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The CD8$^+$ granzyme B$^+$ T-cell subset in peripheral blood from healthy individuals contains activated and apoptosis-prone cells

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SUMMARY

Granzyme B (GrB) has been implicated in induction of apoptosis in target cells. The presence of GrB in peripheral blood CD8$^+$ T cells from healthy individuals was analysed in immunocytochemical and flow cytometric studies. Furthermore, CD8$^+$ GrB$^-$ T cells and CD8$^+$ GrB$^+$ T cells were compared regarding phenotypical characteristics and susceptibility to both spontaneous and Fas-mediated apoptosis. GrB was expressed by approximately one-fifth of CD8$^+$ T cells. Compared with the CD8$^+$ GrB$^-$ T-cell subset, the CD8$^+$ GrB$^+$ T-cell subset contained cells that were relatively more activated and more prone to spontaneous apoptosis. Culturing of cells with immunoglobulin M (IgM) anti-Fas monoclonal antibody had no additional effect on the number of CD8$^+$ GrB$^+$ T cells undergoing apoptosis. We suggest that the presence of CD8$^+$ GrB$^+$ T cells in peripheral blood from healthy individuals results from immune surveillance or contact with infectious agents, and that spontaneous apoptosis of these cells might serve as a mechanism for their eventual clearance.

INTRODUCTION

The granule exocytosis model for lymphocyte-mediated cytotoxicity postulates that target cell recognition triggers a secretory process in which cytoplasmic granules fuse with the effector cell plasma membrane. The cytoplasmic granules contain several constituents, including perforin and the serine proteases granzyme A (GrA) and granzyme B (GrB), which are released into a synaptosomal-like space between effector and target cell. Target cell death is a result of perforin-dependent necrosis and/or perforin/granzyme-mediated apoptosis.[1,2] In the induction of apoptosis, perforin delivers the granzymes to the cytosol of the target cell.[3] Although the biological function of GrA is unclear,[4] compelling evidence supports a crucial role for GrB in induction of target cell apoptosis through activation of the intracellular cascade of caspases.[3,5–7]

There have been conflicting reports concerning the presence of granzymes in peripheral blood lymphocytes (PBL) from healthy individuals. Sunder-Plassman *et al.* reported on the presence of GrA protein in natural killer (NK) cells and a subset of CD8$^+$ T cells using a cytochemical staining method detecting enzymatic activity of GrA.[8] In a flow cytometric study, Berthou *et al.* were unable to detect GrB protein in both NK cells and T cells.[9] Using immunocytochemical methods, Kummer *et al.* detected GrA and GrB proteins in NK cells only.[10] Previously, we reported on detection of GrA and GrB proteins using monoclonal antibodies (mAb) directed against recombinant human granzymes in immunocytochemistry after application of a non-enzymatic antigen retrieval technique. In addition to the presence of both granzymes in CD3$^-$ cells, we detected GrA and GrB in $12 \pm 1\%$ (mean $\pm$ standard error of the mean (SEM)) and $9 \pm 1\%$ of CD3$^+$ T cells, respectively.[11] In the present study, a mAb directed against native human GrB is used to further analyse GrB expression by T cells from healthy individuals. We hypothesized that GrB is expressed by an activated CD8$^+$ T-cell subset. Because apoptosis has been suggested as a mechanism for clearance of activated CD8$^+$ T cells, we additionally analysed the susceptibility of CD8$^+$ GrB$^+$ T cells to both spontaneous and Fas-mediated apoptosis.

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Abbreviations: GrA, granzyme A; GrB, granzyme B.

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MATERIALS AND METHODS

Production and purification of monoclonal antibody GrB-11

The IgG1 mAb GrB-11 was produced by immunization of BALB/c mice with native GrB purified from the human NK cell line YT-INDY.[12] After four intraperitoneal injections,

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blood samples were obtained and anti-GrB antibodies (Ab) were captured with Sepharose-bound goat anti-mouse immunoglobulins (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands) and detected with ^{125}I-labelled GrB. A mouse containing high titres of anti-GrB Ab was injected intravenously with a final dose of GrB and killed. Spleen cells were fused with mouse myeloma Sp2/0-Ag14 cells.[13] Hybridomas producing anti-GrB Ab were subcloned and mAb were purified by affinity chromatography on protein G–Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). The specificity of mAb GrB-11 was amongst others assessed with enzyme-linked immunosorbent assay (ELISA). Purified human serine proteases GrA,[14] GrB, neutrophil elastase (Elastin Products, Pacific, MO), trypsin (Sigma, St. Louis, MO,) and chymotrypsin (Sigma) were coated onto wells of ELISA plates at a concentration of 2 μg/ml and incubated subsequently with biotinylated mAb GrB-11. Plates were developed and binding was expressed as absorption at 450 nm. In immunocytochemistry, mAb GrB-11 was used at a final concentration of 2·5 μg/ml. Phycoerythrin (PE)-labelled mAb GrB-11 was used for flow cytometric analysis at a final concentration of 1·5 μg/ml.

Primary antibodies

Fluorescein isothiocyanate (FITC)-labelled primary mAb were directed against CD3 (Becton Dickinson (BD), San Jose, CA, CD4 (BD), CD8 (BD), CD16 (CLB), CD19 (BD), CD28 (CLB), CD45RA (BD), CD62l (BD), CD69 (BD), HLA-DR (BD), T-cell receptor (TCR)-γδ-1 (BD) and Bcl-2 (Dako, Glostrup, Denmark). R-phycoerythrin-cyanine 5 (RPE-Cy5)-labelled primary mAb were directed against CD3 (Immunotech, Marseille, France) and CD8 (Dako). Unlabelled primary mAb used were IgM anti-Fas mAb (CH-11; Immunotech). IgG1 mAb directed against plant allergens (CLB), FITC- and PE-labelled IgG1 mAb and FITC-labelled IgG2a mAb directed against keyhole limpet haemocyanin (BD), RPE-Cy5-labelled IgG1 mAb directed against a non-biological hapten (Immunotech) and purified myeloma IgM (ICN, Costa Mesa, CA) were used as isotype control.

Immunocytochemistry

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Paque density gradient centrifugation of heparinized blood from 10 healthy individuals selected among the laboratory technicians of our Clinical and Laboratory Immunology Unit and the nursing staff of our Renal Transplant Unit (six males, four females; median age 29 years, range 26–41 years). All selected individuals had stated that they were in good health and without use of any drug medication. PBMC were concentrated on slides by cytocentrifugation. Slides were dried to air, fixed in 4% (v/v) buffered formalin and stained with mAb GrB-11 using the streptavidin–biotin horseradish peroxidase complex technique.

Flow cytometric analysis

Three-colour flow cytometry was employed for phenotypical analysis of GrB expressing cells. PBMC were washed and incubated first with Ab directed against surface markers or appropriate isotype control Ab. Cells that had been incubated with unlabelled IgM anti-Fas mAb or irrelevant IgM Ab were subsequently incubated with FITC-labelled goat anti-mouse F(ab′)$_2$ fragments (CLB), 10% (v/v) normal mouse serum and RPE-Cy5-labelled CD8 mAb. Subsequently, all cells were fixed with buffered formaldehyde acetone (BFA), as described previously.[15] After fixation, cells were washed in saponin detergent composed of phosphate-buffered saline (PBS) supplemented with 0·5% (w/v) bovine serum albumin, 0·01% (w/v) sodium azide, 0·1% (w/v) saponin and 50 mmol/l D-glucose. Subsequently, cells were incubated with mAb directed against intracellular markers or appropriate isotype control mAb. Data acquisition was performed on a fluorescence-activated cell sorter (BD) and a number of $2{\cdot}5\times10^4$ events were analysed. PBL were gated by forward and side scatter parameters.

Cell culture

Cells from six healthy individuals were additionally cultured with either irrelevant IgM Ab or IgM anti-Fas mAb. Culture with irrelevant IgM Ab allowed for detection of spontaneous apoptosis and served as isotype control of culture with IgM anti-Fas mAb. PBMC were first depleted from monocytes through adherence to plastic to prevent clearance of apoptotic cells by phagocytosis. Non-adherent PBL were suspended at 10^6 cells/ml in RPMI-1640 supplemented with 5% fetal calf serum, 20 mmol/l PIPES, 0·02 mmol/l 2-mercaptoethanol, 2 mmol/l L-glutamine plus antibiotics and cultured for 48 hr in a 12-well culture plate in the presence of either irrelevant IgM Ab or IgM anti-Fas mAb (final concentration 1 μg/ml). Binding of IgM anti-Fas mAb was evaluated after culture by incubation with FITC-labelled goat anti-mouse F(ab′)$_2$ fragments and subsequent flow cytometric analysis. In a different context, but parallel to the experiments described in this report, we used irrelevant IgM Ab and IgM anti-Fas mAb in a 9-hr culture to induce apoptosis in isolated peripheral blood granulocytes from 6 healthy individuals.

Apoptosis measurement

Flow cytometric analysis of annexin V binding to phosphatidylserine, exposed on the outer surface of apoptotic cells, was used for quantitative measurement of apoptosis.[16,17] Cultured PBL were incubated with RPE-Cy5-labelled CD8 mAb, washed in HEPES buffer, incubated for 5 min with FITC-labelled annexin V (final concentration 2·5 μg/ml) and washed again in HEPES buffer. Loss of bound annexin V during successive permeabilization and incubation with PE-labelled GrB-11 mAb was prevented by fixation of cells with BFA. Cells washed with HEPES buffer lacking Ca^{2+} ions served as negative control. The ability of intact cells to exclude propidium iodide allows for identification of cells with an intact plasma membrane.[17] Therefore, PBL were also stained with FITC-labelled annexin V and successively incubated for 1 min with propidium iodide (final concentration 50 μg/ml; Sigma), while omitting any successive fixation and permeabilization procedures. Similar methods were used for quantitative measurement of apoptosis in cultured granulocytes.

Statistical analysis

Data are presented as mean ± SEM. Significance was assessed using the Wilcoxon test for matched pairs and the Spearman rank correlation coefficient. A P-value $<0{\cdot}05$ was considered significant.

RESULTS

Characterization of monoclonal antibody GrB-11

Specificity of mAb GrB-11 was shown in immunoprecipitation studies, which showed that this mAb recognized a 32 000 000 MW antigen in lysates of lymphokine-activated killer cells. In radioimmunoassay procedures similar to the assay used for initial screening, mAb GrB-11 did not bind ^{125}I-labelled GrA (less than 3% binding of input), whereas it did bind ^{125}I-labelled GrB (over 50% binding of input). Furthermore, in an ELISA in which wells were coated with various serine proteases, biotinylated mAb GrB-11 only bound to GrB.

Immunocytochemistry

A strong granular staining pattern was detected in up to 20% of PBMC after staining with mAb GrB-11 (Fig. 1a and b). Cells displaying this staining pattern were detected in PBMC from all tested healthy individuals.

Flow cytometric analysis

The use of PE-labelled mAb GrB-11 in combination with the saponin-permeabilization method allowed for a clear distinction between GrB^+ and GrB^- PBL (Fig. 1c and d). Table 1 shows that expression of GrB was detected in the majority of $CD3^-$ $CD16^+$ NK cells and TCR-$\gamma\delta^+$ T cells. In $CD8^+$ T cells, expression was detected in approximately one-fifth of cells. The $CD8^+$ T cell subset within PBL was identified by its bright CD8 staining and consisted for $99{\cdot}0 \pm 0{\cdot}3$% of $CD3^+$ T cells and for $1{\cdot}0 \pm 0{\cdot}5$% of TCR-$\gamma\delta^+$ T cells. Expression in $CD4^+$ T cells was limited to a small percentage of cells.

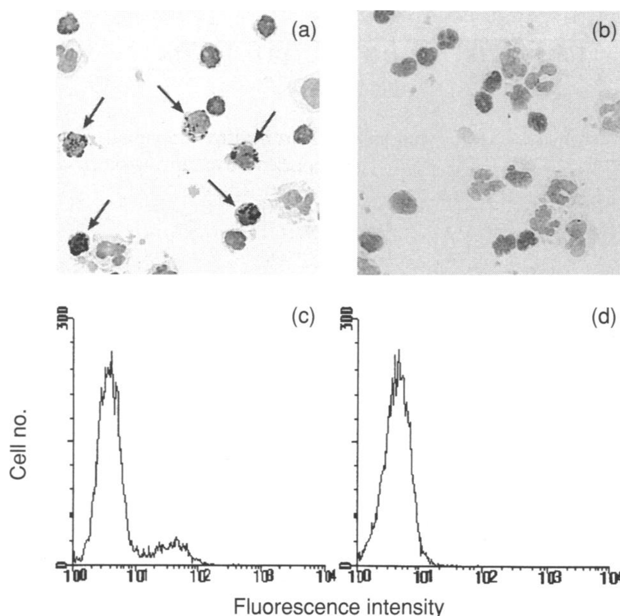

Figure 1. Expression of GrB in peripheral blood from healthy individuals. (a) Immunocytochemical staining showing PBMC displaying a strong granular staining pattern with mAb GrB-11. Arrows indicate GrB^+ cells. (b) Staining was not observed with isotype control IgG1 mAb (magnification (a) and (b): ×560). (c) Flow cytometric detection of GrB^+ PBL using PE-labelled mAb GrB-11. (d) Labelling was not observed with PE-labelled isotype control IgG1 mAb. Data are representative for 10 healthy individuals.

Table 1. GrB expression in PBL and PBL subsets

Subset	Percentage GrB expressing cells in subset*
Peripheral blood lymphocytes	$13{\cdot}8 \pm 1{\cdot}7$
$CD3^-$ $CD16^+$ NK cells	$61{\cdot}3 \pm 4{\cdot}4$
$CD3^+$ T cells	$10{\cdot}8 \pm 1{\cdot}9$
$CD4^+$ T cells	$1{\cdot}9 \pm 0{\cdot}6$
$CD8^+$ T cells	$21{\cdot}7 \pm 2{\cdot}9$
TCR-$\gamma\delta^+$ T cells	$57{\cdot}8 \pm 6{\cdot}8$

*Data represent mean ± SEM from 10 healthy individuals.

Table 2 depicts the expression of activation and differentiation markers and apoptosis-related proteins in the $CD8^+$ GrB^+ and $CD8^+$ GrB^- T-cell subsets. Analysis of CD28 expression revealed that the majority of $CD8^+$ GrB^+ T cells lacked expression of this molecule. In contrast, only a minor proportion of cells in the $CD8^+$ GrB^- T-cell subset lacked expression of CD28. We noted that the percentage of GrB^+ cells in the $CD8^+$ T-cell subset approximated the percentage of $CD28^-$ cells ($21{\cdot}7 \pm 2{\cdot}9$% and $23{\cdot}8 \pm 2{\cdot}8$%, respectively). Further examination of this observation revealed a strong positive correlation between the two parameters ($R = 0{\cdot}90$, $P < 0{\cdot}001$; Fig. 2). The percentage of cells expressing the activation marker histocompatibility leucocyte antigen (HLA-DR) was higher in the $CD8^+$ GrB^+ T-cell subset than in the $CD8^+$ GrB^- T-cell subset. Expression of the very early activation marker CD69 was also higher in the $CD8^+$ GrB^+ T-cell subset, although expression of this marker could be considered minimal in both the $CD8^+$ GrB^+ and the $CD8^+$ GrB^- T-cell subsets. Cells expressing CD45RA and cells expressing CD62L were preferentially found in the $CD8^+GrB^-$ T cell subset. Analysis of expression of apoptosis-related proteins revealed expression of Fas by a high proportion of cells in the $CD8^+$ GrB^+ T-cell subset (Fig. 3a). In addition, a lower mean fluorescence intensity for Bcl-2 was observed in $CD8^+$ GrB^+ T cells, indicating reduced expression as compared to $CD8^+$ GrB^- T cells (Fig. 3b).

Table 2. Expression of activation and differentiation markers and of apoptosis related proteins in the $CD8^+$ GrB^+ T-cell subset as compared to the $CD8^+$ GrB^- T-cell subset

Marker	$CD8^+$ GrB^+ T cells*†	$CD8^+$ GrB^- T cells	*P*-value
$CD28^-$	$70{\cdot}4 \pm 3{\cdot}5$%	$10{\cdot}2 \pm 1{\cdot}4$%	0·005
HLA-DR^+	$36{\cdot}5 \pm 6{\cdot}2$%	$7{\cdot}4 \pm 1{\cdot}4$%	0·005
$CD69^+$	$4{\cdot}6 \pm 0{\cdot}6$%	$2{\cdot}0 \pm 0{\cdot}4$%	0·01
$CD45RA^+$	$41{\cdot}1 \pm 4{\cdot}6$%	$64{\cdot}0 \pm 4{\cdot}5$%	0·01
$CD62L^+$	$44{\cdot}8 \pm 5{\cdot}7$%	$80{\cdot}5 \pm 2{\cdot}3$%	0·005
Fas^+	$73{\cdot}6 \pm 4{\cdot}6$%	$26{\cdot}8 \pm 5{\cdot}3$%	0·01
Bcl-2	$72{\cdot}1 \pm 4{\cdot}5$	$87{\cdot}0 \pm 4{\cdot}5$	0·01

*Data represent mean ± SEM from 10 healthy individuals.

†Expression is presented as percentage of cells expressing a marker of interest in the $CD8^+$ GrB^+ and $CD8^+$ GrB^- T cell subsets. Bcl-2 expression is presented as mean fluorescence intensity.

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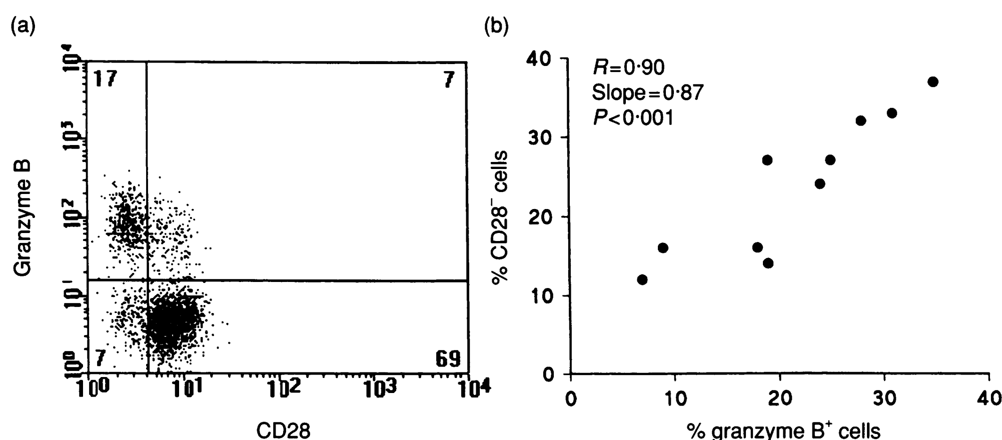

Figure 2. Flow cytometric analysis of expression of GrB and CD28 in the $CD8^+$ T-cell subset from healthy individuals. (a) Representative flow cytometric profile of expression of GrB and CD28 in the $CD8^+$ T-cell subset. (b) Correlation between the percentages of GrB^+ and $CD28^-$ cells in the $CD8^+$ T-cell subset from 10 healthy individuals.

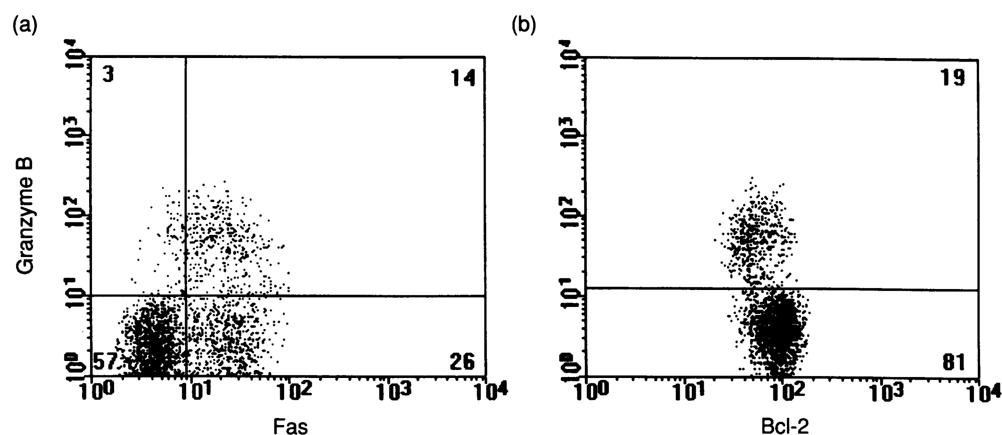

Figure 3. Flow cytometric analysis of expression of GrB, Fas and Bcl-2 in the $CD8^+$ T-cell subset from healthy individuals. (a) Representative flow cytometric profile of expression of GrB and Fas in the $CD8^+$ T-cell subset. (b) Representative flow cytometric profile of expression of GrB and Bcl-2 in the $CD8^+$ T-cell subset.

Apoptosis induction

Results of the apoptosis induction experiments are summarized in Table 3. PBL were cultured for 48 hr with irrelevant IgM Ab or IgM anti-Fas mAb and washed twice. Viability of recovered cells as assessed by trypan blue exclusion was in all cases higher than 95%. Cell loss after culture with irrelevant IgM Ab was higher among $CD8^+$ GrB^+ T cells as compared to $CD8^+$ GrB^- T cells. Binding of annexin V was analysed in cells with forward and side scatter parameters characteristic of viable lymphocytes. The percentage annexin V binding cells was higher in the $CD8^+$ GrB^+ T-cell subset as compared to the $CD8^+$ GrB^- T-cell subset. Annexin V binding PBL had not lost the capacity to exclude propidium iodide, ruling out the possibility that these cells were necrotic. Culturing of PBL with IgM anti-Fas mAb had no additional effect on any of the measured parameters. After culture, binding of IgM anti-Fas mAb was detected preferentially in the $CD8^+$ GrB^+ T-cell subset ($63{\cdot}0 \pm 6{\cdot}5\%$ of $CD8^+$ GrB^+ T cells versus $18{\cdot}0 \pm 6{\cdot}4\%$ of $CD8^+$ GrB^- T cells $P=0{\cdot}01$). The lack of induction of apoptosis in PBL by IgM anti-Fas mAb was not caused by the use of an inappropriate mAb, because Fas-mediated

Table 3. Recovery and annexin V binding of $CD8^+$ -, $CD8^+$ GrB^+ and $CD8^+$ GrB^- T cells after 48 hr of culture in the presence of irrelevant IgM Ab or IgM anti-Fas mAb

Subset	Irrelevant IgM Ab*†	IgM anti-Fas mAb
Recovery		
$CD8^+$ T cells	$70 \pm 7\%$	$73 \pm 8\%$
$CD8^+$ GrB^+ T cells	$52 \pm 9\%$	$52 \pm 7\%$
$CD8^+$ GrB^- T cells	$72 \pm 7\%$‡	$76 \pm 8\%$‡
Annexin V binding		
$CD8^+$ T cells	$4{\cdot}5 \pm 0{\cdot}7\%$	$4{\cdot}8 \pm 0{\cdot}9\%$
$CD8^+GrB^+$ T cells	$13{\cdot}0 \pm 3{\cdot}3\%$	$11{\cdot}7 \pm 3{\cdot}1\%$
$CD8^+GrB^-$ T cells	$3{\cdot}8 \pm 0{\cdot}5\%$‡	$4{\cdot}0 \pm 1{\cdot}0\%$‡

*Data represent mean ± SEM from six experiments.

†Differences between cells cultured with irrelevant IgM Ab and cells cultured with IgM anti-Fas mAb not statistically significant.

‡$P=0{\cdot}03$ for $CD8^+$ GrB^+ T cells versus $CD8^+$ GrB^- T cells.

annexin V binding was detected in approximately one-fifth of granulocytes cultured for 9 hr with IgM anti-Fas mAb.

DISCUSSION

In the present study, we have used a mAb specific for native GrB to demonstrate its presence in peripheral blood NK cells and T cells from healthy individuals. This finding may seem surprising in view of the reported absence of GrB mRNA in resting peripheral blood T cells.[18] However, our observation is consistent with the presence of various other cytotoxic granule constituents in the absence of specific mRNA. Resting peripheral blood T cells do not express specific mRNA for perforin and GrA;[18] however, the presence of perforin and GrA proteins in peripheral blood T cells has been well accepted.[8,9] The reported similarities in *in vitro* requirements for mRNA induction of perforin, GrA and GrB are in support of co-expression of these cytotoxic granule constituents.[18] We detected GrB protein in a subset of CD8^{+} T cells prone to spontaneous apoptosis. Down-regulation of mRNA synthesis is a characteristic of impending apoptosis.[19] Therefore, the apoptosis-prone nature of CD8^{+} GrB^{+} T cells might well explain the reported absence of GrB mRNA.[18]

The CD3^{-} CD16^{+} NK-cell subset is the major lymphocyte subset expressing GrB in peripheral blood. The constitutive expression of GrB by $\approx$60% of NK cells corresponds with the percentage of NK cells with a granular morphology and confirms previous reports describing the expression of GrB at the mRNA level as well as the protein level.[10,20–22] Phenotypical characterization of GrB^{+} T cells revealed GrB expression by TCR-$\gamma\delta^{+}$ T cells, CD8^{+} T cells and a limited number of CD4^{+} T cells. Although GrB transcripts have been detected in TCR-$\gamma\delta^{+}$ T-cell clones,[23] expression of GrB by TCR-$\gamma\delta^{+}$ T cells in peripheral blood has not been reported previously.

The CD8^{+}GrB^{+} T-cell subset preferentially comprised cells lacking CD28 expression, a finding consistent with the granular morphology reported for CD8^{+} CD28^{-} T cells.[24] The strong positive correlation between the percentages of GrB^{+} and CD28^{-} cells in the CD8^{+} T-cell subset indicates that lack of CD28 expression might serve as a surface characteristic indicative for the number of CD8^{+} T cells with cytotoxic potential. This needs further study in pathophysiological conditions such as viral infections, autoimmune diseases and allograft rejection. The importance of CD28 lies primarily in the initiation of CD8^{+} T-cell responses,[25] as CD8^{+} CD28^{+} T cells differentiate into a CD8^{+} CD28^{-} phenotype during subsequent replication.[26] Therefore, we hypothesize that recently activated CD8^{+} T cells reside in the CD8^{+} GrB^{+} CD28^{+} T-cell subset and that these cells will down-regulate CD28 expression in the process of subsequent replication. In view of this hypothesis, the percentage of GrB^{+} CD28^{+} cells in the CD8^{+} T-cell subset ($6{\cdot}3 \pm 0{\cdot}8$%) suggests that a small proportion of circulating CD8^{+} T cells from healthy individuals may have been recently activated, presumably as a result of immune surveillance or contact with infectious agents. This percentage corresponds well with the percentage of GrB^{+} HLA-DR^{+} cells in the CD8^{+} T-cell subset ($7{\cdot}7 \pm 1{\cdot}5$%). CD69 is a very early activation marker expressed rapidly, but transiently, upon T-cell activation,[27] and its expression level in the CD8^{+} T-cell subset ($2{\cdot}4 \pm 0{\cdot}3$%) suggests an even lower number of circulating CD8^{+} T cells in the acute phase of activation.

Naive CD8^{+} T cells display a CD45RA^{+} CD62L^{+} phenotype, while memory CD8^{+} T cells are either CD45RA^{+} CD62L^{-}, CD45RA $^{-}$CD62L^{+} or CD45RA^{-} CD62L^{-}.[28] For each individual, we compared the percentages of CD45RA^{+} cells and CD62L^{+} cells in the CD8^{+} GrB^{+} T-cell subset. Because naive cells are characterized by expression of both markers, we assumed the lower of these two percentages as the highest possible percentage of CD8^{+} GrB^{+} T cells possessing a naive nature. In this way, we calculated that at most $34{\cdot}7 \pm 3{\cdot}6$% of CD8^{+} GrB^{+} T cells display a naive phenotype, consistent with the effector and memory phenotype expected for the majority of these cells.

Apoptosis of activated CD8^{+} T cells has been suggested as a mechanism for clearance of expanded CD8^{+} T-cell populations.[29,30] Apoptosis susceptibility of lymphocytes activated in the course of viral infections is determined by the level of Bcl-2 expression.[29,31] We observed decreased expression of Bcl-2 in the CD8^{+} GrB^{+} T-cell subset of healthy individuals as compared to the CD8^{+} GrB^{-} T-cell subset. Accordingly, we detected selective cell loss and preferential binding of annexin V in this subset upon 48 hr of culture with irrelevant IgM Ab. Such spontaneous apoptosis in culture of lymphocytes activated *in vivo* has been well documented in viral infections and lymphoproliferative disorders.[29,31–33] Although a minor proportion of lymphocytes from healthy individuals has consistently been reported to undergo spontaneous apoptosis in culture,[31,32,34] the phenotype of these lymphocytes has not been characterized. Here, we show that CD8^{+} T cells from healthy individuals prone to spontaneous apoptosis in culture reside preferentially in the CD8^{+} GrB^{+} T-cell subset. The expression pattern of activation and differentiation markers and apoptosis related proteins in this subset strongly resembles the expression pattern of such proteins by CD8^{+} T cells prone to spontaneous apoptosis during viral infections.[31] This implies that increased levels of spontaneous apoptosis in pathophysiologic conditions may represent a homeostatic response by expanded CD8^{+} T-cell populations and do not necessarily reflect abnormalities that contribute to the pathogenesis of such conditions.

Fas–Fas ligand interactions account for the molecular mechanism of activation-induced cell death of T cells activated *in vitro*.[35] Fas is expressed on $\approx$20–40% of peripheral blood CD8^{+} T cells from healthy individuals.[36,37] Peripheral blood T cells from healthy individuals still require several days of *in vitro* stimulation to become susceptible to Fas-mediated apoptosis.[36] We studied susceptibility to Fas-mediated apoptosis of CD8^{+} GrB^{+} T cells from healthy individuals, which we consider a T-cell subset activated *in vivo*. The extent of Fas expression on these cells is indeed similar to Fas expression on peripheral blood T cells from healthy individuals stimulated *in vitro* for 7 days.[36] Yet, culturing of CD8^{+} GrB^{+} T cells with IgM anti-Fas mAb had no additional effect on the number of CD8^{+} GrB^{+} T cells undergoing apoptosis. This resistance of CD8^{+} GrB^{+} T cells from healthy individuals to Fas-mediated apoptosis seems paradoxical in view of their increased susceptibility to spontaneous apoptosis. However, spontaneous apoptosis, which has been attributed to cytokine deprivation, should be considered a pathway towards T-cell

apoptosis distinct from Fas-mediated apoptosis and these pathways have been shown to be differentially regulated.[30]

In conclusion, we have shown that GrB is expressed in PBL from healthy individuals by NK cells, TCR-$\gamma\delta^+$ T cells, $CD8^+$ T cells and a limited number of $CD4^+$ T cells. The $CD8^+$ GrB^+ T-cell subset contains relatively more activated cells and cells prone to spontaneous apoptosis than the $CD8^+$ GrB^- T-cell subset. We suggest that the presence of $CD8^+$ GrB^+ T cells in peripheral blood from healthy individuals results from immune surveillance or contact with infectious agents, and that spontaneous apoptosis of these cells might serve as a mechanism for their eventual clearance.

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REFERENCES

1. Henkart P.A. (1985) Mechansm of lymphocyte-mediated cytotoxicity. *Annu Rev Immunol* **3,** 31.
2. Liu C.-C., Walsh C.M. & Young J.D.-E. (1995) Perforin: structure and function. *Immunol Today* **16,** 194.
3. Froelich C.J., Orth K., Turbov J. *et al.* (1996) New paradigm for lymphocyte granule mediated cytotoxicity: targets bind and internalize granzyme B but an endosomolytic agent is necessary for cytosolic delivery and apoptosis. *J Biol Chem* **271,** 29 073.
4. Ebnet K., Hausmann M., Lehmann-Grube F. *et al.* (1995) Granzyme A-deficient mice retain potent cell-mediated cytotoxicity. *EMBO J* **14,** 4230.
5. Heusel J.W., Wesselschmidt R.L., Shresta S., Russel J.H. & Ley T.J. (1994) Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. *Cell* **76,** 977.
6. Chinnaiyan A.M. & Dixit V.M. (1996) The cell-death machine. *Curr Biol* **6,** 555.
7. Alnemri E.S., Livingston D.J., Nicholson D.W. *et al.* (1996) Human ICE/CED-3 protease nomenclature. *Cell* **87,** 171.
8. Sunder-Plassmann G., Wagner L., Hruby K., Balcke P. & Worman C.P. (1990) Upregulation of a lymphoid serine protease in kidney allograft recipients. *Kidney Int* **37,** 1350.
9. Berthou C., Legros-Maïda S., Soulié A. *et al.* (1995) Cord blood T lymphocytes lack constitutive perforin expression in contrast to adult peripheral blood T lymphocytes. *Blood* **85,** 1540.
10. Kummer J.A., Kamp A.M., Tadema T.M., Vos W., Meijer C.J.L.M. & Hack C.E. (1995) Localization and identification of granzymes A and B-expressing cells in normal lymphoid tissue and peripheral blood. *Clin Exp Immunol* **100,** 164.
11. Wever P.C., Mulder J.B.G., Weening J.J. & Ten Berge I.J.M. (1997) Immunocytochemical detection of granzymes A and B in peripheral blood lymphocytes from healthy individuals after nonenzymatic antigen retrieval. *J Histochem Cytochem* **45,** 467.
12. Su B., Bochan M.R., Hanna W.L., Froelich C.J. & Brahmi Z. (1994) Human granzyme B is essential for DNA fragmentation of susceptible target cells. *Eur J Immunol* **24,** 2073.
13. Hack C.E., Paardekoper J., Smeenk R.J.T., Abbink J.J., Eerenberg A.J.M. & Nijens J.H. (1988) Disruption of the internal thioester bond in the third component of complement (C3) results in the exposure of neodeterminants also present on activation products of C3. An analysis with monoclonal antibodies. *J Immunol* **141,** 1602.
14. Hanna W.L., Zhang X., Turbov J., Winkler U., Hudig D. & Froelich C.J. (1993) Rapid purification of cationic granule proteases: application to human granzymes. *Protein Expr Purif* **4,** 398.
15. Slaper-Cortenbach I.C.M., Admiraal L.G., Kerr J.M., van Leeuwen E.F., von dem Borne A.E.G.K. & Tetteroo P.A.T. (1988) Flow-cytometric detection of terminal deoxynucleotidyl transferase and other intracellular antigens in combination with membrane antigens in acute lymphatic leukemias. *Blood* **72,** 1639.
16. Koopman G., Reutelingsperger C.P.M., Kuijten G.A.M., Keehnen R.M.J., Pals S.T. & van Oers M.H.J. (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* **84,** 1415.
17. Martin S.J., Reutelingsperger C.P.M., McGahon A.J., *et al.* (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* **182,** 1545.
18. Liu C-C., Rafii S., Granelli-Piperno A., Trapani J.A. & Young J.D.-E. (1989) Perforin and serine esterase gene expression in stimulated human T cells. Kinetics, mitogen requirements, and effects of cyclosporin A. *J Exp Med* **170,** 2105.
19. Cohen J.J. (1993) Apoptosis. *Immunol Today* **14,** 126.
20. Lebow L.T. & Bonavida B. (1990) Purification and characterization of cytolytic and non-cytolytic human natural killer cell subsets. *Proc Natl Acad Sci USA* **87,** 6063.
21. Trapani J.A., Klein J.L., White P.C. & Dupont B. (1988) Molecular cloning of an inducible serine esterase gene from human cytotoxic lymphocytes. *Proc Natl Acad Sci USA* **85,** 6924.
22. Salcedo T.W., Azzoni L., Wolf S.F. & Perussia B. (1993) Modulation of perforin and granzyme messenger RNA expression in human natural killer cells. *J Immunol* **151,** 2511.
23. Koizumi H., Liu C-C., Zheng L.M. *et al.* (1991) Expression of perforin and serine esterases by human $\gamma\delta$ T cells. *J Exp Med* **173,** 499.
24. Azuma M., Phillips J.H. & Lanier L.L. (1993) $CD28^-$ T lymphocytes. Antigenic and functional properties. *J Immunol* **150,** 1147.
25. Azuma M., Cayabyab M., Phillips J.H. & Lanier L.L. (1993) Requirements for CD28-dependent T cell-mediated cytotoxicity. *J Immunol* **150,** 2091.
26. Monteiro J., Batliwalla F., Ostrer H. & Gregersen P.K. (1996) Shortened telomeres in clonally expanded $CD28^-CD8^+$ T cells imply a replicative history that is distinct from their $CD28^+$ $CD8^+$ counterparts. *J Immunol* **156,** 3587.
27. Testi R., D'Ambrosio D., de Maria R. & Santoni A. (1994) The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells. *Immunol Today* **15,** 479.
28. Roederer M., Dubs J.G., Anderson M.T., Raju P.A., Herzenberg L.A. & Herzenberg L.A. (1995) CD8 naive T cell counts decrease progressively in HIV-infected adults. *J Clin Invest* **95,** 2061.
29. Akbar A.N., Borthwick N., Salmon M. *et al.* (1993) The significance of low bcl-2 expression by CD45RO T cells in normal individuals and patients with acute viral infections. The role of apoptosis in T cell memory. *J Exp Med* **178,** 427.
30. Akbar A.N. & Salmon M. (1997) Cellular environments and apoptosis: tissue microenvironments control activated T-cell death. *Immunol Today* **18,** 72.
31. Boudet F., Lecoeur H. & Gougeon M.-L. (1996) Apoptosis associated with *ex vivo* down-regulation of Bcl-2 and up-regulation of Fas in potential cytotoxic $CD8^+$ T lymphocytes during HIV infection. *J Immunol* **156,** 2282.
32. Massaia M., Borrione P., Attisano C. *et al.* (1995) Dysregulated Fas and Bcl-2 expression leading to enhanced apoptosis in T cells of multiple myeloma patients. *Blood* **85,** 3679.
33. Lauener R.P., Hüttner S., Buisson M. *et al.* (1995) T-cell death by apoptosis in vertically human immunodeficiency virus-infected children coincides with expansion of $CD8^+$/interleukin-2

receptor^{-}/HLA-DR^{+} T cells: sign of a possible role for herpes viruses as cofactors? *Blood* **86,** 1400.

34. Emlen W., Niebur J. & Kadera R. (1994) Accelerated *in vitro* apoptosis of lymphocytes from patients with systemic lupus erythematosus. *J Immunol* **152,** 3685.
35. Ju S.-T., Panka D.J., Cui H. *et al.* (1995) Fas (CD95)/FasL interactions required for programmed cell death after T-cell activation. *Nature* **373,** 444.
36. Owen-Schaub L.B., Yonehara S., Crump W. L. Iii & Grimm E.A. (1992) DNA fragmentation and cell death is selectively triggered in activated human lymphocytes by Fas antigen engagement. *Cell Immunol* **140,** 197.
37. Miyawaki T., Uehara T., Nibu R. *et al.* (1992) Differential expression of apoptosis-related Fas antigen on lymphocyte subpopulations in human peripheral blood. *J Immunol* **149,** 3753.

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