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ORIGINAL ARTICLE / RESEARCH

The Role of NK and NKT Cells in Patients with Acute Brucellosis

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ABSTRACT

Background: The major role of T cells in *Brucella* immunity is secretion of gamma interferon (IFN-γ) for activation of bactericidal function in macrophages. Natural killer T (NKT) cells are new subtype of T cells and produce IFN-γ very quickly and promptly when they are stimulated. Therefore, NKT cells can play an effective role in immunity of brucellosis.
Aim: It was aimed to evaluate whether natural killer (NK) and NKT cells have an effect on brucellosis immunity and whether there is any difference for these cell percentage between pre- and post-treatment period.
Methods and Material: The study included a total of 40 acute brucellosis cases and 20 healthy subjects. Two-colour flow cytometric analysis was performed on a FACScan flow cytometer, using monoclonal antibodies CD45/CD14, isotype control and CD3/CD16+56.
Results: No statistical significant difference was observed between patient and control groups for NKT cell counts and NK cell percentages. There were no differences between before and after treatment period in terms of NK and NKT cells ratio.
Conclusion: According to our result, acute brucellosis has no effect on increasing of NK and NKT cells. To the best of our knowledge, this is the first study about NKT cells ratio in patients with acute brucellosis.
Key words: Brucellosis, NK cell, CD1-restricted cell, NKT cell

Introduction

Brucellosis is one of the most common bacterial zoonoses in the world, caused by organisms belonging to the genus *Brucella*, facultative, intracellular bacteria, which causes infections both in animals and humans [1]. The response against *Brucella* species involves the whole part of the immune system, from innate to adaptive immunity resulting from stimulation of antigen-presenting cells, NK cells, $CD4^+$ and $CD8^+$ T cells, and B cells [2],[3]. The major role of T cells in *Brucella* immunity is secretion of gamma interferon (IFN-γ) for activation of bactericidal function in macrophages [1]. Moreover, NK cells have been shown to be important for control of infections of intracellular bacterial and protozoan parasites,

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and this role has been shown specifically to relate to their ability to produce IFN-γ [4],[5]. A role for NK cells and cytotoxicity in protective immunity to brucellosis has not been substantiated experimentally [6]. NKT cells are new subtype of T cells, and they produce IFN-γ very quickly and promptly when they stimulated. Therefore, NKT cells can play an effective role on immunity of brucellosis [7].

Patients and Methods

Local ethics committee of Firat University has approved this study, and a written informed consent was obtained from each subject. A total of 45 patients admitted to Firat Medical Center, a tertiary referral University hospital located in East Anatolia, were examined. The presenting signs and symptoms were fever, musculoskeletal system complaints such as generalised aches and pains associated with fatigue, prostration and mental depression. Clinical and laboratory findings revealed acute brucellosis. All patients were started the appropriate treatment. However, five patients were excluded from the study because of non-response to the treatment. The study included 40 acute brucellosis cases (18 males and 22 females; between 17 and 80 years of age, mean ± SE, 37.08 ± 8.76 years).

Brucellosis was diagnosed based on clinical, serologic, bacteriologic and epidemiologic 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 standard tube agglutination (STA) test or Coombs], confirmed by a 2-mercaptoethanol test (2-ME) titre of ≥1/160 in association with compatible clinical findings.

By using BACTEC blood culture system, *Brucella* bacteria were cultured and identified. *Brucella* species were isolated from the blood cultures in 22 cases (55%). The biotyping of the bacteria was performed by H_2S production, urease positivity and dye sensitivity test (20–40 μg/ml basic fuchsine and growth in thionine). All the *Brucella* species were identified as *B. melitensis*. Disease activity was defined by the presence of typical signs and symptoms. The patient's data were recorded to the previously prepared forms.

The control group was composed of 20 healthy subjects (nine male and 11 female subjects), ages ranging between 15 and 90 years (mean ± SE, 39.35 ± 7.53 years). No statistical significant difference was observed between groups' ages. The cases in the control group were STA negative, showed ESR within normal limits and did not have any complaints. Exclusion criteria for the healthy control subjects included acute/chronic diseases, smoking, medication, pregnancy and any abnormalities in renal and liver function tests.

Blood samples were drawn from all patients and healthy subjects for studying NK and NKT cells. Two millilitre venous blood samples were obtained, and sera were separated in both study and control groups. T cells, NK and NKT cells [$CD3^+$, and $CD(16+56)^+$] were established from blood with EDTA. After isolation of the peripheral mononuclear cells, the direct immunofluorescence method was applied. Two-colour flow cytometric analysis was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA), using Becton Dickinson monoclonal antibodies CD45/CD14, isotype control, and CD3/CD16+56, according to the instructions of manufacturer. Cells were fixed with 1% paraformaldehyde and analysed by flow cytometry, after counting 10,000 total events using Cell Quest software (Becton Dickinson). This lymphocyte subpopulation is expressed as percentages of the total number of lymphocytes. The quality criteria involved the frequency above 95% of total lymphocytes in the analysis gate, homogenous $CD45^+$ lymphocyte population (minimum of 2000 events in the gate, CD45 > 95%).

Treatment for brucellosis was started as soon as the diagnoses was established and involved the combination of doxycycline plus rifampin for 45 days. After treatment, same parameters studied prior to start of antibiotics were measured again in all patients. Response criteria to the treatment were improvements of clinical findings with 2-ME tests ≤1/80 after the treatment. Non-responder patients had ongoing complaints such as fever, myalgia and other constitutional symptoms, with 2-ME test measuring ≥1/160 after the treatment.

Comparisons between the different groups were performed by Mann–Whitney U (for between groups) and Wilcoxon Signed Rank Tests (for in groups), and Spearman's correlation analysis

using SPSS 11.0 packet software. $P < 0.05$ was considered as statistically significant.

Results

The most common complaints of the patients were fever (85%) and sweating (72.5%), arthralgia (65%) and malaise (65%) ([Table/Fig 1]). The median of Brucella STA and 2-ME test levels were 1/320 for both, in patients. Mean white blood cell counts of patients were 6.800 ± 2.600, erythrocyte sedimentation rate was 37.75 ± 29.7, and C-reactive protein was 33.53 ± 27.23 ([Table/Fig 2]).

Table/Fig 1

Complaints	n	%
Fever	34	85
Sweating	29	72.5
Arthralgia	26	65
Malaise	26	65
Headache	21	52.5
Lack of appetite (anorexia)	20	50
Weight loss	18	45
Back pain	7	17.5

Complaints of the patients with brucellosis

NK and NKT cells have a particular importance in initiating and regulating the immune response. Here, we evaluated the role of NK and NKT cells in patients with brucellosis during the early phase of infection and their difference in appearance before and after treatment period.

There was no significant correlation between NK and NKT cells ratio and laboratory findings of patients. There were no differences between before and after treatment period in terms of total lymphocytes and NK and NKT cells ratio. $CD3^+$ cells counts were higher at pre-treatment period than post-treatment's levels ($p < 0.001$).

Table/Fig 2

Laboratory signs (n = 30)	Mean ± SD	Minimum	Maximum
White blood cell counts (/mm^3)	6.8 ± 2.6	1.1	10.9
ESR (mm/h)	37.75 ± 29.7	25	111
CRP (mg/l)	33.53 ± 27.23	4	147

Laboratory findings of patients with brucellosis

These cell counts at both pre- and post-treatment period were higher than in healthy subjects ($p < 0.001$ and $p < 0.05$, respectively). No statistically significant difference was observed between patients and controls for NKT cell counts and NK cell percentage. The cell counts and statistical differences are shown in [Table/Fig 3].

Table/Fig 3

	Cases of acute brucellosis (n = 40), (%)		Healthy subjects (n = 20) (%)
	Pre-treatment	Post-treatment	
Total lymphocyte count	34.8 ± 1.9	34.8 ± 1.7	30.1 ± 1.7
CD3+ (T cell)	79.8 ± 1.02[a]	77.4 ± 1.05[b]	73.2 ± 1.6
CD3+/CD(16+56)+ (NKT cell)	4.1 ± 0.4	5.1 ± 0.6	5.9 ± 1.3
CD3/CD(16+56)+ (NK cell)	9.1 ± 0.7	10.7 ± 0.9	11.05 ± 1.5

[a]Vs. post-treatment and control group ($p < 0.001$).
[b]Vs. control group ($p < 0.05$).

Flow cytometric analysis of T cells, NK cells and NKT cells on the peripheral blood of patients with brucellosis in pre and post-treatment periods and healthy subjects

Discussion

Facultative intracellular bacteria, including *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Brucella abortus* and *Salmonella* spp., survive within normal resident macrophages and other non-

professional phagocytes. IFN-γ-producing $CD4^+$ and $CD8^+$ T lymphocytes play an important role in recovery from infection by these organisms [8],[9]. In intracellular bacteria infection such as listeriosis, brucellosis or intracellular protozoan parasitic infections like toxoplasmosis and leishmaniosis, NK cells are known to be crucial for early control of infection [4],[5]. This mechanism is related to production of IFN-γ [4]. Similar effect can be found with the other IFN-γ-producing cells such as NKT cells. NK cells are part of the first line of defence against pathogens and following activation can kill infected targets. However, removal of NK cells in vivo did not alter the ability of mice to contend with *B. abortus* infection [2].

The literature shows that the immune responses are sufficient to control *Brucella* infection, even in the absence of functional NK cell responses [3]. In this study, we did not observe significant difference for NK cell counts between patients and healthy subjects. Our findings related to NK cells in patients with brucellosis supported the results of the previously published articles [2],[3]. Salmeron et al. [10] have found that PBMC from patients with acute brucellar infection showed a significantly depressed NK cell activity, but they have suggested that this depressed activity was not related to a deficient number of NK cells, since the numbers of CD 56^+ and $CD16^+$ cells present in PBMC were similar in patients and controls. They concluded that acute brucellar infection is associated with a deficient cytotoxic activity of NK cells. Dornand et al. [11], on the contrary, show that NK cells are activated by *B. suis*-infected macrophages and that they inhibit the intracellular multiplication of the bacteria by lysing the infected cells, thus suggesting that NK cells could be one actor of the control of *Brucella* development in humans.

The NKT cell is a $CD3^+$ T cell, having one or more markers of the NK cell lineage. This cell type resides predominantly in the liver and responds solely to antigen presentation mediated by class I-like CD1-specific T cells [12],[13]. The NKT cell is especially well poised to perform a detrimental role in that it seems predisposed to IL-4 production [14]. On the other hand, it also appears capable of IFN-γ production, when stimulated by IL-12 from liver perisinusoidal macrophages (Kupffer cells) [15]. The NKT cell induces IL-12 production from antigen-presenting macrophages.

Immune response to brucellosis can be affected by NKT cells, because these cells produce the two most critical cytokines (IL-4 and IFN-γ) [16]. NKT cells responding in a Th1-like manner may upregulate NK cell activity. There are reports that the cell type may also function as a cytolytic cell [17],[18]. These modes of antigen presentation appears critical in the immune response to intracellular bacteria [19]. However, in listeriosis, NKT cells appear to have a detrimental effect, while in tuberculosis, these cells have no effect on the immune response [20],[21].

In our study, this result shows that $CD4^+$ $CD3^+$ T helper cells and $CD8^+$ $CD3^+$ cytotoxic T cells increase in brucellar infections. The higher levels of $CD3^+$ cell counts at pre-treatment period imply that cellular immunity is important to control multiplying of *Brucellae*.

To the best of our knowledge, this is the first study about NKT cells ratio in patients with acute brucellosis. In our study, no statistically significant difference was observed between patient and control groups for NKT cell percentage. Nevertheless, $CD3^+$ T cell counts increase in acute brucellar infections, and further studies are needed for cytotoxic activity of NK and NKT cells.

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