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Interleukin-4 Inhibits Secretion of Interleukin-1β in the Response of Human Cells to Mycobacterial Heat Shock Proteins

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Cellular activation induced by *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) and heat shock proteins (HSP) leads to the production of proinflammatory cytokines such as interleukin-1β (IL-1β) and IL-6. In this study, we found that IL-4 significantly suppressed IL-1β secretion induced by BCG and the 70- and 65-kDa HSP. When exogenous recombinant human IL-4 was added to human mononuclear cells, a dose- and time-related inhibition of the 70-kDa HSP- and BCG-induced IL-1β secretion was observed. IL-1β secretion was maximally inhibited at 24 h of culture, and this inhibitory effect was sustained at a later time point of culture (120 h). In addition, IL-2, another T-cell-derived cytokine acting on monocytes, had no effect on IL-1β secretion induced by either BCG or the 70-kDa HSP, indicating that in these experiments not all cytokines could immunoregulate IL-1β secretion. This inhibitory effect was not due to a cytotoxic effect of IL-4, since the viabilities of human mononuclear cells were comparable in the presence and absence of IL-4. IL-4 was also able to inhibit the secretion of IL-1β by mycobacterium-stimulated cells from three rheumatoid arthritis patients. This inhibitory effect of IL-4 was reversed with a blocking anti-IL-4 antibody. Finally, IL-4 inhibited IL-6 secretion by mycobacterium-activated human cells. These results suggest that IL-4 may be important in the regulation of the immune response to mycobacterial antigens.

Protein and nonprotein products of mycobacteria are potent inducers of the production of proinflammatory cytokines by human cells (24, 25, 33). Heat shock proteins (HSP) are a family of conserved molecules that participate in the folding, unfolding, and translocation of other proteins within the cell (20). However, it is well known that the mycobacterial 70-, 71-, and 65-kDa HSP may play an important role in inflammatory responses by secreting proinflammatory cytokines (14, 18). In addition, it has been shown that T cells from patients with rheumatoid arthritis (RA) are reactive with the mycobacterial 65-kDa HSP (17, 27, 31). RA is an inflammatory disease in which elevated antibody response to the 65-kDa HSP has been observed (30). Furthermore, recent observations have demonstrated that interleukin-1β (IL-1β) acts as a potential mediator of the systemic inflammatory process seen in RA by inducing the expression of adhesion molecules on endothelial cells required for the recruitment of leukocytes to inflammatory sites (3, 28) and/or by stimulating the production of proteases and prostaglandins (6, 16). Thus, it is important to determine the means to control its production. Several investigators have found that the use of IL-1 receptor antagonist or anti-IL-1 antibodies blocks significant amounts of IL-1 (10, 19, 21, 29). Recent studies have demonstrated the immunosuppressor activity of IL-4 on IL-1β secretion by mitogen-stimulated human cells (8, 9, 11). Whether IL-4 has any effect on the mycobacterial induction of proinflammatory cytokines is unknown. In the present study, we examined the effect of IL-4 on IL-1β and IL-6 secretion by human mononuclear cells activated with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) and the 70- and 65-kDa HSP. Our results demonstrated that exogenous recombinant human IL-4 down-regulated the secretion of IL-1β and IL-6 by mycobacterium-activated cells from healthy and RA patients. These findings suggest a possible immunoregulatory role for IL-4 in mycobacterial infection and support the notion of a link between the immune response to mycobacterial infection and autoimmunity.

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MATERIALS AND METHODS

Study subjects. Whole heparinized blood was obtained from 11 BCG-vaccinated healthy volunteers and 3 patients with RA. The diagnosis of patients with RA was done by rheumatologists from the Department of Rheumatology, Instituto Nacional de Cardiología, Ignacio Chavez, México City, Mexico.

Cytokines and reagents. *M. bovis* BCG was donated by J. Ruiz Puente (Instituto Nacional de Higiene, México). The recombinant 71-kDa HSP of *Mycobacterium tuberculosis* and the recombinant 65-kDa HSP of *M. bovis* were a gift from M. Singh (Gene Expression, G.B.F., Braunschweig, Germany) through the United Nations Development Program/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases. The following reagents were purchased from commercial sources: recombinant human IL-2 (Sigma Chemical Co.), recombinant human IL-4 (Amersham International Ltd.,

TABLE 1. IL-1β secretion by human cells in response to mycobacterial antigens

Mycobacterial stimulus	Concn (μg/ml)	Mean concn of IL-1β ± SEM (pg/ml)
Medium		171.8 ± 44.2
BCG	1	610.0 ± 52.4
	5	1,774.5 ± 121.5
	10	848.2 ± 147.0
	30	701.1 ± 98.0
70-kDa HSP	1	789.0 ± 88.0
	5	1,229.0 ± 107.8
	10	925.5 ± 131.0
65-kDa HSP	5	1,106.6 ± 173.3

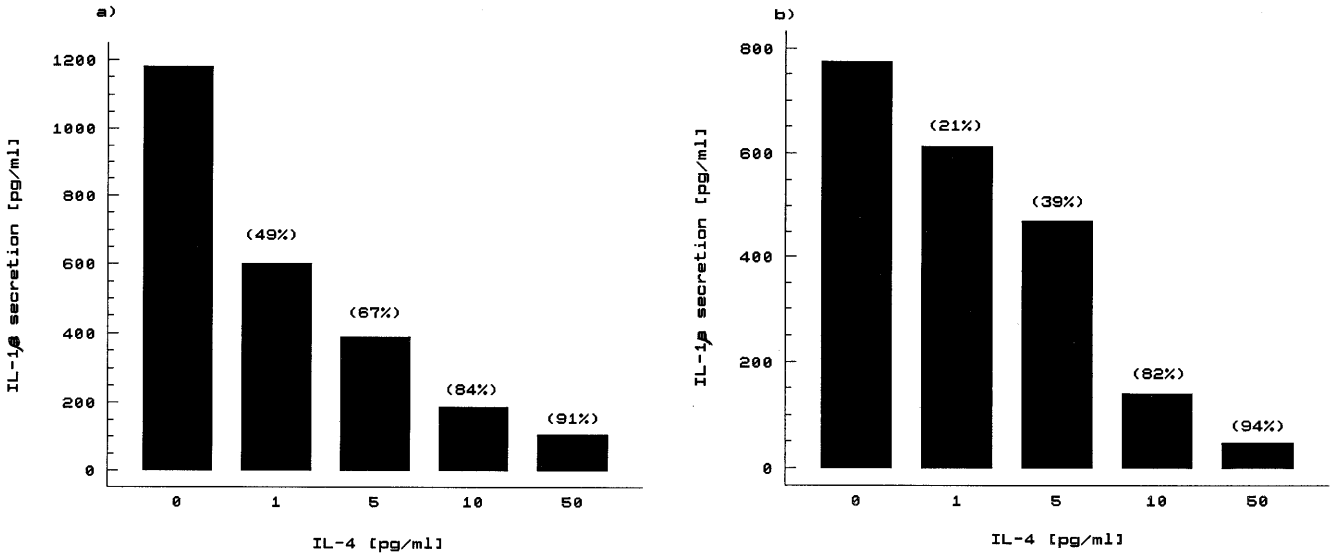


FIG. 1. Effect of treatment with IL-4 on mycobacterium-induced IL-1β secretion. Mononuclear cells (10^6/ml) were incubated in 24-well culture plates with 5 μg of BCG (a) or the 70-kDa HSP (b) per ml in the presence of four different concentrations of IL-4 or with medium alone for 24 h. IL-1β secretion in mycobacterium-activated mononuclear cells was measured by ELISA. Percentages of inhibition relative to those for cultures without IL-4 are indicated. Data are expressed as the means of cultures done in triplicate. Results shown are for PBMC from one of two healthy donors.

Aylesbury, United Kingdom), neutralizing anti-IL-4 monoclonal antibody (Boehringer GmbH, Mannheim, Germany), isotype control immunoglobulin G (Sigma Chemical Co.), and polymyxin B sulfate (Sigma Chemical Co.).

Cell culture and generation of cytokines by human cells activated by mycobacterial antigens. Blood mononuclear cells from healthy donors and patients with RA were obtained by density sedimentation over Histopaque (Sigma Chemical Co.). After being washed, mononuclear cells were suspended at 10^6 cells in 1.0 ml of complete medium (RPMI 1640 medium [Sigma Chemical Co.]) with 2 mM L-glutamine–penicillin (100 U/ml)–streptomycin (100 μg/ml) and 5% heat-inactivated pooled human serum. Cell cultures were stimulated with a dose range of each mycobacterial antigen (BCG and the 70- and 65-kDa HSP) and incubated in 24-well culture plates at 37°C in 5% CO_2–95% air. As a negative control, cells were incubated without mycobacterial antigens. Culture supernatants were harvested 24, 48, and 120 h after stimulation. The cells were then removed by centrifugation, and the supernatants were immediately stored at −20°C until the time of assay. Since polymyxin B is able to inhibit endotoxin-induced cytokine secretion (23), parallel cell cultures were incubated in the presence of polymyxin B (10 μg/ml). Polymyxin B treatment failed to inhibit mycobacterium-induced IL-1β secretion. These data indicate that BCG-induced IL-1β secretion cannot be attributed to the presence of bacterium-contaminating endotoxin. In some experiments, mononuclear cells (10^6/ml) from different donors were incubated with BCG (5 μg/ml) and the 70- or 65-kDa HSP (5 μg/ml) for 1 day in the absence or presence of four different concentrations of IL-4 (1, 5, 10, and 50 pg/ml) or in the absence or presence of IL-2 (50 U/ml). In addition, the effect of IL-4 was reversed with a blocking anti-IL-4 monoclonal antibody (2 ng/ml). Anti-IL-4 alone did not appear to have a significant effect on IL-1β secretion. Cell viability was assessed by trypan blue exclusion.

Quantification of IL-1β and IL-6 in cell culture supernatants by ELISA. Immunologically reactive IL-1β and IL-6 were measured in cell supernatants by commercially available enzyme-linked immunosorbent assay (ELISA) kits (Amersham) according to the method recommended by the manufacturer. Cell supernatants were serially diluted in complete RPMI 1640 medium. One hundred microliters of each dilution was placed in microtiter wells in triplicate. Color intensity was measured as absorbance (optical density) at 450 nm with an automated ELISA plate reader (Labsystems Multiskan MS). The lower limits of detection of these ELISAs were 0.3 and 0.7 pg/ml, respectively, for IL-1β and IL-6.

Statistical analysis. Data were analyzed by Student's *t* test. *P* values of <0.01 were taken as an indication of statistically significant differences between experiments.

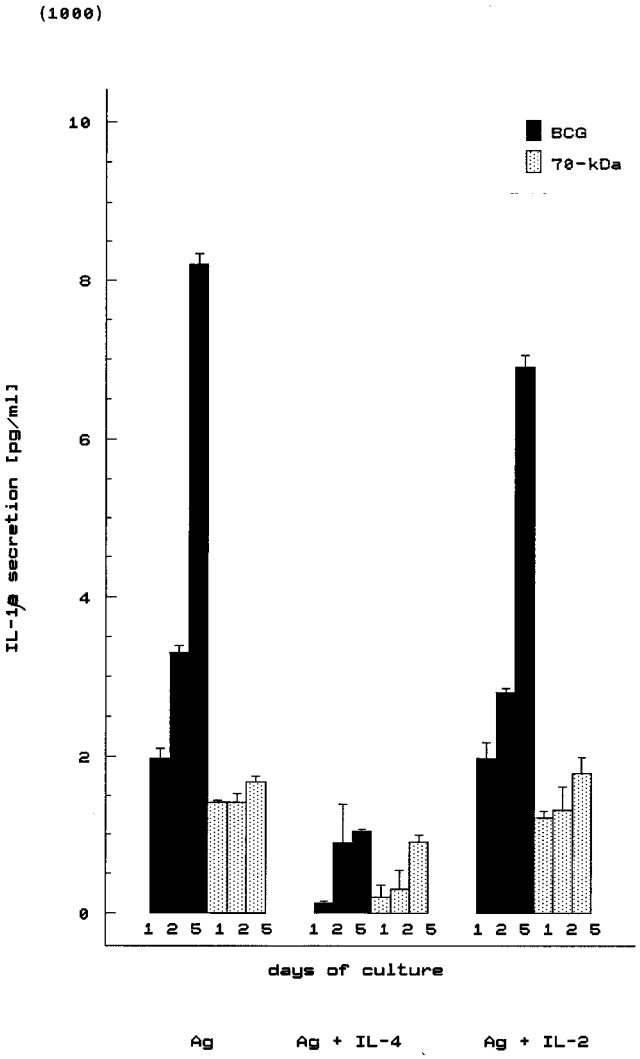
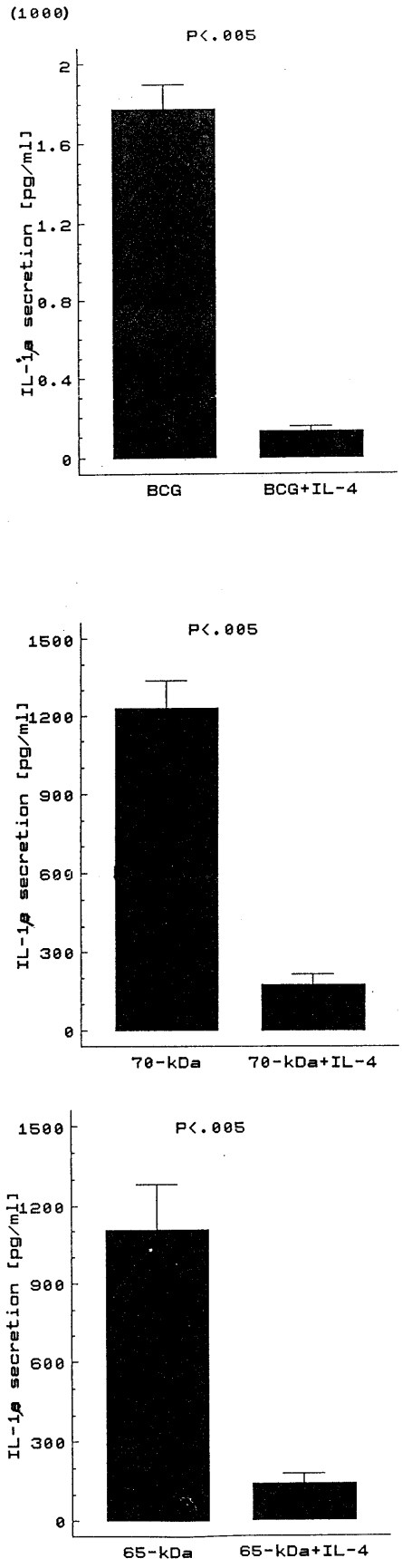


FIG. 2. The inhibitory effect of IL-4 was time dependent. Mononuclear cells (10^6/ml) were stimulated with BCG or the 70-kDa HSP (5 μg/ml) in the absence (Ag) or presence of IL-4 (10 pg/ml) (Ag + IL-4) or IL-2 (50 U/ml) (Ag + IL-2). Supernatants were collected on various days and tested for IL-1β secretion by ELISA. Data represent the means ± SEMs for PBMC from three healthy donors.



RESULTS

Induction of IL-1β by mycobacterium-stimulated mononuclear cells from healthy donors. In preliminary studies, the induction of IL-1β in human cell cultures by the different mycobacterial antigens was examined. Mononuclear cells from 11 healthy donors were stimulated for 24 h with a range of concentrations (1 to 30 μg/ml) of BCG and the 70-kDa HSP or with medium alone, and cell-free supernatants were collected. Although BCG and the 70-kDa HSP induced IL-1β at all concentrations tested, induction was maximal at 5 μg/ml (Table 1). Thus, mononuclear cells from three donors were stimulated for 24 h with the 65-kDa HSP. Compared with unstimulated cultures, the 65-kDa HSP at a concentration of 5 μg/ml induced 6.4-fold higher concentrations of IL-1β for the three donors tested ($P < 0.005$) (Table 1). It is important that the levels of IL-1β induction by BCG and by the 70- and 65-kDa HSP were comparable at 24 h.

Effect of exogenous IL-4 on IL-1β and IL-6 secretion by mycobacterium-stimulated human mononuclear cells. Since IL-4 is a potent immunosuppressive molecule which has been found to inhibit mitogen-induced IL-1β secretion (8, 9, 11), the effect of exogenous IL-4 on IL-1β secretion by human cells stimulated with mycobacterial antigens was studied next. In two donors, BCG and the mycobacterial HSP of *M. tuberculosis* (70 kDa) were used at a concentration of 5 μg/ml to induce IL-1β in the presence of several doses of IL-4 (1, 5, 10, and 50 pg/ml). As shown in Fig. 1, IL-4 inhibited the secretion of IL-1β in a dose-dependent manner. A marked inhibition of IL-1β secretion was seen after treatment of cells with 10 pg of IL-4 per ml (84% inhibition by BCG and 82% inhibition by the 70-kDa HSP) (Fig. 1). This inhibitory effect was significant ($P < 0.001$). It is important that no significant difference was observed when cell cultures were treated with 50 pg of IL-4 per ml. To extend these findings, in three donors the levels of 70-kDa HSP- and BCG-induced IL-1β were both assessed at 24, 48, and 120 h. There was an almost-fourfold increase in the level of BCG-induced IL-1β in cell cultures at 120 h compared with that at 24 h ($P < 0.001$) (Fig. 2). In contrast, the concentration of IL-1β (mean ± standard error of the mean [SEM], 1,095.0 ± 131.4 pg/ml) present in the 70-kDa HSP-activated cell cultures was significant on day 1 and remained high on day 5 (Fig. 2). On the other hand, the maximal down-regulation of mycobacterium-induced IL-1β secretion was reached at 24 h (Fig. 2). In these experiments, all mycobacterial stimuli were used at a concentration of 5 μg/ml, and IL-4 was used at a concentration of 10 pg/ml. The addition of IL-2 to mycobacterium-stimulated mononuclear cells had no significant effect on IL-1β secretion, with *P* values comparable to those for the mycobacterial stimulus alone (Fig. 2). IL-4 was shown to block IL-1β secretion, and this result was extended by 11 experiments in which IL-4-treated mycobacterium-activated mononuclear cells were shown to inhibit IL-1β secretion not only by 70-kDa HSP- and BCG-activated cells but also by 65-kDa HSP-activated cells (Fig. 3). In addition, at the concentration of IL-4 tested, the inhibitory effect of this cytokine did not result from a cytotoxic effect, because the viability of cell cultures was not impaired, as judged by trypan blue exclusion.

FIG. 3. IL-4 inhibits the secretion of IL-1β induced by BCG and two mycobacterial HSP. Mononuclear cells (10^6/ml) were stimulated for 24 h with BCG and the 70- and 65-kDa HSP (5 μg/ml) alone or in the presence of IL-4 (10 pg/ml). IL-1β secretion was measured by ELISA. Data represent the means ± SEMs for PBMC from 11 healthy donors. *P* values were determined by comparisons with cultures stimulated by mycobacterial antigens.

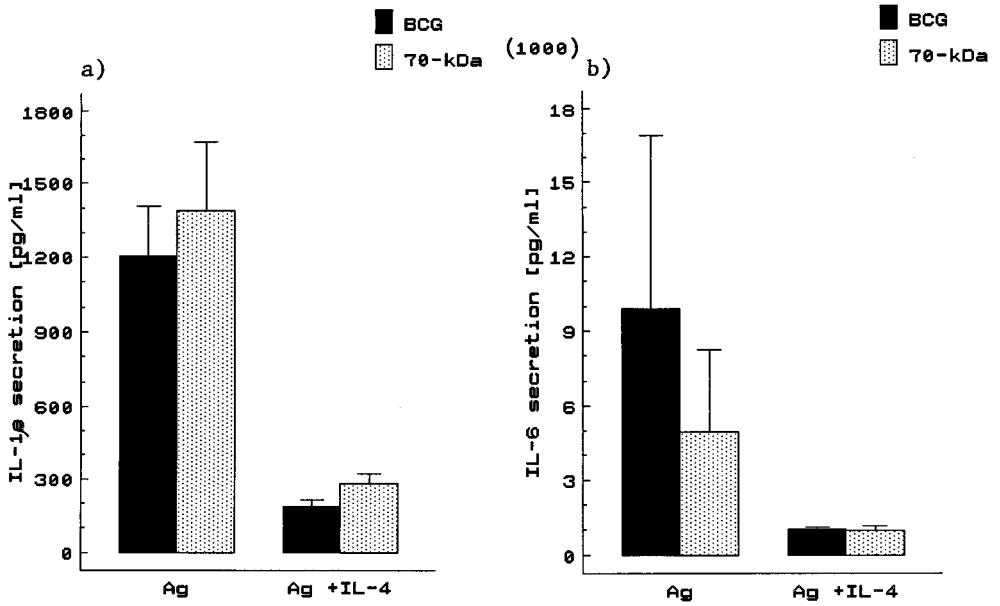


FIG. 4. IL-4 inhibits IL-1β and IL-6 secretion by mononuclear cells from patients with RA. A total of 10^6 mononuclear cells from three different RA patients were cultured for 24 h with BCG or the 70-kDa HSP (5 μg/ml) in the absence (Ag) or presence of IL-4 (Ag + IL-4). IL-1β (a) and IL-6 (b) levels were measured in cell-free supernatants by ELISA.

On the other hand, an association between RA and immune responses to mycobacterial proteins has been reported (17, 27, 31), and elevated levels of IL-1β have been found in response to mycobacterial antigens by testing human cells from the synovial infiltrates of RA patients (14, 18). Therefore, it was of interest to determine the effect of IL-4 on proinflammatory cytokine secretion by mononuclear cells from patients with RA. As shown in Fig. 4a, treatment of mycobacterium-activated cells with IL-4 significantly blocked the secretion of IL-1β by cells from RA patients. To evaluate the specificity of this effect, a neutralizing monoclonal antibody to IL-4 and an isotype control antibody were added to cell cultures that had been stimulated with BCG. Coculture with neutralizing antibody to IL-4 resulted in an 8.1-fold reduction of the effect of IL-4 (Table 2). In contrast, isotype control antibody had no significant effect on the inhibitory activity of IL-4 (Table 2). The dose of anti-IL-4 monoclonal antibody was determined in preliminary experiments (data not shown). Previous studies have demonstrated that IL-1β and IL-6 are likely to play a central role in inflammatory responses (13). Furthermore, IL-6 may also have direct antimycobacterial activity. Therefore, it was of interest to determine whether IL-4 would also modulate mycobacterium-induced IL-6 secretion. As seen in Fig. 4b, IL-4 suppressed the secretion of IL-6 by mononuclear cells activated by both BCG and the 70-kDa HSP.

DISCUSSION

Clinical studies have implicated the activities of IL-1β and IL-6 in the pathology of both mycobacterial infection and RA (12, 22). Recently, it has been demonstrated that proinflammatory cytokine secretion by human cells could be down-regulated by immunosuppressive cytokines such as IL-4 or IL-10 (7, 15, 26). We focused our attention on IL-4, since the response to this cytokine is more persistent than the response to IL-10 (32). In this study, the effect of IL-4 was extended to the inhibition of IL-1β and IL-6 secretion by the HSP- and BCG-stimulated mononuclear cells from healthy donors and patients with RA. These findings support the notion of a link between mycobacterial antigens and RA.

Since IL-4 inhibited IL-1β secretion induced by both whole mycobacteria and two HSP, this effect was not restricted to a particular group of antigens. These data correlate well with observations that IL-4 induced suppression of the lipopolysaccharide-, *Staphylococcus aureus*-, and phorbol myristate acetate-induced IL-1β secretion by human cells (8). On the other hand, in the studies of Donnelly et al. (9), the effect of IL-4 on lipopolysaccharide-treated cells was due to the occupancy of a few IL-4 receptors, suggesting that IL-4 was active at low doses. In our study, IL-4 was active at low concentrations, with maximal inhibition obtained at 50 pg/ml.

The data reported for IL-4 regulation of proinflammatory cytokine secretion by human cells have varied in different studies even though similar doses of IL-4 were used. A possible explanation for this discrepancy is cell isolation technique. Essner et al. (11) demonstrated a significant inhibition of IL-1β secretion when they exposed human monocytes to IL-4 for at least 48 h. Essner et al. obtained cells under adherent conditions, whereas in the present study we used peripheral blood mononuclear cells (PBMC). Our data indicated an inhibitory effect of IL-4 on IL-1β secretion when human cells were exposed to IL-4 for 24 h. Because adherence status has been shown to activate cells (5), this may be the reason the cells in the two studies reached different differentiation.

TABLE 2. Blocking of IL-4 by specific anti-IL-4 antibodies[a]

Treatment of BCG	IL-1β (pg/ml)	*P* value[b]
IL-4 + anti-IL-4	2,900	
IL-4	358	0.005
IL-4 + immunoglobulin G	314	0.005

[a] Results are representative of PBMC from two different donors.
[b] *P* values were determined in comparison to IL-1β secretion in cell cultures stimulated with a mixture of BCG, IL-4, and anti-IL-4 antibody.

Since chronic inflammatory disease has been demonstrated to involve persistent and active proinflammatory cytokine secretion, it was important to elucidate whether the effect of IL-4 on mycobacterium-treated cells was persistent. Our kinetic studies showed that IL-1β progressively accumulated in the cell culture supernatants of mycobacterium-activated human cells. Interestingly, inhibition was clearly seen after a 24-h incubation period and was long lasting. After 5 days, the secretion of IL-1β was blocked approximately 80% by BCG and approximately 40% by the 70-kDa HSP. It should be noted that in the present study, IL-2, another T-cell cytokine, failed to block mycobacterium-induced IL-1β secretion. This finding indicates that the IL-4-induced inhibition of IL-1β secretion was not generalizable to any given interleukin but was specific for IL-4, since an inhibitory anti-IL-4 antibody could prevent the IL-4-induced inhibition of IL-1β secretion.

Because immunosuppressive cytokines often act synergistically in many biological assays, the additive effects of IL-4 and IL-10 in our studies may have been induced. Studies on the cumulative effects of IL-4 and IL-10 are currently being conducted.

It has been recently demonstrated that IL-1β secretion by human cells could be down-regulated with an anti-tumor necrosis factor alpha antibody (4) or an IL-1 receptor antagonist (1, 2). However, considering cytokine regulation, IL-4 is able not only to affect a cascade of proinflammatory cytokines but also to increase amounts of IL-1 receptor antagonist and to inhibit cytokine-induced bone resorption in RA (34).

Considering the inflammatory nature of IL-6 and the pivotal roles of IL-1β and IL-6 in the induction of inflammatory responses, including RA, it was important to elucidate whether IL-4 could also modulate the secretion of IL-6. In our studies, an inhibitory effect of IL-4 on both IL-1β and IL-6 was maintained for at least 24 h. Although we still have to determine whether IL-4 inhibits the secretion of proinflammatory cytokines in vivo, the biological effect on mycobacterium-stimulated human cells of the regulation of IL-1β and IL-6 secretion by IL-4 may be important in the regulation of chronic inflammatory responses.

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