Activated T Lymphocytes Disappear from Circulation during Endotoxemia in Humans

K. S. Krabbe, H. Bruunsgaard, J. Qvist, L. Fonsmark, K. Møller, C. M. Hansen, P. Skinhøj, and B. K. Pedersen*

Department of Infectious Diseases, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark

Received 25 June 2001/Returned for modification 7 November 2001/Accepted 22 January 2002

Seventeen volunteers received an intravenous bolus of endotoxin (2 ng/kg of body weight). Endotoxin-induced lymphopenia was constituted mainly by cells with an immature phenotype (CD45RA⁺ CD45RO⁻) that were less likely to undergo apoptosis (CD28⁺), whereas cells with the highest rates of disappearance were characterized by an activated phenotype (CD45RA⁻ CD45RO⁺) as well as a phenotype linked to apoptosis (CD95⁺ CD28⁻). In conclusion, endotoxin-induced lymphopenia reflects the disappearance from the circulation of activated lymphocytes prone to undergo apoptosis.

The question addressed in the present study was whether apoptosis represented a mechanism contributing to endotoxin-induced lymphopenia. CD28 protects against apoptosis (11), whereas the triggering of CD95 (Fas) leads to apoptosis (7, 8). Furthermore, costimulation via CD28 has been demonstrated to be necessary for optimal proliferation upon stimulation (5, 6, 13). Blood lymphocyte concentration decreases with age (2). Aging is also characterized by an increased number of circulating memory cells, a decreased number of naive cells (5, 12, 16), down regulation of CD28 (3, 4), and an increased expression of CD95 within both CD4⁺ and CD8⁺ cells. Increased apoptosis of T-cell subsets in elderly humans has been demonstrated (1). Thus, the purpose of the present study was to test the hypothesis that apoptosis is involved in endotoxin-induced lymphopenia and that this is more pronounced in elderly subjects.

Volunteers. Eight healthy young subjects aged 20 to 28 years and nine elderly subjects aged 61 to 69 years were included as previously described (9). Subjects were given an intravenous bolus (1 min) of Escherichia coli endotoxin (lot EC-6) at a concentration of 2 ng/kg of body weight.

Flow cytometry analyses. Whole blood was analyzed with a flow cytometer (Epics XL-MCL; Coulter, Hialeah, Fla.). The following antibodies were used: (i) fluorescein-conjugated mononuclear antibodies CD16 (clone 3G8), CD45RA (clone ALB11), and CD28 (clone CD28.2) from Immunotech (Marseille, France) and CD19 (clone HD37) from DAKO (Glostrup, Denmark); (ii) phycoerythrin-conjugated mononuclear antibodies CD95 (clone DX2) from Becton Dickinson (San Jose, Calif.) and CD3 (clone UCHT1) from DAKO; (iii) fluorescein- and phycoerythrin-conjugated mononuclear antibodies CD45RO (clone MT310) and CD8 (clone DK25) from DAKO.

As negative controls, we used fluorescein–phycoerythrin–carboxyamin-5-conjugated immunoglobulin G1 (clone 679.1MC7) and phycoerythrin and Texas Red-conjugated immunoglobulin G2a (clone U7.27) from Becton Dickinson.

Apoptosis. Peripheral blood mononuclear cells were isolated from peripheral blood by density gradient centrifugation (Lymphoprep Nyegaard, Oslo, Norway) on Lymphoprep tubes (Greiner, Frickenhausen, Germany) and washed three times in RPMI medium (Gibco, Grand Island, N.Y.).

Cells (10⁶) were incubated for 20 h in either medium alone or medium with phytohemagglutinin (PHA) (final concentration, 10 µg/ml). Quantification of apoptotic cells in different stages was performed by staining cells with 7-amino-actinomycin D (7AAD) according to the method described by Schmid et al. (14). Staining with 7AAD discriminates living cells from early apoptotic cells and from cells that have lost their membrane integrity (late-apoptotic or dead cells).

After the cells were washed, they were incubated with 20 µg of 7AAD (Sigma)/ml in phosphate-buffered saline for 20 min at 4°C in the dark. Cells were computer analyzed immediately after being stored in their staining solution. Cell debris characterized by a low forward- to side-scatter signal in conjunction with a low 7AAD signal was excluded by gating.

Addition of the surface markers CD4, CD8, and CD45RO was included in the protocol, but they were found to be powerfully down regulated by PHA and thus were not detectable.

Statistical analyses. Parameters were evaluated by an analysis of variance (ANOVA) for repeated measurements (model y = age × t + age + t + K, where t is time). If a significant interaction (age × time) was found (P < 0.05), a two-sample t test for independent groups was used to detect age-related differences in changes from baseline levels.

Flow cytometric analyses. Table 1 demonstrates that the concentrations of all lymphocyte subsets decreased during endotoxemia. The percentages of CD4⁺, CD19⁺, and NK cells changed over time (ANOVA, P < 0.001 in all cases), whereas the percentage of CD8⁺ cells did not (Table 1). Within the CD4⁺ and CD8⁺ subsets, the following subgroups were de-
CD4+ subset. ANOVA revealed that the percentages of naïve CD4+ memory cells and naïve cells (ANOVA, \( P < 0.0005 \)) changed over time (Fig. 1). Furthermore, at baseline, the elderly group showed lower levels of naïve CD4+ cells (\( P = 0.045 \)) and higher levels of memory CD4+ cells (\( P = 0.018 \)) than those in the young group (Table 2). Accordingly, subsequent statistical analyses compared the absolute changes from baseline between the two age groups. ANOVA of data on naïve CD4+ cells revealed a difference in values for age times \( t (P = 0.018) \), which could not be found by Student’s \( t \) tests. In both groups, the percentage of naïve cells within the CD4+ subset increased at \( t = 1.5 \) h (young, \( P < 0.0005 \); elderly, \( P = 0.002 \)) and was back to baseline levels at \( t = 5 \) h (Fig. 1). The percentage of memory cells within the CD4+ T cells in the blood was lower at \( t = 1.5 \) h (\( P < 0.0005 \)) and did not differ from baseline levels at \( t = 5 \) h. No difference between age groups was detected. The percentage of intermediary CD4+ T cells did not change over time, and there were no differences between groups.

CD8+ subset. The percentages of CD8+ memory cells and naïve cells changed over time (ANOVA, \( P < 0.0005 \) in both cases) (Fig. 1, bottom panels). At baseline, the elderly group showed lower levels of naïve CD8+ cells (\( P = 0.045 \)) and higher levels of memory CD8+ cells (\( P = 0.032 \)) and higher levels of memory CD8+ cells (\( P = 0.052 \)) than those in the young group (Table 2). Accordingly, subsequent statistical

### TABLE 1. Mean percentages of CD4+, CD8+, CD19+, and NK cells of total circulating lymphocytes and mean actual concentrations of these subgroups in blood

<table>
<thead>
<tr>
<th>t (h)</th>
<th>Mean % of cells (mean no. of cells/ml) (n = 17)</th>
<th>Mean % of cells (mean no. of cells/ml) (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4+</td>
<td>CD8+</td>
</tr>
<tr>
<td>0</td>
<td>41.1 (768)</td>
<td>24.7 (485)</td>
</tr>
<tr>
<td>1.5</td>
<td>50.8b (403)b</td>
<td>21.8 (189)b</td>
</tr>
<tr>
<td>5</td>
<td>37.9 (163)b</td>
<td>23.7 (106)b</td>
</tr>
</tbody>
</table>

a NK cells are defined as CD56- and/or CD16+ lymphocytes.
b \( P < 0.005 \) in a comparison with baseline values.

c \( P = 0.059 \) in a comparison with baseline values.

![CD4+ naive cells](image1.png)

![CD4+ memory cells](image2.png)

![CD8+ naive cells](image3.png)

![CD8+ memory cells](image4.png)

FIG. 1. Distribution of naïve (CD45RA+CD45RO−), intermediary (CD45RA+CD45RO−), and memory (CD45RA−CD45RO+) T cells in whole blood in the course of human endotoxemia (\( n = 17 \)). Geometric means and 95% confidence intervals are shown. *, \( P < 0.05 \) in a comparison with baseline values.
TABLE 2. Lymphocyte subpopulations at baseline and the effect of age

<table>
<thead>
<tr>
<th>Cells</th>
<th>Lymphocyte subset</th>
<th>% of lymphocyte subset in*: Young subjects (n = 8)</th>
<th>Elderly subjects (n = 9)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+</td>
<td>CD45RA+ CD45RO- naive cells</td>
<td>43.8</td>
<td>26.9</td>
<td>0.045</td>
</tr>
<tr>
<td></td>
<td>CD45RA+ CD45RO+ memory cells</td>
<td>42.3</td>
<td>60.7</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>CD45RA+ CD45RO+ intermedia cells</td>
<td>2.5</td>
<td>2.0</td>
<td>NS^a</td>
</tr>
<tr>
<td>CD8+</td>
<td>CD45RA+ CD45RO- naive cells</td>
<td>61.1</td>
<td>44.7</td>
<td>0.032</td>
</tr>
<tr>
<td></td>
<td>CD45RA+ CD45RO+ memory cells</td>
<td>26.0</td>
<td>36.4</td>
<td>0.052</td>
</tr>
<tr>
<td></td>
<td>CD45RA+ CD45RO+ intermedia cells</td>
<td>4.9</td>
<td>10.1</td>
<td>NS</td>
</tr>
<tr>
<td>CD4+</td>
<td>CD28+</td>
<td>92.4</td>
<td>90.6</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>CD95+</td>
<td>43</td>
<td>71.9</td>
<td>0.007</td>
</tr>
<tr>
<td>CD8+</td>
<td>CD28+</td>
<td>56.6</td>
<td>44.3</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>CD95+</td>
<td>26.8</td>
<td>28.1</td>
<td>NS</td>
</tr>
<tr>
<td>CD3-</td>
<td>CD56+ CD16+</td>
<td>8.2</td>
<td>8.7</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>CD56+ CD16+</td>
<td>61.9</td>
<td>71.3</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>CD56+ CD16-</td>
<td>29.8</td>
<td>19.9</td>
<td>NS</td>
</tr>
</tbody>
</table>

^a Geometrical means are given for CD4 and CD8 subsets. Means are given for NK subsets.

^b NS, not significant.

analyses compared the absolute changes from baseline between the two age groups.

The percentages of CD8+ naïve T cells changed on the basis of differences between age groups (ANOVA age × time, P = 0.034). Thus, at a t of 1.5 h, the increase in the young group was greater than that in the elderly group (P = 0.019), a difference which remained present at a t of 5 h (P = 0.035). When values were analyzed separately, it was revealed that the percentage of naïve cells in the elderly group did not actually change but that the percentage of naïve CD8+ cells in the young group showed a marked increase at t’s of 1.5 and 5 h (P = 0.003 and 0.004, respectively).

There were age-related differences in the increases in CD8+ memory cells over time (ANOVA, P < 0.0005). Thus, at both 1.5 and 5 h, the decrease in CD8+ cells was larger in the young group than in the aged group (P = 0.01 and 0.003, respectively). Similarly to what was found for naïve cells, when the groups were analyzed separately, no change over time was detected in the aged group but there was a marked decrease for the young group from 0 to 1.5 h (P < 0.0005) which remained present at a t of 5 h (P = 0.001 compared to baseline values). The proportion of intermediary CD8+ cells did not change over time, and there were no age-related differences in values.

**CD28 and CD95.** Analysis of cells expressing CD95 showed only minor changes in response to endotoxia. The most remarkable changes were demonstrated within two subgroups, CD28+ and CD95+ CD28- cells.

The percentage of CD4+ and CD8+ cells expressing CD28 changed over time in the course of endotoxia (ANOVA, P = 0.001 and 0.003, respectively) and with no difference between age groups (Fig. 2). Thus, at 1.5 h, the percentage of CD28+ cells had increased significantly compared to baseline values (P = 0.001 for CD4+ and P = 0.009 for CD8+ cells) and was still elevated at 5 h compared to baseline values (P = 0.037 for CD4+ and P < 0.0005 for CD8+ cells).

Simultaneously, the proportion of CD4+ cells that were CD95+ CD28- changed during the period of investigation (ANOVA, P < 0.0005 for CD4+ cells and P = 0.015 for CD8+ cells). Thus, at t’s of 1.5 and 5 h, their percentages were decreased compared to baseline values (P < 0.0005 and P = 0.005 for CD4+ cells and P = 0.005 and P = 0.01 for CD8+ cells).

At baseline, the elderly subjects had more CD4+ cells expressing CD95 than the young subjects (P = 0.007) whereas the young group showed a tendency towards having more CD8+ cells expressing CD28 than the aged group (borderline significant) (Table 2).

**NK cells.** There were significant changes in the distribution of the percentages of NK cell subsets. Changes over time occurred in the proportions of CD3- CD56+ CD16+ and CD3- CD56- CD16+ cells subsets (ANOVA, P < 0.0005 in both cases), whereas no changes over time occurred in the proportion of CD3- CD56- CD16- cells within the NK group.

![FIG. 2. Percentages of CD28+ cells and CD95+ CD28- cells within CD4+ and CD8+ T cells in whole blood in the course of human endotoxia. Geometric means and 95% confidence intervals are shown. * P < 0.05 in a comparison with baseline values.](http://cvl.asm.org/)
Thus, at t’s of 1.5 and 5 h, the percentage of CD3⁻ CD56⁺ CD16⁻ cells had decreased (P < 0.0005 in both cases). At 1.5 and 5 h, the proportion of CD3⁻ CD56⁻ CD16⁺ cells had doubled (P < 0.0005 in both cases). No age-related differences were detected (Table 2).

**Apoptosis.** We were not able to detect changes in levels of unstimulated apoptosis in response to endotoxemia. However, upon stimulation with PHA, changes in apoptotic cells (7AAD, faint staining) occurred (ANOVA, P = 0.012). Thus, baseline cells exhibited apoptotic-cell levels that were 5.8% above unstimulated-apoptotic-cell values. At 1.5 h after endotoxin infusion, the apoptosis rate was reduced to a mean of 2.5% above the unstimulated-apoptosis level and remained at this level at a t of 5 h (Fig. 3).

As for late-apoptotic or dead cells (7AAD, bright staining), the same trend was found, although the importance of this finding may be negligible, since the total proportion of these cells was below 0.5% in all situations. No age-dependent differences were detected.

**Discussion.** The concentration of all lymphocyte subsets declines during the first 5 h of experimental endotoxemia. The major finding of this study was that the cells constituting the lymphopenia were primarily naïve cells and cells expressing CD28 and lacking CD95. Furthermore, decreased activation-induced apoptosis was demonstrated among the cells constituting the lymphopenia.

The finding that fewer cells were prone to undergo apoptosis during endotoxemia than at baseline is compatible with the hypothesis that part of the lymphopenia is a consequence of apoptosis. In support of this, we found a high rate of disappearance of CD95⁻ CD28⁻ cells (memory like), which have been described as being protected from activation-induced apoptosis (11).

In contrast, this study could not demonstrate endotoxin-induced changes in the percentages of CD95⁺ cells within lymphocytes, although it has been suggested that CD95⁻ cells constitute a subpopulation representing naïve cells (5).

We also studied NK cells and found that the relative proportion of NK cells that were CD3⁻ CD16⁺ CD56⁻ was markedly reduced but that the percentage of CD3⁻ CD16⁻ CD56⁺ cells doubled. Sondergaard et al. (15) have proposed that the latter NK subset represents an immature NK cell population, which would also mean that mature NK cells disappear from the circulation more readily than immature cells.

In accordance with the present findings, Lin and coworkers (10) found that monocyte expression of CD95 is reduced during endotoxemia, reaching a nadir at a t of 2 h, when the monocyte concentration is drastically reduced compared to that at baseline. Furthermore, they detected no changes in soluble CD95, indicating that the decrease in CD95 expression is not due to shedding.

Taken together, the present results demonstrate that cells prone to undergo apoptosis—that is, cells with high levels of CD95 expression, cells not expressing CD28, and otherwise mature cells—leave the circulation in response to endotoxin challenge. Furthermore, our results support the hypothesis that some of these cells undergo programmed cell death and/or are redistributed to the spleen and lymph nodes.

The present study shows that aging did not influence endotoxin-induced lymphopenia, although the elderly had a different pattern of CD8⁺ T-cell apoptosis and/or redistribution.

In conclusion, lymphopenia occurs to the same extent in young and elderly individuals during endotoxemia. This is due to the disappearance of activated or mature cells. Thus, relatively more memory cells and cells expressing CD95 but not described as being protected from activation-induced apoptosis (11).

![FIG. 3. Percentages of apoptotic (7AAD, faint staining) and dead (7AAD, bright staining) cells within isolated unstimulated blood mononuclear cells (BMNC) and upon PHA stimulation. Geometric means and 95% confidence intervals are shown. *P < 0.05 in comparison with baseline values.](http://cvi.asm.org/)

Downloaded from http://cvi.asm.org/ on October 29, 2017 by guest
CD28 disappear from the circulation during endotoxin-induced lymphopenia, concomitantly with a reduced rate of activation-induced apoptosis within the cells constituting the lymphopenia. This disappearance may reflect a redistribution of T cells, where mature cells are being removed from circulation in order to encounter a stimulus that will lead some of them to undergo apoptosis.

The excellent assistance of Hanne Villumsen is acknowledged.

REFERENCES