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Various membrane proteins of *Francisella tularensis* induce interferon-γ production in both CD4$^+$ and CD8$^+$ T cells of primed humans

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SUMMARY

Tularaemia is an intracellular infection, which is controlled by the host as a result of an immunospecific T-cell response. A crucial product of the responding T cells is interferon-γ (IFN-γ), which acts by enhancing the microbicidal activity of macrophages. T cells of tularaemia-vaccinated individuals respond *in vitro* to a multitude of protein antigens of the vaccine strain *Francisella tularensis* LVS. In the present study, the responses to four of these antigens were shown to be confined mostly to the CD45RO$^+$ memory T-cell subset. To characterize further the phenotype of the responding cells, purified CD4$^+$ and CD8$^+$ T cells were stimulated with the antigens. CD4$^+$ T cells, but not CD8$^+$ T cells, proliferated and produced IFN-γ. However, when CD8$^+$ T cells were isolated from bulk cultures of lymphocytes, which had been stimulated with antigen for 3 days, they responded to an extent similar to that of CD4$^+$ T cells. Purified CD8$^+$ T cells also responded when they were supplemented with interleukin-2 (IL-2). There was a direct quantitative correlation between the proliferative response of CD4$^+$ and CD8$^+$ T cells and their production of IFN-γ. IL-2 was produced in the cultures, the amounts being higher in the cultures of CD4$^+$ than in those of CD8$^+$ cells. IL-4 was not detected in the culture medium of any of the T-cell subsets. Seventeen human αβ$^+$ CD4$^+$ CD8$^-$ CD3$^+$ T-cell clones, specific to antigens of *F. tularensis*, were raised. When proliferating, these clones did invariably produce IL-2 and IFN-γ but no IL-4. In conclusion, both CD4$^+$ and CD8$^+$ T cells of tularaemia-vaccinated individuals respond with proliferation to various protein antigens of *F. tularensis*, and the proliferative response is strictly associated with IFN-γ production. The CD8$^+$ T-cell response seems to depend on cytokines supplied by proliferating CD4$^+$ T cells.

INTRODUCTION

Francisella tularensis, the causative agent of tularaemia, is a facultative intracellular bacterium. Like the defence to other intracellular bacteria, the host defence to *F. tularensis* depends on cell-mediated immunity. Protection from *F. tularensis* can be acquired by natural infection or by vaccination with the live vaccine strain *F. tularensis* LVS (reviewed in ref. 1). T cells of primed individuals respond to *F. tularensis in vitro* with proliferation[2–4] and production of cytokines such as interleukin-2 (IL-2) and interferon-γ (IFN-γ).[5–7] Moreover, the responding T cells acquire cytotoxic activity.[8] IFN-γ is believed to be of special importance to the host defence. It activates mononuclear phagocytes, i.e. the cells primarily infected with *F. tularensis*, thereby rendering the phagocytes capable of responding with an increased microbicidal activity. Treatment of mice with monoclonal antibody (mAb) to IFN-γ increases their susceptibility to tularaemia.[9]

The ability of various subpopulations of T cells to respond to intracellular bacteria has not been fully elucidated. A predominant involvement of CD4$^+$ T cells is to be expected, because CD4$^+$ T cells recognize exogenous antigens, that is, antigens endocytosed by mononuclear phagocytes. Protein antigen is digested in the endosome of the phagocyte and a complex of peptide and major histocompatibility complex (MHC) class II molecule will be transported to the phagocyte surface for presentation to CD4$^+$ T cells. CD8$^+$ T cells, on the other hand, recognize endogenously synthesized peptides such as viral peptides, in complex with class I molecules of the MHC. Data strongly indicate, however, that exogenous antigens can also be presented by a pathway leading to class I-dependent CD8$^+$ T-cell stimulation.[10–12]

CD4$^+$ T cells of *F. tularensis*-primed humans respond to whole heat-killed cells of *F. tularensis* as well as membrane proteins of the organism.[6,13] Results on the participation of

Abbreviations: APC, antigen-presenting cells; mAb, monoclonal antibody; MHC, major histocompatibility complex; MW, molecular weight; PBMC, peripheral blood mononuclear cells; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

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CD8+ T cells are, however, somewhat contradictory. When, in one study,[13] lymphocytes were stimulated with *F. tularensis* and examined by immunofluorescence analysis for the presence of the activation marker HLA-DR, expression was increased only among CD4+ T cells. However, the levels of soluble CD8 were elevated 2–3 weeks after onset of tularaemia, indicating that CD8+ T cells had been activated. When, in another study,[6] *F. tularensis*-stimulated T cells were radiolabelled with thymidine and separated into CD4+ and CD8+ subsets, one-third of newly synthesized DNA was recovered in the CD8+ cells.

In the present study, purified CD4+ and CD8+ T cells from tularaemia-vaccinated individuals were used. A proliferative response to membrane proteins of *F. tularensis* occurred not only in the CD4+ but also in the CD8+ T cells. The response of the CD8+ T cells depended on help afforded by co-cultivation with CD4+ T cells or by supplementation with IL-2. The proliferative response was associated with IFN-γ production but not with IL-4 production, indicating that a cytokine response similar to that of Th1-clones[14] was induced.

MATERIALS AND METHODS

Antibodies

Simultest Leu-3a/Leu-4, fluorescein-conjugated anti-$\alpha\beta$ T-cell antigen receptor, and fluorescein-conjugated anti-CD3 antibodies were from Becton Dickinson (Mountain View, CA). An antibody directed against the $\gamma\delta$ T-cell receptor was purchased from T Cell Sciences (Cambridge, MA). A monoclonal murine anti-human CD45RO antibody (UCHL1), a sheep anti-mouse Ig antibody, and fluorescein-conjugated $F(ab')_2$ of goat anti-mouse Ig were obtained from Dakopatts A/S (Glostrup, Denmark). Polyclonal rabbit antibodies with neutralizing effects against human IL-2 and IL-4, respectively, were obtained from Genzyme (Cambridge, MA). Dynabeads M-450 coated with monoclonal antibodies directed against human CD4 or CD8 antigens were from Dynal A.S. (Oslo, Norway).

Bacteria

The attenuated vaccine strain *F. tularensis* LVS was supplied by the US Army Research Institute of Infectious Diseases (Fort Detrick, MD).[15] Bacteria were cultivated for 2 days on modified Thayer-Martin agar, containing Gc-Medium Base.[16]

Lymphocyte stimulating agents

As stimulating agents, membrane proteins (10 μg/ml) of *F. tularensis*, heat-killed *F. tularensis* LVS (1×10^5 bacteria/ml), purified protein derivative (PPD) of *Mycobacterium tuberculosis* (10 μg/ml; Statens Seruminstitut, Copenhagen, Denmark), or concanavalin A (Con A, 10 μg/ml; Pharmacia AB, Uppsala, Sweden) were used. Membrane proteins of *F. tularensis* LVS were prepared as previously described.[17] Briefly, after SDS–PAGE, proteins were visualized by treating the gels with 0·25 M potassium chloride. Four major membrane proteins of 67,000, 37,000, 32,000 and 17,000 MW, respectively, were excised, eluted, extensively dialysed and precipitated. They were denoted protein nos. I–IV for convenience. When analysed by SDS–PAGE, each of these eluates was found to contain one major polypeptide. In some eluates, traces of one or a few minor polypeptides were also found, adjacent to the polypeptide purified.

Establishment of cell cultures from peripheral blood mononuclear cells (PBMC)

Heparinized blood samples were obtained from healthy adults (aged 32–46) vaccinated with *F. tularensis* LVS 7–16 years previously. PBMC were prepared by centrifugation on a Ficoll/Metrizoate gradient[18] (Lymphoprep; Nycomed AS, Oslo, Norway) and cultures were established.[2] The culture medium consisted of RMPI-HEPES (Gibco Laboratories, Grand Island, NY), supplemented with 15% pooled human serum, 1% (w/v) of gentamicin, and 2 mM L-glutamine.

For depletion of CD45RO+ T cells, PBMC were incubated with the CD45RO-reactive mouse antibody UCHL1. Thereafter CD45RO+ T cells were allowed to aggregate with microspheres coated with anti-mouse IgG, following the instruction of the manufacturer. The beads were removed using a cobalt samarium magnet. Depleted cells were suspended in culture medium. Each culture (200 μl) contained 3×10^5 PBMC or 3×10^5 PBMC depleted of CD45RO+ T cells. The bead-aggregated CD45RO+ T cells were also used in culture experiments. After incubation at room temperature for 2 hr and agitation, beads were removed and the cells were suspended in culture medium. Each culture (200 μl) contained 3×10^5 CD45RO+ T cells and, as antigen-presenting cells (APC), 3×10^5 irradiated (3000 rads) autologous PBMC. The cell preparations were subjected to flow cytometric analysis using a FACScan flow cytometer and FACScan software (Becton Dickinson). UCHL1 was used as primary antibody and fluorescein-conjugated anti-mouse Ig as secondary antibody. Each analysis comprised 10,000 cells.

Cultures of separated CD4+ and CD8+ T cells were also established. Cells of the two subsets were isolated from PBMC by positive selection by means of coated Dynabeads.[19] Beads ($2{\cdot}1 \times 10^7/1{\cdot}0 \times 10^7$ cells) were added to the PBMC and the mixture was incubated for 30 min at 4° with gentle agitation. Then the beads were removed by use of the magnet. The pellet was resuspended in chilled saline. The presence of bound magnetic beads has been shown not to affect the proliferative response and the cytotoxic activity of T cells.[19] The procedure yielded preparations >97% pure, as estimated by immunofluorescence staining. Each culture (200 μl) contained 3×10^5 CD4+ or CD8+ cells and 3×10^5 irradiated (3000 rads) autologous PBMC.

Assay of DNA synthesis and of IL-2, IL-4, and IFN-γ production of T cells from PBMC

To estimate the proliferative response, cultures were incubated at 37° for 6 days, pulsed for 6 hr with 1·0 μCi [^{3}H]thymidine (18 Ci/mmol; Amersham International, Amersham, Bucks, U.K.), and harvested.

To demonstrate the presence of IL-2, samples (100 μl) were obtained from the medium of the cultures after 72 hr of incubation and added to 100 μl of cell medium containing 8000 cells of the IL-2-sensitive cell line CTLL-2 (obtained from ATCC, TIB-214). The cultures were incubated at 37° for 24 hr, pulsed for the last 4 hr with 1·0 μCi of [^{3}H]thymidine (18 Ci/mmol), and harvested. According to preliminary experiments, a maximal IL-2 response was recorded after 72 hr of incubation. To determine the specificity of the assay, the cultures were incubated in the presence of anti-IL-2. The addition of 20 μl of antiserum (which was found to neutralize 1 U of IL-2/ml) completely inhibited the stimulating effect of all samples.

Samples (100 μl) of culture medium were obtained from the medium of the cultures after 72 hr of incubation and analysed for the presence of IFN-γ by a radioimmunoassay (Centocor, Malvern, PA). Standard samples contained 2·1–45·0 IU/ml. The detection limit was approximately 2·0 IU/ml. The amount of IFN-γ was found to be maximal at 72 hr.

To demonstrate the presence of IL-4, samples (100 μl) were obtained from the medium of the cultures after 1, 2, or 3 days of incubation. The samples were analysed by an ELISA specific for IL-4 (Genzyme) and also by use of CTLL cells. The CTLL cells were incubated for 48 hr with or without anti-IL-4 antibodies. During the last 24 hr, they were pulse-labelled with [^{3}H]thymidine. The detection limit of the ELISA was 90 pg/ml and the detection limit of the biological assay was 20 pg/ml. Standard samples of IL-4 contained 10–3000 pg/ml.

Establishment and testing of T-cell clones

Heparinized blood was obtained from an individual who had been vaccinated 8 years previously with *F. tularensis* LVS. After separation on Lymphoprep, $1{\cdot}0 \times 10^7$ PBMC were mixed in culture medium with 1×10^6 heat-killed *F. tularensis* LVS and incubated at 37° for 6 days. Viable cells were recovered on a Lymphoprep gradient, washed and thereafter cloned as previously described.[20] The experiment was designed to give one cell to every second well together with 1×10^5 irradiated (3000 rads) autologous PBMC, 2×10^4 heat-killed *F. tularensis* LVS or 2 μg of protein no. IV, and 4 U of IL-2 in 200 μl of culture medium. IL-2 was added twice a week and feeder cells and antigen every tenth day. When the cell number reached 1×10^6/well, cultures were transferred for further expansion of each clone. Expanded clones were recovered on Lymphoprep gradients and tested in triplicate or quadruplicate for antigen-induced proliferation. Each culture contained 1×10^5 cloned cells, 1×10^5 feeder cells and antigen (2 μg of one of the purified proteins or 2×10^4 of heat-killed *F. tularensis* LVS) in 200 μl of culture medium. After incubation at 37° for 5 days, cells were pulsed for 6 hr with [^{3}H]thymidine and harvested for assay of DNA synthesis. IL-2 was assayed in samples of the culture medium. In both assays, a clone was considered to respond to an antigen when there were more than 1000 c.p.m. over background counts and the number of counts was at least twice that of the background. Samples of the culture medium were also analysed for the presence of IFN-γ and IL-4. All tests were performed on two occasions with an interval of several months. Irrespective of assay of DNA synthesis or of production of IL-2, IL-4, or IFN-γ, the results were highly reproducible from one occasion to the other ($P < 0{\cdot}001$ according to Fisher's exact test). Clones were saved frozen at −135°.

Statistical evaluation

Student's *t*-test, Pearson's correlation coefficient test, and Fisher's exact test were used.

RESULTS

Response of CD45RO$^+$ T cells to various membrane proteins of *F. tularensis*

Human T cells can be divided into two distinct populations based on expression of different isoforms of the leucocyte common antigen CD45. The RO isoform is believed to represent memory T cells, able to respond to recall antigens.[21-22] Mitogens such as phytohaemagglutin (PHA) and Con A, on the other hand, stimulate naive CD45RO$^-$ T cells at least as efficiently as they stimulate the CD45RO$^+$ memory cells.[21] In the present study, we used four membrane proteins (nos. I–IV) of *F. tularensis* as antigens in T-cell tests. T cells from naturally infected or vaccinated individuals have previously been shown to respond in various degrees to these antigens, whereas non-exposed individuals responded poorly or not at all.[6,17] When PBMC from tularaemia-vaccinated individuals were depleted of CD45RO$^+$ T cells (Fig. 1a, b), the proliferative response and the IL-2 response to the antigens was highly reduced (Table 1). This was not due to a possible inadvertent removal of CD45RO$^+$ APC, because a similar reduction was seen in CD45RO$^+$ T-cell depleted cultures supplemented with irradiated autologous mononuclear APC (data not shown).

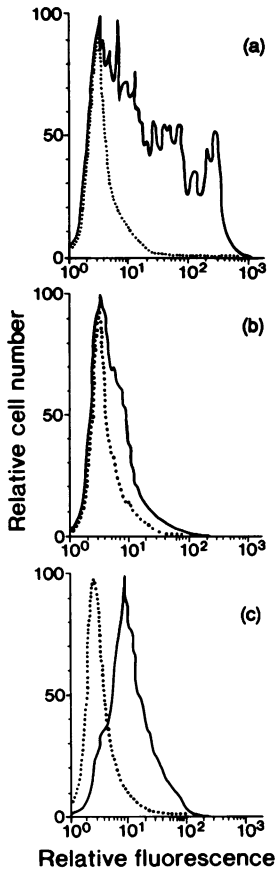

Figure 1. Flow cytometric fluorescence profiles of (a) PBMC, (b) PBMC depleted of CD45RO$^+$ cells and (c) CD45RO$^+$ cells separated by positive selection. Cells were incubated in the presence (——) and absence (· · ·) of the mouse anti-CD45RO mAb UCHL1 and thereafter with fluorescein-conjugated anti-mouse IgG.

CD45RO$^+$ cells were also separated by positive selection and incubated with irradiated autologous APC in the presence of each of the four antigens. The CD45RO$^+$ cells responded to the antigens with proliferation and IL-2 production, although not as strongly as the PBMC (Table 1). The reactivity of the CD45RO$^+$ cells may obviously have been affected by the separation procedure. In fact, a relatively weak fluorescence intensity was recorded in flow cytometric analysis (Fig. 1c). It is

Table 1. Proliferative response and IL-2 response of CD45RO⁺ cells to various antigens of *F. tularensis**

Stimulating agent	Assay	Cells: PBMC	PBMC depleted of CD45RO⁺ cells	CD45RO⁺ cells + irradiated APC	Irradiated APC
Exp. no. 1					
Francisella tularensis LVS					
Protein I	DNA synthesis†	24·0±7·5	2·8±2·0	7·6±3·0	1·1±0·3
	IL-2 production‡	22·1±2·6	2·3±0·4	13·2±1·0	1·3±0·1
Protein II	DNA synthesis	34·0±7·7	3·2±1·7	14·0±4·3	2·4±1·2
	IL-2 production	42·4±2·7	10·4±0·6	21·4±2·1	1·3±0·3
Protein III	DNA synthesis	41·0±6·2	4·0±3·6	13·8±3·8	2·8±2·6
	IL-2 production	30·6±4·6	5·5±1·1	19·4±1·2	2·0±0·1
Protein IV	DNA synthesis	42·3±8·7	3·1±1·7	13·0±4·7	1·5±0·4
	IL-2 production	26·6±2·7	6·9±0·4	27·4±1·6	1·9±0·2
Culture medium	DNA synthesis	5·2±5·2	0·6±0·1	1·7±0·9	1·1±0·1
	IL-2 production	1·4±0·0	1·4±0·2	2·1±0·8	1·4±0·1
Exp. no. 2					
F. tularensis LVS					
Protein I	DNA synthesis	12·3±8·4	5·2±5·1	14·0±7·1	1·0±0·8
	IL-2 production	6·1±1·4	1·7±0·2	7·2±0·5	1·7±0·1
Protein II	DNA synthesis	28·8±13·0	6·7±6·0	13·3±6·0	0·8±0·3
	IL-2 production	5·6±1·3	1·9±0·6	5·4±0·3	2·7±0·4
Protein III	DNA synthesis	36·6±13·6	5·1±1·2	11·3±6·2	2·2±0·9
	IL-2 production	6·5±1·3	2·2±0·5	3·0±0·3	1·6±0·2
Protein IV	DNA synthesis	17·5±6·2	2·9±1·3	10·2±3·1	0·8±0·2
	IL-2 production	4·2±0·4	2·3±0·4	5·2±0·1	1·4±0·3
Culture medium	DNA synthesis	2·8±1·3	0·6±0·2	2·0±0·5	1·7±0·3
	IL-2 production	1·2±0·2	1·8±0·4	0·9±0·2	1·8±0·6

* T cells were from tularaemia-vaccinated individuals.
† Values shown are mean c.p.m. × 10^{-3} ± standard deviation of five cultures.
‡ Values shown are mean c.p.m. × 10^{-3} ± standard deviation of three cultures.

not surprising that affinity separation of CD45RO⁺ cells may interfere with a subsequent recognition of the CD45RO⁺ antigen by the same mAb as used for separation. Taken together, our results indicated that the T-cell response of tularaemia-vaccinated individuals to the *F. tularensis* antigens was confined mostly to the CD45RO⁺ T-cell subset, all antigens thus behaving as expected for recall antigens.

Response of separated CD4⁺ and CD8⁺ T cells to various membrane proteins of *F. tularensis*

CD4⁺ and CD8⁺ T cells were purified by means of antibody-coated magnetic beads and incubated together with irradiated APC and antigen for 6 days. CD4⁺ T cells responded in varying degrees to the four membrane proteins of *F. tularensis* (Table 2). The separated CD8⁺ T cells showed virtually no response to any of the proteins.

The unresponsiveness of CD8⁺ T cells to membrane proteins of *F. tularensis* was found to be reversible. In these experiments, CD4⁺ and CD8⁺ T cells were purified from PBMC, which had been incubated for 3 days in the presence of antigen. When the purified cells were incubated with antigen and irradiated APC for another 3 days, both subsets showed a proliferative response to the antigens (Table 2).

A possible reason for the lack of response of purified CD8⁺ T cells to microbial antigen is a limited access to IL-2 in the cultures, a cytokine which may be afforded by CD4⁺ T cells.[23-25] In accordance with this explanation, the addition of exogenous IL-2 (1 U/ml) was found to render the CD8⁺ T cells fully capable to respond to the membrane proteins (Table 2). CD4⁺ T cells and IL-2-supplemented CD8⁺ T cells showed a similar degree of proliferative response to the proteins.

IL-2, IL-4, and IFN-γ response of CD4⁺ and CD8⁺ T cells of PBMC to membrane proteins of *F. tularensis*

PBMC were incubated for 3 days in the presence of antigen, after which cells of the two subsets were separated from the cultures. CD4⁺ T cells and CD8⁺ T cells were incubated for another 3-day period in the presence of irradiated APC and the antigen. Four individuals responded in varying degrees to the four antigens (Table 3). Altogether, proliferative responses and IFN-γ responses were of similar magnitudes in cultures of CD4⁺ and CD8⁺ cells, whereas IL-2 secretion was lower in cultures of CD8⁺ than in those of CD4⁺ cells. When plotting the results from all these cultures, a quantitative correlation between the magnitude of proliferative response and the IFN-γ content (Fig. 2a) was demonstrated (Pearson correlation coefficient = 0·875, $P < 0·0001$). No quantitative correlation, on the

Table 2. Proliferative response to *F. tularensis* of $CD4^+$ and $CD8^+$ T cells incubated under various conditions*

Stimulating agent	Culture				
	$CD4^+$ cells stimulated for 6 days	PBMC first stimulated for 3 days, $CD4^+$ cells thereafter separated and stimulated for 3 days	$CD8^+$ cells stimulated for 6 days	PBMC first stimulated for 3 days, $CD8^+$ cells thereafter separated and stimulated for 3 days	$CD8^+$ cells stimulated for 6 days in medium supplemented with IL-2
Exp. no. 1					
F. tularensis LVS					
Protein I	7·1±0·4†	3·3±0·2	1·1±0·4	7·8±1·4	8·1±1·2
Protein II	16·2±0·5	12·9±0·7	1·7±0·3	15·0±2·1	11·3±1·9
Protein III	4·4±0·5	3·4±0·4	0·9±0·3	5·4±1·1	4·9±0·6
Protein IV	15·9±2·3	11·0±0·4	1·1±0·2	16·4±2·1	18·5±2·2
Whole bacteria	22·6±2·5	10·8±0·5	1·4±0·2	20·7±2·2	24·0±2·1
Culture medium	0·6±0·1	1·9±0·3	0·9±0·2	1·5±0·3	0·8±0·1
Exp. no. 2					
F. tularensis LVS					
Protein I	1·9±0·7	2·8±0·4	3·3±0·6	3·2±0·3	4·4±1·1
Protein II	11·1±1·4	12·4±1·3	3·6±0·3	8·1±1·0	7·6±0·7
Protein III	2·1±0·4	3·1±1·3	2·5±0·3	2·1±0·9	1·7±0·6
Protein IV	16·4±1·7	12·3±2·1	2·3±0·4	5·6±0·4	6·7±0·5
Whole bacteria	27·3±1·9	18·9±1·5	4·5±0·7	8·7±1·2	12·4±1·1
Culture medium	1·6±0·3	2·7±0·4	2·9±0·6	1·7±0·2	3·4±0·4
Exp. no. 3					
F. tularensis LVS					
Protein I	5·4±0·6	2·9±0·3	0·9±0·1	4·3±0·6	2·9±0·2
Protein II	9·9±0·6	10·2±0·9	1·4±0·3	8·6±0·5	9·8±1·1
Protein III	1·2±0·4	1·8±0·3	0·8±0·4	1·3±0·2	1·9±0·3
Protein IV	13·4±1·6	12·1±0·8	1·2±0·3	10·4±0·9	13·1±1·1
Whole bacteria	29·6±1·8	17·3±2·2	2·7±0·3	30·7±1·4	17·4±0·8
Culture medium	0·9±0·3	1·9±0·3	1·4±0·4	1·7±0·3	2·3±0·3

* T cells were from tularaemia-vaccinated individuals. All cultures were supplemented with irradiated autologous APC.

† Six hours before harvesting, cells were pulsed with [^{3}H]thymidine. Values shown are mean c.p.m. $\times 10^{-3}$ of five cultures.

other hand, was found between proliferation and IL-2 production (Fig.2b).

Cell cultures stimulated with each of the four membrane proteins of *F. tularensis* were also analysed for the presence of IL-4. Neither by a commercial ELISA nor by a bioassay was IL-4 detected in any of the cultures after 3 days of incubation. The bioassay was also applied on all cultures after 2 days and on most cultures after 1 day of incubation, all tests giving negative results. In cultures stimulated with Con A, IL-4 was detected. The detection limit of the most sensitive method, the bioassay, was 20 pg/ml.

IL-2, IL-4, and IFN-γ response of $CD4^+$ T-cell clones to *F. tularensis*

Seventeen T-cell clones specific to antigens of *F. tularensis* were established, five clones in the presence of heat-killed bacteria and 12 clones in the presence of protein no. IV. The clones, all $\alpha\beta^+$ $CD4^+$ $CD8^-$ $CD3^+$, were tested towards protein nos. I–IV and whole cells of *F. tularensis*. The ability of T cells to respond to these antigens has previously been found to be restricted to *F. tularensis*-immunized individuals,[6,17], indicating that none of the antigen preparations acts as a mitogen or a superantigen.

All clones responded to whole cells and to at least one of the proteins of *F. tularensis*. Proliferative responses to appropriate antigens were invariably associated with production of IFN-γ (Fig. 3) and IL-2 (data not shown). The amounts of IFN-γ in the cultures were generally very high, mostly more than 100 IU/ml. No IL-4 was detected.

Ten of the 17 clones responded, as expected, to only one of the four *F. tularensis* proteins, whereas six clones responded to two of the proteins (nos. II and IV) and one clone to three proteins (nos. II, III and IV). This raised doubt on the monoclonal nature of the T-cell clones. Based on calculations of

Table 3. Proliferative response, IL-2 response and IFN-γ response of CD4+ and CD8+ T cells to various membrane proteins of *F. tularensis**

Subject no.	Stimulating agent	Cells					
		CD4+ T cells			CD8+ T cells		
		Proliferation†	IFN-γ‡	IL-2§	Proliferation†	IFN-γ‡	IL-2§
1	*F. tularensis* LVS						
	Protein I	7	5	29	30	22	5
	Protein II	20	32	59	38	44	9
	Protein III	21	14	26	35	31	4
	Protein IV	24	19	28	69	109	11
	Whole bacteria	45	68	65	50	90	56
	Culture medium	1	<2	6	2	<2	7
2	*F. tularensis* LVS						
	Protein I	3	3	36	9	4	4
	Protein II	8	16	58	13	20	7
	Protein III	2	<2	8	4	<2	8
	Protein IV	11	18	16	18	38	7
	Whole bacteria	11	16	13	24	60	10
	Culture medium	2	<2	7	2	<2	6
3	*F. tularensis* LVS						
	Protein I	34	24	8	12	10	10
	Protein II	37	64	18	14	32	12
	Protein III	32	34	18	6	2	7
	Protein IV	25	36	54	15	42	28
	Whole bacteria	55	74	25	20	39	33
	Culture medium	1	<2	5	1	<2	8
4	*F. tularensis* LVS						
	Protein I	5	<2	2	4	<2	2
	Protein II	14	20	5	5	3	1
	Protein III	6	4	5	2	<2	1
	Protein IV	7	18	4	2	<2	2
	Whole bacteria	22	42	6	16	18	2
	Culture medium	1	<2	2	1	<2	1

*After stimulation of PBMC with antigen for 3 days, CD4+ or CD8+ T cells were separated and stimulated with the same antigen in the presence of APC for 3 days.

† Six hours before harvesting, cells were pulsed with [^{3}H]thymidine. Mean c.p.m. $\times 10^{-3}$ of five cultures are indicated. Standard deviations of proliferating cultures were < 18% of the means.

‡ IFN-γ was assayed in samples of culture medium. Mean international units of three cultures. Standard deviations were < 11% of the means.

§ IL-2 was assayed in samples of culture medium by use of CTLL-2 cells. Proliferative responses are indicated as mean c.p.m. $\times 10^{-3}$ of three cultures. Standard deviations were < 16%.

the frequency of originally cloned T cells, the probability of a monoclonal origin of clones raised with whole heat-killed bacteria and protein no. IV was 98·6 and 99·7%, respectively. A monoclonal origin was further supported, when clone ASC9, recognizing protein nos. II and IV, was subcloned. Three clones were obtained, all with the same pattern of recognition as clone ASC9. The ability of discrete antigens of *F. tularensis* to stimulate one and the same T-cell clone has been previously reported.[6]

The uniformity in phenotype and cytokine pattern among the clones generated should be regarded with some caution. All clones originated from one and the same individual and the uniformity may reflect the immune state of this individual. Moreover, the culture conditions for generation of clones may have favoured the growth of CD4+ Th1 cells. Such a bias has previously been suggested as occurring.[26]

DISCUSSION

The results demonstrate that besides CD4+ T cells, separated CD8+ T cells from tularaemia-immune individuals can be stimulated *in vitro* with *F. tularensis* antigen under appropriate culture conditions. This finding is of principal importance. Experiments on mice indicate that CD8+ T cells have a role in the host defence against facultative intracellular bacteria such as *M. tuberculosis*.[27] This is difficult to conciliate with the view that CD8+ T cells only recognize endogenous antigen, i.e. antigen synthesized by the APC. In fact, exogenous proteins have been

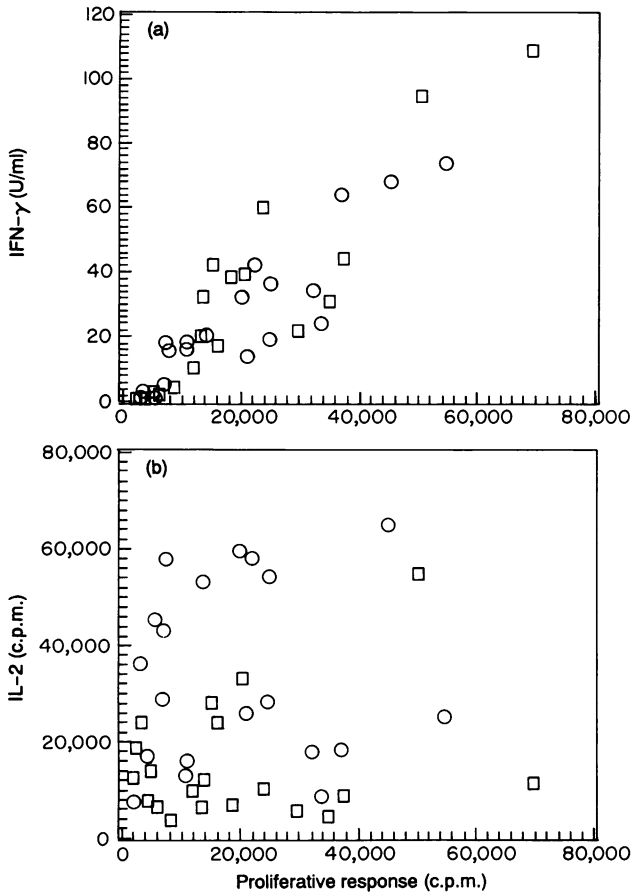

Figure 2. Correlation between proliferative response and (a) IFN-γ response or (b) IL-2 response of CD4+ (○) and CD8+ (□) T cells to various antigens of *F. tularensis*.

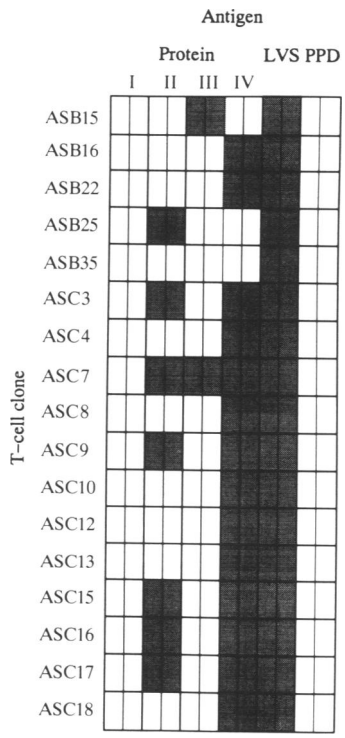

Figure 3. Proliferative response and IFN-γ response of T-cell clones to various proteins and whole bacteria (LVS) of *F. tularensis* LVS and to PPD. Boxes are divided into a left part denoting proliferative response and a right part denoting IFN-γ response. Open boxes indicate values not significantly different from background values ($P > 0.05$). Values significantly above background values are denoted as black boxes ($P < 0.001$). All clones were from the same LVS-vaccinated individual.

shown to induce antigen-specific stimulation of CD8+ T cells.[10-12] By extending the study to include bacterial proteins in tests of several tularaemia-vaccinated individuals, the strict division in endogenous class I and exogenous class II routes is further upset. On the contrary, a general ability of proteins from an intracellular bacterium to induce CD8+ T-cell stimulation is suggested .

The pathway whereby antigenic proteins reach the state of presentation to CD8+ T cells is elusive. The association of antigenic peptides to MHC class I molecules is believed to occur in the endoplasmic reticulum after translocation of the peptides from the cytosol. One possible way of entrance into the pathway would be a passive or active translocation of endosomally located antigenic proteins or proteolytic fragments into the cytosol. There are, however, no data available to suggest such a mechanism except when proteins are inherently specialized in translocation, e.g. diphtheria toxin, human immunodeficiency virus (HIV) Tat protein and listeriolysin. Other posssible pathways for the transport and binding of peptides to class I molecules are, however, even more speculative. Exocytosis of antigenic peptides followed by binding to class I molecules on the cell surface is unlikely because the assembly of class I molecules seems to depend on the presence of peptide already in the endoplasmic reticulum and recycling of class I molecules does not seem to occur.[28]

A requirement of help, besides the presence of APC, for the response of CD8+ T cells to antigens of *F. tularensis* is in agreement with *in vitro* studies of the human T-cell response to other recall antigens such as influenza and tetanus antigen.[29] The *F. tularensis*-induced CD8+ T-cell stimulation seemed to require accessory IL-2. When IL-2 was added to cultures of purified CD8+ T cells, they showed an antigen-specific proliferative response and an IFN-γ response as efficient as that of CD4+ T cells. A failure of CD8+ T cells to satisfy their requirement of IL-2 by autocrine production was supported by the demonstration of lower amounts of IL-2 secreted into supernatant fluids by CD8+ T cells than by CD4+ T cells under conditions when both populations proliferated to a similar extent (Table 3). The CD8+ cells became antigen-reactive, however, when PBMC were incubated for 3 days with antigen. This may be explained by an effect of IL-2 produced by the CD4+ cells, although an influence of other cytokines has not been fully excluded. A prerequisite for IL-2 in antigen-specific induction of IFN-γ secretion by CD8+ T cells has been described with *Listeria monocytogenes*,[23] *M. leprae*,[24] *Toxoplasma gondii*,[25] and allogen as antigen.[30]

The *in vivo* relevance of the ability of CD8+ T cells to secrete IFN-γ in response to *F. tularensis* remains to be studied. Macrophages, the cells primarily believed to be infected by the bacterium, carry class II as well as class I MHC molecules and may thus present antigen both to CD4+ and CD8+ T cells. A predominant role of CD4+ T cells in tularaemia is suggested by the report of class II-restricted host resistance in mice.[31]

However, studies on other facultative intracellular bacteria suggest that CD8+ T cells may also contribute to the host defence, possibly with kinetics different from that of CD4+ T cells. In tuberculosis, a CD8+ T-cell response, primarily cytotoxic, is believed to precede a later CD4+ T-cell response.[32] In accordance, the expression of class II molecules increases when the T-cell response develops. The evidence of macrophages being the only or even the predominant target cells of *F. tularensis* infection is, however, scant.[1] To control a bacterial proliferation in other cells, lacking class II molecules, class I-restricted CD8+ T-cell stimulation would be important. IFN-γ is capable of inducing anti-microbial activity in non-phagocytic cells such as fibroblasts.[33]

The proliferative response of CD4+ and CD8+ T cells to *F. tularensis* was strictly associated with IFN-γ production. Moreover, IL-2, but not IL-4 production, was detected in the cultures. This type of response resembles the response of a subpopulation of CD4+ T-cell clones defined as Th1 cells in studies of murine T cells. Th1 cells produce IL-2, IL-3, granulocyte–macrophage colony-stimulating factor (GM-CSF) and IFN-γ, and mediate macrophage activation, whereas Th2 cells produce IL-3, GM-CSF, IL-4, IL-5 and IL-6, and afford B-cell help. *In vitro* stimulation of murine CD4+ T cells with different sets of microbial antigens[34] or immunization of animals by different procedures[35] have been reported to yield a biased response, favouring either a Th1 or Th2 response. A corresponding Th1/Th2 dichotomy in humans has been questioned, because clones responding to various stimulants including recall antigen have been found to produce IL-2, IFN-γ, as well as IL-4.[36] Recently, however, evidence for a dissociation has been presented.[37–39] Large series of T-cell clones specific to various mycobacterial antigens have shown the same cytokine response as murine Th1 cell clones,[37,39] whereas most CD4+ clones specific to *Toxocara canis* excretory/secretory antigen[37] or to a crude extract of house dust mite[38] had a Th2-like response. We have demonstrated a Th1-like cytokine response in CD4+ T-cell clones as well as in CD4+ T cells of PBMC from tularaemia-vaccinated individuals to various antigens of *F. tularensis*. Moreover, a Th1-like cytokine response to the antigens also occurred in CD8+ T cells of PBMC. Murine CD8+ T-cell clones have been found to respond to alloantigens and mitogens with a Th1 cytokine pattern.[40]

IFN-γ is a key mediator of host defence to facultative intracellular bacteria such as *F. tularensis*. The present demonstration of a quantitative relation between proliferative response to the organism and IFN-γ production suggests that most *F. tularensis*-stimulated T cells, CD4+ or CD8+, may give rise to IFN-γ. The involvement of a wide variety of T-cell reactive proteins in the host defence[6,13] seems appropriate in order to ensure the host of an optimal IFN-γ response in the infected lesion.

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