Changes of Cytolytic Cells and Perforin Expression in Patients with Posttraumatic Stress Disorder

Ivana Škarpa, Gordana Ružaša, Ljiljana Moro, Darko Manestar, Mladen Petrovečki, Daniel Rukavina

Departments of Psychiatry, Otorhinolaringology, Computer Science, and Physiology and Immunology, Rijeka University School of Medicine, Rijeka; and Dubrava University Hospital, Zagreb, Croatia

Aim. To define phenotypic characteristics of cytotoxic T lymphocytes (CTL) and natural killer cells (NK) in peripheral blood, frequency of somatic symptoms, and level of anxiety and depression in 25 patients clinically diagnosed with chronic post-traumatic stress disorder (PTSD).

Methods. Patients were divided into two sub-groups according to the stressor: 18 PTSD patients with the battlefield experience and 7 PTSD patients with battlefield experience who were tortured as the prisoners of war (POW) in Bosnian-Serbian camps. The control group consisted of 15 healthy volunteers matched to the patients by sex and age. We tested all patients using Beck's depression inventory, Spielberger anxiety test, and somatic disturbance list, and analyzed their peripheral blood lymphocytes using flow cytometry with the double fluorescence staining of cell surface antigens (CD3, CD4, CD8, CD16, and CD56) and intracellular cytolytic molecule perforin (P), a mediator of cytolytic action at the molecular level.

Results. All PTSD patients showed a significant level of anxiety, depression, and numerous somatic symptoms. The only significant difference between PTSD patients with and without POW experience was in the anxiety level (median, 71; range 61-79; vs median, 65; range, 49-77). PTSD patients with POW experience had significantly higher levels of CD16+ cells (median, 37%; range, 16-55%) than those without it (median, 12%; range, 5-37%). Double labeling for intracellular P antigen and cell surface antigens showed the highest levels of CD16+P+ (median, 33%; range, 15-40%; vs median, 10%; range, 3-29%) and CD56+P+ (median, 21%; range, 11-40%; vs median 8%; range, 1-30%) cells in PTSD-POW patients.

Conclusion. Chronic PTSD patients who survived concentration camps show the most numerous alterations in PBL phenotype, the highest number of perforin-containing cells, and a significantly higher level of anxiety.

Key words: antigens, CD56; killer cells, natural; membrane, proteins; stress disorders, post-traumatic; T-lymphocytes, cytotoxic; veterans; war

Sufferings during the 1991-1995 wars in Croatia and Bosnia and Herzegovina have left profound consequences on the psychological, endocrine, and immune status of the multiply traumatized and displaced persons (1,2). However, there are few reports which tried to associate immune status of patients with chronic posttraumatic stress disorder (PTSD). PTSD is a relatively new diagnostic entity characterized by symptoms categorized in four groups (A, B, C, and D) (3). Group A refers to the affirmation of the traumatic experience during which the person felt helpless, terrified, or frightened. Group B consists of five symptoms associated with continued re-experience of the traumatic event through pictures, thoughts, or feelings. Group C comprises seven symptoms of avoidance, and group D five symptoms of increased arousal (3). Furthermore, there are two developmental stages of PTSD: acute and chronic. The DSM-IV diagnostic criteria for PTSD allow clinicians to specify whether the disorder is acute (if the symptoms have lasted less than three months) or chronic (if the symptoms have lasted three months or longer). The presence of other disorders and symptoms that PTSD shares with depression, anxiety, and somatoform disorders (4-10) often complicates the diagnosis of PTSD.

In the last 20 years, there has been a tremendous upsurge in the interest in the relationship between psychological condition and immunological functions. However, there is still no decisive evidence about "the real mediator" of the stress reaction, which starts all other biochemical and neurophysiological processes, including cellular and humoral immunity (11). The lymphocytes express membrane receptors for bioactive substances, such as adrenaline, acetylcholine, histamine, endomorphine, ACTH, and several neuropeptides that increase in concentration during stress reaction (10-13). This can help to explain the possible mechanisms that lead to changes, not

www.cmj.hr 551
only in number and phenotype of peripheral blood lymphocytes, but also in their function during or following the stressful event (12,13).

Lymphocyte-mediated cytotoxicity is one of the basic mechanisms of protection against viral and other intracellular parasite infections and of elimination of cells expressing foreign characteristics, such as transplants, tumor antigens, and autoantigens (14). Cytotoxic lymphocytes, both T lymphocytes (CD3+ CD8+ P) and natural killer (NK) cells (CD3CD16/56+ P), are endowed with strong cytotoxic potential (14). For the exertion of their cytotoxic function at the molecular level, cytolytic cells use a cytolytic molecule called pore forming protein, or perforin, and granzymes (serine esterases), which are located in lytic granules of cytolytic cells (15,16).

Our aim was to analyze alterations of two lymphocyte subpopulations responsible for cell-mediated cytotoxicity (CMC) in persons diagnosed with chronic PTSD, especially in relation with the intensity of trauma. The patients were divided into sub-groups according to the stressor: patients with the battlefield experience and patients who, beside having battlefield experience, also experienced the torture as the prisoners of war in detention camps (POW).

Subjects and Methods

Subjects

Twenty-five Croatian soldiers clinically diagnosed with chronic PTSD were examined after undergoing diagnostic procedure based on DSM-IV criteria and giving informed consent. All were men aged between 27 and 48 years (median, 39 years). The duration of their battlefield engagement ranged from 26 to 48 months (median, 34 months). Seven of them spent 120-240 days (median, 135 days) in prison (prisoners of war, POWs, in Bosnian-Serbian prisons). They were divided in two groups, depending on whether they had been prisoners and experienced torture in camps or not. There were 18 PTSD patients with the battlefield experience only, and 7 PTSD patients with battlefield experience and patients who, beside having battlefield experience, also experienced the torture as the prisoners of war in detention camps (POW).

1) PTSD questionnaire according to DSM-IV (3) – a structured diagnostic interview based on DSM-IV criteria, which is commonly used in psychiatric diagnostic procedure.

2) Somatic symptoms questionnaire – a self-assessment questionnaire consisting of 20 items to assess the intensity of somatic symptoms (headache, loss of appetite, feeling of fever, trembling of knees, inability to relax, pressure in the thorax, digestive disturbance, muscular tension, hyperegness to sound, periodical pounding heartbeat, paralysis or feeling "pins and needles", feeling of pressure in the head, increased perspiration, spine symptoms, dizziness, nervousness, feeling of suffocation, tremor of the hands, prostration, tremor of the whole body) on the scale of 0-4 (0 never, 1 rarely, 2 sometimes, 3 frequently, 4 always).

We constructed the questionnaire according to our own experience, with the aim of systematic observation of somatic disturbance in PTSD patients.

3) Spielberger’s State-Trait Anxiety Inventory (STAI) – a self-assessment questionnaire divided in two parts: STAI X-1 and STAI X-2 (17). The first part consists of 20 descriptive statements assessing the state of anxiety at the time of testing on the scale of frequency 1-4 (1 not at all, 2 a little, 3 quite, 4 very much). The second part (STAI-X-2) consists of 20 descriptive statements assessing the anxiety as a personality feature on the scale of frequency 1-4 (1 almost never, 2 sometimes, 3 often, 4 almost always).

4) Beck depression index (BDI-M) – self-assessment scale of 21 items evaluating the intensity of the depression (18). According to their total score on the scale, patients could be classified into five subgroups: 0-3 points – showing complete absence of depression; 4-9 points – showing the depression within normal range; 10-15 points – showing mild depression; 16-19 points – showing moderate depression; 20 or more points – showing profound depression.

The psychiatrist used PTSD questionnaire and somatic symptoms questionnaire, whereas the psychologist applied STAI and BDI-M. Somatic symptoms questionnaire, STAI, and BDI-M have been standardized to the population investigated.

Peripheral Blood Lymphocytes

Heparinized venous blood was diluted and separated on Lymphoprep (Nycomed Pharmaas, Oslo, Norway) for 20 min on 800 G. After the band of cells at the interface was collected and washed twice with RPMI 1640, peripheral blood lymphocytes (PBL) were used for analysis. Viability was assessed by trypan blue and was always around 98%.

Simultaneous Measurement of Cell Surface (CD Markers) and Intracellular Antigen (Perforin) by Flow Cytometry

The method for simultaneous detection of perforin (intracellular antigen) and cell surface antigens is described in detail elsewhere (19). Approximately 10^6 PBLs were fixed in 100 µl phosphate-buffered saline (PBS) containing 1% paraformaldehyde (pH 7.4) for 10 min at room temperature. After being washed twice in fluorescence-activated cell sorting (FACS) buffer, the cells were permeabilized in Saponin buffer (0.1% saponin) (Sigma, Poole, UK) for 20 min at room temperature. Murine monoclonal antibody reacting with human perforin (P) MoAb δC9 (IgG1, purified from Balb/c ascites) (20) was added to the suspension of cells in saponin, at final concentration of 8-10 µg per sample and incubated for 30 min at room temperature. Murine monoclonal antibody reacting with human perforin (P) MoAb δC9 (IgG1, purified from Balb/c ascites) (20) was added to the suspension of cells in saponin, at final concentration of 8-10 µg per sample and incubated for 30 min at room temperature. All further steps, secondary antibody, FITC-conjugated goat-antimouse Ig (Becton Dickenson, San Jose, CA, USA), and rising procedures were performed in Saponin buffer.

Cells surface antigens (CD3, CD4, CD8, CD16, and CD56) were then stained in FACS buffer at a final concentration of monoclonal antibody of 10 µg/ml. (Becton Dickenson) and 100 µL were added to the pelletted cells. After 30-min incubation at +4°C, cells were washed with FACS buffer and stained with avidin-phycocerythrin (20 µg/mL, Becton Dickenson) for 30 min. A minimum of 10^5 cells was analyzed on FACSscan (Becton Dickenson).

Statistical Analysis

Numerical data are presented as median values, with 25th-75th percentiles in parentheses. The difference between groups investigated was calculated with Kruskal-Wallis non-parametric test. If three groups were compared and the difference was
found significant (p<0.05), Mann-Whitney U-test was used as a post-hoc test to establish where the difference exists by comparing the control group with patient groups (three comparisons). The 5%-level of significance was therefore set as a p<0.017 in the U-test. Statistics was done with SPSS software (SPSS for Windows 7.5, SPSS Inc., Chicago, IL, USA).

Results

PTSD and Somatic Symptoms

Patients reported many somatic symptoms. Some patients had only one symptom, whereas others had more of them that appeared simultaneously. All symptoms listed in the test were present in various degrees in all PTSD patients. In the group of patients with POW experience, the most frequent symptoms were inability to relax, nervousness, spine symptoms, feeling of pressure in the head, and hypersensitivity to sound. In the patients without POW experience, the most frequent symptoms were nervousness, inability to relax, feeling of pressure in the head, tremor of the hands, and prostration. However, there were no statistically significant differences between the groups (Table 1).

PTSD and Anxiety

The first 20 items of STAIX-1 questionnaire (scale 0-4) reflected the level of anxiety in patients during the testing. All PTSD patients scored above level 3 (moderate to profound), significantly higher then the control group (score 71 and 65 compared with 38 in controls, p<0.001) (Table 1). Analysis of each patient’s total score showed that scores were significantly higher in the group of patients with POW experience.

The anxiety is one of possible personality features (STAIX-2), which can significantly change as a result of emotional and/or somatic reactions to stress. It was found that anxiety was a significant feature of patients’ personalities. The intensity of anxiety in all patients tested was above level 3 on the scale 0-4 (data not shown), with no statistically significant difference between the two groups of patients.

PTSD and Depression

As described above, the intensity of depression can be evaluated by BDI-M. There are five categories, and the score of 20 or more points shows profound depression. All PTSD patients had a high score on the BDI-M scale (with POW experience; range, 27-87; and without POW experience 30; range, 16-55), compared with the control group (p<0.001), meaning that they suffered from profound depression (Table 1). The difference between the scores in the two patient groups was not statistically significant (Table 1).

Phenotype of PBL in PTSD patients

Phenotype characteristics of T lymphocytes and NK cells are presented in Table 2. Values of CD8+ (p<0.001) and CD16+ (p<0.002) cells were significantly higher in patients than in healthy controls. The highest level of P+ cells was found in group of patients with POW experience (44%; range 19-49%), but did not differ significantly from their level of perforin expression in the group without POW experience (26%; 11-50%).

The increase of CD8+ cells in PTSD patients changed the ratio of CD4/CD8 cells (Table 2), which was significantly lower in patients than in healthy controls (p<0.001).

Characteristics of Perforin-Positive Cells

PBL were double-labeled simultaneously for intracellular P antigen and for a panel of cell surface molecules characterizing T lymphocytes (CD3, CD4, and CD8) and NK cells (CD56 and CD16). Accordingly, double-positive cells (surface marker+ and P+) were represented as a percentage of total PBL counted (Table 3). Significant increase of CD4+ P+ cells was noted.

Table 1. Frequency of somatic symptoms and levels of anxiety and depression in patients with posttraumatic stress disorder (PTSD) during the testing

<table>
<thead>
<tr>
<th>Psychosomatic disturbance</th>
<th>Control (n=15)</th>
<th>PTSD patients</th>
<th>H</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>POW (n=7)</td>
<td>not POW (n=18)</td>
<td></td>
</tr>
<tr>
<td>Somatic symptoms (STAIX-1)</td>
<td>9 (4-15)</td>
<td>56 (49-69)</td>
<td>55 (38-61)</td>
<td>25.08</td>
</tr>
<tr>
<td>Anxiety (BDI-M scale)</td>
<td>38 (25-47)</td>
<td>71 (67-78)</td>
<td>65 (55-66)</td>
<td>21.87</td>
</tr>
</tbody>
</table>

Table 2. Phenotype characteristics of T lymphocytes and NK cells in patients with posttraumatic stress disorder (PTSD) with or without the experience as the prisoners of war (POW)

<table>
<thead>
<tr>
<th>CD marker (%)</th>
<th>Control group (n=15)</th>
<th>PTSD patients</th>
<th>H</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POW (n=7)</td>
<td>not POW (n=18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>63 (53-65)</td>
<td>57 (48-61)</td>
<td>63 (56-69)</td>
<td>1.89</td>
</tr>
<tr>
<td>CD4</td>
<td>34 (33-44)</td>
<td>37 (27-41)</td>
<td>37 (32-43)</td>
<td>0.24</td>
</tr>
<tr>
<td>CD8</td>
<td>22 (17-24)</td>
<td>29 (27-34)</td>
<td>34 (26-38)</td>
<td>19.50</td>
</tr>
<tr>
<td>CD4/CD8</td>
<td>1.8 (1.5-2.2)</td>
<td>1.3 (0.8-1.6)</td>
<td>0.9 (0.9-1.5)</td>
<td>15.81</td>
</tr>
<tr>
<td>CD16</td>
<td>6 (5-25)</td>
<td>37 (27-39)</td>
<td>12 (9-25)</td>
<td>12.22</td>
</tr>
<tr>
<td>CD56</td>
<td>23 (14-28)</td>
<td>20 (14-35)</td>
<td>16 (7-22)</td>
<td>3.74</td>
</tr>
<tr>
<td>Perforin</td>
<td>28 (24-30)</td>
<td>44 (25-47)</td>
<td>26 (17-32)</td>
<td>2.49</td>
</tr>
</tbody>
</table>

1Data are presented with median values with 25th-75th percentiles (in parentheses) of proportion of positive cells in peripheral blood lymphocytes.
2H – statistics of Kruskal-Wallis nonparametric test with p-level of computed significance.
3PTSD-groups that differ significantly from the controls (post-hoc testing, when p<0.05).
4PTSD-groups that differ significantly between each other (post-hoc testing, when p<0.05)
cells in PTSD patients without POW experience was found (5%; range, 3-7%; vs 1.0%; range, 1-2%; p<0.001). There was also significant difference in double-positive NK cells. However, the percentage of CD16 +P+ was higher in PTSD patients with POW experience (33%; range, 15-40%; vs 14%; range, 0.5-24%; p<0.007) and the percentage of CD56+P+ cells was lower in PTSD patients without POW experience (8%; range, 1-30%; vs 15%; range, 12-30%; p=0.01), when compared with healthy controls (Table 3). Significant differences were found when PTSD patients were analyzed according to their POW experience (Table 3). The percentage of CD16+P+ and CD56+P+ cells was the highest in the PTSD patients with POW experience (33% and 21%, respectively) and significantly higher than in PTSD without POW experience group (10% CD16+P+; p=0.007; and 8% CD56+P+; p=0.01).

Table 4 illustrates phenotype characteristics of P+ cells in all PTSD patients. In the healthy control group, P+ cells were predominantly NK cells (86%; range, 42-91% of CD56+ vs 39%; range, 11-72% of CD3+). A change in this ratio was found in PTSD patients with approximately equal percentages of NK and T lymphocytes (48%; range, 6-98% of CD56+ vs 44%; range, 9-93% of CD3+). In all PTSD patients a significant increase in CD4+ cells (10%; range, 3-45% vs 3%; range, 2-8%; p<0.001) and decrease in CD56+ cells (48%; range, 6-98% vs 86%; range, 42-92%; p=0.002) among the P+ cells was found, compared with healthy controls (Table 4).

Table 4. Phenotype characteristics of perforin-positive peripheral blood lymphocytes in patients with posttraumatic stress disorder (PTSD)*

<table>
<thead>
<tr>
<th>CD marker (%)</th>
<th>Control group (n=15)</th>
<th>PTSD (n=25)</th>
<th>H*</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>39.0 (15.6-61.1)</td>
<td>44.4 (32.3-57.7)</td>
<td>0.59</td>
<td>0.442</td>
</tr>
<tr>
<td>CD4</td>
<td>3.5 (2.1-5.8)</td>
<td>10.3 (6.5-17.4)</td>
<td>15.97</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD8</td>
<td>45.0 (19.4-51.4)</td>
<td>44.7 (26.6-55.5)</td>
<td>0.78</td>
<td>0.379</td>
</tr>
<tr>
<td>CD16</td>
<td>67.5 (49.0-75.4)</td>
<td>51.9 (25.0-72.6)</td>
<td>0.98</td>
<td>0.321</td>
</tr>
<tr>
<td>CD56</td>
<td>86.0 (54.9-98.2)</td>
<td>47.8 (25.3-60.8)</td>
<td>10.78</td>
<td>0.002</td>
</tr>
</tbody>
</table>

*Data are presented with median values with 25th-75th percentiles in parentheses of proportion of positive cells in peripheral blood lymphocytes.

**H – statistics of Kruskal-Wallis nonparametric test with p-level of computed significance.

### Discussion

This study shows that the chronic PTSD patients who survived concentration camps have the most numerous alterations in PBL phenotype, the highest number of perforin-containing cells, and a significantly higher level of anxiety. All PTSD patients had a high level of anxiety, depression, and somatic symptoms, compared to healthy controls. However, anxiety level was significantly higher in patients with POW experience than in patients without it. The importance of stressors to the intensity of PTSD symptomatology has already been described (8,11).

Previous research has shown the influence of the war stress and torture on the cellular cytotoxicity (1,2), whereas perforin-mediated cellular cytotoxicity in patients with chronic PTSD was for the first time analyzed in this study. A number of alterations were found, primarily in cells mediating cellular cytotoxicity, both CTL and NK cells. Those cells mostly use perforin pathway at the molecular level (15,16). Perforin can be used as a marker to identify functionally activated cytolytic lymphocytes in vivo (15). Perforin expressing CTL and NK cells are not homogenous cell populations, as suggested previously (21). In all PTSD patients a significant increase in CD4+ cells (10%; range, 3-45% vs 3%; range, 2-8%; p<0.001) and decrease in CD56+ cells (48%; range, 6-98% vs 86%; range, 42-92%; p=0.002) among the P+ cells was found, compared with healthy controls (Table 4).
in patients with POW experience this mechanism is important. Further, increase in CD8+ cells in the population of perforin positive cells points to the possible role of CTL in MHC class II restricted potential in patients with chronic PTSD.

We found significantly more CD8+ PBLs in PTSD patients. The increase in CD8+ cells could be a consequence of increase in a subset of cells that strongly express CD8 antigen but do not contain perforin. These cells are therefore designated as CD8 bright+perforin− (19), and are the most numerous of all CD8+ PBLs (19). The study of Dekaris et al (1) on another detention camp also showed increase in CD8+ cells, and decrease of naïve T3+1 (CD4+CD45RA−) lymphocytes.

Although the most pronounced immunological changes were found in PTSD patients with POW experience, who also had the highest level of anxiety, we cannot directly associate the psychiatric and somatic symptoms with changes in CTL and NK cells in those patients, because the malnutrition to which they were exposed may have disturbed functioning of their immune system (24).

References


Received: March 1, 2001
Accepted: July 23, 2001

Correspondence to:
Daniel Rukavina
Department of Physiology and Immunology
Rijeka University School of Medicine
B. Branchetta 20
51000 Rijeka, Croatia
daniel@medri.hr