI and reactive nitrogen intermediates (RNI) but the extent of the contributions of ROI and RNI may differ from one pathogen to another and one host to another (Adams *et al.*, 1991; Barbior *et al.*, 1975; Miyago *et al.*, 1997). IFN–γ activated macrophages were shown to induce bactericidal activity involving NO and superoxide anion and this killing was found to be inhibited significantly by the antioxidants, SOD and catalase (Miyago *et al.*, 1997). This may imply a similar mechanins is being induced in *C. ruminantium* infected animals in this study, as part of the host defence mechanism.

The produced ROI and RNI may also lead to increased pathology due to the destructive nature of some of their products including peroxynitrite, which is also a potent bactericidal agent (Miyago *et al.*, 1997). Since one mechanism that cells employ to kill parasites is the release of toxic molecules and oxygen radicals (James, 1991), these oxygen radicals released which include the superoxide ion O^{-}_{2}, hydrogen peroxide (H_2O_2), singlet oxygen (O2) and hydroxyl (.OH) radicals can damage cell membranes, unfold or inactivate proteins, degrade nucleic acids and kill cells and eventually parasites. It seems fitting, therefore, to speculate that in these studies, increase in antioxidation enzymes may be resulting in reduction of ROI produced as part of the body defence mechanism, and therefore leading to reduction in *C. ruminantium* killing. TNF–α has been reported to induce production of SOD (Wong *et al.*, 1988) but, although there was elevation of TNF–α after *C. ruminantium* infection, there was no positive correlation between the levels of TNF–α and SOD.

ROI and RNI play important roles in the immune and pathological mechanisms of some infections. Increased antioxidants in the sera of sheep infected with *C. ruminantium* may be a homeostatic response to counteract increased levels of oxidising radicals elevated as a response to *C. ruminantium* infection but this requires further research.

The sheep immunised with the recombinant antigen suffered an acute disease with a fatal outcome inspite of showing higher levels in IFN–γ, NO and reduced infection rate in brain endothelial cells. This may be due to the high virulence of the isolate and may imply that a compromise had to be reached to determine the outcome of a disease, and may be in this case the attributes of virulence outweighed the developing immune responses. The results here also have an indication of higher amounts of *C. ruminantium* DNA in the Welgevonden infections as compared to Kathiani infections, as shown by the size of the bands which were compared with bands obtained using DNA extracted from known amounts on *C. ruminantium* EBs propagated in tissue culture (positive controls), but the results were not conclusive.

Sheep infected with *C. ruminantium* had an elevation of IL-8 and TNF–α levels during infection. In the Welgevonden infections, levels of IL-8 increased gradually after infection, with the infection of naive animals recording significantly higher levels than the immunised animals and with these same animals showing higher infection rates in brain endothelial cells. There was a transient decrease in IL-8 on day 4 in all the infected animals and this was thought to be related to decreased absolute numbers of circulating neutrophils but not monocytes, as counted by Kibor (1997). This is in agreement with other reports in heartwater infections, where a neutropenia occurs during the incubation period (Abdel Rahim and Shommein, 1977), and where the only changes observed in monocytes have been increased evidence of activation

seen as vacuolation of the cells but no changes in the counts (Van Amstel *et al.*,1987). The levels of IL-8 in the Red Maasai remained relatively unchanged during the infection until day 30 when some increase was noted as compared to Dorpers where a significant increase was recorded between day 19 and day 30 corresponding to the febrile reaction and also occurring 3-5 days after there was evidence of infection in these sheep on PCR results.

TNF–α stimulates release of IL-8, among other cytokines. Increase in TNF–α preceded increase in IL-8 in *C. ruminantium* infected sheep. TNF–α also stimulates adhesion of EC, neutrophils, monocytes, eosinophils and basophils by increasing expression of adhesion molecules and increases microvascular permeability (Mathew *et al.*, 1987), one of the main pathological findings of *C. ruminantium* infection. IL-8 is chemotactic to both neutrophils and lymphocytes and induces tissue infiltration by both cells (Taub and Oppenheim, 1993) and also promotes generation of ROI radicals and platelet adhesion factor (Oppenheim *et al.*, 1991). The promotion of ROI production by IL-8 including increase in superoxide anions, may, therefore, explain the noted increase in antioxidation enzymes which may be a consequence of this. It is possible that in combination with the elevated NO levels seen in heartwater, this leads to the release of damaging peroxynitrite produced by reaction of superoxide and NO. Endogenously produced reactive oxygen metabolites also directly induce transcription and translation of IL-8, and therefore a positive feedback between IL-8 and ROI (Taub and Oppenheim, 1993). Increase in IL-8 later in the infection may be, in part, as a result of induction by increased levels in ROI, RNI and activation of macrophages. Increase in GPX and SOD, enzymes which protect against oxidative stress, was seen especially after fever onset. Increasing levels of enzymes involved in antioxidation may have a role in counteracting the cytotoxicity of activated macrophages, and therefore,

these could be mechanisms of cellular resistance to oxidative stress. IL-8 and TNF–α elevation during heartwater infection would therefore imply an increase in oxidising agents and the increase in SOD and GPX levels may then be geared towards protecting cells against oxygen radicals generated by the respiratory burst induced by increased IL-8. Thus, increased IL-8, antioxidising enzymes, TNF–α and nitrite may all be part of the immuno-pathological responses elicited by *C. ruminantium* infection.

Nitric oxide, once referred to as a vital poison inside the immune and inflammatory network, plays an important role in the initiation and development of various inflammatory and immune responses (Dugas *et al.*, 1995; Moncada and Higgs, 1995). An excess of NO production regulates micro-environmental molecule and cell toxicity, the production of pro-inflammatory mediators and induces cell death by apoptosis (Girard and Poitier, 1993). The factors necessary to induce iNOS expression differ between cell types, but a maximal inducing effect is generally obtained by the combination of cytokines and microbial or viral products. Cytokines generated by Th 2 cells reduce production of NO by macrophages while those generated by Th 1 cells (promoters of cellular host defences) induce NO production (Liew *et al.*, 1991a; 1991b). IL-4, IL-8 and IL-10 have been suggested to suppress iNOS expression either at transcriptional level or post-transcriptional level (Liew *et al.*, 1991a). This agrees with our findings in which primary infections had low levels of NO but high levels in IL-8.

Conclusion

This study has shown the possible involvement of reactive oxygen radicals, nitric oxide and cytokines including TNF–α, IL-8 and IFN–γ in the immune response and the pathogenesis of *C. ruminantium* infection. Considerable evidence from this study supported the idea that IFN-γ and NO are protective to *C. ruminantium* infection and may play a major role in cell mediated response in protecting animals against the disease, as seen in animals with prior exposure to *C. ruminantium* infection. Increased IFN-γ levels in immunised sheep infected with the Welgevonden isolate, however, did not alter the fatal outcome of the disease but there was noted reduction in the level of infection in brain endothelial cells in these sheep pointing to a mechanism which reduced dissemination of the infection. Higher levels in the nitrite levels in animals with prior exposure to *C. ruminantium* antigens was also noted with interest and investigated in the following chapter. The resistance observed in the Red Maasai could not be conclusively defined; it could have been due to previous exposure to *C. ruminantium* (acquired resistance), although the PCR results on day 0 were negative for the animal tested in this group making it possible that it may also have been breed-related and therefore innate resistance. However, the resistance in the Red Maasai requires further investigation using more numbers of animals and sheep which are serologically negative to *C. ruminantium* antigens at the beginning of the experiment. Since the cytokine levels were assayed in plasma, they may not reflect the true picture in their biological activities at specific target cells, but act more as indicators of these activities at tissue level. Also, the levels of IFN-γ were expressed as OD values but not real values. Use of standards of known units of IFN-γ can be incorporated in any future assays and the OD converted to units to counteract this.

CHAPTER 4

IN VITRO STUDIES OF *COWDRIA RUMINANTIUM* INFECTIONS IN BOVINE ENDOTHELIAL CELLS: INVOLVEMENT OF NITRIC OXIDE AND INTERFERON GAMMA IN ANTI-*COWDRIA* ACTIVITY

4.1. Introduction

The mechanisms underlying the pathogenesis and resistance to *Cowdria ruminantium* infection have not been fully established. The clinical manifestations and severity of the disease are mainly associated with severe respiratory, cardiac and nervous embarrassment due to increased capillary permeability leading to oedema (Clark, 1962). Whilst increased capillary permeability may explain the marked fall in blood and plasma volume which occur prior to death in heartwater (Clark, 1962.), the cause of such alterations remain obscure. The work reported here was designed to investigate the role of the association between *C. ruminantium* and endothelial cells in the pathogenesis of heartwater and the development of host resistance to this infection.

The approach was prompted by reports that nitric oxide (NO), a powerful vasodilator generated by the endothelium, is intimately related to mechanisms of oedema formation and hyperaemia (Moncada *et al.*, 1988; Moncada *et al.*, 1990; Furchgott and Zawadski, 1980). The multi-functional roles of NO have been been reported by several authors. These include the anti-microbial effects through killing of microorganisms (Adams *et al* 1990; 1991) and also the cytotoxic effects to host cells which contribute to disease pathology (Radi *et al.*, 1991). Cytokines including interferons have been reported to play an important role in resistance to intracellular organisms including *C. ruminantium* (Totte *et al.*, 1993 and 1994; Mahan *et al.*, 1996), *Rickettsia conorii* (Walker *et al.*, 1997), *Plasmodium berghei* (Sequin *et al.*, 1994), *Theileria annulata* (Visser *et al.*, 1995) and *Mycobacterium bovis* (Molloy *et al*, 1994) possibly by NO production.

This study was, therefore, designed to; monitor NO production in cultures of bovine pulmonary endothelial cells (BPEC) infected with *C. ruminantium;* assess the

effect of interferon gamma (IFN-γ) on NO production by BPEC, its effect on *C. ruminantium* and the endothelial cell; assess the effect of an exogenous source of NO on the viability and infectivity of *C. ruminantium* elementary bodies (EBs) to BPEC *in vitro*. Investigation of the effect of the inducible nitric oxide synthase inhibitor, L-NMMA was also carried out.

4.2. Materials and Methods

4.2.1. Nitric oxide production by infected endothelial cells; effect of NO on *C. ruminantium* infection.

A bovine pulmonary endothelial cell line (BPEC) and the Gardel isolate of *Cowdria ruminantium* were obtained and cultured as described previously (see sections 2.1.1. and 2.1.2.). The cells were grown on glass coverslips of about 13 mm in diameter in 4 x 24 well culture plates and infected at confluence with *C. ruminantium* elementary bodies (EBs) as described in section 2.1.4. Assessment of NO production from resting endothelial cells and infected endothelial cells; infected endothelial cells with different concentrations of BorIFN–γ (0, 25, 50, 75 and 100 U/ml) or the NO synthase inhibitor L-NMMA at 1 mM with or without BorIFN-γ as additives, was carried out using the Griess assay method described in section 2.2.1. and 2.2.2. The effects of BorIFN–γ on *C. ruminantium* viability was assessed using fluorescein diacetate staining method as described in sections 2.1.5, while the effect of BorIFN–γ on the endothelial cell viability was assessed using Diff quik and acridine orange staining methods, as described in section 2.1.6.1. The effects of different concentrations (0, 25 50 and 100μM) of an exogenous source of the NO donor molecule, SNAP, on infectivity and viability of *C. ruminantium* was assessed when *C. ruminantium* elementary bodies (EBs) were pre-treated with SNAP prior to infection of endothelial cells or incubated with infected cell

cultures as described in section 2.2.4. The time course effects of SNAP on *C. ruminantium* EBs was carried out by incubating different concentrations of SNAP with EBs in growth medium at 37°C and viability assessed after 4, 6, 24, 48, 72 and 96 hours post-treatment using the fluorescein diacetate staining method. Twelve replicates were done for each treatment.

4.2.2 Data analysis.

The student t-test, Mann Whitney, chi-square and Correlation Coefficient tests were used for statistical data analysis as appropriate and depending on distribution of the data, at 95% confidence intervals.

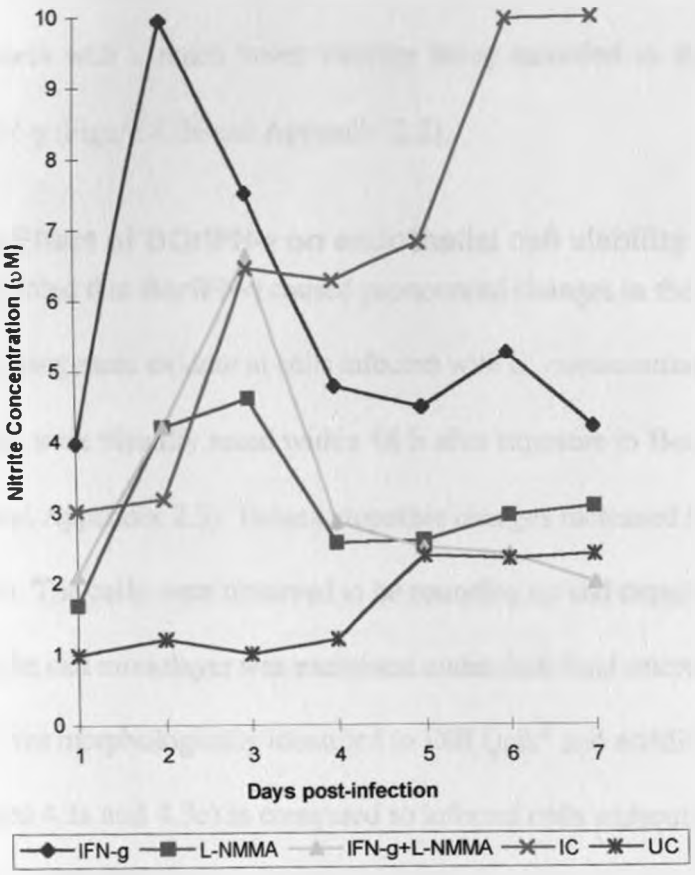
4.3. Results

4.3.1. Nitric oxide production by *C. ruminantium* infected BPEC.

NO levels measured at 24 h intervals revealed a gradual cumulative increase in BPEC infected with *C. ruminantium* and incubated in medium alone (Figure 4.1 and Appendix 2.1). The highest levels were noted at day 7 post infection. The highest NO concentration coincided with the period when the degree of infection was highest as observed in cytospin preparations. Treatment of *C. ruminantium* infected cultures with BorIFN-γ resulted in increased NO production with attainment of peak levels being recorded at 2 days post treatment (Figure 4.1). In the cultures supplemented with the L-arginine analogue L-NMMA, significant inhibition ($P < 0.05$) of NO production was recorded in the absence or presence of BorIFN-γ (Figure 4.1 and Appendix 2.1).

Fig. 4.1.

Nitrite Levels in *C. ruminantium*-infected bovine pulmonary endothelial cell cultures treated with BorIFN-γ and /or with L-NMMA, and infected (IC) and uninfected UC) controls



4.3.2. Effect of BorIFN-γ on *C. ruminantium* viability.

The proportion of viable EBs of *C. ruminantium* in cultures treated with different concentrations of BorIFN-γ was analysed at the end of the experiment. The percentage of viable EBs was significantly lower ($P < 0.05$) in the presence of 100, 75, 50 and 25 U/ml of BorIFN-γ than in the unexposed cultures. The reduced viability was dose dependent with a much lower viability being recorded in the higher concentrations of BorIFN-γ (Figure 4.2a and Appendix 2.2).

4.3.3. Effect of BOrIFN-γ on endothelial cell viability.

It was noted that BorIFN-γ caused pronounced changes in the endothelial cells with the effect being more evident in cells infected with *C. ruminantium*. Significant cellular changes were visually noted within 48 h after exposure to BorIFN-γ ($P < 0.05$)(Figure 4.2b and Appendix 2.3). These cytopathic changes increased in a dose - dependent fashion. The cells were observed to be rounding up and detaching from the culture plate when the cell monolayer was examined under dark-field microscopy. Apoptotic cell death was morphologically identified in Diff Quik^R and acridine orange stained cells (Figures 4.3a and 4.3c) as compared to infected cells without stimulation (control) (Figure 4.3b). The cells revealed nuclear fragmentation, chromatin condensation, indiscernible nuclear organelles, blebbing of the nuclear membrane and presence of dark-staining membrane-bound bodies. There was more evidence of apoptotic cell death in the BorIFN-γ exposed infected cultures (75%) than uninfected treated cultures (50%), where as in the absence of BorIFN-γ, there was an increase of only 5% in the percentage of non-viable cells in infected (12%) as compared to non-infected (7%).

Fig. 4.2a

Viability of *C. ruminantium* EBs in relation to nitrite (NO) levels in bovine endothelial cell cultures treated with different concentrations of BorIFN-γ

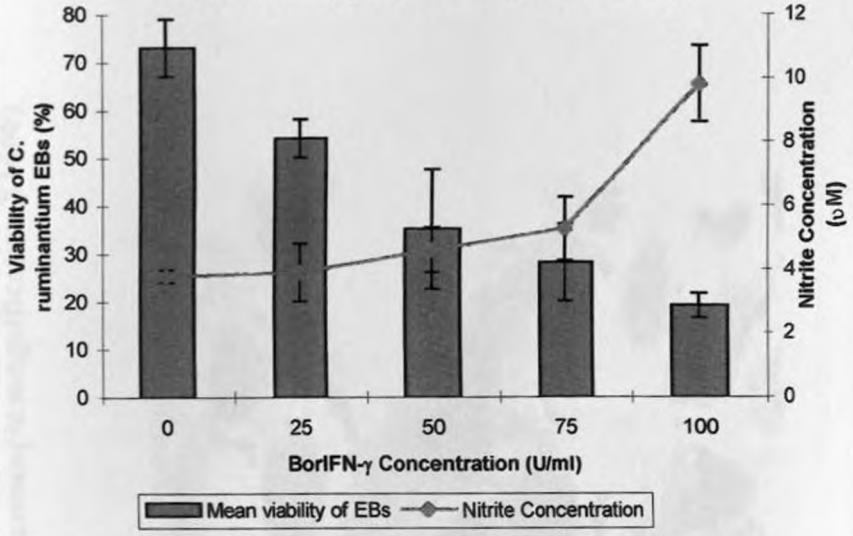


Fig. 4.2b

Cell death of bovine endothelial cells infected or not infected with *C. ruminantium* and treated with different concentrations of BorIFN-γ

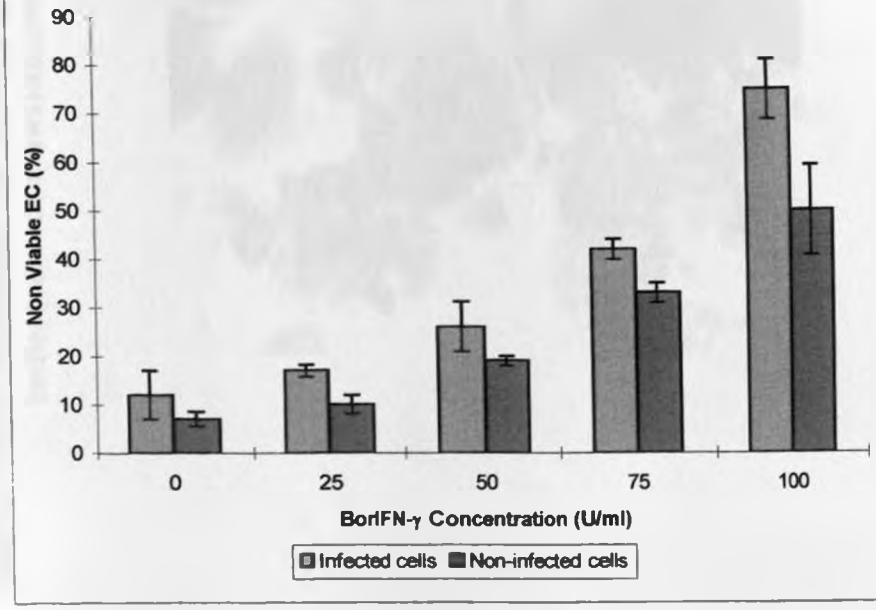


Figure 4.3a

Infected bovine endothelial cells showing apoptotic cell death; blebbing of cell membrane (arrowheads), apoptotic bodies (curved arrows) and nuclear fragmentation (microscopic magnification X1000)

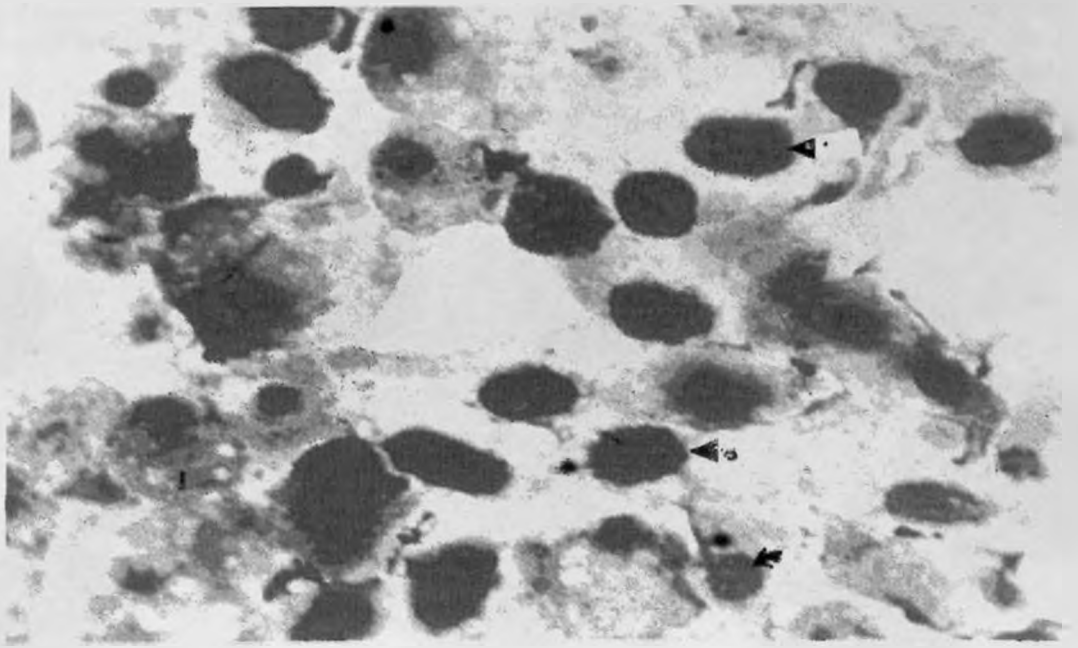


Figure 4.3b.
A photograph showing Giemsa-stained *C. ruminantium* infection of bovine endothelial cells (elementary bodies (EBs) - arrow) 7 days post-infection

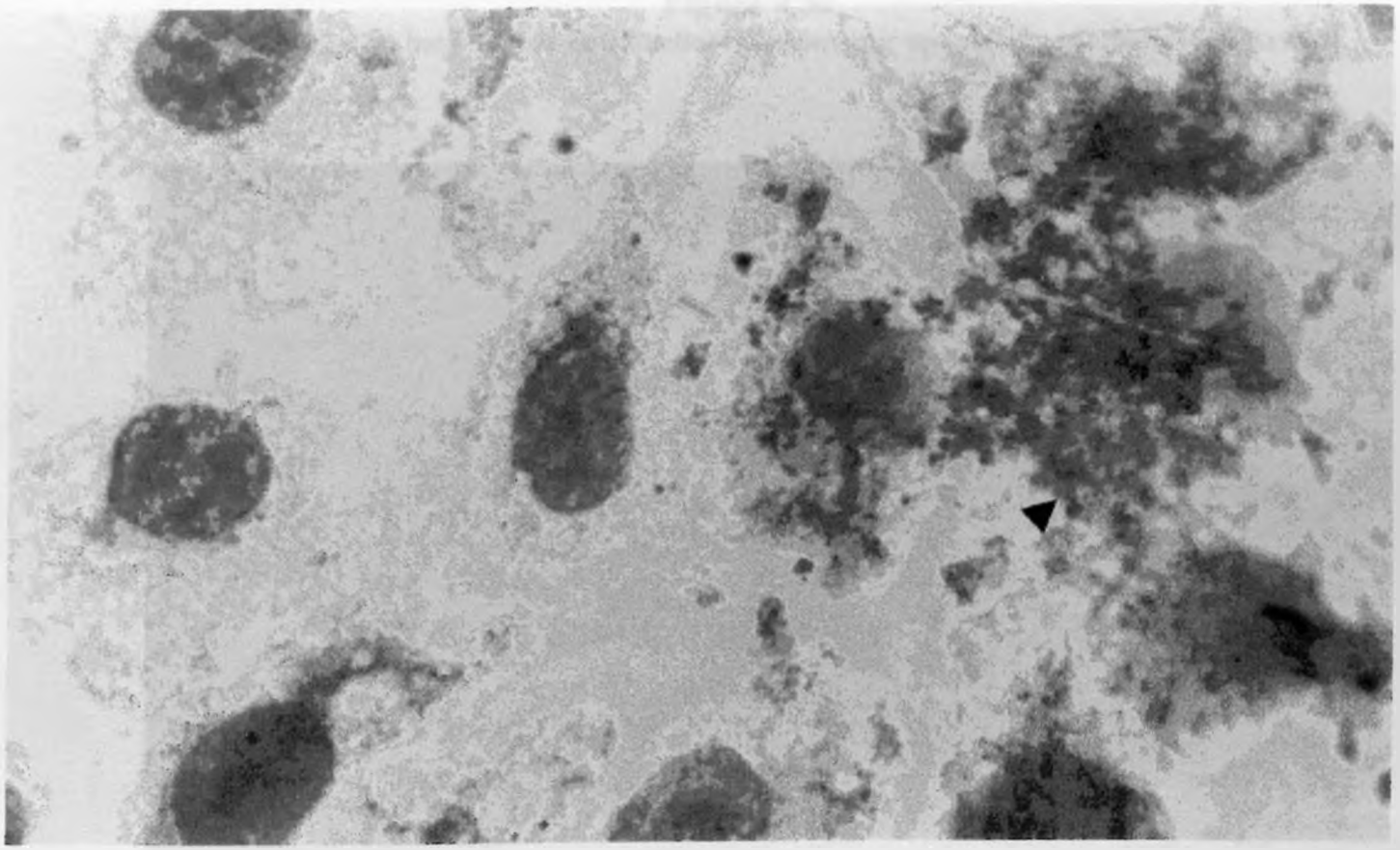
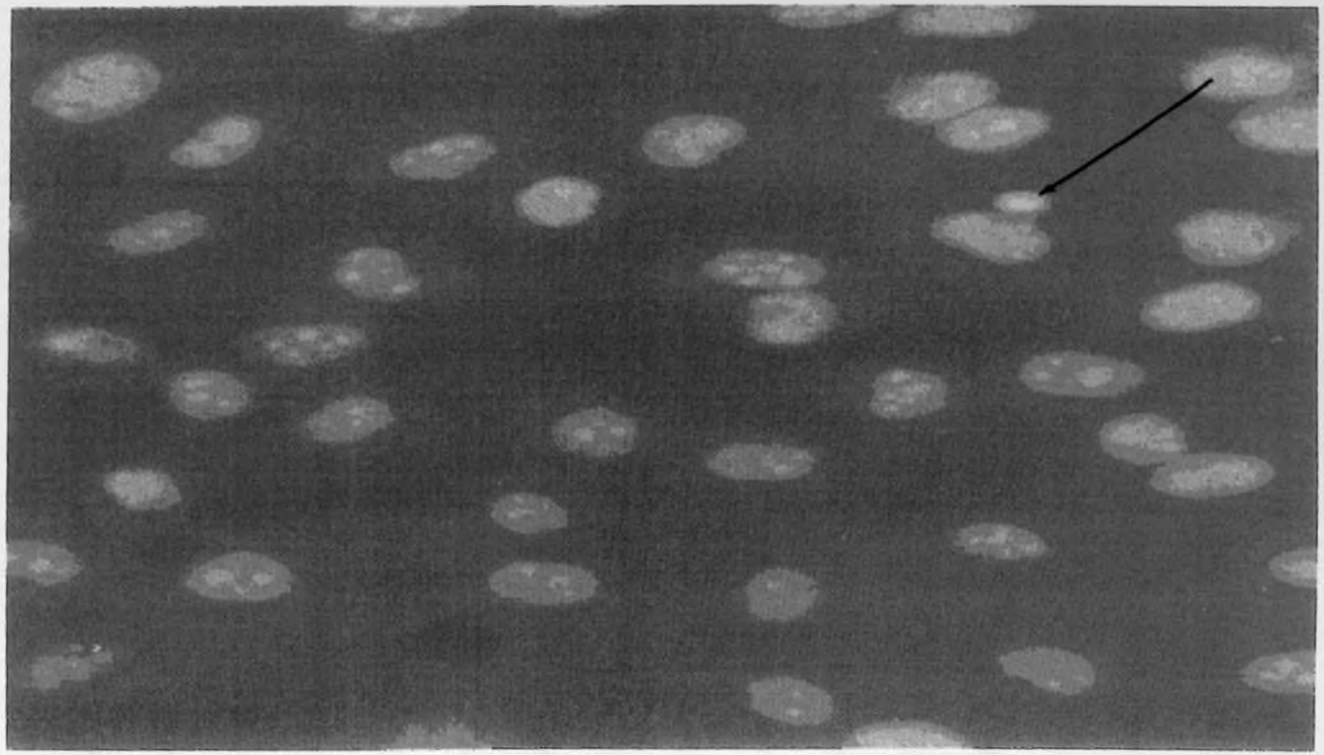


Figure 4.3c

Acridine orange stained bovine endothelial cells showing apoptotic endothelial cell (arrow).



4.3.4. Effect of pre-treatment of *C. ruminantium* EBs with SNAP-generated NO on infectivity.

Significant differences ($p<0.05$) were noted in the number of *C. ruminantium* colonies and the infection rate of endothelial cells at days 2 and 4 post-infection in cultures infected with SNAP pre-treated EBs as compared to cultures infected with non-treated EBs (Figure 4.4a and Figure 4.4b and Appendix 2.4). The number of fluorescing colonies in cultures which had been infected with SNAP pre-treated EBs was reduced as compared to the non-treated controls. This reduction was in a dose dependent manner with lower numbers of fluorescing colonies being recorded in cultures treated with higher concentrations of SNAP (Figure 4.4a). Higher numbers of colonies were recorded on day 4 as compared to day 2 post-infection indicating little or no effect of pre-treatment of EBs on the subsequent multiplication rate of the viable organisms in the cells (Figure 4.4a). The ratio of the number of fluoresceing colonies on day 4 to day 2 had a range of 2-3.4 in all treatments indicating almost similar rates of multiplication had occurred after cell entry (Figure 4.4a). The rate of infection, defined as the percentage of infected endothelial cells, was found to be closely related to the number of fluoresceing colonies, with a higher proportion of infected cells being recorded in cultures infected with EBs not treated with SNAP-generated NO and also on day 4 as compared to day 2 post-infection (Figure 4.4b). The organisms were noticeable by day 2 as small blue-staining closely packed morulae in giemsa stained cells and the morulae were noted to be increased in size and number by day 4 as compared to day 2.

Fig. 4.4a

The mean number of fluoresceing foci in endothelial cells, reflective of infectivity of *C. ruminantium* organisms pre-treated with different concentrations of SNAP

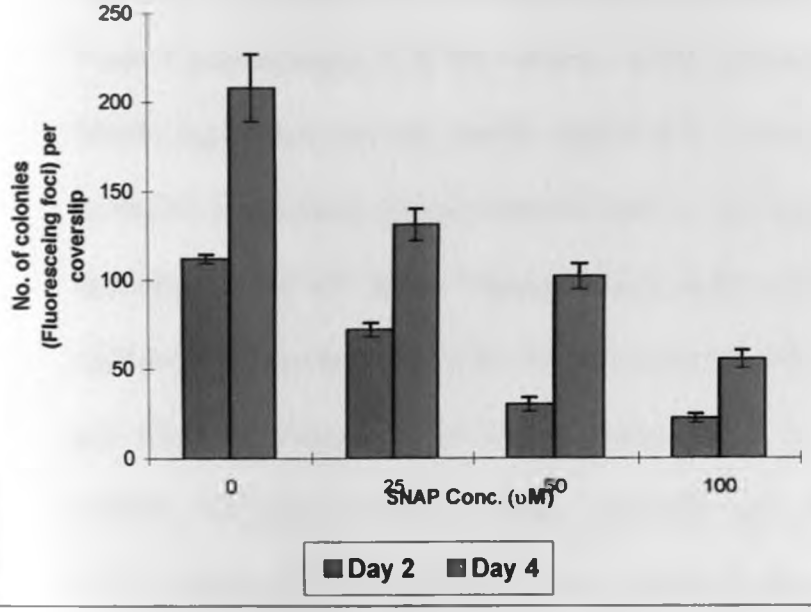
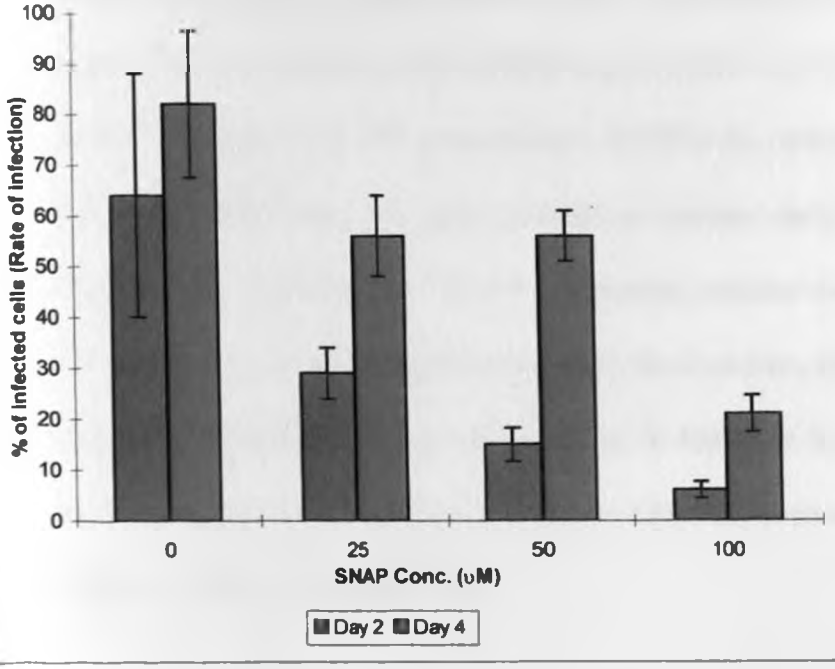


Fig. 4.4b

Rate of infection (%) of endothelial cells assessed on day 2 and 4 post-infection with *C. ruminantium* elementary bodies pre-treated with different concentrations of SNAP



4.3.5. Effect of co-culturing SNAP with *C. ruminantium*-infected cells on the rickettsial viability and growth.

Viability of EBs was assessed in supernatants overlying the cell monolayer on days 1, 2, 4 and 7 post-infection in all the cultures. In the cultures incubated in the presence of SNAP, significantly reduced viability counts of *C. ruminantium* EBs were recorded as compared to untreated control cultures (Table 4.1 and Appendix 2.4). The viability was dose-related with the highest viability counts being recorded in the control cultures without SNAP as compared to the lowest counts recorded in cultures treated with 100 μM SNAP with similar trends being recorded for all the days assessed (Table 4.1). Viability was also assessed in EBs incubated with medium containing different concentrations of SNAP alone the time required for 50% reduction in viability. Time required for 50% reduction in viability of EBs was about 10.5 h in 100 μm SNAP, 24 h in 50 μM of SNAP and 60 hours in 25 μM SNAP.

Cytospin smears prepared on day 7 post-infection showed the number of *C. ruminantium* colonies per field was negatively correlated to the level of SNAP ($r = -0.96$) but was more or less a reflection of the viability counts with a very high correlation coefficient ($r=0.97$). (Figure 4.5a). The concentration of NO in the culture medium did not decline during the experiment, but rather showed an increase during the period of infection (Figure 4.5b). Although the SNAP - containing cultures had higher cumulative final nitrite levels as a result of the NO released by this molecule, the rate of increase of nitrite during the period of infection was found to be higher in heavily infected cultures i.e. those which had been incubated without or with low levels of SNAP, as compared to lightly infected ones. (Figure 4.5a).

Table 4.1. Effect of co-culturing SNAP with *C. ruminantium*-infected cells on the rickettsial viability. Mean viability counts of *C. ruminantium* EBs was assessed on days 1, 2, 4 and 7 post-infection in cultures incubated with different concentrations of SNAP and expressed as percentages +/- standard deviation (SD). Viability was found to be inversely related to the concentration of SNAP and consequently, nitric oxide levels (r=-0.96) but highly correlated with the number of colonies per cover slip (r=0.97) on day 7 post-infection.

SNAP	Viability(%)	SD +/-	Viability(%)	SD +/-	Viability(%)	SD +/-	Viability(%)	SD (+/-)
Conc. (uM)	Day 1		Day 2		Day 4		Day 7	
0	90	8	82	4	75	4.5	82.5	14
25	79	3	68	5	52	4	57.9	7
50	56	7	45	3	28	2.5	25.5	4.2
100	38	5	37	4	20	3	5.4	1

Fig.4. 5a

Mean number of *C. ruminantium* colonies 7 days post-infection, reflective of the infectious yield, in bovine endothelial cell cultures incubated with different concentrations of SNAP

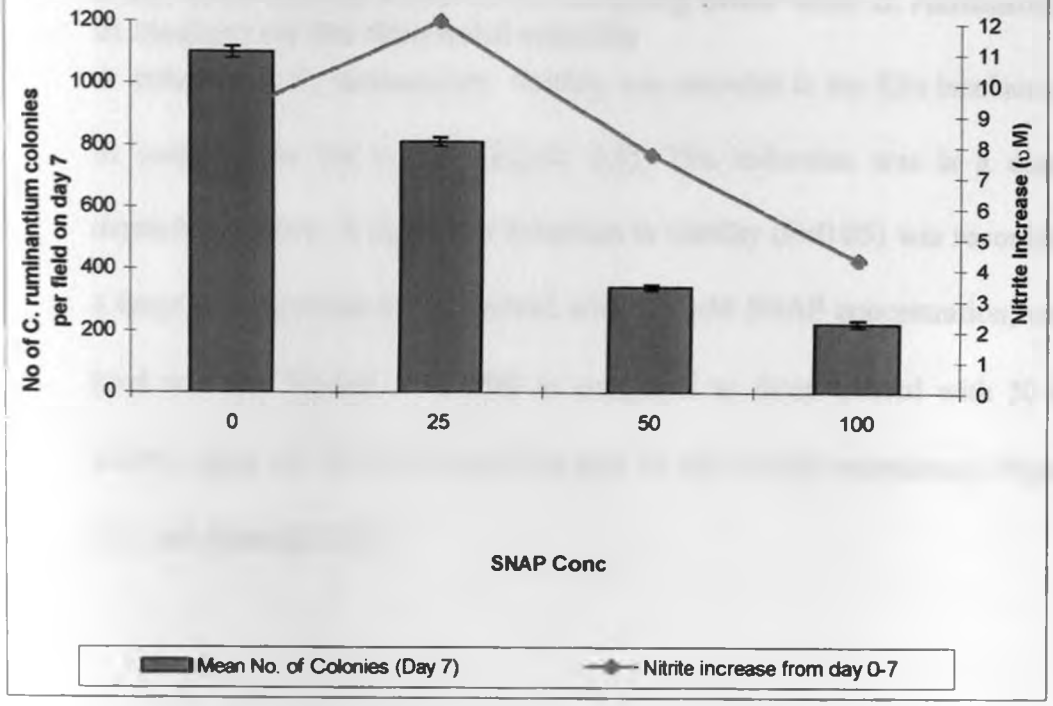
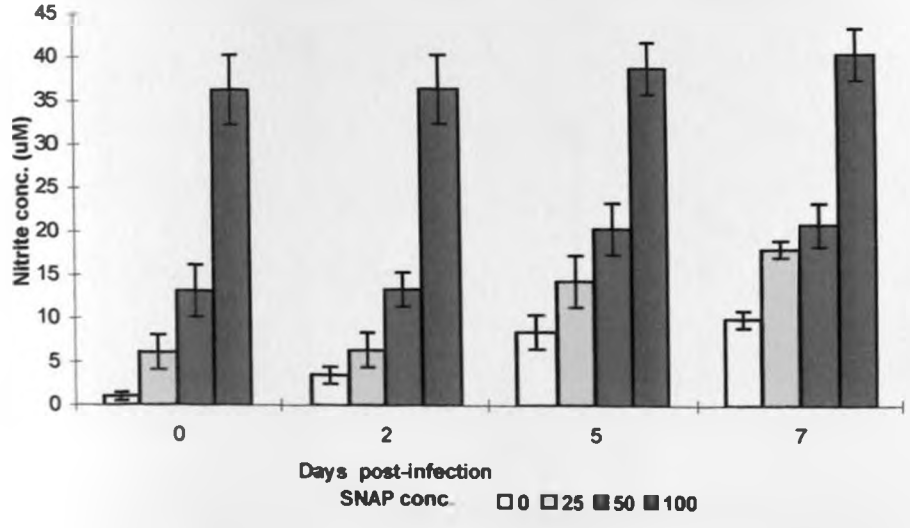


Fig. 4.5b

Nitrite (NO) levels on days 0, 2, 5 and 7 in *C. ruminantium*-infected endothelial cell cultures incubated with different concentrations of SNAP

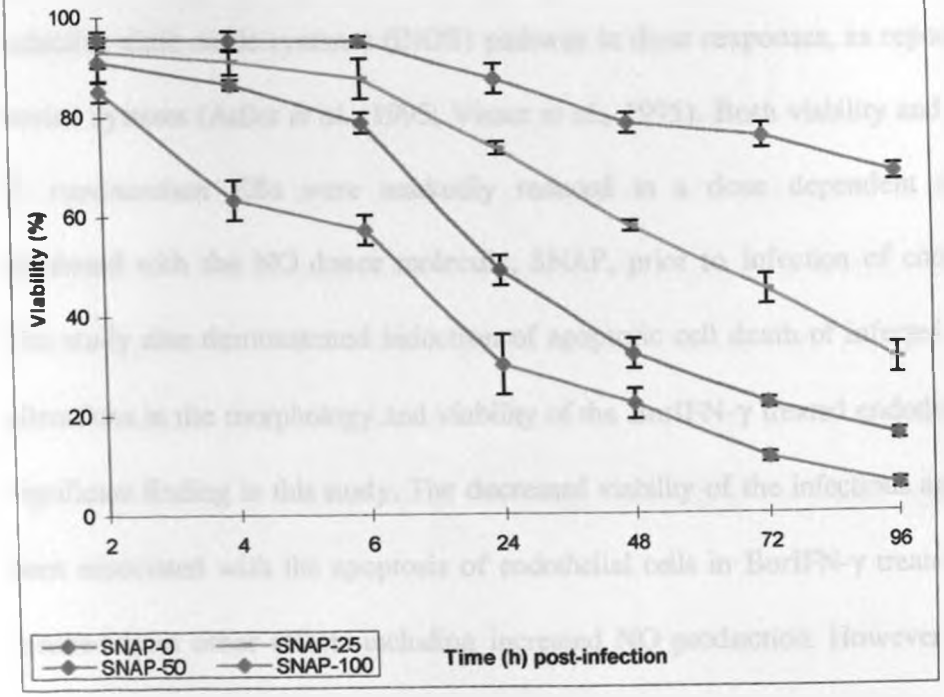


4.3.6. Time course effect of co-culturing SNAP with *C. ruminantium* EBs in medium on the rickettsial viability

A reduction in *C. ruminantium* viability was recorded in the EBs incubated with SNAP as compared to the control (Figure 4.6). This reduction was in a dose and time-dependent fashion. A significant reduction in viability ($P<0.05$) was recorded as early as 4 hours post-infection in EBs treated with 100 μM SNAP concentration, where the NO level was also highest (>35 μM) as compared to those treated with 50 and 25 μM SNAP, where the NO levels were less than 15 and 10 μM respectively (Figure 4.5b and 4.6, and Appendix 2.4).

Fig. 4.6

Reduction of viability, over a period of 96 hours, of *C. ruminantium* elementary bodies (EBs) incubated with different concentrations of SNAP



4.4. Discussion

Previous observations have indicated strongly that the interferon system, and probably other cytokines as well, plays a key role in natural resistance to *C. ruminantium* infections (Totte *et al.*, 1993 and Mahan *et al.*, 1996). This study looked for the production of NO by cultures of resting or BorIFN-γ activated BPEC infected with *C. ruminantium* as well as the effect of the cytokine on the viability of *C. ruminantium* and BPEC *in vitro*. The effect of SNAP as an exogenous source of NO on the viability and infectivity of *C. ruminantium* to BPEC was also examined.

The results of this study demonstrated that infection of resting BPEC with *C. ruminantium* resulted in induction of NO production as compared to uninfected cells. Addition of BorIFN-γ to these cultures resulted in enhancement of the NO production in a dose dependent fashion. Suppression of NO production in both resting and BorIFN-γ - stimulated BPEC by the L-arginine analogue L-NMMA, implied involvement of the inducible nitric oxide synthase (iNOS) pathway in these responses, as reported for other bovine systems (Adler *et al.*, 1995; Visser *et al.*, 1995). Both viability and infectivity of *C. ruminantium* EBs were markedly reduced in a dose dependent fashion when incubated with the NO donor molecule, SNAP, prior to infection of endothelial cells. The study also demonstrated induction of apoptotic cell death of infected cells seen as alterations in the morphology and viability of the BorIFN-γ treated endothelial cells as a significant finding in this study. The decreased viability of the infectious agent may have been associated with the apoptosis of endothelial cells in BorIFN-γ treated cultures, or resulted from other effects including increased NO production. However, the maximal

NO levels induced in 100 U/ml BorIFN-γ exposed cultures was 10 μm (Figure 4.2a) which was much lower than that recorded in 100 μM SNAP in culture medium (35 μM) (Figure 4.4b), yet the rickettsial viability was lower in the BorIFN-γ-treated than in the SNAP-treated cultures.(Figure 4.2a and 4.4a). The inhibition of *Cowdria* growth by IFN-γ in concanavalin A stimulated T cell supernatants has been reported (Mahan *et al.*, 1996) without detectable increases in nitric oxide production. However the activity of IFN-γ was detected by neutralisation and may have been lower than that used in this study. Our findings were consistent with the up-regulation of NO generation with bovine IFN-γ reported for bovine macrophages (Visser et al., 1995; Jungi *et al.*, 1997). TNFα has also been reported to induce NO synthesis in bovine endothelial cells (Umansky *et al.*, 1997) in contrast to bovine macrophages (Visser et al., 1995; Jungi *et al.*, 1997). NO synthesis in bovine macrophages is suppressed by IL-4 but unchanged by IL-10 or TGFβ (Jungi *et al.*, 1997) whereas the latter is reported to increase NO production in bovine aortic endothelial cells (Inoue *et al.*, 1995). Regulation of NO synthesis would therefore appear to differ between bovine endothelial cells and macrophages, but the upregulation by a Th1 cytokine (IFNγ) in both cell types is of interest. Apoptosis in infected cell cultures occurred at a higher rate than in uninfected cells in response to BorIFNγ. It is possible this was associated with NO synthesis, since apoptosis in tumor cells by bovine endothelium derived NO has been reported (Umansky *et al.*, 1997) However cytotoxicity of IFNγ to cells has been reported to occur on exposure to the cytokine alone or in combination with a second signal, including intracellular infectious agents (Hanson, 1991; Walker *et al.*, 1997).

IFN-γ has been reported to have the capacity to directly lyse rickettsial agents or infected cells (Hanson, 1991). We are suggesting here that the killing of *C. ruminantium* in the BorIFN-γ treated cultures was also associated with the cell apoptotic event. Similar findings were reported by Molloy *et al.*, (1994) on viability of intracellular *Mycobacteria bovis* in monocytes *in vitro*. They reported that toxic mediators that killed the monocytes by necrosis had no effect on the viability of the organism while those that killed the cells by apoptosis reduced viability of the organism by 60-70%. It has been suggested that since apoptosis is accompanied by nuclear fragmentation of the cells, the integrity of the genetic material of the intracellular organism may be compromised leading to its killing (Molloy *et al.*, 1994).

The reduced infectivity of *C. ruminantium* in cultures maintained in medium containing SNAP was attributed mainly to the effects of NO upon the organism prior to infection, since the incubation of EB's with SNAP prior to infection reduced the infectivity in a dose dependant manner, but there was no apparent additional reduction of growth by maintenance of the cultures in SNAP. After first appearance of infected endothelial cells on day 2, there was similar increase of infection from day 2 to day 4 (Figure 3a) in treated and untreated cultures. The ratio of the organism per field in the control and the cultures incubated with SNAP (Figure 4a) was not significantly different from the ratio obtained when EBs were treated with the same levels of SNAP prior to infection (Figure 3a). Since the EBs were allowed to infect cell cultures in the presence of SNAP, the dose-dependent reduction in SNAP-maintained cultures can be attributed to the inactivation during infection of the cultures with no significant increase in effect of the SNAP during the 7 day period of experiment. Release of fresh EBs into culture

media, from day 6, is expected from the approximately 6 day cycle of *C. ruminantium* infection *in vitro*, and may explain the increased viability of EBs on day 7 (Fig 4a). Thus, SNAP treatment reduced initial infection rates in the cells (Figure 3a), but the level of infection increased in much the same rate irrespective of initial SNAP concentration in culture medium. Taken together, these findings indicated that the induction of iNOS by IFN-γ may contribute to the anti-*Cowdria* activity manifested in interferon treated cultures of *C. ruminantium* - infected BPEC. The NO level induced by infection of BPEC without any treatment was approximately 10 μM on day 7, with similar levels being recorded in 100 U/ml IFN-γ-treated cultures after 48 hours. This level of NO is intermediate between the 5 μM and 13 μM NO concentration, produced by 25 and 50 μM SNAP concentrations respectively. Since the latter two treatments significantly reduced infection level, it can be concluded that the level of NO released in culture by infection alone or by IFN-γ induction may have an effect on the infectivity of EBs being released and thereby indicate a role of NO in the protective responses to heartwater infection. This study reports for the first time that NO is capable of reducing both viability and infectivity of *C. ruminantium* in BPEC *in vitro*, indicating that NO released by endothelial cells or by other cells such as monocytes may have a role in reducing the infectivity of extracellular EBs and thereby reducing spread of infection. If release of NO during endothelial cell infection occurs *in vivo*, this can be expected to reduce viability of the agent for other endothelial cells and potentially for the arthropod vector. The anti-*Cowdria* effect of BorIFN-γ may involve NO as well as other pathways involving additional factors as the NO generated by cell exposed to interferon was not as high as that generated by 100 μM of SNAP yet the reduction in viability was higher in the

cultures treated by the former. The study shows that *C. ruminantium* organisms are killed by events involving activities on IFN-γ and NO *in vitro*. The findings reported here are comparable with findings of a study carried out on IFN-γ and TNF-α stimulated mouse endothelial cells where it was demonstrated that endothelial cells were capable of killing *Rickettsia conorii* through a mechanism involving NO synthesis (Walker *et al.*, 1997).

Several biological roles of NO have been described by different authors. NO dependent non specific immunity is now accepted both as a general phenomenon involving the reticulo-endothelial systems (Moncada *et al.*, 1993) and as a primary defence mechanism against tumour cells (Woodman, *et al.*, 1991) and several microbial pathogens and a potent agent in non-specific defence mechanisms by upregulating release of inflammatory mediators (Liew *et al.*, 1991a; 1991b). NO production by macrophages and monocytes during response to infection has been correlated with resistance to a wide variety of pathogens *in vivo* including *Mycobacterium bovis* (Walker *et al.*, 1997), *Listeria monocytogenes (*Boockver *et al.*, 1994), *Schistosoma mansoni* (Oswald *et al.*, 1994) *Theileria parva (*Visser *et al.*, 1995*), Plasmodium berghei* (Sequin *et al.*, 1994) *and Histoplasma capsulatum* (Lane *et al.*, 1994). In contrast, NO mediation of immunosupression has been suggested in cattle infected with the rickettsia *Anaplasma marginale* where treatment with an NO inhibitor, aminoguanidine (AG), resulted in lower parasitic levels (Gale *et al.*, 1997). This was thought to be due to immunosuppressive effects of NO, as its neutralisation was beneficial to the host, and the inhibition of NOS by AG was postulated to increase immune effectiveness. The inhibition of peripheral T-cell proliferation by NO in mice has also been reported (Bras *et al.*,

1997). *C. ruminantium* resides in endothelial cells, an association which leads to increased vascular permeability. The underlying cause of this permeability remains obscure but mediators rather than structural changes have been more implicated in the pathogenesis of the disease. Excessive NO production by endothelial cells *in vivo* which are in continuous contact with the blood stream may therefore not only constitute an important role in phenomena such as changes in vascular wall integrity leading to increased vascular permeability. Under other circumstances, NO produced by activated endothelial cells has been found to act as a toxic effector molecule inhibiting various metabolic functions in cells leading to death of the Schistomula of *Schistosoma mansoni* as well as to altered physiology of endothelial cells and increased vascular permeability (Oswald *et al.*, 1994). NO has also been shown to precipitate the loss of intracellular iron resulting in inhibition of certain of the vital enzymes involved in mitochondrial respiration (Busse *et al.*, 1995). This finding suggests that NO may contribute to the mitochondrial changes observed in the alveolar endothelial cells infected with *C. ruminantium* (Prozesky and Du Plessis, 1985a; 1985b).

Conlusion

This study has shown that endogenously produced NO or NO from exogenous source kills *C. ruminantium* in a dose - dependent fashion. Although lack of knowledge limits discussion of the potential role of NO in *C. ruminantium* pathogenesis and immunity, these findings point to a role for NO in resistance to *C. ruminantium* infections and in the pathology of the disease. Toxic mediators have been suggested to be responsible for some unexplained pathology of the disease including increased vascular permeability leading to oedema and also vascular collapse seen in terminal cases

of the disease, consistent with effects of NO production upon the vascular system. In a recent study (unpublished data) we noted that infection of previously exposed African breeds of sheep resulted in higher levels of nitrite in plasma during the clinical response. The induction of NO synthesis by IFN-γ may be an important determinant of the outcome of *C. ruminantium* infection. This is supported by the following sequence of events; rickettsaemia occurs 3-6 days prior to the onset of fever and pathology, but terminal collapse, reduced blood pressure and peak pulmonary oedema occur only a few days after the first immune responses are detectable and when rickettsaemia has declined but endothelial cell infection is highest (unpublished data). On the basis of our results and those cited previously, it is becoming clear that NO maybe an important component in the immune response to intracellular infections and the pathophysiology of heartwater infection.

CHAPTER 5

INTERFERON GAMMA AND ALPHA/BETA ARE INVOLVED IN THE CONTROL OF *COWDRIA RUMINANTIUM* INFECTION IN MICE.

5.1. Introduction:

The principle target cell of *C. ruminantium* is the vascular endothelium but the organism is also found to infect other cells including neutrophils and macrophages. The sequence of events in the response of cells or tissues to *C. ruminantium* infection from the initial stimulus by the aetiological agent to the ultimate expression of the disease are poorly understood. Increased vascular permeability leading to the escape of a protein rich exudate into the interstitium is the hallmark of acute inflammation in heartwater disease (Clark, 1962). It has been shown that *C. ruminantium* causes little damage to parasitised cells (Cowdry 1926, Prozesky and Duplessis 1985b and 1985c). Ultrastructural studies in lungs of sheep, goats and mice infected with the organism have also revealed that the damage to capillary endothelial cells of the alveoli was mild and could not account for the increased vascular permeability, the main pathological finding of the disease (Prozesky and Duplessis 1985b and 1985c). The extent to which pathogenesis is a function of the pathogen, *C. ruminantium,* and the extent to which it is a consequence of the immune responses of the host, is unclear.

Cell-mediated immune responses have been reported as playing key roles in the protective mechanisms against *C. ruminantium* infection, a phenomenon involving T cells and production of inhibitory cytokines (Du Plessis, 1970, 1982; Du Plessis *et al.*, 1991; Mahan *et al.*, 1994). IFN–γ has been demonstrated to have an inhibitory influence on *C. ruminantium* growth *in vitro* (Totte *et al.*, 1993; Mutunga *et al.*, 1998; Mahan *et al.*, 1996). It was also demonstrated that animals that recovered from *C. ruminantium* infection contained higher blood levels of IFN-α compared to those that succumbed to infection and that the inhibitory influence of IFN-α was demonstrated on *Cowdria* growth in *in vitro* studies when added to infected endothelial cultures (Totte *et al.*, 1994).

This study was designed to examine the extent to which IFN-γ, IFN-α and β contribute to the immune responses and the pathogenesis of *C. ruminantium* disease by using mice deficient in IFN-γ or IFN-α/β receptors.

5.2. Materials and Methods

5.2.1. Experimental animals

Three groups of specially bred strains of mice, IFN-γ receptor knockout mice (IFN–γRo/o) (G 129), IFN-α/β receptor knockout mice (IFN–α/βRo/o) (A 129) and wild type control strain of the same genetic background (WT 129) were used in this study. The IFN–γRo/o and IFN–α/βRo/o mice were homozygous for the lack of genes encoding receptors for IFN-γ or IFN-α/β respectively (see section 2.3.1.2.). The groups used were as follows:

1 15 IFN-γ receptor knock out mice (G 129) in which deletion of the α-chain of IFN type II (γ) receptor had occurred (Huang *et al.*, 1993);

2 15 IFN-α/β receptor knock out mice (A 129) in which deletion of IFN type I (α and β) receptor signalling sub unit had occurred (Muller *et. al.*, 1994)

3 15 wild type control strain of the same genetic background (WT 129)

(For individual mice numbers see appendices 3.1). The source and management of these mice before and after infection were as described in sections 2.3.1.2 and 2.3.2. Fifteen mice were used for each group.

5.2.2. Infectious agent

The Welgevonden isolate of *C. ruminantium*, propagated in bovine endothelial cells in tissue culture, as described in section 2.1.4., was used to infect the mice. Each animal was infected with 1 $x10^3$ elementary bodies (EBs) diluted in culture medium after determining viability count by staining the EBs with fluorescein diacetate as described in section 2.1.5. The EBs in culture medium were chilled on ice until all the mice had been infected.

5.2.3. Clinical disease monitoring

The disease was monitored daily by visual examination of the mice as well as recording rectal temperatures as described in section 2.3.2. The mean incubation period in days, based on onset of hypothermia, was determined in the 15 infected mice in each group, while the course of the disease, mortality and morbidity rates were determined in 13 mice (as 2 mice were sacrificed after onset of hypothermia as outlined in the next section). The results were then analysed and compared between groups.

5.2.4. Pathological investigations

Two hypothermic (infected) mice and 1 uninfected mouse were sacrificed on day 7 from each group and lung and brain tissues collected for histopathology and transmission electron microscopy. Brain impression smears were also prepared from these sacrificed mice and stained in giemsa. Pathological investigations, both macroscopically and microscopically, were carried out for the sacrificed mice and the dead mice and any significant changes noted. These included measuring the amount of thoracic oedema fluid using a 2 ml syringe (see section 2.3.3) and the results analysed and compared between the groups. Lung and brain sections were prepared from the sacrificed mice for histopathology and electron microscopy as described in sections 2.3.3.1 and 2.3.3.2 and examined. The histopathological investigation carried out included assessment of inflammatory cell infiltration in the tissues, the distribution and extent of oedema and presence of the rickettsial infectious organisms in the tissues. Assessment of rickettsial burden in lung and brain sections was estimated by counting infectious foci in giemsa stained histopathological sections, viewed under a light microscope using an eyepiece with grids (at a magnification of x1000), as described in section 2.3.3.1. Twenty fields per slide were randomly selected and examined for the presence of the rickettsial organisms and the results expressed as mean +/- standard deviation. The results were subjected to statistically analysis and the results were expressed as tables, charts or photographs.

5.2.5. Data analysis

Students T-test or Mann-Whitney tests and chi-square were used for data analysis and the results expressed at 0.05 level of significance

5.3. Results.

5.3.1. Incubation period, morbidity and mortality rates of infected mice.

The incubation period was taken to be the time from infection to the onset of hypothermia, a characteristic development in mice infected with *C. ruminantium* (Prozesky and Du Plessis, 1985b). The first clinical manifestations of disease included depression, lack of appetite, prostration and closed eyes and these coincided with the onset of hypothermia. Once hypothermia occurred, the mice died within 1-3 days with the mean course of the disease being 1.85 days for both the IFN–γRo/o and IFN–α/βRo/o groups and 2.15 for the control group (Figure 5.1). Significant differences (t-test, $p=0.045$) were shown between the incubation period of IFN–γRo/o mice and the wild type (controls) group but not between IFN–α/βRo/o and IFN–γRo/o (Table 5.1). Significant differences were also seen in the cumulative mortality rates in the IFN receptor deficient mice as compared to the control, with the IFN–γRo/o mice ($x^2=6.89$, $p<0.05$ for day 8,) and to a lesser extent IFN–α/βRo/o mice ($x^2=5.99$, $p<0.05$ for day 8), $p<0.05$) showing higher mortality rates earlier in the disease as compared to the control (Table 5.1, Figure 5.1 and Appendix 3.2).

5.3.2. Pathology

5.3.2.1. Gross pathology

All the sacrificed and dead mice had extensive thoracic oedema, characterised by straw-coloured fluid in the thoracic cavity. The lung and brain tissues were slightly congested in some cases. Lung oedema was found to be more extensive in the IFN–γRo/o mice than in the IFN–α/βRo/o and the control mice, with the IFN–γRo/o mice showing a mean of 1.4 ml of lung oedema fluid as compared to 1.1 in the IFN–α/βRo/o group and 1.0 ml in the control group, although the differences were not significant (t-test, $p=0.34$) (Table 5.1).

Table 5.1

Clinical and pathological observations in interferon gamma and alpha/beta receptor deficient mice (IFN–γRo/o and IFN–α/βRo/o respectively) and the control mice (WT) infected with *C. ruminantium*.

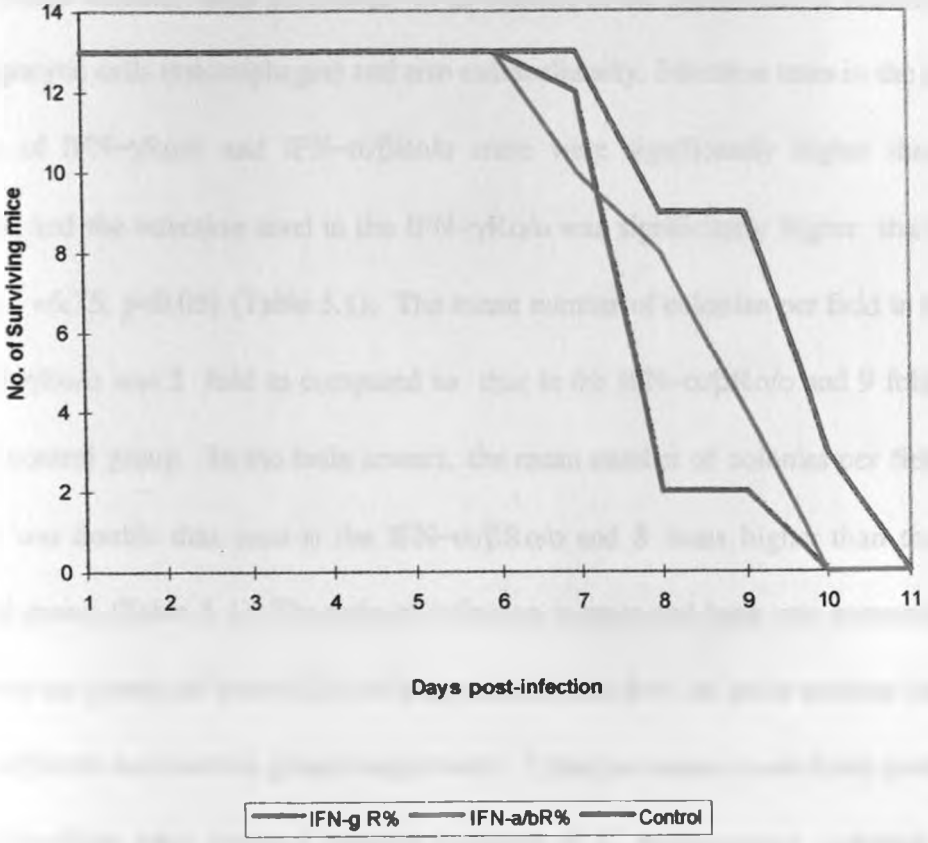
	IFN–γRo/o	IFN–α/βRo/o	WT
Mean infectious dose (ml) (n=15)	0.1	0.1	0.1
Mean incubation Per.(days) (n=15)	6.1+/-0.3	6.5+/-0.5	7.4+/-0.5
Mean course (days) (n=13)	1.85+/-0.4	1.85+/-0.9	2.5+/-0.9
Morbidity rate at day 7(%) (n=13)	87.5	87.5	37.5
Thoracic oedema fluid (ml) (n=13)	1.4	1.1	1.0
Mean No. of organisms (BS) (n=2)	15+/-3	9+/-4	2+/-3
Mean No. of organisms (LS) (n=2)	91 +/- 33	38 +/-12	11+/-6

KEY: TO LEGEND:

1. Mean Incubation per. - Incubation period
2. Mean No. Of organisms (BS) - Mean number of organisms in giemsa stained brain impression smears counted in twenty fields randomly selected at a magnification of X1000.
3. Mean No. Of organisms (LS) - Mean number of organisms in giemsa stained lung histopathological sections, counted in twenty fields randomly selected at a magnification of X1000.
4. n=The number of mice used in calculating each factor.

Fig. 5.1.

Daily number of surviving mice, depicting cumulative mortalitites, in *C. ruminantium*-infected mice deficient in IFN-γ or IFN-α/β receptors, and a control group.



5.3.2.3. Histopathological findings

Fixed histological sections stained in giemsa and haematoxylin and eosin were examined.

The infectious agent was seen in giemsa-stained histopathological lung tissue sections (Figure 5.2) brain impression smears (Figure 5.3 and 5.4) and in cytospin smears of peritoneal lavage fluid (Figure 5.5). Assessment of rickettsial burden in lung and brain sections revealed presence of higher numbers of colonies in the IFN–γRo/o and to a lesser extent IFN–α/βRo/o mice. These colonies were seen singly or in clusters in the cytoplasm of endothelial cells and in phagocytic cells (macrophages) and also extracellularly. Infection rates in the lung and brain tissues of IFN–γRo/o and IFN–α/βRo/o mice were significantly higher than that of the control, and the infection level in the IFN–γRo/o was significantly higher than that in IFN–α/β (x^2 =6.75, $p<0.05$) (Table 5.1). The mean number of colonies per field in the lung tissue of IFN–γRo/o was 3 fold as compared to that in the IFN–α/βRo/o and 9 fold as compared to the control group. In the brain smears, the mean number of colonies per field in the IFN–γRo/o was double that seen in the IFN–α/βRo/o and 8 times higher than that seen in the control group (Table 5.1). The ratio of infection in brain and lung was approximately similar between the groups of mice (9:3:1 in lung sections and 8:4:1 in brain sections for IFN–γRo/o, IFN–α/βRo/o and control groups respectively. Cytospin smears made from peritoneal fluid of the IFN–γRo/o mice revealed massive numbers of *C. ruminantium* colonies seemingly in macrophages, as well as infiltration by macrophages (Fig 5.5). In the IFN–γRo/o there was interstitial pneumonia characterised by extensive infiltration of the lung parenchyma by a mixed population of phagocytic cells with mononuclear cells being more prevalent than neutrophils (Figure 5.6) resulting in thicker alveolar walls as compared to the control group (Figure 5.7 and 5.8.). Pink-staining mass denoting oedema was seen to be more extensive in the IFN–γRo/o group as compared to the control (Figure 5.6., 5.7 and 5.8).

Figure 5.2. Giemsa-stained lung histopathological section of IFN–γ Ro/o mouse showing a colony of *C. ruminantium* organisms (arrow) (X3000)

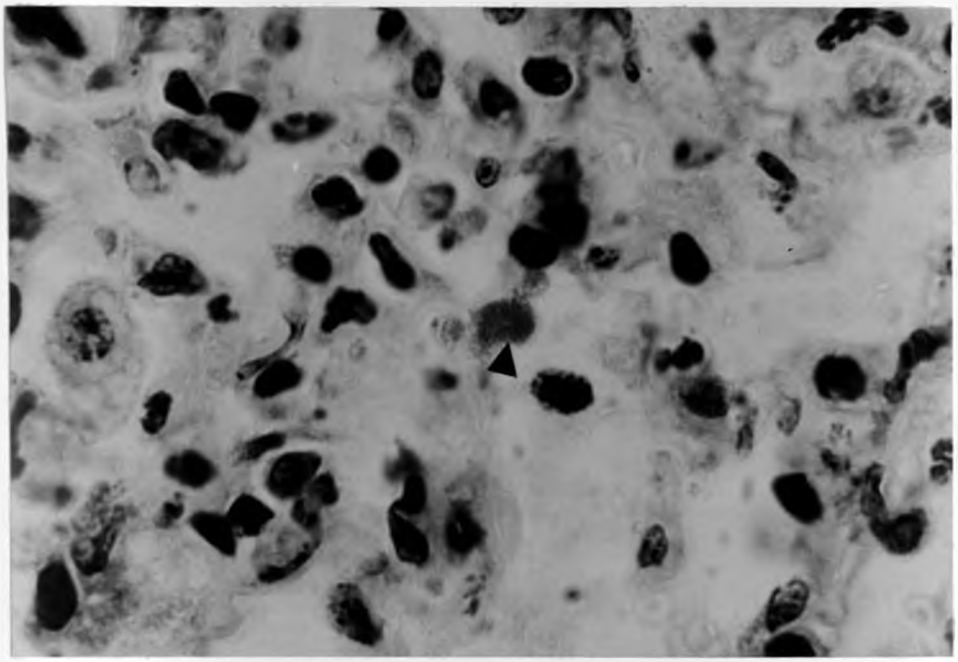


Figure 5.3. Giemsa-stained brain impression smear of an infected IFN–γRo/o mouse showing *C. ruminantium* organisms in the cytoplasm of an endothelial cell of a blood capillary (X3000).

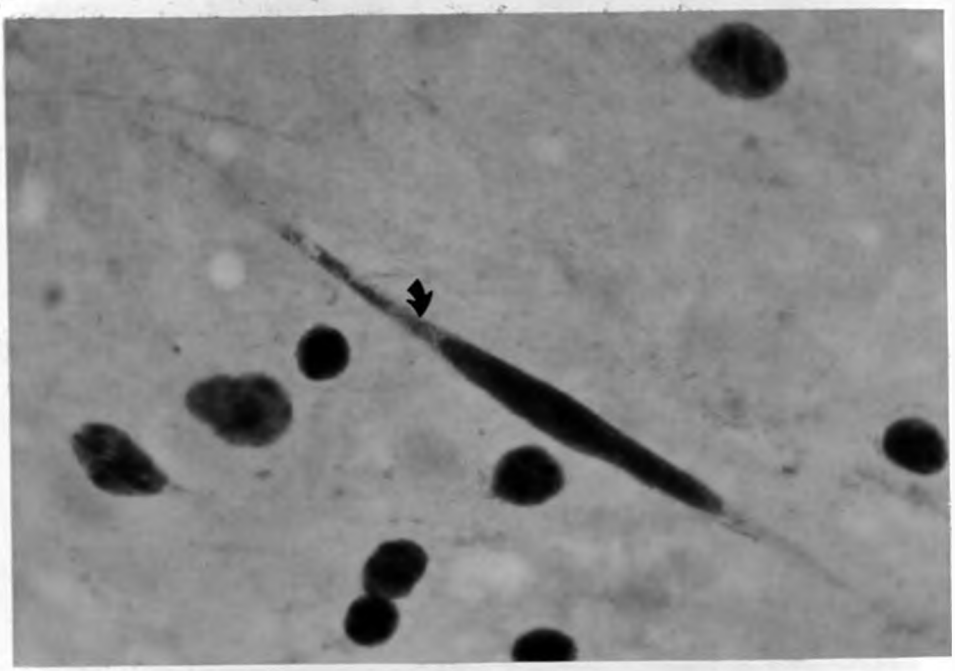


Figure 5.4. Giemsa-stained brain impression smear of an infected IFN–α/βRo/o mouse showing *C. ruminantium* organisms in the cytoplasm of an endothelial cell of a brain blood capillary (X 1500).

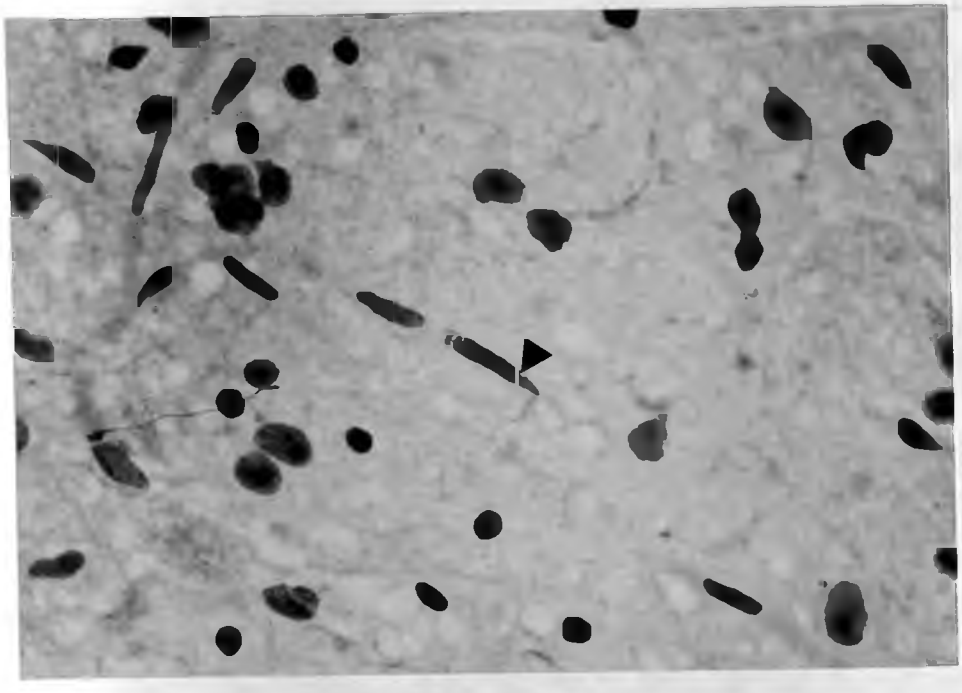


Figure 5.5. Giemsa-stained cytospin smear of peritoneal fluid harvested from IFN–γ Ro/o mice sacrificed on day 7 showing extensive numbers of *C. ruminantium* elementary bodies (arrows) and infiltration by phagocytic cells, predominantly mononuclear cells (X3000).

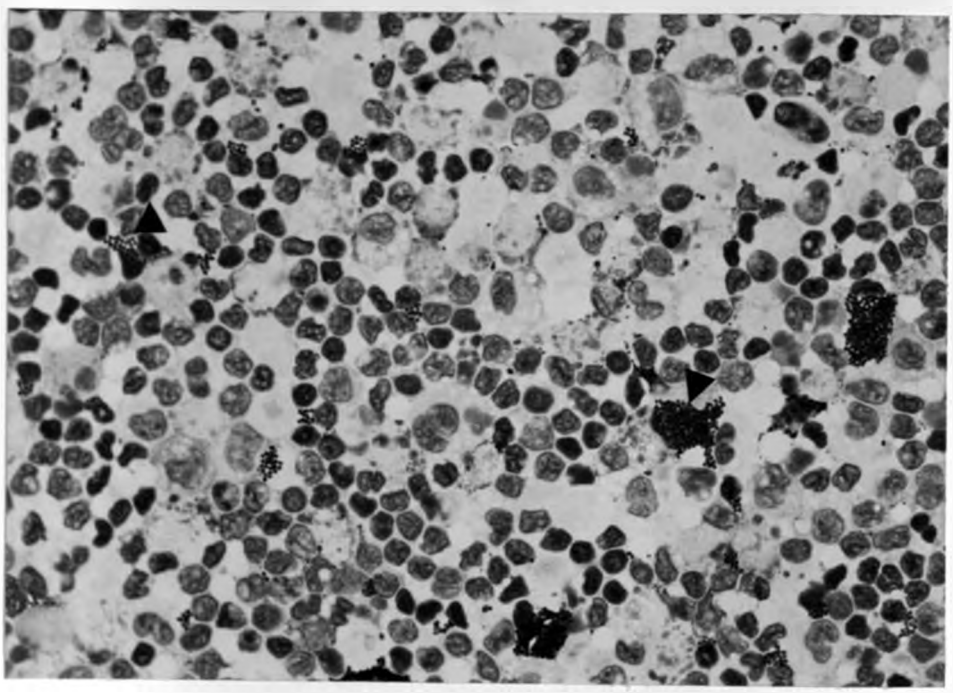


Figure 5.6. Giemsa -stained lung histopathological section of IFN–γ Ro/o mouse. Note the presence of oedema (pink-staining mass) (curved arrow), infection (triangle arrow head) and infiltration by predominantly mononuclear cells (X3000).

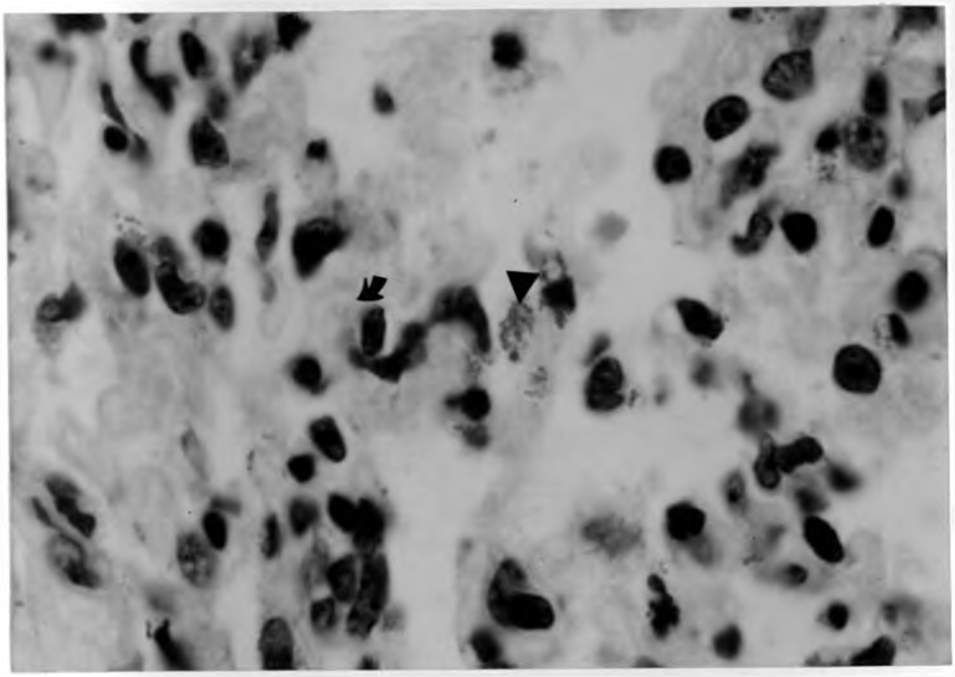


Figure 5.7. Hematoxylin and eosin stained lung histopathological section of IFN–γ Ro/o mouse showing thickened alveolar wall (triangle arrow head) with extensive inflammatory cell infiltration and oedema fluid (arrow) (X300).

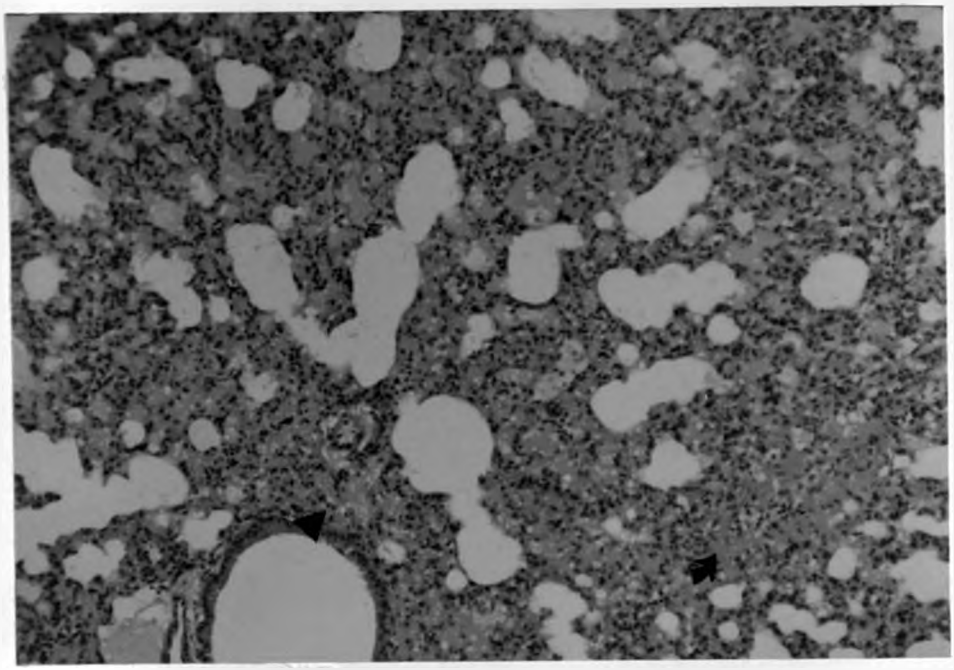
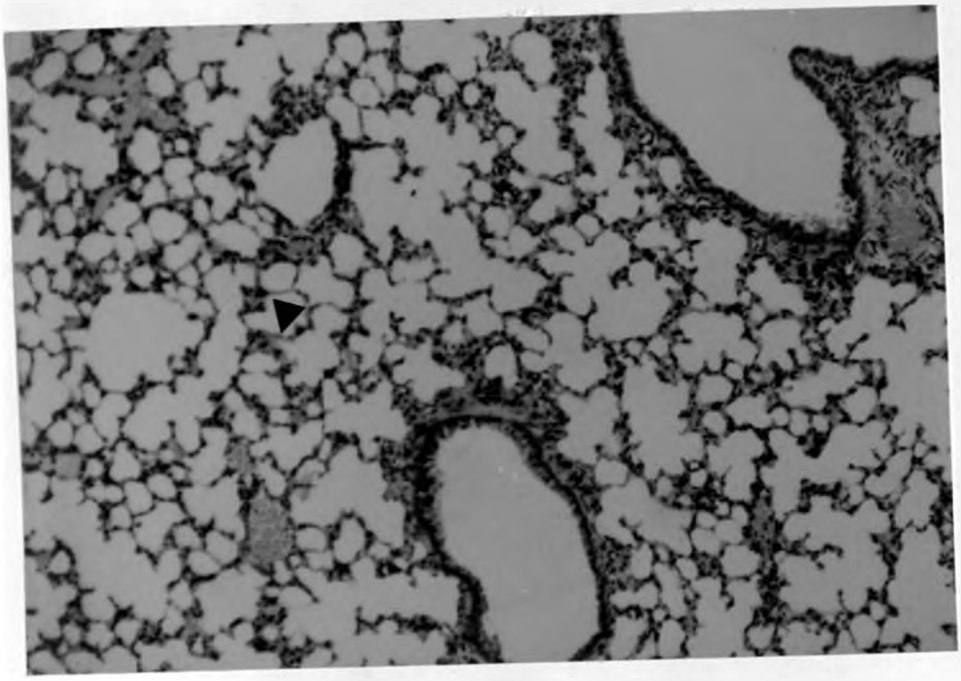
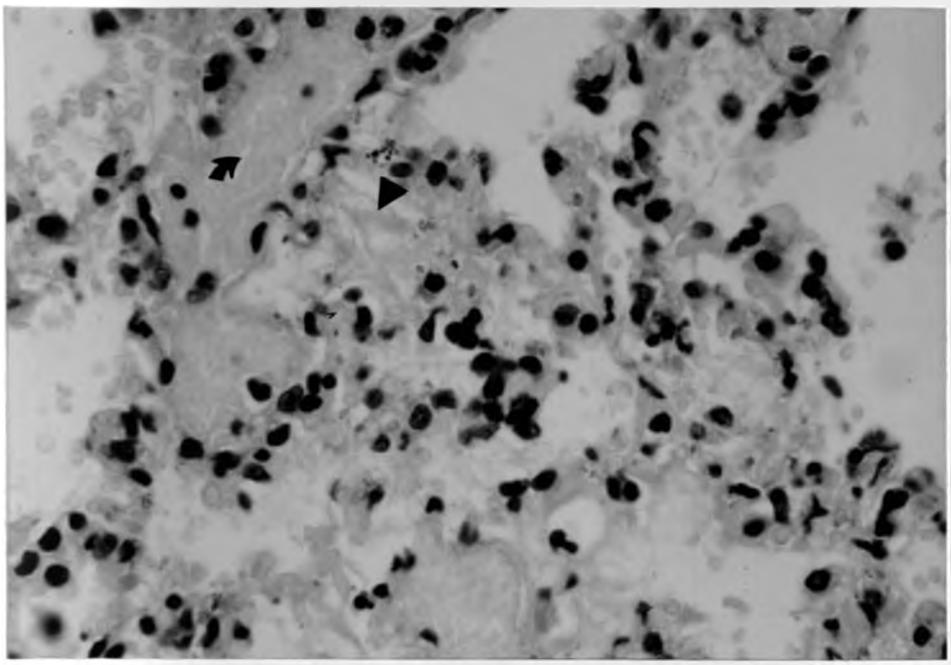


Figure 5.8. Hematoxylin and eosin stained lung histopathological section of control mouse showing less thickened alveolar wall (arrow head) as compared to Figure 5.7. (X300)



Similar histopathological changes were observed in the IFN–α/βRo/o as in IFN-γ Ro/o group, but to a lesser extent. The amount of oedema and interstitial pneumonia (infiltration by inflammatory cells) were lower than in the IFN–γRo/o group and there was marked infectious foci in the lung sections and brain impression smears as compared to the control (Figure 5.9).

Figure 5.9. Giemsa -stained lung histopathological section of IFN–α/β Ro/o mouse. Note the presence of oedema(arrow), infection (arrow head) and phagocytic cell infiltration (X3000).



5.3.2.4. Electron microscopical studies

Ultrastructural studies of lung sections showed infected endothelial cells and macrophages in mice in each group. The main differences noted in the IFN–γRo/o mice and the control group were the forms of the organisms seen. Bizarre looking forms were seen macrophages in the IFN–γRo/o mice (Figures 5.10, 5.11 and 5.12) and also in endothelial cells (Figure 5.11 and 5.12). These forms were in encapsulated vacuoles and were highly pleiomorphic with different shapes ranging from rods, rings, oval, horse-shoe shapes. The organisms were observed to be electron-dense, surrounded by a double membrane and separated by a protein-rich matrix. In the control mice, reticulate forms of the infective agent were seen in lung sections in endothelial cells, and there was evidence of cells dividing by binary fission (Figure 5.13). Haemorrhages and oedema fluid were observed in the lung parenchyma of infected mice (Figure 5.14). Higher numbers of mitochondria in the brain section of IFN–γRo/o mice were seen as compared to the control (Figure 5.15). Lung sections of the IFN–α/βRo/o showed presence of the electron-dense forms of *C. ruminantium* as well as reticulated (dividing) forms in endothelia cells and macrophages (Figure 5.16). Evidence of increased capillary permeability shown by haemorrhages and oedema fluid were also demonstrated in the IFN–α/βRo/o mice (Figure 5.16 and 5.17) and in the control group (Figure 5.18).

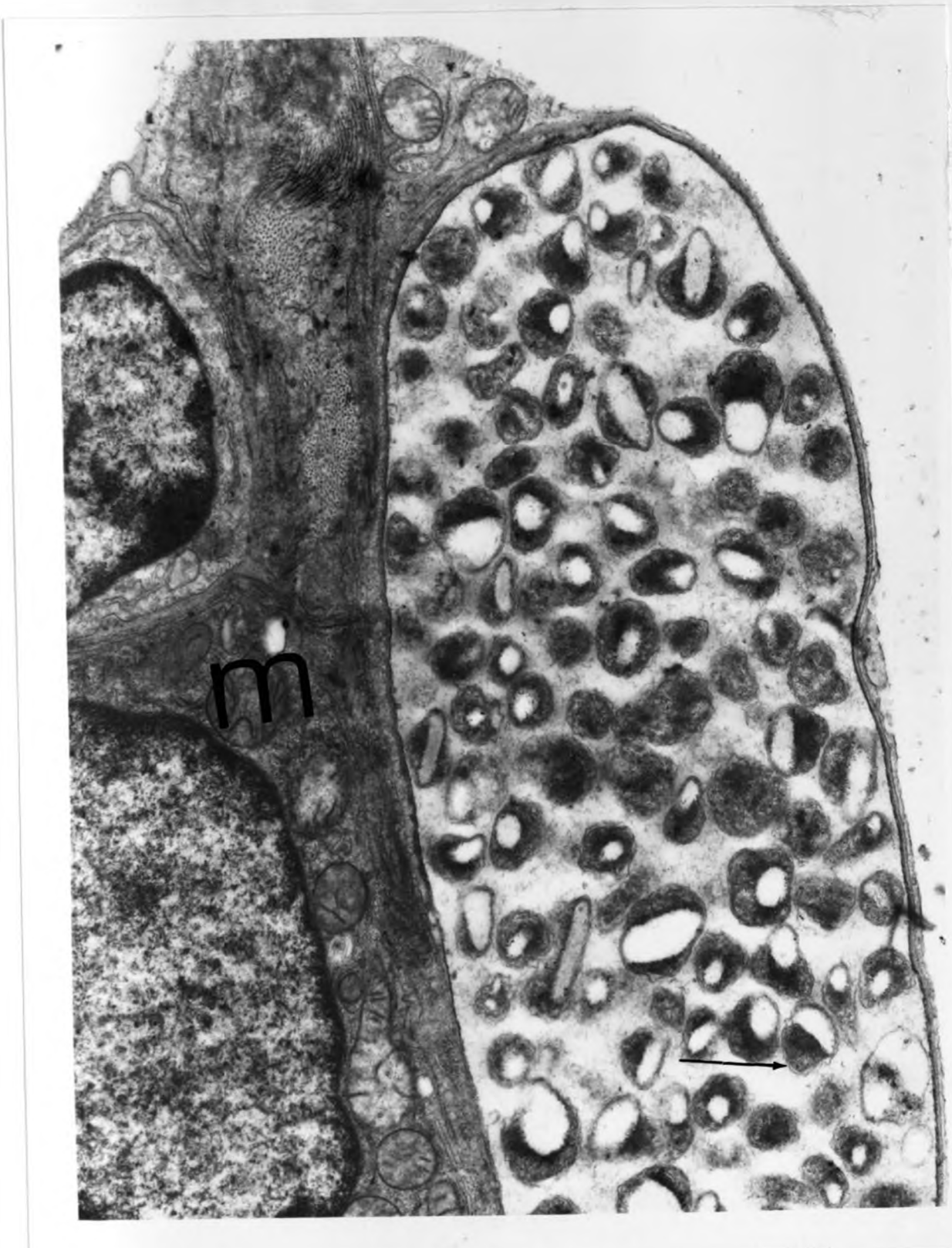

m

Figure 5.11. Electron micrograph of a lung section of an infected IFN-γRo/o mouse. V the two encapsulated colonies of organisms. In one the capsulated vacuole is bulging int lung parenchyma with the cell nucleus pushed to one side although the vacuole wall i continuos with the cell membrane and seems to be in the process of detaching (A). A se encapsulated vacuole seems to have fully detached from the cell (B). Note the presen bizarre forms of the organisms which are electron dense and in different shapes. Also n noted is the oedema fluid in the lung parenchyma (X4125). (Micrograph No. 1572-IF Ro/o).

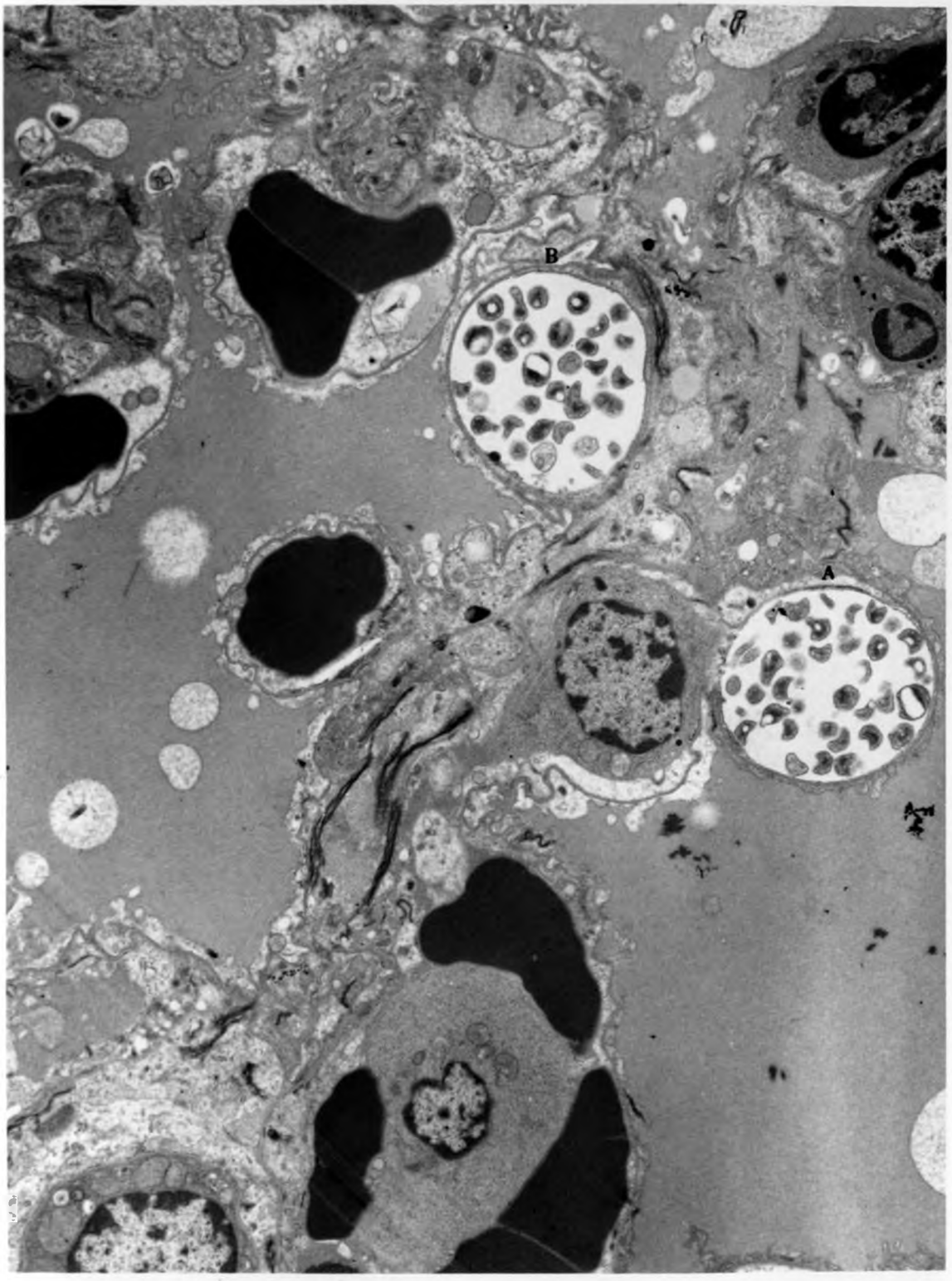

B
A

Figure 5.12. Higher magnification of Figure 5.11 showing the bizarre forms of *ruminantium* which are separated by a protein -rich matrix. The organisms are electron-d and surrounded by two membranes reflecting a late developmental stage. 'Doughnut' sh are probably due to long-axis sectioning of concave elongated *Cowdria* bodies(X24,7 (Micrograph No. 1574-IFN–γ Ro/o)

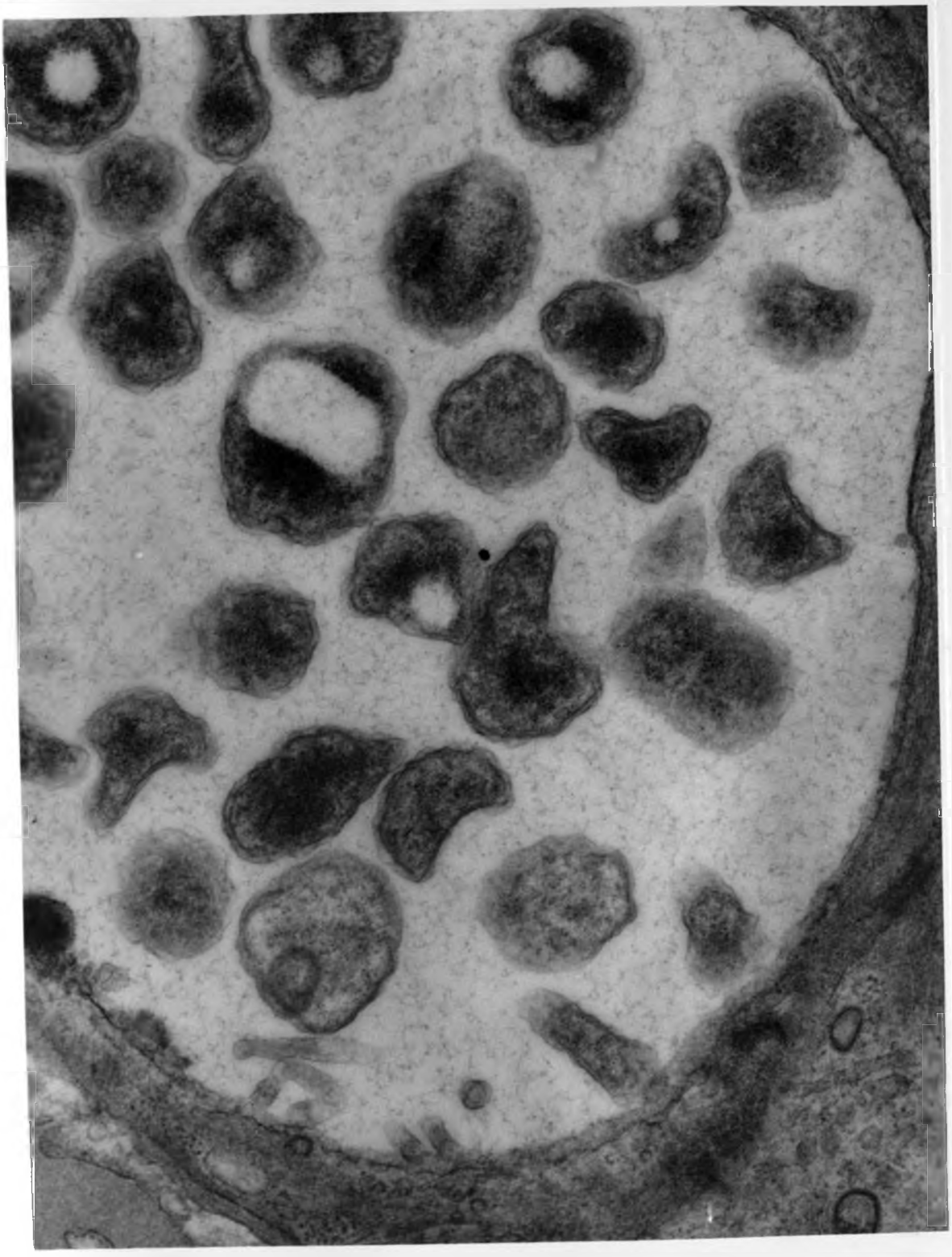
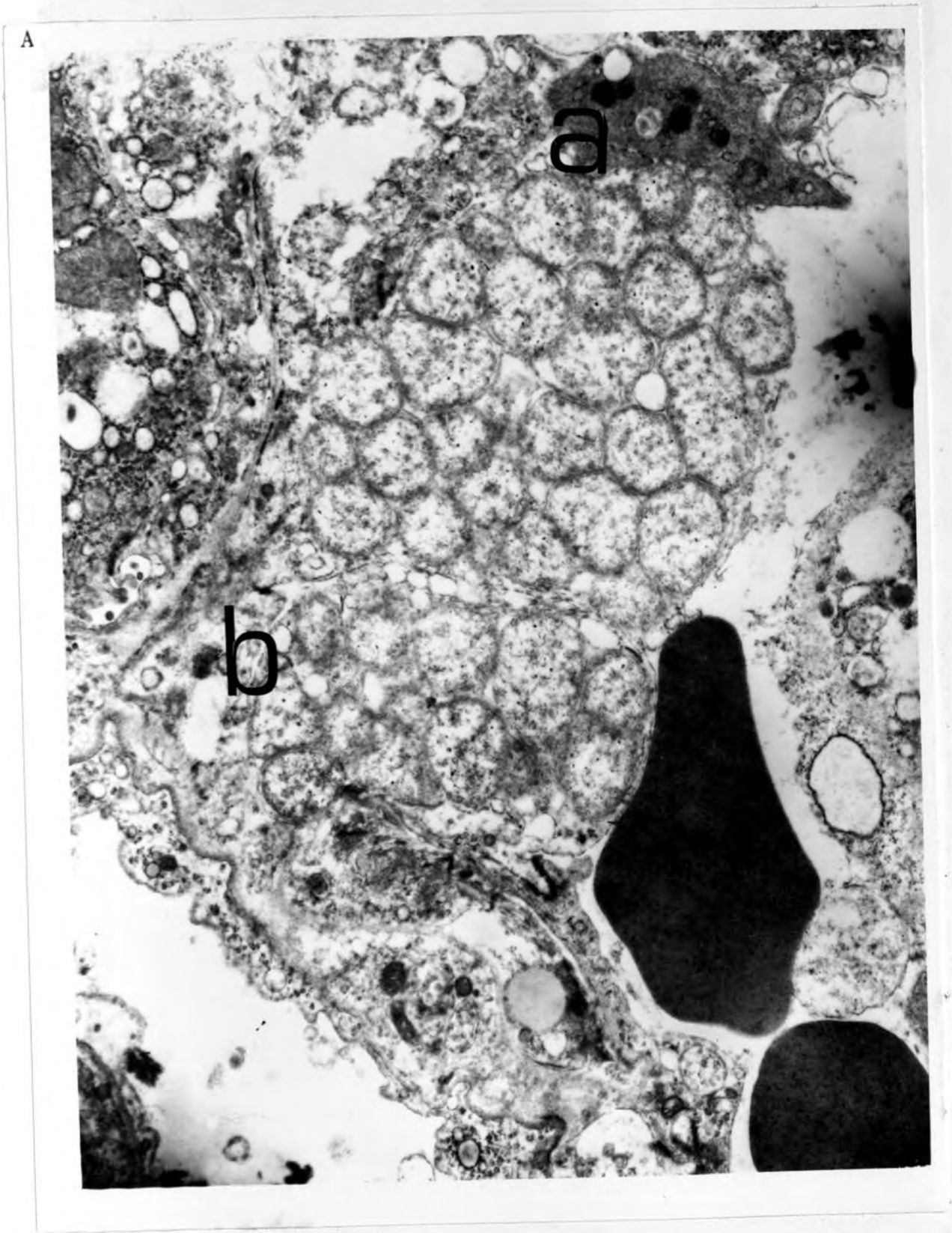


Figure 5.13. Lung of a control mouse infected with *C. ruminantium*. Micrograph A: two colonies adjacent to each other in the cytoplasm of endothelial cell (a and b). The nucleus has been pushed to one side by the bulging cytoplasm, and this seems to have ca narrowing of the capillary. Micrograph B (higher magnification of A); note the retic forms (dividing forms) each surrounded by a double membrane and note one organi dividing by binary fission (arrow) (A-X 9,000 and B-X 24,750). (Micrograph No.1468 1469 - control).


A
a
b

B

Figure 5.14. Lung section of a *C. ruminantium* infected IFN–γRo/o mouse showing ext
haemorrhages (arrow) in the lung parenchyma (X4,125). (Micrograph No. 1578-IFN–γ

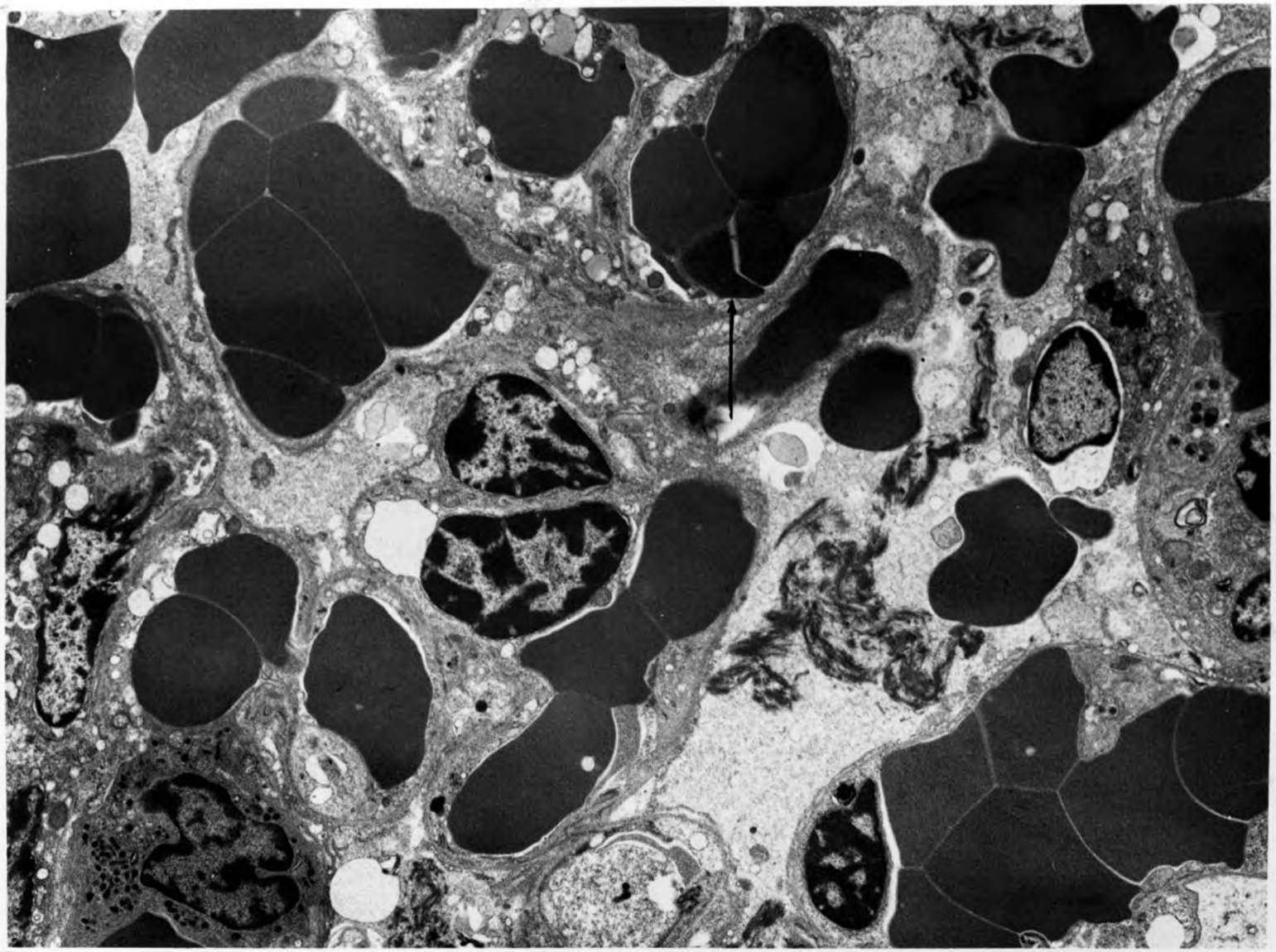


Figure 5.15. Brain of IFN–γ Ro/o infected mouse showing numerous intact mitoch (arrow). (X18,750). (Micrograph No.1513-IFN–γ Ro/o)

Figure 5.15. Brain of IFN–γ Ro/o infected mouse showing numerous intact

(arrow). (X18,750). (Micrograph No.1513-IFN–γ Ro/o)

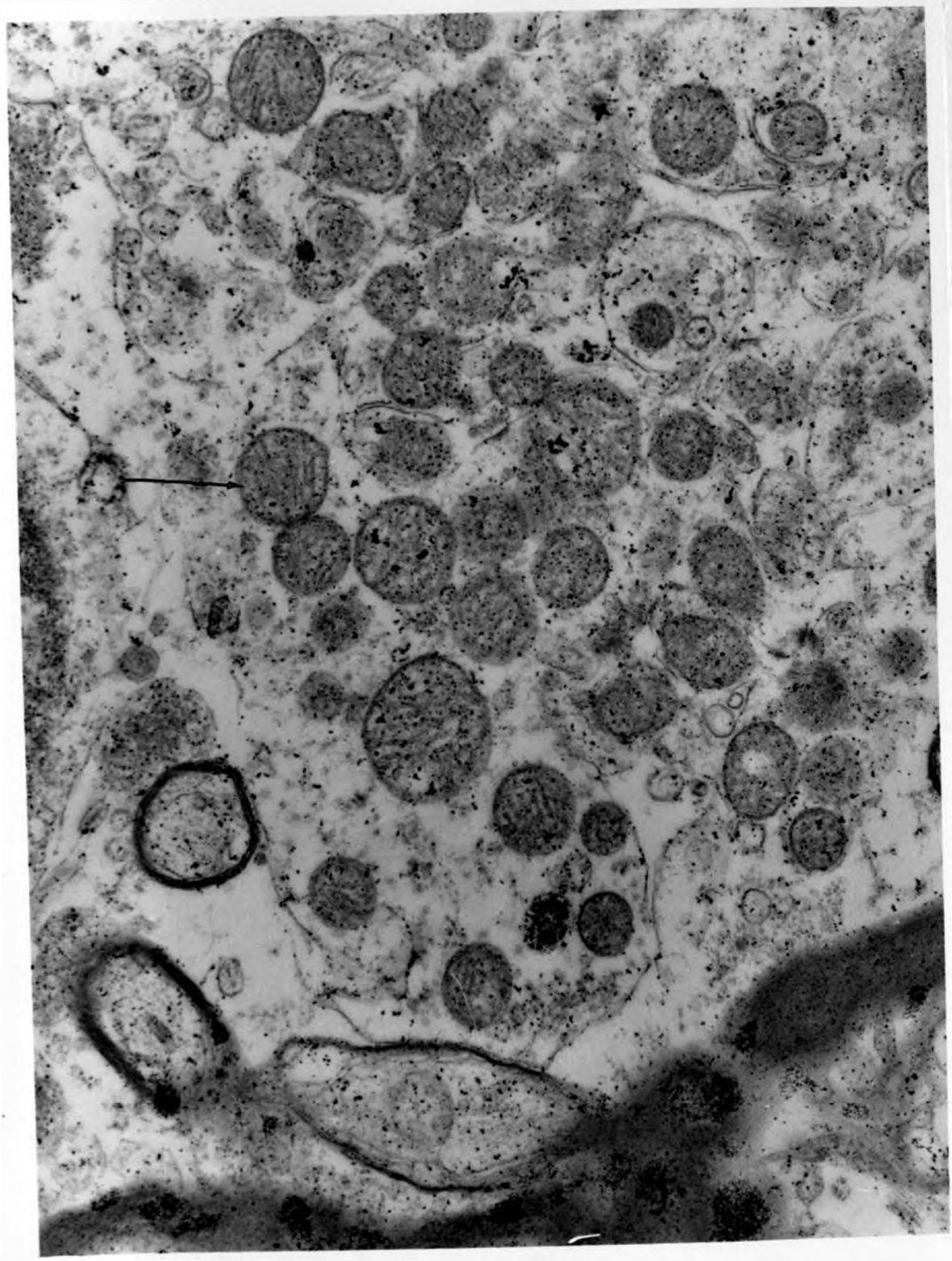


Figure 5.16. Lung of IFN–α/βRo/o infected mouse. A: Note an infected cell with the shape of the cell being altered by the *C. ruminantium* colonies in the cytoplasm and the nucleus being pushed to one side. Two forms of the infectious agent which seem to be separately encapsulated, can be seen, with larger reticulated forms in the upper one (triangle arrow head) and electro-dense forms in the lower ones (curved arrow). B: Presence of the dividing forms of *C. ruminantium* (reticulated forms) can be seen in the infected cell (straight arrow). Note the presence of haemorrhages and oedema fluid in the lung tissue (X6,900). (Micrograph No. 1575- IFN–α/βRo/o)

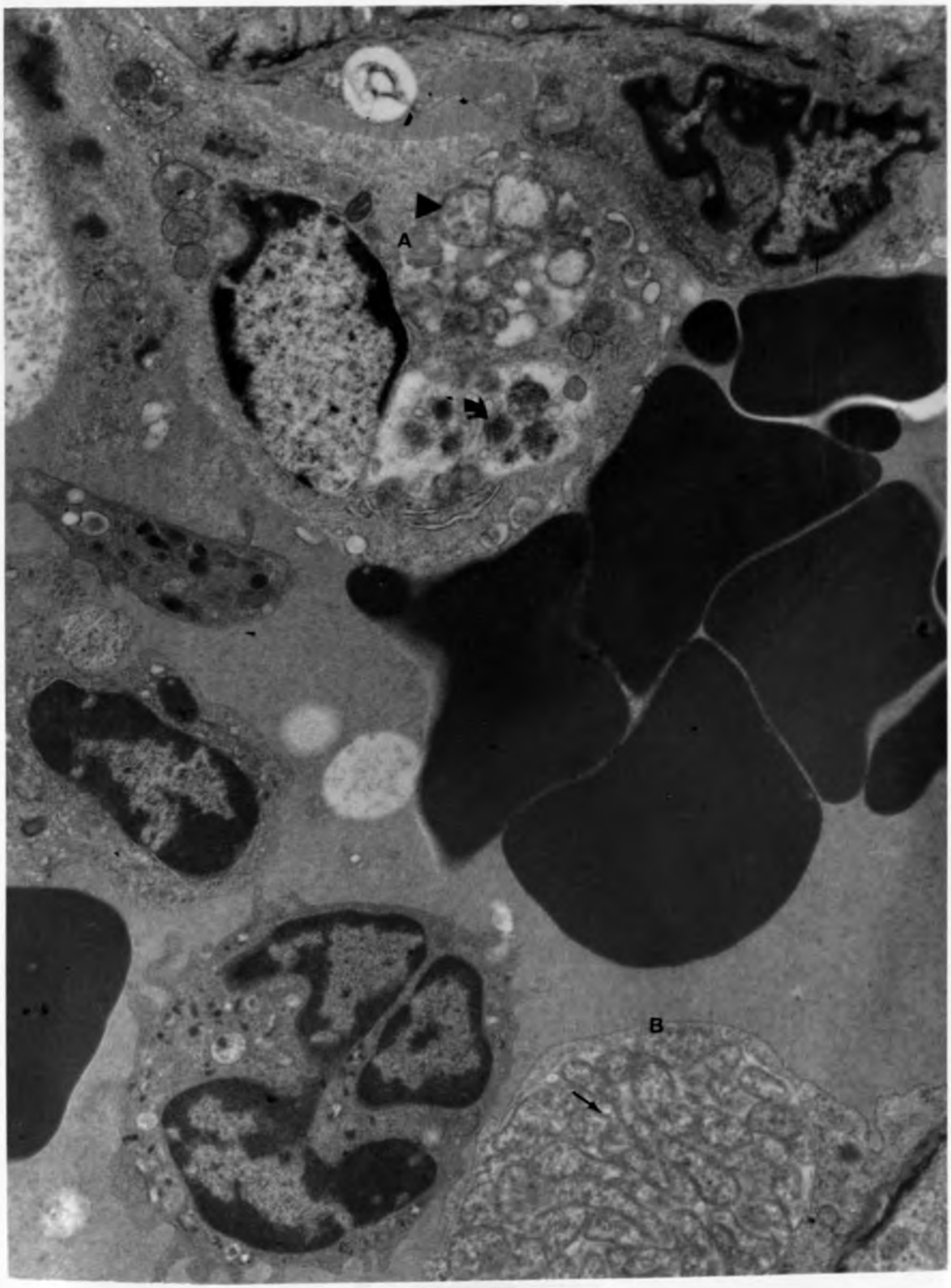

A
B

Figure 5.17. Lung of an infected IFN–α/βRo/o mouse showing increased permeability with seepage of oedema fluid (o) and red blood cells (r) into the alveoli spaces. Note the macrophage (m) in alveolar space (X9,000) (Micrograph No. 1571-IFN–α/βRo/o Lung).

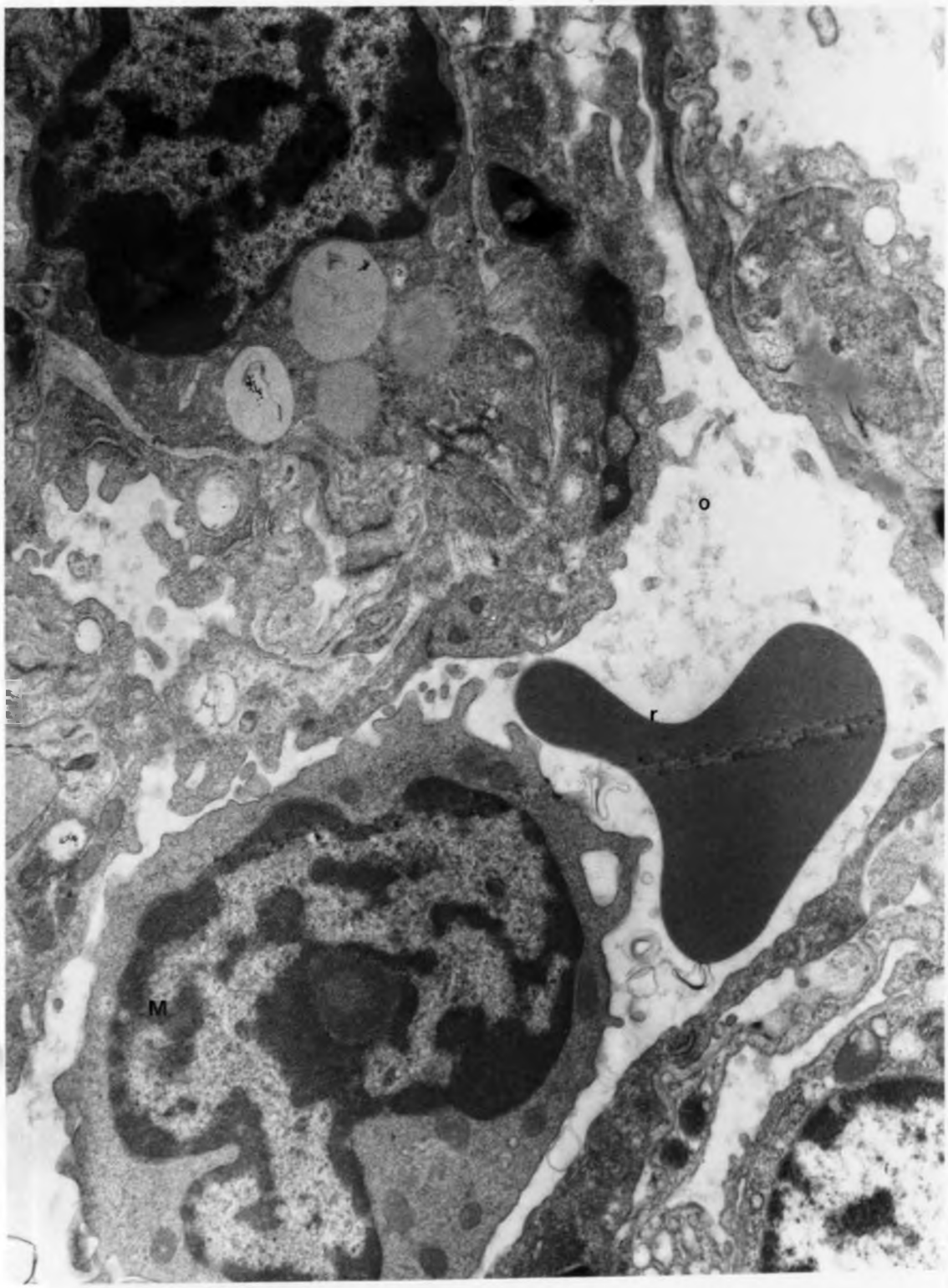
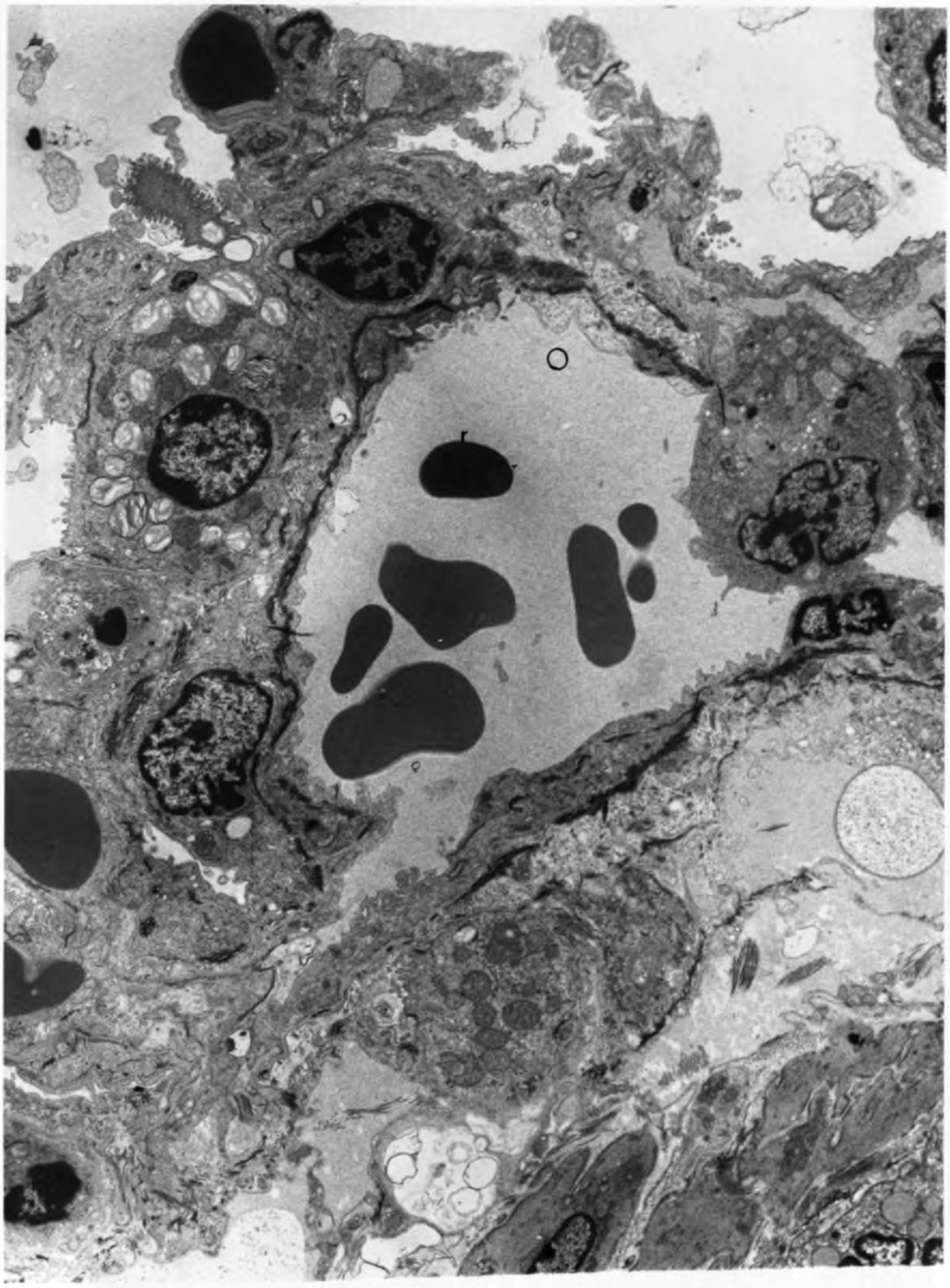

o
r
M

Figure 5.18. Lung of infected control mouse showing disruption of the vascular wa integrity resulting in seepage of red blood cells (r) into lung parenchyma. Note also th presence of oedema fluid (o) in the lung parenchyma (X3,225). (Micrograph No. 1413. -Wi Type)



5.4. Discussion

This study demonstrated that mice lacking in receptors for type I (IFN–α/β) and type II (IFN–γ) interferons had reduced protection from *C. ruminantium* infection. This was shown by significant differences in the clinical and pathological manifestations between the receptor deficient mice and the controls. Infection of the IFN–γRo/o and to a lesser extent, IFN–α/βRo/o mice resulted in an acute explosive disease characterised by shorter incubation period, shorter course of disease, increased thoracic oedema fluid and higher morbidity and mortality rates as a function of time as compared to the controls (Table 5.1 and Figure 5.1). The IFN–γRo/o mice manifested lower resistance to the disease than the IFN–α/βRo/o in that they had shorter mean incubation period, higher levels of thoracic oedema fluid and higher mortality rates. IFN–γRo/o and IFN–α/βRo/o mice showed significantly higher infection rates in lung and brain tissue as compared to mice in the control group. The ease of finding *C. ruminantium* infection in the brain was striking as this is considered a rare finding (Prozesky and Du Plessis, 1985b). In addition, the marked exudation and infection of peritoneal macrophages by *C. ruminantium* in the IFN–γRo/o mice was considered as an unusual feature of infection in mice, but was exploited by Du Plessis (Du Plessis and Malan, 1987) to develop the 'Kumm' antigen using the Kumm strain for use in indirect fluorescent test for serological diagnosis of heartwater. It is tempting to speculate that the Kumm strain, which leads to high levels of infection in peritoneal macrophages and is infective to mice by the intra-peritoneal route, which is not the case for other isolates, is relatively resistant to IFN–γ, allowing higher infection rates in peritoneal macrophages. Furthermore, strain variation to IFN–γ susceptibility has been reported for isolates of *Rickettsia prowazekii* (Turco and Winkler, 1994).

The mice showing higher infection rates revealed a more extensive pathology in lung histopathological sections with the IFN–γRo/o mice showing evidence of increased alveolar oedema (pink-staining mass) and inflammatory cell infiltration than in the control group. The IFN–γRo/o and IFN–α/βRo/o groups also revealed a more thickened alveolar wall which was attributed to a more extensive inflammatory reaction characterised by increased infiltration of the interstitia by predominantly mononuclear cells and also due to distension by oedema fluid (interstitial oedema). However, although the infection rate was up to 8 fold higher in the receptor deficient mice as compared to the control, the oedema fluid was increased but not significantly so. It may therefore be the case that higher infection rate resulted in the earlier occurrence of oedema which resulted in fatal outcome with earlier mortalities in these mice.

Ultrastructural studies in lung and brain sections revealed differences in morphology of the infectious agent in the IFN–γRo/o group, where electron-dense bizarre looking forms of *C. ruminantium* were seen in cytoplasm of endothelial cells and macrophages as compared to the IFN–α/βRo/o and control groups. These forms were highly pleiomorphic ranging from commas, rings, rods and oval shapes, as compared to the consistent observation of reticulate forms seen in the IFN–α/βRo/o and control group. The reasons behind these morphological differences were not conclusively known but one possible explanation for these differences may be determined by the developmental stage of the organism in the disease at the time of death, implying that the infection in the IFN–γRo/o mice may have been predominantly at a later developmental stage (elementary body), while in the control group, the organisms were predominantly at an early developmental stage (reticulate forms) with some seemingly undergoing binary fission. A mixture of these forms of *C. ruminantium* were seen in the IFN–α/βRo/o group further supporting this hypothesis and explaining why the clinical disease and pathology in this group was also intermediate between the two groups. Although these mice

were sacrificed on the same day (day 7) the stages seen on electron microscopy suggest infection may have reached a different developmental stage. This implies that compromised host immune defence mechanisms may result in more rapid developmental cycle with the result that late stage forms predominated in IFN–γRo/o mice whereas dividing reticulate forms predominated in the control mice which had not developed hypothermia on the same day. *C. ruminantium* has a developmental cycle of approximately 5-6 days (Jongejan, 1990) and this implies that the developmental cycle in the IFN–γRo/o mice was more advanced due to less control by the host immune defence mechanisms and may have been in its 'second wave', at the time the sections were made (7 days post-infection), whereby, fresh-released EBs were re-infecting new cells, while in the control there was some mechanism exhibiting a check on the rate of the development of the disease and re-infection rate.

This study agrees with other findings on the role of cell mediated immunity and interferons in disease resistance. Interferons are cytokines that play a complex and central role in the resistance of mammalian hosts to pathogens (Boehm *et al.*, 1997). The inhibition of *Cowdria* growth by IFN–γ has been reported in both *in vitro* and *in vivo* studies (Totte *et al.*, 1993 and 1994, Mahan *et al.*, 1996 and Mutunga *et al.*, 1998). Similar findings have been reported for other infections including other rickettsia diseases. IFN-γ and TNF-α depleted mice infected with a sublethal infectious dose of *Rickettsia conorii* resulted in fatalities in the cytokine-depleted mice but not the controls, with impairment of nitric oxide (NO) being implicated as the main cause of this overwhelming disease (Feng *et al.*, 1994). Walker *et al.*, (1996) reported similar findings in which depletion of IFN–γ and TNF-α receptors was found to convert a sublethal infection of *Rickettsial conorii* into a fatal disease with overwhelming rickettsia growth and decreased nitric oxide synthesis. Liew *et al.*, (1990), reported *in vitro* killing of *Leishmania major* by IFN–γ was through the NO pathway and also showed that

injecting the infected mice with the NO inhibitor, L-NMMA, resulted in 10^4 fold increase in the number of parasites in lesions. IFN–γ was also found to kill *Schistosoma mansoni* parasites through induction of NO production (Oswald *et al.*, 1994). It was also reported that IFN–γ provided by malaria -specific $CD8^+$ cells stimulates liver cells to produce NO for the destruction of infected hepatocytes and the parasites within these cells (Klotz *et al.*, 1995). The absence of IFN–γ receptor may result in reduced NO production, and NO may provide one of the effector mechanisms of controlling cell to cell spread of infection since NO is inhibitory to infection of EBs to endothelial cell *in vitro* (Mutunga *et al.*, 1998).

Antiviral activities of IFN–α/β have also been reported by several reviewers including Svensson *et al.*, (1995) and Haria and Benfield (1995). Higher levels of IFN–α were found in cattle that resisted *C. ruminantium* infection as compared to lower levels found in those that died of the disease (Totte *et al.*, 1994). A similar picture was also reported for *in vitro* studies where IFN–α reduced the yield of *C. ruminantium* (Totte *et al.*, 1994). This inhibitory effect of IFN–α to *C. ruminantium* was found to be limited in that very high concentrations of IFN–α (1000 U/ml) did not completely prevent growth of the *Cowdria* organisms (Totte *et al.*, 1994). This agrees with our findings in this study were IFN–α/βRo/o mice suffered a more acute disease than the control infections, but a milder one as compared to IFN–γRo/o mice. Similar findings were also reported in studies carried out using IFN–α for the treatment of hepatitis B or C in which the results were shown not to be optimal with only less than 20% recovering and relapses occurring once treatment was discontinued (Haria and Benfield, 1995), thus, limited inhibitory effect of IFN–α was further suggested in their work.

Murine macrophages have been reported to be activated by either IFN–γ or IFN–α/β in combination with LPS to induce synthesis of TNF- α and iNOS (Zhou *et al.*, 1995). IFN–γ has been reported to induce induction of IFN–α and IFN-β and its function and response to

be regulated by other cytokines (Boehm *et al.*, 1997). IFN–α/β have been said to be produced early during viral infections and that they have antiviral activity including induction of a Th-1 type immune response (Zhou *et al.*, 1995). Th-1 immune response is important in intracellular infections including *C. ruminantium* infection, and therefore lack of receptors for this cytokine would understandably result in a more severe infection as compared to control animals. IFN–α/β may not completely eradicate the infectious agent but offers a check in the multiplication of the disease, affording the animal longer survival rate. It has been reported that IFN–α/β (type I) and IFN–γ (type II) act through similar but distinct pathways to prime macrophages for the induction of TNF-α and iNOS (Zhou *et al.*, 1995). Both IFN–γ and IFN–α/β induce synthesis and expression of MHC class I antigen but only IFN–γ induces MHC class II antigen expression in murine macrophages (Zhou *et al.*, 1995). Although both IFN–γ and IFN–α/β have antiviral activities, they act to a different extent. It has been shown that these cytokines act through several transcription factors at the gene level , one of which is interferon regulatory factor 1 (IRF-1) (Zhou *et al.*, 1995). IRF-1 was shown to be prerequisite for the induction of the iNOS gene by either IFN–γ or IFN–α/β (Kamijo *et al.*, 1994). Thus it has been generally concluded that type I (IFN–α/β) and type II (IFN–γ) induce in part the same genes, although to a different extent owing to subtle but distinct differences in gene regulation (Shuai, 1994). This may explain the differences in disease severity in the two receptor deficient groups.

Mitochondrial degenerative changes were noted in brain sections in some mice from the control and IFN–α/βRo/o groups but there was not much evidence of these changes in the IFN-γ Ro/o group. One hypothesis for this difference may implicate involvement of NO. IFN–γ activates phagocytic cells including endothelial cells to produce NO which has been reported to suppress mitochondrial respiration by inhibiting electron transport through the respiratory

chain and to decrease mitochondrial membrane potential (Gross *et al.*, 1996). It is probable that induction in mice macrophages of iNOS to produce NO did not occur in the IFN–γRo/o mice, since NO synthesis is upregulated by IFN–γ (Migliorini *et al.*, 1991, Hanson, B. 1991, Oswald *et al.*, 1994, Gale *et al.*, 1997), and that it was with the absence of increased NO and oxidative enzymes that resulted in sparing the mitochondrial degeneration.

Conclusion

It is apparent from this study that *C. ruminantium* is inhibited or killed *in vivo* by events involving the activities of IFN-γ and possibly IFN-α/β. The mechanisms of action are not fully known but the overall picture showed by this study is that, although 'normal' mice undergo a lethal infection with low dose of *C. ruminantium* organisms, there is some control of the infection as compared to the IFN–γ and IFN–α/βRo/o mice, showing that lack of interferons/receptors can result in an explosive disease that has lost control. The killing of various parasites by IFN–γ has been documented. The absence of IFN–γ receptors is of greater effect than loss of IFN–α/β receptor activity. Infection of mice deficient in the receptors for these cytokines resulted in early death, severe pathology and massive rickettsial infection rates in tissues due to a failure of the host defences to destroy intracellular rickettsiae. Absence of IFN-γ and IFN–α/β receptors implies reduction in macrophage and inflammatory cell activation leading to a more severe disease as a result of reduced destruction of the intracellular infectious agent, and perhaps in consequence to release of other macrophage products that may be deleterious to survival. This may also mean there is less NO generation and therefore less anti-*Cowdria* activity. Previous studies have demonstrated the inhibitory effect of interferon gamma (IFN-γ) on *C. ruminantium in vitro* (Mahan *et al.*, 1996; Totte *et al.*, 1994; Mutunga *et al.*, 1998). An infection-limiting response mechanism seems to exist in normal mice infected with *C. ruminantium*. Lack of IFN–γ and IFN–α/β receptors can therefore result in reduced destruction of pathogens by the host

immune mechanisms leading to a more severe pathology due to higher numbers of infectious agent.

Since some control of infection occurred in the control group of mice, factors which control the level of IFN–γ and to a lesser extend, IFN-α/β responses may be of great importance to the outcome of infection. These may include genetic differences between mice but also the upregulation of macrophage activation and upregulation of IFN–γ production by other cytokines such as interleukin 12 (IL-12). The studies in the following chapter (chapter 6) were designed based on this hypothesis. Involvement of IL-12 in the immune response of *C. ruminantium* was studied in the work reported in the next chapter (Chapter 6).

CHAPTER 6

CYTOKINE STUDIES DURING EXPERIMENTAL *COWDRIA RUMINANTIUM* INFECTION OF BALB'C MICE; INVOLVEMENT OF IL-12 IN PROTECTIVE RESPONSES

6.1. Introduction

Heartwater is caused by an obligate intracellular rickettsial organism, *Cowdria ruminantium* which multiplies in endothelial cells and also phagocytic cells including neutrophils (Cowdry, 1925; Logan *et al.*, 1987). The clinical manifestations and severity of the disease are mainly associated with severe respiratory, cardiac and nervous embarrassment mainly due to increased capillary permeability leading to oedema (Clark, 1962; Prozesky and Duplessis, 1985b; 1985c). The pathogenesis and immunologic mechanisms of *C. ruminantium* infection remain obscure and this is a major drawback at a time when developing a vaccine for the disease is of paramount importance due to the massive losses of improved livestock breeds to the disease experienced in the tropics. Cells in the immune system act mainly under the influence of signals from other cells and these activities are regulated by cytokines (Playfair, 1995). Previous studies have highlighted the role played by cell-mediated immune response in *C. ruminantium* infection, which is mediated in part through the production of inhibitory cytokines including inteferons (Totte *et al*; 1993; Mahan *et al.*, 1994; 1996). Cytokines have been found to have widespread and overlapping functions, including influence on the production of reactive nitrogen intermediates like nitric oxide. In order to understand the pathogenesis and non-specific and adaptive immune responses in *C. ruminantium* infections, it is important to investigate the cytokine kinetics induced as a response to the infection. An infection-limiting response mechanism involving IFN-γ activities occurs in normal mice infected with *C. ruminantium* (Chapter 5) and a possible influence upon the production of this cytokine response is interleukin 12 (IL-12).

Interleukin 12 (IL-12) is a pro-inflammatory cytokine produced by phagocytic cells, B cells and other antigen-presenting cells in response to bacteria, bacterial products and intracellular parasites (D'Andrea *et al.*, 1992; Trinchieri, 1993; 1995). IL-12 has been recognised as a central cytokine in early non-specific innate resistance by inducing production of IFN–γ from NK and T cells, which contributes to phagocytic cell activation and inflammation (Kobayashi *et al.*, 1989). IL-12 is known for its role as a promoter of Th 1 cell generation, which are considered central to eradication of intracellular pathogens and acting in antagonism to IL-4, the major promoter of Th 2 responses (Flesch and Kaufman, 1990).

The first experiment described was designed to investigate the cytokine and nitric oxide profiles during *C. ruminantium* infection with special reference to IFN–γ, IL-12p70, IL-4 and IL-10, in an attempt to clarify the host-parasite interactions in the disease. The studies in the second experiment were prompted by the extensive biological effects mediated by IL-12, to examine the role of this cytokine with, and in relation to other cytokines, in *C. ruminantium* infection. This was done by treatment of Balb'c mice with mouse recombinant IL-12 (MrIL-12) given at different doses and different times during the infection. The effects of the cytokine administration upon the clinical disease, pathological and immunological processes of *C. ruminantium* infection were investigated.

6.2. Experiment 1: Cytokine studies in Balb'c mice infected with *C. ruminantium*

6.2.1. Materials and Methods

6.2.1.1. Experimental design

Seventy male 8-10 week old Balb'c mice were used in this study. The source and management of the mice before and after infection was as described in section 2.3.1.1. Six mice (identified as numbers 1-0 to 6-0 -see appendix 4) were sacrificed on day 0, prior to infection, and the spleens removed aseptically and stored in sterile PBS before being weighed and homogenised in CHAPS as described in section 2.3.4. The remaining 64 mice were divided into groups identified as A to M (Table 6.1) and individual mice in each group were given numbers 1 to 6 (see appendix 4). Forty two of the mice were infected with the Welgevonden isolate of *C. ruminantium* each receiving a dose of $1x10^3$ EBs diluted in 200 µl culture medium. The concentration of EBs was determined by fluorescein diacetate staining as described in section 2.1.5. The remaining 22 mice were mock infected with the same volume of culture medium. Six infected and 4 mock infected mice were randomly selected and sacrificed every two days and the spleens removed, weighed and homogenised in CHAPS and stored at -20°C (see appendix 4). The spleen weights were compared between the *Cowdria*-infected and the uninfected controls. The spleen homogenates were used to assay for IL-12p70, IFN–γ, IL-10, IL-4 and nitrite levels and these were compared between infected and mock infected (uninfected) controls and also compared between different days during the disease.

Table 6.1.

GROUP	INFECTED OR UNINFECTED
A	INFECTED
B	INFECTED
C	INFECTED
D	INFECTED
E	INFECTED
F	INFECTED
G	INFECTED
H	UNINFECTED
I	UNINFECTED
J	UNINFECTED
K	UNINFECTED
L	UNINFECTED
M	UNINFECTED

INFECTED = Groups infected with *C. ruminantium*
UNINFECTED = groups mock infected with culture medium (uninfected controls)

6.2.1.2. Clinical disease monitoring

The mice were monitored daily for clinical disease, and by recording rectal temperatures as described in section 2.3.2. The incubation period was considered to be the period of time prior to the onset of hypothermia.

6.2.1.3. Cytokine and nitrite assays:

The levels of IFN–γ, IL-12 p70, IL-4, and IL-10 in the spleen homogenates of all the 56 sacrificed mice were assayed using commercial ELISA kits as described in materials and methods in sections 2.7.1.1, 2.7.1.3, 2.7.1.4 and 2.7.1.5. Nitrite levels were assayed in spleen homogenates using the Griess assay method as described in section 2.2.1.

6.2.2. Results

6.2.2.1. Clinical and pathological findings

The infected mice, with the exception of those sacrificed before disease onset, developed marked hypothermia, with temperatures dropping to below 32°C from day

7 post-infection, followed by development of respiratory distress and general malaise of the affected animals. The mean incubation period was 7.4 days and the mean course of disease was 2.1 days with a range of 1-3 days. Significant differences were noted in the spleen weights between the infected and uninfected controls (Anova, p=0.0002) from day 4 to day 10, with the highest mean spleen weight being recorded on day 6, which was very significantly greater than day 0 spleen weight (t test, p=0.001) (Figure 6.1.a and Appendix 4.1). This weight increase on day 6 was not maintained and had reduced on day 8 (Figure 6.1.a). Only one mouse had showed pleural effusion prior to onset of hypothermia (day 6) but all the mice sacrificed after onset of hypothermia showed marked thoracic oedema (Figure 6.1b and c and Appendix 4.2).

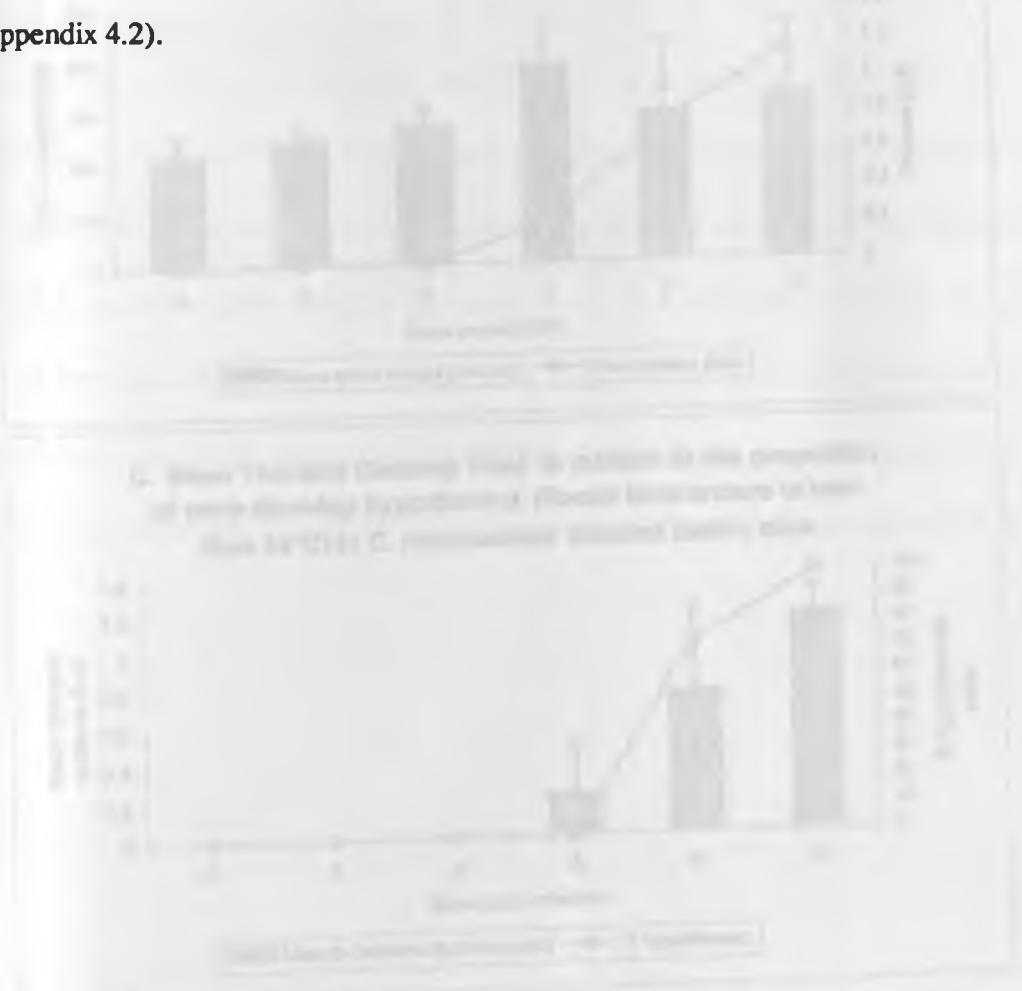
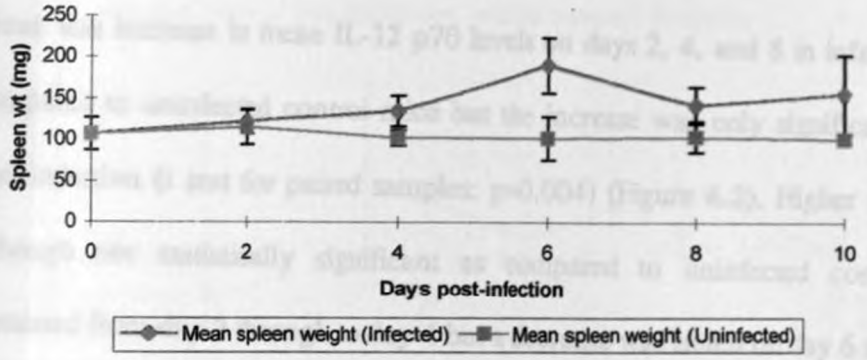
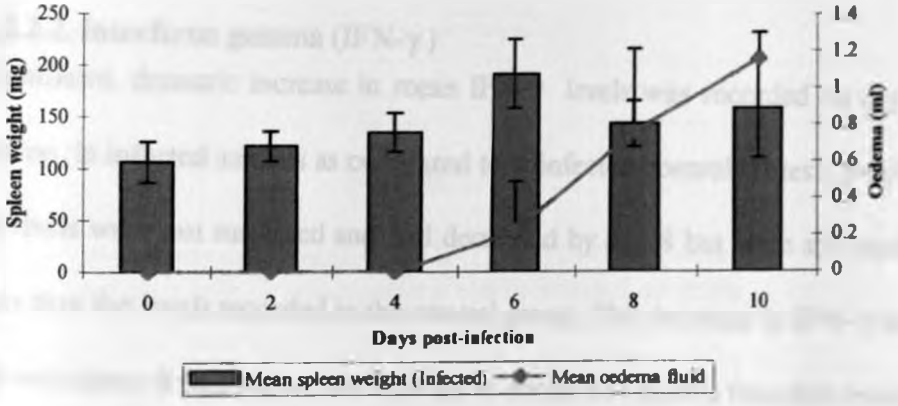


Fig. 6.1.

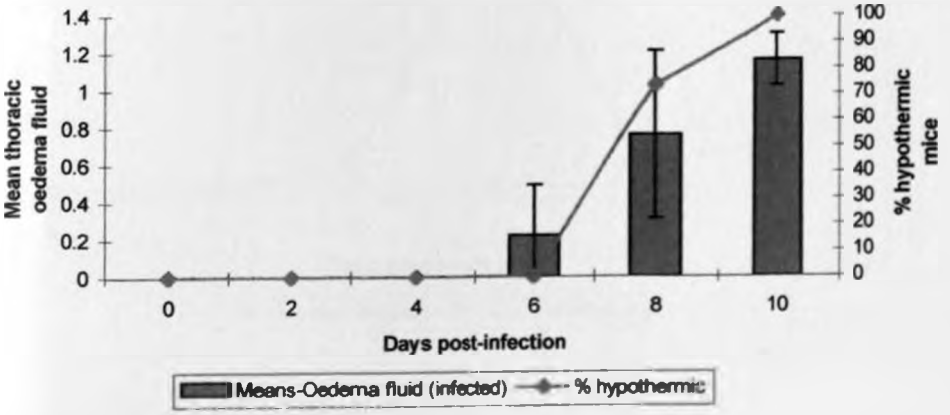
A. Mean Spleen weights (mg) of Balb'c mice infected or not infected with *C. ruminantium*



B. Mean Spleen Weight (mg) in relation to amount of Thoracic Oedema Fluid (ml) in mice infected with *C. ruminantium*



C. Mean Thoracic Oedema Fluid in relation to the proportion of mice showing hypothermia (Rectal temperature of less than 34°C) in *C. ruminantium* infected Balb'c mice



6.2.2.2. Cytokine levels in mice infected with *C. ruminantium*

6.2.2.2.1. Interleukin 12 p70 (IL-12p70)

There was increase in mean IL-12 p70 levels on days 2, 4, and 8 in infected mice as compared to uninfected control mice but the increase was only significant on day 2 post-infection (t test for paired samples: p=0.004) (Figure 6.2). Higher mean levels, although not statistically significant as compared to uninfected controls, were sustained from day 2 through to day 4 but a decrease was noted on day 6 and 10 post-infection at which time the levels were almost the same as those of uninfected control (Figure 6.2 and Appendix 4.4).

6.2.2.2.2. Interferon gamma (IFN-γ)

A significant, dramatic increase in mean IFN–γ levels was recorded on day 6 post-infection in infected animals as compared to uninfected controls (t test; p=0.02). The high levels were not sustained and had decreased by day 8 but were still significantly higher than the levels recorded in the control group. This increase in IFN–γ levels on day 6 was about 8 fold higher (an increase of about 800 pg/ml) than that recorded for uninfected controls and about 4 fold higher than that recorded for infected animals on days 8 and 10 (about 100 and 200 pg/ml respectively) (Figure 6.3 and Appendix 4.3).

Fig. 6.2.

Mean Interleukin 12 p70 (IL-12p70) levels in Balb'c mice infected with *C. ruminantium* and uninfected controls

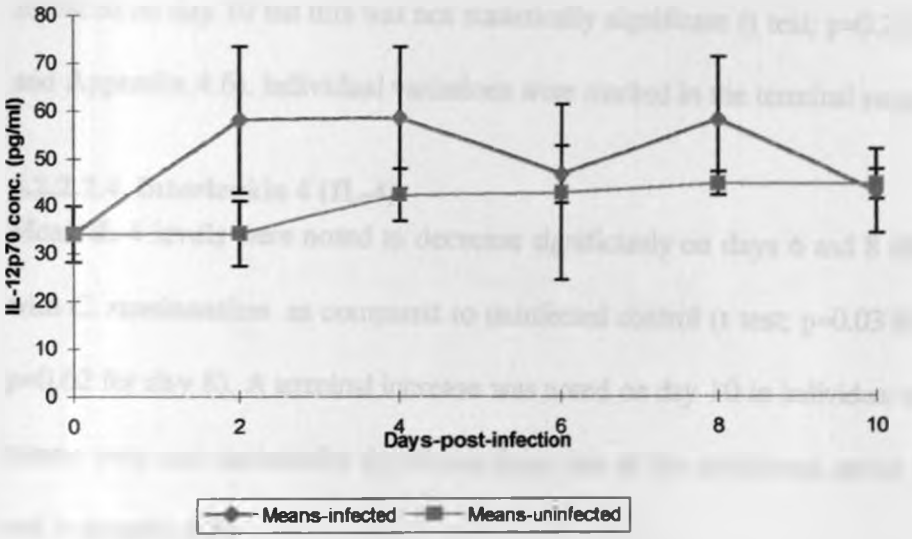
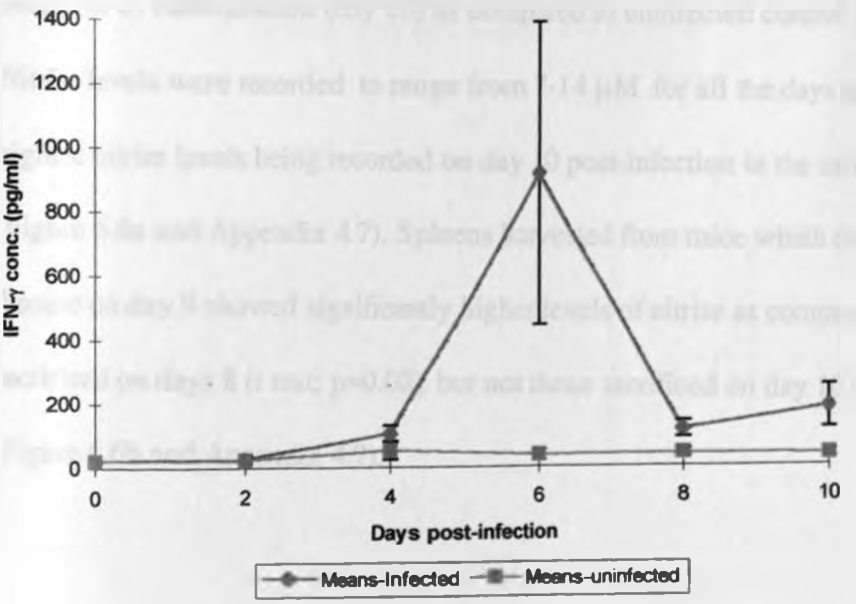


Fig. 6.3.

Mean Interferon gamma (IFN-γ) levels in Balb'c mice infected with *C. ruminantium* and uninfected controls



6.2.2.2.3. Interleukin 10 (IL-10)

There were no significant differences in the mean IL-10 levels between infected and uninfected controls in the course of infection (t test; $p>0.05$). A slight increase was recorded on day 10 but this was not statistically significant (t test; $p=0.22$) (Figure 6.4 and Appendix 4.6). Individual variations were marked in the terminal stages (day 10).

6.2.2.2.4. Interleukin 4 (IL-4)

Mean IL-4 levels were noted to decrease significantly on days 6 and 8 after infection with *C. ruminantium* as compared to uninfected control (t test; $p=0.03$ for day 6 and $p=0.02$ for day 8). A terminal increase was noted on day 10 in individual mice but the means were not statistically significant from that of the uninfected group (Figure 6.5 and Appendix 4.5).

6.2.2.3. Nitrite levels

Significant increase in mean nitrite levels were recorded during the later terminal stages of *C. ruminantium* (day 10) as compared to uninfected control (t test, $p=0.02$). Nitrite levels were recorded to range from 7-14 μM for all the days assayed with the highest nitrite levels being recorded on day 10 post-infection in the sacrificed mice (Figure 6.6a and Appendix 4.7). Spleens harvested from mice which died of the disease on day 9 showed significantly higher levels of nitrite as compared to those sacrificed on days 8 (t test; $p=0.02$) but not those sacrificed on day 10 ($p=0.07$) (Figure 6.6b and Appendix 4.7).

Fig. 6.4.

Mean Interleukin 10 (IL-10) levels in Balb'c mice infected with *C. ruminantium* and uninfected controls

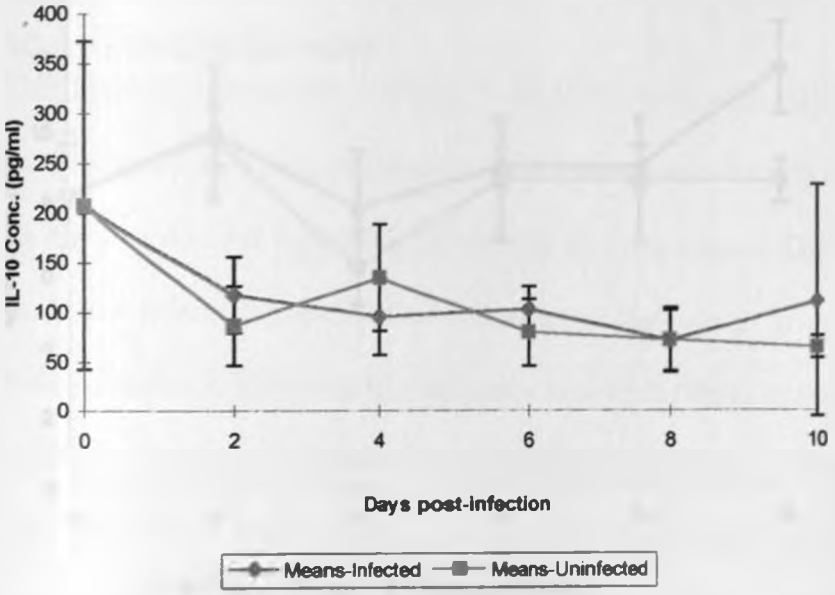


Fig. 6.5.

Mean Interleukin 4 (IL-4) levels in Balb'c mice infected with *C. ruminantium* and uninfected controls

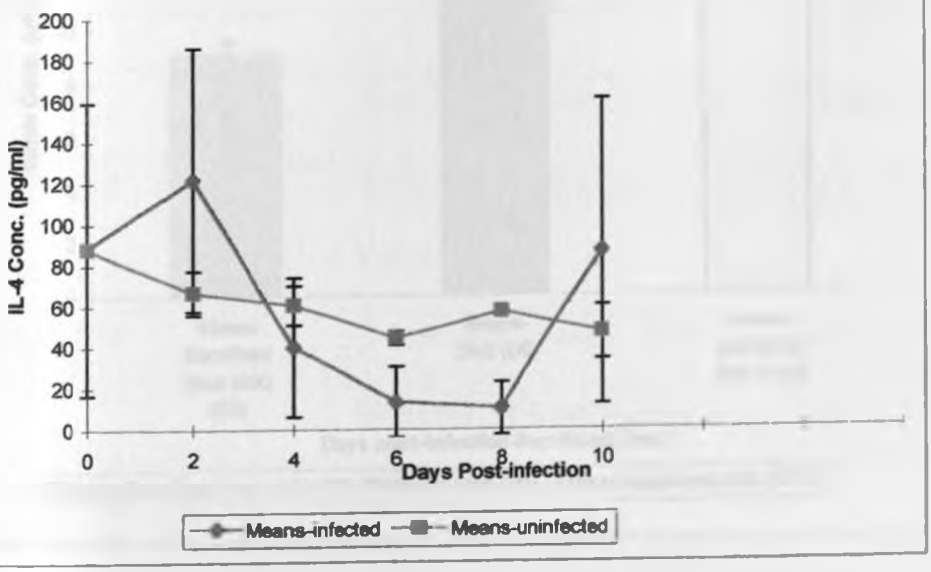
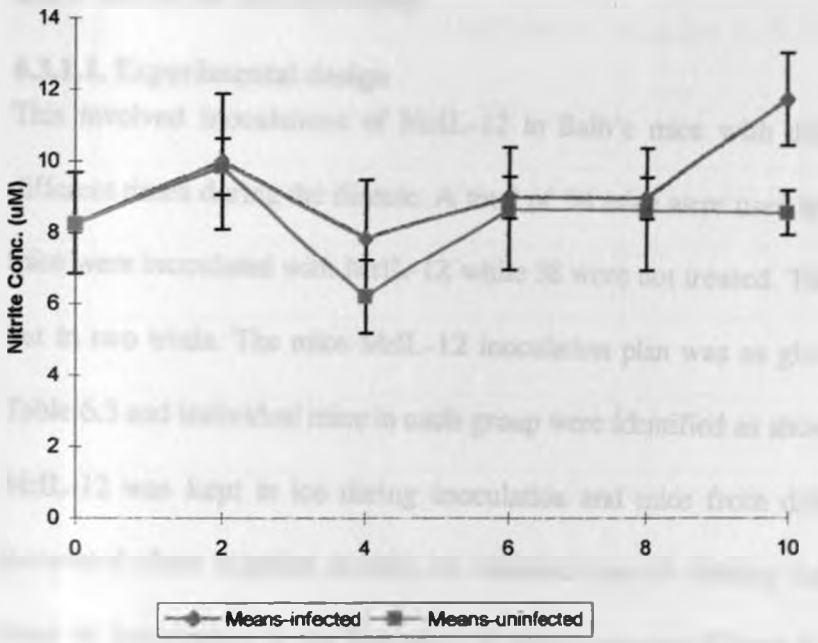
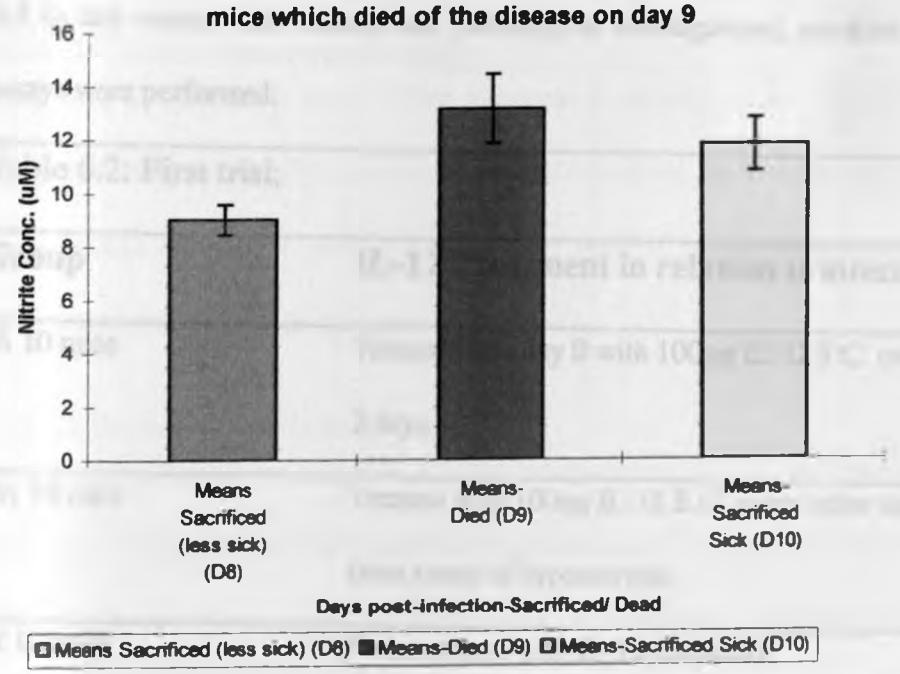


Fig.6.6.

A. Mean Nitrite levels in Balb'c mice infected with *C. ruminantium* and uninfected controls



B. A comparison between nitrite levels in terminally ill, dead and less ill mice: Mean Nitrite levels in *C. ruminantium* infected mice sacrificed on day 8 and 10 as compared to mice which died of the disease on day 9



6.3. Experiment 2: IL-12 inoculations in Balb'c mice

6.3.1. Material and Methods

6.3.1.1. Experimental design

This involved inoculations of MrIL-12 in Balb'c mice with different doses and at different times during the disease. A total of 94 mice were used in this study, sixty six mice were inoculated with MrIL-12 while 38 were not treated. This work was carried out in two trials. The mice MrIL-12 inoculation plan was as given in Table 6.2 and Table 6.3 and individual mice in each group were identified as shown on appendix 5. MrIL-12 was kept in ice during inoculation and mice from different groups were inoculated close together in time, to minimise loss of viability due to differences in times of inoculation. In the first trial, 30 mice were used (Table 6.2) and clinical and pathological investigations and immunoglobulin isotyping of anti-*Candida* antibodies were carried out. In the second trial 64 mice were used and treated as shown in Table 6.3 In this second trial clinical and pathological investigations, cytokine and mitotic assays were performed.

Table 6.2: First trial;

Group	IL-12 Treatment in relation to infection
IA 10 mice	Treated from day 0 with 100ng IL-12 S.C. every 2 days
IIA 10 mice	Treated with 100ng IL-12 S.C. every other day from onset of hypothermia
III 10 mice	No treatment with IL-12 (control)

Table 6.3: 2nd Trial	
Group	**IL-12 Treatment in relation to infection**
IB 18 mice	Treated with 500 ng MrIL-12 S.C. 24 h prior to
	infection and a 2nd inoculation of the same dose 7 days later.
IIB 18 mice	Treated with 500 ng Mr IL-12 S.C. 24 h prior to infection
	only.
III 18 mice	Treated with 0.1 ml PBS 24 h prior to infection .
IV 10 mice	Uninfected controls; 5 mice were inoculated with 500 ng MrIL-12 S.C. 24 h before infection of the test animals; 5 mice were left as uninfected and untreated controls.

Individual mice were identified in each group as shown on appendix 5.

6.3.1.2. Clinical disease monitoring

This was as described for the first experiment whereby rectal temperatures were recorded daily and the following assessed and compared between the groups; days to hypothermia (incubation period), period from onset of hypothermia to death (course of disease) morbidity and mortality rates.

6.3.1.3. Immunoglobulin levels

Blood was collected for sera from 10 mice per group on days 0, and post-infection on days 8, 16 and 24 in animals surviving the infection. The sera was used in enzyme-linked immunosorbent assay for different isotypes of IgG i.e. IgG1, IgG2a, IgG2b and

IgG3 as described in section 2.4.3. The levels were compared between the groups of mice.

6.3.1.4. Pathological studies

Four mice from each group were sacrificed on day 8 post-infection. The spleens were harvested, weighed and homogenised as described in section 2.3.4. and the amount of thoracic oedema fluid (pleural effusions) was measured using a calibrated syringe. This was also conducted for all the other subsequent dead mice. From the sacrificed mice, spleens were collected aseptically into sterile PBS and homogenised as described for the first experiment above, section 6.2.1.1. Lung and brain tissues were also collected from the sacrificed mice and fixed in 4% buffered formalin for histopathology. The tissues were prepared and stained as described in section 2.3.3.1.

6.3.1.5. Cytokine and nitrite assays:

Spleens were collected from mice in groups inoculated with 500 ng/ml MrIL-12 (groups IB and IIB) and the control group on days 8, 10, 11 and 13 post-infection and levels of IFN–γ assayed using a capture ELISA assay. The levels of IFN–γ, IL-12 p70, IL-4, IL-10 and nitrite levels in the spleen homogenates were assayed as previously described in this chapter.

6.3.2. Results

6.3.2.1. Incubation period, course of disease, morbidity and mortality rates

Differences in incubation period, morbidity and mortality rates were noted between the four groups of *C. ruminantium* infected-mice which had also been inoculated with MrIL-12 as compared to the control groups. Groups of mice inoculated with MrIL-12 before or at time of infection (IA, IB and IIB) resulted in increased incubation period, prolonged course of disease and lower morbidity and mortality rates as a function of time, as compared to the group inoculated after disease onset (IIA) and the control groups (III and IV) (Table 6.4 and Appendix 5.1). The highest protection was recorded in the group inoculated with 100ng MrIL-12 from start of infection with booster doses every 2 days post-infection (group IA). This group manifested the longest mean incubation period (10 days) and lowest morbidity and mortality rates, of 80% and 50% respectively. Morbidity and mortality rates were both significantly lower in this group as compared to the group inoculated after disease onset (IIA) and the control group (Table 6.4). Twenty percent of the mice in group IA did not show any evidence of or clinical signs of disease whereas none of the mice in groups IIA and III were resistant to or survived the disease. Administration of 500 ng of MrIL-12 twenty four hours before infection, with or without a booster dose 5 days post-infection (groups IB and IIB respectively) also resulted in increased survival (27 and 18 % respectively) (Table 6.4). A booster inoculation of MrIL-12 given 5 days post-infection (group IB) increased protection of the mice in comparison to the single inoculation, with the mice showing longer mean incubation period, and lower morbidity and mortality rates as compared to group IIB

which was inoculated only once before infection (Table 6.4). Administration of 100 ng of MrIL-12 after disease onset (IIA), however, did not seem to alter the clinical picture of the disease from that of the untreated controls with no significant differences (t test and Chi-square; $p>0.05$) in mean incubation period, mortality and morbidity rates between treated and the untreated controls. There were slight differences in the course of the disease from onset of hypothermia to death between the different groups. The mice inoculated with 500ng MrIL-12 before infection (1B and IIB) recorded the longest course of disease, with a mean of 2 days as compared to the control group which had a mean of 1.4 (Table 6.4) although the differences were not significant. 45% of *C. ruminantium* infected mice in group IA survived the disease while 27% and 18% of mice in groups IB and IIB respectively also survived the disease while none survived in the control group (Table 6.4). Although the survival rate was not very high in groups IB and IIB, the mice inoculated with IL-12 survived the disease longer than those not inoculated (with mean day of death post-infection being day 11 for IB, day 10 for IIB compared to day 8 for control group) (Table 6.4).

Each of the survivors subsequently resisted challenge with 10,000 EBs per mouse indicating an active immune response had occurred, despite the absence of clinical signs in 20% of mice in group IA and IB in primary infection. The challenge inoculum of 10,000 EBs results in 100% mortality in Balb'c mice (Paxton, 1997;. unpublished data).

Table 6.4. Summary of the clinical manifestations in mice treated with MrIL-12 and infected with *C. ruminantium*

Group	I.P.	+/-SD	C.D.	+/-SD	Morb.R.	Killed	Mort. R.	Survival R.
IA	10	1.2	1.5	0.5	8 (80%)	2	4 (50%)	4 (50%)
IIA	7 .3	0.9	1.3	1.2	10 (100%)	2	7 (90%)	1 (10%)
IIIA	7.2	1.0	1.3	0.5	10 (100%)	2	8 (100%)	0 (0%)
IB	9.2	1.2	2	0	15 (83.3%)	4	6 (42.8%)	4 (28.6%)
IIB	8.3	1.3	2	0.6	16 (88.8%)	4	12 (85.7%)	4 (18%)
IIIB	7.0	0.5	1.4	0.5	18 (100%)	4	18 (100%)	0 (0%)

KEY: IA - group inoculated with 100 ng IL-12 from start of infection every 2 days

IIA - group inoculated with 100 ng of IL-12 after disease onset

IIIA - Infected control group

IB - group inoculated with 500 ng of IL-12 twice 24 h pre-infection and 5 days post-infection

IIB - group inoculated with 500 ng of IL-12 24 h before infection

IIIB - infected control group

I.P.- Incubation period in days

C.D.- Course of the disease in days

Morb. R. - Morbidity rate

Mort. R.-Mortality rate

Killed - Number of mice sacrificed

Survival R. - Mice which survived the disease with or without clinical disease

6.3.2.2. Gross pathological findings

The main gross pathological finding in each of the mice which succumbed to the disease was the presence of pleural effusions. Some slight variations were recorded in the amount of fluid between the groups, and, although the amount of oedema fluid was slightly lower in mice inoculated with IL-12 before or at start of infection, the differences were not statistically significant ($p>0.05$) (Figure 6.7. and Appendix 5.1). Significant increase differences in the spleen weights ($p<0.05$) were noted in the groups pre-treated with IL-12 and the control group. The mean spleen weights were 180, 167 and 100 mg for the group inoculated with two doses of 500 ng of IL-12 (IB), for the group which received one dose of 500 ng IL-12 (IIB) and the control group respectively (Figure 6.7).

6.3.2.3. Histopathology

In some lung sections of mice inoculated with IL-12 a more pronounced inflammatory reaction characterised by infiltration of lung interstitia by predominantly mononuclear cells was seen as compared to the control (Figure 6.8 - 6.11). There was increased number of apoptotic cells in the lung and brain sections of mice inoculated IL-12 prior to or at time of infection as compared to the control group, with group IB, which received a double inoculation, showing more apoptotic cells than group IIB (Figure 6.12 and 6.13). Apart from increased weight and size of spleens in mice inoculated with IL-12 (IB and IIB), there were no other gross lesions noted on the spleen, and only slight cellular changes in histopathological sections were discernible.

Fig. 6.7.

Mean Spleen weights and Thoracic odema fluid in *C. ruminantium* infected Balb'c mice inoculated with 500ng MrIL-12 with or without a booster dose (IB and IIB respectively)

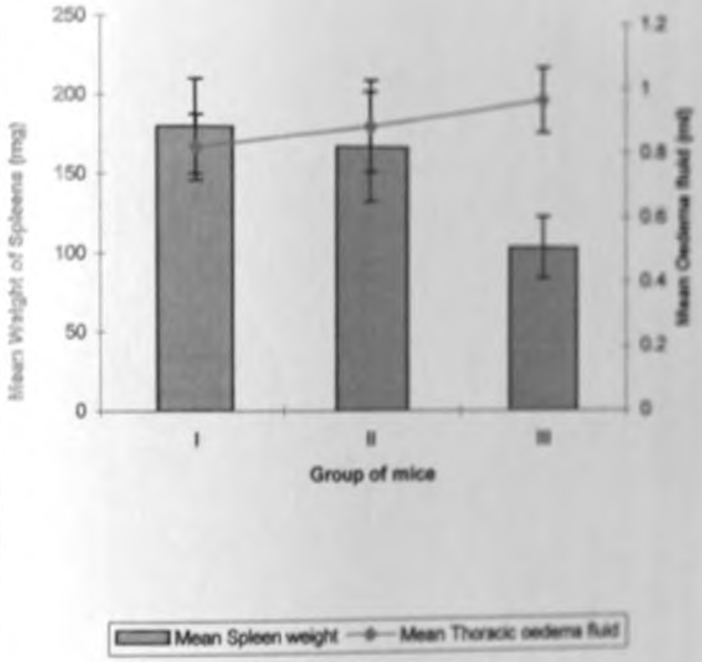


Fig. 6.8.

Giemsa-stained lung histopathological section of a mouse sacrificed on day 8 post-infection. This had been inoculated with 500ng MrIL-12 24 hours prior to infection with a booster dose at 5 days post-infection. Note the infected cell (arrow) and infiltration by phagocytic cells (arrow head) (X 1000).

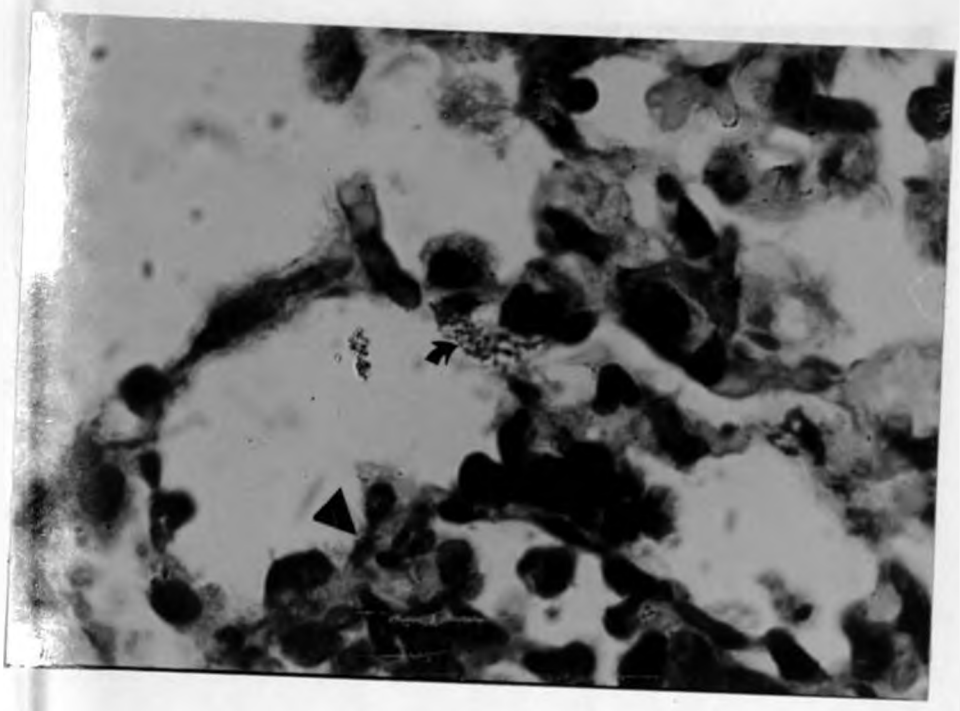


Fig. 6.9.

Giemsa-stained lung histopathological section of a mouse sacrificed on day 8 post-infection. This had been inoculated with 500ng MrIL-12 24 hours prior to infection showing oedema (o), congestion (arrow) and inflammatory cell infiltration (arrow head) (X 3000).

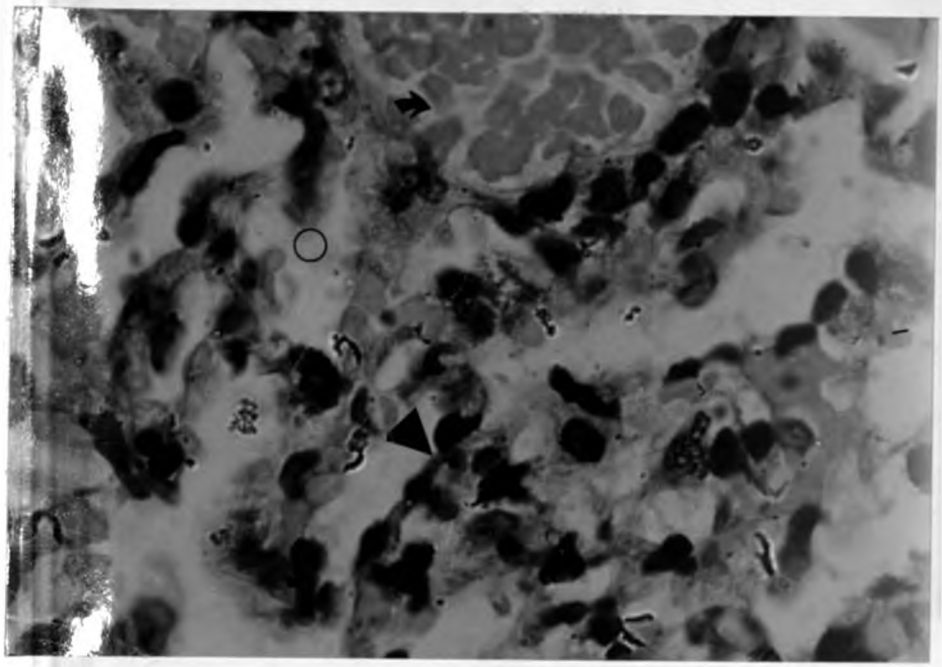


Fig. 6.10.

Giemsa-stained lung histopathological section of a control mouse sacrificed on day 8 post-infection showing infected cell (arrow) and oedema (o). Note the presence of reduced cell infiltration as compared to those inoculated with IL-12 (Fig. 6.8 and 6.9) (X 3000).

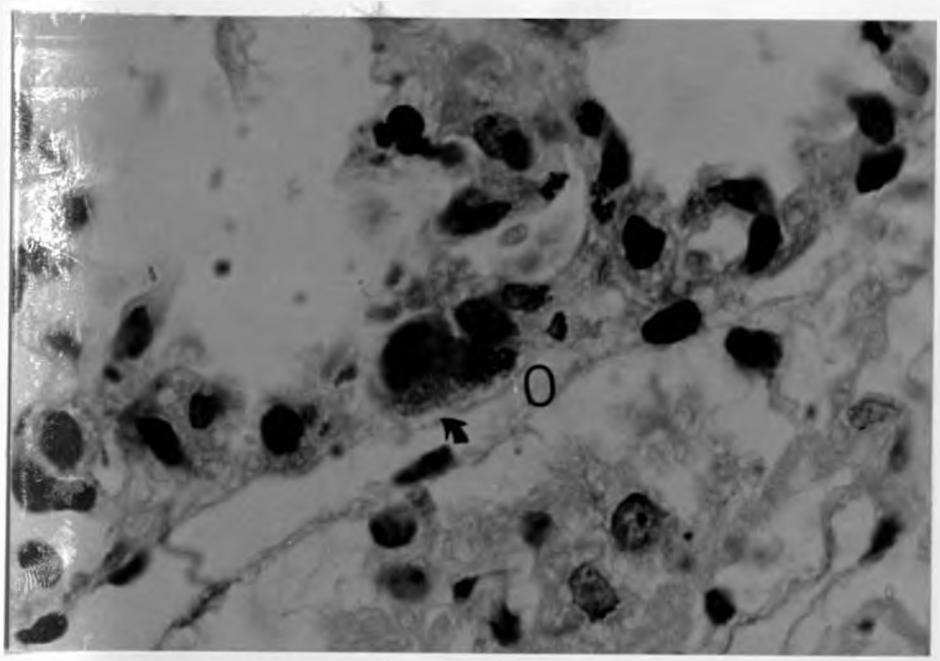


Fig. 6.11.

Hematoxylin and eosin stained lung histopathological section of a *C. ruminantium* infected mouse sacrificed on day 8 post-infection. This mouse had been inoculated with 500ng MrIL-12 24 hours prior to infection. The micrograph shows inflammatory cell infiltration of predominantly mononuclear cells (X1500).

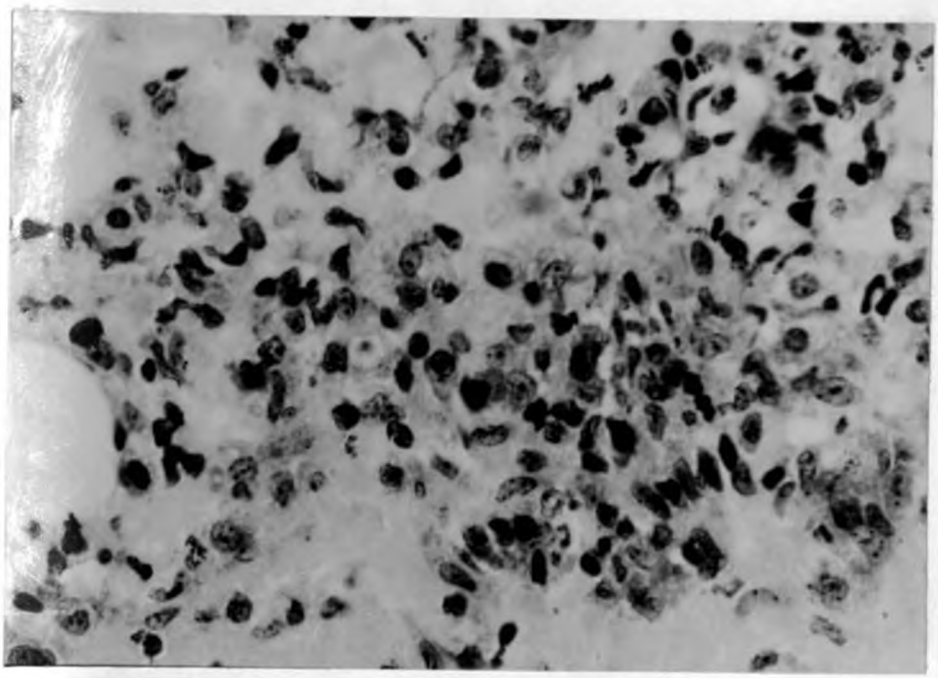


Fig. 6.12.

Giemsa-stained lung histopathological section of a mouse sacrificed on day 8 post-infection. This mouse had been inoculated with 500ng MrIL-12 24 hours prior to infection. The micrograph shows presence of apoptotic cells (arrow), inflammatory cell accumulation in the interstitia (interstitial pneumonia) (arrow head) but less oedema fluid (X3000).

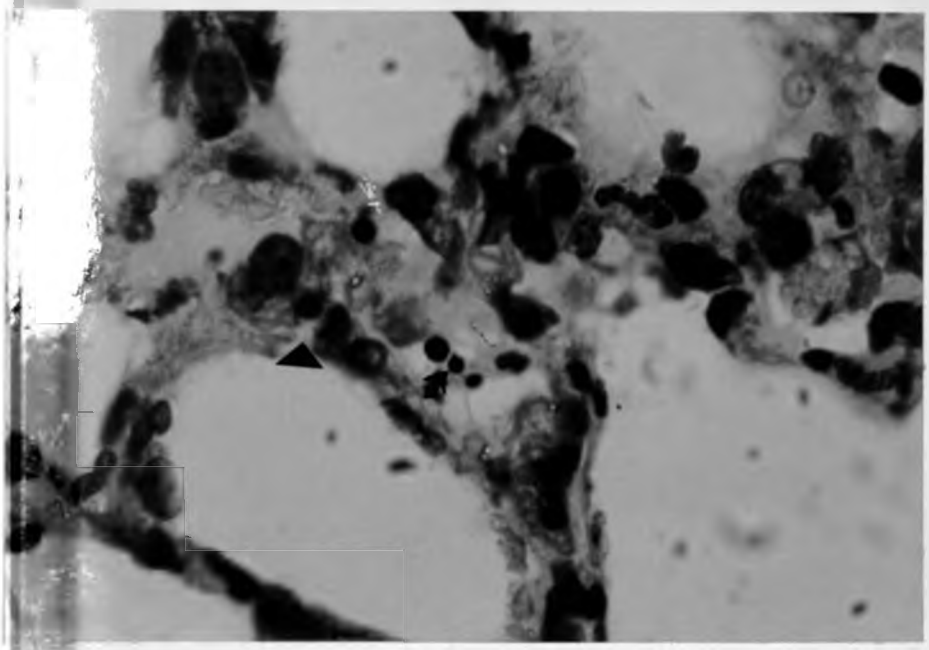
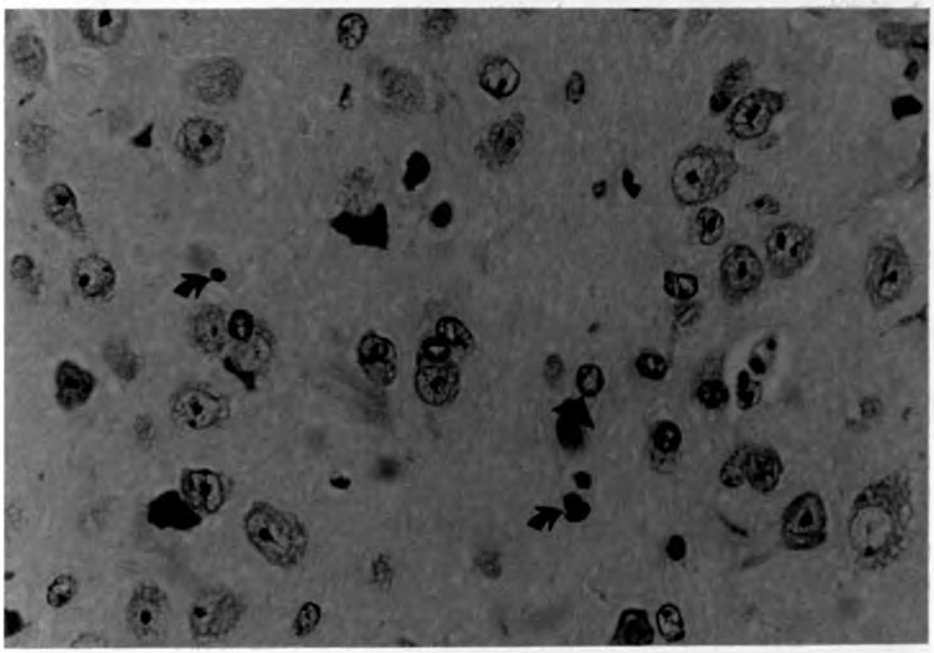


Fig. 6.13.

Hematoxylin and eosin stained brain histopathological section of a mouse sacrificed on day 8 post-infection. This mouse had been inoculated with 500ng MrIL-12 24 hours prior to infection. The micrograph shows presence of apoptotic cells (arrows) (x1500).



6.3.2.4. Infection rate in lung histopathological sections and brain smears

The mean number of *C. ruminantium* colonies per field in sections from mice sacrificed on day 8 were analysed in giemsa stained lung sections by counting colonies in twenty fields per slide. Quantification of infectious agent in showed the mice inoculated with MrIL-12 prior to or at time of infection to have significantly lower infection rates as compared to the control ($x^2=6.24$, $p<0.05$) (Table 6.5). The lowest mean infection rate of 2 colonies per field was recorded in the group IA inoculated with 100 ng of IL-12 every two days as compared to 5 colonies in group IB inoculated with 500 ng twice, 14 in IIB inoculated with 500 ng once, 17 colonies in IIA, the group given 100 ng after disease onset and 18 colonies in the control group (Table 6.5). Non-hypothermic mice sacrificed from group IB, which had received double MrIL-12 inoculation, had very low infection rates with a mean of less than 1 colony per field as compared to the hypothermic mice which had higher infection rates of up to 30 colonies per field (Table 6.5).

Table 6.5. Summary of infection rates in Lung tissue of mice sacrificed on day 8 post-infection.

Group of mice	Mean No. Of Colonies	+/- SD
IA	2.2	1.5
IIA	17.5	4.1
IB	5.2	3.0
IIB	14.6	5.3
III	18.6	9.0

KEY:

IB - group inoculated with 500 ng of IL-12 twice 24 h pre-infection and 5 days post-infection

IIB - group inoculated with 500 ng of IL-12 24 h before infection

III-infected control group

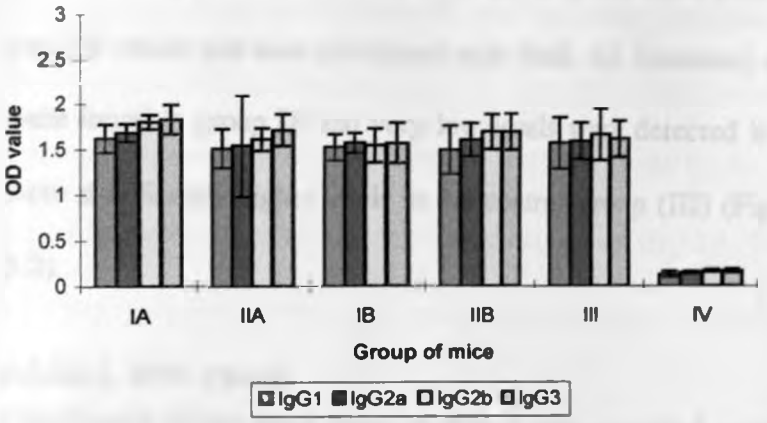
Mean Number of colonies +/- standard deviation (SD) counted in giemsa stained lung sections in twenty fields at a magnification of X1000. Four mice for each group were used.

6.3.2.5. Isotype levels of anti-*Cowdria* immunoglobulins in mice treated with MrIL-12

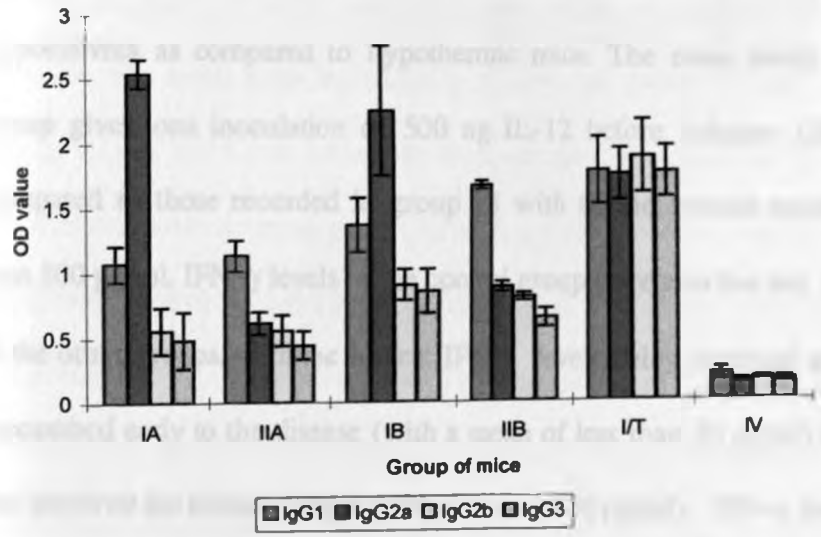
Sera was collected on days 0, 8 and 16 post-infection from survivors and compared with sera obtained from tetracycline-treated controls for anti-*Cowdria* IgG levels by indirect ELISA. Anti-*Cowdria* IgG1, IgG2a, IgG2b and IgG3 levels were determined for the different groups of mice. On day 8 post-infection, IgG levels were recorded for all the infected groups of mice as compared to the uninfected control with very slight differences in levels of different isotypes in all the groups. On day 16 post-infection, however, there were significant differences in IgG1 and IgG2a in these groups with a ratio of 1:2 in group IA and IB and 2:1 in group IIA and IIB whereas the ratio was 1:1 for pooled serum from an infection and treatment group (I/T) used as control, since all mice in group III had died (sera obtained from Kibor, 1997) (Figure 6.14a and b).

Fig. 6.14.

A.Mean Immunoglobulin levels in *C. ruminantium* infected Balb'c mice treated with MrIL-12 at 100ng (IA and IIA) or pre-treated with 500ng with or without a booster dose (IB and IIB), and infected and uninfected controls (III and IV) on day 8



B.Mean Immunoglobulin levels in *C. ruminantium* infected Balb'c mice treated with MrIL-12 at 100ng (IA and IIA) or pre-treated with 500ng with or without a booster dose (IB and IIB), and infected and treated (I/T) and uninfected controls (IV) on day 16



6.3.2.5. Cytokine Levels in *C. ruminantium* infected mice treated with MrIL-12

6.3.2.5.1. IL-12p70 levels

Total IL-12 and IL-12 p70 levels were determined by an ELISA assay for day 9 post-infection. Significantly elevated total IL-12 levels were found in mice in groups IB and IIB which had been inoculated with MrIL-12. However, no detectable IL-12 p70 were found in group IB and very low levels were detected in group IIB, while there were significantly higher levels in the control group (III) (Figure 6.15 and Appendix 5.2).

6.3.2.5.2. IFN–γ levels

Significantly higher mean levels of IFN–γ were recorded in mice inoculated with IL-12 twice (IB) as compared to those inoculated once and the untreated controls ($P<0.05$) (Figure 6.16 and Appendix 5.2). All the animals in this group had IFN–γ levels ranging from 100 -200 pg/ml and a mean of 140 pg/ml. The highest IFN–γ levels in this group were recorded from mice which were sacrificed before onset of hypothermia as compared to hypothermic mice. The mean levels of IFN–γ in the group given one inoculation of 500 ng IL-12 before infection (IIB) were low as compared to those recorded in group IB with all the animals recording values less than 100 pg/ml. IFN–γ levels in the control group were also low but more varied than in the other groups, with the lowest IFN-γ levels being recorded in the mice which succumbed early to the disease (with a mean of less than 50 pg/ml) but high in those that survived the disease longer (with a mean of 80 pg/ml). IFN–γ levels in this group (IIB) and the control group (III) were not significantly different. The most striking differences between these was the trend in which IFN–γ increased or decreased.

Although the mean spleen IFN–γ concentration was significantly higher for group IB mice over the period of 7-13 days than in groups IIB and III, the levels were highest on day 7 and 8 post-infection but remained uniformly high up to day 13 (>120 pg/ml). In group IIB mean IFN–γ levels were highest on day 7, but these levels were about half the mean recorded in group IB, and decreased significantly from day 8 to day 13 when very low mean levels of less than 20 pg/ml were recorded. In contrast, a steady increase of mean levels of IFN–γ were recorded in the control group, with the lowest levels being recorded on day 7 and the highest on day 13. The levels of IFN–γ and apoptotic cell death were found to be highly correlated (r= 0.8) (Table 6.3).

6.3.2.5.3. IL-10 levels

Lower mean spleen levels of IL-10 were recorded over the 6 days in mice in group 1B, the group inoculated with IL-12 twice, 24 hours before infection and 5 days post-infection, as compared to the control mice. The highest mean spleen levels for group IB (100pg/ml) were recorded on day 11 post-infection and the lowest (0 pg/ml) on day 13. In group IIB mice, which had been inoculated with IL-12 once 24 h before infection, the levels were significantly higher than group 1B and the control group (group III) on day 7 after which the levels dropped to about 100 pg /ml. Mean spleen IL-10 levels were lower in group III at day 7 than for the other 2 groups but were significantly higher on day 13 (about 200 pg/ml) as compared to 0 pg/ml in group 1B and about 100 pg / ml in group IIB. The trend of IL-10 in group 1B was the reverse of that of group III with the trend of group II being intermediate between that of group IB and III (Figure 6.17 and Appendix 5.2).

Fig. 6.15.

Mean Spleen IL-12 (total (T) and p70) on days 8 and 9 post-infection in Balb'c mice pre-treated with 500 ng MrIL-12 twice (IB) or once (IIB) and a control, all infected with *C. ruminantium* (n=4 mice per group on day 8 and 3 mice on day 9)

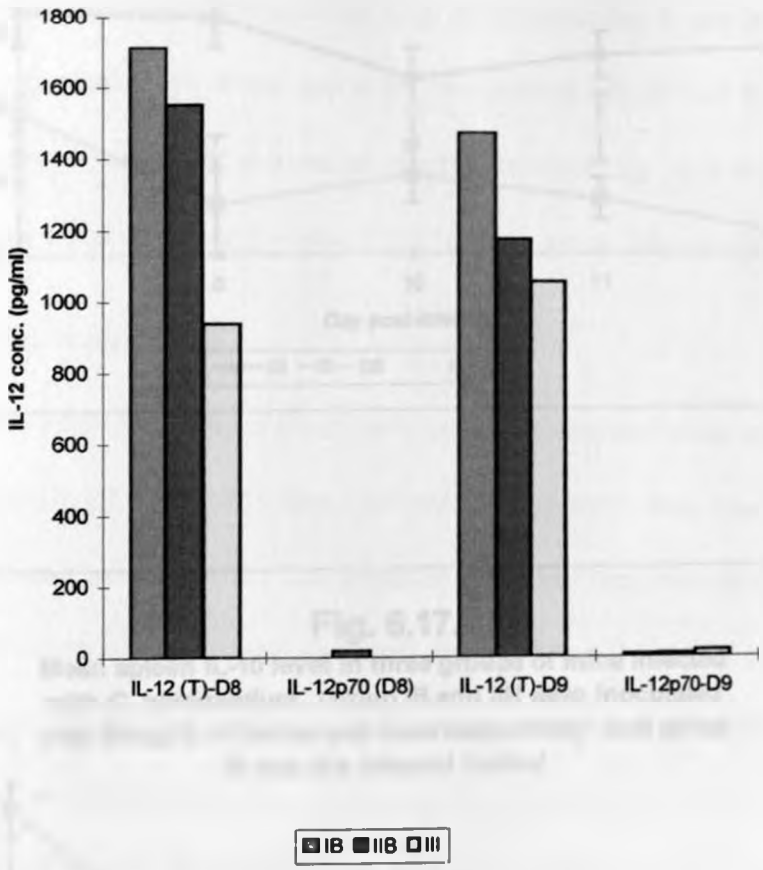


Fig. 6.16.

Mean spleen IFN-γ level in three groups of mice infected with *C. ruminantium*. Group IB and IIB were inoculated with 500ng IL-12 twice and once respectively and group III was the infected control

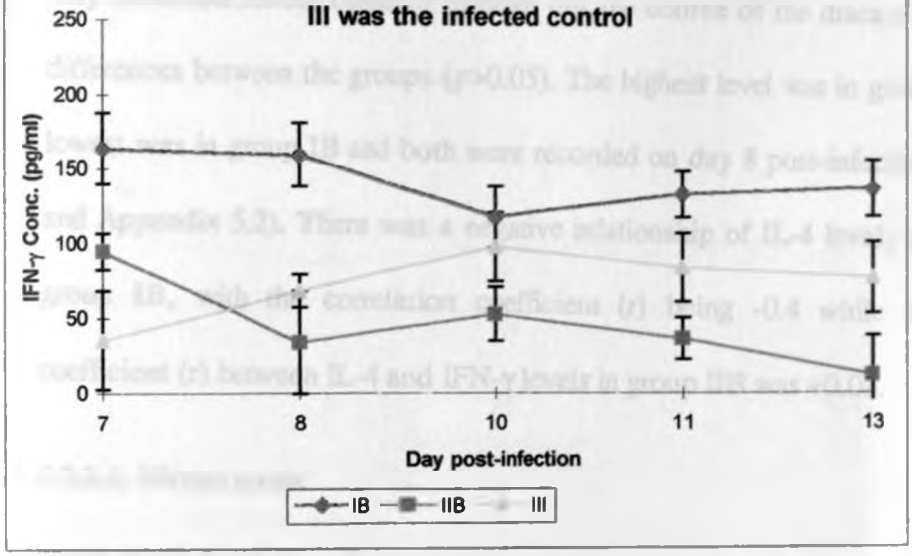
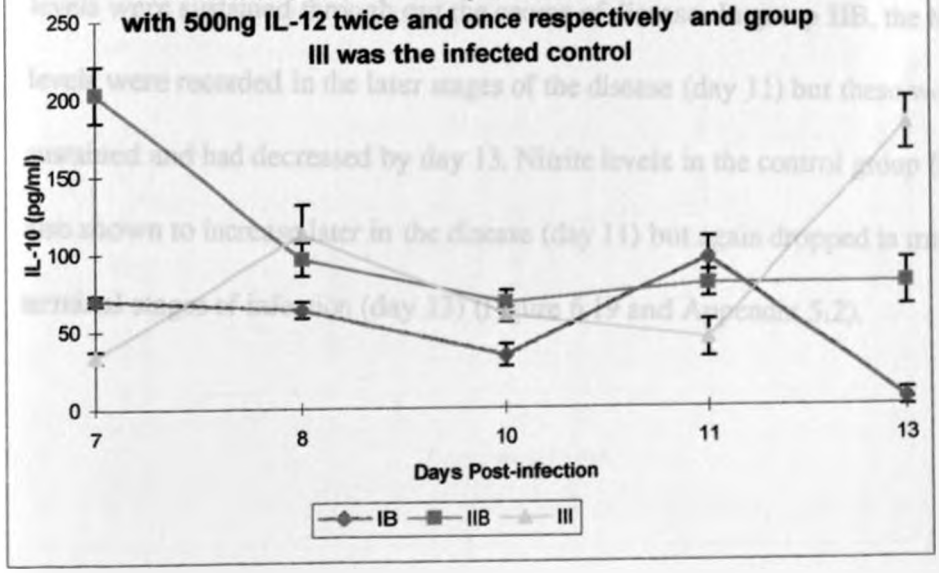


Fig. 6.17.

Mean spleen IL-10 level in three groups of mice infected with *C. ruminantium*. Group IB and IIB were inoculated with 500ng IL-12 twice and once respectively and group III was the infected control



6.3.2.5.4. IL-4 Levels

The levels of IL-4 were much lower than those recorded for IFN–γ and IL-10 and they remained almost constant through out the course of the disease with very little differences between the groups ($p>0.05$). The highest level was in group IIB and the lowest was in group IB and both were recorded on day 8 post-infection (Figure 6.18 and Appendix 5.2). There was a negative relationship of IL-4 levels with IFN–γ for group IB, with the correlation coefficient (r) being -0.4 while the correlation coefficient (r) between IL-4 and IFN-γ levels in group IIB was +0.08.

6.3.2.6. Nitrite levels

Nitrite levels indicative of nitric oxide levels were assayed in the homogenised spleens on days 8, 10, 11 and 13 where applicable. Nitrite levels were found to be significantly higher on day 7 in group IB, which had been inoculated with 500ng MrIL-12 twice as compared to mice in group IIB and the control group, and these levels were sustained through out the course of disease. In group IIB, the highest levels were recorded in the later stages of the disease (day 11) but these were not sustained and had decreased by day 13. Nitrite levels in the control group (III) were also shown to increase later in the disease (day 11) but again dropped in mice in the terminal stages of infection (day 13) (Figure 6.19 and Appendix 5.2).

Fig. 6.18.

Mean Spleen IL-4 levels in three groups of mice infected with *C. ruminantium*. Group IB and IIB were inoculated with 500ng IL-12 twice and once respectively and group III was the infected control

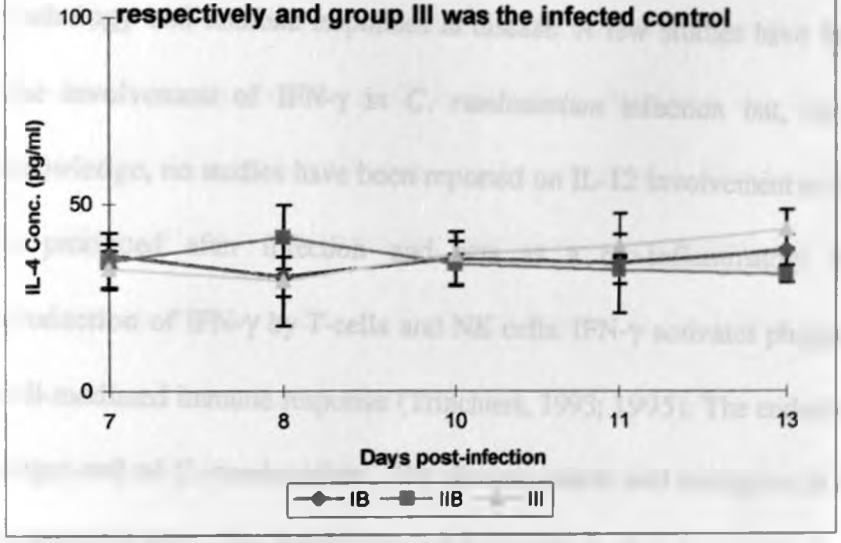
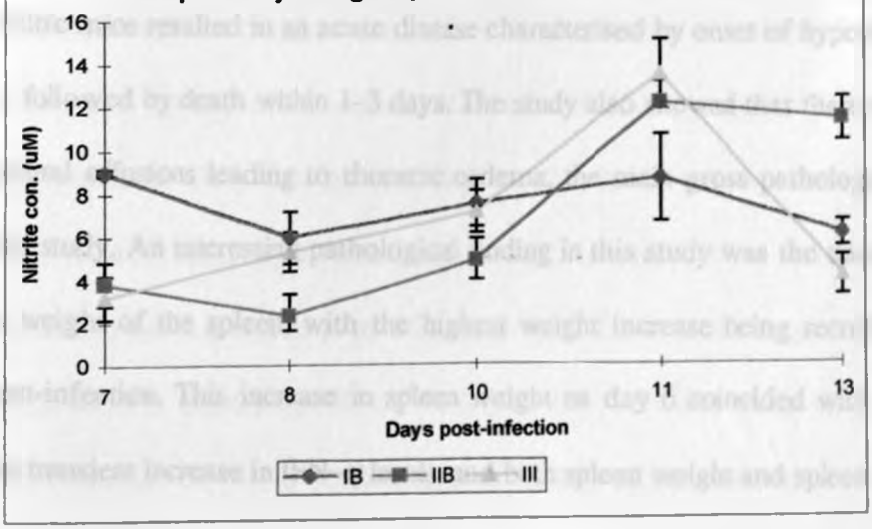


Fig. 6.19.

Mean Spleen Nitrite levels in three groups of mice infected with *C. ruminantium*. Group IB and IIB were inoculated with 500ng IL-12 twice and once respectively and group III was the infected control



6.4. Discussion

In the past much progress has been made in understanding the cellular and molecular mechanisms involved in the regulation of IL-12 and IFN-γ and their role in pathology and immune responses in disease. A few studies have been conducted on the involvement of IFN-γ in *C. ruminantium* infection but, to the best of our knowledge, no studies have been reported on IL-12 involvement in the disease. IL-12 is produced after infection and acts as a pro-inflammatory cytokine eliciting production of IFN-γ by T-cells and NK cells. IFN-γ activates phagocytic cells, hence cell-mediated immune response (Trinchieri, 1993; 1995). The endothelium is the main target cell of *C. ruminantium*. The disease infects and multiplies in the cytoplasm of endothelial cells. The mechanism of immunity to this intracellular parasite has been documented to be primarily cell-mediated, dependent upon T-lymphocytes (Du Plessis, 1982; Du Plessis *et al.*, 1991; 1992; Mahan *et al.*, 1994; 1996; Totte *et al.*, 1993; 1994).

In this study, the first experiment showed that *C. ruminantium* infection of Balb'c mice resulted in an acute disease characterised by onset of hypothermia on day 7, followed by death within 1-3 days. The study also showed that there was increased pleural effusions leading to thoracic oedema, the main gross pathological finding in this study. An interesting pathological finding in this study was the transient increase in weight of the spleen, with the highest weight increase being recorded on day 6 post-infection. This increase in spleen weight on day 6 coincided with the dramatic but transient increase in IFN–γ levels, and both spleen weight and spleen IFN-γ levels

decreased on day 8 post-infection, but were still higher than levels recorded in uninfected controls.

The mechanisms which resulted in increased weight of the spleen and IFN–γ on day 6 require to be established. It is possible, given the 6 day cycle of *C. ruminantium*, that release of *C. ruminantium* organisms into the circulation which occurs 5 to 6 days after infection, may have resulted in filtration of the organisms by the spleen. This may have resulted in induction of proliferation of spleen cells which produce interferons leading to the noted increase in the IFN–γ levels. Alternatively, the infection of antigen presenting cells (APC) in spleen and elsewhere, by the increased numbers of released infectious agent, might result in proliferation in spleen cells, peaking on day 6 and resulting in the noted weight increase, and the loss of cells into circulation, or possibly by apoptotic cell death, to explain the reduction in weight by day 8. It is also possible that the loss of spleen weight and of IFN-γ production is associated with exit of Th 1 $CD4^+$ T cells.

High IL-12p70 levels were noted on days 2 to 4 prior to IFN–γ increase. IL-12 primes cells for production of IFN–γ and is known as a strong inducer of IFN–γ production by NK and T cells (Trinchieri, 1995). IL-4 and IL-10 inhibits cytokine production by Th 1 cells by inhibiting T cell and NK cell functions associated with cell-mediated immunity to intracellular infections while IFN–γ secreted by Th 1 cells inhibits cytokine production by Th 2 cells (IL-10/IL-4) (Trinchieri, 1993; 1995). This suggests that increase in IL-12 on day 2 and 4 in this study may have induced differentiation of IFN–γ producing cells leading to increased production of IFN–γ levels and also increased spleen weight recorded on day 6. Since IL-12 levels were

not sustained beyond day 4, the reduction in IFN–γ between day 6 and 8 may be the consequence, and coincided with the acute onset of the clinical disease with a fatal outcome. It was during this supposedly Th 1 response that IL-4 levels were noted to be reduced in infected mice as compared to uninfected controls, with peak IFN–γ levels coinciding with the lowest IL-4 levels. It is possible that there was induction of a Th-1 response during this period which lead to suppression of Th-2 response but for some reasons this response was not sustained, resulting in lack of sustained high levels of IFN–γ and also reduction of spleen weight.

Increased nitrite levels, indicative of nitric oxide were recorded late in the disease during terminal stages. The spleen homogenates from the dead mice (day 9) showed the highest levels in nitrite levels as compared to mice sacrificed on day 8 and 10. Previous *in vitro* studies demonstrated that *C. ruminantium* infection of endothelial cells induced increased production of nitric oxide (Mutunga *et al.*, 1998) and this may explain the high levels recorded in this study, at a time when the infection level in endothelial cells is likely to be at its highest. Elevated IFN–γ levels on days 8 and 10 (about 3 fold higher than the basal level) could also have induced nitric oxide production.

The first experiment of this study showed that *C. ruminantium* infection of immunocompetent Balb'c mice resulted in induction of a transient IFN-γ production, hence Th 1 immune response, at a time when the disease was already established and this did not influence the course of the disease. The second experiment further supported the findings in the first experiment where early induction of a Th 1 immune response was shown to be more beneficial to the infected mice than a late one. In this study effects of treatment with MrIL-12 in Balb'c mice infected with *C. ruminantium*

were examined and showed, for the first time, that IL-12 given at the start or before an infection increased the survival rate in mice infected with *C. ruminantium* as compared to the controls. The inoculated mice showed elevated resistance to *C. ruminantium* as evidenced by increased incubation period, reduced tissue infection rate as seen in the lung and brain sections and reduced morbidity and mortality rates as a function of time. The resistance was further increased by inoculating the mice with booster doses as shown by groups IA, (given booster doses of 100 ng every two days), and IB (given one booster dose of 500 ng five days post-infection). Inoculating mice with 100 ng of MrIL-12 frequently from start of infection seemed to induce the highest protection with longer mean incubation period and lower mortality rate (IA) as compared to the control group. Mice inoculated with a single dose of 500 ng of MrIL-12 prior to infection showed increased resistance to the disease, where as those given a booster inoculation 5 days post-infection showed a higher survival rate than those inoculated only once (IB and IIB). The protection, however, was not absolute, as evidenced by variations in resistance noted in mice given MIL-12 every 2 days (IA) in which 20% showed evidence of no clinical signs but recovered with solid immunity to a high dose challenge, and 25 % recovered after clinical disease while 50% died of the disease but these had a more prolonged incubation period and reduced infection rates in lung and brain.

Both antibody responses in sera and cytokine assays in spleen homogenates indicated that inoculating mice with MrIL-12 early or before infection induced a Th 1 immune response, a vital protective mechanism in infections by intracellular parasites including *C. ruminantium*. Increased levels of IgG2a as compared to IgG1, IgG2b and IgG3 were observed in group IA inoculated with MrIL-12 at infection as compared to

group IIA and the control group and this increase in IgG2a corresponded to the survival rate of the mice in this group. IFN-γ levels were also found to be significantly elevated ($p<0.05$) in the groups inoculated with 500ng MrIL-12, corresponding to survival rate in these groups (IB and IIB) with the highest IFN-γ levels being recorded in the group given two inoculations of IL-12 (IB). These groups showed an early elevation of IFN-γ as compared to the controls, where higher levels of IFN-γ were recorded late in the disease. As expected, high levels of total IL-12 were also recorded in the groups inoculated with MrIL-12 but the low levels of IL-12 p70 recorded in these groups as compared to the control group were surprising and could require further investigation. IL-10 levels recorded were almost a reverse mirror image of IFN–γ levels , while IL-4 levels were not significantly different between the groups. In this study, we can therefore postulate that IFN–γ and IL-10 levels were regulating each other with the highest levels of IFN–γ and lowest levels of IL-10 being recorded in IB while the lowest IFN–γ levels and highest IL-10 were recorded in the control group.

Nitrite levels indicative of nitric oxide (NO) were found to be significantly elevated in mice inoculated with 500 ng MrIL-12 twenty four hours prior to infection (IB and IIB) as compared to the control group. The group inoculated with IL-12 twice (IB) had higher levels of nitrite concentration than the group given only one inoculation. The most striking feature was the time nitrite levels were highest in each group; the groups inoculated with IL-12 i.e. those manifesting higher protection, showed high nitrite levels earlier in the disease as compared to the control group, suggesting that nitric oxide production early in the infection may be related to

protection of the mice. This agrees with the findings in chapter 4 in which pre-treatment of *C. ruminantium* (EBs) with NO resulted in reduced viability and infectivity of the organism to endothelial cells. The high NO levels recorded especially in IL-12 inoculated mice corresponded to high IFN-γ levels in these groups, pointing to increased induction of inducible nitric oxide synthase (iNOS) by activated macrophages and endothelial cells.

Histopathological sections of mice showed evidence of increased apoptosis in lung sections of mice inoculated with IL-12 as compared to the untreated controls but their significance was not established. There was high correlation between IFN–γ levels and number of apoptotic cells (r=0.8). Thus, high IFN–γ, NO levels and presence of apoptosis were, possibly related as shown previously in an *in vitro* study model in which BorIFN–γ induced NO production and induced increased apoptotic cell death of the infected cells resulting in reduced viability of *C. ruminantium* EBs (Mutunga *et al.*, 1998). In this study, as with the reported *in vitro* one, the increase in NO coincided with decreased rickettsiae in lung tissue and endothelial cells respectively. The high levels of NO recorded, which also corresponded to increased IFN–γ levels and therefore increased protection of the mice to *C. ruminantium*, implies that the production of NO was through the iNOS. This also agrees with results of the reported *in vitro* study where *C. ruminantium* infection of endothelial cells induced production of NO with IFN-γ enhancing this production and L-NMMA, the iNOS inhibitor, blocking this production (Mutunga *et al.*, 1998).

High NO levels in IL-12 inoculated mice may also explain the increased leukocyte infiltration in lungs of mice. In infection of mice with *Rickettsia conorii*,

reduction in the quantity of rickettsia in the endothelial cells was reported to be associated with perivascular infiltration of T-helper and T-cytotoxic lymphocytes (Walker *et al.*, 1997). Therefore, increased leukocyte infiltration in the lungs and spleen of mice inoculated with IL-12, which also corresponded to decreased infection rate was possibly due to induction of innate non-specific cell immune responses which may involve increased NO levels, also known to result in increased leukocyte infiltration (Drapier *et al.*; 1988). NO has also been said to modulate leukocyte adhesion to endothelial cells , an important component of the inflammatory response (Harlan, 1985). This could also have influenced the significant increase in spleen weights and the increased infiltration by inflammatory cells in the treated mice as compared to untreated controls seen in this study. The increased levels of IFN-γ, IL-12, nitrite indicative of NO, and IgG2a in the mice showing resistance to the disease were pointing towards a Th 1 immune response. In this study it seems, as in other murine systems, that induction by IL-12 of IFN-γ by Th 1 cells induced IgG2a and IgG3 production by B cells (Finkelman *et al.*, 1988; Snapper *et al.*, 1992). From these results, it is suggested that production of IFN-γ in high enough levels early in the disease, before development of the primary immune system, reduced the infection development rate in these groups. This resulted in increased survival of the infected mice as seen by prolonged incubation period, lower morbidity and mortality rates as a function of time. On the other hand the control animals had reduced survival rate and also increased IgG1 as compared to IgG2a showing a response associated with a Th 2 type immune response, which may explain the high mortality rate (100%) recorded for this group. Increased IL-12 p70 recorded in the infected mice of the first experiment

and the non MrIL-12-treated control group in the second experiment may be related to the high IFN–γ levels also recorded late in these groups (day 10 and 13) and the response may have come too late to make a noticeable difference to the course and severity of the disease, and already discussed above, may result in increased permeability worsening the pathology.

It was also noted that, although there was increased survival in mice inoculated with IL-12 before or at the start of infection, in the few which died there was severe inflammatory response with increased inflammatory cell infiltration in the lungs culminating in interstitial pneumonia with mononuclear cells being more predominant. The infectious parasites were not in higher numbers in the IL-12 inoculated mice, thus the presence of large numbers of parasites does not appear to be necessary to mediate the pathologic effects observed in these mice. Similar results were reported in IL-10 depleted mice in which early deaths in mice infected with *T. gondii* were found not to be associated with a corresponding increase in parasite burden due to its major role in regulating IFN-γ and IL-12 (Neyer *et al.*, 1997, Candolfi *et al.*, 1995). Similar results were reported for *Plasmodium chabaudi* when IL-10 knock out mice died earlier due to a lethal inflammatory response rather than due to a fulminating parasitaemia (Linke *et al*, 1995). This implies that an immune response to infection can be protective or pathogenic, according to the timing and control of the IL-12 / IFN-γ system. It appears then that in the IL-12 treated mice which died, death was not due to an overwhelming infection but due to some extensive inflammatory reaction. A mechanism exists *in vivo* to limit production of IL-12 or the ability of T and NK cells to respond to it; IL-10 is the best studied inhibitor of IL-12 production (D'Andrea *et al*; 1993). Several reports have indicated that IL-12

itself can induce T cells to produce IL-10 suggesting a model in which IL-12 may limit its own production by induction of its negative regulator IL-10 (Gerosa *et al.*, 1996). Interaction between these cytokines is complex and has important effects on the quality of the immune response. IL-12 has been reported to prime cells for production of IFN–γ and also paradoxically for IL-10 production (Neyer *et al*; 1997). Thus, the increased levels of IL-10 recorded in group IB on day 7 may have been induced by high levels of IL-12. It maybe that the role of IL-10 is therefore a physiological one to mediate deactivation of the innate immune response quickly, to prevent non-specific tissue damage at a time when specific T-cell responses are being generated to eradicate the infection. IL-10 inhibition of macrophage has been said to be selective, rapidly down-regulating pro-inflammatory cytokine synthesis without necessarily inhibiting critical macrophage effector functions needed for bactericidal activity e.g. nitric oxide production (Strassman, 1994) or antigen presentation (Moore *et al.*, 1993). This maybe the explanation then in those mice inoculated with IL-12 which revealed extensive inflammatory reaction which corresponded also with lower IL-10 levels. Thus, the physiologic role of IL-10 is favoured, being to shut off innate immune cell activation by inhibiting IL-12 after specific T-cell immunity has been stimulated. This mechanism has been reported to ensure minimal tissue damage while the infection is specifically cleared (Tripp and Unanue, 1996).

Activation of macrophages represents one of the first events in the innate resistance to intracellular infection. Early non–antigen specific responses of innate resistance are very effective in eliminating pathogens or at least in significantly reducing their ability to multiply (Trinchieri, 1995). Both non-specific innate immune response by macrophages and NK cells, and specific adaptive immune response that

generate specific antigen -specific T-cells are probably important in this disease, as seen in field cases and in planned experiments. The macrophage via its ability to produce IL-12 plays a major role in bridging the innate and specific cellular immune responses in some studied diseases (Trinchieri, 1995).

IL-12 produced during the early inflammatory phases of an infection and IL-12 induced IFN–γ, create an environment in which antigen-specific CD4+ and CD8+ T cells are induced to differentiate into Th 1 cells, producing even higher levels of IFN–γ (Monterio and Trinchieri, 1996; Orange and Biron, 1996). In some viral infections IL-12 has been found to be responsible for early production of IFN–γ mostly by NK cells, where as the late and most efficient production of IFN–γ by T cells appears to be IL-12 -independent (Monterio and Trinchieri, 1996; Orange and Biron, 1996). This may explain why administration of IL-12 after disease onset did not alter the course of the disease from that of the control group.

The study showed the importance on timing of cytokine availability during the disease, as inoculation of MrIL-12 after disease onset did not seem to offer any protection and manifested a clinical picture similar to that of the control group indicating that IL-12 in this case did not influence the final outcome of the disease. Furthermore, the pathological manifestations of some of the mice in this group given MrIL-12 late in the disease, such as thoracic oedema fluid, were more severe than even those of the control group pointing to a possibility that oedema formation was induced by activities of IL-12 in this group at this late stage of the disease. Increased endothelial cell permeability is the hall-mark of *C. ruminantium* disease. Interactions between cytokines and acute inflammatory mediators have been reported to play a key

role in endothelial cell permeability, and this increase in endothelial cell permeability is believed to be the main determinant of immune complex deposition in blood vessel wall (Beyond *et al.*, 1993). IFN–γ was reported to highly alter the endothelial cell shape leading to increased permeability but IL-4 was found to have no effect (Beyond *et al.*, 1993; Stolen *et al.*, 1986). The oedema formation may be associated with IL-12 via its induction of IFN-γ and eventually induction of nitric oxide, a powerful vasodilator which can influence vascular permeability (Furchgott, 1984). In this light, IFN-γ (and IL-12) may also be implicated in elevating the pathology of the disease by leading to increased endothelial cell permeability. The dramatic elevation of IFN-γ on day 6, seen just prior to disease onset in the first experiment, supports this theory. This would indicate that IL-12 activation occurred with the oedema being linked to pro-inflammatory events mediated in part by IL-12.

Conclusion

The importance of the sequence of events at the early stages of infection, as concerns generation of endogenous host mediators including cytokines is hereby underlined as it can determine the outcome of disease. The results of the first experiment in this study demonstrated changes in the weight of spleen, IL-12, IFN–γ, IL-4 and nitrite levels during *C. ruminantium* infection in Balb'c mice and has paved way for more research in the interactions of the cytokines and other mediators in *C. ruminantium* infection. The results in the second experiment demonstrated that IL-12 early in the disease has some immunomodulatory effects on *C. ruminantium* infection in mice by favouring a Th 1 immune response. From the results of this study and the established facts of the functions of IL-12 in literature, we postulate that treatment of mice with IL-12 early in the disease (before or at time of infection) is

beneficial as the specific immune responses are not yet established and this leads into induction of innate non-specific immune response. Treatment with IL-12 late after the disease is established, however, may result in an unwanted increased inflammatory response due to this innate non-specific immune responses. It is suggested here that the protection was through induction of IFN–γ production which then activated macrophages and consequently induced early control of the organisms growth and dissemination. In these mice, a mechanism involving increased IFN–γ, nitric oxide production, suppression of IL-10 and IL-4, and increased IgG2a, together indicative of a Th 1 immune response, came into play.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

The aim of this study was to investigate the pathogenesis and immune responses induced by the host-parasite interactions in *C. ruminantium* infections. The effect of cytokines and nitric oxide levels on the disease, and vice versa, were investigated using both *in vivo* and *in vitro* models.

The pathogenesis of *C. ruminantium*, and especially what culminates in death, remains unclear. Increased vascular permeability resulting in effusions into body systems is the pathological hall mark of the disease. A better knowledge of host-parasite interactions in *C. ruminantium* infection is important in providing a better understanding of the pathogenesis and immune responses of the disease and also for development of effective control measures for the disease. *C. ruminantium* infects and multiplies in endothelial cells and the implication of this host cell-parasite association may be important in understanding the host-parasite interactions as a whole. Also, a knowledge of the immunological mechanisms evoked or inhibited shortly after infection might provide clues regarding the host of responses which determine the course of the disease and that need to be induced or suppressed for effective treatment and control of the disease.

The important role of IFN–γ, and possibly nitric oxide (NO), in the control of *C. ruminantium* infection was a consistent finding in all the chapters of this study. The time factor, when these mediators were made available during the disease, was found to be of paramount importance in the control of infection in mice. Induction of IFN–γ production just before or after clinical disease onset in both ovine and murine experimental infections was shown not to influence the final outcome of disease. This was shown in sheep infected with the Welgevonden isolate of *C. ruminantium* which

suffered a fatal outcome of the disease despite IFN–γ increase recorded after onset of clinical disease (day 10). Red Maasai sheep, which had prior exposure to *C. ruminantium* antigens, showed higher resistance to the disease and also recorded earlier and higher levels of IFN–γ and NO levels as compared to naive infections.

Treatment of *C. ruminantium* infected BPEC with BorIFN–γ resulted in increased production of NO and reduced viability of EBs coupled with induction of apoptotic cell death especially in infected cells. This effect was blocked by the nitric oxide synthase inhibitor, L-NMMA, indicating the induction was through the inducible nitric oxide synthase (iNOS) pathway. Pre-treatment of *C. ruminantium* EBs with the NO donor molecule, SNAP, resulted in reduction in viability and infectivity of the organism to endothelial cells in *in vitro* studies.

In Balb'c mice, very high levels of IFN–γ in spleen homogenates on day 6 post-infection were recorded but these were not sustained and the mice had a fatal outcome of the disease. Treatment of mice with MrIL-12 before or at time of infection was shown to result in increased production of IFN–γ and NO as compared to untreated control, and increased protection as shown by reduced mortality and prolonged course of disease. This increase in IFN–γ and NO in IL-12 treated mice coincided with lower levels of IL-4 and IL-10 as compared to the controls, and also higher levels of IgG2a as compared to IgG1 recorded in the control group. Inoculating mice with IL-12 after disease onset did not influence the disease outcome, in comparison to IL-12 inoculations performed before or at the time of infection.

Further evidence of the protective role mediated by interferons was shown in the *C. ruminantium* infections of IFN–γ and IFN-α/β receptor deficient mice. The

disease was more severe with shorter incubation periods, early deaths and increased infection rates in lung and brain sections as compared to the control groups. Thus, this study also showed that some degree of protection was possibly mediated by the late IFN–γ induction in 'normal' animals, resulting in delayed disease onset and death, as infections of IFN–γ and IFN-α/β deficient mice resulted in a more acute and severe disease than in the controls.

Cell-mediated immune response is important in protection against *C. ruminantium* infections (Du Plessis *et al.*, 1982, 1991 and 1992a; Mahan *et al.*, 1994 and 1996; Totte *et al.*, 1993 and 1994), as is also documented for other intracellular parasites. Du Plessis *et al.*, (1992) showed that transfer of immune spleen cells conferred immunity to DBA mice against challenge with the Kumm strain of *C. ruminantium*. Immune spleen cells depleted of $CD8^+$ cells (Lyt-2^+ T cells) could not confer any immunity to challenge with *C. ruminantium*, whereas depletion of $CD4^+$ T cells (L_3T_4) had no effect on this protection. A later study on the flowcytometric analysis of the peripheral T cell populations in mice immunised by infection and treatment confirmed the above findings, that immunity in heartwater is largely mediated through $CD8^+$ T cells (Du Plessis *et al.*, 1992a). IL-12 induces proliferation of $CD4^+$, $CD8^+$ and NK cells, and induces production of IFN-γ by T cells and NK cells (Nicola, 1994; Trinchieri, 1993). IL-12-induced protection in this study was thought to be mainly due to increased IFN-γ production and, in line with the findings of Du Plessis *et al.*, (1992a), clearence of the infectious agent by $CD8^+$ cells may have been involved in this. One is therefore tempted to speculate that reduction in *C. ruminantium* infection in the IL-12 treated mice was due to a combined effect of IFN-

γ, produced by T cells and NK cells, as well as clearance of the parasite is by $CD8^+$ T cells, but this requires investigating.

IFN-γ has been reported to play a major role in clearance of rickettsial parasite (Turco and Winkler, 1984; Hanson, 1991). Totte *et al.*, (1993 and 1996) reported inhibitory effect of IFN-γ to *Cowdria* growth in endothelial cells *in vitro*. Concanavalin A-stimulated bovine lymphocytes supernatants also inhibited *Cowdria* growth, and this inhibition was abolished in the presence of anti-IFN-γ but not anti-TNF-α, further confirming the inhibitory effect of IFN-γ (Mahan *et al.*, 1994 and 1996). Evidence that IFN-α/β can inhibit rickettsial replication in the host cells has also been reported (Turco and Winkler, 1990; Hanson, 1991), including *C. ruminantium* infections (Totte *et al.*, 1993 and 1994). The latter showed that IFN-α was able to reduce the yield of *Cowdria* organisms but its effects were 10,000 times less efficient than those of IFN–γ, leading them to conclude that IFN-α slows down the course of the disease, allowing the host to mount a protective immune response (Totte *et al.*, 1994). This is in agreement with the results of Chapter 5 where mice deficient in IFN-γ receptors suffered a more severe disease, characterised by shorter incubation periods and early deaths, than those lacking IFN-α/β receptors.

The overall picture of the results discussed here emphasise the importance of an early induction of a Th 1 immune response (and suppression of a Th 2 immune response) for the control of *C. ruminantium* infections. The time factor for the availability of the mediators was found to be of utmost importance in controlling infection. The cytokine micro-environment during the initial phase of an immune response appears to play an important role in determining the outcome of the disease,

as cytokine manipulations have been used to modulate immune responses to a variety of infections, converting susceptible into resistant hosts (Trinchieri *et al.*, 1993 and Wynn and Sher, 199?). The induction of a pathogen-specific $CD4^+$ T helper cell response characterised by production of Th 1 or Th 2 type cytokines determines the resistance or susceptibility of the host to the infecting organism (Wynn and Sher, 1996). Infection of immuno-competent animals with *C. ruminantium* induced some protection by producing increased levels of IFN–γ and NO with higher levels being produced by resistant animals including those inoculated with IL-12 at or before infection. Administration of IL-12 either just before infection or very shortly after exposure to a variety of infectious organisms has proven to be highly effective at eliminating or decreasing infection intensity. IL-12 has been reported to promote the differentiation of $CD4^+$ T cells into Th 1 cells (Stevenson *et al.*, 1995). Animals infected with *Leishmania major* (Spok *et al.*, 1993), *Toxoplasma gondii* (Hunter *et al.*, 1995), *Listeria monocytogenes* (Wagner *et al.*, 1995) *Cryptococcus neoformans* (Clemons *et al.*, 1995), *Plasmodium yoelii* (Sedegah *et al.*, 1994) *Mycobacterium tuberculosis* (Cooper *et al.*, 1995) and *Histoplasma capsulatum* (Zhou *et al.*, 1995) have all benefited from treatment with IL-12 if administered during the initial exposure to the pathogen. IL-12 has been reported to have less of an effect if administered once infections are established (Wang *et al.*, 1994) as was the case in our study, hence the effects of IL-12 must be prominent at the time of antigen priming. As IL-12 promotes Th 1 responses and suppresses Th 2 cell development, this cytokine might be useful as a potential adjuvant for vaccines designed to promote cell-mediated immune responses in *C. ruminantium*.

From the results of mice inoculations with IL-12, it was hypothesised that an early IFN–γ response was beneficial in resolving *C. ruminantium* infections. IL-12 inoculations early in the disease may have led to induction of T-cell subsets which promote Th 1 immune response leading to production of IFN–γ and NO, and possibly $CD8^+$ T cells, and therefore increased killing of *C. ruminantium* organisms preventing dissemination of the infectious agent , hence lower infection rates. The different responses elicited by lethal and non-lethal malaria parasites were found to be due to early IFN–γ responses shown in infections with non-lethal parasites (De Souza *et al.*, 1997). This was further supported by the resistance to *C. ruminantium* infection encountered in Red Maasai sheep in which there was higher IFN–γ levels earlier in the disease as compared the more susceptible Dorper sheep. In the IFN–γ and IFN-α/β deficient mice, early deaths were possibly due to unchecked and faster multiplication rate of the infectious agent as evidenced by the bizarre, and probably, late forms of *C. ruminantium*, in sections of these mice, resulting in overwhelming infectious burden and earlier onset of clinical signs.

An activity of IFN–γ in controlling infection in this study was suggested to partly involve NO production. The killing of organisms by IFN–γ activated macrophages has been reported to involve both reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI), but that the extent of the contributions of ROI and RNI may differ from one pathogen to another and from one host to another (Adams *et al.*, 1991, Barbior *et al.*, 1975, Miyagi *et al.*, 1997). One mechanism used by body defence mechanisms to kill parasites is by release of toxic molecules including oxygen radicals and nitric oxide, (James, 1991). Work has been done to establish the

role for NO in mammalian cell-mediated immunity. Nitric oxide has been postulated to exert anti-microbial effects on pathogenic intracellular micro-organisms (Boockvar et. al 1994, Walker *et al.*, 1997, Liew *et al.*, 1990). IFN–γ and RNI were reported to be central to the immune response against *Mycobacterium tuberculosis* in mice and that this control was not achieved if production of RNI was delayed (Flynn *et al.*, 1995). Green *et al* (1993) showed that increased secretion of NO accompanying *Mycobacterium bovis* BCG administration in mice correlated with non-specific resistance to challenge with the heterologous micro-organisms. Nitric oxide produced during *C. ruminantium* infection seems to be beneficial to the host and is probably part of the animals defence mechanism to the disease, as NO anti-*Cowdria* activity was demonstrated in *in vitro* studies described in chapter 4. The *in vivo* significance of elevated NO is unclear. Experiments were planned to investigate the infection kinetics and pathology in mice in which the iNOS gene had been disrupted, but sufficient numbers of mice were not available because of breeding problems in the colony. Since NO levels tend to be elevated following IFN-γ release, it is not clear if some degree of protection arises from its production *in vivo*, since mice treated with IL-12 had elevated production of IFN–γ and NO, and up to 50% protection as compared to 100% mortality rate in the control group.

The increased resistance seen in Red Maasai sheep cannot be fully explained from these studies. The higher and earlier levels of IFN-γ were thought to be contributory to this resistance, resulting in retarded multiplication rate of the organism, and therefore reduced dissemination of the infectious agent. A method to quantify the level of infection in plasma during the course of the disease may clarify

this; an attempt to use PCR for quantification of infection was tried but the results were not conclusive. Protection or severity of *C. ruminantium* infection and antibody levels were found not to be correlated (Du Plessis, 1984). It has been generally said that antibodies do not play a major role in immunity to *C. ruminantium* infection, as seen in studies where transfer of immune serum or gammaglobulins failed to confer any protection to animals (Alexander, 1931; Du Plessis, 1971 and 1982). But Byrom *et al.*, (1993) showed that hyperimmune serum from mice and cattle had a strong neutralising effect on *C. ruminantium* infection *in vitro* but not *in vivo*. Genetic resistance to *C. ruminantium* was suggested by Matheron *et al.*, (1987), who postulated that populations in heartwater endemic areas developed resistance through natural selection of resistant lines, and even hypothesised that this could be sex-linked genetically. The mechanisms of non-specific resistance to *C. ruminantium* infection of young animals of all breeds is unknown. This resistance of the young has been said to bear no relationship with the immunity of the dam, and that it should not be confused with passive immunity transmitted through colostrum (Neitz and Alexander, 1941). However, some studies have suggested that the resistance of the young is correlated to the prior exposure of the dam (Deeem *et al.*, 1996a), and lately, there has been suggestions that vertical transmission of *C. ruminantium* is possible, further supporting this school of thought (Deem *et al.*, 1996b). The differences in resistance between adults and the young were also suggested to be possibly due to differences in the replication rate of *C. ruminantium* (Du Plessis and Malan 1987). Du Plessis and Malan (1987) found a strong positive relationship between levels of conglutinin in sera of ruminants and resistance to heartwater and

also reported that resistance of wild life to *C. ruminantium* was highly correlated to conglutinin levels.

Increased levels in enzymes involved in antioxidation in sheep infected with *C. ruminantium* were thought to be due to a homeostatic response to counteract increased levels of oxidising radicals produced as part of the body defence mechanism. A possible link between these antioxidising enzymes, NO and IFN–γ may exist but this requires further investigation. On the basis of the results of this study, it is clear that the induction of NO production may be an important component of cell mediated immune response to intracellular infections. Future studies are necessary to investigate the specific role of NO and the target cells of this molecule in the body.

This study showed that the course taken by *C. ruminantium* infection in both *in vivo* and *in vitro* models is dependent on the cytokine / mediators milieu bathing the tissues prior to or during infection. Infection in mice was characterised by a transient high IFN- γ response at about day 6 which was not sustained. It is likely that this response controls *C. ruminantium* to some extent because mice deficient in IFN- γ receptors died earlier in the disease with higher infection levels as compared to the control. Sustained release of IFN- γ occurred in mice treated with MrIL-12 and provided protection in a significant proportion. The failure to sustain IFN- γ in mice may be related to a lack of sustainable IL-12 production.

The importance of a Th 1 type immune response was, therefore, underlined and the possible roles for IL-12 and IFN- γ with involvement of nitric oxide in the killing of the infectious agent suggested. The study showed for the first time the possible roles of nitric oxide in the killing immune response and pathology of the disease and the

importance of availability of IL-12 at time of infection in the development of protective immune response in mice.

Suggested future work:

The study has highlighted some important factors in the pathogenesis and immune response of the disease, which may be taken into consideration when developing better treatment and control regimes for the disease.

It would also be of interest in future studies to investigate if *C. ruminantium* infection by other isolates elicit similar responses; for instance, less virulent strains may be early inducers of IFN–γ and possibly NO.

The kinetics of disease in the young which exhibit some resistance to the disease would also help in clarifying the markers of resistance/susceptibility and may be incorporated in when developing new control measures of the disease. Investigation of breed-related resistance reported in indigenous African breeds needs investigating using animals without prior exposure to *C. ruminantium*.

The role played by NO in the control of the disease needs to be investigated further. Use of animal models with disruption of the iNOS gene would help to clarify this.

Also the effect of NO levels seen terminally may be contributory to pathology, and use of iNOS blockers, like L-NMMA would help to clarify this.

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APPENDICES

Appendix 1:

A. Results of The Red Maasai and Dorper sheep:

1. 1.Temperature Records of The Red Maasai

	Sheep No.							
Day	1	2	3	4	5	6	Mean	SD
2	38.5	39.1	38.4	39.2	38.7	38.6	38.75	0.32710
0	38.7	38.9	39.2	39.1	38.8	38.8	38.916	0.1940
2	38.5	38.4	38.4	38	38.4	39	38.45	0.3209
4	38.6	38.2	39	38.2	38.2	39	38.533	0.393
6	39	39	39.2	39	39	39	39.033	0.0816
8	38.2	38.9	38.1	38.2	38	39	38.4	0.4335
10	38.2	38.7	39.6	39.4	38.5	38.1	38.75	0.6220
12	39.2	39.1	39	39	39.2	39	39.083	0.0983
14	39	39	39	38.2	39	39.8	39	0.5059
16	39.3	39.5	39.5	39.1	38.5	39.6	39.25	0.4086
19	39.3	39	40.6	39	38.9	40.6	39.566	0.8115
22	39.9	39	41	39	38.9	41	39.8	0.9979
26	39.1	39.5		40	39.7		39.575	0.3774
28	39.5	39.7		40.4	39.7		39.825	0.3947
30	40	39.9		39.9	39.7		39.875	0.1258

1.2. Temperature Records of The Dorper

	Sheep No.							
Day	12	15	17	18	19	20	Mean	SD
0	38.9	38.9	38.7	39	39.1	39.1	38.95	0.151658
2	39	39	38.8	39	38.9	38.9	38.93333	0.08165
4	38.2	38.6	38.9	38.7	39	39	38.73333	0.307679
6	39	39.3	39.4	39.1	39.2	39.2	39.2	0.141421
8	39	39	38.3	38.9	38.2	39	38.73333	0.377712
10	38.8	39	39	39	39	38.5	38.88333	0.204124
12	39.4	39.2	39.4	39.4	39.4	39.7	39.41667	0.160208
14	41.5	41.4	39.7	39.5	39.5	39.9	40.25	0.941807
16	41.4	41.2	39.4	39.5	39.4	39.6	40.08333	0.947453
19	40.6	41.5	39.8	41	40.4	40.8	40.68333	0.574166
22	41.2	41.7	39.9	41	40.4	40	40.7	0.715542
26			39.2	40	40	40.3	39.875	0.471699
28			39.7	41.3	40.4	40	40.35	0.695222
30			40	40.4	40.5	40.2	40.275	0.221736

1.3. Antibody and Immunoglobulin (IgG) Levels in Red Maasai and Dorper sheep

1.3.1. IgG1 and IgG2 in Red Maasai and Dorper sheep

IgG1 in Red Maasai and Dorper sheep

	Day					
Sheep No.	0	8	14	19	22	30
1	0.521	0.4	0.453	0.463	0.48	0.781
2	0.23	0.203	0.181	0.168	0.147	0.122
3	0.03	0.15	0.23	0.213	0.2	0.122
4	0.287	0.251	0.224	0.173	0.167	0.154
5	0.587	0.586	0.572	0.55	0.645	0.0635
6	0.049	0.053	0.053	0.055	0.07	0.09
12	0.052	0.05	0.07	0.04	0.055	0.1
15	0.049	0.051	0.051	0.054	0.061	0.111
17	0.058	0.049	0.053	0.055	0.058	0.085
19	0.05	0.052	0.05	0.052	0.055	0.121
20	0.048	0.049	0.051	0.054	0.061	0.111

IgG2 in Red Maasai and Dorper sheep

	Day					
Sheep No	0	8	14	19	22	30
1	0.075	0.095	0.085	0.088	0.089	0.056
2	0.133	0.128	0.112	0.104	0.099	0.072
3	0.056	0.063	0.061	0.075	0.095	0.085
4	0.08	0.084	0.067	0.066	0.056	0.052
5	0.06	0.066	0.064	0.056	0.063	0.061
6	0.056	0.063	0.061	0.048	0.057	0.078
12	0.048	0.057	0.078	0.051	0.057	0.067
15	0.051	0.057	0.067	0.065	0.034	0.05
17	0.048	0.055	0.048	0.051	0.057	0.067
19	0.048	0.048	0.048	0.048	0.057	0.078
20	0.048	0.056	0.047	0.049	0.059	0.091

1.3.2. Antibody Levels (Indirect ELISA results)

Sheep No.	-2	0	4	8	12	16	19	22
1	1.087	1.315	1.205	1.057	.528	.890	.730	1.084
2	.932	1.120	1.003	.465	.227	.682	.730	.690
3	.930	1.020	.955	.985	.900	.679	.804	1.012
4	.951	1.107	1.018	.832	.797	.692	.769	.885
5	1.069	1.109	1.107	.865	.778	.9225	.825	1.040
6	.925	1.427	1.264	.894	.789	.925	1.053	1.106
12	.046	.106	.202	.164	.160	.575	.840	.965
15	.046	.090	.092	.040	.045	.348	.737	.967
17	.055	.065	.072	.051	.031	.058	.054	.200
18	.048	.079	.084	.038	.039	.049	.045	.351
19	.042	.063	.080	.033	.057	.031	.050	.383
20	.063	.097	.093	.027	.079	.067	.076	.042

1.5. Glutathione levels in Red Maasai and Dorpers

GPX in Red maasai

Sheep

1	2	3	4	5	6
81,765	54,005	63,595	36,845	37,850	48,433
73,180	93,880	84,790	51,990	51,600	11,608
74,560	91,333	85,750	61,560	52,170	40,350
76,540	89,170	70,181	56,180	56,600	49,100
70,100	89,760	65,340	54,150	62,170	50,170
68,300	70,140	69,170	61,340	55,310	50,500
66,504	66,170	65,170	62,100	41,600	66,321
63,500	62,340	64,300	61,300	57,160	64,170
61,580	62,580	58,380	83,530	77,220	76,970
61,700	61,511	57,340	82,530	70,120	66,620
63,100	61,810	58,100	59,350	62,300	64,170
60,711	62,510	56,300	62,110	61,170	65,310
61,410	60,100	57,510	61,360	57,180	67,340

GPX in Dorper

SHEEP NO.12	15	17	18	19	20
90,849	119,620	108,260	121,890	108,770	103,970
68,640	140,914	71,670	151,210	114,820	120,760
68,100	141,311	85,700	150,200	107,860	117,720
70,230	130,411	84,100	147,361	115,800	114,760
71,450	126,270	87,500	148,761	113,700	90,600
72,400	110,100	73,130	124,311	100,106	84,440
70,150	90,305	75,750	90,700	97,170	88,560
74,360	91,200	69,120	76,540	94,600	74,130
74,190	74,940	67,630	50,220	75,200	78,480
64,300	71,360	67,510	45,370	60,710	74,110
61,460	68,170	67,750	47,210	59,410	70,310
65,510	66,670	62,400	44,500	60,200	66,510
60,120	62,540	61,720	41,560	55,310	62,560

1.6. Nitrite Levels in Red Maasai and Dorpers

Red Maasai

	Sheep No.					
Day	1	2	3	4	5	6
-2	42.37	32.45	36.1	16.91	31.37	19.28
0	28.79	26.29	31.67	15.02	23.34	33.46
2	26.47	34.95	29.75	16.16	28.16	21.55
4	28.79	34.18	32.35	18.42	25.52	22.4
8	26.57	37.36	24.57	24.61	34.49	38.5
10	21.17	24.8	23.24	23.53	13.23	45.41
12	18.24	24.28	38.51	13.42	21.26	16.25
14	16.63	25.25	28.49	16.63	23.15	20
16	38.9	35.62	32.35	42.46	47.07	36.58
19	37.45	52.08	53.14	57.24	54.48	57.05
21	31.87	44.19	70.24	46.13	71.35	69.5
22	46.88	27.92	72.82	55.58	71.53	63.32
27		34.56		37.16	60.1	
30		51.22		33.02	54.73	

Dorpers

Sheep	12	15	17	18	19	20
Day -2	39.28	31.28	43.71	25.03	16.41	18.32
0	51.89	22.49	24.52	16.75	16.97	27.24
2	16.53	28.46	28.98	17.9	19.83	37.07
4	26.57	39.27	60.37	32.43	52.91	31.67
8	23.95	39.96	38.61	54.1	36.58	42.74
10	20.79	25.12	26.47	20.6	18.93	23.85
12	27.92	30.13	30.23	23.78	19.93	21.95
14	22.59	24.33	30.9	23.58	24.74	28.69
16	116.8	30.61	62.49	33.11	19.35	42.27
19	143.74	44.47	46.3	35.33	38.9	43.15
21	141.3	47.65	47.56	32.73	39.28	43.34
22	115.2	36.55	39.18	29.45	31.29	43.51
27			69.01	84.44	35.03	47.56
30			45.15	115.4	36.87	47.66

1.7. TNF–α levels In Red Maasai and Dorper sheep infected with *C. ruminantium*

Red Maasai

	Day							
Sheep No	0	4	8	10	14	19	22	30
1	0.28	0.34	0.27	0.84	0.38	0.46	0.22	0.33
2	0.52	0.5	0.62	0.51	0.71	0.58	0.68	0.93
3	0.46	0.5	0.97	1.67	0.33	0.59	0.53	
4	0.62	0.67	0.41	0.45	0.75	0.49	1.14	0.71
5	0.23	0.4	0.26	0.87	0.23	0.44	0.24	0.19
6	0.54	0.54	0.63	0.4	0.31	0.54	0.73	
Mean	0.441667	0.491667	0.526667	0.79	0.451667	0.516667	0.59	0.54
Sd	0.154197	0.114615	0.01	0.05	0.221307	0.062823	0.1	0.2

Dorpers

	Day							
Sheep No	0	4	8	10	14	19	22	30
12	0.27	0.63	0.74	0.34	0.56	0.53	0.03	
15	1	0.38	0.36	0.29	0.46	0.27	0.3	
17	0.3	0.3	0.2	0.25	0.24	0.19	0.39	0.35
18	0.28	0.3	0.57	0.61	0.65	0.45	0.37	0.78
19	0.49	0.48	0.33	0.52	0.63	0.49	0.36	0.94
20	0.33	0.33	0.45	0.7	0.56	0.41	0.61	
Mean (D)	0.445	0.403333	0.441667	0.451667	0.516667	0.39	0.343333	0.69
Sd	0.283602	0.130026	0.191355	0.184761	0.151085	0.132665	0.186726	0.305123

1.8. Interferon gamma Levels (OD values) in Red Maasai and Dorpers infected with *C. ruminantium*

	Sheep NO.			
Day	3	6	12	15
-2	0.016	0.014	0	0
0	0.012	0.016	0	0
4	0.008	0.008	0.002	0
8	0.018	0.008	0	0
12	0.011	0.016	0.008	0.002
16	0.011	0.001	0.012	0.001
19	0.011	0.006	0.006	0.018
22	0.017	0.029	0.002	0.009

1.9. Interleukin 8 Levels In Red Maasai and Dorper sheep infected with *C. ruminantium*

Red Maasai

	IL-8 (pg/ml)						
	Day						
Sheep N0.	0	4	8	14	19	22	30
1	3224	5842	1345	5669	5234	5228	11221
2	21664	2923	12156	5858	4567	11889	13158
3	5732	1734	8946	1011	2353	7636	
4	5842	3108	9002	9184	10876	11965	24426
5	4358	4497	8873	8834	7654	2586	2541
6	3036	595	1458	1652	4580	4164	
Means	7309.333	3116.5	6963.333	5368	5877.333	7244.667	12836.5
Median	5045	3015.5	8909.5	5763.5	3466	6432	12836

Dorpers

	Day						
Sheep N0.	0	4	8	14	19	22	30
12	1128	3366	4231	3014	16076	22493	
15	1359	3864	2554	1443	9623	9398	
17	1233	2284	626	1265	15098	1243	15694
18	8142	9830	24782	8809	12890	9712	22886
19	278	4969	9908	4355	14657	4940	8731
20	2763	2376	3855	4512	18765	3827	9025
Means	2483.833	4448.167	7659.333	3899.667	12849.5	8602.167	14084
medians	1296	3615	4043	3684.5		7169	12359.5

1.10. Temperature, SOD, GPX, IL-8 and TNF–α levels of Sheep infected with the Welgevonden isolate of *C. ruminantium*

Sheep No. 67

Day	SOD U/ml	GPX U/ml	IL-8 pg/ml	TNF–α pg/ml	PCV %	Temp (oC)
0	61.6		10600	30	36	39.7
1						39.9
2	150	37.3		0	35	39.9
3						39.2
4	166.6	32.3	4339.9	0	34	39.6
5						39.7
6	174.9	40.3	23900	40	35	39.8
7						39.7
8	166.6	65.6	23500	110	36	40.1
9						41.2
10	133.2	58	21700	10	33	41.6
11						42.2
12	174.9	76.7	12200	0	34	42.5

Sheep No.77

Day	SOD U/ml	GPX U/ml	IL-8 pg/ml	TNF–α pg/ml	PCV %	Temp (oC)
0	18		7338	20	32	39.9
1						39.9
2	166.6	24.2		0	30	39.9
3						39.8
4	174.9	17.7	2792.4	10	33	40
5						39.9
6	141.7	19.2	1728.5	20	31	40
7						41.3
8	158.3	52.5	11400	320	29	41.8
9						41.9
10	191.6	27.3	13600	110	24	42
11						41.7
12	158.3	49.5	6214.5	0	29	41.7

Sheep No. 68

Day	SOD U/ml	GPX U/ml	IL-8 pg/ml	TNF–α pg/ml	PCV %	Temp (oC)
0	57		14100	30	30	39.9
1						40.2
2	133.2	29.8		0	29	39.9
3						39.3
4	174.9	23.7	2867.9	0	30	39.7
5						39.7
6	174.9	29.2	14500	70	28	39.5
7						39.8
8	158.3	62.1	17100	90	27	41.2
9						41.3
10	133.2	45.4	17600	30	21	41.9

11							42.1
12	166.6	100.9	5512	20	33	41.3

Sheep No. 70

Day	SOD U/ml	GPX U/ml	IL-8 pg/ml	TNF-α (pg/ml)	PCV %	Temp (oC)
0	18		7338	120	32	39.9
1						39.9
2	166.6	24.2		40	30	39.9
3						39.8
4	174.9	17.7	2792.4	20	33	40
5						39.9
6	141.7	19.2	1728.5	120	31	40
7						41.3
8	158.3	52.5	11400	320	29	41.8
9						41.9
10	191.6	27.3	13600	160	24	42
11						41.7
12	158.3	49.5	6214.5	100	29	41.7

Sheep 66

Day	SOD U/ml	GPX U/ml	IL-8 pg/ml	TNF-α (pg/ml)	PCV%	Temp (oC)
0			8639.3	20	32	39.8
1						39.7
2	141.7	36.3		0	30	39.8
3						39.3
4	166.6	34.3	1896.36	20	33	39.7
5						39.7
6	166.6	33.8	5070.44	120	31	40.1
7						40.9
8	166.6	73.2	10600	140	29	41.4
9						42.2
10	150	37.8	12400	160	24	41.6
11						42
12	166.6	90.3	3148.8	100	29	41.4

Sheep 69

Day	SOD U/ml	GPX U/ml	IL-8 pg/ml	TNF-α (pg/ml)	PCV %	Temp (oC)
0	51.8		7969.5	0	35	40.1
1						39.8
2	71.1	40.4		0	34	39.9
3						39
4	158.3	33.3	1620.6	20	36	39.4
5						39.7
6	158.3	51.9	1983.2	90	36	39.4
7						40.7
8	150	99.4	5947.3	430	34	41.2
9						41.4
10	133.3	58.5	9742.9	140	34	41.3
11						41.9
12	158.3	76.7	13100	80	30	42.2

Sheep 71

Day	SOD U/ml	GPX U/ml	IL-8 pg/ml	TNF-α (pg/ml)	PCV %	Temp (oC)

0	61.6		10500	0	33	40.1
1						40.1
2		21.2		0	31	39.1
3						39.6
4	158.3	16.6	9406.4	17	36	39.9
5						39.8
6	141.7	19.2	3941	120	31	40.9
7						41.1
8	133.2	35.3	2025.4	120	30	42.1
9						42.5
10	71.1	27.25	5086.1	130	29	42.3
11						41.9
12	150	45.4	1782.9	50	32	42

Sheep 72						
Day	SOD U/ml	GPX U/ml	IL-8 pg/ml	TNF-α (p/ml)	PCV %	Temp (oC)
0			8639.3	0	6905.4	39.8
1						39.7
2	141.7	36.3		0		39.8
3						39.3
4	166.6	34.3	1896.36	20	1710.5	39.7
5						39.7
6	166.6	33.8	5070.44	202	4727.3	40.1
7						40.9
8	166.6	73.2	10600	300	11000	41.4
9						42.2
10	150	37.8	12400	100	12200	41.6
11						42
12	166.6	90.3	3148.8	130	2642.2	41.4

Sheep No 74						
Day	SOD U/ml	GPX U/ml	IL-8 pg/ml	TNF-α (p/ml)	PCV %	Temp (oC)
0	18		7338	0	32	39.9
1						39.9
2	166.6	24.2		0	30	39.9
3						39.8
4	174.9	17.7	2792.4	20	33	40
5						39.9
6	141.7	19.2	1728.5	202	31	40
7						41.3
8	158.3	52.5	11400	560	29	41.8
9						41.9
10	191.6	27.3	13600	300	24	42
11						41.7
12	158.3	49.5	6214.5	130	29	41.7

1.11. Interferon gamma levels in the Welgevonden sheep infected with *C. ruminantium*

Sheep No	Day							
Sheep No	Day							
	mean	sd	Mean	sd	Mean	sd	Mean	sd
	0		6		10		12	
66	0.1125	0.003536	0.1085	0.002121	0.109	0.007778	0.1375	0.003182
67	0.106	0.008485	0.096	0.004243	0.0995	0.001768	0.1085	0.000354
68	0.1275	0.026163	0.105	0.001414	0.096	0	0.136	0.003536
69	0.107	0.022627	0.099	0.001414	0.097	0	0.099	0.002828
70	0.1015	0.014849	0.088	0	0.083	0	0.155	0.00495
71	0.121	0.001414	0.11	0.002828	0.098	0	0.1265	0.001061
72	0.115	0.11	0.1125	0.003536	0.107	0.11	0.1085	0.002121
73	0.112	0.1	0.106	0.008485	0.093	0.099	0.096	0.004243
74	0.146	0.109	0.1275	0.026163	0.104	0.106	0.105	0.001414
75	0.123	0.091	0.107	0.022627	0.1	0.098	0.099	0.001414
76	0.112	0.091	0.1015	0.014849	0.088	0.088	0.088	0
77	0.122	0.12	0.121	0.001414	0.108	0.112	0.11	0.002828

1.12. Nitrite levels Welgevonden infections of Sheep

Primary Sheep infections

		Day				
Sheep No.	0	2	4	8	10	12
67	22.95	23.25	26.55	23.75	33.4	
68	18.05	19.15	19.05	29.4	26.05	27.4
70	27.1	23.05	32.15	49.35	33.8	24.95
73	28.95	24.35	31.1	21.7	40.15	44.1
75	22.26	22.45	29.21	22.05	31.35	32.15
77	26.26	22.45	23.21	39.05	35.35	32.15

Immunised Sheep infections

	Day					
Sheep No.						
66	29.4	23.9	33.8	21.05	17.55	44.85
69	20.1	29.75	33.1	23.7	20.4	40.9
71	32.9	31.9	26.95	30.45	26.45	62.8
72	33	22.35	35.4	29.75	23.55	42.9
74	25.85	28.97	29.31	22.27	24.98	49.86
76	31.85	24.97	34.31	30.75	18.75	45.25

Appendix 2

2.1.Nitrite Levels in Bovine Endothelila cells infected with *C. ruminantium*

Time (day)	Mean NO(υM) - C (UC)	SD	Mean NO (υM)-ilC	SD	Mean NO (υM)-2	SD	Mean NO (υM)-3	SD	Mean NO (υM)-4	SD
0	0.15	0.17	0.42	0.01	0.42	0.01	0.42	0.29	0.42	0.03
1	0.15	0.03	0.75	0.03	2.65	0.18	1.68	0.06	1	0.01
2	0.84	0.18	3.7	0.18	4.77	0.13	4.2	0.53	1.35	0.25
3	1.25	0.2	3.13	0.28	2.94	0.04	3.34	0.41	3.75	0.28
4	1.88	0.18	2.53	0.18	3.57	0.13	1.5	0.18	3.35	0.4
5	1.7	0.2	2.57	0.18	3.08	0.13	2.25	0.18	2.93	0.12
6	1.9	0.13	2.97	0.08	6.73	0.13	5.75	0.06	4.63	0.24
7	1.96	0.2	3.68	0.17	6.67	0.13	3.97	0.06	5.82	0.15
8	0.95	0.13	2.7	0.08	2.7	0.06	2.7	0.18	3.19	0.13
9	1.18	0.18	2.56	0.07	3.34	0.09	1.68	0.06	2.57	0.29
10	1.3	0.06	3.87	0.05	5.36	0.26	2.7	0.06	3.43	0.18
11	1.61	0.29	4.19	0.05	8.96	0.53	4.6	0.06	3.12	0.18
12	1.71	0.18	4.19	0.08	9.41	0.06	4.45	0.06	3.71	0.07
13	2.02	0.18	6.1	0.53	11.45	0.59	4.6	0.13	4.51	0.15
14	2.06	0.18	9.99	0.65	16.58	0.11	8.19	0.02	6.71	0.19
15	1.03	0.13	6.52	0.35	5.66	0.18	5.58	0.05	5.43	0.34
16	1.62	0.18	6.46	0.13	4.83	0.18	3.97	0.02	3.92	0.18
17	1.68	0.13	6.26	0.08	6.74	0.13	5.8	0.13	5.65	0.06
18	2.26	0.13	8.37	0.11	5.62	0.34	5.4	0.53	1.87	0.29
19	2.2	0.13	6.35	0.07	6.87	0.22	6.27	0.06	4.35	0.24
20	1.73	0.09	7.91	0.16	11.07	0.39	8.1	0.06	6.25	0.19
21	1.93	0.2	8.5	0.23	12.07	0.23	8.56	0.13	6.55	0.06

2.2.Viability of *C. ruminantium* Elementary bodies in endothelila cells treated with different concentrations of BorIFN-g

Treatment (IFN-g)	Mean viability of EBs	SD	NO (υM)-24 h	NO (υM) 48 h	SD
0	73	6	2.98	3.8	6
25	54	4	3.12	3.9	4
50	35	12.5	3.36	4.6	12.5
75	28	8	3.8	5.25	8
100	19	2.5	4.9	9.8	2.5

2.3. Endothelial cell death in *C. ruminantium* infected cells treated with different concentraions of BorIFN-g

IFN-G Conc.(U/ml)	Infected cells	SD	Non-infected cells	SD	
0	12	5	7	1.5	
25	17	1.3	10	2	
50	26	5.2	19	1	
75	42	2.1	33	2	
100	75	6.2	50	9.3	

2.4. A. Viability and infectivity of *C. ruminantium* Elementary bodies treated in different SNAP concentratuions

SNAP conc.	Viability-day2					
0	87.5	100	67	67	75	82
25	67	50	60	50	75	67
50	60	42.8	60	25	40	40
100	16.6	33.3	25	40	50	60

SNAP conc.	Viability-day4					
0	71	80	71	78	70	77
25	50	57	50	62.5	50	42
50	25	33	25	25	25	25
100	16.6	20	20	20	25	20

SNAP conc.	infectivity-day2			Mean	SD
0	112	115	110	112	2.5
25	68	73	76	72	4
50	30	26	34	30	4
100	23	20	25	22	2.5

SNAP conc.	Infectivity-day4			Mean infectivity-day4	SD
0	195	200	230	208	18.9
25	140	132	122	131	9
50	96	100	110	102	7.2
100	50	55	60	55	5

B. Viability and infectivity of *C. ruminantium* Elementary bodies treated in different SNAP concentratuions

	Infectivity	Infectivity	Day 2	Day4			Day 7		Day7
SNAP conc.	Day 2	Day 4	Viability	Viability	SD-2	SD-4	Infectivity (7)	SD(Inf7)	Viability
0	112	208	82	75	14	4.3	1100	19	86
25	72	131	64	52	10	7	810	15	65
50	30	102	45	28	13	4	340	9	55
100	22	55	37	20	16	2.7	223	11	45

					Viability	Viabilty	Viability	Viability	
	SNAP	Infectivity (2)	Infectivity (4)	Infectivity (7)	1	2	4	7	Nitrite Conc.
	0	112	200	1100	90	82	75	86	34
	25	70	132	810	79	68	52	65	35
	50	29	100	340	56	45	28	55	37
	100	23	55	223	38	37	20	45	40.1

Appendix 3

3.1. Identification of Individual mice deficient in IFN–γ (gamma), IFN–α/β (alpha beta) receptors and the wild type (WT) (control) groups

gamma 1a	alpha-beta 1a	WT 1a
gamma 2a	alpha-beta 2a	WT 2a
gamma 3a	alpha-beta 3a	WT 3a
gamma 4a	alpha-beta 4a	WT 4a
gamma 5a	alpha-beta 5a	WT 5a
gamma 1b	alpha-beta 1b	WT 1b
gamma 2b	alpha-beta 2b	WT 2b
gamma 3b	alpha-beta 3b	WT 3b
gamma 4b	alpha-beta 4b	WT 4b
gamma 5b	alpha-beta 5b	WT 5b
gamma 1c	alpha-beta 1c	WT 1c
gamma 2c	alpha-beta 2c	WT 2c
gamma 3c	alpha-beta 3c	WT 3c
gamma 4c	alpha-beta 4c	WT 4c
gamma 5c	alpha-beta 5c	WT 5c

3.2. Mortalities as a function of time in IFN-γ and IFN-α/β receptor adeficient mice and infected with *C. ruminantium*

Day	7	8	9	10	11	day 7	ifn-gko	control	total	day 7	dead	not dead	Total
IFN-gR%	1	10	2	0	0	dead	1	0	1	ifn-g	1	12	13
IFN-a/bR%	3	6	3	1	0	not dead	12	13	25	control	0	13	13
Control	0	4	0	6	3	total	13	13	26	total	1	25	26
% dead						Day 8				day 8			
Day	7	8	9	10	11	Dead	11	4	15		11	2	13
IFN-gR%	7.69	76.92	15.38	0	0	not dead	2	9	11		4	9	13
IFN-a/bR%	23	46.15	23.07	7.69	0		13	13	26		15	11	26
control	0	30.7	0	46.15	23.07								

No. of surviving mice													
Day	1	2	3	4	5	6	7	8	9	10	11		
IFN-gR%	13	13	13	13	13	13	12	2	2	0	0		
IFN-a/bR%	13	13	13	13	13	13	10	8	4	0	0		
Control	13	13	13	13	13	13	13	9	9	3	0		

Appendix 4.

4.1. Spleen weights in Balb'c mice infected with *C. ruminantium* and the controls

Spleen wts						
Day	0	2	4	6	8	10
Infected	120	120	120	240	160	200
	120	110	120	180	160	210
	100	150	130	140	160	110
	80	120	170	210	120	160
	130	120	140	200	110	110
	90	110	130	180	150	
Uninfected	100	140	110	100	90	100
	80	110	100	80	110	100
	130	120	110	90	130	110
	90	90	90	140	90	100

4.2. Thoracic oedema in Balb'c mice infected with *C. ruminantium*

Day	Oedema Amount (ml)				
0	2	4	6	8	10
0	0	0	0.5	0.5	1
0	0	0	0	0	1
0	0	0	0	1	1.3
0	0	0	0.6	1.1	1.25
0	0	0	0	1.2	1.3
0	0	0	0.22	0.76	1.17
0	0	0	0.271293	0.449889	0.14

4.3. Interferon gamma levels in Balb'c mice infected with *C. ruminantium*

	absorbance					ifn-g conc
Animal	1	2	MEAN		x 2 dilution	x511,99
A1-Day 2	0.161	0.176	0.1685	0.0465	0.093	47.61507
B2	0.146	0.148	0.147	0.025	0.05	25.5995
C3	0.152	0.147	0.1495	0.0275	0.055	28.15945
D4	0.135	0.146	0.1405	0.0185	0.037	18.94363
E5	0.135	0.159	0.147	0.025	0.05	25.5995
F1	0.136	0.122	0.129	0.007	0.014	7.16786
H1	0.129	0.149	0.139	0.017	0.034	17.40766
I2	0.127	0.157	0.142	0.02	0.04	20.4796
J3	0.163	0.149	0.156	0.034	0.068	34.81532
K4	0.142	0.143	0.1425	0.0205	0.041	20.99159
A2-Day 4	0.178	0.174	0.176	0.054	0.108	55.29492
B3	0.174	0.159	0.1665	0.0445	0.089	45.56711
C3	0.173	0.183	0.178	0.056	0.112	57.34288
D5	0.178	0.183	0.1805	0.0585	0.117	59.90283
E1	0.175	0.165	0.17	0.048	0.096	49.15104
G1	0.707	0.241	0.474	0.352	0.704	360.441
H2	0.154	0.168	0.161	0.039	0.078	39.93522
I3	0.247	0.166	0.2065	0.0845	0.169	86.52631
K5	0.167	0.177	0.172	0.05	0.1	51.199
J4	0.136	0.157	0.1465	0.0245	0.049	25.08751
A3-Day 6	0.945	0.848	0.8965	0.7745	1.549	793.0725
B4	1.082	1.216	1.149	1.027	2.054	1051.627
C5	0.144	0.168	0.156	0.034	0.068	34.81532
D1	1.063	1.125	1.094	0.972	1.944	995.3086
F2	1.342	1.401	1.3715	1.2495	2.499	1279.463
G2	1.365	1.455	1.41	1.288	2.576	1318.886
H3	0.183	0.17	0.1765	0.0545	0.109	55.80691
I4	0.144	0.15	0.147	0.025	0.05	25.5995
L1	0.16	0.16	0.16	0.038	0.076	38.91124
M1	0.142	0.141	0.1415	0.0195	0.039	19.96761
B1-Day 8	0.218	0.217	0.2175	0.0955	0.191	97.79009
D3	0.207	0.207	0.207	0.085	0.17	87.0383
E2	0.246	0.254	0.25	0.128	0.256	131.0694
E3	0.229	0.244	0.2365	0.1145	0.229	117.2457
G3	0.264	0.279	0.2715	0.1495	0.299	153.085
G4	0.217	0.215	0.216	0.094	0.188	96.25412
L3	0.161	0.169	0.165	0.043	0.086	44.03114
L4	0.148	0.156	0.152	0.03	0.06	30.7194
M2	0.144	0.158	0.151	0.029	0.058	29.69542
M5	0.192	0.161	0.1765	0.0545	0.109	55.80691
A5-Day 9	0.253	0.243	0.248	0.126	0.252	129.0215
C2	0.286	0.262	0.274	0.152	0.304	155.645
E4	0.347	0.336	0.3415	0.2195	0.439	224.7636
F3	0.324	0.3	0.312	0.19	0.38	194.5562
F4	0.28	0.271	0.2755	0.1535	0.307	157.1809
G5	0.288	0.281	0.2845	0.1625	0.325	166.3968
A4-Day 10	0.313	0.285	0.299	0.177	0.354	181.2445
B5	0.375	0.371	0.373	0.251	0.502	257.019
C1	0.272	0.315	0.2935	0.1715	0.343	175.6126
D2	0.192	0.205	0.1985	0.0765	0.153	78.33447

D2	0.192	0.205	0.1985	0.0765	0.153	78.33447
F5	0.332	0.346	0.339	0.217	0.434	222.2037
I5	0.149	0.151	0.15	0.028	0.056	28.67144
J2	0.148	0.158	0.153	0.031	0.062	31.74338
J3	0.142	0.158	0.15	0.028	0.056	28.67144
J5	0.177	0.17	0.1735	0.0515	0.103	52.73497
1-0	0.197	0.188	0.1925	0.0705	0.141	72.19059
2-0						
3-0	.					
4-0						
5-0				less blank		
6-0				2.493		
standards				1.521		
IFN-g pg/ml	OD		mean	0.8285		
1600	2.588	2.642	2.615	0.3915		
800	1.651	1.635	1.643	0.1995		
400	0.931	0.97	0.9505	0.0875		
200	0.51	0.517	0.5135	0.052		
100	0.323	0.32	0.3215	0		
50	0.22	0.199	0.2095			
25	0.194	0.154	0.174			
0	0.125	0.119	0.122			
IFN-g pg/ml	OD					
1600	2.493					
800	1.521					
400	0.8285					
200	0.3915					
100	0.1995					
50	0.0875					
25	0.052					
0	0					
	IFN-g					
	Day 2	Day 4	Day 6	Day 8	Day 9	Day 10
A1-Day 2	47.61507	55.29492	793.0725	97.79009	129.0215	181.2445
B2	25.5995	45.56711	1051.627	87.0383	155.645	257.019
C3	28.15945	57.34288	34.81532	131.0694	224.7636	175.6126
D4	18.94363	59.90283	995.3086	117.2457	194.5562	78.33447
E5	25.5995	49.15104	1279.463	153.085	157.1809	222.2037
F1	7.16786	360.441	1318.886	96.25412	166.3968	
H1	17.40766	39.93522	55.80691	44.03114		28.67144
I2	20.4796	86.52631	25.5995	30.7194		31.74338
J3	34.81532	51.199	38.91124	29.69542		28.67144
K4	20.99159	25.08751	19.96761	55.80691		52.73497

4.4. Interleukin 12 levels in Balb'c mice infected with *C. ruminantium*

Animal No	OD-1	OD-2	ODX2	ODX2	IL-12 (X437)	IL-12	MEAN	SD
A1-D2	0.042	0.045	0.084	0.09	36.708	39.33	38.019	1.854034
B2	0.046	0.05	0.092	0.1	40.204	43.7	41.952	2.472045
C3	0.055	0.084	0.11	0.168	48.07	73.416	60.743	17.92233
D4	0.091	0.084	0.182	0.168	79.534	73.416	76.475	4.326079
E5	0.071	0.071	0.142	0.142	62.054	62.054	62.054	0
F1	0.079	0.081	0.158	0.162	69.046	70.794	69.92	1.236023
H1	0.091	0.08	0.182	0.16	79.534	69.92	74.727	6.798125
I2	0.049	0.056	0.098	0.112	42.826	48.944	45.885	4.326079
A2-D4	0.053	0.051	0.106	0.102	46.322	44.574	45.448	1.236023
B3	0.055	0.059	0.11	0.118	48.07	51.566	49.818	2.472045
C3	0.077	0.069	0.154	0.138	67.298	60.306	63.802	4.944091
D5	0.06	0.058	0.12	0.116	52.44	50.692	51.566	1.236023
E1	0.069	0.057	0.138	0.114	60.306	49.818	55.062	7.416136
G1	0.102	0.095	0.204	0.19	89.148	83.03	86.089	4.326079
H2	0.053	0.044	0.106	0.088	46.322	38.456	42.389	5.562102
I3	0.056	0.038	0.112	0.076	48.944	33.212	41.078	11.1242
A3-D6	0.059	0.067	0.118	0.134	51.566	58.558	55.062	4.944091
B4	0.062	0.045	0.124	0.09	54.188	39.33	46.759	10.50619
C5	0.054	0.059	0.108	0.118	47.196	51.566	49.381	3.090057
D1	0.041	0.048	0.082	0.096	35.834	41.952	38.893	4.326079
F2	0.055	0.039	0.11	0.078	48.07	34.086	41.078	9.888181
G2	0.06	0.055	0.12	0.11	52.44	48.07	50.255	3.090057
H3	0.093	0.063	0.186	0.126	81.282	55.062	68.172	18.54034
I4	0.042	0.038	0.084	0.076	36.708	33.212	34.96	2.472045
B1-D8	0.045	0.085	0.09	0.17	39.33	74.29	56.81	24.72045
D3	0.087	0.089	0.174	0.178	76.038	77.786	76.912	1.236023
E2	0.043	0.051	0.086	0.102	37.582	44.574	41.078	4.944091
E3	0.056	0.054	0.112	0.108	48.944	47.196	48.07	1.236023
G3	0.078	0.082	0.156	0.164	68.172	71.668	69.92	2.472045
G4	0.068	0.065	0.136	0.13	59.432	56.81	58.121	1.854034
L3	0.061	0.065	0.122	0.13	53.314	56.81	55.062	2.472045
L4	0.044	0.055	0.088	0.11	38.456	48.07	43.263	6.798125
A4-D10	0.068	0.054	0.136	0.108	59.432	47.196	53.314	8.652159
B5	0.05	0.038	0.1	0.076	43.7	33.212	38.456	7.416136
C1	0.045	0.07	0.09	0.14	39.33	61.18	50.255	15.45028
D2	0.035	0.037	0.07	0.074	30.59	32.338	31.464	1.236023
F5	0.056	0.059	0.112	0.118	48.944	51.566	50.255	1.854034
I5	0.038	0.046	0.076	0.092	33.212	40.204	36.708	4.944091
J2	0.054	0.049	0.108	0.098	47.196	42.826	45.011	3.090057
J3	0.059	0.064	0.118	0.128	51.566	55.936	53.751	3.090057
D-0	0.04	0.047	0.08	0.094	34.96	41.078	38.019	4.326079

Means	Day								
0	2	SD	4	SD	6	SD	8	SD	10
4.326079	38.019	1.854034	45.448	1.236023	55.062	4.944091	56.81	24.72045	53.314
	41.952	2.472045	49.818	2.472045	46.759	10.50619	76.912	1.236023	38.456
	60.743	17.92233	63.802	4.944091	49.381	3.090057	41.078	4.944091	50.255
	76.475	4.326079	51.566	1.236023	38.893	4.326079	48.07	1.236023	31.464
	62.054	0	55.062	7.416136	41.078	9.888181	69.92	2.472045	50.255
	69.92	1.236023	86.089	4.326079	50.255	3.090057	58.121	1.854034	36.708
	74.727	6.798125	42.389	5.562102	68.172	18.54034	55.062	2.472045	45.011

	45.885	4.326079	41.078	11.1242	34.96	2.472045	43.263	6.798125	53.751
STDS	OD								
1600	2.387								
800	1.585								
400	1.005								
200	0.548								
100	0.288								
50	0.147								

4.5. Interleukin 4 levels in Balb'c mice infected with *C. ruminantium*

AnimalNo.		OD-1	OD-2	OD-1-BLANK	OD-2-BLANK	IL-4conc-1	IL-4conc-2	X2 (dilution)	X2	MEAN	SD
1	A1-Day 2	0.977	0.759	0.497	0.279	45.227	25.389	90.454	50.778	70.616	28.05517
2	B2	1.472	1.205	0.992	0.725	90.272	65.975	180.544	131.95	156.247	34.36115
3	C3	1.775	1.778	1.295	1.298	117.845	118.118	235.69	236.236	235.963	0.38608
4	D4	1.341	0.815	0.861	0.335	78.351	30.485	156.702	60.97	108.836	67.69275
5	E5	1.089	0.841	0.609	0.361	55.419	32.851	110.838	65.702	88.27	31.91597
6	F1	0.944	0.806	0.464	0.326	42.224	29.666	84.448	59.332	71.89	17.75969
7	H1	0.855	0.975	0.375	0.495	34.125	45.045	68.25	90.09	79.17	15.44321
8	I2	1.005	0.709	0.525	0.229	47.775	20.839	95.55	41.678	68.614	38.09326
9	J3	0.661	0.877	0.181	0.397	16.471	36.127	32.942	72.254	52.598	27.79778
10	K4	0.85	0.838	0.37	0.358	33.67	32.578	67.34	65.156	66.248	1.544321
11	A2-Day 4	1.452	0.693	0.972	0.213	88.452	19.383	176.904	38.766	107.835	97.67832
12	B3	0.712	0.555	0.232	0.075	21.112	6.825	42.224	13.65	27.937	20.20487
13	C3	0.698	0.595	0.218	0.115	19.838	10.465	39.676	20.93	30.303	13.25542
14	D5	0.564	0.555	0.084	0.075	7.644	6.825	15.288	13.65	14.469	1.158241
15	E1	0.707	0.53	0.227	0.05	20.657	4.55	41.314	9.1	25.207	22.77874
16	G1	0.771	0.569	0.291	0.089	26.481	8.099	52.962	16.198	34.58	25.99607
17	H2	0.473	0.486	-0.007	0.006	-0.637	0.546	-1.274	1.092	-0.091	1.673015
18	I3	0.696	0.468	0.216	-0.012	19.656	-1.092	39.312	-2.184	18.564	29.3421
19	K5	0.772	0.745	0.292	0.265	26.572	24.115	53.144	48.23	50.687	3.474723
20	J4	0.543	0.483	0.063	0.003	5.733	0.273	11.466	0.546	6.006	7.721606
21	A3-Day 6	0.67	0.778	0.19	0.298	17.29	27.118	34.58	54.236	44.408	13.89889
22	B4	0.397	0.65	-0.083	0.17	-7.553	15.47	-15.106	30.94	7.917	32.55944
23	C5	0.592	0.419	0.112	-0.061	10.192	-5.551	20.384	-11.102	4.641	22.26396
224	D1	0.402	0.806	-0.078	0.326	-7.098	29.666	-14.196	59.332	22.568	51.99215
25	F2	0.293	0.322	-0.187	-0.158	-17.017	-14.378	-34.034	-28.756	-31.395	3.73211
26	G2	0.371	0.522	-0.109	0.042	-9.919	3.822	-19.838	7.644	-6.097	19.43271
27	H3	0.629	0.552	0.149	0.072	13.559	6.552	27.118	13.104	20.111	9.909394
28	I4	0.627	0.542	0.147	0.062	13.377	5.642	26.754	11.284	19.019	10.93894
29	L1	0.851	0.636	0.371	0.156	33.761	14.196	67.522	28.392	47.957	27.66909
30	M1	0.684	0.543	0.204	0.063	18.564	5.733	37.128	11.466	24.297	18.14577
31	B1-Day 8	0.781	0.504	0.301	0.024	27.391	2.184	54.782	4.368	29.575	35.64808
32	D3	0.509	0.542	0.029	0.062	2.639	5.642	5.278	11.284	8.281	4.246883
33	E2	0.437	0.317	-0.043	-0.163	-3.913	-14.833	-7.826	-29.666	-18.746	15.44321

34	E3	0.674	0.293	0.194	-0.187	17.654	-17.017	35.308	-34.034	0.637	49.0322
35	G3	0.632	0.572	0.152	0.092	13.832	8.372	27.664	16.744	22.204	7.721606
36	G4	0.456	0.26	-0.024	-0.22	-2.184	-20.02	-4.368	-40.04	-22.204	25.22391
37	L3	0.508	0.692	0.028	0.212	2.548	19.292	5.096	38.584	21.84	23.67959
38	L4	0.932	0.384	0.452	-0.096	41.132	-8.736	82.264	-17.472	32.396	70.524
39	M2	0.511	0.296	0.031	-0.184	2.821	-16.744	5.642	-33.488	-13.923	27.66909
40	M5	0.328	0.314	-0.152	-0.166	-13.832	-15.106	-27.664	-30.212	-28.938	1.801708
41	A5-Day 9	0.562	0.634	0.082	0.154	7.462	14.014	14.924	28.028	21.476	9.265927
42	C2	0.676	0.42	0.196	-0.06	17.836	-5.46	35.672	-10.92	12.376	32.94552
43	E4	1.052	0.54	0.572	0.06	52.052	5.46	104.104	10.92	57.512	65.89104
44	F3	1.391	1.685	0.911	1.205	82.901	109.655	165.802	219.31	192.556	37.83587
45	F4	1.342	1.054	0.862	0.574	78.442	52.234	156.884	104.468	130.676	37.06371
46	G5	1.017	0.231	0.537	-0.249	48.867	-22.659	97.734	-45.318	26.208	101.153
47	A4-Day 10	1.277	1.634	0.797	1.154	72.527	105.014	145.054	210.028	177.541	45.94356
48	B5	0.94	0.624	0.46	0.144	41.86	13.104	83.72	26.208	54.964	40.66713
49	C1	0.637	0.59	0.157	0.11	14.287	10.01	28.574	20.02	24.297	6.048591
50	D2	0.505	0.683	0.025	0.203	2.275	18.473	4.55	36.946	20.748	22.90743
51	F5	1.548	1.12	1.068	0.64	97.188	58.24	194.376	116.48	155.428	55.08079
52	I5	1.354	1.141	0.874	0.661	79.534	60.151	159.068	120.302	139.685	27.4117
53	J2	1.268	1.169	0.788	0.689	71.708	62.699	143.416	125.398	134.407	12.74065
54	J3	1.117	0.978	0.637	0.498	57.967	45.318	115.934	90.636	103.285	17.88839
55	J5	0.888	0.379	0.408	-0.101	37.128	-9.191	74.256	-18.382	27.937	65.50496
56	1-0	0.721	0.51	0.241	0.03	21.931	2.73	43.862	5.46	24.661	27.15431
57	2-0	0.551	1.3	0.071	0.82	6.461	74.62	12.922	149.24	81.081	96.39138
58	3-0	0.85	1.238	0.37	0.758	33.67	68.978	67.34	137.956	102.648	49.93305
59	4-0	0.777	0.957	0.297	0.477	27.027	43.407	54.054	86.814	70.434	23.16482
60	5-0	1.309	1.38	0.829	0.9	75.439	81.9	150.878	163.8	157.339	9.137234
61	6-0	1.571	1.335	1.091	0.855	99.281	77.805	198.562	155.61	177.086	30.37165
STDS											
800	2.143	1.663									
400	1.879	1.399									
200	1.922	1.442									
100	1.561	1.081									
50	1.03	0.55									
25	0.795	0.315									
	Day 2 IL-4	SD	Day 4 IL-4	SD	Day 6 IL-6	SD	Day 8 IL-4	SD	Day 9 IL-4		Day 10 IL-4
	70.616	28.05517	107.835	97.67832	44.408	13.89889	29.575	35.64808	21.476	9.265927	177.541
	156.247	34.36115	27.937	20.20487	7.917	32.55944	8.281	4.246883	12.376	32.94552	54.964
	235.963	0.38608	30.303	13.25542	4.641	22.26396	-18.746	15.44321	57.512	65.89104	24.297

	108.836	67.69275	14.469	1.158241	22.568	51.99215	0.637	49.0322	192.556	37.83587	20.748
	88.27	31.91597	25.207	22.77874	-31.395	3.73211	22.204	7.721606	130.676	37.06371	155.428
	71.89	17.75969	34.58	25.99607	-6.097	19.43271	-22.204	25.22391	26.208	101.153	139.685
	79.17	15.44321	-0.091	1.673015	20.111	9.909394	21.84	23.67959			134.407
	68.614	38.09326	18.564	29.3421	19.019	10.93894	32.396	70.524			103.285
	52.598	27.79778	50.687	3.474723	47.957	27.66909	-13.923	27.66909			27.937
	66.248	1.544321	6.006	7.721606	24.297	18.14577	-28.938	1.801708			

4.6. Inteleukin 10 levels in Balb'c mice infected with *C. ruminantium*

Animal	Animal	OD1	OD2	Mean	pg/ml
1	A1-Day 2	0.07	0.062	0.066	134.6083
2	B2	0.077	0.071	0.074	150.9245
3	C3	0.049	0.035	0.042	85.65984
4	D4	0.099	0.062	0.0805	164.1814
5	E5	0.04	0.026	0.033	67.30416
6	F1	0.055	0.047	0.051	104.0155
7	H1	0.04	0.033	0.0365	74.44248
8	I2	0.041	0.042	0.0415	84.64008
9	J3	0.086	0.052	0.069	140.7269
10	K4	0.025	0.02	0.0225	45.8892
11	A2-Day 4	0.035	0.046	0.0405	82.60056
12	B3	0.043	0.038	0.0405	82.60056
13	C3	0.062	0.072	0.067	136.6478
14	D5	0.024	0.024	0.024	48.94848
15	E1	0.045	0.028	0.0365	74.44248
16	G1	0.065	0.081	0.073	148.885
17	H2	0.066	0.065	0.0655	133.5886
18	I3	0.03	0.065	0.0475	96.8772
19	K5	0.177	0.03	0.1035	211.0903
20	J4	0.042	0.055	0.0485	98.91672
21	A3-Day 6	0.032	0.045	0.0385	78.52152
22	B4	0.039	0.038	0.0385	78.52152
23	C5	0.067	0.063	0.065	132.5688
24	D1	0.046	0.044	0.045	91.7784
25		0.062	0.048	0.055	112.1736
26	G2	0.065	0.058	0.0615	125.4305
27	H3	0.038	0.039	0.0385	78.52152
28	I4	0.032	0.032	0.032	65.26464
29	L1	0.063	0.062	0.0625	127.47
30	M1	0.023	0.025	0.024	48.94848
31	B1-Day 8	0.05	0.048	0.049	99.93648
32	D3	0.068	0.047	0.0575	117.2724
33	E2	0.034	0.027	0.0305	62.20536
34	E3	0.031	0.034	0.0325	66.2844
35	G3	0.027	0.011	0.019	38.75088
36	G4	0.03	0.015	0.0225	45.8892
37	L3	0.036	0.033	0.0345	70.36344
38	L4	0.023	0.017	0.02	40.7904
39	M2	0.028	0.032	0.03	61.1856
40	M5	0.063	0.053	0.058	118.2922
41	A5-Day 9	0.037	0.041	0.039	79.54128
42	C2	0.104	0.027	0.0655	133.5886
43	E4	0.078	0.025	0.0515	105.0353
44	F3	0.048	0.039	0.0435	88.71912
45	F4	0.042	0.044	0.043	87.69936
46	G5	0.029	0.059	0.044	89.73888
47	A4-Day 10	0.024	0.03	0.027	55.06704
48	B5	0.03	0.034	0.032	65.26464
49	C1	0.034	0.042	0.038	77.50176
50	D2	0.013	0.026	0.0195	39.77064
51	F5	0.162	0.153	0.1575	321.2244

52	I5	0.034	0.019	0.0265	54.04728
53	J2	0.041	0.016	0.0285	58.12632
54	J3	0.042	0.026	0.034	69.3436
55	J5	0.042	0.035	0.0385	78.52152
56	1-0	0.081	0.053	0.067	136.6478
57	2-0	0.191	0.197	0.194	395.6669
58	3-0	0.051	0.038	0.0445	90.75864
Day	0	2	4	6	8
IL-10 conc	136.6478	134.6083	82.60056	78.52152	99.93648
Infected	395.6669	150.9245	82.60056	78.52152	117.2724
	90.75864	85.65984	136.6478	132.5688	62.20536
		164.1814	48.94848	91.7784	66.2844
		67.30416	74.44248	112.1736	38.75088
		104.0155	148.885	125.4305	45.8892
Uninfected		74.44248	133.5886	78.52152	70.36344
		84.64008	96.8772	65.26464	40.7904
		140.7269	211.0903	127.47	61.1856
		45.8892	98.91672	48.94848	118.2922
Day	Means-Infected	SD	Means-Uninfected	SD	
0	207.6911	164.4008	207.6911	164.4008	
2	117.7823	38.21992	86.42466	39.74339	
4	95.68748	38.69404	135.1182	53.3761	
6	103.1657	23.60401	80.05116	33.84723	
8	71.72312	30.80733	72.6579	32.83749	
10	111.7657	117.906	65.0097	11.08916	
9	97.38708	19.57036			

Standards

IL-10 (8-1-1998)

OD1	OD2	Mean	pg/ml
1.281	1.418	1.3495	1080
0.424	0.568	0.496	540
0.131	0.126	0.1285	180
0.016	0.016	0.016	60
0.012	0.007	0.0095	30

4.7. Nitrite levels in Bab'c mice infected with *C. ruminantium*

Day 0	Day 2	Day 4	Day 6	Day 8	Day 9	Day 10
7.856	10.736	8.88	8.912	8.912	13.344	12.912
6.768	9.392	8.4	8.832	8.832	11.904	11.632
8.08	10.304	9.568	8.32	8.32	12.256	10.896
10.256	11.328	4.864	8.64	8.64	14.32	10.32
6.928	6.496	7.776	9.872	9.872	12.832	13.376
9.664	11.68	7.696	9.52	9.52	14.272	
	9.376	7.488	11.152	11.152	12.064	12.576
	9.008	4.976	7.568	7.568	9.824	12.528
	10.656	6.128	8.592	8.592	11.952	11.104
	10.352	6.224	7.408	7.408	14.032	11.856

Appendix 5: IL-12 inoculation results

Individual Mice Numbers in different Groups (IA, IIA, IB, IIB, III AND IV)

IA	IIA	III (1st Trial)	IB (Sub groups a, b and c)	IIB (Sub groups a, b and c)	III (2nd Trial) Sub groups a, b and c)	IV (Sub groups a, b and c)
1a	1a	1a	1a	1a	1a	1a
2a	2a	2a	2a	2a	2a	2a
3a	3a	3a	3a	3a	3a	3a
4a	4a	4a	4a	4a	4a	4a
5a	5a	5a	5a	5a	5a	5a
6a	6a	6a	1b	1b	1b	1b
7a	7a	7a	2b	2b	2b	2b
8a	8a	8a	3b	3b	3b	3b
9a	9a	9a	4b	4b	4b	4b
10a	10a	10a	5b	5b	5b	5b
			1c	1c	1c	1c
			2c	2c	2c	2c
			3c	3c	3c	3c
			4c	4c	4c	4c
			5c	5c	5c	5c

5.1 Clinical and pathological manifestations in *C. ruminantium* infected Balb'c mice inoculated with 500 ng IL-12

Group	Mean IP	Mean CD	Mean DOD	Mean Spleen weight	Mean Thoracic odema fluid					
IB	9	2	11	180	0.8					
IIB	8.4	2.2	10.4	168	0.87					
III	6.8	1.5	8.5	105	0.96					
Group of mice	Mean IP(days)	SD	CD (days)	SD	Mean DOD	SD	Mean LOF (ml)	SD	Mean SP.WT	SD
IB	9.2	1.2	2	0	11	1.2	0.8	0.1	180	30
IIB	8.4	1.3	2	0.6	10	1.3	0.86	0.14	167	35
III	6.9	0.5	1.4	0.5	8.3	0.8	1	0.1	100	20
Group	Morb rate				Mort. rate					
Day	6	7	8	9	7	8	9	10		
IB	0	6.6	20	33.3	0	0	0	13		
IIB	0	20	33.3	27	0	13.3	0	47		
III	20	53	6.6	6.6	6.6	48	26.6	0		

KEY: IP=incubation period; CD=course of diseas; DOD=day of death; LOF=lung oedema fluid; SP.WT=spleen weight; Morb =morbidity; Mort=mortality

5.2. Cytokine (IFN-γ , IL-10, IL-4 and Nitrite levels in *C. ruminantium*-infected Balb'c mice inoculated with IL-12

GROUP I				
Mouse No.	IFN-g (pg/ml)	IL-10	IL-4	NO2 (uM)
IA1	96.8	98	32.9	8.63
IA4	163.8	70.2	36.2	8.99
IA5	170.4	93.7	36.1	10.93
IB1	169		33.9	8.9
IB3	148.2		33.7	5.8
IB4	150	33.5	32.5	3.12
IB5	178.7	93.7	25.3	5.86
IC1	137.6	0	38.1	5.99
IC4	87.3	64.4	37.6	9.15
MEAN	144.6444	64.78571	34.03333	7.485556
SD	32.50323	36.47078	3.844802	2.423495
median	150	70.2	33.9	
Group 1				
D7 (mean)	163.8	70.2	36.2	8.99
D8	159.5	63.6	30.6	5.96
D10	117.8	33.5	35.6	7.5
D11	133.6	95.8	34.5	8.6
D13	137.6	0	38.1	5.99
		G1 Stats.	Correlation	IFN/NO
				-0.11492
GROUP II				
Mouse No.	IFN-g (pg/ml)		IL-12p70	IL-10
2A1	68.8			106.8
2C3	12.5		13.86	79
2B3	57			81.9
2B5	34		14.34	96.6
2B4	94.7			203
2C5	63.4			93.7
2B2	44.3			124.4
2C1	41.3			71.7
2B1	42			33.5
2A5	36.5			
MEAN	49.45		14.1	98.95556
SD	21.43302		0.339411	46.48089
median	43.15			93.7
D7	94.7			203
D9	34			96.5
D10	52.8			67.8
D11	36.5			79
D13	12.5			79
GROUP III			IL-12p70	IL-10
Mouse No.	IFN-g 9 pg/mL)			76.1
3A2	23			33.7
3A3	35.5			43.9
3A4	172.8		25.2	181.4
3A5	77.6			87.8
3B4	69		7.2	
3C1	71.2			
3C2	69.8			137.6

3C3	5.36			
3C4	127.8			43.9
3B3	231.9			
MEAN	72.45111		16.2	86.34286
SD	51.83197		12.7	54.9913
Median	70.5			76.1

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Nitric Oxide is Produced by *Cowdria ruminantium*-Infected Bovine Pulmonary Endothelial Cells In Vitro and Is Stimulated by Gamma Interferon

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Nitric oxide (NO) is a labile inorganic free radical produced by NO synthase from the substrate L-arginine in various cells and tissues including endothelial cells. A substantial elevation of nitrite levels indicative of NO production occurred in cultures of *Cowdria ruminantium*-infected bovine pulmonary endothelial cells (BPEC) incubated in medium alone. Exposure of the infected cultures to recombinant bovine gamma interferon (BorIFN-γ) resulted in more rapid production of NO, reduced viability of *C. ruminantium*, and induction of endothelial cell death. Significant inhibition of NO production was noted after addition of the NO synthase inhibitor *N*-monomethyl-L-arginine (L-NMMA), indicating that the increase in production occurred via the inducible NO synthase pathway. Reduction in the infectivity of *C. ruminantium* elementary bodies (EBs) occurred in a dose-dependent manner after incubation with the NO donor molecule *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) prior to infection of endothelial cells. The level of infection in cultures maintained in SNAP was reduced in a dose-dependent manner with significant negative correlation between the final level of infection on day 7 and the level of SNAP ($r = -0.96$). It was established that pretreatment and cultivation of *C. ruminantium* EBs with the NO donor molecule SNAP reduced infectivity to cultures and viability of EBs with the implication that release of NO in vivo following infection of endothelial cells may have an effect upon the multiplication of the agent in the host animal and may be involved in the pathogenesis of heartwater through the effect of this molecule upon circulation.

Heartwater is an important infectious tick-borne disease of domestic and wild ruminants that is caused by an intracellular rickettsial organism, *Cowdria ruminantium*, which infects and multiplies in the cytoplasm of endothelial cells (9, 14). The clinical manifestations and severity of the disease are associated mainly with severe respiratory, cardiac, and nervous embarrassment due to increased capillary permeability, leading to edema (8). While increased capillary permeability may explain the marked fall in blood and plasma volume which occurs prior to death from heartwater infection (8), the cause of such alterations remains obscure. The work reported here was designed to investigate the role of the association between *C. ruminantium* and endothelial cells in the pathogenesis of heartwater and the development of host resistance to this infection.

The approach was prompted by reports that nitric oxide (NO), a powerful vasodilator generated by the endothelium, is intimately related to mechanisms of edema formation and hyperemia (10, 22, 23); that NO is both microbicidal (1, 2, 4) and cytotoxic (27); and that interferons (IFNs) may play an important role in resistance to intracellular viruses and rickettsias, such as *C. ruminantium* (30), possibly by elevating NO production. This work was therefore designed to monitor NO production in cultures of bovine pulmonary endothelial cells (BPEC) infected with *C. ruminantium*, to assess the effect of gamma interferon (IFN-γ) on NO production by BPEC and its effect on *C. ruminantium* and the endothelial cells, and to assess the effect of NO on the viability and infectivity of *C. ruminantium* to BPEC in vitro.

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MATERIALS AND METHODS

Endothelial cells. A BPEC cell line was prepared from vascular tissues, obtained from abattoir-slaughtered cattle, by a modification of the method described by Byrom et al. (7). In brief, short segments of the pulmonary artery were immersed in phosphate-buffered saline containing antibiotics (PBS-A). Collagenase solution at 1 mg/ml in PBS-A was used to detach the cells, which were then cultured in Glasgow minimal essential medium (Gibco Laboratories, Glasgow, United Kingdom) supplemented with 20% heat-inactivated fetal calf serum, 200 mM L-glutamine, 100 IU of penicillin G per ml, and 100 μg of streptomycin per ml.

***C. ruminantium* isolate.** The Gardel isolate of *C. ruminantium* isolated in Guadeloupe (31) was maintained in endothelial cell cultures by using Glasgow minimal essential medium fortified with 10% bovine serum, 20 mM HEPES, 10% tryptose phosphate broth, 20 mM L-glutamine, 100 IU of penicillin G per ml, and 100 μg of streptomycin per ml by previously described protocols (7).

Endothelial cell infection. Glass coverslips of about 13 mm in diameter were sterilized in absolute ethanol for 10 min and then rinsed in sterile PBS before being inserted in four 24-well culture plates (Gibco) with sterile forceps. BPEC were seeded in the plates and incubated at 37°C in 5% CO_2. At confluence, the cells were infected with *C. ruminantium* elementary bodies (EBs) obtained from the supernatant of an infected BPEC culture. The supernatant was centrifuged at 1,500 × *g* to separate endothelial cells and then at 15,000 × *g* to pellet the EBs. The EBs were then resuspended in complete medium, and this was used as the inoculum to infect the cells. The plates were then incubated at 37°C for 4 h, after which all the medium was removed and fresh medium containing 0, 25, 50, 75, or 100 U of bovine recombinant IFN-γ (BorIFN-γ, Novartis Animal Health Inc., Basel, Switzerland) per ml was added to the cells. The effect of the NO synthase inhibitor *N*-monomethyl-L-arginine (L-NMMA) (Sigma, Poole, United Kingdom) at 1 mM was also assessed by culturing infected endothelial cells with or without BorIFN-γ. Twelve replicates were used for each treatment. The other wells were left as uninfected control wells.

Detection of NO generation by *C. ruminantium*-infected BPEC. NO reacts with water in culture medium to produce a stable and quantifiable product, nitrite (NO_2^-), whose concentration can be taken as a measure of NO production by cultured endothelial cells (19). NO_2^- levels were assayed in culture supernatants daily up to day 7 postinfection by the Griess assay method (19, 33). A 100-μl

volume of the test sample was mixed with an equal volume of the Griess reagent (0.1% naphthylethylene diamine · 2HCl and 1% sulfanilamide in 5% phosphoric acid) and incubated at room temperature for 10 min. The optical density value was read at 492 nm on a microplate reader. A standard curve was generated by using doubling dilutions within a range of 0 to 100 μM of sodium nitrite in culture medium. All the reagents, unless otherwise stated, were from Sigma. Samples were tested in triplicate, and the results are expressed as means ± standard deviation. The basal level of NO_2^- in cell-free medium was determined for each assay and deducted from the level in the test samples.

Assay for viable *C. ruminantium* EBs. The viability of *C. ruminantium* EBs was quantified by the fluorescein diacetate staining method. Fluorescein diacetate is taken in by all cells but is hydrolyzed within live cells to produce green fluorescence (28). The culture supernatants were centrifuged at low speed (1,000 × *g*) for 5 min to pellet the endothelial cells before staining. A 90-μl volume of medium from the test plates was mixed with 10 μl of diluted fluorescein diacetate (1:50 in PBS), and the mixture was incubated for 15 min at room temperature in the dark. The mean number of viable EBs was counted by fluorescence microscopy in 10 fields at a magnification of ×1,000, and viability was defined as the count expressed as a proportion of the total *Cowdria* count × 100%.

Assay for viable endothelial cells. The endothelial cell monolayer was examined microscopically daily for any cytopathic changes. On day 7 the medium from the cultures was removed and the cell monolayer on coverslips was stained in Diff Quik (Baxter Diagnostics) by the standard procedure described by the manufacturer, with acridine orange to identify and quantify viable and nonviable endothelial cells. For the acridine orange staining, a modification of a previously described method (25) was used. Briefly, the slides were fixed in 70% ethanol for 10 min, stained with acridine orange (3 μg/ml) for 3 h at room temperature, and viewed under a fluorescence microscope. Apoptotic cells were identified morphologically by condensation of chromatin, nuclear fragmentation, blebbing of the nuclear membrane, and presence of membrane-bound bodies (25). Nonviable cells were counted in five fields at a magnification of ×1,000 and expressed as a mean percentage ± standard deviation.

Assay for the effect of NO pretreatment of *C. ruminantium*. The effect of NO on *C. ruminantium* was assessed by infecting BPEC with EBs pretreated with different concentrations of the NO donor molecule *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) (Affiniti U.K.). *C. ruminantium* EBs were pretreated for 2 h at 37°C with 0, 25, 50, and 100 μM SNAP dissolved in culture medium. The NO level for each concentration of SNAP was determined by the Griess assay method described above. The viability count of the treated EBs was performed before infection by using the fluorescein diacetate staining method as described above. The EBs were then centrifuged at 15,000 × *g* for 20 min and washed in fresh medium before being resuspended in complete medium and used to infect confluent cell monolayers in 24-well culture plates containing coverslips prepared as described above. The plates were incubated at 37°C in 5% CO_2 for 4 h to allow infection of the cells, after which the inoculum was removed from all the wells and replaced with fresh medium. To assess the level and rate of infection, the number of fluorescent foci (colonies) per coverslip and the percentage of infected endothelial cells were determined on days 2 and 4 postinfection by the indirect fluorescent-antibody test and Giemsa staining of endothelial cells on coverslips, respectively. Briefly for the indirect fluorescent-antibody test, the coverslips were fixed in 20% acetone for 10 min. An anti-*Cowdria* 32-kDa monoclonal antibody at 1:200 in PBS was added to the cells on the coverslips and incubated in a humidified chamber at room temperature for 1 h. After three 5-min washes in PBS, the cells were incubated with a biotin-labelled anti-mouse antibody at a dilution of 1:250, incubated for 1 h at room temperature in a humidified chamber, and given another wash. A further 1-h incubation followed the addition of streptavidin-fluorescein isothiocyanate at a 1:300 dilution. The cells were viewed under a fluorescence microscope, and the number of fluorescent foci of infection on the total area of the coverslip was estimated. The infection rate in Giemsa-stained coverslips was assessed, and the number of infected cells per field was expressed as a percentage of the total cell count. Counts were made on four coverslips per treatment, and the mean value was recorded.

Assay for the effect of coculturing NO in *C. ruminantium*-infected cultures. The effect of incubating infected cultures with different concentrations of SNAP on viability and multiplication was assessed. BPEC were cultured on coverslips as described above and infected at confluence with *C. ruminantium* EBs in medium containing 0, 25, 50, and 100 μM SNAP, which was left in the cultures throughout the experiment. The viability of *C. ruminantium* EBs in these cultures was determined on days 1, 2, 4, and 7 postinfection. This was done by taking aliquots from four wells for each treatment, which were then centrifuged at low speed (1,000 × *g*) for 5 min to separate the endothelial cells, and the viability of EBs in the supernatant was determined by the fluorescein diacetate staining method as described above. To estimate the level of infection in cultures at the end of the experiment, cytospin smears were prepared on day 7 postinfection from each of these treatments and stained with Giemsa and a mean estimate of the number of organisms per field was assessed for each treatment. This was taken as a reflection of the multiplication rate of the infectious agent in these cultures.

Data analysis. Student's *t* test, the Mann-Whitney test, the chi-square test, and the correlation coefficient test were used for statistical data analysis as appropriate and depending on the distribution of the data, at 95% confidence intervals.

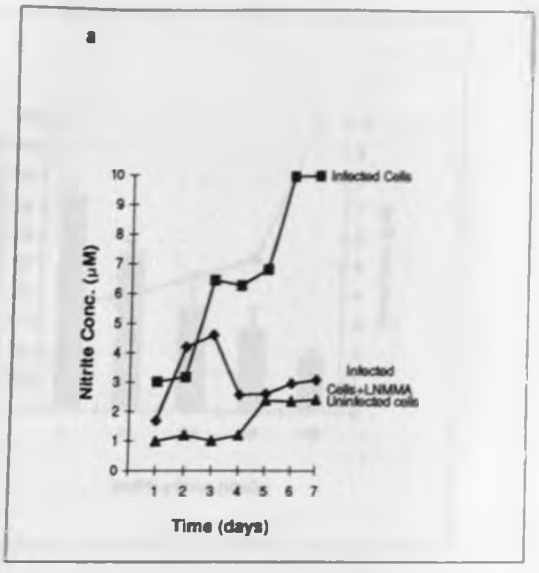


FIG. 1. NO production by *C. ruminantium*-infected BPEC. Nitrite levels indicative of NO were measured daily for 7 days in BPEC cultures infected with *C. ruminantium* in medium alone or supplemented with BorIFN-γ and/or L-NMMA. (a) A significant increase in NO levels was recorded in infected BPEC cultures incubated with medium alone (■) compared to uninfected cells (▲). A significant inhibition of NO production was recorded in cultures supplemented with L-NMMA (◆). (b) In infected BPEC cultures supplemented with BorIFN-γ, a more rapid increase in NO levels was recorded, with peak levels being attained 48 h after exposure (◆). NO production was significantly suppressed in cultures exposed to both BorIFN-γ and L-NMMA (■).

RESULTS

NO production by *C. ruminantium*-infected BPEC. NO levels measured at 24-h intervals revealed a gradual cumulative increase in the number of BPEC infected with *C. ruminantium* and incubated in medium alone (Fig. 1a). The highest levels were noted on day 7 postinfection. The highest NO concentration coincided with the period when the degree of infection was highest, as observed in cytospin preparations. Treatment of *C. ruminantium*-infected cultures with BorIFN-γ resulted in increased NO production, with peak levels being recorded at 2

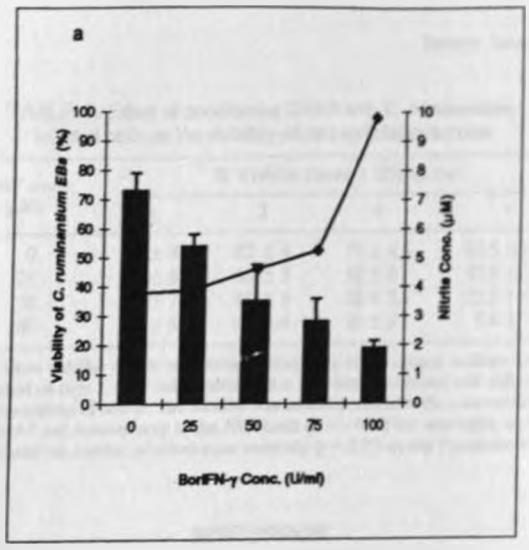
days posttreatment (Fig. 1b). In the cultures supplemented with the L-arginine analog L-NMMA, significant inhibition ($P < 0.05$) of NO production was recorded in the absence or presence of BorIFN-γ (Fig. 1).

Effect of BorIFN-γ on *C. ruminantium* viability. The proportion of viable EBs of *C. ruminantium* in cultures treated with different concentrations of BorIFN-γ was analyzed at the end of the experiment. The percentage of viable EBs was significantly lower ($P < 0.05$) in the presence of 100, 75, 50, and 25 U of BorIFN-γ per ml than in the unexposed cultures. The reduced viability was dose dependent, with a much lower viability being recorded at the higher concentrations of BorIFN-γ (Fig. 2a).

Effect of BorIFN-γ on endothelial-cell viability. It was noted that BorIFN-γ caused pronounced changes in the endothelial cells, with the effect being more evident in cells infected with *C. ruminantium*. Significant cellular changes were noted visually within 48 h after exposure to BorIFN-γ ($P < 0.05$) (Fig. 2b). These cytopathic changes increased in a dose-dependent fashion. The cells were observed to be rounding up and detaching from the culture plate when the cell monolayer was examined under dark-field microscopy. Apoptotic cell death was morphologically identified in acridine orange- and Diff Quik-stained cells. The cells revealed nuclear fragmentation, chromatin condensation, indiscernible nuclear organelles, blebbing of the nuclear membrane, and presence of dark-staining membrane-bound bodies. There was more evidence of apoptotic cell death in the infected BorIFN-γ-exposed cultures (75%) than in the uninfected BorIFN-γ-treated cultures (50%), whereas in the absence of BorIFN-γ, there was an increase of only 5% in the percentage of nonviable cells in infected (12%) compared to noninfected (7%) cultures.

Effect of pretreatment of *C. ruminantium* EBs with SNAP-generated NO on infectivity. Significant differences ($P < 0.05$) were noted in the number of *C. ruminantium* colonies and the infection rate of endothelial cells on days 2 and 4 postinfection in cultures infected with SNAP-pretreated EBs compared with cultures infected with nontreated EBs (Fig. 3). The number of fluorescing colonies in cultures which had been infected with SNAP-pretreated EBs was smaller than that in the nontreated controls. This reduction occurred in a dose-dependent manner, with smaller numbers of fluorescing colonies being recorded in cultures treated with higher concentrations of SNAP (Fig. 3a). Larger numbers of colonies were recorded on day 4 than on day 2 postinfection, indicating little or no effect of pretreatment of EBs on the subsequent multiplication rate of the viable organisms in the cells (Fig. 3a). The ratio of the number of fluorescing colonies on day 4 to that on day 2 had a range of 2 to 3.4 in all treatments, indicating that almost similar rates of multiplication had occurred after cell entry (Fig. 3a). The rate of infection, defined as the percentage of infected endothelial cells, was closely related to the number of fluorescing colonies, with a higher proportion of infected cells being recorded in cultures infected with EBs not treated with SNAP-generated NO and a higher proportion being found on day 4 compared to day 2 postinfection (Fig. 3b). The organisms were noticeable by day 2 as small, blue-staining, closely packed morulae in Giemsa-stained cells, and the morulae were noted to have increased in size and number from day 2 to day 4.

Effect of coculturing SNAP with *C. ruminantium*-infected cells on the rickettsial viability and growth. Viability counts were assessed on days 1, 2, 4, and 7 postinfection in all the cultures. In the cultures incubated in the presence of SNAP, significantly reduced viability counts of *C. ruminantium* EBs were recorded compared with those in untreated control cultures (Table 1). The viability was dose related, with the highest



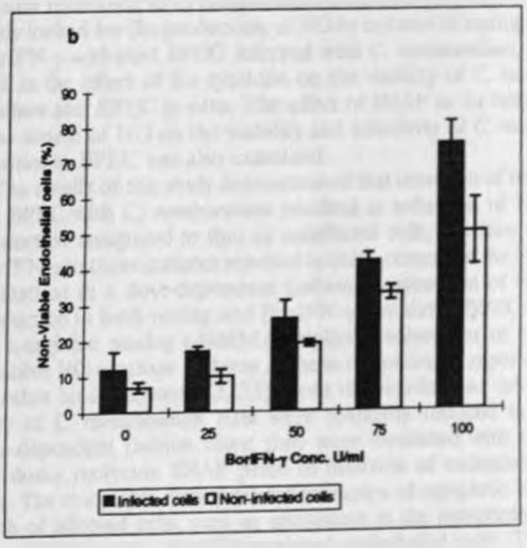
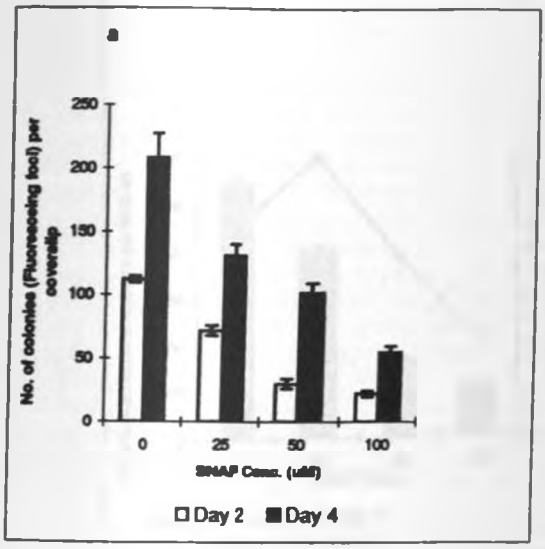


FIG. 2. Effect of BorIFN-γ on *C. ruminantium* and endothelial-cell viability. The proportion of viable *C. ruminantium* EBs in BPEC cultures exposed to different concentrations of BorIFN-γ was assessed on day 7 postinfection by a fluorescein diacetate staining method. (a) A significant reduction in viable EBs was recorded in cultures supplemented with BorIFN-γ, in a dose-dependent manner, with the decrease in viability corresponding to nitrite levels. The bars indicate the mean viability of EBs; lines indicate peak NO levels (at 48 h postinfection). (b) Addition of BorIFN-γ to BPEC cultures induced apoptotic cell death in a dose-dependent fashion. This was assessed morphologically on day 7 postinfection in acridine orange- and Diff Quik-stained cells, and the numbers of nonviable cells were recorded as a percentage. Increased cell death was recorded in infected cells compared to noninfected cells.

viability counts being recorded in the control cultures without SNAP and the lowest counts being recorded in cultures treated with 100 μM SNAP, with similar trends being recorded for all the days assessed (Table 1). Cytospin smears prepared on day 7 postinfection showed that the number of *C. ruminantium* colonies per field was negatively correlated with the level of SNAP ($r = -0.96$) but was more or less a reflection of the viability counts, with a very high correlation coefficient ($r = 0.97$) (Fig. 4a). The concentration of NO in the culture me-



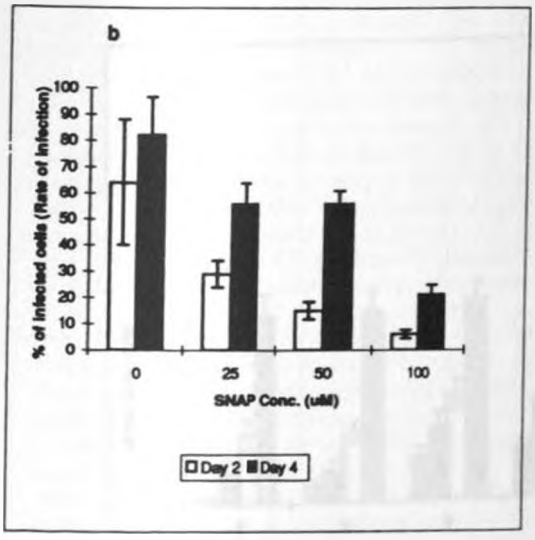


FIG. 3. Effect of pretreatment with SNAP-generated NO on infectivity of *C. ruminantium* EBs. (a) The mean number of fluorescing colonies per coverslip was assessed in cultures infected with SNAP-pretreated EBs on days 2 and 4 postinfection on stained indirect fluorescent-antibody test coverslips. This number, which was a reflection of the infectivity of the pretreated EBs, was found to be inversely related to the SNAP concentration. The number was approximately doubled from day 2 to day 4 irrespective of the SNAP concentration used to treat the EBs, indicating similar multiplication rates of the organism once entry into endothelial cell was gained. (b) The rate of infection (percentage of infected cells) was assessed on days 2 and 4 postinfection in Giemsa-stained cultures infected with SNAP-pretreated EBs. There was an increased rate of infection between days 2 and 4 postinfection in both cultures infected with SNAP-pretreated EBs and the controls.

dium did not decline during the experiment but, rather, increased during the period of infection (Fig. 4b). Although the SNAP-containing cultures had higher cumulative final nitrite levels as a result of the NO released by this molecule, the rate of increase in the nitrite level during the period of infection was higher in heavily infected cultures, i.e., those which had been incubated without SNAP or with low levels of SNAP, than in lightly infected ones (Fig. 4a).

TABLE 1. Effect of coculturing SNAP with *C. ruminantium*-infected cells on the viability of extracellular rickettsiae

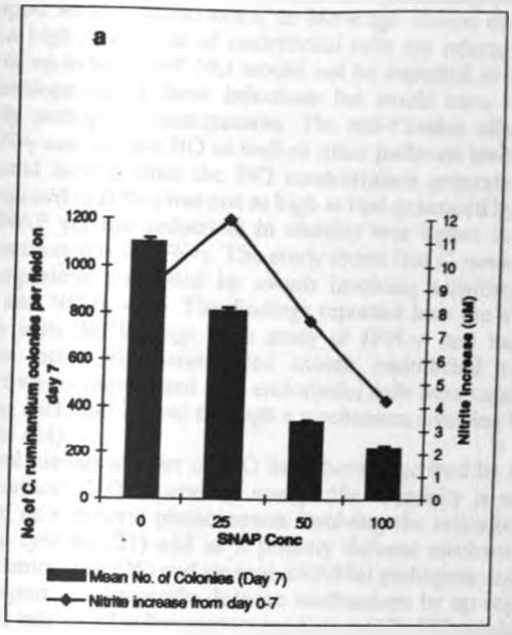
SNAP concn (μM)	% Viability (mean ± SD) on day:			
	1	2	4	7
0	90 ± 8	82 ± 4	75 ± 4.5	82.5 ± 14
25	79 ± 3	68 ± 5	52 ± 4	57.9 ± 7
50	56 ± 7	45 ± 3	28 ± 2.5	25.5 ± 4.2
100	38 ± 5	37 ± 4	20 ± 3	5.4 ± 1

[a] Mean viability counts of *C. ruminantium* EBs in the culture medium were assessed on days 1, 2, 4, and 7 postinfection in cultures incubated with different concentrations of SNAP. The viability was inversely related to the concentration of SNAP and consequently to the NO levels ($r = -0.96$) but was highly correlated with the number of colonies per coverslip ($r = 0.97$) on day 7 postinfection.

DISCUSSION

Previous observations have indicated strongly that the IFN system, and probably other cytokines as well, plays a key role in natural resistance to *C. ruminantium* infections (18, 30). This study looked for the production of NO by cultures of resting or BorIFN-γ-activated BPEC infected with *C. ruminantium*, as well as the effect of the cytokine on the viability of *C. ruminantium* and BPEC in vitro. The effect of SNAP as an exogenous source of NO on the viability and infectivity of *C. ruminantium* to BPEC was also examined.

The results of this study demonstrated that infection of resting BPEC with *C. ruminantium* resulted in induction of NO production compared to that in uninfected cells. Addition of BorIFN-γ to these cultures resulted in enhancement of the NO production in a dose-dependent fashion. Suppression of NO production in both resting and BorIFN-γ-stimulated BPEC by the L-arginine analog L-NMMA implied involvement of the inducible NO synthase pathway in these responses, as reported for other bovine systems (3, 33). Both the viability and infectivity of *C. ruminantium* EBs were markedly reduced in a dose-dependent fashion when they were incubated with the NO donor molecule SNAP prior to infection of endothelial cells. The study also demonstrated induction of apoptotic cell death of infected cells, seen as alterations in the morphology and viability of the BorIFN-γ-treated endothelial cells. The decreased viability of the infectious agent may have been associated with the apoptosis of endothelial cells in BorIFN-γ-treated cultures or may have resulted from other effects including increased NO production. However, the maximal NO level induced in cultures exposed to 100 U of BorIFN-γ per ml was 10 μM (Fig. 2a), which was much lower than that recorded in 100 μM SNAP in culture medium (35 μM) (Fig. 4b), yet the rickettsial viability was lower in the BorIFN-γ-treated cultures than in the SNAP-treated cultures (Fig. 2a and 4a). The inhibition of *Cowdria* growth by IFN-γ in concanavalin A-stimulated T-cell supernatants without detectable increases in NO production has been reported (18). However, the activity of IFN-γ was detected by neutralization and may have been lower than that used in this study. Our findings were consistent with the up-regulation of NO generation with bovine IFN-γ reported for bovine macrophages (15, 33). Tumor necrosis factor alpha has also been reported to induce NO synthesis in bovine endothelial cells (32), in contrast to bovine macrophages (15). NO synthesis in bovine macrophages is suppressed by interleukin-4 but unchanged by interleukin-10 or transforming growth factor β (15), whereas the latter is reported to increase NO production in bovine aortic endothelial cells (13). Regulation of NO synthesis would therefore appear to differ between bovine endothelial cells and macrophages, but the up-



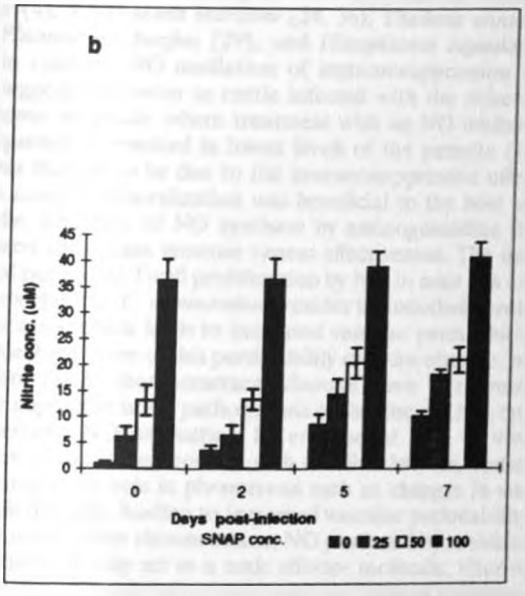


FIG. 4. Effect of coculturing SNAP with *C. ruminantium*-infected cells on rickettsial viability and growth. (a) Cytospin smears were prepared on day 7 postinfection for each treatment, and the mean number of *C. ruminantium* organisms per field was estimated. The count was dose related, with significantly lower counts recorded for 100 and 50 μM SNAP than for 25 μM SNAP and the control. (b) Nitrite levels were measured on days 0, 2, 5, and 7 postinfection for each treatment in cultures in which SNAP was retained throughout the experiment. Nitrite levels increased more in cultures without SNAP than in those containing SNAP, indicating induction of NO production by the infection. The infectious yield in these cultures, a reflection of its growth, was positively related to the increase of nitrite levels, indicating induction of NO production by the organism.

regulation by a Th1 cytokine (IFN-γ) in both cell types is of interest. Apoptosis in infected-cell cultures occurred at a higher rate than in uninfected cells in response to BorIFN-γ. It is possible this was associated with NO synthesis, since apoptosis in tumor cells by bovine endothelium-derived NO has been reported (32). However, cytotoxicity of IFN-γ to cells has been reported to occur on exposure to the cytokine alone or in combination with a second signal, including intracellular infectious agents (12, 20). We are suggesting here that the killing of *C. ruminantium* in the BorIFN-γ-treated cultures was also associated with the apoptotic event. Similar findings were reported by Molloy et al. (20) on the viability of intracellular *Mycobacterium bovis* in monocytes in vitro. They reported that toxic mediators that killed the monocytes by necrosis had no effect on the viability of the organism while those that killed the cells by apoptosis reduced the viability of the organism by 60 to 70%. It has been suggested that since apoptosis is accompanied by nuclear fragmentation of the cells, the integrity of the genetic material of the intracellular organism may be compromised, leading to its killing (20).

The reduced infectivity of *C. ruminantium* in cultures maintained in medium containing SNAP was attributed mainly to the effects of NO upon the organism prior to infection, since the incubation of EBs with SNAP prior to infection reduced the infectivity in a dose-dependent manner. However, the infection increased at a similar rate irrespective of prior SNAP treatment after first appearance of infected endothelial cells on day 2 (Fig. 3). There was no apparent reduction of infection as a result of maintaining the cultures in SNAP; the ratio of the number of organisms per field in the control cultures to that in the cultures maintained in medium containing SNAP (Fig. 4a) was not significantly different from that obtained when EBs were treated with the same levels of SNAP prior to infection (Fig. 3a). Since the EBs were allowed to infect cell cultures in the presence of SNAP, the dose-dependent reduction of infection in SNAP-maintained cultures can be attributed to the inactivation during infection of the cultures, with no significant increase in the effect of the SNAP during the 7-day period of experiment. Indeed, the high correlation ($r = 0.97$) between viability counts of EBs remaining in the medium on day 1 and the level of subsequent *Cowdria* infection in these cultures suggests that the principal effect of SNAP was upon the infectivity of the extracellular organisms. Release of fresh EBs into culture medium, from day 6, is expected from the approximately 6-day cycle of *C. ruminantium* infection in vitro and may explain the increased viability of extracellular EBs on day 7 in cultures maintained in low concentrations of SNAP (25 μM or below [Table 1]). Therefore, SNAP treatment reduced initial infection rates in the cells, but the level of infection increased at much the same rate irrespective of initial SNAP concentration in culture medium.

The induction of inducible NO synthase by IFN-γ may contribute to the anti-*Cowdria* activity manifested in IFN-treated cultures of *C. ruminantium*-infected BPEC. The NO level induced by infection of BPEC without any treatment was approximately 10 μM on day 7, with similar levels being recorded after 48 h in cultures treated with 100 U of IFN-γ per ml. This level of NO is intermediate between the 5 and 13 μM produced by 25 and 50 μM SNAP, respectively. Since the last two treatments significantly reduced the infection level, it can be concluded that the level of NO released in culture by infection alone or by IFN-γ induction may have an effect on the infectivity of released EBs and may thereby indicate a role of NO in the protective responses to heartwater infection. This study reports for the first time that NO is capable of reducing both the viability and infectivity of *C. ruminantium* in BPEC in vitro indicating that NO released by endothelial cells or by other cells such as monocytes may play a role in reducing the infectivity of extracellular EBs and thereby in reducing the spread of infection. If release of NO during endothelial cell infectio

occurs in vivo, this can be expected to reduce the viability of the agent for other endothelial cells and potentially for the arthropod vector; furthermore, in late-stage clinical disease, when a high proportion of endothelial cells are infected, release of endothelial-cell NO would not be expected to affect the development of these infections but would have other, possibly pathogenic consequences. The anti-*Cowdria* effect of BorIFN-γ may involve NO as well as other pathways involving additional factors, since the NO concentration generated by cells exposed to IFN-γ was not as high as that generated by 100 μM SNAP yet the reduction in viability was higher in the cultures treated with IFN-γ. The study shows that *C. ruminantium* organisms are killed by events involving activities on IFN-γ and NO in vitro. The findings reported here are comparable with the findings of a study of IFN-γ- and tumor necrosis factor alpha-stimulated mouse endothelial cells, where it was demonstrated that endothelial cells were capable of killing *Rickettsia conorii* through a mechanism involving NO synthesis (34).

Several biological roles of NO have been described by different authors. NO-dependent nonspecific immunity is now accepted as a general phenomenon involving the reticuloendothelial systems (21) and as a primary defense mechanism against tumor cells (35) and several microbial pathogens and a potent agent in nonspecific defense mechanisms by up-regulating the release of inflammatory mediators (17). NO production by macrophages and monocytes during the response to infection has been correlated with resistance to a wide variety of pathogens in vivo, including *M. bovis* (20), *Listeria monocytogenes* (4), *Schistosoma mansoni* (24, 36), *Theileria annulata* (33), *Plasmodium berghei* (29), and *Histoplasma capsulatum* (16). In contrast, NO mediation of immunosuppression has been suggested to occur in cattle infected with the rickettsia *Anaplasma marginale*, where treatment with an NO inhibitor, aminoguanidine, resulted in lower levels of the parasite (11). This was thought to be due to the immunosuppressive effects of NO, since its neutralization was beneficial to the host and since the inhibition of NO synthase by aminoguanidine was postulated to increase immune system effectiveness. The inhibition of peripheral T-cell proliferation by NO in mice has also been reported (5). *C. ruminantium* resides in endothelial cells, an association which leads to increased vascular permeability. The underlying cause of this permeability remains obscure, but mediators rather than structural changes have been more strongly implicated in the pathogenesis of the disease. Not only may excessive NO production by endothelial cells in vivo, which are in continuous contact with the bloodstream, constitute an important role in phenomena such as changes in vascular wall integrity, leading to increased vascular permeability, but also, under other circumstances, NO produced by activated endothelial cells may act as a toxic effector molecule, altering the physiology of endothelial cells, inhibiting various metabolic functions of cells, and leading to death of pathogens such as the schistomula of *Schistosoma mansoni* (24). NO also precipitates the loss of intracellular iron, resulting in inhibition of certain of the vital enzymes involved in mitochondrial respiration (6). This finding suggests that NO may contribute to the mitochondrial changes observed in the alveolar endothelial cells infected with *C. ruminantium* (26).

This study has shown that NO produced endogenously or from exogenous sources kills *C. ruminantium* in a dose-dependent fashion. Although lack of knowledge limits discussion of the potential role of NO in *C. ruminantium* pathogenesis and immunity, these findings point to a role for NO in resistance to *C. ruminantium* infections and in the pathology of the disease. Toxic mediators have been suggested to be responsible for some unexplained pathology of the disease, including increased vascular permeability leading to edema and also to vascular collapse in terminal cases of the disease, consistent with effects of NO production upon the vascular system. In a recent study (unpublished data), we noted that infection of previously exposed African breeds of sheep resulted in higher levels of nitrite in plasma during the clinical response. The induction of NO synthesis by IFN-γ may be an important determinant of the outcome of *C. ruminantium* infection. This is supported by the following sequence of events: rickettsemia occurs 3 to 6 days before the onset of fever and pathology, but terminal collapse, reduced blood pressure, and peak pulmonary edema occur only a few days after the first immune responses are detectable and when rickettsemia has declined but endothelial-cell infection is highest (unpublished data). On the basis of our results and those cited previously, it is becoming clear that NO may be an important component of the immune response to intracellular infections and the pathophysiology of heartwater infection.

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Interferons gamma and alpha/beta are involved in the pathological and immune response mechanisms of *Cowdria ruminantium* infection in mice.

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It has been suggested that T cells play an important role in the immunity against *Cowdria ruminantium* infections, a phenomenon mediated through the production of inhibitory cytokines. In a previous study we demonstrated the inhibitory effect of interferon gamma (IFN-γ) on *C ruminantium in vitro*. Previous studies also demonstrated that animals recovering from *C ruminantium* infection contained higher blood levels of interferon alpha (IFN-α) as compared to those that succumbed to infection. Armed with these findings, this study was designed to investigate the role played by IFN-γ, IFN-α and interferon beta (IFN-β) in the pathogenesis and immune responses of *C ruminantium* infection. A murine model comprised of three groups of specially bred mice, IFN-γ, IFN-α/β knock-out mice (mice without receptors for IFN-γ and IFN-α/β respectively) and a control group was used. Short incubation period and course of disease were recorded in the IFN-γ and IFN-α/β knock-out mice, with more animals dying in the early stages of the disease. The pathological findings were a reflection of the clinical picture. Grossly, the IFN-γ and IFN-α/β knock-out mice manifested a more extensive thoracic oedema than the control group, a finding possibly linked to higher infection rate in the lungs of the IFN-γ and IFN-α/β knock-out mice as seen in histopathological lung sections. Dot blot hybridization of DNA extracts of lung tissue using digoxigenin-labelled GroEl probe revealed further support for a higher rate of infection in the IFN-γ knock-out, and to a lesser extend, the IFN-α/β knock-out groups as compared to the control group.

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Nitric oxide production in *Cowdria ruminantium*-infected bovine endothelial cells *in vitro*: effect of interferon-γ on the nitric oxide production, the organism and cell viability

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Cowdria ruminantium, the aetiological agent of heartwater, infects and multiplies in the cytoplasm of endothelial cells (EC) (COWDRY, E. V., 1925: *11th–12th Report of the Veterinary Research Union in South Africa*, 161–171). EC generates a powerful vasodilator, nitric oxide (NO), which plays a central role in oedema formation, hypertension and blood pressure (MONCADA, S. *et al.*, 1991, *Hypertension*, 12, 365–372). The objective of this study was to investigate the role of the EC and related mediators in the pathological mechanisms of heartwater.

There was a significant increase in NO levels in infected bovine EC cultures. Exposure of infected cultures to bovine recombinant interferon-γ (BorIFNγ) resulted in more rapid attainment of maximal levels of NO. Morphological evidence of EC apoptosis and decreased viability of the organism were recorded in infected cultures exposed to BorIFNγ. Addition of the NO synthesis inhibitor L-monomethyl-L-arginine (L-NMMA) resulted in suppression of NO production, indicating involvement of the inducible nitric oxide synthase pathway.

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Nitric Oxide And *Cowdria ruminantium* Infection Of Bovine Pulmonary Endothelial Cells *In Vitro*

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Cowdria ruminantium infection of bovine pulmonary endothelial cells resulted in substantial elevation of nitric oxide. Exposure of the infected cultures to bovine recombinant interferon-gamma resulted in more rapid nitric oxide production, reduced viability of the infectious agent and induction of endothelial cell apoptosis in a dose-dependent fashion. Addition of the nitric oxide synthesis inhibitor N-monomethyl-L-arginine resulted in a significant inhibition of nitric oxide production indicating that the increase in production utilises the inducible nitric oxide synthase pathway. Co-cultivation of infected endothelial cells with the nitric oxide donor molecule S -nitroso-N-acetyl DL-penicillamine decreased viability of the infectious agent in a time and dose-dependent fashion.