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NOTES

Cell Response to a Salt-Extractable and Sonicated *Brucella melitensis* 16M Antigen in Human Brucellosis

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We compared the immunological responses of leukocytes taken from healthy negative controls, laboratory workers immunized with the phenol-insoluble French vaccine against brucellosis, patients acutely ill with brucellosis, and patients chronically infected with *Brucella melitensis*. A salt-extractable antigen (protein-rich but with traces of lipopolysaccharide) and a sonicated suspension from *B. melitensis* 16M were used as antigens for in vitro lymphocyte proliferation test. Quantitation of T cells showed that the ratio of $CD4^+/CD8^+$ cells decreased as the condition of the patient deteriorated. An assay to quantitate the cell-mediated immunity showed that the activities of mononuclear cells stimulated with concanavalin A increased as the disease progressed as well.

Brucellosis has been recognized in Latin America since the early 1900s, and it continues to be one of the most prevalent zoonoses in the region (11). It is an important disease because it undermines animal health and productivity. It is also a disease that takes a great toll on humans. It is transmitted from animal reservoirs, their products, or their waste. *Brucella* organisms often invade cells of the reticuloendothelial system, and they may be sequestered in leukocytes, tissues of organs such as the spleen and liver, and bone marrow (21). The internal localization of bacteria may cause even high doses of antibiotics to be ineffective as treatment. Recovery is temporary, with a high incidence of relapses (11). It is necessary to assess the disease status of the patient accurately and to determine whether the patient has both recovered from and cleared the disease. The cell-mediated immunity is considered an integral part of the protection against intracellular pathogens (13). The passive transfer of immune cells in BALB/c mice has demonstrated that the protection against *Brucella abortus* is mediated by $CD4^+$ T cells (1, 2), and more recently, it was also demonstrated that $CD8^+$ T cells play a role in the resistance to *Brucella* infection (1, 3).

In the present study we attempted to measure the in vitro-specific responses of mononuclear cells from patients at different stages of brucellosis, vaccinated laboratory workers, and unexposed healthy volunteers. The antigens used in our study were a salt-extractable antigen (i.e., external antigen) and a sonicated whole-cell suspension (i.e., internal antigen), both obtained from *Brucella melitensis* 16M. The peripheral blood cells examined were $CD4^+$ and $CD8^+$ T cells.

B. melitensis 16M was a kind gift of M. Corbel. Frozen stock cultures were transferred once to Trypticase soy agar and were then grown in Trypticase soy broth (Becton Dickinson, Mexico City, México) supplemented with 12% yeast extract (Oxoid, Unipath Ltd., Basingstoke, United Kingdom) and 30% glucose. Cultures were grown at 37°C for 48 h with continuous shaking (New Brunswick rotary shaker). A cold saline extract was prepared through a modification of the method originally described by Ruiz-Castañeda and Carillo (16). Briefly, formaldehyde-inactivated bacteria were washed with 0.85% saline, resuspended in the same concentration of saline, and finally stirred magnetically with sterile 0.1-mm glass beads at 4°C for 18 h. The cell suspension was then centrifuged at 20,000 × *g*, and the supernatant solution was recovered, filtered, divided into aliquots, and stored at −20°C. This antigen was named RCM-BM. To release internal antigens, nonextracted *B. melitensis* 16M cells were diluted to 10^9 CFU/ml in 0.85% saline, chilled on ice, and sonicated with a cell disrupter (Ultrasonics, Inc.) set at 5 mm of amplitude with three 1-min pulses and then 30-s pauses. The sonicated cells (S-BM) were stored at 4°C. Both antigens were assayed for their protein (12), total carbohydrate (8), nucleic acid (6), and 2-keto-3-deoxyoctulosonic acid (20) contents.

Samples from patients with acute brucellosis and chronic brucellosis were obtained from the Instituto Mexicano del Seguro Social, Mexico City, México. Laboratory workers and healthy vaccinated individuals given the French phenol-insoluble vaccine at different periods of time before the study were included in the study. Healthy donors without a clinical history of brucellosis were included as controls.

Peripheral blood mononuclear (PBMN) cells were isolated from 30 ml of heparinized venous blood by centrifugation at 400 × *g* on Histopaque (density, 1.077 ± 0.001 g/liter; Sigma Chemical Co., St. Louis, Mo.) at room temperature for 30 min. PBMN cells were recovered from the interphase and were washed three times in Alsever's solution by centrifugation. PBMN cells were counted by Trypan blue dye exclusion, and the cell suspension was adjusted to 5×10^7 PBMN cells per ml in RPMI 1640 medium.

To determine the lymphocyte response to antigens, 5×10^5 PBMN cells were added to each of two separate 96-well round-bottom microtiter plates in RPMI 1640 medium (Sigma) containing 2 mM L-glutamine, 1% essential amino acids, 5% fetal bovine serum (Flow Laboratories, McLean, Va.), 100 IU of

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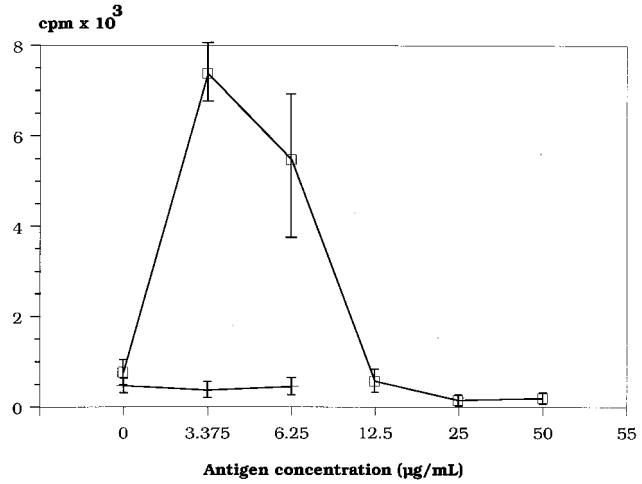

FIG. 1. Effects of different RCM-BM antigen concentrations on PBMN cell proliferation in healthy donors (+) and patients with brucellosis (□). Data represent the mean ± standard deviation counts per minute for triplicate cultures. Results are from three repeated experiments.

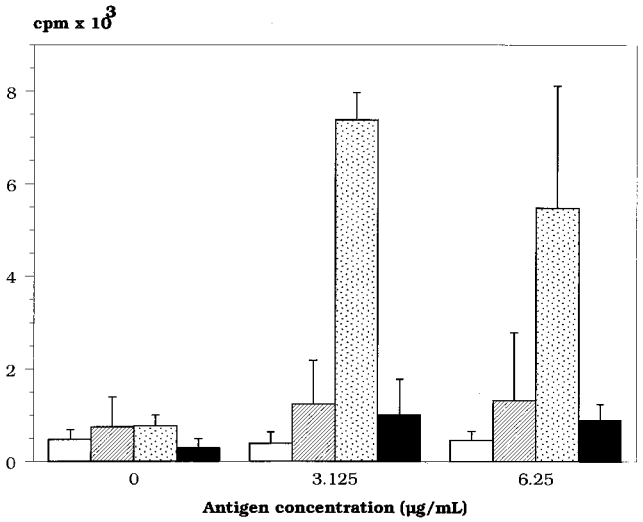

FIG. 2. PBMN cell proliferation in response to RCM-BM antigen in healthy donors (open bars), laboratory workers given the French vaccine (hatched bars), and patients with acute brucellosis (dotted bars) and chronic brucellosis (solid bars). The results are expressed as mean counts per minute for triplicate cultures. Bars indicate standard deviations.

penicillin per ml, and 100 μg of streptomicin per ml (Sigma). Three different concentrations of RCM-BM antigen (3.125, 6.25, and 12.5 μg/ml) and three concentrations of sonicated bacteria (10^5, 10^6, and 10^7 CFU per well) were added in triplicate; three wells without antigen were used as controls. In the second plate, 5 μg of concanavalin A per ml was added in triplicate. Three wells without a stimulus were used as controls. The plates were incubated at 37°C under 5% CO_2. After the 3-day incubation for mitogen and 5-day incubation for antigen, cell cultures were pulsed for 6 h with 0.5 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol; ICN Biomedicals, Inc.) per well. Cells were harvested onto glass fiber mats, and the counts per minute were measured in a liquid scincillation counter (Beckman Instruments, Inc.). Cell proliferation results were expressed as the mean ± standard deviation counts per minute incorporated by triplicate cultures.

On the other hand, PBMN cells were incubated with anti-human CD3, anti-human CD4, and anti-human CD8 mouse monoclonal antibodies (Dakopatts, Denmark) diluted in phosphate-buffered saline (PBS; pH 7.4). Cells were washed with PBS containing 2% bovine serum albumin (PBS-BSA) and were then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Sigma Chemical) diluted in PBS. PBMN cells incubated with conjugate only were used as controls. Cells were washed twice with PBS-BSA and were then mounted on a microscope slide. Cells were counted by phase-contrast microscopy and under UV light by using a Zeiss epifluorescence microscope, and the percentage of fluorescent cells gave an estimate of the T-cell populations.

Statistical differences in lymphocyte proliferation and the T-cell subsets were determined by Student's *t* test.

The salt-extractable antigen was composed mostly of protein, with some nucleic acids (<5%) and traces of lipopolysaccharide.

Figure 1 shows the relationship between RCM-BM antigen concentrations and the proliferative responses of cells from patients with brucellosis. Concentrations of 12.5 μg of RCM-BM antigen per ml or higher did not induce a proliferative response, possibly because of the toxicity of the antigen. Concentrations of 3.12 and 6.25 μg/ml were used in the subsequent experiments. As shown in Fig. 2, only PBMN cells from patients with acute brucellosis responded to RCM-BM. On the other hand, concentrations of S-BM corresponding to 10^5, 10^6, and 10^7 sonicated CFU per well were found to be suitable for lymphoproliferation induction. Figure 3 shows no significant response to S-BM antigen by PBMN cells from patients with acute brucellosis. In contrast, cells from patients with chronic brucellosis did react to S-BM antigen. PBMN cells from neither vaccinated laboratory workers nor healthy donors showed a response when they were stimulated with either of the antigens tested.

Patients with acute brucellosis showed a higher proliferative response than healthy donors to concanavalin A stimulation ($P < 0.01$) (Fig. 4). The responses of patients with chronic bru-

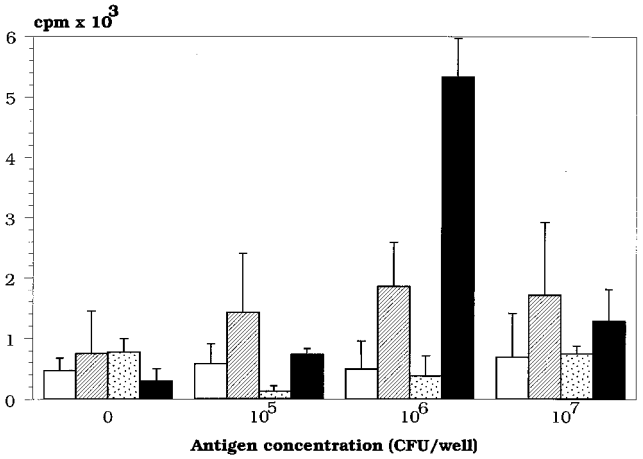

FIG. 3. PBMN cell proliferation in response to 10^5, 10^6, and 10^7 CFU of sonicated *B. melitensis* 16M organisms (S-BM) per ml in healthy donors (open bars), laboratory workers given the French vaccine (hatched bars), and patients with acute brucellosis (dotted bars) and chronic brucellosis (solid bars). The results are expressed as counts per minute for triplicate cultures. Bars indicate standard deviations.

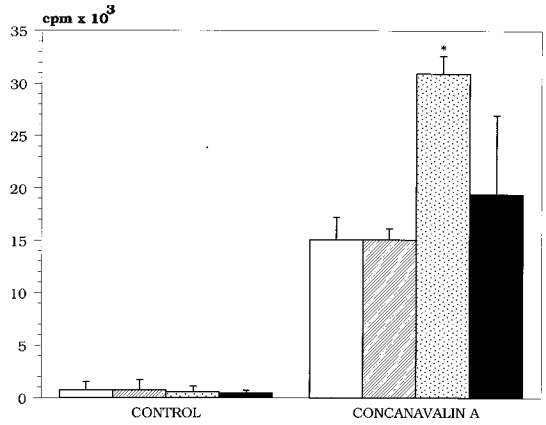

FIG. 4. PBMN cell proliferation in response to the T-cell mitogen concanavalin A (5 μg/ml) in healthy donors (open bars), laboratory workers given the French vaccine (hatched bars), and patients with acute brucellosis (dotted bars) and chronic brucellosis (solid bars). The results are expressed as mean counts per minute for triplicate cultures. Bars indicate standard deviations. *, $P < 0.01$ for acute patients versus healthy and vaccinated individuals.

cellosis was not different from those of healthy donors and the vaccinated group.

The trend shown in Fig. 5 suggests an increase in the number of $CD8^+$ T cells ($P < 0.001$) as the health of the patients with brucellosis deteriorated. No significant differences were noted in $CD8^+$ lymphocyte populations in vaccinated laboratory workers compared with those in healthy donors, although the proportion of $CD4^+$ T cells in vaccinated laboratory workers was between those observed in healthy controls and infected patients. The different groups except the vaccinees had comparable $CD3^+$ T-cell proportions; the vaccinees showed a diminished proportion of $CD3^+$ T cells.

Since *B. melitensis* usually replicates within reticuloendothelial cells, it is likely that immunity will depend on a suitable cell-mediated response by the host, and this response depends on the expression of bacterial antigens in association with molecules of the major histocompatibility complex (MHC) for recognition of the infected cell by the different T-cell subsets.

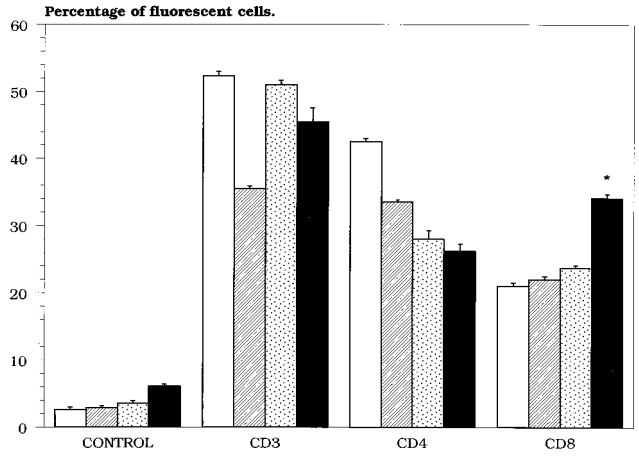

FIG. 5. Percentages of $CD3^+$ lymphocytes and the $CD4^+$ and $CD8^+$ T-cell subsets in PBMN cells from healthy donors (open bars), laboratory workers given the French vaccine (hatched bars), and patients with acute brucellosis (dotted bars) and chronic brucellosis (solid bars). The results are expressed as mean percentage of fluorescence in PBMN cells. Bars indicate standard deviations. *, $P < 0.001$ for chronic patients versus healthy and vaccinated individuals.

For these reasons, we searched for the response and proportion of T-lymphocyte subsets in relation to the clinical states of the patients.

Because of routine contact with cultures of *Brucella* spp. in our laboratory there is a great risk of occupational exposure to *Brucella* antigens. Most laboratory workers have received the French vaccine. So far, none of the 10 vaccinated laboratory workers has developed brucellosis, although the presence of specific antibodies in serum suggests exposure to the bacterium (14). Nevertheless, we did not observe a proliferative response to any antigen tested. The fact that specific lymphocytes are sequestered in lymph nodes may be an explanation for this phenomenon. A great difference in the response to *Brucella* antigens has been observed in vaccinated and infected individuals, and this can be due to differences in the exposure, processing, and presentation of bacterial antigens. This phenomenon has been observed mainly in the antibody response to *Brucella* O polysaccharide, which differs between vaccinated and infected animals. The former appear to have antibodies to the tip epitopes of the O polysaccharide, and the latter appear to have antibodies to the tip and length epitopes (5). Our results suggest a close relationship between the clinical situation of the patients and the kinds of antigens which are recognized by their lymphocytes. Cells from patients with acute brucellosis responded to saline extracts from *B. melitensis* (i.e., predominantly external antigens), whereas lymphocytes from patients with the chronic form of the disease preferentially recognized internal antigens released by sonication. The differences in the responses are puzzling, because both groups of patients have been infected and both groups have been exposed to all of the antigens of the pathogen. However, we do not know whether a change in the pattern of lymphocyte response occurs at some point during the disease or whether these differences arise from the beginning of the disease. A follow-up must be done on these patients. Interestingly, similar salt-extractable protein preparations belonging to the surface of the bacterium, as reported by Tabatabai and colleagues (17, 18), induced protection in lemmings against the virulent *B. abortus* strain 2308. Some components of these kinds of preparations are able to induce the production of cytokines such as interleukin-2 and gamma interferon (IFN-γ) by $CD4^+$ cells and IFN-γ by $CD4^+$ and $CD8^+$ T cells (23). So, the differences in the lymphocyte response to *Brucella* antigens may provide a unique insight into the defense against brucellosis.

Gamazo and colleagues (9, 10) reported that *Brucella* spp. release blebs of outer cell membranes during the exponential phase of growth. This could explain the early response to external antigens in patients with acute brucellosis. For patients with chronic brucellosis, the recognition of internal antigens may be explained on the basis of a turnover of the bacterial structures through the bacterium's intracellular dwelling. Upon comparing the lymphocyte subsets from healthy donors and patients with brucellosis, we observed a significant increase in the $CD8^+$ T-cell subset in the chronic phase of the disease. Thornes et al. (19) observed that after treatment with cyclophosphamide and prednisone, the patients were symptom-free and that the $CD8^+$ T-cell levels decreased to normal values.

Observations made in murine models suggest an association of bacterial antigens with MHC class I molecules. If this were the case for *Brucella* infections, it might explain the increase in $CD8^+$ T cells in *Brucella*-infected humans. In the murine model, $CD8^+$ T cells contribute to protective immunity, although the mechanism for this T-cell-mediated immunity is not well understood yet (1, 15). This population of lymphocytes could destroy MHC class I-expressing infected cells with a

subsequent release of *B. abortus* cells, so they would be available for their elimination by some other mechanisms (i.e., lysis by complement or killing by polymorphonuclear leukocytes). However, a more important role for $CD8^+$ T cells appears to be the IFN-γ production in response to soluble *Brucella* proteins (23), which are responsible for the increase in macrophage activity in mice.

As suggested previously (22), IFN-γ participates in the clearance of *B. abortus* in vivo. Paradoxically, IFN-γ and $CD8^+$ T cells appear to mediate the mechanisms of pathogenesis of brucellosis in mice (4).

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