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Interleukin-10 Downregulates Protective Immunity to *Brucella abortus*

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In vivo neutralization of interleukin-10 (IL-10) with an anti-IL-10 monoclonal antibody resulted in up to 10-fold fewer bacteria in the spleens of BALB/c mice infected with the virulent *Brucella abortus* strain 2308. In vitro neutralization of endogenous IL-10 in brucella antigen-stimulated cultures of splenocytes from infected mice resulted in increased gamma interferon production in these cultures, whereas exogenous recombinant IL-10 inhibited the ability of peptone-elicited peritoneal macrophages to control intracellular brucellae. These data suggest that IL-10 may be downregulating the immune response to *B. abortus* by affecting both macrophage effector function and the production of the protective Th1 cytokine gamma interferon.

Brucella abortus is a gram-negative facultative intracellular bacterium and the causative agent of brucellosis, a chronic disease of cattle, humans, and other species (2, 17). Evidence suggests that the Th1 cytokine gamma interferon (IFN-γ) is important for control of the infection in mice. For instance, macrophages activated by IFN-γ controlled replication of intracellular *B. abortus* strains 19 and 2308 more effectively in vitro (10, 12), and neutralization of endogenous IFN-γ in vivo resulted in a decreased ability of mice to control infection with the attenuated *B. abortus* strain 19 (13). Treatment of mice with recombinant IFN-γ also resulted in an enhanced ability to control infection with the virulent *B. abortus* strain 2308 (21). However, while *B. abortus* is also known to be a strong inducer of the CD4 Th1 cytokine IFN-γ (14, 22, 23), *B. abortus* strains 19 and 2308 nevertheless persist in the spleens of infected BALB/c mice for up to 6 weeks (8) and 6 months (15), respectively. To elucidate the mechanisms which facilitate the establishment of chronic infection despite the probable presence of a protective Th1 response, we evaluated the role of interleukin-10 (IL-10) early in the infection of mice with strain 2308. IL-10 is produced by CD4 Th2 cells, activated macrophages, and Ly-1 B cells (16), and it is known to downregulate Th1 responses and/or to increase susceptibility to a number of bacterial (3, 24) and parasitic (19, 20) infections.

Female BALB/c mice were purchased at 7 to 8 weeks of age from Harlan Sprague Dawley (Indianapolis, Ind.) and used at 8 to 12 weeks of age. Mice were infected intraperitoneally with 5×10^3 CFU of *B. abortus* 2308 in 0.1 ml of phosphate-buffered saline (PBS). At 1 week after infection, mice were sacrificed by cervical dislocation, spleens were removed, and cells were dispersed in PBS. An aliquot of the resulting cell suspension was plated out to determine the number of CFU, and mononuclear cells were separated out from the rest of the suspension by using Ficoll-Paque (Pharmacia, Piscataway, N.J.). To evaluate cytokine production, spleen cells (2×10^5) were cultured in 0.1 ml of complete medium (RPMI 1640 medium with 10% heat-inactivated fetal calf serum [Sigma, St. Louis, Mo.], 50 μg of L-glutamine and 60 μg of gentamicin per ml, and 5×10^{-5} M 2-mercaptoethanol) and stimulated with 0.1 ml of either heat-killed *B. abortus* 2308 at 10^8 CFU/ml or concanavalin A (ConA) at 6.25 μg/ml in microtiter plates. Supernatants were removed after 3 or 5 days of culture and evaluated for either IFN-γ or IL-10, respectively, by a sandwich enzyme-linked immunosorbent assay (ELISA) using monoclonal antibody (MAb) pairs purchased from Pharmingen (San Diego, Calif.). Protocols were as recommended by the manufacturer. To some cultures, rat anti-mouse IL-10 MAb (clone JES5.2A5; Pharmingen) was added at 50 μg/ml at

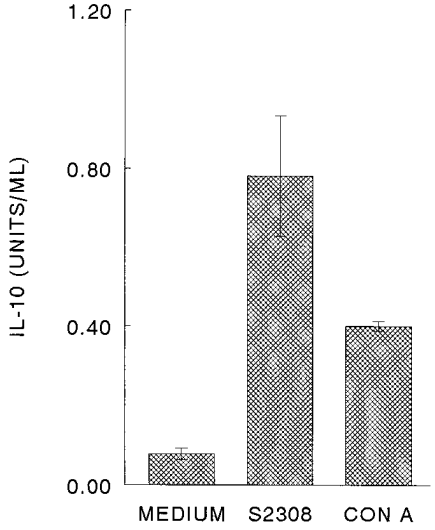

FIG. 1. IL-10 produced by spleen cells from *B. abortus*-infected mice. Spleen cells (2×10^5) were cultured in medium or stimulated with heat-killed *B. abortus* 2308 or ConA. IL-10 was measured in culture supernatants by ELISA, and the concentration was estimated by comparison with a standard curve generated with recombinant IL-10. Each value represents the mean ± the standard error of the mean for cultures from five mice and is representative of data from two experiments performed. Supernatants from the splenocyte cultures were evaluated for IL-10 after 5 days of culture, the time at which maximal IL-10 was detected in both antigen- and mitogen-stimulated cultures in preliminary experiments (data not shown). When data from both experiments performed were analyzed by both Student's *t* test and the Wilcoxon test, cultures stimulated with heat-killed brucellae were found to have produced significantly more IL-10 than medium control cultures ($P \leq 0.004$).

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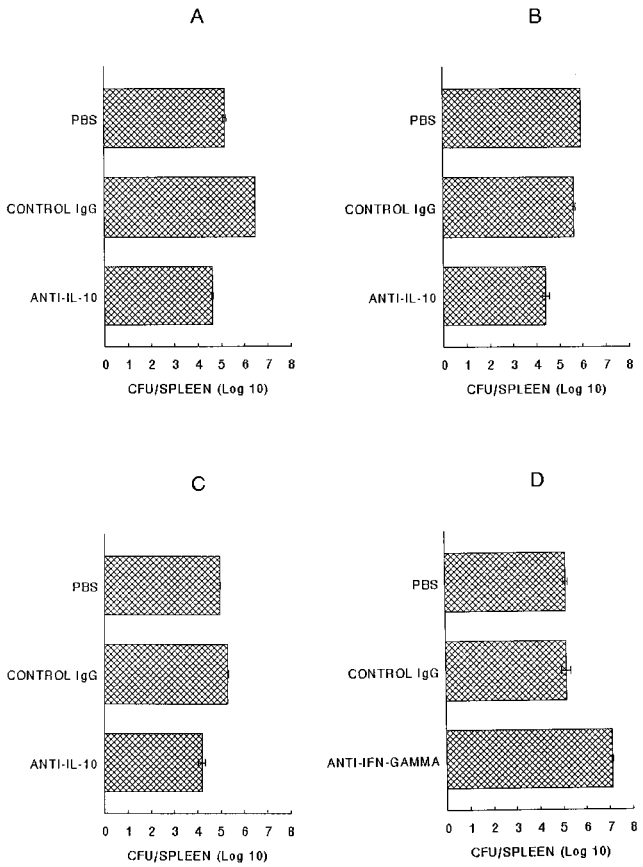

FIG. 2. In vivo neutralization of endogenous IL-10 (experiment 1 [A], experiment 2 [B], and experiment 3 [C] or endogenous IFN-γ (D) in mice infected with *B. abortus* 2308. Mice were injected with either 1 mg of anti-IL-10 MAb or 0.5 mg of anti-IFN-γ MAb at both 24 h before infection and 4 days after infection or with control rat IgG at the same concentration. CFU of *B. abortus* in spleens were enumerated at 1 week after the infection. Each bar represents the mean ± the standard error of the mean for five mice. Anti-IL-10 MAb resulted in a significant decrease in numbers of CFU relative to those in mice receiving control IgG ($P \leq 0.01$ for experiment 1 and $P \leq 0.05$ for experiments 2 and 3 as determined by analysis of variance [ANOVA]; for experiments 2 and 3, $P \leq 0.02$ as determined by the Wilcoxon test). Anti-IFN-γ MAb resulted in a significant increase in numbers of CFU relative to those in controls receiving IgG ($P \leq 0.001$ as determined by ANOVA).

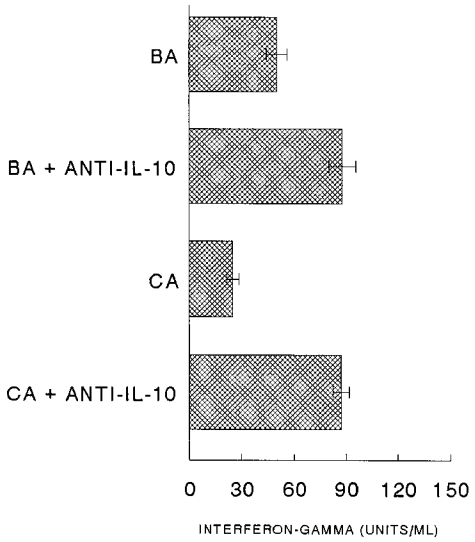

FIG. 3. Neutralization of endogenous IL-10 by addition of MAb to in vitro cultures resulted in higher levels of production of IFN-γ by spleen cells from infected mice. Spleen cells (2×10^5) were stimulated with heat-killed *B. abortus* 2308 (BA) or ConA (CA) in the presence or absence of anti-IL-10 MAb. Concentrations of IFN-γ in supernatants were determined by ELISA. The concentration of IFN-γ in cultures of cells stimulated with medium alone was 1.57 ± 0.14 U/ml, and in cultures stimulated with anti-IL-10 MAb present the concentration was 1.76 ± 0.18 U/ml. Each value represents the mean ± the standard error of the mean for cultures from five mice and is representative of data from two experiments performed. Addition of anti-IL-10 MAb to cultures stimulated with antigen resulted in a significant increase in the amounts of IFN-γ detected in both experiments following stimulation with either heat-killed brucellae (Student's *t* test, $P \leq 0.005$; Wilcoxon test, $P \leq 0.02$) or ConA (Student's *t* test, $P \leq 0.02$; Wilcoxon test, $P \leq 0.02$).

the time of antigen or mitogen stimulation. Recombinant IFN-γ (5 U/ng; purchased from Boehringer Mannheim, Indianapolis, Ind.) and IL-10 (0.5 U/ng; purchased from Pharmingen) were used as standards in the ELISA and were added to cultures of *B. abortus*-infected macrophages where indicated.

For in vivo depletion of cytokines, HB170 (an immunoglobulin G1 [IgG1] rat anti-mouse IFN-γ MAb) and HB10958 (an IgG1 rat anti-mouse IL-10 MAb) were purchased from the American Type Culture Collection (Rockville, Md.). Antibodies were prepared as ascites in pristane-primed nude mice, precipitated by 45% ammonium sulfate precipitation, dialyzed, and assessed by polyacrylamide gel electrophoresis (1). To deplete cytokines, mice were injected intraperitoneally with 1 mg of anti-IL-10 MAb as described previously (9) or with 0.5 mg of anti-IFN-γ MAb in 0.5 ml of PBS at both 24 h before infection and 4 days after infection (5, 13). Equal amounts of purified rat IgG (Sigma) or PBS were injected into control mice as described elsewhere (5).

Peritoneal macrophages were elicted by intraperitoneal injection of mice with 3 ml of 5% Proteose Peptone as described previously (11). Macrophages were harvested and infected with opsonized *B. abortus* 2308 as described previously (10), in either 96-well plates (3×10^4 macrophages per well) or 48-well plates (9×10^4 macrophages per well) at a ratio of 100 bacteria per macrophage. Recombinant cytokines were added at 2 h after infection (100 U of IFN-γ per ml and 1,000 U of IL-10 per ml) in medium containing antibiotics to kill extracellular bacteria. At 48 h after the infection, the cells were washed three times in PBS by centrifugation, supernatants were removed, and macrophages were lysed by adding 0.1% deoxycholate. Serial dilutions of the lysate were plated on Schaedler blood agar, and the CFU were enumerated after incubation at 37°C in 5% CO_2 for 3 days.

IL-10 is known to be produced in mice in response to immunization with heat-killed *B. abortus* 19 (23). Here we first determined whether or not IL-10 was produced by splenocytes following infection of BALB/c mice with live *B. abortus* 2308. Splenocytes taken from mice at 1 week after the infection, a time by which the infection is established (15), produced more IL-10 in vitro when stimulated with heat-killed *B. abortus* 2308 than when stimulated with the T-cell mitogen ConA employed as a control (Fig. 1). However, because in infections with *Listeria monocytogenes* IL-10 is known to be either beneficial or detrimental to the immune response depending upon the phase of the infection (24), we performed further experiments to evaluate the effect of IL-10 early in the infection with *B. abortus* 2308. This was done by neutralizing endogenous IL-10 in vivo by administering the anti-IL-10 MAb HB10958. This resulted in a significant decrease in the numbers of bacteria recovered from the spleen at 1 week after infection (Fig. 2A through C), suggesting that IL-10 was having a detrimental effect on control of brucellae at this time. The level of reduc-

TABLE 1. Effect of IL-10 on the ability of macrophages cultured in medium or activated with IFN-γ to control intracellular *B. abortus* strain 2308

Treatment[a]	CFU per culture (log_{10}) in expt no.[b]:				
	1	2	3	4	5
Medium	5.08	3.02 ± 0.07	3.82 ± 0.06	4.10 ± 0.10	5.14 ± 0.01
IL-10	5.70	3.68 ± 0.03**	4.55 ± 0.04***	4.16 ± 0.03	5.32 ± 0.02*
IFN-γ	2.85	2.23 ± 0.04	2.78 ± 0.08	3.28 ± 0.02	4.23 ± 0.01
IFN-γ + IL-10	3.59	2.14 ± 0.04	3.40 ± 0.03*	2.94 ± 0.06	4.34 ± 0.01**

[a] Macrophages were infected with *B. abortus* 2308 and treated with cytokines (100 U of IFN-γ per ml and/or 1,000 U of IL-10 per ml) at 2 h after infection. Macrophages were lysed and CFU were plated out at 48 h after infection.

[b] Results from five experiments are shown. Each value is the mean ± standard error of the mean for four replicate cultures, except values for experiment 1, which are values for one set of cultures. When experiments were analyzed individually by Student's *t* test, IL-10 was found to have resulted in a significant increase in recovery of CFU at $P \leq 0.05$ (*), $P \leq 0.01$ (**), or $P \leq 0.001$ (***) where indicated (no statistics were obtained for experiment 1). When the data from all five experiments evaluating macrophages cultured in medium were analyzed together, IL-10 was found to significantly decrease the ability of macrophages to control intracellular brucellae by both Student's paired *t* test and analysis of variance (ANOVA) ($P \leq 0.05$). When the data from all five experiments evaluating macrophages activated by IFN-γ were evaluated together, a significant decrease in macrophage control of brucellae in the presence of IL-10 was detected by ANOVA ($P \leq 0.05$) but not by Student's paired *t* test.

tion in the number of bacteria was similar to those reported in other studies (3, 24).

IL-10 is known to affect production of Th1 cytokines, including IFN-γ, by acting on the antigen-presenting capacity of the macrophages (6). As a prelude to evaluating potential mechanisms by which IL-10 could be downregulating control of brucella infections, including downregulation of Th1 cytokine production, we determined whether or not endogenous IFN-γ is important in the control of infections with *B. abortus* 2308. Although the role of IFN-γ in the control of infections with strain 19 has been demonstrated (13), the virulent field strain 2308 is known to cause a considerably more chronic infection in BALB/c mice than strain 19 (8, 15), and the role of endogenous IFN-γ in its control had not been examined previously. In vivo neutralization of IFN-γ resulted in a significant increase in the number of bacteria recovered from spleens at 1 week after infection relative to that for control mice (Fig. 2D), suggesting that endogenous IFN-γ is important in the control of strain 2308.

We therefore next evaluated the effect that neutralization of endogenous IL-10 had on IFN-γ production by spleen cells from mice infected 1 week previously and stimulated with *B. abortus* 2308 in vitro. Neutralization of IL-10 resulted in significantly higher levels of production of IFN-γ by the spleen cells following stimulation with either heat-killed strain 2308 or the mitogen ConA employed as a control stimulus (Fig. 3). This suggests that IL-10 may be downregulating control of *B. abortus* 2308 by suppressing the production of IFN-γ at a time when IFN-γ is crucial to the control of brucellae in vivo. One of the other mechanisms by which IL-10 inhibits IFN-γ production by human lymphocytes is by suppressing the production of IL-12 or natural killer cell stimulatory factor (NKSF) by accessory cells (4). While there is no direct evidence indicating that IL-12 is produced during brucella infections, it is plausible that the above-described mechanism may be responsible in part for the effect reported here.

We have shown previously that *B. abortus* 2308 replicates in non-cytokine-activated macrophages but that IFN-γ enhances the antibrucellacidal capacity of macrophages (10). We evaluated in the present study the effect of IL-10 on the capacities of both non-cytokine-activated and IFN-γ-activated peptone-elicited peritoneal macrophages to control intracellular *B. abortus*. Preliminary experiments indicated that macrophages did not secrete detectable amounts of IL-10 at 24 or 48 h after infection with *B. abortus* 2308 in vitro, as measured by ELISA (data not shown). Addition of either 10 or 100 U of exogenous recombinant IL-10 per ml also had no significant effect on the ability of macrophages cultured in medium or activated with exogenous IFN-γ to control intracellular brucellae when the effects were assessed at 48 h after infection of macrophages (data not shown). However, addition of exogenous recombinant IL-10 at 1,000 U/ml to the cultures inhibited the ability of macrophages cultured in medium or activated with exogenous IFN-γ to control intracellular brucellae, in 4 of 5 and 3 of 5 experiments, respectively (Table 1). Although variability among experiments occurred for reasons that we are unable to explain, similar effects of IL-10 in vivo may contribute to downregulation of the control of *B. abortus* 2308 infections. Furthermore, while the effect of IL-10 on macrophage control of *B. abortus* was modest and required seemingly high concentrations, similar levels of effects of IL-10 on the ability of IFN-γ-activated macrophages to control *Trypanosoma cruzi* (20) and *Schistosoma mansoni* (7) have been previously documented. In those studies, >100 U of IL-10 per ml was also employed.

Svetic et al. have reported elsewhere that heat-killed organisms of the attenuated *B. abortus* strain 19 simultaneously induced production of IFN-γ and production of IL-10 in mice (23). Our results demonstrating that in vivo neutralization of IL-10 and in vivo neutralization of IFN-γ both affected control of the infection imply a similar concomitant production of IL-10 and IFN-γ following infection of mice with a live virulent strain of *B. abortus*, e.g., strain 2308. Moreover, the results indicate that endogenous IL-10 has a detrimental effect on the control of *B. abortus* 2308 infections in BALB/c mice even in the presence of the clearly beneficial IFN-γ. We have also provided in vitro data which suggest that IL-10 can inhibit the anti-brucella effector functions of macrophages as well as decrease the production by spleen cells of the protective cytokine IFN-γ in response to stimulation by brucella antigens. Both of these mechanisms may contribute to the decreased control of infection in vivo when IL-10 is present.

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