ASSESSMENT OF LYMPHOCYTE SUBGROUPS IN CHRONIC BRUCELLOSIS BEFORE AND AFTER IMMUNOTHERAPY

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Abstract- Brucellosis is an infectious disease and it seems that it affects human immune system and can cause acute, subacute and chronic clinical features. Forty patients suffering from chronic brucellosis were studied for $CD3^+$, $CD4^+$,$CD8^+$, $CD19^+$, cells and $CD4^+/CD8^+$ ratio by flow cytometry before and after treatment with antibiotics and immunopotentiators. The results were compared with 15 healthy controls. The patients were divided into three groups: 1) antibiotic, 2) levomisol + antibiotic, and 3) treated with cimetidine + antibiotic. The results showed a significant decrease of B cells ($CD19^+$), $CD4^+$ T cells and $CD4^+/CD8^+$ ratio in comparison with normal subjects before treatment. In the first group, significant decrease of CD4 T cells and CD4/CD8 ratio inversion were seen after immunotherapy. The 3rd group showed the best correction of phenotype quantity. In other words, significant increase of $CD4^+$ T cells, $CD19^+$ B cells, $CD4^+/CD8^+$ratio and decrease of $CD8^+$ T cells were seen with cimetidine immunotherapy.

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Key words: Brucellosis, immunotherapy, cimetidine, levamisole, flow cytometry

INTODUCTION

Brucellosis is a worldwide zoonotic infection and a problem of developing countries. Its course is closely related to the host cellular immunity (1). Different resistance of Brucella species depends on the cellular response mediated by T cells and macrophages and certain cytokines like IL-12, IFN-γ and TNF-α, *in vitro* (2-4). It is shown that brucellosis affects immune response (5,6). It is suggested that potentiation of cellular immune response can control the course of disease because it is observed that in acute cases, there is a normal response to polyclonal mitogens but in chronic cases there is not (7). Based on these observations, Printzis used levamisole and IFN-α in chronic brucellosis patients (8). Cimetidine is a H-2 antagonist which has shown immunoregulatory effects (9-13). In the present study CD3, CD4, CD8, CD19 blood lymphocytes, CD4/CD8 ratio in chronic human brucellosis patients, and the effect of levamisole and cimetidine as immunoregulators were investigated by flow cytometry. Iran is one of the hyperendemic regions of brucellosis. According to the pathogenesis of this disease, it is suggested that immunopotentiators along with antibiotics can be a better therapy and reveal disease mechanism.

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METERIAL AND METHODS

Patients

Forty untreated chronic brucellosis patients (25 male, 15 female; mean age: 3.37±13.2 years) were diagnosed by history, duration of clinical symptoms (more than 1 year) and positive antiglobulin Coombs titers against Brucella antigen (5). The control group comprised of 15 normal subjects (8 male, 7 female,

mean age: 25±3 years). Patients were randomly divided in three groups according to their therapeutic regimen:

1) 21 patients (12 male, 9 female) were treated with conventional antibiotics (doxycycline 200 mg/day and rifampin 600 mg/day) for 6 weeks.

2) 6 patients (5 male, 1 female) who took levamisole Hcl (150 mg/day) as immunotherapy in addition to conventional antibiotics for 6 weeks.

3) 12 patients (7 male, 5 female) who received cimetidine (600 mg/day,3 tablets) as immunotherapy along with conventional antibiotic therapy for 6 weeks.

Flow Cytometric Analysis

Blood samples were collected before and after treatment; 5 ml of whole blood with EDTA (1.5 mg/ml) was collected. Leukocyte counting was done by Coulter counter and absolute number of lymphocytes was estimated. Two milliliters of this blood was used for flow cytometric analysis. Samples were analyzed before 24 hours and were not stored in <4°C because low temperature causes CD4 and CD8 markers to decline. The method described by Jackson and Ormerod(14,15) was used. According to this method 100 µl of whole blood was suspended with specific monoclonal antibody in three separate tubes. After 20 minutes, red blood cells were separated by the lysing buffer and were washed by PBS (1%). Monoclonal antibodies included: Anti CD14, FITC/CD45. RPE (DAKO), Anti CD4. FITC/CD8, RPE (DAKO) and Anti CD3, FITC/CD19, RPE (DAKO). Analysis was done by FACs-can (Becton-Dickinson, Mountain view CA). The resulting percentages were used to estimate the absolute number of lymphocytes and were used for statistical analysis.

The results of flow cytometric analysis of patients before treatment were compared with those of the normal control group by Student t-test. After treatment, the effect of treatment was analyzed in each group by paired t-test.

RESULTS

The results of the lymphocyte subtype analysis of patients before treatment and normal subjects are shown in figure1, and table 1. There was not a significant difference between the percentage of T cells ($CD3^+$) in chronic brucellosis patients and control subjects, but a significant B cell (CD19) difference was found ($p<0.05$). $CD4^+$ T cells have a significant decrease in patients ($p<0.001$). No significant increase of $CD8^+$ T cells was found ($p<0.05$).

These results showed significant decreases of CD4/CD8 ratio of patients ($p< 0.001$).

Comparison of the lymphocyte subtypes of patients, before and after treatment, showed that in patients with antibiotic therapy, the difference in T cell ($CD3^+$) percentage was not significant ($p= 0.55$, paired t-test). In this group, the mean percentage of B cells ($CD19^+$) had a decrease but it was not significant ($p= 0.59$, paired t-test). Significant decrease in $CD4^+$ T cells ($p= 0.04$, paired t-test) and nonsignificant decrease in $CD8^+$ T cells ($p= 0.87$, paired t-test) were found. Mean CD4/CD8 ratio showed significant decline ($p= 0.01$, paired t-test). Results of mean percentages are summarized in figure 2 and table 2.

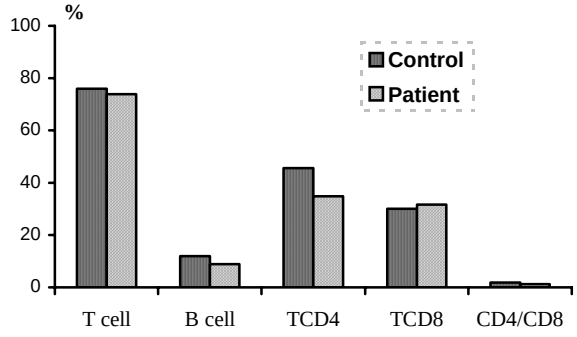

Fig. 1. Average percentage of lymphocytes and ratio of patients and normal subjects

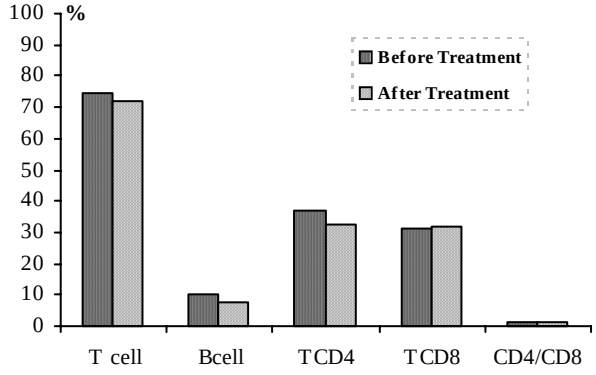

Fig. 2. Average percentage of lymphocytes and ratio of patients before and after treatment with antibiotics

Table 1. The absolute number and percentage of lymphocyte subpopulations in patients and control group

	Lymphocytes/µl	CD3 (%)	CD19 (%)	CD4 (%)	CD8 (%)	CD4/CD8
Control	4750±1400	75.96±6.47	11.93±5.45	45.64±7.21	30.03±5.64	1.82±0.40
Patients	3492.52±1664.16	73.8±10.01	8.95±4.53	34.8±8.49	31.72±8.58	1.19±0.45

Table 2. The absolute number and percentage of lymphocyte subpopulations in patients before and after treatment with antibiotics

	Lymphocytes/µl	CD3 (%)	CD19 (%)	CD4 (%)	CD8 (%)	CD4/CD8
Before treatment	2602.52±808.16	74.38±8.62	10.09±3.17	36.71±7.64	31.38±9.05	1.29±0.48
After treatment	2794.15±774.06	72.19±8.49	7.47±2.73	32.38±7.39	31.85±7.88	1.001±0.24

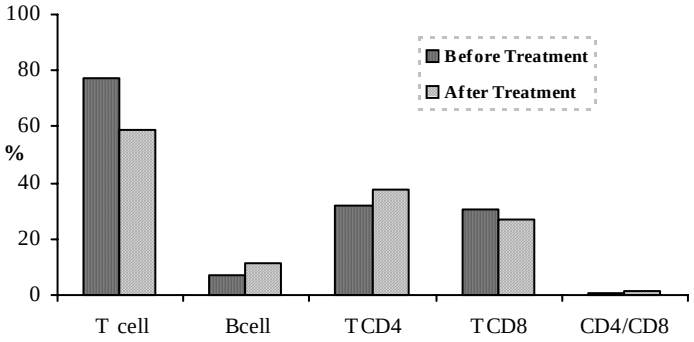

Fig. 3. Average percentage of lymphocytes in patients before and after treatment with antibiotics and levamisole

Table 3. The absolute number and percentage of lymphocyte subpopulations in patients before and after treatment with antibiotics and levamisole

	Lymphocytes/µl	CD3 (%)	CD19 (%)	CD4 (%)	CD8 (%)	CD4/CD8
Before treatment	2992.66±508.44	77.00±6.48	7.00±4.93	31.66±9.79	30.83±6.11	1.00±0.40
After treatment	2313±393.66	58.66±5.98	11.00±4.51	37.66±10.28	27.16±6.49	1.30±0.10

In patients who were treated by levamisole and conventional antibiotics, a significant decline in T cells ($CD3^+$) was found after treatment (p= 0.01, paired t-test, Figure 3, table3). The average percentage of B cells ($CD19^+$) showed an increase but it was not significant (p= 0.05, paired t-test). The increase in $CD4^+$ T cells after treatment was not significant (p=0.26, paired t-test). $CD8^+$ T cells did not show a significant difference after treatment (p= 0.25, paired t-test) and the increased CD4/CD8 ratio was not statistically significant either (p= 0.19, paired t-test).

The third group comprised of patients treated with cimetidine and conventional antibiotics (Fig. 4, Table 4).

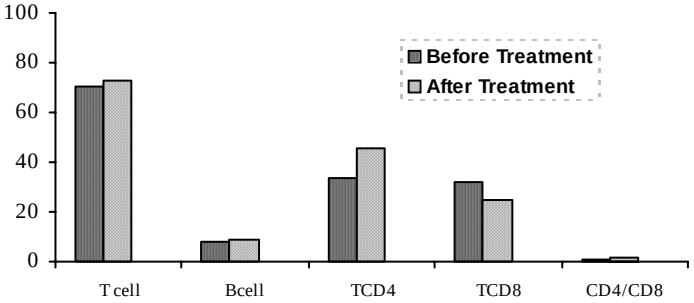

Fig. 4. Average percentage of lymphocytes and ratios in patients, before and after treatment with antibiotics and cimetidine

Table 4. The absolute number and percentage of lymphocyte subpopulations in patients, before and after treatment with antibiotics and cimetidine

	Lymphocytes/μl	CD3 (%)	CD19 (%)	CD4 (%)	CD8 (%)	CD4/CD8
Before treatment	3018.16±581.50	70.66±11.25	8.33±3.65	33.41±9.46	31.75±9.00	1.14±0.41
After treatment	2491.41±879.38	72.75±5.78	8.75±2.48	45.25±10.50	24.41±3.60	1.85±0.52

The increase in mean percentage of T cells ($CD3^+$) and B cells ($CD19^+$) was not statistically significant ($p= 0.6$, paired t-test) ($p= 0.75$, paired t-test). The increase in $CD4^+$ T cells ($p<0.05$) was significant ($p=0.002$, paired t-test) and a significant decrease in $CD8^+$ T cells ($p<0.05$) was also seen ($p=0.009$, paired-test). Mean CD4/CD8 ratio after treatment showed a significant difference ($p=0.002$, paired t-test).

DISCUSSION

Gazapo (1990) et al. found alterations in the immunological index (CD4/CD8 ratio), a decrease in $CD4^+$ T cells, and $CD11b^+$ monocytes, and unchanged $CD20^+$ B cells as brucellosis progresses to chronic stages. They suggested that with relapsing disease, CD4/CD8 ratio would be inverted (7). In 1996, Pourfathollah et al. found significant differences in chronic and subacute patients for $CD8^+$ and $CD4^+$ T cell percentages and CD4/CD8 ratios, which were not significant in acute brucellosis by immunohistochemical method (5). Moreno et al. (1998) reported a nonsignificant difference in HIV-infected patients for agglutinin titer. They later (2002) reported that by measuring CD69 in chronic brucellosis patients, a significant increase was observed in $CD8^+$ T cells and their response to specific antigen(16,17).

In the present study, we did not find a significant change in the mean percentage of $CD3^+$ T cells but the reduction of $CD4^+$ T cells and $CD4^+/CD8^+$ ratio, and the increase in $CD19^+$ B cells were significant. It seems that quantitative alterations in lymphocytes is due to a deviation in cytokines released from leukocytes induced by infection (2,18). It has been suggested that as the disease progresses to chronic stages, suppressor cells are induced to change the aspect of the response, causing a decrease in one subtype of lymphocyte or its function, and an increase in others, so a decline of cellular response and an increase of B cells will be seen. Therefore it is reasonable to use specific immunopotentiators along with conventional treatments to correct these deviations, and study the mechanism of disease and the immune responses involved in it. Printzis et al. (1994) reported an enhancing effect of α-IFN and levamisole on T cell blast formation, with recovery of clinical symptoms in chronic brucellosis patients (8). Pokrovski et al. (1992) showed an enhancing effect of Thymohexin on the DNA repair system and inhibition of DNA sensitivity to destruction by oxygen free radicals (19, 20). Gazapo et al. (1990) suggested that the immunological index (CD4/CD8 ratio) would be inverted as brucellosis evolves to the chronic state (7). The immunomodulatory effect of levamisole has been shown before (8,20). In our study, levamisole caused a nonsignificant increase in $CD4^+$, $CD3^+$ T cells and CD4/CD8 ratio. It is suggested that short-term (1 month) levamisole administration may have been responsible for this failure, which could be overcome by an increased duration of immunopotentiator therapy. The importance of the function of lymphocytes, rather than the quantity of subpopulations is also noteworthy. If the function is improved, quantities will subsequently recover. Cimetidine immuno-therapy causes favorable results, presumably by interfering with the function of $CD8^+$ T cells, and not by their quantity. The antagonistic effect of cimetidine on H2-receptor and inhibition of $CD8^+$ T suppressor secretory compounds was proved by Sahasrabudhe and Schnaper (21,22). It seems that cimetidine corrects the cellular response by improving secretion of specific cytokines, and thus improving subtype quantities. In other words, inhibition of suppressor cytokine(s) by cimetidine causes an up-regulation of T helper cells and may cause a Th_1-Th_2 balance which improves cellular immune responses, and limits infection and symptoms related to cytokines. The

immunomodulatory effect of cimetidine on Th_1 and Th_2 deviations and balance is an important subject which should be further investigated.

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