Analysis of lymphocyte subgroups in Crimean-Congo hemorrhagic fever

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Introduction

Crimean-Congo hemorrhagic fever (CCHF) is a tick-born disease caused by Nairovirus, of the family of Bunyaviridae. It is a potentially fatal disease and is endemic in Africa, the Middle East, Central Asia, and Eastern Europe. Since 2002, large outbreaks have also been seen in the middle, northern, and eastern regions of Turkey during spring and summer seasons. Common clinical signs of CCHF include fever, nausea, headache, diarrhea, myalgia, petechial rash, and bleeding. Leukopenia is also detected in most patients with
CCHF. However, there are limited data on the lymphocyte
subgroups affected by CCHF and their association with mor-
tality of the disease. In the present study, the association
between the lymphocyte subgroups and mortality was inves-
tigated 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. Periph-
eral blood samples were collected in the first 72 hours fol-
lowing 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 anti-
odies, 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 Preven-
tion (CDC).

Peripheral blood samples were collected in tubes contain-
ing ethylenediaminetetraacetic acid (EDTA). Peripheral blood
lymphocyte subgroups were examined using a panel of mono-
clonal 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—ECDF/CD4—FITC/CD8—PE,
CD3—FITC/CD16+56—PE, CD3—FITC/HLA DR—PE, CD3—FITC/CD19—PE (Beckman Coul-
ter). 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 periph-
eral blood lymphocyte subgroups were compared
between the fatal and non-fatal cases. The correlation
between lymphocyte subgroups and viral load was also inves-
tigated.

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 associa-
tion 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>
<thead>
<tr>
<th>Lymphocyte subgroups</th>
<th>Fatal cases (n = 5)</th>
<th>Non-fatal cases (n = 72)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>Mean ± SD value, %</td>
<td>Mean ± SD value, %</td>
<td></td>
</tr>
<tr>
<td>CD19</td>
<td>13 ± 6.3</td>
<td>15.3 ± 9.7</td>
<td>0.600</td>
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<tr>
<td>CD3+CD4+</td>
<td>37 ± 5</td>
<td>35.6 ± 8.6</td>
<td>0.714</td>
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<tr>
<td>CD3+CD8+</td>
<td>32 ± 7</td>
<td>23.2 ± 8</td>
<td>0.017</td>
</tr>
<tr>
<td>CD3—CD16+56+</td>
<td>8.8 ± 8.2</td>
<td>12.8 ± 6.9</td>
<td>0.216</td>
</tr>
<tr>
<td>CD3—HLA DR+</td>
<td>11.4 ± 7.1</td>
<td>18.4 ± 10.9</td>
<td>0.164</td>
</tr>
<tr>
<td>CD3+HLA DR+</td>
<td>13.4 ± 10</td>
<td>9.2 ± 8.1</td>
<td>0.271</td>
</tr>
</tbody>
</table>
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. 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. High levels of neopterin (a marker of cellular immune activation) and increased viral load have also been associated with mortality. It has been suggested that hemophagocytosis occurs in patients with CCHF and has a role in the development of pancytopenia.

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