

Induction and Regulation of Th1-Inducing Cytokines by Bacterial DNA, Lipopolysaccharide, and Heat-Inactivated Bacteria

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Th1 immune responses, characterized by production of gamma interferon (IFN- γ), are associated with protective immunity to viruses and intracellular bacteria. Heat-killed *Brucella abortus* promotes secretion of Th1-inducing cytokines such as interleukin-12 (IL-12) and IFN- γ and has been used as a carrier to induce Th1 responses to vaccines. To explore which bacterial constituents could mediate this response and how it is regulated, murine spleen cells were cultured with *B. abortus* derived DNA, lipopolysaccharide (LPS), or whole killed organisms. Each constituent induced similar, substantial amounts of IL-10. However, only *B. abortus* and *B. abortus* DNA induced high levels of IFN- γ and IL-12. *B. abortus* and *B. abortus* DNA-stimulated IL-12 production was maximal by 6 to 18 h, while IL-10 production steadily accumulated over this time period. These kinetics suggested that IL-10 may eventually downmodulate the Th1-like cytokine response to *B. abortus* and *B. abortus* DNA, which was confirmed by using neutralizing antibody. In the absence of IL-10, *B. abortus* LPS induced strong IFN- γ responses, but IL-12 p70 levels were still undetectable from BALB/c spleen cells. LPS induced IL-12 if the spleen cells were primed with IFN- γ and IL-10 was neutralized, indicating that LPS can stimulate IL-12 production under the most favorable conditions. Responses to *Escherichia coli* LPS and DNA mirrored the responses to *B. abortus* components, suggesting that immune effects observed with these constituents may be generalizable to many microbial species. In vivo experiments demonstrated the same hierarchy of responses for IL-12 production. These findings support the likelihood that microbial components, if used as carriers or adjuvants, can differ substantially in their ability to effect a Th1 response.

Optimal immunity to viruses and intracellular bacteria is typically mediated by the Th1 subset of CD4⁺ T lymphocytes. Th1 cells are characterized by the production of IFN- γ , the ability to help CTL responses, and the promotion of complement-fixing antibody isotypes such as immunoglobulin G2a (4, 15, 39, 44). Interleukin-12, chiefly a product of antigen-presenting cells (APC), is usually critical for the development of Th1 responses (28, 36, 54). In contrast, Th2 cells, which produce interleukin-4 (IL-4), IL-5, IL-6, and IL-10, mediate allergic and some antiparasitic responses (15, 17, 18). Ineffective containment of viral infections, such as human immunodeficiency virus, is correlated with Th2-like responses (10, 55). Thus, antiviral vaccine strategies need to focus upon adjuvants which steer the immune response in a Th1 direction. Intense interest is being directed toward the use of bacterial derivatives which promote Th1-like responses. These include monophosphoryl lipid A (modified lipopolysaccharide [LPS]), as well as plasmids and immunostimulatory oligonucleotides, which contain sequences that mimic the stimulatory properties of bacterial DNA (bDNA) (2, 9, 11, 13, 28, 30, 45, 46, 56, 57). Less-well-defined bacterial preparations from intracellular pathogens, such as soluble toxoplasmosis antigens, soluble listerial antigens, and heat-killed *Brucella abortus* can also be used to stimulate Th1-like responses in mice (16, 26, 47, 49, 51). The purpose of these studies was to directly compare the abilities of different bacterial constituents to stimulate secretion of Th1-inducing cytokines such as IL-12 and gamma in-

terferon (IFN- γ) (27, 34, 36, 54). The requirement for priming with IFN- γ and the sensitivity to IL-10 downregulation was also examined. In this report we demonstrate that *B. abortus* and bDNA, but not LPS, elicit high amounts of Th1-promoting cytokines from spleen cells in vitro and in vivo, even in the absence of priming with exogenous IFN- γ . Similar levels of endogenous IL-10 production are stimulated by all three constituents, and thus the amount of IL-10 in culture cannot account for the inferior IL-12 and IFN- γ induction by LPS in normal mice. These results indicate that bacterial constituents can differ in their ability to trigger the type of innate immune responses which drive the adaptive response in a Th1 direction. In particular, bDNA, and complex bacterial mixtures such as *B. abortus*, are more potent IL-12 inducers than is LPS, although they retain the ability to induce IL-10. These considerations are important for the development of Th1-promoting vaccine adjuvants and carriers which are both effective and safe.

MATERIALS AND METHODS

Mice. IL-10 knockout (KO) mice (on a B10.D2 background) and IFN- γ KO mice (on a BALB/c background) were obtained from the Jackson Laboratory (Bar Harbor, Maine). BALB/c and B10.D2 mice were obtained from Jackson or from the Division of Cancer Treatment, National Cancer Institute (Frederick, Md.). Animals were used according to National Institutes of Health guidelines on animal use and care.

Reagents and antibodies. LPS and DNA from *Escherichia coli* and control eukaryotic herring testis DNA were obtained from Sigma (St. Louis, Mo.). Heat-killed *B. abortus* 1119.3 was kindly provided by Barbara Martin at the U.S. Department of Agriculture (Ames, Iowa). *B. abortus* LPS was purified by butanol extraction as described previously (24). *B. abortus* DNA was extracted from *B. abortus* by using the Qiagen genomic DNA protocol and reagents (Valencia, Calif.). The LPS content of the DNA preparations was determined by using the *Limulus* amoebocyte lysate test (BioWhittaker, Walkersville, Md.), which was performed by Pankaj Amin (Center for Biologics Evaluation and Research, U.S. Food and Drug Administration). The amount of LPS in DNA preparations

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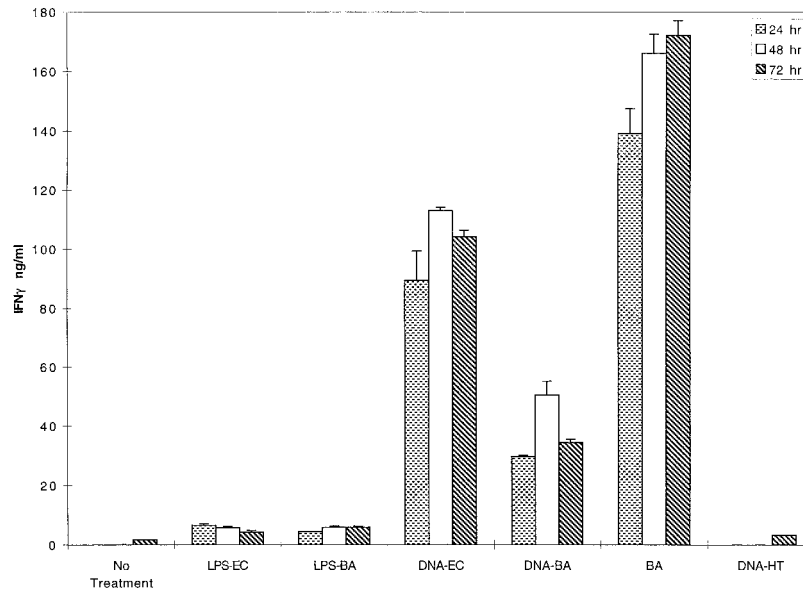


FIG. 1. IFN- γ induction by *B. abortus*, bDNA, and LPS. BALB/c spleen cells were cultured with the indicated stimuli; supernatants were harvested at 24, 48, and 72 h, and supernatants were assayed for IFN- γ by ELISA. LPS was derived from either *E. coli* (LPS-EC) or *B. abortus* (LPS-BA). Controls include cells stimulated with eukaryotic herring testis DNA (DNA-HT) and untreated cells.

ranged from 30 to 300 $\mu\text{g}/\mu\text{g}$ of DNA. Recombinant mouse IFN- γ and anti-IL-10 and anti-IL-12 monoclonal antibodies were obtained from Pharmingen (San Diego, Calif.). For neutralization experiments, antibodies were used at 10 $\mu\text{g}/\text{ml}$ in culture.

Cell preparation and culture. Spleens were removed from mice, and single cell suspensions were prepared by gentle teasing through cell strainers (Becton Dickinson, Franklin, N.J.). Erythrocytes were lysed by using ACK lysing buffer (BioWhittaker) and washed three times in phosphate-buffered saline (PBS) before resuspension in RPMI (Life Technologies, Gaithersburg, Md.). RPMI was supplemented with 10% fetal bovine serum (HyClone, Logan, Utah), penicillin-streptomycin, HEPES buffer, 2-mercaptoethanol, nonessential amino acids, and pyruvate. Spleen cells were cultured in 48-well flat-bottom plates (Costar, Cambridge, Mass.) at a concentration of 10^7 cells/ml. Preliminary experiments determined that this concentration of cells provided optimal IFN- γ production at all time points tested, to LPS, bDNA, and *B. abortus*. Preliminary dose-response analyses were conducted with bDNA concentrations ranging from 0.5 to 100 $\mu\text{g}/\text{ml}$ and LPS concentrations ranging from 3 to 750 $\mu\text{g}/\text{ml}$. Optimal IFN- γ -producing conditions were 25 $\mu\text{g}/\text{ml}$ for bDNA and 30 $\mu\text{g}/\text{ml}$ for LPS; these concentrations were used for all experiments. Cultures were incubated at 37°C with 5% CO₂. Supernatants were harvested and frozen at -20°C for the cytokine assays.

Cytokine ELISA. Cytokine content in supernatants was determined by enzyme-linked immunosorbent assay (ELISA) by using commercial kits for IFN- γ (Life Technologies), IL-10 and IL-12 p70 (Endogen, Woburn, Mass.), and IL-12 p40 (Biosource, Camarillo, Calif.). Samples were assayed in duplicate, and all experiments were performed at least twice. All data shown are from reproducible experiments. Values are expressed as the means with the standard deviations for duplicate samples. The lower limit of detection for IL-12 p70 was 5 pg/ml.

In vivo experiments. BALB/c mice were injected intravenously with *B. abortus* (10^8 organisms), *B. abortus* DNA (100 μg), *E. coli* DNA (100 μg), *B. abortus* LPS (30 μg), low-dose *B. abortus* DNA (0.1 μg), or PBS. Injection volumes were 0.1 to 0.2 ml/mouse. The dose of *B. abortus* was selected because it is nontoxic, suppresses Th2 responses, and induces IL-12 mRNA (49). The 100- μg dose of bDNA has been shown to stimulate IFN- γ secretion in vivo (11). A 30- μg portion of LPS was selected because this is the amount of LPS contained in 10^8 organisms of the *B. abortus* preparation. The 0.1- μg dose of bDNA (low dose) used is the amount of bDNA contained in 10^8 *B. abortus* organisms. At 3 h after injection, spleens were removed and prepared by gentle teasing through cell strainers. After a washing, cells were cultured in RPMI as detailed above at a concentration of 5×10^6 cells/ml in 48-well plates, without further stimulation. Supernatants were harvested at 18 to 24 h for ELISA.

RESULTS

***B. abortus* and bDNA are more potent inducers of IFN- γ secretion than is LPS.** IFN- γ , chiefly a product of T and NK

cells, is an important contributor to the development of primary and recall Th1 responses (4–6). In addition, IFN- γ suppresses Th2 development (21, 40, 42, 44). IFN- γ , secreted in an antigen-nonspecific fashion at the onset of an immune response, enhances macrophage activation and antigen uptake and primes macrophages for IL-12 production (4, 20, 25, 33, 55). Therefore, IFN- γ induction is a measure of potential adjuvant activity by bacterial constituents. The ability of bDNA, LPS, and *B. abortus* to induce IFN- γ was measured in whole spleen cell cultures, which were used in order to provide an environment closest to that seen in vivo. Preliminary dose-response studies indicated that for bDNA and for LPS, 25 to 50 and 30 $\mu\text{g}/\text{ml}$, respectively, were optimal stimulatory doses. *B. abortus* was added at 10^8 organisms/ml, a dose which is stimulatory and nontoxic. The amount of bDNA contained in the dose of *B. abortus* used is 500 ng/ml, which is below the threshold dose of purified bDNA for IFN- γ production. *B. abortus* and bDNA, but not LPS, promoted high levels of IFN- γ secretion (Fig. 1). Similar results were seen in 10 separate experiments. Typically, IFN- γ production by LPS-treated cells was similar to control levels, although in some experiments it exceeded control culture levels. This interexperimental variation may reflect occasional in vivo priming if mice were exposed to microorganisms in the animal care facility. The source of LPS was not a factor, since neither *E. coli* nor *B. abortus* LPS induced high levels of IFN- γ (Fig. 1; see also Fig. 3).

Unprimed spleen cells produce IL-12 p70 in response to *B. abortus* and DNA but not LPS. The greater ability of *B. abortus* and bDNA to induce IFN- γ in vitro, compared to LPS, could be explained by low IL-12 production in response to LPS. Indeed, IL-12 p70 secretion appeared to correlate with subsequent IFN- γ production (Fig. 2). At early time points, both *B. abortus* and bDNA, but not LPS, noticeably increased IL-12 secretion. IFN- γ is typically required to elicit significant amounts of IL-12 from cultured cells exposed to LPS (20, 37). These results show that robust IL-12 secretion, induced by *B. abortus* and bDNA, does not require priming with exogenous IFN- γ .

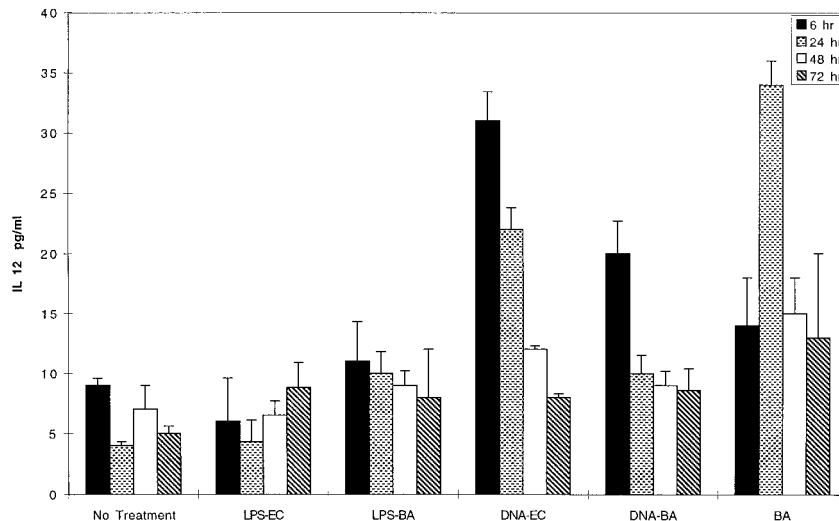


FIG. 2. IL-12 is induced by *B. abortus* and bDNA but not LPS. BALB/c spleen cells were cultured with LPS and bDNA (from *B. abortus* and *E. coli*) and with *B. abortus*. Supernatants were removed at the indicated times and assayed for IL-12 p70 levels by ELISA. Cells cultured with LPS, and untreated cells produced similar constitutive amounts of IL-12 p70, at levels near the detection limits of the ELISA (5 pg/ml). In contrast, bDNA and *B. abortus* both elicited IL-12 production in excess of the control cultures at early time points. The results of one of three similar experiments are shown.

While IL-12 is the most likely cytokine to be responsible for IFN- γ production in this system, other cytokines produced in response to pathogens such as type 1 IFNs and IL-18 (IGIF) can also promote IFN- γ secretion (7, 35, 41, 58). Indeed, human cells secrete IL-18 in response to stimulatory DNA sequences, and *B. abortus* induces type 1 IFNs (15, 48). To confirm that IFN- γ secretion in this system was dependent upon IL-12, cells were cultured in the presence of anti-IL-12 or isotype control antibody. *B. abortus* and bDNA-induced IFN- γ production was completely IL-12 dependent (Fig. 3). These

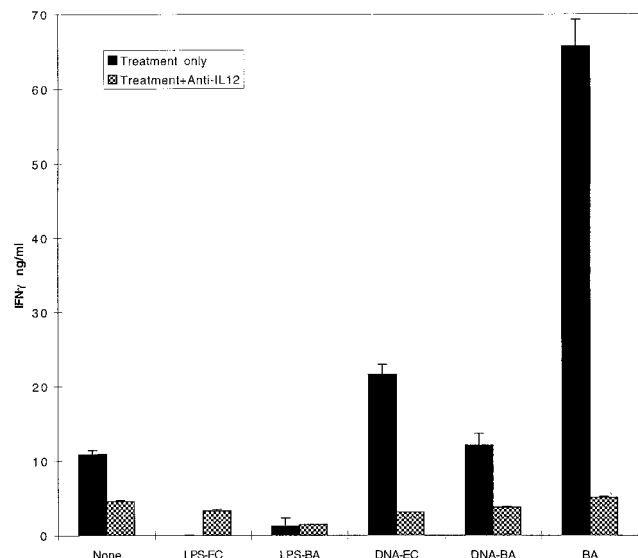


FIG. 3. IFN- γ induction by *B. abortus*, LPS, and bDNA is IL-12 dependent. BALB/c spleen cells were cultured for 72 h with or without anti-IL-12 (10 μ g/ml), and supernatants were assayed by ELISA. More than 90% of the IFN- γ induction by bDNA and *B. abortus* was prevented by anti-IL-12. This experiment is representative of four separate experiments in two strains of mice (BALB/c and B10.D2), which all showed significant reduction of IFN- γ secretion by antibodies to IL-12.

data suggest that other IFN- γ -inducing cytokines do not substitute for the IL-12 requirement to stimulate IFN- γ release.

Removal of IL-10 permits LPS induction of IFN- γ and increases *B. abortus* and bDNA IFN- γ . Interleukin-10 is a potent downregulator of Th1 responses (19, 38, 55). Macrophage-secreted IL-10 attenuates IL-12 and IL-1 secretion, thus limiting the strength of inflammatory reactions and therefore potential damage to host tissues (3, 55). LPS, bDNA, and *B. abortus* can all induce some IL-10 (1, 3, 38, 53). The lack of robust IFN- γ production by spleen cells exposed to LPS could be explained by induction of high IL-10 levels, relative to those induced by *B. abortus* and bDNA. However, all three bacterially derived preparations induced similar quantities of IL-10 (Fig. 4). The kinetics of IL-10 secretion were inversely correlated with those of IL-12 in that, as IL-10 increased, IL-12 levels diminished to baseline amounts by 72 h in *B. abortus* and bDNA cultures. The rate of IFN- γ accumulation decreased between 48 and 72 h in most experiments, consistent with downregulation of IL-12, followed by IFN- γ . Thus, the amount of IFN- γ in supernatants was still elevated at 72 h, reflecting production from earlier timepoints.

Although in this system, similar levels of IL-10 were seen after *B. abortus*, bDNA, and LPS, it was still possible that (potential) LPS-induced IFN- γ secretion is more sensitive to IL-10 downregulation than the pathways used by bDNA and *B. abortus*. To explore this issue, IFN- γ production was determined after incubation of cells with anti-IL-10 (Fig. 5). In the presence of anti-IL-10, LPS consistently induced small amounts of IFN- γ secretion. Anti-IL-10 also increased production of IFN- γ by cells cultured with bDNA and *B. abortus*. To confirm these results, cells from IL-10 KO mice were cultured with LPS or bDNA. Cells cultured with *E. coli* LPS or *B. abortus* LPS produced large amounts of IFN- γ (72.3 \pm 5.6 and 75.2 \pm 2.8 ng/ml, respectively), as did cells cultured with *E. coli* DNA or *B. abortus* DNA (92.5 \pm 1.0 and 73.8 \pm 4.3 ng/ml, respectively). Results in IL-10 KO mice differed from those in normal mouse cultures treated with anti-IL-10. The potency of LPS and bDNA for IFN- γ induction was similar in IL-10 KO spleen cultures, but it was dissimilar in normal mice, with

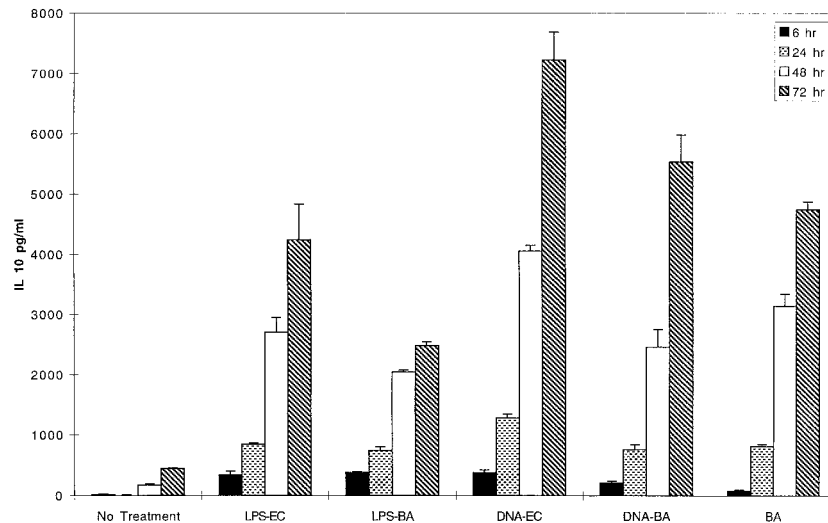


FIG. 4. IL-10 production is not enhanced in LPS cultures. BALB/c spleen cells were cultured with the indicated additives for 6 to 72 h. Supernatants were assayed for IL-10 by ELISA. IL-10 production, like IFN- γ , peaked at the latest time point and showed similar kinetics for all constituents.

bDNA always stimulating more IFN- γ than LPS when spleens were cultured with anti-IL-10. Such results may reflect differences in the reactivity of cell populations in KO mice, which have developed in the complete absence of endogenous IL-10. Overall, however, these results suggest that LPS, while capable of inducing IFN- γ secretion, can only be a potent inducer in the absence of IL-10, whereas the bDNA and pathway of IFN- γ induction can proceed in the presence of IL-10.

Interestingly, even though IFN- γ was increased in LPS- α IL-10-treated cells, no amplification of IL-12 p70 secretion was detected (not shown), suggesting a possible action of another IFN- γ -inducing factor in this setting. To explore this possibility, cells were cultured with LPS- α IL-10 in the presence or absence of α IL-12 (Fig. 5). Coculture with α IL-12 and α IL-10 eliminated the LPS-induced IFN- γ . Thus, small increases of IL-12, which were difficult to detect by ELISA but large

enough to cause IFN- γ secretion, were produced when cells were exposed to LPS in the absence of IL-10.

Role of IFN- γ priming for IL-12 production in response to *B. abortus* bDNA, and LPS. Murine and human macrophages exposed to IFN- γ have enhanced ability to produce IL-12 when stimulated with LPS (14, 25, 55). The mechanism of this priming effect appears to be an IFN- γ -mediated increase of IL-12 p40 mRNA transcription and stability (25, 33). It has not been determined whether bDNA-induced IL-12 is also susceptible to this priming effect. IFN- γ KO mice were used for these experiments to eliminate the possibility of in vivo priming of cells by IFN- γ due to environmental factors. IFN- γ enhanced IL-12 production by cells cultured with bDNA and *B. abortus* to a greater extent than those cultured with LPS (Fig. 6). Only *B. abortus* and bDNA-cultured cells from IFN- γ KO mice produced IL-12 in the presence of anti-IL-10, even without the

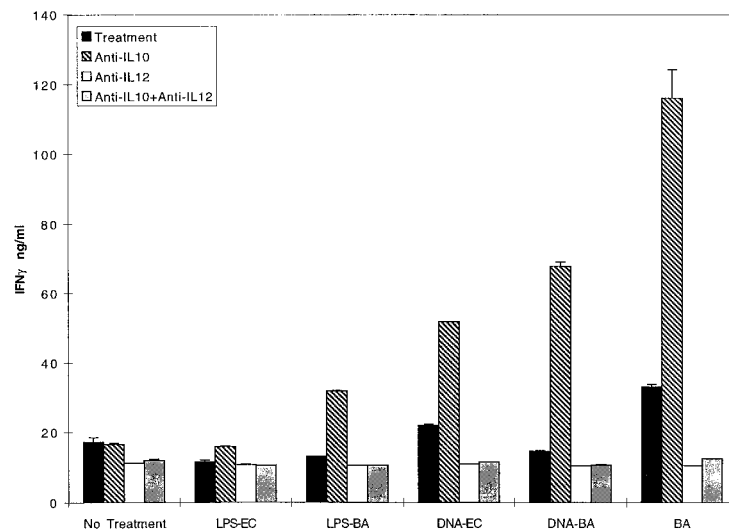


FIG. 5. IFN- γ produced when endogenous IL-10 is blocked is IL-12 dependent. BALB/c spleen cells were cultured with various stimuli in the presence of anti-IL-10, anti-IL-12, or both antibodies (10 μ g/ml). Supernatants were collected at 72 h and analyzed for IFN- γ by ELISA. The results from one of two similar experiments are shown.

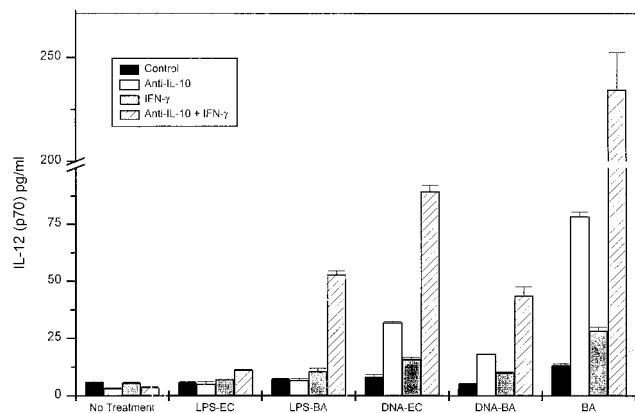


FIG. 6. *B. abortus* and bDNA-induced IL-12 are enhanced by IFN- γ priming, especially in the presence of anti-IL-10. LPS only induced detectable IL-12 with the combination of IFN- γ addition and anti-IL-10. Spleen cells from IFN- γ KO mice (BALB/c background) were stimulated with *B. abortus*, LPS, or bDNA in the presence of IFN- γ (50 ng/ml) and/or anti-IL-10 (10 μ g/ml). LPS, bDNA, or *B. abortus* was added to the culture 10 min after the addition of anti-IL-10 and IFN- γ . IL-12 p70 was measured by ELISA 18 h later. The results of one of three representative experiments are shown. The results in normal BALB/c mice were similar.

exogenous addition of IFN- γ . Furthermore, neutralization of IL-10 was synergistic with the addition of IFN- γ for IL-12 production when cells were cultured with *B. abortus* bDNA, or LPS, but the amount of IL-12 produced was always greatest in *B. abortus* cultures. As in normal mice, anti-IL-10 addition could not suffice to stimulate measurable increases of IL-12 production from LPS-exposed cells. These results emphasize the limited ability of LPS alone to provide a strong Th1-promoting environment, compared to *B. abortus* organisms or bDNA.

***B. abortus* and *B. abortus* DNA induce splenic IL-12 in vivo.** Sher et al. reported that IL-12 can be detected in spleen cell cultures after the injection of a soluble parasite extract (47). To determine whether the superior IL-12-inducing ability of *B. abortus* and *B. abortus* DNA in vitro could be demonstrated in vivo, BALB/c mice were injected intravenously with *B. abortus*, bDNA, or LPS. At 3 h after injection, spleens were removed, and cells cultured without further intervention. *B. abortus*, *B. abortus* DNA, and *E. coli* DNA, but not LPS, stimulated IL-12 secretion from in vivo-treated spleen cells (Table 1). *B. abortus*

TABLE 1. IL-12 production 3 h after intravenous injection of bacterial constituents in BALB/c mice^a

Treatment	IL-12 produced (pg/ml)	
	Expt 1	Expt 2
PBS	35.7 \pm 9.8	8.5 \pm 0.6
<i>B. abortus</i>	234 \pm 68	467 \pm 5.4
<i>B. abortus</i> DNA	104 \pm 32.8	203 \pm 40
<i>B. abortus</i> LPS	9.7 \pm 3.0	17.3 \pm 2.9
<i>E. coli</i> DNA	ND	107 \pm 27.5
<i>B. abortus</i> DNA (low dose)	9.9 \pm 3.6	ND

^a BALB/c mice were injected with *B. abortus* (10^8 organisms), *B. abortus* LPS (30 μ g), *B. abortus* DNA or *E. coli* DNA (100 μ g), or low dose *B. abortus* DNA (0.1 μ g). Three hours after injection, spleens were removed and cultured individually without further stimulation. Supernatants were harvested at 24 h and assayed by ELISA for IL-12 p40. Spleens removed 8 and 24 h after injection produced less IL-12 than that seen at 3 h, although the relative potency of constituents remained the same (data not shown). The results reflect the means \pm the standard error for three mice/treatment. ND, not determined.

given in vivo was consistently the most potent IL-12-inducing substance, whereas when exposure was entirely in vitro, bDNA often stimulated as much IL-12 release as had *B. abortus*. It is possible that bDNA is taken up by other tissues and degraded by DNases before all of it can reach the spleen, which could account for the observed decrease in in vivo potency relative to *B. abortus*.

DISCUSSION

Vaccine adjuvants which foster Th1 responses are likely to be useful in promoting antiviral immunity. LPS derivatives, bDNA-containing vaccines, and synthesized immunostimulatory DNA sequences are under investigation as enhancers of Th1 and cytotoxic T lymphocyte responses for use against viral and parasitic pathogens. All of these bacterial products stimulate the innate immune system, which in turn influences the type of adaptive T-cell response. To compare the abilities of prototypic bacterial constituents to provide a Th1-favoring environment, we studied the cytokine response to bDNA, LPS, and whole heat-inactivated intracellular bacteria (*B. abortus*). In addition, the dependence of IL-12 and IFN- γ responses upon IFN- γ and IL-10 was assessed.

Surprisingly, a direct comparison between LPSs from two different bacteria and bDNA showed that bDNA had superior IL-12- and IFN- γ -inducing capacities, in vitro and in vivo. Unlike this work, most studies which show that LPS induces IL-12 have been done in APC-enriched populations or with the exogenous addition of IFN- γ . LPS clearly can induce measurable IL-12 from activated splenic adherent cells (47) and peritoneal or bone marrow-derived macrophages (50, 52). In some studies, LPS induced IL-12 and IFN- γ production from spleens in vivo, but priming by footpad injection of LPS was required (43). Using immunohistochemical methods, a recent study has shown that intravenous injection of LPS elicits detectable IL-12 p40 in splenic dendritic areas, although to a much lesser degree compared to a parasite protein extract (47). Additional support for poor IL-12-inducing activity in the spleen by LPS is provided by the observation that intraperitoneal injection does not prime for the Schwartzman reaction, which is IL-12 dependent (43). Furthermore, most studies have measured IL-12 p40 rather than the bioactive heterodimer p70. In fact, p40 homodimers can antagonize the action of p70 (22, 23). Overall, these reports are consistent with our findings that LPS elicits little IL-12 p70 from spleen cells.

In vitro, priming of spleen cell cultures by IFN- γ increases IL-12 responses to LPS (20, 55). However, other stimuli, such as viable or heat-killed *M. tuberculosis*, or latex beads, do not require IFN- γ priming for the production of IL-12 by macrophages (31). Our results indicate that bDNA and *B. abortus*-induced IL-12 does not require exogenous IFN- γ priming, since bDNA and *B. abortus* stimulation elicited prompt IL-12 and IFN- γ production from normal spleen cells. In IFN- γ KO mice, *B. abortus* and bDNA alone, still measurably increased IL-12 levels in the absence of IFN- γ , when IL-10 was neutralized. Anti-IL-10 alone did not enhance IL-12 secretion from LPS-treated IFN KO mouse cells, whereas IL-10 neutralization did stimulate IL-12 release from LPS-treated normal mouse cells. The difference between normal and IFN- γ KO mice seen here suggests that constitutive in vivo priming by low levels of environmentally induced IFN- γ occurs in normal mice; this could function to provide a regulated, low level of responsiveness to LPS. In all cases, the combination of anti-IL-10 and exogenous IFN- γ caused the most IL-12 production from IFN- γ KO cells, although *B. abortus* invariably was the most potent stimulator under these conditions. Taken to-

gether, these results support the hypothesis that bDNA- or *B. abortus*-induced IL-12 is less dependent upon IFN- γ priming than is LPS-induced IL-12.

The increased potency of *B. abortus* and bDNA relative to LPS could be explained if these bacterial products stimulate different cell populations or subpopulations to produce IL-12. B cells, monocytes, dendritic cells, and polymorphonuclear leukocytes all respond to LPS, and are all capable of secreting IL-12 (32, 47, 54). CpG motifs, contained in bDNA, directly stimulate B cells and macrophages and promote B-cell production of IL-12 (8, 29, 48). If LPS stimulates different cell populations than does bDNA, these populations may be more sensitive to downregulatory cytokines such as IL-10, may be simply less numerous, or may secrete less IL-12/cell. Our experiments in IL-10 KO mice have suggested that bDNA, *B. abortus*, and LPS-induced IL-12 have similar sensitivities to downmodulation by exogenous IL-10 (not shown). Although IL-10 levels were similar in bDNA and *B. abortus* cultures, *B. abortus* typically stimulated higher IL-12 production. A likely explanation is that a greater number of IL-12-secreting cells initially respond to *B. abortus* than to bDNA. This is supported by recent *in vivo* immunohistochemical analysis of IL-12 expression in *B. abortus*- and bDNA-treated mice, showing more numerous IL-12 secreting cells after treatment with *B. abortus* (49a). Detailed dissection of responding cell populations and the pathways through which they are stimulated should elucidate why bDNA and *B. abortus* are more effective IL-12 and IFN- γ stimulators than LPS.

B. abortus appeared to be similar to bDNA in its ability to induce IL-12 from spleen cell cultures. Like bDNA, it induced IL-12 more effectively than LPS, and this IL-12 production was downregulated by endogenous IL-10 and upregulated by IFN- γ . However, bDNA contained in *B. abortus* is not likely to fully explain the potent effects of *B. abortus*. The amount of bDNA contained in the concentration of *B. abortus* used is close to 500 ng/ml (12), which is well below our minimal stimulatory bDNA concentration of 2.5 μ g/ml. The amount of LPS in the *B. abortus* preparations was 30 to 35 μ g/ml, a level similar to doses of LPS used in these experiments. However, the conclusion that stimulation of IL-12 and IFN- γ secretion by *B. abortus* is caused by constituents other than bDNA is also not entirely proven by these calculations. *B. abortus*, a macrophagotropic organism, may preferentially target and activate APCs and thus could deliver its constituents more effectively with other stimulating signals or in a particularly stimulating configuration to responding cells. Indeed, numerous endeavors in this laboratory to remove bDNA from the *B. abortus* preparations by using DNase digestions coupled with sonication have failed to eliminate PCR-detectable *B. abortus* DNA sequences, suggesting that bDNA is protected from degradation when contained in heat-killed bacteria. However, efforts are under way to identify other immunostimulating compounds in *B. abortus* which can promote Th1 responses.

These studies show that bDNA and LPS differ substantially in their ability to create a Th1-promoting environment *in vitro* and *in vivo*. We also show for the first time that bDNA-induced IL-12 is enhanced by priming with IFN- γ . Although responses to both constituents and to *B. abortus* were regulated by IFN- γ and endogenous IL-10, at optimal doses quantitative differences among them are marked. Further understanding of these differences may lead to the design of more effective Th1-promoting adjuvants, which are needed for protective responses to viral and parasitic diseases.

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