LYMMPHOCYTES WITH KILLER FUNCTION AND ACTIVATED IMMUNOCOMPETENT T LYMPHOCYTES IN THE COURSE OF SALMONELLOSIS – HUMAN TRIALS

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ABSTRACT
The present study was aimed at establishing quantitative changes in the lymphocyte subsets of predominantly killer function and activated immunocompetent T lymphocytes in the peripheral blood of patients in the course of salmonella infection and how these parameters correlated with the disease severity and outcome, in terms of early bacterial clearance. 24 adult patients with culture proven gastrointestinal Salmonellosis were enrolled in the study. They were assigned into groups on the basis of the disease severity (moderate and severe form) and on the presence of bacteria in feces upon discharge (excretors and bacteria free patients). Flowcytometric analysis was used to determine the percentage and absolute count per μl blood of: total killer cells (CD56+), lymphocytes with MHC class I restricted killer function (CD8+CD56+), lymphocytes with MHC non restricted killer function (CD8-CD56+), and activated immunocompetent T lymphocytes (CD3+HLA-DR+). It was found out that lymphocytes with killer function decreased during the acute and convalescent disease stage whereas activated immunocompetent T lymphocytes increased. Established changes in the lymphocyte subsets were similar in patients with moderate and severe disease. Only CD56+ count was significantly (p=0.01) higher in patients with severe salmonellosis. In the acute stage, in patients that were bacteria free upon discharge the level of CD56+ was found significantly (p=0.003) higher when compared to that of the remaining bacteria excretors. It is likely that CD56+ level might indicate early elimination of bacteria from feces.

Introduction
Salmonella bacteria are a major cause of food borne infectious diarrhea. In infants and immunocompromised patients Salmonella infection might be life threatening and even fatal (1, 2, 3). The pathogenesis and control of the infection still remain a challenge to researchers. Information of the immune response to Salmonella infection has been largely gained from experimental animals, mice in particular (4, 5). There is consensus about the importance of T cells for the immune response (4, 5, 6) but the role of the different T cell subsets during the distinct disease stages remains elusive. There have been only a limited number of in vivo studies in humans, focused on the defense mechanism against Salmonella infections (7, 8).

The aim of this study was to establish quantitative changes in the lymphocyte subsets with killer function and activated immunocompetent T lymphocytes in the peripheral blood of patients with Salmonellosis and their relationship with disease severity and early bacterial clearance.
Materials and Methods
The study comprised 24 adult patients (median age 37.83 ± 11.07; age range 19-56 years). All of them presented with gastrointestinal form of Salmonellosis and were admitted to the Clinics of Infectious diseases of the “St. George” University Hospital in Plovdiv. The diagnosis was confirmed by positive stool culture. 18 patients were infected with Salmonella enterica serovar Enteritidis, 5 – with Salmonella enterica serovar Typhimurium and 1 – with Salmonella enterica serovar Agona. Moderate course of infection was observed in 13 patients. Severe disease was seen in the remaining 11 patients. Upon discharge 14 patients were infection free, 10 were still excreting Salmonella bacteria in their feces. All patients were immunocompetent and were treated with antibacterial therapy according to in vitro susceptibility testing.

The intensity of the diarrhea, the presence of general intoxication and dehydration (rated according to the WHO criteria) determined disease severity. Frequent passage of loose stools (5-10/24 h), constitutional complaints and II degree dehydration characterized moderate disease. The presence of marked constitutional complaints, heavy diarrhea (over 10/24 h) and II to III degree dehydration was noted in patients with severe disease.

Control group included 37 healthy persons (median age 40.20 ± 15.32; age range 18-56 years).

Peripheral blood lymphocyte subpopulations studied, included as follows: CD56+ (total killer cells), CD8+CD56+ (lymphocytes with MHC class I molecule restricted killer function), CD8–CD56+ (lymphocytes with MHC unrestricted killer function) and activated immunocompetent T lymphocytes (CD3+HLA-DR+). The percentage and the absolute number of the cells per μl blood were determined. Repeated tests were performed: in the acute phase (day 2 to 6 following disease onset) and during convalescence (day 10 to day 12). Immunophenotyping of lymphocytes was performed using Epics XL-MCL, Coulter (USA) flow cytometer. FITC and RD1 labeled (CD8-FITC/CD56-RD and CD3–FITC/HLA-DR–RD1) monoclonal antibodies were used (Coulter, USA). CD14–RD1/CD45-FITC were employed to control light scattering. Isotyping control was performed using MsIgG1 - FITC/ MsIgG1-RD1 and MsIgG1-FITC/ MsIgM-RD1. DNA cheat, Standard-Brite and Cyto – Trol Conrol Cells were used for quality control.

One factor analysis of variance (ANOVA) was employed to compare lymphocyte subsets in the control and the patient groups. Data are expressed as means ± SEM. Statistical significance was assumed at P ≤ 0.05. Excel software packet was used for statistics.

Results and Discussion
In patients with Salmonellosis when compared to the healthy controls, the percentage (Fig. 1) of total killer cells (CD56+) was significantly reduced in the acute disease stage (p=0.003; the 95% confidence interval /CI/ was 5.22-8.04) and during convalescence (p=0.05; CI was 5.70-7.18). Lymphocytes with MHC restricted killer function (CD8+CD56+) were also reduced at the disease peak but the difference between them and those in the healthy controls was statistically insignificant (p=0.09). Lymphocytes with MHC unrestricted killer function (CD8+CD56+) were significantly reduced in the acute stage (p=0.003, CI 2.86-4.32) and during convalescence (p=0.02, CI 2.55-3.57). A reciprocal tendency was noted in activated immunocompetent T cells. They were elevated significantly in the acute disease phase (p=0.001; CI 4.29-7.71) and during convalescence (p=0.001; CI 3.95-8.32).

Similar dynamics was observed in the absolute lymphocyte counts per μl peripheral blood (Fig. 2). Total killer cells (CD56+) were reduced in the acute phase...
A percentage of lymphocyte subsets (x, ±SEM) in peripheral blood in patients with salmonellosis during ( ) - acute stage (n=24), ( ) - convalescence (n=13) and ( ) - healthy controls (n=37). Significant differences are indicated as single (* p<0.05), double (** p<0.01) or triple (*** p<0.001) asterisks.

Lymphocytes with killer function and activated immunocompetent T cells (cell count/μl) in patients with salmonellosis (x, ±SEM) during ( ) - acute stage, ( ) - convalescence and ( ) - healthy controls. Significant differences are indicated as single (* p<0.05) or double (** p<0.001) asterisks.

(p=0.006; CI 84.14-136.70) and in convalescence (p=0.01; CI 76.88-94.96). Lymphocytes with MHC class I restricted killer function (CD8+CD56+) were decreased in patients with Salmonellosis but the difference was significant only at the disease peak (p=0.02, CI 23.71-44.57), not upon discharge (p=0.2). Lymphocytes with MHC non restricted killer function (CD8-CD56+) were significantly reduced in the acute stage (p=0.006; CI 40.49-66.75) and also in the convalescence (p=0.04; CI 38.65-65.81).
Lymphocyte subsets in the acute stage (Q±SEM) in patients with moderate or severe salmonellosis and excretors or bacteria free at discharge

<table>
<thead>
<tr>
<th>Lymphocyte subsets</th>
<th>Patients</th>
<th>Clinical forms</th>
<th>P</th>
<th>excretors at discharge</th>
<th>bacteria free at discharge</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>moderate</td>
<td>severe</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD56+</td>
<td>%</td>
<td>3.7±0.81</td>
<td>7.27±0.94</td>
<td>0.01</td>
<td>4.35±1.28</td>
<td>7.25±0.87</td>
</tr>
<tr>
<td></td>
<td>count</td>
<td>79±8.11</td>
<td>137±10.13</td>
<td>0.02</td>
<td>82±9.01</td>
<td>129±11</td>
</tr>
<tr>
<td>CD8+ CD56+</td>
<td>%</td>
<td>1.75±0.35</td>
<td>1.99±0.59</td>
<td>0.74</td>
<td>16.2±0.41</td>
<td>20.7±0.53</td>
</tr>
<tr>
<td></td>
<td>count</td>
<td>40.09±8.51</td>
<td>29.15±6.67</td>
<td>0.31</td>
<td>28.41±6.61</td>
<td>38.28±7.82</td>
</tr>
<tr>
<td>CD8- CD56+</td>
<td>%</td>
<td>3.44±0.61</td>
<td>3.72±0.47</td>
<td>0.7</td>
<td>3.58±1.58</td>
<td>3.61±1.92</td>
</tr>
<tr>
<td></td>
<td>count</td>
<td>56±9.22</td>
<td>51.62±9.92</td>
<td>0.75</td>
<td>48.5±10.26</td>
<td>57.29±9.04</td>
</tr>
<tr>
<td>CD3+ HLA-DR+</td>
<td>%</td>
<td>4.42±1.14</td>
<td>6.23±1.63</td>
<td>0.39</td>
<td>4.13±1.09</td>
<td>8.54±1.24</td>
</tr>
<tr>
<td></td>
<td>count</td>
<td>103.82±29.02</td>
<td>159.15±50.62</td>
<td>0.37</td>
<td>118.11±16.12</td>
<td>155.7±42.78</td>
</tr>
</tbody>
</table>

The count of activated immunocompetent T lymphocytes in our patients was elevated at the disease peak (p=0.008; CI 74.21-193.34) and during early convalescence (p=0.002; CI 115.25-158.13).

In the acute disease stage comparison between the lymphocyte populations in patients with different disease severity (Table) did not show considerable differences in terms of quantitative characteristics. Only CD56+ lymphocytes were significantly higher in patients with severe disease. Comparison of the lymphocyte subsets in the acute stage between patients that were bacteria free on discharge and those remaining bacteria excretors showed that the total killer cell level was higher in the former.

According to most experimental animal studies CD4+ T cells are of particular importance for the acquired immune response in Salmonella infection (4, 5, 6). Human trials are scant but they stress that CD4, especially Th1 cells are central for the control of infection (7). However, recently there is increasing evidence that CD8+ lymphocytes also actively participate in the immunity against Salmonella bacteria (4, 8, 9, 10). Raupach et al. (2003) found out that both CD4+ and CD8+ lymphocytes are required for pathogen eradication and that CD4+ and CD8+ T cell dependent immune mechanisms could not compensate for each other (11). Analyzing the kinetics and magnitude of the T cell response in patients with Salmonellosis we established that the level of the lymphocyte with killer function was reduced especially during the acute disease stage. According to other authors Salmonella infection induces a limited CD8+ T cell expansion (4). Different explanations could account for our finding: firstly Salmonella bacteria induced immune suppression that could have hindered the proliferation of T cells (12). Secondly reduced cytotoxic lymphocyte counts are likely to be due to redistribution and adequate location in the intestinal mucosa where they act against antigen-bearing target cells. Experimental models with mice, infected orally with Salmonella revealed similar phenomenon (6). According to recent studies antigen specific cytotoxic T cells in the mucosa and mucosa-associated lymphatic tissues are induced mainly by intracellular pathogens penetrating mucosal surfaces. They limit infection at penetration site and promote clearance of pathogens. (13, 14).

Activation of T cells is specific or non specific occurring upon antigen presentation. Activated T cells enhance cytokine release, expression of surface rest molecules and loss of some surface markers and expression of others. Activation kinetics varies. HLA–DR antigen (class II MHC) is
a marker of activated T cells (15).

Accumulated data showed a significant increase in CD3+HLA-DR+ counts in patients with Salmonella infection during the acute stage. The increase in activated T cells is even more pronounced as accompanied by reduced immunocompetent (CD3+) cells. According to our data, as a percentage of total immunocompetent cells, CD3+HLA-DR+ present - 3.34% in healthy controls, 8.55% in patients during the acute disease stage and 8.33% - in patients during convalescence (data are not shown in the results). These data are consistent with the data presented by other authors (4, 7). They have documented that T cell activation occurs during early disease stage. It has been suggested that virulent Salmonella bacteria cause incomplete T cell activation leading to an activated phenotype of a large fraction of T cells but allowing only a minority of T cells to proliferate (4). More strongly reduced lymphocytes with MHC restricted and MHC unrestricted killer function in patients with severe disease are likely to be attributable to severe intoxication. It is known that T lymphocyte are particularly sensitive to it (4). Another possible explanation is the inhibition of cellular immunity as a result of excess antigens present (high zone immune tolerance) in patients with severe disease (15). However, increased number of total killer cells found in these patients provides no evidence in support of the latter thesis. It is likely that total killer cells contribute to the heavy disease course due to their cytotoxic properties. The absence of statistically significant difference in the studied parameters between patients with moderate and severe disease is probably due to the marked intoxication present in all of them.

Higher CD56+ lymphocyte counts in the acute stage found in patients that were free of infection upon discharge, illustrate their role in the clearance of Salmonella bacteria. This fact provides additional evidence in support of the observation that the largest subset of lymphocytes that were able to kill Salmonella bacteria is likely to be the NK cells (16).

In summary, our study provided evidence that killer cells (CD56+, CD8-CD56+, CD8+CD56+) were reduced in patients with Salmonellosis. In the acute disease stage CD56+ level might be a predictor of early elimination of bacteria from feces.

REFERENCES