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# Depletion of Endogenous Interleukin-10 Augments Interleukin-1β Secretion by *Mycobacterium bovis* BCG-Reactive Human Cells

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**In this study, we found evidence that the interleukin-10 (IL-10) protein is functionally relevant in *Mycobacterium bovis* BCG-induced cytokine synthesis, as neutralization of endogenously synthesized IL-10 in human cells activated with BCG resulted in a two- to threefold increase in the level of IL-1β. When exogenous recombinant human IL-10 was added to human mononuclear cells, a significant reduction of BCG-induced IL-1β secretion was observed. This inhibitory effect was not attributed to a cytotoxic effect, since trypan blue exclusion studies indicated no loss of cell viability in the presence of IL-10, and it was specific, as it was completely abolished in the presence of anti-IL-10 neutralizing monoclonal antibody while an irrelevant antibody used as a control had no effect. Taken together, these are the first studies that demonstrate that the depletion of endogenous IL-10 via anti-IL-10 antibody results in a very significantly enhanced BCG-induced IL-1β secretion and that the addition of exogenous IL-10 to human mononuclear cells stimulated with BCG inhibits IL-1β production. Further experimental work is needed to determine if the neutralization of IL-10 activity via anti-IL-10 antibody indeed enhances cytokine synthesis in vivo. However, the present results may be of importance, since the use of anti-IL-10 antibody could presumably contribute to the protective immunity induced by BCG against tuberculosis via an increase in cytokine synthesis that would amplify antimicrobial systems.**

Tuberculosis remains a major health problem worldwide, due, at least in part, to the emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* and to the greatly increased susceptibility of human immunodeficiency virus-infected individuals to tuberculosis (17, 24, 27). Since 1921, vaccination with the bacillus Calmette-Guérin (*Mycobacterium bovis* BCG) has been the method for preventing tuberculosis (9). An analysis of recent reports in the literature on the efficacy of the BCG vaccine in the prevention of tuberculosis demonstrates that the BCG vaccine prevents 80% of severe forms of tuberculosis (11). However, the use of BCG in humans has led to questions regarding its efficacy across many populations, and it has been reported that although the BCG vaccine is capable of reducing childhood mortality, it has little impact on adult tuberculosis (6). Therefore, advances in new methods that mediate a resistant immune response to *M. tuberculosis* are essential for better control of tuberculosis.

Interleukin-10 (IL-10) is a 35-kDa homodimeric cytokine which is secreted primarily by Th2 cells and macrophages (23). This interleukin is a potent cytokine synthesis-inhibitory factor in lipopolysaccharide- or gamma interferon-activated cells (7, 21, 26). In addition, it has been shown that endogenous IL-10 production by mouse macrophages could block macrophage effector function against *Mycobacterium avium* and that intraperitoneal administration of anti-IL-10 reactive antibody is associated with enhanced resistance to infection with *M. avium* in mice (5). These findings show that IL-10 is a potent downregulator of the immune response implicated in mycobacterial infections.

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It is well known that infection with *M. tuberculosis* is characterized by symptoms, such as chest pain and fever, that are considered to be mediated by proinflammatory cytokines, such as IL-1β (14, 16, 31). It is also known that IL-1β may play an important role in the inflammatory response which effectively eliminates the bacilli (20). In the present study, we examined the effect of IL-10 on IL-1β production by human mononuclear cells activated with BCG.

Our results demonstrate that both endogenously produced IL-10 and exogenous recombinant human IL-10 (rhIL-10) downregulate the production of IL-1β by BCG-reactive human cells.

## MATERIALS AND METHODS

**Subjects.** Heparinized peripheral venous blood was obtained from 12 BCG-vaccinated healthy adult volunteers. None of the donors had any sign of mycobacterial infection at the time of or for at least 6 weeks before analysis.

**Reagents.** rhIL-10, anti-human IL-10 (anti-hIL-10) (goat immunoglobulin G [IgG] neutralizing human IL-10), and rhIL-2 were purchased from Sigma Chemical Co. (St. Louis, Mo.). BCG was kindly provided by J. Ruiz-Puente (Instituto Nacional de Higiene, México) and was used at a concentration of 5 μg/ml. A nonblocking antibody (human gamma globulin) was purchased from Sigma Chemical Co. and was used as a control.

**Cell cultures.** Human peripheral blood mononuclear cells were separated from the heparinized blood samples by density gradient centrifugation on Histopaque (Sigma Chemical Co.). Cells at the interface were washed three times in RPMI 1640 medium (Sigma Chemical Co.), counted, and resuspended at a concentration of $10^6$/ml in complete RPMI 1640 medium supplemented with 5% heat-inactivated (56°C for 30 min) human serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cell viability was determined by trypan blue exclusion. Cells were maintained in a humidified 5% $CO_2$ atmosphere at 37°C and were stimulated in the presence or absence of BCG (5 μg/ml), with or without anti-IL-10 antibody (100 ng/ml). In three different experiments, human mononuclear cells were stimulated with BCG in the presence of incremental doses of rhIL-10 (0.1 to 10 ng/ml) for 24 h (data not shown). rhIL-10 inhibited the production of IL-1β by BCG-stimulated human cells. Maximal inhibition was observed with 10 ng/ml. Higher doses of rhIL-10 (up to 10 ng) did not significantly increase the magnitude of inhibition. Therefore, human mononuclear cells from the 12 BCG-vaccinated donors were incubated

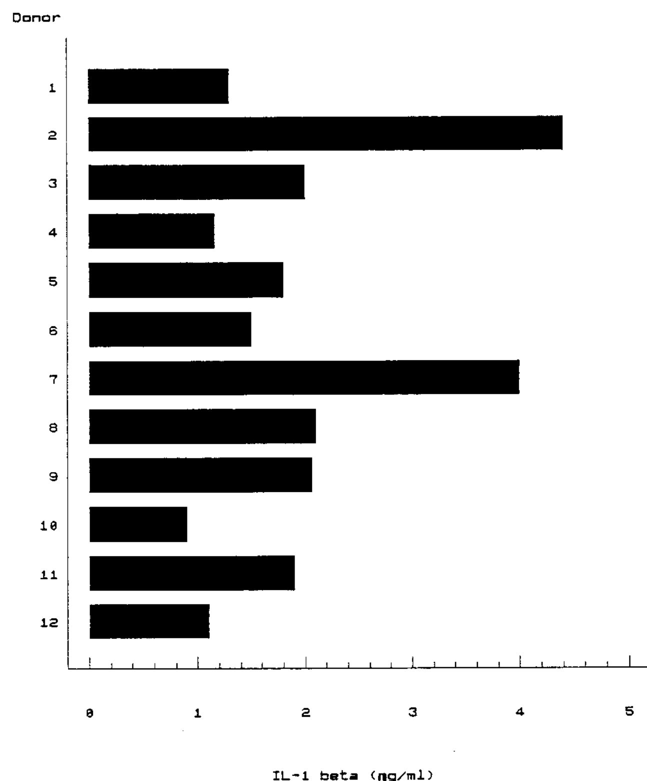


FIG. 1. IL-1β production by BCG-activated human cells. Mononuclear cells from 12 BCG-vaccinated healthy donors were incubated at $10^6$ cells/ml for 24 h with BCG (5 μg/ml). IL-1β concentrations in supernatants were determined by ELISA. The concentration of IL-1β in cultures containing media alone was 0.28 ± 0.057 ng/ml (mean ± SEM).

for 24 h with rhIL-10 (10 ng/ml) or a combination of rhIL-10 and anti-hIL-10 prior to BCG activation. Cell-free supernatants for cytokine determination were harvested, centrifuged, and saved for IL-1β measurement.

**Quantification of IL-1β in cell culture supernatants by enzyme-linked immunosorbent assay (ELISA).** For assay of IL-1β, a commercial immunoassay kit was purchased from Amersham International, Little Chalfont, United Kingdom. Cell-free supernatants were tested after dilution to appropriate concentrations. All data were expressed as nanograms per milliliter. The assay was sensitive to levels of IL-1β of ≥0.3 pg/ml.

**Statistical analysis.** All statistical analysis was done with the Statgraphics program, version 7.1 (Statistical Graphics Co., Rockville, Md.). The significance of the differences between the experimental groups was estimated by Student's $t$ test. Differences were considered significant if $P$ values were <0.01.

## RESULTS

**IL-10 neutralization enhances IL-1β secretion by human mononuclear cells activated with BCG.** In preliminary experiments, we determined the production of IL-1β by human mononuclear cells after stimulation with increasing concentrations of BCG (1, 5, 10, or 30 μg/ml). BCG-induced IL-1β production by cells from three BCG-vaccinated healthy donors was dose dependent, and the maximal production of IL-1β was reached with 5 μg of BCG per ml. When IL-1β secretion by BCG-stimulated mononuclear cells was measured for the 12 BCG-vaccinated healthy donors, BCG-induced IL-1β was detected in all cell culture supernatants (Fig. 1), and IL-1β levels were statistically different from those measured in cultures incubated without BCG ($P < 0.001$).

Since IL-10 is an immunosuppressive cytokine which inhibits the production of IL-1β by lipopolysaccharide-activated cells (7, 21, 26), we examined the ability of anti-hIL-10 antibodies to modulate the BCG-induced IL-1β production by human cells. Mononuclear cells were activated with BCG (5 μg/ml) and cultured for 24 h in the presence or absence of 100 ng of anti-IL-10 antibody per ml. As shown in Fig. 2, blocking IL-10 with the neutralizing goat monoclonal anti-hIL-10 antibody resulted in a significant increase in IL-1β levels for human mononuclear cells activated with BCG, from 1.3 ± 0.30 ng/ml (mean ± standard error of the mean [SEM]) to 3.3 ± 0.1 ng/ml ($P < 0.01$). Anti-IL-10 antibody itself did not induce cytokine secretion (data not shown). The IL-1β levels measured by specific ELISA increased to 234.9% ± 56.0% (mean ± SEM) of the control level (BCG alone). These results indicate that endogenously produced IL-10 suppressed BCG-induced IL-1β release by human cells.

**Addition of exogenous IL-10 to human mononuclear cells activated with BCG inhibits IL-1β secretion.** The finding that neutralization of endogenous IL-10 led to increased IL-1β release prompted us to investigate whether addition of exogenous IL-10 would have any effect on BCG-induced IL-1β production in human cells. Mononuclear cells were stimulated with BCG (5 μg/ml) and cultured for 24 h with or without rhIL-10. Figure 3 demonstrates that addition of 10 ng of rhIL-10 per ml resulted in significantly decreased BCG-induced IL-1β levels, from 1.9 ± 0.29 to 0.78 ± 0.10 ng/ml ($n$ = 12; $P < 0.01$ versus value for cultures without IL-10). At 24 h, IL-1β was reduced to 56.5% ± 5.2% of the control value. This inhibitory effect did not result from cytotoxicity, since cell viability determined by trypan blue exclusion was similar for all culture cells whether or not they were treated with IL-10 (data not shown). In addition, the specificity of the IL-10 inhibition was confirmed by the fact that preincubation of IL-10 with neutralizing goat anti-IL-10 monoclonal antibody blocked the inhibition. As shown in Fig. 4, incubation of human cells with a combination of rhIL-10 and anti-IL-10 monoclonal antibody prior to BCG stimulation resulted in an increase in IL-1β

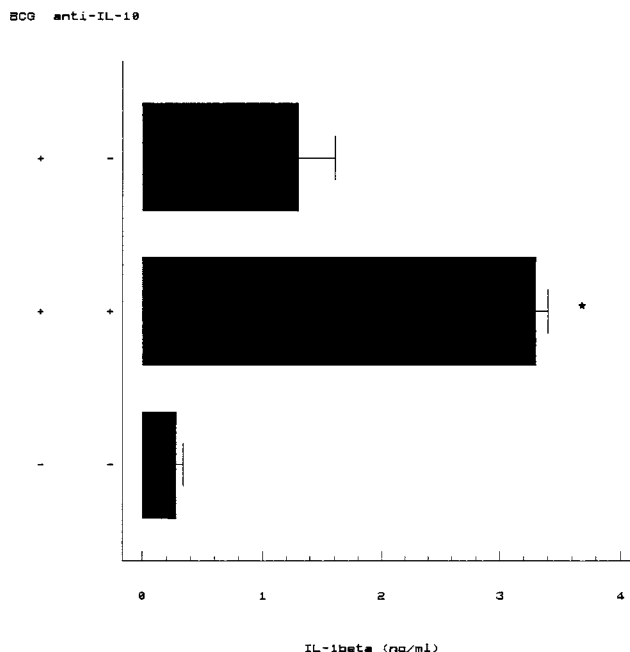


FIG. 2. Effect of anti-IL-10 antibody on production of IL-1β by BCG-stimulated human mononuclear cells. Human cells were incubated at $10^6$ cells/ml for 24 h with BCG (5 μg/ml) alone or with BCG and anti-IL-10 antibody (100 ng/ml). The cell-free supernatant fluid was then assayed for IL-1β content by ELISA. The results are the means ± SEMs for 12 donors. *, $P < 0.01$ compared to value obtained with BCG alone.

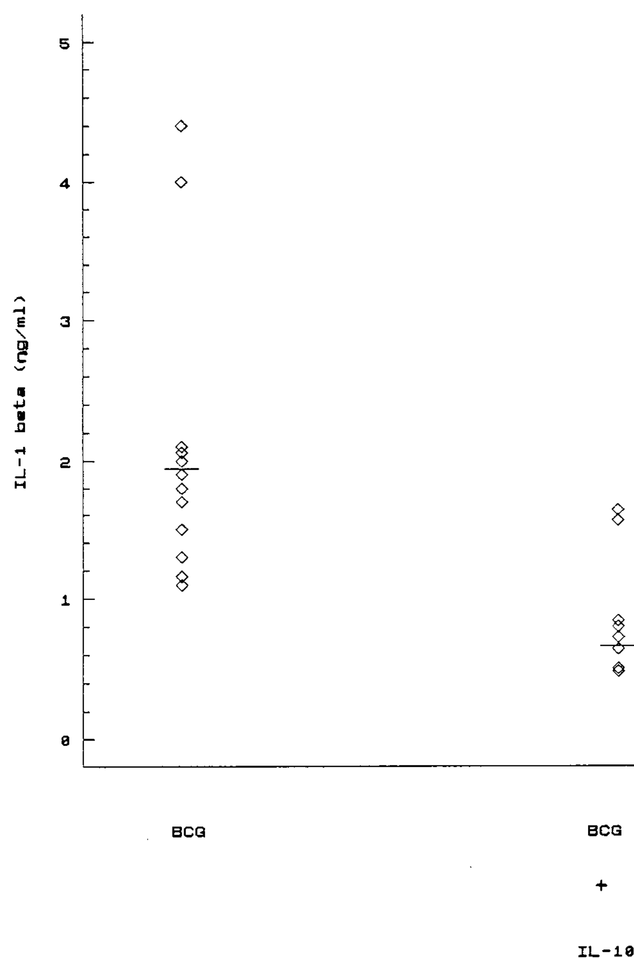


FIG. 3. Effect of IL-10 on production of IL-1β by BCG-stimulated human mononuclear cells. Cells from 12 BCG-vaccinated healthy donors were incubated for 24 h with BCG (5 μg/ml) alone or with BCG and rhIL-10 (10 ng/ml). IL-1β levels were measured by ELISA. Geometric mean values are represented by horizontal lines.

production, while the irrelevant control antibody (IgG) had no effect (1.9 ± 0.29 ng/ml in cultures with anti-IL-10 antibody versus 0.68 ± 0.59 ng/ml in cultures with control antibody [$P < 0.01$]). Finally, addition of IL-2 (10 μg/ml) failed to inhibit IL-1β production by human cells activated with BCG (data not shown), indicating that not any cellular derived interleukin can downregulate IL-1β production.

## DISCUSSION

In this study, we found that the neutralization of endogenously synthesized IL-10 resulted in increased secretion of IL-1β by human mononuclear cells activated with BCG, while the addition of rhIL-10 to these cell cultures markedly suppressed IL-1β secretion. The inhibitory activity of rhIL-10 on BCG-induced IL-1β secretion was indeed due to IL-10, since a neutralizing anti-IL-10 monoclonal antibody completely neutralized this effect. This finding is in agreement with the concept that IL-10 is a potent downregulator for human cells capable of producing cytokines. It is well known that high levels of IL-10 are generated by human cells activated with mycobacterial antigens, such as lipoarabinomannan (1, 2, 8). In this study, the finding that anti-IL-10 antibody upregulated the production of IL-1β by human mononuclear cells activated with BCG (indirect evidence that human mononuclear cells activated with BCG produced IL-10) supports this concept. Also, it is known that cells from tuberculosis patients constitutively secrete high levels of IL-10 (3). This enhanced production of IL-10 may be partly associated with the increased susceptibility of tuberculosis patients to mycobacterial infections and/or with a downregulation of the immune response in humans.

Recent studies have demonstrated that IL-1β levels (both protein and mRNA) are low in mononuclear cells from tuberculosis patients in response to purified protein derivative (19, 28). Since IL-1β is a cytokine that has an important role in the immune response which effectively eliminates *M. tuberculosis* (20), it is possible that as potent inducers of human cellular production of IL-1β, anti-IL-10 antibodies may enhance the protective immunity induced by BCG. The mechanism(s) by which the anti-IL-10 antibodies mediate upregulation of IL-1β may result from a direct effect on IL-10, an indirect effect via increased IL-2 production, and/or an increased antigen pre-

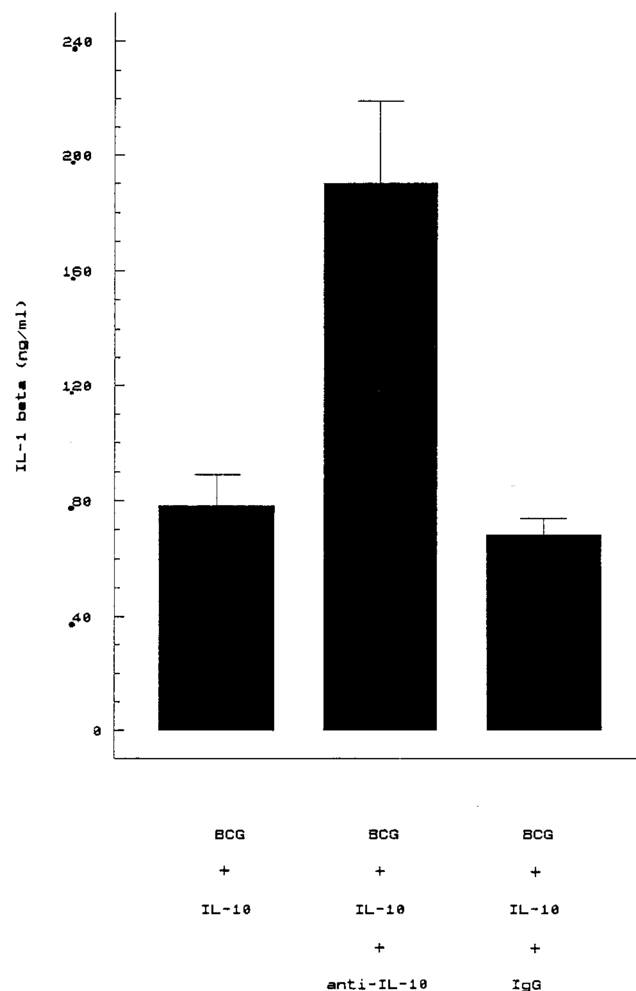


FIG. 4. Neutralizing anti-IL-10 monoclonal antibody significantly reverses the inhibitory effect of rhIL-10 on IL-1β secretion. Mononuclear cells were incubated at $10^6$ cells/ml for 24 h with BCG (5 μg/ml) together with rhIL-10 (10 ng/ml), with a combination of rhIL-10 and anti-IL-10 monoclonal antibody, or with a combination of rhIL-10 and an irrelevant antibody used as a control (human gamma globulin [IgG]; 1 μg/ml) prior to BCG stimulation. IL-1β levels were measured by ELISA. The results are the means ± SEMs for 12 donors.

sentation, since it is known that IL-10 reduces antigen-specific T-cell activation not only by blocking IL-2 production (4, 12) but also by modulating the expression of HLA class II molecules or of accessory and/or adhesion molecules on antigen-presenting cells (13, 15, 18, 32). Studies on the effect of anti-IL-10 antibody on both major histocompatibility complex class II expression (determined by examining expression on the monocytes by flow cytometry) and the production of IL-2 by BCG-stimulated mononuclear cells are currently being conducted to determine the mechanism(s) by which this antibody may enhance the immune response in BCG-activated human cells. It has previously been suggested that the reason for the failed immune response in tuberculosis resides in the interaction of macrophages with the tubercle bacillus and that in humans, a reduction of antigen-presenting ability by mycobacterium-infected macrophages is associated with a reduction in the immune response that controls the infection (6, 18). Recently, it has been demonstrated that monocytes treated with IL-10 do not present *M. tuberculosis* soluble extract to human T lymphocytes (29). Therefore, neutralization of IL-10 activity can result in control of infection and/or protection against reinfection by modulating antigen presentation.

It has been reported previously by workers in this laboratory (22) and others (30) that infection of human cells with mycobacterial antigens results in the induction of transforming growth factor β and IL-4, other immunosuppressive cytokines that also downregulate IL-1β production by human mononuclear cells. In fact, in vitro studies have shown that IL-10 synergizes with IL-4 and transforming growth factor β to inhibit macrophage antimicrobial activity (10, 25). In our study, we could not exclude the possibility that IL-10 synergizes with other cytokines to downregulate IL-1β production. The importance of this study is highlighted by the possibility that coadministration of BCG with multiple doses of anti-IL-10 antibody could presumably contribute to BCG-mediated protection from *M. tuberculosis* infection by enhancing not only the release of cytokines such as IL-1β but also the presentation of BCG.

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