Human Brucellosis Is Characterized by an Intense Th1 Profile Associated with a Defective Monocyte Function

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In animal models, a defective Th1 response appears to be critical in the pathogenesis of brucellosis, but the Th1 response in human brucellosis patients remains partially undefined. Peripheral blood from 24 brucellosis patients was studied before and 45 days after antibiotic therapy. Twenty-four sex- and age-matched healthy donors were analyzed in parallel. Significantly increased levels of interleukin 1β (IL-1β), IL-2, IL-4, IL-6, IL-12p40, gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α), but not of IL-10, in serum and/or significantly increased percentages of samples with detectable levels of these cytokines, measured by enzyme-linked immunosorbent assays (ELISA), were found for untreated brucellosis patients, but these levels were reduced and/or normalized after treatment. Flow cytometry studies showed that the intracytoplasmic expression of IFN-γ, IL-2, and TNF-α, but not that of IL-4, by phorbol myristate-activated CD4+ CD3+ and CD8+ CD3+ T lymphocytes was significantly increased in untreated brucellosis patients and was also partially normalized after antiinfection. The percentage of phagocytic cells, the mean phagocytic activity per cell, and the phagocytic indices for monocytes at baseline were defective and had only partially reverted at follow-up. T lymphocytes from untreated brucellosis patients are activated in vivo and show Th1 cytokine production polarization, with strikingly high serum IFN-γ levels. In spite of this Th1 environment, we found deficient effector phagocytic activity in peripheral blood monocytes.

Brucellosis is a zoonotic disease of worldwide distribution. Despite its control in many countries, it remains endemic in the Mediterranean and Middle Eastern regions (20, 28, 41, 42). Brucella melitensis is the most frequent cause of human brucellosis in these geographical areas (19). In Spain, it has been reported that the majority (more than 97.5%) of isolates were identified as Brucella melitensis (13, 44, 45).

Brucella organisms are facultatively intracellular Gram-negative coccobacilli that reside and replicate in a vacuolar compartment within myelomonocytic cells of the infected host (14, 15, 47). The response to Brucella involves the whole gamut of the immune system, from innate to adaptive immunity (21). In murine models, passive transfer of immune cells resulted in an effective anti-Brucella defensive response mediated by CD4+ and CD8+ T lymphocytes (5, 6, 32, 37, 51, 52). Furthermore, the pattern of T-lymphocyte cytokine secretion is considered to be critical for the effectiveness of the protective anti-Brucella immune response (3, 7). It has been postulated that Th1 cytokines confer resistance, while Th2 cytokines facilitate the development of brucellosis (2, 3, 24, 25, 40, 43, 52). In animal models, gamma interferon (IFN-γ) induces macrophage activation and control of Brucella infection (16, 18, 43). In Brucella-infected mice, administration of recombinant IFN-γ enhances host resistance, resulting in a deep decrease in the number of viable bacteria (51). Moreover, host IFN-γ depletion results in an increase in the number of viable bacteria (17, 37, 52). Several abnormalities in the immune system have been found in human brucellosis (27, 46, 49). It has been found that T and NK lymphocytes show defective functions in brucellosis patients (46, 49). Since mice are naturally resistant to Brucella infections, it is possible to suggest that the immune response elicited by Brucella in humans might have different characteristics. Thus, susceptibility to, or protection from, human brucellosis conferred by T-lymphocyte cytokines has not been established.

In this work, we have further investigated the pattern of T-lymphocyte and monocyte responses to human Brucella infection. We have prospectively studied (i) the levels of Th1, Th2, and regulatory cytokines in serum, (ii) the distribution, activation stage, and pattern of Th1/Th2 cytokine production by T lymphocytes, and (iii) the phagocytic activity of monocytes in a group of brucellosis patients before and after antimicrobial treatment.

MATERIALS AND METHODS

Patients. We studied a group of 24 untreated brucellosis patients at diagnosis and 3 months after the start of antibiotic treatment (gentamicin at 240 mg/day for 21 days and doxycycline at 100 mg every 12 h for 45 days). Diagnosis of brucellosis was established by the isolation of Brucella spp. from blood cultures and/or by serologic tests according to current criteria (41). The exclusion criteria were antibiotic treatment during the 3 months prior to the study; evidence of autoimmune disease or cancer; heart, renal, and/or respiratory insufficiency; and endocarditis and/or neurobrucellosis. To avoid a Th2 bias in the immunologic studies, patients meeting the clinical criteria for atopy were excluded. Also...
excluded were patients who had taken immunosuppressants, immunomodulators, or other drugs considered to modify the immune response within the 3 months prior to the study. Breast-feeding women and subjects under 18 years of age were also excluded. In parallel, we also studied 24 sex- and age-matched healthy volunteers from similar epidemiologic surroundings. The study was approved by the Ethical Committee of Alcalá University and was conducted according to the guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from each patient included in the study. The blood samples were drawn from the patients before the start of antibiotic treatment and 3 months after the treatment was finished. Each patient was studied in parallel with a healthy control. At the end of the antibiotic treatment, all patients were considered to be healthy, without clinical, serological, or bacteriological evidence of relapse.

Study of cytokine levels in serum by ELISA. Concentrations of cytokines were assayed in serum samples using commercially available enzyme-linked immunoasorbent assay (ELISA) kits. Interleukin-2 (IL-2), IL-4, IL-10, IL-12p40, and IFN-γ test kits were purchased from Diaclone (Besançon, France), and IL-1β, IL-18, IL-6, and tumor necrosis factor alpha (TNF-α) were purchased from R&D Systems Inc. (Minneapolis, MN). The results are expressed as picograms per milliliter. The minimum detectable doses of the IL-1β, IL-2, IL-4, IL-10, IL-12p40, IFN-γ, and TNF-α test kits are 0.05, 7.00, 0.11, 0.03, 0.50, 5.00, 0.10, and 0.10 pg/ml, respectively.

Cell separation. Peripheral blood mononuclear cells (PBMC) were obtained as described previously (12). Purified PBMC were resuspended in RPMI 1640 (BioWhittaker Products, Verviers, Belgium) supplemented with 25 mM HEPES (BioWhittaker), 1% penicillin-streptomycin (BioWhittaker), and 10% heat-inactivated autologous serum. Initial cell enumeration was performed by conventional light microscopy using a Neubauer chamber. The viability of fresh PBMC was always greater than 95%.

Flow cytometry studies. Surface and intracellular antigen expression was quantified as described elsewhere (4). Briefly, fresh PBMC were incubated with combinations of fluorescein isothiocyanate (FITC), phycoerythrin (PE), and peridinin chlorophyll protein (PerCP)-labeled monoclonal antibodies (MAb). PerCP-labeled anti-CD3 and FITC-labeled anti-CD4, anti-CD45RA, anti-CD69, and anti-HLA-DR were purchased from Becton Dickinson (San Jose, CA), and PE-labeled anti-CD8, FITC-labeled anti-CD11a, FITC-labeled anti-CD11b, PE-labeled anti-CD25, and PE-labeled anti-CD45RO were purchased from Caltag Laboratories (Burlingame, CA).

To study cytokine production, PBMC were stimulated with 50 ng/ml phorbol-12-myristate-13-acetate (PMA; Sigma Chemical Co., St. Louis, MO) plus 1 μg/ml ionomycin (Calbiochem-Novabiochem, La Jolla, CA) in the presence of 2 μM monensin for 6 h at 37°C. To detect spontaneous and stimulated cytokine production after CD3 and CD8 surface staining, PBMC were fixed and permeabilized with the Fix & Perm Kit (Caltag Laboratories), and the cytokines were detected with FITC-labeled anti-IL-2 (Becton Dickinson), PE-labeled anti-IL-4 (R&D Systems), FITC-labeled anti-IFN-γ (Caltag Laboratories), and PE-labeled anti-TNF-α (R&D Systems).

We also studied the phagocytic activity of monocytes as previously described (30). Each assay was performed in duplicate with 100 μl of cold heparinized whole blood using Phagotest according to the manufacturer's indications (Orion Diagnostica, Finland). The percentages of phagocytes were evaluated by the nonparametric Mann-Whitney U test or Fisher’s exact test, and the longitudinal studies of patient groups were evaluated by a Wilcoxon matched-pair signed-rank test using the Statistical Package for Social Sciences (SPSS) software, version 11.0 (SPSS, Chicago, IL). Statistical significance was set at a P value of <0.05.

RESULTS

Demographic characteristics. The demographic, clinical, and diagnostic characteristics of the patients are listed in Table 1. Twenty-four healthy donors (18 male and 6 female) with a mean age of 35 years (range, 24 to 60 years) were studied in parallel.

Human brucellosis is associated with a systemic Th1 proinflammatory cytokine environment. First, we analyzed the serum cytokine levels in untreated and treated brucellosis patients and in age- and sex-matched healthy controls. Table 2 shows the percentages of serum samples with detectable levels and the mean levels of the cytokines detected in brucellosis patients and healthy controls. For untreated brucellosis patients, we found significantly higher levels of the proinflammatory cytokines IL-1β, IL-2, IL-6, IL-12p40, IFN-γ, and TNF-α in serum and/or significantly higher percentages of samples with detectable levels than those for healthy controls. Especially striking is the increased presence of high levels of IFN-γ in untreated patients. After treatment, significant reductions and normalization of the percentages of patients with detectable levels of IFN-γ and IL-2 in serum were found. Furthermore, the levels of IL-1β, IL-6, IL-12p40, and TNF-α in serum were significantly decreased and normalized after treatment. In contrast, serum IL-10 levels were significantly lower in untreated and treated brucellosis patients than in healthy controls. The percentage of individuals with detectable IL-4 levels in serum was significantly higher for untreated brucellosis pa-
Serum cytokine concentrations for 24 brucellosis patients were measured by ELISA. Twenty-four healthy donors were studied in parallel. The value is statistically different from that for healthy controls. NS, no significant difference.

**TABLE 2.** Serum cytokine levels in untreated and treated brucellosis patients and in age- and sex-matched healthy controls

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Healthy controls (n = 24)</th>
<th>Brucellosis patients (n = 24)</th>
<th>Posttreatment (n = 24)</th>
<th>Pretreatment (n = 24)</th>
<th>Mean concn (range)</th>
<th>P value (pretreatment vs. posttreatment) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>0.18 (0.01–0.32)</td>
<td>0.24 (0.06–0.72)</td>
<td>0.24 (0.08–0.72)</td>
<td>&lt;0.05</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>1.39 (1.00–2.00)</td>
<td>1.36 (0.45–4.56)</td>
<td>1.24 (0.81–2.25)</td>
<td>&lt;0.05</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>IL-12p40</td>
<td>0.00 (0.00–0.00)</td>
<td>0.00 (0.00–0.00)</td>
<td>0.00 (0.00–0.00)</td>
<td>&lt;0.05</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>3.12 (1.25–5.83)</td>
<td>2.00 (BDL–4.25)</td>
<td>1.38 (1.25–4.25)</td>
<td>&lt;0.05</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>12.00 (5.00–25.00)</td>
<td>1.20 (1.00–2.20)</td>
<td>1.80 (1.00–2.50)</td>
<td>&lt;0.05</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>0.00 (0.00–0.00)</td>
<td>0.00 (0.00–0.00)</td>
<td>0.00 (0.00–0.00)</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

Monocytes from untreated brucellosis patients show defective phagocytic function in a proinflammatory environment. Next, the phagocytic activity of monocytes from brucellosis patients before and after treatment was investigated. Figure 2 shows the percentage of phagocytic cells (Fig. 2A), the mean phagocytic activity per cell (Fig. 2B), and the phagocytic indices (Fig. 2C) for monocytes as measured for brucellosis patients and healthy controls. The monocytes of patients with untreated brucellosis had significantly depressed phagocytic activities relative to the levels observed in healthy controls (Fig. 2). The values for these three parameters of phagocytic activity of monocytes increased significantly after treatment but did not reach the values found in healthy donors. In the parallel study performed, we found no significant differences between the two study times in the phagocytic activities of monocytes from healthy controls (data not shown).
In this work, we have demonstrated that T lymphocytes from untreated brucellosis patients are activated in vivo and show a Th1 cytokine production polarization, with a predominance of circulating Th1 cytokines and strikingly high serum IFN-γ levels. In spite of this Th1 environment, we found deficient effector phagocytic activity.

The response of the immune system to Brucella is complex and involves innate and adaptive immunity (2). The focus of our work was to investigate the pattern of circulating cytokines in brucellosis patients. We also investigated the potential involvement of T lymphocytes in generating the abnormal environment of circulating cytokines found in these patients as well the functionality of phagocytic effector mononuclear cells regulated by the cytokines analyzed. Clinically symptomatic human brucellosis patients display a severely abnormal pattern of serum cytokine levels. Interestingly, brucellosis patients show a serum Th1 environment with strikingly high serum IFN-γ levels (levels more than 100 times higher than those found in healthy donors). These levels were dramatically reduced, becoming similar to those found in healthy controls, after antibiotic treatment and clinical and microbiologic cure of the infection. These results agree with a previous report of increased IFN-γ levels in human brucellosis (1, 3, 27, 31). Furthermore, increased IFN-γ concentrations were observed in all the untreated brucellosis patients studied (3), but variability was observed in the levels reached in serum. Different bacteria and/or host-dependent mechanisms may underlie the variability in the serum IFN-γ levels reached in each patient. It is known that several Brucella molecules can induce Th1 responses and that brucellosis patients are heterogeneous in the expression of IFN-γ due to gene polymorphisms (3, 18). Interestingly, the levels of IL-4, a representative Th2 cytokine, measured in untreated brucellosis patients were similar to those in healthy donors (3).

IL-12p40 plays a relevant role in the intracellular defense against bacterial infection, including that against Brucella infection, and in favoring Th1 polarization (1, 3, 29). In accordance with the Th1 environment found in untreated brucellosis patients, increased IL-12p40 levels were also found in these patients. Thus, the increased IL-12p40 levels and the normal IL-10 levels further emphasize the fact that untreated brucellosis patients develop a Th1 environment. The remarkable reduction in the serum IFN-γ and IL-12p40 levels found after the effective treatment of the patients, in the absence of significant variations in the unmodified cytokines, such as IL-4 and IL-10, indicates the reactive character of the Th1 response and the critical role displayed by the Brucella infective microorganisms in its induction. This Th1 environment found in untreated brucellosis patients is associated with a clear increase in the levels of proinflammatory cytokines, such as the monocyte-driven IL-1β and IL-6, as well as TNF-α. This increase in the levels of proinflammatory cytokines in untreated brucellosis agrees with previous reports (3, 35).

The Th1 polarization observed in brucellosis patients was associated with a phenotypically defined activation of both CD3+ CD4+ and CD3+ CD8+ T-lymphocyte subsets. A clear reduction in the percentage of naïve cells was observed in both the CD3+ CD4+ and CD3+ CD8+ T-cell subsets. Concomitant expansion of the effector CD45RA+ CD45RO+ cells in both T-lymphocyte subsets was also observed. Furthermore, the percentages of CD3+ CD4+ and CD3+ CD8+ T lymphocytes expressing cell activation antigens were clearly increased in untreated patients. In agreement with the pattern of evolution of the serum cytokine levels, a significant progression to normalization of the increased activation of T lymphocytes and a recovery of the decreased naïve T-cell compartment were observed after effective antibiotic treatment. The finding of enhanced intracytoplasmic IFN-γ expression in T lymphocytes from brucellosis patients agrees with the increased serum cytokine levels observed (1, 27). We previously found decreased IFN-γ production by phytohemagglutinin (PHA)-stimulated T-lymphocyte-enriched cultures (46). PHA is a monocyte-dependent mitogen (50). Thus, in this work, we tried to avoid this monocyte dependence by using mitogens, such as PMA, that activate T lymphocytes in a monocyte-independent fashion. These results indicate that T lymphocytes can produce large amounts of IFN-γ after adequate monocyte-independent stimulation but fail under monocyte-dependent conditions. It should be emphasized that this increased IFN-γ production by

### DISCUSSION

<table>
<thead>
<tr>
<th>Cytokine expression on CD4 and CD8 subsets</th>
<th>% with cytokine expression (mean ± SD)</th>
<th>Pretreatment (n = 24)</th>
<th>Posttreatment (n = 24)</th>
<th>P (pretreatment vs posttreatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ IL-2+</td>
<td>16.6 ± 6.3</td>
<td>78.1 ± 14.2</td>
<td>27.5 ± 30.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CD8+ IL-2+</td>
<td>6.4 ± 4.2</td>
<td>50.7 ± 21.3</td>
<td>18.9 ± 1.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CD4+ IL-4+</td>
<td>1.8 ± 0.9</td>
<td>1.9 ± 0.1</td>
<td>1.9 ± 1.1</td>
<td>NS</td>
</tr>
<tr>
<td>CD8+ IL-4+</td>
<td>2.3 ± 0.9</td>
<td>4.9 ± 1.1</td>
<td>2.7 ± 0.3</td>
<td>NS</td>
</tr>
<tr>
<td>CD4+ IFN-γ</td>
<td>1.3 ± 0.5</td>
<td>20.5 ± 12.0</td>
<td>11.8 ± 5.9</td>
<td>NS</td>
</tr>
<tr>
<td>CD8+ IFN-γ</td>
<td>4.4 ± 2.3</td>
<td>30.8 ± 22.3</td>
<td>25.2 ± 8.7</td>
<td>NS</td>
</tr>
<tr>
<td>CD4+ TNF-α</td>
<td>5.3 ± 1.9</td>
<td>50.9 ± 16.5</td>
<td>28.3 ± 9.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CD8+ TNF-α</td>
<td>3.6 ± 2.3</td>
<td>61.1 ± 25.1</td>
<td>26.3 ± 12.5</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

* Cytokine expression in peripheral blood T cells from 24 brucellosis patients was determined before (pretreatment) and after (posttreatment) antibiotic treatment. Twenty-four healthy donors were studied in parallel. PBMC were stimulated with PMA plus ionomycin as described in Materials and Methods.

* The value is statistically different (P < 0.05) from that for healthy controls.

* NS, no significant difference.
FIG. 1. Flow cytometry plots depicting the gates for IL-2\textsuperscript{+}, IFN-\gamma\textsuperscript{+}, and TNF-\alpha\textsuperscript{+} CD4\textsuperscript{+} (A) and CD8\textsuperscript{+} (B) T cells from a representative *Brucella* patient before and after treatment and from one of the two studies of his parallel healthy control. PBMC were stimulated with 50 ng/ml PMA plus 1 \mu g/ml ionomycin in the presence of 2 \mu M monensin for 6 h at 37°C. To detect stimulated cytokine production after CD3 (A) and CD8 (B) surface staining, PBMC were fixed and permeabilized, and the cytokines were stained with FITC-labeled anti-IL-2, FITC-labeled anti-IFN-\gamma, and PE-labeled anti-TNF-\alpha monoclonal antibodies.
T lymphocytes from untreated Brucella patients were investigated by stimulation with PMA plus ionomycin, an experimental system commonly employed to study the ability of T cells to secrete cytokines. It could be interesting to determine if a similar pattern of response is obtained after Brucella antigen stimulation. The increased IFN-γ production found in T lymphocytes from untreated brucellosis patients cannot be ascribed to a defined T-cell subset. Further specific analysis of IFN-γ production by αβ T cells and γδ T cells, as well as by different stages of activation/response of T lymphocytes, may provide information about the cellular pathogenic mechanisms of the infection. It has been shown that γδ T cells play a crucial role in defense against Brucella infection and release large amounts of cytokines, particularly IFN-γ (22, 39).

FIG. 2. Phagocytic activities of peripheral blood monocytes measured as percentages of phagocytes positive for FITC-labeled Escherichia coli (A), delta mean phagocytic activity per cell (B), and the phagocytic index (C) in untreated (○) and treated (●) brucellosis patients and in age- and sex-matched healthy controls (△). Delta mean phagocytic activity per cell is the difference between the fluorescence of phagocytes incubated with FITC-labeled E. coli at 37°C and the background fluorescence of negative-control phagocytes incubated with FITC-labeled E. coli on ice. The phagocytic index is the product of the percentage of cells positive for phagocytic activity multiplied by the mean phagocytic activity per cell. Data are shown as individual results (symbols) and means (horizontal lines) for each population indicated.
IFN-γ induces monocyte/macrophage activation and the production of reactive oxygen species, nitrogen intermediates, and cytokines (34, 50). IFN-γ reduces bacterial multiplication inside infected autologous macrophages (7, 52). Furthermore, TNF-α also plays an important role in Th1 cell-mediated immunity and in the phagocyte-dependent antibacterial response (7, 21, 26). In spite of the strikingly increased levels of IFN-γ and the proinflammatory environment, we found deficient effector phagocytic activity in monocytes in untreated brucellosis patients. After invading, *Brucella* spp. come in contact with humoral mediators and are readily ingested by polymorphonuclear leukocytes (PMN) and macrophages (23), and probably by dendritic cells (11, 48). At this stage, it has been proposed that *Brucella* performs several tasks to avoid immediate destruction: first, it circumvents strong activation of the innate immune system; second, the bacterium withstands the direct action of complement and other bactericidal substances; and third, it resists and evades the action of professional phagocytes, such as PMN and monocytes/macrophages (8, 9, 23).

It is interesting that our patients were considered to be healthy, without clinical, serological, or bacteriological evidence of relapse, at 45 days after antibiotic treatment. However, these cured patients show evidence of some remaining immunological disturbances in circulating T-lymphocyte and monocyte compartments but a trend to normalization. A long period of recovery for immune system abnormalities after infection and an inflammatory challenge has been shown previously (10, 27). Long-term studies of treated and cured populations of *Brucella* patients and asymptomatic individuals serologically positive for *Brucella* infections could help to establish the kinetics of full recovery from the immune system abnormalities of previously infected patients. Interestingly, there is evidence of a long-term presence of *Brucella* products in cured patients (13, 36, 38). Furthermore, there is also evidence of immunomodulatory effects of antibiotics (33). Thus, it is possible that the antibiotic therapy given to our patients might affect their immune systems.

The study of *Brucella*-infected patients has limitations for the analysis of the natural and adaptive immune responses to this infectious agent. In patients is not possible to study the early events in the interaction between *Brucella* and the host’s immune system. Moreover, it is difficult to accurately establish the beginning of the infection and the amount of the *Brucella* infective inoculum. The cohort of patients represents a population of nonsynchronized infected individuals. Furthermore, the intensity and pattern of the responsiveness of the immune system to the *Brucella* challenge may differ between individuals. Furthermore, brucellosis patients may represent the failures of the human defensive mechanisms against these bacteria. There is epidemiological evidence of clinically asymptomatic individuals with serological evidence of previous exposure to *Brucella*, especially in regions of endemicity (19). Thus, the immunological findings observed for *Brucella* patients may be understood as the consequences of the persistence of *Brucella* in the host, which has not yet spontaneously eliminated the bacteria.

These clinical factors may shed light on the differences found between the immune response in human brucellosis and in experimental-animal models of this infection. The increased Th1 environment found in patients may be representative of additional immune system failures involved in the resistance of the bacteria. This finding does not contradict the pivotal molecular role of IFN-γ in the early stages of the infection as demonstrated in animal models. The study of the immune system in patients with clinical brucellosis will aid in the understanding of the pathogenesis of the human disease and in the development of innovative therapeutic strategies for the optimal management of these patients.

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