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Analysis of the cells involved in the lymphoproliferative response to *Coxiella burnetii* antigens

A. A. IZZO*, B. P. MARMION* & T. HACKSTADT† *Department of Pathology, University of Adelaide, Adelaide, Australia, and †Rocky Mountain Laboratory, NIAID, Hamilton, Montana, USA

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SUMMARY

Vaccination with an inactivated, whole cell, Q fever vaccine (Q-vax) induces lasting antibody conversion and a positive delayed-type hypersensitivity (DTH) skin reaction in about 60% of recipients but a long-lasting positive lymphoproliferative or mitogenic response to *C. burnetii* antigens with peripheral blood mononuclear cells (PBMC) in 85–95% of subjects. Analysis of the lymphoproliferative response to *C. burnetii* antigens has now been made by fractionation–reconstitution experiments with PBMC from vaccinees, from past infections, and from healthy controls. The major contributor to the response in immune subjects proved to be the T lymphocyte. T cells were stimulated by both the phase I and phase II antigens of two prototype strains of *C. burnetii* and responses were greatly amplified by addition of IL-2. Similar T lymphocyte stimulation profiles were obtained with the 'Priscilla' strain of *C. burnetii* which represents a different biotype of Coxiella isolated from Q fever endocarditis; Q-vax is therefore likely to protect against endocarditis strains. Fractionation–reconstitution experiments with T and B cells from vaccinees and subjects infected in the past, using various antigenic or haptenic fractions from *C. burnetii* indicate that protein, non-lipopolysaccharide components of the organism are responsible for the mitogenic response of immune T cells. However, the role of the lipopolysaccharide in the protective immunogen has still to be defined.

Keywords Q fever antigens and fractions lymphoproliferative responses

INTRODUCTION

Q fever, either as an acute illness or as one of its chronic complications (subacute endocarditis, chronic hepatitis, post-Q fever fatigue syndrome), is a prevalent and costly occupational disease in Australian abattoirs and other groups exposed to cattle, sheep or goats. Clinical trials with a recently developed Q fever vaccine (Q-vax; Commonwealth Serum Laboratories, Melbourne, Australia) have shown that complete protection against natural infection, for a period of at least 5 years, is conferred by one 30-μg dose of a highly purified, formalin-inactivated, whole-cell preparation of *C. burnetii*, Henzerling strain, in the phase I antigenic state (Marmion *et al.*, 1984; Marmion, 1988; Marmion *et al.*, 1990; Shapiro *et al.*, 1990). Rates of conversion to the antibody or skin test positive state after vaccination were low (about 60%) and in sharp contrast to the absolute protection observed against natural infection. Consequently, studies of cellular immunity, as measured by lymphocyte proliferation assays with peripheral blood mononuclear cells (PBMC) and *C. burnetii* phase I and II antigens were undertaken. These showed that positive stimulation indices (LSI) developed 13–15 days after vaccination and were eventually positive in over 85% of vaccinees and remained so for at least 5 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 (Izzo, Marmion & Worswick 1988).

The high protective potency and prolonged cell-mediated immunity (CMI) response with the *C. burnetii* vaccine is unusual for a killed preparation. It is thought that a living vaccine is often required to induce good CMI (but see Crowle (1988) on the requirement for adjuvanticity for effective killed bacterial vaccines).

Interpretation of the *in vitro* correlates of the CMI response to *C. burnetii* offers some potential difficulties. Purified *C. burnetii* lipopolysaccharide (LPS) is mitogenic for the leucocytes of non-immune guinea pigs and stimulates non-specific protection against other organisms (Paquet *et al.*, 1978). Although similar effects have not been demonstrated with human PBMC it seemed desirable to characterize the contributions of B and T lymphocytes to the cellular response following Q fever vaccina-

Correspondence: Professor B. P. Marmion, Department of Pathology, University of Adelaide, G.P.O. Box 498, Adelaide, South Australia 5000.

tion. Our own previous studies, and those of others (Jerrells, Mallavia & Hinrichs, 1975; Ascher, Berman & Ruppanner 1983) on the lymphoproliferative response to *C. burnetii* antigens have used PBMC rather than lymphocyte subsets.

Furthermore, the components (LPS and/or protein) of the organism responsible for specificity in the protective immunogenic response and for stimulating long lasting CMI are not fully defined. Our current studies are directed to these unresolved problems. Here we describe fractionation–reconstruction experiments with PBMC from subjects infected with *C. burnetii* in the past, or vaccinated with Q fever vaccine, along with cells from healthy controls. We also explored T and B lymphocyte responses to whole-cell antigens from phase I and phase II strains of *C. burnetii* of various origins and present preliminary data with antigens extracted from the coxiella with organic solvents and with the cell residues remaining after extraction.

SUBJECTS AND METHODS

Subjects studied

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

A 'low risk' group (group A), consisted of the vaccinated 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 30-μg dose, subcutaneously, of a formalin inactivated, whole-cell vaccine (Q-Vax) from the Henzerling strain of *C. burnetii* in phase I antigenic state, prepared by the method of Ormsbee (1961). Their PBMC were sampled and stimulated with antigen between 5 months and 1 year after vaccination.

A second, 'high-risk' group (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 (group C), comprised 10 persons chosen as negative controls on the basis that they had no previous or current exposure to Q fever. Their PBMC had been tested in the lymphocyte proliferation assay with *C. burnetii* antigens, and gave LSI $< 1{\cdot}2$—below the cut-off point for a positive reaction (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.

For the assays with reconstituted T or B lymphocyte–monocyte mixtures, and solvent extracted antigens and cell residues, or periodate-treated whole cells, PBMC were taken from five subjects: two vaccinees (one inoculated in 1952 with subsequent, periodic skin tests, and the other inoculated in 1985); two subjects infected in 1958, both with positive Q fever CF antibody and positive skin tests; and one control subject with no history of Q fever and a negative antibody test.

Coxiella burnetii *antigens*

Whole-cell preparations. C. burnetii phase I antigen (Henzerling) was a highly purified whole cell, Q fever vaccine prepared from the strain propagated in chick embryo yolk sac and formalin-inactivated (National Drug Company, Philadelphia, PA; 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 IF with specific antisera did not reveal phase II cells (Worswick & Marmion 1985). Neither the National Drug Company vaccine or Q-vax showed any reaction by CF or IF with 64 CF antibody units of a rabbit antiserum against uninfected chick embryo yolk sac suspension.

C. burnetii phase I antigen (Priscilla) was made from an isolate from the placenta of an aborting goat. The strain has special features resembling isolates from human Q fever endocarditis (Samuel *et al.*, 1985; Hackstadt *et al.*, 1985; Hackstadt 1986).

Artificially derived phase II antigen from CSL Nine Mile *C. burnetii* phase I antigen was prepared by treatment with potassium periodate (Schramek, Brezina & Urvolgyi, 1972). The change to phase II serological activity was checked by chess-board titration in CF test with monospecific antisera to phase I and II antigens (Table 1).

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 four 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 non-pathogenic 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, London, England, and prepared by potassium periodate treatment of a phase II antigen with residual phase I cells and also with a diagnostic Nine Mile phase II antigen from CSL.

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 immunized with uninfected yolk sac suspension.

Coxiella suspensions were standardized by adsorption spectrometry at 420 nm (Fiset *et al.*, 1969) and adjusted to a concentration of 1000 μg/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 (Izzo *et al.*, 1988). These optimal dilutions were: for *C. burnetii* phase I (Henzerling), 1·25 μg/ml; for *C. burnetii* phase I antigen (Priscilla), 2·5 μg/ml; and for *C. burnetii* (Nine Mile) phase II, 1·25 μg/ml.

Solvent-extracted preparations. Trichloracetic acid extracts (TCA-E): The method for TCA extraction of *C. burnetii* cells resembled those described by Anacker *et al.* (1962), Brezina & Urvolgyi (1962), Hackstadt *et al.* (1985) and Lukacova *et al.* (1989). It removes a complex of LPS and proteins of variable composition, depending on the conditions of extraction (Lucacova *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 min with periodic mixing. It was then centrifuged at 15000 rev/min for 30 min to deposit the extracted cells. The supernatant fluid

was removed and dialysed for 48 h against two changes of sterile distilled water. The sac contents were concentrated by lyophilization. The extracted cells (TCA residue, TCA-R) were resuspended and washed five to six times with sterile saline.

LPS extract (LPS PE-W) was extracted by the hot phenol–water method as described by Anacker *et al.* (1963) and Brezina, Pospisil & Schramek (1970). One milligram 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 min at 65°C. The mixture was centrifuged at 3000 rev/min 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 h, then re-extracted with 90% phenol at 65°C. After the latter extraction only the aqueous phase was removed, dialysed for 72 h against three changes of sterile water and the sac contents concentrated by lyophilization.

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 & Hesseluis, 1983); and (iii) CF antigen content with, respectively, an early post-infection guinea-pig antibody reacting with *C. burnetii* phase II antigen only; a monospecific, absorbed rabbit antiserum against phase I antigen; and 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, Plackett & Lemcke, 1967) and also as the highest (optimal) antigen titre observed with any of a range of antiserum dilutions (see Table 1).

Preparation of monocyte, T cell and B cell enriched populations from PBMC

PBMC were isolated by centrifugation through a Ficoll/Isopaque gradient (Nycomed, Oslo, Norway) from venous blood samples treated with lithium heparin (1·25 U/ml). Monocytes were isolated from PBMC on a discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden) (Al-Sumidaie, Jones & Young, 1984). A three-step density gradient of iso-osmolar Percoll (1·057, 1·066 and 1·074 g/ml) was prepared and overlayed with $1–2 \times 10^7$ PBMC and centrifuged at 2000 ***g*** for 90 min (Beckman J-6B centrifuge).

The monocyte-enriched population was removed from the interface at 1·057/1·066 g/ml and the lymphocyte population from the 1·066/1·074 g/ml interface. Each cell population was washed twice in sterile saline. The lymphocytes were further depleted of any remaining monocytes by layering over tissue culture grade plastic (Costar, Cambridge, MA) treated with heat-inactivated fetal calf serum (FCS) (Flow Laboratories, North Ryde, New South Wales) for 60 min 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 & Sato (1978). Enrichment for the T cells was done by treating 1·0 ml of lymphocyte suspension ($5–10 \times 10^6$ cells/ml) with 1·0 ml of a monoclonal antibody (MoAb) to human μ chain, FMC HB57 (Flinders Medical Centre (FMC), Bedford Park, South Australia) in culture medium at 4°C for 30 min and then washing twice in 1% v/v FCS in PBS. After the second wash the antibody-sensitized 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 (Selinas, Hawthorn, Victoria). After incubation at 4°C for 70 min, during which time the cells settled on the plates, non-adherent 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 MoAb OKT3 for T cell markers, with FMC 33 for monocyte markers, and with goat anti-human IgG, IgM and IgA (Cappel, Malvern, PA) 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, Li & Crosby, 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 MoAb HB57 would not sensitize B lymphocytes expressing γ, α or other heavy chains and so effect their removal. Consequently, mixtures of MoAb to μ chain and to other B cell markers were compared with the method just described. These mixtures were: (i) HB135 (anti-CD20), HB57 (anti-μ chain), HB60 (anti-γ chain); and (ii) HB135, HB57 and HB43 (anti-γ chain). Analysis by flow cytometry (Epics 750-2, FACS; Coulter, Hileah, FL) of the resulting populations, using a combination of MoAb (HB135, Hb57, FMC63 (anti-CD19), HB60, and HB43) to detect B cells, and a combination of MoAbs anti-CD3 and anti-CD7 to detect T cells, showed that these procedures yielded T cell fraction that were only 1% purer than those obtained with HB57 alone (data not shown).

For B cell enrichment, the suspension of lymphocytes ($6–12 \times 10^6$/ml) were treated in the same general manner, but were initially sensitized with a mixture of OKT3/3A1 MoAbs (FMC) to coat the T cells.

Interleukin-2

Interleukin-2 (IL-2) (Boehringer Mannheim, Sydney, Australia) had been prepared and purified from human lymphocytes stimulated with phytohaemagglutinin (PHA). Dilutions (5 units in 1·0 ml) 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 & Bird 1987).

Lymphoproliferative assay

Lymphoproliferative responses to *C. burnetii* whole-cell or fractionated antigens were performed in cultures with a final concentration of 5×10^5 cells/ml in 200-μl volumes held in 96-well microtitre plates (Nunclon Delta, Nunc, Roskilde, Denmark). Cells were suspended in RPMI 1640 (Flow Laboratories) with HEPES buffer containing 2 mM L-glutamine, 200 U of penicillin/ml, 40 μg of gentamycin/ml and 10% v/v heat-inactivated autologous serum. The unfractionated PBMC, and the reconstituted cell mixtures of monocytes, T and B lymphocytes; monocytes and T lymphocytes; monocytes and B lympho-

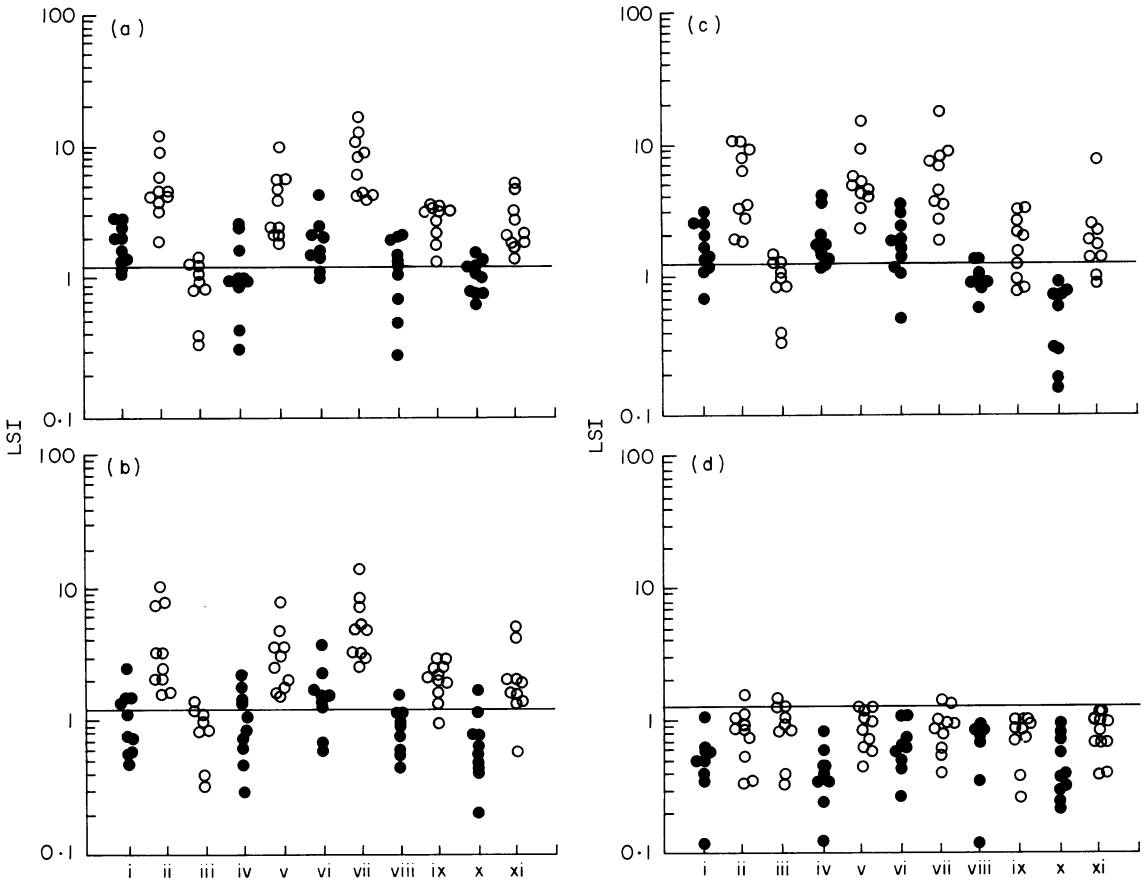

Fig. 1. The lymphocyte stimulation indices for each subject (LSI) within the vaccinated group (group A, $n = 10$). PBMC and various reconstituted lymphocyte fractions were stimulated with *C. burnetii* whole cell Henzerling (a) and Priscilla phase I (b) and Nine Mile phase II antigens (c) and yolk sac suspension (d), with (○) and without (●) exogenous IL-2. The continuous horizontal line represents the cut-off value of 1·2 LSI. i, monocytes + T + B; ii, monocytes + T + B + IL-2; iii, monocytes + T + B + IL-2 (added without prior antigenic stimulation); iv, PBMC; v, PBMC + IL-2; vi, monocytes + T; vii, monocytes + T + IL-2; viii, monocytes + B; ix, monocytes + B + IL-2; x, B + T; xi, T + B + IL-2.

cytes; 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 monocyte + T + B cultures; 80–85% T cells in monocyte + T cultures; 80–85% B cells in monocyte + 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 h with antigen alone, after which the medium was carefully removed and fresh medium added. Cells were then split 1-in-2 by transferring 100 μl to a new well and adding 100 μl 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 U in 200 μl, and the cultures were further incubated for a further 48 h. Cultures not receiving IL-2 were not split but were incubated for 120 h 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, monocytes + T + B and T + B, were set up with added IL-2. Other cell controls (PBMC, monocytes + T + B, T + B) were set up without antigen and without IL-2.

In all assays, cell proliferation was assessed by adding 0·4 Ci ^{3}H-thymidine (specific activity 22 Ci/mmol; Amersham International, Sydney, Australia) 18 h before harvesting cells with an automated cell harvester (Skatron, Lierbyen, Norway). 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 LSI as described by Izzo *et al.* (1988) for each antigen in respect of each cell mixture, as follows: LSI = (mean ct/min of four replicate stimulated cultures − machine background)/(mean ct/min of four replicate unstimulated cultures − machine background).

RESULTS

Reactivity of PBMC and reconstituted mixtures of lymphocytes and monocytes to C. burnetii *whole cell antigens*

Figures 1–3 set out 'scattergrams' of the LSI obtained on antigen stimulation with subjects who were vaccinated (group A), unvaccinated (group B) but with evidence of previous exposure, and that (group C) without previous exposure to *C. burnetii*.

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

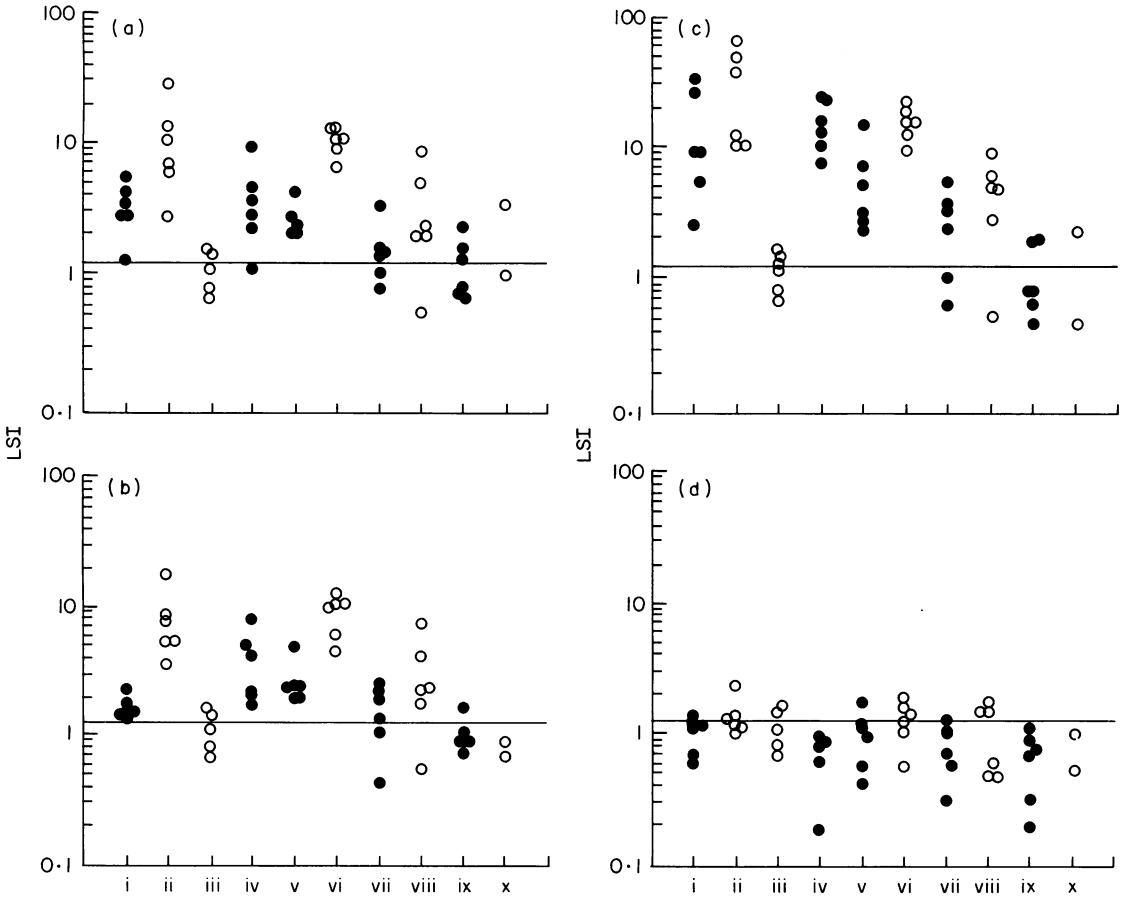

Fig. 2. Lymphocyte stimulation indices (LSI) for each subject within the past infection group (group B, $n=6$). PBMC and various reconstituted lymphocyte fractions, were stimulated with *C. burnetii* whole cell Henzerling (a) and Priscilla phase I (b) and Nine Mile phase II antigens (c) and yolk sac suspension (d), with (○) and without (●) exogenous IL-2. i, monocytes + T + B; ii, monocytes + T + B + IL-2; iii, monocytes + T + B + IL-2 (added without prior antigenic stimulation); iv, PBMC; v, monocytes + T; vi, monocytes + T + IL-2; vii, monocytes + B; viii, monocytes + B + IL-2; ix, B + T; x, T + B + IL-2.

IL-2 was added to the cultures (Fig. 1d, Fig. 2d, Fig. 3d). Among the vaccinees (group A, Fig. 1) detailed comparisons of the means of LSI values with yolk sac antigen showed a small but significant ($P \leqslant 0{\cdot}005$), 1·4–2-fold amplification after addition of IL-2 to those fractions with T cells. The same statistically significant trend was not observed in groups B and C; it presumably represents sensitization 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 (Figs 1 and 2), whereas no such enhanced effect was obtained with the non-immune controls (Fig. 3).

Figure 1 shows that with the vaccinees and *C. burnetii* phase I antigen (Henzerling and Priscilla), the most vigorous responses, either with or without added IL-2, were with the total PBMC, and with reconstituted fractions monocytes + T + B and monocytes + T, whereas monocytes + B showed some, but smaller responses. Mean values and standard deviations with Henzerling phase I antigen were (i) PBMC without IL-2/with IL-2: $1{\cdot}2 \pm 0{\cdot}74/4{\cdot}0 \pm 2{\cdot}5$; (ii) monocytes + T + B: $1{\cdot}9 \pm 0{\cdot}7/5{\cdot}1 \pm 2{\cdot}7$; (iii) monocytes + T: $2{\cdot}2 \pm 1{\cdot}1/8{\cdot}0 \pm 4{\cdot}3$; (iv) monocytes + B: $1{\cdot}3 \pm 0{\cdot}6/2{\cdot}7 \pm 0{\cdot}73$. Differences are all significant at $P \leqslant 0{\cdot}005$ (Student's *t*-test on log-transformed LSI values). The response profile with *C. burnetii* phase I (Priscilla) with the various reconstituted fractions was closely similar to that with Henzerling phase I.

With the *C. burnetii* (Nine Mile) phase II antigen the response profile among the vaccinees was closely similar to that with the phase I antigens. Fractions with T cells were more active; thus monocytes + T with IL-2 gave a mean LSI value of $6{\cdot}4 \pm 4{\cdot}6$, against a mean value of $1{\cdot}8 \pm 0{\cdot}9$ for monocytes + B with IL-2 ($P \leqslant 0{\cdot}005$).

Overall, in the vaccinees, the rank order of mean LSI responses of PBMC and the reconstituted fractions to the three antigens was monocytes + T > monocytes + T + B = PBMC > monocytes + 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.

In group B, subjects with evidence of past infection with *C. burnetii*, the profile (Fig. 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. monocytes + T + B *versus*-

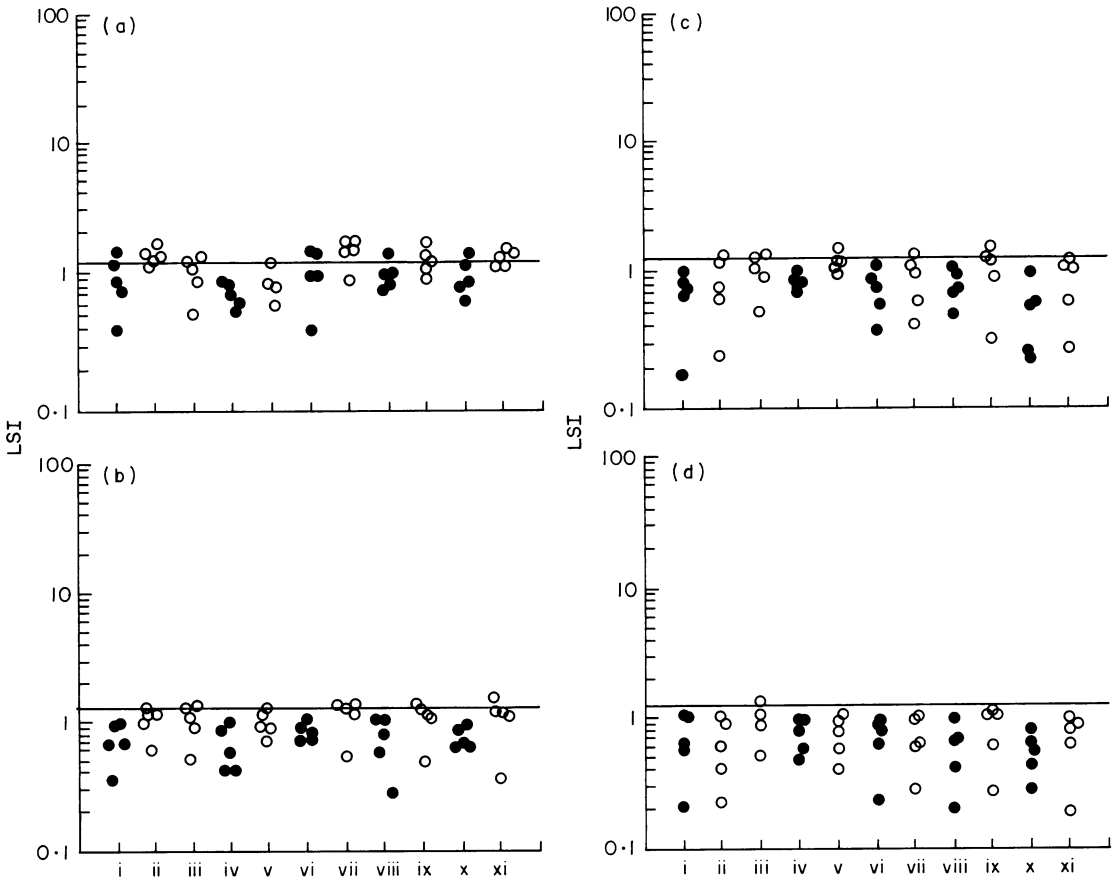

Fig. 3. Lymphocyte stimulation indices (LSI) for each subject in the control group (group C, $n = 5$) without evidence of past infection or immunization against Q fever. PBMC and various reconstituted lymphocyte fractions were stimulated with *C. burnetii* whole cell Henzerling (a) and Priscilla (b) phase I and Nine Mile phase II antigens (c) and yolk sac suspension (d), with (○) and without (●) exogenous IL-2. The horizontal line represents the cut-off value of 1·2 LSI. i, monocytes + T + B; ii, monocytes + T + B + IL-2; iii, monocytes + T + B + IL-2 (added without prior antigenic stimulation); iv, PBMC; v, PBMC + IL-2; vi, monocytes + T; vii, monocytes + T + IL-2; viii, monocytes + B; ix, monocytes + B + IL-2; x, B + T; xi, T + B + IL-2.

Nine Mile phase II antigen gave mean values of 13·9 ± 12·1 and 30·2 ± 23·2, without and with added IL-2). The reconstituted fractions monocytes + B again showed the lower responses on stimulation with antigen relative to fractions containing monocytes and T cells and also showed less amplification with exogenous IL-2.

Finally, in group C, of five subjects with no 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 (Fig. 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.

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

As shown above, T lymphocytes from vaccinees or past infections gave 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 four to six more sugar residues and has a higher molecular weight (Schramek & Mayer, 1982; Baca *et al.*, 1980; Amano, Fukushi & Williams, 1985).

Apart from the variation in the LPS, there appear to be either no or a 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 chick embryo yolk sac (Hackstadt *et al.*, 1985).

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 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 deproteinized 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 non-immune control were tested against a range of dilutions of CSL Nine Mile phases I and II whole cell antigens, periodate-treated Nine Mile phase I antigen, TCA-E and TCA-R and phenol–water extracted phase I LPS (Fig. 4). The phase serological

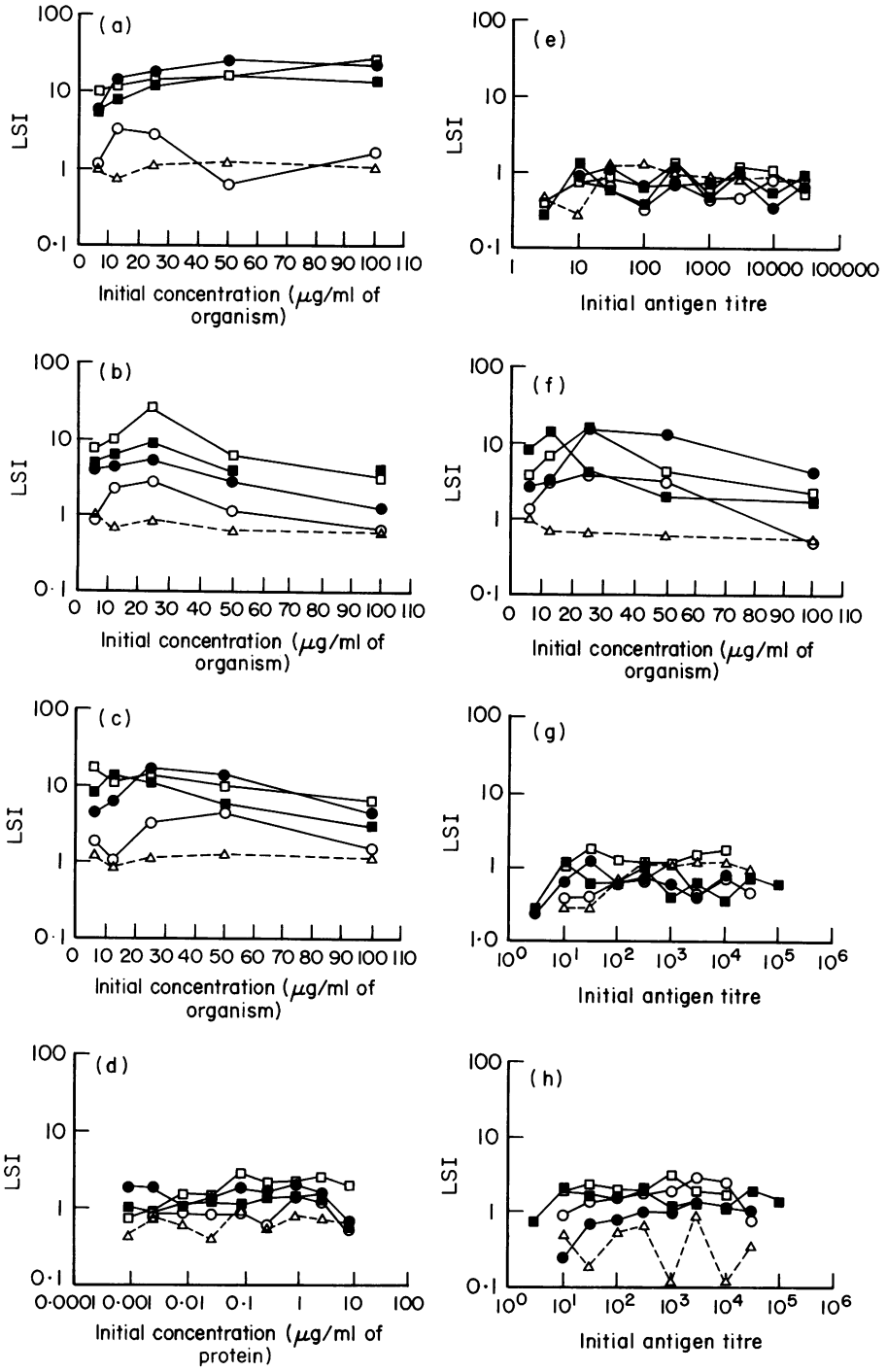

Fig. 4. a–f, T lymphocyte stimulation profiles (LSI, lymphocyte stimulation index) from two vaccinees (■, ●), two subjects with past infection with Q fever (□,○) and one non-immune subject (△) with whole cell and extracted antigens from *C. burnetii*: a, Nine Mile phase I whole cell antigen; b, Nine Mile phase II whole cell antigen; c, Nine Mile phase I antigen, periodate treated; d, Nine Mile phase I antigen, TCA-E; e, Nine Mile phase I antigen, phenol–water phase (water extract); f, Nine Mile phase I antigen, TCA-R. g, h, B lymphocyte stimulation profiles with the five donors and Nine Mile phase I antigen (phenol–water extract, water phase) without (g) and with (h) IL-2.

Table 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

Antigen	Coxiella concentration (μg/ml)	Protein concentration (μg/ml)	Nitrogen concentration (μg/ml)	Complement-fixing activity with antiserum: Guinea pig* phase II CFU	Guinea pig* phase II Antigen titre§	Rabbit† phase I CFU	Rabbit† phase I Antigen titre§	Human‡ phase I CFU	Human‡ phase I Antigen titre§
CSL Nine Mile phase I whole cell	1000	225·8	114·3	0	<4	12	32	13	16
RM RSA516 Nine Mile phase II whole cell	1000	228·1	144·3	35	256	0	<4	0	<4
CSL Nine Mile phase I treated KIO_4	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-R	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

Concentration of coxiellas was determined by spectrophotometry at 420 nm; protein concentration by the Bradford (1976) method; and total nitrogen content by the Dumas method.

* Early post-Q fever infection guinea pig antiserum. Antibody titre against phase I, antigen < 8; phase II, antigen 256.

† Serum from hyperimmunized rabbit, absorbed three times with RM RSA516 Nine Mile phase II antigen. Antibody titre against phase I antigen, 1024; phase II, < 8.

‡ Serum from human chronic Q fever infection; antibody titre with phase I antigen, 128; phase II antigen, 8.

§ Highest (optimal) antigen titre observed with any of a range of antiserum dilutions.

activity of the whole cell antigens and extracted fractions are summarized in Table 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 protein associated with the LPS. TCA-R showed a similar pattern with more phase II activity. Nine Mile phase I whole cells treated with potassium periodate showed, as expected, enhanced phase II activity, but unexpectedly, also enhanced phase I activity. Perhaps removal of some sugar units in the LPS side chains increased access of anti-phase I antibody to other parts of the side chain as well as facilitating access to 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 protein.

The patterns (Fig. 4a–f) of T lymphocyte stimulation with these preparations showed that LPS-rich fractions (LPS P-WE and TCA-E) had little activity compared with whole cells or cell residues. Treatment of Nine Mile phase I whole cell antigen with potassium periodate changed reactivity to the pattern observed with the Nine Mile phase II antigen naturally derived by serial yolk sac passage. Similar patterns were observed with the periodate-treated Priscilla strain (data not shown).

In view of the failure of LPS-PW-E to stimulate T lymphocytes, reconstituted mixtures of B lymphocytes and monocytes from the same five donors were tested with LPS in the presence and absence of IL-2 (Fig. 4b). Low levels of activity were obtained, particularly when IL-2 was added.

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.

DISCUSSION

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 (Jerrells *et al.*, 1975; Ascher *et al.*, 1983; Izzo *et al.*, 1988), or used an *in vivo* skin test to determine delayed hypersensitivity (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 as required antigen-presenting

cells. Exogenous IL-2 gave major amplification with this cell combination. The response of B lymphocytes, even when enriched in a monocytes + B fraction, 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 monocyte + B-enriched fraction from the non-immune comtrol subjects (group C).

The mitogenic responses of T cells from subjects infected in the past were clear cut. 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 facilitating measurement of the responses to *C. burnetii* antigens.

T lymphocytes from vaccinees immunized with the Henzerling strain, *C. burnetii* vaccine (Q-vax) 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 *et al.*, 1985; Hackstadt, 1986; Moos & Hackstadt, 1987), are unlikely to negate the protective value of the CMI response induced by Q-vax. 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 & Hackstadt, 1987; and see below). At the epidemiological level the complete protection given by Q-vax to Australian abattoir workers, many of whom are exposed to infected goats, is in line with the *in vitro* CMI observations and those in guinea pigs.

The conclusion from the results presented, particularly those in Fig. 4, is that the T cell epitopes are associated with the coxiella proteins. The possibility of stimulation of T cells by carbohydrate antigens in tuberculosis 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*. 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 & 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 (i.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 (Kazar *et al.*, 1973; Kishimoto, Rozmiarek & Larson, 1978; Humphries & 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 at the same time by loss variation in cell proteins and conceivably, therefore, in loss of critical T cell epitopes. On the other hand, Hackstadt *et al.* (1985) did not find differences in major protein bands (PAGE) between phase I and II organisms.

Thus far analysis of the protective immunogens of *C. burnetii* cells has established (i) that TCA-E, and the cell residue after TCA extraction, will protect in the guinea pig or mouse model (Anacker *et al.*, 1962); (ii) 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 & Pospisil, 1970). LPS is a hapten; preparations in Freund's incomplete adjuvant do not stimulate antibody formation in rabbits; (iii) that the cell residue (CMR) after chloroform–methanol extraction of the coxiella protects in mice and modifies infection in sheep (Williams, Thomas & Peacock, 1986a; Williams *et al.*, 1986b; Brooks *et al.*, 1986); and (iv) that DMSO extracts of phase I organisms protect in guinea pigs, whereas those from phase II organisms do not (Ormsbee, Bell & Lackman, 1962).

TCA-E, TCA-R and CMR all contain protein and at least traces 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 u.v. absorption (Ormsbee *et al.*, 1962). However, as they provoke phase II antibody in animals (Williams *et al.*, 1986a, 1986b) it is likely that phase II protein(s) are in fact present; indeed Hackstadt *et al.* (1985) found about 25% protein in a DMSO extract of phase I *C. burnetii*.

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 retain good stimulatory mitogenic equivalent to that of phase II whole cells.

These results suggest that coxiella proteins and predominantly those associated with phase II serological activity, are of importance as T cell epitopes and conceivably for protection. Nevertheless extracted protein, free of LPS, has not yet been tested to see whether it will protect. An indication that it may not comes from Kazar & Schramek (1985) who correlated 'protection' against infection assayed in mice, 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 & Schramek's experiments LPS alone again did not protect although (perhaps surprisingly) a positive DTH response was obtained.

From this incomplete evidence it seems probable that the

phase I LPS is part of the protective immunogen of *C. burnetii* 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). Alternatively, the phase I LPS may supply a potent adjuvant for the cell proteins.

Our *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 is indicated by antibody responses after vaccination). However, further definition of the protective efficacy of the cell protein(s) alone is a prerequisite in any attempts to develop alternative vaccines by the rDNA cloning of *C. burnetii* proteins of high mitogenic activity for immune T lymphocytes.

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