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Human Peripheral Blood CD4$^+$ and CD8$^+$ T Cells Express Th1-like Cytokine mRNA and Proteins following In Vitro Stimulation with Heat-Inactivated *Brucella abortus*

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Defining the pattern of lymphokine production associated with *Brucella abortus* is critical for advancing the development of *B. abortus* as a vaccine carrier. In the present study we investigated the ability of heat-inactivated *B. abortus* or lipopolysaccharide from *B. abortus* to induce lymphokine production from purified human T cells in vitro. Gamma interferon (IFN-γ), interleukin-2 (IL-2), IL-4, and IL-5 induction was assayed by mRNA-specific PCR and by enzyme-linked immunosorbent assay and bioassay for protein production. Following depletion of monocytes and B cells, *B. abortus* increased IFN-γ and IL-2 mRNA expression in purified T cells compared with expression in unstimulated cells. In contrast, no IL-5 mRNA expression and only transient low-level IL-4 mRNA expression and no IL-4 protein secretion were detected. Phytohemagglutinin or phorbol myristate acetate plus ionomycin induced mRNA and protein for all these cytokines. Similar results were obtained with LPS purified from *B. abortus*. Removal of NK cells did not reduce lymphokine production, and enriched NK cells did not express IFN-γ mRNA or secrete IFN-γ protein in response to *B. abortus*, indicating that NK cells were not the responding population. Both CD4$^+$ and CD8$^+$ populations produced IFN-γ and IL-2 in response to *B. abortus*. Preincubation of resting T cells with *B. abortus* or LPS from *B. abortus* for 7 days induced their differentiation into Th1-like cells as judged by their subsequent lymphokine response to phorbol myristate acetate plus ionomycin. These results suggest that *B. abortus* can induce differentiation of Th0 into Th1-type cells.

Our understanding of the role of T-cell subsets and cytokines in protective immunity is based on evidence that CD4$^+$ T cells can be separated into subsets on the basis of the profile of the cytokines that they produce. CD4$^+$ Th0 cells have the potential to secrete a mixture of cytokines consisting mainly of interleukin 2 (IL-2), IL-4, and gamma interferon (IFN-γ) (32). It is believed that Th0 cells, after appropriate stimulation, either undergo differentiation into Th1 cells, which secrete IL-2 and IFN-γ, or into Th2 cells, which secrete IL-4, IL-5, and IL-10 (32, 33). It was also found that CD8 cells are capable of cytokine production similar to that of the CD4 Th1-type cells (2).

From a functional standpoint, Th1 cells were demonstrated to be protective in mice with parasitic infections such as leishmaniasis (41), trichinelliasis (22), and schistosomiasis (17, 43). In viral infections, IFN-γ either alone (9, 23, 24) or together with tumor necrosis factor alpha (9, 29, 51) can inhibit virus replication (9) and induce an antiviral state (51). In addition, IFN-γ was shown to promote B-cell switching to immunoglobulin G2a in mice (IgG2a) (46). IgG2a is probably more effective than other mouse IgG isotypes in combating viral infection because it binds complement and Fc receptor with higher affinity (20, 50). On the other hand, Th2 cells produce IL-4, IL-5, and IL-10 and promote higher titers of IgG1 and IgE in mice (32, 48) and of IgE in humans (36, 38).

Knowledge of the immune mechanisms involved in protection against an infectious organism can be used to select a carrier/adjuvant best suited for the activation of the appropriate Th subset. Heat-inactivated *Brucella abortus* possesses certain properties, making it suitable as a carrier in the design of subunit vaccines. As a carrier, *B. abortus* is capable of inducing IgG2a in mice (30, 45), activating neonatal mouse (30) and human (14) B cells, and stimulating human B cells in vitro in the relative absence of T cells (11). Recent data from our laboratory demonstrated that inactivated human immunodeficiency virus type 1 (HIV-1) conjugated to *B. abortus* induced anti-HIV-1-neutralizing antibodies, which were mainly of the IgG2a subclass, in mice (12). Since IgG2a switching requires IFN-γ (46, 47), it was proposed that *B. abortus* stimulates cells to secrete IFN-γ, either directly or by inducing cells to differentiate into IFN-γ-producing cells. These cells could belong to CD4$^+$, CD8$^+$, or NK subpopulations.

In the present report, we examined the effect exerted by *B. abortus* on human T-cell subsets and NK cells in vitro. Our results demonstrated that heat-inactivated *B. abortus* induced IFN-γ secretion from purified human T cells (both CD4$^+$ and CD8$^+$) but not from NK cells. Thus, heat-inactivated *B. abortus* is able to promote a Th1 pattern of T-cell differentiation and could serve as a potential carrier for vaccine development in situations requiring a strong Th1-like response for protection against infection.

MATERIALS AND METHODS

Antigens. Heat-inactivated *B. abortus* was obtained from the U.S. Department of Agriculture, Ames, Iowa, and used at a concentration of 10^8 organisms per ml. The following mitogens were used at the concentrations indicated: phytohemagglutin (Sigma, St. Louis, Mo.), 5 μg/ml; phorbol myristate acetate (PMA) (Sigma) 5 ng/ml, plus ionomycin (Calbiochem-Behring Corp., San Diego, Calif.), 1 μM. Lipopolysaccharide from *B. abortus* (LPS-BA) was purified by butanol

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TABLE 1. Sequences of primer pairs used for PCR amplification in this study

mRNA	5′ sense primer	3′ antisense primer	Size of amplified fragment (bp)
β-Actin	5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′	5′-CTAGAAGCATTGCGGTGGACGATGGAGGG-3′	661
IL-2	5′-ATGTACAGGATGCAACTCCTGTCTT-3′	5′-GTCAGTGTTGAGATGATGCTTTGAC-3′	458
IL-2R p55	5′-TTATCATTTCGTGGTGGGGCAGATGGTTTA-3′	5′-TCTACTCTTCCTCTGTCTCCGCTGCCAGGT-3′	391
IL-4	5′-ATGGGTCTCACCTCCCAACTGCT-3′	5′-CGAACACTTTGAATATTTCTCTCTCAT-3′	456
IFN-γ	5′-ATGAAATATACAAGTTATATCTTGGCTTT-3′	5′-GATGCTCTTCGACCTCGAAACAGCAT-3′	501
IFN-γ for quantitative PCR	5′-GCATCGTTTTGGGTTCTCTTGGCTGTTACTGC-3′	5′-CTCCTTTTTCGCTTCCCTGTTTTAGCTGCTGG-3′	427
IL-5	5′-GCTTCTGCATTTGAGTTTGCTAGCT-3′		
Nested primers			
IFN-γ	5′-ACAAGTTATATCTTGGCTTTTCAGCTCT-3′	5′-CGACCTCGAAACAGCATCTGACTCCTT-3′	476
IL-4	5′-CAACTGCTTCCCCCTCTGTTCT-3′	5′-CTCTCTCATGATCGTCTTTAG-3′	402

extraction as described previously (15). It contained ≤2% protein and ≤1% nucleic acids.

MAbs. OKT1 hybridoma (anti-CD5 monoclonal antibody [MAb]) was obtained from the American Type Culture Collection (Rockville, Md.) and was propagated as ascites in pristane-pretreated BALB/c mice. The MAbs were purified by precipitation in saturated $(NH_4)_2SO_4$ and passage over protein G-Sepharose beads (Pharmacia, Piscataway, N.J.). Purified MAbs to CD3 (OKT3) and CD36 (OKM5) were obtained from Ortho Pharmaceutical Corp. (Raritan, N.J.), and purified MAbs to CD22 (Leu-14) and CD56 (Leu-19) and fluorescein isothiocyanate (FITC)-tagged MAbs to CD3, CD4, CD8, CD14, CD16, CD20, and CD56 (phycoerythrin) were obtained from Becton Dickinson (Mountain View, Calif.).

Cell purification. Heparinized peripheral blood was drawn from healthy donors at the National Institutes of Health Blood Bank or Johns Hopkins Hospital. The interface cells from Ficoll-Hypaque gradient centrifugation were collected. These peripheral blood mononuclear cells (PBMC) were then passed through a nylon wool column, and the nonadherent cell population was subjected to centrifugation on a percoll gradient to remove all activated T cells. The pelleted cells contained 90 to 95% $CD3^+$ T cells with ≤1% monocytes and B cells and ≤10% $CD16^+$ as verified by fluorescence-activated cell sorter (FACS) analysis.

Further enrichment of $CD4^+$ or $CD8^+$ T cells was achieved by negative selection by using either FITC-labeled anti-CD4 or FITC-labeled anti-CD8 MAb (0.25 μg per 10^8 cells) followed by washing and the addition of magnetic beads coated with anti-FITC antibody (Advanced Magnetics Inc., Cambridge, Mass.) at a ratio of 150:1 (beads to cell) (39). Cells were incubated with the beads for 10 min on ice, and the cells binding the magnet were removed. The remaining cells were checked by FACS analysis, and only $CD4^+$ or $CD8^+$ populations of ≥90% purity were used in the experiments.

NK cells were purified as previously described, with some modifications (52). Briefly, PBMC were obtained following Ficoll-Hypaque separation and incubated at 4°C for 30 min with a mixture of mouse IgG MAbs to CD22, CD36, and CD5, which recognize B cells, monocytes, and T cells, respectively. These cells were then incubated with magnetic beads, which were coated with goat anti-mouse IgG (Advanced Magnetics Inc.), at 4°C for 30 min. The bound cells were removed by a magnet. The procedure was performed twice.

Measurement of cytokine secretion. Human purified T cells were maintained at 37°C in a 5% CO_2 atmosphere in RPMI 1640 medium supplemented with L-glutamine and 10% human AB serum (GIBCO BRL, Gaithersburg, Md.) at 10^6 cells per well (200 μl) in 96-well plates for protein assays or in 25-cm^2 flasks (10 ml) for RNA analysis. Supernatants were harvested on days 5 to 7 for IFN-γ and on days 2 or 3 for IL-4. In kinetic studies (data not shown), PMA plus ionomycin elicited peak IL-4 levels from T cells at days 3 and 4, whereas peak IFN-γ levels were detected at days 5 to 7. Human IFN-γ was detected by enzyme-linked immunosorbent assay (ELISA) according to the instructions in the kit (GIBCO BRL), using supernatants neat or diluted 10- or 100-fold. The plates were read at 405 nm, and the IFN-γ concentrations (in picograms per milliliter) were interpolated from the standard curve by using known concentrations of IFN-γ (312.5, 625, 1,250, 2,500, and 5,000 pg/ml) and the V_{max} reader and SOFTmax software (Molecular Devices, Menlo Park, Calif.). To assay IL-4 production, supernatants from the individual cultures were diluted fourfold in full media and added to 5,000 CTh4.s cells (generously provided by William Paul and developed by Immunex, Seattle, Wash.). [^{3}H]thymidine (1 μCi per well) was added at 24 h, the cultures were harvested at 36 h onto glass fiber filter mats (Pharmacia, LKB-Wallac, Gaithersburg, Md.) with a cell harvested (LKB-Wallac), and thymidine incorporation was measured with a Betaplate liquid scintillation counter (LKB-Wallac). The IL-4 concentration (in picograms per milliliter) was interpolated from a standard curve generated by adding human recombinant IL-4 (R&D Systems, Minneapolis, Minn.) at five different concentrations, from 30 to 0.3 pg/ml, diluted serially by half logs. The ELISA for IFN-γ is sensitive at 312.5 pg/ml or higher, and the CTh4.s bioassay can detect 10 pg or more of IL-4 per ml.

RNA isolation and RT PCR analysis. Total RNA was isolated by a standard guanidinium isothiocyanate method (4). For small-scale RNA preparation, the RNAzol B solution (Biotecx Laboratories, Friendswood, Tex.) was used following the manufacturer's protocol. cDNA was produced as previously described (6). Briefly, total cellular RNA (1 μg of the equivalent of 2×10^6 cells) was incubated in buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.2 μg of random primer (Promega Corporation, Madison, Wis.), 20 U of RNasin ribonuclease inhibitor (Promega), and 0.5 mM (each) four deoxynucleoside triphosphates (Perkin-Elmer Cetus Corp., Norwalk, Conn.) for 10 min at 65°C, quickly chilled on ice, and then treated with 200 U of Moloney murine leukemia virus RT (GIBCO BRL) for 1 h at 42°C. Lymphokine-specific primer pairs IFN-γ, IL-2, and IL-4 and primers for IL-2Rα and β-actin were synthesized on a cyclone DNA synthesizer (model 8750; Milligen/Bioseparch, Millipore, Marlboro, Mass.) according to published sequences (8) (Table 1), and purified on a NAP-25 column (Pharmacia LKB). IL-5 primers were purchased from Clontech (Palo Alto, Calif.). 5′ and 3′ primers recognized sequences from the first and last exons, respectively, and are therefore mRNA specific. All primers used were of similar length and contained about 50% G+C bases. Thus, the annealing temperature used was equally optimal for all primers. PCR was performed with the Ampli*Taq* PCR Reagent Kit from Perkin-Elmer Cetus Corp. according to the manufacturer's protocol. Briefly, one half of the RT reaction product was added to 1.25 U of Ampli*Taq* PCR Reagent Kit from Perkin-Elmer Cetus Corp. according to the manufacturer's protocol. Briefly, one half of the RT reaction product was added to 1.25 U of Ampli*Taq* DNA polymerase in 40 μl of buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ in the presence of 400 ng each of the 5′ and 3′ primers, and a mixture of the four deoxynucleoside triphosphates at 0.125 mM each. The reaction was overlaid with a drop of light mineral oil, and PCR was performed in a DNA thermal cycler (Perkin-Elmer Cetus Corp.) for 35 cycles: 45 s of denaturation at 94°C, 45 s of annealing at 60°C, and 2 min of extension at 72°C. The PCR product, which contained IFN-γ- or IL-4-specific primer pairs, was diluted 1:1,000 and subjected to 25 cycles of a second round of PCR by using nested primers (Table 1) and the same concentration of DNA polymerase and the same regimen of amplification as that described for the first round. In all experiments, to ensure that all RT reaction samples initially contained the same amount of RNA, control PCR with primers for mRNA of the "housekeeping" gene β-actin was run in parallel for 25 cycles. Separate experiments were performed to verify the optimal number of β-actin amplification cycles (25 cycles) to detect differences in initial RNA concentrations (data not shown). PCR products were analyzed after electrophoresis of 12 μl of the reaction mix at 75 V for 1.5 h in 2% agarose in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) containing 0.5 μg of ethidium bromide per ml. For calibration of gels, a 123-bp DNA ladder (GIBCO BRL) was used.

Quantitative competitive PCR. To quantify IFN-γ and IL-2 mRNA levels, PCR MIMICs were used (31, 44). These are nonhomologous DNA fragments which bind the primers specific for the corresponding cytokine cDNA, but they bear insertions so that the amplified fragments can be separated from the native cytokine cDNA by electrophoresis. The PCR MIMICs were purchased from Clontech, and the experiments were done following the manufacturer's protocol. Because competitive PCR can only be done with one round of amplification, we increased the starting amount of total RNA to 2 μg and used a higher number of cycles. In brief, 2 μg of total RNA was used as a template for cDNA synthesis and was incubated with Moloney murine leukemia virus RT for 2 h. Ten percent of the RT reaction product was then amplified in the presence of equal volumes of serially diluted MIMIC cDNA with 2 U of Ampli*Taq* polymerase (Perkin-

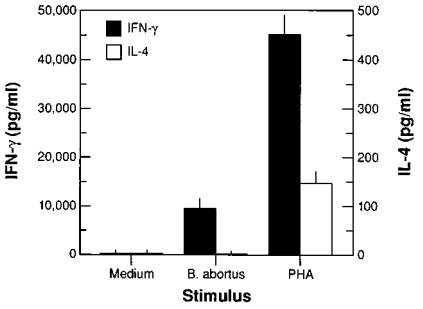

FIG. 1. Effect of *B. abortus* on IFN-γ secretion by human peripheral blood T cells. Human T cells (10^6 per well) were purified from peripheral blood and incubated in quadruplicate cultures with heat-inactivated *B. abortus* (10^8 organisms per ml) or phytohemagglutinin (5 μg/ml). The concentration of IFN-γ secreted was determined on the fifth day of the culture by an IFN-γ-specific ELISA, and IL-4 levels were determined by a bioassay on the third day. The error bars represent the standard errors for four replicate cultures.

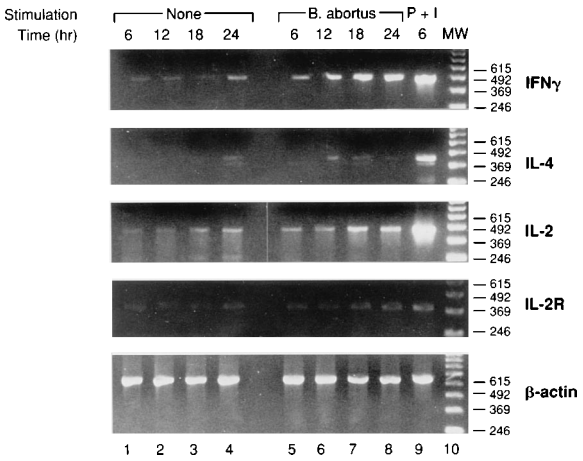

FIG. 2. Kinetics of lymphokine gene activation by *B. abortus*. Total RNA was isolated from human T cells, unstimulated or stimulated by *B. abortus* (10^8 organisms per ml) or PMA (5 ng/ml) plus ionomycin (1 μM) (P+I) for different lengths of time (as indicated), and were analyzed by RT-PCR either with one round of amplification only for IL-2, IL-2Rα, and β-actin or with a second round of amplification with nested primers for IFN-γ and IL-4. Lane MW, molecular weight markers, with base-pair numbers on the right.

Elmer Cetus Corp.) and a 0.4 μM concentration of the 5′ and 3′ primers (Clontech) in a total volume of 50 μl. Six separate PCRs were run for each mRNA samples containing serial dilutions of MIMIC cDNA. A single round of amplification was performed; however, the number of PCR cycles was increased to 45 for IFN-γ and to 40 for IL-2. The PCR product (12 μl) was then resolved in 2% ethidium bromide-agarose gel. Video images of the gels were obtained with a GDS 5000 system (UVP Inc., San Gabriel, Calif.), and the exact quantity of light emitted by ethidium bromide in bands corresponding to the IFN-γ or IL-2 targets or MIMIC DNA was determined by using SW5000 software (UVP Inc., Cambridge, England). The ratio for the amount of emitted light between the target and MIMIC cDNA was then plotted against the reciprocal log of the molar concentration of the corresponding MIMIC cDNA added to the PCR. The lines were drawn by using four data points, and the amount of IFN-γ or IL-2 target cDNA was calculated from the curve for which the ratio of the target to MIMIC equals one. The stimulation index was calculated as a ratio between the determined amounts of cytokine cDNA in samples from stimulated and nonstimulated cells.

Semiquantitative PCR analysis. The difference in cytokine mRNA levels on activation of the cells was estimated with an internal standard derived from PCR amplification of β-actin mRNA. In these calculations, the numbers, expressing the value of DNA in the bands, was obtained as mentioned above by using the GDS 5000 system and SW5000 software. The density of each cytokine band was normalized according to the corresponding β-actin band from the same RT-PCR. The stimulation indices of the activated versus nonactivated cells were obtained by dividing the "normalized" value of each cytokine-amplified product in stimulated cells by that of the nonstimulated cells.

RESULTS

Induction of IFN-γ secretion in the absence of IL-4 production in human T cells by *B. abortus*. Previous work performed in our laboratory (2) and by others (48, 49) suggested that *B. abortus* can induce Th1-like lymphokine production by human PBMC and murine splenocytes. The earlier experiments on human cells did not establish whether *B. abortus* selectively stimulated a Th1-like response, since Th2 cytokines were not measured. To determine, whether *B. abortus* preferentially induced Th1-like cytokines, both Th1 and Th2 cytokine expression was assessed in cells stimulated with *B. abortus*. Cell supernatants were harvested at various days posttreatment and assessed for either IL-4 production, by using the IL-4-dependent CTh4.s cell line in a bioassay, or for IFN-γ secretion, by specific ELISA. As shown in Fig. 1, activation of purified human T cells by *B. abortus* resulted in the generation of IFN-γ (10,000 pg/ml), whereas IL-4 was not detected (i.e., <10 pg/ml) in the same cell cultures. At the same time points, T cells produced IL-4 (150 pg/ml) as well as IFN-γ (45,000 pg/ml) after polyclonal activation with phytohemagglutinin. There was no detectable IFN-γ and IL-4 in the supernatants of unstimulated cells. In 15 separate experiments similar results were obtained. Unstimulated cells varied in their secretion of IFN-γ in amounts from less than 312.5 to 1,425 pg/ml. In each experiment IFN-γ secretion was assessed by dividing the concentration detected in the presence of *B. abortus* by the concentration noted in the absence of *B. abortus*. The mean increase in IFN-γ secretion following *B. abortus* stimulation was 9.5-fold (range, 1.3 to 24.0). In all 15 experiments, IL-4 secretion was <10 pg/ml following *B. abortus* stimulation but ranged from 34 to 232 pg/ml after activation with PMA plus ionomycin. These findings indicated that heat-inactivated *B. abortus* can induce a Th1 cytokine, IFN-γ, but not a Th2 cytokine, IL-4, from purified human T cells in vitro.

Induction of IFN-γ and IL-2 mRNA in human T cells stimulated by *B. abortus*. Although the bioassay for IL-4 was sensitive in the low picogram range, it was possible that *B. abortus* induced IL-4 below the level of detection in this assay. To examine this possibility, the PCR technique was chosen to investigate the kinetics of the cytokine mRNA expression in T cells on activation with *B. abortus*. Human T cells were unstimulated or stimulated with either *B. abortus* or the polyclonal activators PMA and ionomycin. Total RNA was isolated from cells at 6-h intervals during the first 24 h of culture (Fig. 2). Single-round PCR was sufficient for amplification of the cDNA from the housekeeping gene β-actin (25 cycles) and IL-2 and IL-2R cDNA (35 cycles). However, in order to consistently obtain visible bands for IFNγ and IL-4 following PCR amplification, it was necessary to add a second round of amplification (25 cycles) with nested primers, which are presented in Table 1. As shown in Fig. 2, unstimulated T cells produced low or undetectable levels of IFN-γ and IL-4 mRNA during the first 24 h of culture. In contrast, *B. abortus* induced detectable levels of IFN-γ mRNA as early as 6 h after stimulation, which reached a maximum after 18 to 24 h. At the same time points, only transient IL-4 mRNA was detected in cultures stimulated with *B. abortus*, although T cells readily expressed detectable IL-4 mRNA in response to PMA plus ionomycin. The overall levels of IL-4 mRNA expression in the polyclonally activated T cells were somewhat lower than the levels of the IFN-γ mRNA. This observation is in agreement with the findings of a previous study by Lewis et al. (27), who found that the IL-4 gene appeared to be transcribed in adult T cells at a rate

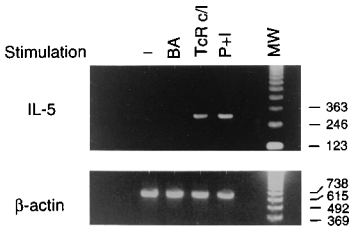

FIG. 3. RT-PCR amplification of IL-5 mRNA from purified T cells activated by *B. abortus*. Total RNA was prepared from human T cells, unstimulated (lane labeled −) or stimulated with *B. abortus* (10^8 organisms per ml) (lane BA), plate-bound anti-T-cell receptor antibody (1 μg/ml) for 18 h (lane TcR c/l), or PMA (5 ng/ml) plus ionomycin (1 μM) for 6 h (lane P+I). PCR was performed by using two rounds of amplification with the same set of primers. Lane MW, molecular weight markers, with base-pair numbers indicated on the right.

markedly lower than that for the IFN-γ and IL-2 genes. However, the kinetics of mRNA induction following PMA plus ionomycin (or concanavalin A) stimulation were found to be similar for IFN-γ, IL-2, and IL-4, peaking at 6 h (27). Even after additional cycles, in the second round of amplification, there was no significant increase in the level of IL-4 mRNA elicited from T cells stimulated by *B. abortus*. Similar kinetics for IL-2 mRNA and IFN-γ mRNA expression were seen in T-cell cultures activated by *B. abortus*. The level of IL-2Rα mRNA was increased only in T cells stimulated with PMA plus ionomycin and not in unstimulated cells or cells stimulated with *B. abortus* (Fig. 2). These results demonstrated that *B. abortus* induces mRNA for IFN-γ and IL-2 and only transiently for IL-4 in human T cells during the first 24 h of culture. These conditions favor the development of Th1 cells.

The ability of *B. abortus* to induce Th2 cytokines was investigated further by adding *B. abortus* to T cells and using RT-PCR to detect IL-5 mRNA. Although polyclonal activation did elicit IL-5 mRNA, *B. abortus* was inactive, even after two rounds of amplification (Fig. 3). The lack of induction of IL-5 mRNA strengthens the notion that *B. abortus* causes the selective differentiation of Th1 cells.

T cells but not NK cells respond to *B. abortus* stimulation. In our regular procedure of T-cell isolation from peripheral blood, there were 5 to 10% of $CD16^+$ cells remaining. These contaminating NK cells may have been responsible for the initial IFN-γ production, thus augmenting IFN-γ production and repressing IL-4 production in T cells after their treatment with *B. abortus*. To test this hypothesis, we used three types of cell populations, prepared from the peripheral blood of each donor: (i) one population containing a mixture of T and NK cells obtained after cell separation on a Ficoll-Hypaque gradient (unseparated), (ii) a T-cell-enriched population depleted of NK cells, and (iii) an NK-cell-enriched population depleted of T cells. The last two populations were obtained by negative selection, as described in Materials and Methods. These different cell populations were prepared from the same donor and were used to assess both the cytokine secretion and mRNA expression (Table 2 and Fig. 4).

Following *B. abortus* stimulation, the unseparated PBMC population (T cells, 69%) induced a threefold increase in IFN-γ secretion (in comparison with unstimulated cells), whereas the increase in IFN-γ secretion in the T-cell-enriched and NK-cell-depleted population (T cells, 95%) was eightfold. In contrast, *B. abortus* did not alter the low level of IFN-γ production by the NK-enriched cells (NK cells, 92%), although PMA plus ionomycin induced detectable levels of IFN-γ in this population (Table 2).

These results were confirmed by RT-PCR. As illustrated in Fig. 4, IFN-γ and IL-2 mRNA were detectable in T cells but not in NK cells after 18 h of incubation with *B. abortus*. In contrast, the polyclonal activators PMA and ionomycin induced an increase in the expression of mRNA for both these cytokines in T cells as well as in NK cells. IL-4 mRNA was not detected in NK cells even after PMA plus ionomycin stimulation, but it was present in T cells after the same stimulation. *B. abortus* induced low levels of IL-4 mRNA (Fig. 4). These data strongly suggest that the main population responding to *B. abortus* activation by cytokine secretion is T cells and not NK cells.

TABLE 2. *B. abortus*-induced IFN-γ production from T cells but not from NK cells

Stimulation	IFN-γ (pg/ml) produced in cell population type indicated[a]		
	Unseparated	T-cell enriched	NK-cell enriched
None	3,545	820	456
B. abortus[b]	10,448	6,540	364
PMA + ionomycin[c]	177,000	85,217	17,217

[a] Culture supernatants were harvested from quadruplicate wells on days 5 to 7, and the amount of IFN-γ produced was measured by ELISA. The results represent the means. The standard errors were 15% or less of the means. The proportion of T cells and NK cells in culture was determined by flow cytometry analysis using antibodies to CD3 and CD56. In the T-cell-enriched population (95% T cells), 5% of the $CD3^-$ cells did not express NK-cell markers. Similarly, in the NK-cell-enriched population (92% T cells), the $CD56^-$ cells did not express T-cell markers.

[b] *B. abortus* was added at 10^8 organisms per ml.

[c] PMA and ionomycin were added at 5 ng/ml and 1 μM, respectively.

Analysis of relative changes in IFN-γ and IL-2 mRNA levels by competitive PCR. Competitive PCR was used to quantify the increase in IFN-γ and IL-2 mRNA expression in T cells activated by *B. abortus* (37, 44). In this method, competitive template DNA (MIMIC) with an insertion or deletion is added to the target test cDNA at decreasing concentrations. Both are amplified by the same primer pairs, but the amplified fragments are distinguished on agarose gel and quantitation is

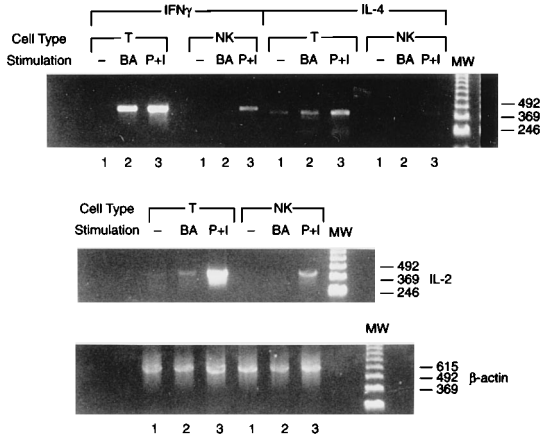

FIG. 4. Effects of *B. abortus* on IFN-γ and IL-2 transcription in T cells depleted of NK cells. T and NK cells were isolated from peripheral blood and enriched by negative selection. Cells (10^6 per well) were placed in quadruplicate cultures either in the absence of any stimulus for 18 h (lanes 1) or stimulated with *B. abortus* (BA) (10^8 organisms per ml) for 18 h (lanes 2) or with PMA (5 ng/ml) plus ionomycin (1 μM) (P+I) (lanes 3) for 6 h. Total RNA was obtained by RNAsol extraction, and RT-PCR was performed, with two rounds of PCR amplification for IFN-γ and IL-4 and one round of PCR amplification for IL-2 and β-actin. Lanes MW, molecular weight markers, with base-pair numbers indicated on the right.

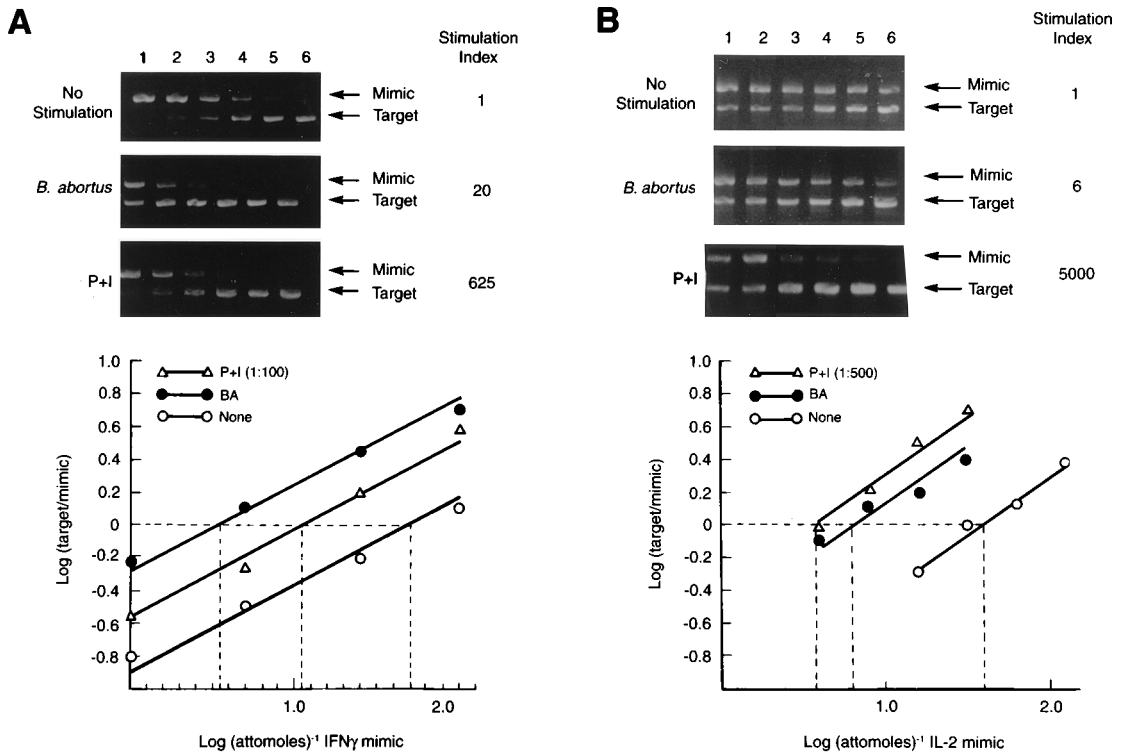

FIG. 5. Quantitation of IFN-γ and IL-2 mRNA expression in T cells after *B. abortus* activation by competitive PCR. T cells were cultured with *B. abortus* (BA) (10^8 organisms per ml) for 18 h or with PMA plus ionomycin (P+I) for 6 h. Video images of electrophoretically resolved, ethidium bromide-stained PCR products derived from target sequence (lower band: 494 bp for IFN-γ and 458 bp for IL-2) and the competitive template-MIMIC (upper band: 630 bp for IFN-γ and 630 bp for IL-2) are shown. (A) Quantitation of IFN-γ mRNA. The RT-PCR products (target cDNA) either neat or diluted 1:100 (from cells stimulated with PMA and ionomycin) were amplified in the presence of a fivefold dilution of competitive (MIMIC) cDNA. Lanes 1 to 6, 1, 0.2, 0.04, 0.008, 0.0016, and 0.00032 amol of competitive cDNA, respectively. (B) Quantitation of IL-2 mRNA. The RT-PCR products (target cDNA) either neat or diluted 1:500 (from cells stimulated with PMA plus ionomycin) were amplified in the presence of a twofold dilution of competitive (MIMIC) cDNA. Lanes 1 to 6, 0.25, 0.125, 0.0625, 0.03125, 0.015625, and 0.0078125 amol of competitive cDNA. The graphs were plotted to determine the concentrations of target cDNA as described in Materials and Methods. The stimulation indices were calculated from these concentrations by comparing the results from unstimulated and stimulated cells. These data are representative of four similar experiments.

based on determination of the MIMIC concentration which results in a 1:1 ratio of cDNA-MIMIC amplification. The advantage of using competitive PCR is that it is not necessary to assay PCR products exclusively during the exponential phase of amplification (37, 44). A fixed concentration of cDNA derived from 2 μg of total RNA was amplified in the presence of a fivefold serial dilution of IFN-γ MIMIC and a twofold serial dilution of the IL-2 MIMIC. The starting concentration of IFN-γ MIMIC was 1 amol (1 amol is equal to approximately 600,000 molecules), and the starting concentration of IL-2 MIMIC was 0.25 amol. Figure 5A shows that the IFN-γ mRNA concentration can be estimated by visually noting that equimolar amounts of the products (MIMIC and native cDNA) are achieved for unstimulated cells by adding 0.04 to 0.008 amol and for *B. abortus*-stimulated cells by adding 1 to 0.2 amol of IFN-γ MIMIC (Fig. 5A, lanes 3 and 4, and 1 and 2, respectively). For IL-2 mRNA quantitation, 625×10^{-4} to 12×10^{-4} amol of IL-2 MIMIC was required to achieve 1:1 amplification with cDNA for unstimulated cells, while 25×10^{-2} to 12×10^{-2} amol was required to achieve the same ratio with cDNA from *B. abortus*-stimulated cells (Fig. 5B, lanes 3 and 4 and 1 and 2, respectively). From the known molar quantity of the competitive PCR MIMIC, the number of target DNA molecules added to the PCR mixture was calculated from the graphs presented in Fig. 5; it was determined that *B. abortus* stimulation resulted in a 20-fold increase in the amount of IFN-γ (Fig. 5A) and a 6-fold increase in the amount of IL-2 mRNA (Fig. 5B). Because the levels of IFN-γ and IL-2 RT reaction product (cDNA) from cells activated by PMA plus ionomycin was much higher than the cDNA in the MIMIC stock solution (1 amol/1 μl), we diluted the former cDNA 100-fold for the IFN-γ PCR and 500-fold for IL-2 PCR (Fig. 5). The stimulation indices of IFN-γ and IL-2 mRNA production in PMA plus ionomycin-stimulated cells were calculated to be 625 and 5,000, respectively (Fig. 5). Regression analysis was performed on the data, and r^2 was ≥0.94 for all the lines shown in Fig. 5. We were unable to conduct similar competitive PCR analyses for IL-4 and IL-5 mRNA since the levels of amplified DNA material in the *B. abortus*-stimulated cultures were undetected or too low to compete with MIMIC DNA (37).

Both $CD4^+$ and $CD8^+$ T cells respond to *B. abortus*. Purified populations of T cells contain $CD4^+$ and $CD8^+$ T cells, both of which are capable of producing IFN-γ (31, 32). In order to investigate which T-cell subpopulation could be activated in vitro by *B. abortus*, the T cells were separated by negative selection into $CD4^+$ and $CD8^+$ cells. As shown in Fig. 6, unseparated, $CD4^+$, and $CD8^+$ T cells all expressed elevated levels of IFN-γ mRNA after stimulation by *B. abortus*. After normalization relative to β-actin mRNA, the stimulation indices for IFN-γ, compared with those for unstimulated cells, were calculated to be 5-fold for unseparated T cells, 10-fold for the $CD4^+$-enriched population, and 2-fold for the $CD8^+$-enriched population. The levels of IL-2 mRNA were also upregu-

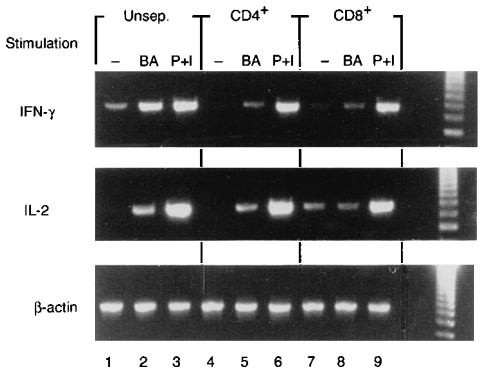

FIG. 6. PCR amplification of cytokine mRNA from $CD4^+$ or $CD8^+$ cells activated with *B. abortus*. Unseparated T cells (Unsep.) or T cells enriched for $CD4^+$ or $CD8^+$ by negative selection were cultured with (10^6 cells per well) either no stimulation or stimulation with *B. abortus* (BA) (10^8 organisms per ml) for 18 h or PMA and ionomycin (P+I) for 6 h. Total RNA was isolated with RNAzol B and amplified as described in the legend to Fig. 2.

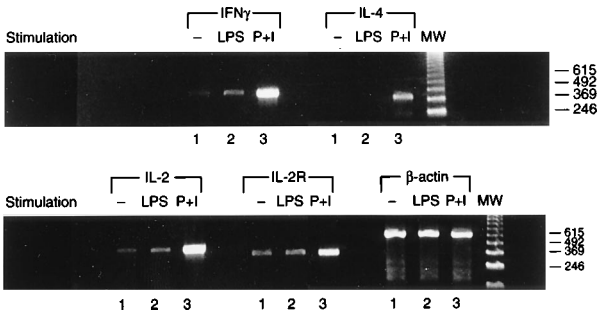

FIG. 7. Effect of LPS-BA on expression of cytokine mRNA. T cells were incubated in culture without antigen (lanes 1), with LPS-BA (10 μg/ml) (lanes 2), or with PMA and ionomycin (P+I) (lanes 3) for 18 h. RT-PCR was performed on total RNA for IFN-γ, IL-4, IL-2, IL-2Rα, and β-actin as described in the legend to Fig. 2. Lanes MW, molecular weight markers, with base-pair numbers indicated on the right.

lated by *B. abortus* in unseparated (8-fold increase) and $CD4^+$ (40-fold increase) T cells. In contrast, $CD8^+$ T cells expressed similar levels of normalized IL-2 mRNA with and without *B. abortus* stimulation (Fig. 5). PMA plus ionomycin induced expression of mRNA for both cytokines in unseparated T cells and in the $CD4^+$ and $CD8^+$ cells, with higher stimulation indices in the $CD4^+$ subpopulation than in the $CD8^+$ subpopulation. These data were confirmed by testing supernatants of $CD4^+$ and $CD8^+$ T cells, stimulated by *B. abortus*, by ELISA. The results, presented in Table 3, indicate that *B. abortus* induced IFN-γ secretion in both cell subsets, but the $CD4^+$ T cells produced more IFN-γ than the $CD8^+$ T cells did ($P < 0.001$, Student's *t* test).

LPS-BA induces IFN-γ mRNA in vitro in T cells. LPS from several gram-negative bacteria have been shown to stimulate NK cells and macrophages. In an attempt to identify the biologically active component of *B. abortus*, it was of interest to determine whether LPS-BA could stimulate T cells in a manner similar to *B. abortus*. As shown in Fig. 7, LPS-BA induced IFN-γ and IL-2 mRNA in T cells. In contrast, the LPS-BA-stimulated T cells, similar to those activated with inactivated BA, did not express IL-4 mRNA (Fig. 7). Similar to *B. abortus*, from which it was derived, LPS-BA did not alter IL-2Rα mRNA in T cells (Fig. 2 and 7). These findings were further confirmed by ELISA. In five separate experiments, LPS-BA alone did not induce IFN-γ protein release, but the addition of IL-2 had a synergistic effect. In each experiment IFN-γ secretion was assessed by dividing the concentration detected in the presence of LPS-BA plus IL-2 by the concentration noted in the presence of IL-2 alone. The mean increase in IFN-γ following LPS-BA plus IL-2 stimulation was 7.3-fold (range, 2.8 to 11.7). The results of a representative experiment are shown in Fig. 8. LPS-BA did not elicit detectable quantities of IL-4 in the presence or absence of IL-2. In contrast, IL-4 was secreted by the same cells in response to PMA and ionomycin.

These results suggest that the LPS component of the *B. abortus* membrane possesses properties similar to those of the whole bacteria in that it activates T cells directly to release Th1-like cytokines. The requirement for IL-2, however, suggests that the LPS stimulation is less potent than that of the intact organism.

In vitro priming with *B. abortus* or LPS-BA changes the phenotype of cells responding to PMA plus ionomycin. Human T cells were shown to secrete both IL-4 and IFN-γ in response to PMA plus ionomycin following 3 days in culture (Fig. 9). That is, the phenotype of the responding cells is Th0 or a mixture of Th1 and Th2 cells (or both). In order to determine the effect of priming cells with *B. abortus* or LPS-BA on subsequent stimulation with polyclonal activators, human T cells were first stimulated with either *B. abortus* or LPS-BA for 6 days. They were then washed and placed in culture with PMA

TABLE 3. Effect of *B. abortus* on secretion of IFN-γ by T-cell subsets

Expt	IFN-γ (pg/ml) in T-cell subset[a]			
	$CD4^+$		$CD8^+$	
	Unstimulated	*B. abortus*-stimulated	Unstimulated	*B. abortus*-stimulated
1	10	12,610	10	3,210
2	170	21,690	270	4,040

[a] The production of IFN-γ by unstimulated or *B. abortus*-stimulated (10^8 organisms per ml) cells was determined by ELISA on the 7th day of culture. The results represent the means of quadruplicate cultures. The standard errors were 15% or less of the means. $CD4^+$ and $CD8^+$ cells were negatively selected by magnetic beads and contained ≤5% of the depleted subset.

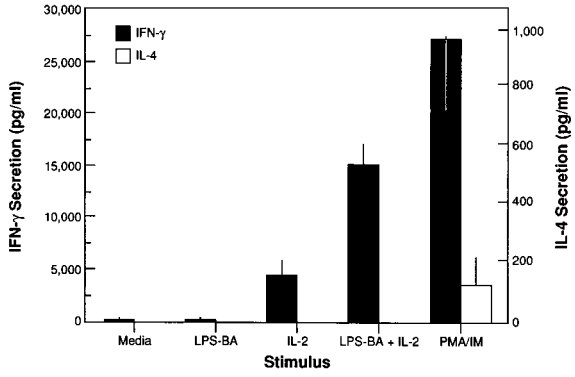

FIG. 8. LPS-BA induces secretion of IFN-γ from human T cells in the presence of exogeneous IL-2. Purified human T cells were cultured in quadruplicate cultures in the presence or absence of LPS-BA (10 μg/ml). IL-2 (20 U/ml) was added either alone or together with LPS-BA. PMA (5 ng/ml) and ionomycin (1 μM) (P+I) were added together to separate cultures. IFN-γ was assayed by ELISA using supernatants from day 7 of culture. IL-4 was measured by bioassay using supernatants from day 3. The error bars represent the standard errors for four replicate cultures.

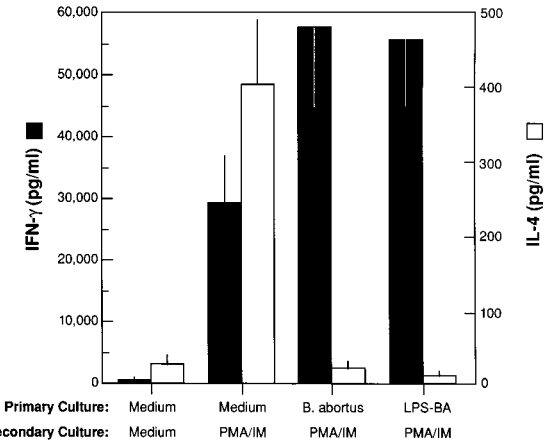

FIG. 9. In vitro priming with *B. abortus* or LPS-BA changes the pattern of cytokines produced by human T cells stimulated by PMA and ionomycin. Human T cells were cultured in medium alone or stimulated with *B. abortus* (10^8 organisms per ml) or LPS-BA (10 μg/ml) for 6 days, washed thoroughly, and then restimulated with PMA (5 ng/ml) and ionomycin (1 μM) (IM) for 3 days. The supernatants were assayed for IL-4 and IFN-γ. The error bars represent the standard errors for four replicate cultures.

and ionomycin for 3 days, and their supernatants were assayed for cytokine content. As can be seen from the results presented in Fig. 9, both *B. abortus* and LPS-BA altered the cytokine profile of the responding population in that mainly IFN-γ secretion was seen, with minimal or no IL-4 detected in the supernatants after PMA plus ionomycin stimulation. Thus, *B. abortus* and LPS-BA, in the absence of antigen-presenting cells, induced-differentiation from a Th0 phenotype or a Th1-Th2 mixed population to a predominantly Th1-type phenotype (which could include $CD8^+$ cells) and/or suppression of IL-4-producing cells. Since *B. abortus* does not induce vigorous cell proliferation, it is unlikely that it simply induced the expansion and differential survival of Th1-like cells during the first 6-day culture.

DISCUSSION

This study demonstrates that *B. abortus* and LPS-BA induce human T cells to differentiate into a Th1-like phenotype, characterized by IFN-γ and IL-2 but no IL-4 secretion. PCR techniques, including quantitative methods, were employed to confirm these findings. The signals delivered induced differentiation of T cells, as evidenced by the finding that primary stimulation with *B. abortus* or LPS-BA was followed by a predominant Th1-like response to a subsequent polyclonal activation with PMA plus ionomycin. This pattern of cytokine secretion occurred following primary in vitro stimulation of enriched T cells (>95%) following depletion of antigen-presenting cells and NK cells, suggesting that *B. abortus* and LPS-BA can trigger human T cells directly. Although monocytes and B cells are depleted, we cannot exclude the possibility that they played a role. It is unlikely that small numbers of dendritic cells contributed to the response since dendritic cells have limited capacity to phagocytose particles such as *B. abortus* cells. Furthermore, in murine studies, dendritic cells could activate T cells to proliferate but were incapable of stimulating a Th1 response (28).

In this study, the T cells were stimulated in vitro without prior exposure to *B. abortus*. In this aspect, this study differs from previous studies of humans in which the T cells were derived from the PBMC of individuals who had been exposed to microbial or allergenic antigens in vivo, for which selection and differentiation took place during prolonged and repeated antigen exposure (36, 38).

Receptors for LPS have been identified on murine macrophages as well as B and T cells (26), and they may be involved in the pathway utilized by *B. abortus* and LPS-BA. Additional evidence indicating that stimulation of T cells by *B. abortus* involves a unique pathway comes from recent experiments showing that major histocompatibility complex class II knockout mice (16), deficient in mature $CD4^+$ Th cells, produced high titers of IgG antibodies against HIV-1-derived peptide conjugated to *B. abortus*, whereas they were unable to produce any antibodies against the same peptide linked to the T-dependent carrier KLH (10a).

B. abortus does not appear to induce an anergic state. Despite the fact that *B. abortus* did not induce significant T-cell proliferation (data not shown), the stimulated populations produced both IFN-γ and IL-2. Furthermore, cells primed in vitro with *B. abortus* responded to secondary in vitro activation with PMA plus ionomycin by secreting higher levels of IFN-γ and lower levels of IL-4 than did unprimed cells. As previously defined (reviewed in reference 19), anergy implies the lack of both cell proliferation and cytokine production. Thus, we conclude that *B. abortus* did not induce anergy.

Although no IL-4 protein was detected in response to *B. abortus*, by using a bioassay that can detect 10 pg of IL-4 per ml, *B. abortus* stimulation did induce transient expression of IL-4 mRNA, as detected by PCR. Recently, it was demonstrated that both IFN-γ- and IL-4-producing effector T cells have a common precursor, which expresses the IL-4 gene (21). Therefore, the transient IL-4 mRNA expression observed in our experiments suggests that *B. abortus*, in addition to Th1 cells, activated Th0 cells, which, in turn, differentiated into Th1 cells, as evidenced by the phenotype of the cytokine secretion seen after 1 week of culture, i.e., IFN-γ but no IL-4. IL-5 mRNA, which is expressed in Th2 cells, was not detected following *B. abortus* stimulation, despite the fact that IL-5 mRNA was found following polyclonal activation of these cells. This supports the conclusion that *B. abortus* causes Th0 cells to differentiate into Th1 cells. The switch to Th1 cells was further confirmed by showing that T cells stimulated for 6 days with either *B. abortus* or LPS-BA responded to subsequent polyclonal activation by secreting mainly IFN-γ.

In addition to the expression of IFN-γ mRNA, IL-2 mRNA expression was also increased after *B. abortus* stimulation. Like IFN-γ, IL-2 is a product of Th0 and Th1 cells. Previous attempts to detect IL-2 protein secretion in the culture supernatants following activation with *B. abortus*, were unsuccessful (2). This was probably because IL-2 was being produced in limited amounts. The addition of exogenous IL-2 to the cultures caused a synergistic increase in the amount of IFN-γ secreted. Furthermore, treatment with the anti-Tac antibody did reduce the amount of IFN-γ released by *B. abortus*-stimulated human T cells (2). However, IL-2Rα mRNA was not increased following *B. abortus* stimulation, which is in agreement with our previous observation showing that activation of human T cells with *B. abortus* does not result in an increase of IL-2Rα (CD25) expression on the cell surface (2). Also, *B. abortus* does not induce appreciable increases in T-cell proliferation as measured by [^{3}H]thymidine uptake (10b). Thus, the ability of *B. abortus* to elicit IL-2 production probably potentiates its effect on IFN-γ secretion but does not promote proliferation of the responding cells because of the suboptimal amount of IL-2 secreted and the lack of induction of IL-2Rα. Taken together, these data suggest that *B. abortus* induces Th0

cells to differentiate, with minimal proliferation, into Th1 cells, which secrete IFN-γ.

Induction of IFN-γ secretion in these experiments by *B. abortus* may have been a consequence of stimulation of $CD4^+$, $CD8^+$, or NK cells. The NK cells were excluded on the basis of experiments in which NK cells were enriched by negative selection and found to be nonresponsive to *B. abortus* in terms of IFN-γ mRNA expression and protein secretion. On the other hand, NK-cell-depleted T cells expressed IFN-γ mRNA and secreted IFN-γ in response to *B. abortus*. Both $CD4^+$ and $CD8^+$ T cells were capable of responding to *B. abortus*. The ability of $CD8^+$ T cells to secrete IFN-γ in response to *B. abortus* may explain our earlier findings that mice depleted of $CD4^+$ T cells retained their IgG humoral responses to HIV-*B. abortus* (12) and to V3 peptide-*B. abortus* (13) immune conjugates. These responses were predominated by antibodies of the IgG2a isotype. It was previously shown that isotype switching to IgG2a following *B. abortus* stimulation in vivo was dependent on IFN-γ (10).

The ability of *B. abortus* to stimulate human $CD8^+$ T cells to secrete IFN-γ suggested that persons with impaired $CD4^+$ T-cell function, such as that which occurs following HIV-1 infection (5, 25) would retain at least some ability to produce IFN-γ in response to *B. abortus*. These data are in agreement with our previous observations that *B. abortus* induced IFN-γ secretion from HIV-infected patients at different stages of disease, including those with reduced $CD4^+$ T-cell counts (2).

In an attempt to identify the active component in the cell wall of *B. abortus* that was responsible for stimulating cells of the immune system, we purified LPS-BA and tested its function in several ways. Previously, we had shown that LPS-BA is much less toxic than LPS from *Escherichia coli* in causing fever in rabbits and lethality in mice (15). Furthermore, it was much less potent in stimulating IL-1β and tumor necrosis factor alpha secretion from human monocytes in vitro (15). These monokines are implicated in the pathogenesis of endotoxic shock (3, 7). On the other hand, LPS-BA was capable of acting as a carrier for trinitrophenyl in antibody responses, and as such it behaved as a T-independent type 1 carrier, similar to *B. abortus* (1). In the current study we found that, similar to *B. abortus*, LPS-BA was capable of inducing IFN-γ mRNA and protein secretion but did not induce IL-4 mRNA or protein secretion. However, in terms of IFN-γ protein secretion, exogenous IL-2 was required for LPS-BA but not for *B. abortus*-activated T cells. There are several possible explanations for these differences. Cell wall-associated LPS-BA may be more effective than soluble LPS because of the ability of the former to cross-link LPS receptors on the T cells or because LPS assumes a unique conformation in the bacterial cell wall which is not present in the soluble form. Alternatively, molecules other than LPS on the surface of the bacterium may play a role in T-cell activation.

In conclusion, we have developed a novel in vitro system which can be used to study the differentiation of human T cells into Th1 cells by primary in vitro stimulation. This was evidenced by the demonstration that *B. abortus* was capable of inducing human T cells to transcribe IFN-γ mRNA and secrete IFN-γ protein following depletion of antigen-presenting cells and NK cells. In the same cultures, no IL-4 protein and only transient levels of IL-4 mRNA were detected. These findings lend support to the use of *B. abortus* as a vaccine carrier in situations in which a Th1 pattern of cytokine secretion would be beneficial. Such is probably the case in human lepromatous leprosy (38) and following certain parasitic infections in mice (18, 41). Allergic states which are secondary to IL-4 production by Th2 cells (36, 42) would potentially be alleviated by promoting allergen-specific Th1 cells. Although a Th2 bias has been implicated in the progression to AIDS, this hypothesis remains controversial (5). Protection against many viral infections requires a combination of high titers of neutralizing antibodies and cytotoxic T-cell responses, which together enable the immune system to eliminate virus-infected cells and cell-free virus. Activation of Th1 cells probably also favors cytotoxic T-cell development (35). Recently, we found that peptides, containing both B-cell and cytotoxic T-cell epitopes, conjugated to *B. abortus* can evoke neutralizing antibodies as well as cytotoxic cells against HIV-1 in normal mice, as well as in mice depleted of $CD4^+$ cells (24a). These properties of *B. abortus* enhance its potential as a carrier in the design of subunit- or peptide-based vaccines.

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