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Localization and identification of granzymes A and B-expressing cells in normal human lymphoid tissue and peripheral blood

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SUMMARY

Cytoplasmic granules from activated natural killer (NK) and cytotoxic T lymphocytes (CTL) contain a pore-forming protein, perforin, and several homologous serine proteinases called granzymes. Expression of these proteins correlates with the cytolytic potential of cytotoxic lymphocytes. Using a panel of MoAbs specific for human granzyme A and B, respectively, expression of these proteinases in non-pathological lymphoid tissue and peripheral blood lymphocyte (PBL) subpopulations was investigated. Using immunohistochemistry and double stainings, the phenotype of granzyme-expressing cells in lymphoid tissue was investigated. Granzyme-positive cells were detected in all lymphoid tissues tested. No large differences in the number and distribution between granzyme A- and granzyme B-positive cells were observed. The highest number of positive cells was located in the red pulp of the spleen. Significant numbers were detected in tonsil, lymph nodes, liver and thymus. Low numbers were present in the lamina propria of non-inflamed stomach, small intestine and colon. Phenotypic analysis and cell sorting showed that most of the granzyme-positive cells in lymphoid tissue and PBL consisted of $CD3^-CD16^+CD56^+$ lymphocytes. Hardly any granzyme-positive $CD3^+CD8^+$ CTL were present in peripheral blood. The synthesis of granzyme A as well as B by both $CD3^-CD16^+$ $CD56^+$ and $CD3^+CD8^+$ cells in peripheral blood was increased upon IL-2 stimulation. These results indicate that in normal lymphoid tissue the predominant cytolytic cell population is formed by the NK cells, and activated CTL are rare.

Keywords granzyme A granzyme B lymphoid tissue immunohistochemistry

INTRODUCTION

Knowledge about the molecular pathways involved in the process of cytotoxicity mediated by cytotoxic T cells (CTL) and natural killer (NK) cells, has rapidly expanded. The cytoplasm of activated human CTL and NK cells contains specialized, so called cytotoxic granules. The major components of these cytotoxic granules are the protein 'perforin' [1,2], proteoglycans [3], and a unique family of highly homologous serine proteases, termed 'granzymes'. Because of its property to form pores in membranes, perforin is believed to play a central role in T lymphocyte and NK cell-mediated cytolysis (reviewed in [4,5]).

Seven different granzymes (granzymes A–G) have been isolated and cloned from mouse CTL and NK cells [6,7]. In contrast, in man full length cDNAs as well as the corresponding proteins of only two serine proteases, granzyme A and B, have been identified [8–10]. A third granzyme protein (granzyme 3) has been identified only at the protein level [11]. In addition, a full-length cDNA of another granzyme, H, has been cloned, but a corresponding protein has not yet been described [12,13].

The precise role of the granzymes in T cell- and NK cell-mediated responses is poorly understood. Observations that target cell lysis by CTL clones and isolated granules is inhibited by pretreatment with serine protease inhibitors [14–16], and that granzyme A is able to cause DNA fragmentation in target cells [17,18], suggest that granzymes may be involved in the cytotoxicity process itself. Recently, a critical role for mouse granzyme B in DNA fragmentation and apoptosis has been reported using mice in which the granzyme B gene was deleted [19]. On the other hand, other functions such as degradation of extracellular matrix [20,21] and myelin basic proteins in myelin membranes [22], and activation of pro-urokinase [23] have been ascribed to granzymes. Irrespective of their physiological role, expression of granzymes and perforin correlates with the

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cytolytic potential of cytotoxic cells [24–27], as has been shown in autoimmune diseases and transplant rejection. Therefore, the presence of these proteins in cytotoxic cells can be used to identify cytotoxic cells *in vitro* as well as *in vivo* (reviewed in [28]).

Surprisingly, little data are available on expression of granzymes A and B proteins by different lymphocytic subpopulations in normal lymphoid tissue, and only limited data on that in peripheral blood under physiological conditions. Freshly isolated peripheral blood NK cells as well as $\gamma\delta$ T cells [29–31] constitutively express perforin in agreement with their functional cytolytic potential. In contrast, unstimulated $CD3^+CD8^+$ CTL express only marginal levels of perforin and granzymes A and B mRNA, but synthesis is rapidly induced after IL-2 stimulation [32].

Recently, we developed specific MoAbs against recombinant human granzymes A and B also reacting with the natural proteins [33]. Using these MoAbs we present the results of the expression of granzymes A and B in various lymphoid and non-lymphoid tissues and in peripheral blood mononuclear cells (PBMC) under physiological conditions.

MATERIALS AND METHODS

Monoclonal antibodies

Two panels of MoAbs were raised against recombinant granzyme A and B, respectively. These MoAbs specifically reacted with the appropriate proteins in an immunoblot of IL-2-stimulated PBMC [33]. Specifically, the MoAbs GrA-8 and GrB-4 were used to detect granzymes A and B in acetone-fixed fresh frozen tissue, and the MoAbs GrA-6, GrB-7 and GrB-9 in case of paraffin-embedded, formalin-fixed tissue. All MoAbs were purified from hybridoma culture supernatant by protein G affinity-chromatography (Pharmacia Fine Chemicals, Uppsala, Sweden). Antibodies were biotinylated using long chain biotinyl-N-hydroxysuccinimide ester sulfonacid (Pierce Chemical Co., Rockford, IL) following the manufacturer's instructions.

MoAbs with the prefix CLB were obtained from our institute, the other MoAbs, unless indicated otherwise, from Becton Dickinson (San Jose, CA). The different T cell subsets and NK cells in lymphoid tissue were characterized by immunohistochemistry using MoAbs against Leu-4 (CD3), Leu-3a + 3b (CD4) and Leu-2a (CD8), MoAb CLB-FcR gran1 (against FcRIII (CD16), the low-affinity IgG receptor [34]), MoAb anti-Leu-7 against CD57 (expressed on a small subset of NK cells and T cells), and MoAb 733.24 against CD56 (NKH1 marker, kindly provided by Dr E. Roosnek, Department of Transplantation, Hospital Cantonal, Genève, Switzerland). The cellular distribution of the antigen recognized by 733.24 was similar to that for MoAb anti-Leu-19, which latter MoAb is directed against CD56 [35]. In addition, in immunoprecipitation experiments with a human NK cell line, MoAb 733.24 bound a major protein band with an apparent mol. wt of 220 000 on SDS–PAGE, and some minor protein bands migrating slightly faster, thus supporting that MoAb 733.24 is directed against CD56 [35]. Flow cytometric analysis by two-colour fluorescence using PE-labelled anti-Leu-19 and FITC-labelled 733.24, revealed that the same subset of lymphocytes, most probably NK cells, was recognized by either MoAb. MoAb CLB-gran/2 was used to detect the neutrophil marker CD15.

NK cells were purified from PBMC by absorption with MoAbs CLB-T3 (anti-CD3), CLB-CD14 (anti-CD14), CLB-CD19 (anti-CD19) to deplete T cells, monocytes and B cells, respectively. Cells were sorted using FITC-labelled anti-Leu-3a (CD4), PE-labelled anti-Leu-2a (CD8) and FITC-labelled 733.24 (anti-CD56).

Tissue selection and cell preparation

Specimens of reactive lymphoid tissues (lymph node 3, spleen 3, tonsils 3, thymus 2, liver 2 and various parts of the gastrointestinal tract (stomach, duodenum, ileum, colon and sigmoid)) were used. Most tissue specimens were randomly collected from patients undergoing surgery, some from patients that had died from a non-lymphoid, non-immune disorder, and underwent autopsy. Representative portions of the same tissue specimen were either snap-frozen in liquid nitrogen and stored at −70°C until use, or fixed immediately in 4% buffered formalin for up to 10 h and subsequently embedded in paraffin. No fresh frozen specimens were available of gastrointestinal tract tissues. PBMC were obtained from six healthy donors by Percoll (Pharmacia) density gradient centrifugation.

Separation of lymphoid subsets and generation of lymphokine-activated killer cells

From six healthy donors $CD8^+$ and $CD4^+$ lymphocyte subsets were obtained by sorting on a FACStar using FITC-labelled anti-CD4 and PE-labelled anti-CD8 MoAb. Purity was always assessed by re-analysis of the sorted population, and was always > 95%. NK cells were purified according to two methods:

1 From three donors PBMC were incubated with saturating amounts of anti-CD3, anti-CD14 and anti-CD19 MoAbs to deplete $CD3^+$ T cells, monocytes and B cells. After three washes with Iscove's modified Dulbecco's medium, supplemented with 5% (v/v) heat-inactivated (30 min at 56°C) fetal calf serum (FCS), cells were incubated with goat anti-mouse IgG-coated magnetic particles (Dynabeads M450; Dynal, Oslo, Norway) and the cells bound to the beads were removed by magnetic separation. This depletion procedure with magnetic beads was then repeated for the remaining cell population. The cell population selected in this way was > 93% positive for CD16 or CD56, and negative for CD3, CD14 and CD19, as determined by FACS analysis (donors 1–3, Table 1). Of these cells, 53% expressed low levels of CD8.
2 From PBMC of three other donors $CD56^+$ cells were isolated by sorting on a FACStar using FITC-labelled 733.24 in combination with PE-labelled anti-CD8. Purity of $CD56^+$ cells varied between 82% and 95% as determined by re-analysis of the sorted cells (donors 4–9, Table 1).

Cytospins of the isolated $CD4^+$, $CD8^+$ and NK cell subpopulations were fixed on slides with 4% (w/v) paraformaldehyde for 10 min at room temperature, washed twice with PBS pH 7·4, permeabilized using methanol 100% v/v (2 min at room temperature) and stored at −20°C.

Lymphokine-activated killer (LAK) cells were obtained by culturing PBMC at an initial concentration of $0{\cdot}5 \times 10^6$ cells/ml for 7 days in Iscove's modified Dulbecco's medium supplemented with 5% (v/v) heat-inactivated FCS, streptamycin, penicillin, β-mercaptoethanol (β-ME) and 1000 U/ml IL-2 (Chiron Corp., Emeryville, CA). Paraffin-embedded cell

Table 1. Percentages of $CD3^+CD4^+$ T helper and $CD3^+CD8^+$ T cytotoxic lymphocytes and $CD3^-CD56^+$ natural killer (NK) cells expressing granzyme A (GrA) or B (GrB) proteins. Subsets were isolated as described in Materials and Methods.

	CD4		CD8		NK	
Donor	GrA	GrB	GrA	GrB	GrA	GrB
1	0	0	0	0	50	50
2	0	0	0	0	50	50
3	0	0	0	< 1	60	60
4	0	0	< 1	< 1	50	25
5	ND	ND	0	0	75	25
6	ND	ND	< 1	< 5	40	25
7	ND	ND	0	0	30	30

blocks of unstimulated and IL-2-stimulated PBMC were prepared after overnight fixation in 4% (v/v) buffered formalin.

Immunohistochemical techniques

Paraffin sections from lymphoid tissues and PBMC/LAK cells were deparaffinized and rehydrated. After washes with distilled water and PBS, sections were treated twice with target unmasking fluid (Kreatech, Amsterdam, The Netherlands) for 5 min at 90°C as described [36], to enhance reactivity of the anti-granzyme MoAbs. Slides were washed again with PBS, and incubated with methanol containing 0·3% (v/v) H_2O_2 (Sigma, St Louis, MO) for 30 min to inactivate endogenous peroxidase. To block non-specific binding, the sections were pre-incubated for 10 min with normal rabbit serum, 1 : 50 diluted in PBS 1% (w/v) bovine serum albumin (PBS-B). Next, sections were incubated with GrA-6 or GrB-9 MoAb (20–50 μg/ml in PBS-B) for 1 h at room temperature, washed three times with PBS and incubated for 30 min with biotinylated polyclonal rabbit anti-mouse Fab_2 immunoglobulin (Dakopatts, Copenhagen, Denmark), 1 : 500 diluted in PBS containing 10% (v/v) normal human serum (PBS–NHS). Sections were washed with PBS and subsequently incubated for 30 min with streptavidin–biotin peroxidase complex (Vector Elite Kit, Burlingame, CA) diluted in PBS–NHS, according to the manufacturer's instructions. The slides were then stained for 3 min with 30 mg diaminobenzidine (Sigma) in 100 ml PBS containing 20 μl 30% (v/v) H_2O_2. As a control, sections were incubated with an irrelevant MoAb of the appropriate subclass.

Double staining

To identify the phenotype of granzyme-expressing cells in lymphoid tissue a sequential immunohistochemical technique was used. Serial 4-μm cryostat sections were mounted on poly-L-lysine slides, air dried and fixed in acetone at room temperature for 10 min. The slides were then rinsed with PBS, preincubated with normal rabbit serum for 10 min, and then incubated with the primary MoAb (directed against different CD markers) for 1 h at room temperature. After another washing procedure with PBS, the slides were incubated for 60 min with rabbit anti-mouse IgG (Dakopatts; 1 : 25 diluted in PBS–NHS), washed with PBS, and then incubated for 60 min with immune complexes of a MoAb against alkaline phosphatase and alkaline phosphatase as an antigen (APAAP, Dakopatts; 1 : 50 diluted in PBS–NHS). To improve the sensitivity of the procedure, the slides were incubated for a second time with the rabbit anti-mouse and APAAP immune complexes for 15 min, subsequently. Bound alkaline phosphatase was then visualized by addition of naphtol AS-MX phosphate (Sigma) and fast blue BB in 0·2 M Tris–HCl pH 8·5. Endogenous alkaline phosphatase activity present in the tissues was blocked by addition of 1 mM levamisole to the reaction mixture. Subsequently, these slides were incubated with biotinylated MoAb GrA-8 or GrB-4. Binding of these MoAbs was detected using the streptavidin–biotin peroxidase method. Briefly, slides were preincubated with 10% (v/v) normal mouse serum for 15 min, to prevent binding of the biotinylated MoAb to the rabbit anti-mouse IgG used. Slides were then incubated for 60 min with biotinylated GrA-8 or GrB-4 diluted in PBS-B, washed with PBS and incubated for 25 min with 0·1% (w/v) sodium azide, 0·3% (v/v) H_2O_2, to block endogenous peroxidase activity. The binding of the biotinylated MoAbs was detected using the streptavidin–biotin–peroxidase method described above. Peroxidase activity was visualized using amino-ethyl-carbozole (Sigma).

Cytospins of isolated lymphocyte subsets were air dried and stained for granzyme expression as described above using the biotinylated MoAbs GrA-8 and GrB-4 in combination with the streptavidin–biotin–peroxidase method. Peroxidase activity was detected using diaminobenzidine.

RESULTS

Detection of granzymes A and B by immunohistochemical staining

To determine the potential use for immunohistochemical studies the MoAbs against granzyme A and B were tested on both acetone-fixed and on paraffin-embedded, formalin-fixed PBMC and LAK cells. MoAbs GrA-8 and GrB-4 detected granzyme A and B antigens in acetone-fixed cells, whereas MoAbs GrA-6, GrB-7 and GrB-9 preferentially reacted with formalin-fixed, paraffin-embedded cells. Pretreatment of the formalin-fixed tissue with target unmasking fluid (TUF) was necessary to enhance reactivity and to reduce non-specific background staining of the antibodies. Equal results were obtained by pretreatment with 10 mM sodium citrate pH 6·0 for 10 min at 100°C. Figure 1 shows formalin-fixed, paraffin-embedded LAK cells stained with MoAb GrA-6 (Fig. 1a), GrB-7 (Fig. 1b) and a control, irrelevant MoAb (Fig. 1c). More than 60% of the LAK cells expressed granzyme A or B. In general the staining pattern was granular and cytoplasmic, in agreement with the granular localization of the granzymes [8]. These cells were used as positive control in our immunohistochemical experiments. When tested on unstimulated PBMC, GrA-6 (Fig. 1d) and GrB-7 MoAbs detected low levels of granzyme A and B, respectively, in only 5–10% of the cells. MoAb GrB-9 showed similar staining patterns. In addition, GrB-9 was able to detect granzyme B in CTL by immuno-electronmicroscopy (not shown). In these experiments it appears that this MoAb bound to the dense core and internal vesicles associated with the cytotoxic granules, in agreement with the subcellular localization of the granzyme B antigen [37]. Thus, the MoAbs obtained could be used to detect expression of granzymes A or B *in vivo* in freshly frozen, acetone-fixed or in formalin-fixed, paraffin-embedded tissue.

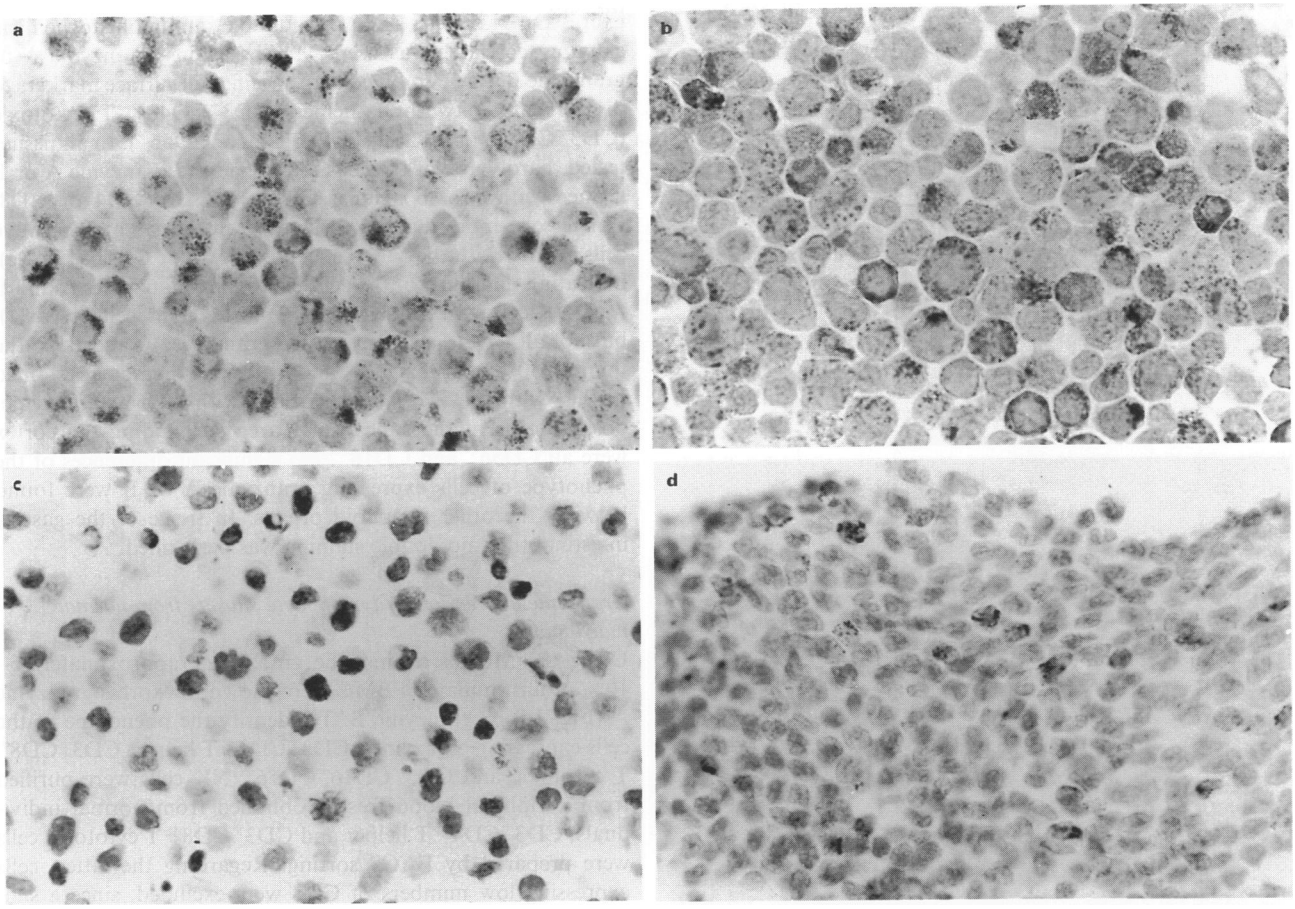

Fig. 1. Immunohistochemical staining of lymphokine-activated killer (LAK) cells (a–c) and fresh peripheral blood mononuclear cells (PBMC) (d) with MoAbs GrA-6 and GrB-7. Sections of paraffin-embedded cells were deparaffinized and treated with target unmasking fluid (TUF) and incubated with MoAb GrA-6 (60 μg/ml; a and d), GrB-7 (20 μg/ml; b) and a control MoAb (60 μg/ml; c). Peroxidase is detected in brown using diaminobenzidine (mag. a–d ×130).

Localization and identification of granzyme-expressing cells in normal lymphoid tissue

To study the distribution and localization of granzyme-expressing cells in lymphoid organs, paraffin sections were stained with GrA-6, GrB-7 or GrB-9. Granzyme A- as well as granzyme B-expressing cells were detected in all lymphoid organs, although the number of granzyme-positive cells varied among different organs studied. As in LAK cells, staining with anti-granzyme MoAbs revealed a cytoplasmic, sometimes granular pattern (Fig. 2a). Granzyme A and granzyme B-positive cells were similarly distributed in the different organs; in addition, the numbers of granzyme A- and granzyme B-expressing cells were the same within each tissue investigated. Generally, the staining intensity of GrA-6 was less intense than that of GrB-7 or GrB-9. The largest number of cells expressing granzymes A and B was found in the spleen (Fig. 2a–c) and tonsil (Fig. 3b), whereas the smallest number occurred in the gastrointestinal tract (Fig. 3d). Generally, the distribution and the number of granzyme-expressing cells found was identical in several specimens of the same lymphoid organ.

In the spleen granzyme-expressing cells were located in sinuses of the red pulp (Fig. 2b). No positive cells were found in the main T and B cell areas: the periarteriolar lymphoid sheath and the germinal centre. In the liver, positive cells were scattered throughout the tissue and located in the sinusoids between the liver cords and within the portal triads.

In tonsils, large numbers of granzyme A and B-positive cells were found in the interfollicular area, especially around high endothelial venules. Positive cells did also infiltrate the epithelium of the tonsil crypts (Fig. 3b). In lymph nodes a small but significant number of positive cells was observed in medullary sinus, medullary cords and the subcapsular sinusoids (Fig. 3a). In both organs localization of granzyme A-positive cells was identical to that of granzyme B-expressing cells, suggesting that both granzymes were produced by the same cell population. No positive cells were observed in the outer cortex and in the follicle centra. Some granzyme-producing cells were also observed in the thymus. Positive cells were located in the medulla (Fig. 3c) and in the subcapsular area, surrounding the cortex and the interlobular septa. Sporadically, a granzyme-positive cell was found in the thymic cortex.

In the gastrointestinal tract only few granzyme A or B-positive cells were found. Mainly, these cells were scattered throughout the lamina propria at the base of the crypts, and sometimes around the lymph follicles of Peyer's patches in the ileum. Although few positive cells were found in the lamina

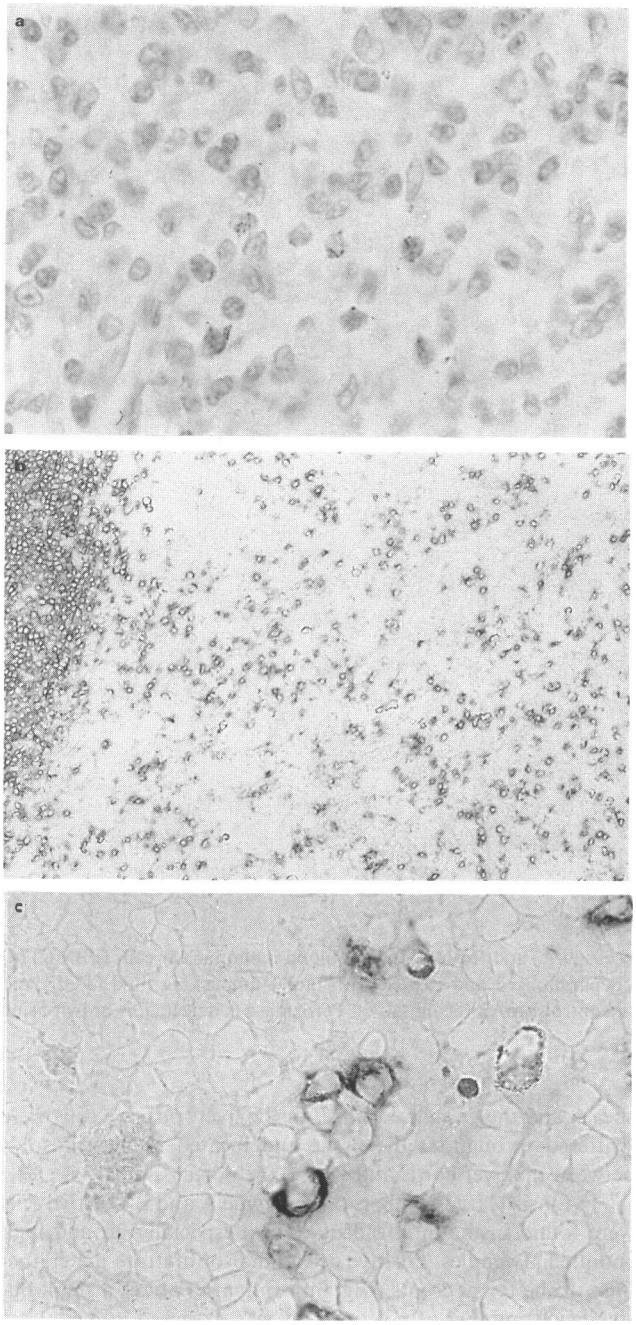

Fig. 2. Granzyme A and B-positive cells in the spleen. Granzyme B-positive cells are detected by immunostaining on target unmasking fluid (TUF)-treated, paraffin-embedded sections (a, mag. ×160), using MoAb GrB-7 (detected in brown using DAB). A double immunohistochemical staining was used to identify the phenotype of granzyme A-expressing cells in freshly frozen, acetone-fixed, tissue. A peroxidase technique (red) was used to detect granzyme A (using MoAb GrA8) and alkaline phosphatase to detect CD3 (b, mag. ×130) or CD56 (c, mag. ×160).

propria of the villi (Fig. 3d), no granzyme-postive intraepithelial lymphocytes were found.

Identification of granzyme-positive cells in lymphoid tissue

Granzyme A and B mRNA have been detected in activated CTL and NK cells [6]. To identify the phenotype of the cells expressing granzyme A or B, a double immunohistochemical staining technique was used. MoAbs against granzyme A or granzyme B were combined with specific cell surface markers to discriminate between T helper ($CD3^+CD4^+$), T cytotoxic ($CD3^+CD8^+$) or NK cells ($CD3^-CD16^+CD56^+$). In all tissues tested, hardly any of the cells that expressed granzyme A or B stained with MoAb against CD3 (Fig. 2b), CD4 or CD8. Conversely, more than 95% of the granzyme A or B-positive cells stained with the MoAbs against CD16 or CD56 (Fig. 2c), indicating that most of the cells expressing granzymes A and B were NK cells. On the other hand, most $CD56^+$ cells (> 90%), but not all $CD16^+$ cells, expressed granzymes A and B. In the thymus, 50% of granzyme A and B-positive thymocytes stained neither with the T cell markers nor with CD56. Granzyme-positive cells in the subcapsular area surounding the cortex were all $CD56^+$ and $CD16^+$. No significant differences of the phenotype of cells expressing granzyme A or B were found between the other lymphoid organs. In tissue of the gastrointestinal tract no double staining was performed.

Granzyme expression by lymphocyte subsets from normal individuals

Using the MoAbs against the granzymes, approximately 5–10% of unstimulated PBMC were shown to express granzyme A (Fig. 1d) or granzyme B. To identify the phenotype of the cells expressing granzymes, $CD3^+CD4^+$ T helper, $CD3^+CD8^+$ T cytotoxic and $CD3^-CD16^+CD56^+$ NK cells were purified from peripheral blood freshly obtained from normal individuals. $CD3^+CD4^+$ T helper and $CD3^+CD8^+$ T cytotoxic cells were prepared by FACS sorting. Regarding the latter, cells expressing low numbers of CD8 were excluded, since a subpopulation of $CD3^-CD8^{low}CD16^+$, representing NK cells, have been described [35]. Table 1 represents the expression of the granzymes A and B by the purified lymphocyte subpopulations of the normal donors. None of the $CD4^+$ T cells and hardly any of the $CD8^{high}$ T cells expressed detectable levels of granzyme A or B. At the most, up to 2% of the $CD8^{high}$ cells from some donors were positive for granzyme B.

In contrast, a varying amount of NK cells, isolated by an immunoabsorption procedure or FACS sorting, expressed granzyme proteins: 30–80% expressed granzyme A and 25–60% expressed granzyme B, depending on the donor (Table 1). Each purified NK cell preparation contained granzyme-positive cells as well as granzyme-negative cells. No difference in morphology between these cells was observed. In the positive control, consisting of cytospins of LAK cells, more than 60% of these cells showed a strong granular staining pattern.

After stimulation with IL-2, granzyme A and B proteins were detected in more than 60% of the activated PBMC (Fig. 1a,b). This LAK cell population consisted of 70% $CD3^+$, 55% $CD8^+$ and 40% $CD16^+/CD56^+$ cells as determined by FACS analysis. Clearly, LAK cells consist of a heterogeneous lymphocyte population consisting of $CD3^+$ CTL as well as $CD3^-$ NK cells. In addition, some non-cytotoxic cells, e.g. non-cytolytic T helper cells and B lymphocytes, are present. This heterogeneous cytotoxic cell population as well as the presence of non-cytotoxic cells explains the non-uniform staining pattern and the fact that not all cells are granzyme-positive.

From these experiments we conclude that granzyme A and B proteins are constitutively synthesized *in vivo* by a subpopulation of NK cells, and not by unstimulated $CD3^+CD8^+$

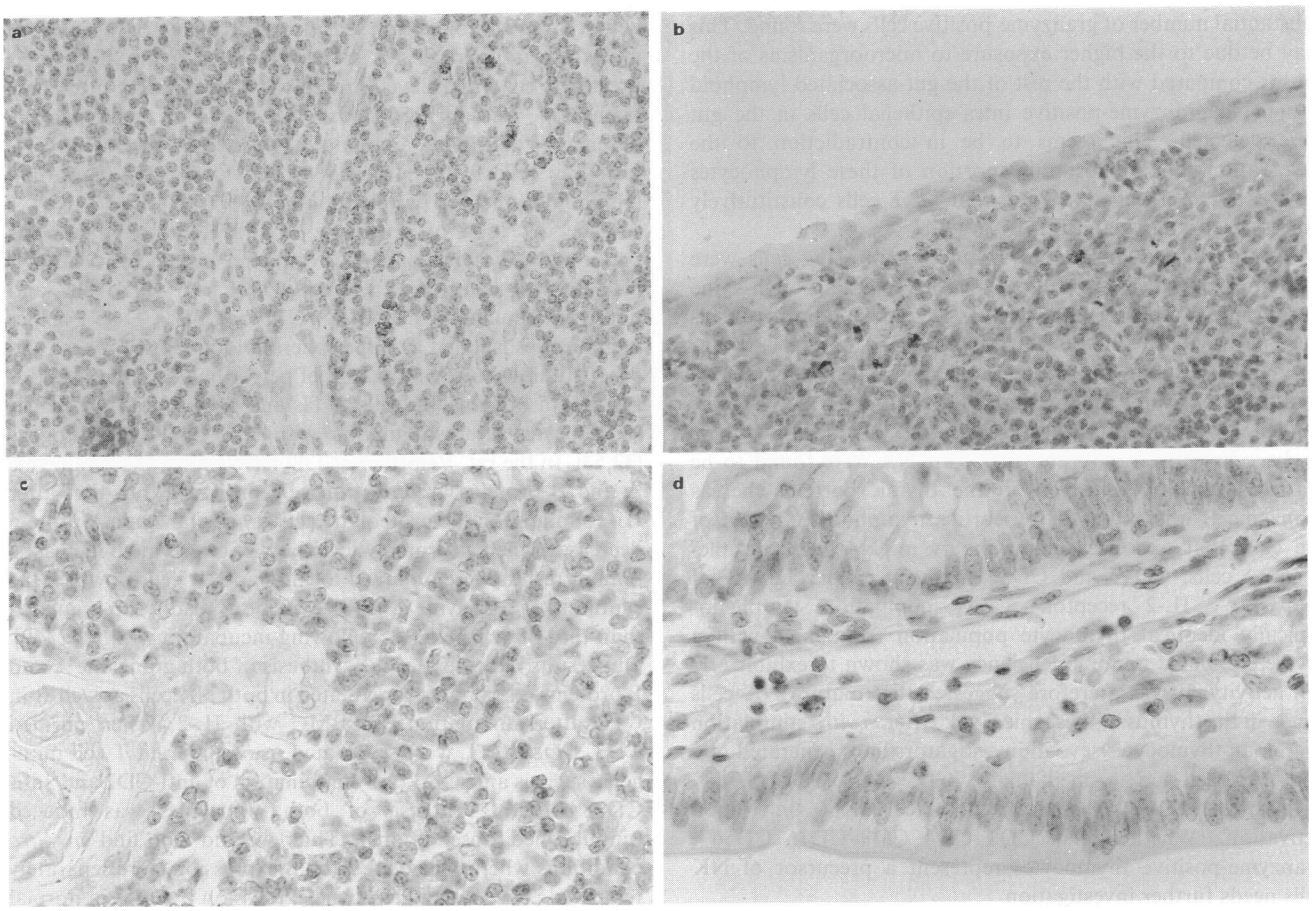

Fig. 3. Immunoperoxidase staining (brown) of granzyme B-positive cells in different lymphoid tissues using MoAb GrB-7. Granzyme B-positive cells were detected in lymph nodes (a, mag. ×100), tonsil (b, mag. ×100), thymus (c, mag. ×130) and ileum (d, mag. ×130).

cells, and that synthesis of these enzymes can be induced in both subsets by IL-2.

DISCUSSION

In this study the presence and distribution of granzyme A and B-expressing cells in lymphoid organs and peripheral blood was investigated. In addition, the phenotype of these cells was determined by double immunohistochemical staining techniques and cell sorting. The expression of both granzyme A and granzyme B protein was investigated because it has been reported in humans and in rats that expression of granzyme A and B mRNA by cytotoxic cells is not regulated in the same way [32,38,39]. Our findings indicate that in man under physiological conditions most cells expressing granzyme A *in vivo* also express granzyme B, and that NK cells are the major cell subset expressing these granzymes.

The panel of MoAbs [33] contained MoAbs which detect both granzyme antigens in acetone-fixed fresh frozen material as well as in TUF or sodium citrate-treated, formalin-fixed paraffin-embedded tissue. The use of MoAbs is often severely hampered by fixation procedures which may impair the structure of the epitope recognized. As formalin is very often used as a fixative in routinely paraffin-embedded tissue samples, it was important that the MoAb reacted with granzymes in formalin-fixed tissue. Treatment of the paraffin slides with TUF or sodium citrate, however, appeared to be essential to retrieve the epitope recognized by the MoAbs. Incubation with proteolytic enzymes, used to retrieve immune reactivity, did not result in an enhanced reactivity.

In all lymphoid organs tested, both granzyme A- and granzyme B-positive cells were detected. With the exception of the tonsil, granzyme-positive cells were mainly located outside the major T and B cell areas, for example in the red pulp and marginal zone of the spleen and medullary cords and sinuses of lymph nodes. The highest frequency of granzyme-positive cells occurred in the spleen; lower but still significant amounts were detected in tonsil, liver, lymph node and thymus. In the gastrointestinal tract a positive cell was found only sporadically. This localization and distribution is similar to the organ and tissue distribution of NK cell activity and markers previously described in rat [40–42] and in man [42,43]. The results obtained with double immunohistochemical staining confirmed that the majority of granzyme A- and B-positive cells, as detected in reactive lymphoid tissues, bear the NK cell phenotype, i.e. $CD3^-$, $CD16^+$ and $CD56^+$. An occasional granzyme-expressing cell was $CD3^+$ or $CD8^+$, and none was $CD4^+$, indicating that in these tissues NK cells constitutively express granzymes. Remarkably, tonsils were the only part of the gut-associated lymphoid tissue where a

substantial number of granzyme-positive cells were found. This may be due to the higher exposure to microorganisms of the tonsils compared with the rest of the gut-associated lymphoid tissue. No granzyme-positive intra-epithelial cells in the gut were detected. This seems to be in contradiction to the observation that a substantial portion of these lymphocytes consists of $\gamma\delta$ T cells [44], and that $\gamma\delta$ T cells constitutively express granzyme A and B mRNA [30].

In the thymus granzyme A- and B-expressing cells were predominantly located in the thymic medulla, although some were found in the cortex as well. All granzyme-positive cells were negative for CD3, CD4 and CD8, and only a part was $CD56^+$. These findings are in partial agreement with a previous study where expression of granzyme A and B mRNA was studied in the thymus of 6–8-week-old mice [45]. In this study, cells expressing granzyme A mRNA were found to be scattered almost exclusively in the thymic cortex, whereas granzyme B was expressed by cortical lymphocytes. Most of the cells that expressed granzyme A mRNA were double-negative cells, $CD4^-$ and $CD8^-$ thymocytes, particularly of the $CD3^-$, IL-2 receptor-negative population. A phenotypically identical thymocyte population from mice with a severe combined immunodeficiency was shown to exhibit NK cell activity. It was therefore suggested that mature NK cells reside in the thymus of these mice [46]. After *in vitro* stimulation of human thymocytes several investigators have generated NK and LAK cell activity mediated by $CD3^-CD4^-CD8^-CD56^+$ thymocytes [47,49]. These cells acquired CD56 only upon activation. Whether the $CD3^-CD4^-CD8^-CD16^-CD56^-$, granzyme-positive thymocytes represent a precursor of NK cells needs further investigation.

Unexpectedly, we observed that the MoAbs GrA-6, GrA-8 and especially GrB-9 also stained some granulocytes in a granular pattern (results not shown). With MoAb GrB-7 a positive granulocyte was observed only sporadically. GrB-7 and GrB-9 each recognized a different epitope on granzyme B. This reactivity with granulocytes in tissues was not due to aspecific Fc interactions, since GrB-9 Fab_2 fragments showed a similar reactivity with granulocytes. We suppose that GrB-9 and the granzyme A MoAbs cross-react with homologous serine proteases in the granulocytes. For example, the neutrophil-associated serine protease cathepsin G is 56·1% homologous to human granzyme B [7]. In a series of experiments not shown here, we have excluded the possibility that this reaction with granulocytes was due to production of granzymes by these cells. For example, MoAb GrB-9 did not recognize a specific protein band on immunoblots made from purified granules from granulocytes.

When lymphocyte subsets isolated from peripheral blood were tested for expression of granzymes, similar results were obtained compared to lymphoid tissue. Granzymes A and B were predominantly expressed by NK cells, not by $CD3^+CD4^+$ T helper cells. In two donors $< 2\%$ of $CD3^+CD8^+$ CTL expressed granzyme B. This could be due to contamination with $CD8^+$ NK cells, or to cells representing a subpopulation consisting of $\gamma\delta$-CTL or a $CD11b^+$ subset of $CD8^+$ $\alpha\beta$-CTL which both, in addition to NK cells, constitutively express perforin mRNA and protein [29,30,50]. The constitutive expression of cytolytic molecules by NK cells present in peripheral blood as well as in lymphoid tissue is in agreement with observations that NK cells have spontaneous cytotoxic activity, i.e. without prior sensitization, against virus-infected and tumour cells [51,52]. In contrast, CTL are present as precursor cells and develop cytolytic activity only after help from $CD4^+$ cells during an immune response. In this respect the absence of detectable amounts of granzyme proteins in CTL probably reflects the absence of activated CTL in normal lymphoid tissue and peripheral blood.

The detection of granzyme A and B proteins supports previous studies in which perforin and granzyme A and B mRNA were detected in resting $CD3^+CD16^+$ NK cells, and not in unstimulated $CD3^+CD8^+$ CTL or $CD3^+CD4^+$ T helper lymphocytes isolated from peripheral blood [32,53]. These and other studies showed induction of perforin, granzyme A and B mRNA in human NK cells, CTL and sometimes in T helper lymphocytes after stimulation with IL-2 or T cell mitogens [32,53]. In the rat, Velotti *et al.* [38] have shown that granzyme A and B proteins are constitutively produced, but that after IL-2 stimulation the proteolytic activity of granzyme A protein decreases, whereas proteolytic activity of granzyme B increases. Similar results were obtained for human NK cells in which the synthesis of granzyme B mRNA is more readily up-regulated than that of granzyme A, following incubation with IL-2 [39]. Our results indicate that the synthesis of both granzyme A and B proteins is strongly up-regulated in both NK cells as well as in CTL after stimulation of PBMC with IL-2. When purified $CD3^+CD8^+$ CTL were stimulated using different T cell mitogens, such as anti-CD3 or a combination of anti-CD2 and anti-CD28 MoAb, synthesis of both granzymes was induced (unpublished observations). Thus we did not find *in vitro* conditions where granzyme A or B expression were separately regulated. The lack of granzyme expression by CTL in normal lymphoid tissue was not due to technical failures. CTL infiltrating the liver in patients infected with hepatitis C virus (C. Bronkhorst, unpublished observations) and CTL infiltrating acutely rejected renal allograft tissue [54] stained with MoAbs against granzyme A and granzyme B. Moreover, we were able to detect granzyme B expression by several peripheral T cell lymphomas ($CD3^+CD8^+$) using the same immunohistochemical methods as described in this study [55].

Using a rat MoAb anti-mouse perforin Nakata *et al.* detected perforin protein in more than 95% of freshly isolated human NK cells [29]. In contrast, we found only 25–60% of the isolated peripheral NK cells, depending on the donor, staining with the anti-granzyme A and B MoAbs. Some cells showed marked granular staining, whereas others in the same preparation were hardly, if at all stained. This difference was not due to contamination with other cells, since the preparations used consisted at least 85% NK cells. We therefore favour the explanation that NK cells express heterogeneous levels of granzyme proteins, and that low levels of granzyme proteins are not detected by the MoAb used. Support for this hypothesis is provided by observations in electronmicroscopic studies of the ultrastructure of purified NK cells. It was demonstrated that purified NK cells could be divided into different morphological subpopulations, including one which represents approximately 40% of the NK cells and which expresses NK cell markers but has an agranular morphology [56]. Thus, this observed absence of cytotoxic granules is consistent with our findings that some cells with apparent NK cell phenotype do express lower levels of granzymes. However, for the lymphoid tissues apparently the sensitivity of the methods used was

sufficient, since virtually all $CD56^+$ cells were also granzyme-positive.

In conclusion, we show that most cells that express granzymes under physiological conditions in lymphoid tissues are NK cells. These findings are consistent with the concept that NK cells *in vivo* are capable of mediating cytotoxicity without prior stimulation, whereas CTL require prior activation.

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REFERENCES

1 Lowrey DM, Hameed A, Lichtenheld M *et al.* Isolation and characterization of cytotoxic granules from human lymphokine (interleukin 2) activated killer cells. Cancer Res 1988; **48**:4681–8.
2 Lichtenheld MG, Olsen JK, Lu P *et al.* Structure and function of human perforin. Nature 1988; **335**:448–51.
3 MacDermott RP, Schmidt RE, Caulfield RP *et al.* Proteoglycans in cell-mediated cytotoxicity. Identification, localization, and exocytosis of a chondroitin sulfate proteoglycan from human cloned natural killer cells during target cell lysis. J Exp Med 1985; **162**:1771–87.
4 Krahenbuhl O, Tschopp J. Debate: the mechanism of lymphocyte-mediated killing: perforin-induced pore formation. Immunol Today 1991; **12**:399–403.
5 Doherty PC. Cell-mediated cytotoxicity. Cell 1993; **75**:607–12.
6 Jenne DE, Tschopp J. Granzymes, a family of serine proteases released from granules of cytolytic T lymphocytes upon T cell receptor stimulation. Immunol Rev 1988; **103**:53–71.
7 Jenne DE, Masson D, Zimmer M *et al.* Isolation and complete structure of the lymphocyte serine protease granzyme G, a novel member of the granzyme multigene family in murine cytolytic T lymphocytes. Evolutionary origin of lymphocyte proteases. Biochemistry 1989; **28**:7953–61.
8 Krahenbuhl O, Rey C, Jenne D *et al.* Characterization of granzymes A and B isolated from granules of cloned human cytotoxic T lymphocytes. J Immunol 1988; **141**:3471–7.
9 Gershenfeld HK, Hershberger RJ, Shows TB *et al.* Cloning and chromosomal assignment of a human cDNA encoding a T cell- and natural killer cell-specific trypsin-like serine protease. Proc Natl Acad Sci USA 1988; **85**:1184–8.
10 Trapani JA, Klein JL, White PC *et al.* Molecular cloning of an inducible serine esterase gene from human cytotoxic lymphocytes. Proc Natl Acad Sci USA 1988; **85**:6924–8.
11 Hameed A, Lowrey DM, Lichtenheld M *et al.* Characterization of three serine esterases isolated from human IL-2 activated killer cells. J Immunol 1988; **141**:3142–7.
12 Meier M, Kwong PC, Fregeau CJ *et al.* Cloning of a gene that encodes a new member of the human cytotoxic cell protease family. Biochemistry 1990; **29**:4042–9.
13 Haddad P, Jenne D, Tschopp J *et al.* Structure and evolutionary origin of the human granzyme H gene. Int Immunol 1991; **3**:57–66.
14 Hudig D, Haverty T, Fulcher C *et al.* Inhibition of human natural cytotoxicity by macromolecular antiproteases. J Immunol 1981; **126**:1569–74.
15 Hudig D, Allison NJ, Kam C-M *et al.* Selective isocoumarin serine protease inhibitors block RNK-16 lymphocyte granule-mediated cytolysis. Mol Immunol 1989; **26**:793–8.
16 Hudig D, Allison NJ, Pickett TM *et al.* The function of lymphocyte proteases. Inhibition and restoration of granule-mediated lysis with isocoumarin serine protease inhibitors. J Immunol 1991; **147**:1360–8.
17 Hayes MP, Berrebi GA, Henkart PA. Induction of target cell DNA release by the cytotoxic T lymphocyte granule protease granzyme A. J Exp Med 1989; **170**:933–46.
18 Shiver JW, Su L, Henkart PA. Cytotoxicity with target DNA breakdown by rat basophilic leukemia cells expressing both cytolysin and granzyme A. Cell 1992; **71**:315–22.
19 Heusel JW, Wesselschmidt RL, Shresta S *et al.* Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. Cell 1994; **76**: 977–87.
20 Simon MM, Simon HG, Fruth U *et al.* Cloned cytolytic T-effector cells and their malignant variants produce an extracellular matrix degrading trypsin-like serine proteinase. Immunology 1987; **60**:219–30.
21 Froelich CJ, Zhang X, Turbov J *et al.* Human granzyme B degrades aggrecan proteoglycan in matrix synthesized by chondrocytes. J Immunol 1993; **151**:7161–71.
22 Vanguri P, Lee E, Henkart P *et al.* Hydrolysis of myelin basic protein in myelin membranes by granzymes of large granular lymphocytes. J Immunol 1993; **150**:2431–9.
23 Brunner G, Simon MM, Kramer MD. Activation of pro-urokinase by the human T cell-associated serine proteinase HuTSP-1. FEBS Lett 1990; **260**:141–4.
24 Gershenfeld HK, Weissman IL. Cloning of a cDNA for a T cell-specific serine protease from a cytotoxic T lymphocyte. Science 1986; **232**:854–8.
25 Garcia-Sanz JA, Velotti F, MacDonald HR *et al.* Appearance of granule-associated molecules during activation of cytolytic T-lymphocyte precursors by defined stimuli. Immunology 1988; **64**:129–34.
26 Schmid J, Weissmann C. Induction of mRNA for a serine protease and a β-thromboglobulin-like protein in mitogen-stimulated human leucocytes. J Immunol 1987; **139**:250–6.
27 Garcia-Sanz JA, MacDonald HR, Jenne DE *et al.* Cell specificity of granzyme gene expression. J Immunol 1990; **145**:3111–8.
28 Griffiths GM, Mueller C. Expression of perforin and granzymes *in vivo*: potential diagnostic markers for activated cytotoxic cells. Immunol Today 1991; **12**:415–9.
29 Nakata M, Smyth MJ, Norihisa Y *et al.* Constitutive expression of pore-forming protein in peripheral blood $\gamma\delta$ T cells: implication for their cytotoxic role *in vivo*. J Exp Med 1990; **172**:1877–80.
30 Koizumi H, Liu CC, Zheng LM *et al.* Expression of perforin and serine esterases by human gamma/delta T cells. J Exp Med 1991; **173**:499–502.
31 Smyth MJ, Strobl SL, Young HA *et al.* Regulation of lymphokine-activated killer activity and pore forming protein gene expression in human peripheral blood $CD8^+$ T lymphocytes. Inhibition by transforming growth factor-beta. J Immunol 1991; **146**:3289–97.
32 Liu CC, Rafii S, Granelli-Piperno A *et al.* Perforin and serine esterase gene expression in stimulated human T cells. J Exp Med 1989; **170**:2105–18.
33 Kummer JA, Kamp AM, van Katwijk M *et al.* Production and characterization of monoclonal antibodies raised against recombinant human granzymes A and B and showing cross reactions with the natural proteins. J Immunol Methods 1993; **163**:77–83.
34 Huizinga TWJ, Kleijer M, Roos D, von dem Borne AEGKr. Differences between FcRIII of human neutrophils and human K/NK lymphocytes in relation to the NA antigen system. In: Knapp W, Dörken W, Gilks WR, Rieber EP, Schmidt RE, Stein H, von dem Borne AEGKr, eds. Leucocyte typing IV. Oxford: Oxford University Press, 1989: 582–5.
35 Lanier LL, Le AM, Civin CI *et al.* The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. J Immunol 1986; **136**:4480–6.
36 van den Berg FM, Claessen N, Mulder J *et al.* Target unmasking fluid (TUF) for immunohistochemistry in (archival) paraffin-embedded tissue. Eur Clin Laboratory 1993; **2**:24–25.

37 Peters PJ, Borst J, Oorschot V *et al.* Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. J Exp Med 1991; **173**:1099–109.
38 Velotti F, Palmieri G, D'Ambrosio D *et al.* Differential expression of granzyme A and granzyme B proteases and their secretion by fresh rat natural killer cells (NK) and lymphokine-activated killer cells with NK phenotype (LAK-NK). Eur J Immunol 1992; **22**:1049–53.
39 Salcedo TW, Azzoni L, Wolf SF *et al.* Modulation of perforin and granzyme messenger RNA expression in human natural killer cells. J Immunol 1993; **151**:2511–20.
40 Rolstad B, Herberman RB, Reynolds CW. Natural killer cell activity in the rat: V. The circulation patterns and tissue localization of peripheral blood large granular lymphocytes (LGL). J Immunol 1986; **136**:2800–8.
41 van den Brink MRM, Palomba ML, Basse PH *et al. In situ* localization of 3.2.3.$^+$ natural killer cells in tissues from normal and tumor-bearing rats. Cancer Res 1991; **51**:4931–6.
42 Reynolds CW, Ward JM. Tissue and organ distribution of NK cells. In: Lotzova E, Herberman RB, eds. Immunobiology of NK cells, Vol. I. Boca Raton: CRC Press, 1986: 63–71.
43 Perussia B, Starr S, Abraham S *et al.* Human natural killer cells analysed by B73.1, a monoclonal antibody blocking Fc receptor functions. I. Characterization of the lymphocyte subset reactive with B73.1. J Immunol 1983: **130**:2133–41.
44 Vroom TM, Scholte G, Borst J. Tissue distribution of human $\tau\delta$ T cells: no evidence for general epithelial tropism. J Clin Pathol 1994; **44**: 1012–7.
45 Held W, MacDonald HR, Mueller C. Expression of genes encoding cytotoxic cell-associated serine proteases in thymocytes. Int Immunol 1990; **2**:57–62.
46 Garni-Wagner BA, Witte PL, Tutt MM *et al.* Natural killer cells in the thymus. Studies in mice with severe combined immune deficiency. J Immunol 1990; **144**:796–803.
47 Michon JM, Caligiuri MA, Hazanow SM *et al.* Induction of natural killer effectors from human thymus with recombinant IL-2. J Immunol 1988; **140**:3660–7.
48 Ramsdell FJ, Golub SH. Generation of lymphokine-activated killer cell activity from human thymocytes. J Immunol 1987; **139**:1446–53.
49 Hori T, Spits H. Clonal analysis of human $CD4^-CD8^-CD3^-$ thymocytes highly purified from postnatal thymus. J Immunol 1991; **146**:2116–21.
50 Young LHY, Joag SV, Lin PY *et al.* Expression of cytolytic mediators by synovial fluid lymphocytes in rheumatoid arthritis. Am J Pathol 1992; **140**:1261–8.
51 Whiteside TL, Heberman RB. The role of natural killer cells in human disease. Clin Immunol Immunopathol 1989; **53**:1–23.
52 Trinchieri G. Biology of natural killer cells. Adv Immunol 1989; **47**:187–376.
53 Lu P, Garcia-Sanz JA, Lichtenheld MG *et al.* Perforin expression in human peripheral blood mononuclear cells: definition of an IL-2-independent pathway of perforin induction in $CD8^+$ T cells. J Immunol 1992; **148**:3354–60.
54 Kummer JA, Wever, PC, Kamp AM *et al.* Expression of granzyme A and B proteins by cytotoxic lymphocytes involved in acute renal allograft rejection. Kidney Int 1994; 1995; **47**:70–77.
55 de Bruin PC, Kummer JA, van der Valk P *et al.* Granzyme B expressing peripheral T-cell lymphomas: neoplastic equivalents of activated cytotoxic T-cells with preference for mucosa associated lymphoid tissue (MALT) localization. Blood 1994; **84**:3785–91.
56 Lebow LT, Bonavida B. Purification and characterization of cytolytic and noncytolytic human natural killer cell subsets. Proc Natl Acad Sci USA 1990; **87**: 6063–7.