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CLINICAL INVESTIGATION

Lymphocyte Subpopulations in Patients With Acute Brucellosis

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Abstract: The aim of this work was to evaluate changes in lymphocyte subpopulations, especially helper and cytotoxic T cells, in acute brucellosis patients undergoing treatment. Forty-three acute brucellosis patients were included in the study. Twenty healthy subjects served as controls. Total lymphocytes and the $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$ and CD $(16+56)^+$ subpopulations were counted by two-color flow cytometric analysis. The $CD4^+$ counts in patients before and after treatment were not statistically different ($p = 0.7$), but healthy subjects had significantly more of these cells ($p = 0.001$ and $p = 0.001$ compared to pre- and post-treatment patients, respectively). The $CD8^+$ counts in acute brucellosis patients decreased after treatment ($p = 0.004$), but remained higher in both pre- and post-treatment samples than in healthy subjects ($p = 0.001$ and $p = 0.01$ respectively). Neither the total leukocyte counts nor the numbers of cells in any subpopulation correlated with blood culture results (positive or negative). No statistically significant differences in the patients' $CD4^+$ T cell counts were observed between the pre-and post-treatment periods, and the count was higher in healthy subjects. Counts of $CD8^+$ T cells increased in acute brucellosis patients, and although they decreased after treatment they remained higher than in the controls. In view of this increase, it was concluded that $CD8^+$ T cells could be the major component in immunity against brucellosis.

Key Words: lymphocyte subpopulations, brucellosis, treatment.

Introduction

Brucellosis is one of the most important zoonoses affecting human welfare and livestock health worldwide. The disease is caused by bacteria of the genus *Brucellae*, which comprises several species differing in host affinity and virulence (1).

Cellular immune responses are a critical part of the host's defense against intracellular bacterial infections (2). The response against *Brucellae* spp. involves the whole gamut of the immune system from innate to adaptive immunity. In brucellosis, the different arms of the immune system, namely antigen-presenting cells, NK cells, $CD4^+$ and $CD8^+$ T cells and B cells, act together to provide a coordinated response (3). However, the primary mechanism of control involves cell-mediated immunity rather than antibodies, although some immunity against re-infection is provided by serum immunoglobulins (3-5). T-cell counting is the standard method for evaluating the cellular immune response against intracellular pathogens. Previous studies on murine models have shown that cytokine-secreting $CD4^+$ T cells are the fundamental components of immunity against *Brucella*. Nevertheless, it is known that $CD8^+$ T cells are very important in protective immunity (4).

Our aim in this study was to evaluate lymphocyte subpopulations in acute brucellosis patients during the pre- and post-treatment periods. We also investigated whether changes in the numbers of these cells correlated with blood culture results.

Material and Methods

The forty-three patients included in the study were admitted to our hospital with fever and musculoskeletal system dysfunction evidenced by generalized aches and pains and associated with fatigue, prostration and mental depression. Clinical and laboratory findings supported a diagnosis of brucellosis. The control subjects were 20

healthy persons (11 men and 9 women) with no history of *Brucella* infection. The ages of the patients and controls were 18-64 years (36.42 ± 14.5 years) and 19-60 years (36.6 ± 12.8 years) respectively.

The controls had negative STA and 2-ME test results, showed an erythrocyte sedimentation rate (ESR) within normal limits and had no complaints. Exclusion criteria for the healthy control subjects included acute/chronic diseases, smoking, alcohol consumption, medication, pregnancy, abnormalities in renal and liver function tests, and other bacterial infections.

Brucellosis was diagnosed on the basis of clinical, serological, bacteriological and epidemiological data. The diagnostic criteria were: isolation of a *Brucella* species from blood culture (BACTEC 9050, Becton-Dickinson Diagnostic Instrument System, Sparks, USA) or a single *Brucella* titre of ≥ 1/160 (by a standard tube agglutination test or Coombs), confirmed by a 2-mercaptoethanol (2-ME) test titre of ≥ 1/160, in association with compatible clinical findings. A 2-ME test ≥ 1/160 suggested ongoing infection. Using the BACTEC system, *Brucellae* were cultured and identified. *Brucella* species were isolated from the blood cultures in 21 cases (48.8%). The bacteria were biotyped by H_2S production, urease activity, and a dye sensitivity test (20–40 µg/ml basic fuchsine and growth in thionine). All the *Brucellae* were identified as *B. melitensis*. Disease activity was defined by the presence of typical signs and symptoms and a 2-ME titre ≥ 1/160. The patients' data were recorded on prepared forms.

Questionnaires concerning symptoms were completed by the specialist. Ethical consent was granted by the Local Ethics Committee. All participants were informed about the purposes of the study. Before treatment, blood samples (5 ml) were drawn from all consenting patients and healthy subjects, treated with EDTA and subjected to flow cytometry to evaluate the lymphocyte subpopulations. After isolation of the peripheral mononuclear cells, a direct immunofluorescence method was used. Two-color flow-cytometric analysis was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using a Becton Dickinson Simultest IMK-Lymphocyte kit: CD45/CD14, isotype control, CD3/CD19, CD3/CD4, CD3/CD8 and CD3/CD16+56. All lymphocyte subpopulations were measured in all blood samples by the same method. The cells were fixed with 1% paraformaldehyde and a total of 10.000 total events was recorded using CellQuest software (Becton Dickinson). Each lymphocyte subpopulation count was expressed as a percentage of the total number of lymphocytes. Criteria of quality included a greater than 95% total lymphocyte frequency in the analysis gate and a homogenous $CD45^+$ population.

Therapy, using one combination of doxycycline plus rifampin for 45 days, was initiated as soon as the diagnosis of brucellosis was established. After treatment, the lymphocyte subpopulations of all the patients were evaluated again as described above. Responses to treatment were indicated by (a) improvements in clinical findings and (b) 2-ME tests with titres ≤ 1/80. Non-response to the treatment was identified by ongoing complaints such as fever, myalgia and other constitutional symptoms, and by a 2-ME titre ≥ 1/160.

The data were expressed as arithmetic means with standard deviations. A Mann-Whitney U test for between-groups, a Wilcoxon Signed Rank Test for in-groups, and Spearman's correlation analysis, were all performed using the SPSS 12.0 packet software. $P < 0.05$ was considered statistically significant.

Results

Forty-three patients were included in the study. The mean ages of the patients and control subjects were 36.42 ± 14.5 years (range 18-64) and 36.6 ± 12.8 (range 19-60) respectively. The patient group consisted of 19 men and 24 women, and the control subjects comprised 11 men and 9 women.

The most common mode of disease transmission was consumption of un-pasteurized cheese and uncooked meat (51.2%). Symptoms were first noticed between 5 and 75 days after infection (mean ± SD = 24.1 ± 17.3). Thirty-six patients had fever on admission (83.7%). The most common complaints were weakness (95.3%), arthralgia (88.4%), fever (83.7%), sweating (74.4%), lethargy (48.8%) and headache (46.5). The most frequently affected organ was the liver, as indicated by the elevation of liver enzymes in the plasma (21.1%). Abdominal ultrasonographies revealed splenomegaly and hepatomegaly in eight (18.6%) and four (9.3%) patients, respectively. The most commonly affected joints were the waist and knee (18.6% for both). The physical and laboratory findings are shown in Table 1.

Table 1. Physical and laboratory findings in brucellosis patients

Findings	Mean ± Std. Deviation
Temperature on admission	37.4 ± 1.1
Pulse (per minute)	86.2 ± 7.9
Hemoglobin (g/dl)	12.6 ± 1.8
Hematocrit (%)	36.7 ± 5.2
Erythrocyte sedimentation rate (mm/h)	36.7 ± 29
C- reactive protein (g/dL)	31.0 ± 26.1
Rheumatoid Factor (g/dL)	9.2 ± 37.7
White Blood Cell Count (cells/mm^3)	6888 ± 2647
*PMNL Count (%)	51.70 ± 11.07
*Lymphocyte Count (%)	38.23 ± 11.38
*Monocyte Count (%)	9.30 ± 4.31
Brucella Standard Tube Agglutination (1/titre)	344 ± 286
2-Mercaptoethanol test (1/titer)	225 ± 196

* In peripheral blood

Total lymphocyte and natural killer (NK) cell counts were higher in patients both before and after treatment than in healthy subjects; treatment had no significant effect on the counts ($P > 0.05$). In contrast, the $CD3^+$ and $CD8^+$ cell counts in the patients were higher before than after treatment, but even after treatment they remained higher than in the healthy subjects. However, the $CD19^+$ levels and the $CD4^+/CD8^+$ ratio were higher in the healthy subjects than the patients and higher in the patients after treatment than before. Treatment did not affect the $CD4^+$ cell count but healthy subjects had more of these cells. These results are summarized in Table 2.

Counts of the T cell population and its subsets in patients with positive blood cultures were not statistically different from those with negative blood cultures (Table 3).

Discussion

In most infections, including those by *Brucella* species, the different arms of the immune system, namely antigen presenting cells (APCs), NK, T and B cells, act together to provide a coordinated response (2). NK cells are part of the first line of defense against pathogens and following activation can kill infected targets. However, removal of NK cells *in vivo* does not alter the ability of mice to contend with *B. abortus* infections. This result shows that the immune response is sufficient to control *Brucella* infection even in the absence of functional NK cells (6). In

Table 2. Percentages of T cells and subsets, B cells and Natural Killer cells in peripheral blood samples from patients with brucellosis before and after treatment and from healthy subjects

		N	Mean (%)	SD (%)	P
Total Lymphocyte count	Pre-treatment	43	34.48	12.02	b
	Post-treatment	43	34.88	10.80	c
	Healthy subjects	20	30.13	7.65	
$CD3^+$	Pre-treatment	43	79.88	6.52	a, b
	Post-treatment	43	77.42	6.53	c
	Healthy subjects	20	73.24	7.14	
$CD3^+CD4^+$	Pre-treatment	43	35.56	7.30	b
	Post-treatment	43	36.23	7.46	c
	Healthy subjects	20	43.36	6.01	
$CD3^+CD8^+$	Pre-treatment	43	36.70	7.65	a, b
	Post-treatment	43	32.80	7.07	c
	Healthy subjects	20	27.17	7.01	
$CD3^+CD4^+/CD3^+CD8^+$	Pre-treatment	43	1.03	0.39	a, b
	Post-treatment	43	1.17	0.42	c
	Healthy subjects	20	1.69	0.47	
$CD3\text{-}CD19^+$	Pre-treatment	43	7.26	3.83	a, b
	Post-treatment	43	10.13	3.63	c
	Healthy subjects	20	11.86	3.26	
$CD3^-CD(16+56)^+$	Pre-treatment	43	9.05	4.85	
	Post-treatment	43	10.52	5.18	
	Healthy subjects	20	11.03	6.70	

N: number of patients or healthy subjects; SD: standard deviation; a: $P < 0.05$ between pre- and post-treatment; b: $P < 0.05$ between pre-treatment and healthy subjects, c: $P < 0.05$ between post-treatment and healthy subjects.

Table 3. Counts of T cell population and its subsets in patients with positive and negative blood cultures

Cells		Culture Negative Mean ± SD	Culture Positive Mean ± SD	P
Total Lymphocyte count	Pre-treatment	34.1 ± 14.1	32.15 ± 8.7	0.92
	Post-treatment	34.7 ± 12.5	33.6 ± 8.12	0.99
$CD3^+$	Pre-treatment	78.1 ± 7.5	81.6 ± 5.1	0.13
	Post-treatment	75.9 ± 6.8	78.5 ± 6.3	0.4
$CD3^+CD4^+$	Pre-treatment	34.2 ± 5.6	37.2 ± 9.2	0.27
	Post-treatment	36.0 ± 6.5	35.8 ± 7.3	0.78
$CD3^+CD8^+$	Pre-treatment	36.5 ± 7.2	35.9 ± 7.3	0.65
	Post-treatment	32.0 ± 7.6	32.3 ± 6.8	0.98
$CD3^+CD4^+/CD3^+CD8^+$	Pre-treatment	1.0 ± 0.4	1.1 ± 0.5	0.28
	Post-treatment	1.2 ± 0.5	1.2 ± 0.4	0.68
$CD3^-CD19^+$	Pre-treatment	8.4 ± 4.3	6.5 ± 3.3	0.12
	Post-treatment	10.7 ± 3.9	9.1 ± 3.0	0.32
$CD3\text{-}CD(16+56)^+$	Pre-treatment	9.1 ± 4.8	8.7 ± 4.4	0.81
	Post-treatment	11.5 ± 6.2	10.7 ± 4.3	0.86

the present study, we observed no differences in NK cell counts between patients, either before or after treatment, and healthy subjects. These results are consistent with the findings described above (6).

Three main types of mechanisms are involved in the adaptive immune response in brucellosis. First, IFN-γ produced by $CD4^+$, $CD8^+$ and γδ T cells activates the bactericidal activity of the macrophages, hampering the intracellular survival of *Brucella*. Second, the cytotoxic $CD8^+$ and γδ T cells kill the infected macrophages. Third, Th1-type antibody isotypes such as IgG2a and IgG3 opsonize the pathogen to facilitate phagocytosis. The significance of $CD4^+$ and/or $CD8^+$ T cells in *Brucella* immunity has been controversial. Araya *et al.* (5) reported that both T-cell populations are important. However, Oliveira *et al.* (7) reported that major histocompatibility complex (MHC) class I-deficient mice, which have no $CD8^+$ T cells, control the infection more slowly than do wild-type mice, while MHC class II-deficient mice, defective in $CD4^+$ T cells, control the infection at a similar rate to wild-type mice. These results suggest that $CD8^+$ T cells play a critical role although, the role of $CD4^+$ T cells in brucellosis should not be ignored (3). Moreno-Lafont *et al.* (8) reported an increase in the percentage of $CD8^+$ T-cells in the peripheral blood of patients chronically infected with *Brucella* spp. In addition, they found increased numbers of *Brucella* antigen-specific $CD8^+$ T cells. Gazapo et al. (9) observed both a decreased percentage of $CD4^+$ lymphocytes and an increase in $CD8^+$, hence an inverted $CD4^+/CD8^+$ ratio, at the time of diagnosis. They also evaluated the $CD3^+$ and $CD20^+$ subpopulations in the second month after treatment and observed no changes during the follow-up period.

In this study, we found results consistent with many of the publications mentioned above; peripheral blood $CD8^+$ T cell counts were higher in patients than in healthy subjects. In addition, the patients' $CD8^+$ T cells counts decreased with treatment. The $CD4^+/CD8^+$ ratios were inverted between the pre- and post-treatment periods. These findings suggest that the first step in the development of immunological protection against brucellosis is an increase in $CD8^+$ T cell numbers. This contrasts with the findings of Moreno-Lafont *et al.* (8).

Many serum passive-transfer experiments suggest that humoral immunity is significant in murine brucellosis (10). Hoffmann *et al.* (11) indicated that high levels of antibody block the complement-mediated killing of *B. abortus*, which would make resistance to the disease dependent on cell-mediated immunity. Gazapo *et al.* (9) indicated that $CD20^+$ cells did not change in brucellosis patients during the follow-up period. In our study, the $CD19^+$ cell levels in healthy subjects were significantly higher than those in acute brucellosis patients; they were higher before than after treatment in the patients.

We found no literature in English addressing the correlation between blood culture results and T lymphocyte subset counts. We found no significant differences between patients with positive and negative

blood cultures from the point of view of total T cell and T-cell subset counts.

In conclusion, $CD8^+$ T cell counts increased in acute brucellosis. However, these counts decreased after treatment, though not as far as the levels in healthy control subjects. The $CD4^+$ T cells counts were higher than in healthy subjects. $CD19^+$ and NK cell counts increased after treatment. Blood culture results did not correlate with the lymphocyte subpopulation counts.

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