A STUDY OF CELL-MEDIATED IMMUNITY IN SUBJECTS VACCINATED AGAINST Q FEVER AND AFTER Q FEVER INFECTION

ANGELO ANTONIO IZZO

B.Sc (Hons)

Division of Medical Virology
Institute of Medical and Veterinary Science
Adelaide
South Australia

Department of Microbiology and Immunology
Department of Pathology
University of Adelaide
Adelaide
South Australia

A Thesis submitted to the University of Adelaide in fulfillment of the requirement for the degree of Doctor of Philosophy

August
1991

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University.

To the best of my knowledge and belief, this thesis contains no material previously published or experimental results obtained by another person, except where due reference is made in the text of the thesis.

In accordance with the University of Adelaide regulations, I give my consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

ANGELO A IZZO

ACKNOWLEDGEMENTS

I wish to express my immense gratitude to Professor B.P. Marmion for his help and guidance, and in particular for his editorial skills in the preparation of this thesis. Thanks also go to Dr Graham Mayrhofer (Department of Microbiology and Immunology), my other supervisor, for his professional guidance, and to Mr David Worswick for advice and serological expertise.

I would like to take this opportunity to formally thank Professors C.J. Burrell and B. Vernon-Roberts for allowing me to use the facilities of the Division of Medical Virology (IMVS) and the Department of Pathology (University of Adelaide).

I am grateful to Dr T. Hackstadt (NIAID, Rocky Mountain Laboratory, Hamilton, U.S.A.) for supplying me with the necessary antigens for my experiments. Also to Medical Staff of the abattoirs, with special thanks to Sister P. Mitchell and the staff at the South Australian Meat Corporation for providing me with specimens, and to the innumerable people who assisted me in so many ways.

My grateful thanks go to Mrs J. Maylin and Mrs D. Campbell for their onerous and often time-consuming task of typing the many drafts and the finished product.

Finally, I would like to thank my parents for their encouragement and support in my studies, and my wife Judy, to whom I dedicate this thesis, for her inspiration, support and patience during this period of my life.

LIST OF CONTENTS

PREFACE

The causative organism of Q fever, originally named *Rickettsia burnetii* and now known as *Coxiella burnetii*, was isolated, quite independently and at about the same time (mid 1930's), by Derrick and colleagues from patients with "abattoir fever" in Brisbane, Queensland, Australia, and by Cox and colleagues from ticks (*Dermacentor andersoni*) collected at Nine Mile Creek, Hamilton, Montana by staff from the nearby Rocky Mountain laboratory - a well established centre of excellence for rickettsial studies originally set up to investigate Rocky Mountain Spotted Fever.

Work in the 5 or so years after the initial isolation of the Q fever organism in these two widely separated parts of the world established that the Australian and American strains of *C.burnetii* were closely similar, both biologically and antigenically, and that both caused a severe fever and sometimes pneumonitis and hepatitis in man. Also that the organism was widely distributed in ticks and small bush animals in Queensland (see Historical and Epidemiological sections).

Sporadic cases and small outbreaks of Q fever in American laboratories handling the Q fever organism and outbreaks of Q fever in abattoirs and meat processing plants in Chicago and Texas illustrated the similarity of the epidemiology of Q fever in America and in Australia.

Extensive outbreaks of Q fever among Allied and Axis troops during the

Mediterranean campaigns of the 1940's then revealed that Q fever was not limited to the USA and Australia. Studies after the war, and particularly during the 1950's in North and South California, showed that in addition to reservoirs of Q fever infection in arthropods and small bush animals identified earlier, domestic animals such as cattle, sheep and goats were also infected and that the organism could be transmitted among them by aerosol or by ingestion, independently of arthropod vectors.

The distribution of Q fever in now recognised to be essentially world-wide, although its prevalence varies considerably from country to country depending partly on the patterns of animal husbandry, the size of the population and the mix of domestic animals and the level of awareness and interest in the disease among physicians and public health workers.

In Australia Q fever, as an acute disease and because of certain chronic sequelae, is an important cause of morbidity among meatworkers and in the agricultural industry in general; the consequent expense to the industry and to Health Services is substantial. Studies of its clinical complications, pathogenesis, immunopathology, and particularly of its prevention by vaccination are therefore highly appropriate in the Australian environment.

In addition to these practical considerations, *C.burnetii* is a highly successful intracellular parasite, able to grow in macrophages, to modulate the immune response of the host, and to establish chronic infections. It therefore merits study in its own right and for the light that understanding of its interaction

with the host may throw on pathogenetic mechanisms with *Mycobacteria*, *Listeria*, *Legionella* spp. and other intracellular pathogens.

CHAPTER 1

INTRODUCTION AND BACKGROUND TO Q FEVER

In view of the orientation of this thesis, greater attention has been given to the biology of the organism, particularly its antigenic composition, and to the pathogenesis of infection and the immune response of the host, than to the epidemiological and ecological aspects of Q fever.

1.1 Historical aspects of Q fever

It is possible to argue that studies of the Q fever organism - as distinct from the disease - began in America in 1926 when Noguchi (1926), while examining *Dermacentor andersoni*, the wood tick involved in the transmission of Rocky Mountain Spotted Fever (*Rickettsia rickettsii*), discovered a "filter-passing virus" transmissible to guinea pigs but not cultivable in cell-free bacteriological media. The organism produced a febrile reaction similar to that of spotted fever in guinea pigs but convalescent animals were not protected when challenged with *R.rickettsii*.

While these observations served to establish Noguchi's agent as different from *R.rickettsii*, they do not throw any light on the nature of the organism he had isolated or establish its identity - or otherwise - with *R.diaporica* or *R.burnetii* (see below).

Nevertheless, an initially similar set of observations on a "filter-passing virus" was made a decade later by Davis and Cox (1938) when they isolated an agent from *D.andersoni* collected at Nine Mile Creek, Montana. The tick

suspensions were inoculated into guinea pigs and produced fever. Suspensions of spleen tissue from infected animals produced fever in nonimmune guinea pigs and also a marked inflammatory thickening of the skin on subcutaneous or intradermal injection. They also found that the organism retained infectivity after passing through a Berkefeld filter - unusual behaviour for a rickettsia.

Cox (1939, 1940) in further studies examined inflammatory exudates (peritoneal, perisplenic or perihepatitic) from guinea pigs taken during fever and found numerous minute extracellular and intracellular, pleomorphic, coccobacillary organisms, which suggested that the infectious agent was a rickettsia rather than a virus. The organism was also found as cytoplasmic microcolonies in infected spleen cells; the microcolonies were sometimes large enough to push the nucleus to the edge of the cell cytoplasm. The organism grew in modified Maitland-type tissue cultures consisting of minced chick embryonic tissues in human ascitic fluid. Cox (1939) called the organism *Rickettsia diaporica* because its infectivity was retained on passage through Berkefeld filters. It did not grow in cell-free media, but remained viable over long periods of time. *R.diaporica*, Cox found, grew more readily in yolk sac fragments than in other tissues of developing chick-embryo (Cox & Bell 1939). This observation led him to use the intact chick embryo yolk sac for the growth of large numbers of rickettsiae for antigen and vaccine production (Cox 1940).

The significance of *R.diaporica* for human infection was uncertain at this stage although observations on clinical Q fever in Australia were to come together with Cox's observations after some laboratory infections in the latter's

laboratory (see below).

In Australia, at about the same time (1934-1936) as Cox's studies, but quite independently, E.H. Derrick began to investigate an unusual febrile disease in abattoir workers in a large metropolitan abattoir in Brisbane, Queensland, Australia. Derrick (1937) described the principal symptoms and clinical features associated with the abattoir fever, viz: severe headache, rigors, sweats, photophobia and conjunctival congestion, low pulse rate, and a maintained or remittent fever. Derrick inoculated guinea pigs with blood or urine from febrile patients and produced a characteristic fever in the animals about 7-10 days after inoculation. After isolation from a patient the agent could be transmitted serially in guinea pigs with blood taken at the height of fever or with suspensions of liver or spleen. Once an animal had recovered from the fever, challenge with the original inoculum, or with inocula from strains isolated from other (similar) patients, or from passage lines of the agent maintained in guinea pigs, showed that the convalescent animals were immune to reinfection and did not develop a fever. This provided a specific, if very laborious method of identifying new isolates of the agent from what Derrick now termed Q (for query) fever. It also allowed cross-comparison of the new agent with strains of endemic (murine) typhus and other rickettsiae and eventually also constituted a method for testing vaccine efficacy.

Derrick was unable to see an organism in stained smears and sections of the tissues or inflammatory exudates from Q fever-infected guinea pigs. (In retrospect, he seems to have been rather unfortunate in that the Australian isolates of *C.burnetii* were less virulent for guinea pigs than the Nine Mile

strain studied by Cox who had detected rickettsias in organ smears without difficulty). Given the absence of organisms on microscopy, and negative cultures on bacteriological media, Derrick concluded that a virus might be present, and so sent infected guinea pig liver to Macfarlane Burnet at the Walter and Eliza Hall Institute in Melbourne. Burnet and Freeman (1937), Burnet (1938) and Burnet and Freeman (1938 a&b) inoculated the material sent by Derrick into guinea pigs, mice, rats, monkeys, onto the chorioallantoic membrane (CA) of developing chick embryos and into tissue cultures. Their mouse inoculations provided a significant advance. The mice developed enlarged livers and spleens. Histological sections and impression smears from these organs showed cells (probably macrophages) with cytoplasmic microcolonies or inclusions of organisms typical of rickettsias - namely, tiny rods less than 1.0mu in length and about 0.3mu in width - as short rods or coccoid forms.

The abundant growth of the rickettsia in the mouse spleen allowed the preparation of semipurified suspensions for seroagglutination tests (Burnet and Freeman 1938a) and the titration of antibody in human sera, those from immunised laboratory animals, and the detection of antibody in animals captured in the bush.

Efforts were made by the workers in Brisbane and Melbourne to classify the new rickettsia and establish biological or antigenic similarities with other rickettsias (Burnet *et al.* 1939). No antigenic cross-reactions were found with the rickettsias of endemic typhus, scrub typhus, tick-bite fever, Rocky Mountain spotted fever, Heartwater (ovine), tick-borne fever (ovine), Bovine

Rickettsiosis and Rickettsial infection of hares. Other investigations established that sera from human Q fever cases or animals did not agglutinate Proteus X strains.

After these initial clinical definitions of Q fever, the isolation of the organism and its characterisation as a rickettsia, Derrick (1939) proposed that the organism be called *Rickettsia burnetii*. Some years later, in 1948, when the differences between *R.burnetii* and other rickettsiae became more apparent (e.g., cell morphology, variable gram staining, maintenance cycles in animals not dependent on arthropods, filterability and heat resistance, lack of cross reactions with *Proteus* spp. and failure to stimulate Weil-Felix agglutinins), Cornelius B. Philip proposed that the organism be placed in a separate genus to other rickettsias. This genus he named Coxiella in honour of Herald Cox's contributions with *R.diaporica*. The type species is *Coxiella burnetii* (Derrick). The separation of *C.burnetii* as a genus was later substantiated by differences in genome size, G + C ratios and lack of nucleotide sequence homology with the typhus, spotted fever or other groups of the rickettsias.

However, Derrick's early searches for other rickettsias related to the Q fever organism were not entirely fruitless as a relationship was found with the organism isolated by Cox and Davis from ticks at Nine Mile Creek.

The convergence of the Australian experience with clinical Q fever, the isolation of the organism from patients and its identification as a rickettsia finally meshed with the American experience with *R.diaporica* as a result of a laboratory infection with the latter organism.

Dr Rolla Dyer from the central National Institutes of Health (NIH) at

Bethesda Md. visited Dr Cox's laboratory in NIH's Rocky Mountain Laboratory at Hamilton, Montana to see the technique of growing large numbers of rickettsias in the chick embryo yolk sac just developed by Cox. The organism under yolk sac passage was the Nine Mile strain of *R.diaporica*. After his visit, Dyer came down with an acute fever which he recognised as resembling that of Australian Q fever (Dyer 1938,1939). Blood samples taken during the illness were inoculated into guinea pigs and a rickettsia isolated. Cross-challenge experiments in guinea pigs using an Australian Q fever strain in the form of infected mouse spleen provided by Burnet showed that it and the Dyer strain of *C.burnetii* (as it then became), were antigenically similar or identical.

Comparisons of the Dyer isolate and the Australian Q fever strains continued in both Australia and the USA. Despite similarities, there were several differences between the two strains. In the USA, cross challenge experiments between the "Q" fever strain from Australia and the Dyer strain provided consistent evidence for the identity of protective immunogen(s) of the two organisms; convalescent sera from Australian and American infections agglutinated suspensions of *R.burnetii*. However, in other aspects, including animal susceptibility, fever and mortality in guinea pigs, the Nine Mile (Dyer) strain appeared to be more virulent than those from Australia.

In Australia, Burnet and Freeman found the Dyer strain to be of greater pathogenicity in monkeys and guinea pigs than the local "Q" fever strains.

Burnet and Freeman also inoculated the Dyer strain onto chorioallantoic membrane (CA) of chick embryos (Burnet & Freeman 1941a).

It produced well-marked focal lesions, which contrasted with the local strains which multiplied only slightly and failed to produce any lesions.

A historically interesting feature of these experiments in relation to the subject of this thesis is the finding that antisera from human beings, bandicoots, rabbits or guinea pigs did not prevent the formation of foci caused by the Q fever rickettsia on the chorioallantoic membrane.

This lack of neutralising activity on the CA membrane was in marked contrast to the neutralisation of typical viruses such as those of the pox group (e.g., vaccinia, ectromelia) or influenza virus adapted to the CA membrane. On the other hand, when suspensions of the Q fever rickettsia were mixed with antisera and inoculated into guinea pig or mice, disease-modifying effects were obtained. In guinea pigs this took the form of suppression of the febrile response. In mice multiplication of the rickettsia in the spleen was reduced with a concomitant decrease in spleen size and weight. In each instance the animals were resistant to reinfection on subsequent challenge. Despite modification of the disease, infection was not completely prevented (i.e., the organism persisted in the animals at low levels), so that 'protection' rather than 'neutralisation' is a more appropriate description of the phenomenon.

These early observations mark the beginning of an understanding of the nature of immunity in Q fever and the development of techniques for its investigation some of which are still in use today.

In essence, Burnet and colleagues had shown that antibody would modify infection in animals with an intact immune system whereas in an animal - chick embryo - without an immune system, 'protection' or

'neutralisation' did not take place. The interaction between antibody and cell-mediated immunity involved in resistance to *C.burnetii* was subsequently explored in depth by other workers and is detailed in later chapters.

CHAPTER 2

ECOLOGY OF Q FEVER AND ITS TRANSMISSION TO MAN

2.1 Reservoirs of Q fever infection

The widespread distribution of Q fever, involving most if not all countries in the world has been well documented (Berge & Lennette 1953a, Derrick 1953, Marmion 1953, Stoker and Marmion 1955, Kaplan and Bertagna 1955, Babudieri 1959). The widespread prevalence of Q fever has been attributed to the ability of the organism to combine an obligate intracellular mode of existence with broad tissue trophisms. It grows, for example, equally well in the intestine of a tick, the reproductive tract of a cow, or the respiratory tract of man. To these attributes for survival may be added a resistance to heat, drying and sunlight when passing between hosts, which would be remarkable in a vegetative bacterium and which has led to much interest in morphological spore forms found in some cultures of *C.burnetii* as a possible explanation for its hardiness.

2.2 Maintenance of Q fever in the wild: *C.burnetii* in arthropods and feral animals

In the wake of the isolation of the Q fever organism in Queensland, and its identification as a rickettsia, Derrick and colleagues, probably influenced by analogies with typhus and other rickettsias, extended their observations on the hypothesis that the source of human infection was probably a natural maintenance cycle of the organism in small bush animals and their arthropod

ectoparasites (Derrick *et al.* 1939, Derrick and Smith 1940, Derrick *et al.* 1940). These studies uncovered serological evidence of infection with *C.burnetii* in various species of bush rats and the bandicoot (*Isoodon torosus* and other species). Strains of *C.burnetii* were isolated from bandicoots as well as from their tick, *Haemaphysalis humerosa*, but not from other ectoparasites (Smith and Derrick 1940, Derrick and Smith 1940). Laboratory experiments showed that *C.burnetii* would survive through each instar of *H.humerosa* and suggestive evidence of transovarian passage was also obtained (Smith 1940a).

C.burnetii was also isolated from the bush tick, *Ixodes holocyclus*, a vector of Australian (North Queensland) tick typhus in man, and it was shown that it could be infected from bandicoots carrying *C.burnetii* (Smith 1942).

It was proposed that *C.burnetii* was probably indigenous to the Queensland bush, with its primary cycle occurring in several species of ticks and their bandicoot and other small bush animal hosts.

Ticks taken from Australian cattle were not found to be infected. Thus, the postulated link - e.g., by inhalation of tick faeces on the hides of cattle (Derrick *et al.* 1942) - between infected ticks, cattle and Q fever in abattoir workers was not supported.

It also appeared that the tick-bandicoot cycle, although probably a reality in terms of the maintenance of the organism in the wild, was not of great importance for infection of man. Bandicoots and ticks on Moreton Island, off the coast near Brisbane, had a high rate of infection but military personnel exercising in the area did not become infected with Q fever. The tick-bandicoot cycle constitutes a model for maintenance of the organism in

other feral animals and their ectoparasites (reviewed Stoker and Marmion 1955, Marchette 1982, Lang 1990).

Berge (1959), in reviewing the pattern of Q fever world wide, concluded that ticks are not significant for human infection.

Reviews by Stoker and Marmion (1955), Babudieri (1959) and Marchette (1982) conclude that over 50 species of tick have been shown to be infected in nature; 17 could be infected experimentally. Transovarial transmission has been shown in 15. Persistence of the Coxiella in ticks may be prolonged, thus Davis (1943) showed that the Nine Mile strain of *C.burnetii* survived in *Ornithodorus moubata* and *O.hermsi* for 670 and 979 days respectively.

2.3 ***Coxiella burnetii* in wild animals and birds worldwide**

Many species of small wild mammals and rodents have evidence of infection with *C.burnetii* (Babudieri 1959, Stoker and Marmion 1955, Marchette 1982). Some of these feral animals were also parasitized by infected ticks, thus implying the existence of a small mammal-tick reservoir of infection with perhaps potential for transmission of infection to domestic animals pastured in nearby areas. A few selected examples are given to illustrate the variability of the findings and the incomplete nature of the evidence.

Thus, some evidence of transmission of infection from feral animals to domestic animals was adduced by Sidwell *et al.* (1964) in a comprehensive study of native fauna in Utah, U.S.A. *C.burnetii* was isolated or serological evidence of infection was found in rabbits, foxes, coyotes and in various species of rodents, as well as in ticks, fleas, lice and mites. An increase in the

prevalence of antibodies to *C.burnetii* in cattle occurred at the same time as the prevalence of infection in the wildlife.

Elsewhere in the USA, in the Bitter Root Valley in Western Montana, Burgdorfer *et al.* (1963) concluded that *C.burnetii* was maintained in nature in two cycles co-existing independently, namely among wild animals and their haematophagous arthropods on the one hand, and among domestic livestock on the other. Four strains of *C.burnetii* were isolated from wild rodents trapped in areas in the Bitter Root Valley inaccessible to livestock and seldom visited by man.

In northern California, Enright *et al.* (1971) found the prevalence of antibodies to *C.burnetii* varied among wild animals and was probably influenced by whether they were carnivorous or not; infection by the respiratory route might occur in carnivores while eating infected animals and in herbivores while foraging in areas contaminated by livestock.

Elsewhere, outside the USA, Czechoslovakian workers found a high incidence of antibodies to *C.burnetii* in small animals such as *Mus musculus* and *Microtus analis* (mice and voles) in a study in east Slovakia (Rehacek *et al.* 1976).

After the early Australian studies, a possible maintenance cycle involving kangaroos (*Megaleia rufa, Macropus major*) was also uncovered (Pope *et al.* 1960). Thirteen strains of *C.burnetii* were isolated by mouse and guinea pig inoculations from more than two thousand *Amblyomma triguttatum* ticks collected in western Queensland. Of these isolates, nine came from ticks collected from kangaroos, two from ticks from goats and two from ticks from

sheep. Antibodies were detected in kangaroos, and *C.burnetii* was isolated from one of the animals. It was considered that transovarial transmission of infection in ticks might not be a prerequisite for maintenance of infection in the wild as observations suggested that there was sufficient overlap of annual tick generations for transmission to occur from instar to instar, with the rickettiaemia produced in kangaroos by the last of the infected adults serving as a source of infection for the larvae of the next generation. Airborne infection from nearby cattle or sheep was however also possible.

In the U.S.S.R. Karalin (1960) reported that the main vector of *C.burnetii* in foci of Q fever in the wild was an ixodiid tick. The presence of *C.burnetii* was identified in twenty-two tick species in the U.S.S.R., sixteen of which were ixodiid ticks. Warm-blooded feral animals in these foci of Q fever also played an important role because they were not only hosts for different stages of ticks, but also reservoirs of infection for the ticks. *C.burnetii* was found in eighteen species of mammals (gerbils, squirrels, marmots) and three species of birds.

Proreshnaya *et al.* (1960) also studied the natural foci of Q fever with particular reference to their ecological and geographical features in the northern part of Kirgizia (U.S.S.R.). Infection with *C.burnetii* was identified in mammals such as suslik, great jerboa, long-eared hedgehog and polecat. Tick species *Hyalomma plumbeum* and *Dermacentor marginatus* were also carriers of the organism.

In contrast, studies in Armenian Soviet Socialist Republic (Tarasevic *et al.* 1976), east Slovakia (Rehacek *et al.* 1976) and south Bohemia (Rehacek *et al.* 1977), on the natural foci of *C.burnetii* and other rickettsias, did not

produce clear evidence on transmission cycles between ticks and natural fauna. *C.burnetii* was not isolated from ticks in these areas, and antibodies to *C.burnetii* were limited to only a few species, including foxes and hares. On the contrary, the prevalence of antibodies in humans and domestic cattle from these regions suggested that transmission occurred by direct contact among animals and by human contact with infected domestic animals or their excrement.

Italian workers (Babudieri and Muscovici 1952) found that pigeons and geese may be naturally infected with *C.burnetii.* Zhmeava and Pchelkina (1957) reported the isolation of *C.burnetii* from suspensions of chicken brain and liver inoculated into guinea pigs.

Overall, although *C.burnetii* has been isolated from a few species of birds, suggesting that some may be infected in nature, too few isolates have been made to allow correlations with infection in wild populations (Marchette 1982). It may be that these creatures act as "short-term" or "secondary" hosts for the coxiella and carry it into domestic or other environments well away from domestic ruminants, their products, or fomites directly contaminated by them (Marmion and Stoker 1958).

2.4 ***Coxiella burnetii* in domestic animals**

The original observations of Derrick *et al.* (1942) on Q fever in abattoir and dairy farm workers pointed to cattle as a source of infection, although the mechanism whereby this occurred was quite obscure.

In the U.S.A., in the period just after the work at the Rocky Mountain

Laboratory and the preliminary work on the definition of maintenance cycles in wild animals and ticks, there were again indications of cattle as a source of infection. These came, for example, during an outbreak of Q fever among stock handlers and slaughterhouse workers in Amarillo, Texas, U.S.A.. Topping *et al.* (1947), Irons and Hooper (1947) and Irons *et al.* (1947) noted that cattle were apparently infectious for abattoir workers even though they did not appear diseased.

The mechanisms and exact source of infection for those in contact with the cattle remained obscure although the circumstances of the infection of Dr Dyer at the Rocky Mountain Laboratory, those of the laboratory outbreaks of Q fever at N.I.H. and the general pattern of infection of Allied troops during the Balkan campaigns of the early '40s (e.g., bivouacing in lofts with dusty hay etc.) all suggested that airborne infection with the coxiella was a strong possibility.

The matter was brought into sharper focus by the investigations of Huebner and colleagues in the Los Angeles area in the late 1940s. The suburban areas of the city had expanded rapidly and overran so-called dairy farms which were little more than restricted feed lots (concreted areas holding cattle on which dung, urine, placentas and birth fluids accumulated). In the warm climate these excreta and tissues dried out and on windy days (Santa Anna wind) the dust was blown over the surrounding housing estates in which numerous cases of Q fever developed.

Investigations by Caminopetros in Greece (Caminopetros 1948) had shown that *C.burnetii* was excreted in the milk of infected goats. Huebner and

his colleagues (Huebner *et al.* 1948) followed this lead, and showed that a significant proportion of cattle in the Los Angeles area were shedding coxiellas in the milk. This represented an additional, although probably less numerically important, mode of infection for those local residents who consumed raw milk.

The source of the organism in dust and for other modes of airborne infection was finally identified, and an analogy with brucellosis extended, when Luoto and Huebner (1950) isolated *C.burnetii* from bovine placentas and showed that the number of organisms was, on occasion, over 10^{10} guinea-pig ID50 per gram of placental tissue.

Conversely, Lennette and his colleagues (1951) at Berkeley, northern California found that the main sources of infection for man were sheep and sometimes goats rather than cattle. *C.burnetii* was isolated from ovine placentas and milk and, as with cattle, the numbers of coxiellas in placentas was sometimes considerable. The workers at Berkeley conducted systematic experiments on routes of infection in sheep and showed that immunologically naive animals could be infected by the respiratory route, undergo a mild infection with (probable) localisation of the organism in the liver; an organ from which the placenta was later seeded when the animal became pregnant. Extensive multiplication in the placental trophoblastic cells took place during the last weeks of the pregnancy. Air-sampling studies (see below) demonstrated that at parturition, infected ewes liberated coxiellas into the air; these, in turn, were a source of infection for other sheep in the vicinity, for human beings in contact, and also for contamination of fomites for transfer

of infection and later liberation into the air at some distance from the source of contamination.

The presence of the organism in bovine, ovine and caprine placentas and other products of conception has been confirmed by many workers (reviewed by Lang 1990).

The rôle of infected milk in producing human infection has been more controversial than that of airborne infection from parturient animals. The recovery of *C.burnetii* from raw milk in southern California (Huebner *et al.* 1948) and the epidemiological studies of Huebner and Bell (1951) supported the view that milk was a source of infection. Marmion and Stoker (1950), Marmion *et al.* (1953) and Marmion and Stoker (1958) in studies in Britain showed that persons consuming raw milk more often had antibody than those who had a pasteurised milk supply. A number of clinical cases of Q fever were detected in which sources of infection other than with infected raw milk could not be found.

The infective titre of *C.burnetii* in milk for guinea pigs is generally low - 100 to 1000 guinea pig ID50 per ml - and whey antibody was shown to protect mice against Q fever infection. It might be supposed that exposure to small numbers of the organism, mixed with protective antibody, mostly resulted in a subclinical immunising infection rather than overt illness. Other authorities have suggested that the generation, and inhalation of aerosols while consuming infected milk, leads to infection via the respiratory rather than the ailmentary route. The point is perhaps somewhat theoretical. Pasteurisation of milk is effective and is mandatory to prevent infection, even if subclinical,

as some persons exposed to infected raw milk have subsequently developed serious complications such as chronic endocarditis.

On the other hand, surveys (e.g., Benson *et al.* 1963) of institutional groups known to have an infected raw milk supply have not revealed clinical Q fever, although increased rates of antibody might be present.

C.burnetii has been isolated from the milk of goats in many countries of the world. Camels are reared for dairy purposes in some countries (e.g., Pakistan) and camel milk may serve as a source of infection for man (see Lang 1990). Sheep also excrete *C.burnetii* in milk and although this might lead to human infection in uncured cheese made from sheep's milk, this does not appear to be a significant source.

2.5 Infection in other animal species as a source for man

Horses and mules may have serological evidence of infection and occasional Q fever cases have been described among those in contact.

Dogs may be infected from ticks, after eating infected placentas or by airborne exposure. The organism is excreted in the canine milk and placenta and may be a source of infection for man. Cats may be infected by eating infected mice and excrete the organism at parturition. Twelve or more small outbreaks from parturient cats have been described in eastern Canada; other studies have shown that cats are readily infected by the subcutaneous or oral routes or by contact with infected cats (Marrie 1990a).

2.6 Airborne infection of man

As indicated in the preceding sections, the epidemiology of human Q fever has consistently pointed to infection by the respiratory tract as the major mode of infection with ingestion of infected raw cow or goat milk as a lesser, but real risk factor.

The theme of airborne infection, sometimes with most unexpected variations, has been a common element to the dust-born outbreaks (Santa Anna fever) around the Los Angeles 'dairies', the exposure (to 'Balkan grippe') of Allied troops in the barns and farmhouses of the Italian and Greek campaigns in WW2, the infection of workers (and visitors) in abattoirs as far apart as Texas, Queensland, New South Wales and South Australia, to Swiss outbreaks among residents along routes used by sheep to move from high Alpine to lower pastures, to the unlikely circumstances of infection of party goers and poker players exposed to parturient cats.

The unexpected variations to the theme mostly relate to the long term survival of the coxiella on fomites such as clothing, hair, straw or other objects which can transfer the organism to locations at a distance from its source, with liberation of the infected dust and so infection of persons in the distant microenvironment.

More systematic experimental and epidemiological observations were provided by air sampling studies in the vicinity of infected cattle or sheep. Thus DeLay *et al.* (1950) demonstrated *C.burnetii* in the dust of places contaminated with the body secretions or excreta of infected livestock. Samples were collected with an impinger air sampler at a dairy farm in

Southern California where cases of Q fever had occurred and the organism was isolated in guinea pigs. Similarly, *C.burnetii* was also isolated from air samples collected on a sheep ranch in northern California where human cases of Q fever had occurred. Positive results were obtained with air samples from areas in which sheep had been kept up to 30 days previously, indicating persistence of the organism in the environment.

2.7 Laboratory infections with *C.burnetii*

Q fever holds pride of place as a cause of accidental infections in laboratory workers, followed closely by hepatitis viruses and then the typhus group of rickettsiae (Pike and Richard 1979).

This unenviable performance rests on several factors. Cultivation of *C.burnetii* in the chick embryo yolk sac or in cell culture produces suspensions of high infectivity titre which may be dispersed into the air during the process of blending the yolk sacs or handling the cell cultures. The infective dose for man by the respiratory tract is small. The resistance of the organism to inactivation by commonly used disinfectants, phenol or cresol based, may lead to delicate equipment, which cannot be autoclaved, remaining infected after immersion in such disinfectant.

In the early years, laboratory outbreaks of Q fever occurred in Melbourne, Brisbane and at the Rocky Mountain Laboratory. A little later an outbreak of pneumonitis occurred at the National Institutes of Health (Hornibrook and Nelson 1940). Fifteen cases of a pneumonic illness, subsequently identified by Dyer *et al.* (1940) as Q fever, occurred in a building

in which work with *C.burnetii* was in progress. It was notable that the workers who were closely concerned with handling the organism escaped infection - presumably because they were immune from previous subclinical or unrecognised infection - whereas those in adjacent rooms or other parts of the building became infected, probably because of the dispersal of the organism in infected dust through the air conditioning system - a mode of dissemination of the organism to be frequently observed in abattoir outbreaks. In passing, this outbreak drew attention to the pneumonic presentation of Q fever which was well described and investigated by roentgenograms of the infected subjects.

A Q fever outbreak with similar features occurred in a U.S. Army laboratory in conjunction with the investigation of soldiers with 'Balkan Grippe' from the Mediterranean campaigns in Italy and Greece during the closing stages of the 1940-45 war (Robbins and Rustigan 1946).

These early outbreaks were followed by others at the National Institutes of Health, with features similar to that described by Hornibrook and Nelson (1940) (Spicknall *et al.* 1947, Huebner 1947).

Three to four decades later Q fever is still an occupational illness at the NIH (Bayer 1982) and also in research institutes and medical schools although in the context of the use of sheep as experimental animals for studies of foetal physiology and experimental surgery (Hall *et al.* 1982, Curet and Paust 1972, Schachter *et al.* 1971, Meiklejohn *et al.* 1981).

2.8 Experimental airborne infection

Apart from the documentation of the reality of airborne Q fever by air sampling in the vicinity of infected animals in the field, a series of experiments on airborne infection were conducted with guinea pigs or human volunteers (Tiggert and Benenson 1956, Benenson 1959), primarily as part of studies of protective effect of Q fever vaccine.

Tiggert *et al.* (1961) exposed guinea pigs to graded doses of an aerosol of the AD strain of *C.burnetii*, originally isolated from cows milk. It was found that (statistically) one *C.burnetii* cell was capable of infecting a guinea pig, an infective dose comparable with that by the intraperitoneal route.

The incubation period - i.e., time between exposure to the aerosol and onset of fever - was inversely proportional to the dose given and the duration of fever was likewise related to dose.

Pneumonia was not found in the guinea pigs and it was concluded that the respiratory tract served more as a portal of entry rather than a target organ.

Somewhat earlier, Tiggert and Benenson (1956) reported exposure of human volunteers to aerosols of the AD strain of *C.burnetii*. As with the guinea pigs, the incubation period to onset of fever was proportional to the dose given, being as short as 10 days with a dose of 150,000 guinea pig infective doses [one dose (GPID50) is that which produced a seroconversion in 50% of inoculated guinea pigs]. Around 10 GPID50 were required to infect man. About half the inoculated volunteers showed small lung lesions on chest X-ray. In a group vaccinated with a formalin-inactivated preparation of

C.burnetii, Henzerling strain, disease was prevented after exposure to aerosols with doses, respectively of 150 and 150,000 GPID50.

In summary, therefore, a substantial body of evidence exists that the major mode of infection for man is inhalation of particles containing *C.burnetii* either as dried dust or as freshly dispersed liquid droplets. Ingestion of infected raw milk runs a poor second as a mode of infection and infection by skin penetration (mainly tick bite) is a rare event indeed. Person-to-person spread is unusual presumably because the pneumonitis of Q fever is infrequently accompanied by production of sputum or increased respiratory secretions and coughing which would disperse the organism from the respiratory tract. Nevertheless, family or hospital ward outbreaks have been recorded and examination of the Q fever infected lung at autopsy is a hazard.

Given the small dose of organisms required to infect, and the stability of the organism, most features of the endemicity, and outbreaks of the disease can be understood in terms of the airborne transfer of the coxiella from the infected parturient animal (cattle, sheep, goats, cats, rabbits, horses etc), or fomites contaminated by them. Isolation of the organism from the air in the vicinity of infected lambing sheep or calving cattle provides both an explanation of the infection of human bystanders and of nonimmune animals in the vicinity, with eventual recrudescence of infection in the latter when they in turn become pregnant.

In view of the difficulty of protecting the respiratory tract from the inhalation of infected dust or droplets, even in the laboratory, it is clear that an effective vaccine is mandatory for protection of 'at risk' groups working with

infected domestic animals or with the organism in the laboratory.

CHAPTER 3

CLINICAL ASPECTS OF Q FEVER IN MAN

3.1 Early studies and clinical presentations

Derrick and his clinical colleagues in Brisbane defined many, if not most, of the clinical symptoms and signs of human Q fever in the original investigations in the 1930's (reviewed Derrick 1973).

In a study of 273 cases, diagnosed in Brisbane between September 1935 and June 1947, he noted that the incubation period ranged from 13 to 26 days with a few isolated instances when it may have been over 35 days. The incubation period in both man and laboratory animals is dose-dependent and the protracted incubation periods may represent infection with a small dose of *C.burnetii*, or the presence of antibody in the inoculum along with the coxiella. Duration of fever ranged from 5 to 57 days with a median of 10 and an average of 13.5 days. The commonest fever pattern was a rapid ascent of temperature for 2 to 4 days, then a plateau sometimes broken by remissions and then defervescence by rapid lysis; 5 to 14 days fever in total. Some 28% of cases had a biphasic course. The first phase was that just described with a return to normal temperature and then a second phase (so-called secondary phase) lasting one to 19 days, in which the temperature was lower and intermittent. The distinction between a primary and secondary febrile phase is of interest in that *C.burnetii* was readily isolated from the blood in the primary phase (indicating a rickettsiemia) whereas it was not isolated during the secondary phase or after the 15th day from onset in patients with prolonged fever. In

contrast, *C.burnetii* was isolated from the urine during both the febrile phase and in early convalescence (up to 19 days). [The change of pattern around 15 days from onset, when antibody and the cell mediated-immune response is developing raises the interesting possibility that the secondary phase of fever noted by Derrick arises from production of cytokines (e.g., TNFα.) in the emerging immune response rather than from effects of the organism *per se*].

Derrick (1973) also noted the hepatic damage, progressing as far as overt jaundice in some patients. Powell (1961) studied 72 patients in Brisbane and found overt jaundice in 3, liver enlargement in 47 and some impairment of function in 61. Needle biopsy revealed "focal inflamation" in 14 patients sampled by liver biopsy - presumably the small granulomas now well recognised (see Marrie 1990b). A relationship was observed between prolonged fever and hepatic involvement. With the greater availability of sensitive liver function tests, it is now well recognised that Q fever in Australian patients is frequently accompanied by raised aminotransferase levels or, in particular, by raised alkaline phosphatase values.

Derrick and colleagues also noted the severe headache, photophobia and conjunctival congestion, the drenching sweats and the loss of weight (sometimes up to 3 stone) during the acute illness. Sometimes the headache and neurological signs were severe enough to suggest cerebral involvement such as meningoencephalitis or cerebellar ataxia. However, attempts to isolate *C.burnetii* from the CSF or brain biopsy in such cases have not been successful.

In contrast to the hepatic presentation in Australian Q fever cases,

American and European experience has placed more emphasis on pneumonia as a presenting, or even diagnostic feature. Derrick (1973) speculated that the difference resulted from the strain differences or lower virulence of local strains of *C.burnetii.* Powell (1961) confirmed the rarity of lung involvement in Queensland Q fever cases. All 72 patients had clear radiographs; only 3 had evidence of pneumonia; one had a pleural effusion; 7 had blood ~~strained~~ stained sputum. Cough was, however, present in 46% and ronchi or basal crepitations were frequent. The organism was not isolated from the pleural fluid.

Recognition of respiratory involvement in Q fever may have been influenced by the experience of infectious disease physicians looking after American service personnel suffering from "atypical pneumonia" in the large outbreaks in training camps, studied by the Commission on Acute Respiratory Disease in the early 1940's (Dingle *et al.* 1944). These were predominantly caused by Eaton Agent (*Mycoplasma pneumoniae*) and later 'Balkan grippe' (Q fever). Outbreaks of Q fever at the National Institutes of Health were also recognised to be accompanied by pneumonia (Hornibrook and Nelson 1940); Q fever and psittacosis (Binford and Hauser 1944) were other differential diagnoses for "atypical pneumonia - etiology unknown".

The prevalence of "Balkan grippe", "Mediterranean Q fever", or "atypical pneumonia" among Allied and Axis troops in the Greek and Italian campaigns of WW2 was the subject of intense clinical and laboratory study.

Outbreaks took the form of a febrile disease which closely resembled primary atypical pneumonia. Following the lead of Hornibrook and Nelson (1940), chest radiographs located pulmonary consolidation; patchy areas

involving a small portion of a lung lobe and with a homogeneous, "ground glass" appearance. *C.burnetii* was isolated by guinea pig inoculation and was positive two to eight days after onset of illness in the majority of the cases studied. Complement fixation tests were perfomed on patient sera using the the Henzerling strain isolated from a soldier in one of the regiments stationed in Italy (Robbins and Ragan 1946; Robbins *et al.* 1946 a,b).

C.burnetii isolated in guinea pigs from cases of Mediterranean Q fever (Robbins *et al.* 1946b) provided representative strains of *C.burnetii* prevalent in Italy. In guinea pig cross-protection tests, these isolates were antigenically identical.

Symptoms varied in severity and suddeness of onset, and included headache, fever, malaise, muscular aches, chills, cough, pleuritic pain and anorexia. The radiographic lung pictures taken of some of the patients revealed lesions at multiple sites around the lung, considered to be characteristic of Mediterranean Q fever.

In a number of these studies laboratory confirmation was obtained with a seroagglutination test. However, Robbins and Reagan's work with the complement fixation test, as distinct from the agglutination reaction, revealed a phenomenon which was later, with the discovery of antigenic phase variation, to prove of considerable importance.

The complement fixation test with Henzerling strain antigen and sera from soldiers infected in the Mediterranean outbreaks revealed Q fever antibody at high titre. When the sera were tested with antigen from the American (Dyer) strain (Nine Mile) they were negative even though sera from

patients infected with the Nine Mile strain (i.e., in the USA) reacted well with the Henzerling strain.

Further examination of the discrepancy showed that early convalescent phase sera from the patients reacted well with Henzerling antigen but not with the "Dyer" antigen, whereas late convalescent phase sera (after 70th day from onset) reacted well with both antigens. Similar effects were obtained with early and late post-infection guinea pig sera.

C.burnetii antigens were thus classified in terms of reactivity as either "Henzerling type" or "Dyer type". It was concluded that Henzerling strain antigens contained a serologically active fraction which was absent or present only in small amounts in the American strain. The correct explanation was to prove quite different.

It should be emphasised that the difference between the two categories of antigens was apparent in complement fixation tests - agglutination reactions were satisfactory with either type of antigen.

After these early clinical descriptions of acute Q fever there have been numerous publications on the clinical aspects of acute Q fever which have not, in fact, significantly modified the overall picture from the early Australian and American studies. In more recent reviews, the clinical manifestations of acute Q fever have been described as influenza-like (Baca and Paretsky 1983). After an incubation period ranging from 14 to 39 days, with an average of 20 days (Sawyer *et al.* 1987), symptoms are characterized by fever, pneumonia, headache, muscle cramps, arthralgia, coughing and general debility (Dupuis *et al.* 1983,1987). Repeated rigors, with spikes of fever to 104°F have also been

reported (Sawyer *et al.* 1987). Other symptoms may include retrobulbar pain, general malaise, myalgia and chest pain. Subclinical infections are common, with infection being confirmed by detection of specific anti-*C.burnetii* antibodies (Fiset *et al.* 1969, Peacock *et al.* 1979).

Most recently, Marrie (1990c) reviewed the main clinical presentations of Q fever with some illustrative case histories. There is also a useful comparison of the frequency of symptoms and signs with two other infections - namely, *Mycoplasma pneumoniae* and *Legionella pneumophila.* Symptom profiles were remarkably similar in the three conditions, but Q fever cases showed a higher frequency of fever, rigors, headache and confusion. *Legionella* spp. infections were in the older age groups and had a higher death rate.

3.2 Complications associated with acute Q fever

Unusual presentations of Q fever, or complications in convalescence, include optic neuritis, thyroiditis, pericarditis or myopericarditis, peripheral arterial or venous thrombosis, bone marrow necrosis and persistent granulomatosis. A number of workers have described patients, serologically confirmed as Q fever, with extensive granulomatous hepatic infiltration (Derrick 1937, Clark *et al.* 1951, Powell 1961, Pellegrin *et al.* 1980) either with pulmonary involvement (Sawyer *et al.* 1988) or without (Dupont *et al.* 1971). These reports confirm the occurrence of hepatic involvement in patients with Q fever and support the view that liver damage may be common but covert in many cases. Derrick (1937) and Clark *et al.* (1951) reported liver involvement during the second or third week of illness. Dupont *et al.* (1971) found liver abnormalities

commenced prior to the onset of clinical illness and persisted for longer than a month after exposure, in spite of appropriate antimicrobial therapy. In view of these atypical presentations, Sawyer *et al.* (1988) suggested that Q fever may occur more frequently than suggested by existing surveillance based on a concept of Q fever as an atypical pneumonia. They recommended that a diagnosis of Q fever be considered in patients with a respiratory or influenza-like illness during periods of low influenza activity and also in patients with abnormal liver function tests when serological evidence for hepatitis A, B or other viral hepatitis was absent.

Bone marrow granulomas associated with granulomatous hepatitis have also been reported (Okun *et al.* 1979, Brada and Billingham 1980, Travis *et al.* 1986) in Q fever. Okun *et al.* (1979) reported several "doughnut type" lesions - thought to be characteristic of Q fever (Srigley *et al.* 1985) - in bone biopsy and aspirates of cells from bone marrow of a patient with acute Q fever.

Clinical reports have also featured pleuropericardial lesions (Caughey 1977) and gastroenteritis (Lim and Kang 1980) as modes of presentation of acute Q fever. Biggs *et al.* (1984) reported a patient with acute Q fever who developed hyponatraemia associated with inappropriate secretion of anti-diuretic hormone. The patient failed to respond to extensive antibiotic therapy and subsequently developed chronic Q fever. Lipton *et al.* (1987) and Janigan and Marrie (1983) described the presence of a pulmonary mass in Q fever infection, with the roentgenographic appearance of a pseudotumour.

It is clear from these reports that the protean presentations of Q fever, taken together with the CNS involvement, require the inclusion of Q fever

antigen in serological 'battery' testing irrespective of the organ system involved clinically.

3.3 Laboratory diagnosis of acute Q fever

Serological diagnosis of Q fever infection has been employed since the initial work of Burnet and Freeman (1937,1938a), Burnet *et al.* (1939) and Bengtson (1941a). In these instances agglutination or complement fixation tests were employed to determine antibody levels in either infected animals or humans. The antigens employed were prepared from yolk sac suspensions or infected mouse spleens, and used to determine antibody titres irrespective of the antigenic phase of the organism. The discovery of phase variation in *C.burnetii* (Stoker 1953, Stoker and Fiset 1956) permitted these assays to be refined further, with the classification of antibody as either Phase I or Phase II, depending on reactivity with either the Lipopolysaccharide or the protein components of the organism (details Chapter 6).

Q fever agglutination reactions are expensive in the amount of antigen required and exacting to read. They have the advantage that both the early IgM antibody response and the later IgG response is detected. In the period 1950 to 1980 the complement fixation test was used by most laboratories, although CF antibody rises slowly during the course of the disease in step with the IgG response.

The search for a more rapid serodiagnostic method for Q fever increased the numbers and types of assay employed in different laboratories. These assays range from the capillary-agglutination test used by Luoto (1956) and

agglutination-resuspension test of Ormsbee (1964) to the more recently introduced immunofluorescence assay for determination of Q fever antibody in the IgM, IgG and IgA subclasses (Murphy and Hunt 1981, Hunt *et al.* 1983, Peacock *et al.* 1983, Dupuis *et al.* 1985, Peter *et al.* 1985, Worswick and Marmion 1985, Raoult *et al.* 1988). The immunofluorescence techniques not only allow detection of immunoglobulin isotype, but in all instances are more sensitive than the complement fixation test. Immunofluorescence assays detect antibody for a longer period of time and are not complicated by the nonspecific anticomplementary activity, a complication of complement fixation assays. Variations on the principle of immunofluorescence assays, but with different detection systems, include radioisotope precipitation tests (Tabert and Lackman 1965, McKiel and Millar 1968) and the enzyme-linked immunosorbent assay (Schmeer *et al.* 1984, Doller *et al.* 1984, Kovacova *et al.* 1987).

3.4 Longer term complications of Q fever

The original descriptions of Q fever leave an impression of an unpleasant, often severe but self-limiting illness with full recovery and little deficit. This view still largely prevails with physicians who have limited contact with Q fever patients.

Less common manifestations or complications of the disease include hepatitis (Derrick 1937), thrombophlebitis and pulmonary infarction, pleural effusion, oesophagitis, pericarditis and pancreatitis (Huebner *et al.* 1949, Clark *et al.* 1951, Marmion *et al.* 1953), as well as rarer complications described

earlier.

The first indications that an attack of Q fever, or even a subclinical infection, might have more long-term pathological consequences, emerged with the recognition that a small number of Q fever patients subsequently developed signs and symptoms resembling subacute bacterial endocarditis. Blood cultures were consistently sterile for bacteria, but Q fever complement-fixation tests were positive. Clinically, the signs and symptoms of Q fever endocarditis are similar to those of subacute bacterial endocarditis and include persistent, generally low grade fever, finger-clubbing, anaemia, heart murmurs and splenomegaly. The complication commonly, but not invariably, affects patients whose heart valves have been damaged by rheumatic fever or are congenitally malformed. Enlargement of the liver is commoner than in bacterial endocarditis. The disease is a generalised infection, with *C.burnetii* being isolated from vegetations on the heart valves, from the blood, liver and spleen.

The possibility of a chronic endocarditis caused by *C.burnetii* was raised by Marmion *et al.* (1953) in their discussion of Q fever cases in the United Kingdom and in the light of the description by Huebner and colleagues, of two patients with valve lesions and serological evidence of infection with *C.burnetii*; however, isolation attempts were not made from the valve lesions.

In the late 1950's a chance arose to investigate the matter in depth and joint reports by Robson and Shimmin (1959) and Andrews and Marmion (1959) described the clinical, pathological and bacteriological findings of a patient with chronic Q fever with symptoms and signs of endocarditis. The patient, an engineer, had an acute illness resembling Q fever while in Panama. He

appeared to recover, but subsequently 9-12 months later developed symptoms and signs of subacute endocarditis. Blood cultures were sterile for bacteria but *C.burnetii* was isolated and high titres of Q fever CF antibody were present. The patient died and autopsy revealed a cerebral haemorrhage, and a distorted aortic valve with vegetations containing microcolonies of *C.burnetii.* Infectivity titres for guinea pigs were higher in the valve material; spleen, liver and lung were much less infective. It was also found that sera from the patient reacted with Phase I CF antigen, an observation which was to prove of great value as indication of chronic infection.

Other endocarditis patients were described by Marmion (1962). Delaney and Roberts (1975) drew attention to chronic hepatic disease in Q fever endocarditis. Their patient presented with Q fever endocarditis and a moderate degree of hepatosplenomegaly. Liver function was abnormal and the histological examination revealed numerous granulomas in the liver tissue.

Turck *et al.* (1976) reviewed sixteen cases of chronic Q fever either from their own records or from the literature. The majority of patients presented with symptoms of infective endocarditis but two presented primarily with liver disease. All patients had evidence of liver involvement and in one, this led to death from cirrhosis. Again, liver function tests were abnormal and histological examination of the liver showed mononuclear cell infiltration of

*Amendment - "Raised levels of IgG and IgM occurred frequently, and less frequently IgA.

fever antibody titres were high with both Phase I and Phase II antigens. Thrombocytopaenia was also a common feature. However, Varma *et al.* (1980)

found thrombocytopaenia was found in only three of eight chronic Q fever endocarditis patients. The gamma globulin fraction of serum was elevated in the majority of patients, as reported by Turck *et al.* (1976). Kimbrough *et al.* (1979) also found high levels of IgG and IgA antibody against *C.burnetii* but low levels of IgM antibody in their patient with Q fever endocarditis.

Willey *et al.* (1979) described a patient with a silent cryptic Q fever infection with a large ventricular aneurysm. The patient had high levels of complement fixing antibody to Phase I antigen, a feature which had, up until then, been found only in patients with valvular disease. The patient was unique also in having none of the usual clinical features associated with Q fever endocarditis, despite raised antibody titres to *C.burnetii* Phase I antigen 1:200. Fergusson *et al.* (1985) described seven patients in whom chronic Q fever was detected serologically during routine screening on admission for cardiac catheratisation. None had clinical evidence of endocarditis, hepatitis or other foci of infection. Seroconversion following inoculation of cardiac tissue into guinea pigs indicated the presence of *C.burnetii* infection.

With the increased awareness of chronic Q fever the number of reported cases has increased so that this and associated complications of Q fever can no longer be considered rare, as originally thought (Marmion 1962). Examples of this have been demonstrated in reports by Lumio *et al.* (1981), Haldane *et al.* (1983), Raoult *et al.* (1987) and Perez-Fontan *et al.* (1988). A good review is given by Raoult *et al.* (1990).

3.5 Recrudescent Q fever in pregnant women

Isolation of *C.burnetii* from the placenta or curettage samples of women previously infected with Q fever has been described by Syrucek *et al.* (1958), Babudieri (1959) and Derrick (1973). The organism apparently is reactivated during human pregnancy, as with domestic animals (Marchette 1982). Infants born to mothers with recrudescent Q fever have been normal, even though there was evidence for intrauterine infection (Fiset *et al.* 1975). Riechman *et al.* (1988) reported a case of chronic Q fever in a woman in the 21st week of pregnancy. At birth, the placenta was necrotic and *C.burnetii* was isolated. The baby was not infected.

3.6 Post Q fever fatigue syndrome

A less well recognised sequel of acute Q fever infection is the Q fever fatigue or debility syndrome (QDS). Symptoms of QDS can be compared, on a limited basis, to those of chronic fatigue syndrome. Chronic fatigue syndrome (CFS) is a term proposed to describe a collection of symptoms of which disproportionate muscle fatigue, particularly after exercise, predominates. Other accompanying symptoms include sore throat and mild fluctuating lymphadenopathy and neuropsychological disturbances such as depression, insomnia and difficulties with concentration (Gin *et al.* 1989). Many clinical, virological and immunological investigations have been reported on CFS, describing minor differences between patients and control healthy subjects (Lloyd *et al.* 1989, Gold *et al.* 1990).

QDS patients have had proven Q fever, diagnosed by Q fever antibody

titres during acute and convalescent phases of illness. The pattern of Phase I antibody titres is not indicative of chronic infection (endocarditis), and during relapse of symptoms, antibody titres remain low and unchanging.

A recent retrospective survey of acute Q fever cases and matched controls showed that about 20% are affected by the syndrome (Shannon and Marmion, personal communication). Other investigators such as Meiklejohn and Lennette (1950) have made reference to a protracted period of debility that may follow acute illness. Spelman (1982) commented on some patients in whom original symptoms resolved without specific treatment, but were later treated because of a recrudescence of their symptoms. Whether these cases were QDS is unknown; however this sequel of Q fever requires further investigation to obtain better definition of symptoms, incidence and dysfunction.

CHAPTER 4

CELL BIOLOGY OF *COXIELLA BURNETII*

Coxiella burnetii, as other members of the tribe Rickettsiae, is a small rod-like or coccobacillary organism, about 1.0μm long and 0.3-0.7μm wide. The cells are less pleomorphic, particularly as regards to length, than those of other rickettsias, although "replicative" and "spore" forms have been described recently and depart from the mean size. So far, it has not been grown in cell-free media and is an obligate intracellular bacterium which grows in one of the cytoplasmic compartments of a wide variety of arthropod or vertebrate cells in culture. In the intact animal the macrophage phagolysosome appears to be the preferred site for its replication; the metabolic enzymes of the coxiella are uniquely adapted, in terms of pH optima, to function in that potentially hostile environment.

It can also replicate in the phagolysosomes of nonprofessional phagocytes, in the placental trophoblastic cells and in (as yet unidentified) cells in the mammary gland (all of obvious importance for the life cycle of the organism). However, its interaction with cells of the monocyte-macrophage lineage and their various equivalents (alveolar macrophages, Kupffer cells, microglia, mesangial cells etc), either in the resting (unactivated) state, or at various stages of immune activation, is of central importance to the immunopathogenesis of Q fever.

4.1 The *Coxiella burnetii* cell

The cells of *C.burnetii* stain gram-negative, or gram-positive when alcoholic iodine is used as a mordant (Gimenez 1965). Electron microscopy of thin sections of the cell shows a cell wall 7-10nm thick with 3 layers; with higher resolution 5 layers may be detected as with *E.coli*. There is no well-developed capsule, as found with other rickettsiae, but with ruthenium red staining a 20nm thick layer of fibrillary material can be demonstrated.

The cell envelope is made up of lipopolysaccharide layer, then a periplasmic space and a peptidoglycan layer and finally the cytoplasmic membrane of the cell, 5-7nm thick, with a typical trilaminar structure.

Despite the generally regular coccobacillary appearance of *C.burnetii* seen in yolk smears or smears from infected vertebrate cells, there are morphological variations which have attracted much attention recently, although their biological significance is not fully resolved.

Earlier, workers (Kordova and Rehacek 1959, Kordova and Brezina 1963) suggested that the filterability of *C.burnetii* (*R.diaporica*) might depend on cells substantially smaller than the majority seen in stained smears.

Wiebe *et al.* (1972) found two morphological cell types in electron microscope preparations of an early yolk sac passage of *C.burnetii* Nine Mile strain. One cell type was small, compact, and rod-shaped or slightly 'corkscrewed', with very dense nucleoid. The other cell type was larger, more rounded and the DNA filaments were dispersed through the cytoplasm of the cell. The two forms could be separated by centrifugation on a cesium chloride gradient; two bands were seen, in the upper band the mean size of cells was

200x600nm, and in the lower band, 310x80nm.

The cells in each band were viable as determined by infectivity of chick embryos. The phenomenon appeared to be partly dependent on the media through which cells were centrifuged, since gradients of different osmotic pressures modified the number of bands and the size of the cells. The authors postulated that the differences in the two cell populations were due either to partial degradation by host cell enzymes and therefore that one was a degenerate form, or perhaps that the cell types represented two stages in a complex developmental cycle. The former explanation had been advanced previously by several groups (Burton *et al.* 1978, Nermut *et al.* 1968).

The observations were extended by McCaul and Williams (1981) and McCaul *et al* (1981) who described a life cycle of *C.burnetii*, with growth by binary fission as with bacteria and also an unequal cell division consistent with the formation of endogenous spores. These conclusions were based mainly on electron microscopic observation of ultrathin sections of *C.burnetii*. The 'life cycle' involved two distinct morphological cell types, designated large cell variants (LCV) (3.0μm dia.) and small cell variants (SCV) (1.0μm dia.). Differences between the two cell variants included (a) a wider periplasmic space in LCV, and (b) a much greater electron density of the cytoplasm in SCV. The LCV was highly susceptible to osmotic shock, whereas the SCV was more stable and therefore considered (possibly) to be a compact, resistant variant coxiella cell suited for extracellular existence and transmission from host to host in a hostile environment. These extrapolations from the morphological, electromicroscopic observations echo the conclusions advanced for chlamydias

in which the large cells are an intracellular replicative form whereas the small dense and compact elementary particle is an extracellular transport form.

McCaul and Williams (1981) also observed the development of "endospore" within one pole of the LCV within which the SCV was recognizable by a dense central nucleoid region surrounded by granular material.

The formation of an endospore was initiated by asymmetrical septation, then the involvement of membrane-like material during sporogenic differentiation with an eventual morphological resemblance between the differentiated spore and the small resting cell (McCaul and Williams 1980).

The significance of these morphological observations for the biology of *C.burnetii* and in particular for the immune response in Q fever (the subject of this thesis), remains uncertain. The SCV were shown to have metabolic activity as evinced by CO_2 formation from glutamate, and were infectious for chick yolk sac and mice.

The term 'spore' has, in concept, been transferred from sporing organisms such as *Clostridia* spp. or *Bacillus* spp. and has implications of extreme resistance to heat and disinfectant. However, the endospores of *C.burnetii* have not yet been shown to be as robust as those of sporogenic bacteria although the coxiella is somewhat more heat resistant than most vegetative bacteria although readily inactivated at temperatures at which bacterial spores survive. It has also been suggested that an SCV might be related to the ability of *C.burnetii* to resist intrinsic and acquired immune responses in the host and to initiate chronic infection. These are attractive notions but at present little experimental evidence is available about the

antigenic specificities of endospores and their capacity, or that of SCV, to initiate an acute or chronic colonisation of macrophages.

As reported (McCaul and Williams, 1981) the coxiella endospore does not contain dipicolinic acid, a prominent constituent of bacterial spores. In view of this and other biological uncertainties, an appropriate description of SCV would be "sporelike" (McCaul - personal communication).

4.2 Genome of *C.burnetii*

Early studies of the nucleic acids in *C.burnetii* (e.g., Smith and Stoker 1951) demonstrated the presence of both RNA and DNA. The base composition and a lack of 5-methyl-cytosine residues in the DNA indicated the organisms resembled the bacteria and the larger animal viruses.

Subsequent studies of the fine structure of the *C.burnetii* cells revealed that inside the cell there are electron dense ribosomes, 7-20nm diam, resembling those of bacteria. The cytoplasm of the cell may also contain electronlucent spheres, 4-40nm in diameter, granules and what appear like intracytoplasmic membranous organelles, which may however be (sectioned) invaginations of the cytoplasmic membrane.

The single DNA chromosome is separated from the ribosome-containing cytoplasm with strands reaching out to the cytoplasmic membrane. The genome size is now considered to be 1.04×10^9 molecular mass and has a higher guanine plus cytosine content - 42.7% - than other rickettsias which range from 29-33% for the typhus and spotted fever group and around 38-39% for *Ro.quintana* and the related vole vesicular agent.

Earlier reports of the size of the genome varied. One view considered the DNA to be a duplex with a molecular weight of approximately 1.8×10^7 daltons and a G + C molecular content of 42.9%. Subsequently, however, Myers *et al.* (1980) using a procedure based on the initial rate of renaturation of heat-denatured DNA and not on the isolation of an intact genome reported the molecular weight to be 104×10^7 daltons, with a G + C molecular content of 42.7%.

In studies directed primarily at the detection of genomic differences between coxiellas in the Phase I and Phase II antigenic states (defined and discussed in Chapter 6), O'Rourke *et al.* (1985) compared the restriction endonuclease (Hae 111) digestion patterns of genomes of strains of *C.burnetii* strains isolated in America and Europe. Differences between strains were detected and in extension of these studies, Vodkin *et al.* (1986) distinguished four different patterns among the American and European strains. Comparisons of the restriction enzyme maps of Phase I and II organisms showed deletions of stretches of DNA from the latter which may be connected with the loss variations in LPS structure which determine the differential survival of organisms in the two antigenic phases in animals with an intact immune system.

4.3 Extrachromosomal genetic information in *C.burnetii*

The discovery of plasmids in *C.burnetii* is of fairly recent origin and its significance for the biology of the organisms in terms of the type of information encoded is not yet clear. Samuel *et al.* (1983) isolated a plasmid from Nine

Mile strains, in both the Phase I and Phase II antigenic state.

On agarose gel electrophoresis a 22.5kb band was observed which was considered to be a single plasmid. However based on restriction enzyme mapping analysis, this DNA band appeared to be of $2.4x10^7$ daltons molecular weight (36kbp) and was designated QpHI. Further restriction enzyme mapping and cross-hybridisation experiments did not reveal significant differences in the plasmids from organisms in Phase I or II. It was concluded that the plasmid played no part in directing the phase antigen shift. The authors speculated that the plasmids coding information might provide functions involved in cell viability, e.g., in rendering the organisms resistant to hydrolytic enzymes in the macrophage phagolysosome, or providing some other metabolic function.

Plasmids from other strains of *C.burnetii* were then examined and those from sources such as ticks and cattle, from various geographical areas, had plasmids similar to QpHI. On the other hand, Samuel *et al.* (1985) when examining the DNA from various isolates from different disease states, found another plasmid in *C.burnetii*, designated QpRS, with a genome 2-3Kbp larger than that of QpHI. Although there was substantial sequence homology between the genomes of the two plasmids (excluding the region comprising the extra 2-3Kbp extra coding capacity) there were differences in restriction enzyme patterns. A point of much interest was that QpRS appear to be characteristic of strains of *C.burnetii* isolated from chronic endocarditis - at least in the USA - although a strain (Priscilla) from the aborted conceptus of a goat had a similar plasmid. Some strains of *C.burnetii* do not have a

episomal plasmid but probe analysis indicates that the plasmid DNA is integrated into the *C.burnetii* chromosome.

Various genes of the plasmids have been expressed in *E.coli* but the function of the gene products has not yet been determined. As the lipopolysaccharides of the prototype and endocarditis/Priscilla strains show some antigenic differences in Western blots with hyperimmune rabbit antisera (see Chapter 6) it may be speculated that the proteins specified by the extra coding information in QpRS specify enzymes in the carbohydrate transferase system or at some other step in the synthesis of the CHO side chains of the LPS.

4.4 Ribosomes and rRNA

Burton *et al.* (1975) provided clear illustrations of ribosomes in thin section electron micrographs of *C.burnetii*. Subsequent analysis by Thompson *et al.* (1971) and Baca *et al.* (1974) of the rickettsial ribosomal RNA from highly purified organisms indicated the presence of a 23S and 16S species, as well as 5S RNA. The base ratios (A+U/G+C) for the rRNA were 0.85 for the 16S and 0.90 for the 23S; values comparable to that of other prokaryotic organisms. Ribosomes of *C.burnetii*, when isolated and characterised, were shown to have an overall sedimentation constant of 70S, comprising two subunits of 50 and 30S.

4.5 Biochemical composition and intermediate metabolism of *Coxiella burnetii*

A detailed account of the biochemical composition and metabolic pathways of *C.burnetii* is not relevant to the purpose of this thesis. Details of the composition of the lipopolysaccharide (LPS) and the LPS- and membrane-associated proteins are however pertinent to serological and cell-mediated immunity in Q fever and are reviewed at greater length under 'cell envelope'.

As far as intermediate metabolism of *C.burnetii* is concerned, numerous investigations of the biochemical pathways of *C.burnetii* have identified a large number of intrinsic enzymatic activities (Paretsky *et al.* 1958, Myers and Paretsky 1961, Paretsky *et al* 1962, Matthies *et al.* 1963, Mallavia and Paretsky 1963). Disrupted preparations of organisms, freed from host tissue enzymes, synthesize glucose-6-phosphate from glucose via a phosphate-hexokinase pathway, oxidize glucose-6- phosphate, synthesize citrate, hydroxymethylate glycerine and serine, utilise carbamyl phosphate in synthesizing cetrulline from ornithine and orotate from aspartate. A unique form of folate was also demonstrated in *C.burnetii* but its function is unknown.

The enzyme, 6-phosphogluconic acid dehydrogenase has also been detected (McDonald and Mallavia 1971); its presence together with the oxidation of glucose-6-phosphate suggest that the organism catabolizes glucose via the hexose monophosphate shunt. Enzymes of the Embden-Meyerhof pathway have also been identified (McDonald and Mallavia 1971), implying that glucose can be converted to pyruvate with the concomitant production of

ATP. End products from the glycolytic pathway are further metabolised via the Krebs cycle and the electron-transport chains involving cytochrome.

Ormsbee and Peacock (1964) demonstrated the presence of an operative Krebs cycle in intact cells, but found pyruvate to be the only substrate stimulating respiration and then at rates much less than in other rickettsias. The rate of oxidation was influenced by permeability factors; as in the previous investigations, oxygen uptake in disrupted cells increased presumably due to the increased access of enzyme to substrate. On the other hand, Paretsky (1968) found that disrupted *C.burnetii* cells contained glucose-6-phosphate dehydrogenase and other enzymes of the Embden-Meyerhof pathway as well as the enzymes of the Krebs cycle.

The contradictions between the work with intact and disrupted organisms was resolved by Hackstadt and Williams (1981(a),(b); 1983) who showed that the minimal glutamate metabolism observed and absence of glucose metabolism by whole organisms was the result of incubation cell-substrate mixtures at pH levels beyond the optima for the *C.burnetii* enzymes. As an obligate intracellular parasite adapted to growth in the phagolysosome of the macrophage, *C.burnetii* has enzymes (and a membrane transport system) adapted to the acidic environment in the phagolysosome. The enzymes of the Embden-Meyerhof pathway and the pentose phosphate pathway activity function optimally at pH 4-5 in *C.burnetii* in contrast to optima at higher pH levels found in *Chlamydia* spp. which multiply in the cytoplasm of vertebrate cells.

In summary, *C.burnetii* possesses the enzymic machinery for synthesis

of its own DNA and RNA, structural proteins, complex lipids and lipopolysaccharides. It is able to synthesise ATP via the Krebs cycle and is not dependent on host cell ATP, although it can utilise this as a supplementary energy source. Given this metabolic self-sufficiency, it is difficult to discern the constraints which make it an obligate intracellular parasite. Evolution to existence at acid pH in the phagolysosome may be one factor. It is also suggested that *C.burnetii* may be unable to make its cell wall peptidoglycan as it lacks the capacity to synthesise glucosamines and N-acetylglucosamine which presumably are supplied by the host cell.

CHAPTER 5

PROPAGATION OF *COXIELLA BURNETII* IN LABORATORY SYSTEMS

In view of the wide range of hosts - arthropods, small and large bush animals, domestic animals, man - that experience a natural Q fever infection, it is perhaps not surprising that *C.burnetii* can be grown without difficulty in a number of laboratory animals or in systems such as the chick embryo or in cultured tissues or cells.

For the primary isolation of the organism from field or clinical samples most use has been made of guinea pigs, hamsters or mice. Although the chick embryo yolk sac is of great value for the production of large amounts of the coxiella for antigenic or biochemical studies, or for vaccine production, it is of less value for the primary isolation of strains because of the hazards of bacterial contamination; ideally inocula should be free of bacteria as the choice of antibiotics to control contamination is limited to penicillin. Filtration to exclude bacteria may reduce the number of coxiellas present and so reduce the efficiency of cultivation.

Monolayer cell cultures are now being used for primary isolation and are also of value for propagation of the organism after an initial passage in guinea pigs. Special cell lines (e.g., macrophages) have been used in studies of the life cycle and cell-coxiella interactions.

5.1 Q fever infection in guinea pigs, hamsters, monkeys and mice

Derrick's classical paper on Q fever (Derrick 1937) showed that guinea pigs

were susceptible to Q fever after inoculation with blood or urine from human patients, or blood or organ suspensions from other infected guinea pigs. After a 2 to 18 days incubation period (depending on the infectivity level of the inoculum), a febrile period of 1 to 8 days, usually 4 to 6 days, was observed with temperatures over 104°F (40°C) - the upper level of normal temperature for a guinea pig.

Guinea pigs are not unduly disturbed by the infection. Apart from the fever, a roughened coat, loss of appetite and a failure to gain weight are the main manifestations. Mortality is usually negligible, although some American strains (e.g., Nine Mile) produce a higher death rate; Australian strains and those from endocarditis patients are mostly of low virulence.

Limited titrations of the organism in blood and liver from infected guinea pigs showed higher titres in the liver. The organism was not isolated from guinea pig urine by Derrick but later work, in which guinea pigs were inoculated with larger numbers of organisms in infected yolk sac or with different strains, showed that the coxiella is sometimes excreted in the urine - a hazard for animal house personnel who have not been vaccinated.

Autopsy of guinea pigs during the fever reveals an enlarged spleen and a variable, usually minimal, enlargement of the liver. There is no scrotal reaction or lymph node enlargement. Other workers (Johnson *et al.* 1976, 1977) working with the Russian M44 living attenuated vaccine strain described myocarditis, pericarditis, hepatitis, kidney involvement and granulomatous/necrotic lesions in the liver (disconcerting properties in a living attenuated vaccine strain).

In terms of histopathological changes in infected guinea pigs, Lillie (1942) examined the brain, part of the spinal column, and the entire thoracic and abdominal viscera of guinea pigs at specific time intervals after intraperitoneal infection with either the X strain of *C.burnetii* or the M strain (isolated from a fatal human case at the NIH). The heart showed interstitial lesions with lymphocytic infiltrations beneath the endocardium and diffusely around vessels in the epicardium.

Lymphocytic infiltrations were also observed in the lung, pancreas, spleen, bladder, prostate, seminal vesicles and central nervous system (CNS). Predominant lesions at different stages after inoculation were epitheloid granulomata, which were observed in the heart, lungs, liver, spleen, kidneys, bladder, prostate, seminal vesicles, lymph node and CNS. Lillie concluded that each strain produced essentially similar types of histological lesions, but with differing intensity.

Guinea pigs develop antibody (and cell-mediated immunity) 20-30 days after experimental infection. The development of antibody between a preinoculation serum sample and one taken in late convalescence is often taken as an 'isolation' of *C.burnetii* without subculture and further identification of the organism.

As Derrick showed originally, after recovery from the fever and associated illness, guinea pigs do not become febrile on challenge with a suspension of *C.burnetii* and this 'cross-protection' test has been much used for comparison of isolates and assay of vaccine fractions. Guinea pigs are also protected against fever if Phase I antibody is mixed with the infective inoculum.

The biochemical analogues of the histological liver changes just mentioned were reported by Kishimoto's group (Powanda *et al.* 1978) who found raised blood glucose, aminotransferases and alkaline phosphatase, 3-5 days post-infection, followed by hypoglycaemia and hypophosphaemia as the hepatomegaly and splenomegaly developed. There were also changes in the levels of certain plasma proteins (e.g., ceruloplasmin) and trace metals thought to reflect the action of factors released from the activated macrophages (e.g., as evidenced by the widespread granuloma formation).

Paretsky and his colleagues in a series of reports (summary in Baca and Paretsky 1983) extended Kishimoto's observations and noted extensive fatty infiltration in the guinea pig liver 3 days post-infection, mainly triglycerides, and concurrently mobilisation of lipids from fat depots elsewhere in the body. Liver glycogen diminished, there was a marked stimulation of RNA and protein synthesis in hepatic cells along with increased cortisol levels and polyamine concentrations. The liberation of tumour necrosis factor (see Chapter 7) from activated macrophages is of probable relevance to these effects.

The inoculation of guinea pigs with lipopolysaccharide (LPS) from Phase I *C.burnetii* produced hyperthermia, weight loss, hepatomegaly, elevated plasma and liver protein synthesis and leucocytosis - i.e., all changes mirroring those just described in active Q fever infection.

The persistence of *C.burnetii* in guinea pigs after recovery from acute infection is pertinent to persistent infection in man and to recrudescent infection in domestic animals and man.

Parker and Steinhaus (1943) isolated *C.burnetii* from various organs of

guinea pigs previously inoculated with American or Australian strains of the coxiella. With the American strains, liver was positive 15 days after defervescence, and lung and brain after 40 days. With the Australian strains brain and kidney were positive after 5 and 110 days; contrary to earlier statements from Australian workers, the coxiella was present in the urine during this prolonged carriage. Other authors have observed carriage of the organism for as long as 130 or even 526 days (Reczko 1950; Germer 1954).

Pregnancy in previously infected guinea pigs may reactivate a latent infection with excretion of the organism in the placenta as in cattle, sheep and goats, and sometimes man. Multiple cortisone injections and whole body irradiation will also reactivate latent infection in guinea pigs and mice (Sidwell *et al.* 1964 a,b).

Hamsters. These animals are reported to be more sensitive than guinea pigs to small doses of some strains of *C.burnetii* isolated from naturally infected rodents (Stoenner and Lackman 1960).

Mice. Q fever infection in mice was extensively studied by Burnet and Freeman (1937). Seven to 10 days after infection the mice were found to have markedly enlarged livers with a pale opalescent appearance. Sometimes small haemorrhages were scattered through the organ and other workers have observed necrotic foci. The spleen was also considerably enlarged, engorged and deep red in colour. Impression smears from the cut surfaces of the spleen show numerous cells (macrophages) with intracytoplasmic colonies of *C.burnetii.* Histological examination (Perrin and Gengtson 1942) of mice inoculated intranasally or intraperitoneally with the X strain of *C.burnetii*

showed pneumonia with large mononuclear cells and lymphocytes and granulomatous lesions in the spleen, liver, adrenal glands and kidney, as with guinea pigs. These workers found, in contrast to the experience of Burnet and Freeman (1937), that detection of the rickettsia in mononuclear phagocytes was more difficult unless the organism had been passaged several times through mice.

Other laboratory animals. Rabbits and monkeys are susceptible to infection and the latter have been used as experimental models for infection and vaccine studies in man (Gonder *et al.* 1979).

Cynomologous monkeys (*Maccaca fascicularis*), exposed to infectious aerosols, developed fever, anorexia, dyspnoea, after 4-7 days. *C.burnetii* was isolated from blood stream during days 7-13 with pneumonitis from day 16. Histopathological changes included pneumonia and granulomatous hepatitis. The kinetics of the serological response to Phase I and II antigens resembled that of man.

5.2 *C.burnetii* in chick embryos

Reference has already been made to the differences in growth patterns of various strains of *C.burnetii* on the CE chorioallantoic membrane (Chapter 1, and Burnet and Freeman 1941a). The yolk sac entodermal cell is optimal for the growth of the organism either *in situ* in the wall of the yolk sac or as explant cultures *in vitro* (Weiss and Pietryk 1957). The organism proliferates in large vacuoles in the cell producing little apparent damage. After inoculation the organism remains in the yolk sac for 6 or so days but

eventually the infection spills over to involve the chorioallantoic membrane and gut, and finally the amniotic membrane, liver, spleen, muscles and heart - a generalised infection of the embryo (Leyk and Scheffler 1975). The replication of the organism in the CE yolk sac has been studied by electron microscopy by Anacker *et al.* (1964) and Khavkin *et al.* (1981).

5.3 Growth of *C.burnetii* in cultured cells

Tick cell cultures. Cultures of *Hyalomma asiaticum* tick cells of supported massive multiplication of *C.burnetii* (Nine Mile Strain, Phase II), from the seventh day after inoculation (see Rehacek and Brezina (1964) and Yunker *et al.* (1970)). This supported previous *in vivo* work (Kordova and Rehacek 1959) that ticks are a convenient medium for cultivation of *C.burnetii*. Growth of organisms in ticks was detectable from day eight after inoculation; suspensions of infected organs produced morphologically characteristic forms of *C.burnetii* on subinoculation into the chick embryo yolk sac.

Growth of *Coxiella burnetii* in cultured cells. As stated in Chapter 1, in the first work on Q fever, Cox and Bell (1939) used Maitland type cultures of CE tissues to grow *C.burnetii*. Chick entodermal cells (Weiss and Pietryk 1957) were another early cell culture system.

Since the early days, *C.burnetii* has been cultured in L929 cells (mouse fibroblasts), green monkey cells, arthropod (tick and mosquito) cells, and primary macrophage cultures and several macrophage cell lines (J774 and P388D1) (Baca *et al.*, 1981).

Growth patterns of *C.burnetii* in vertebrate cell culture. Pospisil (1965) used a stable continuous cell line from monkey kidney to propagate *C.burnetii*, Nine Mile Phase II strain *in vitro*. Kinetics of growth in these cells were comparable to those in the CE yolk sac, but the coxiellas were easily seen in cell vacuoles. Propagation of the infected cell line was achieved for 14 passages with a persistent infection in which *C.burnetii* was transferred into each of the daughter cells at cell division. However after the fifth passage the number of organisms decreased markedly and could only be demonstrated by inoculation into chick embryo yolk sac (Pospisil 1965, 1966). From the sixth passage, cells multiplied at a slower rate and infected cells were increasingly difficult to detect. It was concluded that infection of a cell did not always alter the cell or its metabolism, to an extent that it prevented infected cells from growing or dividing.

Difference in the multiplication of Phase I and Phase II *C.burnetii* in cells was found by Brezina *et al.* (1969). Whereas Phase I and II organisms did not differ markedly in their multiplication pattern in primary chick embryo cells, Phase I strains multiplied to lower titres (in terms of TCID50/ml) than Phase II in HeLa, L929 cells and monkey kidney cells. With a culture of *C.burnetii* containing a mixture of both Phase I and II cells, multiplication resembled that of a Phase II strain in cultures, thus implying a dominance of one phase over the other. Ormsbee *et al.* (1978), in examining the limits of rickettsial infectivity, showed a profound difference in the infectivity of *C.burnetii* that was correlated with the phase of the organism. Phase I *C.burnetii*, Nine Mile strain was the most infective of any rickettsia tested. Mouse L cells were found to be

more refractory to infection with *C.burnetii* Phase I cells, in contrast to the infectivity of the coxiella in mice, in which the difference between Phase I and II infectivity is 3000 fold in favour of the former.

Overall, these investigations were at variance with the findings of Kordova and Brezina (1963), who found no difference in the dynamics of multiplication between Phase I and II coxiella in Detroit 6 cells, human amnion cells and HeLa cells.

Burton *et al.* (1978) examined the ultrastructural consequences of *C.burnetii* infection in L-cells and Vero cells. L cells took up *C.burnetii* into their cytoplasm through phagocytic activity. Filamentous material was observed at the point of contact between the host cell and the organism. Coxiellas inside some cytoplasmic vacuoles appeared in a state of disintegration. In both cell types, *C.burnetii* proliferated within cytoplasmic vacuoles, however Vero cells contained organisms that were more pleomorphic than those in L cells. Also in the former instance coxiellas resembled mitochondria in size, with vacuoles crowded with whorled-membranous elements of various sizes and configurations. The nuclei of infected L cells were pushed to one side, with the remainder of the cell as a thin layer of cytoplasm stretched around a large vacuole. No consistent difference was noted between infected and uninfected cells in the appearance of the rough endoplasmic reticulum.

Persistently-infected L929 cells had diminished amounts of acid phosphatase and a reduced lysosomal response, a feature not seen with Vero cells. In contrast, during short-term infection, an abundance of

acid-phosphatase-containing vacuoles were present in infected L cells. Akporiaye *et al.* (1983) extended and confirmed these observations in a persistently infected murine macrophage cell line (J774) with *C.burnetii*, Nine Mile Phase I strain. Because the acid pH within vacuoles enclosing rickettsiae was identical to the pH of lysosomes of uninfected cells, it was concluded that fusion occurred between coxiella-containing phagosome and the lysosome. No significant difference was observed in the activities of acid phosphatase and β-galactosidase and lysosome activities were elevated in the former.

Further investigations by Baca *et al.* (1985) and Baca and Crissman (1987) on persistently infected L929 cells demonstrated that despite an enormous parasite burden, these cells remained essentially unperturbed in respect of cell cycle progression, generation time and genome stability.

Analysis of the contents of cellular RNA and protein, the RNA to DNA, and RNA to protein ratios of cells, in relation to the phase of the cell cycle, showed that there was no significant difference between normal and infected cells. It was concluded that in long-term persistent infection, cells are in a state of balanced growth.

These observations led to a model for persistent infection of host cells (Roman *et al.* 1986). The model stated that infected cells could undergo mitosis and cytokinesis. Irrespective of whether a cell was lightly or heavily infected, asymmetric division then produced one daughter cell which inherited the parasite containing vacuole while the other remained parasite-free. However, if there was more than one infected vacuole then both daughter cells would be infected. The former situation might be the key to the maintenance

of persistent infection.

Growth of *C.burnetii* in macrophages. Baca *et al.* (1981 a,b) studied the fate of *C.burnetii* Phase I and II in five macrophage-like tumour cell lines (PV-S-IR, J774, P388DI, WEHI-3, WEHI-274). All cell lines could be infected with Phase I coxiellas; some cultures contained a higher percentage of infected cells than others. Two cell lines of the five became persistently infected with Phase I organisms, as distinct from Phase II infection in which all five were persistently infected. Both Phase I and II organisms proliferated within vacuoles and eventually heavily infected cells contained one large vacuole that occupied most of the volume of the cell, with the nucleus pushed to one side. When P388DI cells were infected with cloned Phase I organisms, phase-loss variation was observed after approximately 200 days, after which 50% of the rickettsiae were in Phase II.

Phase II organisms did not revert to Phase I, possibly indicating the antigenic stability of Phase II organisms.

Little *et al.* (1983), working with guinea-pig peritoneal macrophages, reported that phagolysosome fusion and subsequent degradation of digested Phase I *C.burnetii* was achieved only if the coxiella was opsonized with Q fever immune serum. Ferencik *et al.* (1984) then postulated that the survival of virulent Phase I *C.burnetii* in professional phagocytes was due to their failure to activate phagocytic metabolism, particularly superoxide production and hexose-monophosphate shunt in the absence of specific Phase I antibodies. Their study utilised human polymorphonuclear leukocytes (rather than

macrophages) interacting with *C.burnetii* and was in accord with previous findings of Wisseman *et al.* (1967), Brezina and Kazar (1965) and Kishimoto *et al.* (1976).

CHAPTER 6

THE IMMUNE RESPONSE TO *COXIELLA BURNETII*

6.1 *C.burnetii* antigens and serological reactions

It was apparent from Derrick and Burnet's early work with the Q fever organism that there was probably only one antigenic type of *C.burnetii*. Thus, Derrick found that infection of guinea pigs with one strain conferred resistance to the production of fever (and other disease manifestations) on subsequent challenge of the convalescent animals with other strains. This strong cross-protection between strains applied not only to those isolated from Q fever patients and animals in Australia, but also to cross-challenge experiments with Australian and the American isolates of *C.burnetii*.

Burnet and Freeman's development of a simple seroagglutination test with suspensions of *C.burnetii* purified from infected mouse spleen, for reactions with human antisera, and antisera prepared in laboratory animals, reinforced the impression of the antigenic homogeneity of *C.burnetii* isolates. In retrospect, it is recognised that the antigens from mouse spleen would have been in the Phase I antigenic state - to be explained below - and further that the agglutination test would measure all classes of immunoglobulin, thus avoiding a selectivity which gave rise to puzzling results with the complement fixation test and antigens propagated in the yolk sac of the chick embryo from isolates originating from different animal hosts or hosts in different geographic locations.

Propagation of *C.burnetii* in the chick embryo yolk sac yielded large

amounts of coxiella suspension for serological tests and greatly facilitated the application of the complement fixation test to the serodiagnosis of acute Q fever cases. At the time the CF test was widely used for serological testing in Virology and the Q fever CFT fitted well with laboratory practice. Marked and puzzling differences in seroreactivity with various CF antigens and Q fever antibody then became apparent. For example, Topping *et al.* (1946) demonstrated complete reciprocal cross-immunity between five strains of *C.burnetii* in guinea pig protection experiments. On the other hand, CF tests carried out with the same strains indicated the presence of more than one antigen, with quantitive variations in at least one antigenic component shared by all the strains. In reciprocal absorption tests, antibodies from antisera against strains from Italy and the Balkans were more difficult to remove than those reacting with strains from Panama and America.

Complement fixation studies by various workers (Robbins *et al.* 1946b, Topping *et al.* 1946, Wolfe and Kornfeld 1949) with the Dyer strain on the one hand, and on the other hand the use of the Henzerling strain of *C.burnetii* for serodiagnosis by CFT of acute Q fever in American abattoirs, those in the NIH laboratory outbreaks, of some patients with "Balkan Grippe" or Mediterranean Q fever, and - a little later - for the laboratory confirmation of cases in southern California, revealed two broad patterns of reactivity. The Henzerling or Nine Mile antigens, prepared from strains of *C.burnetii* given multiple passages in the chick embryo yolk sac, fixed complement effectively with early and late convalescent sera from human Q fever cases, and with early (10-15 days) post-infection sera from infected guinea pigs. In contrast, antigen

prepared from yolk sac-propagated lines of the "Dyer" strain showed either no, or only poor complement-fixing ability with early sera but reacted well with late guinea pig sera. Other isolates showed similar differences and strains were classified either as Nine Mile type ("good CF fixers") or Dyer type ("poor CF fixers").

The reason for this puzzling behaviour was identified by Stoker and Fiset in three, now classical papers (Stoker 1950,1953; Stoker and Fiset 1956). They found that a strain (Christie) isolated from the blood of a Q fever patient in the outbreak of Q fever at the Royal Cancer Hospital, London (Stoker 1950, 1953) when first grown in the CE yolk sac did not fix complement with the convalescent serum from the patient Christie even though his serum had significant antibody titres with standard CF diagnostic antigens from the Nine Mile or Henzerling strains; i.e., the Christie strain was "Dyer" type. After 5 serial passages in the CE yolk sac, the Christie strain gave antigens showing modest reactions with Christie convalescent serum but by passage 9 antigen titres were comparable in complement-fixing potency (i.e., in "block" or "chessboard" titrations) to the prototype Henzerling antigen.

Further investigations established that the differences in antigen potency were not simply due to smaller numbers of coxiellas in the early egg passage harvests. Significantly, in the light of later findings, it was found that other serological techniques - e.g., conglutinating complement-absorption test, antiglobulin sensitisation test - showed antigen/antibody union with the early egg passage antigen and the Christie antisera; this was also found to be so with agglutination tests with whey from the milk of a Q fever infected cow. In

other words, the phenomenon was essentially a failure of antibody formed in early convalescence to fix complement with early passage antigen, not a failure of antibody to bind to the antigen.

The early egg passage antigens with this pattern of low reactivity by complement fixation - typified by the Dyer strain - were said to be in Phase I (Phase I antigen) whereas the later passage, reactive antigens were said to be in Phase II (Phase II antigen).

The phenomonen of Phase variation on serial CE yolk sac passage proved to be reversible. If a Phase II strain - such as the standard Nine Mile strain used for CF antigen - was inoculated into guinea pigs, mice or other immunologically competent animals and subsequently the strain was reisolated and cultured in the CE yolk sac, it was found to have regained Phase I characteristics in the complement fixation test.

Laboratory animals inoculated with Phase I coxiella produced antibody to both Phase I and Phase II antigens, the latter rising earlier in convalescence, whereas the highly egg-adapted Phase II strains produced only Phase II antibody. The phase variation phenomenon was considered to be a loss variation, on CE yolk sac passage, of a surface antigenic component characteristic of Phase I, unmasking a deeper Phase II antigen. Fiset (1957) found that treatment of Phase I coxiellas with potassium periodate 'unmasked' Phase II CF reactivity whereas the Phase II antigen was susceptible to protease digestion.

It thus appeared that strains of *C.burnetii*, when isolated from naturally infected hosts - sheep, cattle, ticks, man - were in Phase I, as were those

Table 6.1: Summary of the major properties of *Coxiella burnetii* cells, or extracted fractions, in the Phase I or Phase II antigenic state.

***C.BURNETII* CELL OR SUBFRACTION**			
PROPERTY	**PHASE I**	**PHASE II**	**REFERENCE**
Buoyant density	d = 1.33g/cm^3	d = 1.22g/cm^3	Hoyer *et al* 1963
Attachment to cells in culture	+	+++ (effected by coxiella proteins)	cited in Baca and Paretsky 1983
Surface staining by ruthenium red	++ (20nm 'fuzzy' layer)	+ to - (variable reports)	Burton *et al* 1975 Ciampor *et al* 1972
Complement fixing activity with guinea pig antisera	Antibody present in late sera	Antibody present in early and late sera	
Infectivity for guinea pigs (dose causing infection)	1 - 5 organisms	10^7 organisms (cloned strains)	Ormsbee *et al* 1978
Surface anionic binding sites	Absent	Present	Krauss *et al* 1977
Phase variation in cultured cells (i) L929 cells (ii) Macrophage P388D1	Phase I to Phase II Phase II maintained		Baca *et al* 1981 (b)

Table 6.1 (cont): Summary of the major properties of *Coxiella burnetii* cells, or extracted fractions, in the Phase I or Phase II antigenic state.

***C.BURNETII* CELL OR SUBFRACTION**			
PROPERTY	**PHASE I**	**PHASE II**	**REFERENCE**
Suspension stability of whole cells	High	Low (spontaneous aggregation)	Fiset *et al* 1969
Phagocytosis without opsonins	±	+++++	Kazar *et al* 1975b; Kishimoto and Walker 1976
Lipopolysaccharide (i) LPS: content (per 1gm wet wt of organism)	5.8 ± 0.7mg	0.55 ± 0.17mg	Baca and Paretsky 1974b; Baca *et al* 1980
(ii) LPS: gelation of *Limmulus* amoebocyte lysates	+	+	Schramek and Brezina 1976; Hackstadt *et al* 1985
(iii) LPS: induction of DNA synthesis in or activation of non-immune guinea pig leucocytes or macrophages	+	- (?)	Paquet *et al* 1978; Kelly 1977
(iv) LPS: toxicity for chick embryos	- (>80µg/embryo)	-	Hackstadt *et al* 1985

Table 6.1 (cont): Summary of the major properties of *Coxiella burnetii* cells, or extracted fractions, in the Phase I or Phase II antigenic state.

***C.BURNETII* CELL OR SUBFRACTION**			
PROPERTY	**PHASE I**	**PHASE II**	**REFERENCE**
(v) LPS: tumour regression (with mycobacterial glycolipid)	++	-	Kelly *et al* 1976
(vi) CHO % dry wt KDO-like % dry wt Protein Phosphorous	33-48 0.49 2.6 3.2	16-20 0.33 3.4 3.4	Baca and Paretsky 1974b Baca *et al* 1980 Chan *et al* 1976 Schramek and Brezina 1976
(vii) LPS: Hyperthermia in guinea pigs (160µg/pig)	++	-	Baca and Paretsky 1974a
(viii)LPS: Lipid A	+	+	Schramek *et al* 1978; Schramek and Galanos 1981
(ix) LPS: Dermal Schwartzman reaction	++	-	Paquet *et al* 1978; Schramek and Brezina 1976

carried in laboratory animals such as guinea pigs, mice and hamsters. Fiset (1957) proposed that immune reactions in animals selected for Phase I organisms and eliminated Phase II organisms; he produced some evidence that Phase I antibody would effect such selection in the CE yolk sac, although other workers (Brezina *et al*, 1969) could not confirm this finding.

The discovery of phase variation had a considerable impact on Q fever research as many important biological properties of the organism were found to be related to the presence of Phase I antigen. These have been mentioned in various sections of the Introduction, but are summarised in tabular form at this point (Table 6.1); details of the chemical composition of LPS and the cell envelope follow in a later section.

Eventually, with increasing knowledge of the biology and fine structure of *C.burnetii*, it was recognised that phase variation is equivalent to smooth-rough change in enteric organisms such as *Salmonella spp.* or *E.coli.*

Phase I strains of *C.burnetii*, when first isolated from infected human beings, animals or ticks, have a lipopolysaccharide (LPS) with its full complement of sugar units in the side chains. On growth in the yolk sac, variants emerge with truncated side chains and these overgrow the Phase I organisms perhaps because the synthesis of shorter sugar side chains is metabolically more economical and gives a survival advantage. Phase II organisms are not eliminated by immune mechanisms in the yolk sac as they would be in an immunocompetent animal. After a number of serial yolk sac passages, the Phase II organisms become dominant enough in the population for it to react overall as a Phase II antigen in the complement fixation test. The

number of passages required to reach this stage varies widely between different strains. Herzberg and Urback (1951) found that some of their strains only converted to Phase II after many passages. Berge (1959) (pers. comm. cited in Fiset 1959) found that one strain that was still nonreactive (i.e., still in Phase I) after 68 egg passages. Presumably the "Dyer" strain is another which is slow to show the loss variation.

Although a *C.burnetii* suspension grown in the yolk sac may appear to be in one phase, organisms in the other phase are often present. Phase II preparations thus characterised by CF, when examined with Phase I antiserum by immunofluorescence not infrequently show Phase I organisms, and the organisms in the two phases may be separated by density gradient centrifugation (Hoyer *et al.* 1963). It appears, therefore, that yolk sac cultures of Phase II organisms (by CF test), when inoculated into guinea pigs 'become' Phase I by the proliferation and overgrowth of the residual Phase I organisms that are present; the Phase II organisms initially present being eliminated by the host's immune system.

This explanation is further supported by the finding that a 'pure' culture of Phase II *C.burnetii* obtained by subculturing single plaques produced in monolayers of CE fibroblasts and propagating the clone in CE yolk sac contains no residual Phase I cells and cannot be passed serially in guinea pigs (i.e., it is nonpathogenic) although antibody may be stimulated in the first animals inoculated. (Such 'plaque-purified' or 'cloned' Phase II preparations have been used in the studies described in this thesis).

While the explanation of the host-controlled phase variation in

immunocompetent or incompetent hosts is well described in terms of survival and selection, the exact immune mechanisms involved are only partly defined. *In vitro* experiments indicate that unopsonised Phase II organisms are more readily phagocytosed (by polymorphonuclear leucocytes and macrophages) than Phase I organisms.

The presence of antibody to Phase I antigen greatly enhances uptake and destruction of *C.burnetii* by phagocytes (Wisseman *et al*, 1967, Kishimoto and Walker 1976, Kishimoto *et al.* 1976, 1977). It is also suggested that acute phase reactants (Kindmark and Williams 1990) - normal globulin and complement components (C3b, C4 and C8) - are absorbed by the organism and may facilitate phagocytosis. Nevertheless, antibody to Phase I antigen greatly facilitates the ingestion and destruction of at least some of the coxiellas by macrophages (see below for contradictory evidence on the latter point).

The working hypothesis is, therefore, that the Phase I state is maintained by the immune pressure in the infected host which eliminates Phase II variants as they arise.

There are some problems with this hypothesis. For example, strains of *C.burnetii* isolated from ticks appear to be in the Phase I state but ticks do not have an immune system comparable to that in mammals, although macrophage-like cells are present. Admittedly, isolation of the organism from ticks has generally involved passage of the organism through guinea pigs, hamsters or mice which would select Phase I strains. Presumably the demonstration of Phase II organisms in ticks - if they occur - would require direct IF staining of haemolymph or intestinal smears and/or isolation of the

coxiella in cell culture. This has not been done as far as I am aware.

Again, a direct test of the "immune pressure" hypothesis does not seem to have been made. A population of Phase II organisms does not passage serially in guinea pigs. Kazar *et al.* (1971) showed that Phase II organisms can be converted to Phase I in mice treated with cyclophosphamide, which do not form antibody. However, it is possible that macrophages and other components of the cell-mediated arm of the immune response were still intact in the cyclophosphamide-treated mice and that selection of Phase I organism was effected by them. There is compelling evidence that the limitation and clearance of infection is finally dependent on the cell-mediated immune system although antibody plays some part. This is well illustrated in experimental infection of athymic (nude) mice and their heterozygous euthymic litter mates. Infected euthymic mice cleared *C.burnetii* from the blood and spleen within 14 days. In athymic mice on the other hand, *C.burnetii* could be isolated from blood and spleen for at least 60 days (Kishimoto *et al.* 1978b).

This summary of the basis and significance of antigenic phase variation in *C.burnetii* may be concluded by returning to the opening statement of the chapter - namely, that there appears to be a single antigenic type of *C.burnetii*. Clearly when the antigenic composition of strains from various hosts, in different geographical locations, are compared, due account has to be paid of the antigenic phase of the organism; in essence, strains in Phase I have to be compared.

Fiset *et al.* (1971) examined four strains of *C.burnetii* obtained from different parts of the world. Phase I antigens were found to be identical, as

antibodies against each Phase I antigen were absorbed by homologous and heterologous Phase I antigens. However with Phase II antigens, there were qualitative differences in the antigenic determinants of various strains. Some Phase II strains also appeared to have antigenic determinants more readily available to antibodies than did others, possibly due to variable masking of the Phase II antigen by Phase I antigen, or perhaps more probably, by the variable proportions of Phase I and Phase II organisms in preparations at different yolk sac passage levels.

The conclusion from Fiset's study was that the Phase I LPS of *C.burnetii* strains is of a single antigenic type, at any rate as measured by conventional cross-absorption, serological assays - and that the variability between strains might reside in the Phase II cell proteins. It is possible that the somewhat contradictory reports on differences between strains and serological reactions found by various workers (e.g., Berge and Lennette 1953; Stoker *et al.* 1955) may rest partly on difference in phase antigen state and partly on differences in Phase II protein. This suggestion is reinforced by the fact that analysis of restriction enzyme patterns of the genomic DNA of strains isolated from different sources and locations suggests a strain heterogeneity which might be reflected in variation in Phase II proteins.

Despite the homogeneity of Phase I LPS as judged by cross-protection tests and conventional serological tests, recent studies by Hackstadt (1986) have revealed some subtle differences in Phase I LPS antigenic determinants in strains from different sources. LPS was extracted from prototype strains such as Henzerling and Nine Mile and from a number of strains isolated from

patients with chronic endocarditis, and also from a strain (Priscilla) isolated from the aborted conceptus of a goat. The LPS from these strains was run on SDS-PAGE and transferred to nitrocellulose for immunoblotting with rabbit antisera to the prototype Nine Mile Phase I strain or to Priscilla.

The LPS from Phase I strains from acute infections or other sources (designated 'California, 'Ohio', 'El Tayeb', 'Panama', 'Africa') reacted with the

*Amendment - "whereas the rabbit serum to the Priscilla strain reacted with the homologous antigen and the endocarditis strains.

These differences between the LPS of the two groups were observed with the rabbit antisera but not with broader reacting antisera from human subjects infected in the past. Moreover, cross-protection experiments in guinea pigs showed no difference between Priscilla and the prototype strains (Moos and Hackstadt, 1987). It is concluded that stringent antigenic analysis for small differences between the LPS of different strains of *C.burnetii* probably requires immunoblot analysis of LPS separated from the cells, a different circumstance to antigenic analysis with suspensions of the whole *C.burnetii* cell. It is possible that immunoblot analysis with anti-Phase I LPS and rabbit antisera absorbed with various strains would reveal a number of epitopes in the LPS sugar chains. LPS with truncated sugar chains, extracted from Phase II organisms, is said to react as Phase I even though the immunodominant terminal sugars have been lost (Baca et al. 1980); the finer details of epitope heterogeneity with Phase I LPS require further investigation.

Antibody to *C.burnetii* can be measured by a wide variety of serological techniques. These include tube agglutination, enhanced agglutination by

antiglobulin treatment of antibody-sensitised coxiella cells or by centrifugation of serum-suspension mixtures, agglutination in cups of microtitre plates, radioimmunoprecipitation and other forms of radioimmunoassay. Antibody is also measured by complement fixation, by immunofluorescence on microdots of coxiella fixed to microscope slides, often in small wells in a Teflon coated slide, and by enzyme immunoassay. The latter techniques have the advantage that antibody in various immunoglobulin classes can be measured. IgM antibody to *C.burnetii* Phase II antigen appears early in infection followed by IgM antibody to Phase I and by IgG antibody to Phase II and a little later and variably by IgA antibody to Phase II (for details see Elisberg and Bozeman 1979 and Worswick and Marmion 1985).

In chronic infection, IgM antibody is mostly at low titre or absent, whereas antibody titres in the IgG and IgA classes are high to both Phase I and II antigens (summary: Worswick and Marmion 1985).

Serological reactions with the LPS and cell envelope proteins account for the specific antigenic reactivity of *C.burnetii*. However, the lipid A of the LPS shares antigenic determinants with lipid A of some other organisms. Antiserum to *Salm. minnesota* has been used to detect lipid A as it was unmasked by acid hydrolysis of the sugar residues of the core and side chains of the LPS (Schramek and Galanos 1981). Cross reactions also occur with the lipid A of chlamydia and conceivably with *Legionella* spp. It is possible that with diagnostic antigens made from *C.burnetii* strains with degenerate LPS, serological cross-reactions might occur with antibody from subjects infected with salmonella or chlamydia in the past; it seems important to use antigens

with an intact LPS without exposed lipid A.

6.2 Protective effects of antibody and of vaccines from whole cells of *C.burnetii* or separated fractions or extracts.

In the early work with *C.burnetii*, Burnet and Freeman, probably influenced by the importance of the measurement of neutralising antibody in virus infections, developed a neutralising antibody test for *C.burnetii*. Although this is of little practical value for laboratory serodiagnosis of Q fever, it has provided valuable pointers to the function of antibody in the pathogenesis of Q fever infection and also a way of measuring protective efficacy of antigens and antibody.

Burnet and Freeman (1938b) titrated an infective suspension of *C.burnetii* in groups of mice and killed them some 5-7 days after inoculation. At this time the spleen was enlarged (probably due to the accumulation of lymphoblastic B cells; Khavkin 1972) and the spleen weights were related to inoculum size. Stained impression smears from the spleen showed coxiellas also in numbers related to inoculum size.

Admixture of *C.burnetii* antiserum with a standard dose of the coxiella, modified the disease in the mouse, reducing spleen size and the number of organisms detectable in the spleen smears. The mice were infected despite the modification of the disease so that 'neutralisation' is a less appropriate term than 'protection' (against disease).

Abinanti and Marmion (1957) modified the mouse protection model by titrating the content of coxiellas in the mouse spleen as complement fixing antigen rather than assessing infection by counting organisms in spleen

smears. They further found that good protection was given by antibody against Phase I antigen but substantially less by antibody against Phase II antigen.

These findings with the mouse protection model have been confirmed by other workers (Ormsbee *et al.* 1968; Fiset and Ormsbee 1968; Kazar *et al.* 1973; Cracea *et al.* 1968). Some workers adhered to the measurement of CF antigen in the mouse spleen as described by Abinanti and Marmion (1957); others (Peacock *et al.* 1979) estimated the numbers of coxiellas in the mouse spleens by plaque titrations in CE fibroblasts. Kazar *et al.* (1973) subtitrated spleen suspensions in chick embryo yolk sac.

Cracea *et al* (1968) compared titres of protective and complement fixing antibody in guinea pigs vaccinated with Phase I or Phase II antigens. The vaccinated guinea pigs were challenged (febrile response) with viable *C.burnetti* at varying intervals after vaccination. It was found that resistance conferred by Phase I vaccine to challenge correlated well with the development of protective antibody measured in the mouse protection test and with Phase I CF antibody. Phase II vaccine did not prevent fever on challenge and protective antibody did not develop. Cracea *et al.* (1968) made an attempt to develop a neutralising antibody test in the CE yolk sac. However, Phase I antibody did not inhibit growth of the organism. Similar results were obtained by Kazar *et al.* (1973) who found that Phase I antiserum did not reduce the proliferation of the organism in the CE yolk sac. However, antiserum to Phase II inhibited the growth of Phase II organism in cultures of chick embryo cells and increased the growth of Phase I organisms. Phase I antibody decreased growth

of *C.burnetii* in the spleen of healthy mice but had a much reduced effect in mice treated with cyclophosphamide. Multiplication of Phase II organisms was increased in cyclophosphamide treated mice.

Peacock *et al.* (1979) analysed the protective antibody response after an intradermal booster dose of *C.burnetii* Phase I vaccine and found, using a mouse neutralisation test, that most of the protective activity of the serum was associated with the IgM fraction of the serum and that Phase I IgM antibody would reduce multiplication of *C.burnetii* in the mouse spleen when mixed with the challenge inoculum. It was noted that CF antibody (mostly IgG) was not readily detected in these sera but microagglutination or microimmunofluorescence (IgM) assays showed good titres of Phase I antibody.

6.3 Cell-mediated immunity to *Coxiella burnetii* and its measurement in Q fever cases and in vaccinees

The importance of cell mediated immunity (CMI) in resistance to infection with intracellular organisms and resolution of infection is generally recognised.

The previous sections described the protective effect of antibody to Phase I antigen *C.burnetii* when mixed with living organisms and given to mice or guinea pigs. It was found, however, that the antiserum had to be given before inoculation, or mixed with it; passive immunisation after inoculation was not effective. Furthermore, to be protected the inoculated animal had to have an intact cellular immune system; that is 'protection' was achieved as a cooperative effect between the antibody transferred and the cellular immune system of the inoculated animal.

The experimental model was studied, for example, by Humphries and Hinrichs (1981) who examined the role of antibody during infection with *C.burnetii*. Passive transfer of immune serum 24 hours before challenge with viable *C.burnetii* (Nine Mile Phase I) significantly altered the growth of coxiellas in infected mice. In mice not given antiserum, the numbers of organisms outside of phagocytes decreased 7 days after infection, and by day 10 all rickettsiae were intracellular, with clearance of these organisms beginning during the second week of infection.

In contrast, animals given antiserum cleared the challenge dose within one week of infection. From these observations it was concluded that antibody did not directly affect the course of infection, but accelerated immunological responses (i.e., CMI) that finally controlled the intracellular replication of the organism.

In a series of papers, Kazar *et al.* (1971, 1973, 1975a) explored the role of antibody from a different angle by examining the capacity of antisera against *C.burnetii* to protect mice immunosuppressed with cyclophosphamide (CPA) so that their own antibody production was greatly impaired. Phase I and Phase II strains of *C.burnetii* were used and the proliferation of the coxiella in mice was assessed by staining impression smears of spleen. Antibody responses in the mice were suppressed and there was enhanced multiplication of the organism and increased mortality.

When the Phase I *C.burnetii* inoculum was pretreated with antiserum with antibody to Phase I antigen both CPA and untreated mice were protected to the same degree. However, serum with Phase II antibody, or serum without

antibody did not protect against Phase I organisms. Interestingly, although Phase II organisms multiplied in CPA mice the Phase II antiserum only slightly reduced their multiplication. Phase II organisms 'converted' to Phase I in CPA mice in the absence of an antibody response by the mice.

Similar investigations with CPA treatment in guinea pigs were made by Ascher *et al.* (1980). CPA treated animals, immunised with a purified, formalin-inactivated particulate Henzerling strain, Phase I vaccine, in Freund's incomplete adjuvant, were found to have an increased skin test response lasting several weeks, as well as an enhanced *in vitro* lymphocyte mitogenic response to both *C.burnetii* Phase I and II antigens. Antibody response was reduced, if not totally abolished in these animals, yet they were protected when challenged with living coxiellas, perhaps because delayed type hypersensitivity and a capacity for T lymphocyte mitogenesis was retained.

Meantime, as mentioned previously, Kishimoto's group had shown that the pattern of infection with *C.burnetii* was quite different in euthymic and in athymic (nude) mice with prolonged carriage of the organism in the latter. This is considered in greater detail below in the section on CMI.

Further dissection of the CMI response to *C.burnetii* followed various paths. It was recognised that the macrophage is of central importance as a host cell for *C.burnetii* and the effect was investigated of opsinising antibody, on the one hand, and macrophage activation by immune T lymphocytes and macrophage activators, such as interferon γ, on the other hand. Finally, various *in vivo* or *in vitro* correlates of CMI were sought. The former comprised skin sensitivity tests in laboratory animals or human beings. The latter

centered on macrophage migration inhibition assays in infected laboratory animals and lymphocyte mitogenesis or stimulation in immune human subjects.

Some results from these approaches are now summarised.

Victor *et al.* (1952) found that *C.burnetii* was phagocytosed by polymorphonuclear leucocytes in the presence of Q fever specific opsinins (antibody). The experiments were done before the differences between Phase I and II antigens, and their antisera, was recognised and the antibody specificity of the opsinin is unknown. Wisseman *et al.* (1967) examined the interaction of polymorphonuclear leucocytes with other rickettsiae as well as *C.burnetii.* With the latter he found that Phase I organisms were significantly resistant to phagocytosis as compared with the Phase II. Phagocytosis of Phase I organisms ~~were~~ was markedly enhanced by the presence of Q fever opsinins (antibody), whereas the Phase II organisms were unaffected. Brezina and Kazar (1963, 1965), in the course of studies attempting to relate the phase variation phenomenon of *C.burnetii* to its virulence, also found that Phase I organisms were less easily phagocytosed. Kazar *et al.* (1975b) later extended this work to show that not only was there a quantitative difference between Phase I and II in the number of organisms within a cell, but also that Phase I phagocytosis was increased in the presence of serum containing antibody to Phase I and not Phase II.

In studies with macrophages, as distinct from polymorphonuclear leucocytes Wisseman *et al.* (1967), Brezina and Kazar (1963, 1965) and Kishimoto *et al.* (1976) all concluded that there was a rapid breakdown of

opsinised *C.burnetii* in macrophages. The experience of Hinrichs and Jerrells (1976) is at variance with this conclusion. They found that while pretreatment of *C.burnetii* suspensions with Q fever antibody (prepared in guinea pigs, not specified as to Phase, but probably a mixture of Phase I and II antibodies) certainly increased the phagocytosis of Phase I coxiella by peritoneal macrophages from non-immune guinea pigs, the extent of subsequent intracellular multiplication did not differ significantly ~~significant~~ from that of coxiella pretreated with nonimmune sera. They found however that replication was contained in macrophages activated with culture supernatants (i.e. lymphokines) either from immunocytes from lymphnodes or spleen cells of immune animals, stimulated with antigen *in vitro*, or those from nonimmune animals stimulated with concanavalin A.

Kishimoto and Walker (1976), in the same year, again found, that immune serum increased phagocytosis. However contrary to the findings of Hinrichs and Jerrell (1976), *C.burnetii* cells pretreated with Phase I antibody declined in numbers in the macrophages over 5 days in culture, whereas those treated with nonimmune serum, increased substantially. Peritoneal macrophages collected from animals immunised IP with formalin-killed *C.burnetii* Phase I vaccine were able to kill Phase I organism which had been pretreated with normal, nonimmune serum. [The authors recognise that this last finding is difficult to interpret. The macrophages had been collected 10-12 days after the last IP injection of vaccine. It is possible that persistence of the vaccine antigen in peritoneal macrophages, and the linked CMI response, meant that they were already activated at the time of harvest. Furthermore

although steps had been taken to remove nonadherent cells (i.e., lymphocytes), a few (1% of cells) large lymphocytes adhering to macrophages were seen in the stained smears made to assess the degree of replication of coxiella in the macrophage].

In another report at variance with other workers, Downs (1968) found that Phase I and II organisms multiplied to the same extent in monocytes from nonimmune or immune animals.

Antibody might have a rôle as cytophilic antibody on macrophages, or in antibody-dependent, cell-mediated cytotoxicity (ADCC). Koster *et al* (1984) investigated the latter with a mouse macrophage tumour cell, J774, infected with Nine Mile Phase I strain as a target. Specific cytotoxicity was achieved in the presence of immune human sera, together with effector cells from nonimmune cell donors. Cytotoxicity was dependent on Fc receptors on cells and varied with the titre of antiserum employed. Because it was uncertain whether *C.burnetii* antigen was incorporated into the macrophage cell membrane, it was postulated that during initial cell infection, some antigen remains on the cell membrane after endocytosis, or that small amounts of soluble antigen may leak out of infected cells during late stages of infection, thus making them targets for ADCC.

In extension of the work on the development of the CMI response in Q fever, Kishimoto and Burger (1977) and Kishimoto *et al.* (1977, 1978a) used the macrophage migration inhibition assay (MMI) to chart CMI developing in the guinea pigs after, respectively, exposure to small particle aerosols of viable *C.burnetii* and particulate inactivated Phase I vaccines. In the former they

detected MMI 3 days post-exposure before the emergence of an antibody response, and lasting for a maximum of 42 days. In the latter, when they compared the MMI response with another *in vitro* correlate of cell mediated immunity - the lymphocyte transformation assay - they found that after one vaccine dose the MMI activity was at a peak one week post-vaccination but was short-lived, whereas the lymphocyte response developed more slowly but persisted at low levels for a longer period (35 days after vaccination).

Systematic investigations of the components of the immune response to other rickettsiae (e.g., *R.typhi*) had already shown (summary Wisseman 1978) that antibody would opsonise the organisms and assist their breakdown in macrophage phagolysosomes. Adoptive transfer of T (but not B) lymphocytes from immune to nonimmune animals conferred protection against challenge with living organisms.

In experiments on similar lines, Kazar *et al.* (1977) immunised mice with live Phase I or Phase II *C.burnetii* or with TCA extracts. Adoptive transfer of resistance to nonimmune mice occurred only with spleen cells harvested from mice given live Phase I *C.burnetii*. This transfer could not be effected with peritoneal exudate cells from mice given live Phase I, or killed Phase I organisms. [The inabilty to transfer resistance with peritoneal exudate cells may have been due to the low proportion of lymphocytes in the exudate, as compared with that in the spleen suspension].

Macrophage activation is known to be an important factor for the destruction of intracellular parasites. Activation is mediated by T cells and their secreted products, lymphokines. The work of Hinrich and Jerrells (1976)

on the effects of lymphokines and immune lymphocytes on the replication of the organism in guinea pig peritoneal exudate macrophages has already been described.

It is recognised that macrophage activation occurs in a series of steps. Intermediate levels of activation may be achieved by stimulation with LPS or with suspensions of organisms such as *Corynebacterium parvum.*

Macrophages activated thus far may have significant biological effects but not destroy coxiellas. Full activation for the destruction of *C.burnetii* requires lymphokines from activated T lymphocytes, and interferon γ in particular. Evidence for this came from several directions.

6.4 Effects of interferon on the multiplication of *Coxiella burnetii.*

Kazar (1966) found interferon-like activity in sera from mice after intravenous administration of *C.burnetii.* The activity, measured by the *in vitro* inhibition of viral cytopathic effect, was maximal at 3 to 5 hours after inoculation.

Hahon and Kozikowski (1968) assayed the interferon (not speciated) induced in cell cultures (L929 cells and McCoy cells) with a partially purified suspension of the KD strain of *C.burnetii.* In L929 cells, the organism multiplied but did not induce measurable amounts of interferon. However multiplication of *C.burnetii* in McCoy cells was not achieved, but significant amounts of interferon were induced.

Increasing knowledge of the interferon system identified the main categories of IFNα, IFNβ and IFNγ and the cloning of the genes to produce pure, single species of interferon proteins facilitated definitive experiments of

their effects.

Winkler and Turco (1985) conducted a series of experiments with mouse L929 cells and *R.prowazekii* and showed that conditioned media from mouse spleen cells stimulated with pan mitogens, such as conconavalin A, Staphylococcal enterotoxin A, *C.parvum* or with *R.prowazekii* (spleen cells from immunised mice), all contained a substance which inhibited growth of the rickettsia and cleared infection from the cells. The activity was neutralised by antibody against murine IFNγ. Virus-induced interferon (IFNα) had no effect. Conditioned media from five cloned murine T cells lines, sensitised to bovine serum albumin, and stimulated with this antigen or stimulated with conconavalin A, likewise inhibited growth of *R.prowazekii* in L929 cells and in the murine macrophage line RAW 264. Finally purified murine IFNγ had the same, species-specific antirickettsial effect; no effect was obtained with murine recDNA IFNγ in human foreskin fibroblasts.

Similar experiments by Turco *et al.* (1984) showed that replication of *C.burnetii* was inhibited in mouse fibroblast cells by IFNγ and by other interferons.

It is clear that the formation of IFNγ is of central importance and the "final common pathway" for macrophage activation and the destruction of intracellular pathogens, including *C.burnetii*. It follows that protective immunisation should produce a state of lymphocyte sensitisation or memory that will lead to secretion of IFNγ when the infective agent is encountered after immunisation. This efficacy has been investigated in respect of Q fever vaccine and the results constitute part of this thesis.

6.5 Measurement of cell mediated immunity in man: *in vivo* and *in vitro* correlates.

Despite the clear indication that CMI is of central importance in resistance to rickettsiae and to *C.burnetii*, most attention has been given to measurement of antibody after infection or vaccination as an index of immunity. This reflects, in part, the fact that measurement of CMI is more laborious than measurement of antibody and partly the fact that there is often a partial, but by no means complete, correlation between formation of antibody and CMI.

Moreover, levels of antibody may decay more rapidly than CMI. In laboratory animal models, antibody may be formed on vaccination with certain rickettsial antigens but protection is not conferred unless CMI (measured by skin test) is stimulated by inoculation of the vaccine with adjuvant.

Consequently measurement of CMI in human subjects, particularly vaccinees, is of some importance.

Probably the earliest attempts to measure CMI after infection or vaccination with *C.burnetii* were made by the group at the Rocky Mountain Laboratory, Hamilton, Montana. Lackman *et al.* (1962) gave an intradermal dose of 0.02 complement fixing units as part of a prevaccination 'screen' test. Reactions (erythema, oedema, pain, induration) were read after 48 hours and considered to be similar to DTH in tuberculosis. It was however noted that induration took 5-7 days to develop and results might be slight or negative at 48 hours. The authors also noted that a positive skin was slow to develop after vaccination and might take some months, recalling a similar observation made by Babudieri (1959).

The skin test method was used subsequently by Luoto *et al.* (1963) who found that 80-90% of volunteers inoculated subcutaneously became skin test positive 40 months after vaccination.

Bell *et al.* (1964b) found that 80% of 60 vaccinees given 1 to 3 doses of 10 CFU of inactivated *C.burnetii* intradermally developed a positive skin test after vaccination. In neither of these studies was there more than a general correlation between the development of antibody and a positive skin test; the most sensitive antibody assay was the radioimmunoprecipitation test and the mouse protection test.

It may be noted at this point that emphasis on the various stages of the skin test response and their interpretation has changed slightly since Lackman *et al*'s (1962) initial observations. Two stages are generally observed in an immune subject. Within 48 hours there is often a red erythematous flare with some oedema of the skin test site and perhaps some irritation and pain. The flare may fade and by 5-7 days after injection an indurated, more circumscribed area is present. This has a definite edge and the skin over it may be red-blue in colour. The later reaction, which is considered to be a small granuloma, is taken as an index of immunity or hypersensitivity.

This two-stage reaction has been explored in sensitised guinea pigs by Ascher and colleagues (1983 a,b,c). Guinea pigs were immunised with Phase I and II *C.burnetii* whole cell vaccines, trichloroacetic acid (TCA) extract (TCA-E) and residue (TCA-R) of TCA-extracted cells of Henzerling Phase I strain of *C.burnetii* and the chloroform-methanol extract (CME), and cell residue (CMR) of Ohio strain Phase I cells, all in Freund's incomplete adjuvant. They were

then skin tested with the same preparations.

Whole cell preparations, TCA-E and TCA-R all produced a reaction (DTH) within two days and the reaction increased (in terms of skin thickness) up to day 8 after intradermal inoculation. Interestingly, the Phase II antigen preparation also produced induration. On the other hand, CMR gave an early (1-3 days) reaction but no late indurated, 'granulomatous' reaction.

Other correlates of CMI, measured *in vitro* are the macrophage migration, inhibition (MMI) test and the lymphocyte stimulation or mitogenic assay. There appear to be no reports on the use of MMI to measure CMI to *C.burnetii* in human subjects. Kishimoto and Burger (1977) reported that peritoneal macrophages from guinea pigs exposed to *C.burnetii* showed migration inhibition 3 days after exposure and at a time when there was no circulating antibody to *C.burnetii* Phase I antigen. The same group (Kishimoto *et al.* 1976) found that an inactivated *C.burnetii* Phase I vaccine induced a CMI response in guinea pigs as measured by lymphocyte stimulation and MMI. As stated earlier, Ascher and colleagues also demonstrated positive lymphocyte stimulation responses in their guinea pig experiments.

Heggers *et al* (1974) also used the guinea pig and MMI to assess the CMI response to whole cells of *C.burnetii* Phase I, a trichloroacetic acid extract (a complex of LPS and protein, see next chapter) and also the extract after treatment to remove either the carbohydrate or the protein. Peritoneal macrophages from normal, Q fever non-immune guinea pigs showed no inhibition of macrophage migration with any of the antigens. Macrophages from guinea pigs immunised with inactivated whole cells or TCA extract

showed greatest inhibition (80-90%) when exposed to whole cell or TCA-E; rather surprisingly the degree of inhibition either by deproteinated TCA-E, or the preparation with the carbohydrate removed, showed essentially the same degree of inhibition - 55% - with macrophages from animals given whole cells and 77% with those from animals given TCA-E. (It appears that the various treated preparations were not assayed serologically to see if the antigens had in fact been modified; the chemical analyses did however indicate the carbohydrate and protein had been removed).

MMI estimations were also used to chart the development of the inhibition response after infection of the guinea pigs. This was present at 14 days after infection and rose to a peak by 21 days. There was a response to all antigens, greatest with whole cell antigen and least with the TCA-E with the protein removed. (As the macrophage inhibiting factor is elaborated by T lymphocytes it is presumed that the harvests of peritoneal macrophages also contain significant numbers of lymphocytes which react with antigen presented by the macrophages).

The authors concluded that the carbohydrate determinants of *C.burnetii* are significant in MMI as well as the proteins and draw attention to the role of carbohydrates as reactive antigens in tuberculin sensitivity. They noted that *M.tuberculosis* (BCG) culture filtrates, 97.5% CHO, were reactive both as skin test antigens and in MMI (Godfrey *et al.* 1969).

Slightly later in the same laboratory, Jerrells *et al.* (1975) examined 11 subjects by lymphocyte stimulation assays - 4 cases of Q fever, 3 exposed to *C.burnetii* in the laboratory, one Q fever vaccinee, one subject with a borderline

skin test, and two controls. A whole blood stimulation assay was used but it is assumed that reactions were given by the lymphocytes.

Subjects who had had Q fever or been exposed to the organism all showed raised lymphocyte stimulation ratios with whole cell *C.burnetii* Phase I, or II antigen prepared by TCA-E from Phase I organisms. The controls gave essentially negative reaction profiles.

Deproteinated TCA-E did not stimulate lymphocytes from the past Q fever cases or exposed laboratory workers and similar negative stimulation ratios were obtained with TCA-E from *C.burnetii* Phase II organisms. The latter result is puzzling considering that whole cell Phase II organisms stimulated and the TCA-E II would be an extract of the proteins and residual lipopolysaccharide.

Ascher and colleagues (1983b) also evaluated the immunological responses to an inactivated *C.burnetii* Phase I vaccine, Henzerling strain (National Drug Co - NDCO) in 74 subjects. Peripheral blood mononuclear cells were separated and stimulated with Phase I (National Drug Co. vaccine) and a Phase II vaccine undiluted or diluted in one in 70. Antibody was measured by FIAX immunofluorescence in an automated fluorometer and also by complement fixation. Skin tests were done with a 1 in 382 dilution of the NDCo vaccine giving a dose of 20ng. The volunteer subjects had had contact with Q fever but all were CF negative before skin testing. 38% of the subjects were negative in the skin test and the remainder had varying types of reaction; 49% with longer lasting erythema and induration. There was an overall correlation (r=0.6 to 0.7) between skin test diameter, lymphocyte stimulation

index (LT) and FIAX positivity. It was noted, however, that some skin test negatives were LT positive; a larger number were skin test positive, but FIAX IF antibody negative.

Fifteen subjects were vaccinated, 12 with 6µg of vaccine; 6 with 30µg. Of this group 15 had LT assays performed before and after vaccination, and 10 showed an increase or conversion in stimulation index, mostly to Phase II antigen.

Other studies of lymphocyte mitogenic responses in a larger series of Q fever vaccinees have been published (Izzo *et al.* 1988) but the data forms part of this thesis.

Kazar *et al.* (1984) surveyed skin test reactivity in subjects convalescing from Q fever and vaccinees who had been given a "chemovaccine", prepared by TCA-extracton of *C.burnetii* organisms. In both groups, skin testing with dilute TCA-extract was superior for the detection of immunity than antibody detection (microagglutination), irrespective of the time interval after infection or vaccination. In assessments of post vaccination immunity, there was a high degree of correlation between the skin test and the *in vitro* lymphocyte reaction assay (the latter employed TCA-extracted antigen to stimulate lymphocytes).

Gajdesova and Brezina (1989) continued these investigations of Q fever CMI in convalescent subjects and vaccinees. Individuals vaccinated with the chemovaccine from 2 months to 7 years before testing responded *in vitro* lymphocyte proliferation assays (LSI) only to Phase I *C.burnetii* antigen. The response gradually decreased with time. When compared with skin tests the LSI results were similar to those seen by Ascher *et al* (1983b). In the

convalescent Q fever patients, lymphocyte transformation was detected in all of the cases with a Phase I antigen. Approximately half were reactive to Phase II antigen at several concentrations; the skin test was positive in a majority of cases. It was concluded that antibody response alone was insufficient to induce protective immunity, effective vaccines should stimulate cell mediated immunity, as measured by *in vivo* skin testing or *in vitro* lymphocyte stimulation.

In summary, the reports in the literature indicate that cell-mediated immunity plays an important part in resistance of laboratory animals to disease from *C.burnetii* infection. It is probable that the same situation applies to man.

The degree of activation of the macrophage, the primary host cell for *C.burnetii*, appears crucial to the outcome. There is some conflict in the literature on the point of whether a resting macrophage can destroy *C.burnetii* cells opsinised by Q fever Phase I antibody. There is little doubt that a macrophage activated by gamma interferon (IFNγ) can do so.

There are discrepant results and lack of information on the antigens of *C.burnetii* which stimulate CMI. Macrophage-migration inhibition studies in guinea pigs seem to suggest that both the proteins and the LPS CHO determinants of the organism, and particularly the former, activate macrophages (presumably via the T lymphocytes that are present in peritoneal macrophage harvests). Studies with spleen cells or peripheral blood lymphocytes suggest that the proteins of the organism but not the LPS CHO provide critical T cell epitopes.

The role of antibody to Phase I antigen is unclear. It provides 'protection' in mice with an intact cellular immune system, but its precise function is unknown. It facilitates rapid uptake of the organism, and may facilitate rapid presentation of antigens along with class II MHC for stimulation of T lymphocytes, or there may be some other function.

In this chapter, the antigenic composition of strains of *C.burnetii* have been considered and an account of assay methods for measurement of antibody and cell mediated immunity to *C.burnetii* has been given.

Much of the work on the composition of the cell envelope of *C.burnetii* has been done with the objective of relating its structure to immunological function and pathogenesis of Q fever rather than from the standpoint of the structure of the organism as such. Consequently, the structure of the cell envelope is now reviewed next to the section on antigenic structure and activity as protective immunogens.

CHAPTER 7

THE CELL ENVELOPE OF *COXIELLA BURNETII*: LIPOPOLYSACCHARIDE AND PROTEINS: IMMUNOLOGICAL IMPLICATIONS

The lipopolysaccharide and cell envelope proteins of *C.burnetii* are of central importance for the immune response in Q fever and in its modification or modulation.

7.1 Lipopolysaccharide of *Coxiella burnetii*

Once electron microscope studies of *C.burnetii* had established that the cell envelope of the organism had features resembling that of *E.coli*, the way was open to explore the composition of the lipopolysaccharide (LPS) by fractionation and biochemical analyses with techniques well validated with *E.coli* and other enteric commensals or pathogens. A major thrust of the work extending from about 1962 to the present time, was directed to detecting differences in the LPS from *C.burnetii* in Phase I or Phase II antigenic states which had been defined by Stoker and Fiset a few years before (reviewed in Chapter 6) and explaining antigenic differences in terms of a biochemical composition. Part of the motive force for the work arose from the need to prepare less reactogenic vaccines and therefore to define the protective immunogen free from reactogenic components.

With this end in view, Anacker and his colleagues at the Rocky Mountain Laboratory (Anacker *et al.* 1962) prepared cell walls, cytoplasm (protoplasm) from disrupted coxiella and also trichloroacetic acid extracts (TCA-E) by the

method of Boivin *et al* (1933). These were tested for serological activity, assayed in the guinea pig-fever model for protective efficacy, and also titrated for capacity to produce a delayed hypersensitivity reaction in the skin of sensitised rabbits.

Whole organisms and the separated cell envelopes were highly protective immunogens whereas the cell cytoplasm had little or no protective activity. TCA-E was also protective but weight for weight, significantly less - about 10 times - so than the whole cell preparation or the cell envelopes. At about the same time, TCA-E was also found to be serologically reactive (and protective) by Brezina and Urvolgyi (1962). Subsequent work showed that the TCA-E was a complex of LPS and approximately nine proteins, the exact composition depending on the conditions of extraction (see below).

The following year, Anacker and his colleagues (Anacker *et al.* 1963) reported work with phenol-water extracts of TCA-E prepared by the method of Westphal *et al* (1952) for extraction of endotoxin.

The aqueous phase of the phenol-water extraction retained the Phase I serological activity at about the same titre as the TCA-E but unlike the latter it had no Phase II reactivity (presumably due to separation of the proteins which comprise or make a major contribution to Phase II serological reactivity). Guinea pig protection tests showed that whole cells and TCA-E were protective as before, but the water phase of the phenol-water extract - the LPS - had no protective activity and did not provoke antibody on guinea pig inoculation (i.e., it was a hapten). The phenol-water extract did not produce skin reactions in sensitised rabbits and it was surmised that the protein components of whole

cells and of the TCA-E was responsible for the delayed hypersensitivity reactions.

Chemical analysis of whole cells, TCA-E and phenol-water extract showed that the latter had 3-4 times less nitrogen, and approximately 4 times more carbohydrate than the whole cell. The residual nitrogen in the extracted LPS is presumably from nonprotein sources such as amino sugars or complex lipids.

Once again, Brezina *et al* (1965), working on parallel lines, also found that phenol-water treatment of *C.burnetii* extracted serologically active, haptenic material and that it was not protective in mice unless a prolonged course of immunisation was given (Brezina and Pospisil 1970, Brezina *et al.* 1970).

Ormsbee *et al* (1962) used two nonaqueous organic solvents, dimethylsulphoxide (DMSO) and dimethylacetamide (DMAC), to extract serologically- active material from the whole cell of a Phase I strain (Ohio 314) and a Phase II strain (Nine Mile, 17th yolk sac passage) of *C.burnetii.*

DMSO extracts of Phase I organisms had high serological CF activity with phase I antisera, a reduced nitrogen content, and protected guinea pigs against developing fever on infective challenge. Extracts from Phase II organisms were much less active in terms of complement fixing units per mg N, or dry weight, and also much less potent in protection. Similar results were obtained with dimethylacetamide; neither extract produced a hypersensitivity reaction in the skin of a hypersensitised rabbit.

The DMSO and DMAC extracts were said to be free of protein as judged

by UV spectroscopy. This aspect is somewhat confusing. The extracted material would be largely LPS and clearly there is some conflict with the results of Anacker *et al.* (1962, 1963). The DMSO and DMAC extracts behaved more like TCA-E; the presence of at least small amounts of Phase II antigen (protein) may be deduced from the formation of CF antibody in those guinea pigs given 234µg of DMSO extract of Nine Mile Phase II organisms. It may be surmised that these amounts of protein were sufficient in the hapten-carrier relationship to stimulate protective immunity.

Some years later, Hackstadt *et al.* (1985) repeated the DMSO extractions of Nine Mile Phase I coxiella and found that in terms of percent dry weight, the extract contained 23% of protein whereas the water phase of the phenol-water extraction contained <1%. The DMSO extracted more material in total than TCA - 32mg per 100mg dry weight of *C.burnetii* Phase I cells as against 7.1mg percent.

After these early observations, which had the great merit that protective efficacy was measured in animals, in addition to assays for serological activity, several groups of workers in the USA and a collaborative group in Slovakia and Germany have explored the chemical composition of the LPS in terms of the sugar units in the side chains, the presence of Lipid A, the composition of the core and the nature of the associated fatty acids.

As the lipopolysaccharide (LPS) of *C.burnetii* resembles that of gram-negative enteric bacteria, Phase I-II variation was considered in terms of a smooth-to-rough variation, dependent on the length of the polysaccharide side chains (Baca and Paretsky 1974a,b, Chan *et al.* 1976, Schramek and

Brezina 1976, Schramek and Galanos 1981, Baca *et al.* 1980, Schramek and Mayer 1982, Amano and Williams 1984).

Investigations were complicated by the fact that some preparations of *C.burnetii* reacting as Phase II in overall serological tests, contained a residue of Phase I cells. For accurate analyses it was necessary to work with a cell population in either Phase I or Phase II and to take cognisance of the fact that LPS of intermediate structure between Phase I and Phase II is sometimes encountered.

Initial attempts at chemical characterisation of LPS from both Phase I and Phase II organisms revealed a variety of sugars and fatty acids characteristic of gram-negative organisms. Sugars such as vibritol, xylitol, mannitol, sorbitol, D-glycero-D-mannoheptose, plus several unidentified sugar residues were detected on chromatography by several groups (Baca and Paretsky 1974 a,b, Schramek and Mayer 1982, Schramek and Brezina 1976, Dahlman *et al.* 1986, Chan *et al.* 1976, Amano and Williams 1984). Schramek *et al.* (1985) identified 6-deoxy-3-C-methyl-hexose and 3-L-(hydroxymethyl) - pentose as two unusual sugars located at the terminal positions of the side chains of the LPS. Fatty acids (isomyristic, palmitic and B-hydroxy-myristic acids) were also detected in Phase I LPS.

Glucosamine, a component of Lipid A, is present and a keto-deoxyoctonic acid (KDO)-like compound was isolated from Phase I LPS (Schramek and Mayer 1982, Amano *et al.* 1987). Amano and Williams (1984) also found a Lipid A in *C.burnetii* which was considered to be different in chemical composition and structure from the gram-negative bacterial Lipid A.

At first, chemical comparison of Phase II LPS with that of Phase I by Baca *et al.* (1980) concluded that the sugars present in Phase I LPS were also detected in Phase II LPS, but with some ~~quantative~~ quantitative and qualitative differences.

A reexamination of the matter by Schramek and Mayer (1982) using strains strictly in Phase I (early egg passage) or Phase II (late egg passage, plaque purified) brought out clear differences between the two phases with Phase I LPS having at least eight sugar units in the side chain whereas Phase II had two. Studies by Amano and Williams (1984), in general, supported those of Schramek and Mayer (1982).

In summing up their studies over several years on composition of sugar chains in *C.burnetii* LPS, Schramek *et al* (1985) conclude that mild acetic acid hydrolysis (1% acetic acid, 100°C, 15-60min) markedly reduces the serological reactivity of Phase I antigen in parallel with or even before the exposure of the core and Lipid A (detected by serological cross-reactivity with antiserum to *Salm.minnesota* Lipid A). During this reduction of activity two unusual sugars - (1) 6 deoxy-3-C- methylhexose and (2) a 3-C-(hydroxymethyl)pentose - are liberated. Comparisons with authentic standards and by various thin layer and gas chromatographic techniques showed sugar 1 (X in the paper) to be 6-deoxy-3-C- methylgulose (i.e., hexose in the pyranose formation; designated *virenose*, because previously isolated from the anti-tumour antibiotic virenomycin) and sugar 2 (Y in the paper) to be 3-C(hydroxymethyl)-lyxose (pentose in furanose form, designated *dihydro-hydroxystreptose* as previously isolated from hydroxystreptomycin). Both sugars, branched at carbon 3, are probably in terminal positions on the sugar chains as judged by products

liberated on acid hydrolysis after methylation. The sugars have not been found so far in prokaryote LPS although branched sugars have been found in *Yersina pseudotuberculosis*.

The structure of the two branched sugars with two axial OH groups in C1 conformation, permits oxidation with periodate which destroys Phase I serological activity to some extent. Both sugars have structural features which increase the rate coefficient for mild acid hydrolysis of the glycosides.

The 'rough' LPS from *C.burnetii* fully in Phase II was considered to have truncated sugar chains with 2 neutral sugar units - D-mannose and D-glycero-D-mannose-heptose (Schramek and Mayer 1982).

The findings of the other main group in the field - J.C. Williams and colleagues at Fort Detrick - presented a more complex picture (Amano *et al.* 1987). They reported the presence of a unique disaccharide, galactosaminouronyl-(1-6)-glucosamine with a molecular wt of 354 in Phase I LPS which was not described by Schramek and Mayer (1982). They did, however, support the latters' conclusions that virenose and dihydrohydroxystreptose were present. Smooth LPS contained all three sugars and gave a major 10.5KDa band on polyacrylamide gel electrophoresis (PAGE). Heterogeneity was observed in the PAGE patterns of LPS from the nine strains of *C.burnetii* examined. Phase I strains gave LPS with a variable number of bands between 8.2 and 17.2 or 23KDa markers. Phase II LPS was uniformly at the bottom of the gel around 2.6-4.5KDa, a position also occupied by rough LPS from *Salm.typhimurium*. The 'ladder' effect observed with LPS from enteric organisms was observed to only a limited extent with Phase I LPS, particularly

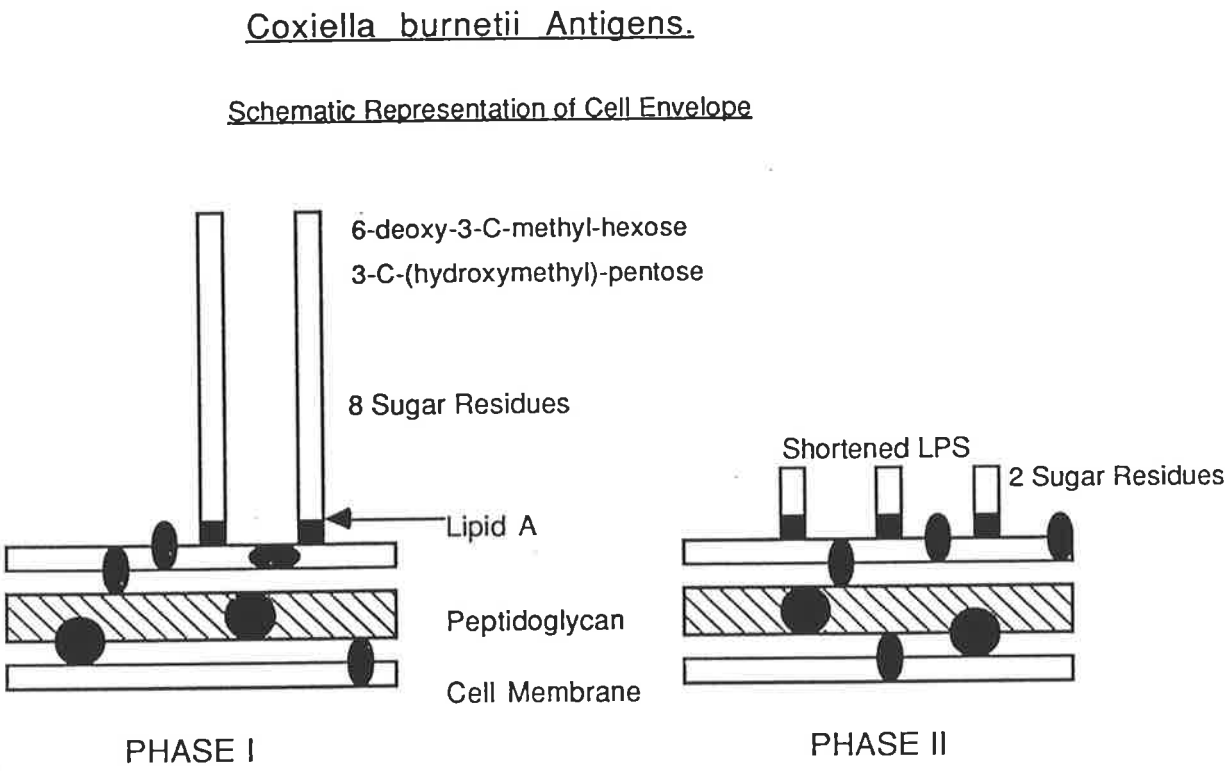


Figure 7.1. Schematic representation of cell envelope of C.burnetii in Phase I and Phase II antigenic state. Dense black ovoids or spheres represent cell proteins.

with a concentration of 17.5% acrylamide in the gel.

These authors also noted that the KDO-like component of *C.burnetii* was different from that of enteric organisms with dissimilar chromatographic mobilities and resistance to hydrolysis by acetic acid and HCL. Content of KDO was variable between strains at 18-54nmol/mg dry wt, with one strain giving a very low value at 4. The presence of glucosamine and B-hydroxymyristic acid indicated the presence of Lipid A. The antigenic specificity of the disaccharide, galactosaminuronyl-(1-6)-glucosamine, was not determined and its contribution to the Phase I antigen state remains to be determined.

Overall, the results from the two groups are in line with the earlier findings of Baca *et al.* (1980) who found that total carbohydrate in Phase I LPS was approximately twice that in Phase II LPS although levels of KDO, protein and phosphorous were similar.

The pattern of differences between the cell envelopes of Phase I and Phase II organisms are depicted diagramatically in Figure 7.1

Most recently, other important contributions to the analysis of *C.burnetii* LPS and protein have been made by Hackstadt and colleagues at the Rocky Mountain Laboratory, Hamilton, Montana, USA.

These studies have been directed more to the comparison of band patterns of proteins and LPS from Phase I and Phase II organisms on PAGE and by immunoblot with various antisera to *C.burnetii.* Hackstadt *et al.* (1985) compared the patterns of Phase I and II LPS with that of solubilised and Proteinase K treated whole cells. Silver stained PAGE gels of Phase I LPS

showed semicontinuous bands over the region from 10KDa to 25KDa and immunoblot showed components in the same areas but also extending up to the 43KDa zone. Phase II LPS, both by silver staining and immunoblot, showed a compact band at 2-6KDa.

[The wide 'spread' of Phase I LPS through the gel, not detected by silver staining, but only apparent by immunoblot has clear implications for analysis of band slices of the gel undertaken to define the immunological activity of proteins].

The striking differences in band patterns between Phase I and II LPS contrasts with the polypeptide profiles obtained by these workers with Nine Mile Phase I and II organisms. Both phases showed closely similar polypeptide patterns with numerous strong bands between 10 and 18KDa, again around 25KDa and above 43KDa. There were some bands around 18KDa in Phase I preparations which were not present in Phase II but this was attributed to the changed comigration patterns arising from the differences in LPS. The overall pattern was confirmed in autoradiographs of radioiodinated surface proteins of the two phases, labelled by the lactoperoxidase method (Other valuable observations in this paper include endotoxin estimations).

Further studies by Hackstadt (1986) brought out the interesting fact that Phase I LPS from prototype strains of *C.burnetii* (Nine Mile) and strains isolated from cows milk or ticks, differed from that isolated from strains from endocarditis patients and a strain (Priscilla) from an aborted goat placenta. The differences were most marked by immunoblot and some of the faster migrating bands were shared between the two groups. The findings

presumably have implications for differences in sugar unit composition but this point was not investigated.

7.2 Endotoxic and other physiological effects of *Coxiella burnetii* LPS

Several groups of workers have reported that Phase I LPS will gel amebocyte lysates from the horseshoe crab (*Limulus polyphenus*) (Paquet *et al.* 1978, Hackstadt *et al.* 1985). Baca *et al.* (1980) quantified the reactions with Phase I and II LPS and found that *E.coli* LPS and Phase I LPS had similar concentration-response curves whereas Phase II LPS had a flatter response curve and was active only at much stronger concentrations.

Phenol-water extracts of Phase I *C.burnetii* have been shown to induce pyrexia and weight loss when injected into laboratory animals (Baca and Paretsky 1974a, Schramek and Brezina 1976). Biochemical changes in the animals included an enlarged liver with increased lipid content, and an increased incorporation of orotate into the 18S and 28S species of RNA in the liver. An increase of cortisol levels in plasma and liver were also observed. Changes induced by LPS were in many ways similar to those seen during Q fever infection, leading to the belief that endotoxic effects during Q fever are due to release in the host of a toxic LPS complex.

Subsequent studies by Amano *et al.* (1987) in galactosoamine-treated mice demonstrated at least three endotoxic strengths amongst LPS from nine *C.burnetii* strains in both Phase I and Phase II. Endotoxic activity was not related to antigenic phase and it was therefore proposed that components such as lipid A mediated toxicity. Nevertheless, overall, the toxicity of *C.burnetii* was

much less than that of *E.coli* and *S.typhimurium* in similar systems, a phenomenon reported previously by Schramek and Galanos (1981) in comparisons with *S.minnesota*. Hackstadt *et al.* (1985) found that in the chicken embryo model, *C.burnetii* Phase I LPS was 3,000 times less toxic by weight than LPS from *Salmonella* spp.

C.burnetii LPS resembles other endotoxins in inducing the Shwartzman reaction in rabbits (Schramek and Brezina 1976, Paquet *et al.* 1978). In the Shwartzman reaction a small dose of endotoxin is injected in the skin of a rabbit and a mild inflammatory reaction occurs. Twenty four hours later, the same dose of endotoxin is injected intravenously and the original skin injection site becomes haemorhagic within a few hours; histologically there are leucocyte and platelet thrombi, particularly in venules. It seems likely that this is one manifestation of the interaction of LPS with the macrophage which results in the liberation of various cytokines such as TNF and IL-1, a process augmented by the 'priming' of the macrophage with IFNγ. The TNF liberated plays an important part in production of shock, gastrointestinal haemorrhage, fever, transient leukopenia followed by leucocytosis, hyperglycemia, haemorrhagic necrosis of tumours (hence TNF, tumour necrosis factor), various circulatory disturbances and vascular hyperreactivity to adenergic drugs. The IL-1 also acts centrally to produce fever.

On a less acute basis, TNF also plays a significant part in granuloma formation and in alteration of lipid metabolism and inhibition of lipid storage in fat storage cells (hence cachexia and cachexin).

7.3 *C.burnetii* cell proteins and peptidoglycan

Hackstadt and his colleagues (Hackstadt *et al.* 1985) extracted *C.burnetii* polypeptides by boiling purified whole cells in Laemmli buffer with or without 2-mercaptoethanol, electrophoresis on a 12.5% sodium dodecyl sulphate - polyacrylamide gel and staining with Coomassie blue or silver nitrate. Bands were also detected by autoradiography after surface labelling of the proteins with ^{125}I by the lactoperoxidase method. The Coomassie blue stained gels showed at least 30 protein bands of varying definition and intensity. Major groupings of intense bands were seen around and between the 14.3 and 18.4 KDa molecular wt markers, around 25.7 KDa (particularly in the radio-iodinated preparations) and at and above 43 KDa. The differences between the profiles for Phase I and Phase II cells was not striking although there were some bands - probably 2-4 in number - present in the preparations from Phase I cells which were not seen in those from Phase II cells. These differences were more evident in the iodinated preparations. The differences were not interpreted as differences in the species of proteins *per se*, but as mentioned previously as due to differences in the comigratory patterns with the different LPS from the two antigen phases.

Digestion of the solubilized, cell lysates with proteinase K appeared to remove all protein bands leaving LPS bands with the characteristically different migration patterns of Phase I, Phase II and intermediate (Cr) LPS.

Using similar SDS-PAGE techniques Novak and Brezina (1989) investigated the protein and LPS profiles of 8 strains of *C.burnetii*, all in Phase I, and of different geographic origin.

There were 18 distinct proteins (Zones: 77, 60, 57, 55 (double line) 49, 46, 44, 27, 20.3 to 18.5, 17.3, 16.2, 15.7, 13 and 12.5 KDa) shared between all strains. In general, the groupings - i.e., numerous bands 12 to 20 KDa, around 27 and 44 to 77 KDa - were not too dissimilar to the findings of Hackstadt *et al.* (1985). In total, counting distinct and indistinct bands, about 40 bands were visualised in gels stained with Coomassie blue and 60 in those stained with silver nitrate.

Studies by Williams and his colleagues since 1981 have explored rather different aspects of *C.burnetii* cell protein composition. Williams (1981) examined radioiodinated surface protein, separated on PAGE, and found at least 35 components of molecular mass, 150-11.8 KDa.

In studies directed to the composition of the cell wall of whole cells of *C.burnetii*, and cellular variants - viz: large cell variants (LCV), small cell variants (SCV) and endospores - the population of *C.burnetii* cells was first fractionated into the various forms, preparations checked by electron microscopy, and chemical analyses performed. Whole cells had a total protein composition of around 50-57% of dry weight. The amino acid or amino sugar composition resembled those found in gram-negative bacterial cell walls, with muramic acid, glucosamine and meso-diaminopimelic acid; the content of muramic acid was highest in small cell, compact variants. The proteins associated with the peptidoglycan were resistant to digestion with trypsin, proteinase K and protease VI. (Presumably these proteins are not extracted in whole cell lysates as Hackstadt *et al.* (1985) found all proteins to be digested by Proteinase K).

The large cell variants (LCV) contained less peptidoglycan (PG) than small cell variants (2 versus 32%) and there was evidence of less cross-linking in the PG of LCV as compared with SCV. Examination of the various fractions as immunogens in rabbits indicated that the proteins associated with the PG stimulated only a Phase II antibody response, whereas material from the various 'infraforms' stimulated both Phase I and II antibody, indicating the presence of residual LPS which was also signified by the presence of KDO-like compound in all fractions.

The fatty acid composition of the cell wall comprised saturated, unbranched fatty acids C16, C18 and C20, 25-29% and branched chain fatty acids, anteiso C15 and C17, 66-72%. These fatty acid profiles resembled those of some gram-positive bacteria and *Legionella* spp. Once proteins are removed from the peptidoglycan the latter is sensitive to lysozyme (muramidase) (Amano and Williams 1984).

Examination of *C.burnetii* proteins by cloning genome fragments and expressing gene projects is at an early stage.

The impetus to do so is partly related to the possibility that one or more cell proteins might be suitable as a subunit vaccine (Williams and Stewart 1984).

Vodkin and Williams (1988) prepared a gene library from the DNA of *C.burnetii* in the cosmid vector pHC79. Colonies of *E.coli* transfected with the cosmid were found to react with *C.burnetii* antisera from immunised mice, a subject vaccinated against Q fever and a subject with chronic Q fever endocarditis, ~~all reacted with a particular *E.coli* colony~~.

Isolation of this clone showed expression of two serological reactive proteins of 62 KDa and 14 KDa molecular mass. (In view of the large number of proteins in the *C.burnetii* cell and the reaction of many of them with *C.burnetii* antisera in immunoblots, it is perhaps surprising that only one seroreactive clone was obtained from the library).

Sequencing of the gene showed two ORF coding for proteins of 10.5 and 58.5KDa. An associated transcriptional control resembled that of a heatshock (protein) promoter. Further sequence comparisons revealed substantial homology (~50%) with sequences for heat shock proteins in *M.tuberculosis* and *M.leprae* where they are immunodominant proteins; also heatshock protein in *Pseudomonas aeruginosa.*

The 62KDa protein is immunogenic in mice and its use as a subunit vaccine is suggested. However, evidence of protective ability (as distinct from immunodominance) was not produced and appears not to have been published during the 3 years since. (The matter of the protective immunogen forms part of the subject matter of this thesis and is considered in the Discussion sections of the "Results" chapters and in the General Discussion).

Other publications from the group (Williams *et al.* 1984) identified a 29.5 KDa surface protein on Phase II *C.burnetii* reacting in the immunoprecipitation test with a mouse monoclonal antibody. This protein was apparently masked in Phase I organisms but became accessible after removal of some of the LPS layer with chloroform-methanol.

Other studies of *C.burnetii* proteins have been connected with the extraction of LPS - protein complexes from the organism for use as subunit

vaccines. Slovak workers and others in Eastern Block countries have made much use of trichloroacetic acid (TCA) extracts of the organism as a chemovaccine. Lukacova *et al.* (1989) made a systematic survey of the composition of the extracted material with 10% TCA and various times and temperatures of extraction. Forty-five minutes extraction at 0°C gave the maximum yield of material (16%) with the highest levels of protein (16%) and sugars (40%). PAGE examination showed nine or so strong protein band mainly between 14 and 24 KDa and around 55 to 60 KDa. Hackstadt et al. (1985) found similar polypeptide profiles in TCA extracts and also 20 or more bands in material extracted with dimethylsulphoxide. The properties of cells and material extracted with chloroform methanol are considered in the section on vaccines.

7.4 Conclusions: serological reactivity and components of *C.burnetii*

As an overall generalisation it is correct to say that the Phase I antigen of *C.burnetii* is the lipopolysaccharide (LPS) and the Phase II antigen is one or more of the proteins associated with the LPS or other parts of the cell wall of the organism.

LPS extracted by the hot phenol-water method from Phase I organisms fixes complement or reacts in other serological techniques (EIA, gel diffusion) with antisera from hyperimmunised rabbits or other experimental animals; also with late convalescent sera from infected guinea pigs or with human patients suffering from chronic Q fever endocarditis.

Early post infection sera from guinea pigs, or early convalescent phase

sera from acute Q fever cases in human beings, do not fix complement with Phase I organisms or Phase I LPS. The proteins of coxiella are more immunogenic than the LPS and the first CF antibody response is to the former, and may be detected by Phase II organisms which allow access of complement fixing IgG antibody to the *C.burnetii* proteins, without steric hindrance from the longer LPS sugar chains found in the Phase I organisms.

This central pattern underlying the Phase I to II antigenic change is less clear cut in some details and requires qualification.

Hackstadt *et al.* (1985) showed that LPS from Phase I and Phase II organisms reacted quite differently in immunoblots developed with antisera from rabbits hyperimmunised with Phase I or II whole cells. Phase I LPS showed numerous reactive bands between 4.3 and 43 KDa and beyond, whereas Phase II LPS showed one homogenous band between 3 and 6 KDa reacting only with Phase II antiserum.

Baca *et al.* (1980) found that Phase II LPS did not react with early post infection guinea pig antisera in the complement fixation test but did react in the CFT with a rabbit antiserum to Phase I and II. Double immunodiffusion tests (Ouchterlony) showed reactions of identity between Phase I and Phase II LPS. This evidence conflicts with the findings of Hackstadt *et al.* (1985) but may be due to technical factors arising from the different sensitivities of immunoblot and complement fixation techniques.

The distinction between Phase I and Phase II antigenic states is also influenced by the type of serological assay and the immunoglobulin class of antibody. Complement fixation with sera from acute Q fever and Q fever

vaccinees mostly measures IgG antibody. In order to fix complement, IgG molecules need to bridge two antigenic determinants and the union between IgG Phase II antibody and Phase II cell proteins is hindered by Phase I LPS side chains. On the other hand, IgM antibody to Phase I antigen is formed early after infection or immunisation and may be demonstrated by agglutination (Fiset and Ormsbee 1968) or immunofluorescence assay (Worswick and Marmion 1985). The bound IgM antibody does not fix complement (Worswick and Marmion 1985, Schmeer *et al.* 1984).

It seems probable that many of the cell proteins are immunogenic and probably act as "Phase II" antigens. Thus Hackstadt *et al.* (1985) found that whole cell lysates from Phase I and Phase II organisms reacted by immunoblot and gave bands around 16 KDa, 18.4 KDa, 25.7 KDa and a series of faint bands up to and beyond 43 KDa. These reactivities were removed by proteinase K digestion. It does not follow, of course, that all of these proteins would be accessible at the surface of Phase II organisms used in a complement fixation test.

Williams *et al.* (1984) prepared monoclonal antibodies (mAB) against chloroform-methanol extracted *C.burnetii* cells. Two mAB were eventually obtained, one directed against Phase I LPS, the other against a Phase II protein of 29.5KDa. The implication was that the latter was "the" Phase II protein but this seems unlikely in view of Hackstadt's results.

In summary, therefore, Phase II serological reactivity is predominantly that of the *C.burnetii* cell proteins, particularly when assayed by complement fixation with early post infection or early post immunisation antisera. The organism - designated overall as Phase II antigen mosaic - has components of

LPS which share epitopes with Phase I LPS. However, this relationship needs further definition, particularly investigation of possible changes in LPS band reactivity after periodate or periodic acid treatment.

Phase I serological reactivity appears to be exclusively an attribute of LPS side chains and is removed by treatment with periodic acid oxidation of the terminal sugar units or hydrolysis of the glycosidic linkages with weak acid.

CHAPTER 8

CELLULAR IMMUNE REACTIONS TO *COXIELLA BURNETII* IN COMPARISON WITH THOSE TO OTHER INTRACELLULAR MICROBIAL PATHOGENS

Coxiella burnetii resembles other intracellular microbial organisms whose survival is dependent on the ability to replicate in mononuclear phagocytes. It replicates in the phagolysosome of the latter, as do some other obligate intracellular pathogens, and is thereby protected from the host's humoral and some cellular-immune reactions, thus creating a special problem for the immune host (Nacy *et al.* 1981, 1982). Other intracellular pathogens include *Mycobacterium* spp, *Brucella* spp, *Listeria monocytogenes*, chlamydias, rickettsias, *Legionella pneumophila*, *Leishmania*, *Trypanosoma* and *Toxoplasma*. These pathogens have been described as granulomatous, - i.e., eliciting granulomatous tissue responses - resulting in cyclic systemic disease with a pattern of chronic course and accompanied by a delayed-type hypersensitivity to the antigens of the organism (Hahn and Kaufmann, 1981). Detailed histological investigations of the lesions induced by the various *C.burnetii* strains, in animal models (Ascher *et al.* 1983) and human patients (Srigley *et al.* 1985) reveal that this description is also applicable to *C.burnetii*.

8.1 Immunological aspects of intracellular infections

The involvement of the various components in the immune response to *C.burnetii* and other intracellular pathogens is not limited to one or two classes

of cells. Hahn and Kaufmann (1981) point out that members of distinct T cell subsets do not exert their function in an isolated way. They are linked in a homeostatic system consisting of regulatory loops in which one cell type modifies the activity of others. Thus, the qualitative outcome of an immune response is determined by interactions among members of different T cell subsets, macrophages and humoral factors.

The importance of T cells in resolving infection with intracellular organisms was highlighted in the work on mycobacteria in athymic mice. Mycobacterial infections in such mice induce a chronic illness, with persistence of bacteria in internal organs and the absence of granulomatous lesions (McGregor *et al.* 1973, North 1974). Development of antimycobacterial immunity is mediated by immunologically-committed thymus-derived lymphocytes and the actual immune reactions eliminating the organisms are executed by activated macrophages, with each part being dependent on the other for the expression of an effective immune response. This need for cooperation between mononuclear phagocytes and specific T lymphocytes in the immunity to intracellular bacterial infections has been shown - e.g., in the experimental model for *Listeria* spp., in which mice were pretreated to eliminate mononuclear phagocytes and then injected with sensitised (immune) spleen cells. After this adoptive transfer they were, however, unable to resist infection (Hahn 1975). These experiments indicated that bone marrow was an essential element in cell-mediated antibacterial immunity, since the immunity mediated by specifically committed lymphocytes was ultimately dependent upon the cells whose precursors came from the bone marrow.

The production of lymphokines from specifically activated T cells was demonstrated to be a necessary part of the cell-mediated immunity to facultative or obligate intracellular organisms. This has been shown *in vitro* by Kostiala *et al.* (1976) and Jungi and McGregor (1977) and *in vivo* by Salvin *et al.* (1973) and Kaufmann *et al.* (1975).

8.2 Lymphocyte activation

The activation of antigen specific T lymphocyte subsets, whether of the helper (CD4+) or suppressor (CD8+) type in anti-coxiella immunity is not well understood. It is known that T cells mediate the immune response, but the subset involved is still unknown. Protection mediated by either CD4 or CD8 subset must involve the activation of macrophages.

It is reasonable to believe that T lymphocytes expressing either the CD4+ or the CD8+ marker form an integral part in the chain of reactivity (to microbial antigens), since recent experiments on the quaternary complex involved in T cell activation suggest that CD4 and CD8 molecules may provide an activation signal to resting T cells as soon as the T cell-antigen-receptor (TCR) is in close proximity to CD4 or CD8 during ligand binding (Emmrich 1988). The TCR recognises peptide fragments from foreign antigens in association with Major Histocompatibility Complex (MHC)-encoded molecules. Since the TCR is associated on the surface of T cells with the CD3 antigen, in a TCR-CD3 molecular complex, the primary pathway of antigen-mediated activation proceeds via this complex, consequently triggering lymphokine production, the expression of new surface markers, blastoid formation and cell

division.

It is likely that a wide range of cells become activated during infection. This is exemplified by the work of Carl and Dasch (1986a,b) who characterised a population of human lymphocytes which were positive for CD3 and CD8 cell markers and capable of lysing typhus group rickettsia-infected target cells. These cells, derived from PBMC of immune individuals after stimulation with antigens of typhus rickettsias or by stimulation with IL-2, were classified as lymphokine-activated killer (LAK) cells. Monoclonal antibodies (mAB) directed against the CD3 antigen were able to inhibit lysis. Other workers, in similar systems, using different mAB have shown that mAB directed against the CD8 can inhibit target recognition by cytotoxic lymphocytes (Meuer *et al.* 1982, Moretta *et al.* 1984). Lysis of typhus rickettsia infected cells (targets) by cytotoxic effector cells was not HLA-restricted, and were presumably LAK cells rather than cytotoxic lymphocytes. Lysis was not inhibited by mAB recognising specific epitopes of the CD4 or CD8 surface antigens; i.e., the epitopes recognised were not involved in the recognition of T cells for cytolysis.

Mustafa *et al.* (1986) generated T cell clones from BCG-immunised healthy individuals. The majority of these clones grew in the presence of BCG, together with adherent antigen presenting cells (APC) and exogenous IL-2. Several clones proliferated with BCG and APC while another group did not, being unable to produce IL-2 in response to BCG. Therefore vaccination of human beings with BCG led to activation of T cells of various phenotypes. A phenotypic analysis of the clones showed that all but one were CD4+. Mustafa and Godal (1987) then produced CD4+ T cell clones specific for mycobacterial

antigens from BCG-vaccinated healthy subjects. These clones exhibited specific killing of monocyte-enriched adherent cells with mycobacterial antigens. Some CD4+ T cells showed a functional suppressor activity that inhibited BCG-induced *in vitro* proliferation.

8.3 The role of CD8 positive T lymphocytes

The role of CD8+ T lymphocytes as an important mediator during intracellular infections is only now being recognised. The involvement of CD8+ antigen in the interaction with the T cell receptor is now known to be an important factor in the activation of T cells (Emmrich 1988).

The localisation of CD8+ T cells in lesions caused by intracellular pathogens is coming under further study. The function of these cells in such situations, whether in a cytotoxic or suppressor role is still unclear. In particular, in respect of *C.burnetii*, an understanding of the function of CD8+ lymphocytes remains unknown. It is therefore necessary to examine the role of such cells in infections with other intracellular pathogens.

Modlin *et al.* (1986) examined lepromatous lesions from patients infected with *M.leprae*, and found CD8+ suppressor lymphocytes in juxtaposition to macrophages containing bacilli. The CD8+ cells in the blood of such patients functioned as suppressor cells. In *in vitro* assays these workers found that T cells from lepromatous lesions had antigen-specific suppressor activity.

Modlin *et al.* (1988) showed that T cells of the CD4+ subset were predominant in tuberculoid lesions, being of the human T helper phenotype

(CD4+4B4+2H4-). They showed a mitogenic response to *M.leprae in vitro*, producing IFN-γ. Conversely, in lepromatous granulomas, bacilli-filled macrophages were associated with both CD4+ and CD8+ T cells with the latter predominating. However, the CD4+ subset in these lesions was 2H4+ (suppressor/inducer) and the CD8+ cells were also of the suppressor subset. Therefore, the CD8+ suppressor cells may contribute to the functional depletion of *M.leprae*-reactive (IFN-γ forming) CD4+ lymphocytes from the immunological repertoire.

8.4 Subsets of CD4 positive T lymphocytes

Functional subsets of CD4+ T lymphocytes in mice and probably in man have been divided on the pattern of cytokine secretion into two non-overlapping subtypes (Mosmann *et al.* 1986, Cherwinski *et al.* 1987). The classification of helper-inducer and suppressor-inducer CD4 cells originated from work based on reponses to antigen receptor-mediated or lectin-mediated stimulation. The two subsets have been designated (a) Th1, which produces IL-2 and IFN-γ and uses IL-2 as an autocrine factor, and (b) Th2 which secretes IL-4 (B cell stimulatory factor 1) and uses IL-4 as its autocrine growth factor, rather than IL-2 (Mosmann *et al.* 1986; Fernandez-Botran *et al.* 1986, Lichtman *et al.* 1987). These patterns have been confirmed with panels of T helper clones and have thus far, established the criteria for separation of murine T helper subtypes. However there is increasing evidence that other cytokine secretion patterns exist and increasing controversy over whether the Th1 and Th2 phenotypes represent two distinct categories of mature T helper cells, or

whether they represent different stages of a maturation lineage, e.g., naive T cells (encompassing suppressor/inducer subsets) and memory T cells (encompassing helper/inducer subsets) (Paul 1989, Sanders *et al.* 1988). Monoclonal antibodies used to differentiate T lymphocytes have also been employed to define the two major subtypes. The suppressor-inducer subset is identified with CD45R mABs. Helper-inducers are CD45R negative but express the UCHL-1 antigen, which is part of the CD45 complex (leucocyte common antigen T200) (Beverley 1987). T cell subsets classified by the CD45 surface antigen have been functionally associated with particular disease states (Morimoto *et al.* 1985, Rose *et al.* 1985).

Barnes *et al.* (1989) have shown that cells in pleural fluid from patients with tuberculous pleuritis are enriched with CD4+CDW29+ T lymphocytes, which represent the memory T cells. These cells were concentrated at the site of disease activity, and produced IFN-γ. On the other hand, PBMC from such patients produced significantly less IFN-γ; production of this lymphokine *in vivo* was at the site of disease activity. As mentioned above, Modlin *et al.* (1988) found the opposite pattern in lepromatous lesions, i.e., a predominance of cells expressing CD45R (as defined by mAB 2H4) indicating suppressor-inducer or naive T cells. Thus, the subtype of T lymphocyte inducing or not inducing IFN-γ is reflected in progressive disease (lepromatous leprosy) or the nonprogressive "hypersensitivity" type of lesion in which bacteria are few.

In leishmaniasis in mice, the two subtypes of CD4+ T cells have been implicated as either host-protective or disease-promoting. The activation of a specific sub-population of CD4+ T cells may play an important role in the

production of IFN-γ, an essential lymphokine for the activation of infected macrophages for destruction of the parasite (Liew 1989). Scott *et al.* (1988) in determining the nature of T cells contributing to either susceptibility or resistance to leishmania in mice, established T cell lines that were reactive against protective and non-protective soluble leishmanial antigens. These T cell lines differed in the types of lympokine produced after mitogen and antigen stimulation, the "protective" clones produced IL-2 - IFN-γ, whereas the "nonprotective" produced IL-4 or IL-5. These findings were later supported by Heinzel *et al.* (1989) using *L.major* infected mice to examine lymphokine production at the mRNA level.

8.5 Macrophage activation - capacities for activation

For macrophages to attain functional maturation, certain organelles, structural components and metabolic products have to be present (Adams and Hamilton 1984). The intracellular equipment can be divided into three categories; first, the vacuolar system, encompassing phagosomes, pinosomes, endosomes, primary/secondary lysosomes and phagolysosomes; second, the cytoskeletal apparatus which include filaments and tubules that participate in phagocytic and secretory function, including the proteins actin, myosin, accumentin and gelsolin (Stossel 1981); and, third, the mitochondrial system, the associated respiratory chain, the enzymes of the Embden-Meyerhoff path and hexose monophosphate shunt (Axline 1970).

The activation of macrophages is a two-signal process involving priming of the cell with a stimulus, thus producing a noncytotoxic state, which enables

the cell to respond to other trigger stimuli (Meltzer 1981). The priming signal can be provided *in vivo*, as demonstrated by Ruco and Meltzer (1978) when they examined the immune response to BCG infection in mice, and *in vitro* by Meltzer (1981) during experiments in which murine macrophages were treated with lymphokines at a concentration not inducing cytotoxicity. In both situations primed macrophages when treated further with lymphokines, developed a strong microbicidal and tumoricidal activity, after only brief exposure to the second signal. Macrophages exposed to a trigger signal alone, or before the priming stimulus, did not express cytotoxic functions. Nacy *et al.* (1984) postulated that the signals which regulate macrophage activity may come from IFN-γ, as this factor was obtained from the conditioned media from fractionation of the EL-4 thymoma cell line which had biological, physicochemical and antigenic properties similar to those of IFN-γ.

8.6 The role of monocytes/macrophages in the intracellular killing of pathogens

In the complex network of the immune system, the induction of an immune response *in vivo* involves a series of events resulting in mononuclear cell proliferation, mononuclear and granulocyte cell infiltration and the induction of effector activities, all directed at eliminating the invading organism. Activation of macrophages is essential for the successful resolution of *C.burnetii* infection.

Macrophage activation is a selective and stringently controlled set of alterations of various physiological capacities and properties, which culminates

in an enhanced ability to kill intracellular microbial pathogens (Mackaness 1970, Adams and Hamilton 1987).

The killing of intracellular pathogens is related to the macrophage's ability to secrete reactive oxygen intermediates (Nathan 1982). These include superoxide (O_2-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH), produced during the reduction of oxygen to water (Klebanoff 1980). Together with the other factors secreted by macrophages, these products influence the degree and chronicity of infection, besides facilitating the induction of an antigen-specific immune response. Other factors include iron-binding proteins (e.g., transferrin) which exerts an antimicrobial effect. Complement enhances the binding capacity of phagocytic leukocytes to microbes and the subsequent secretion of cytotoxic factors. Lysozyme acts on organisms already killed by complement (Nathan 1983). Neutral proteases, such as collagenase and elastase, are secreted in a two-step process (Gordon 1978).

The growth of obligate intracellular pathogens within macrophages reflects the environment in the host cell which provides ~~substates~~ substrates required by these organisms. The complex pattern of enhanced expression of proteins in macrophages is accompanied by an equally complex pattern of diminished expression of other proteins (Adams and Hamilton 1987). It is suggested that failure to produce the latter may limit the growth of parasites which require them for their own metabolism (Nathan 1983).

8.7 Interferon γ as the mediator for macrophage activation in antimicrobial mechanisms

The parallel between *C.burnetii* and other intracellular pathogens illuminates or suggests how the former, which replicates within the phagolysosome of phagocytic cells can evade the host's immune mechanisms. Apart from the many antibody studies in humans, the role of soluble mediators viz., cytokines has received only limited attention.

The importance of such factors was first shown by Nathan *et al.* (1983) who found that effective cell-mediated immunity to intracellular pathogens required "macrophage activating factor" (MAF) which turned on the macrophage oxidative metabolism and antimicrobial activity. With *Toxoplasma gondii*, it was concluded that IFN-γ was the potent activator. Monoclonal antibodies directed against IFN-γ inhibited the cytopathic effect of assay; the only activator consistently detected in the medium of specific antigen or mitogen stimulated human leucocytes was IFN-γ.

Murray *et al.* (1983) demonstrated that enhanced oxidative and antiprotozoal activity, two closely correlated and key markers of lymphokine-induced macrophage activation, were both dependent on IFN-γ. When they used both a crude preparation or a pure recombinant IFN-γ, normal macrophages were activated *in vitro* to a leishmanicidal state, an activity that could be abrogated with monoclonal antibody specific for IFN-γ. Furthermore, by using monocytes derived from a chronic granulomatous disease patient, IFN-γ activated the oxygen - independent pathway to produce an antileishmanial state.

This work was consolidated by other workers (Wisseman and Waddell 1983), who showed that treatment of human umbilical vein endothelial cells in culture with a lymphokine preparation containing human IFN-γ ~~containing~~ lymphokines caused the death of intracellular *R.prowazekii.* Turco and Winkler (1983) found that IFN-γ inhibited the growth of *R.prowazekii* in mouse cells and then repeated these experiments and showed the same occurred with *C.burnetii* (Turco *et al.* 1984).

Nathan *et al.* (1984) continued previous studies on the identification of IFN-γ as the factor responsible for inducing activation of macrophages by comparing its action with that of other cytokines. Among a collection of mediators including IFN-γ, IFN-β, colony stimulating factor (CSF) type 1, granulocyte macrophage-CSF, pluripotent-CSF, migration inhibition factor, Interleukin-2 (IL2) and Tumour Necrosis Factor (TNF), only IFN-γ enhanced both H_2O_2 secretion and antitoxoplasma activity of human macrophages. Hoover *et al.* (1985) reported that IFN-γ induced human monocyte microbicidal activity against intracellular *L.donovani.*

8.8 Other mediators involved in intracellular killing

The importance of soluble factors within the immune response has been extensively examined under many pathogenic circumstances. The hormone-like molecules which can affect various cell functions enable communication between different cell types. Therefore many cytokines which are released by T lymphocytes have been tested *in vitro* for their ability to activate macrophages for anti-microbial activity.

The importance of IFN-γ as a macrophage activating factor for the enhancement of H_2O_2 production as an anti-microbial mechanisms was established. However, further investigations using other cytokines to produce anti-microbial mechanisms produced varying results. Reed *et al.* (1987) induced a microbiostatic state in mouse peritoneal cells after treatment with recombinant murine granulocyte/macrophage-colony stimulating factor (GM-CSF) before or after uptake of *Trypanisoma cruzi.* It was postulated that after microbial infection, macrophage activation mediated by GM-CSF occurred more quickly than that obtained with IFN-γ which was dependent on recruitment and clonal expansion of antigen-specific T cells in an infective focus. These results contradicted previous reports of Nathan *et al.* (1984), but are in accord with those of Handman and Burgess (1979) who found that GM-CSF could also inhibit *Leishmania tropica* in mouse peritoneal macrophages. Esparza *et al* (1987) reported that human recombinant lymphotoxin (LT) and recombinant (rec.) tumour necrosis factor (TNF) acted synergistically with IFN-γ on mouse macrophages to induce schistosomacidal activity, but were inactive alone.

Pathological effects induced by TNF have been investigated in relation to intercellular parasites (Playfair and Tavern 1987). TNF secretion by macrophages in response to invasion has been demonstrated to correlate with their activation, thereby mediating the pathological changes that accompany the invasion by a foreign organism. The discovery of TNF as a transmembrane protein on monocytes capable of killing target cells by either cell-to-cell contact or local release of TNF secretory component has led to the postulate that

different forms of TNF may induce different physiological responses (Kriegler *et al.* 1988).

Work by Clark (1979) showed ~~in~~ mice given an intravenous injection of killed *C.burnetii* recovered from infection with falciparum malaria and were demonstrated to have TNF in their serum. Bate *et al.* (1988, 1989) demonstrated that killed parasites as well as heat soluble antigens induced the secretion of TNF in mice, both *in vitro* and *in vivo*. It was postulated that these antigens may have induced TNF directly, but this was not the sole mechanism of production. It is probable that the major mechanism by which malaria induced TNF secretion was through the action of IFN-γ, since the treatment of macrophages with IFN-γ increased the amount of TNF produced after stimulation with malarial antigens.

The action of TNF in activating macrophages for killing of intracellular microorganisms was found to be selective for the infectious agent. De Titto *et al.* (1986) using rec. TNF with mouse peritoneal macrophages found that intracellular multiplication of *T.cruzi* in cultured macrophages was inhibited, but had no effect on *T.gondii*. No inhibition was evident when *T.cruzi* was used to infect fibroblasts, therefore suggesting that TNF sensitivity was related to the nature of both the parasite and the host cell.

Mice infected with *Myco.bovis* (BCG strain) formed granulomas which represented the assembly of a new tissue with a topological organisation and a functional finality (Kindler *et al.* 1989). Granuloma formation was used to study TNF release by activated macrophages resulting from BCG infection. Despite forming large numbers of granulomas disseminated in various organs,

TNF was not detectable in the blood of infected animals, however TNF mRNA and protein accumulated in parallel to the expansion and decline of the granulomas. It was proposed that TNF released by macrophages in BCG granulomas act locally in the microenvironment and involve autoamplification leading to accumulation and differentiation of macrophages into highly bactericidal epithelioid cells.

8.9 The use of recombinant interferon-γ for the treatment of chronic infection in humans

As a result of *in vitro* experiments, indicating that IFN-γ was a macrophage-activating factor in humans, trials were begun in patients with lepromatous leprosy using recombinant IFN-γ. These trials attempted to improve features of the disease, associated with inadequate macrophage activation and with a deficient production of endogenous IFN-γ (Nathan *et al.* 1986, Kaplan *et al.* 1987). Such patients had no intrinsic defect in the ability of their macrophages to respond to macrophage-activation factor, but were anergic, upon skin testing with *M.leprae*. Patients given intradermal injections of rec-IFN-γ, developed induration and erythema at the site of inoculation within 24 to 48 hours. Upon biopsy these lesions contained an increased infiltration of mononuclear leucocytes at the dermis, half of which were mononuclear phagocytes and the remainder T cells positive for either CD8 or CD4 markers. There was also a concomitant increase HLA-DR expression of on all cells in the dermis and epidermis, an increase in keratinocyte proliferation and a decrease

in the number of Langerhans cells. However, the skin test response to IFN-γ was not as prolific as that observed to the tuberculin response of similar diameter (i.e. not as firm). Peripheral blood monocytes from these patients were able to respond to both PMA and *M.leprae* with a boosted H_2O_2 secretory capacity. Therefore a systemic effect of IFN-γ was induced despite failure to detect it in the serum after local administration. The bacterial index at the site of inoculation was approximately tenfold lower than in pre-innoculation samples in some of the treated patients. In subsequent studies (Kaplan and Cohn, 1986) patients were given two series of three injections of rec-IFN-γ. The initial dose induced a preparatory effect for the second dose which was considerably enhanced as indicated by the diameter of the induration, while a third produced a relatively smaller response indicative of either hyporesponsiveness or the generation of suppressor factors. An infiltration of monocytes and T cells was observed with a predominance of CD4+ lymphocytes. These levels remained elevated for 21 days in half the patients examined. The bacterial index was decreased in the majority of patients (from 5 fold to 1000 fold), but only after repeated injections of the rec-IFN-γ. A six month, post-treatment examination indicated that this decrease was persistent. Mononuclear cells persisted at the site of inoculation and the presence of epithelioid cells and multinucleated giant cells indicative of an active lesion demonstrated that rec-IFN-γ evoked cellular components that persisted long after the lymphokine had been cleared from the site.

Intradermal rec-IFN-γ treatment was also used with patients suffering from either American cutaneous leishmaniasis (ACL, *L.braziliensis*) or Oriental

cutaneous leishmaniasis (OCL. *L.tropica*) (Harms *et al.* 1989). Doses given at the site of lesions were found to induce aggregations of lymphocytes and macrophages between numerous plasma cells in the dermis. Dermal macrophages expressed increased levels of HLA-DR, there was an influx of CD4 and CD8 cells into the site of inoculation and an increased number of Langerhans cells strongly expressing HLA-DR. The presence of cells expressing CD25 (Tac-IL-2 Receptor light chain) indicated an effective interaction with cells and lymphokines involved in cell-mediated immunity. Overall, lesions from both groups of patients were cleared of parasites, and in many of the OCL cases, there was complete resolution of treated lesions, possibly due to the higher treatment dose.

8.10 Defective immune response due to intracellular microbial infection

The pathogenic states exhibited after intracellular microbial infection in humans vary widely. These range from subclinical infection, to acute clinical infections, to chronic persistence of the organism within the host. The malfunctions which induce persistent infection may be either an innate response or limitation of the host that is highlighted by the infection, or are induced by the organism as a direct consequence of its interaction with the immune system.

8.11 The absence of lymphokine production correlated with a defect in cell-mediated immunity

Chronic granulomatous disease associated with Leprosy is a chronic illnesses

that has been well documented (Cohn and Kaplin 1986). The clinical manifestations of leprosy vary depending on the immune status of the host, from the more severe lepromatous disease where leprosy bacilli multiply to high levels within the cell, to the tuberculoid form at the other end of the scale where only rare bacilli are seen. Haregewoin *et al.* (1983) reported that, although lepromatous T cells failed to produce IL-2 after exposure to *M.leprae* they can respond by proliferating to the antigen in the presence of IL-2 rich, T cell conditioned media. This suggested that the lack of response was due to a deficiency in the production of IL-2 and not a lack of *M.leprae* -reactive T cells. The majority of cells proliferating *in vitro* in this system were of the CD4+ subset, hence immunological competence could be restored in such patients.

These results were augmented by Nogueira *et al.* (1983) in a similar group of patients. PBMC from these patients failed to generate IFN-γ in response to specific *M.leprae* antigen or to the mitogen Concanavalin A (Con A), despite showing proliferative responses. However PBMC stimulated with IL-2 plus *M.leprae* antigen, induced significant levels of IFN-γ. Histologic examination of skin biopsies of cutaneous lesions revealed that low IFN-γ expression was associated with a low CD4/CD8 ratio. Hence, it was concluded that CD8+ T cells influenced IL-2 production, a necessary but not sufficient element in the chain of events resulting in IFN-γ production. Kaplan *et al* (1985) then found that specific hyporesponsiveness of T cells to *M.leprae* antigen could be reversed *in vitro* by the addition of exogeneous IL-2 which enhanced pre-sensitised T cell proliferation and IFN-γ production. Absence of IL-2 production was not responsible for an anergic state since non-responders

(i.e. with no lymphocyte proliferation or IFN-γ production) showed no enhanced activity with exogeneous IL-2, hence it was concluded that nonresponsiveness was non-reversible.

In a study of patients with newly diagnosed active tuberculosis Onwubalili *et al.* (1985) found that IFN-γ responses of PBMC to tuberculin antigen were depressed in a proportion of these cases. Low responders also produced lower levels of IFN-γ and IFN-α to mitogenic stimulation in comparison to other patients in this group. Since there was no significant clinical difference between responder types, it was speculated that a reduced response was attributable to either a smaller proportion of responding cells or a deficiency of some accessory cell factor such as macrophage presentation or IL-2, or the presence of circulating suppressor monocytes and T cells.

Examination of the lepromatous leprosy lesions by immunohistologic techniques (Volc-Platzer *et al.* 1988) also indicated that immunologic dysfunction was localised at sites of lesions. Lesions were found to contain dendritic cells which stained for IL-1 but were devoid of cells staining for IFN-γ and only a few cells stained for IL-2R. Reinforcing the previous work of Nogueira *et al.* (1983) and Kaplan *et al.* (1985), it was concluded that macrophage function was not defective, but was attempting to overcome impaired T helper/inducer activation by increased IL-1 production.

8.12 Mononuclear cell-mediated immune suppression

Suppression of the immune response during either active acute or chronic infection has been associated with the presence of circulating suppressor cells.

Ellner (1978) documented the presence of adherent suppressor, cell activity *in vitro* that was specific for PPD-induced proliferation in tuberculin-low responders who were diagnosed as having active pulmonary tuberculosis. Adherent mononuclear cells were identified as monocytes (or a subset of monocytes) that mediated their activity via cellular interaction and not by a factor produced in the media by such cells (e.g., prostaglandins). Since these low responder patients were hyporeactive when skin-tested, it was postulated that suppressor adherent cells may have a role in the depression of DTH in tuberculosis.

Immune suppression has been reported to occur in *Coxiella burnetii* infection during the acute phase (Kishimoto and Gonder 1979) in experimentally infected cynomolgous monkeys' and during chronic endocarditis (Koster *et al.* 1985a,b) in humans. In the latter case nonspecific suppression occurred *in vitro*, mediated by a mechanism involving a CD8+T cell-monocyte interaction, in which lymphokines stimulated the production of prostaglandin E2 by monocytes.

Ottenhoff *et al* (1986) suggested the presence of a suppression inducing determinant on a 36Kd MW *M.leprae* protein. This protein reacted with T suppressor clones as well as with T helper clones derived from a borderline lepromatous patient. A model for the mechanism of action proposed that T suppressor cells, suppressed *M.leprae* reactive T helper cells when both suppressor and helper epitopes for T helper cells, were expressed on the same protein. Therefore T helper specific epitopes which exist on proteins shared with *M.leprae* (i.e. from other mycobacterial species) induce T helper responses

against non-*M.leprae* epitopes. Suppression by cross-reactive T suppressor cells can then be evaded irrespective of the presence of a cross-reactive suppressor epitope on that same molecule.

CHAPTER 9

VACCINES AGAINST INTRACELLULAR PATHOGENS

The development of a vaccine for the prevention of Q fever was greatly aided by the discoveries of phase variation in *Coxiella burnetii* (Stoker and Fiset, 1956) and that inactivated Phase I organisms were about 300 times more potent than equivalent preparations of Phase II coxiella in protecting laboratory animals against experimental infection (Ormsbee *et al.* 1964). Early attempts in preventing infection among laboratory workers in the USA involved either a course of injections (Smadel *et al.* 1948, Meiklejohn and Lennette 1950) or a single dose vaccine (Lackman *et al.* 1962) made from semi-purified inactivated suspensions of whole coxiellas. In these instances adverse systemic and local reactions were a characteristic of vaccination, due mainly to a lack of understanding of the antigenic nature of *C.burnetii* in provoking delayed type hypersensitivity reactions after infection with the organism or in recipients of Q fever vaccine. Later examination of the vaccines used in these early trials were found to be, in the majority of cases, *C.burnetii* in the Phase II state although containing some Phase I cells (Ormsbee *et al* 1962).

The basis of Q fever vaccine development in modern times has ~~centered~~ centred on two main areas. First the emergence of the low dose, highly purified, whole cell inactivated Phase I vaccines, which, with the inclusion of pre-vaccination testing for both Q fever specific antibody and skin test proved less reactogenic. The use of such vaccines has proven to be both safe and highly protective

(Marmion *et al.* 1984). Alternatively, as the result of early American experience with vaccines of semi purified organisms or chemical extracts, work has been directed to the development of subunit vaccines, either by extraction techniques, or by recombinant gene technology. Examples of subunit vaccines include, chloroform-methanol-residue vaccine and trichloroacetic acid-extracted vaccine both of which have been used on a limited basis, either in animals or in man.

What then, makes a vaccine effective, and are all the Q fever vaccines developed, capable of protecting individuals from infection? Ultimately, the efficacy of a vaccine can only be assessed by its ability to protect individuals from Q fever after natural exposure. Nevertheless, an increased understanding of how T cells recognise antigens and how they interact with many components of the immune system would facilitate the development of vaccines to ensure the greatest efficacy and safety possible.

Examples of vaccine development and the difficulties in determining a protective antigen for use in vaccination can be demonstrated with *M.leprae* and *M.tuberculosis*. Kaufmann (1988) states that demonstration of T cell epitopes in several recombinant proteins should not be taken as proof that these proteins are of particular importance for protection. This statement has been supported by other workers endeavouring to develop vaccines against microbial pathogens. Faced with the problem that these organisms elicit a wide range of immune responses (eg., macrophage maturation and activation, granuloma formation, hypersensitivity reaction, generation of cytotoxic cells and production of antibody), some of which may contribute to protective

immunity, others which are probably irrelevant while yet again others may favour the pathogen. The dilemma also arises in developing a vaccine by using genomic libraries of DNA derived from the organism. Screening an expression library for the putative polypeptide vaccine may only identify those polypeptides recognised by antibodies and may not take into account proteins with T cell epitopes.

Lamb and Young (1987), by using T cell clones have overcome this problem, and have developed a means of identifying T cell epitopes in *M.tuberculosis*. In a procedure involving T cell recognition of antigens fractionated by polyacrylamide gel electrophoresis, they analysed the contribution of antigens of different molecular weight to the overall polyclonal T cell response to a mycobacterial extract. Another group (Kilgus *et al.* 1989) working with *Plasmodium falciparum* developed a competition assay that has allowed the examination of different peptides for their capacity to bind to different HLA class II proteins. With the expectation that protective immunity against malaria was likely to be mediated via class II and class I-MHC-restricted T cells and B cells, the assay was based on the binding of different peptides to the same MHC protein which would then compete with each other. Using T cells of known MHC restriction, recombinant peptide antigens derived from the circumsporozoite protein and the whole protein as the competitor peptide, they were able to identify the stimulator peptide and assess its ability to induce primary *in vitro* responses of T cells.

The importance of assessing cell-mediated immunity to purified protein preparations is also demonstrated in experiments by Mehra *et al.* (1989) to

characterize the immunodominant *M.leprae* cell wall associated proteins at the T cell level as a candidate vaccine against leprosy. T lymphocyte lines and clones derived from lesions of tuberculoid leprosy patients or peripheral blood of healthy contacts of lepromatous patients were stimulated with cell wall protein for inducing proliferation. This enabled them to monitor the purification and preservation of antigenicity of their protein purifications.

Studies into the development of vaccines against intracellular pathogens, in particular, the use of subunit vaccines, based on a single peptide, must be cautiously monitored for their ability to induce cell mediated immunity and in particular their ability to protect against infection in laboratory animals or naturally ~~naturaly~~ exposed human beings.

CHAPTER 10

OVERVIEW OF AIMS AND SCOPE OF THESIS PROJECT

The studies reported below were undertaken in an attempt to define the nature of the immune response to, and the critical protective immunogens of a highly efficacious killed Q fever vaccine developed with the Commonwealth Serum Laboratories (Qvax: CSL).

The vaccine is a highly purified, formalin-inactivated whole cell preparation prepared from the Henzerling strain of *C.burnetii* in an early egg passage; i.e., in the Phase I antigenic state. This vaccine has been given in a single dose of 30µg, subcutaneously, to more than 6000 abattoir workers and no cases of Q fever have been observed among them when they had had time (10-15 days) to develop immunity after inoculation. A few (8-10) Q fever cases were observed among subjects inoculated during the second half of the incubation period of a natural attack of Q fever. That is, the subject was already infected before vaccination was given (Marmion *et al.* 1990).

In contrast to the experience of the vaccinees, there were high incidence rates of Q fever among unvaccinated workers in the same abattoir, or among visitors to it; statistically highly significant incidence ratios were obtained with the two groups (Marmion *et al.* 1990). A numerically limited, double-blind, placebo-controlled trial, on a sequential analysis design, compared Qvax and influenza vaccine and confirmed the high protective efficacy of the Q fever vaccine (Shapiro *et al.* 1990). Cohort analysis of the experience of the vaccinated subjects in the South Australian abattoirs indicated that the

protection lasted for at least five years.

Such high protective efficacy is very unusual for a killed vaccine; inactivated whole cell suspensions of rickettsias such as *R.typhi* or *R.prowazekii* do not protect laboratory animals unless given in adjuvant.

Measurement of the antibody response to Qvax in vaccinated subjects, by various serological techniques (CF, immunofluorescence, RIA) showed short lived antibody formation in 80% of them during the first 2-3 months, predominantly in the IgM fraction; this declined with time to an overall rate of 50-60%, the predominant antibody then being in the IgG fraction (Marmion *et al.* 1990, Worswick and Marmion 1985). Skin test conversion rates in vaccinated subjects were also about 60%.

The paradoxical contrast between the complete protection against Q fever given by the vaccine in naturally exposed abattoir populations and the rather unimpressive antibody and skin test response raised doubts in the minds of the regulatory authorities who had to consider marketing approval for the vaccine. Such authorities are accustomed to judging vaccine efficacy in terms of antibody formation because such assays are a simple and labour saving method of measurement.

However, as described in detail in earlier chapters, immunity to intracellular pathogens, and to *C.burnetii* in particular, is heavily dependent on cell-mediated immunity although antibody to Phase I antigen plays a part in protection by mechanisms yet to be defined.

In the light of these uncertainties and the procedural constraints in the licencing process, it was decided to investigate certain *in vitro* correlates of cell

mediated immunity - namely, lymphocyte stimulation or mitogenesis on exposure of peripheral blood mononuclear cells (PBMC), exposure to *C.burnetii* antigens and the capacity of lymphocytes from vaccinees to form gamma interferon (IFNγ); a lymphokine of central importance for macrophage activation and destruction of intracellular *C.burnetii.*

Initial studies of lymphocyte stimulation assays with peripheral blood mononuclear cells showed that a high proportion (>85%) of vaccinees developed positive, low level lymphocyte stimulation indices (LSI) some 10-15 days after inoculation and that these lasted for up to five years (the longest period of sampling) (results detailed in Chapter 11). The LSI responses to a range of *C.burnetii* antigens were qualitatively, but not quantitatively similar to those observed with PBMC from persons who had had clinical or subclinical Q fever in the past.

Further studies of the lymphocyte stimulation assay were then undertaken to confirm that the mitogenic response was a property of T-lymphocytes with monocytes as requisite antigen presenting cells and that the responses obtained were not a nonspecific or polyclonal response of B-lymphocytes. This showed that T cells predominated in the mitogenic response and that CD4 positive and CD8 positive cells both reacted with *C.burnetii* antigen. As the extent of the LSI responses with T cells appeared to be limited by the amount of IL-2 formed in the reaction mixtures, the effect of exogenous recDNA IL-2 was investigated; this enhanced the LSI values in a specific fashion. It would be expected (from theory) that the epitopes stimulating the T-lymphocyte responses and memory would be associated with

the proteins of the coxiella. However, a role for the LPS of the organism could not be entirely discounted in view of the interaction of lipophosphoglycans of *Leishmania major* - another intracellular pathogen - with T-lymphocytes. Other carbohydrate determinants, including those of vegetable mitogens and glycolipids from *Mycobacteria* spp. react with T-lymphocytes (Discussion, Chapter 16). LSI determinations were therefore made with T cells and whole cells of *C.burnetii* and with various fractions containing cell protein, protein-LPS complexes, LPS alone and LPS with modified sugar chains. These are described in Chapter 13.

Although a positive stimulation response with T cells from vaccinees to *C.burnetii* antigens was a useful indicator of active cellular immunity, it was important to know whether the lymphocytes sensitised would respond to antigenic challenge by forming IFNγ. As explained in Chapters 6 and 8, IFNγ is the final, effective mediator of macrophage activation and the destruction of intracellular coxiellas as with other intracellular pathogens. It is known that one subset of CD4 positive lymphocytes does not form IFNγ but secretes lymphokines which stimulate B cell activity (Chapter 8). Presumably both CD4 positive subsets would show a mitogenic response although only one would form IFNγ. Consequently, the acquisition of the capacity of T-lymphocytes from vaccinees to form interferon, and IFNγ in particular, after vaccination and Q fever infection was investigated (Chapter 14).

The observations, which in general showed that the *C.burnetii* proteins were the major stimulating antigens for T-lymphocytes, were extended by separating the cell proteins by PAGE, transferring the bands to nitrocellulose

and using solubilised preparations of the transferred proteins to stimulate T-lymphocytes. This showed that although there are certain proteins which were more stimulatory than others, there was no immunodominant protein and most of the cell proteins had some effect. This finding is of substantial potential significance for attempts to prepare "2nd generation" vaccines by cloning an immunodominant protein, or a few proteins, and expressing them in a prokaryotic or eukaryotic cell. Results are described in Chapters 13 and 14.

Finally, it is recognised that in some human subjects and in laboratory animals, Q fever infection has a marked immunomodulatory or immunosuppressive effect and T-lymphocyte mitogenesis may be suppressed or diminished. This may be mediated by a prostaglandin E2 suppression of lymphocyte activity. In Chapter 14 some preliminary evidence is presented on inhibition of IFNγ formation by *C.burnetii* Phase I antigen and its reversal by Peroxicam, an inhibitor of prostaglandin synthesis.

The results point to the possibility that Phase I LPS down regulates IFNγ production by restriction of IL-2 formation when it interacts with the monocyte. Aside from these inhibitory effects *in vitro*, Q fever infection or large doses of killed suspensions of the coxiella, produce granulomatous changes and inhibition of CMI responses in mice. It was important to establish that doses of Qvax, in the dose/weight ratio used in man, did not produce such effects in mice and this proved to be so (Chapter 15).

Each of these experimental stages of the project is described in a separate chapter with its own specific and detailed introduction, the related

material and experimental methods, the results and a discussion of the findings.

In a final chapter - 16 - there is a general discussion, a summary of conclusions and some comments about the significance of the results described for further work.

RESULTS

CHAPTER 11

STUDIES OF CELL MEDIATED IMMUNITY IN HUMAN SUBJECTS GIVEN Q FEVER VACCINE

11.1 Introduction

Inactivated whole cell Q fever vaccines have been used since the late 1940's to protect laboratory workers handling living *Coxiella burnetii* (Smadel *et al.* 1948, Meiklejohn and Lennette 1950).

Vaccine regimens have since been improved, first, with the identification of the Phase I antigen of *C.burnetii* as the protective immunogen (Ormsbee *et al.* 1964); second, with the more effective removal of yolk sac contaminants from the vaccine (Ormsbee 1961); and third with the realisation (Benenson 1959), that the periodic severe reactions at the vaccine inoculation site, which sometimes included a sterile abscess (Bell *et al.* 1964a), were immunologically mediated and signified prior sensitisation to the organism. Prevaccination testing of subjects for antibody and for skin reactivity to *C.burnetii* and restriction of vaccination to those subjects who showed no reactivity to either test, removed the problem of serious reactions at the inoculation site.

Low dose, highly purified, inactivated whole cell vaccines in the Phase I antigen configuration have been used for a number of years at the Rocky Mountain Laboratory, Hamilton, Montana, USA and appeared to protect (Philip R., personal communication via Ormsbee R.A.). The serological, skin test and

cellular immune responses to such vaccines in volunteers have been described by Luoto *et al.* (1963), Bell *et al.* (1964b) and Ascher *et al.* (1983a).

Despite the long experience with inactivated whole cell Q fever vaccines in laboratory workers, little use has been made of them to protect industrial populations exposed to Q fever, such as abattoir workers. In 1981, in the face of increased prevalences of Q fever in South Australian abattoirs, a vaccine programme was commenced in which volunteers from among abattoir workers and from some non-exposed groups were given one subcutaneous injection of 30μg of a highly purified, formalin-inactivated Phase I vaccine from *C.burnetii* (Marmion *et al.* 1984).

By 1990, these trials have involved 4 abattoirs in South Australia and over 6,000 abattoir and other workers have been vaccinated, with only two untoward local reactions; no cases of Q fever have been detected among the vaccinated other than in 10 individuals who were inoculated during the incubation period of a natural infection. On the other hand, more than 100 Q fever cases have been detected in unvaccinated workers in or among visitors to the same working environments (Marmion *et al.* 1990).

Preliminary observations (Marmion *et al.* 1984, 1990, Worswick and Marmion 1985) on the serological response to this vaccine, as measured by complement fixation or immunofluorescence in abattoir workers with *C.burnetii* Phase I and Phase II antigens, indicated that some 56% of workers in one abattoir, and 64% in another, seroconverted after vaccination. The contrast between the high rate of protection given by the vaccine against natural infection, and the modest rate of seroconversion provoked by the vaccine,

highlighted the need to investigate the use of other *in vitro* markers or correlates of vaccine-induced immunity.

There is compelling evidence from animal models that the various components of cellular immunity are of central importance in resistance to and recovery from infection with *C.burnetii*, as with other intracellular organisms (Kishimoto and Walker 1976, Kishimoto *et al.* 1976, 1977, 1978a,b, Kishimoto and Burger 1977, Kishimoto and Gonder 1978). Studies of cell mediated immunity to Q fever in man have, of necessity, been less extensive, but lymphocyte mitogenic responses to *C.burnetii* antigens have been demonstrated - sometimes in the absence of antibody - in subjects with post infection or vaccine-induced immunity (Ascher *et al.* 1983b, Jerrells *et al.* 1975). The subsets of lymphocytes involved in these reactions and the degree of similarity between lymphocyte sensitisation from natural infection or from vaccine-induced immunity, has not, as far as I am aware, been defined. Nor ~~is~~ has the relationship between a natural or an artificially-induced immunity and the capacity to produce various cytokines been investigated, although there is mounting evidence that the mediator of macrophage activation - IFNγ - also inhibits the growth of the coxiella in mouse fibroblasts (Turco *et al.* 1984).

In view of the dichotomy between antibody formation and resistance to natural infection observed in our vaccine trials, and also because of the gaps in the information in the detailed analysis of cellular immunity induced by Q fever vaccine in man, a series of investigations have been undertaken of cellular immunity in vaccinated and nonvaccinated subjects. The results of the initial stages of this study, which deal with the lymphoproliferative

response of peripheral blood cells to *C.burnetii* antigens, are presented in this chapter.

11.2 Materials and Methods

Preparation of lymphocytes. Venous blood samples treated with Lithium-Heparin (125U/ml) (Disposable Products, Adelaide, South Australia) were diluted with an equal volume of sterile saline, or medium RPMI-C: RPMI 1640 (Flow Laboratories, Virginia, USA) with HEPES buffer containing 200 mM L-glutamine, penicillin (200U/ml) and gentamycin (40µg/ml). The blood was layered onto Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) and centrifuged at 2000 rpm for 20 minutes (Boyum 1968). The peripheral blood mononuclear cells (PBMC) at the interface were collected and washed twice in sterile saline. Samples containing red cells were treated with Tris-buffered ammonium chloride (0.83% w/v) for 15 minutes at room temperature to lyse the red blood cells.

Coxiella burnetii antigens and mitogens.

(a) Antigens: *C.burnetii* Phase I, Henzerling strain prepared from infected yolk sacs of embryonated chicken eggs, formalin-inactivated and used for Q fever vaccination (The National Drug Company, Pennsylvania, USA: Code No. NDBR 105 Lot 4/5) (A gift from Ormsbee RA.). The antigen was diluted in sterile phosphate buffered saline (S-PBS) and stored at 4°C. This preparation corresponds to the Q fever vaccine (Qvax) prepared by the Commonwealth Serum Laboratories (Melbourne, Victoria, Australia) which used Henzerling

strain, Phase I.

C.burnetii Phase II, Nine Mile antigen prepared from *C.burnetii* mainly in Phase II, with convertion of residual Phase I cells by treatment with potassium periodate (Fiset *et al.* 1969). (The Standards Laboratory, Central Public Health Laboratory, London, England) (A gift from Bradstreet P.). This antigen was also diluted in S-PBS and stored at 4°C.

Small amounts of a *C.burnetii* Phase II antigen, Nine Mile strain, prepared by serial passage (RSA 516, Nine Mile 90EP/ITC/4EP) in chick embryos were available as a gift from Peacock M., Rocky Mountain Laboratory, Hamilton, Montana, USA. This antigen was from a strain permanently in Phase II and non-pathogenic for guinea pigs. Its pattern of reactivity with PBMC was comparable to that of the Colindale antigen (see Results section). Yolk sac antigen control for Q fever tests was that supplied by Commonwealth Serum Laboratories. The concentrations of these specific or control antigens for use in the lymphocyte stimulation assays were determined by testing a range of dilutions of the reagent against PBMC from subjects immune to Q fever or sensitive to yolk sac protein.

(b) Mitogen: Purified Phytohaemagglutinin (PHA-P) (Wellcome Research Laboratories, Beckenham, England) was used as a known positive stimulant of T cells. PHA was made up in S-PBS and stored in 100µg/ml aliquots at -20°C until used at a predetermined concentration.

Skin tests. Intradermal inoculation of 0.02µg of coxiellae as described previously (Marmion *et al.* 1984, 1990).

Proliferation assay. PBMC at a concentration of $5x10^5$ cells/ml were cultured in medium containing RPMI-C supplemented with 10% heat-inactivated autologous serum. All cultures were carried out in 96-well microtiter plates with flat-bottom wells (Nunclon Delta, Nunc, Denmark). Each antigen and mitogen was tested in four replicate wells. A set of control wells was also included which contained no antigen/mitogen. Cultures with antigen were incubated for 144 hours, those with mitogen for 96 hours; both at 37°C in a humid atmosphere with 5% v/v CO_2. Proliferation of PBMC in response to antigen/mitogen was measured by pulsing the cultures with 0.4µCi [^{3}H]thymidine (sp. act. 22Ci/mmol; Amersham International, Sydney, Australia) per well for 18 hours before harvesting cells with an automated cell harvester (Skatron, Lierbyen, Norway) onto glass fibre filters. Radioactivity incorporated into cell DNA was counted in a liquid scintillation counter (Beckman, LS 2800, Sydney, Australia). The extent of proliferation was determined by calculating a lymphocyte stimulation index (LSI) for each antigen and mitogen. The formula for LSI is: (mean counts per minute of 4 replicate stimulated cultures - machine background)/(mean counts per minute of 4 replicate unstimulated cultures - machine background).

Serological analysis. All specimens received for investigation by PBMC proliferation to *C.burnetii* antigens, were examined for specific antibody titers to *C.burnetii* using the complement fixation (CF) test and the immunofluorescence (IF) test as previously described (Worswick and Marmion

1985).

Study groups. The subjects investigated in this study are divided into two main categories:

(i) **Low risk**: the staff of institutions conducting physiological research that involves the periodic handling of animals that may possibly be infected with Q fever organisms, or those who make periodical visits to the SAMCOR abattoir complex to obtain animal specimens for their research. This category contains staff from three separate institutions, from two different states of Australia. The total for this group was 46; 20 had pre- and postvaccination samples (at 0.3 to 2 months after inoculation); 13 had prevaccination samples only, and 13 had postvaccination samples only.

(ii) **High risk**: this comprised two groups of workers at the SAMCOR abattoir complex and made up of (a) a group of 81 abattoir workers enrolled in the vaccine programme starting in 1981 and given one s.c. injection of 30µg of inactivated *C.burnetii* Phase I organisms (Henzerling strain). The subjects had been bled and skin tested before vaccination and were negative for CF and IF antibody, and also in the skin test. However, prevaccination samples were not available for lymphocyte proliferation studies. Samples for such studies were obtained from subsets of the group at various intervals from 2 to 5 years after vaccination. And (b) a group of 25 abattoir workers at SAMCOR who had been enrolled in the vaccination programme but not vaccinated because they

had either a positive CF or IF test for antibody, or a positive skin test, or both.

Statistical analysis. The Student t-test was used to determine the statistical significance of difference between experimental LSI values. The paired t-test used for examining pre- and postvaccination LSI from the same individuals and the unpaired t-test when analysing pre- and postvaccination LSI within a population.

Regression and correlation analysis was performed to determine the correlations between the parameters involved in determining cell-mediated immunity (e.g., skin test and lymphocyte proliferation assay).

11.3 Results

Optimal antigen concentrations for measurement of LSI. The optimal concentrations of *C.burnetii* antigens for use in the LSI tests were determined by taking the PBMC of seven subjects who were known to have been previously exposed to Q fever antigens, either by natural infection or vaccination, and testing them against a series of concentrations of each antigen: 2.50, 1.25, 0.625, 0.313 µg/ml.

PBMC from five subjects who were considered unlikely to have come into contact with Q fever antigens were tested in an identical manner.

The mean values and standard errors for LSI response for these two groups at each antigen concentration is shown in Figure 11.1(a) and 11.1(b) for Phase I and Phase II antigens, respectively. From these curves the optimal concentrations of antigen were taken as 1.25µg/ml for both Phase I and Phase

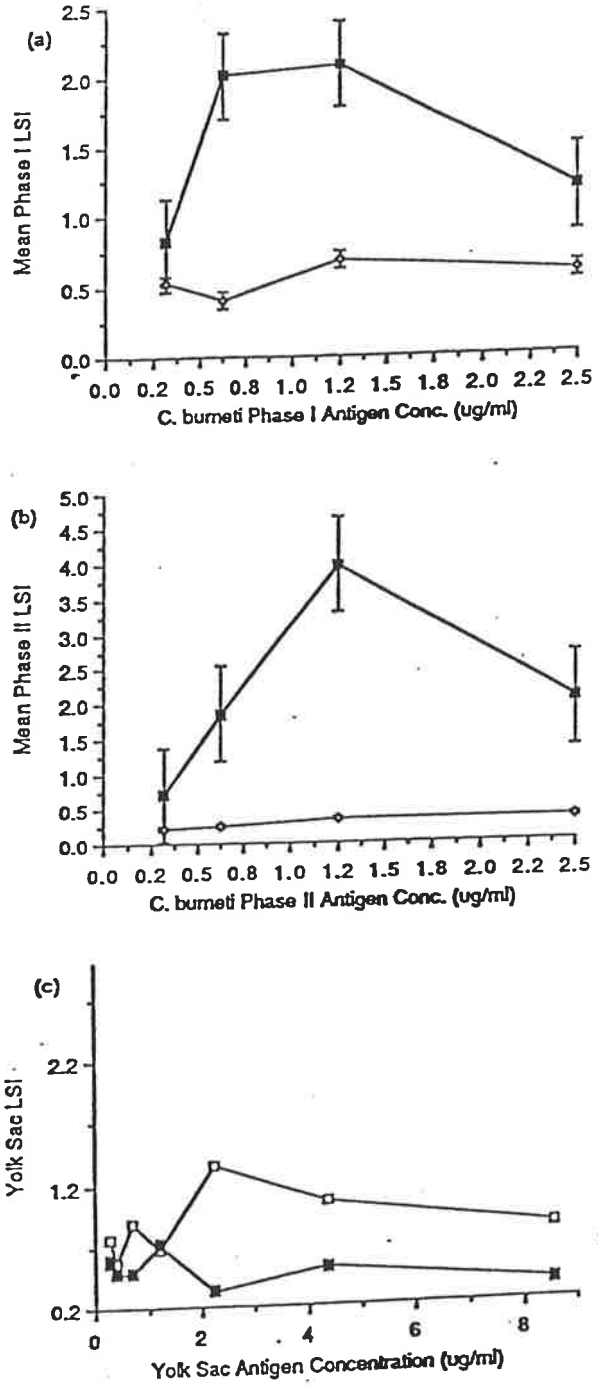
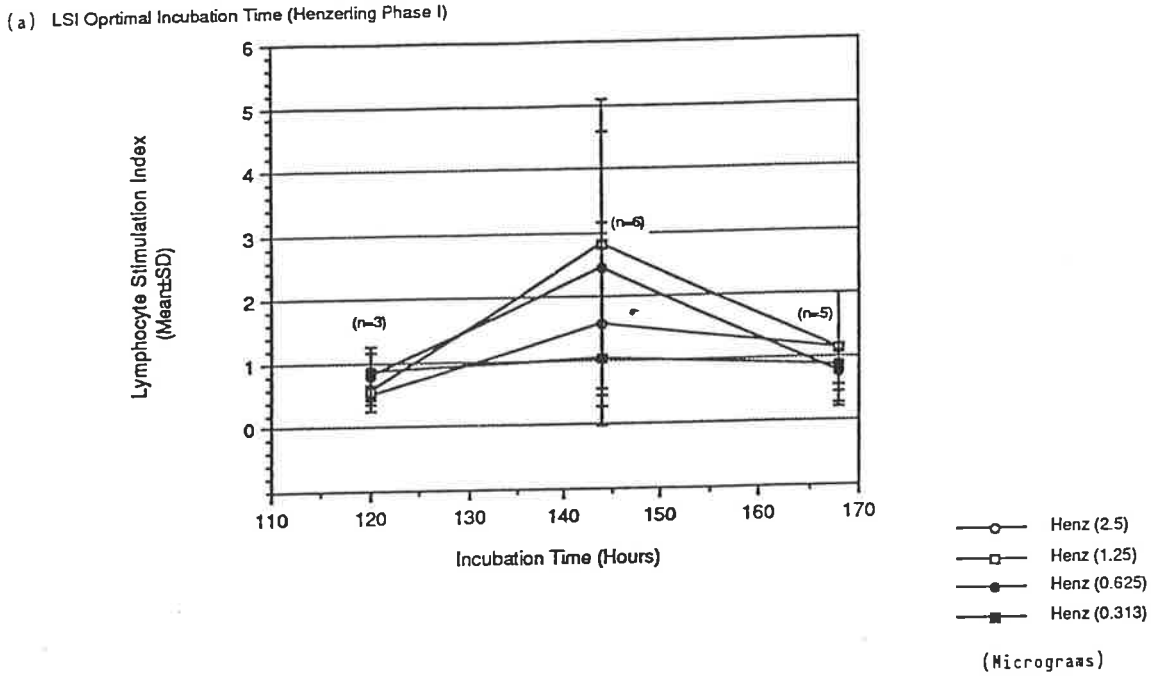


Figure 11.1 (a),(b),(c). Determination of optimal antigen concentrations for measurement of LSI values. The mean LSI response of PBMC to C.burnetii of a group of seven subjects immune to Q fever (■) and of five non-immune subjects (◇) is plotted against (a) a range of concentrations of C.burnetii Phase I and (b) Phase II antigens. Similarly, in (c) the yolk sac antigen response is determined with PBMC from an individual with an allergy to eggs (□), coplotted with the responses of cells from non-allergic individual (■).



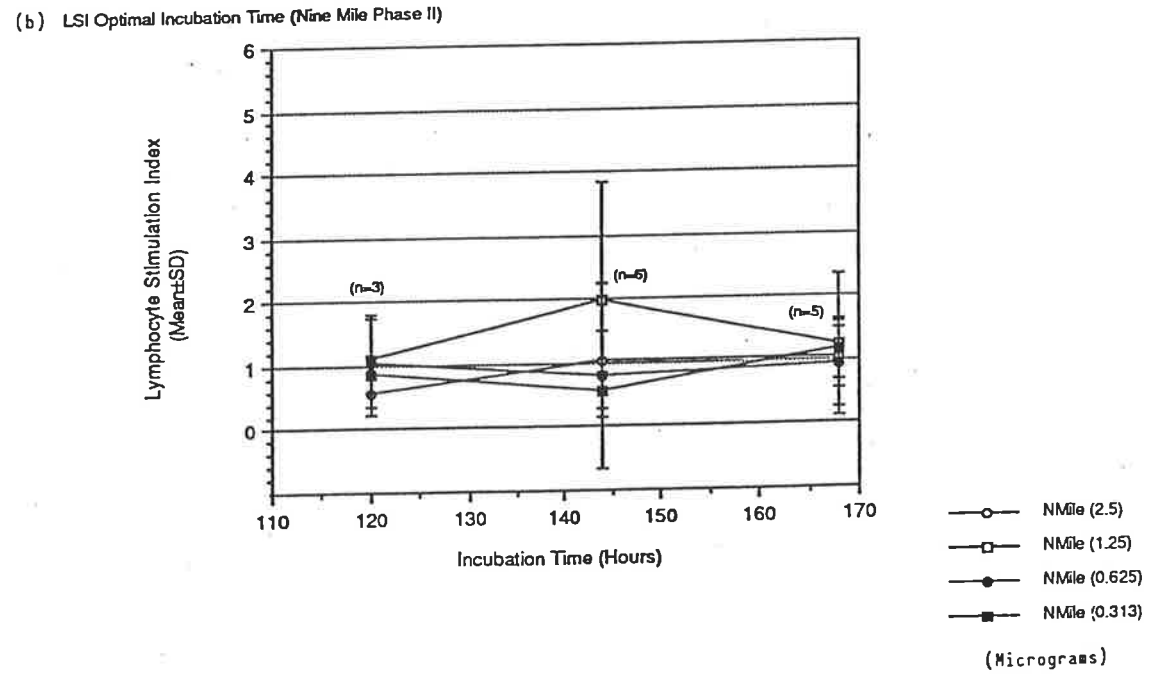


Figure 11.2 (a),(b). Determination of optimal time for *in vitro* measurement of LSI values. The mean LSI response of PBMC from 6 vaccinated subjects, to several concentrations of (a) *C.burnetii* Phase I antigen and (b) *C.burnetii* Phase II antigen, is plotted against time in culture.

II antigen.

The YS antigen was used at a concentration of 2.1µg/ml as this gave an LSI value greater than 1 with the cells of an individual who had an allergy to eggs. The latter also shows the cell responses of a non-allergic subject (Figure 11.1(c)).

The PHA-P concentration was determined for each batch received before use as the positive stimulant for the PBMC. The LSI value obtained for all subjects tested was greater than 100.

Assessment of optimal incubation time for the *in vitro* LSI assay. To determine the optimal time for the development of the *in vitro* LSI assay, PBMC from six vaccinees were cultured with *C.burnetii* whole cell antigen at time periods of 120, 144 and 168 hours. Figure 11.2(a) and (b), represent the mean (±SD) of the LSI over various concentrations of Henzerling Phase I and Nine Mile Phase II organisms, respectively. The optimal time for the incubation of reaction mixtures to give an elevated LSI with PBMC from previously sensitised individuals was 144 hours. Variation in LSI response, as revealed by the SD values, may reflect the variation between human subjects to respond to antigenic stimulation.

Comparison of lymphocyte stimulation indices with PBMC from 5 vaccinees and two preparations of Nine Mile Phase II antigens. Figure 11.3 presents the LSI values obtained when PBMC from 5 vaccinees were stimulated with (a) periodate-treated Nine Mile Phase II antigen (Colindale) and (b) Nine Mile Phase

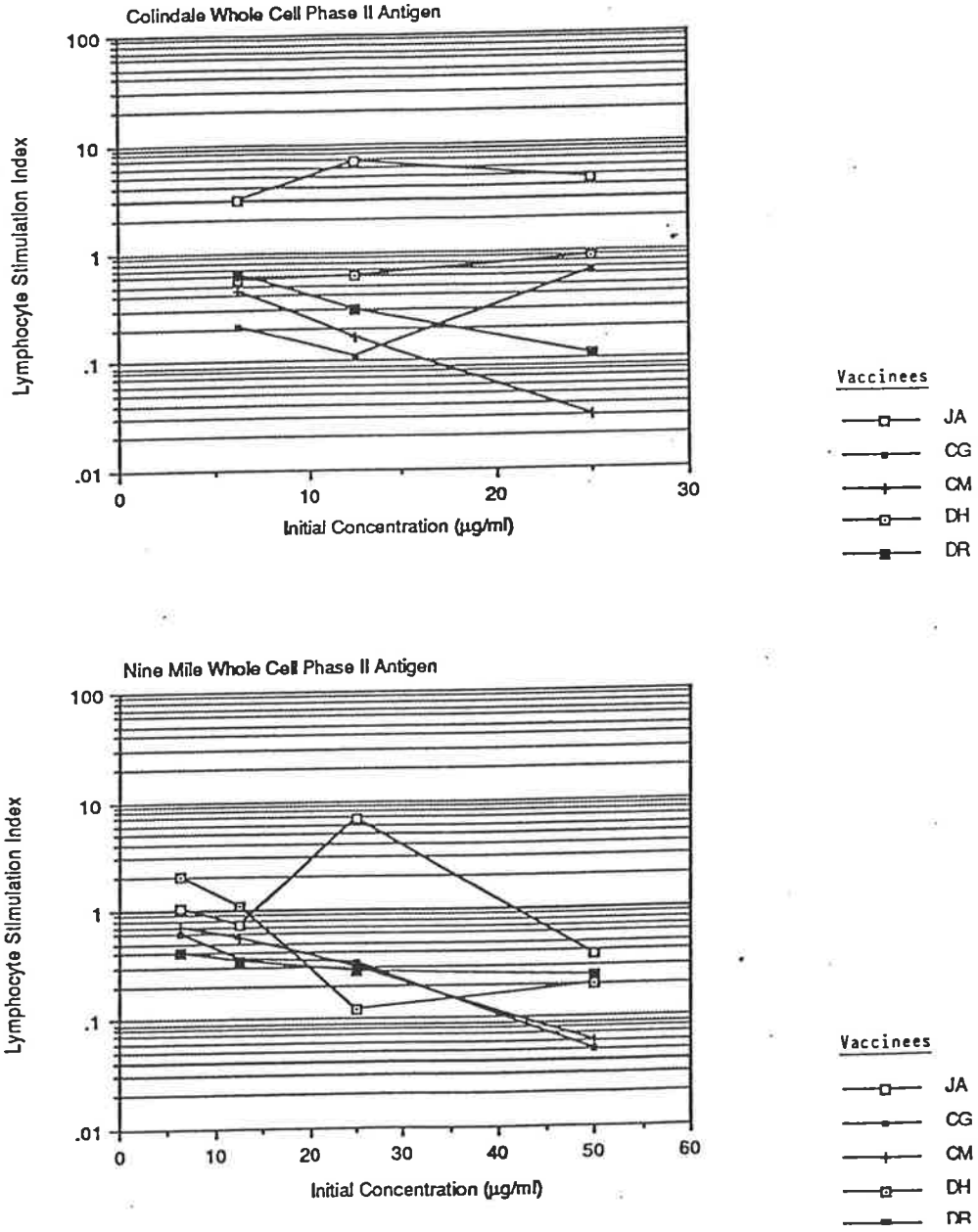


Figure 11.3. Comparison of lymphocyte response profiles to Nine Mile Phase II antigens from two different sources: (a) Colindale Laboratories, England, and (b) Rocky Mountain Laboratory, U.S.A.

II antigen (Rocky Mountain) given multiple egg passages and plaque-purified. The response pattern among the vaccinees to either antigen is similar in terms of the number of values above the level of 0.1 and below 10.0.

Determination of specific and significant levels of response in lymphocyte stimulation assays with *C.burnetii* antigens.

It was evident not only from previous work (Ascher *et al.* 1983b, Jerrells *et al.* 1975) but also from our initial experiences with the lymphocyte stimulation assays with *C.burnetii* antigens that the LSI obtained from PBMC samples were mostly grouped in the range 1 to 10, with smaller numbers in the range 10 to 100; values above 100 were rare, even in subjects who had experienced overt or subclinical infection with *C.burnetii* in the past. Such a distribution of values - in contrast to the values over 200-300 obtained with the panstimulator, PHA - might (presumably) be expected considering the small numbers of specific antigen-sensitive lymphocytes in the total population of cells (but see results of enhancement with IL-2 in Chapter 12).

Given these small LSI values, what value ('cut-off') should be used to determine that which is 'positive' and 'negative'? This could be determined either by ascertaining the range of responses to a dissimilar, unrelated antigen (in this instance, yolk sac 'antigen') to the one providing the immune stimulation, or, alternatively, that to the specific stimulating antigens in persons who have not been exposed and who are non-immune; or both approaches might be adopted. The problem with the use of LSI from supposedly non-immunes is that contact with *C.burnetii* resulting in

subclinical infection is commoner than might be supposed as judged by antibody tests of medium sensitivity such as the complement fixation test. This is illustrated by the findings of Tabert and Lackman (1965) who observed that antibody detected by radioimmunoprecipitation was up to 3 times commoner in occupationally exposed persons than CF antibody. Furthermore, it now appears that antibody tests such as complement fixation, immunofluorescence, micro-agglutination etc. are less sensitive than LSI. There is therefore no easy way of independently checking the actual immune state of those considered to be immunologically 'virgin' because of their apparent lack of contact.

The specificity and magnitude of the responses to *C.burnetii* antigens, and to the Q fever vaccine in particular, could also be validated by measuring the response to vaccination in subjects shown to be 'negative' (i.e., below the cut-off value) before vaccination.

All of these approaches were adopted in our preliminary calibration of the lymphocyte stimulation assays.

In the first, the values for LSI given with the dissimilar control antigen - yolk sac - and PBMC from 183 subjects (namely: 'high-risk' (SAMCOR) vaccinated or non-vaccinated, in total, 106; 'low-risk' non-vaccinated or pre/postvaccination, in total, 77) were combined from some 45 assay runs. The total sample gave a mean LSI of 0.48 with a standard deviation of 0.37. The significance or 'cut-off' level was therefore taken as the mean plus 2SD = 1.2; this value is used in all figures and tables.

In the second, the specificity and magnitude of response were assessed

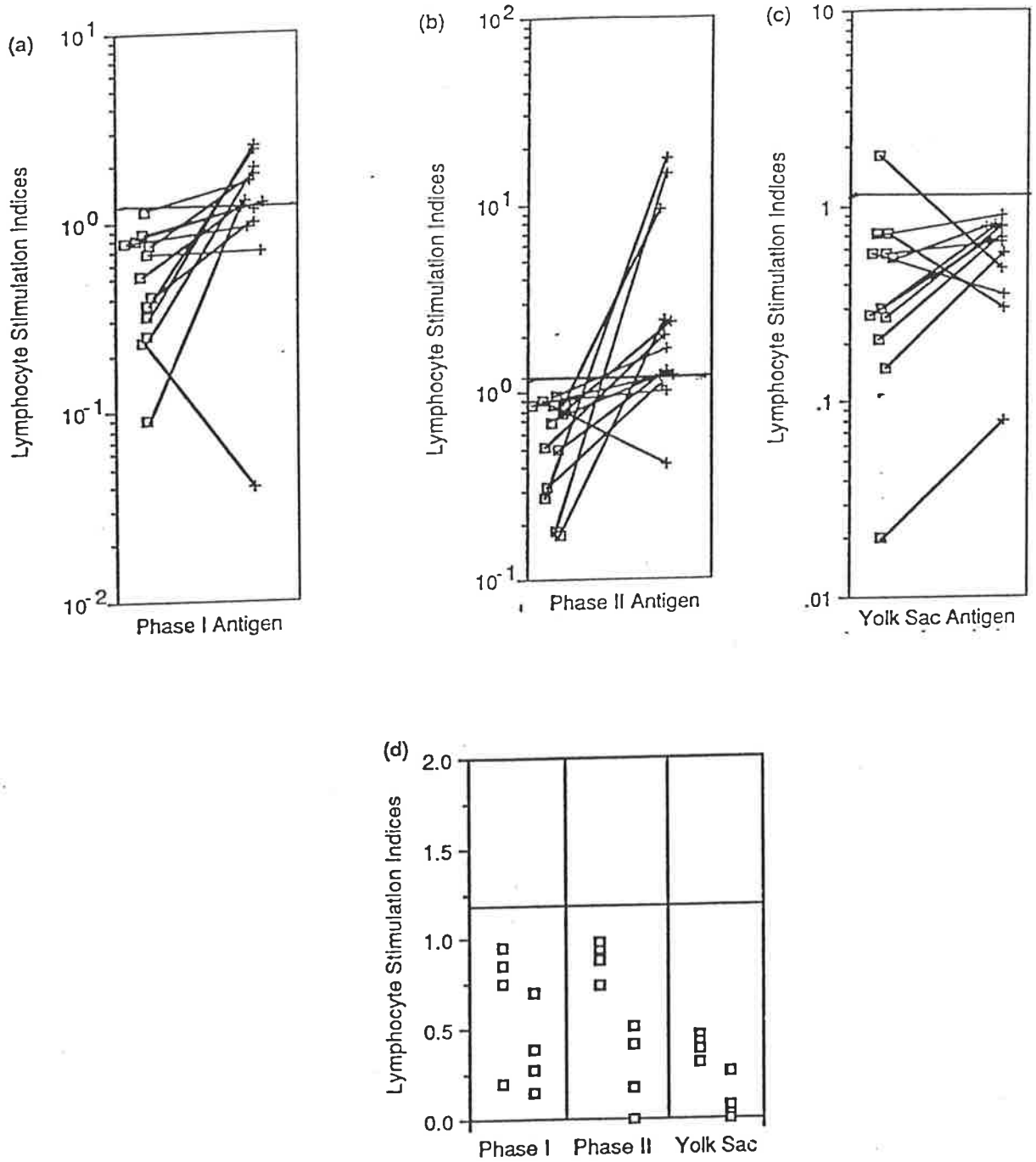


Figure 11.4 (a),(b),(c),(d). Scattergrams of the LSI obtained with (a) C.burnetii Phase I and (b) II antigens and (c) to yolk sac control antigen in 13 subjects from the 'low-risk' population whose prevaccination samples were antibody and LSI negative. The horizontal line across the scattergram represents the 1.2 'cut-off' level derived from replicate observation with yolk sac. Panel (d) shows results with 8 laboratory workers with no occupational exposure to Q fever.
(NB: □ = prevaccination, + = postvaccination).

by analysing the response to vaccine of a subset of 13 subjects from the low exposure group with pre- and postvaccination samples who, prevaccination, were negative in the CF test (<2.5 with *C.burnetii* Phase I and II antigens) and in the IF test (<10 with both antigens) and who had LSI <1.2 with both antigens. The pre and postvaccination LSI values to the two antigens and yolk sac control of this group is shown in Figure 11.4 (a),(b),(c),(d).

It will be seen from this scattergram that - as selected - all the prevaccination samples were below the 1.2 cut-off level with the two *C.burnetii* antigens and, with one exception, with the yolk sac antigen. The postvaccination samples showed a substantial conversion, most marked with the Phase II antigen (Figure 11.4 (b)). All postvaccination samples remained under the cut-off level with the yolk sac control (Figure 11.4 (c)). In total, 85% of the vaccinated subjects converted to a 'positive' LSI with one or other antigen, or both. Student t-test analysis of the differences in the degree of response between pre- and postvaccination LSI values gave $0.0005<p\leq0.005$ for Phase I and $0.01<p\leq0.025$ for Phase II but $0.1<p\leq0.38$ for the yolk sac control. Analysis of these variables with Student t-test showed that postvaccination LSI values with Phase I and II antigens were significantly greater than with the yolk sac control (Phase I: $p\leq0.0005$, Phase II: $0.01<p\leq0.025$). Figure 11.4 (d) also shows the range of LSI obtained with the 3 antigens with cells from a group of 8 laboratory workers considered to have no occupational or environmental exposure to Q fever and who were not vaccinated. It will be seen that the LSI values in all subjects were below the 'cut-off' level.

Table 11.1: Relation between frequency of immune markers after vaccination, and the presence or absence of such markers in prevaccination samples from 20 'low risk' subjects.

PREVACCINATION STATE	POSTVACCINATION STATE LSI +ve* OR Ab +ve#		TOTAL SUBJECTS TESTED
LSI +ve* or Ab +ve#	6 (86%)	4 (57%)	7 (100)
LSI -ve/Ab -ve	11 (85%)	5 (38%)	13 (100)
	17 (85%)	9 (45%)	20 (100)

* Lymphocyte stimulation index (LSI) positive = a value above the cut-off point of 1.2 with *C.burnetii* Phase I and/or II antigens, with a value < 1.2 with control yolk sac suspension.

Antibody positive = serum collected at the same time as the peripheral blood mononuclear cells which showed either CF antibody to *C.burnetii* Phase I or II antigens at a serum dilution of ≥ 2.5 or IF antibody ≥ 10, or both

Table 11.1 summarises the frequency of positive LSI and antibody markers in the sample of 20 'low-risk' subjects, from whom pre- and postvaccination samples were available, and compares the 7 subjects who had either positive LSI or CF or IF antibody before vaccination with 13 who were LSI-antibody negative. The proportion in both groups with positive LSI after vaccination was virtually identical - 85-86%; antibody markers were slightly commoner after vaccination (57% versus 38%) in those with (presumptive) evidence of previous exposure or sensitisation, but the numbers are small, and statistical significance is not attained as analysed by the Student t-test ($0.05<p\leq0.1$). The significance of the prevaccination immune markers is considered in the Discussion.

Development and persistence of immune markers at intervals after vaccination in a 'low-risk' population. Table 11.2 summarises the results with 32 vaccinees, from whom postvaccination samples were available; these were sampled in groups at different times after vaccination. Eighty-seven percent of vaccinees had positive LSI by two weeks after vaccination, the earliest positive value (in a subject negative in a prevaccination specimen) being observed at 9 days after vaccination. However, in this subgroup of 8 subjects, only 12% had CF or IF antibody. In a subgroup of 10 subjects examined 2-4 weeks after vaccination, the frequency of positive LSI was similar, but 80% of subjects had antibody.

In the subgroup of 14 subjects sampled at varying intervals from 5 to 96 weeks, the proportion with antibody was again low - 14% - but that for LSI was

Table 11.2: Frequency of immune markers (CF and IF antibody and lymphocyte stimulation indices to *C.burnetii* Phase I and Phase II antigens) at various time intervals (weeks) from vaccination in subjects in a 'low-risk' group.

	WEEKS POSTVACCINATION									ALL TIME INTERVALS		
	<2			2-4			5-96					
LSI*:	**+**	**-**	**Sub total**	**+**	**-**	**Sub total**	**+**	**-**	**Sub total**	**+**	**-**	**Total**
ANTIBODY*:												
+	1	0	1 (12)	6	2	8 (80)	1	1	2 (14)	8	3	11 (34)
-	6	1	7	2	0	2	11	1	12	19	2	21
SUB TOTALS	7 (87)	1	8 (100)	8 (80)	2	10 (100)	12 (86)	2	14 (100)	27 (84)	5	32 (100)

* Values as defined in Table 1

over 85%.

The general pattern of response after vaccination in the 'low-risk' group therefore appeared to be, first, appearance, around 9 days after vaccination, of significantly raised LSI followed by a short lived prevalence of antibody between 2 and 4 weeks after vaccination. Overall, 84% of subjects had persisting, positive LSI values (the longest being observed at 96 weeks after vaccination in this 'low-risk' group) but only 34% had antibody by the tests (CF and IF) of medium sensitivity employed.

These findings after vaccination of the 'low-risk' population are considered to validate the lymphocyte proliferation assay, along with antibody measurements by various techniques, as a legitimate means of assessing, *in vitro*, the immunity induced in abattoir workers.

Sequential analysis of lymphocyte proliferation responses after vaccination. The development of lymphocyte proliferation responses after vaccination was examined in seven low-risk vaccinees over a period of time. Before vaccination, each individual was assessed serologically by complement fixation test for Q fever antibody and by Q fever specific skin test. PBMC collected from each individual were cultured with whole cell *C.burnetii* antigens; Henzerling Phase I and Nine Mile Phase II, and with yolk sac antigen. PBMC were also cultured with the mitogen PHA.

Figures 11.5 (a),(b),(c),(d) graph the lymphocyte stimulation indices (LSI) for each subject against time (days) post-vaccination. The LSI for both Phase I and II antigens was elevated after vaccination in 6 subjects; the seventh

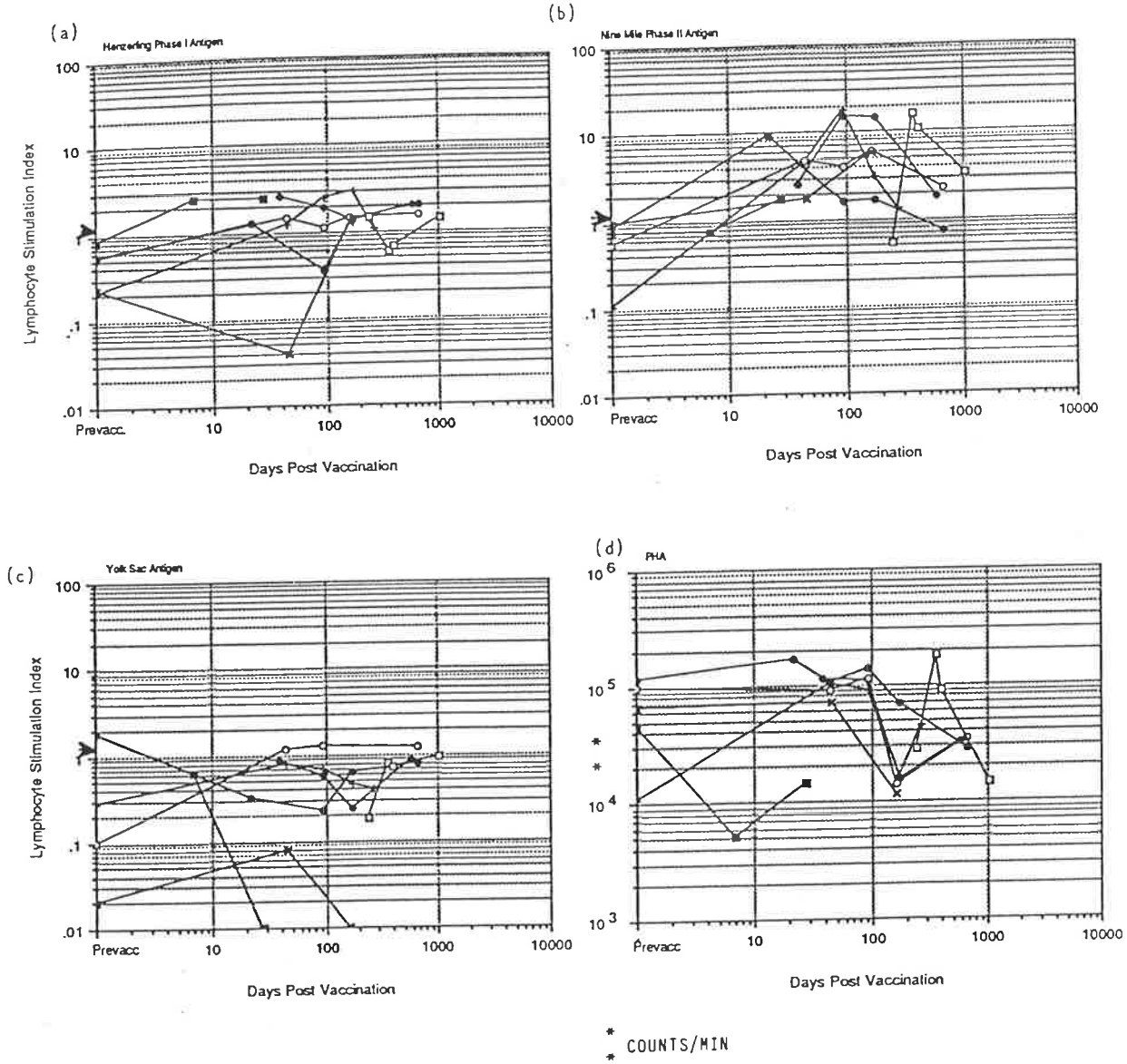


Figure 11.5 (a),(b),(c),(d). Curves show the sequential lymphocyte stimulation indices of PBMC from 7 vaccinees when stimulated with (a) Henzerling Phase I, (b) Nine Mile Phase II and (c) yolk sac antigen suspension, at various times after vaccination. Note that panel (d) shows mitogenic responses in the same individuals after PHA stimulation and plots 'counts-per-minute' against days post-vaccination. ➤ Cut off point (see text).

reacted only with Phase II antigen. In all instances the Nine Mile Phase II whole cell antigen detected a sharp rise in LSI immediately after vaccination, which then gradually declined over time. With the Henzerling Phase I antigen, the LSI values gradually increased over time from vaccination, indicating a slower response to Phase I than to Phase II antigens. The LSI values with yolk sac antigen were negative and validated the specificity of the *C.burnetii* reactions with the lymphocytes.

Mitogenic response curves for each individual, though variable from test to test, were within the normal range and indicated that there was no immunosuppressive effect (see Damrow *et al.* 1981) induced by vaccination with Qvax.

Immune markers in a high exposure population. This part of the study was undertaken to answer particular questions in relation to vaccination policy in the abattoir. Namely, if abattoir workers - particularly new recruits suspected to be non-immune and at risk of clinical Q fever - are 'screened' for Q fever antibody and skin tested, and then vaccinated if negative, what immune markers can be detected after vaccination which correlate with their apparent resistance to natural infection? And further, how long after vaccination can these markers be detected?

Some additional points should be noted. First, it is not claimed that all subjects who are antibody/skin test negative are in fact non-immune and susceptible to Q fever although the attack rate of natural infection is substantially higher in this group as compared with other abattoir workers

Table 11.3: Frequency of immune markers at various times after vaccination in 81 high-risk subjects.

IMMUNE MARKERS (IM) BEFORE VACCINATION, ANTIBODY (Ab) STATUS AFTER VACCINATION*	NUMBER OF SUBJECTS (%) WITH IMMUNE MARKERS AT STATED TIMES AFTER VACCINATION					
	20-40 MONTHS			41-60 MONTHS		
	LSI+	LSI-	SUBTOTAL	LSI+	LSI-	SUBTOTAL
IM PRESENT						
Ab POSITIVE	19	1	20 (90)	7	0	7 (100)
Ab NEGATIVE	2	0	2	0	0	0
SUBTOTAL	21 (95)	1	22 (100)	7 (100)	0	7 (100)
IM ABSENT						
Ab POSITIVE	19	2	21 (77)	10	0	10 (40)
Ab NEGATIVE	6	0	6	14	1	15
SUBTOTAL	25 (92)	2	27 (100)	24 (96)	1	25 (100)

NOTE: The groups at the various times after vaccination are not the same cohorts of individuals repeatedly sampled in successive years but are different groups sampled at the stated times.

* The presence of immune markers before vaccination was defined as a positive test by CF, IF or RIA or, for those subjects in whom these tests were negative, a positive skin test. The absence of these markers was defined as a negative CF, IF, or RIA and a negative skin test. LSI: lymphocyte-stimulation index; +: positive; -: negative.

with antibody or a positive skin test (Marmion *et al.* 1984, 1990). Second, it is not claimed that when an antibody/skin test negative subject is vaccinated, and tested subsequently, the evidence of immunity found relates solely to vaccine and is not affected by periodic natural exposure in the abattoir (see Discussion).

In practical terms, prevaccination screening is done, first, to exclude subjects who because of prior immune sensitisation may react adversely to vaccination, and second, to exclude those who by virtue of this sensitisation are obviously immune and do not require vaccination and might suffer adverse reactions if given it.

Groups of workers at the SAMCOR abattoir who had been vaccinated in 1981 and in subsequent years, were sampled for antibody, and the LSI to *C.burnetii* Phase I and II antigens determined (Table 11.3).

Note that the groups at the various time intervals after vaccination are not the same cohort of individuals, repeatedly sampled in successive years, but different groups sampled at the stated time from vaccination.

The 81 subjects were divided into those who had immune markers before vaccination (n=29) and those who were negative (n=52; Table 11.3). In the former group, 90-95% were antibody or LSI positive 20-40 months after vaccination, and essentially the same pattern was found in those subjects who were sampled 41-60 months after vaccination. In the group without immune markers before vaccination, 77% had antibody, and 92% had a positive LSI 20-40 months after vaccination. By 41-60 months after vaccination, only 40% had antibody, but 96% had a positive LSI. Thus the pattern of response in

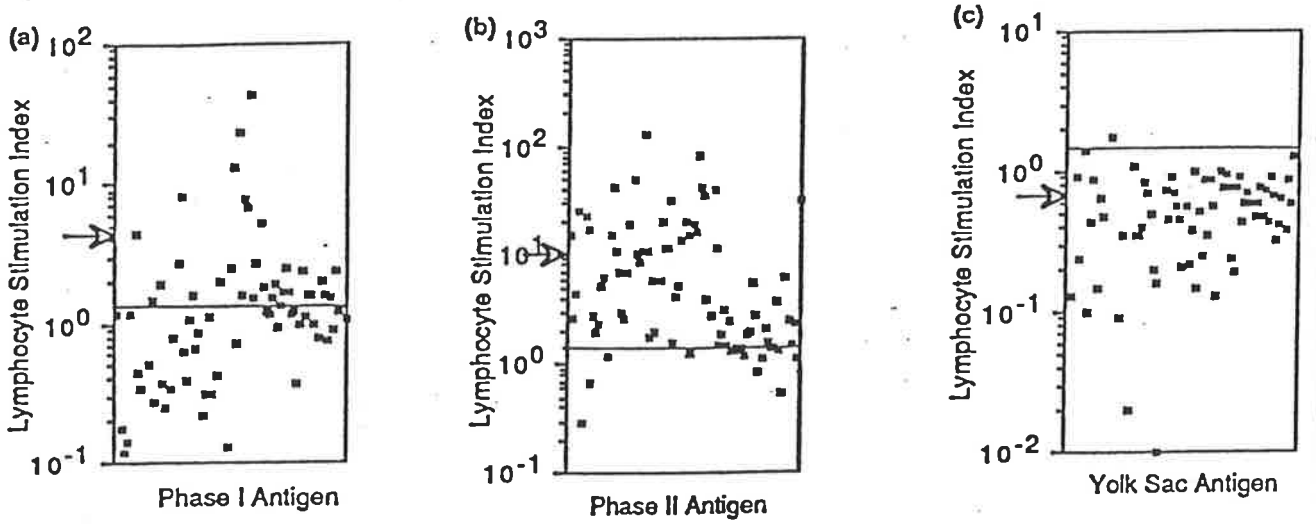


Figure 11.6 (a),(b),(c). Scattergrams of LSI obtained in a group of 81 abattoir workers with (a) C.burnetii Phase I (b) Phase II antigens and (c) yolk sac control antigen, sampled at varying intervals after vaccination. The horizontal line is 1.2 'cut-off' value. The arrows indicate the mean LSI value for each antigen.

those abattoir workers without immune markers before vaccination resembled that in the low-risk group (Table 11.2), but titers of antibody and positive LSI values were somewhat lower in the latter group (i.e., 84% for LSI and 34% for antibody). Student's t-test analysis of the responses to the separate antigens for the low-risk group and the initially negative high-risk group showed no significant difference for the LSI values in the two groups ($0.1<p\leq0.38$).

It is surmised that these differences arise either from the inclusion, in each group of vaccinees, of subjects who although antibody and SKT negative were in fact 'primed' to *C.burnetii* and responding more vigorously or, alternatively, that the vaccinees in the 'high risk' group were stimulated by natural exposure to maintain raised LSI and antibody (see Discussion).

Further analysis of LSI within the 'high risk' population. Figure 11.6 (a),(b),(c) presents the individual LSI to *C.burnetii* Phase I and Phase II antigens, and yolk sac antigen, in the total group of 81 vaccinated workers at the SAMCOR abattoir. With one exception, all values with yolk sac antigen were below the 'cut off' level of 1.2 (horizontal line). Values with Phase II antigen were higher (mean=11.59, SD=19.16) than those with Phase I (mean=2.25, SD=5.57). LSI in both of the latter groups were significantly higher than those with yolk sac antigen (Phase I vs yolk sac antigen $p\leq0.005$; Phase II vs yolk sac antigen $p\leq0.0005$).

Figure 11.7 (a),(b) shows the distribution of LSI in groups of vaccinees sampled at intervals after vaccination. With Phase II antigen, values were higher in the groups sampled within 30-40 months after vaccination when

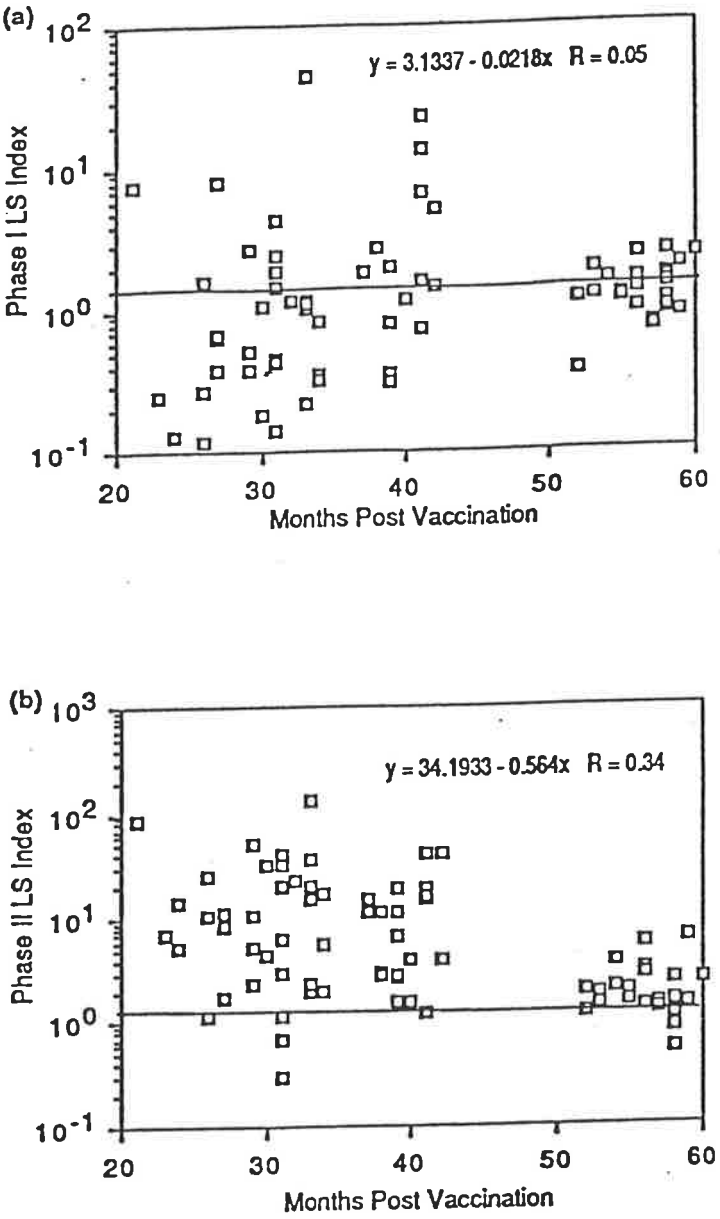


Figure 11.7. Relation between lymphocyte stimulation indices and time since vaccination in subgroups of 81 subjects in a 'high-risk' group. The continuous horizontal line represents the 'cut-off' value of 1.2 LSI.

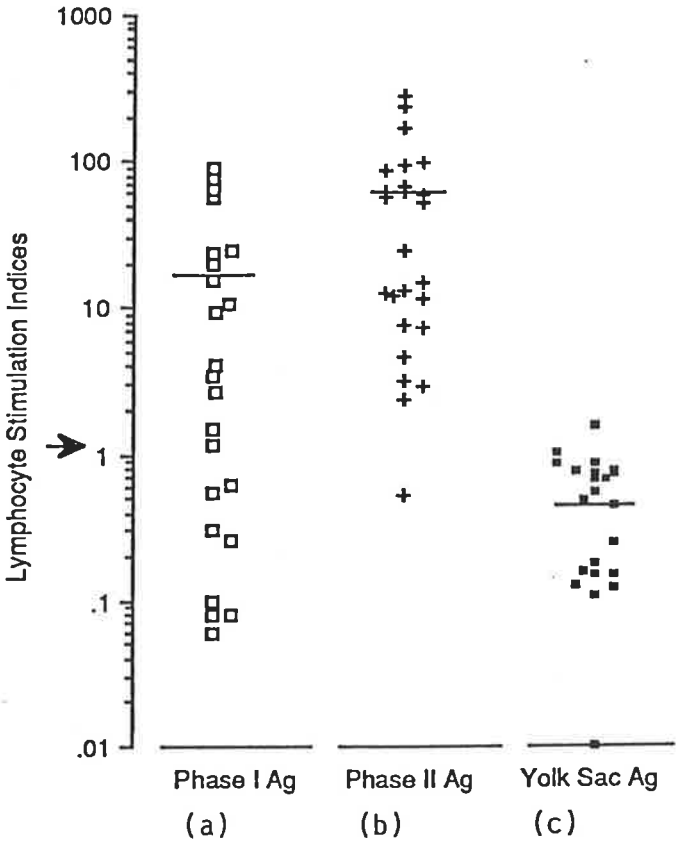


Figure 11.8 (a),(b),(c). Scattergrams of LSI to C.burnetii Phase I antigen (a), Phase II antigen (b), and yolk sac antigen (c), in 25 subjects in 'high-risk' population excluded from vaccination because of positive antibody or skin test. The arrow is the 1.2 'cut-off' value. The horizontal lines indicate the mean LSI value for each antigen.

compared with 50-60 months ($r=0.34$: $p \leq 0.005$). The results with Phase I antigen, on visual inspection, suggest the same pattern but the scatter of observations is larger and there are more values under the 'cut-off' level and a significant correlation was not obtained ($r=0.05$: $p \leq 0.25$).

Patterns of lymphocytic proliferative responses and skin test responses in vaccinated and unvaccinated, sensitised subjects in a 'high risk' population. During the initial 18 months of the clinical trial of vaccine, (Marmion *et al.* 1984) no Q fever cases were observed in abattoir workers who, on enrolment in the trial, had CF antibody at a titer of 10 or higher, or who were skin test positive. This pattern of resistance has been maintained during the period 81-86; in particular, no individual with a clearly positive skin test has developed clinical Q fever (Marmion *et al.* 1990). Given this experience, it was of interest to determine LSI in a group of enrolled but unvaccinated workers with antibody and/or a positive skin test. Twenty five workers from the 'high-risk' abattoir population were available with all three measurements. Figure 11.8 shows the distribution of results with the two Q fever antigens and the control yolk sac. Overall, the patterns resembled those obtained with vaccinated abattoir workers (Figure 11.3); but the mean values for Phase I and Phase II antigens were higher [16.25 (SD=26.41) and 56.99 (SD=72.41) respectively].

Figure 11.9 (a),(b) shows the curves for LSI versus skin test diameter (mm) in the same immune unvaccinated group. There is a general tendency for LSI to be related to skin test diameter, particularly with Phase II antigen,

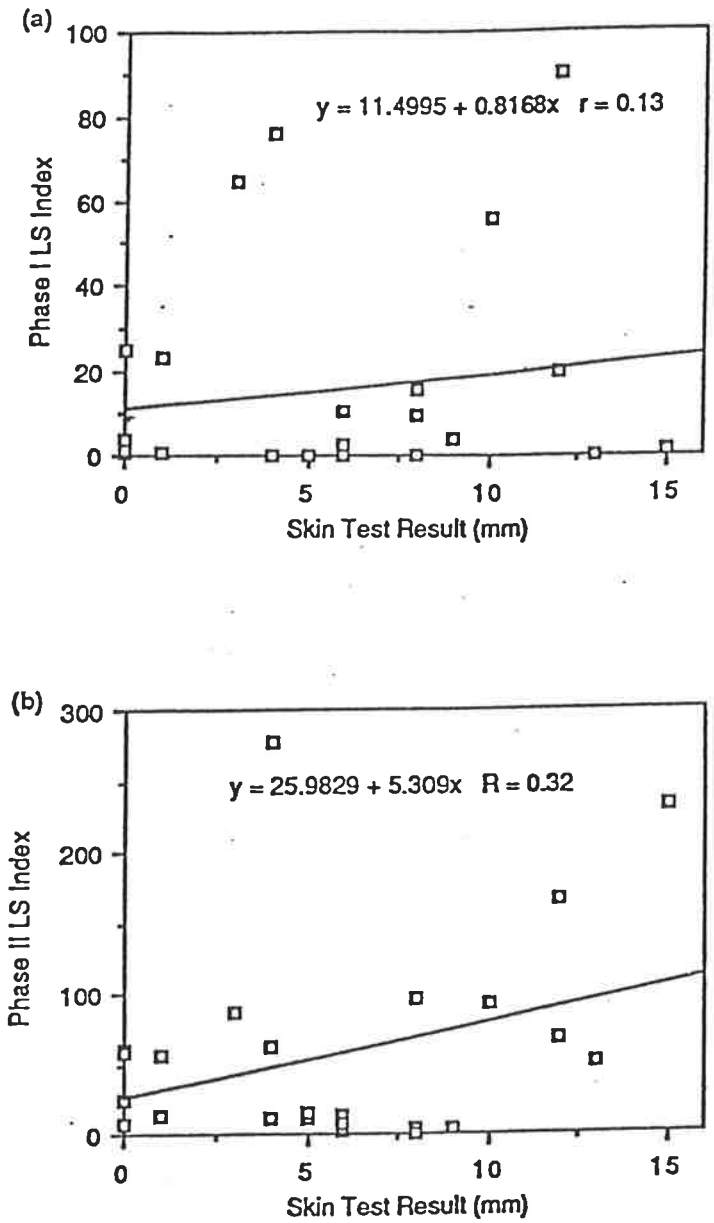


Figure 11.9 (a),(b). Relation between lymphocyte stimulation indices with C.burnetii Phase I antigen (a) and Phase II antigen (b), and skin test diameter (mm) in 24 subjects excluded from vaccination because of evidence of past Q fever infection.

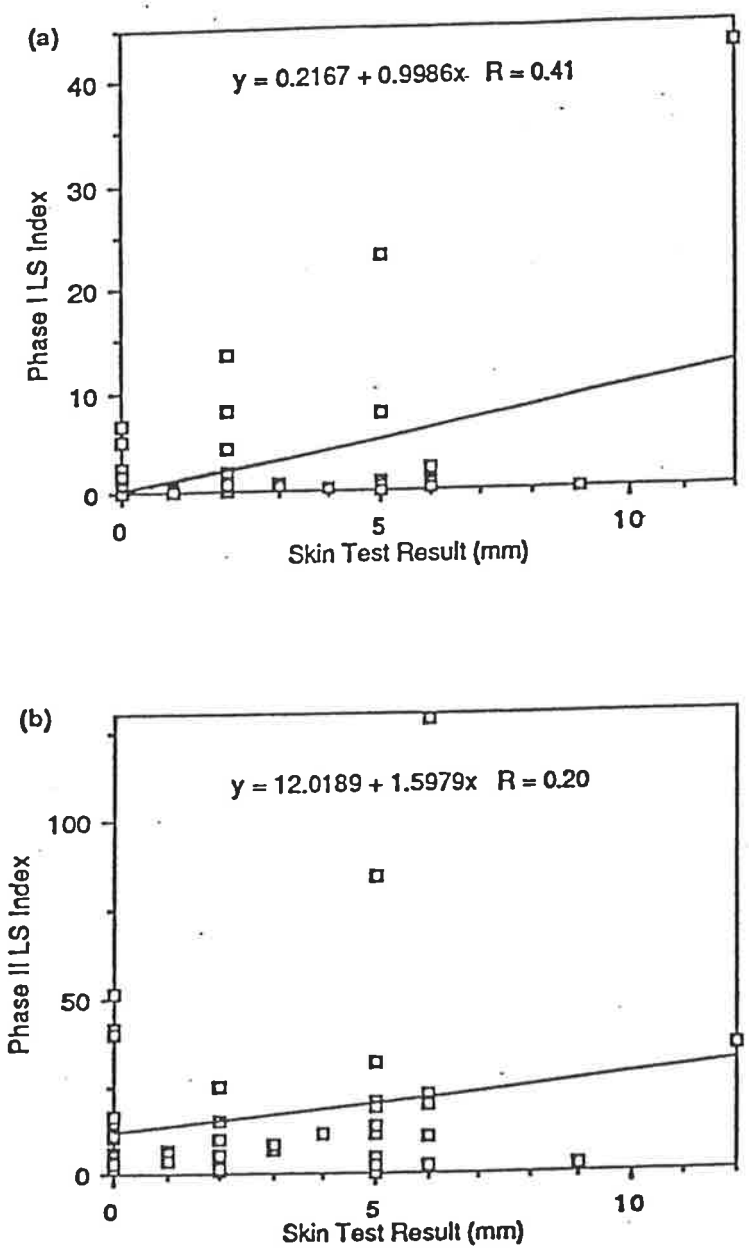


Figure 11.10 (a),(b). Relation between lymphocyte stimulation indices and skin test diameter in 52 vaccinated subjects from the 'high-risk' population; C.burnetii Phase I antigen (a) and Phase II antigen (b).

but the correlations are poor (Phase I vs skin test diam, r=0.13, $p \leq 0.25$; Phase II vs skin test diam, r=0.32, $p \leq 0.1$). This contrasts with the results obtained with Phase I antigen in vaccinated workers in this population. The correlation of the skin test with the LSI of each antigen in the vaccinated subjects is given in Figure 11.10 (a,b). There is a stronger correlation with the Phase I LSI response (r=0.41 $0.0001 < p \leq 0.005$) than with the Phase II LSI response (r=0.20 $0.10 < p \leq 0.25$). However, it is clear that in either instance positive LSI values may be obtained with a negative or equivocal skin test.

11.4 Discussion

The results establish that a single dose of 30µg of an inactivated whole cell Q fever vaccine will induce cell-mediated immunity in recipients as judged by lymphocyte proliferation assays (LSI) with PBMC and *C.burnetii* Phase I and II antigens. In a 'low-risk' population (i.e., one either not, or infrequently exposed to Q fever infection), 85% of subjects given the single dose of vaccine converted from LSI negative to positive and subsequently remained positive for weeks or months. With the dose of vaccine used, antibody response was less impressive; while 75-80% of subjects had a positive CF or IF antibody response 2-4 weeks after vaccination, the frequency of antibody was lower - 35-45% - in subjects sampled at and after 5 weeks. Positive values for LSI were higher and more frequent against *C.burnetii* Phase II, as compared with Phase I antigen. Conversion from LSI negative to positive took place within 9-15 days after vaccination, before CF or IF antibody was detected. The timing of this conversion to LSI positive is of considerable interest in relation to the

development of the protective immunity induced by the vaccine. During the vaccine trial up to 1986, eight cases of Q fever were observed among vaccinees, all in subjects clearly vaccinated during the incubation period (c 21 days) of a natural infection with *C.burnetii*; the periods of time between vaccination and onset of illness were 1,5,7,9,11,11,11 and 13 days respectively; no cases were observed at a longer period after vaccination. This suggests that immunity to natural infection is induced by vaccine after some 13 days, which agrees very well with the time of LSI conversion.

In a high-risk population of abattoir workers, who were CF and IF antibody and skin test negative before vaccination, but not tested for LSI response prevaccination, a higher proportion - 95% - were found to have positive LSI at various time intervals up to 4-5 years after vaccination. The frequencies of CF and/or IF antibody were also higher at the late stage after vaccination - around 60% - as compared with the 'low risk' population - 35-45%.

There are two possible explanations for the differences between responses to vaccination in the 'high' and 'low risk' groups. The first is that despite the prevaccination testing, a number of subjects were sensitised to Q fever antigens at levels detectable only by lymphoproliferative response and may therefore have responded with a higher and prolonged LSI and antibody formation.

The second explanation is that repeated natural exposure after vaccination has boosted the lymphocyte sensitisation initially induced by vaccine. It should be noted, however, that over a period of time, antibody

prevalence and cellular sensitivity to Phase II antigen steadily declined after vaccination (Table 11.3 and Figure 11.7). This is evidence against any substantial booster effect from repeated natural exposure or to antigen from an abortive infection.

The results described in this paper are broadly compatible with those published earlier by Ascher *et al.* (1983b). These authors used somewhat different criteria for skin test reactivity but, in line with our observations, they found a good general correlation with antibody and lymphoproliferative responses; nevertheless, some skin test negative subjects were antibody or LT positive. Vaccination with a similar Phase I Henzerling vaccine, in doses of either 6 or 30μg, produced a lymphoproliferative response to Phase II antigen in about two thirds of their vaccinated subjects; responses to Phase I antigen were less common. About the same proportion developed low titers of CF antibody. Their study group was too small to judge the persistence of positive lymphocyte responses after vaccination.

Jerrells *et al.* (1975) used a somewhat different lymphocyte proliferation assay to that in the present work. Nevertheless, the size of the lymphocyte stimulation ratios in their very small number of vaccinated or skin tested subjects corresponds to those we have observed, as do the values found in their subjects who had had Q fever in the past.

Gajdesova and Brezina (1989) examined the lymphoproliferative responses in individuals given a vaccine prepared by extraction of highly purified Phase I organisms with trichloroacetic acid (Q fever "chemovaccine"). Their results indicated that proliferative responses were directed mainly

against the Phase I antigen (in contrast to findings in Adelaide: see Chapter 16), and that these responses gradually decreased with time. When lymphocyte proliferation was compared to skin test response in a subset of the subjects, the results were similar to those found by Ascher *et al.* (1983b) in that high lymphocyte responsiveness was not well correlated with positivity in the skin test.

The studies in Adelaide have not found that conversion to skin test positive after vaccination is a useful indicator of vaccine immunogenicity in the 'high-risk' subjects at the SAMCOR abattoir. Only 31 of 52 subjects in the 'high-risk' group converted skin test positive, even to a larger skin test dose of 0.1µg of the coxiellae. These observations differ from those of Luoto et al. (1963) and Bell *et al.* (1964) who found that over 80% of their vaccinees given one dose of vaccine converted by skin test after a period of 40 weeks and 96% developed antibody detected by radioimmunoprecipitation assay. The dose of vaccine used in these trials was however, 7 times greater than that used in our trials; the latter was chosen on the basis of the dose conferring immunity on volunteers challenged with aerosols of *C.burnetii* (Ormsbee RA., personal communication).

The variations and partial correlations between lymphocyte proliferative responses, skin test reactivity and antibody formation might be expected in the light of the different classes or subsets of the lymphocyte compartment involved in humoral responses (B lymphocytes), skin test reactivity (T-helper-DTH) and lymphoproliferative responses (T-suppressor and T-helper, and conceivably, B-lymphocytes; but see Chapter 12).

CHAPTER 12

ANALYSIS OF THE CELLS INVOLVED IN THE LYMPHOPROLIFERATIVE RESPONSE TO *COXIELLA BURNETII* ANTIGENS

12.1 Introduction

The preceding chapter described studies of cellular immunity, as measured by lymphocyte proliferation assays with peripheral blood mononuclear cells (PBMC)and *C.burnetii* Phase I and II antigens in vaccinees and persons who had had Q fever in the past. These showed that positive stimulation indices (LSI) developed around 13 days after vaccination and were eventually positive in over 85% of vaccinees and remained so for at least five years. A qualitatively similar lymphoproliferative response, but of greater magnitude, was observed in subjects infected clinically or subclinically in the past; healthy subjects with no previous exposure to Q fever were negative (Chapter 11 and Izzo *et al.* 1988).

The high protective potency and prolonged CMI response with the *C.burnetii* vaccine is unusual for a killed preparation; it is thought that a living vaccine is mostly required to induce good cell-mediated immunity (CMI); but see Crowle (1988) on the requirement for adjuvancity for effective killed bacterial vaccines. The protective responses with the Q fever vaccine (Qvax: CSL) differ from those obtained with, for example, other killed rickettsial vaccines which require adjuvantation in order to protect (Murphy *et al.* 1978, 1979, 1980).

Interpretation of the findings with *in vitro* correlates of the CMI response

to *C.burnetii* (e.g., lymphocyte stimulation assays) offers some potential difficulties. Purified *C.burnetii* lipopolysaccharide (LPS) is mitogenic for the leucocytes of nonimmune guinea pigs and stimulates nonspecific protection against other organisms (Paquet *et al* 1978). Although similar effects have not been demonstrated with human PBMC it seemed desirable for this and other reasons to characterise the contributions of B- and T-lymphocytes to the cellular response following Q fever vaccination. Previous studies in our laboratory, and those of others (Jerrells *et al.* 1975, Ascher *et al.* 1983) of the lymphoproliferative response to *C.burnetii* antigens, have used PBMC or whole blood rather than lymphocyte subsets.

The experiments now described are directed to these unresolved problems. The present chapter describes fractionation - reconstruction experiments with peripheral blood mononuclear cells from subjects infected with *C.burnetii* in the past, or vaccinated with Q fever vaccine, along with cells from healthy controls.

12.2 Materials and Methods

Subjects studied. In the fractionation - reconstitution experiments with PBMC three groups of human subjects were studied, one vaccinated and two unvaccinated.

A 'low-risk' group, (A), consisted of the staff of medical laboratories vaccinated because they visit the local abattoirs to obtain animal specimens for their research, but do so infrequently and only for short periods. There were ten subjects who had been vaccinated with one 30µg dose, subcutaneously,

of a formalin inactivated, whole-cell vaccine (Qvax) from the Henzerling strain of *C.burnetii* in Phase I antigenic state prepared by the method of Ormsbee (1961). Their peripheral blood mononuclear cells (PBMC) were sampled and stimulated with antigen between five months and one year after vaccination.

A second, 'high-risk' or past infection group, (B), of six subjects worked full-time in a local abattoir. They had been enrolled in the vaccination programme and pretested (Marmion *et al.* 1984), but had been excluded from vaccination because they had evidence of previous infection (clinical or subclinical) as evidenced by the presence of either Q fever antibody or a positive skin test with diluted vaccine, or both.

A third group, (C), comprised ten persons chosen as negative controls (NC) on the basis that they had no known previous or current exposure to Q fever. Their PBMC had been tested in the lymphocyte proliferation assay with *C.burnetii* antigens, and gave LSI <1.2 - i.e., below the 'cut-off'point for a positive reaction (see Chapter 11 and Izzo *et al.* 1988); they were also negative for Q fever antibodies as determined by complement-fixation (CF) and immunofluorescence (IF) tests. They had not, however, been skin tested in order to avoid 'priming' them with a small dose of antigen.

Coxiella burnetii antigens. (i) *C.burnetii* Phase I antigen (Henzerling) was a highly-purified whole cell, Q fever vaccine prepared from the strain propagated in chick embryo (CE) yolk sac and formalin-inactivated (National Drug Company, Philadelphia, Pennsylvania, Code No. ND BR 105, lot 4/5, gift from R.A. Ormsbee and prepared by the method of Spicer *et al* (1970). It was at low

passage level and repeated examination by immunofluorescence with specific antisera did not reveal Phase II cells (Worswick and Marmion 1985). Neither the National Drug Co vaccine or Qvax showed any reaction by CF or IF with 64 CF antibody units of a rabbit antiserum raised against uninfected chick embryo yolk sac suspension.

(ii) *C.burnetii* Phase I antigen (Priscilla) was made from an isolate from the placenta of an aborting goat. The strain has unusual features - small differences in the antigenic determinants in the LPS when compared with those of standard prototype and a plasmid with additional, unique coding sequences not found in the 'generality' of *C.burnetii* plasmids. In this it resembles isolates from human Q fever endocarditis (Samuel *et al*, 1985, Hackstadt *et al.* 1985, Hackstadt 1986).

(iii) Artificially-derived Phase II antigen from CSL Nine Mile *C.burnetii* Phase I antigen was prepared by treatment with potassium periodate (Schramek *et al*, 1972). The change to Phase II serological activity was checked by 'chessboard' titration in CF test with monospecific antisera to Phase I and II antigens (Table 13.1).

(iv) Naturally-derived *C.burnetii* Phase II antigen (Nine Mile) was a gift from M. Peacock, and prepared from a strain given 90 egg passages (90EP), cloned once in cell culture, (1TC) and then passaged 4 times in eggs (4EP) (designation, Rocky Mountain Laboratory, Hamilton, Montana, RSA516 Nine Mile

90EP/1TC/4EP). The strain is permanently in Phase II (i.e., does not contain residual Phase I organisms) and is nonpathogenic for guinea pigs. Stimulation patterns with peripheral blood mononuclear cells were closely similar to those exhibited with a Nine Mile Phase II antigen obtained from the Central Public Health Laboratories, Colindale, London and prepared by potassium periodate treatment of a Phase II antigen to modify residual Phase I cells in the suspension, and also with a diagnostic Nine Mile Phase II antigen from CSL (see Chapter 11).

(v) A suspension of uninfected yolk sac was used as a negative control antigen and had a titre of 5 against an antiserum from a rabbit immunised with uninfected yolk sac suspension.

Coxiella suspensions were standardised by absorption spectrometry at 420 nm (Fiset *et al.* 1969) and adjusted to a concentration of 1000µg/ml. The optimal dose of each antigen for use in the lymphoproliferative assays was determined by titration against aliquots of reactive lymphocytes; the dilution giving the highest LSI value was selected for use in the test proper (see Izzo *et al.* (1988) and Chapter 11). The optimal dilutions were for *C.burnetii* Phase I (Henzerling): 1.25µg/ml; for *C.burnetii* Phase I antigen (Priscilla): 2.5µg/ml; and for *C.burnetii* (Nine Mile) Phase II 1.25µg/ml.

Preparation of monocyte, T cell and B cell enriched populations from PBM cells. Peripheral blood mononuclear cells (PBMC) were isolated by

centrifugation through a Ficoll/Isopaque gradient (Nycomed, Oslo, Norway) from venous blood samples treated with lithium heparin (1.25 units/ml). Monocytes (Mo) were isolated from PBMC on a discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden) (Al-Sumidaie *et al.* 1984). A three-step density gradient of iso-osmolar Percoll - 1.057, 1.066 and 1.074µg/ml - was prepared and overlayed with 1-2x10^7 PBMC and centrifuged at 2200 x g for 90 minutes (Beckman J-6B centrifuge). The Mo-enriched population was removed from the interface at 1.057/1.066µg/ml and the lymphocyte population from the 1.066/1.074µg/ml interface. Each cell population was washed twice in sterile saline. The lymphocytes were further depleted of any remaining Mo by layering over tissue culture-grade plastic (Costar, Cambridge, MA) treated with heat-inactivated foetal calf serum (FCS; Flow Laboratories, North Ryde, New South Wales) for 60 minutes at 37°C. This purified population was divided into two portions from which the T cell-enriched and B cell-enriched populations were derived by negative selection, i.e., by 'panning' as described in principle by Wysocki and Sato (1978). Enrichment for the T cells was done by treating 1.0ml of lymphocyte suspension (5-10x10^6 cells/ml) with 1.0ml of a monoclonal antibody to human µ chain, FMC HB57 (mAB) (Flinders Medical Centre (FMC), Bedford Park, South Australia) in culture medium at 4°C for 30' and then washing twice in 1% v/v FCS in PBS. After the second wash the antibody-sensitised cell suspension was made up to a total volume of 5.0 ml and poured over a polystyrene petri dish (Disposable Products, Adelaide, South Australia) that had been coated with 10 µg/ml of an affinity-purified sheep anti-mouse IgG (Selinus, Hawthorn, Victoria). After incubation at 4°C for 70

minutes, during which time the cells settled on the plates, nonadherent cells were then resuspended by gentle rocking of the plate. The supernatant fluid with the suspended cells was aspirated and the cells centrifuged out and washed twice in PBS/FCS.

Initially, checks of the cell fractions for purity were made by counting cells stained by direct or indirect immunofluorescence. Cells from each fraction were stained in suspension with mAB OKT3 for T cell markers, with FMC 33 for monocyte markers, and with goat antihuman IgG, IgM and IgA (Cappel, Philadelphia, USA) for B cell markers. T cell populations of >95% purity were obtained as assessed by these techniques. Esterase activity (α naphthylbutyrate and chloroacetate) of the isolated subpopulations was also determined (Yam *et al.* 1971). The monocyte fractions were found to be between 90% and 95% esterase positive, whereas not more than 5% of the lymphocyte fractions reacted.

In theory, the use of the anti-μ chain mAB HB57 would not sensitise B-lymphocytes expressing γ, α or other heavy chains and so effect their removal. Consequently, mixtures of mAB to γ chain and to other B cell markers were compared with the method just described. These mixtures were (a) HB135 (anti-CD 20), HB57 (anti-μ chain), HB60 (anti γ chain) and (b) HB135, HB57 and HB43 (anti γ chain). Analysis by flow cytometry (Epics 750-2, fluorescence activated cell sorter, Coulter Hileah FL USA) of the resulting populations, using a combination of mAB (HB135, HB57, FMC63 (anti-CD19), HB60, and HB43) to detect B cells, and a combination of mAB anti-CD3 and CD7 to detect T cells, showed that these procedures yielded T

Figure 12.1 Flow cytometry analysis of separated lymphocyte populations after 'panning' with various combinations of antibodies to T and B lymphocyte cell markers (see text).

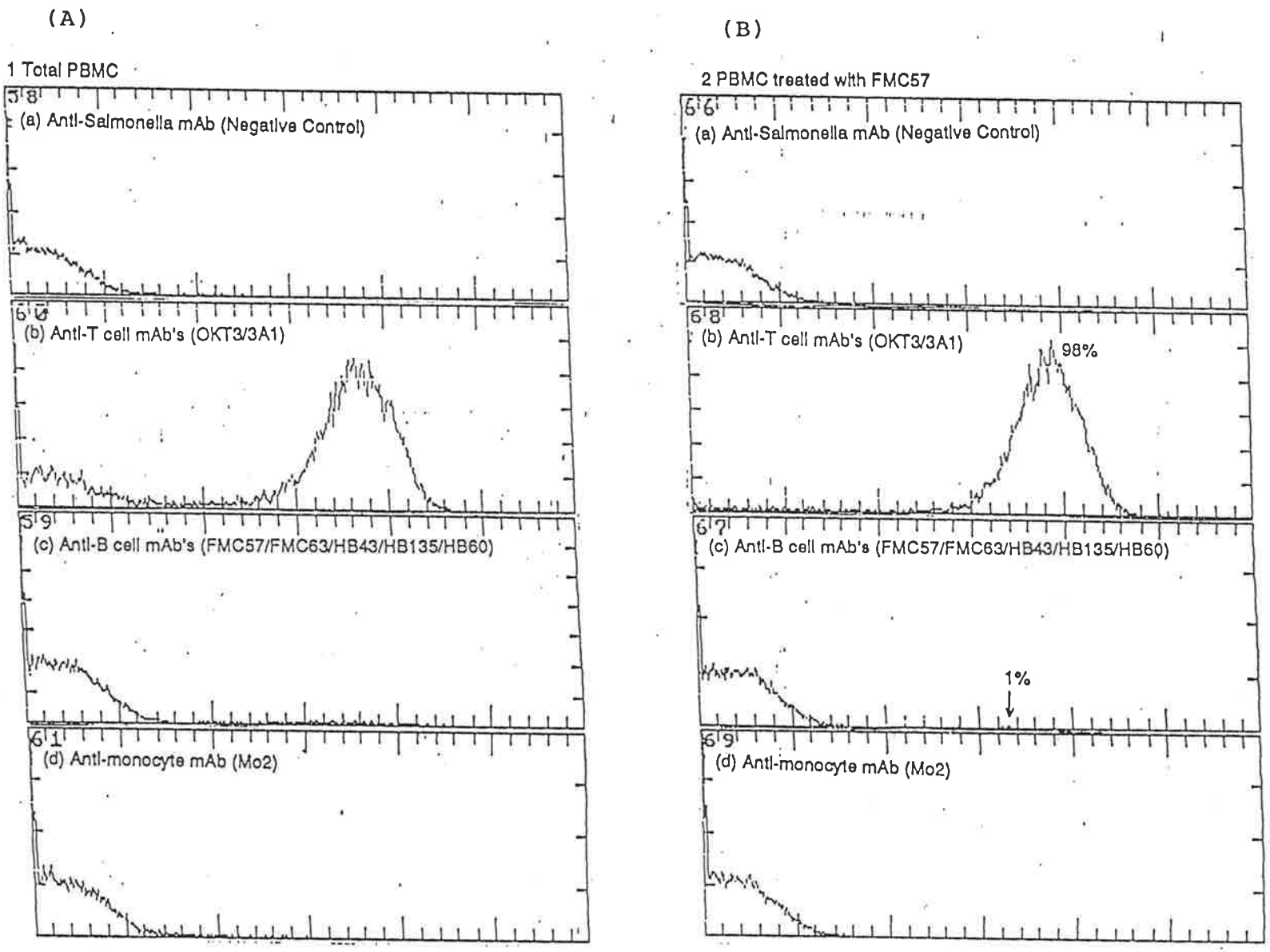
(A) Unfractionated PBMC - distribution patterns with (a) Anti salmonella mAB (negative control); (b) T cells, (mAB OKT3/3A1); (c) B cells (mAB FMC57/FMC63/HB43/HB135/HB60); (d) Monocytes (mAB Mo2).

(B) PBMC fractionated with mAB FMC57 - ditto (a) negative control; (b) T cells; (c) B cells; (d) Monocytes.

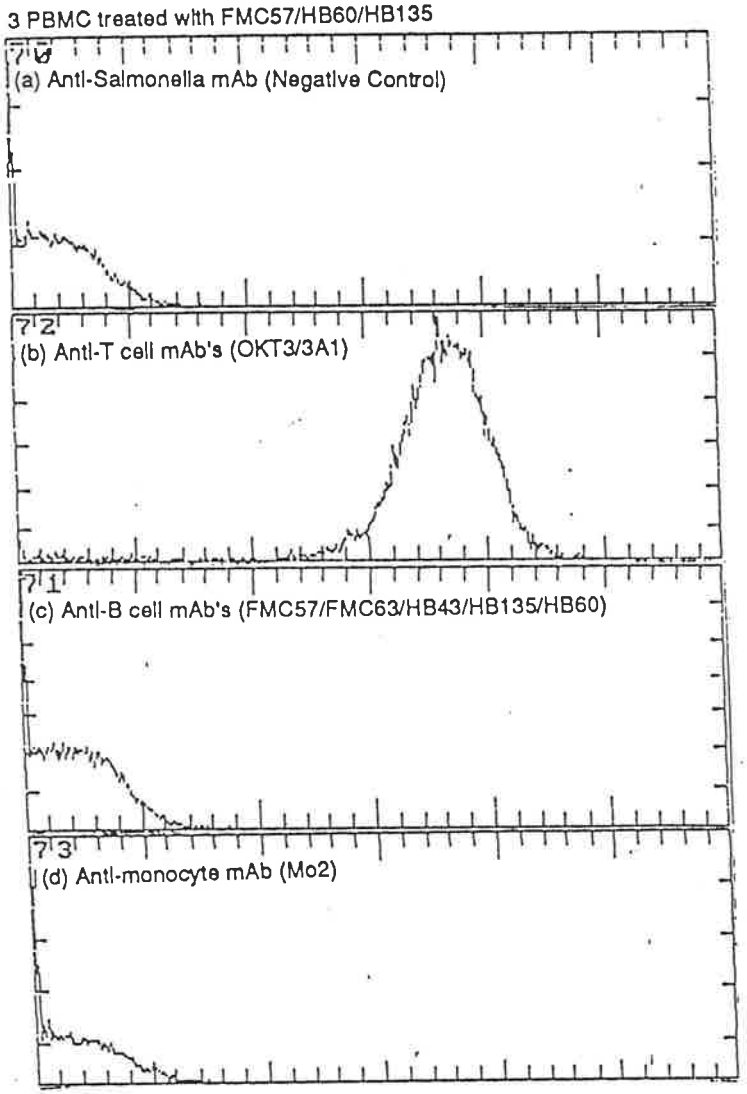
(C) PBMC fractionated with mAB FMC57/HB60/HB135 - ditto (a) negative control; (b) T cells; (c) B cells' (d) Monocytes.

(D) PBMC fractionated with mAB FMC57/HB43/HB135 - ditto (a) negative control; (b) T cells; (c) B cells; (d) Monocytes.

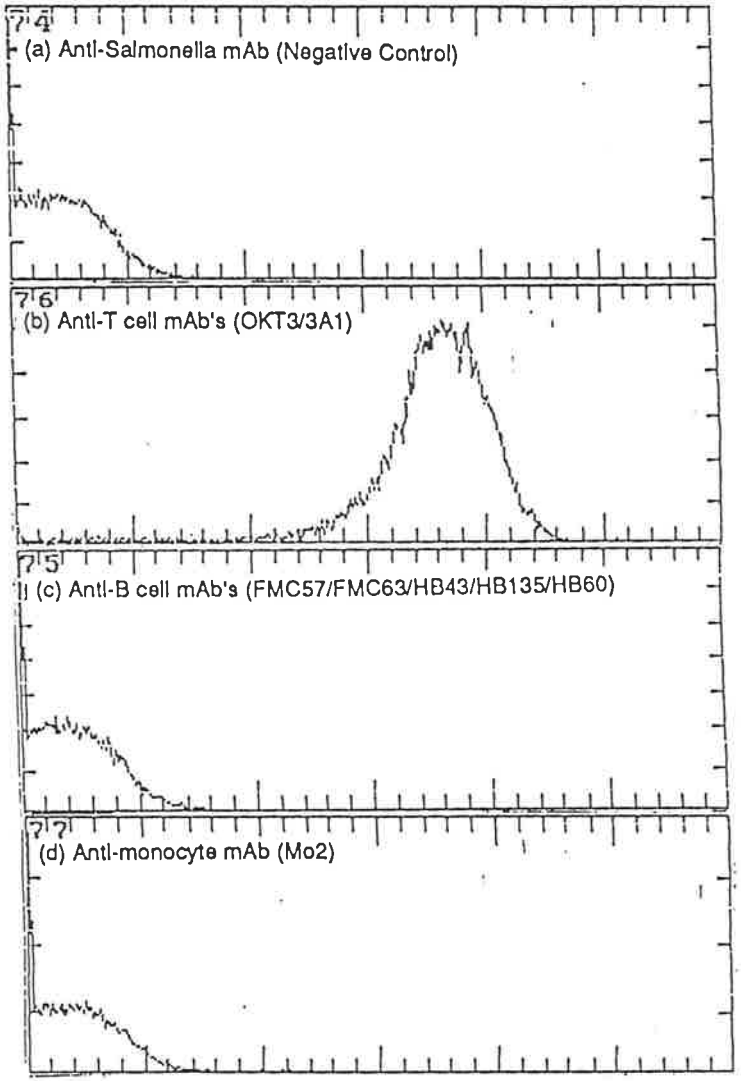
(E) (1) PBMC unfractionated (E) or fractionated with mAB FMC57/HB43/FMC63 (F), or fractionated with mAB FMC57/HB60/FMC63, (G) or fractionated with mAB FMC57/HB43, (H) or fractionated with FMC57/HB6, (I) with the same flow cytometry sorting antibodies as in A to D.

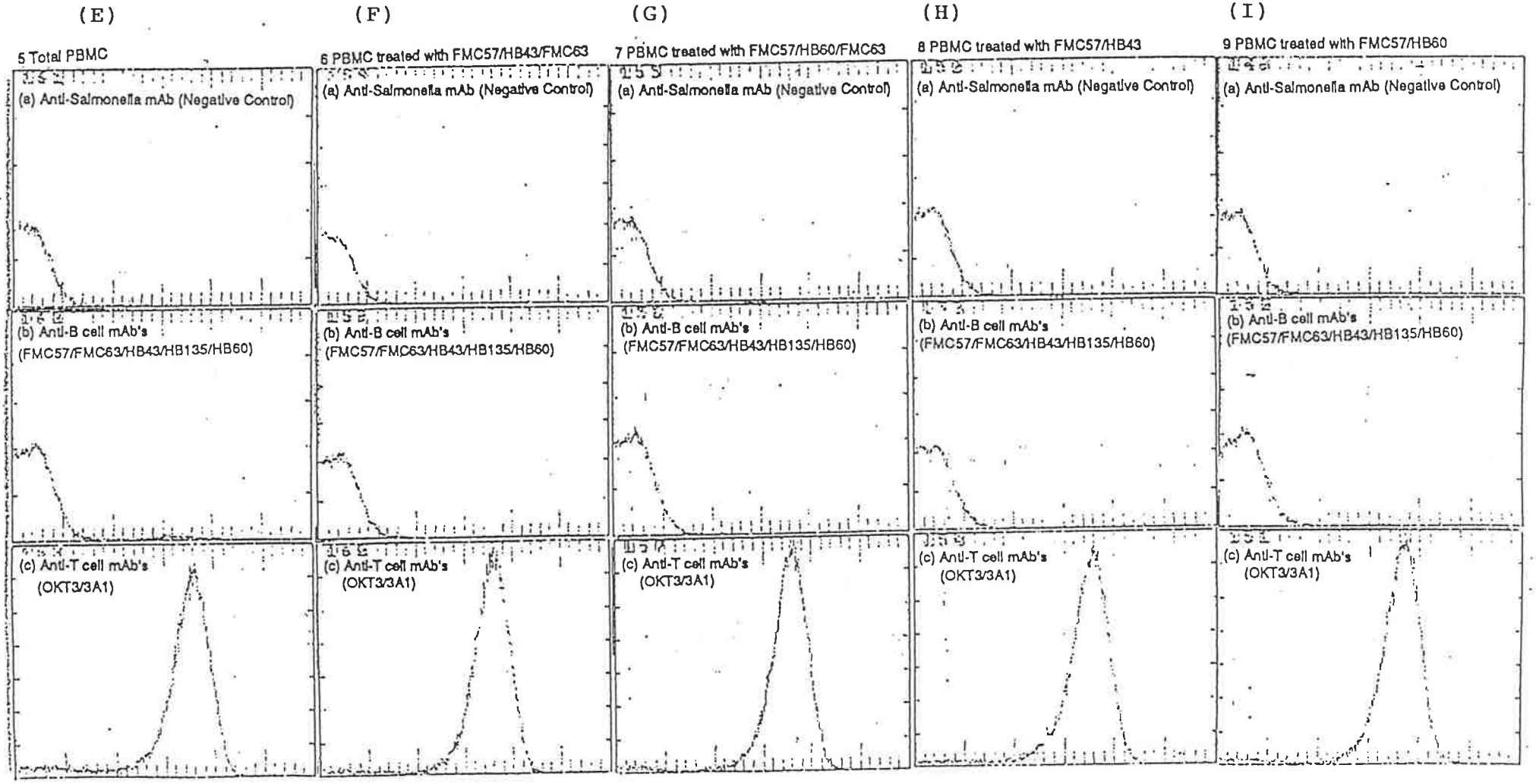

(A)
1 Total PBMC
(a) Anti-Salmonella mAb (Negative Control)
(b) Anti-T cell mAb's (OKT3/3A1)
(c) Anti-B cell mAb's (FMC57/FMC63/HB43/HB135/HB60)
(d) Anti-monocyte mAb (Mo2)
(B)
2 PBMC treated with FMC57
(a) Anti-Salmonella mAb (Negative Control)
(b) Anti-T cell mAb's (OKT3/3A1)
98%
(c) Anti-B cell mAb's (FMC57/FMC63/HB43/HB135/HB60)
1%
(d) Anti-monocyte mAb (Mo2)

(C)


3 PBMC treated with FMC57/HB60/HB135
(a) Anti-Salmonella mAb (Negative Control)
(b) Anti-T cell mAb's (OKT3/3A1)
(c) Anti-B cell mAb's (FMC57/FMC63/HB43/HB135/HB60)
(d) Anti-monocyte mAb (Mo2)

(D)


4 PBMC treated with FMC57/HB43/HB135
(a) Anti-Salmonella mAb (Negative Control)
(b) Anti-T cell mAb's (OKT3/3A1)
(c) Anti-B cell mAb's (FMC57/FMC63/HB43/HB135/HB60)
(d) Anti-monocyte mAb (Mo2)


(E)
(F)
(G)
(H)
(I)
5 Total PBMC
6 PBMC treated with FMC57/HB43/FMC63
7 PBMC treated with FMC57/HB60/FMC63
8 PBMC treated with FMC57/HB43
9 PBMC treated with FMC57/HB60
(a) Anti-Salmonella mAb (Negative Control)
(b) Anti-B cell mAb's (FMC57/FMC63/HB43/HB135/HB60)
(c) Anti-T cell mAb's (OKT3/3A1)

cell fractions that were only 1% purer than those obtained with HB57 alone. Methods are given in the next section and the evidence for this is given under Results and in Figure 12.1.

In an initial experiment to further fractionate T-lymphocytes into CD4+ and CD8+ subsets, 'negative panning selection' was employed with mAB OKT4 and OKT8 (FMC).

For B cell enrichment, the suspension of lymphocytes (6-12x10^6/ml) were treated in the same general manner, but were initially sensitised with a mixture of OKT3/3A1 (anti-CD7) mABs (FMC) to coat the T cells.

Flow cytometric analysis of PBMC populations: Immunofluorescence staining. Cells for analysis by flow cytometry were washed twice in saline plus 0.1% Sodium azide (Ajax Chemicals, Auburn, Australia) (Sal/NaN_3). Viable cells were counted and the concentration adjusted to 1.2x10^7/ml in Sal/NaN_3.

50µl of the cell suspension plus 10µl normal rabbit serum (NRS) were added to 3DT tubes (Disposable Products, Adelaide, Australia). The monoclonal antibody (mAB) was then added. When the mAB preparation was the cell culture media from a hybridoma cell line, 50µl of undiluted fluid was added. The tubes were incubated for 30 min at 4°C. Cells were washed by adding 2ml of cold Sal/NaN_3 to each tube and then centrifuged at 2000rpm for 5 min (LKB Midi Spin).

This step was repeated and 50µl of rabbit anti-mouse Ig-FITC conjugated (Silenus, Melbourne, Australia) was added. The optimal concentration of the conjugate had been previously determined and was diluted in Sal/NaN_3. Tubes

were incubated for 30 min at 4°C and washed as above.

Cells were then resuspended in 1ml "FACSFIX" and stored at 4°C until analysed by flow cytometry on an Epics 750-2 fluorescence activated cell sorter (Coulter Hileah FL., USA). Cells were gated for cell analysis of the lymphocyte populations. Figure 12.1 reproduces the distribution patterns of the separated cells.

Interleukin-2. Interleukin-2 (IL-2) (Boehringer Mannheim, Sydney, Australia) had been prepared and purified from human lymphocytes stimulated with phytohaemagglutinin. Dilutions (5 units in 1.0ml) were prepared in phosphate buffered saline (PBS), pH 7.2 and aliquots stored at -20°C. The activity of the preparation was evaluated in a Concanavalin A-stimulated PBMC blast assay (Gearing and Bird 1987).

Determination of optimal Interleukin-2 concentration after stimulation with *Coxiella burnetii*. $5x10^5$ peripheral blood mononuclear cells (PBMC) per ml from sensitised individuals were cultured with two concentrations of different *C.burnetii* strains in 200µl RPMI-1640 + 10% v/v heat inactivated autologous serum + 20mM HEPES + $5x10^{-5}$M 2-Mercaptoethanol (2-ME). Cultures were incubated at 37°C in 5% v/v CO_2 for 72 hours, after which 100µl of media was removed. Human purified Interleukin-2 (IL-2) was then added to each culture at concentrations of 5, 10, 20 and 40 units/ml in 100µl RPMI-1640 + 10% v/v heat inactivated FCS + 20mM HEPES + $5x10^{-5}$M 2-ME. Cultures were incubated for a further 96 hours during which at the final 16 hours, 20µl of

0.4μCi[^{3}H]-Thymidine (Amersham, Sydney, Australia) was added. Cells were harvested and proliferation measured and expressed as the LSI.

Lymphoproliferative assay. Lymphoproliferative responses to *C.burnetii* whole-cell or periodate-treated antigens were performed in cultures with a final concentration of $5x10^5$ cells/ml in 200μl volumes held in 96 well microtitre plates (Nunclon Delta, Nunc, Denmark). Cells were suspended in RPMI-1640 (Flow Laboratories) with HEPES buffer containing 2mM L-glutamine, 200U of penicillin/ml, 40μg of gentamicin/ml and 10% v/v heat inactivated autologous serum. The unfractionated PBMC, and the reconstituted cell mixtures of monocytes, T- and B-lymphocytes (Mo+T+B); monocytes and T-lymphocytes (Mo+T); monocytes and B-lymphocytes (Mo+B); and T- and B-lymphocytes (T+B) were tested with and without added IL-2. The ratio of cells within each reconstituted mixture was: 15-20% monocytes in all cultures, 56-60% T cells and 24-30% B cells in Mo+T+B cultures; 80-85% T cells in Mo+T cultures; 80-85% B cells in Mo+B cultures, and 75-80% T cells and 20-25% B cells in T+B cultures. Cells that were to receive IL-2 were cultured for 72 hours with antigen alone, after which the medium was carefully removed and fresh medium added. Cells were then split 1-in-2 by transferring 100μl to a new well and adding 100μl of medium to each of the old and new wells. IL-2 was added to each of the cultures to give a final concentration of 5 units in 200μl, and the cultures were incubated for a further 48 hours. Cultures not receiving IL-2 were not split but were incubated for 120 hours without interruption. All cultures were incubated at 37°C in a humid atmosphere with 5% v/v CO_2. As

selected controls for cell proliferation in the absence of antigen, reconstituted mixtures, Mo+T+B and T+B, were set up with added IL-2. Other cell controls (PBMC, Mo+T+B, T+B) were set up without antigen and without IL-2.

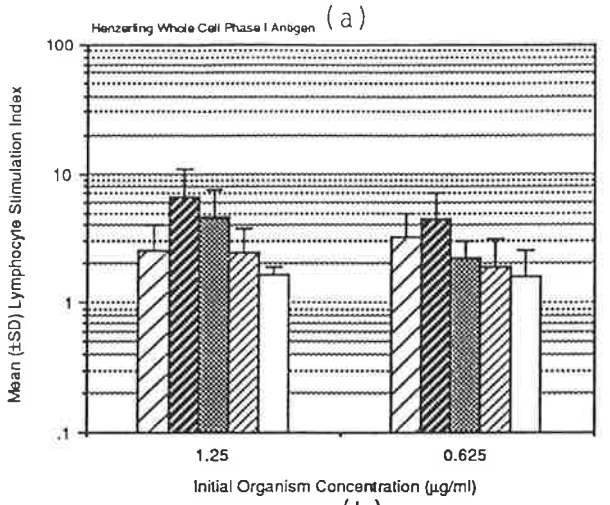
In all assays, cell proliferation was assessed by adding 0.4μCi [^{3}H] thymidine (sp. act. 22Ci/mmol; Amersham International, Sydney, Australia) 18 hours before harvesting cells with an automated cell harvester (Skatron, Lierbyen, Norway)with transfer of cells to glass fibre filters. The two IL-2 treated cultures for each antigen-stimulated cell mixture were combined and treated as one from this stage onwards. Incorporation of [^{3}H] thymidine into cell DNA was counted in a liquid scintillation counter (Beckman, LS2800, Sydney, Australia). Values for the degree of cell proliferation were determined by calculating the lymphocyte stimulation index (LSI) as described by Izzo *et al.* (1988) for each antigen in respect of each cell mixture, as follows:

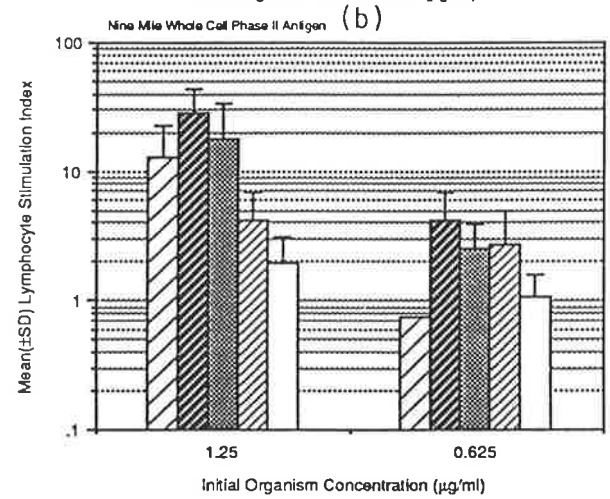
LSI = (mean cpm of four replicate stimulated cultures minus machine background) / (mean cpm of four replicate unstimulated cultures minus machine background).

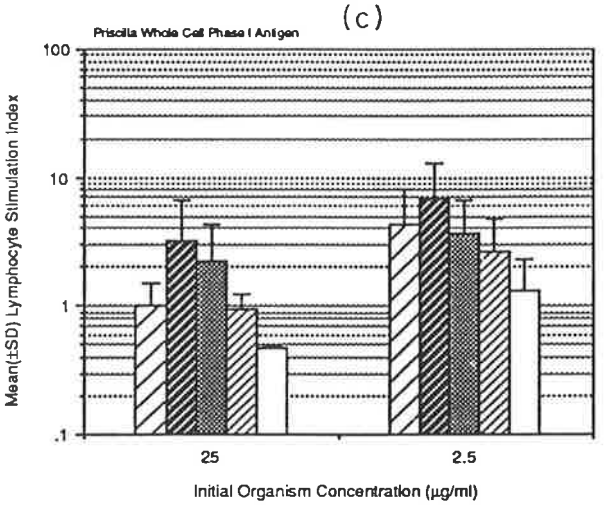
12.3 Results

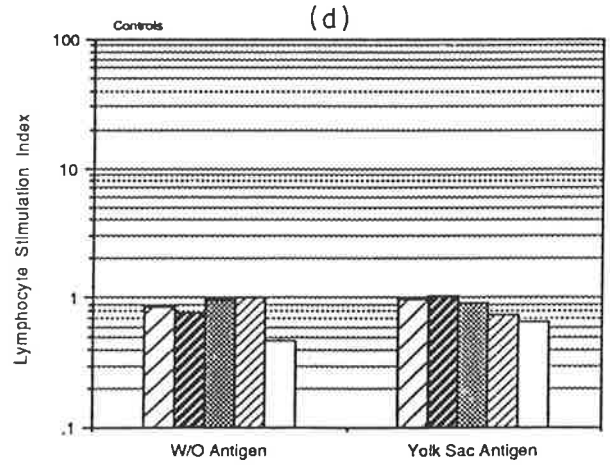
Separation of B and T-lymphocytes and levels of purity obtained by various methods. PBMC isolated from heparinised blood were subjected to panning as described in the "Materials and Methods" section "Preparation of monocyte, T cell and B cell enriched populations from PBM cells" using various combinations of monoclonal antibodies (mAB) against B cell markers. Resulting non-adherent populations were then analysed by Flow Cytometry to

Figure 12.2 (a),(b),(c),(d). Mean LSI values (bar = one standard deviation) with peripheral blood mononuclear cells, three C.burnetii antigens and a control yolk sac antigen, and various concentrations of recDNA IL-2; (a) Henzerling whole cell Phase I antigen at 1.25 and 0.625 μg/ml, without IL-2 (left hand histogram) and with concentrations of 5,10,20 and 40 units/ml; (c) and (b) similarly but with Priscilla whole Phase I and Nine Mile whole cell Phase II antigen; (d) LSI values without (w/o) prior C.burnetii stimulation, or from stimulation with yolk sac control antigen.


(a)
Henzerling Whole Cell Phase I Antigen
Mean (±SD) Lymphocyte Stimulation Index
100
10
1
.1
1.25
0.625
Initial Organism Concentration (µg/ml)


(b)
Nine Mile Whole Cell Phase II Antigen
Mean(±SD) Lymphocyte Stimulation Index
100
10
1
.1
1.25
0.625
Initial Organism Concentration (µg/ml)


(c)
Priscilla Whole Cell Phase I Antigen
Mean(±SD) Lymphocyte Stimulation Index
100
10
1
.1
25
2.5
Initial Organism Concentration (µg/ml)


(d)
Controls
Lymphocyte Stimulation Index
100
10
1
.1
W/O Antigen
Yolk Sac Antigen

assess the efficiency of negative selection for B cells.

Histograms (Figure 12.1) produced from fluorescence activated cell sorter show cells gated for total lymphocyte scatter, analysed for the various sub-populations. The results show that the use of mAB FMC57 produced a 98% pure T cell population with 1% of B cells (Figure 12.1 (B), and that the various other combinations of antibody (C to I) offered no further advantage.

Determination of optimal Interleukin-2 concentration after stimulation with *Coxiella burnetii*. Figure 12.2 (a),(b),(c),(d) sets out the LSI values obtained with PBMC from 5 subjects (3 vaccinees and 2 past infections), with two concentrations of *C.burnetii* antigens (Henzerling whole cell Phase I; Priscilla whole cell Phase I and Nine Mile Phase II) and with yolk sac control antigen; all tested against a range of IL-2 concentrations. The results are expressed as histograms of mean values (bar shows standard deviation) for each IL-2 dilution.

It will be seen (Figure 12.2 (a),(b),(c)) that there was a consistent increase in mean LSI values with 5 units of IL-2/ml, somewhat more marked with the various concentrations of Nine Mile Phase II antigen, than with the two Phase I antigens (Henzerling or Priscilla).

Higher concentrations of IL-2 - up to 40U/ml - inhibited rather than enhanced ~~enhancing~~ proliferation, perhaps because of toxicity or conceivably because too rapid proliferation of the cells outstripped the supply of nutrients or other growth factors.

Panel (d), 'controls', in Figure 12.2 shows that prior stimulation with yolk

Table 12.1: Mean values for lymphocyte stimulation indices (LSI) obtained with unfractionated or various reconstituted fractions of peripheral blood mononuclear cells from 10 vaccinated subjects (group A) which were stimulated in culture with 3 C.burnetii antigens and a control yolk sac suspension.

CELLS OR FRACTIONS	PRISCILLA (PHASE 1)			HENZERLING (PHASE 1)			NINE MILE (PHASE 2)			YOLK SAC SUSPENSION (CONTROL)		
	No IL-2[a]	IL-2[b]+	p[c]	No IL-2	IL-2+	p	No IL-2	IL-2+	p	No IL-2	IL-2+	p
PBMC	1.092[d] (±0.608)	3.298 (±1.951)	0.0005	1.218 (±0.748)	3.997 (±2.556)	0.0005	1.974 (±0.991)	6.002 (±3.723)	0.0005	0.429 (±0.190)	0.880 (±0.279)	0.0005
Mo+T+B	1.079 (±0.589)	4.267 (±3.213)	0.0005	1.879 (±0.670)	5.104 (±2.691)	0.0005	1.773 (±0.749)	5.791 (±3.603)	0.0005	0.482 (±0.269)	0.824 (±0.368)	0.005
Mo+T	1.653 (±0.846)	5.763 (±3.501)	0.0005	2.163 (±1.140)	7.998 (±4.296)	0.0005	1.838 (±0.907)	6.416 (±4.621)	0.0005	0.606 (±0.326)	0.885 (±0.326)	0.005
Mo+B	0.942 (±0.346)	2.121 (±0.664)	0.0005	1.274 (±0.646)	2.736 (±0.733)	0.0005	0.983 (±0.218)	1.824 (±0.903)	0.01	0.621 (±0.324)	0.791 (±0.274)	0.01
T+B	0.724 (±0.436)	2.200 (±1.380)	0.1	1.051 (±0.287)	2.670 (±1.250)	0.0005	0.550 (±0.282)	2.177 (±1.963)	0.0005	0.494 (±0.260)	0.819 (±0.276)	0.025

a = no exogenous IL-2 added to culture.
b = exogenous IL-2 added 3 days after addition of antigen.
c = P value for the differences between cultures with and without added IL-2 determined by Student t-test on the log transformed LSI values.
d = Values expressed as Mean Lymphocyte Stimulation Index (± Standard Deviation).

Table 12.2: Mean values for lymphocyte stimulation indices (LSI) obtained with unfractionated or various reconstituted fractions of peripheral blood mononuclear cells from 10 subjects (group B) with antibody or skin test reactivity indicating previous infection with C.burnetii which were stimulated with 3 C.burnetii antigens and a control yolk sac suspension.

CELLS OR FRACTIONS	PRISCILLA (PHASE 1)			HENZERLING (PHASE 1)			NINE MILE (PHASE 2)			YOLK SAC SUSPENSION (CONTROL)		
	No IL-2[a]	IL-2[b]+	p[c]	No IL-2	IL-2+	p	No IL-2	IL-2+	p	No IL-2	IL-2+	p
PBMC	3.893[d] (±2.425)	NT		3.978 (±2.920)	NT		15.597 (±6.831)	NT		0.558 (±0.381)	NT	
Mo+T+B	1.607 (±0.311)	7.877 (±4.983)	0.005	3.293 (±1.438)	11.705 (±8.852)	0.01	13.905 (±12.177)	30.225 (±23.225)	0.005	0.973 (±0.288)	1.357 (±0.485)	0.01
Mo+T	2.660 (±1.088)	9.063 (±3.171)	0.005	2.665 (±0.832)	10.615 (±2.403)	0.0005	5.775 (±4.645)	15.529 (±4.529)	0.005	0.970 (±0.452)	1.263 (±0.427)	0.1
Mo+B	1.573 (±0.784)	3.048 (±2.398)	0.05	1.612 (±0.869)	3.437 (±2.944)	0.05	2.677 (±1.767)	4.869 (±2.869)	0.05	0.803 (±0.323)	1.028 (±0.584)	0.1
T+B	1.035 (±0.310)	2.840 (±3.069)	0.375	1.257 (±0.624)	2.230 (±1.739)	0.4	1.077 (±0.620)	1.355 (±1.237)	0.1	0.648 (±0.351)	0.755 (±0.318)	0.1

a = no exogenous IL-2 added to culture.
b = exogenous IL-2 added 3 days after addition of antigen.
c = P value for the differences between cultures with and without added IL-2 determined by Student t-test on the log transformed LSI values.
d = Values expressed as Mean Lymphocyte Stimulation Index (± Standard Deviation).
NT = Not Tested

Table 12.3: Mean values for lymphocyte stimulation indices (LSI) obtained with unfractionated or various reconstituted fractions of peripheral blood mononuclear cells from 10 subjects with no serological or skin test evidence of infection.

CELLS OR FRACTIONS	PRISCILLA (PHASE 1)			HENZERLING (PHASE 1)			NINE MILE (PHASE 2)			YOLK SAC SUSPENSION (CONTROL)		
	No IL-2[a]	IL-2[b]+	p[c]	No IL-2	IL-2+	p	No IL-2	IL-2+	p	No IL-2	IL-2+	p
PBMC	0.652[d] (±0.272)	0.992 (±0.198)	0.05	0.692 (±0.130)	0.916 (±0.263)	0.005	0.836 (±0.091)	1.144 (±0.193)	0.01	0.746 (±0.202)	0.766 (±0.282)	0.4
Mo+T+B	0.714 (±0.248)	1.032 (±0.261)	0.05	0.898 (±0.376)	1.340 (±0.189)	0.05	0.676 (±0.298)	0.814 (±0.413)	0.1	0.690 (±0.348)	0.642 (±0.341)	0.375
Mo+T	0.848 (±0.132)	1.134 (±0.343)	0.1	1.014 (±0.416)	1.418 (±0.325)	0.1	0.718 (±0.274)	0.868 (±0.358)	0.05	0.692 (±0.287)	0.698 (±0.309)	0.1
Mo+B	0.736 (±0.317)	1.082 (±0.357)	0.05	0.916 (±0.165)	1.242 (±0.286)	0.1	0.782 (±0.224)	1.034 (±0.451)	0.1	0.586 (±0.296)	0.790 (±0.351)	0.05
T+B	0.738 (±0.152)	1.057 (±0.414)	0.1	0.956 (±0.306)	1.727 (±0.172)	0.05	0.508 (±0.299)	0.814 (±0.372)	0.05	0.544 (±0.202)	0.686 (±0.311)	0.1

a = no exogenous IL-2 added to culture.
b = exogenous IL-2 added 3 days after addition of antigen.
c = P value for the differences between cultures with and without added IL-2 determined by Student t-test on the log transformed LSI values.
d = Values expressed as Mean Lymphocyte Stimulation Index (± Standard Deviation).

sac antigen did not lead to lymphocyte stimulation and that PBMC (from the one vaccinee tested) did not lead to proliferation. In other words, prior antigen stimulation was a pre-requisite for IL-2 action - as would be expected from its known mode of action.

Reactivity of PBMC and reconstituted mixtures of lymphocytes and monocytes to *C.burnetii* whole cell antigens. Tables 12.1, 12.2 and 12.3 set out the results of the three groups (subjects who were vaccinated (group A), unvaccinated (group B) but with evidence of previous exposure, and (group C) without previous exposure to *C.burnetii*) as the mean LSI values obtained with the unfractionated PBMC, or with various reconstituted mixtures of mononuclear cells, after stimulation with 3 *C.burnetii* antigens, or with the yolk sac 'control' suspension. Data is also included on the enhancing effect of the addition of IL-2 to each of the cultures of unfractionated PBMC or the reconstituted mixtures.

Figures 12.3, 12.4 and 12.5 set out the same data as 'scattergrams' of the LSI obtained on antigen stimulation; this permits easier inspection of the reaction profiles than consideration of the tabulated results.

In all three groups most of the responses to the yolk sac control antigen were below the cut-off level of 1.2 even when the IL-2 was added to the cultures (lower right panels in Figures 12.3, 12.4 and 12.5). Among the vaccinees (group A, Figure 12.3) detailed comparisons of the means of LSI values with yolk sac antigen showed a small but significant ($p \leq 0.005$), 1.4 to 2 fold amplification after addition of IL-2 to those fractions with T cells (see

Figures 12.3, 12.4, 12.5. Profiles of lymphocyte stimulation indices with three C.burnetii antigens and yolk sac control antigen and various fractions - reconstituted cell mixtures, with or without IL-2 - in 12.3 a group of vaccinees from a 'low-risk' population, 12.4 subjects who had had Q fever infection in the past, 12.5 subjects with no evidence of immunity to Q fever.

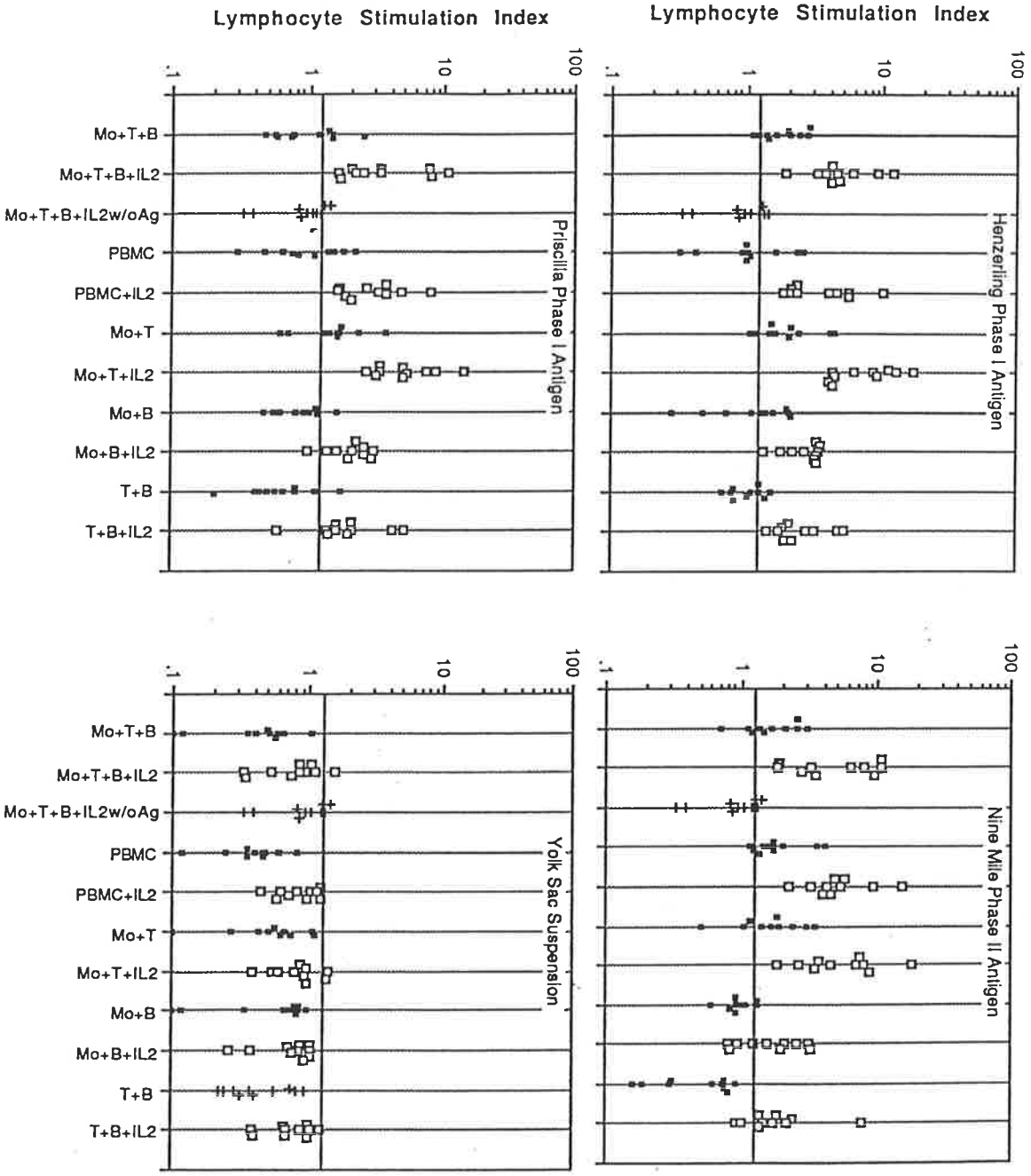
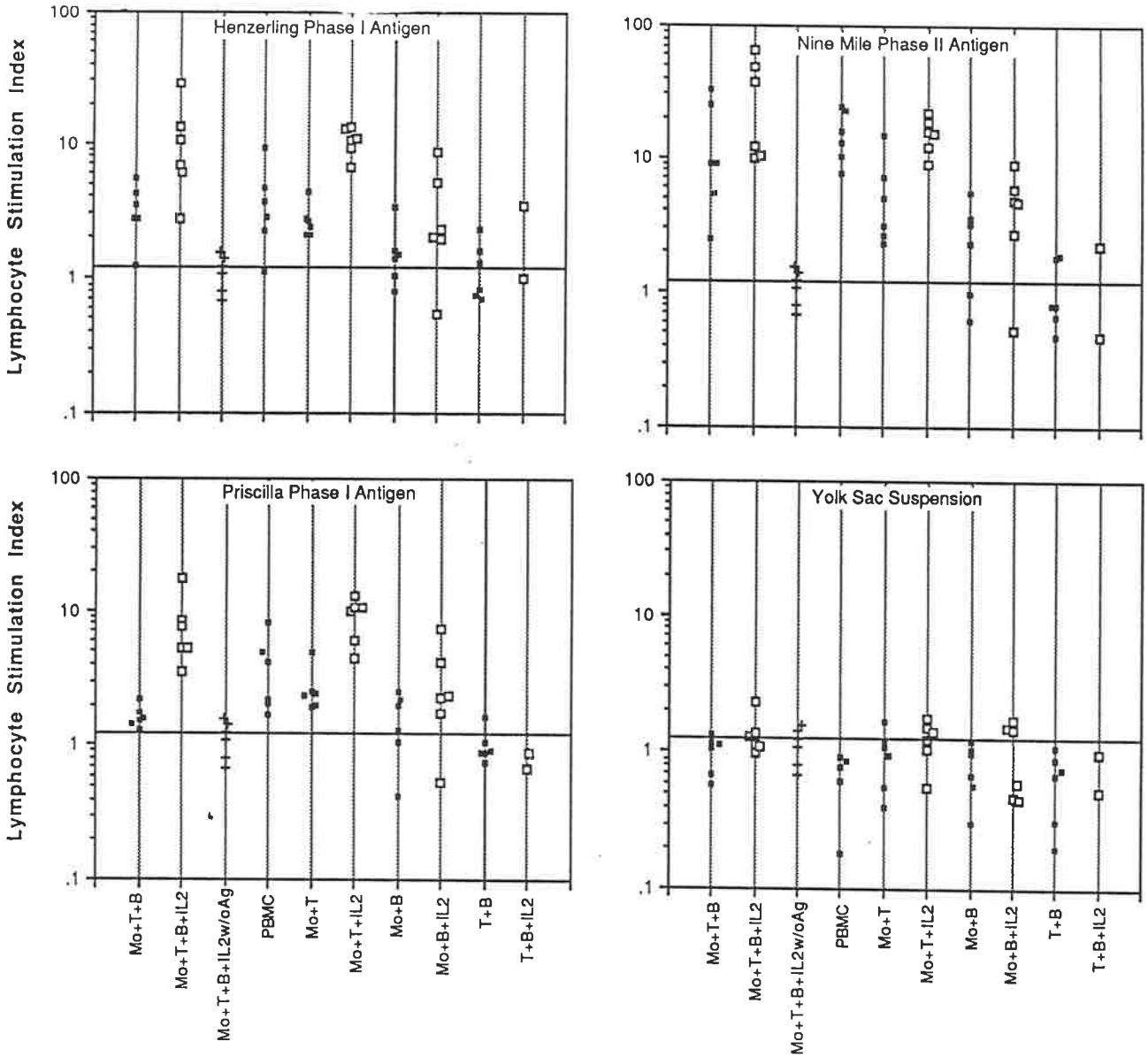


Figure 12.3 (Vaccinees) (Group A)

Figure 12.4 (Past Infection) (Group B)



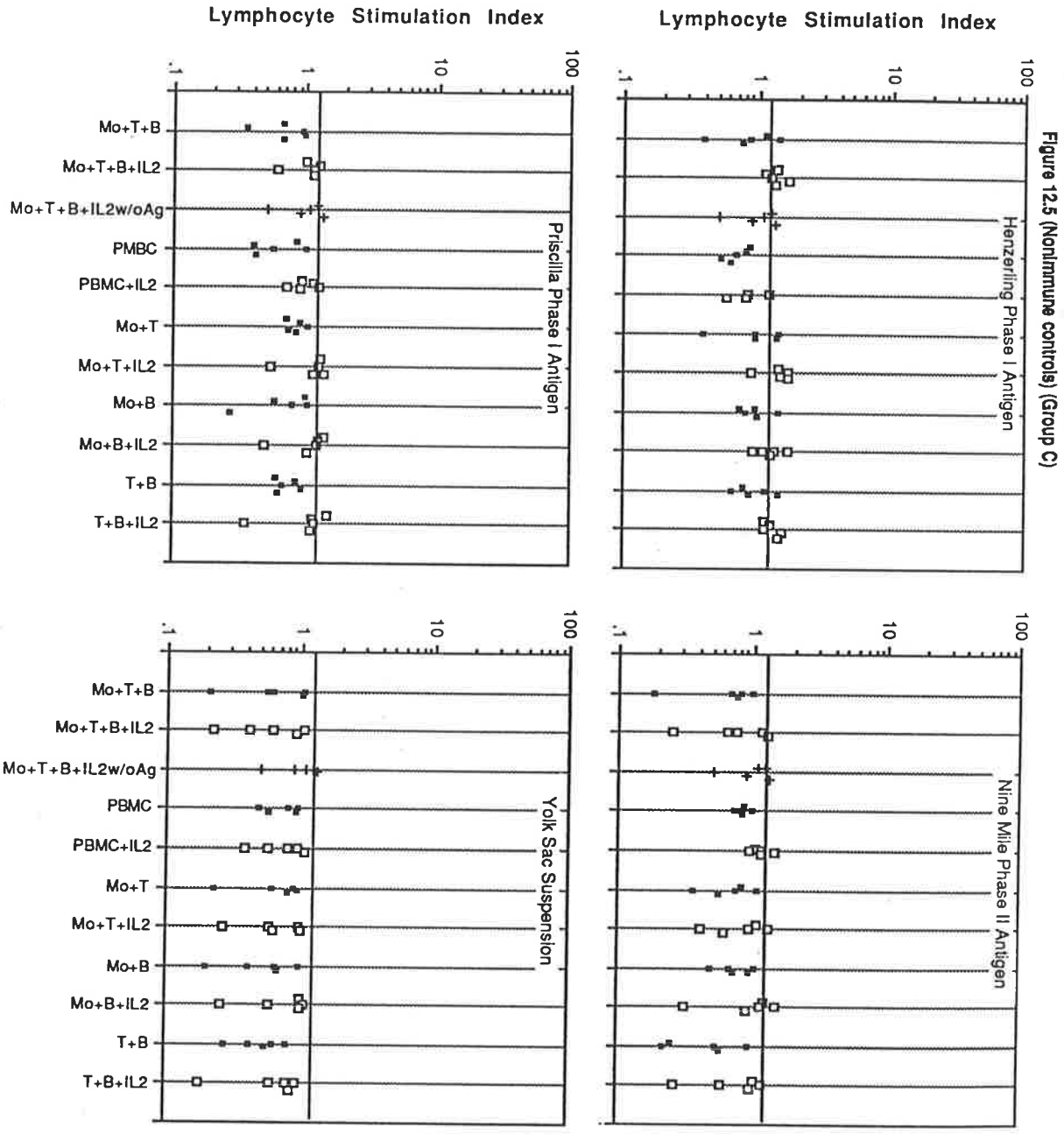


Figure 12.5 (Nonimmune controls) (Group C)

Table 12.1). The same statistically significant trend was not observed in groups B and C; it presumably represents sensitisation of the vaccinees to minor amounts of residual yolk sac protein in the vaccine despite the failure to detect the latter by serological tests.

Overall, in groups A and B, reactions to *C.burnetii* (Henzerling and Priscilla) Phase I, and Nine Mile Phase II whole cell antigens were enhanced by exogenous IL-2 to values greatly in excess of the cut off level and of the values obtained with yolk sac control antigen (Figures 12.3 and 12.4, Tables 12.1 and 12.2), whereas no such enhanced effect was obtained with the nonimmune controls (Figure 12.5). The mean LSI values from group C, with the various cell mixtures without IL-2, - i.e., negative control subjects - were also all below the 1.2 cut-off value with all three *C.burnetii* antigens and generally within the same range as the values obtained with the control yolk sac suspension (Figure 12.5 and Table 12.3).

Figure 12.3 shows that with the vaccinees and *C.burnetii* Phase I antigen, (Henzerling and Priscilla), the most vigorous lymphoproliferative responses - either with or without added IL-2 - were with the total PBMC, and with reconstituted fractions Mo+T+B and Mo+T, whereas Mo+B showed some, but smaller, responses. Mean values and standard deviations were (i) PBMC, without IL-2/with IL-2: 1.2 ± 0.74/ 4.0 ± 2.5; (ii) Mo+T+B: 1.9 ± 0.7/5.1 ± 2.7; (iii) Mo+T: 2.2 ± 1.1/8.0 ± 4.3; (iv) Mo+B: 1.3 ± 0.6/ 2.7 ± 0.73. Differences are all significant at $p \leq 0.005$ (student t test on log transformed LSI values; Table 12.1). The response profile with *C.burnetii* Phase I (Priscilla) with the various reconstituted fractions was similar to that with Henzerling Phase I.

With the *C.burnetii* (Nine Mile) Phase II antigen the response profile among the vaccinees was essentially similar to that with the Phase I antigens. Fractions with T cells were again more active; thus Mo+T with IL-2, gave a mean LSI value of 6.4 ± 4.6, against a mean value of 1.8 ± 0.9 for Mo+B with IL-2; $p \leq 0.005$. There was a steady progression from the mean LSI value with PBMC down to the value obtained with T+B cells. Comparison of the values of the reconstituted mixtures with the value for PBMC showed that, with the exception of the pair Mo+T+B/Mo+T, where $p \leq 0.1$, the differences are all significant at $p \leq 0.0005$ (Table 12.1).

The combination Mo+B gave lower proportions with groups A and B - indicating that part of the lymphoproliferative response is presumably due to proliferation of B cells. The enhancing effect of exogenous IL-2 was less marked with this combination; IL-2, normally from activated T-lymphocytes, plays a part in B cell proliferation along with several other lymphokines (Jelinek *et al.* 1986, Hamblin, 1988).

The mixture T+B gave low responses with Nine Mile Phase II antigen and there was some enhancement with IL-2. It is presumed that in the absence of monocytes, B cells are acting as antigen-presenting cells for T cells (Chesnut *et al.* 1982).

Overall, in the vaccinees, as seen in Table 12.1, the rank order of mean LSI responses of PBMC and the reconstituted fractions to the 3 antigens was Mo+T>Mo+T+B=PBMC>Mo+B>T+B.

The similarity of T cell responses to Phase I and Phase II antigens, given the differences in the LPS composition of the two antigenic states, suggests T

cell responses to common, non-LPS components of the coxiella (see later chapters and Discussion).

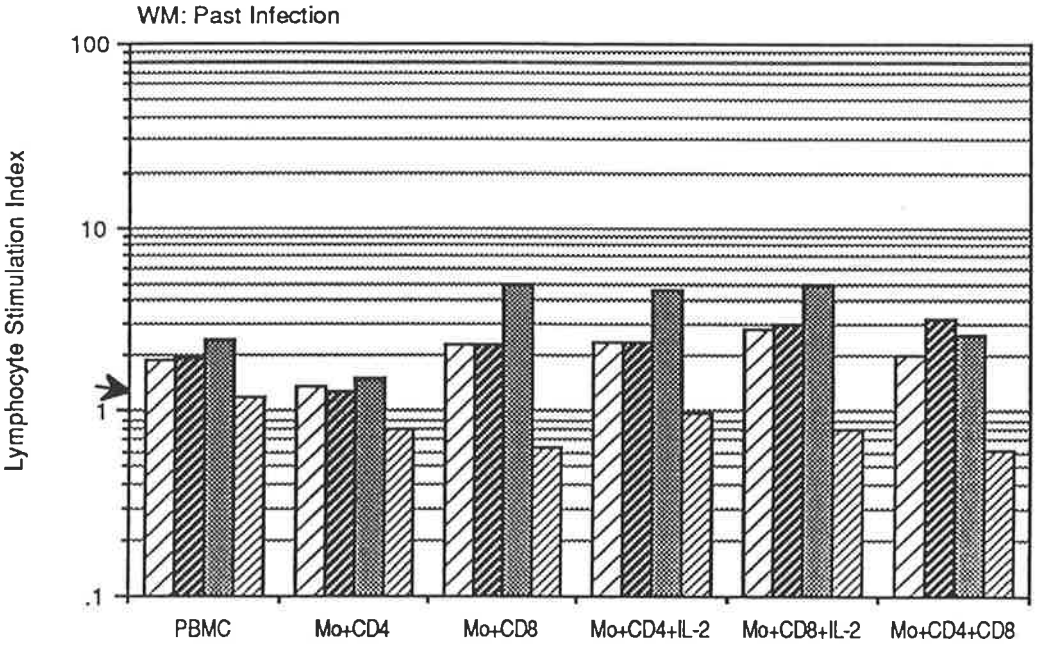
In group (B) - subjects with evidence of past infection with *C.burnetii* - the profiles (Figure 12.4 and Table 12.2) of responses to both Phase I and II antigens was similar to that in the vaccinees (group A) in terms of differences between cell fractions but the magnitude was much greater in absolute terms (e.g., Mo+T+B versus Nine Mile Phase II antigen gave mean values of 13.9 $\pm$ 12.1 and 30.2 $\pm$ 23.2, without and with added IL-2 respectively). The reconstituted fractions Mo+B and T+B again showed the lower responses on stimulation with antigen relative to fractions containing monocytes and T cells (significantly different from PBMC at $p<0.0005$) and also showed less amplification with exogenous IL-2.

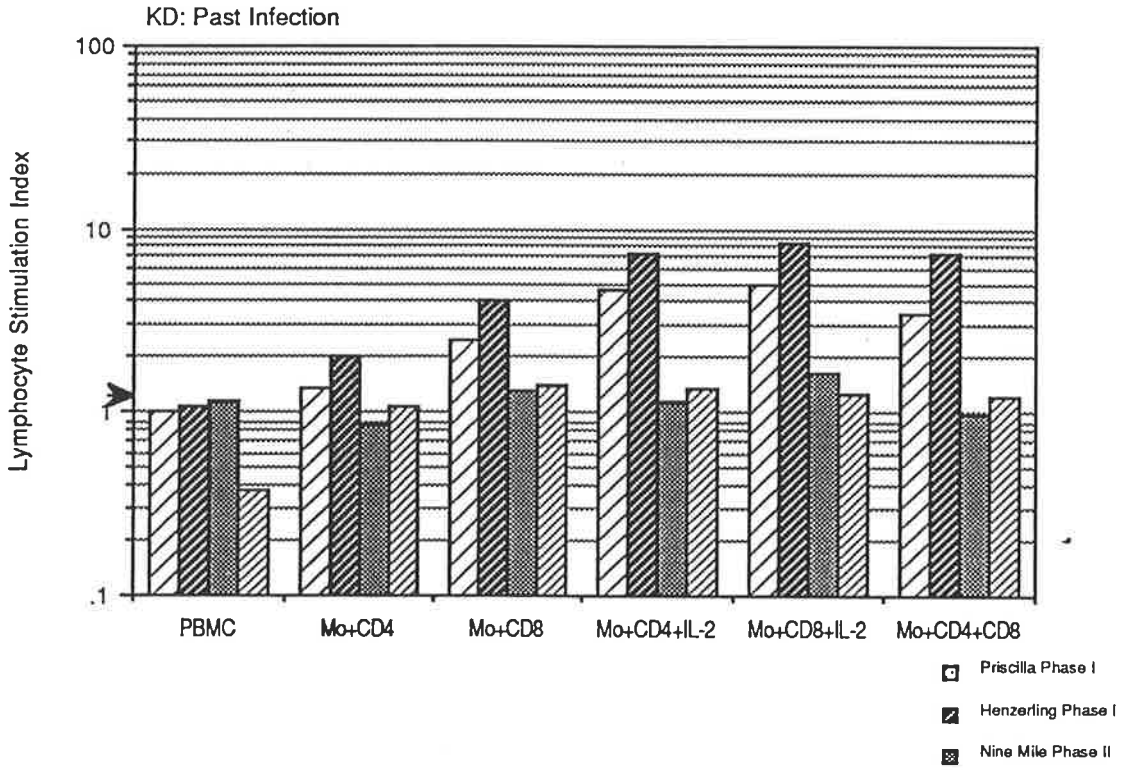
Finally, in the group (C) of 5 subjects with no evidence of past exposure to *C.burnetii*, PBMC and all reconstituted cell fractions without IL-2 gave scattergrams of LSI values with the three *C.burnetii* antigens which were at or below the cut-off value of 1.2; with minimal amplification by exogenous IL-2 (Figure 12.5, Table 12.3).

The differences in the profiles in group (C) on the one hand, as compared with those in groups (A) and (B) on the other hand, clearly indicate the specificity of the responses.

Mitogenic reactions of T-lymphocyte subclasses with *C.burnetii* antigens. There is a view (see Chapter 8) that with intracellular bacteria the main activator of intracellular killing, particularly with macrophages, is the T-helper cell (CD4

Figure 12.6. Profiles of lymphocyte stimulation indices obtained with PBMC and fractionated CD4+ and CD8+ lymphocytes reconstituted with monocytes as antigen presenting cells. The two subjects WM and KD had had Q fever in the past. The arrow marks the cut-off point of 1.2 LSI. The antigens are, reading from left to right, in each group of histograms, Henzerling Phase I, Priscilla Phase I, Nine Mile Phase II, Yolk Sac (control).


WM: Past Infection
Lymphocyte Stimulation Index
100
10
1
.1
PBMC
Mo+CD4
Mo+CD8
Mo+CD4+IL-2
Mo+CD8+IL-2
Mo+CD4+CD8


KD: Past Infection
Lymphocyte Stimulation Index
100
10
1
.1
PBMC
Mo+CD4
Mo+CD8
Mo+CD4+IL-2
Mo+CD8+IL-2
Mo+CD4+CD8
Priscilla Phase I
Henzerling Phase I
Nine Mile Phase II

positive) and probably one particular subset of CD4 positive which elaborates IFNγ among other lymphokines. This T helper cell would recognise antigen presented with Class II MHC antigens. CD8 +ve T-lymphocytes, on the other hand, are thought to recognise cellular or viral antigens displayed on the host cell membrane with Class I MHC antigens.

It was therefore of interest to determine whether lymphocyte proliferative responses to *C.burnetii* were restricted to CD4+ lymphocytes or whether CD8+ cells would also respond. CD4+ and CD8+ lymphocytes were separated from the PBMC of two subjects with past Q fever infection and stimulated with *C.burnetii* antigens. Figure 12.6 shows the LSI values obtained and it is clear that both subsets of T-lymphocyte show a mitogenic response. The question of whether this is accompanied by the elaboration and release of IFNγ is considered in Chapter 14.

12.4 Discussion

In general, the understanding of immunity to rickettsial infections which has accrued from animal experiments with typhus rickettsias (Murphy *et al.* 1978, 1979, 1980), and with the rickettsia of Q fever (Kishimoto *et al.*, 1977) suggests that antibody plays a part in facilitating phagocytosis and destruction of extracellular organisms - although there is some inconsistency between the results of various workers - and possibly also via antibody-dependent cell-mediated cytotoxicity reactions (Koster *et al*, 1984).

Other studies showed that although antibody may modify the course of infection in an animal, organisms are not eliminated, nor can complete

protection be transferred by passive immunisation with antibody. Protection was, however, conferred by adoptive transfer of immune T-lymphocytes or by immunisation with a vaccine which is sufficiently immunogenic, or has been adjuvanted, so as not only to produce antibody, but also a positive DTH reaction by skin test.

Previous studies of cellular immunity after Q fever infection or vaccination in man have assayed the *in vitro* lymphoproliferative response of unfractionated PBMC to *C.burnetii* antigen (Ascher *et al.* 1983b, Jerrells *et al.* 1975, Izzo *et al.* 1988), or used an *in vivo* skin test to determine delayed hypersensitivity (e.g. Bell, *et al.* 1964).

The present study of vaccinees and subjects infected in the past indicates that the cellular immune response to *C.burnetii*, as measured by the proliferation of circulating blood lymphocytes on antigen challenge, is predominantly effected by T-lymphocytes with monocytes (Mo) as required antigen presenting cells. Exogenous IL-2 gave major amplification with this cell combination. Limited, preliminary experiments with further subdivision of T cells into CD4+ and CD8+ subsets indicated that both subsets showed a mitogenic response to *C.burnetii* antigens (see also Chapter 14 on IFNγ responses).

The response of B-lymphocytes, even when enriched in an Mo+B fraction, though present, was consistently less marked. The possibility of a nonspecific polycolonal stimulation of B cells by *C.burnetii* LPS, as a component of the total PBMC response, is excluded by the negative reactions of the Mo+B enriched fraction from the nonimmune control subjects (group C).

The mitogenic responses of T cells from subjects infected in the past were clear cut and striking. Those from vaccinees were less vigorous, although specific, presumably reflecting a less intense T cell memory induced by vaccine. Exogenous IL-2 was particularly valuable in amplifying the response with the latter and facilating measurement of the responses to *C.burnetii* antigens.

T-lymphocytes from vaccinees immunised with the Henzerling strain, *C.burnetii* vaccine (Qvax) gave essentially the same reaction patterns with Priscilla Phase I whole cell antigen as with the prototype strains of *C.burnetii*, Henzerling and Nine Mile.

This is reassuring as it suggests that the serological differences demonstrated in the extracted LPS of the Priscilla strain of *C.burnetii* - isolated from a goat placenta and biologically representative of isolates from human endocarditis - when compared with LPS extracted from the prototype Nine Mile and Henzerling strains (Hackstadt 1986, Moos and Hackstadt 1987), are unlikely to negate the protective value of the CMI response induced by Qvax. In Hackstadt's laboratory, the differences in LPS were observed with rabbit antisera on Western blots of the LPS and were not so evident with other species of antisera or in conventional serological comparisons of strains by CF or EIA. PAGE analysis of proteins from Priscilla and Nine Mile strains revealed similar patterns. Priscilla and prototype strains cross-protect against infection in the guinea pig fever model (Moos and Hackstadt 1987 and see below Chapter 16). At the epidemiological level the complete protection given by Qvax to Australian abattoir workers, many of whom are exposed to infected

goats, is in line with the *in vitro* CMI observations in our laboratory and those in guinea pig cross-protection experiments in Hackstadt's laboratory.

CHAPTER 13

ANALYSIS OF ANTIGENIC COMPONENTS OF *COXIELLA BURNETII* THAT INDUCE LYMPHOPROLIFERATION

13.1 Introduction

Coxiella burnetii has a cell wall closely resembling that of a gram-negative bacterium, with a lipopolysaccharide (LPS) as an outer membrane, a protein-peptidoglycan layer and then the cytoplasmic membrane of the cell (Chapter 7). Organisms with their full complement of sugar residues in the LPS (i.e., Phase I) are more virulent and are more potent protective immunogens than their Phase II counterparts which have undergone a loss variation of sugar residues from the LPS. The LPS of Phase I organisms and in particular its lipid A component have been suggested as mediators of adverse side reactions after vaccination with Phase I whole cell vaccines which seem to be related to granuloma formation. Efforts to reduce these reactions led to experimental separation of antigenic components of Phase I organisms. The first analyses were those of Colter *et al.* (1956) and Anacker *et al.* (1962) and used sonication and solvent extraction respectively.

Protective immunogenicity was associated with the cell envelope and not cell protoplasm. Extraction of Phase I cells with trichloroacetic acid yielded a protective extract and a residue which was also protective. LPS extracted by the phenol-water method was not protective although Phase I antigen activity was retained on serological testing.

Ormsbee *et al.* (1962) extracted protective immunogens and serologically

reactive fractions with dimethyl sulphoxide or diacetamide from Phase I organisms; these extracts were at least 200 times less reactive in producing DTH reactions in sensitised rabbbits than intact coxiellas. Extracts from Phase II cells were much less protective.

Trichloroacetic acid extracts of Phase I coxiellas have also been used by Czechoslovakian workers as a "chemovaccine" (Brezina and Urvolgyi 1962, Kazar *et al.* (1982). Such extracts contain a Phase I LPS:protein complex which retains its serological reactivity and protective immunogenicity in laboratory animals when challenged with live *C.burnetii.* It appeared to protect laboratory and factory workers against occupational Q fever.

The residue (CMR) left after extraction of Phase I *C.burnetii* cells with chloroform-methanol is also being developed as a vaccine. Chloroform-methanol extraction is thought to remove a factor inducing granulomas as well as an "immunosuppressive complex" postulated to be associated with whole cell Phase I organisms (Williams *et al.* 1986a, Waag and Williams 1988). CMR preparations contain coxiella cell proteins and residual LPS and protect mice and sheep against experimental infection with *C.burnetii.* Their protective efficacy in man is presently unknown.

In the two preceding chapters (Chapter 11 and 12) it was shown that past infection or vaccination with an inactivated whole cell vaccine generates a long lasting T-lymphocyte sensitisation or T cell memory and that the epitopes concerned are probably those of the proteins or non-LPS component of the coxiella. The latter preliminary conclusion is based on the observation that Phase II organisms, or Phase I organisms in which the immunodominant

sugars of the LPS had been modified by periodate treatment, provoke a similar, sometimes greater lymphoproliferative response with immune T cells to that given by Phase I coxiellas in which the LPS is intact.

General evidence (see Introductory Chapters 6 and 8) emphasises the importance of cell-mediated immunity in resistance to recovery from *C.burnetii* infection. On the other hand, the earlier work with whole coxiella cells and extracts and animal protection experiments underlined the importance of both Phase I LPS and protein. There is a need to integrate information from cell component analyses, assay of serological reactivity, lymphocyte stimulation assays and animal protection experiments in an attempt to define the essential elements of the protective immunogen, and, in particular the role of the LPS.

The results presented in this chapter explore T- and B-lymphocyte responses to whole-cell antigens from Phase I and Phase II strains of *C.burnetii* of various origins and those with antigens extracted from the coxiella with organic solvents and with the cell residues remaining after extraction. Results are also presented on the stimulatory activity of the separated proteins of the coxiella.

13.2 Materials and Methods

Coxiella burnetii whole cell antigens.

(a) Coxiella suspensions were standardised by adsorption spectrometry at 420 nm (Fiset *et al.* 1969) and adjusted to a concentration of 1000µg/ml. The optimal dose of each antigen for use in the lymphoproliferative assays was determined by titration against replicate preparations of reactive lymphocytes;

the antigen dilution giving the highest LSI value was selected for use in the test proper (see Izzo *et al.* (1988)). These optimal antigen dilutions were, for *C.burnetii* whole cell (WC) Phase I (Henzerling): 1.25µg/ml; for *C.burnetii* WC Phase I antigen (Priscilla): 2.5µg/ml; and for WC *C.burnetii* (Nine Mile) Phase II 1.25µg/ml.

Naturally-derived, high yolk sac passage *C.burnetii* Phase II antigen (Nine Mile) was a gift from M. Peacock, and prepared from a strain given 90 egg passages (90EP), cloned once in cell culture, (1TC) and then passaged 4 times in eggs (4EP) (designation, Rocky Mountain Laboratory, Hamilton, Montana, RSA516 Nine Mile 90EP/1TC/4EP). The strain is permanently in Phase II (i.e., it does not contain residual Phase I organisms) and is nonpathogenic for guinea pigs. Stimulation patterns with peripheral blood mononuclear cells were closely similar to those obtained with a Nine Mile Phase II antigen obtained from the Central Public Health Laboratories, Colindale, London and prepared by potassium periodate treatment of a Phase II antigen to modify ~~with~~ any residual Phase I cells in the preparation, and also similar to those with a diagnostic Nine Mile Phase II antigen from CSL.

(b) Solvent-extracted preparations from *C.burnetii*

Trichloroacetic acid extracts (TCA-E). The method for trichloroacetic acid (TCA) extraction of *C.burnetii* cells resembled those described by Anacker *et al.* (1962), Brezina and Urvolgyi (1962), Hackstadt *et al.* (1985), Lukacova *et al.* (1989). It removes a complex of lipopolysaccharide and proteins of variable composition depending on the conditions of extraction (Lukacova *et al.* 1989).

One milligram of *C.burnetii* whole cells, Phase I Nine Mile strain (CF diagnostic antigen Phase I antigen CSL) was suspended in 2.0 ml of 10% v/v TCA and held at 0°C for 45 minutes with periodic mixing. It was then centrifuged at 15,000 rpm for 30 minutes to deposit the extracted cells. The supernatant fluid was removed and dialysed for 48 hours against two changes of sterile distilled water. The sac contents were concentrated by lyophilisation. The extracted cells (TCA residue - TCA-R) were resuspended and washed 5-6 times with sterile saline.

(c) LPS extract from *C.burnetii*. (LPS PE-W) was extracted by the hot phenol-water method as described by Anacker *et al.* (1962) and Brezina *et al.* (1970). One mgm of CSL *C.burnetii* Phase I Nine Mile whole cells was suspended in 1.0 ml of 90% v/v phenol-water mixture and held for 30 minutes at 65°C. The mixture was centrifuged at 3,000 rpm to separate phenol and water layers, and the interface 'fuzz' was removed. The phenol layer and the insoluble residue were re-extracted with an equal volume of water. The combined water extracts were dialysed against sterile water for 24 hours, then re-extracted with 90% phenol at 65°C. After the latter extraction only the aqueous phase was removed, dialysed for 72 hours against 3 changes of sterile water and the sac contents concentrated by lyophilisation.

Fraction TCA-E, the extracted cell residue, TCA-R, and LPS PE-W were assayed for (i) total protein (Bradford 1976); (ii) total nitrogen by the Dumas method (Kersten and Hesseluis 1983) and (iii) CF antigen content with respectively, an early post-infection guinea pig antibody reacting with

Table 13.1: Concentrations of organisms, protein and nitrogen content and patterns of serological reactivity of whole cell and extracted fractions of *C.burnetii* used in lymphocyte stimulation assays.

ANTIGENIC PREPARATIONS				COMPLEMENT-FIXING ACTIVITY WITH ANTISERUM					
				GUINEA PIG[4] Phase II		RABBIT[5] Phase I		HUMAN[6] Phase I	
ANTIGEN	COXIELLA CONC.[1] (µg/ml)	PROTEIN CONC.[2] (µg/ml)	NITROGEN CONC.[3] (µg/ml)	CFU[7]	AT[8]	CFU	AT	CFU	AT
CSL Nine Mile Phase I WC	1000	225.8	114.3	0	<4	12	32	13	16
RM RSA516 Nine Mile Phase II WC	1000	228.1	144.3	35	256	0	<4	0	<4
CSL Nine Mile Phase I treated K104	1000	264.4	-	27	128	25	128	33	256
CSL Nine Mile Phase I TCA-E	N/A	90.0	-	4	16	39	512	30	512
CSL Nine Mile Phase I TCA-Residue	1000	251.6	76.0	9	64	43	512	30	512
CSL Nine Mile Phase I LPS (PE-W)	N/A	0.63	-	0	<4	38	512	31	1024

(1) Concentration of coxiellas determined by spectrophotometry at 420nm.
(2) Protein concentration by Bradford (1976) method.
(3) Total nitrogen content by Dumas method.
(4) Early post Q fever infection guinea pig antiserum. Antibody titre against Phase I antigen <8, Phase II antigen 256.
(5) Serum from hyperimmunised rabbit, absorbed x3 with RM RSA516 Nine Mile Phase II antigen. Antibody titre against Phase I antigen 1024, Phase II <8.
(6) Serum from human chronic Q fever infection; antibody titre Phase I antigen 128, Phase II antigen 8.
(7) Complement-fixing units (see text).
(8) Highest (optimal) antigen titre observed with any of a range of antiserum dilutions.
N/A = not applicable - = not tested

C.burnetii Phase II antigen only, with a monospecific, absorbed rabbit antiserum against Phase I antigen and finally with a chronic infection serum (human) reacting predominantly against *C.burnetii* Phase I antigen. Whole cell antigens and fractions were titrated in 'chessboard' fashion against the antisera and the results expressed as complement fixing units (CFU: = total number of cups in plate with 3 or 4+ fixation of complement; Marmion *et al* 1967) and also as the highest (optimal) antigen titre (AT) observed with any of a range of antiserum dilutions (see Table 13.1).

(d) Separation of *Coxiella burnetii* proteins by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Coxiella burnetii cell preparations. Preparations from *C.burnetii* Phase I and II cells were obtained as a gift from Dr T. Hackstadt (NIH, Rocky Mountain Laboratories, MA, USA) as whole cell lysates in Laemmli dissociation buffer (Laemmli 1970) at a concentration of approximately 2mg/ml. Each cell lysate was divided into 30µl lots and stored at -20°C. Before use the preparations were allowed to thaw, boiled for 30 seconds, cooled and then centrifuged for 5 minutes at 13000rpm in a microfuge (Hettich Mikroliter 2020, Tuttlingen, Germany).

Preparation of SDS-PAGE. Polyacrylamide gels were prepared as 8 mini-gels, measuring approximately 90x103 mm (Matsudaira and Burgess, 1978). The apparatus, the glass plates, spacers and combs were washed in a mild

detergent solution (Pyroneg, Diversey, NSW, Australia), rinsed in distilled water (DW) and finally rinsed with a 70% (v/v) ethanol-water solution. Polyacrylamide gels were made by a modification of the method of Laemmli. Acrylamide-bisacrylamide (30:0.8) was prepared by dissolving 30gm of acrylamide (Sigma No. A-8887, St. Louis, MO, USA) and 0.8gm of N,N'-Methylenebisacrylamide (Electrophoresis Grade, Pierce, Rockford, IL, USA) in 100ml of DW. The solution was filtered through Whatman No. 1 filter paper (Whatman Ltd, England) and stored at 4°C in a dark bottle. A 10% (w/v) SDS stock solution was prepared by dissolving 10gm of SDS (Lauryl, Sequanal Grade, Pierce, Rockford, IL, USA) in 100ml of DW. Ammonium persulphate (1.5% w/v), (Analar, BDH Chemicals, Vic, Australia) was prepared fresh just before use. N,N,N',N'-tetramethylethylenediamine (TEMED, BDH Chemicals, Poole, England) was used as supplied. An SDS-discontinuous buffer system was employed, with a final acrylamide concentration in the resolving gel at 12.5%. The gel mixture (50ml) was prepared according to the formula: Acrylamide:bisacrylamide 20.8ml; Resolving gel buffer 6.25ml; 10% SDS 0.50ml; 1.5% Ammonium persulphate 2.50ml; DW 15.75; TEMED 0.025ml.

The resolving gel buffer (3.0M Tris-HCl pH8.8) was prepared by mixing 36.3gm of Tris[hydroxymethyl]aminomethane (Tris, Trizma Base, Sigma No. T-1503, St. Louis, MO, USA) and 48.0ml of Hydrochloric Acid (HCl, Analytical Reagent, Univar, Ajax Chemicals, NSW, Australia) and brought to a 100ml final volume with DW. The buffer was filtered through Whatman No. 1 filter paper and stored at 4°C.

The stacking gel mixture (20ml) was prepared according to the formula:

Acrylamide:bisacrylamide 2.50ml; Stacking gel buffer 5.00ml; 10% SDS 0.20ml; 1.5% Ammonium persulphate 1.00ml; DW 11.30ml; TEMED 0.015ml.

The stacking gel buffer (0.5M Tris-HCl pH6.8) was prepared by dissolving 6.0gm of Tris in 40ml of DW, titrating to pH 6.8 with 1M HCl and then bringing the volume to 100ml with DW. The solution was filtered and stored as above.

Reservoir buffer stock (0.25M Tris, 1.92M Glycine, 1% SDS pH 8.3) was prepared by dissolving 30.3gm of Tris, 144.0gm of Glycine (Analar, BDH Chemicals, Vic, Australia) and 10.0gm of SDS in DW which was then brought to 1000ml. The 10x stock solution was diluted further in DW prior to use.

12.5% gels for SDS-PAGE, that were 0.5mm thick, with 10 wells were prepared and stored in a humid chamber at 4°C.

Before use, the wells were washed with DW and all the water removed. The samples and molecular weight markers were loaded into wells with a Hamilton syringe (Hamilton Co., Nevada, USA). A current at 50V was passed through the gel until the dye from the Laemmli buffer had migrated to the stack/resolving gel interface, at which time the current was increased to 150V. When the dye reached the bottom of the gel the current was switched off, the gel removed from the apparatus and glass plates and treated as follows.

Those gels to be stained directly were fixed for 1-2 hours in a solution of DW:Methanol:Acetic Acid (5:5:2) at room temperature (RT) with shaking and then stained with Coomassie Brilliant Blue R-250 solution (Bio-Rad, NSW, Australia). Coomassie blue dye, prepared and filtered just prior to use, was dissolved in DW:Methanol:Acetic Acid to give a final concentration of 0.1%.

Gels were stained overnight at RT with agitation and then destained with DW:Ethanol:Acetic Acid (7:2:1) until the gel had cleared.

Molecular weight markers. Prestained high and low range SDS-PAGE standards were purchased from Bio-Rad (NSW, Australia). Each standard was divided into aliquots and stored at -20°C, until used, at which time they were treated in the same manner as the antigen. Each standard contained (molecular weights in kilodaltons (kDa)):

Protein	Low	High
Myosin	-	205
β-galactosidase	-	116.6
Phosphorylase B	110	-
Bovine Serum Albumin	84	77
Ovalbumin	47	46.5
Carbonic anhydrase	33	-
Soybean Trypsin Inhibitor	24	-
Lysozyme	16	-

Transfer of electrophoresed *C.burnetii* proteins onto nitrocellulose filter. Gels from which proteins were to be transferred to nitrocellulose were removed from the glass plates and washed 3x10 minutes in transfer buffer (25mM Tris, 192mM Glycine pH8.3). The gel was then slid onto a glass plate. A filter paper (Whatman No. 1) that had been slowly dipped into transfer buffer was layered over the gel, ensuring that there were no air bubbles between the gel and filter

paper. The gel with the filter paper was slid off the glass plate and turned over so that a nitrocellulose (NC) membrane (Schleicher and Schuell GmbH, Dassel, Germany) could be layered over the gel. After a second buffer soaked filter paper was placed over the NC, the completed 'sandwich' was then placed between two Scotch Brite pads (pot scourers) inside a support that was inserted into the transfer chamber containing the buffer. The alignment was such that the gel was closest to the cathode and the NC closest to the anode. A 60V current was passed through the chamber for 4 hours, after which the gel and NC were removed and examined for transfer of the proteins.

Assessment of efficiency of transfer of proteins. To determine the efficiency of transfer and localisation of protein bands on the NC, filters were stained by the method described by Hancock and Tsang (1983) using india ink. NC were washed 4x for 10 minutes each in approximately 250ml of Phosphate Buffered Saline-Tween 20 solution (PBS-Tween: 0.15M NaCl in 0.01M KH_2PO4/Na_2HPO_4pH7.4 plus 0.3% Tween 20 BDH Chemicals, Poole, England) at 37°C with agitation. The NC was then stained with Pelikan fount india drawing ink for fountain pens (Pelikan AG, Hanover, Germany), prepared by adding 2µl of ink into 200ml of PBS-Tween. The NC was stained overnight at 37°C with agitation, then rinsed with DW and dried between filter paper.

The gel was stained with Coomassie blue dye to assess whether any protein bands remained in the gel.

Preparation of fractionated antigen bound to nitrocellulose particles. The method used for preparation of antigen-coated NC particles was a combination of that described by Abou-Zeid *et al.* (1987) and Lee *et al.* (1989). NC to be used for assessing lymphoproliferative responses were stored at -20°C within filter paper and aluminium foil. The membrane was cut with a clean scalpel into approximately 40mm^2 pieces. The resulting 22 strips plus an NC membrane control without protein, were each placed into vials to which 1ml of dimethyl sulphoxide (DMSO, Ajax Chemicals, Sydney, Australia) was added. Vials were incubated for 6 hours at room temperature with periodic agitation. Reprecipitation of fine antigen-bearing particles was achieved by the addition of an equal volume of 50mM carbonate-bicarbonate buffer, pH 9.6. The buffer was added dropwise at a constant rate (approximately 1ml/minute), while the vial constantly agitated mixed with a vortex at its highest speed. The particles were washed three times with RPMI-1640 (plus 20mM HEPES, 200U/ml Penicillin, 100µg/ml Gentamicin) by centrifuging in a microfuge for 15 minutes. The resulting pellet was resuspended in 800µl of RPMI-1640 and then aspirated through a 26 guage needle (Terumo, Melbourne, Australia) to ensure total resuspension of fine antigen-bearing particles. The vials were stored at either 4°C or -20°C depending on time of use.

Lymphoproliferation assay. For the assays with reconstituted T or B-lymphocyte- monocyte mixtures, and solvent extracted antigens and cell residues, or periodate-treated whole cells, and separated proteins, PBMC were taken from 5 subjects - viz; two vaccinees (one, BPM, inoculated 1952 with

subsequent, periodic skin tests and the other AI in 1985); two subjects (JD and DM) infected with Q fever in 1958, both with positive Q fever CF antibody and positive skin tests; and, finally, one control subject (LS) with no history of Q fever and a negative antibody test. These were separated into T- and B-lymphocyte and monocyte preparations and reconstituted as described in Chapter 12.

13.3 Results

Reactivity of reconstituted mixtures of immune T- and B-lymphocytes and monocytes to antigenic fractions from *C.burnetii*.

As shown in Chapter 12, T-lymphocytes from vaccinees or past infections gave qualitatively similar stimulation profiles with *C.burnetii* Phase I and Phase II antigens. A major difference between the two antigenic phases is the number of sugar units in the LPS side chains; Phase I LPS contains 4-6 more sugar residues and has a higher molecular weight (Schramek and Mayer 1982, Baca *et al* 1980, Amano *et al* 1985).

Apart from the variation in the LPS, there appear to be either no or much less striking loss-variations in the patterns of protein bands on SDS-PAGE with strains of *C.burnetii* in Phase II derived by extensive serial passage in the CE yolk sac (Hackstadt *et al.* 1985, and Discussion).

It was therefore of interest to determine, first, whether a whole cell preparation of *C.burnetii* Phase I, in which the LPS sugar chains had been modified by periodate treatment to generate an artificial Phase II serological

Figure 13.1 (a),(b),(c),(d),(e),(f). Profiles of T-lymphocyte stimulation responses with two vaccinees (BPM and AI), two subjects (JD and DM) with past Q fever and one non-immune subject (LS) and various C.burnetii antigens. The latter were Nine Mile Phase I whole cells; Nine Mile Phase II whole cells; Nine Mile Phase I whole cell, periodate treated; Nine Mile Phase I cells, TCA extract, and extracted cell residue; Nine Mile Phase I phenol water extract (LPS).
x———x LS

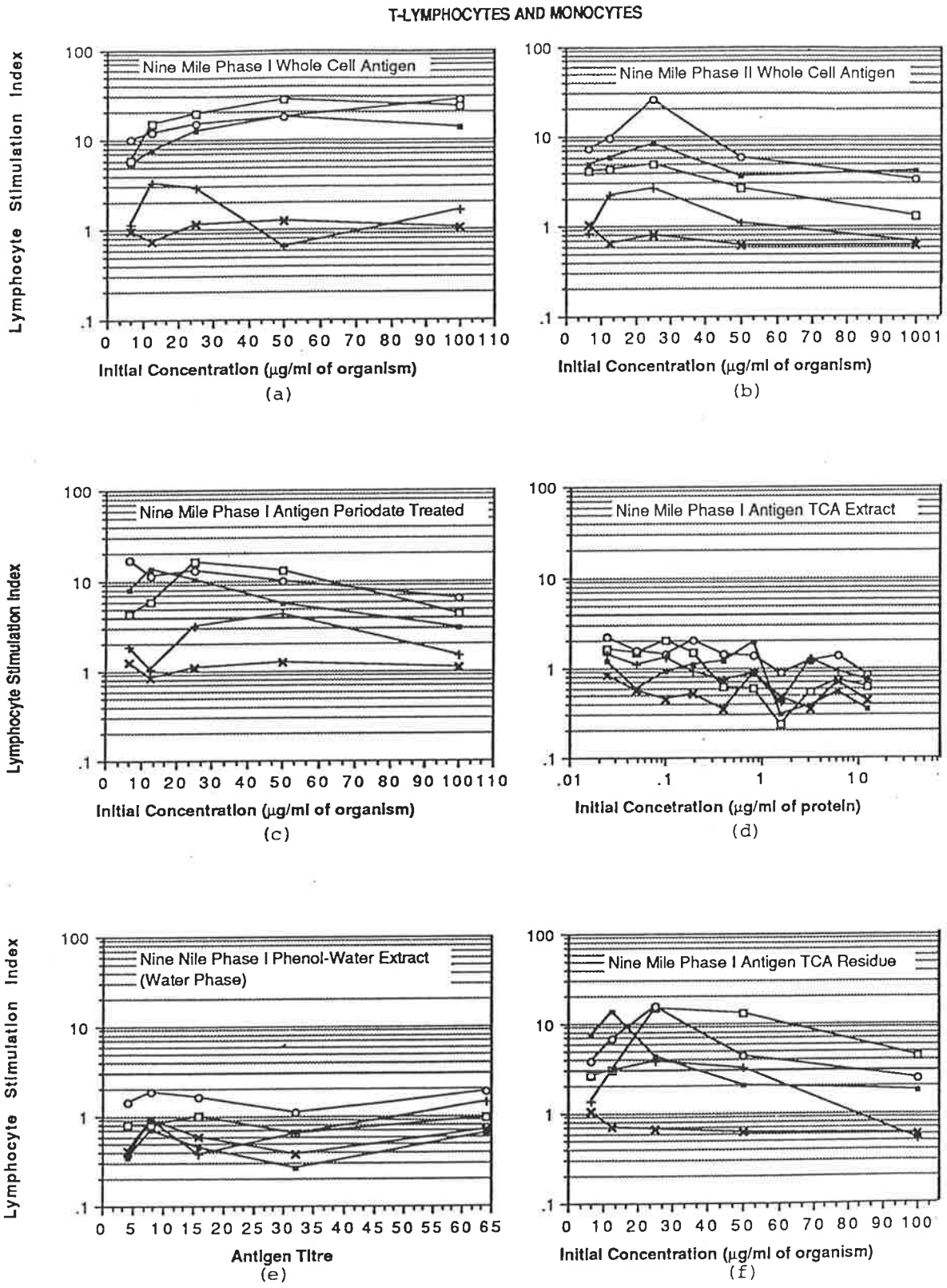


Figure 13.1 (a) (b) (c) (d) (e) (f)

state - but without modification of the cell protein - would exhibit a different reaction profile with T-lymphocytes from vaccinees, or past infection. And, second, to determine whether deproteinised LPS, extracted complexes of LPS and protein, and cell residues with no, or reduced LPS, but containing the bulk of the cell protein would stimulate immune T-lymphocytes.

For these purposes T-lymphocyte-monocyte mixtures from the two vaccinees, two past infection subjects and one nonimmune control were tested against a range of dilutions of CSL Nine Mile Phase I and II whole cell antigens, periodate-treated Nine Mile Phase I antigen, TCA extract and TCA-extracted cell residue and Phenol-water extracted Phase I LPS (Figure 13.1 (a),(b),(c),(d),(e),(f)). The Phase serological activity of the whole cell antigens and extracted fractions are summarised in Table 13.1. In brief, LPS-PE-W, essentially protein free, reacted as a Phase I antigen only. TCA-E reacted predominantly as Phase I antigen, with some Phase II activity presumably due to the proteins associated with the LPS. TCA-R showed a similar pattern with more Phase II activity. Nine Mile Phase I whole cells (WC) treated with potassium periodate showed, as expected, enhanced Phase II activity, but unexpectedly, also enhanced Phase I activity. Perhaps alteration of some (?terminal) sugar units in the LPS side chains increased access of anti-Phase I antibody to other parts of the LPS sugar chain as well as facilitating access of antibody to the Phase II proteins. The increased protein value for the periodated preparation given by the Bradford method may also represent better access of the Comassie blue dye to the underlying proteins.

The patterns of T-lymphocyte stimulation responses with these

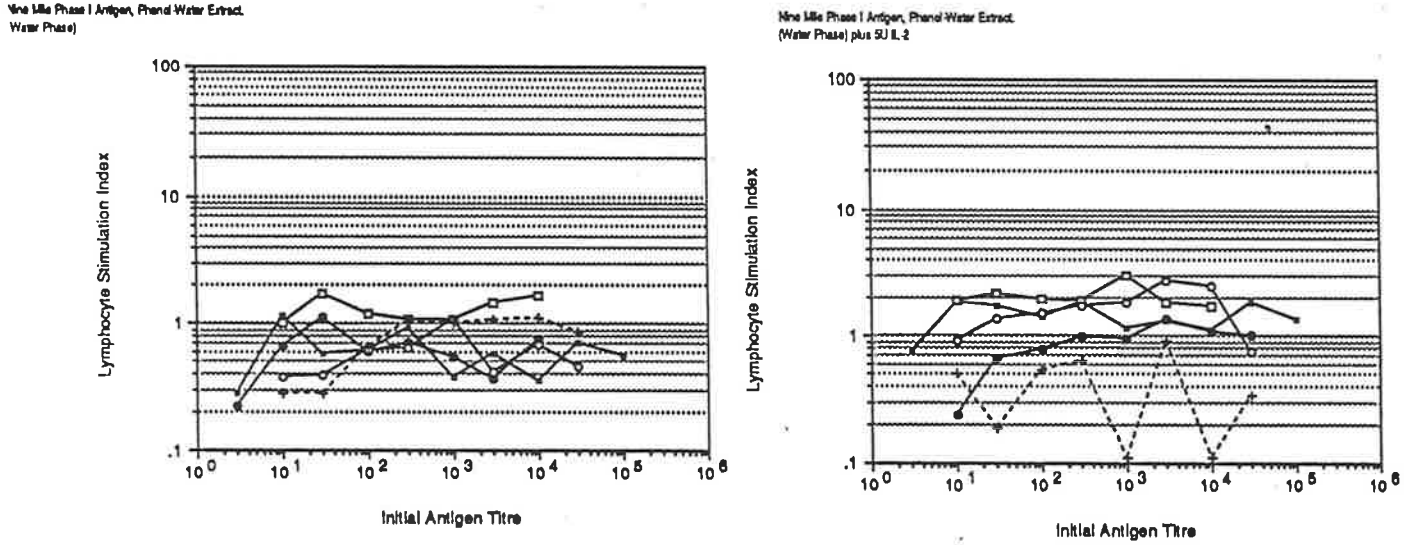


Figure 13.2 (a),(b). Profiles of B-lymphocyte stimulation responses with cells from two vaccinees (BPM and AI), two subjects (JD and DM) with past Q fever infection and one non-immune subject (LS) and LPS from Nine Mile Phase I organisms.

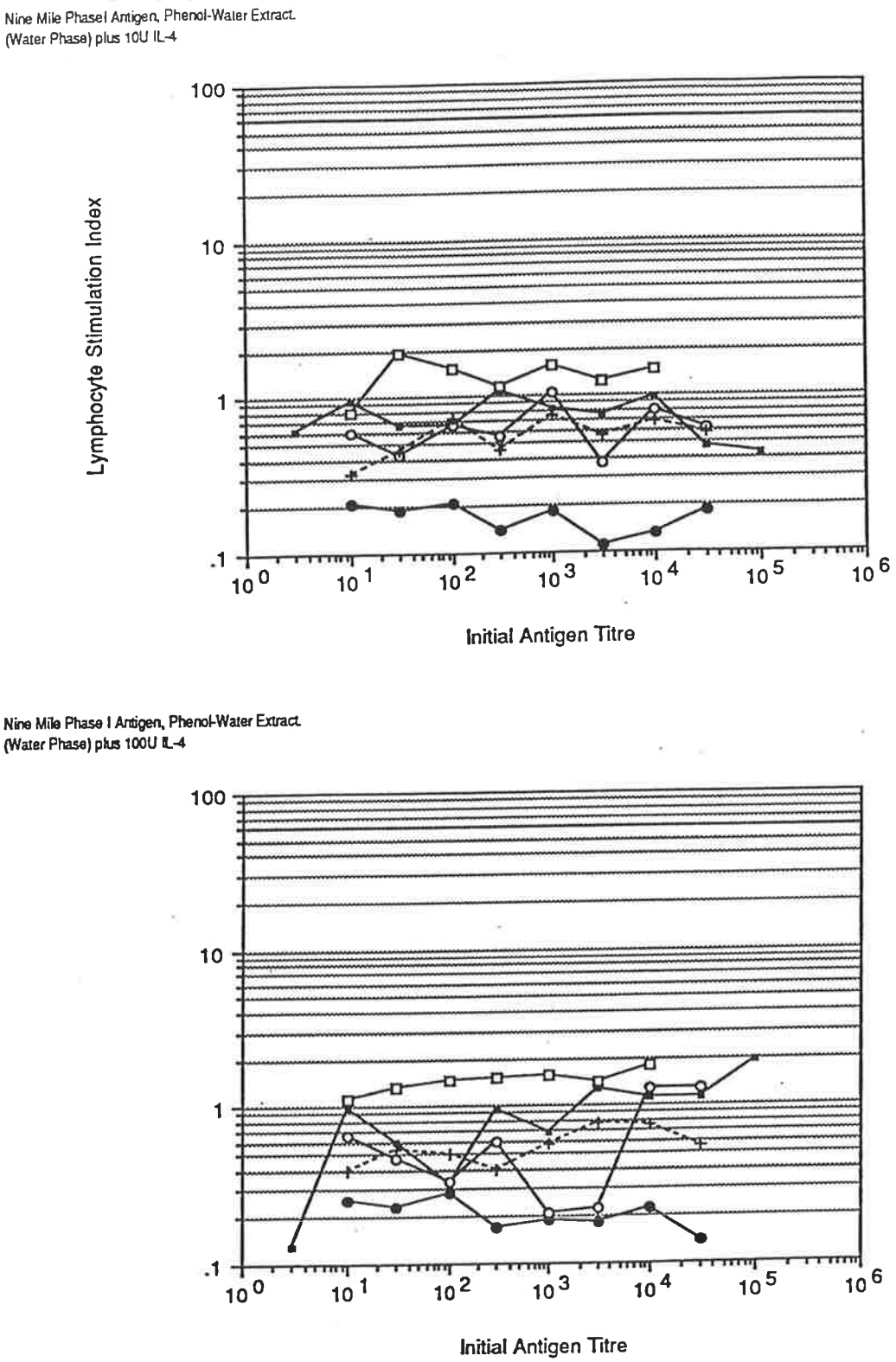


Figure 13.3. Profile of lymphocyte stimulation indices with separated B-lymphocytes from the same panel of cell donors as in Figure 13.2 with supplementation with IL-4 antigen (LPS) in the water phase of a phenol water extract of Nine Mile Phase I antigen.

preparations showed that LPS-rich fractions (LPS P-WE and TCA-E) (Figure 13.1 (d) and (e)) had little activity compared with WC or cell residues. Treatment of Nine Mile Phase I WC antigen with potassium periodate changed reactivity to the pattern observed with the Nine Mile Phase II antigen naturally derived by serial yolk sac passage (Figure 13.1 (a), (b) and (c)). Similar patterns were observed with periodate-treated Priscilla strain WC (data not shown).

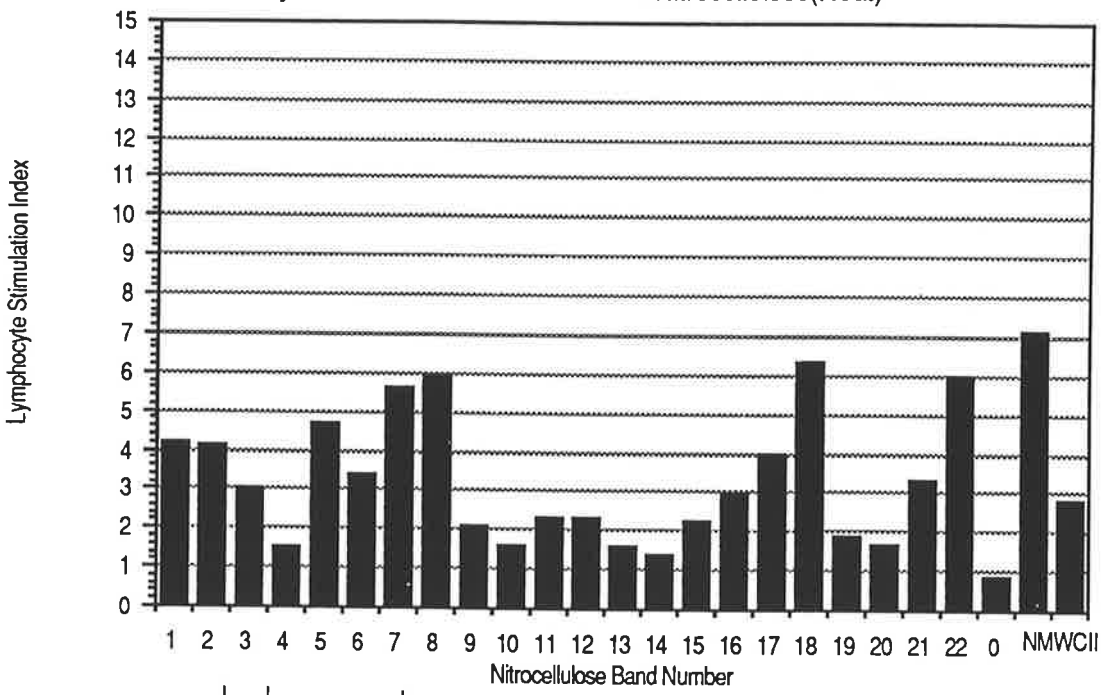
B-lymphocytes from the 5 donors showed no or only slight (one of 5) raised LSI against the *C.burnetii* Phase I LPS (Figure 13.2 (a)). Addition of IL-2 to the system increased the stimulation indices above the cut-off level with cells from 4 of the donors while the non-immune control remained negative. Addition of 10 or 100 units of IL-4 also produced a meagre increase but less than that with IL-2 (one of 5 donors; Figure 13.3).

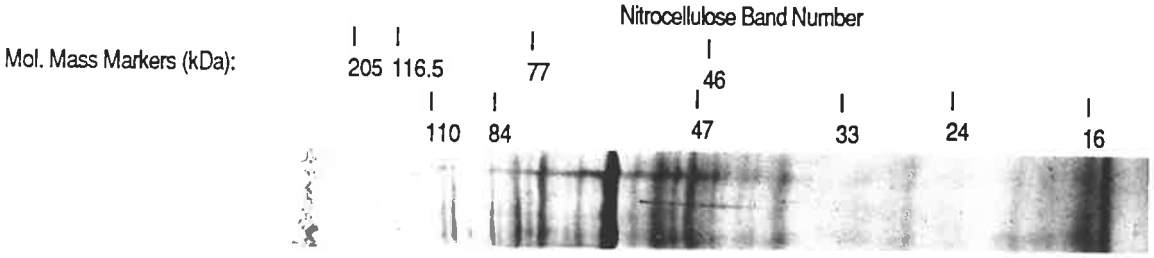
Overall, it is evident that the major T-lymphocyte stimulating activity resides with the whole cell or extracted cell residue (e.g. TCA-R), rather than with the LPS. That is, the proteins of the coxiella provide critical T cell epitopes.

As outlined in the introductory Chapters 6 and 7, PAGE analysis of the coxiella proteins reveals 40-60 bands of variable intensity. Serological analysis by Williams *et al.* (1984) led to the conclusion that a 27.5KDa protein was the immunodominant Phase II antigen although other workers, particularly Hackstadt *et al.* (1985) found that many of the proteins separated on PAGE would react with rabbit antisera to *C.burnetii* or human Q fever convalescent sera.

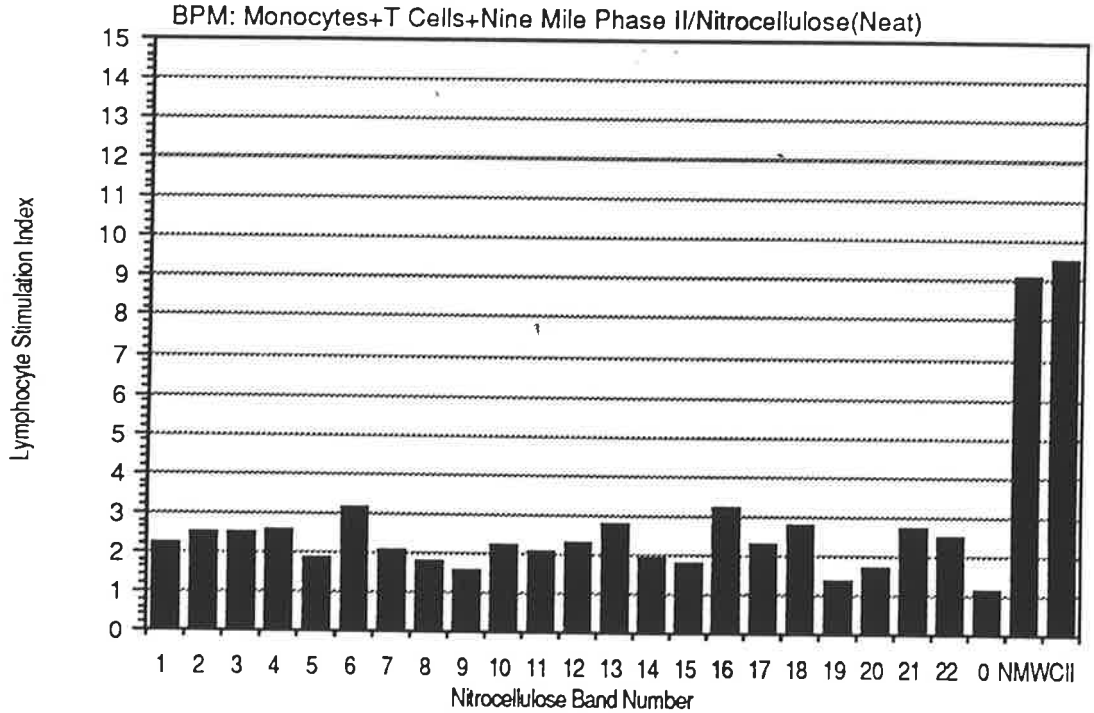
Figure 13.4 (a),(b),(c),(d),(e). Profiles of lymphocyte stimulation assay responses with C.burnetii proteins separated by PAGE, transferred to nitrocellulose and redispersed for antigen stimulation. 0 = no antigen, nitrocellulose only. NMWCII = Nine Mile whole cell preparation, proteins diluted 1 in 1 or 1 in 3. (b) Vaccinee BPM, (a) Vaccinee AI, (c) past infection, subject JD, (d) past infection DM, (e) negative control, nonimmune subject LS.

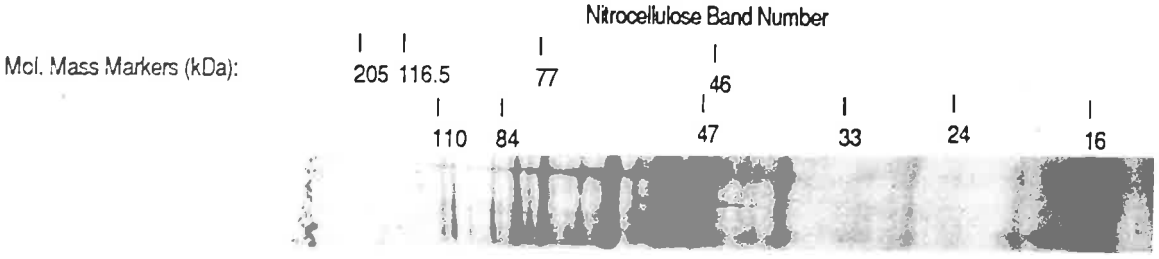
AI: Monocytes+T Cells+Nine Mile Phase II/Nitrocellulose(Neat)

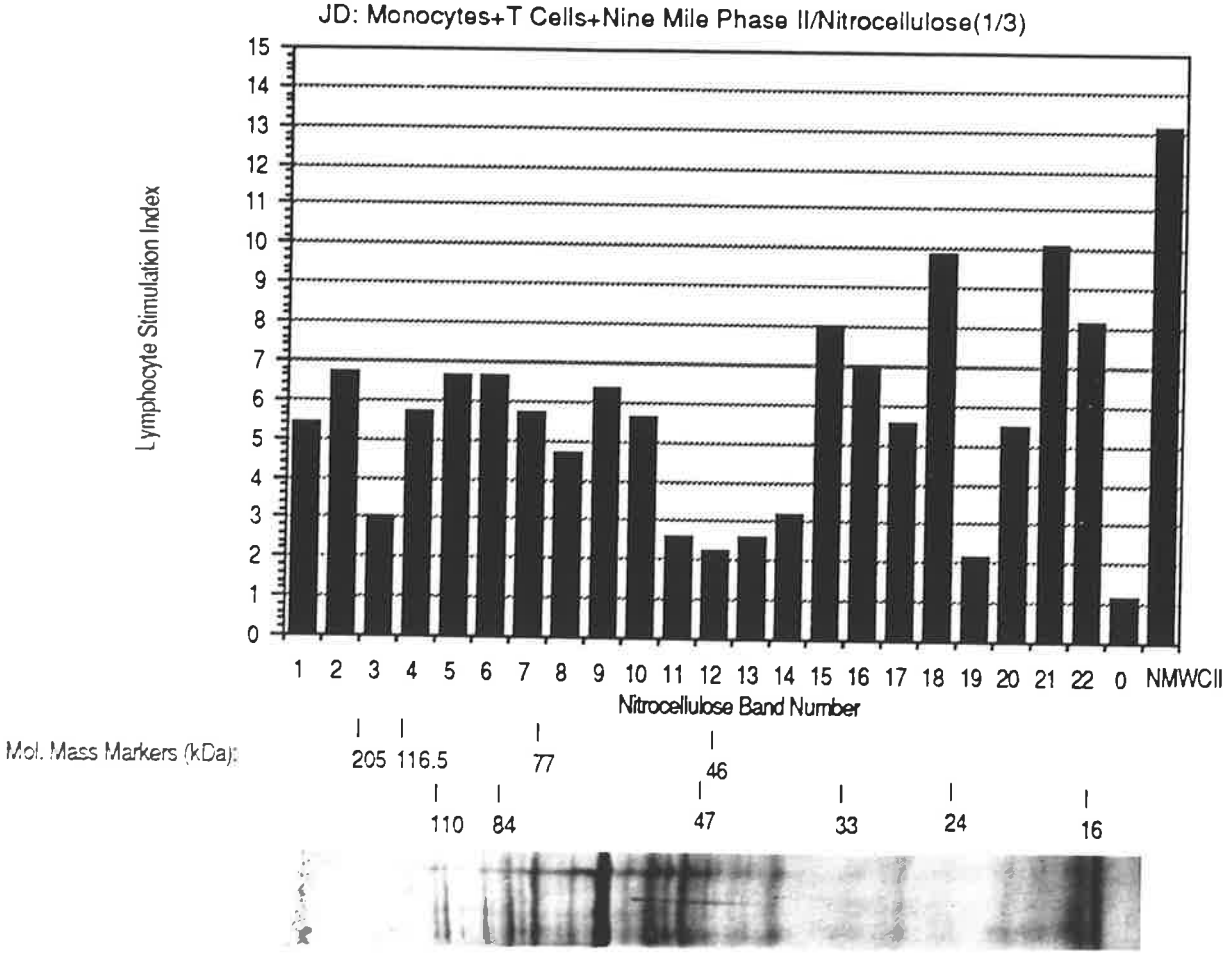


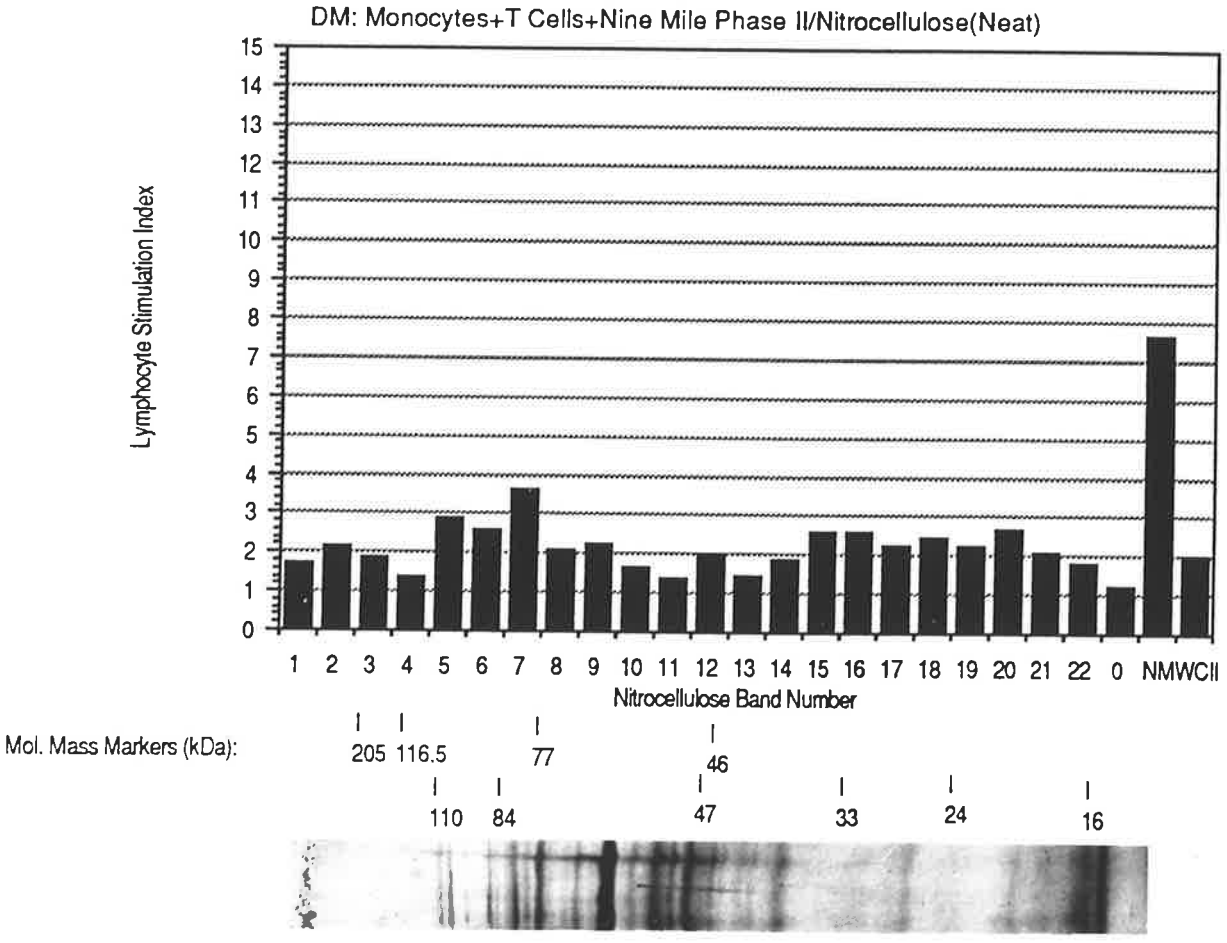


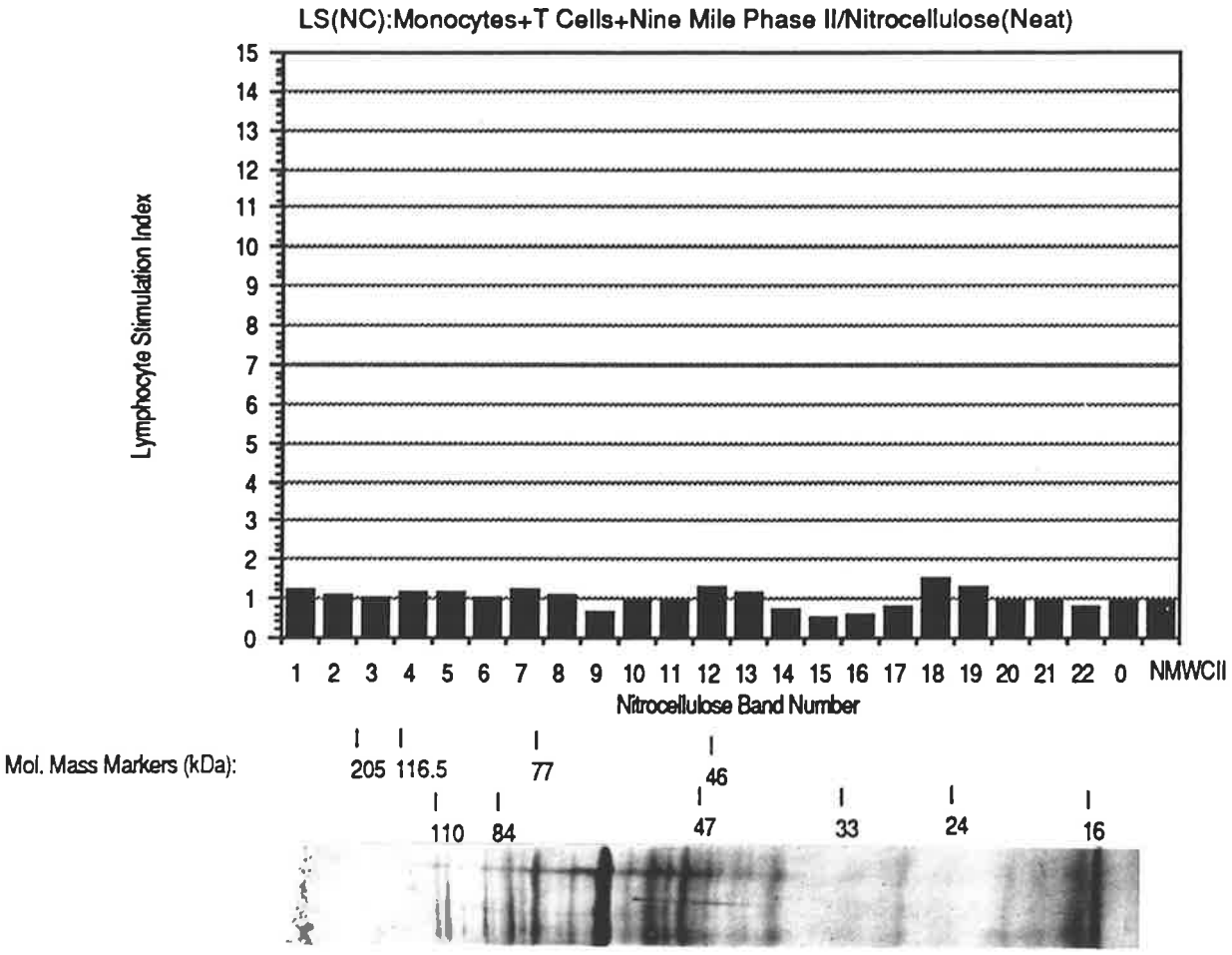
BPM: Monocytes+T Cells+Nine Mile Phase II/Nitrocellulose(Neat)






JD: Monocytes+T Cells+Nine Mile Phase II/Nitrocellulose(1/3)
Lymphocyte Stimulation Index
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 0 NMWCII
Nitrocellulose Band Number
Mol. Mass Markers (kDa):
205 116.5 77 46 110 84 47 33 24 16


DM: Monocytes+T Cells+Nine Mile Phase II/Nitrocellulose(Neat)
Lymphocyte Stimulation Index
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 0 NMWCII
Nitrocellulose Band Number
Mol. Mass Markers (kDa):
205 116.5 77 46 110 84 47 33 24 16


LS(NC):Monocytes+T Cells+Nine Mile Phase II/Nitrocellulose(Neat)
Lymphocyte Stimulation Index
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 0 NMWCII
Nitrocellulose Band Number
Mol. Mass Markers (kDa):
205
116.5
77
46
110
84
47
33
24
16

It was therefore of much interest to know whether a similar pattern of broad reactivity would obtain with T-lymphocytes and the various cell proteins or whether there was indeed a single or a few immunodominant proteins which were highly stimulating for T-lymphocytes.

Accordingly, cell lysates from Nine Mile Phase II (from T. Hackstadt) were separated in PAGE and the transferred proteins in nitrocellulose dispersed to stimulate T-lymphocytes from the 5 donors used for the previous experiments. Nitrocellulose-protein dispersions were tested neat and diluted one in three.

Profiles of T-lymphocyte responses with PAGE-separated proteins from *C.burnetii*. Figure 13.4 (a), (b), (d) and (e) shows a selection of the LSI profiles with the separated proteins (reactions with neat lysate, or one in three dilutions, were chosen on the basis of maximum effect). Cells from the non-immune control (LS) showed no or few reactions above the cut-off level with neat or one in three protein dispersion (Figure 13.4 (e)). The highest value - 1.3-1.5 - was with bands 18 and 19.

Cells from the two vaccinees, BPM and AI, showed positive reactions with all protein bands, in each case less than the response with the unfractionated whole cell lysate. In particular, with T-lymphocytes from AI, bands around 77KDa (band numbers 5 to 8), around 24 KDa (band numbers 17 and 18) and 16KDa (bands 21 and 22) stood out as peaks in the spectrum of responses. These findings were mirrored, by the profiles from the past infection subjects, particularly JD (Figure 13.4 (c) and (d)).

The overall conclusion is that most cell proteins from the coxiella are

recognised by immune T-lymphocytes and that the most powerful stimulant is in fact the unfractionated cell lysate representing the whole antigen mosaic. Nevertheless, the more potent stimulating proteins, or groups of proteins, will be analysed in a mouse protection test (see Discussion Chapter 16) to confirm that in fact they do or do not have protective efficacy in the absence of LPS.

13.4 Discussion

The conclusion from the results presented, particularly those in Figure 13.1 (a) and (b), is that the T cell epitopes are associated with the coxiella proteins. The phenomenon of stimulation of T cells by carbohydrate antigens from *Mycobacteria* has been reviewed (Crowle 1988) and a recent paper (Moll *et al.* 1989) describes T cell activation and lymphokine production by lipophosphyoglycan from the intracellular parasite *Leishmania major*. From the observations described above it seems that a similar mechanism does not appear to operate with *C.burnetii* LPS.

Experimental models for measuring "protective" immunity are either suppression of fever in guinea pigs (as used for example by Ormsbee *et al.* 1964) or reduction of the multiplication of *C.burnetii* in the mouse spleen (Abinanti and Marmion, 1957). "Protection", in this sense of disease modification, can be effected in both animal models by antibody against Phase I antigen (i.e., antibody against the sugar chains of the LPS) when mixed with inoculum, but not by antibody against Phase II (.e., against the proteins associated with the LPS or membrane of the organism). However, such "protection" by Phase I antibody is effective only in animals with intact cellular

immune system and does not operate in nude or immunosuppressed mice, chick embryos or cell culture (Kishimoto *et al.* 1978b; Kazar *et al.* 1973, Humphries and Hinrichs 1981).

The protective immunogenicity of LPS and protein from *C.burnetii* is only partly defined. In the guinea pig model Ormsbee *et al.* (1964) found that a vaccine prepared from *C.burnetii* Phase I organisms was, organism for organism, 100-300 more protective than one from Phase II organisms. It has been tempting to assume therefore that the difference in protective efficacy between Phase I and Phase II vaccines is due to the phase, loss-variation, in LPS CHO structure. However it is also possible that the change to the Phase II antigenic state on prolonged yolk sac passage of *C.burnetii*, is accompanied *pari passu*, by loss variation in cell proteins and conceivably, therefore, in loss of critical T cell epitopes. However, investigations and discussion of this matter by Hackstadt *et al.* (1985) led to the conclusion that there were no striking differences between the PAGE protein patterns of Phase I and Phase II organisms and that what differences were observed might be due to the change in LPS-protein co-migration following the loss variation in Phase I LPS on change to Phase II LPS.

Thus far analysis of the protective immunogens of *C.burnetii* cells by various workers in the field has established (a) that TCA-E, and the cell residue after TCA extraction, will protect in the guinea pig or mouse model (Anacker *et al.* 1963); (b) that LPS extracted by the phenol-water method (LPS-PE-W), either from TCA-E or whole cells, is not protective in guinea pigs (Anacker *et al*, 1963) or in mice unless multiple doses are administered (Brezina and

Pospisil 1970). LPS is a hapten; preparations in Freunds incomplete adjuvant do not stimulate antibody formation in rabbits; (c) that the cell residue (CMR) after chloroform-methanol extraction of the coxiella protects in mice and modifies infection in sheep (Williams *et al.* 1986a, Brooks *et al.* 1986) and (d) that dimethylsulphoxide (DMSO) extracts of Phase I organisms protect in guinea pigs whereas those from Phase II organisms do not (Ormsbee *et al.* 1962).

TCA-E, TCA-R and CMR all contain protein and variable amounts of LPS; the latter as evidenced by the stimulation of Phase I antibodies in inoculated animals. As stated, LPS-PE-W, protein-free, neither stimulates antibody or protects. The DMSO extracts, which do protect, were said to be mainly LPS and to be free of protein by UV absorption (Ormsbee *et al.* 1962). However as they provoke Phase II antibody in animals (Williams *et al.*, 1986a) it is likely that Phase II protein(s) are in fact present in small amounts. Indeed recent observations by Hackstadt *et al.* (1985) indicate that DMSO extracts of *C.burnetii* cells contain around 25% protein.

Our results with T-lymphocyte stimulation assays and coxiella fractions are broadly in line with the protection data. LPS-PE-W does not stimulate T cells; TCA-E and TCA-R are mitogenic. Nine Mile and Priscilla Phase I antigens modified by periodate treatment to destroy the dominant carbohydrate epitopes of the LPS retain good stimulatory mitogenic equivalent to that of Phase II whole cells.

These results suggest that coxiella proteins and in particular those associated with Phase II serological activity, are of importance as T cell

epitopes. This conclusion is reinforced by the analysis of PAGE separated proteins in the T-lymphocyte stimulation assay (Figure 13.4). This might suggest that proteins, stimulating CMI, would protect. However, extracted protein, free of LPS, has not yet been tested to see if it will protect. An indication that it may not comes from Kazar and Schramek (1985) who correlated 'protection' against infection assayed in mice, with serological response, and CMI as measured by DTH response in the mouse footpad. TCA-E vaccine had a significant protective efficacy although somewhat lower than that of whole cells; it also stimulated Phase I and II antibody and a positive DTH response. After treatment with potassium periodate, the protective efficacy of TCA-E was greatly reduced although a positive DTH reaction was still obtained. In Kazar and Schramek's experiments LPS alone again did not protect although (perhaps surprisingly) a positive DTH response was obtained.

From this wide range of observations it seems probable that the Phase I LPS is part of the protective immunogen of *C.burnetii* along with the associated proteins acting as antigenic determinants for generation of T helper cell memory, or cytotoxic T cells - i.e., the carrier - hapten relationship postulated earlier by Anacker *et al.* (1963), but see detailed discussion in Chapter 16.

The current *in vitro* studies of PBMC from Q fever vaccinees show features compatible with the earlier protection data. *C.burnetii* proteins stimulate T-lymphocytes and some B cell mitogenesis is observed with LPS (and, of course, is indicated by antibody responses after vaccination). Further

definition of the protective efficacy of the cell protein(s) alone is however desirable in attempts to develop alternative vaccines by the recDNA cloning of *C.burnetii* proteins of high mitogenic activity for immune T-lymphocytes. It is also necessary to understand why an antibody response to Phase I LPS is an essential part of the immunity to *C.burnetii*.

CHAPTER 14

ANALYSIS OF CYTOKINE ACTIVITY IN *COXIELLA BURNETII* - LYMPHOCYTE INTERACTIONS

14.1 Introduction

As indicated earlier, immune mechanisms involved in the final elimination of *C.burnetii* in the human host are dependent on *C.burnetii*-reactive T-lymphocytes. *C.burnetii*, as an obligate intracellular parasite replicating within phagolysosomes of monocytes/macrophages, is protected from direct antibody-dependent clearing mechanisms; instead processes involving macrophage activation are central to the intracellular destruction of the organism. The balance between immune mediators and modulators produced by T-lymphocytes, and the degree of macrophage activation, may determine the extent, severity and resolution of the infection. Therefore in characterising the protective mechanisms of a Q fever vaccine, it was important to show that interferon-γ (IFNγ), the final activator of macrophages, and mediator of intracellular killing of the coxiella in professional and nonprofessional phagocytes, is produced by T-lymphocytes from Q fever vaccinees, when stimulated by *C.burnetii*. The finding of IFNγ would indicate, by inference, that the "right" (protective) category of T helper cells on stimulation was sensitised (see below).

Although T-lymphocytes from vaccinees exhibit a proliferative response when stimulated with *C.burnetii* antigens, it does not necessarily follow that the response would be accompanied by an effective generation and secretion

of IFNγ. In mice, certain subsets of T helper cells produce lymphokines stimulating B cell replication and differentiation while others produce IFNγ (Liew 1989; Lichtman *et al.* 1987).

Experiments in mice with *Leishmania major*, an intracellular pathogen, as is *C.burnetii*, have shown that different *Leishmania* vaccine preparations and different routes of inoculation with vaccine, stimulate one or other helper T cell subset and thereby enhance or suppress the disease on challenge of the immunised mice with living *Leishmania major* organisms. There is some debate as to whether analogues of the two helper T cell subsets in mice, are also found in the human CD4+ cell compartment although some workers have produced evidence that this is probable (Chapter 8).

Apart from the differential effects of lymphokine species liberated by different subsets of CD4+ cells, the mitogenic and linked lymphokine response of CD8+ lymphocytes or other non-CD4+ subsets of lymphocytes, to antigen might inhibit or suppress IFNγ production by regulation via a prostaglandin (e.g., PGE_2) pathway (Koster *et al.* 1985b).

Measurement of the secretion and action of IFNγ could be made in various ways. For example, IFNγ has been shown, *in vitro*, to inhibit the growth of *C.burnetii* in either guinea pig macrophages or murine monocyte-like cell lines (Hinrichs and Jerrells 1976, Turco *et al.* 1984).

While intracellular destruction of the coxiella in cells provides a direct and striking demonstration of IFNγ efficacy, setting up the system with human monocytes presents some difficulties. Consequently the studies reported below were limited to showing that PBMC or separated T-lymphocytes from vaccinees

or subjects infected in the past produce interferons, and IFNγ in particular; also to an investigation of possible inhibition of IFNγ production depending either on the type of antigen (e.g., Phase I versus Phase II whole cells or components such as PAGE separated proteins), or the subsets of T-lymphocyte involved.

Assays of IFNγ were compared with the lymphoproliferative responses (LSI) of PBMC from the same subjects to assess the correlation between the two *in vitro* correlates of cell mediated immunity.

14.2 Materials and Methods

Subjects. These comprised two groups: (a) For experiments to determine whether inoculation with Q fever vaccine (Qvax) induced a T-lymphocyte sensitisation capable of generating interferon, and IFNγ in particular, on stimulation of PBMC or separated T-lymphocytes with *C.burnetii* antigen. Samples of blood were collected pre- and post vaccination from a group of up to 23 volunteers in the "low-risk" category - i.e., laboratory workers who visited abattoirs to collect samples for research purposes but were not regularly exposed to infection. Prevaccination samples were collected from most but not all subjects. The group corresponded to the "low-risk" category described in Chapter 12. Interferon assays were ~~done~~ conducted on conditioned media harvested around the time of the lymphocyte stimulation assays and held at -70°C until tested.

Secondly, PBMC or separated T-lymphocytes and monocytes were taken from smaller panels of vaccinated subjects or nonimmune controls; in

particular from a panel of two vaccinees (AI and BPM), two past infections (DM and JD) and one non-immune control (LS), for various, more detailed analytical experiments on the effects of antigen fractions and T-lymphocyte subsets on IFNγ formation and the generation of inhibitors of IFNγ production.

Isolation and stimulation of Peripheral Blood Mononuclear Cells (PBMC). PBMC from heparin-treated whole blood were harvested by centrifugation over a Ficoll Hypaque gradient, as described in Chapters 11 and 12. Isolated PBMC were cultured at a density of $2x10^6$ cells/ml in a 1.0ml volume in RPMI-1640 supplemented with 10% v/v heat-inactivated autologous serum, 20mM HEPES buffer, 2mM L-Glutamine, $5x10^{-5}$M 2-Mercaptoethanol, 200U penicillin/ml and 40µg gentamicin/ml. All cultures were performed in 24-well tissue culture plates (Nunclon Delta, Nunc, Denmark). Antigen or mitogen (100µl) was added to each well at a previously determined optimal concentration. PMBC without antigen or mitogen were also included as a control for each subject. Cultures were incubated for 24 hours at 37°C in a humid atmosphere with 5% v/v CO_2. Supernatant fluids were harvested and immediately stored at -70°C.

Monocyte isolation by adherence to gelatin-coated plastic. The method employed for the isolation of monocytes from PBMC was that described by Jones *et al.* (1989). $75cm^2$ tissue culture flasks (Costar, Cambridge, Mass., USA) were coated with 10ml of a 30mg/ml solution of gelatin (BDH Chemicals Ltd, Poole, England). The flasks were incubated for 2 hours in a 37°C incubator and then the gelatin was removed by suction. Finally the flasks

were incubated overnight at 55°C, then stored at room temperature. PBMC from heparin-treated whole blood were harvested by centrifugation over Ficoll Hypaque gradient, as described in Chapter 12. Plasma from the gradient was saved and 10ml was added to a gelatin-coated flask, after which it was incubated at room temperature for at least 1 hour. The plasma was removed and the flask washed two times with sterile PBS. Approximately $2x10^7$ PBMC were resuspended in 15ml RPMI-1640 + 10% FCS and added to the gelatin-coated flask. The flask was incubated for 90 minutes at 37°C, with 5% CO_2. Nonadherent cells (NAC) were initially washed off with excess sterile saline and then several times with RPMI-1640 + 10% FCS. NAC were kept for later use. Adherent cells (AC) were treated with 10ml of cold 10mM disodium ethylenediaminetetra-acetate (Analytic Reagent, Ajax Chemicals, Auburn, Australia) in Hanks' balanced salt solution, at 4°C for 10 minutes. To remove as many AC as possible, the flask was tapped on the heel of the hand and then any remaining cells were scraped off with a sterile cell scraper (Nunc, Roskilde, Denmark). AC were transferred to silicon-treated (Coatasil, Ajax Chemicals, Auburn, Australia) sterile glass tubes and washed twice. Viable cells were counted by the Trypan Blue method.

Analysis of nonadherent cells (NAC) for monocyte contamination by flow cytometry. In initial experiments NAC were analysed for monocytic contamination by using monoclonal antibodies (mAB) directed against cell surface markers. The mAB employed were: (i) Negative mAB (anti-salmonella); (ii) T cell (OKT3/3AI); (iii) B cell (FMC57/FMC63/HB135/HB43/HB60); (iv)

FIGURE 14.1

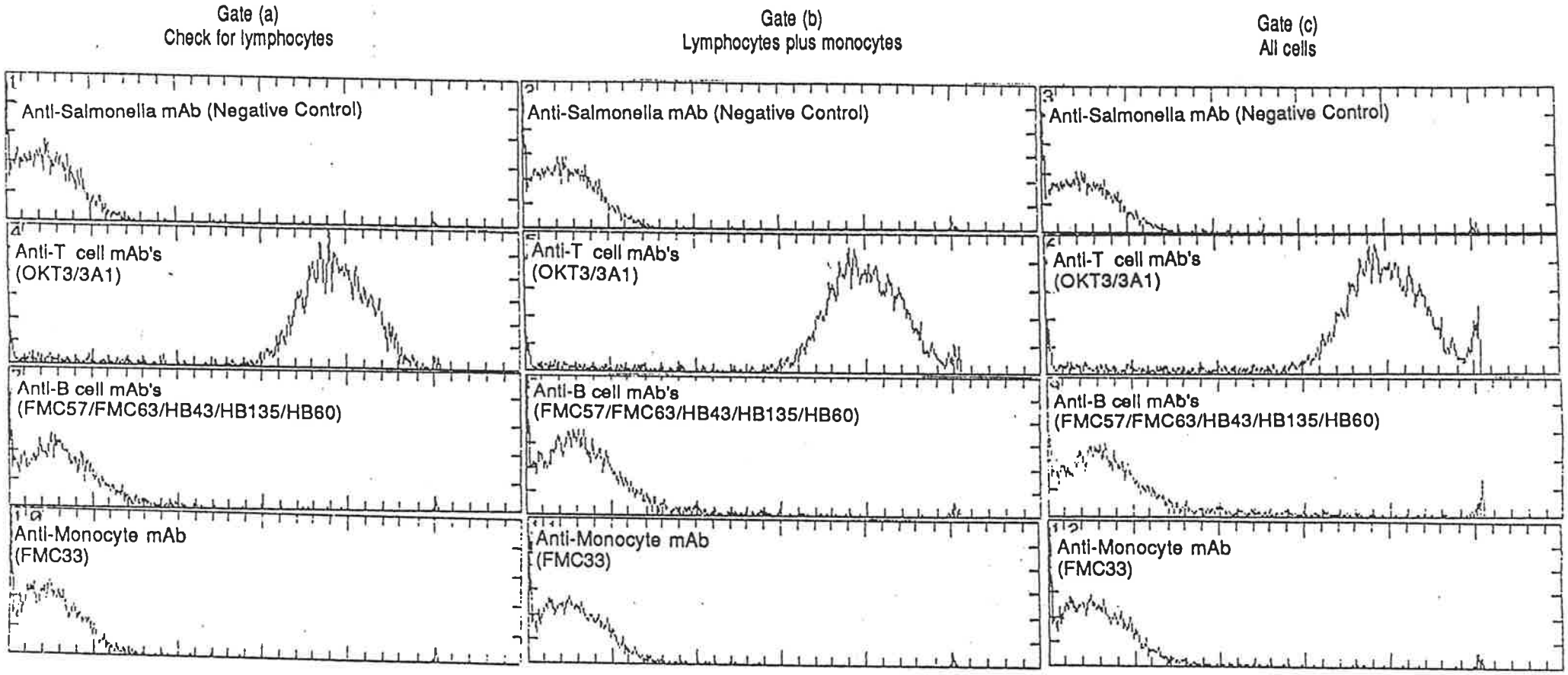


Figure 14.1 (a),(b),(c). Flow cytometry analysis of nonadherent cells fractionated from PBMC. This separation of T-lymphocytes and monocytes was done as a quality control for the cell suspensions used in the interferon gamma experiments.

Monocyte (FMC33).

The mAB are described in Chapter 12. Content and proportions of NAC were analysed by flow cytometry (Epics 750-2, fluorescence activated cell sorter, Coulter Hileah, Flo., USA) using three gatings. The first (a) to examine the lymphocyte population, the second (b) for lymphocytes plus monocytes and the third (c) for all possible cells within the NAC. Figure 14.1 shows the purity of the subpopulations obtained.

Monocyte stimulation. Monocytes were resuspended in RPMI-1640 + 10% FCS + 20mM HEPES at a concentration of $2x10^5$/ml. Depending on the volume, cells were cultured in either 96-well microtitre plates in 200µl/antigen or in 24-well tissue culture plates in 500µl/antigen. The antigens and formats were:

(a) Priscilla Phase I LPS; a water phase from phenol-water extraction (PRIS-PE-W)

(b) Priscilla Phase I Whole Cell, periodate-treated (PRIS-KIO_4)

(c) Henzerling Phase I LPS; a water phase from phenol-water extraction (HENZ-PE-W)

(d) Nine Mile Phase II Whole Cell (NMILE WCII)

(e) Unstimulated (UNST).

PRIS-PE-W and HENZ-PE-W were prepared from 5ml of organisms at 1mg/ml by the phenol extraction described previously in Chapter 13. The resultant water phase was freeze-dried and resuspended into 500µl sterile distilled water. These antigens were added to monocyte cultures to give a final dilution

of one in 10. PRIS-KIO_4 and NMILE WCII were added to monocyte cultures to give a final concentration of 10 and 5µg/ml respectively. Cultures were incubated at 37°C, 5% CO_2. Monocyte conditioned media (MonoCM) was harvested at 24 hours and stored at -70°C.

Determination of lymphoproliferative response. PBMC were treated in either one or other of two ways: (a) as described previously (Chapter 12 and Izzo, *et al* 1988), or (b) cultured for IFNγ induction and then resupplemented with RPMI-1640 (as above, except with 10% v/v FCS) and incubated upto 144 hours, at which time 200µl replicate volumes were transferred to 96-well microtitre plates (Nunclon Delta, Nunc, Denmark), pulsed with 0.4µCi of [^{3}H]thymidine (specific activity, 22Ci/mmol; Amersham International, Sydney Australia), incubated a futher 18 hours and then harvested. Radioactivity incorporated into DNA was counted in a liquid-scintillation counter (Beckman, LS2800, Sydney, Australia). The extent of proliferation was determined by calculating the lymphocyte stimulation index as described previously (Chapter 11 and Izzo *et al.* 1988).

Analysis of augmentation and inhibition of IFN-γ after stimulation with *C.burnetii*. PBMC were examined under various conditions for their ability to produce IFN-γ after stimulation with *C.burnetii* antigens. Initial experiments involved the unsupplemented, total PBMC from vaccinees and these produced rather low levels of IFN-γ on antigen stimulation. To enhance IFNγ production low levels of IL-2 were added to cultures together with the *C.burnetii* antigens.

The IL-2 concentration used was suboptimal for proliferation as determined and illustrated in Chapter 12 and was equal to 1-2U/ml.

In experiments to identify inhibitors of IFN-γ, an assay system comprised PBMC depleted of B cells and enriched for T cells and monocytes by the method described in Chapter 12, using the mAB combination FMC57/HB43/FMC63.

C.burnetii antigens. The strains of *C.burnetii* were those used previously (Chapters 11 and 12). They were (a) two prototype strains: the Phase I Henzerling strain from which the vaccine was prepared and the Phase II Nine Mile strain and (b) the Phase I Priscilla strain, isolated from placentitis in a goat, representative of strains isolated from chronic Q fever endocarditis (Hackstadt 1986).

Interferon-γ Enzyme Immunoassay (EIA). Conditioned media were assayed directly for IFN-γ by antigen capture-EIA using a commercial kit (Commonwealth Serum Laboratories, Melbourne, Australia). The procedure was that prescribed in the manual, with the exception that conditioned media was allowed to incubate at room temperature for 90 min.

Bioassays for total IFN and IFN α and IFNγ. Conditioned media were assayed for total interferon by inhibition of viral cytopathic effect (CPE). The indicator system was the GM2504 cell (Human fibroblast cell line; a gift from Dr. R. Harris, University of South Australia) together with Semliki Forest Virus (SFV).

Cells were seeded in DMEM supplemented with 10% v/v FCS, 1% v/v non-essential amino acids (Sigma, St. Louis, MI), 2mM L-glutamine, 200U penicillin/ml, 40µg gentamicin/ml and 20mM HEPES into a 96-well flat-bottomed microtitre plate (Disposable Products, Adelaide, South Australia) in 100µl volumes (1-2x10^5cells/ml). Plates were incubated overnight at 37°C in a humid atmosphere with 5% v/v CO_2. To each well, 100µl of media was then added after which 100µl of conditioned media was added to duplicate wells and diluted in half-log dilutions. A recombinant IFN-γ of known unitage (a gift from Biogen, Geneva, Switzerland; Batch 10M31) was used as a laboratory standard. Dilutions of the standard were included in each plate to calibrate the inhibition of viral CPE. Infected and non-infected cell controls (i.e., without IFN) were also included on each plate. Plates were incubated overnight as above. Media from each well was removed, the monolayers washed with Phosphate-buffered-saline (pH7.2) and then resupplemented with 150µl DMEM media with reduced serum (1% v/v FCS). Semliki Forest Virus, diluted to 100$TCID_{50}$ ($TCID_{50}$ = a dose producing CPE in 50% of inoculated cultures) in the assay media was added to each well except the noninfected control wells. Plates were incubated for 48-72 hours as above, after which time the cytopathic effect was assessed for each well by crystal violet dye uptake. Interferon titres were expressed as the reciprocal of the highest dilution of the test supernatants which protected 50% of the target cells from viral destruction. Titres were converted into units per ml by comparison with the standard IFNγ from Biogen.

Weissmann's crystal violet stain. For determination of cytopathic effect in the IFN bioassay, cells monolayers were treated with Weissmann's stain, prepared as a stock solution to a total volume of 155 ml with the following reagents: 0.25 gm NaCl (Analytical Reagent, Ajax Chemicals, Aust.); 0.75 gm Crystal Violet (G.T. Gurr Ltd. London, England); 100 ml Distilled Water; 50 ml 96% v/v Ethanol (Analytical Reagent, Ajax Chemicals, Aust.); 5 ml 35% Formaldehyde (Analytical Reagent, Ajax Chemicals, Aust.). The solution was stirred overnight with a magnetic flea over a magnetic stirrer.

Recombinant Human Interferon (rhIFNγ). rhIFNγ was obtained as a gift from Biogen (Geneva, Switzerland, Batch 10M31). A vial contained 0.01 mg of lyophølised powder, equivalent to $1.3x10^6$ units/ml of IFNγ. IFNγ was reconstituted in a 1.0 ml volume of DMEM + 1% FCS, and subsequently diluted 100-fold to obtain a concentration of $1.3x10^4$ units/ml. This preparation was further diluted to give a final concentration of 100 units/ml, which was then divided into 1 ml lots and stored at -70°C until used as a standard in each IFN bioassay run.

rhIFNα_2 was obtained as a gift from the Schering Corporation (Kenilworth, N.J., USA), as a lyophølised powder, product name 'Intron A' (Lot 11891-106/K) which contained $1x10^7$ International Units (IU) of interferonα_2, stabilised with human albumin. The rhIFNα_2 was diluted 100-fold in DMEM + 1% FCS and stored at -70°C at either this concentration or diluted further to 200 IU/ml, in 1.0 ml lots for direct use in the IFN bioassay.

Antibody preparations against interferons. Antibodies against either rhIFN-γ or rhIFNα_2 were obtained from various sources. Polyclonal antibody, prepared in sheep against human IFNα_2 was a gift from the Division of Medical Virology (Institute of Medical and Veterinary Science, Adel., Aust.), and was sufficient to inhibit 10,000 IU of IFNα_2. Polyclonal antibody, prepared in the horse against human IFNα_2 was purchased from Boehringer Mannheim (Sydney, Aust.), neutralised 10,000 units of IFNα_2. Polyclonal antibody, prepared in the rabbit against human IFNγ was purchased from Genzyme (Boston, MA); it neutralised 10,000 units of IFNγ. The specificity of each antibody preparation was confirmed in homologous and heterologous neutralisation assays in the IFN bioassay using Hep-2 cells with Semliki Forest Virus (SFV).

Preparation of Semliki Forest Virus (SFV) stocks. SFV was obtained from Dr Paul Hertzog (Department of Biochemistry, Monash University, Melbourne), where it had been grown in a Hep-2 cell line (Human epidermoid carcinoma tissue). For propagation of SFV, GM2504 or Hep-2 cells were grown in DMEM + 10% FCS + 20mM HEPES + 1% nonessential amino acids (Sigma, St. Louis, MO) to confluency in a 75 cm^2 tissue culture flask (Costar, Cambridge, Mass.).

Monolayers were washed 2x with sterile PBS; pH 7.2, and then 100 $TCID_{50}$ of SFV in DMEM + 1% FCS was added to the culture. Cultures were incubated at 37°C in 5% CO_2 (v/v) atmosphere. Virus growth was monitored daily by inspection of the monolayer for CPE with an inverted microscope (Nikon, Diaphot, Japan). SFV was harvested when the monolayer culture displayed between 90% to 100% CPE. Cell debris was removed by

centrifugation of the culture media at 600xg (LKB Bromma 2161 Midispin R). 1 ml lots were stored at -70°C.

To determine the $TCID_{50}$ of the new batch of SFV, virus was titrated in an assay that was similar to that described for the IFN bioassay. Briefly, GM2504 cells were harvested and 100µl aliquots dispensed into a 96 well microtitre plate (Disposable Products, Adelaide, Aust.). Cells were allowed to reach confluence, after which SFV was added in serial log dilutions from 10^{-1} to 10^{-12}, in DMEM + 1% FCS. Plates were incubated as in the IFN bioassay and the extent of CPE determined by Weissmann's Stain. The $TCID_{50}$ and $100TCID_{50}$ were calculated from the Karber formula for determination of the 50% endpoint:

$LogTCID_{50} = L-d(S-0.5)$ where

L = Negative log of the lowest dilution

d = The difference between log dilution steps

S = Sum of proportion of 'positive' tests,

eg., monolayer cultures showing viral CPE.

Immunofluorescence for peripheral blood mononuclear cell-associated IFNγ. Gelatin-coated glass slides were used. 1% gelatin solution was prepared - 0.25gm of gelatin powder (BDH Chemicals, England) in 25ml DW was boiled until the gelatin had completely solubilized. The solution was cooled slightly and the pH of the solution adjusted to pH 7.4. Slides were individually dipped for 10-15 seconds, so that both sides were coated. The excess gelatin was blotted off and the gelatin on the slides allowed to set by standing in a slide

rack at room temperature (R.T.). Gelatin was fixed to the slides with Formalin vapour, by placing the slide rack into a staining dish containing paper tissue dampened with Formalin and sealing the dish. Slides were fixed overnight at R.T. The slide rack was then removed and the slides aerated in a ventilated hood. Slides were stored in a dry slide box.

Peripheral blood mononuclear cells for immunofluorescence. Peripheral blood mononuclear cells (PBMC) stimulated with Q fever antigen or mitogen were removed from culture and washed 2x in excess sterile saline. Cells were then resuspended in 500µl saline and viable cells counted with 1% Trypan Blue. The volume was then adjusted to a final cell concentration of $5x10^5$/ml in saline. Cell spots were prepared on 1% Gelatin-coated slides mounted into a Cytospin centrifuge (Shandon, Australia) with filter-card and cell-chamber apparatus. 200-250µl of PBMC was added to the cell-chamber, then centrifuged at 500 rpm, 5 min., R.T. Slides were allowed to dry, fixed in acetone (BDH, Analar): methanol (Ajax, Analytical Reagent), 1:1 v/v, (dehydrated with anhydrous Calcium Sulphate) at -70°C for 10 min. and then dried. If immunofluorescence was not performed immediately after fixation, slides were stored in a black box with dessicant at -70°C.

Immunofluorescence procedure. Slides were removed from -70°C storage, and allowed to reach room temperature. Cells were rehydrated with PBS for 10 min., R.T. PBS was then removed and 50µl 20% normal goat serum (NGS) in PBS added to cell spots. Slides were incubated for 30 min., R.T. in a humid

Table 14.1: Details of total interferon, interferon alpha and gamma by bioassay, interferon gamma by EIA, and lymphocyte stimulation assays, pre and postvaccination in 23 subjects in the 'low risk' category (see text) vaccinated with Q fever vaccine (Qvax).

		PREVACCINATION										POSTVACCINATION															
		IFN -EIA (CSL)$			LSI%			BIOASSAY TOTAL IFN				IFN -EIA (CSL)$				LSI%				BIOASSAY TOTAL IFN				BIOASSAY IFN α#				BIOASSAY IFN γ#			
		ANTIGENS			ANTIGENS			ANTIGENS				ANTIGENS				ANTIGENS				ANTIGENS				ANTIGENS				ANTIGENS			
NO.	VACCINATED SUBJECT	QI[c]	QII	QIP	QI	QII	QIP	QI	QII	QIP	DAYS POST VACC	QI[c]	QII	QIP	QIIP	QI	QII	QIP	QIIP	QI	QII	QIP	QIIP	QI	QII	QIP	QIIP	QI	QII	QIP	QIIP
1	SCHOPPE	< 2	< 2	ND	3.22	2.81	ND	< 6	< 6	ND	9	<2	<2	ND	-	1.59	1.52	ND	-	<6	<6	ND	-	<6	<6	ND	-	<6	<6	ND	-
											1216	4	11	4	8	0.58	0.65	0.67	1.50	3	60	5	32	3	10	5	6	3	18	5	6
2	FANNING	< 2	2	ND	0.87	0.69	ND	< 6	6	ND	9	<2	4	ND	-	1.28	1.21	ND	-	<6	126	ND	-	<6	13	ND	-	<6	32	ND	-
3	BROGAN	13	4	ND	0.32	0.91	ND	>13	>13+	ND	12	<2	<2	ND	-	2.56	1.02	ND	-	<5	<5	ND	-	<5	<5	ND	-	<5	<5	ND	-
4	CAMERON	(<2)*	-	ND	-	-	ND	<4*	-	ND	12	<2	2	ND	-	0.95	1.81	ND	-	<5	<4	ND	-	<5	<4	ND	-	<5	<4	ND	-
5	HUFFAM	< 2	<2	ND	0.29	0.59	ND	<4	<4	ND	12	<2	<2	ND	-	1.63	2.16	ND	-	<4	<4	ND	-	<4	<4	ND	-	<4	<4	ND	-
											1317	2	2	2	2	0.63	0.50	0.56	1.88	6	6	13	10	6	6	13	10	6	6	6	6
6	HENDERSON	< 2	<2	ND	2.71	2.18	ND	< 6	< 6	ND	14	<2	91	ND	-	2.22	2.34	ND	-	<4	50	ND	-	<4	<4	ND	-	<4	40	ND	-
7	REINBOTH	< 2	<2	ND	1.14	0.50	ND	<6	<6	ND	14	<2	<2	ND	-	1.15	1.29	ND	-	<6	<6	ND	-	<6	<6	ND	-	<6	<6	ND	-
8	PAPAZOGLOU	ND	<2	ND	0.41	0.86	ND	-	<6	ND	14	<2	5	ND	-	1.00	0.91	ND	-	<6	64	ND	-	<6	13	ND	-	<6	32	ND	-
9	LAGNADO	< 2	<2	ND	0.43	0.57	ND	<4	<4	ND	18	<2	10	ND	-	0.69	0.78	ND	-	<6	80	ND	-	<6	20	ND	-	<6	8	ND	-
10	RAEBURN	<2	<2	ND	0.68	0.86	ND	<6	<6	ND	18	<2	30	ND	-	0.69	1.23	ND	-	<6	>126	ND	-	<6	20	ND	-	<6	20	ND	-
11	GIBSON	<2	<2	ND	0.75	0.77	ND	<6	<6	ND	18	<2	9	ND	-	1.77	2.34	ND	-	<6	>200	ND	-	<6	20	ND	-	<6	80	ND	-
12	KRISHNAN	<2	<2	ND	0.92	1.16	ND	<6	<6	ND	24	<2	<2	ND	-	0.65	1.29	ND	-	<6	<6	ND	-	<6	<6	ND	-	<6	<6	ND	-
13	KUMARATILAKE	<2	<2	ND	0.09	0.17	ND	<6	<6	ND	24	<2	<2	ND	-	1.21	2.41	ND	-	<10	<10	ND	-	<10	<10	ND	-	<10	<10	ND	-
14	QUIN	< 2	<2	<2	0.73	1.64	1.83	<6	<6	<6	32	<2	<2	<2	-	1.33	11.91	1.39	-	<10	<6	<6	-	<10	<10	<6	-	<10	<6	<6	-
											158	<2	<2	<2	6	0.66	0.67	0.74	0.79	-	-	-	-	-	-	-	-	-	-	-	-
15	SKENE	<2	<2	<2	0.36	0.48	0.34	<6	<6	<6	32	<2	2	<2	-	1.11	1.10	0.67	-	<6	<6	8	-	<6	<8	<8	-	<6	<8	<8	-
16	HAYNES	<2	5	<2	0.70	0.80	1.32	<6	>20	<6	40	<2	14	<2	-	4.38	6.57	3.44	-	<8	>200	10	-	<8	10	10	-	<8	126	<6	-
17	WHITE (8/6/89)	< 2	<2	<2	0.56	0.29	1.88	<6	<6	<6	40	<2	<2	<2	-	1.89	3.12	3.40	-	<6	<13	<13	-	<6	<6	<13	-	<6	<6	<13	-
											189	4	4	4	9	0.97	1.13	1.34	-	13	13	13	10	13	13	13	10	13	13	13	10
18	WRIGHT	(10)*	-	-	-	-	ND	20*	-	-	59	2	8	ND	-	1.66	2.00	ND	-	13	13	-	-	6	<4	-	-	5	5	-	-
19	OEHLER	< 2	<2	<2	ND	ND	ND	-	<6	-	72	<2	<2	<2	-	1.97	1.22	6.19	-	<5	<6	<6	-	<5	<6	<6	-	<5	<6	<6	-
												2	-	2	3	0.85	0.84	1.00	1.29	6	-	6	10	6	-	6	10	6	-	-	6
20	JILBERT	<2	<2	2	1.58	1.07	0.39	<6	<6	<6	645	<2	4	<2	-	0.38	1.20	0.35	-	<13	80	<5	-	<13	8	<5	-	<13	26	<5	-
21	JOLLY	(<3)*	-	-	ND	ND	ND	6*	-	-	895	<3	3.5	<3	-	0.04	1.08	1.27	-	6	64	6	-	6	20	6	-	6	20	6	-
22	WORSWICK	(<3)*	-	-	ND	ND	ND	6*	-	-	1690	<3	3.5	<3	-	0.76	0.49	1.17	-	4	64	4	-	4	12	4	-	4	20	4	-
23	LINDSCHAU	(<3)*	-	-	ND	ND	ND	6*	-	-	1610	<3	4	<3	-	0.33	0.67	1.22	-	5	100	5	-	5	16	5	-	5	50	5	-

* Specimens not available: value with post vacc unstimulated cells.
\# Neutralised with polyclonal antibody; horse anti-human-interferon gamma (Boehringer Mannheim) or Sheep anti-human-interferon Alpha-2 (Schering) (see text)
\+ Unstimulated cells also showed a positive reaction (50 units) ?subject had intercurrent infection.
© Final dilution in reaction mixture.
£ QI; Henzerling Phase I antigen, QII; Nine Mile Phase II antigen, QIP; Priscilla Phase I antigen.
$ Picograms 1ml. Cut-off point mean + 4xSD of replicate determination on fluid phase from unstimulated cells.
% LSI = lymphocyte stimulation index. Values above 1.2 are taken as positive.
ND = Not Done

chamber. NGS was removed, and 50µl of primary antibody (rabbit anti-human IFNγ (Genzyme, Boston, MA)) diluted in 1% NGS/PBS at a predetermined optimal dilution was then added. Slides were incubated 120 min., R.T. in a humid chamber. The antibody solution was washed off with PBS, and the slides placed into a slide rack, washed 3x in excess PBS (5 min/wash) and allowed to dry. 50µl FITC-conjugated-secondary antibody (sheep anti-rabbit immunoglobulin, affinity-purified (Selinas, Melbourne, Australia) diluted in 1% NGS/PBS at a predetermined optimal dilution was then added and incubated for 30-45 min., R.T. in a humid chamber. Slides were washed as above, then 2x in excess DW and allowed to dry. For mounting, a solution of p-phenylenediamine (1ng/ml) (Sigma, St Louis, MO)/Glycerol:PBS (pH 8.0), was freshly prepared, spotted onto cell spots and covered with a coverslip. Slides were stored at 4°C, in the dark until read. A fluorescence microscope with phase contrast was used to examine cells. Between 100 and 200 cells per cell population were counted.

14.3 Results

Formation of total IFN and IFNα and IFNγ by vaccinees in a "low risk" group. Table 14.1 sets out the individual data - namely, lymphocyte stimulation indices, and interferon assayed by enzyme immunoassay (IFNγ-EIA) or bioassay - on PBMC from 23 vaccinees in the low risk category.

Interferon estimations were made with conditioned media harvested at 24 hours. The enzyme immunoassay for IFNγ developed by CSL had a lower level of detection of 2 picograms/ml as measured with the recDNA IFNγ from

Table 14.2: Summary of lymphocyte proliferative responses, total interferon and interferon γ responses on *C.burnetii* antigen stimulation of PBMC from 23 vaccinees in 'low risk' group (outside abattoir).

ASSAY	ANTIGENS	PERCENT POSITIVE (N = 23)
LSI	*C.burnetii* antigens, PI and PII	78%
BIOASSAY		
Total IFN	*C.burnetii* antigens, PI and PII	68%
IFNγ	*C.burnetii* antigens, PI and PII	58%
IFNγ - EIA	*C.burnetii* antigens, PI and PII	75%

Biogen. The bioassay with GM 2504 cells had a detection limit of 6 units/ml total IFN.

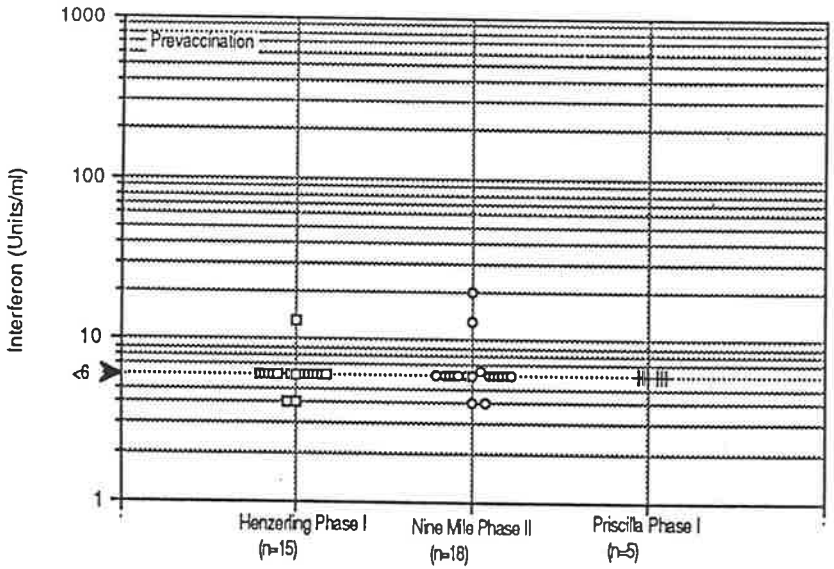
The results are summarised in Table 14.2. The IFNγ-EIA detected IFNγ in conditioned media from 75% of the postvaccination PBMC samples from the inoculated subjects; three subjects were positive in prevaccination samples although their LSI values were below the cut-off value (1.2). Sixty-eight percent of postvaccination samples were positive in the bioassay for total IFN; there was a low correlation ($r=0.16$; $p=\geq 0.45$) between the EIA and bioassay which would be expected as more than one species of interferon are stimulated from PBMC by *C.burnetii* antigens. When the bioassay was made specific for IFNγ; after neutralisation with antiserum to IFNα, there were 58% reactors, all of whom were also positive in the IFNγ-EIA. Some postvaccination specimens had been taken 9-12 days after inoculation and were negative. Resampling of these subjects at a longer period after inoculation showed a conversion, but one subject (Huffam) still remained negative at 1317 days after inoculation.

Seventy-eight percent of vaccinees developed a positive LSI value after vaccination but there was no correlation between the amounts of secreted IFNγ and the size of the LSI value ($r=0.03$; $p<0.25$).

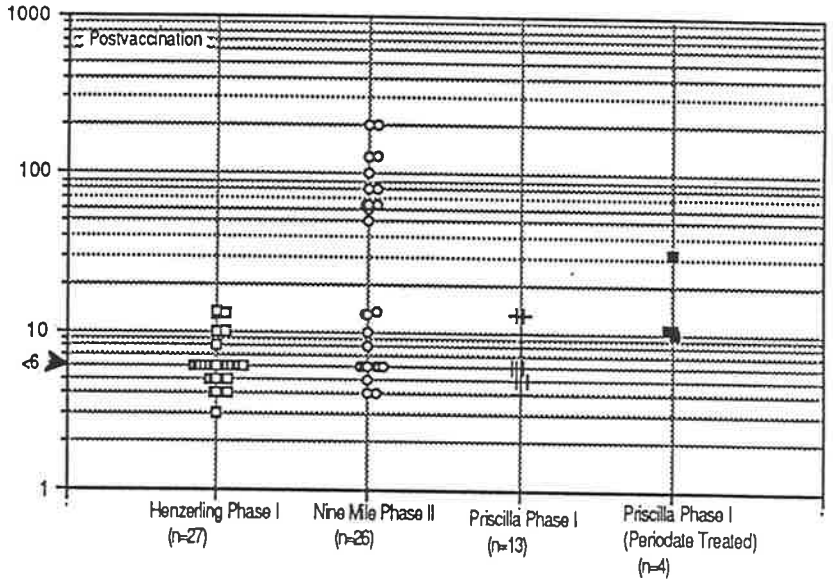
For further analysis the LSI and interferon values obtained, pre- and post v accination, in the group of 'low risk' vaccinees have been set out in a series of scattergrams. A range of Phase I and Phase II antigens was used to stimulate the PBMC.

Figure 14.2 (a) and (b) shows the values for total interferon by bioassay, before (a), and after (b) vaccination. Figure 14.2 (c) shows the distribution of

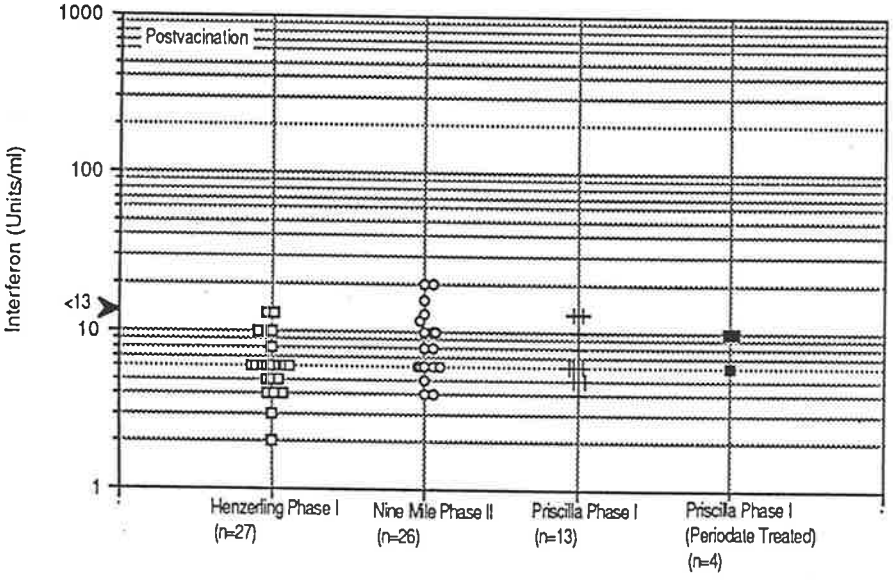
Figure 14.2 (a) (b) (c) (d) (e) and (f). PBMC from vaccinees in 'low risk' group (N~23), pre- and post-vaccination, stimulated with various *C.burnetti* Phase I and II antigens. Conditioned media assayed for total interferon IFNα and IFNγ by bioassay. Panels (a) and (b) pre- and post-vaccination samples, total interferon (units/ml) by bioassay. (c) Bioassay for IFNα, post-vaccination samples. (d) bioassay for IFNγ , post-vaccination samples. Panels (e) and (f) LSI values, pre- and post-vaccination, on same general range of specimens.



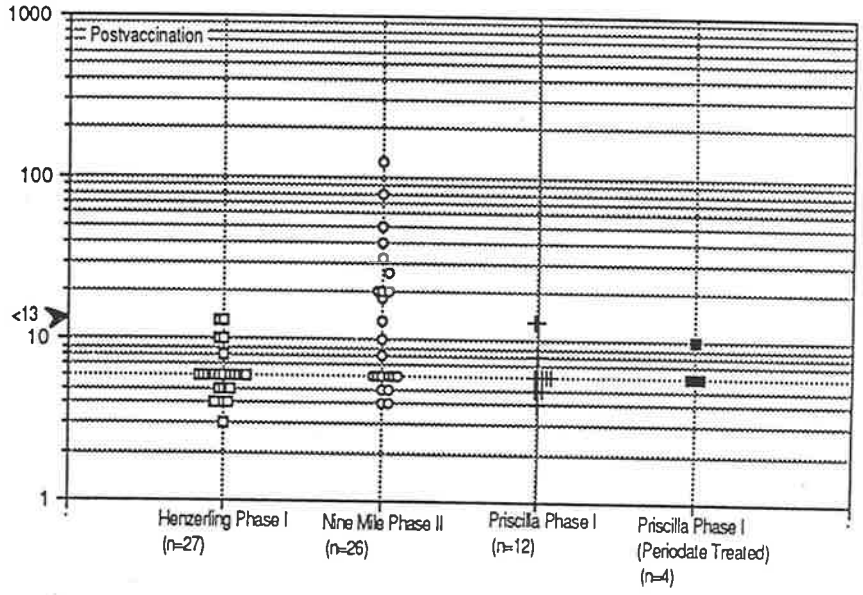
(a) Total interferon (pre-vacc)



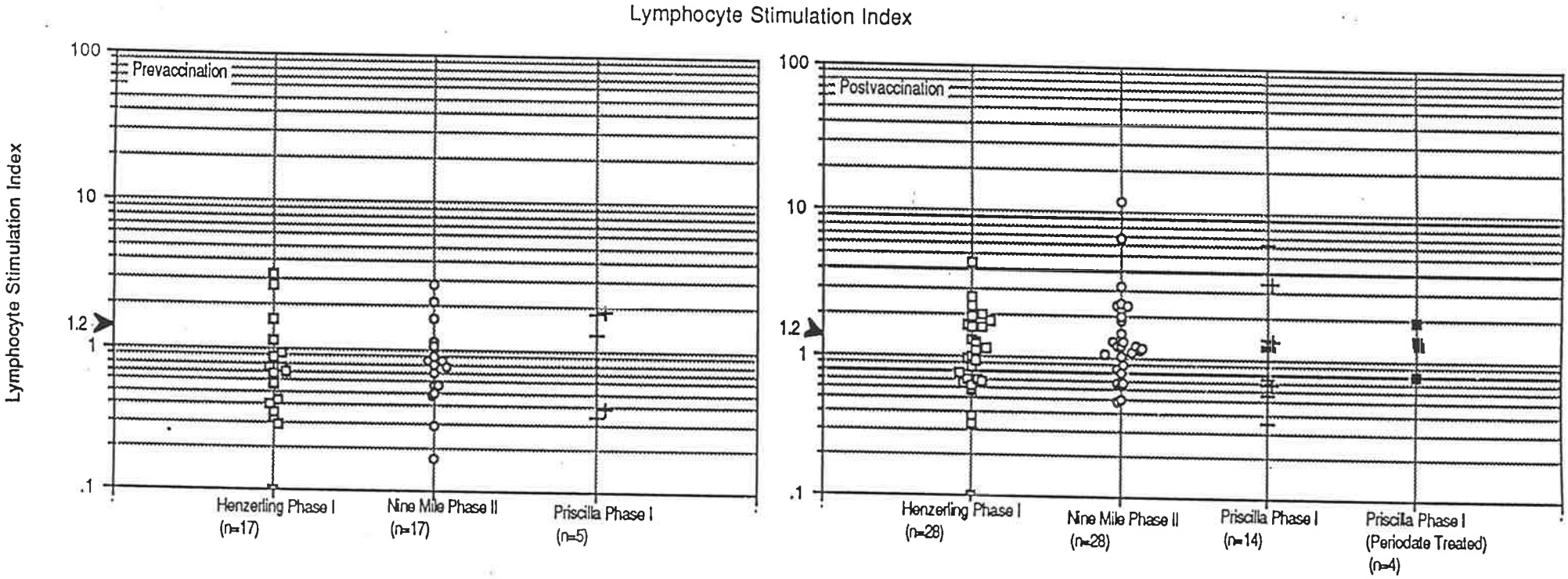
(b) Total Interferon (post-vacc)



(c) IFNα (post-vacc)



(d) IFNγ (post-vacc)



(e) LSI (pre-vacc)

(f) LSI (post-vacc)

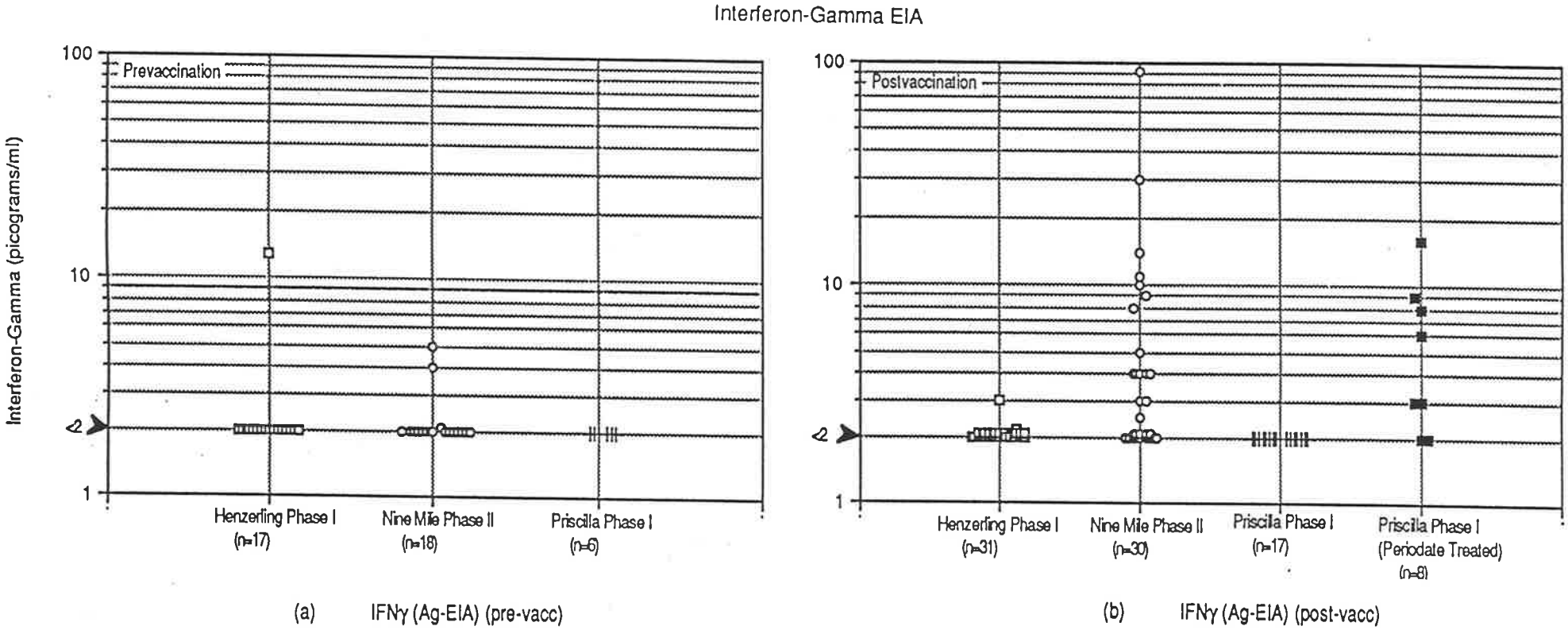


Figure 14.3 (a) and (b). PBMC from vaccinees in 'low risk' group (N~23), pre- and post-vaccination, stimulated with various *C.burnetii* Phase I and II antigens. Conditioned media assayed for IFNγ by Ag-EIA (CSL). (a) pre-vaccination samples, (b) post-vaccination.

values after neutralising the CM from postvaccination samples with anti-IFNγ to make the assay specific for IFNα; similarly (d) shows postvaccination values specific for IFNγ. It will be seen that the most vigorous response, postvaccination, in terms of total interferon, was to Nine Mile Phase II antigen. The Phase I antigens - Henzerling and Priscilla - produced ~~with~~ a poor total IFN, and IFNγ response.

Figure 14.2 (e and f) shows the LSI values, pre and postvaccination, for the same group of subjects although there are small differences in the totals in each of the subgroups as not all samples were tested by all techniques. The pattern of responses is different to that observed for interferon formation by bioassay in that the post vaccination LSI values to Henzerling Phase I and Nine Mile Phase II did not show as marked a differential response between the two antigens as observed for the interferons. In other words mitogenesis was stimulated with Phase I antigens with immune IFN production. A similar, indeed clearer, differential response was observed when the IFNγ was assayed with the CSL IFNγ-EIA. Figure 14.3 (a) and (b) shows that most of the prevaccination samples, with 3 exceptions, were negative (at <2 picograms IFNγ) whereas after vaccination there was a vigorous response to Nine Mile Phase II antigen in which 15 of the 30 samples were positive, whereas 30 or 31 samples were negative with Henzerling Phase I antigen. Although it is likely the protein composition of the Henzerling and Nine Mile antigens are similar (although their LPS are different) it could be argued that nevertheless it was sufficiently different to explain the differential effect between the Henzerling and Nine Mile strains. However differences in protein composition would not

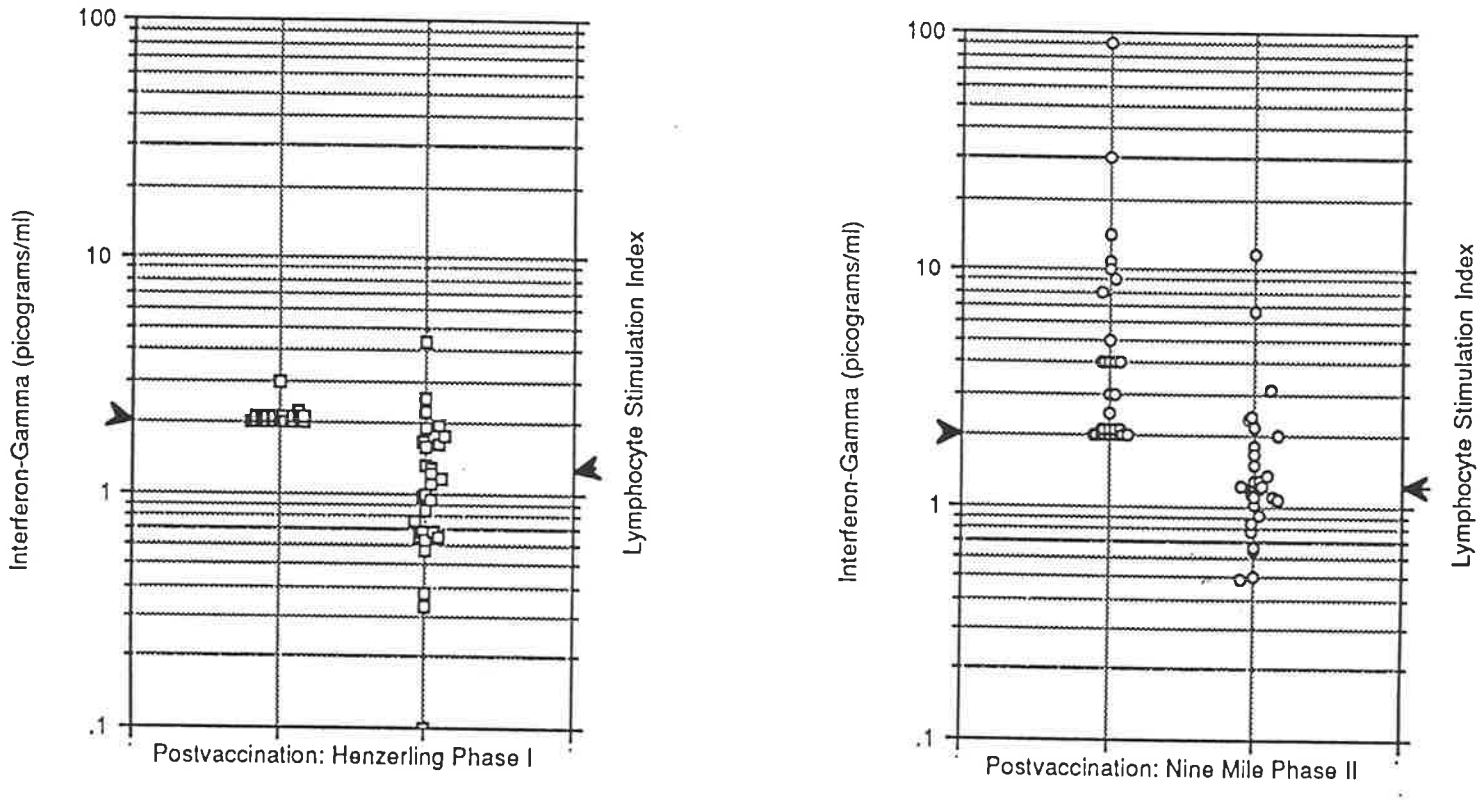
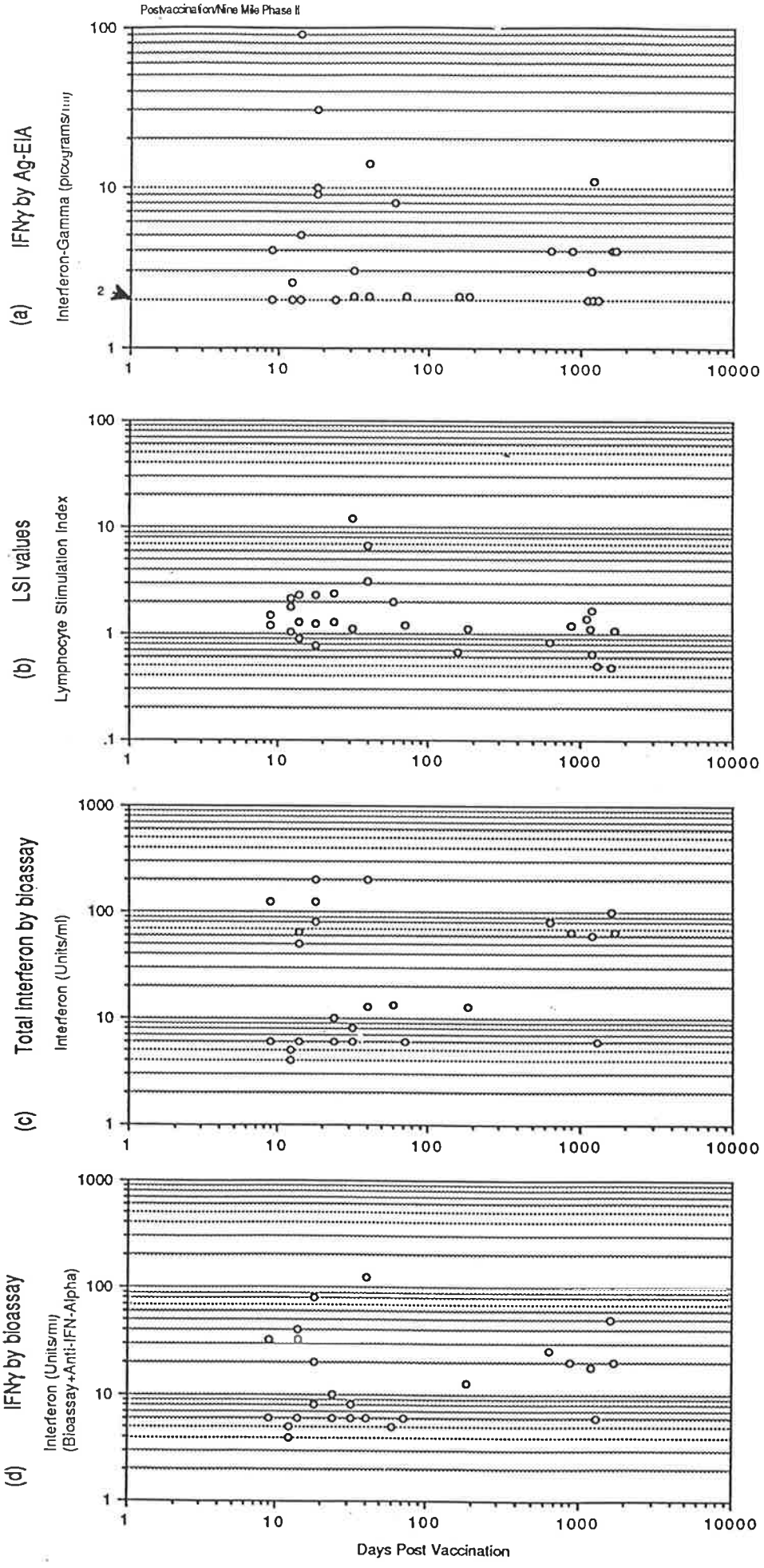


Figure 14.4. Comparison of distribution of LSI values and values for IFNγ, assayed by Ag-EIA (CSL), with PBMC from vaccinees in 'low risk' groups, after stimulation with *C.burnetii* Henzerling Phase I and Nine Mile Phase II antigens.

Figure 14.5. LSI and interferon values at various times after vaccination in the 'low risk' group (N=23). (a) IFNγ by Ag-EIA (CSL), (b) LSI values, (c) total interferon by bioassay, (d) IFNγ by bioassay.


Postvaccination/Nine Mile Phase II
(a) IFNγ by Ag-EIA
Interferon-Gamma (picograms/ml)
100
10
2
1
1
10
100
1000
10000
(b) LSI values
Lymphocyte Stimulation Index
100
10
1
.1
1
10
100
1000
10000
(c) Total Interferon by bioassay
Interferon (Units/ml)
1000
100
10
1
1
10
100
1000
10000
(d) IFNγ by bioassay
Interferon (Units/ml)
(Bioassay+Anti-IFN-Alpha)
1000
100
10
1
1
10
100
1000
10000
Days Post Vaccination

exist between the Priscilla Phase I and periodate treated (artificial Phase II) antigens as the only modification is in the sugar chains of the LPS. Yet the periodate treated Priscilla Phase I antigen gives a good IFNγ response not shown by the corresponding Phase I antigen (Figure 14.3 (b)).

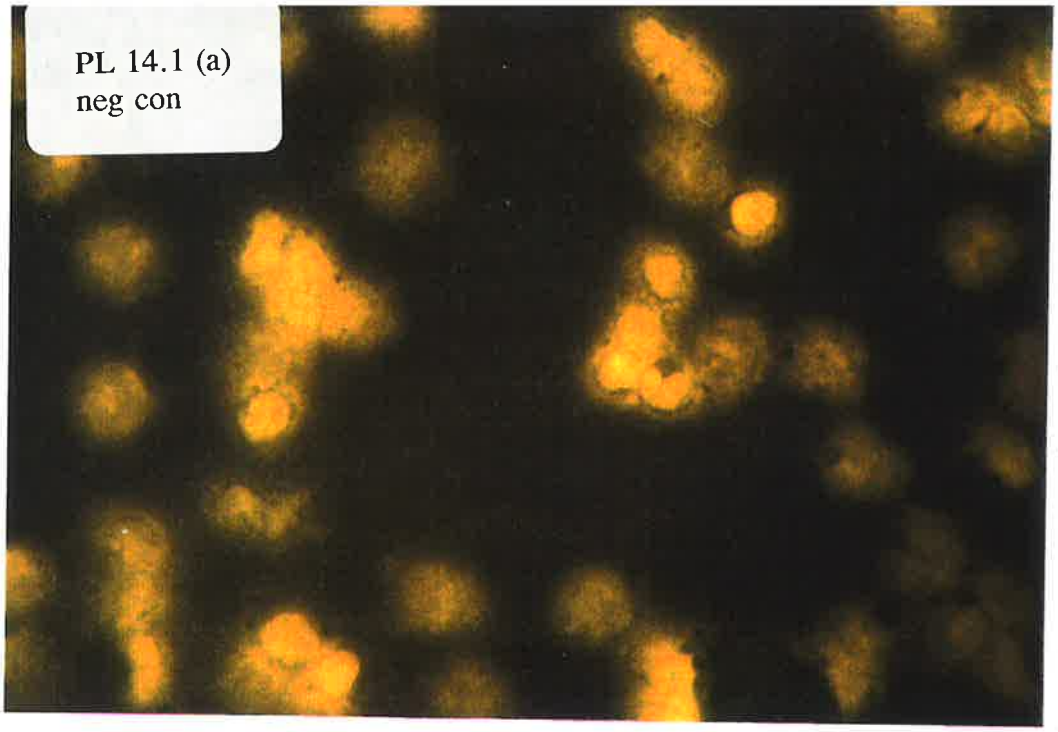
Finally, Figure 14.4 co-plots, for direct comparison, the IFNγ values by Ag-EIA with the LSI values, obtained with Henzerling Phase I antigen and with Nine Mile Phase II. Again it can be seen, as with the comparisons between bioassay and LSI, that although there was a mitogenic response to both Phase I and II antigens, slightly more marked with the latter, there was a major difference in IFNγ response between Phase I and II antigens.

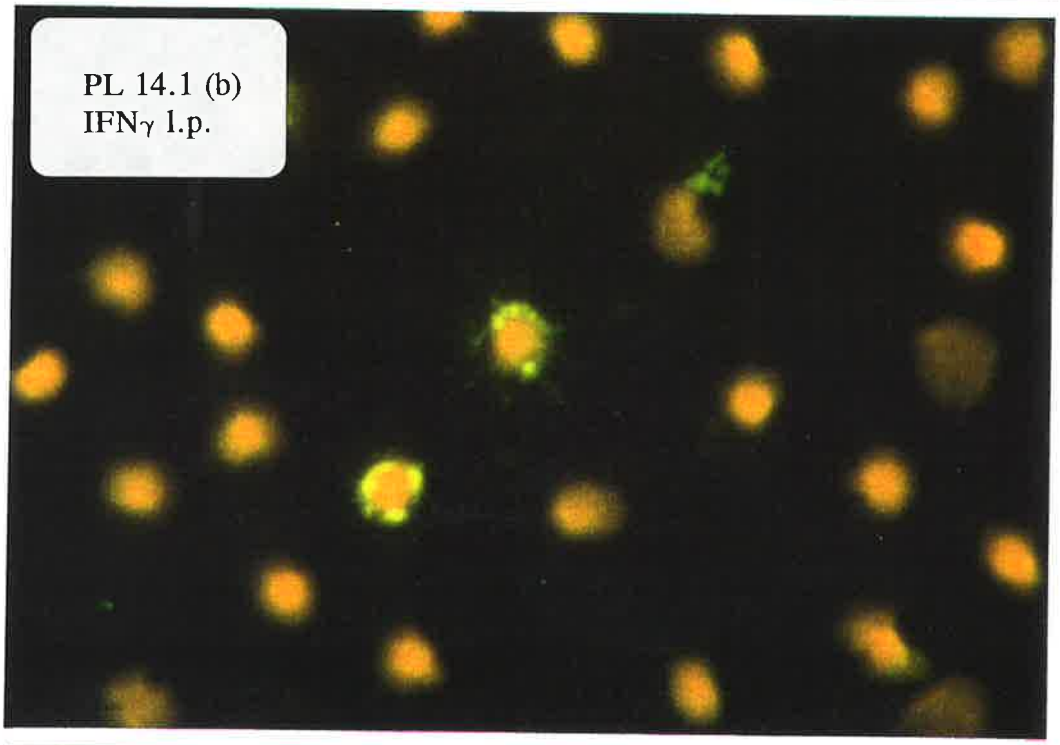
Figure 14.5 (a),(b),(c),(d) illustrates the patterns in the 23 "low-risk" subjects for total interferon and IFNγ and LSI values over time after vaccination. Raised IFNγ values were seen as early as 10-12 days after vaccination and were present at 1000 or more days after vaccination. There was a general similarity between the profiles for IFN and LSI, the higher values were between 10 and 100 days after vaccination with a downward trend later (on 100-1000 days). However, as stated earlier, the correlation between the absolute values in the two assays was low.

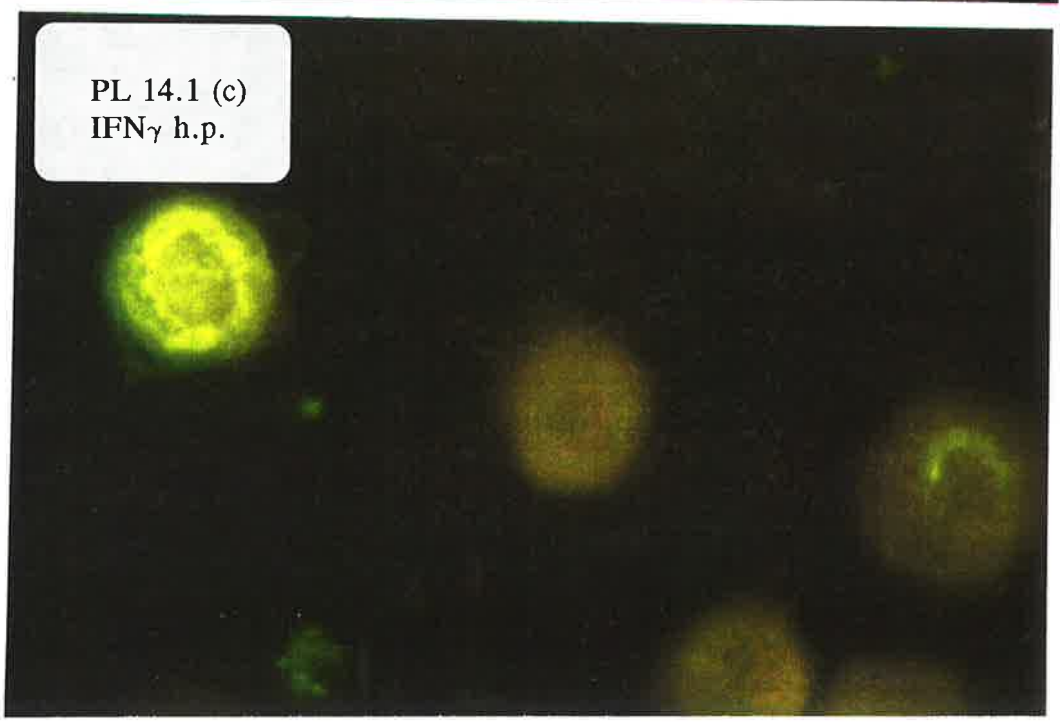
As already noted, 5 of the 23 subjects did not develop the capacity to secrete IFNγ, as assayed by IFNγ-EIA or by bioassay, and the responses to the Phase I antigens was poor.

This failure might be explained as a limitation of sensitivity of the detection methods, or as a failure to secrete interferon into the fluid phase of the cultures or as an inhibition of IFNγ formation in PBMC from some

Plate 14.1 (a), (b), (c). Cell preparations from PBMC of vaccinated subjects stained by immunofluorescence with antiserum to IFNγ. All cell preparations were counterstained with p-phenylenediamine which gives an orange fluorescence which contrasts sharply with the green fluorescence of the fluorescein. (a) negative control, PBMC without antiserum to IFNγ. (b) low power view of cells stained with antiserum to IFNγ. (c) higher power preparation showing details of a strong and weakly staining cell positive of IFNγ.


PL 14.1 (a)
neg con


PL 14.1 (b)
IFNγ l.p.


PL 14.1 (c)
IFNγ h.p.

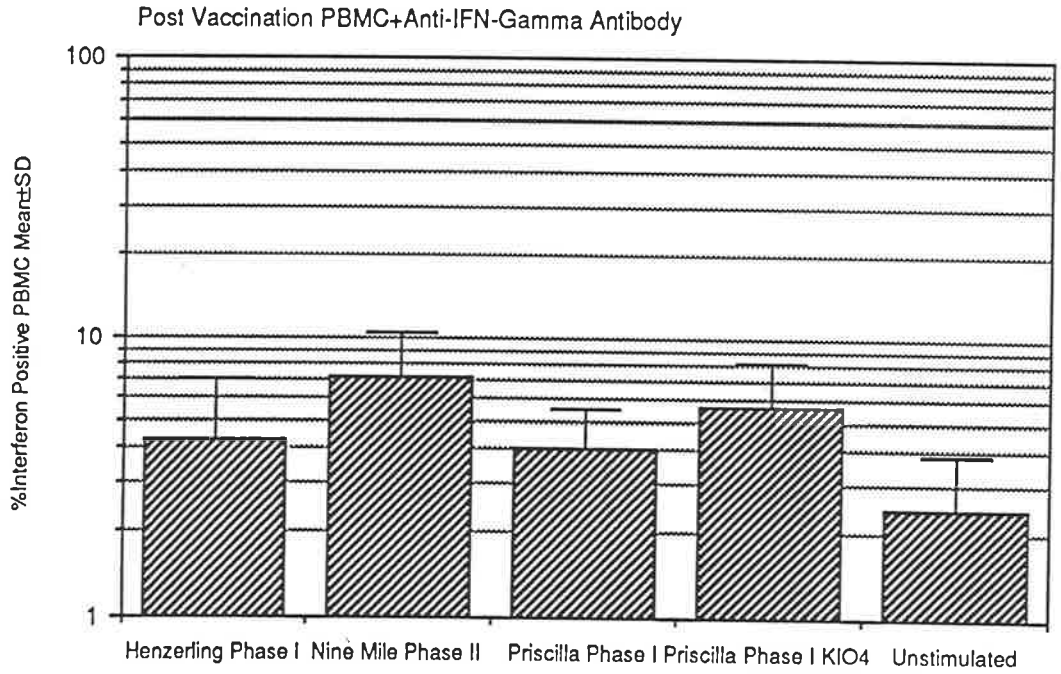


Figure 14.6. PBMC from vaccinees in 'low risk' group who had not responded by formation of detectable IFNγ in Con Med on stimulation with various *C.burnetii* antigens. Histograms show proportion of total staining by IF for intracellular IFNγ (see text for details).

subjects, or with some types of antigens. Accordingly, tests were made for intracellular IFNγ in single cells from the stimulated PBMC. Plate 14.1 (a),(b),(c) illustrates the type of immunofluorescence staining obtained with a polyclonal rabbit anti-human IFNγ (GENZYME, Boston, USA) antiserum. A cytoplasmic or vesicular fluorescence, was observed, perhaps indicating accumulation of the IFNγ in the Golgi apparatus of the cell. The cells are counterstained with p-phenylaminediamine. Figure 14.6 summarises the percentage of positive cells observed in vaccinees whose PBMC were stimulated with various Phase I, periodate treated or II antigens. All those vaccinees who had been negative for extracellular IFNγ had cell bound IFNγ by IF. It may also be noted that, as with assays of extracellular IFNγ, reactions (in this instance expressed as % of cells positively stained) were most frequent with Nine Mile Phase II and Priscilla Phase I periodate-treated antigens, as compared with the two unmodified Phase I antigens.

In light of the apparent inhibition of IFN-γ formation in cultures stimulated with *C.burnetii* Phase I antigens when compared those stimulated with Phase II antigens, it was of interest to see if the addition of IL-2 would enhance the secretion of IFNγ in the test systems in the same manner as it had stimulated responses in the lymphocyte stimulation assays (Chapter 12).

For these experiments PBMC from five vaccinees (SP, AI, RK, SH, JK) were used together with cells from SM, a negative control subject with no serological or CMI evidence of exposure to *C.burnetii*. The vaccinees were those who had not secreted IFNγ in the survey of the 'low risk' group (Table 14.1) and were all negative for IgM or IgG antibody when tested on microdots of

Table 14.3: Effect of a limiting concentration of IL-2 on amounts of IFNγ present in conditioned media from PBMC of 5 previously unresponsive vaccinees. Also on the percentage of cells stained with antiserum to IFNγ by immunofluorescence.

		WITHOUT INTERLEUKIN-2			WITH INTERLEUKIN-2		
STIMULATING ANTIGEN	SUBJECT	IFN-GAMMA EIA (picogm/ml)	LSI	%IFN-POSITIVE CELLS	IFN-GAMMA EIA (picogm/ml)	LSI	%IFN-POSITIVE CELLS
Priscilla Phase I	SP	<3	1.80	4	35	2.34	10
	AI	<3	6.40	8	90	6.89	10
	RK	<3	1.18	4	25	0.46	4
	SH	<2	1.54	3	20	1.38	11
	JK	<2	2.39	5	75	2.71	6
	SM (Neg Con)	<2	0.96	3	<2	0.66	2
Priscilla Phase I ($K10_4$)	SP	16	1.38	6	75	2.73	13
	AI	<3	1.27	6	350	4.51	12
	RK	<3	1.52	6	100	0.78	5
	SH	<2	1.92	3	15	1.55	4
	JK	<2	1.18	4	140	1.37	7
	SM (Neg Con)	<2	0.73	1	<2	0.66	1
Henzerling Phase I	SP	<3	1.24	5	22	1.75	7
	AI	<3	4.15	7	38	5.75	10
	RK	<3	1.27	4	13	0.36	5
	SH	<2	1.74	3	2	1.36	10
	JK	<2	1.07	5	13	2.20	6
	SM (Neg Con)	<2	0.70	2	<2	0.41	2
Nine Mile Phase II	SP	<3	1.66	5	23	4.46	13
	AI	<3	9.13	10	65	15.19	15
	RK	<3	1.11	5	24	0.84	6
	SH	<2	1.65	5	17	1.45	5
	JK	<2	1.37	4	25	2.30	6
	SM (Neg Con)	<2	0.86	2	<2	0.52	1
None (Unstimulated)	SP	<3		3	<3		3
	AI	<3		4	<3		3
	RK	<3		2	<3		3
	SH	<2		2	<2		2
	JK	<2		2	<2		..
	SM (Neg Con)	<2		2	<2		1

coxiella antigen by IF. In contrast to the protocol used in experiments in Chapter 12, IL-2 was used at a limiting concentration (1-2 units) so as to prevent complications from possible prior activation of T cells resulting from unrelated stimuli in the donor of the cells. This stimulation might lead to expression of IL-2 receptors and raised "background" levels of activity in cultures without *C.burnetii* antigens. In the conditioned media from cells with added IL-2 there was a striking increase in the amount of IFNγ detected by the IFNγ-EIA with both Phase I and II antigens (Table 14.3 and Figure 14.7). No change was noted with the negative control. There was also a (somewhat inconsistent) increase in the percentage of cells staining for IFNγ, less than the increase of cell-free interferon (Table 14.3). IL-2 therefore appears to increase secretion of the lymphokine and to overcome the (apparent) inhibition of IFNγ formation with Phase I antigens. LSI values showed some increase but at the limiting dilution of IL-2 used these were less than the type of response illustrated in Chapter 13.

Particular attention is drawn to the striking increase of IFNγ secretion in cells stimulated with Priscilla Phase I periodate treated antigen in the presence of IL-2 (Table 14.3 and Figure 14.7, panel (b)). The difference between this antigen preparation and the Priscilla Phase I WC antigen rests simply in the alteration to the terminal sugars of the LPS. Concentrations of all other antigenic components - e.g., in particular, the cell proteins - should be the same. The change in IFNγ levels induced by the modification of the LPS suggests that the complete Phase I LPS produces an inhibition of lymphoproliferative response and, more strikingly, inhibition of IFNγ

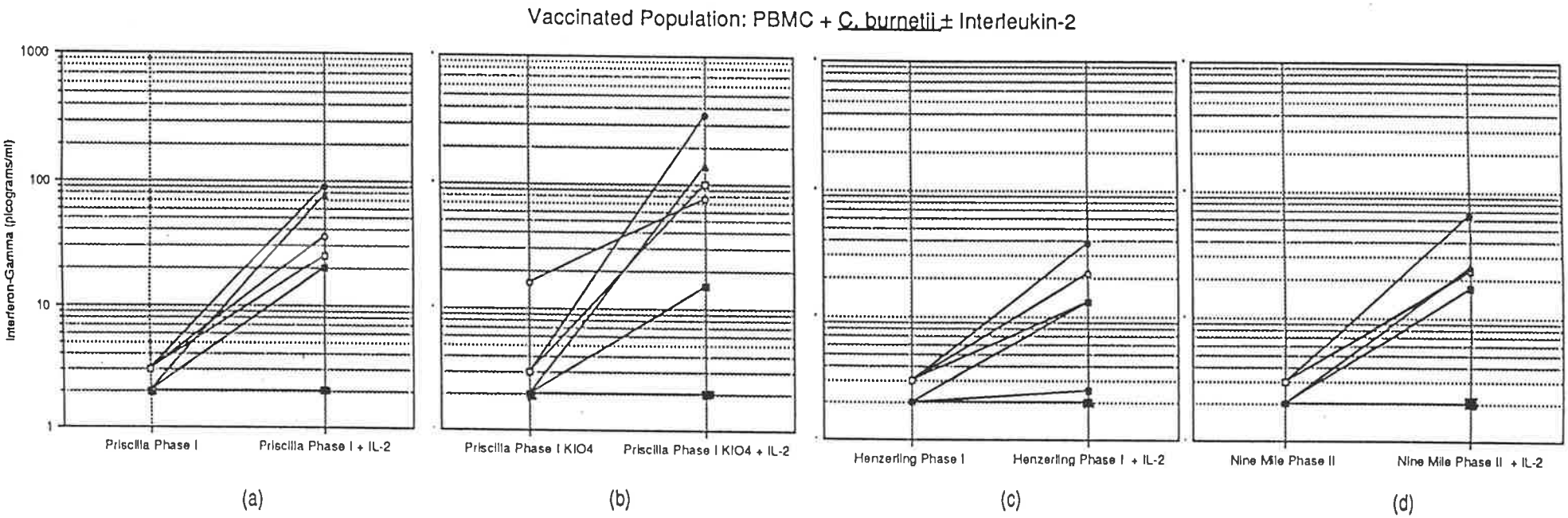


Figure 14.7. Changes in values for IFNγ production or secretion after addition of IL-2 to reaction mixtures. PBMC from 5 vaccinees and one unvaccinated, nonimmune control subject were stimulated with (a) Priscilla Phase I wc antigen, (b) Priscilla Phase I treated with potassium periodate, (c) henzerling Phase I antigen and (d) Nine Mile Phase II antigen. Values for the negative control subject are shown with the larger black square points at the level 2 picograms/ml on the graphs.

production.

It has been established by other workers that LPS can induce, directly or indirectly, the secretion of prostaglandin E_2 from monocytes. PGE_2 is known to inhibit the mitogenic response in T-lymphocytes and to limit the action of IL-2 (see Discussion).

Because of the experimental observations and the published reports further experiments were undertaken on the inhibition or augmentation of IFNγ in response to *C.burnetii* stimulation.

Inhibition or enhancement of IFNγ responses on antigen stimulation of immune T-lymphocytes with *C.burnetii* antigens. In the light of data just presented, namely, that PBMC or T-lymphocytes from vaccinated subjects, or those infected in the past, show a lymphoproliferative response to *C.burnetii* Phase I antigen, but only low levels of cell-free IFNγ and that these levels were enhanced by addition of IL-2 to the reaction mixtures, it was suspected that interactions between Phase I LPS and monocyte or T-lymphocyte induced an inhibitor for IFNγ formation by T-lymphocytes. Indeed Koster *et al.* (1985b) in an investigation of T-lymphocyte "anergy" (= diminished lymphoproliferative response), in subjects with chronic Q fever endocarditis, concluded that T-lymphocyte mitogenic responses were inhibited by a prostaglandin-mediated inhibition following an interaction of *C.burnetii* antigen with CD8+ lymphocytes and liberation of PGE_2 from monocytes.

This pointer relating to lymphoproliferative response was followed up for IFN-γ responses in the experiments now described. PBMC or T-lymphocytes

Table 14.4: Levels of IFNγ detected in conditioned media from monocytes and T cells from 3 vaccinees (AI, BPM, SP) stimulated with *C.burnetii*, Priscilla strain Phase I antigen, with various additives in the reaction mixtures.

		PICOGRAMS/ML OF IFNγ IN CONDITIONED MEDIA FROM VACCINEES		
INDICATOR SYSTEM	**SUPPLEMENT ADDED TO INDICATOR ASSAY**	**AI**	**BPM**	**SP**
Priscilla PhI antigen + Monocytes and T cells	None	3.5	4.5	35
	IL-2	230	18	300
	IL-2+Piroxicam (50μM)	350	17	425
	IL-2+PGE2 (10)	9.5	2	170
	(100)	55	NT	210
	(1000)	110	NT	240
	Autologous Con. Med. from Monocytes + Priscilla PhI LPS + IL-2 (10)	35.5	10.5	215
	Autologous Con. Med. from Monocytes + Henzerling PhI LPS + IL-2 (10)	55	13.5	NT
Monocytes and T cells, but no antigen	None	<2	<2	<2
Monocytes and T cells, but no Priscilla PhI antigen	IL-2	<2	<2	<2

(10) = dilution of one in ten etc. of specified supplement NT = not tested

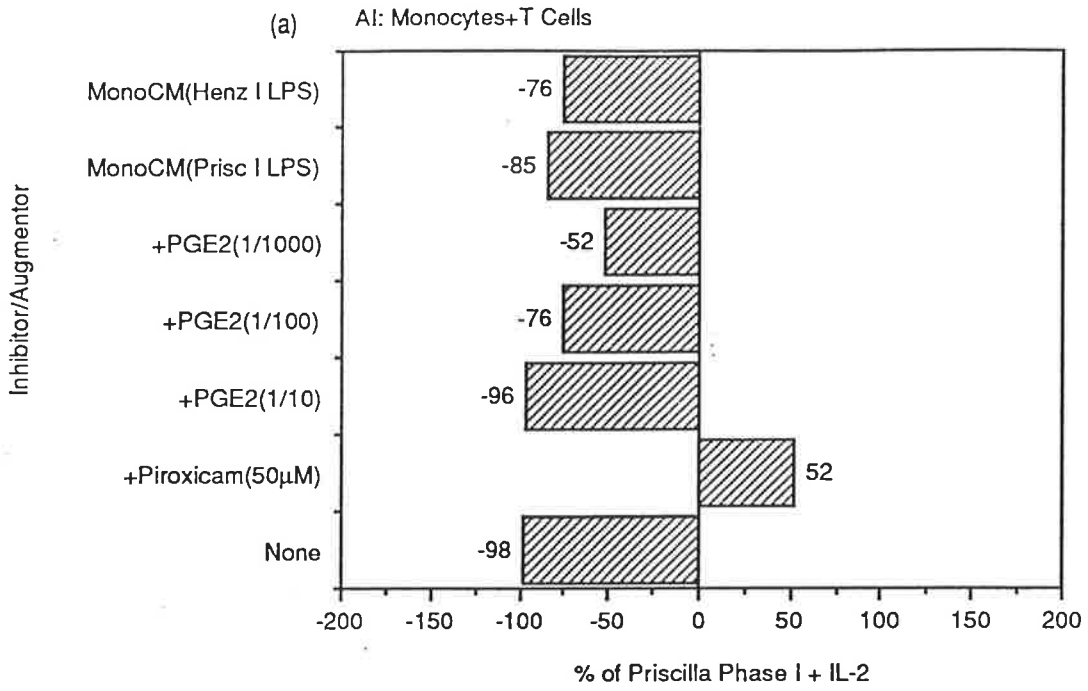
plus monocytes were examined from 3 vaccinees and two subjects with past infections. The only consistent patterns were obtained with the 3 vaccinees - AI, BPM and SP. T cell-monocyte combinations from the two past infection subjects showed either pre-existing activation or a tight suppression which confused experimental manipulation.

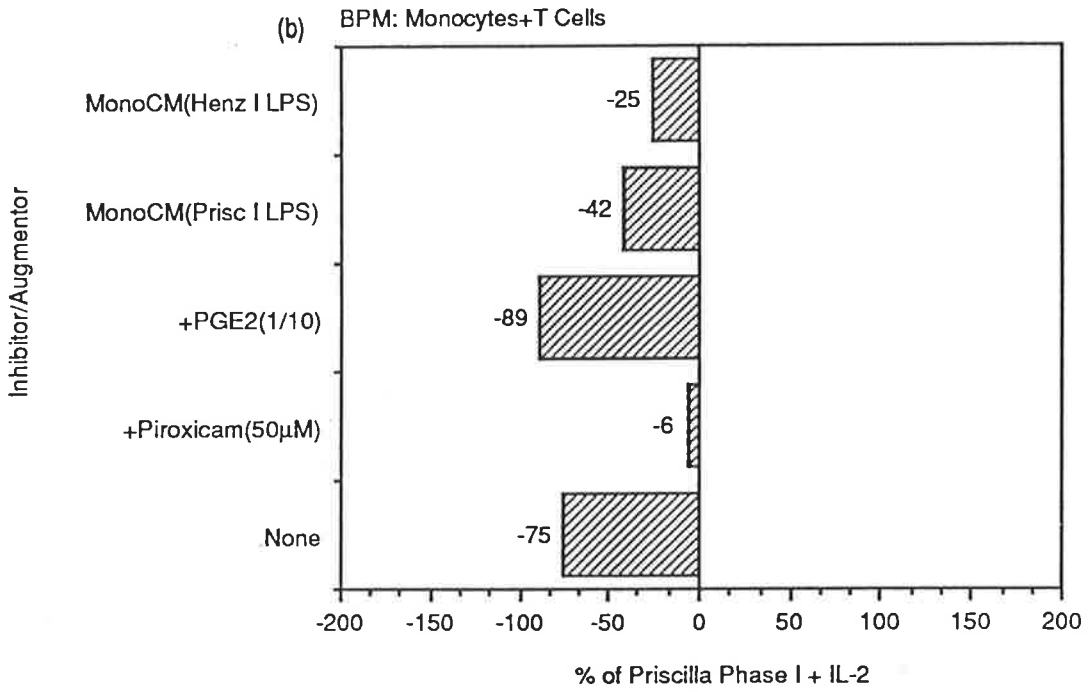
Table 14.4 summarises the amounts (picograms/ml) of IFNγ found in conditioned media (Con Med) from the 3 vaccinees when various supplements were added to the reaction mixture of an indicator system consisting of Priscilla Phase I antigen, monocytes and T cells. The results given are mean values from two experimental runs as there was some variation between results from the same subject in different experiments.

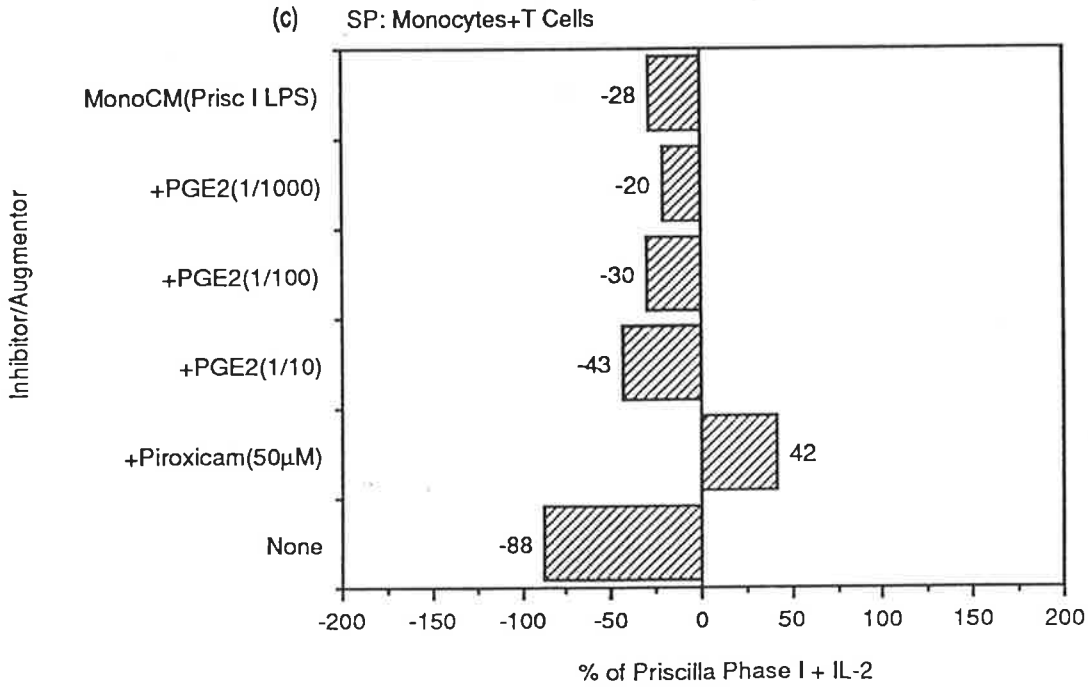
It will be seen that modest levels of IFNγ were found in Con Med from the indicator system from each subject, without added IL-2. The maximum value - 35 picograms/ml - was obtained with cells from SP.

The addition of IL-2, as illustrated previously, produced a striking increase in the amount of IFNγ present. The addition of 50μM of Piroxicam, (Sigma), an inhibitor of prostaglandin cycloxygenase or PGE_2 synthetase further increased the amount of IFNγ in Con Med from AI and SP, but not BPM. Addition of a one in 10 to one in a 1000 dilution of PGE_2 produced a dose-related reduction in IFNγ secretion. Addition of a one in ten dilution Con Med from autologous monocytes stimulated with LPS (i.e., water phase of phenol-water extract of *C.burnetii*) from Priscilla Phase I or Henzerling Phase I *C.burnetii* also reduced the secretion of IFNγ to an amount approximately equivalent to one in 100 prostaglandin dilution.

Figure 14.8 (a), (b), (c). Production/secretion of IFNγ from mixtures of monocytes and T cells from 3 vaccinees, stimulated with *C.burnetii* Priscilla Phase I wc antigen in presence of various supplements (PGE_2, Piroxicam, conditioned media from monocytes exposed to Phase I LPS from Priscilla or Henzerling Phase I antigen). Results are expressed as a proportion (%) of the IFNγ value obtained with autologous monocytes, T cells stimulated with Priscilla wc Phase I antigen, with added IL-2.


(a)
AI: Monocytes+T Cells
Inhibitor/Augmentor
MonoCM(Henz I LPS)
MonoCM(Prisc I LPS)
+PGE2(1/1000)
+PGE2(1/100)
+PGE2(1/10)
+Piroxicam(50μM)
None
-76
-85
-52
-76
-96
52
-98
-200
-150
-100
-50
0
50
100
150
200
% of Priscilla Phase I + IL-2


(b)
BPM: Monocytes+T Cells
Inhibitor/Augmentor
MonoCM(Henz I LPS)
MonoCM(Prisc I LPS)
+PGE2(1/10)
+Piroxicam(50μM)
None
-25
-42
-89
-6
-75
-200
-150
-100
-50
0
50
100
150
200
% of Priscilla Phase I + IL-2


(c)
SP: Monocytes+T Cells
Inhibitor/Augmentor
MonoCM(Prisc I LPS)
+PGE2(1/1000)
+PGE2(1/100)
+PGE2(1/10)
+Piroxicam(50μM)
None
-28
-20
-30
-43
42
-88
-200
-150
-100
-50
0
50
100
150
200
% of Priscilla Phase I + IL-2

However direct estimations of PGE_2 from the Con Med of the monocytes stimulated with Phase I LPS from Priscilla or Henzerling strains did not show elevated levels. Either the assay was not sensitive enough or the inhibitory factors elaborated by the monocytes are perhaps prostaglandin-like mediators other than PGE_2 - the matter is not resolved and is under further investigation.

It is concluded at this stage that direct stimulation of monocytes with *C.burnetii* LPS does not provoke monocytes to produce PGE_2 through the LPS receptor (CD14) although an (unidentified) inhibitor of IFNγ is produced. It is possible that a combination of monocytes and separated CD8 T-lymphocytes, as used by Koster *et al.* (1985a) might have produced PGE_2 but this experiment remains to be done.

The results in Table 14.4 are expressed graphically in Figure 14.8 (a),(b),(c) by relating the degree of enhancement or inhibition in the various reaction mixtures to the value obtained with Priscilla Phase I WC antigen, monocytes, T cells and added IL-2. Calculations were made according to the formula

$$\% \text{ Inhibition (or enhancement)} = \frac{\text{IFN-}\gamma \text{ treated} - \text{IFN-}\gamma \text{ untreated}}{\text{IFN-}\gamma \text{ untreated}} \times 100$$

It will be seen that when depicted in this fashion, the pattern of reactions is consistent - Phase I LPS conditioned media reduced the production or secretion of IFNγ as did PGE_2, whereas Piroxicam reversed the process.

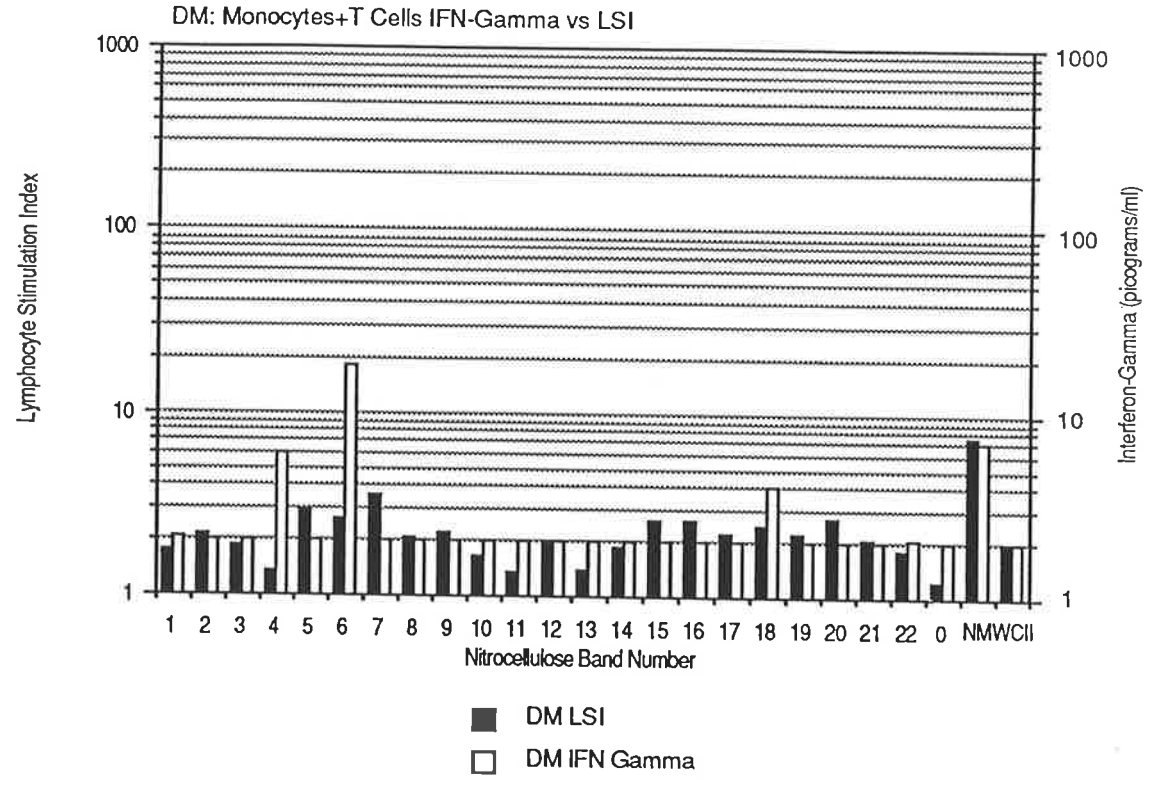
Table 14.5: IFNγ (picograms/ml) from CD4+ and CD8+ T-lymphocytes after stimulation with various *C.burnetii* antigens, cells taken from past infection subjects: WM, KD, JD.

T-cell/antigen mixtures	Picograms IFNγ/ml in conditioned media from cells of: WM	KD	JD
Mo+CD4+, Priscilla Phase I	4	3	3
Mo+CD4+, Henzerling Phase I	11	3	3
Mo+CD4+, Nine Mile Phase II	80	60	7
Mo+CD4+, Yolk Sac	<3	<3	NT
Mo+CD8+, Priscilla Phase I	<3	<3	<3
Mo+CD8+, Henzerling Phase I	7	6	<3
Mo+CD8+, Nine Mile Phase II	80	60	<3
Mo+CD8+, Yolk Sac	<3	<3	<3
Mo+CD4+, no antigen	<3	5	NT
Mo+CD8+, no antigen	<3	5	NT
Mo+CD4+,CD8+, no antigen	NT	5	<3
CD4+,CD8+, no antigen	NT	<3	NT

NT = not tested

Production of IFNγ from CD4+ and CD8+ lymphocytes. In Chapter 13 it was reported that both separated CD4+ and CD8+ positive T-lymphocytes from two subjects infected with Q fever in the past showed mitogenic responses to *C.burnetii* antigen. Because of the uncoupling of mitogenic response and IFNγ formation observed with Phase I antigens, just described, the stored conditioned media from the experiments with CD4+ and CD8+ from past infection subjects KD and JD was examined for IFNγ along with that from WM, a subject also infected with Q fever in the past (Table 14.5). It will be seen that CD4+ positive lymphocytes from all three subjects formed IFNγ when stimulated by Nine Mile Phase II antigen. The CD4+T cells responded poorly or not at all when stimulated with the two Phase I antigens. Similar differential patterns to Phase I and Phase II antigens were shown by the CD8+ T cells. Note that JD, who was a poor IFNγ producer, had shown a positive lymphoproliferative response (Chapter 13).

Stimulation of interferon γ secretion by separated proteins from *C.burnetii*. In Chapter 13 it was reported that most of the proteins separated by PAGE from Nine Mile Phase II whole cell lysates reacted positively in the lymphocyte stimulation assay. Some values were higher than others but none of the separated proteins gave indices approaching those obtained with the unfractionated lysate from whole cells. It remained possible that there might be a difference between the ability of individual proteins to provoke secretion of IFNγ on the one hand and a lymphoproliferative response on the other. Figure 14.9 coplots the IFNγ and LSI values in two subjects infected with Q



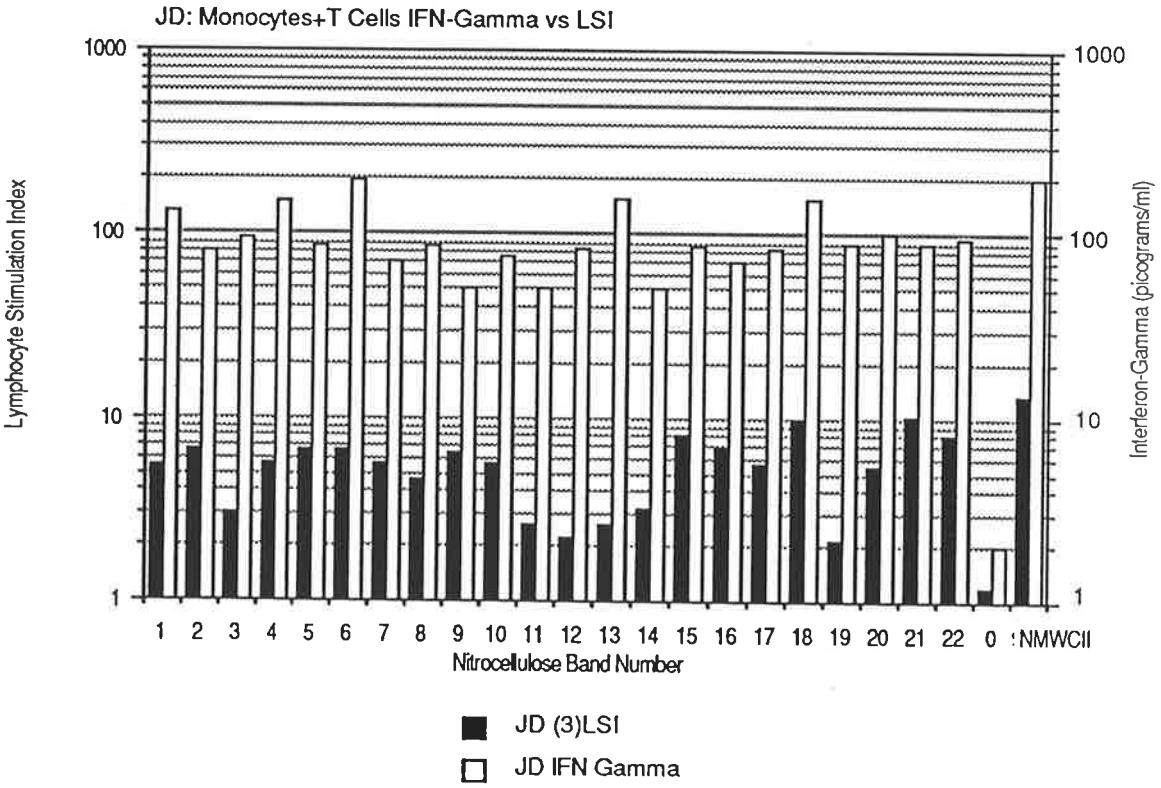


Figure 14.9. Co-plot of values for lymphocyte stimulation indices and IFNγ formed on stimulating monocytes and T lymphocytes from two subjects (DM and JD) infected in the past with Q fever, with the PAGE-separated bands of proteins from a lysate of Nine Mile Phase II *C.burnetii*.

fever in the past. It will be seen that most protein bands stimulated IFNγ; and that the responses were vigorous. There was some indication of greater IFNγ formation from proteins around 70-80 KDa and 28 KDa.

14.4 Discussion

The following conclusions may be drawn from the experiments described in this chapter.

Inoculation of subjects with the inactivated Q fever vaccine (Qvax) induces a T-lymphocyte sensitisation which leads to the generation of IFNγ when PBMC from the vaccinees are stimulated with *C.burnetii* antigens.

As outlined earlier, a principal objective of the experiments described in this chapter was to ascertain whether the T-lymphocytes sensitised by immunisation were those producing IFNγ and not other lymphokines stimulating a B-lymphocyte response and, from analogy with mouse models, likely to produce disease-enhancement rather than resistance. The results obtained from the examination of PBMC from a substantial number of vaccinees indicates that the Q fever vaccine produces these required effects; a result which matches its high protective efficacy against natural infection in the field.

The particular subsets (CD4+ or CD8+) of T-lymphocytes producing IFNγ in reactions with cells from vaccinees was not ascertained directly. However, subfractionated T-lymphocytes from 3 subjects infected with Q fever in the past showed that both CD4+ and CD8+ T-lymphocytes produce IFNγ on stimulation with *C.burnetii* antigens. Overall, it is reasonable to conclude that

both CD4+ and CD8+ T-lymphocytes from vaccinees would behave in a similar fashion but formal experiments to confirm the point would be useful.

The second point to emerge from the experiments was that the majority if not all of the proteins of *C.burnetii* have epitopes which stimulate T cells, thereby producing a proliferative response and IFNγ release into the conditioned media.

The stimulus (mitogenesis: IFNγ production) provided by lysate from the whole cell (WC) preparation of Nine Mile Phase II antigen exceeded that of its component proteins providing support for the notion that a whole cell vaccine is likely to prove of higher protective efficacy in terms of cell-mediated immune response than a "split vaccine" made from one or a number of individual proteins. This point is to be investigated experimentally (see General Discussion Chapter 16).

A third, most interesting conclusion, from the experiments is that T-lymphocytes from immune subjects show a poorer lymphoproliferative response and less IFNγ production when stimulated with Phase I than with Phase II antigens. This trend, in terms of LSI responses, has been evident in the Results described in previous chapters but the pattern with IFNγ is clearer and more striking.

It is presumed, according to current theory, that the killed *C.burnetii* antigens in the reaction mixtures would be taken up by monocytes, degraded and their peptides presented to T-lymphocytes along with Class II MHC antigens (and, it would appear, with Class I MHC antigens). That is, the LPS Phase I antigen would be separated from the *C.burnetii* proteins. Why then is

there a difference between lymphoproliferative and IFNγ responses to the Phase I and II antigens? One possible explanation is that the protein composition of Phase I and II antigens is different. However, most workers (e.g., Hackstadt *et al.* 1985) consider that there are at most only minor differences, although it might be argued that the comparison - in the present experiments - of Henzerling Phase I antigen and Nine Mile Phase II antigen - two different strains - might introduce minor strain related differences in cell proteins. However, the differential effects on mitogenic response and IFNγ formation obtained with Priscilla Phase I antigen and the periodate treated antigen (Priscilla K104) derived from it would not be complicated by differences in protein composition as the action of the periodate is limited to modifying some sugars in the side chains of the LPS. Moreover, as shown, all proteins, not just a few immunodominant proteins, extracted from the *C.burnetii* cell produce an IFNγ response. Thus minor differences in protein compositions between strains are unlikely to be significant.

As an alternative explanation for the differences between reactions with Phase I and Phase II antigens it is postulated that the complete Phase I LPS antigen has an inhibitory action on lymphocyte mitogenesis and interferon γ production. This appears to be offset by the addition of IL-2 to the reaction mixtures. The inhibition of IFNγ formation by Phase I LPS is apparently mediated by a limitation of endogenously-generated IL-2 in the system. The addition of Piroxicam, an inhibitor of prostaglandin synthesis, further offsets the inhibition by Phase I LPS whereas addition of PGE_2 to the system produces a similar inhibition to that of LPS. Furthermore, conditioned media from

monocytes stimulated with Phase I LPS from Henzerling or Priscilla Phase I strains contains an inhibitory factor (not finally identified as PGE_2) which down regulates IFNγ production by immune T-lymphocytes.

The implications of the action of Phase I LPS in down-regulating IFNγ production, both for the virulence of the organism and for the rôle of antibody to the Phase I LPS in producing protection (or disease modification), are considered in the General Discussion, Chapter 16.

CHAPTER 15

IMMUNOSUPPRESSIVE AND IMMUNOSTIMULATING EFFECTS OF WHOLE CELL Q FEVER VACCINE IN MICE

15.1 Introduction

Immunomodulation by Q fever infection, vaccines and coxiella cell fractions. Kishimoto and Gonder (1979) demonstrated suppression of *in vitro* lymphocyte responses during acute infection of cynomologous monkeys with *C.burnetii*, Henzerling strain. There was a significant reduction in the lymphoproliferative response of peripheral lymphocytes by PHA, during the early convalescence from Q fever, even with normal lymphocyte counts. In contrast, the efficiency of CMI, as measured by macrophage migration-inhibition assays during the acute and convalescent stages of infection, was unaffected. The antibody response, as an indicator of normal B cell function, was also intact in the animals as judged by assays of antibody by microagglutination, immunofluorescence and complement fixation. The suppression of blastogenesis occurred only after an acute Q fever infection and not after vaccination with killed *C.burnetii* Phase I whole cells. Kishimoto and Burger (1977) had previously shown that PBMC from guinea pigs vaccinated with a killed Phase I whole cell vaccine had, if anything, increased stimulation indices when cultured with PHA.

Immunological and biological comparisons in laboratory animals between whole cell and chloroform-methanol extracted preparations of *Coxiella burnetii* Phase I organisms. As part of a study to improve Q fever vaccines and remove reactogenicity, Damrow *et al.* (1981) postulated that the major side effects associated with the Q fever whole cell (WC) vaccine were the result of immunosuppression induced by the inoculum. C57BL/10ScN mice were used as a model for testing humoral and cell-mediated responses. Administration of substantial doses of inactivated *C.burnetii* - in fact well in excess of those used in vaccine regimens - resulted in a severe depression of the *in vitro* lymphocyte transformation (mitogenic) response to various panmitogens. It was believed the immunosuppression associated with the inoculation of *C.burnetii* whole cells was mediated via the macrophage.

The same group (Williams and Cantrell, 1982) extended these observations, in C57BL/10ScN endotoxin nonresponder mice, and compared the effects of various concentrations of formalin-inactivated WC from *C.burnetii* Ohio strain, Phase I, with those of a chloroform-methanol extracted cell residue (CM-R) and extract (CM-E) from the Ohio strain. The pathological effects of the killed coxiella WC cells in mice included splenomegaly and hepatomegaly with necrosis and granulomatous inflammation. These effects were similar to those observed with viable *C.burnetii*. Such features were not present in mice given CM-R or CM-E. There were also marked increases in total spleen cells (i.e., in spleen weights) and in the blastogenic activity of splenocytes harvested from mice given 100 and 300µg doses of killed WC vaccine, whereas no increase in spleen weight or blastogenic responses was observed with 100 or 1000µg of

CM-R. Substantial proportion of mice given 1200µg of killed WC died, with a clear dose-response relationship; this did not occur in mice given an equivalent doses of CM-R. Lymphoproliferation studies showed that WC vaccines suppressed the mitogenic response of PBMC to ConA, PHA and PWM, but responses were not suppressed when mice were given with 300µg of CM-R.

Antibody responses to the different preparations was dose-dependent. Phase II antibody persisted until day 21 in mice given CM-R or killed WC. Levels of Phase I antibody were lower in mice given CM-R as compared with those inoculated with WC. Neither Phase I nor Phase II antibody were induced with CM-E. The authors suggested that the CM-E may contain an adjuvant-like component and also that this component is responsible for the immunosuppression and deleterious tissue reactions. The particular component in CM-E was not identified but the extract had a high content of fatty acids.

The Williams group also examined the ability of different strains and fractions of *C.burnetii* in the Phase I state to induce and elicit skin and *in vitro* lymphoproliferation in guinea pigs (Ascher *et al.* 1983c). The strains used were formalin-inactivated Phase I Henzerling WC vaccine, γ-irradiated Phase I WC Ohio strain, the soluble component (TCA-E) of trichloroacetic acid- extracted Henzerling strain and the cell residue after extraction, and the extract and cell residue of the CM-treated Ohio strain. Animals were given 10µg doses in Freund's incomplete adjuvant into each footpad and then skin tested. Both the Henzerling and Ohio WC strain elicited similar delayed type hypersensitivity (DTH) reactions, while the TCA-E and TCA-residue fractions

of each strain produced both an immediate and DTH reaction. On the other hand, the CM-R and CM-E did not induce either type of reaction. In the lymphoproliferation assay with PBMC from immune animals, it was found that the response was almost identical for CM-cell residue, from Henzerling and Ohio strains. Because histological examination of mice given the CM residue fraction did not reveal lesions, they concluded that the substances causing the granulomas was removed by the CM extraction.

Williams *et al.* (1986a) also compared the protective effect of killed WC vaccine and CM-R vaccine in C57BL/10 ScN mice. CM-R vaccines were claimed to be more effective than WC vaccines in preventing infection in mice challenged with a lethal dose of Phase I *C.burnetii.* Mice injected with WC were found to be immunosuppressed when their splenocytes were tested with ConA, PHA and PWM. From this, they concluded that the WC was either less mitogenic than CMR or that it contained components that decreased the activity or viability of mouse splenocytes. Again, with the WC preparations, there was an increase of spleen weight in the mice that was time-dependent and dose-dependent. The antibody response elicited with each vaccine preparation was also found to be dose-dependent. Phase II antibody was detected 3 days post-injection with both preparations, over a range of several doses; Phase I antibody was detected 7 and 14 days post-injection of either 100 or 1000μg of WC and CMR-vaccine with a continued increase to day 35. At a lower dose (10μg), Phase I antibody was produced by WC vaccines but not with CM-R vaccines. The failure of the latter, it was speculated, was due to the loss of adjuvant-active components extracted from WC by CM treatment.

When CM-R was reconstituted with CM-E in CM, the effects induced in mice became similar to those induced by the WC vaccine.

Biochemical and physiological analysis of the toxic effect of *C.burnetii.* Earlier work by Brezina *et al.* (1965) in guinea pigs showed that the Phase I antigen of the *C.burnetii* L35 strain, extracted with TCA, produced a pyrexial reaction within 5-7 hours, that was proportional to the dose administered intraperitoneally. When this antigen was treated with hyperimmune serum containing complement-fixing antibodies against Phase I and II, the pyrogenic effect as well as the protective efficacy of the Phase I antigen were neutralised. This neutralisation was shown to be due to the presence of Phase I antibodies. It was also found that the Phase I TCA-E retained its pyrogenic effect after extraction with phenol-water mixture (transforming it into a hapten by removing Phase II proteins from TCA-E) thus indicating that the pyrogenic effect was related to the LPS rather than the proteins of the organism. Injection of guinea pigs with Phase I antigen TCA-E induced an immunity to the pyrogenic activity of whole cell *C.burnetii* suspension. From all of this, they concluded that the Phase I antigen was the factor responsible for the virulence of the Q fever organism. Kazar *et al.* (1986) extended the work of the Williams group by comparing the protective effects of different types of Q fever vaccines in guinea pigs; Phase I WC *C.burnetii*, a CM extracted cell residue and TCA-E were used. The highest degree of protection was achieved with the WC vaccine, irrespective of the route of immunisation and of intraperitoneal or aerosol challenge.

Other workers have been concerned with the toxic effect of *C.burnetii* in animals. Baca and Paretsky (1974a,b) partially characterised the product of a phenolic extract of LPS from *C.burnetii* that had toxic properties in guinea pigs, producing many of the biochemical changes observed during active infection (see also earlier chapters).

Immunomodulatory effects of *C.burnetii*. The most recent studies by the Williams group (Waag and Williams, 1988) have revealed the presence of a so-called "immunosuppressive complex" (ISC) associated with the Phase I cell which is resistant to chemical and enzyme treatment. The ISC was inactivated and rendered accessible by CM extraction during the production of CM-R and CM-E. ISC was considered to contain the carbohydrate residues attached to the cell by disulphide bonds and was a stuctural feature of the Phase I strains; Nine Mile, Henzerling, Nine Mile 514 and Ohio. The extracted ISC, when reconstituted with the two fractions (CM-R and CM-E) before injection into mice, restored suppressive properties to the level of WC preparations. The exact chemical nature of ISC and that of its place in the cell envelope is difficult to envisage from the data presented.

Current study of a possible immunosuppressive nature of the formalin-inactivated Q fever Phase I whole cell vaccine

In the light of all of these observations it was clearly necessary to search for any immunosuppressive qualities of the Phase I whole cell formalin-inactivated Q fever vaccine, made from the Henzerling strain and employed in the vaccine

trial with human subjects in South Australia. In considering the implications of the findings by the William group a point of central importance was that the doses given to mice and guinea pigs in their studies, were far above - on a dose/weight basis - that given as vaccine to human subjects in the South Australian trials. In the latter each nonimmune individual received a 30µg dose of whole cell vaccine. On a proportional basis to the average body weight of a human (approximately 80 kilograms), a corresponding dose for an 18-20 gram mouse would be 7.5 nanograms of vaccine; the doses given to mice by Williams and his group were considerably in excess of this amount - 10 to 300µg. The question we wished to answer was whether a dose around 7 nanograms would produce immunosuppressive effects or gross pathological or histological effects in mice. Investigations directed to this problem are now described.

15.2 Materials and Methods

Antigen preparation. Formalin-inactivated *Coxiella burnetii*, Henzerling Phase I strain is used for the Q fever vaccine. The dose given in humans is 30µg in a 0.5ml volume, subcutaneously. The concentrations used in mice to bracket the equivalent vaccine concentration in relation to body weight of mice were 30ng, 15ng, 7.5ng, 3.75ng (the 7.5ng concentration is an equivalent dose, in mice, to that given in humans). A 10µg dose was also used as a positive control to correspond to the lower level of the dose range used in previous experiments by Williams and colleagues. All vaccine dilutions were made in sterile saline.

Mice. Male C3H mice were used in this experiment. Mice were divided at random into 6 groups designated A, B, C, D, E, F, each consisting of 15 mice. All except group F were inoculated subcutaneously. Group A acted as a control group and received 1ml of sterile saline. Group B received a 3.75ng dose. Group C, 7.5ng, Group D, 15ng, Group E, 30ng, Group F, 10µg, but intraperitoneally to match Williams *et al*'s experiments. Mice were sampled at 3, 14, 21 days after inoculation for groups A, C, F and at 16 and 22 days for Groups B, D and E. At each sampling time, five mice were anaesthetised, bled, and then killed to remove the liver and spleen. All procedures were performed using aseptic techniques. A portion of pooled blood from each group was placed into a tube containing heparin for isolation of lymphocytes for the lymphoproliferative assay. The liver and spleen were weighed and the size measured. Samples from livers and spleens from each group were also fixed in 10% formalin-saline solution for histological examination. One spleen from each group was used for the blastogenic assay. Pooled blood from each group was also allowed to clot, and the serum used in the complement fixation and immunofluorescence tests for antibodies to Q fever antigens.

Peripheral Blood Mononuclear Cell (PBMC) isolation. The method used was that of Chi and Harris (1978), with some modifications. Briefly, 2ml of Ficoll-Hypaque gradient (Pharmacia, Uppsala, Sweden) was added to a 10ml plastic test tube. Whole blood, diluted with RPMI-1640 (supplemented with L-glutamine, penicillin and gentamycin) at a ratio of 1:3 (vol:vol) was then layered over the gradient. The tube was centrifuged at 400 rpm in a bench top

centrifuge (MSE), for 25 minutes at room temperature. The cells at the interface plus the Ficoll-Hypaque were removed without disturbing the red cell layer. An excess volume of RPMI-1640 was then added to the cells and centrifuged at 3000 rpm, for 10 minutes. This step was repeated and the final cell pellet resuspended in 1ml of RPMI-1640.

Lymphoproliferative assay. PBMC from each group of mice were tested against several concentrations of phytohaemagglutinin (PHA) (four replicates for each concentration). The optimal concentration of PHA had been determined previously with mouse PBMC. 200µl aliquots of PBMC diluted to a concentration of $5x10^5$ cells/ml in RPMI-1640 supplemented with 10% heat inactivated FCS were dispensed into 96 well microtitre plates (Nunclon Delta, Nunc, Denmark). An unstimulated control was also included for each group. Cells were incubated for 96 hours, at 37°C in a humidified 5% CO_2 incubator, after which 20µCi of [^{3}H]-thymidine (sp. act. 22Ci/mmol; Amersham Int. Sydney) was added to each well and incubated for a further 18 hours. Cells were harvested in an automatic cell harvestor (Skatron, Lierbyen, Norway) onto glass fibre filters which were then dried, placed into scintillation fluid and counted on a β-counter (Beckman, LS 2800, Sydney, Australia). The mean counts per minute (cpm) were calculated from the four replicates and a stimulation index (SI) was calculated as:

SI = (mean cpm of stimulated cultures - cpm of machine background)/(mean cpm of unstimulated cultures - cpm of machine background).

Preparation of spleen cell suspension. A spleen from a mouse in each group was excised asceptically and placed into a petri dish, on ice. The spleen was minced with fine scissors for two minutes. Two mls of RPMI-1640 (without FCS) was added to the spleen suspension and it was repeatedly aspirated through a pasteur pipette for two minutes. The suspension was then repeatedly aspirated through an 18 gauge needle for two minutes. This step was then repeated with a 21 gauge needle, and finally with a 22 gauge needle. The suspension was placed into a 10ml centrifuge tube, to which RPMI-1640 was added upto the 10ml mark and allowed to stand at room temperature (RT) for 5 to 10 minutes (allowing the debris to settle). The supernatant fluid was removed, placed into a new centrifuge tube and centrifuged at 1500 rpm for 10 minutes at RT. The supernatant fluid was discarded and the pellet resuspended into 10ml of red blood cell lysing solution (0.8% NH_4Cl; 0.1% EDTA; 0.01% KH_2PO_4). The tube was centrifuged as above and the pellet resuspended in 10ml of RPMI-1640 supplemented with 10% FCS. The centrifugation step was repeated 3-4 times, as above, until the cell pellet became progressively white. The cell pellet was finally resuspended into 10ml of RPMI-1640 (containing 10% FCS).

Blastogenesis assay. The assay employed was that described by Cantrell and Wheat (1979) with some minor modifications. Spleen cell concentrations were adjusted to $5x10^5$ cells/ml and 200µl was dispensed into each well in 96 well microtitre plates. Each spleen cell suspension from each group was tested in 8 replicate wells. 20µl of 20.Ci/ml [^{3}H]-thymidine was added to each well.

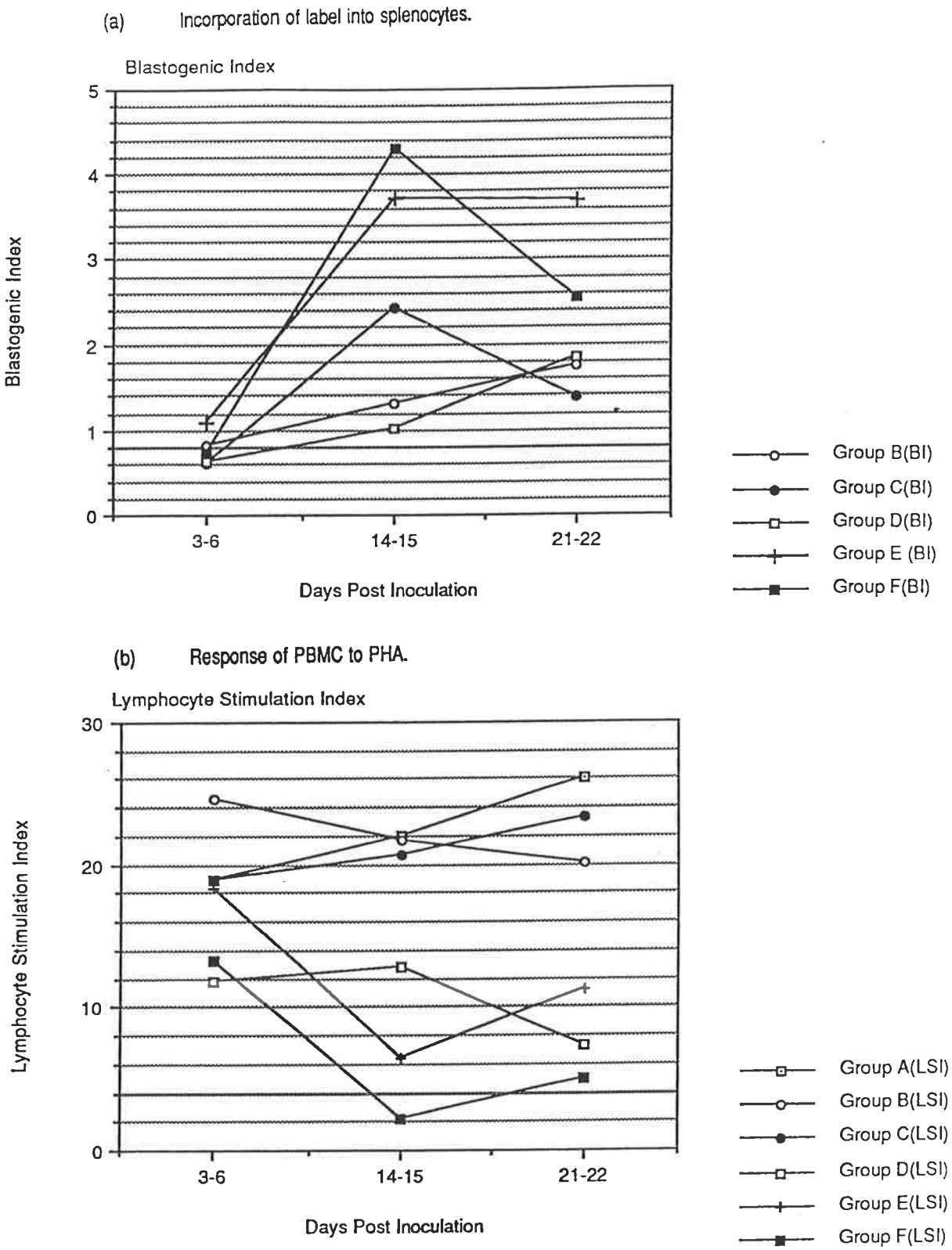


Figure 15.1 (a), (b). Blastogenic indices and stimulation indices in mice given Q vax. (a) Blastogenic indices (BI) obtained with splenocytes harvested at various intervals after the inoculation of graded doses of Q vax. Group C received a vaccine dose equivalent on a weight ratio bases to that used in man. Note that the BI measures the *in vivo*, endogenous cellular immune response to the antigenic inoculum. Specific antigen is not added to the *in vitro* cultures of the splenocytes. (b) lymphocyte stimulation indices, induced by the pan mitogen, PHA, with PBMC harvested at the same time intervals.

Cells were cultured for 18 hours at 37°C in a humidified CO_2 (5%) incubator. Cells were then harvested with the automated Skatron harvester onto glass fibre filters which were dried and placed into scintillation fluid and counted on the β-counter. The mean cpm was calculated from the eight replicate wells for each group and a blastogenic index (BI) was calculated as follows:
BI = (cpm of test spleen cells - machine background)/(cpm of normal spleen cells - machine background)

Serological tests. Clotted blood specimens from each group of mice were tested for complement fixing antibody and immunofluorescent antibody specific for Q fever antigen. The method used was that of Worswick and Marmion (1985).

Histology. Liver and spleen specimens from each group, fixed in 10% Formol saline, were embedded and sections cut and kindly examined in the Division of Tissue Pathology, Institute of Medical and Veterinary Science.

15.3 Results

Blastogenic indices in the various groups of mice. Figure 15.1 (a) shows the blastogenic indices (BI) obtained at various time periods after inoculation of the different doses of vaccine. At 14-15 days the highest BI was with Group F which had been given 10μg of vaccine. Group C - which had received a dose corresponding to the Q fever vaccine dose for human subjects, showed an intermediate response. Overall there was a good dose-response relationship between the BI value and inoculum size, except for Group D (15.0ng) which

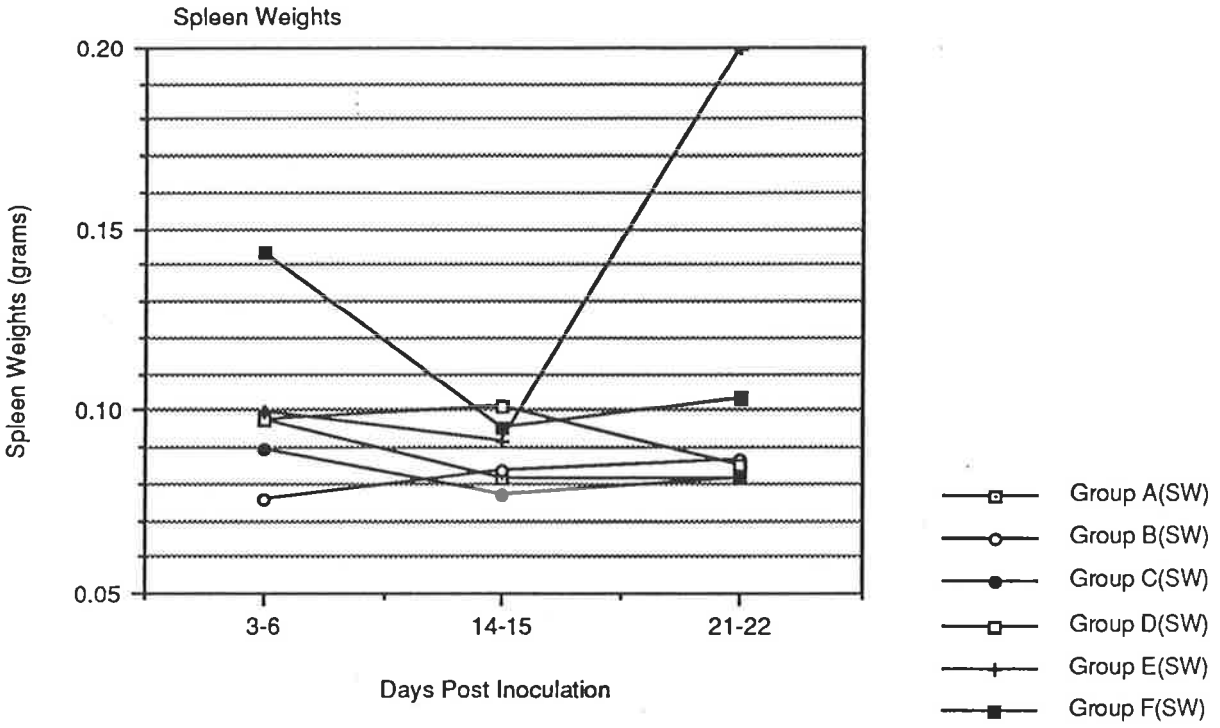
was out of line with the other groups, perhaps because of the sampling variation involved in testing only one spleen per group.

Responses in lymphoproliferative assay. Mitogenic responses to PHA were essentially the inverse of those obtained in the blastogenic assay and were most depressed with the 10µg dose (Figure 15.1 (b), Group F), whereas Group C (equivalent dose to that used in human beings) showed responses essentially the same as Group A which had not received vaccine. The 15 and 30ng doses showed some depression, intermediate between the values obtained with 3.75 and 7.5ng on the one hand and 10µg, Group F, on the other.

With both measurements the values at 14-16 days after inoculation discriminated best between the groups.

Spleen and liver weights. Responses are shown in Figures 15.2 (a) (b). At 14-15 days there was little difference in spleen weight between all groups. Group C and Group A (control) showed similar liver weights.

Tissue responses. No gross pathological lesions were identified in spleens and livers of any group of mice. Histologically, no difference was noted between Group A (control) and Group C in the livers or in the spleens at 14-15 days. At 21 days after inoculation, Group E and Group F showed increased numbers of band cells and neutrophil polymorphs in the liver. No discrete granulomas were observed in Groups A to E although in liver sections from Group E occasional collections of mononuclear cells were present.



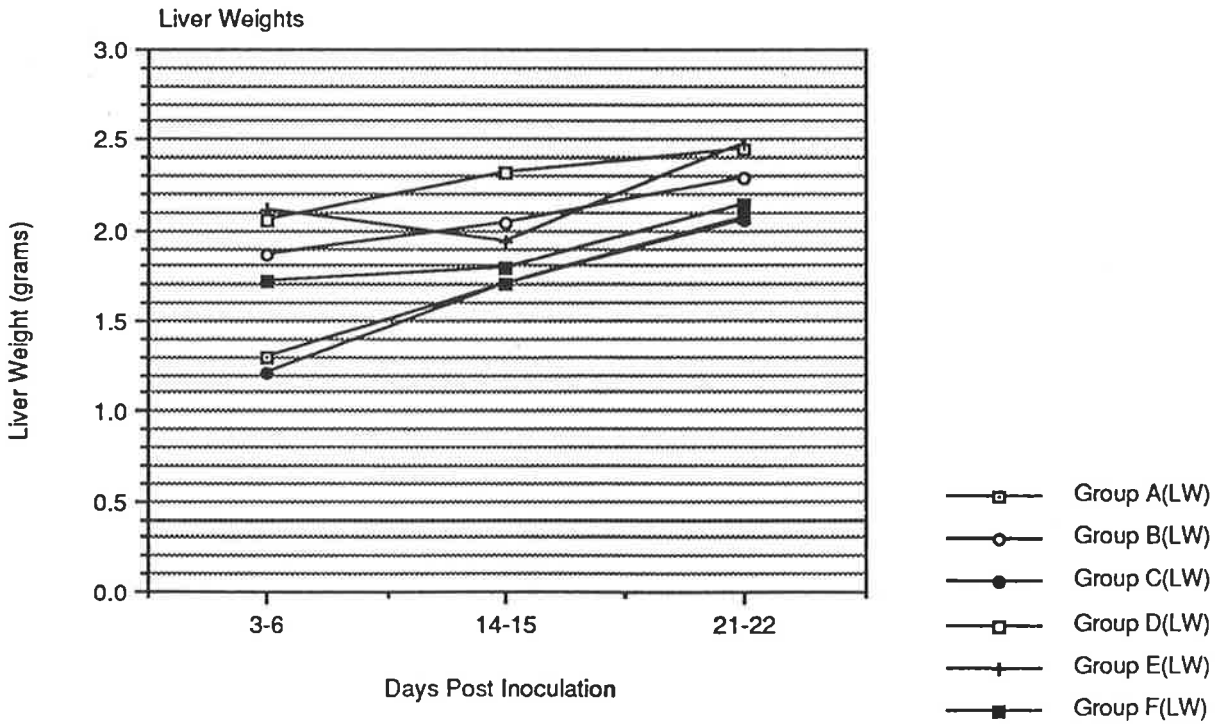


Figure 15.2. Spleen and liver weights in groups of mice at varying intervals after inoculation with varying doses of Qvax. Group C received a dose equivalent on a dose-weight basis to that given to human subjects. Group F were given a higher dose known to produce an effect in the experiment of other workers.

Table 15.1: Q fever antibody responses measured by immunofluorescence or complement fixation, at various times after inoculation, of groups of mice given various doses of an inactivated whole cell Q fever vaccine, *C.burnetii* Henzerling strain, Phase I antigenic state (see text).

Immunofluorescence assay titres of mice inoculated with various doses of killed *C.burnetii* at the following days (d) after inoculation:

Group	3-6d	Phase I antigen 14-15d	21-22d
A	<5	<5	<5
B	<5	<5	<5
C	<5	<5	<5
D	<5	<5	<5
E	<5	<5	<5
F	<5	<5	10
		Phase II antigen	
A	<5	<5	<5
B	<5	<5	<5
C	<5	<5	<5
D	<5	<5	<5
E	<5	<5	<5
F	<5	10	10

Complement fixation assay titres:

Group	3-6d	Phase I antigen 14-15d	21-22d
A	<5	<5	<5
B	<5	<5	<5
C	<5	<5	<5
D	<5	<5	<5
E	<5	<5	<5
F	<5	<5	<5
		Phase II antigen	
A	<5	<5	<5
B	<5	<5	<5
C	<5	<5	<5
D	<5	<5	<5
E	<5	<5	<5
F	<5	<5	10

Serological responses are set out in Table 15.1. The only group to develop antibody (IF or CF) was Group F, at 21 days after inoculation.

15.4 Summary and Conclusions

It appears that the inactivated Q fever vaccine (Qvax), when given to mice in doses around or equivalent to those used to vaccinate human subjects, does not produce gross, or histologically detectable lesions in liver or spleen and does not depress the mitogenic response of mouse lymphocytes to the lectin PHA. Blastogenic activity was observed in the spleen, and was broadly dose-related. Such a response to antigenic challenge is to be expected, although antibody was not detected at 21 days after inoculation by assays of intermediate sensitivity. Larger doses - 10μg - i.e., at the bottom of the range used by Williams and colleagues, produced some suppression of panmitogenic response to PHA and some minor cytological changes in liver or spleen. Antibody was also stimulated. None of the vaccine doses equivalent to those employed in human subjects produced the widely-dispersed, small granulomas which are a characteristic response to large doses of killed organisms or to infection with living organisms. That said, it is noted that the subcutaneous inoculation of inactivated *C.burnetii* WC vaccine does produce a small local granuloma when inoculated intradermally or subcutaneously in human subjects who are already immune. The experiments described in this chapter appear to exclude any general, immunosuppressive effect by the vaccine doses employed with human subjects. The conclusions by Williams and his colleagues from the use of "unphysiological" doses of WC vaccine leave

unsettled the matter of whether the "immunosuppressive" effects observed in their mice are merely a temporary regulatory, homeostatic brake on antibody production after a large dose of antigen, or whether there is a longer term, more serious immunosuppression which would modify host response to infection with other agents.

CHAPTER 16

GENERAL DISCUSSION: CONCLUSIONS: FUTURE DIRECTIONS

The thesis describes work designed to clarify various aspects of the cell-mediated immunity (CMI) in human subjects after a single subcutaneous dose of 30 micrograms of a formalin-inactivated, whole cell, *C.burnetii* vaccine, prepared from the Henzerling strain in Phase I antigenic state.

The purpose of this last Chapter is to draw together the findings described in the Results Chapters 11 to 15, to integrate them, and to compare and contrast crucial findings with existing information in the literature. In particular it is hoped to draw out the rôle of the Phase I LPS in protective immunity (as demonstrated by earlier workers in guinea pigs or mice given the various types of Q fever vaccine). The preceding Chapters - cross referenced in this Chapter - should be consulted for details of the experimental results and discussion of some other issues of a more subsidiary importance.

The starting point of the work on CMI was that studies with the vaccine in abattoir workers indicated a complete protection against natural infection yet only 55-65% of subjects developed or retained an antibody response in the year after vaccination (Marmion *et al* 1984).

In an attempt to resolve the apparent contradiction between protection and antibody responses, studies of CMI - as measured by *in vitro* proliferative responses with peripheral blood mononuclear cells to *C.burnetii* antigens, or by skin tests with diluted vaccine - were undertaken and the patterns in the vaccinated were compared with those in human subjects immune from

previous clinical or subclinical Q fever infection.

The findings (detailed and discussed in Chapter 11) revealed that ~85% of an infrequently exposed, "low risk" population, and ≥95% of a highly exposed, "high risk" population (abattoir workers) developed a positive lymphocyte stimulation index (LSI) to Phase I or II antigens, or both, after vaccination. The conversions took place about 9 to 15 days after inoculation and the majority of subjects remained positive for up to 5 years or more (the longest time tested). These high and maintained rates of CMI response again contrasted with the antibody formation rates which in these particular study groups showed a short-lived, 70-80% positive response, most marked in the IgM immunoglobulin class, in the two to three weeks after inoculation. This fell thereafter to 35-45% in the "low risk" group and to about 60% in the "high risk" group. The differences in antibody and CMI responses between the two groups suggested that some of the "high risk" group, although antibody and skin test negative before vaccination, may nevertheless have been primed to *C.burnetii* antigen. Indications that this was possible emerged from the finding of a positive LSI in subjects who were skin test and antibody negative (see also Ascher *et al* 1983b). Alternatively, perhaps repeated exposure of the 'high risk' subjects to *C.burnetii* in their working environment, may have reinforced their immunity from time to time. Overall, the *in vitro* CMI data fitted well with the complete protection observed in the abattoir workers and with its establishment 10 to 15 days after vaccination (see detailed discussion: Chapter 11).

The *in vitro* CMI studies also established that the PBMC proliferative

responses, both in vaccinees and in the post-infection subjects, were somewhat more marked with the Phase II antigen rather than with Phase I. (See, for example, Figures 11.4 a & b, 11.6 a & b and 11.7 a & b). Similar rather striking trends were observed with cells from post-infection subjects tested with Priscilla Phase I, Henzerling Phase I, and Nine Mile Phase II antigens as part of the work in Chapter 12, see Figure 12.2. The difference in mitogenic responses between the two antigens was puzzling. In the conventional view of a lymphocyte proliferation assay the stimulating antigens - in this instance *C.burnetii* Phase I or Phase II cells - are taken up by monocytes in the mixture, broken down, and their peptides presented in juxtaposition to MHC II antigens on the monocyte surface for recognition by immune T lymphocytes. In this process the LPS would be dissociated from the proteins and so a simple steric hindrance of access by LPS to Phase II antigen (proteins), familiar from whole cell and antibody mixtures, would not operate. On this model, one would expect, therefore, that if the protein composition of Phase I and Phase II cells were the same, then the T cell mitogenic responses would be the same - but this was not what was observed. It is true that not all workers agree that the protein composition of Phase I and Phase II cells are identical, or that the proteins composition of Phase II cells from different strains is the same (reviewed Chapters 6 and 7). As restriction enzyme maps of the genomes of different strains do vary, this might be reflected in different protein composition. This particular reservation has been offset in the present work by the use of organisms in Phase I but treated with periodate salt to open the sugar rings in the side chains of the LPS so as to modify the biological

behaviour of the Phase I LPS, without altering the protein composition of the coxiella cells. It should also be noted that, endocarditis strains apart, the LPS of Phase I strains is considered to be antigenically identical (Chapter 6), so that differences in LSI profiles with different standard, prototype Phase I strains are not explicable in terms of LPS differences.

Differences between PBMC proliferative responses to the two Phase antigens has not been extensively investigated by other workers, but a similar trend maybe seen in the results described by Jerrells *et al* (1975). These workers found that five of their small group of nine subjects with various forms of exposure to Q fever infection or Q fever antigen in the past had higher LSI values with Phase II antigen than with Phase I, though the differences were not substantial. Similarly, Ascher *et al*, (1983b) also found that numerically more subjects reacted positively in the LSI test with Phase II antigen than with Phase I (see Figure 2 of their paper). Our results, and those of the workers just quoted, are in marked contrast to a report by Gajdesova and Brezina (1989) who, in a study of *in vitro* lymphoproliferative responses in vaccinees given "Chemovaccine" (a protein-LPS complex [TCA-E] extracted from *C.burnetii* Phase I organisms with trichloroacetic acid) found that the responses were more striking with Phase I rather than with Phase II antigen. The reasons for this difference are not immediately apparent. TCA-E contains fewer proteins than the whole cell - about nine versus >50 bands on PAGE. Our own observations with Phase I TCA-E and a small group of vaccinees showed a poorer mitogenic response to this extract when used as a stimulating antigen (Chapter 12, Figure 13.1 (d)). It is presumed that the absence, from the

"Chemovaccine", of the bulk of the proteins making up the Phase II whole cell antigen preparation gives a narrower T-cell response directed to those proteins which are more firmly associated with Phase I LPS and which co-partition with it during the extraction process.

Overall, our results (Chapter 11) are compatible with those of Jerrells *et al*, (1975) and Ascher *et al*, (1983b) in the sense that they observed quantitatively similar, rather small, specific LSI responses to Q fever antigen amongst vaccinees or postinfection subjects. However, in neither report was data given on the time of conversion of LSI after vaccination or the duration of the immunity as measured by LSI. It is considered that the present investigations (also published, Izzo *et al*, 1989 - see Appendix) have advanced knowledge and established useful comparative standards for the future laboratory assessment of human immune responses to any "second generation" vaccine (e.g. the chloroform-methanol extracted whole cell residue developed by the Williams group and the United States Army).

In studies in Adelaide, the skin test as a measure of response to vaccination was disappointing in that only about 60% of subjects became skin test positive after inoculation. There was a general, although low correlation, between LSI value and skin test diameter which was more marked with Phase II than Phase I antigen. Similar trends, but with higher correlation coefficients were observed by Ascher *et al* (1983b) in their comparison of LSI values and skin test diameter. It should however be noted that the latter were observations in post-infection subjects rather than in vaccinees. In all three studies substantial numbers of LSI positive/skin test negative subjects were

observed, and, in addition, Jerrells *et al* (1975) infrequently found antibody in those subjects with a positive LSI.

It is also noted that the utility of skin test to measure vaccine immunity fell well short of that observed in the earlier studies of Bell *et al*, (1964) but in the latter a larger dose of vaccine was employed -- about seven times that in the present investigations.

These initial studies of the proliferative responses to *C.burnetii* Phase I and Phase II antigens of PBMC from immune subjects, either vaccinated or exposed in the past, were extended in various ways in the next stage of the work. First, the class of lymphocytes involved in the proliferative response was determined, second, it was ascertained whether the rather meagre LSI values could be specifically augmented with IL 2 and third it was determined whether the immune lymphocytes responded to the LPS or the proteins - or both - in the whole cell antigens employed in the early studies.

It could not be assumed that the lymphoproliferative response was solely or predominantly due to T-lymphocytes. Previous workers Jerrells *et al* (1975) and Ascher *et al* (1983b), had used either cultures of whole blood obtained by venepuncture, or mixtures of mononuclear cells separated from red cells and polymorphonuclear leucocytes. It was possible (although perhaps numerically unlikely given the small numbers of B-lymphocytes in peripheral blood) that the proliferative responses observed with such preparations were those of B-, rather than T-lymphocytes and that the tests simply constituted an indirect measure of antibody (B cell response), or even a nonspecific response of B-lymphocytes to *C.burnetii* LPS as has been observed with guinea pig B-

lymphocytes (Paquet *et al*, 1978). These possibilities were explored by blood mononuclear cell fractionation-reconstitution ("enrichment") experiments starting from PBMC, and testing with various combinations of T-lymphocytes and monocytes (as antigen presenting cells), B-lymphocytes and monocytes etc., with *C.burnetii* antigens. (Chapters 12 and 13). These experiments established that the major proliferative response to *C.burnetii* whole cell antigens resides in the T-lymphocyte compartment (with monocytes as antigen presenting cells) and that this response is specifically enhanced by the addition of IL2 to the reaction mixtures. The enhancement appeared to be specific in that (i) it did not occur with immune cells in the absence of antigen (ii) cells from nonimmune subjects in the presence of antigen did not show a response or enhancement (iii) only a minor response occurred to the control yolk sac antigen and that only in vaccinees who had probably received traces of yolk sac antigen when given the vaccine; there were no reactions to yolk sac antigen with cells from subjects naturally infected with *C.burnetii* in the past. Other limited fractionation experiments showed that proliferative responses could be obtained with both CD4+ and CD8+ cells from subjects infected with Q fever in the past. However, the proliferative response to *C.burnetii* antigens was not completely restricted to T-lymphocytes. Lower level responses were observed with the separated and enriched suspensions of B-lymphocytes and monocytes; and these B cell responses were also enhanced by IL2 and to a lesser extent by IL4. As far as I can determine this is the first time that classes of immune lymphocytes proliferating in response to the *C.burnetii* antigens ~~has~~ have been ascertained. The findings underline the need to use

ERRATUM

Page 252 lines 17 and 18

Should read... plant mitogens or lectins, such as PHA, are proteins which bind to carbohydrate receptors and... activate T cells with IFNγ production; ...

importance to determine whether there was any proliferative reaction of T-lymphocytes with Phase I LPS. It is well established (see Chapters 13 and Table 16.1) that Phase I LPS is essential in a vaccine to stimulate protective immunity, but it was already evident from the work up to this stage that PBMC or T-lymphocytes responded at least as vigorously to Phase II WC antigen (i.e., without Phase I LPS determinants) as to Phase I WC antigen. Although the T-cell receptor recognises short peptides presented in juxtaposition to Class I or II MHC on the surface of the macrophage it could not be assumed that a potential LPS-T cell interaction, resulting in T cell activation and cytokine liberation, might not take place through receptors other than the T cell receptor for peptides. Plant mitogens or lectins, such as PHA, are carbohydrates and activate T cells with IFNγ production; carbohydrates from *Mycobacteria* spp and a phosolipoglycan from *Leishmania major* also activate T cells and induce cytokine production.

Chapter 13 describes experiments with T-lymphocytes and monocytes from immune and non-immune subjects and WC or antigen fractions (TCA-E, TCA-R, cell proteins separated on PAGE and extracted LPS [P-W-E]) from the coxiella.

Significant conclusions from this stage of the work are that (i) as before, Phase II WC were at least as mitogenic as Phase I WC and sometimes more so; (ii) WC preparations in which the LPS had been modified by treatment with potassium periodate and which would have identical protein composition to the unmodified Phase I cells, were equally mitogenic for T lymphocytes; (iii) that the PAGE-separated proteins from lysates of Nine Mile Phase II cells all showed some, although a variable mitogenic activity with immune T lymphocytes. There was no clear-cut immunodominant protein although with T cells from some donors gave larger stimulation indices with proteins ca. 77KDa and 27KDa and 16KDa, than with other proteins. In no instance however was there a separated protein which gave a greater LSI value than the total unfractionated lysate from the Nine Mile Phase II cells. [The lysate was prepared from Nine Mile Phase II cells because, in PAGE separations, Phase I LPS tends to be spread throughout the gel, whereas Phase II LPS localises at the bottom of the gel (Hackstadt, 1983). There was therefore less chance of LPS contamination of the individual protein bands on transfer to nitrocellulose, with possible modification by LPS of LSI or IFNγ responses on dispersal and stimulation of lymphocytes (see also below)].

Finally, (iv) preparations of semi-purified LPS extracted from *C.burnetii* Phase I cells by the phenol-water method showed no stimulatory activity with immune T lymphocytes when tested over a wide range of dilutions.

These Phase I LPS preparations were demonstrably free of protein but would contain nucleic acids and carbohydrates, in addition to the LPS.

It therefore appeared from these experiments that the stimulatory

epitopes for immune T cells were associated with the proteins of the coxiella and that the Phase I LPS, whether associated with coxiella cell, or modified in its sugar chain structure by periodate oxidation or extracted by the phenol-water method, played no direct-stimulatory-part in T lymphocyte proliferative responses.

Although *C.burnetii* cell or antigen fractions (such as LPS, TCA-E, TCA-R, periodate treated WC) have been extensively tested in mouse or guinea pig protection models (see Chapter 7 and Table 16.1), only three very limited studies appear to have been made with the fractions and *in vitro* correlates of CMI. In particular no previous tests have been made with Phase I LPS extracted by the phenol-water method.

Some comparable information was obtained by Jerrells *et al,* (1975) who set up various antigen fractions with whole blood cultures from 3 subjects who had had clinical Q fever in the past, and 3 subjects who had become immune by exposure to *C.burnetii* in the laboratory. The fractions used were TCA-E from Phase I organisms (TCA-EI), that from Phase II organisms (TCA-EII), TCA-EI treated with protease to remove the associated protein and leave the carbohydrate (TCA-EI CHO) and TCA-EI treated with weak acid to hydrolyse the glycosidic linkages between the sugar residues of the LPS, while retaining the proteins (TCA-EI-Pro). TCA-EI showed vigorous proliferative responses (i.e., significant LSI) with cells from all six subjects. TCA-EI CHO showed much lower mitogenic activity, whereas TCA-EI-Pro gave LSI values in 4 of the 6 subjects which were the same as those with TCA-E1. The results with TCA-EII were anomalous (in terms of the hypothesis being developed in this Discussion)

in that LSI were no better than those with TCA-EI CHO. It may be speculated that TCA extraction from Phase II whole cells removes less protein, either because the protein copartitions more efficiently with Phase I LPS when Phase I cells are being extracted, and less efficiently with the small amounts of altered LPS in Phase II cells which do not operate as an effective carrier when Phase II cells are being extracted. Alternatively, the trichloroacetic acid might be more damaging to the cell proteins in the absence of Phase I LPS. Jerrells *et al* (1975)do not give any analytical data (e.g., PAGE analysis of proteins) on their preparations so it is not possible to decide between these alternatives. It may be noted at this point that other colleagues from Jerrells laboratory (Heggers *et al*, 1974) had tested similar derivatives of TCA-E in the macrophage-migration inhibition test with cells from immune guinea pigs and obtained rather different results to those just described with the lymphocyte stimulation assay and human mononuclear cells - namely that TCA-EI CHO was often equally inhibitory as TCA-EI Pro (see below for other explanations). So different techniques used as *in vitro* correlates of CMI may yield different results.

In the final stages of the work described in the thesis PBMC or separated T lymphocytes from immune subjects were tested for their ability to form and excrete IFNγ on stimulation with various *C.burnetii* antigens.

The assays were done for two main reasons. The first was that IFNγ is known to be the final mediator of activation of cells, particularly macrophages, for the destruction of intracellular *C.burnetii* and it was desirable to show that T lymphocytes from vaccinees had acquired that ability after vaccination. The

second reason, linked with the first, was the specific point that the ability of an immune T lymphocyte to respond *C.burnetii* antigens by mitogenesis might not be a guide to its performance in IFNγ formation. For example, a subset of CD+ lymphocytes (at any rate in mice) may show a mitogenic response on antigen stimulation but cytokines stimulating B lymphocytes, rather than IFNγ, are formed and liberated.

Initially interferon was assayed by inhibition of viral CPE by conditioned media from cell stimulation experiments ('bioassay'). Specificity for IFNα and IFNγ was obtained by neutralisation with anti-IFNα or anti-IFNγ. The technique proved somewhat less sensitive than an antigen-capture EIA for IFNγ developed by CSL, although the two techniques gave compatible results.

PBMC from the 'low risk' group of 23 vaccinees, stimulated with various *C.burnetii* antigens, showed that conditioned media from 75% of subjects were positive by the CSL IFNγ-EIA as compared with 56% in the bioassay (Chapter 15, Table 15.1).

Induction of the ability of PBMC to form IFNγ was observed 10-15 days after vaccination, as we observed earlier with the lymphoproliferative response. A point of interest was that PBMC from vaccinated subjects whose conditioned media were negative in the IFNγ-EIA or bioassay, were nevertheless found to have IFNγ positive cells by immunofluorescence, in numbers significantly above the background number with unstimulated cultures. It was thought that this reflected either a greater sensitivity of the IF technique for IFNγ detection, or that the IFNγ remained cell-bound and was not liberated into the conditioned medium.

During the course of these studies on IFNγ formation by lymphocytes from Q fever immune subjects an observation emerged that finally gave some insight into the puzzling phenomonen that lymphoproliferative responses were, in general, more vigorous with Phase II antigen than Phase I. It was found (Chapter 14) that although mononuclear cells from the 'low risk' group of vaccinees gave a mitogenic response with both Phase I and II antigens, only the conditioned media from the latter contained IFNγ. It was further demonstrated that all of the PAGE-separated proteins from Nine Mile Phase II cells were capable of stimulating IFNγ production, as well as a mitogenic response.

In otherwords, the presence of the Phase I LPS appeared to inhibit IFNγ production. It was further found, in a strictly homologous system using different antigens from the same strain of *C.burnetii* - Priscilla - that mononuclear cells stimulated with either Priscilla Phase I WC antigen or the same antigen after periodate treatment, yielded IFNγ only in the CM from the reaction mixtures with periodated antigen, implying that modification of the (terminal) sugars in the Phase I LPS had removed or diminished the inhibitory effect.

The inhibition of IFNγ in reaction mixtures with Phase I WC antigen, was reduced by addition of IL-2, although this supplement also increased the amount of IFNγ produced in response to Phase II WC antigen (Figures 14.7, 14.8, Tables 14.3, 14.4).

In further attempts to define the inhibitory effect of Phase I LPS it was found that the down-regulation of IFNγ production was mimicked by prostaglandin E2 and antagonised by Piroxicam, an inhibitor of

prostaglandin synthetase. An inhibitory effect was also obtained by CM from monocyte cultures exposed to LPS extracted from Priscilla Phase I WC or Henzerling Phase I WC (Tables 14.3, 14.4). However tests of these CM did not detect PGE2 by radioimmunoassay (Hart. P., personal communication) and the mediator may be some other prostaglandin-like substance.

These observations on differential IFNγ responses to *C. burnetii* Phase I and II antigens have not been reported elsewhere as far as I am able to ascertain. Reports which may touch on the same phenomenon are those of Koster *et al* (1985a) and (1985b). These workers used inhibition of lymphocyte mitogenesis (not IFNγ formation) as an index of "immunosuppression" and observed that WC Phase I preparations appeared to mediate suppression of mitogenic responses, both to *C.burnetii* antigens and to Candida, via an antigen specific suppressor T cell (?CD8+) which after reacting with antigen (presented to the cells in an undefined fashion) secreted a factor (?lymphokine) which induced monocytes to synthesise PGE2, thereby inhibiting lymphocyte mitogenesis supposedly by suppression of endogenous IL-2 production.

It is also possible that the reports of diminished lymphocyte proliferative responses to the panmitogen PHA during acute Q fever in monkeys (Kishimoto and Gonder 1979), and those in mice given large doses of inactivated *C.burnetii* cells in the experiments by Williams and his colleagues (see Chapter 15 and Figure 15.1 (b) for an example in our own work) may also represent a *C.burnetii* Phase I antigen-mediated suppression acting through the monocyte and effected by restriction of IL-2 formation by T lymphocytes.

It is clear that further experiments are required to identify the mediators

Table 16.1: Protective properties, immune T lymphocyte stimulatory activity and stimulation of IFNγ by various *C.burnetii* antigens and subfractions.

REAGENT OR MATERIAL	COMPOSITION	PROTECTIVE EFFICACY (MICE, GUINEA PIGS)	T LYMPHOCYTE RESPONSES		
			MITOGENESIS	IFNγ SECRETED	IFNγ CELLULAR
C.burnetii wc Phase I	Proteins, LPS I peptidoglycan etc	++++	++	±	+
C.burnetii wc Phase II	as above, incomplete LPS II	±	++++	++++	++
C.burnetii wc Phase I Periodate treated	as above, modified LPS I	+	++++	+++	+
Phase I cells extracts, organic solvents (DMSO, TCA-E)	Proteins, LPS I	++++	+→+++	UK	UK
Phase II cells extracts (DMSO, TCA-E)	Protein modified LPS II	±	+++	UK	UK
Phase I cells Chlor. methanol residue	Protein modified LPS I	+++	++++	UK	UK
Phase .II cell lysate PAGE separated proteins	Proteins No LPS	- (predicted)	++++	++++	++
Phenol water extract Phase I organisms	Phase I LPS only	-	-	UK	UK
Phase I antibody Phase II antibody	.. ..	++++ ±	? Up regulation of IFNγ ? Down regulation of IFNγ (i.e. effect of LPS-1 not neutralised)		

UK = unknown .. = not applicable

liberated on the Phase I LPS-monocyte interaction and the details of the circuit down-regulating IFNγ production. Lymphocyte proliferation response-limitation from restriction of endogenous IL-2 production has been a background theme in much of the experimental work described in the thesis.

Although the precise details of the mechanism by which *C.burnetii* Phase I LPS down-regulates IFNγ production by immune T lymphocytes are unclear, it is considered that the phenomonen is well enough established in general by the work described in Chapter 14 to permit an attempt to interpret the respective rôles of *C.burnetii* Phase I LPS and cell proteins (Phase II antigens) in the immune response to the organism. The concept is that two components of the immune response to infection or vaccination are required (i) an antibody to Phase I LPS among other actions to 'neutralise' the immunomodulatory (IFNγ-suppressive) effect of Phase I LPS and (ii) a T lymphocyte response to generate IFNγ to terminate intracellular infection by the coxiella. The ability of Phase I LPS to interfere with the process activating macrophages - the principal host cell for *C.burnetii* - has an obvious survival value for the organism and implications for chronic infection by the coxiella. An antigen-driven immunosuppression also has implications for superinfection with other organisms after Q fever. Finally it also has significance for vaccine formulation and future vaccine development.

Previous work on the protective properties of various whole cell and solvent-fractionated *C.burnetii* preparations was reviewed in detail in Chapters 6 and 7 and is summarised in Table 16.1, along with the T lymphocyte findings resulting from the work described in this thesis.

Key observations to be reconciled in advancing the concept of a dual component immune response are that (1) WC vaccines with Phase I LPS are 100-300 times more protective in laboratory animals, weight-for-weight, than those from wc Phase II organisms; (2) WC Phase I vaccines, treated with periodate, are reduced in protective ability; (3) antibody to Phase I LPS protects whereas that to Phase II proteins does not; (4) the cell proteins ("Phase II antigen") stimulates IFNγ production whereas Phase I LPS down-regulates it.

It thus appears that the Phase I LPS, (and probably antibody to it), is an essential target for protective immunity.

It is therefore suggested that the immune response to the Phase I LPS (either in natural infection, or after vaccination) is required, among other actions to block the-down regulation of IFNγ production following the interaction of the Phase I LPS with the monocyte. If we accept the findings of Kishimoto and Walker (1967a) and Kishimoto *et al* (1977) (see Chapter 6, section 6.3) it seems that antibody to Phase I LPS facilitates phagocytosis and destruction of *C.burnetii* by macrophages. The antibody to Phase I LPS may in effect block the down-regulation of IL2 production and of IFNγ production by combining with the LPS epitopes which otherwise interact with macrophage receptors to produce prostaglandin-like substances which interfere with the development of the immune T-lymphocyte response and macrophage activation for destruction of the organism. However Kishimoto *et al* (1977) findings imply that antibody alone, in macrophages free of lymphocytes (but perhaps already partly activated - see Chapter 6), can destroy the organism.

It seems therefore that a vaccine containing both Phase I LPS and Phase

II proteins - most easily provided as an inactivated whole cell vaccine which also has the advantage of adjuvant properties - will continue to be required. Also that, for example, recombinant DNA vaccine prepared by expressing some of the coxiella proteins, without LPS, is unlikely to be effective. The failure of inactivated vaccines prepared from Phase II whole cells to protect make this prediction credible; Phase II vaccines do not provide the necessary antibody response to Phase I LPS. Nevertheless we propose to test in a mouse protection system the protective efficacy of various fractionated proteins, with and without adjuvant, with and without added Phase I LPS, and with and without Phase I antibody. This work is planned for the near future.

Further work is also required to reconcile the properties of the poorly-defined "immunosuppressive complex" described by Waag and Williams (1988) with the mechanism of Phase I LPS suppressing IFNγ production through a monocyte-T lymphocyte mechanism just proposed. Clarification of this aspect of *C.burnetii* host interaction may hold the key to the pathogenesis of chronic Q fever endocarditis and to conditions such as the post Q fever fatigue syndrome which seems to have an as yet undefined basis in defective immunoregulation.

BIBLIOGRAPHY

Abinanti, F.R. and Marmion, B.P. (1957) Protective or neutralising antibody in Q fever.
Am. J. Hyg. 66: 173-195.

Abou-Zeid, C., Filley, E., Steele, J. and Rook, G.A.W. (1987) A simple new method for using antigens separated by polyacrylamide gel electrophoresis to stimulate lymphocytes *in vitro* after converting bands cut from Western blots into antigen-bearing particles.
J. Immunol. Met. 98: 5-10.

Adams, D.O. and Hamilton, T.A. (1984) The cell biology of macrophage activation.
Ann. Rev. Immunol. 2: 283-318.

Adams, D.O. and Hamilton, T.A. (1987) Molecular transductional mechanisms by which IFNG and other signals regulate macrophage development.
Immunol. Rev. 97: 5-27.

Akporiaye, E.T., Rowatt, J.D., Aragon, A.A. and Baca, O.G. (1983) Lysosomal response of a murine macrophage-like cell line persistently infected with *Coxiella burnetii*.
Infect. & Immun. 40: 1155-1162.

Al-Sumidaie, A.M., Jones, D.L. and Young, H.L. (1984) Characterisation of the under-agarose method for quantifying migration of highly purified human monocytes.
J. Immunol. Meth. 75: 129-140.

Amano, K. and Williams, J.C. (1984) Sensitivity of *Coxiella burnetii* peptidoglycan to lysozyme hydrolysis and correlation of sacculus rigidity with peptidoglycan-associated protein.
J. Bacteriol. 160: 989-993.

Amano, K.-I., Fukushi, K. and Williams, J.C. (1985) Electron microscopic studies of lipopolysaccharides from Phase I and Phase II *Coxiella burnetii*.
J. Gen. Microbiol. 131: 3127-3130.

Amano, K.-I., Williams, J.C., Missler, S.R. and Reinhold, V.N. (1987) Structure and biological relationships of *Coxiella burnetii* lipopolysaccharides.
J. Biol. Chem. 262: 4740-4747.

Anacker, R.L., Lackman, D.B., Pickens, E.G. and Ribi, E. (1962) Antigenic and skin-reactive properties of fractions of *Coxiella burnetii.*
J. Immunol. 89: 145-153.

Anacker, R.L., Haskin, W.T., Lackman, D.B., Ribi, E. and Pickens, E.G. (1963) Conversion of the Phase I antigen of *Coxiella burnetii* to hapten by phenol treatment.
J. Bacteriol. 85: 1165-1170.

Anacker, R.L., Fukushi, K., Pickens, E.G. and Lackman, D.B. (1964) Electron microscopic observations of the development of *Coxiella burnetii* in the chicken yolk sac.
J. Bacteriol. 88: 1130-1138.

Andrews, P.S. and Marmion, B.P. (1959) Chronic Q fever. 2. Morbid anatomical and bacteriological finding in a patient with endocarditis.
Brit. Med. J. 2: 983-988.

Ascher, M.S., Jahrling, P.B., Harrington, D.G., Kishimoto, R.A. and McGann, V.G. (1980) Mechanisms of protective immunogenicity of microbial vaccines: effects of cyclophosphamide pretreatment in Venezuelan encephalitis, Q fever and tuleraemia.
Clin. Exp. Immunol. 41: 225-236.

Ascher, M.S., Berman, M.A., Parker, D. and Turk, J.L. (1983a) Experimental model for dermal granulomatous hypersensitivity in Q fever.
Infect. & Immun. 39: 388-393

Ascher, M., Berman, M. and Ruppanner, R. (1983b) Initial Clinical and Immunologic Evaluation of a New Phase 1. Q fever Vaccine and Skin Test in Humans.
J. Infect. Diseases 148, 2: 214-222.

Ascher, M., Williams, J.C., and Berman, M. (1983c) Dermal Granulomatous Hypersensitivity in Q fever: Comparative Studies of the Granulomatous Potential of Whole Cells of *Coxiella burnetii* Phase 1 and Subfractions.
Infect. & Immun. 42, 3: 887-889.

Axaline, S.G. (1970) Functional biochemistry of the macrophage.
Seminars in Hematology 7: 142-160

Babudieri, B. and Muscovici, C. (1952) Experimental and natural infection of birds by *Coxiella burnetii.*
Nature 169: 195-196.

Babudieri, B. (1959) Q fever - A zoonosis. In: Advances in Veterinary Science. (Ed. Brandly CA and Jungherr EL), Academic Press p 81-182.

Baca, O.G., Hersh, R.T. and Paretsky, D. (1974) Ribosomes and ribonucleic acids of *Coxiella burnetii.*
J. Bacteriol. 166: 441-446.

Baca, O.G. and Paretsky, D. (1974a) Some physiological and biochemical effects of a *Coxiella burnetii* lipopolysaccharide preparation on guinea pigs.
Infect. & Immun. 9: 939-945.

Baca, O.G. and Paretsky, D. (1974b) Partial chemical characterization of a toxic lipopolysaccharide from *Coxiella burnetii.*
Infect. & Immun. 9: 959-961.

Baca, O.G., Martinez, I.L., Aragon, A.S. and Klassen, D. (1980) Isolation and partial characterization of a lipopolysaccharide from phase II *Coxiella burnetii.*
Can. J. Microbiol. 26: 819-826.

Baca, O.G., Aragon, A.S., Akporiaye, E.T., Martinez, I.L. and Warner, N.L. (1981a) Interaction of *Coxiella burnetii* with macrophage-like tumor cell lines. In: Rickettsiae and Rickettsial Diseases. (Ed. Burgdorfer W. and Anacker R.L.) Academic Press Inc., New York p 91-101.

Baca, O.G., Akporiaye, E.T., Aragon, A.S., Martinez, I.L., Robles, M.V. and Warner, N. L. (1981b) Fate of phase I and phase II *Coxiella burnetii* in several macrophage-like tumor cell lines.
Infect. & Immun. 33: 258-266.

Baca, O.G. and Paretsky, D. (1983) Q fever and *Coxiella burnetii*: a model for host-parasite interactions.
Microbiological Reviews 47: 127-149.

Baca, O.G., Scott, T.O., Akporiaye, E.T., DeBlassie, R. and Crissman, H.A. (1985) Cell cycle distribution patterns and generation times of L929 fibroblast cells persistently infected with *Coxiella burnetii.*
Infect. & Immun. 47: 366-369.

Baca, O.G. and Crissman, H.A. (1987) Correlation of DNA, RNA and protein content by flow cytometry in normal and *Coxiella burnetii*-infected L929 cells.
Infect. & Immun. 55: 1731-1733.

Barnes, P.F., Mistry, S.D., Cooper, C.L., Pirmez, C., Rea, T.H. and Modlin, R.L. (1989) Compartmentalization of a CD4+ T lymphocyte subpopulation in tuberculosis pleuritis.
J. Immunol. 142: 1114-1119.

Bate, C.A.W., Tavern, J. and Playfair, J.H.L. (1988) Malarial parasites induce TNF production by macrophages.
Immunology 64: 227-231.

Bate, C.A.W., Tavern, J. and Playfair, J.H.L. (1989) Soluble malarial antigens are toxic and induce the production of tumour necrosis factor *in vivo.*
Immunology 66: 600-605.

Bayer, R.A. (1982) Q fever as an occupational illness at the National Institutes of Health.
Public Hlth Rep. Wash. 97: 58.

Bell, F.J., Lackman, D.B., Meis, A. and Hadlow, W.J. (1964a) Recurrent reaction at site of Q fever vaccination in a sensitized person.
Military Medicine 591-595.

Bell, F.J., Luoto, L., Casey, M. and Lackman, D.B. (1964b) Serologic and skin test responses after Q fever vaccination by the intracutaneous route.
J. Immunol. 93: 403-408.

Benenson, A.S. (1959) Q fever vaccine: efficacy and present status. Symposium on Q fever. (Ed. Smadel, J.E.). **Walter Reed Army Institute of Medical Science Publication No. 6,** US Government Printing Office, Washington, D.C. 47-60.

Bengtson, I.A. (1941) Complement fixation in "Q" fever.
Proc. Soc. Exp. Biol. & Med. 46: 665-668.

Benson, W.W., Brock, D.W. and Mather, J. (1963) Serologic analysis of a penitentiary group using raw milk from a Q fever infected herd.
Pub. Hlth Rep. 78: 707-710.

Berge, T.O. and Lennette, E.H. (1953) Q fever: a study of serological relationships among strains of *Coxiella burnetti* (Derrick)
Amer. J. Hyg. 57: 144-169.

Berge, T.O. and Lennette, E.H. (1953a) World distribution of Q fever: Human, animal and arthropod infection.
Am. J. Hygiene 57: 125-143.

Berge, T.O. (1959) Public health importance of Q fever. Symposium on Q fever. (Ed. Smadel, J.E.). **Walter Reed Army Institute of Medical Science Publication No. 1,** US Government Printing Office, Washington, D.C. 15-19.

Beverley, P.C.L. (1987) Human T cell subsets.
Immunol. Lett. 14: 263-267.

Biggs, B.A., Douglas, J.G., Grant, I.W.B. and Crompton, G.K. (1984) Prolonged Q fever associated with inappropriate secretion of anti-diuretic hormone.
J. Infect. 8: 61-63.

Binford, C.H. and Hauser, G.H. (1944) An epidemic of a severe pneumonitis in the Bayou region of Louisiana. III Pathological observations. Report of autopsy on two cases with a brief note on Psittacosis and Q fever.
Public Health Reports 59: 1363-1374.

Boivin, A., Mesrobeanu, I. and Mesrobeanu, L. (1933) Technique pour la préparation des polysaccharides microbiens spécifiques.
Compt. rend. Soc. de biol. 113: 490-492.

Böyum, A. (1968) Isolation of mononuclear cells and granulocytes from human blood.
Scandinavian Journal of Clinical Laboratory Investigation 97: 77-89.

Brada, M. and Billingham, A.J. (1980) Bone-marrow necrosis and Q fever.
Brit. Med. J. 281: 1108-1109.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principles of protein dye binding.
Annal. Biochem. 72: 248-254

Brezina, R. and Urvolgyi, J. (1962) Study of the antigenic structure of *C.burnetii*. I. Extraction of Phase I antigenic component obtained by means of trichloroacetic acid.
Acta Virol. 6: 84-88.

Brezina, R., Kazar, J. (1963) Phagocytosis of *Coxiella burnetii* and the phase variation phenomenon.
Acta Virol. 7: 476.

Brezina, R., Kazar, J. (1965) Study of the antigenic structure of *Coxiella burnetii*. IV. Phagocytosis and opsonization in relation to the phases of *C.burnetii*.
Acta Virol. 9: 268-274.

Brezina, R., Schramek, S. and Urvolgyi, J. (1965) Study of the antigenic structure of *Coxiella burnetii*. III. Pyrogenic effect of Phase I antigen in experimental guinea pigs.
Acta Virol. 9: 180-185.

Brezina, R., Kazar, J. and Pospisil, V. (1969) Difference in the multiplication of Phase I and II *Coxiella burnetii* in cell culture.
Acta Virol. 13: 455.

Brezina, R., Pospisil, V. and Schramek, S. (1970) Study of the antigenic structure of *Coxiella burnetii*. VII. Properties of phenol extracted Phase I antigenic component.
Acta Virol. 14: 295-301.

Brezina, R. and Pospisil, V. (1970) Study of the antigenic structure of *Coxiella burnetii*. VIII. Immunogenicity of phenol-extracted Phase I antigenic component.
Acta Virol. 14: 302-306.

Brooks, D.L., Ermel, R.W., Franti, C.E., Ruppanner, R., Behymjer, D.E., Williams, J.C. and Stephenson, J.C. (1986) Q fever vaccination of sheep; challenge of immunity in ewes.
Am. J. Vet. Res. 47: 1235-1238.

Burgdorfer, W., Pickens, E.G., Newhouse, V.F. and Lackman, D.B. (1963) Isolation of *Coxiella burnetii* from rodents in Western Montana.
J. Infect. Dis. 112: 181-186.

Burnet, F.M. and Freeman, M. (1937) Experimental studies on the virus of "Q" fever.
Med. J. Aust. 2: 299-305.

Burnet, F.M. (1938) Tissue culture of the Rickettsia of "Q" fever.
Aust. J. Exp. Biol. Med. Sci. 16: 219-224.

Burnet, F.M. and Freeman, M. (1938a) The Rickettsia of "Q" fever: Further experimental studies.
Med. J. Aust. 1: 296-298.

Burnet, F.M. and Freeman, M. (1938b) "Q" fever: factors affecting the appearance of Rickettsiae in mice.
Med. J. Aust. 2: 1114-1116.

Burnet, F.M., Freeman, M., Derrick, E.H. and Smith, D.J.W. (1939) The search for immunological relationship between "Q" fever and other Rickettsioses.
Med. J. Aust. 2: 51-54.

Burnet, F.M. and Freeman, M. (1941a) Studies of the X strain (Dyer) of *Rickettsia burnetii*. I. Chorioallantoic membrane infections.
J. Immunol. 40: 405-419.

Burnet, F.M. and Freeman, M. (1941b) Studies of the X strain (Dyer) of *Rickettsia burnetii*. II. Guinea pig infections, with special reference to immunological phenomena.
J. Immunol. 40: 421-437.

Burton, P.R., Stuechemann, J. and Paretsky, D. (1975) Electron microscopy studies of the limiting layers of the rickettsia *Coxiella burnetii.*
J. Bacteriol. 122: 316-324.

Burton, P.R., Stueckemann, J., Welsh, R.M. and Paretsky, D. (1978) Some ultrastructural effects of persistent infections by the Rickettsia *Coxiella burnetii* in mouse L cells and Green Monkey Kidney (Vero) cells.
Infect. & Immun. 21: 556-566.

Caminopetros, J. (1948) L Q fever en Grece: le lait, source de l'infection pour l'homme et les animaux.
Ann. Parasitol., Paris 23: 107-118.

Cantrell, J.L. and Wheat, R.W. (1979) Antitumor activity and lymphoreticular stimulation properties of fractions isolated from *Corynebacterium parvum.*
Cancer Research 39: 3554-3563.

Carl, M. and Dasch, G.A. (1986a) Characterization of human cytotoxic lymphocytes directed against cells infected with typhus group rickettsiae: evidence for lymphokine activation of effectors.
J. Immunol. 136: 2654-2661.

Carl, M. and Dasch, G.A. (1986b) Lymphokine-Activated- Killer-Mediated Lysis of Cells Infected with Typhus Group Rickettsiae Can be Inhibited by OKT3 Monoclonal Antibody.
Infect. & Immun. 53: 226-228.

Caughey, J.E. (1977) Pleuropericardial lesion in Q fever.
Brit. Med. J. 278: 1447.

Chan, M.L., McChesney, J. and Paretsky, D. (1976) Further characterization of a lipopolysaccharide from *Coxiella burnetii.*
Infect. & Immun. 13: 1721-1727.

Cherwinski, H.M., Schumacher, J.H., Brown, K.D. and Mosmann, T.R. (1987) Two types of mouse helper T cell clones. III Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies.
J. Exp. Med. 166: 1229-1244.

Chesnut, R.W., Colon, S.M. and Grey, H.M. (1982) Requirement for the processing of antigen by antigen presenting B cells. I. Functional comparison of B cell tumors and macrophages.
J. Immunol. 129: 2382-2388.

Chi, D.S. and Harris N.S. (1978) A simple method for the isolation of murine peripheral blood lymphocytes.
J. Immunol. Meth. 19: 169-172.

Ciampor, F., Schramek, S. and Brezina, R. (1972) Electron microscopy of ruthenium red-stained Phase I and II *Coxiella burnetii.*
Acta Virol. 16: 503-506.

Clark, W.H., Lennette, E.H., Railsback, D.C. and Romer, M.S. (1951) Q fever in California VII. Clinical features in one hundred and eighty cases.
Arch. Intern. Med. 88: 155-167.

Clark, I.A. (1979) Resistance to *Babesia* spp. and *Plasmodium* spp. in mice pretreated with an extract of *Coxiella burnetii.*
Infect. & Immun. 24: 319-325.

Colter, J.S., Brown, R.A., Bird, H.H. and Cox, H.R. (1956) The preparation of a soluble immunizing antigen from Q-fever rickettsiae.
J. Immunol. 76: 270-274.

Cox, H.R. (1939) Studies of a filter-passing infectious agent isolated from ticks. V. Further attempts to cultivate in cell-free media. Suggested classification.
Public Health Reports 54: 1822-1827.

Cox, H.R. and Bell, E.J. (1939) The cultivation of *Rickettsia diaporica* in tissue culture and in the tissues of the developing chick embryo.
Public Health Rep. 54: 2171-2178.

Cox, H.R. (1940) *Rickettsia diaporica* and American Q fever.
Am. J. Trop. Med. 20: 463-469.

Cox, H.R. (1941) Cultivation of Rickettsiae of the Rocky Mountain spotted fever, typhus and Q fever groups in the embryonic tissues of developing chicks.
Science 4: 399-403.

Cracea, E., Dumitrescu, S., Mihancea, N., Novac, S. and Zarnea, G.H. (1968) Study of protective antibodies in Q fever vaccination.
Zeit. f. Immunitatforch., Aller. U. Klin. Immunol. 136: 1-9.

Crowle, A.J. (1988) Immunisation against tuberculosis. What kind of vaccine?
Infect. & Immun. 56: 2769.

Curet, L.B. and Paust, J.C. (1972) Transmission of Q fever from experimental sheep to laboratory personnel.
Amer. J. Obst. Gynae. 114: 566.

Dahlman, O., Garegg, P.J., Mayer, H. and Schramek, S. (1986) Synthesis of three 3-C-Hydroxymethylpentose with the D-ribo, D-xylo and L-lyxo-configuration. Identification of the latter with a monosaccharide isolated from phase I *Coxiella burnetii* lipopolysaccharide.
Acta Chem. Scand. B 40: 15-20.

Damrow, T.A., Cantrell, J.L. and Williams, J.C. (1981) Modification of immune competence in mice by Q fever vaccine. In: **Rickettsiae and Rickettsial Diseases,** (Ed. W. Burgdorfer, R.L. Anacker), Academic Press, N.Y. 115-124.

Davis, G.E. and Cox, H.R. (1938) A filter-passing infectious agent isolated from ticks. I. Isolation from *Dermacentor andersoni*, reactions in animals, and filtration experiments.
Pub. Hlth Rep. 53: 2259-2267.

Davis, G.E. (1943) American Q fever. Experimental transmission by the argasid tick *Ornithodorus moubata* and *O.hermsi.*
Pub. Hlth Rep. 58: 984-987.

Delaney, J.C. and Roberts, H.L. (1975) Q fever endocarditis and chronic liver involvement.
Practitioner 214: 243-245.

DeLay, P.D., Lennette, E.H. and DeOme, K.B. (1950) Q fever in California. II. Recovery of *Coxiella burnetii* from naturally-infected air-borne dust.
J. Immunology 65: 211-220.

Derrick, E.H. (1937) "Q" fever, a new fever entity: clinical features, diagnosis and laboratory investigation.
Med. J. Aust. 2: 281-299.

Derrick, E.H. (1939) *Rickettsia burnetii*: The cause of "Q" fever.
Med. J. Aust. 1: 14.

Derrick, H.E., Smith, D.J.W., Brown, H.E. and Freeman, M. (1939) The role of the bandicoot in the epidemiology of "Q" fever: A preliminary study.
Med. J. Aust. 1: 150-155.

Derrick, E.H. and Smith, D.J.W. (1940) Studies in the Epidemiology of "Q" fever. 2. The isolation of three strains of *Rickettsia burnetii* from the bandicoot *Isoodon Torosus.*
Aust. J. Exp. Biol. Med. Sci. 18: 99-102.

Derrick, E.H., Smith, D.J.W. and Brown, H.E. (1940) Studies in the epidemiology of "Q" fever. 6. The susceptibility of various animals.
Aust. J. Exp. Biol. Med. Sc. 18: 409-413.

Derrick, E.H. Smith, D.J.W. Brown, H.E. (1942) Studies in the epidemiology of "Q" fever. 9. The role of the cow in the transmission of human infection.
Aust. J. Exp. Biol. Med. Sc. 20: 105-110.

Derrick, E.H. (1953) The epidemiology of Q fever: a review.
Med. J. Aust. 1: 245.

Derrick, E.H. (1973) The course of infection with *Coxiella burnetii.*
Med. J. Aust. 1: 1051-62

De Titto, E.H., Catterall, J.R. and Remington, J.S. (1986) Activity of recombinant tumor necrosis factor on *Toxoplasma gondii* and *Trypanosoma cruzi.*
J. Immunol. 137: 1342-1345.

Dingle, J.H., Abernethy, T.J., Badger, G.F., Buddingh, G.J., Feller, A.E., Langmuir, A.D., Ruegsegger, J.M., Wood Jr, W.B. (1944) Primary atypical pneumonia, etiology unknown. (Part III).
American Journal of Hygiene 39: 269-336.

Doller, G., Doller, P.C. and Gerth, H.J. (1984) Early diagnosis of Q fever: Detection of Immunoglobulin M by radioimmunoassay and enzyme immunoassay.
Eur. J. Clin. Microbiol. 3: 550-553.

Downs, C.M. (1968) Phagocytosis of *Coxiella burnetii,* phase I and phase II by peritoneal monocytes from normal and immune guinea pigs and mice.
Zbl. Bakt. I. Orig. 206: 329-343.

Dupont, H.L., Hornick, R.B., Levin, H.S., Rapoport, M.I. and Woodward, T.E. (1971) Q fever hepatitis.
Ann. Int. Med. 74: 198-206.

Dupuis, G., Peter, O., Petite, J., Vouilloz, M. and Peacock, M.G. (1983) Q fever outbreak - Switzerland.
MMWR 33: 355-361.

Dupuis, G., Peter, O., Peacock, M., Burgdorfer, W. and Haller E. (1985) Immunoglobulin responses in acute Q fever.
J. Clin. Microbiol. 22: 484-487.

Dupuis, G., Petite, J., Peter, O. and Vouilloz, M. (1987) An important outbreak of human Q fever in a Swiss Alpine Valley.
Int. J. Epidemiol. 16: 282-287.

Dyer, R.E. (1938) A filter-passing infectious agent isolated from ticks. IV. Human infection.
Pub. Hlth Rep. 53: 2277-2282.

Dyer, R.E. (1939) Similarity of Australian "Q" fever and a disease caused by an infectious agent isolated from ticks in Montana.
Pub. Hlth Rep. 54: 1229-1237.

Dyer, R.E., Topping, N.H. and Bengtson, I.A. (1940) An institutional outbreak of pneumonitis II. Isolation and identification of causative agent.
Pub. Hlth Rep. 55: 1945-1954.

Elisberg, B.L. and Bozeman, F.M. (1979) "The Rickettsiae" In: **Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections** (Ed. E.H. Lennette and N.J. Schmidt) American Public Health Association Wash. p 1061-1108

Ellner, J.J. (1978) Suppressor adherent cells in human tuberculosis.
J. Immunol. 121: 2573-2579.

Emmrich, F. (1988) Cross-linking of CD4 and CD8 with the T-cell receptor complex: quaternary complex formation and T-cell repertoire selection.
Immunol. Today 9: 296-300.

Enright, J.B., Franti, C.E., Behymer, D.E., Longhurst, W.M., Dutson, V.J. and Wright, M.E. (1971) *Coxiella burnetii* in a wildlife-livestock environment. Distribution of Q fever in wild mammals.
Am. J. Epidem. 94: 79-90.

Esparza, I., Mannel, D., Ruppel, A., Falk, W. and Krammer,P.H. (1987) Interferon-G and lymphotoxin or tumor necrosis factor act synergistically to induce macrophage killing of tumor cells and schistosomula of *schistosma mansoni.*
J. Exp. Med. 166: 589-594.

Ferencik, M., Schramek, S., Kazar, J. and Stefanovic, J. (1984) Effect of *Coxiella burnetii* on the stimulation of hexose monophosphate shunt and on superoxide anion production in human polymorphonuclear leukocytes.
Acta Virol. 28: 246-250.

Fergusson, R.J., Shaw, T.R.D., Kitchin, A.H., Matthews, M.B., Inglis, J.M. and Peutherer, J.F. (1985) Subclinical chronic Q fever.
Quart. J. Med. 57: 669-676.

Fernandez-Botran, R., Sanders, V.M., Oliver, K.G., Chen, Y.-W., Krammer, P.H., Uhr, J.W. and Vitetta, E.S. (1986) Interleukin 4 mediates autocrine growth of helper T cells after antigenic stimulation.
Proc. Natl. Acad. Sci. USA 83: 9689-9693.

Fiset, P. (1957) Phase variation of *Rickettsia (Coxiella) burnetii.* Study of the antibody response in guinea pig and rabbits.
Canadian J. Microbiol. 3: 435-445.

Fiset, P. (1959) Serological diagnosis, strain identification and antigenic variation. In: **Symposium on Q fever.** (Ed. J.E. Smadel), Walter Reed Army Institute of Medical Science, Pub. No. 6, U.S. Government Printing Office, p 28-33.

Fiset, P. and Ormsbee, R.A. (1968) The antibody response to antigens of *Coxiella burnetii.*
Zbt. Bact. I Orig. 206: 321-329.

Fiset, P., Ormsbee, R.A., Silberman, R., Peacock, M. and Spielman, S.H. (1969) A microagglutination technique for detection and measurement of rickettsial antibodies.
Acta Virol. 13: 60-66.

Fiset, P., Wike, D.A., Pickens, E.G. and Ormsbee, R.A. (1971) An antigenic comparison of strains of *Coxiella burnetii.*
Acta Virol. 15: 161-166.

Fiset, P., Wisseman, C.L. Jr. and El Butani, Y. (1975) Immunological evidence of human fetal infection with *Coxiella burnetii.*
Am. J. Epidemiol. 101: 65-69.

Gajdesova, E. and Brezina, R. (1989) Cell mediated immune response to *Coxiella burnetii* antigens in Q fever convalescents and vaccinees.
Acta Virol. 33: 474-481.

Gearing, A.J.A. and Bird, C.R. (1987) Production and assay of interleukin-2. **In: Lymphokines and Interferons, A Practical Approach.** (Ed. M.J. Clemens, A.G. Morris and A.J.H. Gearing), IRL Press, Washington, p 291-301.

Germer, W.D. (1954) Das Q fieber bein meerweinchen.
Arch Ges. Virus Forschung 5: 336-344.

Gimenez, D.F. (1965) Gram staining of *Coxiella burnetii.*
J. Bacteriol. 90: 834-835.

Gin, W., Christiansen, F.T. and Peter, J.B. (1989) Immune function and the chronic fatigue syndrome.
Med. J. Aust. 151: 117-118.

Godfrey, H.P., Baer, H. and Chaparas, S.D. (1969) Inhibition of macrophage migration by a skin-reactive polysaccharide from BCG culture filtrates.
J. Immunol. 120: 1465-1473.

Gold, D., Bowden, R., Sixbey, J., Riggs, R., Katon, W.J., Ashley, R., Obrigewitch, R.M. and Corey, L. (1990) Chronic fatigue. A prospective clinical and virologic study.
J.A.M.A. 264: 48-53.

Gonder, J.C., Kishimoto, R.A., Kastello, J.D. and Pederson, C.E. (1979) Cynomologous monkey model for experimental Q fever infection.
J. Infect. Dis. 139: 191.

Gordon, S. (1978) Regulation of enzyme secretion by mononuclear phagocytes: studies with macrophage plasminogen activator and lysozyme.
Fed. Proc. 37: 2754-2758.

Hackstadt, T. and Williams, J.C. (1981a) Biochemical stratagems for obligate parasitism of eukaryotic cells by *Coxiella burnetii.*
Proc. Natl. Acad. Sci. USA 78: 3240-3244.

Hackstadt, T. and Williams, J.C. (1981b) Stability of the adenosine 5'-triphosphate pool in *Coxiella burnetii*: Influence of pH and substrate.
J. Bacteriol. 148: 419-425.

Hackstadt, T. and Williams, J.C. (1983) pH Dependence of the *Coxiella burnetii* glutamate transport system.
J. Bacteriol. 154: 598-603.

Hackstadt, T., Peacock, M.G., Hitchcock, P.J. and Cole, R.L. (1985) Lipopolysaccharide variation in *Coxiella burnetii*: Intrastrain heterogeneity in structure and antigenicity.
Infect. & Immun. 48: 359-365.

Hackstadt, T. (1986) Antigenic variation in the lipopolysaccharide of *Coxiella burnetii* isolates.
Infect. & Immun. 52: 337-340.

Hahn, H. (1975) Requirement for a bone marrow-derived component in the expression of cell-mediated antibacterial immunity.
Infect. & Immun. 11: 949-954.

Hahn, H. and Kaufmann, S.H.E. (1981) The role of cell-mediated immunity in bacterial infection.
Rev. Infect. Dis. 3: 1221-1250.

Hahon, N. and Kozikowski, E.H. (1968) Induction of interferon by *Coxiella burnetii* in cell culture.
J. Gen. Virol. 3: 125-127.

Haldane, E.V., Marrie, T.J., Faulkner, R.S., Lee, S.H.S., Cooper, J.H., MacPherson, D.D. and Montague, T.J. (1983) Endocarditis due to Q fever in Nova Scotia: Experience with five patients in 1981-1982.
J. Infect. Dis. 148: 978-985.

Hall, C.J., Richmond, S.J., Caul, E.O., Pearce, N.H. and Silver, I.A. (1982) Laboratory outbreak of Q fever acquired from sheep.
The Lancet i: 1004.

Hamblin, A.S. (1988) "Lymphokines".
IFL Press, Oxford p 35-52.

Hancock, K., and Tsang, V.C. (1983) Indian ink staining of proteins on nitrocellulose paper.
Anal. Biochem. 133: 157-162.

Handman, E. and Burgess, A.W. (1979) Stimulation by granulocyte-macrophage colony-stimulating factor of *Leishmania tropica* killing by macrophages.
J. Immunol. 122: 1134-1137.

Haregewoin, A., Godal, T., Mustafa, A.S., Belehu, A. and Yemaneberhan, T. (1983) T-cell condition media reverse T-cell unresponsiveness in lepromatous leprosy.
Nature 303: 342-344.

Harms, G., Zwingenberger, K., Chehade, A.K., Talhari, S., Racz, P., Mouakeh, A., Douba, M., Nakel, L., Naiff, R.D., Kremsner, P.G., Feldmeier, H. and Bienzle, U. (1989) Effects of intradermal gamma-interferon in cutaneous leishmaniasis.
Lancet 1: 1287-1292.

Heggers, J.P., Mallavia, L.P. and Hinrichs, D.J. (1974) The cellular immune response to antigens of *Coxiella burnetii*
Canadian J. Microbiol. 20: 657-662.

Heinzel, F.P., Sadick, M.D., Holaday, B.J., Coffman, R.L. and Locksley, R.M. (1989) Reciprocal expression of interferon G or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets.
J. Exp. Med. 168: 59-72.

Hemmingson, S.M., Woolford, C., van der Vies, S.M., Tilly, K., Dennis, D.T., Georgopoulos, C.P., Hendrix, R.W. and Ellis, R.J. (1988) Homologous plant and bacterial proteins chaperone oligomeric protein assembly.
Nature 333: 330-334.

Herzberg, K. and Urback, H. (1951) Untersuch migen mit europaichen Q Fieber - antigenen 1.
Mitteilung Ztschr. f. Immunitat sf un Exper. Therap. 108: 376-388.

Hinrichs, D.J. and Jerrells, T.R. (1976) In vitro evaluation of immunity to *Coxiella burnetii.*
J. Immunol. 117: 996-1003.

Hoover, D.L., Nacy, C.A. and Meltzer, M.S. (1985) Human monocyte activation for cytotoxicity against intracellular *Leishmania donovani* amastigotes: induction of microbicidal activity by interferon-G.
Cell. Immunology 94: 500-511.

Hornibrook, J.W. and Nelson, K.R. (1940) An institutional outbreak of pneumonitis I. Epidemiological and clinical studies.
Public Health Reports 55: 1936-1944.

Hoyer, B.H., Ormsbee, R.A., Fiset, P. and Lackman, D.B. (1963) Differentiation of phase I and phase II *Coxiella burnetii* by equilibrium density gradient sedimentation.
Nature 197: 573-574.

Huebner, R.J. (1947) Report of an outbreak of Q fever at the National Institute of Health. II Epidemiological features.
Amer. J. Publ. Health 37: 431-440.

Huebner, R.J., Jellison, W.L., Beck, M.D., Parker, R.R. and Shepard, C.C. (1948) Q fever studies in Southern California 1. Recovery of *Rickettsia burnetii* from raw milk.
Public Hlth Rep 63: 214-222.

Huebner, R.J. and Bell, J.A. (1951) Q fever studies in Southern California. Summary of current results and a discussion of possible control measures.
JAMA 145: 301-305.

Humphries, R.G. and Hinrichs, D.J. (1981) Role for antibody in *Coxiella burnetii* infection.
Infect. & Immun. 31: 641-645.

Hunt, J.G., Field, P.R. and Murphy, A.M. (1983) Immunoglobulin responses to *Coxiella burnetii* (Q fever): single-serum diagnosis of acute infection, using an immunofluorescence technique.
Infect. & Immun. 39: 977-981.

Irons, J.V. and Hooper, J.M. (1947). Q fever in the United States. II. Clinical data on an outbreak among stock handlers and slaughterhouse workers.
J. American Med. Assoc. 133: 815-818.

Irons, J.V., Murphy Jnr, J.N. and Wolfe, D.M. (1947). Q fever in the United States. III. Serologic observations in an outbreak among stock handlers and slaughterhouse workers.
J. American Med. Assoc. 133: 819-820.

Izzo, A.A., Marmion, B.P. and Worswick, D.A. (1988) Markers of cell-mediated immunity after vaccination with an inactivated, whole-cell Q fever vaccine.
J. Infect. Dis. 157: 781-789.

Janigan, D.T. and Marrie, T.J. (1983) An inflammatory pseudotumor in the lung in Q fever pneumonia.
N. Engl. J. Med. 308: 86-88.

Jelinek, D.F., Splawski, J.B. and Lipsky, P.E. (1986) The roles of interleukin 2 and interferon-γ in human B cell activation, growth and differentiation.
Eur. J. Immunol. 16: 925-932.

Jerrells, T.R., Mallavia, L.P. and Hinrichs, D.J. (1975) Detection of long-term cellular immunity to *Coxiella burnetii* as assayed by lymphocyte transformation.
Infect. & Immun. 11: 280-286.

Johnson, J.W., Eddy, G.A. and Pederson, C.E. (1976) Biologic properties of the M44 strain of *Coxiella burnetii.*
J. Infect. Dis. 133: 334-338.

Johnson, J.W., McLeod, C.G., Stookey, J.L., Higbee, G.A. and Pederson, C.E. (1977) Lesions in guinea pigs infected with *Coxiella burnetii* strain M44.
J. Infect. Dis. 135: 995-998.

Jones Jr., F. and Paretsky, D. (1967) Physiology of Rickettsiae. VI. Host-independent synthesis of polyribonucleotides by *Coxiella burnetii.*
J. Bacteriol. 93: 1063-1068.

Jones, B.M., Nicholson, J.K., Holman, R.C. and Hubbard, M. (1989) Comparison of monocyte separation methods using flow cytometric analysis.
J. Immunol. Meth. 125: 41-47.

Jungi, T.W. and McGregor, D.D. (1977) Generation of macrophage chemotactic activity *in situ* in Listeria-immune rats.
Cell. Immunol. 33: 322-339.

Kaplan, M.M. and Bertagna, P. (1955) The geographical distribution of Q fever.
Bull. Wld. Hlth. Org. 13: 829-860.

Kaplan, G., Weinstein, D.E., Steinman, R.M., Levis, W.R., Elvers, U., Patarroyo, M.E. and Cohn, Z.A. (1985) An analysis of in vitro T cell responsiveness in lepromatous leprosy.
J. Exp. Med. 162: 917-929.

Kaplan, G. and Cohn, Z. (1986) Regulation of cell-mediated immunity in lepromatous leprosy.
Lepr. Rev. 57, Suppl. 2: 199-202.

Kaplan, G., Nusrat, A., Sarno, E.N., Job, C.K., McElrath, J., Porto, J.A., Nathan, C.F. and Cohn, Z.A. (1987) Cellular responses to the intradermal injection of recombinant human G-interferon in lepromatous leprosy patients.
American J. Pathology 128: 345-353.

Kaplan, G., Mathur, N.K., Job, C.K., Nath, I. and Cohn, Z.A. (1989) Effect of multiple interferon G injections on the disposal of *Mycobacterium leprae.*
Proc. Natl. Acad. Sci. USA 86: 8073-8077.

Karalin, B.E. (1960) The geographico-ecological analysis of foci of Q fever.
J. Microbiol. Epidemiol. Immunobiol. 31: 1597-1604.

Kaufmann, S., Weber, L. and Hahn, H. (1975) Macrophage inhibiting activity in serum and central lymph of Listeria-immune mice.
Eur. J. Immunol. 5: 799-800.

Kaufmann, S.H.E. (1988) CD8+T lymphocyte in intracellular microbial infection.
Immunol. Today 9: 168-174.

Kazar, J. (1966) Interferon-like inhibitor in mouse sera induced by rickettsiae.
Acta Virol. 10: 277.

Kazar, J. (1969) Multiplication of *Coxiella burnetii* in virus-infected and interferon-treated cell cultures.
Acta Virol. 13: 346-348.

Kazar, J., Brezina, R. and Mayer, V. (1971) Study on the effect of cyclophosphamide on experimental rickettsial infection.
Acta Virol. 15: 499-509.

Kazar, J., Brezina, R., Kovacova, E. and Urvolgyi, J. (1973) Testing in various systems of the neutralizing capacity of Q fever immune sera.
Acta Virol. 17: 79-89.

Kazar, J., Brezina, R., Schramek, S., Urvolgyi, J., Pospisil, V. and Kovacova, E. (1974) Virulence, antigenic properties and physiochemical characteristics of *Coxiella burnetii* strains with different chick embryo yolk sac passage history.
Acta Virol. 18: 434-442.

Kazar, J., Brezina, R., Skultetyova, E., Kovacova, E. and Schramek, S. (1975a) Phase II to phase I conversion of *Coxiella burnetii* in immunosuppressed mice.
Acta Virol. 19: 359-363.

Kazar, J., Skultetyova, E. and Brezina, R. (1975b) Phagocytosis of *Coxiella burnetii* by macrophages.
Acta Virol. 19: 426-431.

Kazar, J., El. Najdawi, E., Brezina, R. and Schramek, S. (1977) Search for correlates of resistance to virulent challenge in mice immunised with *Coxiella burnetii.*
Acta Virol. 21: 422-430.

Kazar, J., Brezina, R., Palanova, A., Rvrda, B. and Schramek, S. (1982) Immunogenicity and reactogenicity of a Q fever chemovaccine in persons professionally exposed to Q fever in Czechoslovakia.
Bull. Wrld. Hlth. Org. 60: 389-394.

Kazar, J., Schramek, S. and Brezina, R. (1984) The value of skin testing in "Q" fever convalescents and vaccinees as indicator of antigenic exposure and inducer of antibody recall.
Acta Virol. 28: 134-140.

Kazar, J. and Schramek, S. (1985) Relationship between delayed hypersensitivity reaction and resistance to virulent challenge in mice immunised with different *Coxiella burnetii* antigenic preparations. **In: Rickettsiae and Rickettsial Diseases,** Proc 3rd International Symposium (Ed. Kazar, J.) Slovak Acad. Sciences, p 282.

Kazar, J., Votruba, D., Propper, P. and Schramek, S.l (1986) Onset and duration of immunity in guinea pigs and mice induced with different Q fever vaccines.
Acta Virol. 30: 499-506.

Kelly, M.T., Granger, D.L., Ribi, E. Milner, K.C., Strain, S.M. and Stoenner, H.G. (1976) Tumor regression with Q fever rickettsiae and a mycobacterial glycolipid.
Cancer Immunology and Immunotherapy 1: 187-191.

Kelly, M.T. (1977) Activation of guinea pig macrophages by Q fever rickettsiae.
Cell. Immunol. 28: 198-205.

Kersten, W.J. and Hesseluis, G.U. (1983) Rapid automatic, high capacity Dumas determination of nitrogen.
Microchemical Journal, 28: 120-126.

Khavkin, T. (1972) Comparative study of infectious processes induced by *Rickettsia burnetii* and *Rickettsia prowazekii.* Doctoral Dissertation (Sci Med) Res. Inst. Exp. Med. and Pasteur Inst. Leningrad URRR 1972 **Cited in: Q fever - The Disease,** Volume 1 (Ed. Marrie T.J.) CRC Press 1990.

Khavkin, T., Sukhinin, V. and Amosen Kova, N. (1981) Host parasite interaction and development of intraforms in chicken embryos infected with *Coxiella burnetii* via the yolk sac.
Infect. & Immunity 32: 1281.

Kilgus, J., Romagnoli, P., Guttinger, M., Stuber, D., Adorini, L. and Sinigaglia, F. (1989) Vaccine T-cell epitope selection by a peptide competition assay.
Proc. Natl. Acad. Sci. USA 86: 1629-1633.

Kimbrough, R.C., Ormsbee, R.A., Peacock, M., Rogers, W.R., Bennetts, R.W., Raaf, J., Krause, A. and Gardener, C. (1979) Q fever endocarditis in the United States.
An. Int. Med. 91: 400-402.

Kindler, V., Sappino, A.P., Grau, G.E., Piguet, P.-F. and Vassalli, P. (1989) The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection.
Cell 56: 731-740.

Kindmark, C.O. and Williams, J.C. (1990) Interaction between *Coxiella burnetii* and C reactive protein. **Cited in** Waag DM, **Q fever the disease.** (Ed. T.J. Marrie) CRC Press.

Kishimoto, R.A., Veltri, B.J., Canonico, P.G., Shirley, F.G. and Walker, J.S. (1976) Electron microscopic study on the interaction between normal guinea pig peritoneal macrophages and *C.burnetii.*
Infect. & Immun. 14: 1087-1096.

Kishimoto, R.A. and Walker, J.S. (1976) Interaction between *Coxiella burnetii* and guinea pig peritoneal macrophages.
Infect. & Immun. 14, 3: 416-421.

Kishimoto, R.A. and Burger, G.T. (1977) Appearance of cellular and humoral immunity in guinea pigs after infection with *Coxiella burnetii* administered in small-particle aerosols.
Infect. & Immun. 16: 518-521.

Kishimoto, R.A., Veltri, B.J., Shirey, F.G., Canonico, P.G. and Walker, J.S. (1977) Fate of *Coxiella burnetii* in macrophages from immune guinea pigs.
Infect. & Immun. 15, 2: 601-607.

Kishimoto, R.A., Johnson, J.W., Kenyon, R.H., Ascher, M.A., Larson, E.W. and Pedersen, C.E. (1978a) Cell mediated immune response of guinea pig to an inactivated phase 1 *Coxiella burnetii* vaccine.
Infect. & Immun. 19, 1: 194-198.

Kishimoto, R.A., Rozmiarek, H. and Larson, E.W. (1978b) Experimental "Q" fever infection in congenitally nude mice.
Infect. & Immun. 22, 1: 69-71.

Kishimoto, R.A. and Gonder, J.C. (1979) Suppression of PHA-stimulated lymphocyte transformation in cynomolgus monkeys following infection with *Coxiella burnetii*.
Can. J. Microbiol. 25: 949-952.

Klebanoff, S.J. (1980) Oxygen metabolism and the toxic properties of phagocytes.
Ann. Intern. Med. 93: 480-489.

Kordova, N. and Rehacek, J. (1959) Cultivation of ultrafiltrable particles of *Coxiella burnetii* in the tick organism.
Folia Micro. 4: 275-279.

Kordova, N. and Brezina, R. (1963) Multiplication dynamics of Phase I and II *Coxiella burnetii* in different cell cultures.
Acta Virol. 7: 84-87.

Koster, F.T., Kirkpatrick, T.L., Rowatt, J.D. and Baca, O.G. (1984) Antibody-dependent cellular cytotoxicity of *Coxiella burnetii*-infected J774 macrophage target cells.
Infect. & Immun. 43: 253-256.

Koster, F.T., Williams, J.C. and Goodwin, J.S. (1985a) Cellular immunity in Q fever: specific lymphocyte unresponsiveness in Q fever endocarditis.
J. Infect. Dis. 152: 1283-1289.

Koster, F.T., Williams, J.C. and Goodwin, J.S. (1985b) Cellular immunity in Q fever: modulation of responsiveness by a suppressor T cell-mediated circuit.
J. Immunol. 135: 1067-1072.

Kostiala, A.A., McGregor, D.D. and Lefford, M.J. (1976) The mediator of cellular immunity. XI. Origin and development of MIF producing lymphocytes.
Cell Immunol. 24: 318-327.

Kovacova, E., Gallo, J., Schramek, S., Kazar, J. and Brezina, R. (1987) *Coxiella burnetii* antigens for detection of Q fever antibodies by ELISA in human sera.
Acta Virol. 31: 254-259.

Krauss, H., Schieffer, H.-G. and Schmatz, H.-D. (1977) Ultrastructural investigations on surface structures involved in *Coxiella burnetii* phase variation.
Infect. & Immun. 15: 890-896.

Kriegler, M., Perez, C., DeFay, K., Albert, I. and Lu, S.D. (1988) A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF.
Cell 53: 45-53.

Lackman, D.B., Bell, E.J., Bell, J.F. and Picken, E.G. (1962) Intradermal sensitivity testing in man with a purified vaccine for Q fever.
Amer. J. Public Health 52: 87-92.

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

Lamb, J.R. and Young, D.B. (1987) A novel approach to the identification of T-cell epitopes in Mycobacterium tuberculosis using human T-lymphocyte clones.
Immunology 60: 1-5.

Lang, G.H. (1990) Coxiellosis (Q fever) in animals. **In: Q fever Volume 1 The Disease.** (Ed. T.J. Marrie) CRC Press, Boca Raton, p 24-42.

Lee, S.P., Stoker, N.G., Grant, K.A., Handzel, Z.T., Hussain, R., McAdam, K.P.W.J. and Dockrell, H.M. (1989) Cellular immune responses of leprosy contacts to fractionated *Mycobacterium leprae* antigens.
Infect. and Immun. 57: 2475-2480.

Lennette, E.H., Dean, B.H., Abinanti, F.R., Clark, W.H., Winn, J.F. and Holmes M.A. (1951) Q fever in California. V. Serologic survey of sheep, goats and cattle in three epidemiologic categories, from several geographic areas.
American J. Hygiene 54: 1-14.

Leyk, W. and Scheffler, R. (1975) Multiplication of *Coxiella burnetii* in different tissues and organs of embryonated eggs.
Zentralbl. Bakteriol. Parasitenkd. Infect. Hyg. Abt. 1 Orig. Reihe A. 231 519-524.

Lichtman, A.H., Kurt-Jones, E.A. and Abbas, A.K. (1987) B-cell stimulatory factor 1 and not interleukin 2 is the autocrine growth factor for some helper T lymphocytes.
Proc. Natl. Acad. Sci. USA 84: 824-827.

Liew, F.Y. (1989) Functional heterogeneity of CD4+ T cells in leishmaniasis.
Immunol. Today 10: 40-45.

Lillie, R.D. (1942) Pathological histology in guinea pigs following intraperitoneal inoculation with the virus of "Q" fever.
Public Health Reports 57: 296-306.

Lim, K.C.L. and Kang, J.Y. (1980) Q fever presenting with gastroenteritis.
Med. J. Aust. 1: 327.

Lipton, J.H., Fong, T.C., Gill, M.J., Burgess, K. and Elliot, P.D. (1987) Q fever inflammatory pseudotumor of the lung.
Chest 92: 756-757.

Little, J.S., Kishimoto, R.A. and Canonico, P.G. (1983) Intracellular fate of Phase I *Coxiella burnetii* in guinea pig peritoneal macrophages.
J. Reticul. Soc. 33: 331-334.

Lloyd, A.R., Wakefield, D., Boughton, C.R. and Dwyer, J.M. (1989) Immunological abnormalities in the chronic fatigue synrome.
Med. J. Aust. 151: 122-124.

Lukacova, M., Brezina, R., Schramek, S. and Pastorek, J. (1989) Chemical composition of Phase I *Coxiella burnetii* soluble antigen prepared by trichloroacetic acid extraction.
Acta Virol. 33: 75-80.

Lumio, J., Penttinen, K. and Pettersson, T. (1981) Q fever in Finland: Clinical, Immunological and Epidemiological finding.
Scand. J. Infect. Dis. 13: 17-21.

Luoto, L. and Huebner, R.J. (1950) Q fever studies in Southern California IX Isolation of Q fever organisms from paturient placentas of naturally infected dairy cows.
Public Hlth Rep. 65: 541-544.

Luoto, L. (1956) A capillary-tube test for antibody against *Coxiella burnetii* in human, guinea pig and sheep sera.
J. Immunol. 77: 294-298.

Luoto, L., Bell, J.F., Casey, M. and Lackman, D.B. (1963) Q fever vaccination of human volunteers. I. The serologic and skin-test response following subcutaneous injections.
Am. J. Hyg. 78: 1-15.

Mackaness, G.B. (1970) The monocyte in cellular immunity.
Seminars Hemat. 7: 172-184.

Mallavia, L. and Paretsky, D. (1963) Studies on the physiology of rickettsiae. V. Metabolism of carbamyl phosphate by *Coxiella burnetii.*
J. Bacteriol. 86: 232-238.

Marchette, N. (1982) *Coxiella burnetii* - its origin and distribution. **In: Ecological Relationships and Evolution of the Rickettsiae. CRC Press,** Boca Raton, Florida Chapter 10: 65-95.

Marmion, B.P. (1953) World-wide Q fever.
The Lancet 2: 616-617.

Marmion, B.P., Stoker, M.G.P., McCoy, J.H., Malloch, R.A. and Moore, R.A. (1953) Q fever in Great Britain. An analysis of 69 sporadic cases, with a study of the prevalence of infection in humans and cows.
The Lancet 1: 503-510.

Marmion, B.P. and Stoker, M.G.P. (1958) The epidemiology of Q fever in Great Britain. An analysis of the findings and some conclusions.
Brit. Med. J. 2: 809-816.

Marmion, B.P. (1962) Subacute rickettsial endocarditis: an unusual complication in Q fever.
J. Hyg., Epid., Micro. & Immunol. 6: 79-84.

Marmion, B.P., Plackett, P. and Lemcke, R.M. (1967) Immunochemical analysis of *Mycoplasma pneumoniae.* I. Methods of extraction and reaction of fractions from *M.pneumoniae* and from *M.mycoides* with homologous antisera and antisera against *Streptococcus MG.*
Aust. J. Exp. Biol. Med. Sci. 45: 163-187.

Marmion, B.P., Ormsbee, R., Kyrkou, M., Wright, J., Worswick, D., Cameron, S., Esterman, A., Feery, B. and Collins W. (1984) Vaccine Prophylaxis of Abattoir-Associated "Q" fever.
The Lancet II: 1411-1414.

Marmion, B.P., Ormsbee, R.A., Kyrkou, M., Wright, J., Worswick, D.A., Izzo, A.A., Esterman, A., Feery, B. and Shapiro, R.A. (1990) Vaccine prophylaxis of abattoir-associated Q fever: eight years' experience in Australian abattoirs.
Epidemiol. Infect. 104: 275-287.

Marrie, T.J. (1990a) Epidemiology of Q fever. **In: Q fever: The Disease Volume 1.** (Ed. T.J. Marrie) CRC Press, Boca Raton, Flo., USA, p 50-66.

Marrie, T.J. (1990b) Q fever hepatitis. **In: Q fever: The Disease. Volume 1** (Ed. T.J. Marrie) CRC Press, Boca Raton, Flo., USA, p 172-176.

Matsudaira, P.T. and Burgess, D.R. (1978) SDS microslab linear gradient polyacrylamide gel electrophoresis.
Anal. Biochem. 87: 386-396.

Matthies, M.S., Silverman, M. and Paretsky, D. (1963) Studies on the physiology of rickettiae. IV. Folic acids of *Coxiella burnetii.*
J. Bacteriol. 85: 37-41.

McCaul, T.F., Hackstadt, T. and Williams, J.C. (1981) Ultrastructural and biological aspects of *Coxiella burnetii* under physical disruption. **In: Rickettsiae and Rickettsial Diseases,** (Ed. W. Burgdorfer, R.L. Anacker), Academic Press, N.Y. 267-280.

McCaul, T.F. and Williams, J.C. (1981) Developmental cycle of *Coxiella burnetii*: structure and morphogenesis of vegetative and sporogenic differentiations.
J.Bacteriol. 147: 1063-1076.

McDonald, T.L. and Mallavia, L. (1971) Biochemistry of *Coxiella burnetii*: Embden-Meyerhof pathway.
J. Bacteriol. 107: 864-869.

McGregor, D.D., Hahn, H.H. and Mackaness, G.B. (1973) The mediators of cellular immunity V. Development of cellular resistance to infection in thymectomized irradiated rats.
Cell. Immunol. 6: 186-199.

McKiel, J.A. and Millar, A.M. (1968) Serodiagnosis of Q fever with special emphasis on the radioisotope precipitation test.
Can. J. Microbiol. 14: 721-726.

Mehra, V., Bloom, B.R., Torigian, V.K., Mandich, D., Reichel, M., Young, S.M.M., Salgame, P., Convit, J., Hunter, S.W., McNeil, M., Brennan, P.J., Rea, T.H. and Modlin, R.L. (1989) Characterization of *Mycobacterium leprae* cell wall-associated proteins with the use of T lymphocyte clones.
J. Immunol. 142: 2873-2878.

Meiklejohn, G. and Lennette, E.H. (1950) Q fever in California. I. Observations on vaccination of human beings.
American J. Hygiene 52: 54-64.

Meiklejohn, G., Reimer, L.G., Graves, P.S. and Helmick, C. (1981) Cryptic epidemic of Q fever in a medical school.
J. Infect. Dis. 144: 107-113.

Melby, P.C., and Sacks, D.L. (1989) Identification of antigens recognised by T cells in human leishmaniasis: analysis of T cell clones by immunoblotting.
Infect. & Immunol. 57: 2971-2976.

Meltzer, M.S. (1981) Macrophage activation for tumor cytotoxicity: characterization of priming and trigger signals during lymphokine activation.
J. Immunology 127: 179-183.

Meuer, S.C., Schlossman, S.F. and Reinherz, E.L. (1982) Clonal analysis of human cytoxic T lymphocytes: T4 and T8 effector T cells recognize products of different major histocompatibility complex regions.
Proc. Natl. Acad. Sci. USA. 79: 4395-4399.

Mikelvie, P. (1980) Q fever in a Queensland meatworks.
Med. J. Aust. 1: 590-593.

Modlin, R.L., Mehra, V., Wong, L., Fujimaya, Y., Chang, W.-C., Horwitz, D.A., Bloom, B.R., Rea, T.H. and Pattengale, P.K. (1986) Suppressor T lymphocytes from lepromatous leprosy skin lesions.
J. Immunol. 137: 2831-2834.

Modlin, R.L., Melancon-Kaplan, J., Young, S.M.M., Pirmez, C., Kino, H., Convit, J., Rea, T.H. and Bloom, B.R. (1988) Learning from lesions: Patterns of tissue inflammation in leprosy.
Proc. Natl. Acad. Sci. USA 85: 1213-1217.

Moll, H., Mitchell, G.F., McConville, M.J. and Handman, E. (1989) Evidence of T-cell recognition in mice of a purified lipophosphoglycan from *Leishmania major.*
Infect. & Immun. 57 (11): 3349-56.

Moos, A. and Hackstadt, T. (1987) Comparative virulence of intra- and interstrain lipopolysaccharide variants of *Coxiella burnetii* in the guinea pig model.
Infect. & Immun. 55: 1144-1150.

Moretta, A., Pantaleo, G., Mingari, M.C., Moretta, L. and Cerottini, J.C. (1984) Clonal heterogenicity in the requirement for T3, T4 and T8 molecules in human cytolytic T lymphocyte functions.
J. Exp. Med. 159: 921-934.

Morimoto, C., Letvin, N.L., Distaso, J.A., Aldrich, W.R. and Schlossman, S.F. (1985) The isolation and characterization of the human suppressor inducer T cell subset.
J. Immunol. 134: 1508-1515.

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

Murphy, A.M. and Hunt, J.G. (1981) Retrospective diagnosis of Q fever in a country abattoir by the use of specific IgM globulin estimation.
Med. J. Aust. 2: 326-327.

Murphy, J.R., Wisseman, C.L. and Fiset, P. (1978) Mechanisms of immunity in typhus infection: some characteristics of *Rickettsia mooseri* infection in guinea pigs.
Infect. & Immun. 21: 417-424.

Murphy, J.R., Wisseman, C.L. and Fiset, P. (1979) Mechanisms of immunity in typhus infection: adoptive transfer of immunity in *Rickettsia mooseri.*
Infect. & Immun. 24: 387-393.

Murphy, J.R., Wisseman, C.L. and Fiset, P. (1980) Mechanisms of immunity in typhus infection: analysis of immunity to *Rickettsia mooseri* infection of guinea pigs.
Infect. & Immun. 27: 730-738.

Murray, H.W., Rubin, B.Y. and Rothermel, C.D. (1983) Killing of intracellular *Leishmania donovani* by lymphokine-stimulated human mononuclear phagocytes. Evidence that interferon-G is the activating lymphokine.
J. Clin. Invest. 72: 1506-1510.

Mustafa, A.S., Kvalheim, G., Degre, M. and Godal, T. (1986) Mycobacterium bovis BCG-induced human T-cell clones from BCG-vaccinated healthy subjects: antigen specificity and lymphokine production.
Infect. & Immun. 53: 491-497.

Mustafa, A.S., and Godal, T. (1987) BCG induced CD4+ cytotoxic T cells from BCG vaccinated healthy subjects: relation between cytotoxicity and suppression *in vitro.*
Clin. Exp. Immunol. 69: 255-262.

Myers, W.F. and Paretsky, D. (1961) Synthesis of serine by *Coxiella burnetii.*
J. Bacteriol. 82: 761-763.

Myers, W.F., Baca, O.G. and Wisseman Jr., C.L. (1980) Genome size of the rickettsia *Coxiella burnetii.*
J. Bacteriol. 144: 460-461.

Nacy, C.A., Leonard, E.J. and Meltzer, M.S. (1981) Macrophages in resistance to rickettsial infections: characterization of lymphokines that induce rickettsiacidal activity in macrophages.
J. Immunol. 126: 204-207.

Nacy, C.A. and Meltzer, M.S. (1982) Macrophages in resistance to rickettsial infection: strains of mice susceptible to the lethal effects of *Rickettsia akari* show defective macrophage rickettsicidal activity.
Infect. & Immunol. 36: 1096-1101.

Nacy, C.A., Oster, C.N., James, S.L., and Meltzer, M.S. (1984) Activation of macrophages to kill rickettsiae and Leishmania: dissociation of intracellular microbicidal activities and extracellular destruction of neoplastic and helminth targets.
Contemp. Top. Immunobiol. 13: 147-170.

Nathan, C.F. (1982) Secretion of oxygen intermediates: role in effector functions of activated macrophages.
Fed. Proc. 41: 2206-2211.

Nathan, C.F. (1983) Mechanism of macrophage antimicrobial activity.
Trans. Roy. Soc. Trop. Med. Hyg. 77: 620-630.

Nathan, C.F., Murray, H.W., Wiebe, M.E. and Rubin, B.Y. (1983) Identification of interferon-G as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity.
J. Exp. Med. 158: 670-689.

Nathan, C.F., Prenderggast, T.J., Wiebe, M.E., Stanley, E.R., Platzer, E., Remold, H.G., Welte, K., Rubin, B.Y. and Murray, H.W. (1984) Activation of human macrophages. Comparison of other cytokines with interferon-G.
J. Exp. Med. 160: 600-605.

Nathan, C.F., Kaplan, G., Levis, W.R., Nusrat, A., Witmer, M.D., Sherwin, S.A., Job, C.K., Horowitz, C.R., Steinman, R.M. and Cohn, Z.A. (1986) Local and systemic effects of intradermal recombinant interferon-G in patients with lepromatous leprosy.
New England J. Med. 315: 6-15.

Nermut, M.V., Schramek, S. and Brezina, R. (1968) Electron microscopy of *Coxiella burnetii* in phase I and II.
Acta Virol. 12: 446-452.

Nogueira, N., Kaplan, G., Sarno, E.N., Kushner, P., Granalli-Piperno, A., Vieira, L., Gould, V.C., Levis, W., Steinman, R., Yip, Y.K. and Cohn, Z.A. (1983) Defective G interferon production in leprosy. Reversal with antigen and interleukin 2.
J. Exp. Med. 158: 2165-2170.

Noguchi, H. (1926) A filter-passing virus obtained from *Dermacentor andersoni.*
J. Exp. Med. 44: 1-10.

North, R.J. (1974) T cell dependence of macrophage activation and mobilization during infection with *Mycobacterium tuberculosis.*
Infect. & Immun. 10: 66-71.

Norvak, M. and Brezina, R. (1989) Comparison of protein and lipopolysaccharide patterns of several *Coxiella burnetii* strains in Phase I.
Acta Virol 33: 172-176.

Okun, D.B., Sun, N.C.J. and Tanuka, K.R. (1979) Bone marrow granulomas in Q fever.
A.J.C.P. 71: 117-121.

Onwubalili, J.K., Scott, G.M. and Robinson, J.A. (1985) Deficient immune interferon production in tuberculosis.
Clin. Exp. Immunol. 59: 405-413.

Ormsbee, R.A. (1961) A method of purifying *Coxiella burnetii* and other pathogenic rickettsiae.
J. Immunol. 88: 100-108.

Ormsbee, R.A., Bell, E. J. and Lackman, D.B. (1962) Antigens of *Coxiella burnetii* I. Extraction of antigens with nonaqueous organic solvents.
J. Immunol. 88: 741-749.

Ormsbee, R.A. (1964) An agglutination-resuspension test for Q fever antibodies.
J. Immunol. 92: 159-166.

Ormsbee, R.A., Bell, E.J., Lackman, D.B. and Tallent, G. (1964) The influence of phase on the protective potency of Q fever vaccine.
J. Immunol. 92: 404-412.

Ormsbee, R.A. and Peacock, M.G. (1964) Metabolic activity in *Coxiella burnetii.*
J. Bacteriol. 88: 1205-1210.

Ormsbee, R., Peacock, M., Talleng, G. and Munoz, J.J. (1968) An analysis to the immune response to rickettsial antigen in the guinea pig.
Acta. Virol. 12: 78-82.

Ormsbee, R., Peacock, M., Gerloff, R., Tallent, G. and Wike, D. (1978) Limits of rickettsial infectivity.
Infect. & Immun. 19: 239-245.

O'Rourke, A.T., Peacock, M., Samuel, J.E., Frazier, M.E., Natvig, D.O., Mallavia, L.P. and Baca, O. (1985) Genomic analysis of phase I and II *Coxiella burnetii* with restriction endonucleases.
J. Gen. Microbiol. 131: 1543-1546.

Ottenhoff, T.M.H., Elerink, D.G., Klaster, P.R. and de Vries, R.R.P. (1986) Cloned suppressor T cells from a lepromatous leprosy patient suppress *Mycobacterium leprae* reactive helper T cells.
Nature 322: 462-464.

Palmer, S.R. and Young, S.E.J. (1982) Q fever endocarditis in England and Wales, 1975-1981.
The Lancet 1: 1448-1449.

Paquet A., Rael, E.D., Klassen, D., Martinez, I. and Baca. O.G. (1978) Mitogenic and protective activity associated with a lipopolysaccharide from *Coxiella burnetii.*
Can. J. Microbiol. 24: 1616-1618.

Paretsky, D., Downs, C.M., Consigli, R.A.and Joyce, B.K. (1958) Studies in the physiology of rickettsiae. I. Some enzyme systems of *Coxiella burnetii.*
J. Infect. Dis. 103: 6-11.

Paretsky, D., Consigli, R.A. and Downs, C.M. (1962) Studies on the physiology of rickettsiae. III. Glucose phosphorylation and hexokinase activity in *Coxiella burnetii.*
J. Bacteriol. 83: 538-543.

Paretsky, D. (1968) Biochemistry of rickettsiae and their infected hosts, with special reference to *Coxiella burnetii.*
Zent. Bakt. Parasit. Infekt. und Hyg. 206: 283-291.

Parker, R.R. and Steinhaus, E.A. (1943) American and Australian "Q" fever: persistence of the infectious agents in guinea pig tissues after defervescence.
Public Health Reports 58: 523-527.

Paul, W.E. (1989) Pleiotropy and redundancy: T cell-derived lymphokines in the immune response.
Cell 57: 521-524.

Peacock, M.G., Fiset, P., Ormsbee, R.A. and Wisseman, C.L. (1979) Antibody response in man following a small intradermal inoculation with *Coxiella burnetii* Phase I vaccine.
Acta Virol. 23: 73-81.

Peacock, M.G., Philip, R.N., Williams, J.C. and Faulkner, R.S. (1983) Serological evaluation of Q fever in humans: Enhanced Phase I titres of immunoglobulin G and A are diagnostic for Q fever endocarditis. **Infect. & Immun.** 41: 1089-1098.

Pellegrin, M., Delsol, G., Auvergnat, J.C., Familiades, J., Fauve, H., Guiu, M. and Voigt, J.J. (1980) Granulomatous hepatitis in Q fever. **Human Path.** 11: 51-57.

Perez-Fontan, M., Huarte, E., Tellez, A., Rodriguez-Carmona, A., Picazo, M.L. and Martizez-Aera, J. (1988) Glomerular nephropathy associated with chronic Q fever. **Am. J. Kidney Dis.** 11: 298-306.

Perrin, T.K. and Gengtson, I.A. (1942) The histopathology of experimental Q fever in mice. **Public Health Rep.** 57: 790-798.

Peter, O., Dupuis, G., Burgdorfer, W. and Peacock, M. (1985) Evaluation of the complement fixation and indirect immunofluorescence tests in the early diagnosis of primary Q fever. **Eur. J. Clin. Microbiol.** 4: 394-396.

Pike, R.M. and Richard, J.E. (1979) Prevention of laboratory infections. **In: Diagnostic procedures for Viral, Rickettsial & Chlamydial infections** (Ed. E.H. Lennette & N.J. Schmidt) 5th Edition, American Public Health Association, Washington Ch. 2 p.49.

Playfair, J.H.L. and Taverne, J. (1987) Antiparasitic effects of tumour necrosis factor *in vivo* and *in vitro*. **1987 Tumour Necrosis Factor and Related Cytotoxins, Wiley, Chichester.** (Liba Foundation Symposium 131): 192-205.

Pope, J.H., Scott, W. and Dwyer, R. (1960) *Coxiella burnetii* in kangaroos and kangaroo ticks in Western Queensland. **Aust. J. Exp. Biol.** 38; 17-28.

Pospisil, V.F. (1965) Propagation of *Coxiella burnetii* in cultures of a monkey kidney stable cell line. **Acta Virol.** 9: 188-189.

Pospisil, V.F. (1966) Persistant infection of cell cultures with *Coxiella burnetii*. **Acta Virol.** 10: 542-548.

Powanda, M.C., Machotka, S.V. and Kishimoto, R.A. (1978) Metabolic sequelae of infection in the guinea pig. **Proc. Soc. Exp. Biol. & Med.** 158: 626-630.

Powell, O.W. (1961) Liver involvement in "Q" fever.
Aust. Ann. Med. 10: 52-58.

Proreshnaya, T.L., Rapoport, L.P., Erdoshenko, V.G. and Kilhator, E.A. (1960) A study of natural foci of Q fever in Kirgizia (USSR).
J. Microbiol. Epidemiol. Immunobiol. 31: 1613-1618.

Raoult, D., Etienne, J., Massip, P., Iacono, S., Prince, M.A., Beaurain, P., Benichou, S., Auvergrat, J.C., Mathieu, P., Bachet, Ph. and Serradimigni, A. (1987) Q fever endocarditis in the south of France.
J. Infect. Dis. 155: 570-573.

Raoult, D., Urvolgyi, J., Etienne, J., Roturier, M., Puel, J. and Chaudet, H. (1988) Diagnosis of endocarditis in acute Q fever by immunofluorescence serology.
Acta Virol. 32: 70-74.

Raoult, D., Raza, A. and Marrie, T.J. (1990) Q fever endocarditis and other forms of chronic Q fever. **In: Q fever: The Disease, Volume 1.** (Ed. T.J. Marrie) CRC Press, Boca Raton, Flo., USA. p 180-197.

Reczko, E. (1950) Ueber Ausscheidung und Verweildenier des Q fiebers - Erregers bei experimentall infizierten meerscheinchen.
Zentral. Bacteriol. Parasitenk Abt. 1 Orig. 157: 81-84.

Reed, S.G., Nathan, C.F., Pihl, D.L., Rodricks, P., Shanebeck, K., Conlon, P.J. and Grabstein, K.H. (1987) Recombinant granulocyte/macrophage colony - stimulating factor activates macrophages to inhibit *Trypanosoma cruzi* and release hydrogen peroxide. Comparison with interferon-G.
J. Exp. Med. 166: 1734-1746.

Rehacek, J. and Brezina, R. (1964) Propagation of *Coxiella burnetii* in tick tissue culture.
Acta Virol. 8: 380-381.

Rehacek, J., Zupancicova, P.A.C., Brezina, R., Urvolgyi, F., Koracova, E., Tarasevic, L.V., Jablonskaja, V.A., Pospisil, R. and Baloghova, D. (1976) Rickettsioses Studies. 2. Natural foci of rickettsioses in east Slovakia.
Bull. World Health Organ. 53: 31-38.

Rehacek, J., Vosta, J., Tarasevic, I.V., Brezina, R., Jablonskaja, V.A., Platnikova, L.F., Fetisova, N.F. and Hanak, P. (1977) Rickettsioses Studies. 3. Natural foci of rickettsioses in south Bohemia.
Bull. World Health Organ. 55: 455-461.

Riechman, N., Raz, R., Keysary, A., Goldwasser, R. and Flatau, E. (1988) Chronic Q fever and severe thrombocytopenia in a pregnant woman. **Am. J. Med.** 85: 253-254.

Robbins, F.C., Gauld, R.L. and Warner, F.B. (1946a) Q fever in the Mediterranean area: report of its occurrence in Allied Troops. II. Epidemiology
American Journal of Hygiene 44: 23-50.

Robbins, F.C. and Ragan, C.A. (1946) "Q" fever in the Mediterranean area: report of its occurrence in Allied Troops. I. Clinical features of the disease.
American Journal of Health 44: 6-22.

Robbins, F.C. and Rustigian, R. (1946) Q fever in the Mediterranean area: report of its occurrence in Allied Troops. IV. A laboratory outbreak.
American Journal of Hygiene 44: 64-71.

Robbins, F.C., Rustigan, R., Snyder, M.J. and Smadel, J.E. (1946b) Q fever in the Mediterranean area: report of its occurrence in Allied Troops. III. The etiological agent.
American Journal of Hygiene 44: 51-63.

Robson, A.O. and Shimmin, C.D.G.L. (1959) Chronic Q fever. 1. Clinical aspects of a patient with endocarditis.
Brit. Med. J. 2: 980-983.

Roman, M.J., Coriz, P.D. and Baca, O.G. (1986) A proposed model to explain persistent infection of host cells with *Coxiella burnetii.*
J. Gen. Micro. 132: 1415-1422.

Rose, L.M., Ginsberg, A.H., Rothstein, T.L., Ledbetter, J.A. and Clark, E.A. (1985) Selective loss of a subset of T helper cells in active multiple sclerosis.
Proc. Natl. Acad. Sci. USA 82: 7389-7393.

Rosenberg, M. and Kordova, N. (1962) Multiplication of *Coxiella burnetii* in Detroit-6 cell cultures: an electron microscope study.
Acta Virol. 6: 176-180.

Ruco, L.P. and Meltzer, M.S. (1978) Macrophage activation for tumor cytotoxicity: development of macrophage cytotoxic activity requires completion of a sequence of short-lived intermediary reactions.
J. Immunol. 121: 2035-2042.

Salvin, S.B., Younger, J.S. and Lederer, W.H. (1973) Migration inhibition factor and interferon in the circulation of mice with delayed hypersensitvity.
Infect. & Immun. 7: 68-75.

Samuel, J.E., Frazier, M.E., Kahn, M.L., Thomashow, L.S. and Mallavia, L.P. (1983) Isolates and characterization of a plasmid from phase I *Coxiella burnetii.*
Infect. & Immun. 41: 488-493.

Samuel, J.E., Frazier, M.E. and Mallavia, L.P. (1985) Correlation of plasmid type and disease caused by *Coxiella burnetii.*
Infect. & Immun. 49: 775-779.

Sanders, M.E., Makgoba, M.W. and Shaw, S. (1988) Human naive and memory T cells: reinterpretation of helper-inducer and suppressor-inducer subsets.
Immunol. Today 9: 195-199.

Sawyer, L.A., Fishbein, D.B. and McDade, J.E. (1987) Q fever: Current concepts.
Rev. Infect. Dis. 9: 935-946.

Sawyer, L.A., Fishbein, D.B. and McDade, J.E. (1988) Q fever in patients with hepatitis and pneumonia: Results of laboratory-based surveillance in the United States.
J. Infect. Dis. 158: 497-498.

Schachter, J., Sug, M. and Meyer, K.F. (1971) Potential danger of Q fever in a University hospital environment.
J. Infect. Dis. 123: 301-304.

Schmeer, N., Krauss, H. and Wilske, B. (1984) Serodiagnosis of Q fever in man - detection of non-complement-fixing IgM antibodies by enzyme-linked immunosorbent assay (ELISA).
Immun. Infekt. 12: 245-251.

Schramek, S., Brezina, R. and Urvolgyi, J. (1972) A new method of preparing diagnostic Q fever antigen.
Acta Virol. 16: 487.

Schramek, S. and Brezina, R. (1976) Characterization of an endotoxic lipopolysaccharide from *Coxiella burnetii.*
Acta Virol. 20: 152-158.

Schramek, S., Brezina, R. and Kazar, J. (1978) Influence of mild acid hydrolysis on the antigenic properties of Phase I *Coxiella burnetii.*
Acta Virol. 22: 302-308.

Schramek, S. and Galanos, C. (1981) Lipid A component of lipopolysaccharides from *Coxiella burnetii.*
Acta Virol. 25: 230-234.

Schramek, S. and Mayer, H. (1982) Different sugar compositions of lipopolysaccharides isolated from Phase I and pure Phase II cells of *Coxiella burnetti.*
Infect. & Immun. 38: 53-57.

Schramek, S., Radziejewska-Lebrecht, J. Mayer, H. (1985) 3-C-Branched aldoses in lipopolysaccharide of phase I *Coxiella burnetii* and their role as immunodominant factors.
Eur. J. Biochem. 148: 455-461.

Scott, P., Natovitz, P., Coffman, R.L., Pearce, E. and Sher, A. (1988) Immunoregulation of cutaneous leishmaniasis. T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens.
J. Exp. Med. 168: 1675-1684.

Shapiro, R.A., Siskind, B., Schofield, F.D., Stallman, N., Worswick, D.A. and Marmion, B.P. (1990) A randomised, controlled, double-blind, cross-over, clinical trial of Q fever vaccine in selected Queensland abattoirs.
Epidem. & Infect. 104: 267-273.

Sidwell, R.W., Lundgren, D.L., Bushman, J.B. and Thorpe, B.D. (1964) The occurrence of a possible epizootic of Q fever in fauna of the Great Salt Lake Desert of Utah.
Am. J. Trop. Med. 13: 754-762.

Sidwell, R.W., Thorpe, B.D. and Gebhardt, L.P. (1964a) Studies of latent Q fever infection I. Effects of whole body irradiation on latently infected guinea pigs, white mice and dear mice.
Am J. Hyg. 79: 113-124.

Sidwell, R., Thorpe, B.D. and Gebhardt, L.P. (1964b) Studies of latent Q fever infections II. Effects of multiple cortisone injections.
Am J. Hyg. 79: 320-327.

Smadel, J.E., Snyder, M.J. and Robbins, F.C. (1948) Vaccination against Q fever.
American J. Hygiene 47: 71-81.

Smith, D.J.W. (1940) Studies in the epidemiology of Q fever. 3. The transmission of Q fever by the tick *Haemaphysalis humerosa.*
Aust. J. Exp. Biol. Med. Sci. 18: 103-11.

Smith, D.J.W. and Derrick, E.H. (1940) Studies in the epidemiology of Q fever. 1. The isolation of six strains of *Rickettsia burnetii* from the tick Haemaphyalis humerosa.
Aust. J. Exp. Biol. Med. Sci. 18: 1-8.

Smith, D.J.W. (1942) Studies in the epidemiology of Q fever. 10. The transmission of Q fever by the tick Ixodes holocyclus (with notes on tick-paralysis in Bandicoots).
Aust. J. Exp. Biol. Med. Sci. 20: 213-217.

Smith, J.D., Stoker, M.J.P. (1951) The nucleic acids of *Rickettsia burnetii.*
Brit. J. Exp. Path. 32: 433-441.

Spelman, D.W. (1982) Q fever. A study of 111 consecutive cases.
Med. J. Aust. 1: 547-553.

Spicer, D.S., DeSanctis, A.M. and Beiler, J.M. (1970) Preparation of highly purified concentrates of *Coxiella burnetii.*
Proc. Soc. Exp. Biol. & Med. 135: 706-708.

Spicknall, C.F., Huebner, R.J., Finger, J.A. and Blocker, W.P. (1947) Report of an outbreak of Q fever at the National Institutes of Health. I Clinical features.
Ann. Internal Medicine 27: 28-40.

Srigley, J.R., Geddie, W.R., Vellend, H., Van Nostrand, A.W.P., Palmer, N., Edwards, V.D. and Phillips, M.J. (1985) Q fever. The liver and bone marrow pathology.
Am. J. Pathol. 9: 752-758.

Stoenner, H.G. and Lackman, D.B. (1960) The biological properties of strains of *C.burnetii* isolated from rodents in Utah.
Am. J. Hyg. 71: 45-51.

Stoker, M.G.P. (1950) Q fever in Great Britain: the causative agent.
Lancet 2: 616-620.

Stoker, M.G.P. (1953) Variation in complement fixing activity of *Rickettsia burnetii* during egg adaption.
J. Hyg. Camb. 51: 311-321.

Stoker, M.G.P. and Marmion, B.P. (1955) The spread of Q fever from animals to man. The natural history of a Rickettsial disease.
Bull. Wld. Hlth. Org. 13: 781-806.

Stoker, M.G.P., Page, Z, and Marmion, B.P. (1955) Problems in the diagnosis of Q fever by complement fixation tests.
Bull. Wrld. Health Org. 13: 807-827.

Stoker, M.G.P. and Fiset, P. (1956) Phase variation of the Nine Mile and other strains of *Rickettsia burnetii.*
Can. J. Micro. 2: 310-321.

Stossel, T.P. (1981) Actin filaments and secretion: the macrophage model. **In: Methods in Cell Biology** vol 23: 215-229

Syrucek, L., Sobeslavesky, O. and Gutvirth, I. (1958) Isolation of *Coxiella burnetii* from human placentas.
J. Hyg. Epidemiol. Mikrobiol. Immunol. 2: 29-35.

Tabert, G.G. and Lackman, D.B. (1965) The radioisotope precipitation test for studying Q fever antibodies in human and animal sera.
J. Immunol. 94: 959-965.

Thompson, H.A., Baca, O.G. and Paretsky, D. (1971) Presence of ribosomal ribonucleic acid in the rickettsia *Coxiella burnetii.*
Biochem. J. 125: 365-366.

Tiggert, W.D. and Benenson, A.S. (1956) Studies on Q fever in man.
Trans. Assoc. Am. Phys. 69: 98-104.

Tiggert, W.D., Benenson, A.S. and Gochenour, W.S. (1961) Airborne Q fever.
Bacteriol. Rev. 25: 285-293.

Topping, N.H., Shepard, C.C. and Huebner, R.J. (1946) Q fever. An immunological comparison of strains.
Am. J. Hyg. 44: 173-182.

Topping, N.H., Shepard, C.C. and Irons, J.V. (1947) Q fever in the United States I. Epidemiological studies of an outbreak among stock handlers and slaughterhouse workers.
J.A.M.A. 133: 813-815.

Travis, L.B., Travis, W.D., Li, C-Y. and Pierre, R.V. (1986) Q fever. A clinicopathologic study of five cases.
Arch. Pathol. Lab. Med. 110: 1017-1020.

Turck, W.P.G., Howitt, G., Turnerg, L.A., Fox, H., Longson, M., Matthews, M.B. and Das Gupta, R. (1976) Chronic Q fever.
Quart. J. Med., New Series XLV: 199-217.

Turco, J. and Winkler, H.H. (1983) Cloned mouse interferon-G inhibits the growth of *Rickettsia prowazekii* in cultured mouse fibroblasts.
J. Exp. Med. 158: 2159-2164.

Turco, J., Thompson, H.A. and Winkler, H.H. (1984) Interferon-G inhibits growth of *Coxiella burnetii* in mouse fibroblasts.
Infect. & Immun. 45: 781-783.

Varma, M.P.S., Adgey, A.A.J. and Connolly, J.H. (1980) Chronic Q fever endocarditis.
Br. Heart J. 43: 695-699.

Victor, J., Raymond, R., Valliant, J., Wagner, J.C. and Pollack, A.D. (1952) Studies on opsonins in Q fever.
J. Exp. Med. 95: 61-70.

Vodkin, M.H., Williams, J.C. and Stephenson, E.H. (1986) Genetic heterogeneity among isolates of *Coxiella burnetii*.
J. Gen. Microbiol. 132: 455-463.

Vodkin, M.H. and Williams, J.C. (1988) A heat shock operon in *Coxiella burnetii* produces a major antigen homologous to a protein in both Mycobacteria and Escherichia coli.
J. Bacteriol. 170: 1227-1234.

Volc-Platzer, B. Stemberger, H., Luger, T., Radaszkiewicz, T. and Wiedermann, G. (1988) Defective intralesional interferon-gamma activity in patients with lepromatous leprosy.
Clin. Exp. Immunol. 71: 235-240.

Waag, D.M. and Williams, J.C. (1988) Immune modulation by *C.burnetii*: characterization of a Phase I immunosuppressive complex differentially expressed among strains.
Immunopharmicol. Immunotoxicol. 10: 231.

Weiss, E. and Pietryk, H. (1957) Growth of *Coxiella burnetii* in monolayer cultures of chick embryo entodermal cells.
J. Bacteriol. 72: 235.

Welsh, H.H., Lennette, E.H., Abinanti, F.R. and Winn, J.F. (1951) Q fever in California. IV. Occurrence of *Coxiella burnetii* in the placenta of naturally infected sheep.
Public Health Reports 66: 1473-1477.

Westphal, O., Luderitz, O. and Bister, F. (1952) Uber die extraktion von bakterien mit phenol/wasser.
Zeit. Naturforsch 76 148-155.

Wiebe, M.E., Burton, P.R. and Shankel, D.M. (1972) Isolation and characterization of two cell types of *Coxiella burnetii* phase I.
J. Bact. 110: 368-377.

Willey, R.F., Matthews, M.B., Peutherer, J.F. and Marmion, B.P. (1979) Chronic cryptic Q fever infection of the heart.
The Lancet 2: 270-272.

Williams, J.C. (1981) Iodination of *Coxiella burnetii* cells: optimal conditions for the iodination of exposed surface proteins. **In: Rickettsiae and Rickettsial Diseases,** (Ed. Burgdorfer W. and Anacker R.L.) Academic Press NY pp 473-482.

Williams, J.C. and Cantrell, J.L. (1982) Biological and immunological properties of *Coxiella burnetii* vaccines in C57BL/10SCN endotoxin-nonresponder mice.
Infect. & Immun. 35: 1091-1102.

Williams, J.C., Johnston, M.R., Peacock, M.G., Thomas, L.A., Stewart, S. and Portis, J.L. (1984) Monoclonal antibodies distinguish phase variants of *Coxiella burnetii.*
Infect. & Immun. 43: 421-428.

Williams, J.C. and Stewart, S. (1984) Identification of immunogenic proteins of *C.burnetii* variants.
Microbiology-1984 American Society for Microbiology p 257-262.

Williams, J.C., Damrow, T.A., Waag, D.M. and Amano, K.I. (1986a) Characterisation of a Phase I *Coxiella burnetii* chloroform-methanol residue vaccine that induces active immunity against Q fever in C57 BL/10Sc N Mice.
Infect. & Immun. 57: 851-858.

Williams, J.C., Thomas, L.A. and Peacock, M.G. (1986b) Identification of Phase-specific antigenic fractions of *Coxiella burnetii* by enzyme-linked immunosorbent assay.
J. Clin. Microbiol. 24: 929-934.

Winkler, H.H. and Turco, J. (1985) Role of lymphokine (Interferon) in host defense against *Rickettsia prowazekii* **In: Microbiology-1984** American Society of Microbiology, Washington p 273-276.

Wisseman Jr., C.L., Fiset, P. and Ormsbee, R.A. (1967) Interaction of Rickettsiae and Phagocytic Host Cells V.Phagocytic and opsonic interaction of phase 1 and phase 2 *Coxiella burnetii* with normal and immune leukocytes and antibodies.
J. Immunol. 99: 669-674.

Wisseman, C.L. (1978) Prevention and control of rickettsial diseases with emphasis on immunoprophylaxis. **In: Proceedings of the 2nd International Symposium on Rickettsiae and Rickettsial Diseases,** (Ed. J. Kazar, R.A. Ormsbee, I.N. Tarasevich) Veda, Bratislava.

Wisseman Jr, C.L. and Waddell, A. (1983) Interferon-like factors from antigen - and mitogen-stimulated human leukocytes with antirickettsial and cytolytic actions on *Rickettsia prowazekii.* Infected human endothelial cells, fibroblasts, and macrophages.
J. Exp. Med. 157: 1780-1793.

Wolfe, D.M. and Kornfeld, L. (1949) The application of a quantitative complement fixation method to a study of Q fever strain differentiation.
J. Immunol. 61: 297-306.

Worswick, D. A. and Marmion, B.P. (1985) Antibody responses in acute and chronic Q fever and in subjects vaccinated against Q fever.
J. Med. Microbiol. 19: 281-296.

Wysocki, L.J. and Sato, V.L. (1978) 'Panning' for lymphocytes: a method for cell selection.
Proc. Natl. Acad. Sci. 75: 2844-2848.

Yam, L.T., Li, C.Y. and Crosby, W.A. (1971) Cytochemical identification of monocytes and granulocytes.
Am. J. Clin. Pathol. 55: 283-290.

Yunker, C.E., Ormsbee, R.A., Cory, J. and Peacock, M.G. (1970) Infection of insect cells with *Coxiella burnetii.*
Acta Virol. 14: 383-392.

Zhmaeva, Z.M. and Pchelkina, A.A. (1957) Domestic birds as carriers of the rickettsia of Q fever in the Turkmen-kala S.S.R.
J. Microbiol. Epidemiol. Immunobiol. 28: 347-349.

APPENDIX

A.A. Izzo, B.P. Marmion and D.A. Worswick (1988) Markers of Cell-Mediated Immunity After Vaccination with an Inactivated, Whole-Cell Q Fever Vaccine. *The Journal of Infectious Disease, v. 157 (4), pp. 781-789, April 1988*

A.A. Izzo, B.P. Marmion and T. Hackstadt (1991) Analysis of the cells involved in the lymphoproliferative response to Coxiella burnetii antigens.
Clinical & Experimental Immunology, v. 85 (1), pp. 98–108, July 1991

B.P. Marmion, R.A. Ormsbee, M. Kyrkou, J. Wright, D.A. Worswick, A.A. Izzo, A. Esterman, B. Feery and R.A. Shapiro (1990) Vaccine prophylaxis of abattoir-associated Q fever: eight years' experience in Australian abattoirs.
Epidemiology and Infection, v. 104 (2), pp. 275-287, April 1990