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The Effect of the Doxycycline-Rifampicin and Levamisole Combination on Lymphocyte Subgroups and Functions of Phagocytic Cells in Patients with Chronic Brucellosis

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Key Words
Brucellosis, chronic · Doxycycline · Levamisole · Lymphocyte subgroups · Outcome, clinical · Phagocytic function · Rifampicin

Abstract
Background: Brucellosis is one of the important health problems for both humans and animals in Turkey since agriculture and stock raising appears to be the most important means of subsistence. Investigations on the pathogenesis of brucellosis reveal that the etiologic agent can survive in phagocytic cells, and cell-mediated immunity plays an important role in immunity against bacteria. ***Methods:*** In this study, we investigated whether supplementation of levamisole, a well-known antihelminthic agent with immune-stimulating activity to conventional antibiotic therapy, would improve the anergy against *Brucella*. ***Results:*** The results of our study reveal that a 6-week course of levamisole as a supplement to conventional antibiotic therapy in chronic brucellosis is not superior to conventional antibiotic treatment alone with respect to lymphocyte subgroup ratios and phagocytic function. ***Conclusion:*** In chronic brucellosis, levamisole administered as a supplement concomitantly with conventional antibiotic therapy has no immunostimulating effect on the basis of the lymphocyte subgroups ratios measured and the ability of phagocytosis in the present study. Further large clinical and laboratory trials are necessary to investigate the immunological and physiological effects of levamisole on T_{H1} subtypes and cytokine secretion.

Introduction

Brucellosis is one of the five common bacterial zoonoses worldwide and is caused by organisms belonging to the genus *Brucella*, i.e. gram-negative, non-spore-forming, facultative, intracellular bacteria [1]. The genus *Brucella* consists of seven species depending on the antigenic variation and the primary host: *Brucella melitensis* (sheep and goats), *B. suis* (hogs), *B. abortus* (cattle), *B. ovis* (sheep), *B. canis* (dogs), *B. neotomae* (wood rats) and *B. maris* (marine mammals) [2, 3].

The origins of the diverse clinical manifestations in *Brucella*-infected human beings and animals have not been clearly elucidated. However, there is no doubt that the augmentation of *Brucella* replication in the host is ascribed to these symptoms. This increase in *Brucella* numbers in the host is mainly due to their ability to avoid

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the killing mechanisms and proliferate within macrophages like other intracellular pathogens. For example, *Brucella* organisms not only resist killing by neutrophils following phagocytosis [4, 5] but also replicate inside macrophages [6] and nonprofessional phagocytes [7]. Additionally, survival in macrophages, which is considered to be responsible for the establishment of chronic infections, enables bacteria to escape the extracellular mechanisms of host defense such as complement activation and antibody reactions.

Innate immunity is composed of nonspecific immune responses in the early stage of infection, before adaptive immunity, mediated by clonal selection of specific lymphocytes, has become established. The basic components of innate immunity are complement, neutrophils, natural killer cells and macrophages. The ultimate roles of the innate immune system in *Brucella* infection in vivo are to reduce to the initial number of bacteria without memorization and to provide the environment for Th1 immune responses in the host [8].

Adaptive immune responses composed of αβ CD4+ and CD8+ T cells, γδ T cells, B cells and cytokines are critical for the memory function, which plays the key role in vaccination. In brucellosis, adaptive immune responses can be classified into three mechanisms. First, interferon (IFN)-γ produced by CD4+, CD8+, and γδ T cells activates the bactericidal function in macrophages to hamper the intracellular survival of *Brucella*. Second, cytotoxicity of CD8+ and γδ T cells kills the infected macrophages. Third, Th1-type antibody isotypes such as IgG2a and IgG3 opsonize the pathogen to facilitate phagocytosis [8].

The bactericidal phase of brucellosis coincides with the onset of cell-mediated immunity. Control of infection depends on specifically involved T lymphocytes that excrete lymphokines, which in turn activate the bactericidal mechanisms of the macrophages [9].

Ahmed et al. [10] found significantly higher levels of IL-12 and IFN-γ in the serum of patients with brucellosis compared with patients without brucellosis. These data indicate that there is induction of Th1-type cytokines during human brucellosis. However, Rodriguez-Zapata et al. [11, 12] ascertained that T lymphocytes from patients with acute brucellosis have defective IFN-γ production and a defective proliferative response to membrane mitogenic signals, which disappeared after antibiotic treatment. Taken together, all these findings may suggest anergy due to a defect in the proliferative response of T lymphocytes and their cytokine secretion in chronic brucellosis.

After demonstration of the established role of cell-mediated immunity in the pathogenesis of brucellosis, immune-stimulating agents, such as IFN-α and levamisole, which stimulate cell-mediated immunity, have been successfully used in the treatment of chronic brucellosis [12–14], where antibiotic therapy alone appears to be inadequate [15, 16].

The phenylimidothiazole derivative levamisole is essentially an antihelminthic agent. Levamisole is assumed to stimulate cell-mediated immunity by oxidation of pioneer molecules of the soluble immune response suppressor, a protein-structured lymphokine that is thought to be responsible for the suppressor effects of activated T_S lymphocytes on the immune system [17–19]. Several studies including patients with chronic brucellosis demonstrate the good therapeutic effect of levamisole on clinical outcome, and laboratory results could probably be attributed to the enhancement of both T-cell function and monocyte phagocytosis [13, 18].

In the present study, we investigated the efficacy of an immunostimulating agent, levamisole, in combination with conventional antibiotic therapy in the treatment of chronic brucellosis by comparing it with conventional antibiotic therapy alone on the basis of lymphocyte subgroups and phagocytic function. To our knowledge, this is the first study which investigates the efficacy of levamisole in the therapy of chronic brucellosis using these methods.

Materials and Methods

A total of 49 cases were included in the study. Patients with chronic brucellosis (determined clinically and serologically) for at least 1 year [9] who did not respond to standard antibiotic therapy constituted the study groups; group A (immune-stimulation group, n = 17) comprised 11 males and 6 females and group B (standard therapy group, n = 22) 13 males and 9 females. The diagnosis of chronic brucellosis was confirmed by standard tube agglutination (STA) test. Five-milliliter blood samples were obtained from patients in groups A and B for the STA test with rivanol into red-stoppered tubes (Becton-Dickinson Vacutainer Systems, Belliver Industrial Estate, Plymouth, UK) just before the commencement of the therapy. Patients in group A received a combined antibiotic therapy consisting of 200 mg/day doxycycline and 600 mg/day rifampicin plus levamisole (40 mg/day for the first 2 weeks, 80 mg/day for the next 2 weeks and 120 mg/day for the last 2 weeks, Ketrax® 40-mg tablets, Zeneca-Abdi Ibrahim, Istanbul, Turkey) for a period of 6 weeks. Group B received only a standard antibiotic therapy (doxycycline and rifampicin). Ten healthy subjects (6 males and 4 females) were chosen as the control group (group C). The study was approved by our local ethic committee, and informed consent was obtained from the study participants.

Table 1. Comparison of lymphocyte subgroups of groups A and C before treatment (means ± SD)

	CD19	CD3 HLA DR	CD16+56	CD4/CD8
Group A (n = 17)	9.4 ± 3.4	15.9 ± 9.0	15.2 ± 6.2	1.15 ± 0.5
Group C (n = 10)	8.2 ± 1.4	8.2 ± 4.4	3.6 ± 2.9	1.42 ± 0.8
p value	0.231	0.006	<0.001	0.365

To determine the lymphocyte subgroups and to measure the phagocytic function, for each procedure 5 ml of blood were obtained into yellow-stoppered acid citrate dextrose solution B-containing tubes (Becton-Dickinson Vacutainer Systems, Meylan, France) and into green-stoppered heparin and lithium-containing tubes (Vacuette, Greiner Labortechnik, Frickenhausen, Germany), respectively, before, at the end of the 1st week and after the treatment. Blood samples were stored in ice-filled containers and immediately transferred to the immunology laboratory.

Before the treatment, blood was sampled for the STA test from patients in group B. At the end of therapy, blood samples were obtained to assess lymphocyte subgroups and phagocytic function.

Blood samples were obtained only once from cases in group C to determine the lymphocyte subgroups and phagocytic function.

STA Test with Rivanol

An antigen containing a suspension of bacteria in phenol, generated from the *B. abortus 99S* strain and inactivated by means of heat (Pendik Veterinary Research Institute, Istanbul, Turkey) was used. A 4‰ solution of rivanol (6,9 ethoxy acridine) was used to decompose the IgM antibodies that might be existing in the patient serum. The titrations were found >1/160 in both groups.

Determination of Lymphocyte Subgroups

A Simultest IMK-Plus kit (Becton-Dickinson Immunocytometry Systems, San Jose, Calif., USA) was used to determine the lymphocyte subgroups. Fluorescein-isothiocyanate- and phycoerythrin-marked monoclonal antibodies were present in the kit. The study was carried out by the aid of a FACScalibur Flow Cytometry device and a FACScalibur Simulset program (Becton-Dickinson).

Determination of Phagocytotic Function

Phagocytotic function was assessed using the PHAGOTEST® kit (Orpegen Pharma, Heidelberg, Germany) and the FACScalibur Flow Cytometry device, CELL Quest program (Becton-Dickinson).

Statistical Evaluations

The data obtained were entered into a PC using the Windows 95/Excel 5.0 package program. The Mann-Whitney U test was used to compare the independent groups, and Wilcoxon's t test was used to compare the pre- and post-treatment values of each group. A p value < 0.05 was accepted as significant.

Table 2. Comparison of the ratios of phagocytosis between groups A and C before treatment

	Monocytes (%)	Granulocytes (%)
Group A (n = 17)	65.2 ± 25.8	87.8 ± 15.3
Group C (n = 10)	74.9 ± 6.7	96.6 ± 2.5
p value	0.194	0.05

Results

Values before Treatment

When the pre-treatment lymphocyte subgroup values were compared between groups A and C, there were no significant differences between the mean ratios of B lymphocytes with a CD19 surface marker ($p > 0.05$; table 1). Comparing the mean ratios of activated T lymphocytes expressing a CD3 HLA marker on their surfaces, values of group A were significantly higher than those of group C ($p < 0.05$; table 1). The mean CD16/CD56 ratio of natural killer cells in group A was significantly higher than that in group C ($p < 0.05$; table 1). When the ratios of the T_C and T_S numbers (CD4/CD8) were compared, no significant differences were found between groups A and C ($p > 0.05$; table 1). No statistically significant differences between the numbers of monocytes and granulocytes were found when determining phagocytic function before treatment between both groups ($p > 0.05$; table 2).

Lymphocyte Subgroups

When the values of lymphocyte subgroups at the end of weeks 1 and 6 were compared with those before treatment in group A, there were no statistically significant differences ($p > 0.05$; table 3).

Phagocytic Function

When the ratios of phagocytosis at the end of weeks 1 and 6 were compared with those before the treatment in group A, there were no statistically significant differences ($p > 0.05$; table 4).

Table 3. Comparison of lymphocyte subgroups (means ± SD) before and at the end of weeks 1 and 6 of treatment in group A (n = 17)

	Before treatment	Week 1	p value	Week 6	p value
CD19	9.4 ± 3.4	9.2 ± 3.6	0.884	8.5 ± 4.6	0.572
CD3 HLA DR	15.9 ± 9.0	16.1 ± 10.1	0.971	11 ± 4.9	0.06
CD16+56	15.2 ± 6.2	17.1 ± 7.6	0.449	17.5 ± 10.2	0.473
CD4/CD8	1.15 ± 0.5	1.24 ± 0.5	0.552	1.24 ± 0.5	0.602

Table 4. Comparison of the ratios of phagocytosis before and at the end of weeks 1 and 6 of treatment in group A (n = 17)

	Before treatment	Week 1	p value	Week 6	p value
Monocytes, %	65.2 ± 25.8	71.2 ± 20.3	0.512	63.4 ± 20.4	0.845
Granulocytes, %	87.8 ± 15.3	86.5 ± 17.3	0.837	85.2 ± 24.9	0.765

Table 5. Comparison of the lymphocyte subgroups between groups A and B at the end (week 6) of the treatment (means ± SD)

	CD19	CD3 HLA DR	CD16+56	CD4/CD8
Group A (n = 17)	8.5 ± 4.6	11 ± 4.9	17.5 ± 10.2	1.24 ± 0.5
Group B (n = 22)	6.9 ± 3.5	13.8 ± 8.3	12.1 ± 5.3	1.22 ± 0.4
p value	0.297	0.210	0.09	0.915

Table 6. Comparison of the phagocytic function between groups A and B at the end of treatment

	Group A (n = 17)	Group B (n = 22)	p value
Monocytes, %	63.4 ± 20.4	68.6 ± 13.2	0.452
Granulocytes, %	85.2 ± 24.9	89.4 ± 12.5	0.609

Lymphocyte Subgroups and Phagocytic Function

A comparison of the values of lymphocyte subgroups at the end of the treatment showed that the mean ratios of activated lymphocytes were not significantly different between the groups ($p < 0.05$; table 5).

There were also no statistically significant differences between the mean values of the two groups comparing the phagocytic function at the end of the 6th week of treatment ($p > 0.05$; table 6).

Discussion

The finding that T lymphocytes from patients with brucellosis have defective IFN-γ production and an impaired proliferative response to membrane mitogenic signals (before patients respond to antibiotic treatment [11, 12]) indicates that chronic brucellosis is an anergic status due to a defect in the proliferative response of T lymphocytes and their cytokine secretion. In order to improve this anergy, immunostimulating drugs, e.g. IFN, that possess a restricted efficacy when administered to elderly patients, and which are highly expensive and also display severe side effects, have been used [12–14]. In our study, we used levamisole, which is an inexpensive drug and has no serious side effects or contraindications compared with other immunostimulating agents. To our knowledge, this is the first study which compares the efficacy of conventional antibiotic therapy versus conventional antibiotic therapy plus levamisole.

At the beginning of our study, patients in group A and in the control group, who had no obvious complaints, were compared. Thus, changes in lymphocyte subgroups

and in their phagocytic function were scrutinized in patients with chronic brucellosis.

The higher ratios of activated T lymphocytes in group A compared to the control group both before and at the end of treatment suggest that T lymphocytes tend to increase in number due to the antigenic stimulus of infection in patients with chronic brucellosis. However, they are quite incapable of removing the infectious agent hidden within the phagocytic cells since there is an inadequate cytokine secretion. The lack of significant differences in the CD4/CD8 ratios before treatment between groups A and C and during and after the combined therapy, and the higher ratio of activated T lymphocytes in group A in comparison with the control group also suggest that CD8+ T lymphocytes also increased in number. However, the increased number of CD8+ T lymphocytes should have consisted of T_S cells, and the T_{H2}-subtype immune response should have occurred since chronic infection continued simultaneously.

No significant differences were determined between groups A and C with respect to the phagocytic ability of monocytes and granulocytes before the treatment. Similarly, pre-treatment values were not significantly different compared with those obtained at the end of weeks 1 and 6 within group A. These findings are in accordance with the results of the lymphocyte subgroups in the present study because IL-2 and IFN-γ secretions of T_H lymphocytes would diminish and phagocytes would not be stimulated in the absence of an adequate T_{H1} subtype response [10, 13]. The absence of an increase in the (post-treatment) phagocytic function despite combined therapy in group A reflects the inability of levamisole to increase the T_{H1} response. The lack of significant differences in the post-treatment lymphocyte subgroup ratios and phagocytic ability of monocytes and granulocytes between groups A and B further support this hypothesis.

Levamisole administered as a supplement concomitantly with conventional antibiotic therapy in chronic brucellosis has no immunostimulating effect on the basis of the ratios of lymphocyte subgroups measured and the phagocytic function in the present study. Further large clinical and laboratory trials are necessary to investigate the immunological and physiological effects of levamisole on T_{H1} subtypes and cytokine secretion.

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