

Granzymes A and B Are Targeted to the Lytic Granules of Lymphocytes by the Mannose-6-Phosphate Receptor

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Abstract. To investigate the question of whether lytic granules share a common biogenesis with lysosomes, cloned cytolytic T cell lines were derived from a patient with I-cell disease. The targeting of two soluble lytic granule components, granzymes A and B, was studied in these cells which lack a functional mannose-6-phosphate (Man-6-P) receptor-mediated pathway to lysosomes. Using antibodies and enzymatic substrates to detect the lytic proteins, I-cells were found to constitutively secrete granzymes A and B in contrast to normal cells in which these proteins were stored for regulated secretion. These results suggest that granzymes A and B are normally targeted to the lytic granules of activated lymphocytes by the Man-6-P receptor.

In normal cells, the granzymes bear Man-6-P residues, since the oligosaccharide side chains of granzymes A and B, as well as radioactive phosphate on granzyme A from labeled cells, were removed by endoglycosidase H (Endo H). However, in I-cells, gran-

zymes cannot bear Man-6-P and granzyme B acquires complex glycans, becoming Endo H resistant.

Although the levels of granzymes A and B in cytolytic I-cell lymphocytes are <30% of the normal levels, immunolocalization and cell fractionation of granzyme A demonstrated that this reduced amount is correctly localized in the lytic granules. Therefore, a Man-6-P receptor-independent pathway to the lytic granules must also exist. Cathepsin B colocalizes with granzyme A in both normal and I-cells indicating that lysosomal proteins can also use the Man-6-P receptor-independent pathway in these cells. The complete overlap of these lysosomal and lytic markers implies that the lytic granules perform both lysosomal and secretory roles in cytolytic lymphocytes. The secretory role of lytic granules formed by the Man-6-P receptor-independent pathway is intact as assessed by the ability of I-cell lymphocytes to lyse target cells by regulated secretion.

LYTIC granules of cytotoxic lymphocytes and natural killer cells are specialized secretory organelles containing a unique set of proteins involved in cell-mediated killing. These granules provide a mechanism of directed regulated secretion in response to a signal generated upon specific recognition of the target by the cytotoxic cell. Upon recognition, the granules reorient within the cell and move towards the point of contact where they fuse with the plasma membrane and release their contents. This event results in the destruction of the target cell. The granules contain a small number of proteins which appear to be involved in the destruction process. The major components of the lytic granules are proteoglycans, a number of serine proteases termed granzymes of which three have been identified in human lymphocytes and eight in mouse lymphocytes, and the lytic protein perforin (for review see Tschopp and Nabholz, 1990). These granules contain all of the components required for killing and isolated granules are able to cause target cell lysis *in vitro* (Henkart et al., 1984; Podack and Konigsberg, 1984). Exactly how these proteins are selectively sorted and packaged into the lytic granules is not known.

Several recent studies have suggested that the lytic granules are closely related to lysosomes and that the two organelles may share a common biogenesis. A number of lysosomal enzymes have been colocalized with perforin and granzymes in the lytic granules in human, mouse, and rat cells, and the cation-independent mannose-6-phosphate (Man-6-P)¹ receptor has been identified in some of the granules (Burkhardt et al., 1990; Peters et al., 1991). Furthermore, lytic granules have been identified as slightly acidic organelles with a pH of 5.5, comparable to that of the prelysosomal compartment (Burkhardt et al., 1990; Masson et al., 1990). Furthermore, the uptake of exogenous proteins has demonstrated that the lytic granules, like lysosomal compartments, can be reached by the endocytic pathway (Burkhardt et al., 1990). These observations demonstrate that lytic granules are endocytic compartments and may share a common biogenesis with lysosomes.

In a rat natural killer cell line it has been demonstrated that

1. *Abbreviations used in this paper:* BLT, *N*-benzyloxy-carbonyl-L-lysine-thiobenzylester; Endo H, endoglycosidase H; Man-6-P, mannose-6-phosphate; PNGase F, peptide-*N*-glycosidase F.

one of the granzymes is modified with a Man-6-P group, suggesting that this protein might use the Man-6-P receptor pathway to be sorted to the cytolytic granules (Burkhardt et al., 1989). Direct functional evidence for this sorting mechanism in the formation of lytic granules is lacking. To examine this possibility, cloned cytotoxic T cell lines from a patient with I-cell disease (mucopolipidosis II) were derived. These patients exhibit a genetic autosomal defect for the enzyme (*N*-acetylglucosaminyl-phosphotransferase) responsible for the phosphorylation of mannose which occurs on some glycoproteins in the *cis*-Golgi compartment and results in the formation of Man-6-P oligosaccharide (Kornfeld and Kornfeld, 1985; von Figura and Hasilik, 1986). As a consequence of the I-cell defect, soluble lysosomal proteins are not phosphorylated and cannot be recognized by the Man-6-P receptor which would normally sort them into the lysosomal pathway.

Previous studies have demonstrated the absence of the phosphotransferase in various cell types including lymphoid tissues of I-cell patients (Haubeck et al., 1985; Little et al., 1987; Waheed et al., 1982). However, lymphocytes as well as hepatocytes and Kupffer cells are exceptional in I-cell disease patients in showing nearly normal levels of lysosomal enzymes (Glaser et al., 1974; Owada and Neufeld, 1982; Waheed et al., 1982), and it has been suggested that an alternative pathway exists for the delivery of proteins to lysosomes in these cell types. Studies using B and natural killer cell lines clearly demonstrate that the I-cell defect is present in these cell types either by increased secretion of lysosomal enzymes or by the absence of Man-6-P on these enzymes (Haubeck et al., 1985; Little et al., 1987). Consequently, Man-6-P receptor-dependent delivery of lysosomal enzymes should be defective in such cells.

By deriving T cell lines from a patient with I-cell disease, it was possible to test the hypothesis that the soluble lytic granule proteins granzymes A and B, like soluble lysosomal enzymes, might also require the Man-6-P modification for correct sorting to the lytic granules. These studies suggest that granzymes A and B are normally sorted to the lytic granules via the Man-6-P pathway, but that an alternative Man-6-P receptor-independent pathway to the lytic granules also exists.

Materials and Methods

Antisera

Antibodies against human granzyme A were raised by immunizing NZW rabbits with lytic granules from the dense fraction obtained after Percoll fractionation of activated human cytolytic clones as described below. Rabbits were primed with 0.5 mg of immunogen, emulsified in complete Freund's adjuvant, and boosted 2 and 5 wk after the initial priming with 0.5 mg immunogen emulsified in incomplete Freund's adjuvant. One rabbit responded with an antiserum that specifically recognized human granzyme A by immunostaining and immunoblotting.

Antibodies against recombinant human granzyme B were provided by J. Tschopp and prepared as previously described (Masson and Tschopp, 1987). Anti-human cathepsin B was purchased from The Binding Site, Birmingham, U.K., as a sheep antiserum and biotinylated using standard procedures (Harlow and Lane, 1988) for use in double-labeling immunostaining.

All second-stage antibodies were purchased from Southern Biotechnology, Birmingham, AL. These were goat anti-mouse and goat anti-rabbit IgG conjugated to either Texas red or fluorescein as described for immunostaining or goat anti-mouse kappa light chain coupled to horseradish peroxidase in the case of immunoblotting using chemiluminescence.

Radioactive second-stage antibodies labeled with ^{125}I were purchased from Amersham Intl., Buckinghamshire, U.K.

Cell Culture

Human T cell clones were maintained in RPMI, 100 U/ml rIL-2, 5% human serum. T cell clones were derived essentially as previously described (Lanzavecchia, 1985). Peripheral blood lymphocytes from a patient diagnosed with I-cell disease were isolated. The clinical diagnosis of this patient indicated increased serum levels of the following lysosomal enzymes (normal control values in parentheses): β -hexosaminidase 208 mU/mg (14), arylsulfatase 5.9 mU/mg (0.3), α -*N*-acetylglucosaminidase 0.45 mU/mg (0.16), and β -galactosidase 0.43 mU/mg (0.4). The enzyme levels from fibroblast cell lysates derived from the same patient were correspondingly decreased compared with normal controls (in parentheses): β -hexosaminidase 22 mU/mg (215), arylsulfatase A 1.1 mU/mg (13.5), and β -glucuronidase 0.4 mU/mg (2.2). Lymphocytes were purified over Histopaque 1077 (Sigma Immunochemicals, St. Louis, MO) and plated at 10^6 /ml with irradiated allogeneic lymphocytes at the same concentration in RPMI medium containing 5% human serum (Red Cross, Switzerland), 100 U/ml of rIL-2 (Hoffmann-La Roche, Nutley, NJ), and 1 μ g/ml phytohem-agglutinin (Burrighs Wellcome, Dartford, U.K.). After expansion, cells were sorted by FACS[®] after staining with anti-CD4 or anti-CD8 antibodies (Becton-Dickinson Immunocytometry Sys., Mountain View, CA) and cloned at 0.2 cells per well. All cells used in this study were CD8⁺ cells. Normal T cell clones were a gift from A. Lanzavecchia and were generated in the same way (Lanzavecchia, 1985).

Enzymatic Assays

Glucosaminidase was assayed as described (Scalera et al., 1980). Briefly, samples were diluted to 200 μ l in 200 mM citric acid, pH 4.5, 0.2% Triton X-100, and incubated in the presence of 8 mM *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide (Sigma Chem. Co.) for 30 min at 37°C. The reaction was stopped by the addition of 1 ml 67 mM NaCl, 83 mM Na₂CO₃, 33 mM glycine, pH 10.5, and the absorbance read at 405 nm.

Enzymatic assays for granzymes A and B were carried out as previously described (Odake et al., 1991; Pasternack et al., 1986). Granzyme A was assayed by addition of 180 μ l of 0.2 M Tris-HCl, pH 8.1, 2×10^{-4} M *N*-benzyloxy-carbonyl-L-lysine-thiobenzylester (BLT), 2.2×10^{-4} M 5,5'-dithio-bis(2-nitrobenzoic acid) to 20 μ l of lysate (0.1 M Tris HCl, pH 8, 0.1% Triton X-100). The absorbance at 405 nm was read over 30 min at 37°C on a microplate reader (Molecular Devices Corp., Menlo Park, CA). Protein concentrations from total cell lysates prepared as described below were determined using the protein assay and specific activities calculated from the rate of this reaction and this value (Bio Rad Labs., Hercules, CA). 1 U is defined as the amount of enzyme activity required to produce a change in absorbance at 405 nm of 1 OD at 37°C.

Granzyme B was assayed using essentially the same procedure, the substrate being 0.1 mM Boc-Ala-Ala-Asp-thiobenzylester in 0.3 mM 5,5'-dithio-bis(2-nitrobenzoic acid), 1 mM EDTA, 0.05% Triton X-100. 100 μ l of lysate was mixed with 100 μ l substrate and the rate of change of absorbance at 405 nm determined using the kinetic plate reader.

For each time point cell lysates from two independently derived clones were assayed for activity. For the normal cell lysates these clones were derived from two separate individuals. All I-cell clones were derived from a single individual, but 2-4 independently isolated CD8⁺ clones derived from this individual were tested for each time point shown.

Glycosidase Treatments

Total cell lysates were prepared by washing cells three times in PBS, resuspending at 10^7 /ml in 0.1 M Tris, pH 8, 0.1% Triton X-100, vortexing, and lysing on ice for 30 min. Lysates were then centrifuged in a microfuge at 4°C for 15 min and the soluble fraction stored at -20°C before further analysis.

Samples in 0.1% Triton X-100 treated with peptide-*N*-glycosidase F (PNGase F) (Boehringer Mannheim, Germany) were first denatured in 0.5% SDS at 95°C for 5 min, the SDS was quenched by addition of a twofold excess of nonionic detergent, and the enzyme was added for 12-16 h at 37°C. Samples treated with endoglycosidase H (Endo H) were incubated at 37°C for 12-16 h in 9 mM CaCl₂ and 50 mM sodium acetate, pH 5.5, in the presence of 1 mM PMSF.

Metabolic Labeling and Immunoprecipitation

Normal T cells were labeled with [^{32}P]orthophosphate (>1,000 Ci/mmol,

Amersham Intl.) or [³⁵S]methionine for 3 h at 37°C, at concentrations of 1–2 × 10⁷/ml and 0.5 mCi [³²P]orthophosphate/ml in phosphate-free medium, or 250 μCi/ml [³⁵S]methionine in methionine-free medium, both containing 10% dialyzed FCS and 100 U/ml rIL-2. The cell pellet was lysed in 1 ml of 150 mM NaCl, 50 mM Tris-HCl, pH 8, 1 mM MgCl₂, 2% NP-40, and 0.1 mM PMSF for 30 min on ice and precleared by incubation with 10 μl normal rabbit serum, and then protein A-Sepharose (Pharmacia, Uppsala, Sweden) each for 30 min. Granzyme A was precipitated with rabbit antiserum overnight and then for 30 min with protein A-Sepharose. A preimmune serum from the same rabbit was used as a negative control. Precipitates were washed six times with PBS, 0.1% NP-40 and eluted in 20 mM Tris, pH 7, 0.5% SDS at 95°C. All precipitations and washes were carried out at 4°C. For Endo H treatment, both untreated and treated samples were adjusted to 1% NP-40, 0.1 mM PMSF and incubated at 37°C either with or without the addition of Endo H for 4–5 h. Samples were electrophoresed on a 10% polyacrylamide gel under reducing conditions as described below.

Electrophoresis and Immunoblotting

SDS-PAGE was carried out in 10% acrylamide gels under nonreducing conditions. 12% nonreducing acrylamide gels were used for glycosidase-treated samples. Gels were transferred to nitrocellulose (Schleicher & Schuell, Inc., Germany) using the Trans-Blot apparatus in 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3 (Bio Rad Labs). Filters were then probed using either radioactive or chemiluminescent antibody detection as indicated. Filters were blocked for 2 h in 1% casein, 150 mM NaCl, 50 mM Tris-HCl, pH 8, 0.1% sodium azide. The primary antibody was diluted in casein buffer and incubated 2–16 h at room temperature. Filters were washed twice in PBS for 5 min, and incubated with ¹²⁵I-labeled anti-rabbit antibody at 10⁵ cpm/ml for 1 h. Filters were then washed three times in PBS for 10 min each, blotted dry, covered with cellophane film, and exposed using an intensifying screen and XAR-5 photographic film (Eastman Kodak, Rochester, NY) at –70°C for 12–72 h. For chemiluminescent detection the procedure was essentially the same except that the blocking buffer contained 5% nonfat dried milk, the wash buffer was PBS, 0.1% Tween 20, and a horseradish peroxidase-labeled anti-Ig second antibody was used (Southern Biotechnology or Amersham Intl.). Filters were developed for 1 min in chemiluminescent detection reagents (Amersham Intl.), and exposed for times varying between 15 s and 5 min using Hyperfilm ECL (Amersham Intl.).

Transfection

Transient transfections were carried out using DEAE-Dextran as previously described (Guan and Rose, 1984). Briefly, COS-7 cells were plated at 6–7 × 10⁵ per 100-mm dish and transfected the following day with 10 μg of DNA in pSRaSD7 (kindly provided by Dan Denney, Stanford University) in 10 mM Tris-HCl, 150 mM NaCl, pH 7.4, containing 500 μg/ml DEAE-Dextran (Pharmacia). This vector contains the SRα promoter (Takebe et al., 1988), and is otherwise based on the cDNA expression vector previously described (Okayama and Berg, 1983). After 30 min at 37°C, the DNA solution was replaced with medium containing 100 μM chloroquine. Cells were incubated an additional 3 h before the medium was replaced with normal culture medium (Dulbecco's MEM, 10% FCS). Cells were assayed by immunostaining after 2 d.

Immunofluorescence

Cells were adhered to glass slides coated with 50 μg/ml poly-L-lysine (Sigma Immunochemicals), fixed in 2% paraformaldehyde (Polyscience, Niles, IL) in PBS for 45 min at room temperature, and quenched in three changes of 50 mM ammonium chloride. Cells were permeabilized for 20–30 min in dilution buffer (PBS, 0.01% saponin, 0.25% gelatin) containing 0.1% NP-40, rinsed in dilution buffer, and stained with the primary antibody in dilution buffer for 30–45 min. After extensive washing in dilution buffer, cells were stained with fluorescent conjugated second antibodies for 30–45 min, washed extensively in dilution buffer, and then PBS before mounting in 90% glycerol/PBS containing 2.5% DABCO (1,4-Diazabicyclo[2.2.2]octane) (Fluka Chemie AG, Buchs, Switzerland) antileaching reagent. Cells were stained with antisera at a 1:100 dilution for anti-granzyme A and a 1:50 dilution for biotinylated sheep anti-human cathepsin B antisera (The Binding Site), followed by a 1:100 dilution of a stock solution of either anti-rabbit FITC or streptavidin-Texas red (Southern Biotechnology), respectively. Appropriate controls without first stage or with the rabbit preimmune serum at the same dilution were always included. All samples were examined using the MRC 600 confocal microscope (BioRad

Labs). When comparisons were made between cell types and for negative control samples, care was taken to use the exact same settings of the microscope for the laser intensity and confocal detection.

Cell Fractionation

Typically 5 × 10⁷–10⁸ cells were fractionated. Cells were washed three times in PBS and once in 50 mM Hepes, pH 7.2, 90 mM KCl. Cells were resuspended in a minimum of 6 ml of Hepes/KCl or fivefold the volume of the pellet, whichever was greater. Cells were disrupted at 4°C by passing them through the ball bearing cell homogenizer (Balch and Rothman, 1985) 20 times using a ball bearing of 8.006 mm diam. Lysis was verified under the microscope when the lysis buffer appeared grainy owing to the presence of organelles. Nuclear material was spun out at 900 g (2,100 rpm in a Heraeus Sepatech centrifuge) for 4 min at 4°C. The pellet was washed once in 4 ml Hepes/KCl buffer and added to the soluble fraction to make a final volume of 10 ml. Using a stock of 90 ml Percoll (Pharmacia) with 10 ml 500 mM Hepes, pH 7.2, 900 mM KCl, a Percoll step gradient was formed by underlaying 15.9 ml 39% Percoll with 12.1 ml 90% Percoll. All dilutions of Percoll were made in 50 mM Hepes, pH 7.2, 90 mM KCl. The soluble cell lysate was then overlaid onto this gradient and centrifuged at 40,000 g (15,000 rpm using a TST 28.38 Kontron rotor) for 30 min at 4°C. The top 10 ml of soluble lysate that did not enter the gradient was removed and discarded, and 1-ml fractions were collected from the top of the gradient. Samples of each fraction were assayed for granzyme A activity: 35 μl of a 1:10 dilution of each fraction were added to 200 μl of 1 mM BLT, 1 mM 5,5'-dithio-bis(2-nitrobenzoic acid) in 300 mM Tris, pH 8.1, and the absorbance at 405 nm was read after 20 min. For fractions used for immunization, granules were lysed by addition of NaCl to 1.5 M, and Percoll was removed by centrifugation at 200,000 g (45,000 rpm in the SW50.1 rotor) for 2.5 h at 4°C. The granule fractions were then stored at –20°C.

Cell Cytotoxicity

Cytotoxicity of T cells was determined at 14 d after stimulation using anti-CD3 antibody bound via the Fc receptor of target cells as previously described (Lanzavecchia et al., 1988). Target cells were coated with anti-CD3 antibody by addition of purified antibody at 1 μg/ml immediately before addition. Since only the target cells express the Fc receptor, only these cells become coated with antibody. The antibody is directed against an invariant component of the T cell receptor complex which is able to recognize and activate T cells (Lanzavecchia et al., 1988). Briefly, varying ratios of stimulated cells were added to 10⁴ ⁵¹Cr-labeled P815 target cells in a final volume of 200 μl RPMI, 10% FCS at 37°C for 4 h. Plates were spun gently at 1,000 rpm for 5 min and 100 μl aliquots counted using a gamma counter. The percentage of specific target lysis was calculated as equal to (experimental release – spontaneous release)/(total release – spontaneous release). Spontaneous release was <10%.

Results

I-Cell Lymphocytes Contain Reduced Levels of Granzymes

The cell lines derived were first analyzed for the presence of *N*-acetyl-β-D-glucosaminidase, a typical soluble lysosomal enzyme. Lysates from four different clones were assayed. I-cell lines were found to contain reduced levels of glucosaminidase activity (9.8 mU/mg, standard error 0.9) compared with normal cell lines (27.2 mU/mg, standard error 2.9). Granzymes are serine proteases with well-defined substrate specificities (Odake et al., 1991). To determine the amount of these proteins present in normal and I-cell clones, chromogenic substrates were used to measure the specific activities of these enzymes in cell lysates. The specific activities of granzyme A and B vary at different time points after T cell stimulation during which period the cells are synthesizing the lytic granule components (Podack and Kupfer, 1991). In normal T cells, granzyme A activity increases with time after stimulation from 50 mU/mg at day 6 after stimulation to 150 mU/mg at day 14 after stimulation. In the I-cell

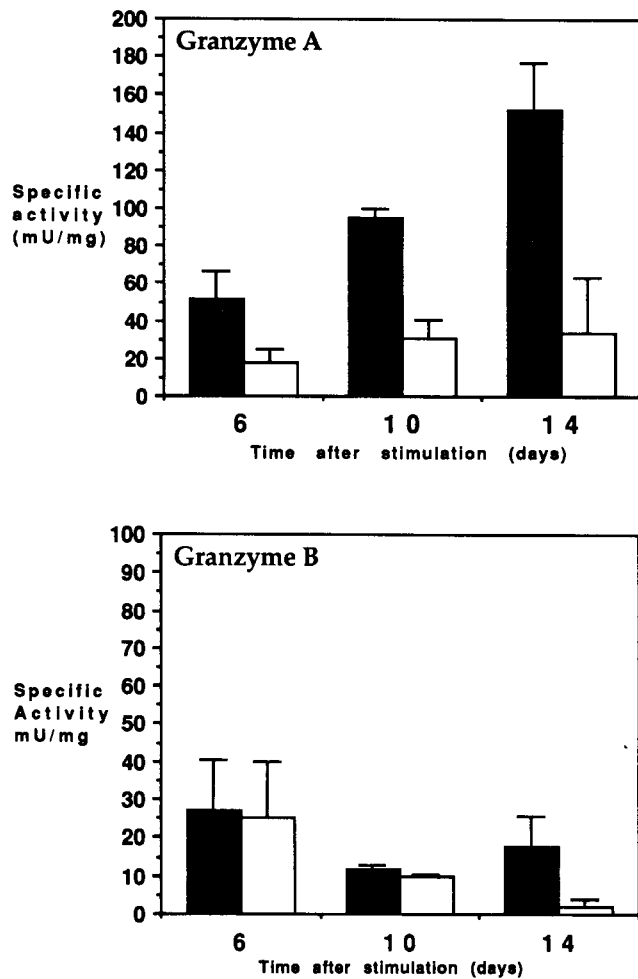


Figure 1. The specific activities of granzymes A and B at different times after stimulation. Black bars represent values from normal cells; white bars, values from I-cells. Standard error bars are shown.

clones, the level of granzyme A is much lower at all three time points analyzed and only increases from 18 mU/mg at day 6 after stimulation to 30 mU/mg at day 14 (Fig. 1). For granzyme B the specific activity varied only between 12 and 27 mU/mg at the three different time points. In I-cells the level of granzyme B seems to decrease with time after stimulation, from a level of 25 mU/mg, equivalent to that of the normal cells at day 6 after stimulation, to a level of only 2 mU/mg well below that of the normal cells at day 14 (Fig. 1). This assay, using the synthetic substrate for granzyme B, is both less sensitive and more variable than the assay for granzyme A using the synthetic substrate BLT. However, with both substrates it is clear that by 14 d after stimulation, when cells are effective killers, the I-cells contain significantly lower levels of both granzymes A and B than the normal cells. This suggests either that the I-cells produce lower levels of the granzymes or that these proteins are being secreted from the cell rather than correctly packaged and stored.

I-Cell Cytotoxic Lymphocytes Secrete Granzymes Constitutively

To distinguish whether the reduced levels of granzyme A and

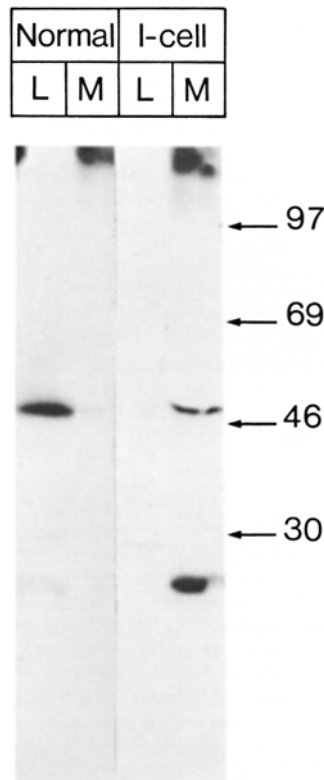


Figure 2. Immunoblot of normal and I-cell lysates (L) and supernatants (M) with anti-granzyme A antiserum, and chemiluminescent detection. Both normal and I-cells were lysed at 10^7 cells/ml and 100 μ l of lysate was loaded in each lane. Supernatants were taken from equal numbers of cells grown in media between days 5 and 7 after stimulation. Arrowheads mark the relative positions of molecular mass markers (kD).

B in the I-cells were due to a reduced biosynthesis or secretion from the cell, the culture media from the T cell clones were examined for the presence of granzyme A and B by immunoblotting. To avoid possible contamination from the initial target cell destruction in the first 2–3 d after stimulation, cells were cultured for at least 5–10 d after stimulation, washed, and resuspended in new culture medium without target cells. The cells were then cultured for two additional days before the supernatant was collected, and cells from the same culture were washed and lysed using 0.1% Triton X-100 for analysis. Fig. 2 shows that I-cells secrete granzyme A, whereas normal cells do not. Granzyme A is a 27-kD monomer which forms a homodimer with an apparent molecular mass of 50 kD (Hameed et al., 1988; Krahenbuhl et al., 1988). Virtually all of the granzyme A is found as a homodimer in normal cell lysates, and only a faint band represents the monomeric granzyme A. Granzyme A is not detectable in the supernatant of normal cells as either a monomer or a dimer. Loading an equal number of I-cells (equivalent to 10^6 cells), the granzyme A dimer is barely visible in the I-cell lysate. In contrast, both the monomer and dimer forms of granzyme A are clearly present in the culture media of I-cells. This demonstrates that while granzyme A is retained as a dimer in normal cells, it is constitutively secreted from I-cells as both monomer and dimer. It should be noted that in other experiments (not shown) using increased amounts of I-cell lysate, a faint band corresponding to the granzyme A dimer could be detected by immunoblotting. This is consistent with the data from Fig. 1 in which granzyme A activity can be detected by the more sensitive enzymatic assay.

Fig. 3 shows a similar immunoblot using an antiserum which detects granzyme B. Granzyme B is detected as a 32-kD band in the normal cell lysate, but is not detectable in

I	N	I	N	-
Lysate		Medium		

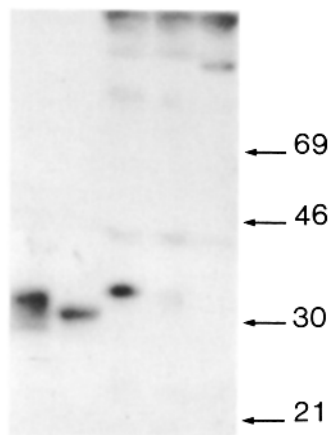


Figure 3. Immunoblot of normal (N) and I-cell (I) cell lysates and culture media from days 10–12 after cell stimulation and the medium alone (–) with anti-granzyme B antiserum and radioactive detection. Lysates were prepared from cells grown for 12 d after restimulation. The culture media were harvested from equal numbers of cells grown at the same concentration between days 10 and 12 after restimulation. An additional lane containing only the culture medium was run as a negative control for possible background derived from the serum. Arrowheads mark the relative positions of molecular mass markers (kD).

the cell medium. However, in the I-cells the major band detected is a 35-kD form which is present in both the cell lysate and in the cell medium. This suggests that, in I-cells, granzyme B is constitutively secreted from the cells, in contrast to normal cells in which this protein is stored for regulated secretion. The fact that granzyme B migrates with an apparently higher molecular mass of 35-kD in I-cells compared with normal cells suggests that this protein is differently processed in the two cell types. Lower molecular mass forms of 32-kD and 30-kD are also detected in the I-cell lysate, consistent with the three forms representing different stages of glycosylation. It is the highest molecular mass, 35-kD form which is constitutively secreted from the I-cells.

To understand the basis for these size differences, glycosidase treatments were used to investigate the processing of granzyme B in normal and I-cells. PNGase F digestion, which cleaves all sugar moieties from the granzyme B, demonstrates that the higher molecular mass form of granzyme B is the result of the different processing of this protein in these cells (Fig. 4). After PNGase F digestion, all three bands detected in the I-cell lysate are reduced to a single band of 27 kD, as is the 32-kD band found in the normal cell lysate. This confirms that the 30-, 32-, and 35-kD forms represent three different glycosylation states of the same protein.

An important point is that the carbohydrate on granzyme B is sensitive to digestion with Endo H in normal cells, but resistant in I-cells (Fig. 5). The 27-kD form found in normal cells is Endo H sensitive, which is consistent with the concept that addition of Man-6-P in the *cis*-Golgi compartments prevents further glycosylation of these side chains in the medial and *trans*-Golgi compartments (von Figura and Hasilik, 1986). Consequently, the phosphorylated oligosaccharide side-chains of granzyme B remain as high mannose residues which are sensitive to Endo H digestion. In contrast, the 30-kD, 32-kD, and 35-kD forms found in I-cells are all unaffected by Endo H treatment. This shows that the oligosaccharide side chains of granzyme B are not phosphorylated in the *cis*-Golgi compartment of I-cells, but are modified to become complex glycans, which are resistant to Endo H. The fact that it is not possible to detect any Endo

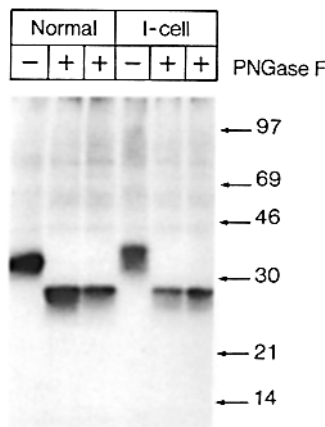


Figure 4. Immunoblot showing PNGase F treatment of normal and I-cell lysates immunoblotted and detected with anti-granzyme B and radioactive detection. Each sample was digested with two different concentrations of enzyme to ensure complete digestion. Arrowheads mark the relative positions of molecular mass markers (kD).

H-sensitive component in the I-cell granzyme B is important as this demonstrates that the phosphotransferase defect in these I-cell lines is complete, since any granzyme B undergoing phosphorylation would result in an Endo-H sensitive form.

Granzyme A Bears Man-6-P Residues in Normal Lymphocytes

The data obtained from the I-cells suggests that granzymes A and B are normally targeted to the granules by the Man-6-P receptor. This predicts that in normal cells granzymes A and B must bear Man-6-P residues in their side chains. To confirm this biochemically, granzyme A was immunoprecipitated from cells labeled with radioactive phosphate. Fig. 6 *a* shows a normal T cell clone labeled at 6 d after stimulation with inorganic [³²P]orthophosphate and precipitated with a rabbit antiserum to granzyme A. The immune serum (but not the preimmune serum) precipitates a phosphate-labeled band of 30 kD under reducing conditions corresponding to the granzyme A monomer. Endo H treatment removes the oligosaccharide side chain containing the radioactive phosphate and the granzyme A monomer can no longer be seen. That this is not due to degradation of the granzyme A by addition of Endo H is confirmed by an identical Endo H treatment of granzyme A immunoprecipitated from cells labeled with [³⁵S]methionine (Fig. 6 *b*): in these samples Endo H treatment results in a decrease in molecular mass from 30

Normal			I-cell			Endo H
-	+	+	-	+	+	

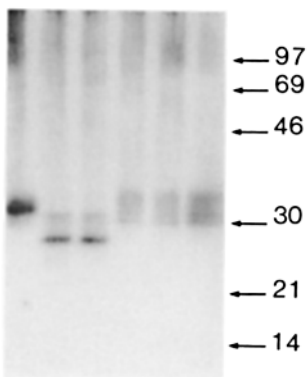


Figure 5. Immunoblot showing Endo H digestion of granzyme B in cell lysates of normal and I-cells. Lysates were digested in two different concentrations of enzyme to ensure complete digestion. The control samples (–) were treated identically except that no enzyme was added. Arrowheads mark the relative positions of molecular mass markers (kD).

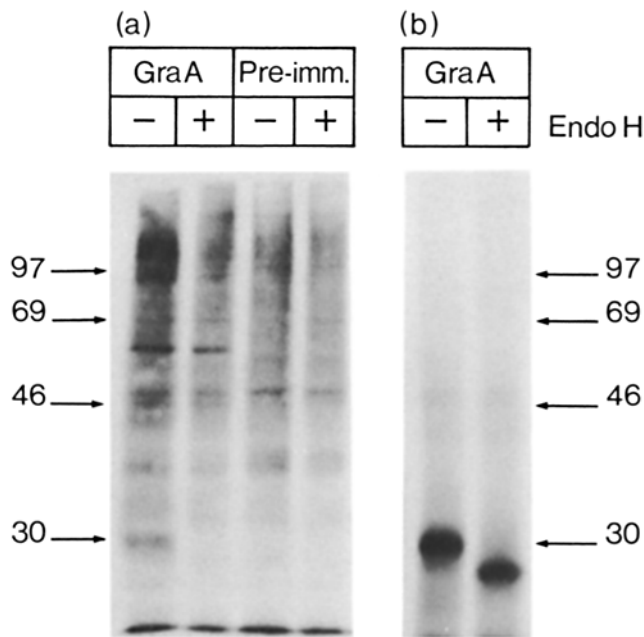


Figure 6. (a) Autoradiograph of ^{32}P -labeled T cell clone, precipitated with an antiserum against granzyme A or the preimmune serum and run under reducing conditions. Precipitates were either treated (+) or untreated (-) with Endo H. Each lane represents a precipitate from 5×10^6 labeled cells. (b) [^{35}S]Methionine-labeled T cell clone precipitated and Endo H treated as above. Arrowheads mark the relative positions of molecular mass markers (kD).

to 27 kD, consistent with the loss of high mannose sugars, but with no loss of intensity of the immunoprecipitated band. Together these results confirm that, in normal cells, the oligosaccharide side chains of granzyme A are high mannose and furthermore bear a phosphate addition.

A Man-6-P Receptor-Independent Pathway to the Lytic Granules Also Exists

Although the I-cells show reduced levels of granzymes, these proteins are still detectable within the cell lysates (Fig. 1). The antiserum to granzyme A was used to determine the in-

tracellular localization of this protein by immunofluorescence. As shown in Fig. 2, this antiserum detects the 27-kD monomer and 50-kD dimer of granzyme A by immunoblotting. The staining specificity of this antiserum is shown in Fig. 7, where permeabilized COS-7 cells were transiently transfected with the human granzyme A cDNA in order to verify the staining specificity of this antiserum. Transfected cells stain with the antisera whereas untransfected cells in the background do not. The staining pattern indicates that in transiently transfected COS-7 cells the majority of the protein is in the ER and Golgi region. Fig. 8 demonstrates intracellular staining with this antisera on permeabilized activated lymphocytes at 4 and 13 d after stimulation. All pictures shown in Fig. 8 were taken using the confocal MRC-600 microscope (Bio Rad Labs.) under identical settings. By 13 d after stimulation both normal and I-cells show a punctate cytoplasmic staining for granzyme A suggesting that even in the I-cells a certain amount of the granzyme A is correctly localized within the lytic granules of the cells. Quantification of the intensity of staining in the granules for each type revealed a mean fluorescence intensity of 133 for granules in normal cells, but only 86 for granules in I-cells. Taken together with the fact that there are fewer granules per cell in I-cells compared with normal cells, this is consistent with the biochemical data that show reduced levels of granzyme A in I-cells.

The nature of the exact endocytic compartments in which granzyme A was detected in the I-cells was further investigated by subcellular fractionation of normal and I-cell granules on a Percoll gradient (Fig. 9). Granzyme A activity was measured by the enzymatic cleavage of BLT from different fractions of a Percoll density gradient taken from equal numbers of normal and I-cells at 12 d after stimulation. This method has been demonstrated to provide a reliable separation of the lytic components from other mitochondrial, cytosolic, and plasma membrane markers (Masson et al., 1985; Millard et al., 1984). In normal cells the majority of the granzyme A activity is found in the dense granule peak (fractions 14–18). A lower amount of granzyme A activity is found in these same fractions in the I-cells, demonstrating that a certain amount of granzyme A can be correctly localized within the cells to an organelle of the same density as

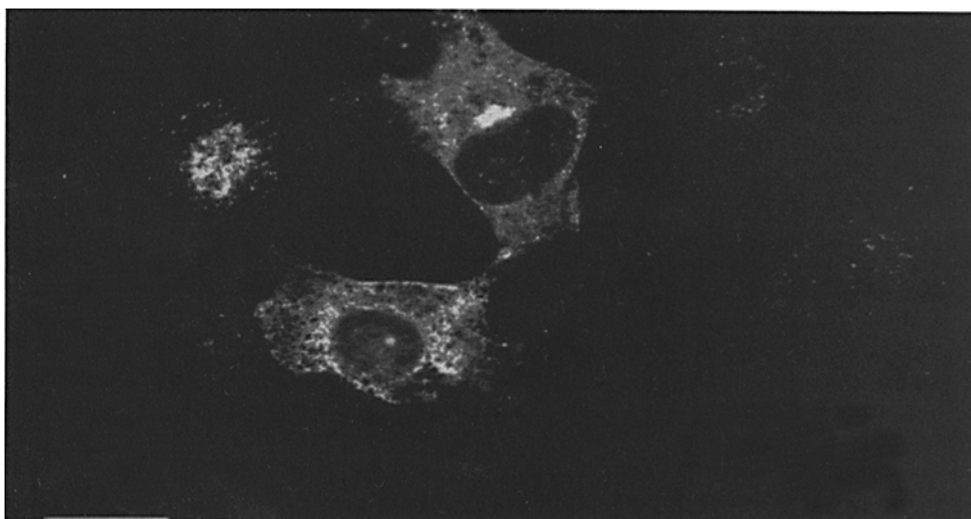


Figure 7. COS-7 cells transiently transfected with human granzyme A, stained after permeabilization with the rabbit anti-granzyme A antiserum and an anti-rabbit FITC antibody, and photographed by confocal microscopy using a $63\times$ objective. Bar, 10 μm .

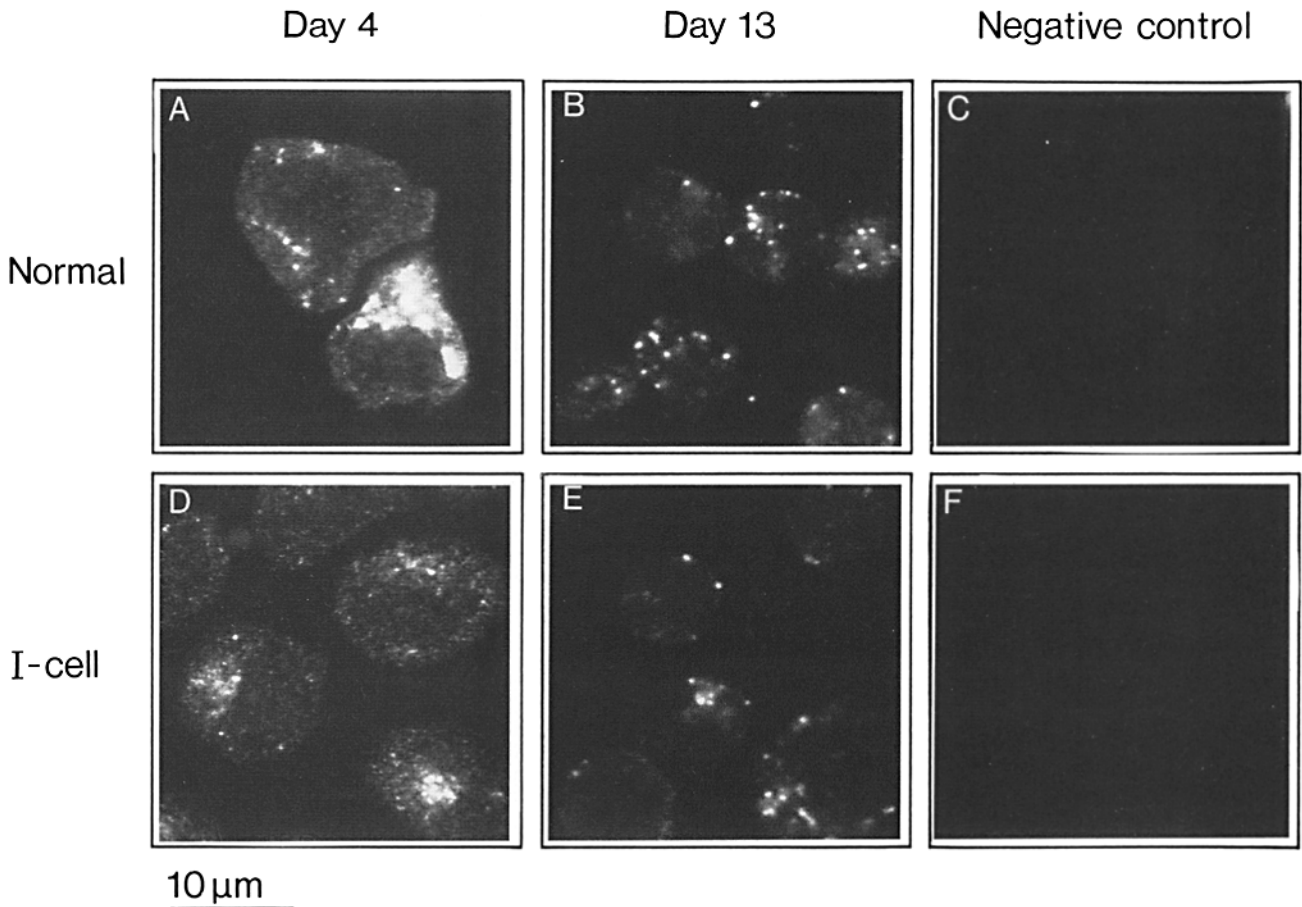


Figure 8. Expression of granzyme A in normal (*A* and *B*) and I-cell (*D* and *E*) T cell clones at different stages after stimulation. Cells were permeabilized and stained as described (see Materials and Methods) at 4 d (*A* and *D*) and 13 d (*B* and *E*) after stimulation. The same dilution of preimmune serum was used to stain both normal and I-cells in the negative controls (*C* and *F*, respectively).

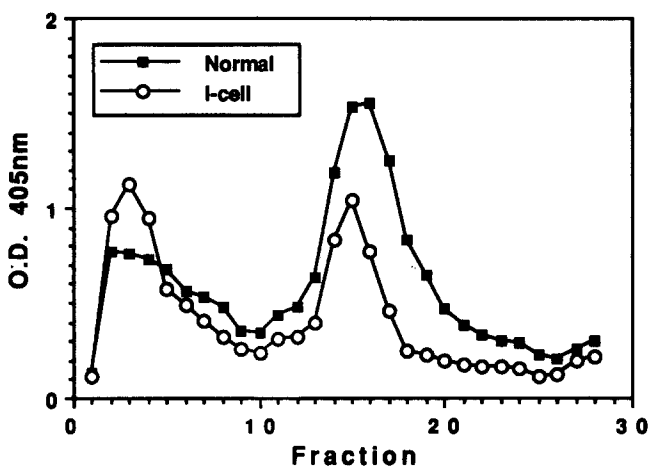


Figure 9. Granzyme A activity of different fractions from a Percoll density fractionation of equal numbers of cells from normal and I-cell cultures at 12 d after stimulation. 1 in 10 dilutions of each fraction were made and assayed for granzyme A activity using the synthetic substrate BLT as described. A single endpoint reading for absorbance at 405 nm was made after 20 min at room temperature. The absorbance at 405 nm was read after 20 min and plotted against the fraction number.

the normal lytic granule. However, an equivalent amount of the I-cell granzyme A activity appears to be localized within a lighter density fraction (fractions 2–5), compared with a smaller amount in normal cells. These lighter vesicles could possibly correspond to an earlier stage in the biogenesis of the lytic granule or smaller vesicles in the constitutive secretory pathway.

These results indicate that, despite the fact that I-cell lymphocytes secrete granzymes A and B, a small amount of these granzymes is able to be correctly localized in the lytic granules. One possibility is that the lytic granule components in the I-cells are taken up exogenously from the medium. To be sure that the granzyme A activity detected represented endogenous granzyme and not exogenous protein taken up via the endocytic pathway, the cells were grown in the presence or absence of 5 mM Man-6-P. Normal cells grown in the presence of 5 mM Man-6-P showed a level of granzyme A activity (54 mU/mg) slightly below that of normal cells grown in the absence of Man-6-P (64 mU/mg). I-cells exhibited lower specific activities of granzyme A compared with normal cells and this activity was not affected by the presence (20 mU/mg) or absence (19 mU/mg) of Man-6-P. This indicates that granzyme A is reaching the lytic granules of I-cells from the biosynthetic and not the endo-

Cathepsin B Granzyme A

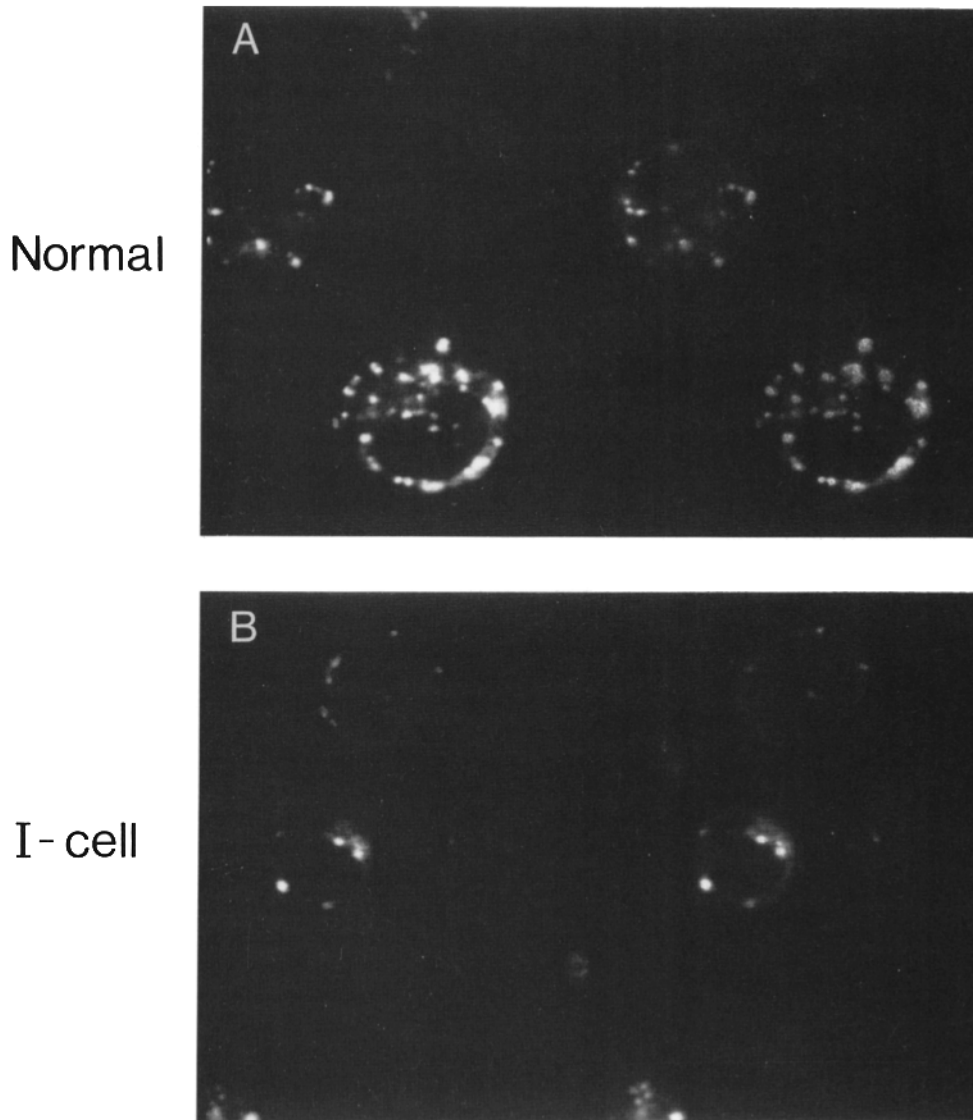


Figure 10. Colocalization of granzyme A and cathepsin B in (A) normal and (B) I-cell granules. Cells were permeabilized and stained for granzyme A and cathepsin B as described (see Materials and Methods), and examined using the MRC-600 confocal microscope with the Krypton Argon laser. Signals from fluorescein detecting granzyme A (right) and Texas red detecting cathepsin B (left) are displayed simultaneously for the same group of cells.

cytic Man-6-P receptor route. Although the possibility that external granzyme A is being internalized via a Man-6-P-independent pathway cannot be excluded, the data are consistent with the idea that a Man-6-P receptor-independent pathway to the lytic granules also exists in these cells.

Lytic granules are known to co-stain with lysosomal markers such as the cathepsins (Bonifacino et al., 1989; Burkhardt et al., 1990; Peters et al., 1989). Lytic granules co-stain with cathepsin B in both the normal and I-cells (Fig. 10). All granzyme A structures stain with cathepsin B, and all cathepsin B structures stain with granzyme A in both cell types, indicating a complete overlap of lytic and lysosomal compartments in the lymphocytes at 13 d after stimulation in both I-cells and normal cells. (This overlap can be verified by overlaying all pixels from the fluorescein and Texas red channels using the "merge" program on the Bio-Rad 600 confocal microscope.) This indicates that in lymphocytes the Man-6-P receptor-independent pathway is utilized by both lysosomal and lytic granule components.

I-Cell Cytotoxicity is Not Impaired

To determine whether regulated secretion of the I-cell granules could occur, the ability of I-cells to lyse targets was examined. Target cell lysis by normal and I-cell CD8⁺ lymphocytes was compared. To equalize the strength of the signal triggering the two types of T cells to kill, the target cells were coated with an antibody to the CD3 molecule in the T-cell receptor complex, which is identical in all T cells (unlike the variable, antigen-T cell receptor). Cross-linking of this receptor triggers T cells to release their lytic granules and kill the targets contacted (Lanzavecchia et al., 1988). By examining the degree of cell lysis of the targets at different effector (T cell)-to-target ratios it is possible to assess the effectiveness of the killer cells. Fig. 11 plots the amount of target cell lysis at different effector-to-target cell ratios for T cells at 14 d after activation. The cytotoxicity of the I-cells is not impaired and even at a 1:1 ratio these cells can lyse 40% of the target cells. These results are consistent with the

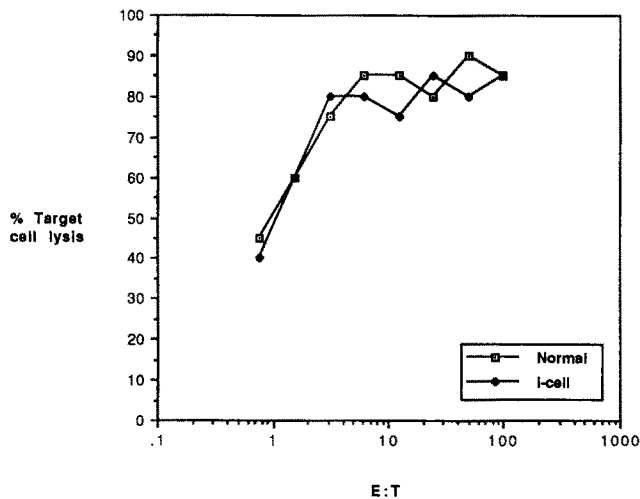


Figure 11. The percentage of specific target cell lysis of normal versus I-cells after a 4-h incubation of P815 targets coated with anti-CD3 antibody plotted against different effector-to-target (*E/T*) ratios used. Cytolytic lymphocytes were assayed at 14 d after stimulation.

idea that some of the lytic proteins are correctly localized in lytic granules within the I-cells, and that these granules can be exocytosed by triggering via the T cell receptor complex as in a normal cytolytic T cell.

Discussion

The results presented here demonstrate that granzymes A and B can be targeted to the specialized lytic granules of cytolytic lymphocytes by two different mechanisms. The major mechanism involves recognition by the Man-6-P receptor. However, the present results suggest that a Man-6-P receptor-independent pathway also exists.

The Man-6-P receptor pathway has been well characterized as the lysosomal sorting pathway for soluble lysosomal proteins (Kornfeld, 1990). Proteins destined for the lysosome are selectively recognized by the phosphotransferase responsible for addition of Man-6-P during glycosylation. These proteins are recognized by the Man-6-P receptor in the Golgi apparatus and shuttled to the lysosome by this receptor. In I-cells the phosphotransferase is defective or missing and, consequently, cells are unable to add the Man-6-P oligosaccharide during processing and the lysosomal proteins cannot be recognized by the Man-6-P receptor. In these cells, lysosomal proteins are constitutively secreted (Neufeld et al., 1975).

Studies on the lytic granules which demonstrated the colocalization of lysosomal and lytic markers in cytolytic cells (Bonifacino et al., 1989; Burkhardt et al., 1989; Peters et al., 1991), and the phosphorylation of one of the granzymes in a rat cell line (Burkhardt et al., 1989) led to the suggestion that the granzyme might be targeted to the lytic granules by the lysosomal pathway. By deriving T cell clones from a patient with I-cell disease it was possible to test the hypothesis that granzymes A and B are targeted to the lytic granules of these cells by the Man-6-P receptor. The finding that granzymes A and B are secreted from lymphocyte clones derived from a patient with I-cell disease suggests that these proteins are behaving exactly like soluble lysosomal

proteins and that their targeting normally relies upon the recognition of a Man-6-P. It is known from the cDNA sequences of these proteins that they contain potential glycosylation sites. Granzyme A, which forms a dimer, contains one glycosylation site per chain and granzyme B, which is a monomer, contains two glycosylation sites (Gershenfeld et al., 1988; Schmid and Weissmann, 1987). According to the data presented in this paper, the single granzyme A site and at least one of the carbohydrate structures of granzyme B must be modified with a Man-6-P. In the case of granzyme A, direct biochemical evidence of a phosphate-containing oligosaccharide side chain is shown (Fig. 6). This data is consistent with previous studies on a rat natural killer cell line, RNK-16, which also demonstrated a phosphate-labeled Endo H-sensitive granzyme in these cells (Burkhardt et al., 1989).

The fact that granzyme B is differently processed in normal and I-cells supports the idea that Man-6-P is also normally added to this protein during processing, and that in I-cells the Man-6-P modification is not possible. However, as previously noted in fibroblasts (Hasilik and von Figura, 1981), the glycosylation site can still be modified by other side chains. In the case of granzyme B, this results in a glycosylated protein with a higher molecular weight, which becomes Endo H resistant, indicating that the protein has acquired complex carbohydrate structures. The fact that treatment with PNGase F, which removes all of the carbohydrates, reduces both the I-cell and normal cell granzyme B to the same size protein supports this conclusion. This difference in processing is also important as it demonstrates that the phosphotransferase defect is complete in the I-cell lines derived. Since any granzyme B which was modified with a Man-6-P would remain Endo H sensitive, the absence of Endo H-sensitive forms of granzyme B in the I-cell lysate demonstrates the absence of detectable phosphotransferase activity in these cells. All three forms of granzyme B identified by immunoblotting in the I-cell lysate (30, 32, and 35 kD) remain resistant to Endo H treatment (Fig. 5) demonstrating that all three intermediate forms possess complex sugars.

Granzymes A and B are soluble lytic granule components and the data demonstrate that they can be targeted to the lytic granules by the same mechanism used to target soluble lysosomal proteins to lysosomes. Membrane-bound components of the lytic granules also appear to reach the lytic granules via a lysosomal targeting system: a recently identified protein, TIA-1, which can induce DNA fragmentation in target cells, has been found to contain the carboxy-terminal lysosomal targeting motif (Tian et al., 1991) responsible for directing membrane-bound lysosomal proteins such as lamp-1 and Igp 120 (Chen et al., 1988; Howe et al., 1988) to the lysosomes. The finding that the lytic granules use lysosomal targeting mechanisms to target their lytic proteins to the lytic granules strongly supports the idea that these granules are modified lysosomes. There is a great deal of evidence in favor of this view. Lysosomal proteins are released during cell-mediated killing (Zucker et al., 1983) and, furthermore, a correlation between the presence of lysosomal proteins and killing in natural killer cells, which also contain lytic granules, has been noted (Shau and Dawson, 1984, 1985). Several studies have demonstrated the colocalization of lysosomal and lytic proteins and the fact that both can be

reached via the endocytic as well as biosynthetic pathways (Bonifacino et al., 1989; Burkhardt et al., 1990; Peters et al., 1991). Furthermore, EM studies have shown that lytic granules with a pH-sensitive tracer have an acidic pH of 5.5 (Burkhardt et al., 1990; Masson et al., 1990) similar to that of prelysosomes or lysosomes.

Two important features distinguish the lytic granules from lysosomes: (1) only the lytic granules contain lytic proteins, and (2) these lytic granules are secretory storage granules that can be released upon stimulation of the cell. Cytolytic lymphocytes only develop lytic granules after activation and it is not clear whether these are preexisting organelles which become filled by the newly synthesized lytic proteins or whether the lytic granules are newly derived organelles. However, as a result of T cell activation, a new organelle (the lytic granule) containing both lysosomal and lytic proteins appears, and this organelle can be exocytosed in response to signaling via the T cell receptor complex. In general, lysosomes do not undergo exocytosis, although exceptions have been described. Several studies have reported the constitutive secretion of lysosomal enzymes from different cell lines. Macrophages, which are phagocytic cells of the immune system, secrete lysosomal enzymes independently of external stimuli (Schnyder and Baggiolini, 1978). In osteoclasts, lysosomal enzymes are also constitutively secreted and the colocalization of the cation-independent Man-6-P receptor with the lysosomal proteins along the exocytic pathway implies that this receptor is involved in the secretion of these proteins (Baron et al., 1988). There are also a few examples of regulated secretion of lysosomal enzymes: polymorphonuclear leukocytes, which are involved in the inflammatory responses of the immune system, store lysosomal proteins in azurophilic granules which are released during phagocytosis by these cells (Bretz and Baggiolini, 1974). Cathepsin B has also been shown to be secreted via the regulatory secretory pathway in rat exocrine pancreatic cells (Tooze et al., 1991), although it is not clear whether the Man-6-P receptor is involved in this trafficking.

The secretion of lysosomal enzymes is unusual, but illustrates that the sorting of lysosomal from secretory proteins need not be absolute in every cell type. Furthermore, the sorting between the regulated and constitutive secretory pathways can vary for a given protein. There are now several examples of proteins that, under different physiological conditions, can be secreted by both regulated and constitutive routes. For example, in lactating mammary epithelial cells caseins and other milk proteins can be secreted not only constitutively but also via a calcium-dependent regulatory pathway in response to ionomycin (Turner et al., 1992). In converse, proteins normally secreted via the regulatory pathway can be secreted constitutively under conditions of hyperstimulation, as has been demonstrated for insulin released from immature granules of pancreatic cells (Arvan et al., 1991). These examples suggest that the segregation between the constitutive, regulatory, and lysosomal routes occurring in the *trans*-Golgi network (Griffiths and Simons, 1986; Orci et al., 1987; Tooze et al., 1989) may not be rigid for a given protein and that the route taken may vary. This is certainly the case in the activated cytolytic cell where both lysosomal and secretory proteins are targeted to the same compartment. In activated cytolytic cells, it appears that the positive lysosomal sorting signal (Man-6-P recognition) can be uti-

lized to direct lytic proteins to a lysosomal structure which is nevertheless a regulatory secretory organelle. Exactly where and indeed whether the lytic granule diverges from a lysosome during biogenesis in order to become a regulatory secretory organelle is still unclear. From the present results it is not possible to distinguish between the possibilities that these lysosomal and regulatory secretory proteins fail to separate in the TGN, or that they separate in the TGN and merge in a later endocytic compartment.

The data provide evidence for two mechanisms for sorting granzymes to the lytic granules, one of which is the Man-6-P receptor sorting signal used for sorting soluble proteins to lysosomes. However, even when the Man-6-P receptor pathway is inoperable, as it is in I-cells, the reduced levels of granzymes retained within the cells are nevertheless correctly localized. Therefore, an alternative Man-6-P receptor-independent pathway to the lytic granules must also exist. This alternative route appears to be less efficient than the Man-6-P receptor-mediated sorting since I-cells contain only 20% or less of the granzyme A and B levels of normal cells by day 14 after stimulation (Fig. 1).

Interestingly, cathepsin B is also able to reach the lytic granules in the I-cells, suggesting that a Man-6-P receptor-independent pathway also exists for these proteins in cytolytic lymphocytes. It is important to note that there is no evidence for this Man-6-P receptor-independent pathway in the majority of other cell types (Kornfeld, 1990). The question of whether such a route exists for lysosomal enzymes was raised some time ago in reports that noted nearly normal levels of lysosomal enzymes in certain cell types from I-cell patients including hepatocytes and lymphocytes (Owada and Neufeld, 1982; Waheed et al., 1982). These data were derived from whole tissues and it was not clear whether the cells were able to increase their enzyme levels by means of very efficient endocytosis or whether the newly synthesized enzymes were directed to the lysosomes by an alternative signal. In a recent report (Rijnboutt et al., 1991), it has been demonstrated that in hepatocytes the unglycosylated form of cathepsin D, produced by growing the cells in the presence of tunicamycin, could be correctly transported to lysosomes. The authors were able to demonstrate a transient membrane association of procathepsin D in an intermediate endosomal compartment before the lysosome, suggesting a specific Man-6-P receptor-independent sorting association. The fact that an alternative pathway exists in cytolytic lymphocytes not only for the lytic proteins but also for the lysosomal enzymes raises the possibility that the lysosomal and regulated secretory pathways are merged in these cells. The exact nature of the regulated secretory sorting signal is not known, but sorting seems to involve a selective aggregation of the secretory proteins in the *trans*-Golgi network (Huttner and Tooze, 1989). Whether such an aggregation occurs in the formation of lytic granules is not known.

The regulated secretion of lytic proteins by cytolytic cells can be detected by the ability of these cells to lyse targets. Both normal and I-cells lyse target cells, indicating that regulated secretion of the lytic granules is similar in these two cell types (Fig. 11). These results agree with earlier studies which demonstrated normal levels of natural killer cell activity in I-cell patients (Haubeck et al., 1985). Although the constitutive secretion of granzymes from activated I-cells might be expected to have deleterious effects and lead

to nonspecific target cell destruction, this is unlikely since it has been demonstrated that both direct cell contact and T cell receptor stimulation are required for killing to occur (Lanzavecchia, 1986). This would explain why I-cell patients appear to have normal cytotoxic responses.

The lytic granules are specialized secretory organelles, produced upon T cell activation. Morphologically they appear to be a cross between a lysosome and a regulatory secretory granule (Burkhardt et al., 1989; Peters et al., 1991). Furthermore, the lytic proteins can be sorted to these secretory granules by virtue of lysosomal targeting signals. This strengthens the idea that the biogenesis of these organelles is related. Conventional lysosomes are rarely found in activated cytolytic lymphocytes as demonstrated by Fig. 10, which shows that all endocytic vesicles containing cathepsin B also contain granzyme A. This suggests that the lytic granules perform both lysosomal and secretory roles in the activated T cell.

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