Spontaneous and Glucocorticoid-Induced Apoptosis in Human Mature T Lymphocytes

By Mauro Brunetti, Nicola Martelli, Antonella Colasante, Mauro Piantelli, Piero Musiani, and Francesca B. Aiello

Glucocorticoid (GC)-induced apoptosis is a well-recognized physiologic regulator of murine T-cell number and function. We have analyzed its mechanisms in human mature T cells, which have been thought to be insensitive until recently. Peripheral blood T cells showed sensitivity to GC-induced apoptosis soon after the proliferative response to a mitogenic stimulation, and were also sensitive to spontaneous (i.e., growth factor deprivation–dependent) apoptosis. CD8+ T cells were more sensitive to both forms than CD4+ T cells. Acquisition of sensitivity to GC-induced apoptosis was not associated with any change in number or affinity of GC receptors. Both spontaneous and GC-induced apoptosis were increased by the macromolecular synthesis inhibitors, cycloheximide (CHX) and puromycin. A positive correlation between the degree of protein synthesis inhibition and the extent of apoptosis was observed. Interleukin-2 (IL-2) IL-4, and IL-10 protected (IL-2 > IL-10 > IL-4) T cells from both forms of apoptosis in a dose-dependent manner. Our data suggest that spontaneous and GC-induced apoptosis regulate the human mature T-cell repertoire by acting early after the immune response and differentially affecting T-cell subsets.

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MATERIALS AND METHODS

Cell preparation. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood obtained from healthy donors by Ficoll-Hypaque (Pharmacia Biotech, Brussels, Belgium) density-gradient centrifugation, as follows: (1) freshly prepared T cells were obtained by treatment with anti-CD16 (Becton Dickinson) and anti-CD14 (Immunotech, Marseilles, France) MoAb plus complement. After Ficoll separation, cells (5 × 10^6/mL) were cultured in medium supplemented at the onset of the culture with 1 ng/mL rIL-2 (Peprotech Inc, Rocky Hill, NJ). At day 10, cells were harvested and layered on a Ficoll-Hypaque (Pharmacia) gradient to remove dead cells. They were greater than 90% CD3+ by cytofluorometry. (2) Three-day or 7-day cultured T cells were obtained by stimulating PBMC (10^6/mL) with OKT3 (10 ng/mL). At day 3 or 7, purified T cells were obtained by treatment with anti-CD16 (Becton Dickinson) and anti-CD14 (Immunotech) MoAb plus complement. Ficoll separation was performed to remove dead cells. Cells were greater than 90% CD3+ (4) CD4+ and CD8+ subsets were separated from stc T cells by immunomagnetic bead depletion. Magnetic beads coated with anti-CD4 MoAb or with anti-CD8 MoAb and a magnetic particle concentrator (MPC-1) (all from Dynal, Oslo, Norway) were used in accordance with the manufacturer’s recommendations. The procedure was performed twice. Ficoll separation was performed to remove dead cells. The purified populations were greater than 90% CD4+ or CD8+. Various treatments of T cells were performed. T cells (2 × 10^6/mL) were cultured in the presence of absence of rIL-1β, rIL-2, rIL-4, recombinant tumor necrosis factor alpha (rTNFα), recombinant tumor growth factor beta (rTGFβ), rIL-10, recombinant interferon gamma (rIFNγ) (Peprotech), Dex (Sigma Chemical, St Louis, MO), cycloheximide (CHX) Sigma), or puromycin (Sigma) at times and concentrations as indicated. The culture medium was RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 2

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mmol/L L-glutamine (GIBCO Laboratories, Grand Island, NY), 100 U/ml penicillin (GIBCO), 100 μg/mL streptomycin (GIBCO), and 10% (vol/vol) heat-inactivated fetal calf serum (Biochrom). Cell viability was determined both by light microscopy (trypan blue exclusion test) and by flow cytometry after staining with propidium iodide ([PI] µg/mL; Sigma).

Surface antigen quantitation by flow cytometry. One million cells were stained with the following fluorescein isothiocyanate−conjugated MoAb: anti-CD3, anti-CD4, and anti-CD8 (Dakopatts, Glostrup, Denmark). Cells stained with nonreacting fluorescein isothiocyanate−and phycoerythrin-conjugated MoAb (Dakopatts) served as controls for nonspecific staining. All staining and washing procedures were performed at 4°C in phosphate-buffered saline−bovine serum albumin 1% supplemented with 0.1% NaN3. The samples were analyzed on a FACStar (Becton Dickinson).

Measurement of apoptosis. Apoptosis was measured in the following three ways. (1) Morphology: May-Grünwald-Giemsa-stained cells were considered morphologically apoptotic when they displayed loss of volume, chromatin condensation, and/or nuclear fragmentation. (2) Flow cytometry: Cells fixed in 70% cold ethanol and stained with a PI (50 µg/mL in phosphate-buffered saline; Sigma) solution containing RNAse (50 µg/mL; Pharmacia) were incubated in the dark at room temperature for 15 minutes and kept at 4°C in the dark overnight before analysis with a FACStar flow cytometer (Becton Dickinson) equipped with an argon-ion laser. PI fluorescence was detected through a 585/40-band pass filter and registered in a logarithmic scale. The percentage of apoptotic cells (subdiploid DNA peak in < 2 x 10^10 channels) was calculated with a specific FACStar research software (Becton Dickinson). Since morphologic evaluation and flow cytometry rendered similar apoptosis percentages, only the latter are presented to avoid redundancy. (3) DNA electrophoresis: DNA fragmentation was determined by a modification of the method of Sellins and Cohen.10 Briefly, cells were collected by centrifugation, and pellets were lysed with 0.5 mL hypotonic lysis buffer, pH 7.5 (10 mmol/L Tris buffer, 1 mmol/L EDTA, and 0.2% Triton X-100). The lysates were centrifuged at 13,000g, and the supernatants containing fragmented DNA were collected. DNA was precipitated overnight at −20°C in isopropanol and 0.5 mol/L NaCl, air-dried, and resuspended in 10 mmol/L Tris and 1 mmol/L EDTA. Loading buffer (1:10 vol/vol) was added to the samples before heating at 65°C for 10 minutes. DNA was visualized by staining with ethidium bromide.
SPONTANEOUS AND GC-INDUCED T-CELL APOPTOSIS

Fig 3. Analysis of spontaneous and Dex-induced apoptosis in unfractionated, CD4+, CD8+ stc T cells. DNA fluorescence was analyzed at time 0 and after 24 hours of culture in medium alone or with Dex (10^{-7} mol/L). Percentages of apoptotic cells were determined as in Fig 1. Results are from one of eight experiments.

RESULTS

Dex-induced apoptosis of peripheral blood T cells. Freshly prepared T cells showed minimal apoptosis after 24 hours of culture in medium and were largely insensitive to

Table 2. Effect of CHX and Puromycin on Spontaneous and Dex-Induced Apoptosis

<table>
<thead>
<tr>
<th>Culture Condition</th>
<th>Protein Synthesis (% inhibition)</th>
<th>Apoptosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>0</td>
<td>28.2 ± 3.1</td>
</tr>
<tr>
<td>CHX (μg/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>7.2 ± 2.4</td>
<td>28.7 ± 4.5</td>
</tr>
<tr>
<td>0.5</td>
<td>51.2 ± 3.8</td>
<td>36.3 ± 2.7</td>
</tr>
<tr>
<td>1</td>
<td>67.3 ± 1.4</td>
<td>45.1 ± 5.4</td>
</tr>
<tr>
<td>5</td>
<td>87.2 ± 5.2</td>
<td>53.3 ± 4.4</td>
</tr>
<tr>
<td>Puromycin (μg/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>11.0 ± 2.3</td>
<td>29.5 ± 3.4</td>
</tr>
<tr>
<td>1</td>
<td>55.2 ± 5.8</td>
<td>41.1 ± 1.4</td>
</tr>
<tr>
<td>5</td>
<td>84.1 ± 4.6</td>
<td>54.3 ± 4.0</td>
</tr>
<tr>
<td>10</td>
<td>90.2 ± 2.1</td>
<td>84.6 ± 5.2</td>
</tr>
<tr>
<td>Dex 10^{-7} mol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>49.5 ± 4.0</td>
<td></td>
</tr>
<tr>
<td>Dex + CHX 0.05 μg/mL</td>
<td></td>
<td>48.3 ± 3.1</td>
</tr>
<tr>
<td>Dex + CHX 0.5 μg/mL</td>
<td></td>
<td>55.2 ± 3.3</td>
</tr>
<tr>
<td>Dex + CHX 1 μg/mL</td>
<td></td>
<td>60.3 ± 3.2</td>
</tr>
<tr>
<td>Dex + CHX 5 μg/mL</td>
<td></td>
<td>72.7 ± 6.2</td>
</tr>
<tr>
<td>Dex + puromycin 0.1 μg/mL</td>
<td></td>
<td>49.3 ± 2.2</td>
</tr>
<tr>
<td>Dex + puromycin 1 μg/mL</td>
<td></td>
<td>63.5 ± 4.0</td>
</tr>
<tr>
<td>Dex + puromycin 5 μg/mL</td>
<td></td>
<td>76.1 ± 6.6</td>
</tr>
<tr>
<td>Dex + puromycin 10 μg/mL</td>
<td></td>
<td>90.1 ± 4.3</td>
</tr>
</tbody>
</table>

The stc T cells were cultured for 18 hours in medium alone or with Dex (10^{-7} mol/L) in the presence or absence of CHX or puromycin at the indicated concentrations. Measurement of protein synthesis was made by 3H-Leu uptake. Results are the mean ± SD of three experiments.

GC receptor-binding studies. Radioligand-binding studies were performed on whole cells as reported previously. Aliquots of 10^6 cells were incubated in borosilicate glass tubes at 37°C with six concentrations (1.5 to 30 nmol/L) of [6,7-{^3}H(N)]-Dex (specific activity, 39.2 Ci/mmol; NEN Research Products, Boston MA) in the presence or absence of 1,000-fold excess of unlabeled Dex (Sigma). All values were corrected for nonsaturable binding at each concentration. The apparent equilibrium kd and the concentration of receptor sites were obtained by Scatchard analysis.

Measurement of protein synthesis. Inhibition of protein synthesis in stc T cells by CHX or puromycin was assessed by culturing cells (10^6/mL) in leucine-free medium, with or without these drugs at the indicated concentrations, following a 2-μCi/well pulse of L-[4,5-{^3}H(N)]-leucine (specific activity, 60.0 Ci/mmol; NEN). Cells were harvested at 1, 6, and 18 hours, and proteins were precipitated by trichloroacetic acid. 3H-Leucine uptake was measured by liquid scintillation counting.

Statistical analysis. Results are the mean ± SD. Student’s t-test for paired data was performed for statistical analysis.

Table 1. GC Receptor Sites Per Cell and kd in Freshly Prepared and stc T Cells

<table>
<thead>
<tr>
<th>T Cells</th>
<th>Sites Per Cell</th>
<th>kd (nmol/L)</th>
<th>No. of Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly prepared*</td>
<td>6,091 ± 779</td>
<td>9.4 ± 2.8</td>
<td>6</td>
</tr>
<tr>
<td>stc*</td>
<td>6,280 ± 1,445</td>
<td>8.8 ± 2.9</td>
<td>6</td>
</tr>
<tr>
<td>stc CD4+†</td>
<td>5,692 ± 229</td>
<td>8.9 ± 0.8</td>
<td>4</td>
</tr>
<tr>
<td>stc CD8+†</td>
<td>5,454 ± 469</td>
<td>8.2 ± 2.5</td>
<td>4</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± SD. No significant differences were observed in sites per cell and kd values between freshly prepared and stc T cells or between CD4+ and CD8+ stc T cells.

* GC receptor sites per cell and kd values were evaluated on freshly prepared and stc T cells from the same donor.

† GC receptor sites per cell and kd values were evaluated on CD4+ and CD8+ stc T cells from the same donor.
A

\[ \text{Cell number} \]

\begin{tabular}{c|c|c|c}
 & Medium & IL-2 & DEX + IL-2 \\
\hline
1600 & 28\% & 7\% & 11\% \\
700 & 52\% & 1900 & \\
\end{tabular}

\[ \text{Log PI fluorescence} \]

\[ 10^{-7} \text{ mol/L Dex (Fig 1). The stc T cells were in the G0/G1 phase of the cell cycle (data not shown) and showed less than 10\% apoptotic cells (Fig 1). After culture in medium alone for 24 hours, apoptosis significantly increased (Fig 1) (} P < .001) \text{ due to growth factor deprivation.}^{5,12,13} \text{ We will refer to this as spontaneous apoptosis. DEX induced apoptosis in about half the cells (Fig 1). In eight subjects, spontaneous and DEX-induced apoptosis mean percentages were 27.1\% \pm 4.7\% (range, 20\% to 35\%) and 50.5\% \pm 8.3\% (range, 38\% to 65\%) (} P < .001). \text{ We next investigated whether acquisition of sensitivity to DEX-induced apoptosis was an early event. Three-day cultured T cells were largely insensitive (data not shown). This was expected, since activated T cells are insensitive during the proliferative phase.}^{8,15,16} \text{ Seven-day cultured T cells were highly sensitive (Fig 1); they were in the G0/G1 phase (data not shown). Spontaneous and DEX-induced apoptosis percentages were 24.3\% \pm 3.7\% and 49.2\% \pm 5.8\%, respectively. The effect of DEX was dose-dependent (Fig 2).}

We then investigated whether DEX-induced apoptosis was confined to CD4\(^+\) or CD8\(^+\) T-cell subsets. The CD4/CD8 ratio was not modified after the short-term culture. Analysis of surface markers of stc T cells before and after DEX showed a reduction of the CD8\(^+\) percentage and a relative increase in the CD4\(^+\) percentage (data not shown), suggesting that the latter were less sensitive or insensitive to DEX. CD8\(^+\) and CD4\(^+\) cells purified by immunomagnetic bead depletion showed different sensitivity to apoptosis (Fig 3). Spontaneous and DEX-induced apoptosis percentages were higher in CD8\(^+\) cells. The spontaneous apoptosis percentage was 27.4\% \pm 2.9\% for CD4\(^+\) and 42.1\% \pm 3.5\% for CD8\(^+\) cells, and the DEX-induced apoptosis percentage was 45.1\% \pm 2.6\% for CD4\(^+\) and 58.7\% \pm 4.9\% for CD8\(^+\) cells.

\[ \text{GC receptor analysis.} \text{ We investigated whether a difference in the number and/or affinity of GC receptors correlated with the different sensitivity to DEX-induced apoptosis. Freshly prepared and stc T cells (unfractionated, CD4\(^+\), and CD8\(^+\)) displayed similar GC receptor number and affinity (Table 1). Similar results were observed with 7-day cultured T cells (data not shown).}

\[ \text{Effects of CHX and puromycin.} \text{ CHX and puromycin were used as inhibitors to determine whether DEX-induced apoptosis is dependent on macromolecular synthesis. These inhibitors increased the spontaneous apoptosis of stc T cells (Table 2). The increase of DNA fragmentation was confirmed by gel electrophoresis (data not shown). A positive dose-dependent correlation between the degree of protein synthesis inhibition and the extent of spontaneous apoptosis was observed. Both inhibitors increased DEX-induced apoptosis (Table 2).} \]
**DISCUSSION**

Most studies of GC-induced apoptosis have been performed in mice because human cells were thought to be insensitive. The recent demonstration of sensitivity of human mature T cells awaits further investigation. This report shows that stc, not freshly isolated, T cells are indeed sensitive to GC-induced apoptosis. This occurs soon after the proliferative response to a mitogenic stimulation, suggesting that it may serve as an early negative regulatory mechanism shortly after the onset of the immune response. Similarly, an early endogenous GC-induced apoptosis limits mature T-cell expansion in mice consequent to superantigen stimulation.

Both the CD8+ and to a lesser extent the CD4+ subpopulations are sensitive to spontaneous and GC-induced apoptosis. It has been reported that human CD4+ and CD8+ activated T cells can undergo spontaneous apoptosis, and a high spontaneous apoptosis of CD8+ T cells has been attributed to a preferential activation of this subpopulation in certain infections. GC-induced apoptosis of CD8+ T cells might per se be more sensitive to spontaneous and GC-induced apoptosis. Our results may contribute to explaining the previously reported differences in GC inhibition of proliferative and functional responses of helper and suppressor T lymphocytes.

Recent study has shown that T-cell resistance to inhibition by GCs in asthmatic patients correlates with a decreased affinity of their receptors. No correlation with receptor number or affinity emerged in our results, as in other studies showing no correlation with the degree of GC sensitivity of lymphoid cells and GC receptor number and affinity. Recent data suggesting that cell cycle–dependent posttranslational modifications of GC receptors account for GC resistance may provide an explanation for these divergencies.

Three types of apoptotic mechanisms have been described on the basis of protein synthesis requirements. Inhibition of protein synthesis can induce or block apoptosis, or it can have no effect. This may depend on the cell type, its degree of maturation, and the type of apoptotic stimulus. We have observed that stc T-cell spontaneous apoptosis is increased by macromolecular synthesis inhibitors. There is a positive correlation between the degree of protein synthesis inhibition and the extent of spontaneous apoptosis. This suggests that continuous synthesis of some protein is required to maintain viability. We have observed that blockade of protein synthesis increases GC-induced apoptosis. This has also been demonstrated in murine mature T cells. On the contrary, GC-induced apoptosis of both murine and human thymocytes is blocked by protein synthesis inhibitors. Thus, the differential sensitivity of such apoptosis to protein synthesis inhibitors enables a distinction to be drawn between the mature (peripheral) and immature (thymic) compartments.

A balance between death and survival regulates the immune response by limiting overreactions and establishing an immunologic memory. Cytokines play an important role in this balance; some rescue human activated T cells from spontaneous apoptosis. However, the position with respect to GC-induced apoptosis has not been investigated. We have...

**Effect of cytokines.** We tested the effect of IL-1β, IL-2, IL-4, IL-10, TNFα, TGFβ, and IFNγ on stc T-cell apoptosis. IL-2 greatly inhibited spontaneous and Dex-induced apoptosis (Fig 4A). This was confirmed by DNA electrophoresis (Fig 4B). IL-2, IL-4, and IL-10 protected (IL-2 > IL-10 > IL-4) unfractionated, CD4+, and CD8+ stc T cells from spontaneous and Dex-induced apoptosis in a dose-dependent manner (Fig 5). The maximum effect was observed at 2 ng/mL for IL-2 and at 20 ng/mL for IL-4 and IL-10. These cytokines similarly inhibited stc T-cell apoptosis induced by 10⁻⁶ mol/L Dex (data not shown). IL-1β, TNFα, TGFβ, and IFNγ were not protective (data not shown).
found that IL-2, IL-10, and IL-4 protect stc T cells from spontaneous and GC-induced apoptosis. The mechanisms involved are unknown. Human T cells in G2/M phase are resistant to GC-induced apoptosis. An IL-2–induced entry into this phase may explain its protective effect. IL-10 does not induce proliferation of human activated T cells (and data not shown), and its protective effect may therefore depend on a different mechanism. Whatever the mechanism, B cells, monocytes, and keratinocytes can produce IL-10, as can T cells, suggesting that T-cell survival can be positively influenced by these cells. It has been shown that IL-4 selectively rescues murine Th2 clones from GC-induced apoptosis. The development of human Th2 in vitro requires interaction. Sensitivity to antigen-driven or CD95-mediated apoptosis increases slowly and gradually upon repeated stimulation and does not seem restricted to a specific phase of the cell cycle. No difference in CD4+ and CD8+ cells differentially, and are protein synthesis–independent. Most studies of normal mature T cells concern antigen-driven apoptosis, which is in turn linked to CD95 receptor/ligand interaction. Sensitivity to antigen-driven or CD95-mediated apoptosis increases slowly and gradually upon repeated stimulations and does not seem restricted to a specific phase of the cell cycle. No difference in CD4+ and CD8+ sensitivity has been reported. Antigen-driven apoptosis is protein synthesis–dependent, consistent with the de novo synthesis of CD95 and its ligand. Interestingly, GCs down-regulate CD95 mRNA expression. Thus, the mechanisms underlying spontaneous and GC-induced apoptosis seem to be dissimilar to those underlying surface receptor–driven apoptosis. All of these forms may act coordinately in vivo in a time-spaced regulation of the strength and degree of specificity of the T-cell repertoire.

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Spontaneous and glucocorticoid-induced apoptosis in human mature T lymphocytes

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