Monocyte and Lymphocyte Apoptosis Resistance in Acute and Chronic Brucellosis and Its Possible Implications in Clinical Management

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This study evaluated the level of susceptibility of monocytes and lymphocytes to spontaneously induced and CH11-induced apoptosis in 16 patients with Brucella infection. The expression of some immunological and apoptotic markers was evaluated. Before therapy, monocytes showed a high level of resistance to spontaneously induced or CH11-induced apoptosis in all patients. In patients with acute infection, this resistance persisted for 10–20 days after treatment was initiated, then decreased; in chronically infected patients, it persisted after 45 days of treatment. Lymphocytes were also more resistant to CH11-induced apoptosis. The level of activated CD8+ T lymphocytes was high in patients with acute infection. The data indicate that the CD95-mediated apoptotic pathway is not involved in CH11 resistance. Lymphocytes are not infected by Brucella, so their resistance to apoptosis may be due to a soluble factor released by infected monocytes. The evaluation of levels of susceptibility to CH11-induced apoptosis in monocytes may be used to test the effectiveness of the therapy.

Brucellosis is a zoonotic infection in domestic and wild animals that is caused by organisms of the Brucella genus. Humans become infected by ingesting unpasteurized dairy products, being in direct contact with infected animals, or inhaling infectious aerosols [1]. The distribution of this disease is worldwide, and areas of high endemicity include the Mediterranean, the Middle East, Latin America, and Asia [2–5]. Patients frequently experience relapse, even with treatment, and the disease often becomes chronic (i.e., a clinical manifestation of >6 months duration). Relapse is not always due to antibiotic resistance, but can be correlated to other causes [6].

Brucellae are facultative intracellular parasites that are able to survive within mononuclear phagocytes (monocytes or macrophages) [7]. These cells play an important role in the dissemination of brucellae to other organs, such as the spleen, brain, and heart, and to the bones, and they allow the parasite to escape the extracellular immune mechanisms of the host (i.e., complement and antibodies) [8]. The control of Brucella infection is mediated by a cell-mediated immune response mainly involving activated antigen-presenting cells, CD4+ and CD8+ T cells [9, 10]. Brucellae are recognized and phagocytized by antigen-presenting cells. The internalized bacteria are processed, and this results in an expression of bacterial peptides on the antigen-presenting cell surface membrane in association with major histocompatibility complex (MHC) class 1 and class 2 molecules [11]. The MHC peptide complex is recognized, via a T cell receptor, by CD4+ and CD8+ T cells. This interaction induces the activation of these subclasses of lymphocytes. Activated CD4+ T cells secrete cytokines (IL-2 and IFN-γ from Th1 and IL-4 and IL-5 from Th2 cells) that stimulate the differentiation of CD8+ T cells in cytotoxic T cells. Cytotoxic
T cells play a major role in killing infected targets. They kill infected cells by inducing apoptosis, which is Fas-Fas ligand or granzyme mediated [12], by recognizing Brucella peptides and MHC class 1 molecules.

Apoptosis, also known as programmed cell death, is a physiological cell suicide mechanism that controls cell numbers in metazoan tissues [13, 14]. Moreover, it plays an important role in the development of the immune response [15, 16]. The activation of programmed cell death can occur via 2 different pathways: the extrinsic pathway, in which cell death receptors are activated by specific ligands [17–19], and the intrinsic pathway, in which mitochondria are primarily involved [20]. The Fas-Fas ligand–mediated extrinsic pathway represents the main way, in which mitochondria are primarily involved [20]. The mechanism by which cytotoxic cells kill infected or heterologous cells. Recent in vitro studies provide evidence that Brucella infection inhibits spontaneously occurring apoptosis in human monocytes by inducing the overexpression of the A1 gene, a member of the antiapoptotic bcl-2 family [21].

In this study, we evaluated the level of susceptibility of monocytes and lymphocytes of patients affected with acute or chronic Brucella infection to apoptosis induced by a Fas-activating monoclonal antibody (CH11). Our aims were to understand the implications of this event during the course of acute and chronic infection and to assess the modifications to apoptosis induced by a specific treatment.

**PATIENTS AND METHODS**

**Patients.** The study group was comprised of 16 patients (10 children and 6 adults, with a mean age of 21 years). They were admitted to the Institute of Infectious Pathology and Virology of the University of Palermo and the Reference Center for Brucellosis Control in Sicily, Italy, from July 2001 to February 2002.

All the patients had similar clinical manifestations of brucellosis: a tube agglutination titer test (Wright test) result of ≥1:160 or a 4-fold rise in the titer level between 2 samples collected within 15–30 days of each other. The diagnosis also included the identification of Brucella species by blood culture and/or peripheral blood PCR. Ten patients had acute disease and 6 had chronic brucellosis.

All patients received a 6-week (for adults) or a 3-week (for children) regimen of both rifampin (20 mg/kg per day, up to a maximum total dose of 1200 mg/kg) and doxycycline (5 mg/kg per day, up to a maximum total dose of 200 mg/kg). Rifampin was administered intravenously for the first 3 weeks because intravenous preparations yield higher and less-variable concentrations of rifampin [22]. Mononucleated cells were harvested from each patient before treatment and 10, 20, 30, 45, and 60 days after the therapy had begun.

**Cell surface phenotypic characterization.** Flow cytometry was performed on peripheral whole-blood samples with a FACScan Becton Dickinson flow cytometer after staining with the following monoclonal antibodies: CD3, CD4, CD8, CD61, CD38, and CD95. A 2- or 3-color panel of monoclonal antibodies was used to measure each lymphocyte subset.

**Cell culture.** Freshly isolated PBMCs were seeded in 1.91 cm² wells (tissue culture plates; Costar) at a density of 1 × 10⁶ cells/mL in RPMI 1640 (Gibco) containing 10% fetal calf serum (Gibco), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), and 2 mmol/L L-glutamine (Sigma Chemical) in a 5% CO₂ atmosphere at 37°C.

**Apoptosis evaluation.** Apoptosis was induced by exposing freshly isolated PBMCs to 0.5 μmol/L of Fas-agonistic monoclonal antibody CH11 for 24 h in a complete culture medium. Spontaneously occurring apoptosis in monocytes and lymphocytes was also evaluated after 72 h of culture in complete culture medium. Apoptosis was detected by the Annexine-V-Fluos staining kit (Roche Molecular Biochemicals). The cells (10⁶) were washed with PBS and centrifuged at 200 g for 5 min. The cell pellet was suspended in 100 μL of staining solution containing an annexine-V-fluorescein labeling reagent and fluorescein isothiocyanate–conjugated anti-CD61 (for monocyte detection) or anti-CD3 (for lymphocyte detection) MoAb (Becton Dickinson) and then incubated for 15 min at 20°C. Lymphocytes that were positive for annexine V/CD61 or annexine V/CD3 were evaluated by flow cytometry (Becton Dickinson).

**Statistical analysis.** The data were tested for statistical significance by means of Prism software (GraphPad).

**RESULTS**

**Apoptosis in monocytes.** Fresh PBMC isolated from patients with Brucella infection and from healthy donors (i.e., the control group) were cultured in complete medium. After 72 h of culture, the percentage of noninfected monocytes with spontaneously occurring apoptosis was 48% (figure 1). On the other hand, monocytes isolated from infected patients were markedly resistant to spontaneously programmed cell death, and, after 72 h, only 10% of the cells were observed to be apoptotic (figure 1).

The freshly harvested PBMC of healthy donors and infected patients was cultured for a period of 24 h in the presence of 0.5 μg/mL CH11, an anti-Fas activator monoclonal antibody. CH11 induced apoptosis in >60% of normal monocytes, whereas apoptosis occurred in ≤10% of monocytes obtained from infected patients (figure 1).

Figure 2 shows the percentage of monocytes with CH11-induced apoptosis in samples obtained from patients with Brucella infection 10, 20, 30, and 45 days after treatment had started. The level of susceptibility to apoptosis increased markedly 10–20 days after the beginning of treatment in the mono-
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Figure 1. Apoptosis in monocytes from Brucella-infected (infected group) and uninfected persons (control group). Apoptosis was induced in vitro by means of 72 h of culture in complete medium (spontaneously induced apoptosis, SA) or by 24 h of exposure to CH11 (CH11). There were 16 infected patients and 16 healthy donors. SDs are indicated by bars.

cytes of most patients who were affected by acute infection. Of note is the fact that monocytes isolated from chronically affected patients showed a slow rise in the level of apoptosis susceptibility, and, after 1 month of therapy, the percentage of monocytes with apoptosis induced by CH11 was ≈40% (figure 2). Generally, 1 month after the commencement of therapy, monocytes of patients with acute infection showed a level of susceptibility to CH11-induced apoptosis similar to that of monocytes obtained from healthy donors (60%–70% of monocytes). In samples obtained from chronically infected patients, the percentage of monocytes demonstrating CH11-induced apoptosis was not higher than 45%, even 2 months after treatment had been started (figure 2).

Apoptosis in lymphocytes. Lymphocytes isolated from patients with Brucella infection were more resistant to CH11-induced apoptosis than were lymphocytes from healthy donors. However, as shown in figure 3, the difference between the levels of CH11-induced apoptosis susceptibility of lymphocytes from infected and from noninfected patients was lower than that observed among monocytes. This difference in levels of apoptosis susceptibility of lymphocytes disappeared after 30 days of therapy, whereas a slight but significant ($P < .01$) difference was still observed in the level of apoptosis susceptibility of the lymphocytes from chronically infected patients after 45 days of therapy (figure 3).

Lymphocyte subpopulations. In >50% of patients with acute or chronic Brucella infection, the number of lymphocytes per milliliter of blood and the percentage of CD4$^+$ T lymphocytes were slightly lower than those found in the control group. Generally, this percentage of patients with lower levels increased 20 days after starting treatment. No important quantitative modifications in CD8$^+$ T lymphocytes subpopulations were observed (data not shown).

The mean percentage of activated CD8$^+$ T lymphocytes (CD8$^+$CD38$^+$) (± SD) in samples obtained from acutely infected patients (26% ± 6.6% of lymphocytes) was higher than that observed in samples obtained from the control group of healthy persons (5.4% ± 2.3%) and decreased progressively after treatment was initiated. Patients with chronic Brucella infection only showed a slight increase in the level of CD8$^+$CD38$^+$ T lymphocytes before treatment (figure 4).

Expression of the cell death receptor Fas. In contrast to the course of apoptosis susceptibility in monocytes, the level of expression of the cell death receptor Fas (CD95) was high before treatment and decreased in the weeks after therapy was initiated. As shown in figure 5, the percentage of CD95-expressing monocytes decreased by ~20% after 1 month of therapy. Samples obtained from chronically infected patients showed higher levels of CD95$^+$/CD61$^+$ cells than those obtained from acutely infected patients both before treatment was initiated and after its completion.

DISCUSSION

Several in vitro studies have shown that intracellular bacteria that infect monocytes, macrophages, and other cells are able to protect host cells against the induction of apoptosis by different stimuli. *Mycobacterium tuberculosis* and *Mycobacterium bovis* seem to inhibit monocyte apoptosis by inducing the production of TNF-$\alpha$ [23, 24]. Moreover, it has been observed that HeLa cells infected with chlamydiae and endothelial cells infected with *Rickettsia rickettsii* become resistant to pro-
programmed cell death [25, 26]. This strategy, adopted by several bacteria, provides protection against intracellular pathogens associated with immune attacks, thereby allowing an optimal multiplication of bacteria in host cells. Although antibiotic therapy quickly leads to clinical improvement, the infection persists in some patients, who experience relapse and the establishment of chronic infection after several months. Currently, the mechanisms by which infection becomes chronic are uncertain [27–29].

Recent studies have shown that Brucella infection inhibits spontaneously occurring apoptosis in human monocyte cell lines cultured in vitro [21]. In order to understand whether the status of apoptosis resistance in Brucella host cells could play a role in the establishment of chronic infection, we studied the level of susceptibility to apoptosis induced by an anti-Fas activator monoclonal antibody (CH11) in the monocytes and lymphocytes of patients with acute or chronic Brucella infection.

We observed that the monocytes and lymphocytes of patients affected with an acute Brucella infection were highly resistant to apoptosis. Moreover, they showed high levels of activated CD8+ T cells. Increased levels of both apoptosis resistance and CD8 activation disappeared after ~20 days of therapy. Although rifampin is unable to induce apoptosis, previous observations have revealed that doxycycline is able to induce programmed cell death [30]. However, the concentration of doxycycline needed to induce apoptosis in cells (15 μg/mL) is several times higher than that achieved in the serum of treated patients (2.5 μg/mL) [30]. This suggests that the “normalization” of apoptosis susceptibility levels of monocytes and lymphocytes of patients with Brucella infection is unrelated to the potential apoptotic effect of rifampin and doxycycline administered to infected patients.

We did not observe important increases in levels of CD8+ T cell activation in patients with chronic infection before therapy, and the levels of apoptosis resistance persisted for several months after the therapy had begun. This suggests that the chronic status of Brucella infection could be due either to a lasting increase in the level of apoptosis resistance among monocytes or to a low-grade cytotoxic lymphocyte activation during an infectious period. As reported above, the ability of the immune system to block the infection is correlated with the activity of cytotoxic cells acting against Brucella antigen–presenting cells (monocytes and macrophages). Therefore, the high and lasting level of apoptosis resistance of monocytes containing living brucellae and the low level of activation among cytotoxic cells may cooperate in creating a condition of chronic infection that responds only in part to a specific therapy.

It is interesting to note that the percentage of monocytes expressing CD95 was higher before therapy and decreased slightly but progressively during and after treatment. These data are in contrast with the higher levels of apoptosis resistance observed among monocytes obtained from patients before treatment, and they suggest that the mechanism of apoptosis resistance in monocytes is unrelated to the levels of Fas expression. The high percentage of monocytes expressing Fas may be explained as an attempt by cells to increase their level of susceptibility to programmed cell death induced by cytotoxic T cells, with the aim of eradicating the infection from host cells. Therefore, an overexpression of CD95 in monocytes may
play a role in the recovery phase of the infection, as shown by the observed decrease in the number of CD61+/CD95+ cells after therapy. This decrease occurred in acutely infected patients but not in chronically infected patients.

It has been suggested that the diminished production of Th1 cytokines may contribute to T cell unresponsiveness in chronic human brucellosis [27]. Moreover, Brucella abortus genes, which are involved in chronic infection, have recently been identified [29]. We suggest that chronic Brucella infection could have multiple causes, including a high level of resistance to cytokotoxic lymphocyte–induced apoptosis and a low level of activated CD8+ T cells, in addition to other genetic causes and a reduced production of Th1 cytokines.

Currently, we do not know how brucellae induce resistance to apoptosis in the host cells of acutely or chronically infected patients. Previous experimental research involving a continuous cell line (THP-1 cells) infected in vitro with specific clones of Brucella suis (B. suis 503, GFP–B. suis, and others) revealed specific overexpression of the A1 gene. This is a member of the bcl-2 family that is implicated in the survival of hematopoietic cells [21]. However, the observation that both invaded and noninvaded monocytes in vitro were protected against apoptosis [21] and our in vivo data (which showed an apoptosis-resistant status in lymphocyte cells not invaded by Brucella) indicate that one or more mediators released during infection could be involved in the phenomenon.

As is well known, no clinical, serological, or culture data are available for the prognosis of brucellosis. A high level of apoptosis resistance among monocytes and lymphocytes during and after therapy may therefore represent an index of chronic illness, suggesting to the clinician, in some cases, that a change in therapy is required.
