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Variation in interferon-gamma responses to *Coxiella burnetii* antigens with lymphocytes from vaccinated or naturally infected subjects

A. A. IZZO & B. P. MARMION *Department of Pathology, University of Adelaide, Adelaide, South Australia*

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SUMMARY

Previous work in our laboratory has shown that lymphocytes from persons vaccinated with a formalin-inactivated Phase I Q fever vaccine (Q-Vax CSL Ltd) show a mitogenic response to *Coxiella burnetii* antigens. The mitogenic response is the sum of that from various subsets of $CD4^+$, T helper cells, $CD8^+$ T cells and probably B cells. It does not distinguish between T helper cell responses leading to formation of interferon-gamma (IFN-γ)—a cytokine responsible for clearing intracellular infection with *C. burnetii* organisms—and responses of other T cell subsets which may produce disease-enhancing cytokines. The present study analyses (i) the capacity of Q-Vax to induce T cell sensitization which leads to IFN-γ responses on antigen stimulation, and (ii) the immunomodulatory, (down-regulatory) effects of the Phase I lipopolysaccharide (LPS) of the organism, which interacts with monocyte/macrophages to limit IL-2 production and production of IFN-γ by sensitized T lymphocytes.

Keywords Q fever vaccine interferon-gamma lipopolysaccharide immunomodulation

INTRODUCTION

Human Q fever is a costly and highly debilitating infection, lasting 2–6 weeks; it is the major zoonosis in the Australian livestock industry. During an acute infection any organ may be involved, but the lungs and liver are most often affected [1]. Infrequent chronic sequelae include valvular or mural endocarditis or chronic hepatitis [1]; more often there is a disabling debility or fatigue syndrome of uncertain pathogenesis which may last for months or years after the initial illness. Human infection is mainly via the respiratory route and by airborne spread of the causative organism, *Coxiella burnetii*, from infected cattle, sheep or goats which may excrete the coxiella in the products of conception, faeces or milk.

Q fever vaccination is the only practicable preventive method and is highly effective [1,2]. Previous studies in our laboratory have shown that 80–90% of human subjects vaccinated with one 30-μg dose of a formalin-inactivated, whole cell, *C. burnetii* vaccine (Q-Vax, CSL Ltd) in the Phase I antigenic state develop sensitized T lymphocytes which give a mitogenic response to whole cell or other preparations of *C. burnetii* containing the proteins of the organism, but not to the extracted, purified, Phase I lipopolysaccharide (LPS). Positive T lymphocyte reactivity (i.e. a raised lymphocyte stimulation index (LSI)) appears 10–15 days after vaccination and is maintained for at least 5 years [3,4]. On the other hand, antibody responses in subjects vaccinated against Q fever are modest and transient, at least as measured by commonly used, somewhat insensitive techniques [2,3].

Measurement of the LSI is widely used as an *in vitro* correlate of T cell immunity to *C. burnetii* [5–7]. Nevertheless, such assays are likely to be only an indirect measure of the *in vivo* mechanisms—i.e. activation of macrophages and other cells by interferon-gamma (IFN-γ)—actually central to the resolution of intracellular infection by *C. burnetii* [8].

Q fever vaccine-induced, or post-infection immunity at the level of the whole animal appears to depend on two responses: (i) development of T lymphocyte reactivity to the proteins (peptides) of the organism [9,10], and (ii) B lymphocyte reactivity (antibody; B cell memory) to the intact, complete Phase I LPS, i.e. LPS with a full complement of sugar residues in the side chains ([11]; see Discussion for details and further references).

The lymphocyte stimulation assay, as used on separated peripheral blood mononuclear cells (PBMC), has the limitation that the stimulation index is the sum of the proliferative responses of various subsets of $CD4^+$ and also of $CD8^+$ T lymphocytes with perhaps a small contribution from B lymphocytes [4]. It is further limited as an *in vitro* correlate of cellular immunity by the recent recognition [12–15] of subsets of CD^+ T cells in mice or man which may show a mitogenic response to antigen, but yet elaborate different cytokines which may either enhance or diminish resistance to infection (see murine leishmaniasis model [16–18] and mycobacteria [19]).

A.A.I. current address: Trudeau Institute, PO Box 59, Saranac Lake, New York 12983, USA.

Correspondence: B. P. Marmion, Department of Pathology, University of Adelaide, Adelaide, S. Australia 5000.

The present study explores IFN-γ formation by T lymphocytes from subjects vaccinated, or previously infected with *C. burnetii*; its modulation with exogenous IL-2; also the effects of *C. burnetii* Phase I LPS as a down-regulator of IFN-γ production. It also defines the range of coxiella proteins that stimulate T lymphocyte proliferation and IFN-γ formation.

SUBJECTS AND METHODS

Subjects studied

The first group comprised 23 volunteers from among 'low risk' laboratory workers [3,4]. Subjects were negative for Q fever antibody by complement fixation (CF) and immunofluorescence (IF) before vaccination and also skin test negative [3]. Pre- and post-vaccination samples of PBMC were taken after inoculation with Q-Vax, and the PBMC, or separated T lymphocytes with autologous monocytes as antigen-presenting cells (APC), were stimulated with various *C. burnetii* antigens.

The second group comprised a small panel of three vaccinees (A.I., S.P. and B.P.M.), two subjects who had had Q fever in the past, and one non-immune control subject. Lymphocytes from this panel were used for detailed analytical experiments in Table 3 and Fig. 4.

C.burnetii *antigens*

The Henzerling, Nine Mile and Priscilla strains of *C. burnetii* were those used previously [3,4].

Isolation and stimulation of PBMC

Separation of PBMC, T lymphocytes and monocytes, and the LSI assays were as described [3,4]. Cultures for the preparation of conditioned media (CM) were performed in 24-well tissue culture plates (Nunclon Delta, Nunc, Roskilde, Denmark) with 2×10^6 cells/ml. Antigen or mitogen (100 μl) was added to each well at a previously determined optimal concentration. PBMC without antigen or mitogen were also included as a control for each subject. Cultures were incubated for 24 h at 37 C in a humid atmosphere with 5% v/v CO_2, after which supernatant fluids were harvested and stored at -70 C. These cultures were then resupplemented with RPMI 1640 (plus 10% v/v fetal calf serum (FCS)) and incubated for up to 144 h, at which time 200 μl replicate volumes were transferred to 96-well microtitre plates (Nunclon Delta, Nunc), pulsed with 0·4 μCi of ^{3}H-thymidine (specific activity, 22 Ci/mmol; Amersham International, Sydney, Australia), incubated for a further 18 h, harvested and counted and the LSI calculated as described [3].

Isolation of monocytes by adherence to gelatin-coated plastic

Monocytes were isolated [20] from PBMC in gelatin (BDH Chemicals, Poole, UK)-coated 75-cm^2 tissue culture flasks (Costar, Cambridge, MA). Autologous plasma from Ficoll–Hypaque gradients was used to treat the gelatin-coated flasks. The plasma was removed and the flasks were washed twice with sterile PBS. Approximately 2×10^7 PBMC in 15 ml RPMI 1640 + 10% FCS were then added to each gelatin-coated flask and incubated for 90 min at 37 C, with 5% CO_2. Non-adherent cells (NAC) were initially washed off with sterile saline and then several times with RPMI 1640 + 10% FCS. NAC were kept for later use. Adherent cells (AC) were eluted with 10 ml of cold 10 mM disodium ethylenediaminetetraacetate (Analytic Reagent, Ajax Chemicals, Auburn, Australia) in Hanks' balanced salt solution (HBSS) at 4°C for 10 min; the flask was tapped on the heel of the hand to remove AC and then any remaining cells were scraped off with a sterile cell scraper (Nunc). AC were transferred to silicon-treated sterile glass tubes (Coatasil, Ajax Chemicals) and washed twice. Viable cells were counted by the trypan blue method.

Monocyte stimulation

Monocytes were resuspended in RPMI 1640 + 10% FCS + 20 mM HEPES at a concentration of 2×10^5/ml in tissue culture plates. The antigens or controls were: (i) Priscilla Phase I LPS; the water phase from phenol-water extraction (PRIS wc P1-PE-W); (ii) Priscilla Phase I whole cell, periodate-treated (PRIS wc P1-KIO_4); (iii) Henzerling Phase I LPS; the water phase from phenol-water extraction (HENZ wc P1-PE-W); (iv) Nine Mile Phase II whole cell (NMILE wc P11); (v) no antigen: unstimulated (UNST).

LPS extracts from Priscilla Phase I cells (PRIS wc P1-PE-W) and Henzerling Phase I cells (HENZ wc P1-PE-W) were prepared by the phenol extraction method [4]. The resulting water phase was freeze-dried and resuspended into 500 μl sterile distilled water. These antigens were added to monocyte cultures to give a final dilution of 1 : 10. Periodate-treated Priscilla Phase I cells (PRIS wc P1-KIO_4) and NMILE wc P11 were added to monocyte cultures to give final concentrations of 10 and 5 μg/ml respectively. Cultures were incubated at 37°C with 5% CO_2. Monocyte-conditioned media (CM-MO) were harvested at 24 h and stored at -70°C.

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 IFN-γ production, IL-2 (1–2 U/ml) at a concentration suboptimal for proliferation was added to cultures together with the *C. burnetii* antigens. In experiments to identify inhibitors of IFN-γ, an assay system of PBMC depleted of B cells with MoAb FMC57/HB43/FMC63 and enriched for T cells and monocytes was used [4].

IFN-γ enzyme immunoassay

Conditioned media from PBMC cultures with and without *C. burnetii* antigens were assayed directly for human IFN-γ by an antigen capture enzyme immunoassay (EIA) using a commercial kit (CSL Ltd, Melbourne, Australia); in the assay conditioned media were allowed to incubate at room temperature for 90 min.

Bioassays for total IFN, IFN-α and IFN-γ in conditioned media from antigen-stimulated PBMC cultures and controls

Conditioned media were assayed for total IFN by inhibition of viral cytopathic effect (CPE). The indicator system was the GM2504 cell (human fibroblast cells; a gift from Dr R. Harris, University of South Australia) together with Semliki Forest Virus (SFV; a gift from Dr Paul Hertzog, Monash University, Melbourne, Australia). CM was added to duplicate wells and diluted in half-log dilutions. Dilutions of a recombinant IFN-γ of known unitage (Biogen, Geneva, Switzerland; Batch 10M31) 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. SFV, diluted to $100TCID_{50}$, was added to each well except for the non-infected control wells, and the cytopathic effect later assessed in each well by uptake of crystal violet dye by cells. IFN titres were expressed as the highest dilution of the supernatant fluid under test which protected 50% of the target cells from viral destruction. Titres were converted into U/ml by comparison with the standard IFN-γ from Biogen.

The component of IFN-γ in the fluid was determined by neutralization with a polyclonal antibody to IFN-α2 raised either in sheep or horse (Boehringer Mannheim, Sydney, Australia) and able to neutralize 10 000 U of IFN-α2. Similarly a polyclonal antibody against human IFN-γ (Genzyme, Boston, MA) sufficient to neutralize 10 000 U of IFN-γ was used to assess IFN-α levels in the bioassay. Sera were monospecific for each IFN.

Immunofluorescence detection of IFN-γ in PBMC

PBMC stimulated with Q fever antigen or mitogen were examined by immunofluorescence for IFN-γ broadly as described [21] for IL-1α and β. For examination, slides with cell spots were removed from −70 C storage, allowed to reach room temperature and rehydrated with PBS. Normal goat serum (NGS; 20%) in PBS was added to cell spots, and incubated for 30 min at room temperature in a humid chamber. NGS was removed, and primary antibody (rabbit anti-human IFN-γ (Genzyme)) diluted in 1% NGS/PBS at a predetermined optimal dilution was added. Slides were incubated for 120 min at room temperature in a humid chamber. The antibody was washed off with PBS. FITC-conjugated secondary antibody (sheep affinity-purified anti-rabbit immunoglobulin (Selinas, Melbourne, Australia)), diluted in 1% NGS/PBS at a predetermined optimal dilution, was then added and incubated for 30–45 min at room temperature in a humid chamber. Slides were washed as above, then twice in distilled water, dried and mounted in a solution of *p*-phenylenediamine (1 ng/ml) (Sigma, St Louis, MO)/glycerol:PBS (pH 8·0). A fluorescence microscope with phase contrast was used to examine cells. Between 100 and 200 cells per cell population were counted.

Separation of C. burnetii *proteins by SDS–PAGE*

A preparation of whole cell lysate from *C. burnetii* from Nine Mile Phase II cells in Laemmli dissociation buffer [22] at a concentration of ~2 mg/ml was kindly provided by Dr T. Hackstadt (NIH, Rocky Mountain Laboratories, Hamilton, MT). This was run on minigels (90 × 103 mm) as an SDS-discontinuous buffer system with 12·5% final acrylamide concentration in the resolving gel [23]. Gels were either stained with coomassie blue, or the protein bands transferred to nitrocellulose (NC).

The separated *C. burnetii* proteins were transferred to nitrocellulose membrane by electrophoresis; the efficiency of transfer was assessed by staining the NC with indian ink [24], and the gel with coomassie blue. Antigen-coated NC particles were prepared [25,26] with solubilization of the NC strips with dimethylsulphoxide (DMSO; Ajax Chemicals, Sydney, Australia), precipitation of the antigen-coated particles with 50 mM carbonate-bicarbonate buffer, pH 9·6, washing of the precipitate with RPMI 1640 and final dispersion, after centrifugation, by repeated aspiration through a 26 G needle.

Table 1. Lymphocyte mitogenic responses, total IFN, IFN-α and IFN-γ responses after *Coxiella burnetii* antigen stimulation of peripheral blood mononuclear cells (PBMC) from 23 vaccinated 'low risk' subjects (outside the abattoir)

	Response to stimulatory antigen¶ values pre- and post-vaccination				
		Increase with			
Assay	No change	Phase I (%)	Phase II (%)	Phase I or Phase II (%)	Total (%)
Lymphocyte mitogenesis*	7	10 (43)	12 (52)	16 (69)	23 (100)
IFN-γ† (antigen-EIA)	5	4 (17)	17 (73)	18 (78)	23 (100)
Total IFN (bioassay)	7	5 (21)	15 (65)	16 (69)	23 (100)
IFN-α‡ (bioassay)	8	8 (34)	12 (52)	15 (65)	23 (100)
IFN-γ§ (bioassay)	6	8 (34)	16 (69)	16 (69)	23 (100)

* Lymphocyte stimulation index: values above 1·2 taken as positive.

† Antigen-enzyme immunoassay (EIA) for IFN-γ, values above 2 pg/ml taken as positive.

‡ Positive readings remaining after neutralization with polyclonal rabbit anti-human IFN-γ.

§ Positive readings after neutralization with sheep anti-human IFN-α2.

¶ *Coxiella burnetti* Phase I, Henzerling or Priscilla strain: Phase II, Nine Mile strain.

RESULTS

Formation of total IFN, IFN-α and IFN-γ by vaccinees in a 'low risk' group

Table 1 summarizes the responses—increased values or no change, pre/post-vaccination—in LSI, and the values for various IFN species assayed by EIA (IFN-γ-EIA; CSL Ltd.) or bioassay (total, IFN-α, IFN-γ) with PBMC from the 23 vaccinees in the low risk category. Antibody titres are not given, as responses to vaccine are low level and transient [2,3].

The EIA (antigen-EIA; CSL Ltd.) for IFN-γ had a detection limit of 2 pg/ml as measured with the recDNA IFN-γ from Biogen. The bioassay with GM 2504 cells had a detection limit of 6 U/ml of total IFN.

The antigen-EIA for IFN-γ detected increased values for IFN-γ over the baseline value (< 2 pg/ml) in 78% of the post-vaccination PBMC samples from the inoculated subjects when responses to either or both Phase I and Phase II antigens are considered (Table 1). Three subjects were IFN-γ-positive in prevaccination samples; two did not show an increased value post-vaccination, and the third increased three-fold.

Total IFN values measured by bioassay were positive in 69% of post-vaccination samples from vaccinees. Although total IFN comprises both IFN-α and γ, there was a reasonable correlation between positive responses by antigen-EIA for IFN-γ and those for total IFN (χ^2_1 (association) = 8·9, $P < 0{\cdot}01$). When the bioassay was made specific for IFN-γ by neutralizing the CM with antiserum against IFN-α, 69% of subjects had a

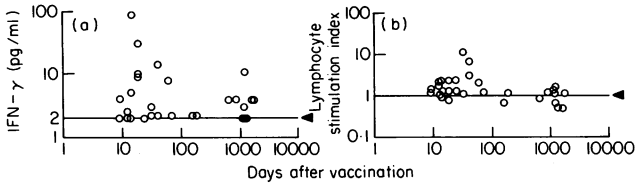

Fig. 1. Interferon-gamma levels in conditioned media (CM) from peripheral blood mononuclear cells (PBMC) stimulated with *Coxiella burnetti* antigens (a) and lymphocyte stimulation indices with *C. burnetii* Phase II antigen (b) in specimens taken at various times after Q fever vaccination in the low risk group ($n = 23$). Cutoff points for the two tests are shown by black arrow.

positive response. All the latter were also positive by the antigen-EIA for IFN-γ, although the antigen-EIA detected two extra positives (Table 1).

In general terms it will be seen that about half (43% and 52% respectively) of the subjects developed positive LSI responses against Phase I or Phase II antigens. However, in the IFN-γ assays, there were two to four times more responders to Phase II than to Phase I antigen (see below).

Overall the proportion of vaccinees with a positive LSI was 69%, less than the value (78%) for IFN-γ measured by antigen-EIA for IFN-γ. There was little overall correlation between IFN-γ and LSI responses (χ^2_1 (association) = 1·8, $P > 0{\cdot}1$), or between the amounts of secreted IFN-γ and the size of values for the LSI ($r = 0{\cdot}03$; $P \geq 0{\cdot}25$) (data not shown).

Q fever antigen stimulation of PBMC from nine of the 23 vaccinated subjects gave negative results for IFN-γ up to 40 days after inoculation. Five of the nine were resampled at 100 or more days after inoculation, and PBMC from four had become positive in the IFN-γ EIA-antigen.

Figure 1a, b sets out IFN-γ values by antigen-EIA, and those for the LSI on stimulation with Nine Mile Phase II antigen, at various times after vaccination. Raised IFN-γ values with CM from antigenically stimulated PBMC were seen as early as 10–12 days after vaccination, and were still present with cells collected at 1000 or more days after vaccination. There is a general similarity between the profiles for IFN-γ and LSI. Again, however, the correlation between the absolute values in the two assays was low.

Figure 2a, b compares size of the lymphoproliferative responses (LSI values), and of the IFN-γ responses (antigen-EIA) to various Phase I and Phase II antigens, as scattergrams of the individual observations. The total numbers in each subgroup stimulated with a particular antigen vary, because not all samples were tested with all antigens. Also, all values are included and so the totals in some subgroups are in excess of the 23 subjects in the experimental group as a whole. Comparisons of Henzerling Phase I and Nine Mile Phase II *C. burnetii* antigens in LSI and IFN-γ assays were done at the same time points with replicates of PBMC collected from the individuals making up the sample.

The scattergrams of the post-vaccination LSI show roughly the same proportion of positive responses to Phase I and II antigens, with those to Phase II being slightly more vigorous (Fig. 2a).

In contrast, the pattern of IFN-γ responses was quite different. Prevaccination samples were negative except for cells from the three subjects mentioned earlier (data not shown).

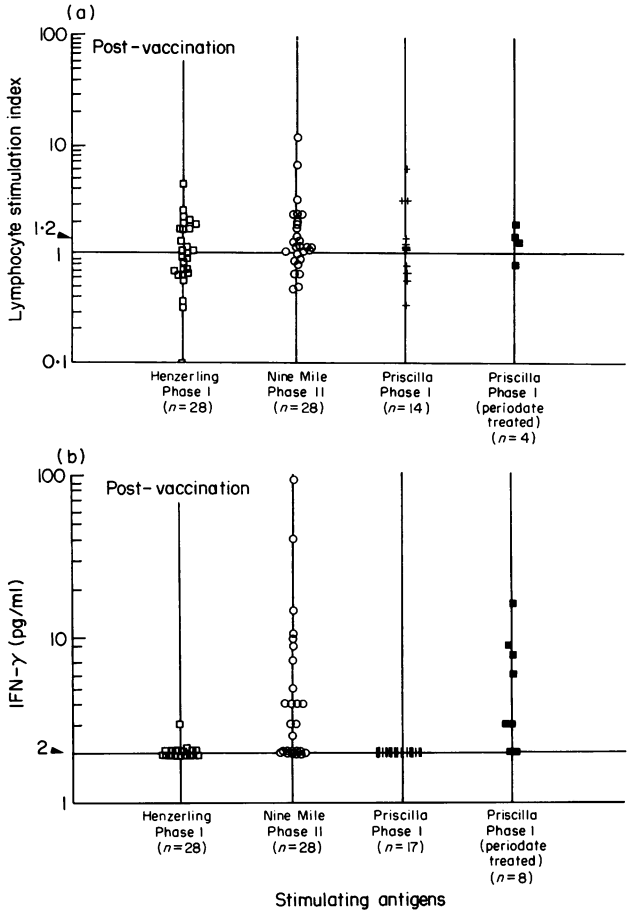

Fig. 2. Scattergrams of lymphocyte stimulation indices (a) and IFN-γ values (b) with various *Coxiella burnetii* antigens and post-vaccination peripheral blood mononuclear cells (PBMC) from the 23 vaccinees in the low risk group

Post-vaccination, there was little response to Henzerling Phase I antigen or to Priscilla Phase I antigen. With Nine Mile Phase II antigen, however, 15/28 samples were positive, seven at or above 7 pg/ml.

The differences in IFN-γ responses with the various antigens might in theory be due to differences in the proteins of the Henzerling and Priscilla strains on the one hand, and those of the Nine Mile strain on the other. However, the differences between the three strains in the polypeptide band patterns on PAGE are not considerable [27]. In any event, as we show below, most if not all proteins from Nine Mile Phase II cells are capable of provoking IFN-γ formation by immune T lymphocytes.

Comparisons were therefore made of the IFN-γ responses to Priscilla Phase I wc antigen treated with KIO_4 (to modify the sugar chains) with the corresponding untreated preparation. The difference between the two stimulating antigens then rested solely in the sugar chains of the LPS; the protein composition of the preparations should be identical. Periodate treatment modified the capacity of the Priscilla Phase I antigen to stimulate IFN-γ formation to a profile closely resembling that of the Nine Mile Phase II (Fig. 2b). The changes in serological reactivity induced by periodate treatment are illustrated elsewhere [4].

The presence of Phase I LPS in the assay system appears, therefore, to inhibit IFN-γ formation by T lymphocytes (see Discussion).

Detection of cell-associated IFN-γ in Q fever-immune lymphocytes and the effect of exogenous IL-2 on IFN-γ secretion

As shown in Table 1, post-vaccination PBMC from five of the 23 'low risk' vaccinees did not show a post-vaccination increase of IFN-γ in the antigen-EIA. Apart from a failure to synthesize any IFN-γ, it was possible either that IFN-γ was not released from the stimulated lymphocytes into the fluid phase of the medium (CM), which was assayed subsequently, or that, if it was released, then assay methods were too insensitive. Accordingly, PBMC from the five subjects were stimulated with coxiella antigens and examined by immunofluorescence. Cytoplasmic or vesicular staining was seen in lymphocytes with a distribution which resembled that of IL-1β [21] (not shown). Cells treated with a serum without antibody to IFN-γ were uniformly negative by IF.

Figure 3 shows the mean values by IF for the proportion of positive to total cells obtained with the five subjects, and *C. burnetii* antigens. One to two per cent of cells from the five subjects, not stimulated with antigen (unstimulated:UNST), nevertheless stained by IF; presumably normal background activity of cells activated by other antigens. There were modest but significant increases in proportions of positive cells in all antigen-stimulated cultures (unstimulated:UNST *versus* Henzerling Ph I $P<0{\cdot}01$; UNST *versus* Nine Mile Ph II $0{\cdot}02>P>0{\cdot}01$; UNST *versus* Priscilla Ph I $0{\cdot}02>P>0{\cdot}01$; UNST *versus* Priscilla K104 $P<0{\cdot}01$). As before, responses to Phase I antigens were lower than to Phase II. Therefore, subjects negative by assays on CM nevertheless formed cell-associated IFN-γ.

Next, IL-2 was added to the reaction mixtures in an attempt to increase the synthesis or secretion of IFN-γ, in view of the enhancement of LSI by exogenous IL-2 in the coxiella-T cell system [4].

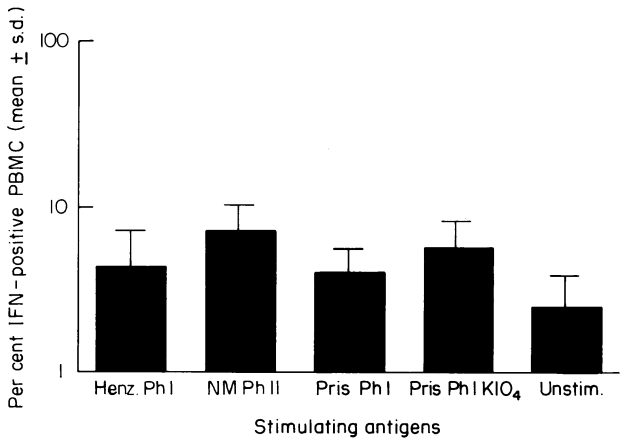

Fig. 3. Immunofluorescence examination of peripheral blood mononuclear cells (PBMC) from vaccinees in 'low risk' group who had not responded by liberation of detectable IFN-γ into conditioned media on stimulation with various *Coxiella burnetii* antigens. Histograms show per cent of the total tested which stained by immunofluorescence (IF) for intracellular IFN-γ (see text for details).

Table 2 sets out the mean values, before and after addition of IL-2 (1–2 U), for IFN-γ (antigen-EIA), IF-positive cells and LSI obtained with cells from five vaccinees, initially negative with Henzerling and Nine Mile antigens. The LSI were only slightly increased in those cultures with IL-2. The amount of IFN-γ in CM by antigen-EIA was, however, consistently increased by IL-2 supplementation, the highest being in cultures stimulated with Priscilla Phase I-KIO_4-treated antigen. The proportion of IF-positive cells with cell-associated IFN-γ was slightly increased by IL-2, but to a lesser extent than the increases in the fluid phase of the culture.

IFN-γ formation, therefore, and its secretion by Q fever-immune T lymphocytes, appears to be more vigorous on exposure of the T cells and their APC to *C. burnetii* cells with a modified (Phase II-like) rather than a Phase I LPS, particularly when the reaction mixtures are supplemented with IL-2.

Table 2. Mean values for cell-free and cell-associated IFN-γ, and the related lymphocyte stimulation index (LSI) values, in cultures of peripheral blood mononuclear cells (PBMC) from five vaccinated subjects with and without IL-2 (1-2 U)

	Without IL-2			With IL-2		
	Mean IFN-γ values			Mean IFN-γ values		
Antigen	Antigen-EIA, pg/ml	Per cent IF-positive cells	Mean LSI	Antigen-EIA, pg/ml	Per cent IF-positive cells	Mean LSI
Henzerling Phase I	<2	4·8	1·9	17·6	7·6	2·3
Nil	<2	2	0·7	<2	2	0·4
Nine Mile Phase II	<2	5·8	3·0	31	9	4·8
Nil	<2	2	0·9	<2	1	0·5
Priscilla Phase I	<2	4·8	2·7	49	8·2	2·7
Nil	<2	3	0·9	<2	1	0·6
Priscilla Phase I-KIO_4	3·2	5	1·4	136	8·2	2·7
Nil	<2	1	0·7	<2	1	0·6

EIA, Enzyme immunoassay; IF, immunofluorescence.

However, all of the whole cell antigen preparations used in the above experiments contained residual, if modified LPS. Consequently, we investigated whether the proteins of the coxiella, when completely separated from LPS, would exhibit increased LSI or IFN-γ values; also, whether there were any individual cell proteins which were significantly more stimulatory for immune T lymphocytes than others.

Profiles of T lymphocyte responses with SDS–PAGE-separated proteins from C. burnetii

A whole cell lysate of Nine Mile Phase II organisms was separated into protein bands by SDS–PAGE, transferred to nitrocellulose and dispersed for cell stimulation. Separated T lymphocytes were prepared from two vaccinees, two subjects infected by *C. burnetii* in the past, and from one unvaccinated subject with no serological evidence of exposure. These were stimulated with the dispersed antigens with autologous monocytes as APC.

In summary (details not shown), the LSI profiles indicated that dispersions from the great majority of protein bands stimulated immune T cells from both vaccinees and past-infection subjects. With all immune subjects the most vigorous response was with the unfractionated *C. burnetii* cell lysate, which gave LSI of 7–13 or more—larger values than seen in most of the assays described earlier. Cells from the control subject gave values at or below the cut-off point (data not shown).

As an illustration, Fig. 4 shows combined histograms for LSI and IFN-γ values with T cells from a subject who had had Q fever in the past. All protein bands stimulated the subject's T cells to give IFN-γ responses at or above 50 pg/ml in the antigen-

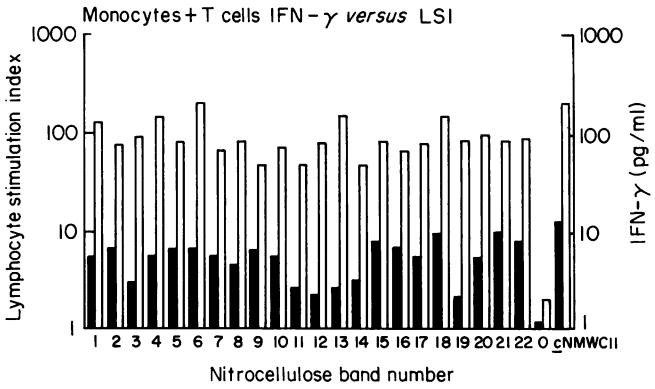

Fig. 4. Profile of lymphocyte stimulation indices (■), and amounts (pg/ml) of IFN-γ (□) liberated into conditioned media, in assays of separated *Coxiella burnetii* proteins with monocytes and T lymphocytes from a subject with a history of Q fever.

EIA; the whole cell lysate (cNMWCII, right of Fig. 4) gave a value of 110 pg/ml. Both the LSI and IFN-γ responses were clearly greater than those obtained with whole cell preparations of Nine Mile Phase II with its residual LPS present (see Table 2). These high readings with the separated proteins, free from LPS, are in accord with the contention that the presence of LPS in the reaction system inhibits the formation of IFN-γ.

Inhibition or enhancement of IFN-γ on antigen stimulation of immune T lymphocytes with C. burnetii *antigens*

From the results just presented, it was suspected that interactions between *C. burnetii* Phase I LPS and macrophages or

Table 3. Inhibiting or enhancing effect of various supplements on the production or liberation of IFN-γ from T lymphocytes taken from three vaccinated subjects and stimulated with *Coxiella burnetti* Phase I antigen (strain Priscilla)

Indicator system	Supplement added to indicator system	IFN-γ in conditioned media from reaction mixture of subject (pg/ml)		
		A.I.	B.P.M.	S.P.
Priscilla Phase I antigen	None	3	4	35
plus monocytes, plus T cells	IL-2	230 (S)	18 (S)	300 (S)
from indicated vaccinee	IL-2 plus:			
	Piroxicam (50 μM)*	350 (+52)	17 (−5)	425 (+41)
	PGE_2 (10)	9 (−96)	2 (−88)	170 (−43)
	1. CM-MO, Priscilla PI, LPS (10)	35 (−84)	10 (−44)	215 (−28)
	2. CM-MO, Henzerling PI LPS (10)	55 (−76)	13 (−27)	ND
	3. CM-MO, Priscilla PI WC-KIO_4 (10)	150 (−34)	22 (+22)	290 (−3)
	4. CM-MO, Priscilla PI LPS KIO_4 (10)	130 (−43)	ND	ND
	5. CM-MO, Nine Mile PII WC (10)	120 (−47)	ND	ND
	6. CM-MO, No antigen (10)	260 (+13)	20 (+11)	220 (−26)
Monocytes + T cells, no antigen	—	<2	<2	<2
Monocytes + T cells, no antigen	IL-2	<2	1	2

1–6. Conditioned media (CM) from separated autologous monocytes stimulated with 1. lipopolysaccharide (LPS) extracted from Priscilla Phase I strain, added to indicator system at a dilution of 1 : 10; 2. ditto Henzerling Phase I LPS; 3. Priscilla Phase I whole cells treated with potassium periodate; 4. LPS extracted from Priscilla Phase I whole cells and treated with potassium periodate; 5. CM from monocytes treated with Nine Mile Phase II whole cell antigen; 6. CM from monocytes without coxiella antigen stimulation.

(−*x*), (+*x*), per cent decrease or increase of IFN-γ production compared with reaction mixture supplemented with IL-2 alone; (S), standard for this comparison.

* Dilution of supplement added.

ND, Not done; CM-MO, monocyte-conditioned media.

monocytes might induce an inhibitor of IFN-γ formation by T lymphocytes. Table 3 summarizes the results of a series of experiments with T cells from three vaccinees A.I., B.P.M. and S.P. The basic indicator system consisted of Priscilla Phase I antigen with autologous monocytes as APC and T lymphocytes from the vaccinated subject under investigation. The reaction mixtures were supplemented with a variety of substances described in detail in the footnotes to Table 3. The indicator system alone produced low levels (3 and 4 pg/ml) in the CM with T cells from A.I. and B.P.M., although S.P. was less inhibited and gave a value of 35 pg/ml. Addition of 1–2 U of IL-2, in agreement with experiments described in Table 2, greatly increased the amount of IFN-γ detected. Addition of 50 μM of Piroxicam (Sigma), an inhibitor of prostaglandin synthesis, further increased the amount of IFN-γ detected by about 40–50% over that given by mixtures with IL-2—the reference standard (S)—in mixtures from A.I. and S.P.; that from B.P.M. showed virtually no change. Addition of PGE_2 (a 1:10 dilution (w/v)) sharply reduced production of IFN-γ by around 90% in A.I. and B.P.M., and by 43% in S.P.; the reductions were less, and in proportion, with a series of dilutions of PGE_2 (latter data not shown).

The addition to the indicator system of CM-MO, prepared by exposing separated autologous monocytes to phenol-water-extracted LPS from Priscilla Phase I and Henzerling Phase I whole cell preparations, at a dilution of 1:10, consistently reduced IFN-γ production by 27–44% of the corresponding standard in the instance of cells from B.P.M. and S.P., and up to 76–84% in reaction mixtures from A.I. On the other hand, CM-MO from monocytes exposed to Priscilla Phase I whole cells treated with periodate (PRIS wc PI KIO_4) or to the LPS first extracted and then treated with potassium periodate, produced a smaller reduction (34–43%) with mixtures from A.I., and essentially no reduction with those from B.P.M. or S.P. CM from monocytes exposed to Nine Mile Phase II wc antigen (CM-MO Nine Mile PII wc) also reduced the production of IFN-γ to about the same extent ($\sim 40\%$) as the periodate-treated Phase I antigens, but much less than with the CM from monocytes stimulated with complete Phase I LPS (observations available for A.I. only).

These results suggest that *C. burnetii* Phase I LPS interacts with monocytes/macrophages to produce an inhibitor of the production/liberation of IFN-γ from T lymphocytes ($CD4^+$ or $CD8^+$, or both: unpublished experiments show that separated $CD8^+$ lymphocytes also produce IFN-γ when stimulated with *C. burnetii* Phase II antigen).

DISCUSSION

This study shows that after inoculation with an inactivated whole cell Q fever vaccine (Q-Vax CSL Ltd) a high proportion ($> 75\%$) of vaccinees develop sensitized PBMC or T lymphocytes which form IFN-γ on stimulation *in vitro* with *C. burnetii* antigens, particularly that of Phase II. The ability to form IFN-γ was detected 10–15 days after inoculation, as with the lymphoproliferative response [1–3], and was present for at least 1000 days after inoculation (Fig. 1a, b).

IFN-γ was produced by both $CD4^+$ and $CD8^+$ T lymphocytes from Q fever-immune individuals (data not shown). Most of the separated proteins of the *C. burnetii* cell stimulated the formation of IFN-γ. There appeared to be no unique protein acting as a highly potent stimulator of IFN-γ, which might be cloned as a protein vaccine to substitute for the whole cell preparation.

Bioassay for IFN-γ was less sensitive than the antigen capture assay, although results of the two were concordant (Table 1). Subjects whose PBMC failed to form cell-free IFN-γ on antigenic stimulation nevertheless formed cell-associated IFN-γ as detected by IF, particularly when stimulated with natural or artificial Phase II antigens (Fig. 3, Table 2). IFN-α, which has immunoregulatory effects [28] and which produces systemic symptoms when administered to human beings [29], was also produced on stimulating immune PBMC with coxiella antigens (Table 1).

Overall, the results indicate that the Q fever vaccine induces a state of cellular immunity with the production of IFN-γ on antigenic challenge rather than other cytokines which enhance B lymphocyte responses, or restrict macrophage activation. The latter cytokines might, from analogy with experimental murine leishmaniasis [16–18], produce disease enhancement rather than resistance. This satisfactory conclusion fits with the high protective efficacy of the vaccine against natural infection [1,2].

Our earlier observations [3,4] with lymphoproliferative responses with coxiella antigens in naturally infected or vaccinated subjects showed that they were generally greater with Phase II than with Phase I antigen. Also, endogenous IL-2 synthesis in the system appeared to be restricted, as the addition of IL-2 specifically increased LSI.

In the present work with IFN-γ formation, the dichotomy in responses with the two phase antigens of *C. burnetii*, and their enhancement with IL-2, was even more clearcut than with the lymphoproliferative responses. Thus, with the PBMC from the low risk group of 23 vaccinees, the IFN-γ response, both in terms of frequency of conversion to produce IFN-γ after vaccination, and its amplitude, was strikingly greater with Phase II than with Phase I antigen (Table 2, Fig. 2a, b).

The differences are not explicable in terms of a strain-based variation in protein composition of the two stimulating phase antigens, as these are small [27]. In any case, as shown (Fig. 4), most coxiella proteins stimulate IFN-γ when presented to immune T cells. Furthermore, modification of Phase I LPS sugar chains with potassium periodate to form an artificial Phase II antigen sharply increases IFN-γ synthesis in the *in vitro* test system, even though the protein compositions remain the same (see Priscilla antigens, Fig. 2b).

The presence of Phase I LPS in the assays has therefore a marked down-regulatory action on both LSI and IFN-γ formation. This is offset by the addition of IL-2. The effect was mimicked by an authentic preparation of PGE_2 and antagonized to some extent by Piroxicam, an inhibitor of prostaglandin synthesis (Table 3). The interaction of monocytes either with naturally derived Phase II antigen, or extracted Phase I LPS modified by periodate, also produced a CM with some down-regulatory activity, but substantially less than that of Phase I LPS or PGE_2 (Table 3).

These observations mesh with current views on the immunoregulatory outcomes of other bacterial LPS–macrophage interactions. There are receptors for various components of LPS on macrophages, including CD14 [30]. Engagement of these receptors, CD14 in particular, generates prostaglandins in the E series, and also regulatory cytokines such as transforming growth factor-beta (TGF-β) [31–35]. Such products diminish

LSI and IL-2 synthesis by T cells, but an effect on IFN-γ formation by human T cells has not been reported before, as far as we are aware.

IL-2 and IFN-γ are products of the Th1 subset of CD4$^+$ T cells, and the effect of the suppressor products from macrophages is presumed to change the balance in favour of the Th2 subset, with a potential for up-regulation of IL-4, IL-5 and IL-10 and to a variable degree IgI and IgE synthesis, or even, with a marked 'tilt', formation of rheumatoid factor (RF) [36–38].

The following implications may be deduced for immunopathogenic concepts of Q fever. *Coxiella burnetii* Phase I cells, which multiply in the phagolysomes of macrophages, would have a survival advantage by down-regulating IFN-γ production, which otherwise would activate the host cell and generate bactericidal metabolites. Coxiellas in Phase II, on the other hand, do not fully restrict the IFN-γ response, perhaps because their truncated LPS engages receptors other than CD14 on the macrophage, and so are more readily eliminated. Note that, *in vivo*, *C. burnetii* fully in Phase II is avirulent, and cannot be passed serially in guinea pigs [1].

Antibody to Phase I antigen, but not Phase II, protects against the major effects of Q fever infection in a mouse model [39]. Early studies by Ormsbee *et al.* [11] showed that Phase I antigen was critical for protection. Recent protection experiments in mice (Marmion *et al.*, 1993, unpublished) with Phase I antisera absorbed with periodate-treated Phase I whole cells suggest that the protective antibody is directed at the terminal sugars of the LPS. It may be surmised, therefore, that one among a number of functions of Phase I antibody (e.g. opsonization, ADCC, etc.) may be to block the critical LPS macrophage–receptor interaction by Phase I cells, preventing down-regulation of IFN-γ. In this context it may be noted that a periodate-treated Phase I whole cell vaccine is less protective for mice than an untreated one, and presumably does not provoke the necessary blocking antibody ([40] and Marmion *et al.*, 1993 unpublished).

Finally, there are hints (Table 3) that the LPS associated with the Priscilla strain may restrict IFN-γ production more than the Henzerling strain. Priscilla is representative of endocarditis strains which differ slightly in their LPS antigens compared with those of the non-endocarditis strains, such as Henzerling [27]. This difference might be reflected in the strength of their interactions with critical macrophage receptors for restriction of IFN-γ formation. Some patients with Q fever endocarditis exhibit an antigen-driven, prostaglandin-mediated, inhibition of T lymphocyte mitogenesis [41]. Perhaps significantly, the great majority exhibit high antibody titres to Phase I and II antigens, accompanied by RF—features which suggest a strong tilt to Th2 T cell activity.

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