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Differential Activation of *Brucella*-Reactive CD4$^+$ T Cells by *Brucella* Infection or Immunization with Antigenic Extracts

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In order to induce acquired cellular resistance to facultative bacterial pathogens, infection with live organisms is required. We have previously demonstrated that spleen cells from *Brucella*-infected mice produced gamma interferon (IFN-γ) and interleukin-2 (IL-2) in response to *Brucella* antigens in vitro, while spleen cells from mice immunized with soluble *Brucella* proteins (SBP) produced substantial amounts of IL-2 but no detectable amount of IFN-γ. In this study, we further analyzed the response of T cells from *Brucella*-infected mice and SBP-immunized mice and demonstrated that CD4$^+$-enriched cells from SBP-immunized mice also produced significant amounts of IL-4, which was not detected in bulk cultures of spleen cells from infected mice. Limiting dilution analysis showed that infection resulted in a higher precursor frequency of IFN-γ-producing CD4$^+$ T cells and a lower precursor frequency of IL-4-producing CD4$^+$ T cells, while immunization with SBP resulted in a higher precursor frequency of IL-4-producing cells and a very low frequency of IFN-γ-producing cells. The precursor frequencies of IL-2-producing cells for the two groups were similar. Furthermore, IFN-γ-producing CD4$^+$ T cells from infected donor mice were capable of mediating resistance against challenge infection in recipient mice, but IL-4-producing CD4$^+$ T cells from immunized mice failed to do so. These results indicate that the form of antigen has a profound influence on the outcome of the immune response. The results are discussed in light of the supposed dichotomy between Th1 and Th2 cytokine responses.

Antigen-specific induction of acquired cellular resistance to intracellular bacteria can be achieved by immunization of animals with viable bacteria, including *Brucella* species (7, 19), *Mycobacterium tuberculosis* (13, 18), *Listeria monocytogenes* (3, 14), and *Salmonella* species (4). In contrast to the effectiveness of immunization with viable bacteria, killed bacteria seem to be incapable of inducing acquired cellular resistance. Why this is so is still not clear. It is assumed that different T-cell subpopulations are activated by viable and killed bacteria (11, 18, 24).

A recent advance in understanding the regulation of the specific immune response to pathogens was the identification of subpopulations of murine CD4$^+$ T helper lymphocytes. Murine CD4$^+$ T cells have been divided into at least two subsets (Th1 and Th2) on the basis of the cytokine profile they secrete upon antigen stimulation (16). Th1 cells characteristically secrete interleukin-2 (IL-2) and gamma interferon (IFN-γ), while Th2 lymphocytes typically produce IL-4, IL-5, and IL-10. Th1 CD4$^+$ cells are responsible for macrophage activation and attraction of inflammatory effector cells and therefore have been suggested to play a role in acquired cellular resistance. Adoptive transfer of CD4$^+$ T cells prepared from the spleens of mice infected with *Brucella* or *Listeria* species or *M. tuberculosis* can transfer resistance to challenge and an accelerated inflammatory response (2, 5, 10, 17). The protective property of CD4$^+$ T cells was shown to depend upon the ability to produce IFN-γ since transfer was abolished by inclusion of monoclonal antibody to IFN-γ (9, 26).

Previous studies by us and others (11, 24, 28) revealed that lymphocytes from mice infected with living bacteria produced high levels of IFN-γ in response to in vitro stimulation with antigens and belong to a Th1-like subset. On the other hand, lymphocytes from mice injected with heat-killed bacteria or bacterial extracts produced very little or no IFN-γ, although the same population of cells produces substantial amounts of IL-2. It is not clear whether low IFN-γ production by T cells in mice immunized with nonliving vaccine resembled a Th2 response, since IL-4 was not detected in bulk spleen cell cultures. In this report, we not only extend, by both bulk culture and limiting dilution culture, the finding that infection results in IFN-γ-producing Th1-like cells but also demonstrate that CD4$^+$ T cells from mice immunized with soluble *Brucella* proteins (SBP) were preferentially activated to produce the characteristic Th2 cytokine IL-4. Thus, immunization with SBP drives T-cell activation toward a pathway which is different from infection with living bacteria, at least partly accounting for the difference in protective efficiency.

MATERIALS AND METHODS

Mice. CBA mice were bred by pedigreed brother-sister mating in the Department of Microbiology, University of Melbourne, Melbourne, Australia. They were housed under conditions of isolation and fed sterile pellets and water to maintain their infection-free status.

***Brucella* antigens.** *Brucella abortus* 19, an attenuated vaccine strain, 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 mice. Preparation of SBP and subfractions has been described elsewhere (27). Briefly, hot saline extracts of *Brucella* organisms were precipitated with ammonium sulfate (50% saturation). Resultant proteins after dialysis against 0.01 M phosphate buffer (pH 7.2) were used as SBP. SBP were also further fractionated with DEAE-Sepharose, and three subfractions were prepared. Pool 1 had a small number of 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. All SBP and subfractions contained residual endotoxin according to the *Limulus* hemolysate assay. However, they did not elicit detectable amounts of IFN-γ or IL-4 from cells of naive mice.

Infection and immunization. To establish infection, mice were injected intraperitoneally with 5×10^5 *B. abortus* 19 organisms. To immunize mice with SBP, they were given one intraperitoneal injection of 120 μg of SBP in phosphate-

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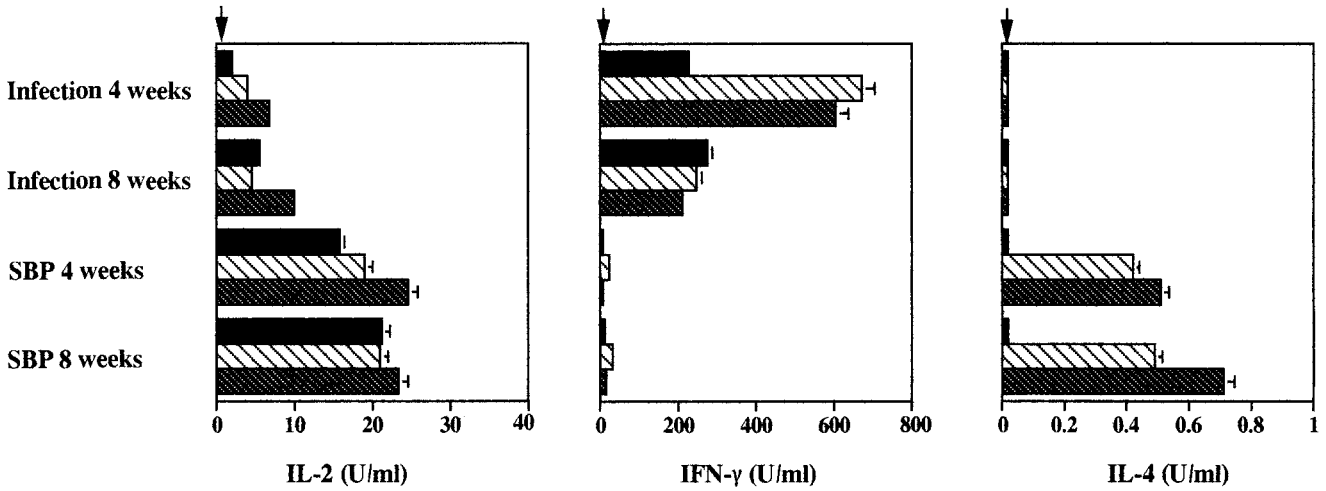

FIG. 1. Lymphokine production by lymphoid cells from infected and SBP-immunized mice. Mice were either injected with 5×10^5 *B. abortus* organisms or with 120 μg of SBP in alum adjuvant plus 10^9 *Bordetella pertussis* organisms and again with 120 μg of SBP without adjuvant 2 weeks before sacrifice. Spleen cells (■) were cultured at 2×10^6 cells per ml with 60 μg of SBP per ml in 2-ml volumes. T cells (▨) and $CD4^+$-enriched T cells (▨) were cultured with 60 μg of SBP per ml plus APC. Supernatants were collected at 24 h after initiation of culture for IL-2 assay or at 72 h for IFN-γ and IL-4 assays. Data shown for cultures with added SBP are the means and standard deviations of triplicate cultures. The limits of detection (indicated by arrows) were 0.1 U of IL-2 per ml, 1 U of IFN-γ per ml, and 0.06 U of IL-4 per ml. All cultures without SBP restimulation were under these limits.

buffered saline, complete Freund's adjuvant (CFA) (F-5881; Sigma, St. Louis, Mo.) or alum adjuvant and 10^9 heat-killed *Bordetella pertussis* organisms (Commonwealth Serum Laboratories) in 0.2 ml of normal saline. In some experiments (as indicated), mice were injected again with 120 μg of SBP without adjuvant 2 weeks before sacrifice.

Tissue and cell preparation. Mice infected or immunized for various times were sacrificed in a CO_2 chamber, and their spleens were removed and pooled within experimental groups. Spleens were teased through an 80-mesh stainless steel sieve into Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). The spleen cell suspension was centrifuged at $800 \times g$ for 7 min, suspended in Tris-buffered 0.83% ammonium chloride to lyse erythrocytes, underlaid with 1 ml of FCS to remove debris, and centrifuged again. Cells were suspended again in DMEM-FCS.

Preparation of T cells and $CD4^+$ T cells has been described previously (27). Briefly, T-cell-enriched splenocytes were nonadherent cells from two passages through nylon wool columns. $CD4^+$ subset-enriched T cells were prepared by negative panning with anti-CD8 monoclonal antibody (3.168)-coated petri dishes. To each plate, 5 ml of nylon wool-purified T cells (8×10^6 cells per ml) in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered DMEM and 5% FCS was added; plates were incubated flat at 4°C for 30 min, swirled, and left for 40 min. Nonadherent cells were then gently washed off with 10 ml of medium. The panning procedure was repeated once. The resultant populations were >90% $CD4^+$ and <2% $CD8^+$ by fluorescence microscopy.

Adoptive transfer of resistance to *B. abortus*. To assess the protective activity of T cells, 4×10^7 $CD4^+$ T cells from mice infected with *B. abortus* or immunized with SBP in alum adjuvant for 8 weeks were injected intravenously into naive recipient mice together with 10^6 *B. abortus* organisms. The numbers of viable bacteria in spleens and livers were determined 10 days later by plating dilutions of homogenates onto horse blood agar plates.

Culture for cytokine production. Bulk culture of spleen cells was carried out with 4×10^6 cells in the presence or absence of 60 μg of SBP per ml in 2-ml volumes in 24-well tissue culture trays. Purified T cells and $CD4^+$ subset cells (2×10^6 cells) were cultured with or without SBP in 2-ml volumes in the presence of 4×10^6 irradiated (2,500 rads) uninfected spleen cells as antigen-presenting cells (APC). Cells were cultured for 24 h for IL-2 accumulation and for 72 h for IFN-γ and IL-4 accumulation.

Limiting dilution cultures. The limiting dilution assay has been described previously (15). Graded numbers of purified $CD4^+$ cells (250 to 4,000 cells per well; 44 wells per dose) were cultured in 200 μl of DMEM–10% FCS with 5×10^5 APC and 120 U of recombinant mouse IL-2 (Boehringer GmbH, Mannheim, Germany) per ml in the presence or absence of antigen (60 μg of SBP per ml) on round-bottomed 96-well plates (Nunclon, InterMed, Roskilde, Denmark). Each plate included eight control wells that contained no $CD4^+$ cells. Cells were cultured for 12 to 14 days and scored visually for lymphocyte growth. Wells were washed twice and restimulated with 10 μg of concanavalin A (Boehringer GmbH) per ml to induce maximal lymphokine synthesis. Supernatants were collected 24 h later and assayed for lymphokine secretion.

Cytokine bioassays. Cytokine titers were determined as described elsewhere (12) by comparing dose-response curves with titrations of recombinant cytokines in proliferation assays with cytokine-responsive cell lines. The cell lines used were CTLL for IL-2, WEHI-279 for IFN-γ, and CT.4S for IL-4. The specificities of IL-4 and IFN-γ assays were checked with anti-IL-4 and anti-IFN-γ monoclonal antibodies. Recombinant murine IL-2 was purchased from Boehringer GmbH, while murine IL-4 was prepared from supernatants of Sf9 cells transfected with the IL-4 gene (provided by AMRAD Corp., Hawthorn, Australia). IFN-γ standard was supplied by the National Institutes of Health, Bethesda, Md.

Statistics. The statistical significance of bacterial count data was determined by Student's *t* test. Estimated frequencies of responding cells in limiting dilution cultures were assessed by the maximum-likelihood method from the Poisson distribution relationship between the number of T cells cultured and the logarithm of the fraction of negative wells (6).

RESULTS

Cytokine production by $CD4^+$ T cells from infected and immunized mice. In our previous work (28), bulk spleen cell culture was used to evaluate lymphokine production by *Brucella*-reactive T cells. It was found that spleen cells from 4-week-infected mice produced high levels of IFN-γ and low titers of IL-2 in response to in vitro stimulation with SBP, while spleen cells from SBP-immunized mice produced substantial amounts of IL-2 but no IFN-γ. IL-4 could not be detected from cultures of spleen cells from either source. In this study, nylon wool nonadherent spleen cells (T cell enriched) and subsequent negatively selected $CD4^+$ T cells were cultured with SBP in the presence of irradiated uninfected syngeneic spleen cells as APC. As well as confirming our previous findings, the results also showed that separated $CD4^+$ T cells from SBP-immunized mice but not from infected mice produced significant amounts of IL-4 after 72 h of culture with SBP in vitro (Fig. 1). Although the cell line (CT.4S) used in the IL-4 bioassay responds to a high concentration of IL-2, the activity in culture supernatants was proved to be IL-4 by neutralization with monoclonal anti-IL-4 antibody.

It is notable that IL-4 could not be detected in supernatants of bulk cultures of spleen cells from SBP-immunized mice, although T cells and $CD4^+$ subset cells isolated from the same source produced detectable amounts of IL-4. This was apparently due to consumption of IL-4 by spleen cells, as evidenced by the removal of added IL-4 from cultures of unirradiated uninfected and infected spleen cells (Table 1). Irradiated un-

TABLE 1. IL-4 consumption in cultures of spleen cells[a]

IL-4 added (U/ml)	IL-4 (U/ml)						
	Medium	Infected spleen cells		Uninfected spleen cells		Irradiated uninfected spleen cells	
		− Antigen	+ Antigen	− Antigen	+ Antigen	− Antigen	+ Antigen
0	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06
0.35	0.21 ± 0.01	<0.06	<0.06	<0.06	<0.06	0.20 ± 0.04	0.17 ± 0.02
1	0.48 ± 0.02	<0.06	0.30 ± 0.07	<0.06	<0.06	0.36 ± 0.02	0.46 ± 0.10
3.15	2.39 ± 0.12	0.09 ± 0.02	0.36 ± 0.04	0.48 ± 0.12	0.20 ± 0.04	1.57 ± 0.18	1.47 ± 0.37

[a] Spleen cells (2×10^6 cells per ml) from different sources in 2-ml volumes were incubated with the indicated concentrations of IL-4 for 72 h. Culture supernatants were then assayed for remaining IL-4 activity. Data are the means and standard variations of triplicate cultures. Uninfected spleen cells were from untreated C57B/10 mice. Some were irradiated for 2,500 rads. Infected spleen cells were cells from mice infected intraperitoneally with 5×10^5 *B. abortus* organisms for 4 weeks. Added IL-4 was murine recombinant IL-4.

infected spleen cells, used in T-cell cultures as APC, removed only minimal amounts.

Effects of adjuvants on *Brucella*-specific immune response. Adjuvants used in immunization have been demonstrated to affect the outcome of the immune response to ovalbumin (25). Alum adjuvant favored IL-4 production, and CFA favored IFN-γ production. To test whether the alum adjuvant used in this study biased the immune response, mice were injected with SBP without adjuvant, SBP with CFA, or SBP with alum adjuvant and *Bordetella pertussis* organisms. Lymphokine production by spleen cells from the different groups was analyzed just 2 weeks after injection. The results indicated that alum adjuvant and CFA enhanced IL-2 production, but both failed to induce IFN-γ production (Fig. 2). Cells from 2-week-infected mice produced IFN-γ and low titers of IL-2 but no IL-4.

To further exclude the possibility that IL-4 production by T cells from mice immunized with SBP was merely attributable to alum adjuvant, lymphokine production by T cells from mice injected with SBP alone was analyzed. The results showed that T cells from SBP-immunized mice produced IL-2 and IL-4, although the titers were relatively low (Fig. 3).

Limiting dilution analysis of $CD4^+$ T cells from infected and immunized mice. Limiting dilution assay, adapted from the method described by Morris et al. (15), allowed the precursor frequency of antigen-specific, lymphokine-producing T cells to be estimated. $CD4^+$ T cells were cultured with APC with or without SBP in medium that contained recombinant IL-2. After 10 to 14 days, cultures were scored visually for lymphocyte growth and then restimulated with concanavalin A for 24 h to induce maximal lymphokine synthesis. This procedure allows detection of cytokine production by viable clones and results in similar frequencies of cytokine producers as restimulation with antigen and APC, without the complication of cytokine production by APC (15).

Limiting dilution assays were performed with $CD4^+$ T cells from *B. abortus*-infected, SBP-immunized, and uninfected mice. For both growth and lymphokine production, the relationship between the number of cells cultured and the logarithm of the fraction of negative cultures was linear and intercepted the *y* axis near the origin (Fig. 4). $CD4^+$ T cells from unprimed mice showed very low frequencies of growth and IFN-γ-producing clones compared with those of $CD4^+$ T cells from *B. abortus*-infected mice and SBP-immunized mice, indicating that SBP was not acting as a nonspecific mitogen for T cells. Without SBP in limiting dilution cultures, all categories of T cells had extremely low frequencies of growth and lym-

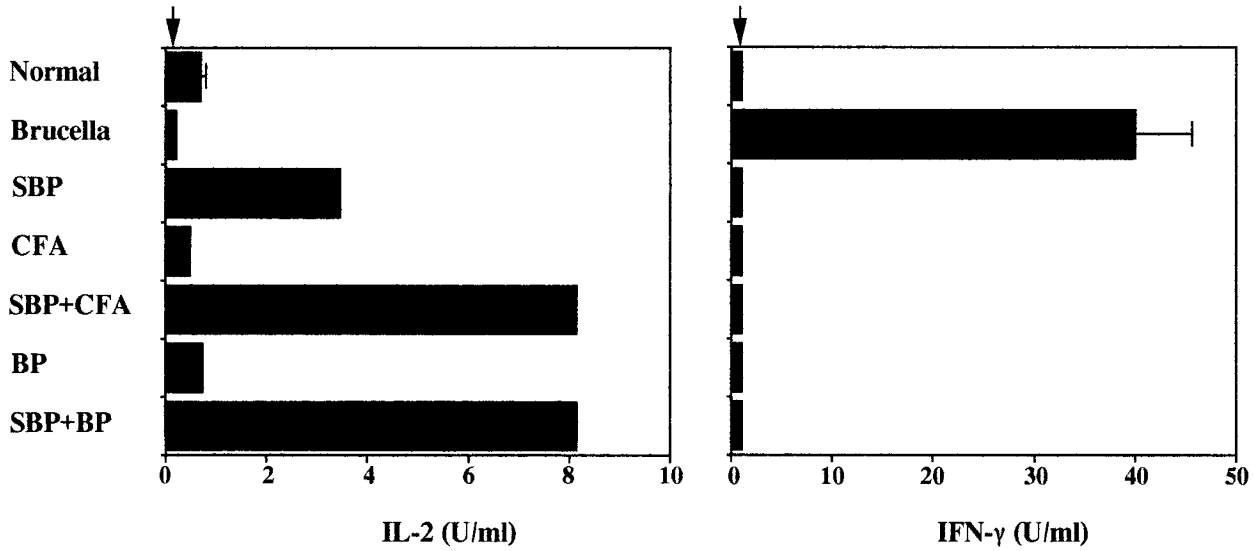

FIG. 2. Lymphokine production by spleen cells of mice with different adjuvants. Mice were injected intraperitoneally with 5×10^5 *B. abortus* 19 organisms or with 120 μg of SBP without adjuvant, with CFA, or with alum adjuvant plus 10^9 *Bordetella pertussis* organisms (BP). Mice were sacrificed 2 weeks after injection for spleen cell culture. Supernatants were collected 24 h after initiation of culture for IL-2 assay or 72 h after initiation for IFN-γ assay. Data shown for cultures with added SBP are the means and standard deviations of triplicate cultures. The limits of detection (indicated by arrows) were 0.1 U of IL-2 per ml and 1 U of IFN-γ per ml. All cultures without SBP restimulation were under these limits.

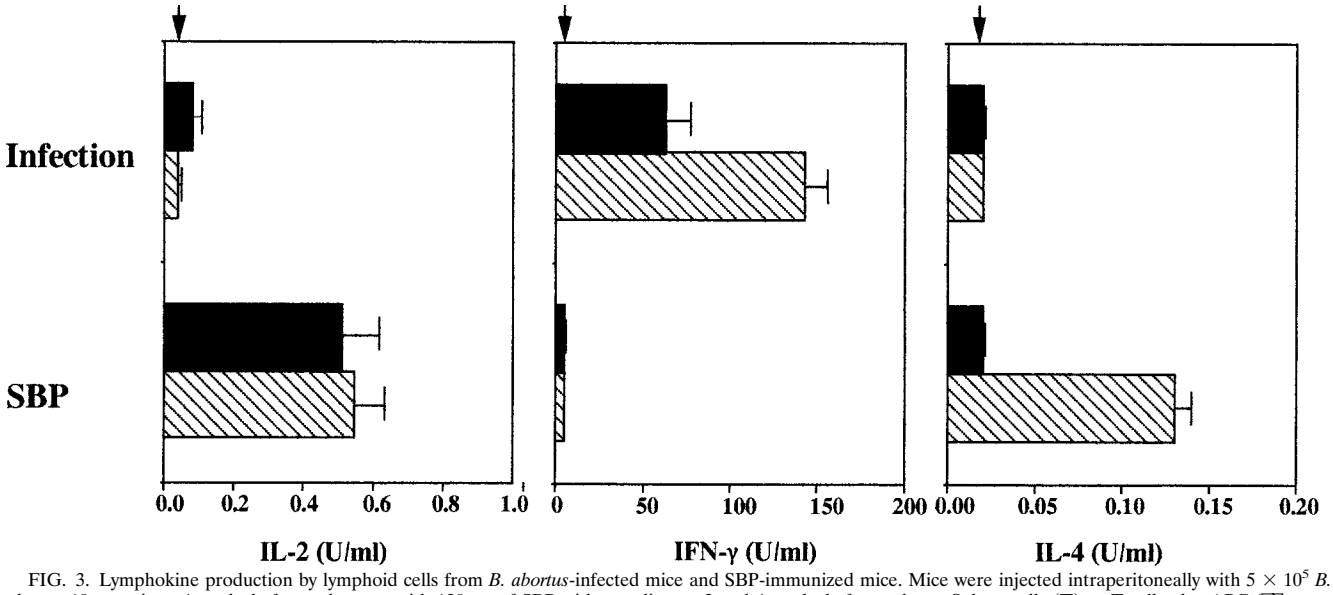

FIG. 3. Lymphokine production by lymphoid cells from *B. abortus*-infected mice and SBP-immunized mice. Mice were injected intraperitoneally with 5×10^5 *B. abortus* 19 organisms 4 weeks before culture or with 120 μg of SBP without adjuvant 2 and 4 weeks before culture. Spleen cells (■) or T cells plus APC (▭) were cultured with 60 μg of SBP per ml, and supernatants were assayed for IL-2, IFN-γ and IL-4. Data shown for cultures with added SBP are the means and standard deviations of triplicate cultures. The limits of detection (indicated by arrows) were 0.1 U of IL-2 per ml, 1 U of IFN-γ per ml, and 0.02 U of IL-4 per ml. All cultures without SBP restimulation were under these limits.

phokine production. In most experiments, no *Brucella*-reactive T cells were demonstrable at 8×10^3 T cells per well in the absence of antigen (data not shown). Figure 4 also shows that most clones produced one cytokine or the other. However, a very small proportion of clones did not produce detectable amounts of lymphokines and a very small proportion of wells with no visible clones contained lymphokines.

Preferential activation of lymphokine-producing $CD4^+$ T cells was confirmed by limiting dilution assay. As shown in Fig. 4 and Table 2, there were larger numbers of *B. abortus*-reac-

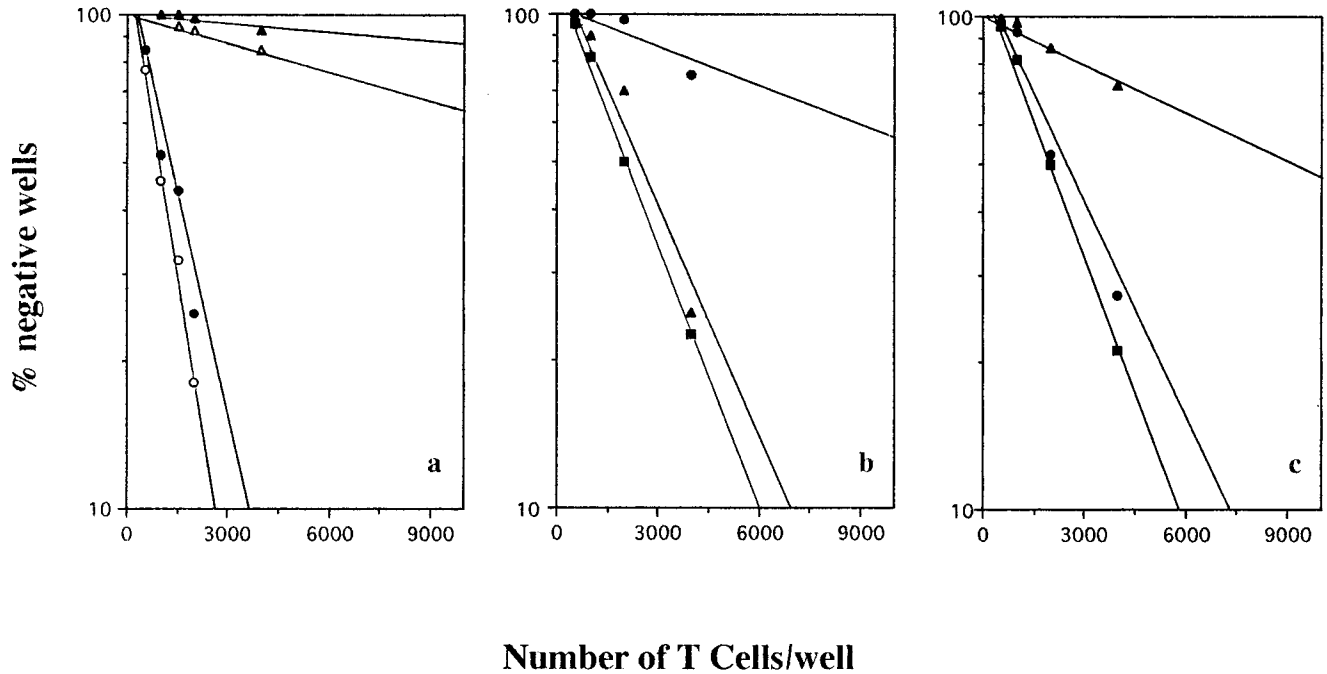

FIG. 4. Limiting dilution analysis of lymphokine-producing $CD4^+$ T cells responding to SBP. $CD4^+$ T cells were cultured at limiting dilution with SBP. (a) Precursor frequency of growth of $CD4^+$ T cells from 8-week-infected mice (○) or uninfected (△) and IFN-γ production of $CD4^+$ T cells from 8-week-infected mice (●) or uninfected mice (▲). (b) Precursor frequency of growth (■), IFN-γ production (▲), and IL-4 production (●) of $CD4^+$ T cells from 8-week-infected mice. (c) Precursor frequency of growth (■), IFN-γ production (▲), and IL-4 production (●) of $CD4^+$ T cells from mice injected 8 weeks earlier with SBP in alum adjuvant plus *Bordetella pertussis* organisms and boosted 2 weeks earlier with SBP without adjuvant.

TABLE 2. Precursor frequency analysis of lymphokine-secreting T cells[a]

$CD4^+$ T-cell type	Reciprocal of precursor frequency	
	IFN-γ	IL-4
B. abortus infected		
4 wk	6,008 (4,458–8,099)	41,889 (18,895–92,862)
8 wk	4,178 (3,214–5,431)	30,312 (15,320–59,974)
SBP immunized		
4 wk	16,681 (10,535–26,413)	4,640 (3,493–6,166)
8 wk	14,527 (9,278–22,745)	3,642 (2,798–4,743)

[a] $CD4^+$ T cells from spleens of *B. abortus*-infected mice and SBP-immunized mice were cultured in limiting dilution as described in the legend to Fig. 1. Each result is expressed as the reciprocal of precursor frequency, and the numbers in parentheses indicate 95% confidence limits. Data are for one of three repeated experiments. All reciprocal frequencies of lymphokine-producing T cells cultured without antigen were >100,000. All reciprocal frequencies of lymphokine-producing T cells from unprimed uninfected mice cultured with or without antigen were >100,000. The goodness of fit for all data was tested, and all probabilities were >0.01, indicating that data conform with the Poisson model.

TABLE 3. Lymphokine production by $CD4^+$ T cells to fractionated SBP[a]

In vitro stimulus	IL-2 (U/ml)		IFN-γ (U/ml)		IL-4 (U/ml)	
	Infection	SBP	Infection	SBP	Infection	SBP
None	<0.1	<0.1	<1	<1	<0.06	<0.06
SBP	0.6 ± 0.1	5.3 ± 1.8	51 ± 7	<1	<0.06	0.91 ± 0.02
Pool 1	0.4 ± 0.1	3.6 ± 0.7	41 ± 7	<1	<0.06	0.66 ± 0.06
Pool 2	0.4 ± 0.1	3.5 ± 0.8	70 ± 8	<1	<0.06	0.99 ± 0.08
Pool 3	0.8 ± 0.1	2.3 ± 0.1	61 ± 5	<1	<0.06	0.74 ± 0.10

[a] Mice were injected either with 5×10^5 *B. abortus* organisms (infection) or with 120 μg of SBP in alum adjuvant plus 10^9 *Bordetella pertussis* organisms and again with 120 μg of SBP without adjuvant 2 weeks later. $CD4^+$ T cells prepared from mice 4 weeks after initial injection (10^6 cells per ml) were cultured on 96-well flat-bottom plates with 60 μg of SBP or a subfraction (pool 1, pool 2, or pool 3) per ml in the presence of 2×10^6 irradiated uninfected spleen cells per ml. Culture supernatants were collected at 24 h for IL-2 assay and at 72 h for IL-4 and IFN-γ assays. Data are the means and standard deviations of triplicate cultures.

tive, IFN-γ-producing $CD4^+$ T cells from *B. abortus*-infected mice (precursor frequency, approximately 1/5,000) than from SBP-immunized mice (1/15,000) (at both 4 and 8 weeks). On the other hand, the precursor frequency of IL-4-producing $CD4^+$ T cells was higher for mice immunized with SBP (1/4,000) than mice infected with *B. abortus* (1/35,000). The estimated precursor frequencies of IL-2-producing cells ranged from 1/10,000 to 1/15,000 and were similar for *B. abortus*-infected mice and SBP-immunized mice (data not shown). Although exact estimated frequencies varied from experiment to experiment, the discrepancy in the precursor frequencies of IFN-γ- and IL-4-producing T cells between *B. abortus*-infected and SBP-immunized mice was observed in all experiments.

Cultures of $CD4^+$ T cells (1,000 cells per well) from mice immunized with SBP 8 weeks earlier showed that about 10% of cultures had viable lymphocytes, giving a probability of 95% that they were clonal. IL-2 was produced by 66% of these positive cultures, and IL-4 was produced by 62% of positive cultures. About 44% of positive cultures produced both IL-2 and IL-4 (data not shown). The frequency (44%) of observed IL-2 and IL-4 double-producer cultures among all lymphokine-producing cultures was far higher than the predicted frequency of coincidence of an IL-4 producer and an IL-2 producer clone in one well (2%). Therefore, production of IL-2 and IL-4 in cultures of $CD4^+$ T cells from SBP-immunized mice is most likely due to a single precursor cell. On the other hand, there were very few double producers of IFN-γ and IL-4 after either infection or immunization with SBP.

Responses of $CD4^+$ T cells from infected and immunized mice to fractionated SBP. To test whether individual *Brucella* protein antigens preferentially elicited different immune responses, lymphokine production by isolated $CD4^+$ T cells from *Brucella*-infected mice and SBP-immunized mice in response to fractionated SBP was analyzed (Table 3). Although there were differences in the amount of lymphokine produced by $CD4^+$ T cells in response to different fractions of antigens, no fraction stimulated a unique spectrum of lymphokines. Pool 2 stimulated the highest level of IFN-γ by T cells from infected mice but also stimulated the highest IL-4 production by T cells from SBP-immunized mice.

Adoptive transfer of acquired cellular resistance with $CD4^+$ T cells from infected and immunized mice. To determine whether $CD4^+$ T cells from infected and immunized mice act differently in vivo, splenic T cells were passively transferred to syngeneic recipient mice and the recipient mice were challenged with *Brucella* organisms. Bacteria in the spleens and livers of recipient mice were enumerated 10 days postchallenge. The results show that only T cells from infected mice conferred marked protection against *Brucella* challenge (Table 4). There was no difference in bacterial numbers between mice which had received unprimed T cells and those which had received T cells from SBP-immunized mice. When splenocytes from infected recipient mice were assessed for the ability to produce IFN-γ, cells from mice which had received T cells from infected donor mice produced a higher level of IFN-γ than cells from mice which had received unprimed T cells or T cells from SBP-immunized donors (Table 4).

DISCUSSION

In this study, we have shown a clear dichotomy between the cytokine response induced by active infection with *B. abortus* and that induced by immunization with bacterial extracts. Earlier reports by us and others (11, 24, 28) showed that bulk cultures of spleen cells from mice injected with killed bacteria or bacterial extracts produced IL-2 exclusively (no IFN-γ or IL-4), in contrast to cells from mice infected with live intracellular bacteria, which produced IL-2 and IFN-γ but no IL-4. Production of IL-2 without detectable IL-4 and IFN-γ production by lymphoid cells from mice injected with soluble antigens led to two suggestions. Either these cells have not been fully activated and represent a intermediate activation stage (IL-2

TABLE 4. Adoptive transfer of resistance and IFN-γ production by different donor cells[a]

Donor cell type	Log_{10} *Brucella* organisms[b]		IFN-γ (U/ml)[c]	
	Spleen	Liver	Medium	SBP
B. abortus infected	6.30 ± 0.13[d]	4.91 ± 0.20[d]	10.9 ± 0.4	161.4 ± 16.6
SBP immunized	7.48 ± 0.17	5.40 ± 0.15	20.1 ± 1.8	72.4 ± 3.4
Unprimed	7.43 ± 0.09	5.53 ± 0.33	12.8 ± 0.9	63.6 ± 22.3

[a] Donor mice were injected either with 5×10^5 *B. abortus* organisms or with 120 μg of SBP in alum adjuvant plus 10^9 *Bordetella pertussis* organisms 8 weeks before adoptive transfer.

[b] Bacterial numbers in recipient mice are the log means and standard deviations of five mice in each group.

[c] IFN-γ production by spleen cells from recipient mice was assessed in 24-h bulk cultures. Data are the means and standard deviations of triplicate cultures from each sample.

[d] $P < 0.001$, compared with groups which had received $CD4^+$ T cells from unprimed or SBP-immunized donors.

production only), or they have already acquired the capacity to produce other lymphokines, such as IL-4, but it was undetected for technical reasons. Uninfected and immune spleen cells were shown here to actively consume added IL-4. On the other hand, irradiated spleen cells, the common source of APC in T-cell cultures, were much less active in consuming IL-4. Thus, by modifying cell culture conditions so that T cells or $CD4^+$ subset cells were isolated and cultured with irradiated uninfected spleen cells as APC, IL-4 production was detected in supernatants of cultures of T cells, but only from SBP-immunized mice. Limiting dilution assays, by measuring the frequencies of clonogenic antigen-specific T cells, also indicated that there was a higher precursor frequency of IFN-γ-producing $CD4^+$ T cells and a lower precursor frequency of IL-4-producing $CD4^+$ T cells among these clonogenic cells from *B. abortus*-infected mice than among those from SBP-immunized mice. Therefore, the results reported here indicate that infection induced a Th1-like response (IL-2 and IFN-γ production). On the other hand, immunization with protein antigens induced a response which was different from a typical Th1 or Th2 response in that both IL-2 and IL-4 were produced, seemingly in many cases by the same clone.

The functional differentiation of T cells between mice with infection and those with SBP immunization happened quite early, becoming quite clear by 2 weeks. From our results, such a difference was not due to the influence of the adjuvant employed. Both adjuvants used in this study, alum and CFA, increased the magnitude of the response compared with that induced by immunization with antigen without adjuvant but had no influence on the type of immune response. All SBP-immunized groups, regardless of adjuvant, showed little or no IFN-γ production. This contrasts with the report (25) that culture of spleen cells obtained from mice immunized with ovalbumin in alum adjuvant elicited strong IL-2 and IL-4 responses while T cells from mice immunized with ovalbumin in CFA generated strong IL-2 and IFN-γ production but virtually no IL-4.

It has been nicely demonstrated for murine leishmaniasis that T cells from mice immunized with different parasite antigens produced either IFN-γ or IL-4 upon in vitro restimulation by soluble parasite antigens (20, 21). Therefore, the antigenic epitope(s) which elicits protective immune responses could be identified and selected for vaccine development. However, in this study, three fractions of SBP shared similar activities in terms of stimulating lymphokine production by T cells, although they displayed different efficiencies. This was similar to findings that various polypeptides of *Francisella tularensis* stimulated IFN-γ production by T cells from infected mice (23).

As $CD4^+$ T cells from infected and SBP-immunized mice produced different lymphokines in vitro, the in vivo protective activities of these cells were of interest. Adoptive transfer of protection against *Brucella* infection is at least partly dependent on IFN-γ (26). In this study, only $CD4^+$ T cells from mice infected with viable *B. abortus* organisms were capable of transferring resistance; $CD4^+$ T cells from SBP-immunized mice were not. Moreover, spleen cells from mice which had received $CD4^+$ T cells from infected donors showed enhanced production of IFN-γ compared with that of mice which had received unprimed or SBP-primed T cells. However, IFN-γ production by spleen cells from mice which had received IL-4-producing $CD4^+$ T cells from SBP-immunized donor mice was not diminished, compared with that from uninfected spleen cells, suggesting that either the negative signal (IL-4) to downregulate IFN-γ production was too weak or the positive signal (IL-12 and other cytokines produced during challenge infection in recipient mice) was dominant. A possible reason for a weak IL-4 signal could be insufficient reactivation of Th2-like cells by infecting bacteria, although SBP are clearly released during infection since all IFN-γ production by cultured cells from infected mice was elicited by using SBP as recall antigen.

Taken together, the significance of the findings in this study is that immunization with nonliving vaccine may not only induce a weak immune response but also fail to induce a desirable, protective immune response. Successful adoptive transfer of resistance with T cells from infected mice but not with T cells from immunized mice further demonstrated the superiority of live vaccine. However, by employing suitable cytokines, the immunogenic efficiency of nonliving, subunit vaccine might be greatly enhanced. A key cytokine in this respect may be IL-12, which has been shown in vitro (8) and in vivo (22) to favor the induction of a Th1-like IFN-γ response. We have recently shown that elimination of IL-12 by injection of monoclonal antibody before infection with *B. abortus* both exacerbates infection and abolishes the induction of IFN-γ-producing T cells (26a). A recent report that vaccination of BALB/c mice with leishmanial antigens and IL-12 promoted the development of leishmania-specific $CD4^+$ Th1 cells (1) is encouraging.

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