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Analysis of lymphocyte subgroups in Crimean-Congo hemorrhagic fever

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KEYWORDS
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Summary
Objectives: This study examined the association between lymphocyte subgroups and mortality in patients with Crimean-Congo hemorrhagic fever (CCHF) in Turkey.
Methods: During the spring and summer of 2007, peripheral blood was collected from hospitalized patients with suspected CCHF. Lymphocyte subgroups were characterized by fluorescence-activated cell sorting. CCHF cases were confirmed by detecting viral RNA by PCR and/or IgM antibodies by ELISA. Lymphocyte subgroups were compared between fatal and non-fatal cases. The correlation between lymphocyte subgroups and viral loads was also investigated.
Results: Seventy-seven confirmed cases of CCHF were included in this study (five cases were fatal (6.5 %)). No differences in lymphocyte subgroups were found between fatal and non-fatal cases, except for significantly higher CD3+CD8+ T cells in the fatal cases ($p = 0.017$). A positive correlation between viral load and CD3+CD8+ T cells was also detected ($p = 0.044$). There was no correlation between other lymphocyte subgroups and viral load.
Conclusions: Higher levels of CD3+CD8+ T lymphocytes were detected in fatal compared to non-fatal CCHF cases. Despite this cytotoxic immune activation, a fatal outcome could not be prevented. We hypothesize that high viral load and other factors may influence this outcome, although more studies are required to explain the pathogenesis of CCHF.
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Introduction

Crimean-Congo hemorrhagic fever (CCHF) is a tick-born disease caused by *Nairovirus*, of the family of *Bunyaviridae*.[1] It is a potentially fatal disease and is endemic in Africa, the Middle East, Central Asia, and Eastern Europe.[2—4] Since 2002, large outbreaks have also been seen in the middle, northern, and eastern regions of Turkey during spring and summer seasons.[5—11] Common clinical signs of CCHF include fever, nausea, headache, diarrhea, myalgia, petechial rash, and bleeding.[4] Leukopenia is also detected in most patients with

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CCHF. However, there are limited data on the lymphocyte subgroups affected by CCHF and their association with mortality of the disease. In the present study, the association between the lymphocyte subgroups and mortality was investigated in patients with CCHF.

Patients and methods

This prospective study was conducted at Ankara Numune Education and Research Hospital, a referral and tertiary-care hospital in Turkey. The local medical ethics committee approved the study, and all patients included in the study gave informed consent. During the spring and summer seasons of 2007, patients were admitted to the hospital with a possible case of CCHF if they had epidemiological risk factors (residence in an endemic area and/or history of a tick-bite) and had clinical signs and laboratory findings (fever, nausea and vomiting, headache, diarrhea, myalgia, petechiae, bleeding, leukopenia, thrombocytopenia, and elevated levels of transaminase, lactate dehydrogenase, and creatine phosphokinase) compatible with CCHF. Peripheral blood samples were collected in the first 72 hours following admission to detect lymphocyte subgroups, and were tested within two hours after the collection of the samples.

CCHF cases were confirmed by a serum PCR test to detect CCHF viral RNA and/or the presence of CCHF-specific serum IgM antibodies determined by ELISA. Confirmed cases of CCHF were included in the study. The other possible causes of infections such as brucellosis, salmonellosis, viral hepatitis, TORCH infections, leptospirosis, and Mediterranean spotted fever were excluded by serological tests. In all of the patients, blood cultures were negative and no focal infection sites were detected.

The TaqMan-based 1-step reverse transcriptase PCR assay was used for the detection and quantification of CCHF viral RNA.[12] To detect the CCHF-specific serum IgM antibodies, in-house ELISA tests were performed at the national reference laboratory; the antigens used in the tests were obtained from the Centers for Disease Control and Prevention (CDC).

Peripheral blood samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA). Peripheral blood lymphocyte subgroups were examined using a panel of monoclonal antibodies (MoAbs) by lysed whole blood technique. The following combinations of mouse antihuman MoAbs were used for two- or three-color staining: CD45—FITC/CD14—PE, CD3—ECD/CD4—FITC/CD8—PE, CD3—FITC/CD16+56—PE, CD3—FITC/HLA DR—PE, CD3—FITC/CD19—PE (Beckman Coulter). Phycoerythrin (PE), fluorescein isothiocyanate (FITC), phycoerythrin—Texas Red (ECD)-labeled Mouse IgG of the appropriate isotypes was used as negative control in all of the studies. Data were analyzed using Coulter system CXP software for the Beckman Coulter FC-500. Lymphocytes were gated in CD45—FITC versus side scatter plot (10 000 events in this gate were acquired) and these were further analyzed for expression of CD3 (T cells), CD19 (B cells), CD3+CD4+ (T helper cells), CD3+CD8+ (T cytotoxic cells), CD3—CD16+56+ (natural killer cells), and CD3—HLA DR+ and CD3+HLA DR+ (active T cells).

The clinical course and laboratory data of the patients were recorded prospectively on individual forms. The peripheral blood lymphocyte subgroups were compared between the fatal and non-fatal cases. The correlation between lymphocyte subgroups and viral load was also investigated.

Statistical analyses were performed using SPSS 10.0 (SPSS Inc., Chicago, Illinois) statistical package. The Kolmogorov—Smirnov test was used to check the normality of distribution. For continuous variables, differences were compared with the Student's *t*-test. The Chi-square test or Fisher's exact test was used for comparisons between groups. The association between viral load and T lymphocyte subtypes was assessed by Pearson correlation coefficient.

Results

Seventy-seven confirmed cases with positive serum PCR and/or IgM ELISA tests for CCHF, were included in the study. Five of the 77 subjects with CCHF (6.5%) died, while the remaining 72 patients (93.5%) survived.

Of the 77 patients included in this study, 60 (77.9%) had CCHF viral RNA detected by PCR and 69 (89.6%) had CCHF-specific IgM antibodies detected by ELISA. Most of these cases (79.2%) handled livestock, and half of them (53.2%) had a history of tick-bite. All of the patients were living in endemic areas within Turkey and 38 (49.4%) of them were male. The mean age of the patients was 44.3 (range 15—50) years and mean duration of symptoms was 6 (range 1—20) days.

Lymphocyte subgroups were studied for all of the patients. The difference in the lymphocyte subgroups was not statistically significant between fatal and non-fatal cases, except for CD3+CD8+ T cells (Table 1). Cytotoxic T cells (CD3+CD8+) were significantly higher in the fatal cases ($p = 0.017$). A correlation between viral load and CD3+CD8+ T cells was also detected. As the viral load increased, CD3+CD8+ T cells increased ($p = 0.044$) (Figure 1). There

Table 1 Lymphocyte subgroups in the fatal and non-fatal cases.

Lymphocyte subgroups	Fatal cases ($n = 5$) Mean ± SD value, %	Non-fatal cases ($n = 72$) Mean ± SD value, %	*p*-Value
CD3	71.2 ± 7	60.9 ± 12.1	0.066
CD19	13 ± 6.3	15.3 ± 9.7	0.600
CD3+CD4+	37 ± 5	35.6 ± 8.6	0.714
CD3+CD8+	32 ± 7	23.2 ± 8	0.017
CD3—CD16+56+	8.8 ± 8.2	12.8 ± 6.9	0.216
CD3—HLA DR+	11.4 ± 7.1	18.4 ± 10.9	0.164
CD3+HLA DR+	13.4 ± 10	9.2 ± 8.1	0.271

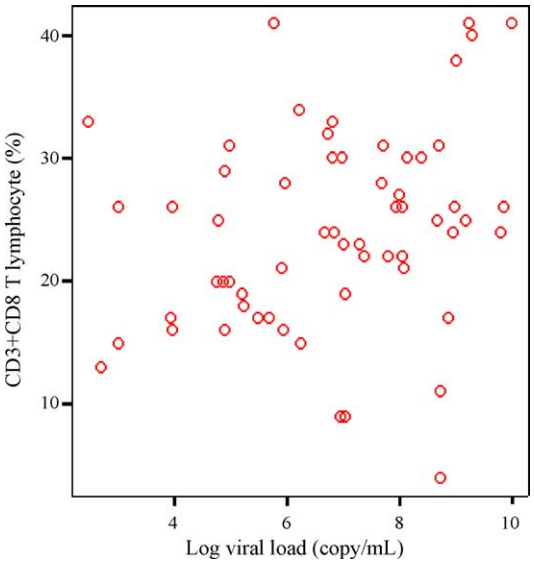

Figure 1 Correlation between viral load and CD3+CD8+ T cells. As the viral load increases, CD3+CD8+ T cells increase ($p = 0.044$).

was no correlation between the other lymphocyte subgroups and viral load ($p > 0.05$) (Table 2).

Discussion

CCHF is a severe disease with a reported mortality rate of 3–30%. Activation of immune responses and vascular damage have been detected in hemorrhagic fever virus infections.[13]

There are limited data on the pathogenesis of CCHF. It has been demonstrated that interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) are the cytokines mostly induced during the infection, and levels have been found to be higher in patients with fatal CCHF.[14,15] High levels of neopterin (a marker of cellular immune activation) and increased viral load have also been associated with mortality.[11,16] It has been suggested that hemophagocytosis occurs in patients with CCHF and has a role in the development of pancytopenia.[7]

Leukopenia is common in CCHF. However, data on the lymphocyte subgroups affected by CCHF and their association with mortality are limited. We found only one published study examining the lymphocyte subgroups in CCHF. In this study, natural killer (NK) cells were significantly higher in patients with severe CCHF, and a positive correlation was found between NK cell counts and serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and activated partial thromboplastin times (aPTT).[17] Therefore, it was suggested that elevated NK cell counts may serve as a prognostic marker in patients with CCHF. However in our study, although NK cells were less increased in fatal cases, there was no statistically significant difference between the fatal and non-fatal cases. We considered that the innate immune system might be suppressed in fatal cases leaving insufficient time for the activation of adaptive immunity. However, the numbers of fatal or severe cases are too low in both studies for any definite conclusions to be drawn. Thus we need more studies with higher numbers of patients.

Several studies have examined the lymphocyte subgroups in patients with other types of hemorrhagic fevers. In the lethal mouse Ebola model of Bradfute et al., it was shown that there is an increase in the expression of the activation and maturation marker CD44 in both CD4+ and CD8+ T cells late in infection, which suggests that there is lymphocyte activation despite apoptosis.[18] Furthermore, although functional immune responses occur in this murine model, the infection still kills the host. Several possible explanations were provided as to why this immune response was insufficient for survival.[18] First, the response may occur too late to control the rapidly replicating virus and the number of CD3+CD8+ T cells could be too low to control the high viral titers. Second, the immune response could be dampened by altered cytokine production. Third, the damage done by infection could be too severe to be overcome at the time the adaptive immune response is activated. In dengue virus infection, another model of hemorrhagic fever, an increase in CD3+CD8+ T lymphocytes and CD4/CD8 inversion has been detected.[19] The inversion was found to be significantly higher in dengue hemorrhagic fever and dengue shock syndrome than the dengue fever. In the present study, the levels of CD3+CD8+ T lymphocytes were higher in fatal CCHF cases than non-fatal cases. This shows that cytotoxic immune responses were activated in CCHF. However, despite this activation, a fatal outcome could not be prevented. We hypothesize that the high viral load, as well as other additional factors, might have influenced the outcome.

In conclusion, the pathogenesis of CCHF remains unclear. This study suggests that the cytotoxic T cell response increases lymphocyte subgroups in fatal CCHF cases, and that this correlates with the elevated viral load. However, the T cell response may be too late or insufficient to prevent death. More studies are required to explain the pathogenesis of CCHF.

Conflict of interest: No conflict of interest to declare.

Table 2 Correlation coefficients between viral load and lymphocyte subtypes.

	Lymphocyte subtype				
	CD3+CD4+	CD3+CD8+	CD3−CD16+56+	CD3−HLA DR+	CD3+HLA DR+
Log_{10} viral load					
Pearson correlation	−0.157	0.261	−0.142	−0.028	0.190
p-Value	0.230	0.044	0.278	0.834	0.147

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