

Cytokine Response of T-Cell Subsets from *Brucella abortus*-Infected Mice to Soluble Brucella Proteins

YIFAN ZHAN,* JI YANG, AND CHRISTINA CHEERS

Department of Microbiology, The University of Melbourne, Parkville, Victoria 3052, Australia

Received 4 January 1993/Accepted 16 April 1993

Hot saline extracts of *Brucella abortus* 19 were separated by successive differential precipitation with 50 and 70% ammonium sulfate, yielding fractions SBP50, with predominantly 36-kDa proteins and a number of medium-sized proteins (26 to 33 kDa), and SBP70, with 14-kDa and lower-molecular-mass proteins. Both fractions stimulated specifically proliferation and cytokine production by spleen cells from brucella-infected mice, although the activity of SBP50 was much higher than that of SBP70. Further separation of SBP50 by a DEAE-Sepharose column resulted in three distinct subfractions which were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The three subfractions were analyzed for their abilities to induce lymphocytes to proliferate and produce cytokines. The three subfractions were all active but with characteristic differences in magnitude. Subfraction 1 stimulated moderate proliferation, high interleukin 6 (IL-6) production, and relatively low production of gamma interferon (IFN- γ). Subfraction 2 was the strongest stimulus for proliferation and production of IL-6 and IFN- γ , while subfraction 3 stimulated moderate cell proliferation, a high level of IFN- γ , and a low level of IL-6. IL-2 production stimulated by the three subfractions was similar. SBP50 and all three subfractions stimulated purified T cells of both CD4⁺ and CD8⁺ subsets to produce IFN- γ . The production of IFN- γ by CD8⁺ T cells to brucella antigens was enhanced with exogenous IL-2.

It has been established in a murine model that protective acquired immunity to *Brucella abortus*, a facultative intracellular bacterium, involves T-cell-dependent activation of macrophages. Both CD4⁺ and CD8⁺ T cells contribute to protection against brucella infection (1, 23). Although the mechanism of T-cell-mediated immunity is not completely understood, cytokines such as gamma interferon (IFN- γ) produced by T cells are believed to be major mediators of macrophage activation. In vitro treatment of macrophages with recombinant IFN- γ enhanced killing of *B. abortus* (15) and other intracellular organisms (12, 26). Injection of recombinant IFN- γ into mice enhanced resistance to *B. abortus* (32) and *Listeria monocytogenes* (18), while injection of antibody to IFN- γ exacerbated infection with these intracellular bacteria (7).

Brucella organisms, perhaps more than other intracellular bacteria, induce the formation of antibodies. This can give some protection against secondary infection (8) but is not believed to be a major contributor to recovery from primary infection (8, 23). The vaccine presently used in cattle is attenuated *B. abortus* 19, which has limited effectiveness and causes diseases in cattle and humans (9, 39). No safe vaccine is available for humans. Therefore, there is a need to search for improved, possibly recombinant, vaccines to induce acquired cellular resistance.

The study of brucella protein antigens which could activate T cells has been attempted for both potential development of vaccines and diagnostic use (4, 11, 31, 33, 35). Outer membrane proteins from *B. abortus* have been isolated, characterized (35), and tested for their ability to stimulate a cellular response measured by lymphocyte blastogenesis (2). These proteins, combined with adjuvant, conferred some protection against brucella challenge in a murine model (21). Proteins prepared by salt extraction (11, 33) have also been demonstrated to induce protective immunity. However, the

immune mechanisms evoked by these preparations of brucella protein antigens have not been well defined. The lymphocyte response was evaluated only by proliferation, which does not differentiate between the various biological functions of T and B cells. Furthermore, within the CD4⁺ subpopulation of T cells, different subpopulations apparently secrete different profiles of lymphokines, which may determine the bias of the immune response towards cell-mediated immunity (Th1 cells) or antibody production (Th2 cells) (22). It is possible that those T cells which do not produce protective cytokines may exacerbate infection (29, 34). It is by no means clear whether certain antigens favor one subset of T cells or the other. Furthermore, given the importance of CD8⁺ T cells (1, 23), it is desirable that the antigen(s) should also stimulate this population. Thus, defining the brucella antigens which are able to stimulate T-cell activation in terms of cytokine production will be a first step towards the development of a more effective vaccine.

In the experiments described here, we fractionated a crude antigen preparation from *B. abortus* and tested the abilities of a number of fractions to stimulate T-cell activation by cell proliferation and cytokine production. The response of T-cell subsets (both CD4 and CD8) to these fractionated antigens was also investigated.

MATERIALS AND METHODS

Infection of mice. CBA mice were bred by pedigree brother-sister mating in the Department of Microbiology, University of Melbourne. They were housed under conditions of isolation and fed sterile pellets and water to maintain their infection-free status. *B. abortus* attenuated vaccine strain 19 was originally obtained from the Commonwealth Serum Laboratories (Melbourne, Australia) and maintained by weekly subculture on horse blood agar. Cultures were periodically renewed from freeze-dried stock to maintain a constant level of virulence for the mice. For infection, mice

* Corresponding author.

were injected intravenously with 5×10^5 *B. abortus* 19 cells from a 24-h culture on horse blood agar.

Preparation of soluble brucella protein (SBP). Viable *B. abortus* organisms (5 ml of a suspension of 10^{10} /ml) were added to 800 ml of sterile Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) and incubated at 37°C on a shaker platform for 48 h. Bacteria were harvested and washed once with saline. Hot saline extracts were obtained by suspending organisms in saline and autoclaving at 132°C (138 kPa) for 15 min. The autoclaved suspension was centrifuged at $12,000 \times g$ for 15 min, and the supernatant was collected and precipitated with ammonium sulfate (50% saturation). After centrifugation at $8,000 \times g$ for 15 min, the precipitate was dissolved in 0.01 M phosphate-buffered saline (PBS) (pH 7.2) and dialyzed against 0.01 M phosphate buffer (pH 7.2) for 48 h. This preparation was designated SBP50. The supernatant from 50% ammonium sulfate precipitation was further precipitated with ammonium sulfate to 70% saturation. The resultant pellets were dissolved in PBS and dialyzed as described above. This preparation was termed SBP70. Both preparations were centrifuged at $12,000 \times g$ to remove insoluble material and sterilized by filtration. The protein content was determined by A_{280} .

Fractionation of SBP with DEAE-Sepharose. Fractionation was performed at room temperature. SBP50 (5 ml of a solution of approximately 2 mg of protein per ml) was applied to a 20-ml column of DEAE-Sepharose (Pharmacia LKB, Uppsala, Sweden) equilibrated with 0.1 M NaCl–0.01 M phosphate buffer (pH 7.2) at a flow rate of 0.5 ml/min. The column was then washed with 50 ml of the starting buffer (0.1 M NaCl, 0.01 M phosphate buffer, pH 7.2). Bound proteins were washed out in a stepwise fashion with 0.3, 0.5, and 1 M NaCl in 0.01 M phosphate buffer (pH 7.2). Each step of elution contained 50 ml (2 ml per fraction). The protein concentrations were assayed by A_{280} . All samples were sterilized by filtration and tested for activities, including stimulation of proliferation and cytokine production by spleen cells from *B. abortus*-infected and normal mice. Control cultures of unseparated SBP50 diluted in elution buffer (1 M NaCl, 0.01 M phosphate buffer, pH 7.2) were included to rule out a possible effect of salt. Thereafter, peak fractions were pooled, concentrated, and dialyzed. The concentrations of three pools of proteins were assayed by Hartree's method (14).

SDS-PAGE. The stacking and separating gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) consisted of 5 and 12.5% acrylamide, respectively. Samples were heated at 100°C for 2 min in 0.05 M Tris buffer (pH 6.5) containing 2% SDS, 10% 2-mercaptoethanol, and 10% glycerol. Electrophoresis was carried out at 15 V/cm. The gels were visualized after being stained with Coomassie brilliant blue R-250 (Bio-Rad, Richmond, Calif.) and calibrated with low-molecular-weight standard mixtures (Bio-Rad).

Preparation of T-cell populations. T-cell-enriched splenocytes were prepared by two passages through nylon wool columns (16). Subset-enriched T cells were prepared by negative panning (19). CD4⁺ T cells were negatively selected with anti-CD8 monoclonal antibody (3.168) (27)-coated petri dishes (90-mm diameter; Disposable Products, Adelaide, Australia). CD8⁺ T cells were negatively selected from anti-CD4 monoclonal antibody (GK1.5) (10)-coated dishes. Coating of petri dishes was achieved by incubating with antibody (0.1 ml of a solution of 10 mg/ml of ammonium sulfate-precipitated ascitic fluid) in 10 ml of PBS. Immedi-

ately before use, plates were washed five times with PBS. Onto each plate, 5 ml of nylon wool-purified T cells at 8×10^6 /ml in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered Dulbecco's modified Eagle's medium plus 5% fetal calf serum was pipetted, and plates were incubated flat at 4°C for 30 min, swirled, and left for 40 min longer. Supernatants were decanted, and plates were washed gently twice with 5 ml of cold PBS. Supernatants and two washings were pooled, spun down, and resuspended in 5 ml of HEPES-buffered Dulbecco's modified Eagle's medium plus 5% fetal calf serum. The panning procedure was repeated once.

Culture for cell proliferation and cytokine production. For proliferation, 2×10^5 spleen cells were cultured with or without antigen in a 0.2-ml volume for 3 days. On the third day, cultures were pulsed with 1 μ Ci of [³H]thymidine for 6 h. Cells were then harvested onto glass fiber filters (ENZ Diagnostics, Inc., New York, New York) which were placed into vials containing scintillation fluid, and incorporated radioactivity was measured by liquid scintillation counting in a Packard beta-counter. For cytokine production by splenocytes, 4×10^6 cells were cultured with or without antigen in a 2-ml volume. Supernatants were harvested 24 h later and assayed for cytokine level. Purified T cells and subtypes were cultured at 2×10^6 cells with or without antigen in a 2-ml volume in the presence of 4×10^6 irradiated normal spleen cells as antigen-presenting cells. For blocking lymphokine release (10), anti-CD4 (GK1.5) or anti-CD8 (3.168) antibody (described above) was added to cultures at a 1/200 dilution. Cultures were incubated for 24 h for assay of interleukin 2 (IL-2) and 72 h for assay of IFN- γ or IL-6.

Cytokine bioassays. Serial dilutions of culture supernatants were incubated with appropriate cytokine-responsive cell lines in Dulbecco's modified Eagle's medium with 10% fetal calf serum in flat-bottomed microtiter wells. Titration of the appropriate standard cytokine was included in each assay, and this standard curve was compared with the test titration to calculate international units by ratio. IFN- γ titers were compared with an international reference standard (National Institutes of Health, Bethesda, Md.; catalog number Gg 02-901-533). IL-2 was standardized against recombinant human IL-2 purchased from Cetus Corp. (Emeryville, Calif.). IL-6 was standardized against recombinant mouse IL-6 (specific activity, 2×10^8 IU/mg; the generous gift of R. Simpson, Ludwig Institute, Melbourne, Australia). The cell lines used were WEHI-279 for IFN- γ (25), CTLL for IL-2 (13), and 7TD1 for IL-6 (36). For IL-6 and IFN- γ , specificity was checked by assaying in the presence and absence of neutralizing monoclonal antibody.

RESULTS

Spleen cell response to ammonium sulfate-precipitated fractions of SBP. Both SBP50 and SBP70 were tested for their abilities to stimulate proliferation and cytokine production by spleen cells from mice which were infected with *B. abortus* for 6 to 8 weeks and from normal mice. Brucella-immune spleen cells proliferated in response to both antigens and produced IL-2 and IFN- γ (Fig. 1). In contrast, normal spleen cells proliferated to a lesser degree and produced no detectable IL-2 or IFN- γ . SBP50 stimulated more [³H]thymidine uptake and cytokine production by immune spleen cells than did SBP70. This may be related to the different pattern of protein bands seen in SDS-PAGE (Fig. 2), although specific proteins corresponding to the observed activities were yet to be identified. SBP50 had a 36-kDa

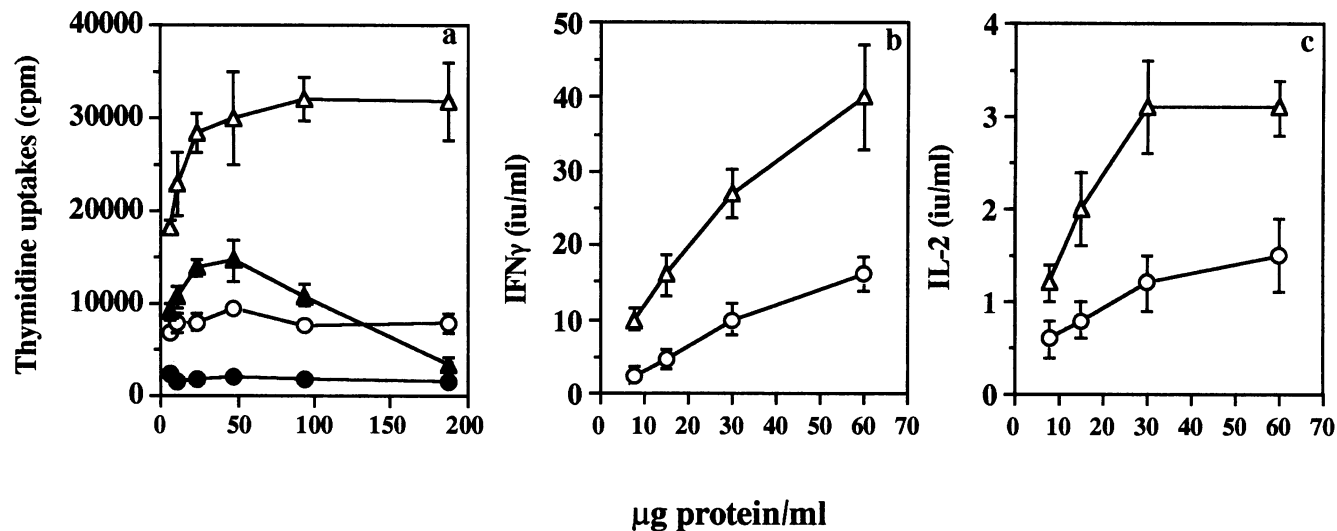


FIG. 1. Brucella-immune spleen cells (open symbols) and normal spleen cells (solid symbols) were stimulated with SBP50 (Δ , \blacktriangle) or SBP70 (\circ , \bullet) at the indicated concentrations. Data from one representative experiment of three are shown and are expressed as the mean and standard deviation from triplicate cultures at each point. (a) Thymidine uptake by spleen cells. (b) IFN- γ production by spleen cells. (c) IL-2 production by spleen cells. Normal spleen cells did not produce IFN- γ or IL-2.

dominant band and four minor bands (26, 28, 31, and 33 kDa), while SBP70 had a 14-kDa dominant band and several minor bands.

Proliferation and cytokine responses to fractionated SBP50. To identify the antigens which were likely to stimulate a cell-mediated immune response from a complex of brucella proteins, we used ion-exchange chromatography to fractionate SBP50 (Fig. 3). Figure 3a shows the elution profile achieved by increasing the NaCl gradient stepwise from 0.1 to 1 M. Three peaks were observed by measuring the protein content of each fraction by A_{280} . This pattern was found to be reproducible in several separations. From each fraction, 5 μ l was added to 0.2-ml spleen cell cultures to measure proliferation, and 20 μ l was added to 2-ml cultures to measure cytokine production. The peak activity generally correlated with peaks of eluted proteins (Fig. 3). All three

peaks stimulated specific proliferation and cytokine production by spleen cells from brucella-infected mice. These results were reproducible with the same batch of separated antigen and with repeated separations.

After initial observation of the activities of each fraction, they were combined into three pools, termed pools 1, 2, and 3, on the basis of the peaks observed. Each pool was concentrated, and the protein content was estimated by Hartree's method. Pool 1 contained 14% of the total protein, pool 2 contained about 60% of the total protein, and pool 3 contained about 26% of the total protein. Sizes and numbers of proteins in each pool were checked by SDS-PAGE separation (Fig. 2). Pool 1 had a few low-molecular-mass bands (about 14 kDa), pool 2 had two medium-molecular-mass bands (26 and 28 kDa), and pool 3 had a dominant 36-kDa band.

The three pools of protein were tested for their abilities to stimulate proliferation and cytokine production by spleen cells. On the basis of protein concentration, pool 2 proteins were the most active at inducing cell proliferation, while pool 1 and pool 3 showed similar but lower activities (Fig. 4). Proliferation by normal cells was measurable but low. Stimulation of IFN- γ production by brucella-primed spleen cells was similar for pool 2 and pool 3 but lower for pool 1 (Fig. 5). No IFN- γ was detected in supernatants of normal cells. In contrast, IL-6 titers in cultures from brucella-immune mice stimulated by pool 1 and pool 2 were similar, while the IL-6 titer stimulated by pool 3 protein was comparatively low. Normal spleen cell cultures produced much lower titers of IL-6, varying with antigen concentration from 150 to 1,000 U. Titers were similar for the three pools. IL-2 titers were low in all cultures and similar among the three fractions (results not shown).

Cytokine production by T-cell subsets to SBP. As shown above, SBP stimulated the production of T-cell-derived cytokines by immune spleen cells. In the following experiments, an investigation of the relative contributions of T cells and subsets was undertaken. SBP was found to stimulate unseparated T cells and CD4⁺ and CD8⁺ T cells from

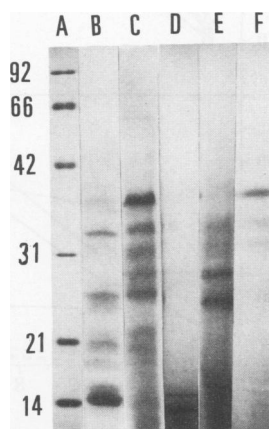


FIG. 2. SDS-PAGE (12% polyacrylamide) analysis of SBP. Lanes: A, protein molecular weight standards; B, SBP70; C, SBP50; D, pool 1 fractions from SBP50 (obtained by DEAE-Sepharose separation); E, pool 2 fractions from SBP50; F, pool 3 fractions from SBP50. Numbers indicate molecular weights in thousands.

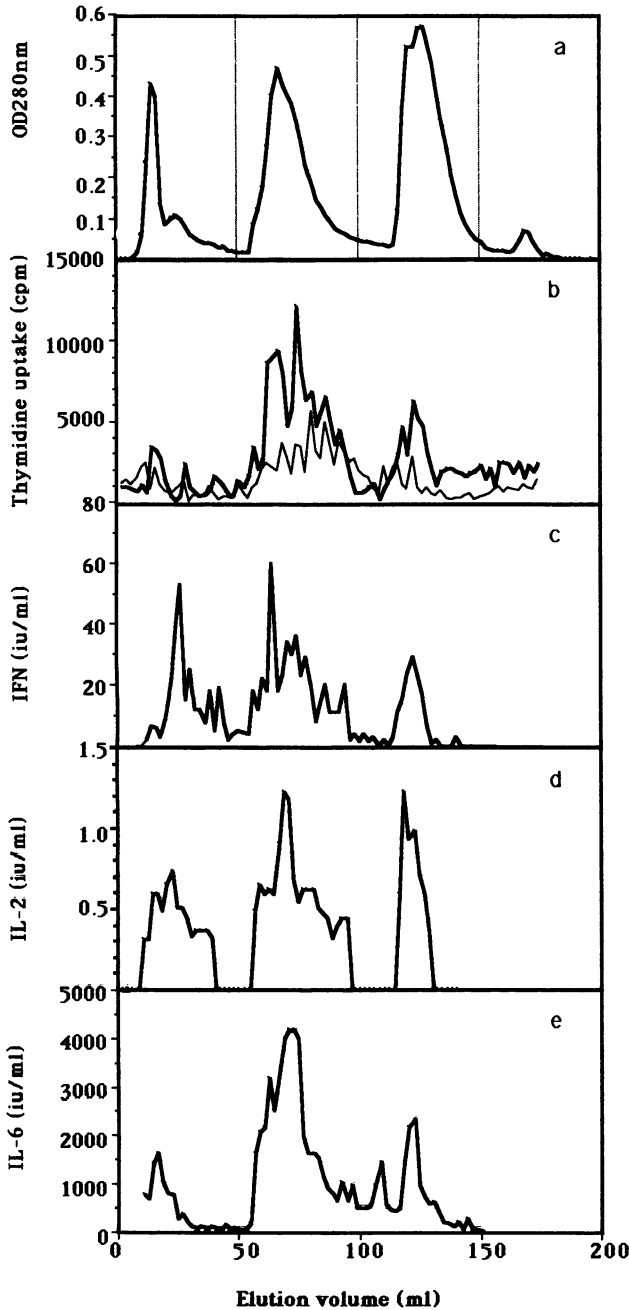


FIG. 3. Proliferation and cytokine production by spleen cells induced by SBP50 fractionated by a DEAE-Sepharose column. Fractions were tested for relative protein content (a) (optical density at 280 nm) and ability to stimulate proliferation (b) and production of IFN- γ (c), IL-2 (d), and IL-6 (e) of spleen cells from brucella-infected mice (thick line) or normal mice (thin line). The results are from one of three repeated assays and are shown as data from duplicate cultures. No IFN- γ and IL-2 and minimal amounts of IL-6 were detected in cultures of spleen cells of normal mice.

infected mice to produce cytokines. Unseparated T cells and CD4⁺ T cells produced both IL-2 and IFN- γ , while CD8⁺ T cells produced lesser amounts of IFN- γ and no IL-2 (Fig. 6). The CD4⁺ T cells, prepared by depletion of CD8⁺ cells, produced very high titers of IL-2 and IFN- γ . This was a consistent observation in repeated experiments and may

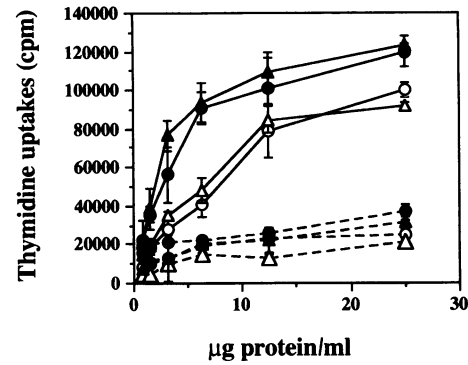


FIG. 4. Spleen cell proliferation of brucella-infected mice and normal mice. Three pooled fractions were tested for their abilities to stimulate the proliferation of spleen cells from brucella-infected mice (solid lines) and normal mice (dashed lines). Symbols: ○, pool 1; ●, pool 2; △, pool 3; ▲, control SBP50. Data from one representative experiment of three are shown and are expressed as the mean and standard deviation of triplicate cultures at each point.

reflect a suppressor activity of CD8⁺ cells, perhaps related to removal of IL-2 from culture by the IL-2 receptor on CD8⁺ cells (20). Inclusion of anti-CD4 or anti-CD8 antibody in the culture significantly reduced ($P < 0.01$) IFN- γ production by unseparated T cells and completely abrogated production by CD4⁺ or CD8⁺ cells, respectively, confirming the role of both subsets in IFN- γ production. T cells and subsets from normal mice did not produce significant amounts of IFN- γ (data not shown). As shown in Table 1, CD8⁺ cells produced more IFN- γ when they were cultured with exogenous IL-2. Table 1 also shows that all three pools of antigens stimulated T cells and CD4⁺ and CD8⁺ T-cell populations to produce IFN- γ . The amount of IFN- γ pro-

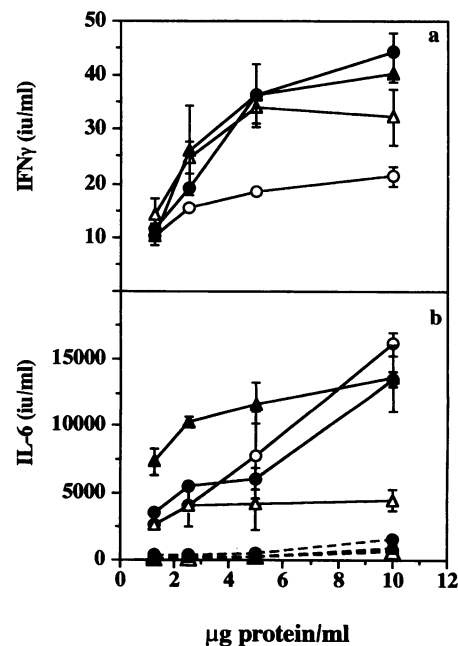


FIG. 5. IFN- γ (a) and IL-6 (b) production by spleen cells from brucella-infected mice stimulated by three pooled fractions. For the key to symbols, see Fig. 4. In the culture supernatants of normal cells, no IFN- γ was detected.

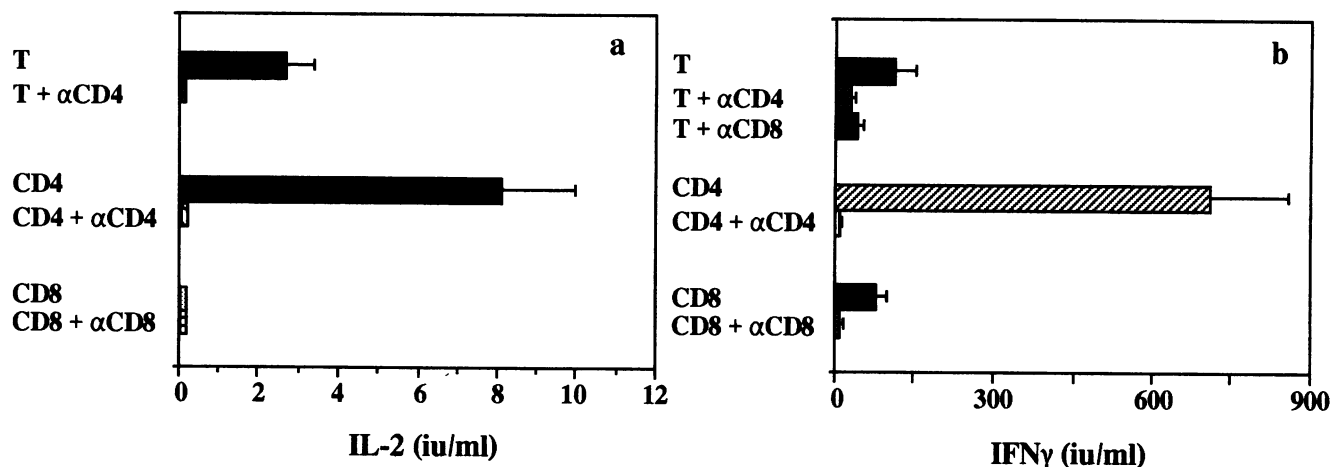


FIG. 6. IL-2 (a) and IFN- γ (b) production by T cells and subsets. T cells (twice nylon wool passaged) and CD4- or CD8-enriched T cells (2×10^6 /ml) in the presence of 2×10^6 irradiated normal spleen cells per ml as antigen-presenting cells were cultured with $60 \mu\text{g}$ of SBP50 per ml as antigen in a 2-ml volume. Anti-CD4 or anti-CD8 antibodies (α) were added to the indicated cultures. The supernatants were harvested 24 h later (for IL-2) and 72 h later (for IFN- γ). Data are typical of three repeated experiments.

duced in response to the individual fractions was less than that produced in response to unfractionated antigen, perhaps because each fraction stimulated only a limited population of T cells.

DISCUSSION

Protection against experimental infection with *B. abortus*, and presumably against natural infection, is mediated by CD4⁺ and/or CD8⁺ T cells (1, 23) and to a lesser extent by antibody (8). Since IFN- γ plays a prominent role (15, 32), it is presumably the Th1 subset of CD4⁺ T cells (22) which must be activated. Because earlier studies of T-cell activation by purified brucella antigens have concentrated simply on ability to induce proliferation by T cells (2), we undertook to isolate an immunodominant protein(s) which would evoke suitable cytokines by cultured T cells from infected immune mice.

Simple methods of extraction were used: hot saline extracts of whole bacteria were separated first by ammonium sulfate cuts and then by stepwise elution from a DEAE-Sephacose column. By using spleen cell cultures in microtiter wells, it was possible to measure proliferation and

cytokine production induced by each individual fraction, producing a very detailed profile which was reproducible between experiments. This was a more satisfactory method than cellular immunoblotting (38) in our hands, and it could be adapted to any separation resulting in soluble fractions.

All preparations stimulated proliferation of spleen cells from normal mice, although proliferation by immune cells was stronger. This may well have been influenced by contaminating endotoxin, stimulating B-lymphocyte mitogenesis (30). It confirmed the fact that proliferation was not a particularly satisfactory assay of specific activity. However, lymphokine production was not observed among spleen cells from normal mice, and therefore this was a useful assay of specific immune responsiveness.

Far from there being a single or limited number of proteins which could elicit IFN- γ production from cultured T cells of immune mice, all preparations stimulated IFN- γ production by both CD4⁺ and CD8⁺ populations. The strong response of CD8⁺ cells to soluble proteins, particularly in the presence of IL-2, is contrary to the expectation that live organisms must enter the cytosol of antigen-presenting cells in order to be presented by class I major histocompatibility complex molecules to CD8⁺ cells (6). However, there is precedent in the literature for CD8⁺ cells responding to soluble bacterial antigen (5, 24, 37). The fact that CD8⁺ cells are capable of producing substantial amounts of IFN- γ suggests that they may not be confined to acting as lytic cells releasing sequestered bacteria for destruction by active macrophages, as has been suggested (17), but may be involved in macrophage activation alongside the CD4⁺ cells.

While all the antigenic fractions could elicit IFN- γ , different fractions displayed different efficiency and different lymphokine profiles. Of the pools eluted from DEAE-Sephacose, pool 2, which contained two bands of approximately 26 and 28 kDa on SDS-PAGE, had the highest activity per microgram of protein in eliciting overall cytokine production. Pool 1, with a number of low-molecular-mass (approximately 14-kDa) bands, stimulated less IFN- γ but more IL-6. Pool 3, with a single dominant 36-kDa band, stimulated much less IL-6 but a strong IFN- γ response. We have shown that IL-6 is the product of both CD4⁺ T cells from brucella-infected mice and non-T splenic cells, proba-

TABLE 1. IFN- γ production by brucella-primed T cells and subsets^a

| Antigen | IFN- γ (IU/ml) produced by: | | | |
|---------|------------------------------------|--------------|---------------|-------------------|
| | T cells | CD4 cells | CD8 cells | |
| | | | Medium alone | IL-2 ^b |
| SBP50 | 67 \pm 7.2 | 360 \pm 47 | 12 \pm 4.2 | 86 \pm 11.3 |
| Pool 1 | 14 \pm 2.1 | 7 \pm 2 | 2.3 \pm 1.0 | 23 \pm 4.7 |
| Pool 2 | 18 \pm 4.0 | 18 \pm 3.7 | 3.0 \pm 1.0 | 38 \pm 7.3 |
| Pool 3 | 11 \pm 3.7 | 10 \pm 2.4 | 7.9 \pm 2.3 | 23 \pm 4.0 |
| Medium | <1 | <1 | <1 | <1 |

^a Cultures of 10^6 T cells per ml plus 2×10^6 irradiated normal spleen cells per ml as antigen presenting cells in the presence of SBP (60 $\mu\text{g}/\text{ml}$) were incubated for 72 h in a 2-ml volume. Supernatants of cultures were tested for IFN- γ . Data represent the means and standard deviations of triplicate cultures. A single representative of three similar experiments is shown.

^b Recombinant mouse IL-2 was added at 100 U/ml.

bly macrophages (28). It is notable that we did not detect IL-4 in the supernatants of these cells derived from infected mice (data not shown). IL-4 is accepted as the marker of the Th2 subpopulation of CD4⁺ T cells, the converse of the IFN- γ -producing Th1 cells, which are believed to mediate acquired cellular resistance to intracellular bacteria (22). Whether precise antigenic specificity determines the ability to stimulate different lymphokine profiles or whether these differences relate to broad physicochemical differences between the proteins of the various fractions is not clear. Clarification will have to await the purification and determination of antigenic epitopes. Undoubtedly, for each fraction there are multiple antigenic epitopes stimulating multiple clones of T cells.

While none of these fractions has yet been completely purified, these results suggest that a number of proteins, rather than a single immunodominant moiety, are likely to have the desired characteristic of stimulating IFN- γ production. Furthermore, it may be possible to choose proteins which favor the desired lymphokine profile when a recombinant vaccine is designed. It is likely that any vaccine will have to be based on a live carrier organism, since live organisms favor induction of the required cell-mediated immunity (3). Nevertheless there is always the hope that with more advanced adjuvants, and with the choice of the right antigenic epitopes, a satisfactory nonliving vaccine could be manufactured, eliminating all the variables of live vaccines. Such considerations apply to immunization against not only *B. abortus*, but also the other intracellular bacteria, for example, *Mycobacterium tuberculosis* and *Mycobacterium leprae*.

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