PROTEOGLYCANS IN CELL-MEDIATED CYTOTOXICITY

Identification, Localization, and Exocytosis of a Chondroitin Sulfate Proteoglycan from Human Cloned Natural Killer Cells during Target Cell Lysis

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Natural killer (NK)[1] cell activity (1, 2) is mediated by a small but heterogeneous population of peripheral blood mononuclear cells (PBMC), which are morphologically classified as large granular lymphocytes (LGL). Although not completely elucidated, the mechanisms of target cell lysis by different types of NK cells are believed to be similar (2, 3). The initial binding of the NK effector cell to the target cell occurs through the recognition of a determinant on the target cell by a specific receptor on the effector cell (2, 3). Perturbation of a separate NK cell membrane structure (4) leads to activation of the NK cell by a calcium-dependent process, resulting in an increase in phospholipase A_2 activity. Target cell lysis, which is also calcium-dependent, is postulated to result from the deposition on the plasma membrane of lytic molecules (5, 6) exocytosed with the secretory granules of the effector cell. After this event, the calcium-independent lethal hit stage of cytotoxicity occurs (7) with the formation of transmembrane channels on the target cell (8, 9).

Dourmashkin et al. (8) observed by electron microscopy that in cell-mediated cytolysis, human peripheral blood leukocytes formed ring-like structures with an internal diameter of 15 nm on the target cell. Henkart and Henkart (9) observed that formation of the ring structures was due to material (designated as cytolysin)

[1] *Abbreviations used in this paper:* ΔDi-4S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose; EBV, Epstein-Barr virus; GnHCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; LCM, lymphocyte-conditioned medium; LGL, large granular lymphocytes; NK, natural killer; PBMC, peripheral blood mononuclear cells; TSG, buffer containing 0.1 M Tris-HCl, 0.1 M sodium sulfate, and 4 M GnHCl, pH 7.0.

that was released with the cytoplasmic granules of the effector cell. They and their associates also showed (10, 11) that isolated granules from rat LGL leukemia cells mediated cellular cytotoxicity with the concomitant formation of ring-structured lesions. Podack and Dennert (12), using cloned mouse NK cells as effectors and both YAC-1 cells and rabbit erythrocytes as targets, demonstrated the formation of two types of membrane lesions with inner diameters of either 16 ± 2 nm (~160 Å) or ~5 nm (~50 Å) on the targets. NK cells (12) are believed to interact with susceptible targets to assemble monomeric precursors into polymerized complexes that probably contain hydrophobic and hydrophilic domains, thereby allowing the formation of transmembrane channels and target cell lysis (12, 13).

Proteoglycans are glycoconjugates that consist of a polypeptide core with covalently linked oligosaccharides and glycosaminoglycan side chains (14). Although most of the proteoglycans in mammals are located in extracellular matrices and are protease-susceptible, IgE-Fc-bearing cells contain protease-resistant proteoglycans in their secretory granules (15, 16). Heparin proteoglycans are present in the secretory granules of rat (15) and mouse (17) serosal mast cells, and human lung (18) and skin (19) mast cells. Chondroitin sulfate E proteoglycan and chondroitin sulfate di-B proteoglycan are present in the granules of interleukin 3–dependent mouse bone marrow–derived mast cells (17, 20) and in rat basophilic leukemic cells (16), respectively. The negative charge characteristics of these intracellular proteoglycans make them particularly well suited as storage and carrier molecules for basically charged proteases and other proteins that are present in secretory granules and are exocytosed in response to a specific stimulus. We investigated the human NK clone JT_B18, which has the plasma membrane phenotype of human peripheral blood NK cells (21), and a defined target cell specificity (22), to determine whether it possesses proteoglycans in its granules. We determined that this cloned human NK cell contains a cell-associated chondroitin sulfate A proteoglycan which is localized to the granules of the cell and is specifically exocytosed when the NK cell lyses susceptible tumor cells.

Materials and Methods

Materials. RPMI 1640, fetal calf serum, 2-mercaptoethanol, L-glutamine, nonessential amino acids, penicillin, and streptomycin were obtained from Gibco Laboratories, (Grand Island, NY); PD-10 gel filtration columns, Sepharose CL-4 B and CL-6 B were from Pharmacia Fine Chemicals (Piscataway, NJ); [^{35}S]sulfate (sp act ~4,000 Ci/mol) was obtained from New England Nuclear (Boston, MA); Hydrofluor was from National Diagnostics (Somerville, NJ); *Streptomyces griseus* pronase (77 U/mg) and Zwittergent 3–12 were purchased from Calbiochem-Behring Corp. (San Diego, CA); ultrapure guanidine hydrochloride (GnHCl) was from Schwarz/Mann (Spring Valley, NY); phytohemaggluti-nin was supplied by Burroughs Wellcome (Research Park, NC); phorbol myristate acetate and porcine heparin were obtained from Sigma Chemical Co. (St. Louis, MO); and *Staphylococcus aureus* V8 protease (570 U/mg), shark chondroitin sulfate C, whale chon-droitin sulfate A, and chondroitinase ABC and AC were purchased from Miles Labora-tories (Elkhart, IN).

Culture of a Human Cloned NK Cell Line. The method for the generation of human cloned NK cells has been described (22, 23). Cloned JT_B18 cells derived from PBMC were cloned at 1 cell/well on a feeder layer of allogeneic irradiated (5,000 rad) PBMC and allogeneic irradiated (5,000 rad) Epstein-Barr virus (EBV)-transformed B cells in

medium containing 2 μg/ml of phytohemagglutinin. The JT$_B$18 NK cloned cell line was selected for its ability to lyse K562 target cells, and the clone was expanded by culture in RPMI 1640 supplemented with 1% penicillin, 1% streptomycin, 1% sodium pyruvate, 20% human AB serum (enriched medium) and 10–15% (vol/vol) lymphocyte-conditioned medium (LCM). The culture medium was replaced every 3 d. LCM was produced by stimulating whole PBMC at a concentration of 2.5 × 10^6 cells/ml for 2 h with 5 μg/ml phytohemagglutinin, 5 ng/ml phorbol myristate acetate and 5,000 rad–irradiated EBV-transformed B cells (0.5 × 10^6 cells/ml) (21–24). The activated PBMC were then washed four times to remove the mitogens, and were resuspended in RPMI 1640 supplemented with 2.5% human AB serum. After 40 h of incubation at 37°C, the cells were pelleted at 500 g, and the LCM was sterilized by passage through 0.45 μm filters and stored at −70°C until it was used for cloning or subcloning the JT$_B$18 cells (21–24). All NK cells used in these studies were subcloned at least four times at 100 cells/well on a feeder layer of allogeneic irradiated PBMC plus irradiated EBV-transformed B cells. The phenotype of the cloned JT$_B$18 NK cells is characteristic of peripheral blood NK cells: T3$^-$, T11$^+$, NKH1$^+$, and NKH2$^-$ (22). After subcloning procedures, both the phenotype and the cytotoxic function of this cell line remained stable.

Human NKH1$_A$$^+$ LGL were obtained by first staining PBMC with anti-NKH1$_A$ antibody and mouse Fab IgG labeled with fluorescein isothiocyanate, and then separating the NKH1$_A$$^+$ cells from the NKH1$_A$$^-$ cells by use of the cell sorter (Epics V; Coulter Electronics, Hialeh, FL) (21).

Microscopic and x-ray Energy-dispersive Analysis of Cloned NK Cells. For transmission electron microscopy, preparations of LGL (4.0–5.5 × 10^5 cells) were fixed with 2% glutaraldehyde in 0.1 M cacodylate, pH 7.2, and were stored for 1 wk or 17 h in 7% sucrose in 0.1 M cacodylate, pH 7.2. The cells were pooled, pelleted in a Microfuge B (Beckman Instruments, Palo Alto, CA), postfixed in 1% OsO$_4$ in acetate veronal buffer, stained in block with uranyl acetate, dehydrated in graded ethanols and propylene oxide, and embedded in Epon 812. JT$_B$18 (2 × 10^6 cells/ml of enriched medium) were fixed by the addition of an equal volume of mixed aldehydes in cacodylate for 45 min at 4°C (25), pelleted and processed as above for transmission microscopy. For x-ray energy-dispersive spectroscopy, the JT$_B$18 cells were fixed in aldehydes (25), pelleted, dehydrated, and embedded. Thin sections were cut with silver-gold interference colors for transmission microscopy and with gold interference colors for x-ray analysis. Sections were picked up on naked copper grids, and were stained with uranyl acetate and lead citrate for transmission microscopy, and with uranyl acetate for x-ray analysis. Grids were examined in a JEOL 100C/ASID electron microscope equipped with a Kevex 7000 x-ray analysis system. x-ray specimens were observed in the scanning$_e$ transmission mode at an accelerating voltage of 80 kV using a probe diameter of <100 Å (26). Elemental spectra were recorded from granules, cytoplasm, nuclei, and the epoxy resin over 100-s counting intervals. The sulfur window, 2.24–2.42 keV, consisted of 10 channels (20 eV/channel) centered around the characteristic sulfur emission (K$_\alpha$ = 2.32 keV). Background values were determined by a program that averaged the two channels immediately adjacent to the high and low limits of the sulfur window and extrapolated linearly between the two values. Sulfur peaks (P_s) were considered to be significantly above background (Bg) when $P_s > Bg + 3\sqrt{2Bg}$ (see 27 for discussion of this analysis). Three separate preparations of JT$_B$18 cells were examined by transmission microscopy and two preparations by x-ray analysis.

Radiolabeling of NK-cloned Cells and Characterization of Cell-associated Proteoglycans. JT$_B$18 cells were incubated at 37°C for 24 h at a concentration of 2 × 10^6 cells/ml of enriched medium containing 10% LCM and 100 μCi/ml of [^{35}S]sulfate. Radiolabeled cloned NK cells were sedimented at 400 g for 5 min, the supernatants were removed and saved, and the cells were washed once with medium. The cell-associated ^{35}S-labeled proteoglycans were extracted by the addition of 50 μl of 1% (vol/vol) Zwittergent 3–12, followed 30 s later by 450 μl of 4 M GnHCl (17). Heparin or chondroitin sulfate A glycosaminoglycan (1 mg) was added to each extract as a carrier to minimize nonspecific absorption and loss of the radiolabeled proteoglycans during their isolation. The 24 h culture medium supernatants, which contained the ^{35}S-labeled proteoglycans released

during the radiolabeling, were made 4 M in GnHCl by the addition of an equal volume of 8 M GnHCl. Solid CsCl was added to each sample to give a final density of 1.4 g/ml, and the cell extracts and supernatants were centrifuged separately in a Beckman L2-65B ultracentrifuge at 95,000 g for 40–48 h (17). The bottom half of each gradient, which contained the ^{35}S-labeled proteoglycans, was dialyzed (3,000 M_r cut-off dialysis tubing) sequentially against 1 M sodium acetate for one exchange, and then against ammonium bicarbonate for five exchanges to remove the GnHCl and free [^{35}S]sulfate.

To determine the hydrodynamic size of the ^{35}S-labeled NK cell proteoglycan, the samples were dialyzed, lyophilized, and suspended in 500 μl of H_2O, and portions were applied to Sepharose CL-4 B columns (0.6 × 120 cm) that were equilibrated in and eluted with 4 M GnHCl, 0.1 M Tris-HCl, 0.1 M Na_2SO_4, pH 7.0 (TSG buffer), containing 25 μg/ml of heparin glycosaminoglycan carrier (17). A sample of each 0.5-ml column eluate fraction was added to 0.5 ml of 70% (vol/vol) ethanol and 12.5 ml of Hydrofluor, and the radioactivity was quantitated on a Searle 6880 Beta Counter. For protease suscepti-bility studies, ~1 μg of the density gradient–purified ^{35}S-labeled proteoglycan was sus-pended in 100 μl of Hanks' balanced salt solution, and was incubated with 1 mg of pronase, or with 3 or 100 U of S. aureus V8 protease for 1 h at 37°C. The digests were made 4 M in GnHCl and the ^{35}S-labeled proteoglycans were analyzed for their hydrody-namic size by Sepharose CL-4 B gel filtration. To determine their approximate size, glycosaminoglycans were liberated from purified ^{35}S-labeled JT_B18 proteoglycans by β-elimination in 30 μl of 0.5 M NaOH for 16 h at 4°C followed by neutralization with 30 μl of 0.5 M acetic acid. Each sample was then chromatographed on a 0.7 × 100-cm column of Sepharose CL-6 B that had been equilibrated with TSG containing 50 μg/ml heparin. 0.5-ml fractions were collected and analyzed for radioactivity. The relative molecular weights (M_r) of the glycosaminoglycan side chains were estimated by comparing the K_{av} values to published K_{av} values for glycosaminoglycans of known M_r (28).

The ^{35}S-labeled disaccharides were identified by enzymatic digestion of the ^{35}S-labeled proteoglycan, followed by high performance liquid chromatographic (HPLC) analysis of the digests. After the addition of chondroitin sulfate C (100 μg) and chondroitin sulfate A (100 μg) carriers, ^{35}S-labeled NK cell proteoglycans were incubated at 37°C for 60 min with 0.4 U of chondroitinase ABC or with 0.4 U of chondroitinase AC, as previously described (17, 29). The extent of digestion was assessed by Sephadex G-25/PD-10 gel filtration chromatography of a 250-μl portion of each digest on separate 1.5 × 5.5 cm columns, which were equilibrated and eluted with TSG; the amounts of radioactivity eluting in the void volume and in the included volume of each column were determined. For HPLC analysis of the chondroitinase digests, liberated ^{35}S-labeled disaccharides were separated from undegraded proteoglycans, glycosaminoglycans, degradative enzymes, and contaminating macromolecules by an 80% ethanol extraction. Each digest was diluted with four volumes of absolute ethanol, cooled to 0°C for 2 h, and centrifuged in a Beckman Microfuge at 8,000 g for 5 min. The supernatants were decanted, dried over nitrogen, and resuspended in the HPLC solvent composed of 70% acetonitrile/methanol (3:1, vol/vol) and 30% 0.5 M ammonium acetate/acetic acid, pH 5.3. HPLC was per-formed on an Altex Model 322 system (Altex Scientific, Berkeley, CA) using a 4.6 × 250-mm Whatman Partisil-10 PAC amino-cyano–substituted normal-phase silica column with a 4.6 × 24-mm precolumn containing the same packing (Whatman Inc., Clifton, NJ), as previously described (30). The elution of the ^{35}S-labeled disaccharides was characterized relative to authentic disaccharide standards: 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-ene-pyranosyluronic acid)-4-O-sulfo-D-galactose (ΔDi-4S), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose (ΔDi-6S), and 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4,6-di-O-sulfo-D-galactose (ΔDi-diS$_E$) (30).

Cytotoxicity Assays.   Cytotoxicity assays with human tumor lines used as targets were performed according to a standard chromium-release method (21–24). The K562 cell line was established from a patient with chronic myelogenous leukemia and the KG1 cell line from a patient with acute myeloblastic leukemia. REX, Molt4, and CEM are human T cell leukemic cell lines, and Laz156 is an EBV-transformed B cell line. Chromium-release assays were performed in triplicate using V-bottom microtiter plates containing

RPMI 1640, 5% normal human AB serum, and 1% penicillin-streptomycin at various effector/target cell ratios with 5,000 target cells/well.

Exocytosis of ^{35}S-labeled Proteoglycan from NK Effector Cells During Interaction with Target Cells. After being labeled with [^{35}S]sulfate, the cloned NK cells were centrifuged and washed once to remove most of the unincorporated [^{35}S]sulfate. 2.5 × 10^4 ^{35}S-labeled NK cells were incubated in quadruplicate with K562 or KG1 cells in V-bottom microtiter plates for 30 min to 4 h. The number of ^{35}S-labeled NK effector cells (2.5 × 10^4) was held constant, and the number of K562 or KG1 target cells was varied to obtain effector/target cell ratios ranging from 10:1 to 0.125:1. After incubation in the microtiter plates, 150-μl supernatants from two wells containing the same target cells were pooled, resulting in two 300-μl duplicate samples for each target cell. Each 300-μl duplicate supernatant sample was mixed with 25 μl of chondroitin sulfate A carrier and 250 μl of 8 M GnHCl and applied to separate Sephadex G-25/PD-10 columns, which were equilibrated and eluted with TSG to determine the total macromolecular radioactivity in the supernatants and extracts. Eight 0.5-ml fractions were collected. After the addition of 0.5 ml ethanol and 12.5 ml Hydrofluor to each fraction, ^{35}S-radioactivity was quantitated with a β-scintillation counter. The net percent release of ^{35}S-labeled proteoglycan from the NK effector cell was calculated as 100 × (experimental − minimal)/(maximal − minimal) where experimental is the amount of ^{35}S-radioactivity (cpm) released from the effector cell in the presence of target cells; minimal is the amount of the radioactivity (cpm) nonspecifically released when target cells were not present; and maximal is the total amount of ^{35}S-labeled proteoglycan (cpm) extractable from the ^{35}S-labeled effector cells. The maximal content was determined by lysing the ^{35}S-labeled NK cells with 1% Nonidet P-40 detergent, and quantifying the total amount of ^{35}S-labeled proteoglycan. The minimal release in eight experiments was 8.5 ± 4.5% (mean ± SD) of the maximal release.

Results

Ultrastructural Analysis of LGL and the Cloned JT_B18 NK Cell. LGL obtained by fluorescence-activated cell sorting of NKH1$_A$-stained human PBMC were homogeneous in appearance, with individual cells measuring 6–10 μm in diameter (Fig. 1). The cells were mononuclear with a cytoplasm rich in organelles. Mitochondria, Golgi apparatus, and 0.1–0.2 μm electron-dense granules tended to cluster in the cell where the nucleus was indented. The granules varied in number from cell to cell, were homogeneous in electron density, and did not contain parallel tubular arrays (Fig. 1, inset). JT_B18 cells measured 6–10 μm in diameter, and appeared to be similar to the LGL in the distribution and diversity of their subcellular organelles (Fig. 2). Their granules were small and homogeneous in electron density (Fig. 2, inset). The nucleus was occasionally invaginated and consequently appeared to surround portions of the cytoplasm.

Incorporation of [^{35}S]Sulfate into Cloned JT_B18 Cells and Characterization of Their Proteoglycan. As assessed by PD-10 chromatography under dissociative conditions, the JT_B18 cells incorporated [^{35}S]sulfate into total, cell-associated, and released macromolecules at linear rates of 622, 476, and 146 cpm/h/10^6 cells, respectively (Fig. 3). After 24 h of culture, only 23% of the total ^{35}S-labeled macromolecules were recovered in the culture medium in this experiment, while in a second experiment, 26% of the total ^{35}S-labeled macromolecules were recovered in the culture medium after 12 h of culture. Upon CsCl density-gradient centrifugation under dissociative conditions, 80% of the ^{35}S-labeled macromolecules in both the cell extract and in the culture medium sedimented to the bottom half of the gradient, indicating the presence of proteoglycan-like molecules with high buoyant densities. Gel filtration on Sepharose CL-4 B under
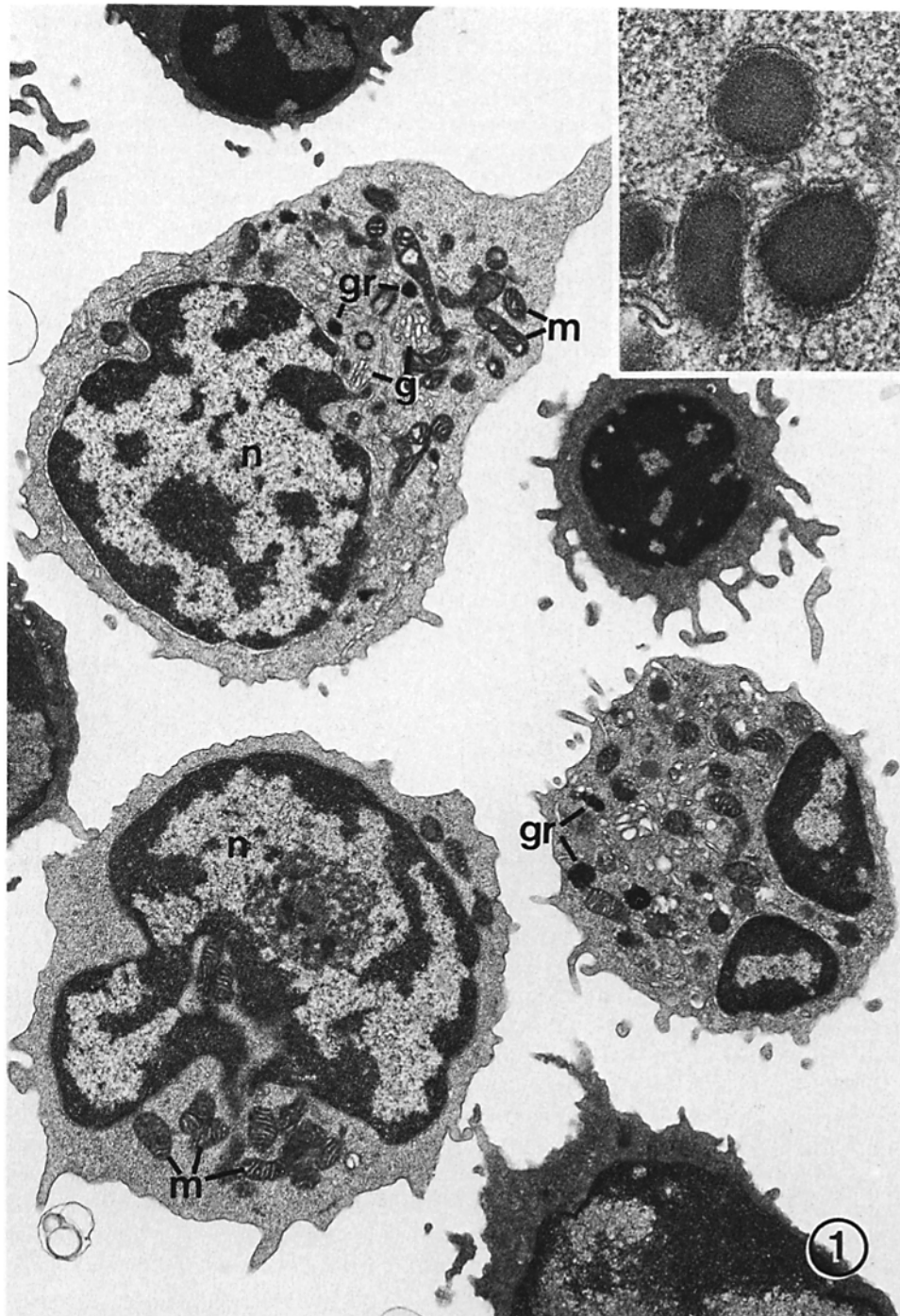
FIGURE 1.   Transmission electron micrograph of human LGL isolated by fluorescence-activated cell sorting. The cells contain a single nucleus (*n*). Most organelles, including mitochondria (*m*), Golgi apparatus (*g*), and granules (*gr*), are clustered near the nuclear indentation. The inset is a high magnification of the granules showing their homogeneous electron-dense structure. × 10,500. Inset, × 64,000.
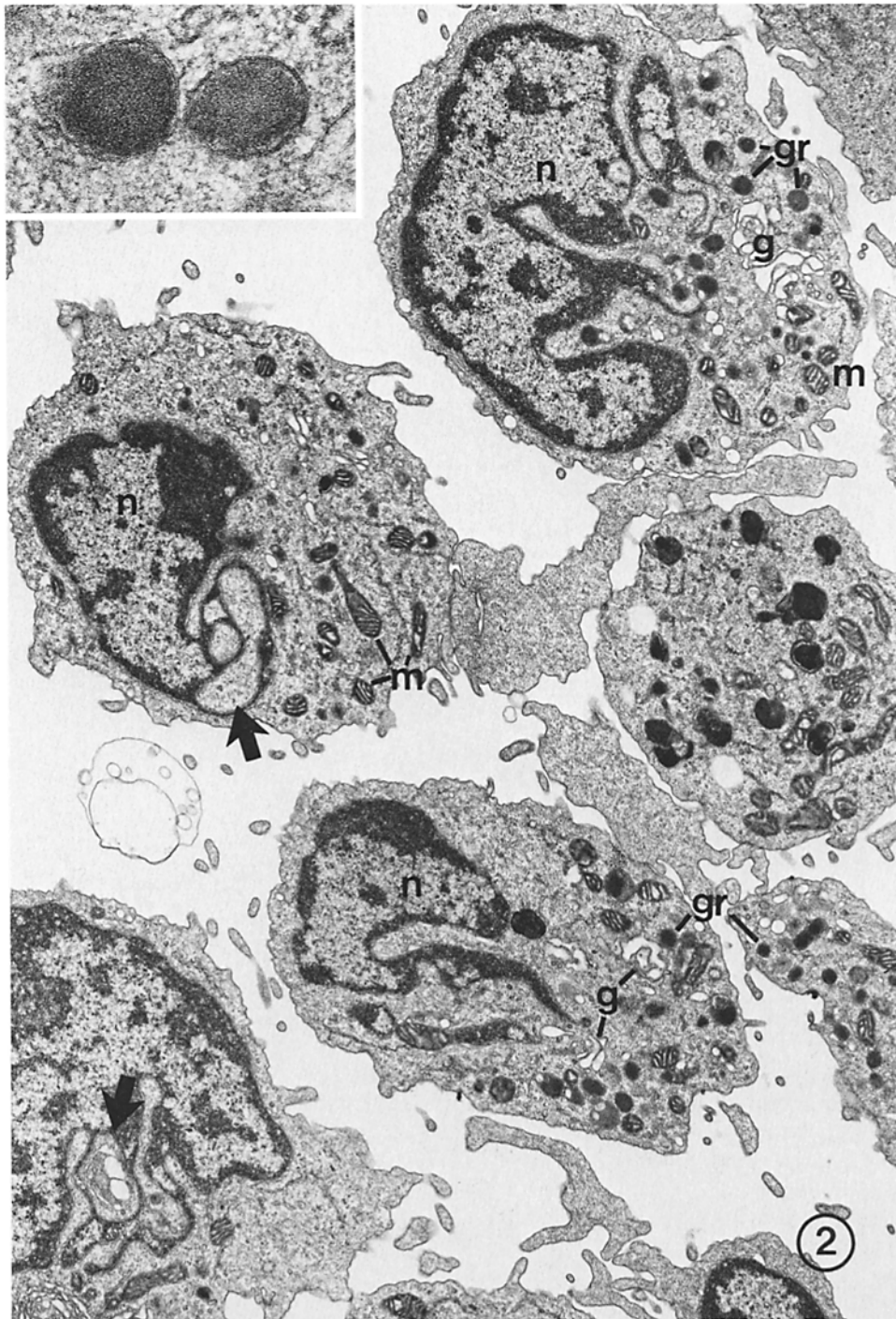
FIGURE 2.   Transmission electron micrograph of JT$_B$18 cells. The nuclei (n) are deeply invaginated and appear to surround portions of the cytoplasm in areas (arrows). Mitochondria (m), Golgi apparatus (g), and granules (gr) are clustered as in the LGL. The granules are shown at higher power in the inset, and appear to be similar to those shown in Fig. 1 inset. × 9,600. Inset, × 78,000.

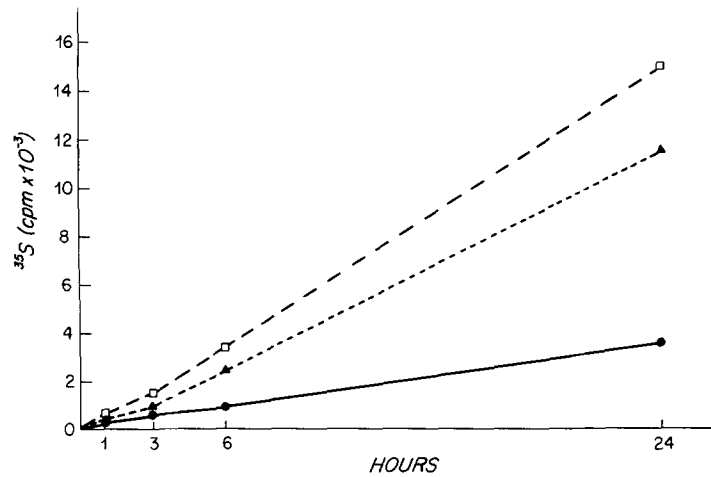FIGURE 3. Kinetics of [^{35}S]sulfate incorporation into macromolecules by cultured JT$_B$18 cells. 10^6 cells were cultured at a density of 2×10^6 cells/ml of standard culture medium containing 100 μCi of [^{35}S]sulfate and the total ($\square$), cell-associated ($\blacktriangle$) and released ($\bullet$) radiolabeled macromolecules were quantified at defined intervals.
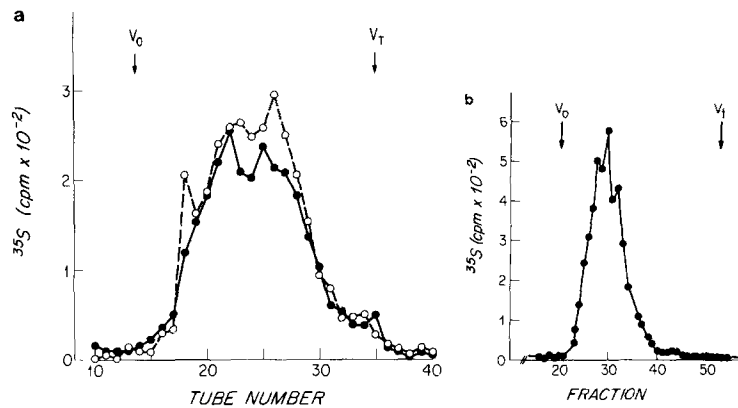

FIGURE 4. (a) Sepharose CL-4 B chromatography of ^{35}S-labeled, density gradient–purified cell-associated proteoglycans synthesized by JT$_B$18 cells and filtered before ($\bullet$) and after (O) incubation with pronase. (b) Sepharose CL-6 B chromatography of ^{35}S-labeled NK cell glycos-aminoglycans. V_o and V_t mark the excluded and total volumes of the columns, respectively.

dissociative conditions revealed that the cell-associated and the secreted ^{35}S-labeled proteoglycans from the cloned NK cells exhibited a predominant poly-dispersed peak with K_{av} values of 0.47 ± 0.02 (mean $\pm$ SD, $n = 3$) and 0.42 ± 0.05 (mean $\pm$ SD, $n = 3$), respectively, corresponding to ~200,000 M_r (Fig. 4 A). The hydrodynamic size of the ^{35}S-labeled NK cell proteoglycan was not altered by incubation of the ^{35}S-labeled NK proteoglycan with either pronase ($n = 2$) (Fig. 4A) or S. aureus V8 protease ($n = 3$) (data not shown) before the gel filtration. The glycosaminoglycans liberated from the ^{35}S-labeled proteoglycan filtered on Sepharose CL-6 B with a K_{av} of 0.31 ± 0.02 (mean $\pm$ SD, $n = 3$) (Fig. 4B), indicating ~50,000 M_r.

When the ^{35}S-labeled cell-associated macromolecules were treated with chon-droitinase ABC or chondroitinase AC, 89% (mean; $n = 3$) and 92% (mean; $n =$
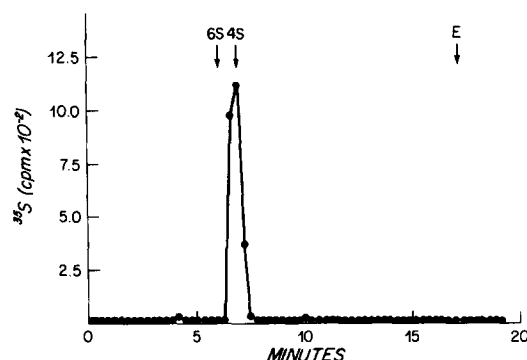
FIGURE 5. HPLC analysis of a chondroitinase AC digest of density gradient–purified ³⁵S-labeled proteoglycan from JT$_B$18 cloned cells. The retention times of standard disaccharides are indicated.

2) of the starting material, respectively, were digested to ³⁵S-labeled disaccharides, as assessed by a shift of radioactivity from the void volume to the included volume on Sephadex G-25/PD-10. Duplicate samples, digested with either chondroitinase ABC ($n = 2$) or chondroitinase AC ($n = 1$) (Fig. 5) and analyzed for ³⁵S-labeled disaccharides by HPLC, revealed in each instance a single radioactive peak eluting at 6.9 min corresponding in its retention time with the unsaturated standard disaccharide ΔDi-4S. That ΔDi-4S was generated by treatment with either chondroitinase ABC or chondroitinase AC indicated that most of the disaccharides in the chondroitin sulfate chain contained the glucuronic acid isomer rather than the iduronic acid isomer of the uronic acid. Thus, the glycosaminoglycans of the protease-resistant proteoglycan of the human NK cell were predominantly chondroitin sulfate A (chondroitin-4-sulfate).

Granule Localization of the Cell-associated Proteoglycan. x-ray energy-dispersive spectroscopy was performed on the granules, nucleus, and cytoplasm of the JT$_B$18 cells, and on the Epon embedding matrix. The sections were stained with uranyl acetate before analysis in order to visualize the granules. The Epon contained only chlorine; the cytoplasm chlorine and uranium; the nucleus phosphorus, uranium, and chlorine; and the granules sulfur, uranium, and chlorine (Fig. 6). The granule sulfur peaks were significantly (>99%) above the background in 30 of the 36 granule spectra collected. In contrast, only 2 of 17 spectra from the cytoplasm, 1 of 16 spectra from the Epon, and 0 of 16 spectra from the nucleus contained a sulfur peak.

³⁵S-Labeled JT$_B$18 cloned cells treated with chondroitinase ABC in the presence or absence of 1 mg of chondroitin sulfate C carrier released <1% of the total cell-associated ³⁵S-radioactivity in each experiment ($n = 3$) during a 15-min incubation period (data not shown). In contrast, the exogenous chondroitin sulfate C was >99% ($n = 2$) digested to unsaturated disaccharides, indicating that the enzyme was not inhibited by the presence of the NK cells. That the ³⁵S-labeled proteoglycans from intact NK cells were not susceptible to digestion provides additional evidence that these proteoglycans were located intracellularly and, in conjunction with the x-ray energy-dispersive analysis, within secretory granules of the cloned NK cells.
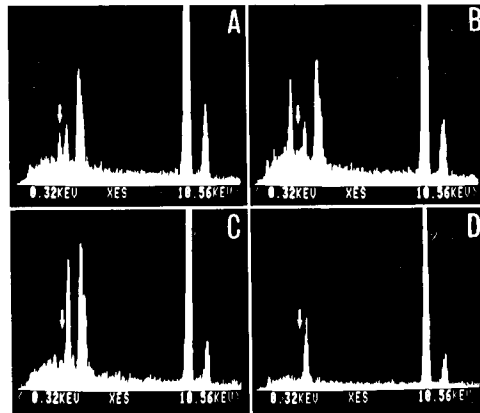
FIGURE 6. Representative x-ray spectra from JT_B18 cells. (A) granules; (B) nucleus; (C) cytoplasm; (D) Epon embedding resin. In all spectra, the arrow marks the position of the sulfur emission. In each spectrum, the peaks reading from left to right represent (A) sulfur, chlorine, uranium, and two copper peaks; (B) phosphorus, chlorine, uranium, and two copper peaks; (C) chlorine, uranium, and two copper peaks; (D) chlorine and two copper peaks. Chlorine is a contaminant in the resin, and copper is present in the support grid. Uranium is from the uranyl acetate stain. However, the sulfur and phosphorus peaks are specific to the granular proteoglycan and the nuclear DNA, respectively. All spectra are unsmoothed, and are displayed with the same vertical scale, which has a maximum of 256 counts per channel.
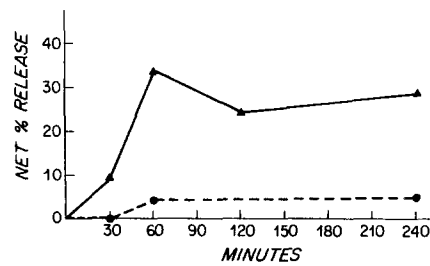

FIGURE 7. Time-dependent net percent exocytosis of ^{35}S-labeled proteoglycans from JT_B18 cells incubated at 0.5:1 ratios with K562 (▲) or KG1 (●) targets for defined intervals. The spontaneous release values for ^{35}S-labeled proteoglycan during the 30-, 60-, 120-, and 240-min time intervals were 1, 2, 3, and 3%, respectively.

Exocytosis of ^{35}S-labeled Proteoglycans Triggered Specifically by Sensitive Targets. When the cloned ^{35}S-labeled JT_B18 cells were incubated from 30–240 min with susceptible targets such as K562 cells, ^{35}S-labeled proteoglycans were exocytosed, whereas incubation with resistant targets, such as KG1 cells, did not result in incremental proteoglycan release (Fig. 7). The net percent of ^{35}S-labeled proteoglycans released increased rapidly during the first 60 min of culture, with a plateau after 60 min of incubation, as shown in the representative experiment in Fig. 7, performed at a 0.5:1 effector/target cell ratio. A similar effect of incubation time was observed at effector/target cell ratios from 0.5:1 to 5:1 in two other experiments, and no further release was observed in one experiment with an 8-h incubation period (data not shown). The exocytosis of ^{35}S-labeled proteoglycans from these human NK cells was dependent upon the effector/target cell ratio (Fig. 8), with a maximum net percent of the radiolabeled
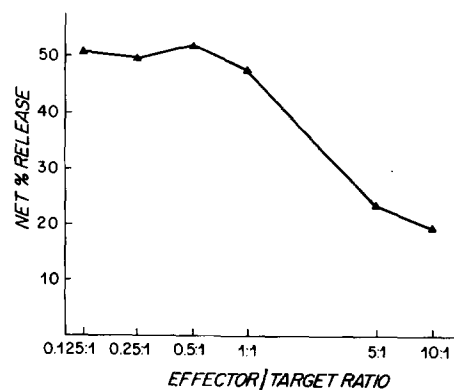
FIGURE 8.   Effect of different effector/target cell ratios on the net percent exocytosis of ^{35}S-labeled proteoglycans from the JTB18 cell incubated with K562 targets for 4 h.

proteoglycan being exocytosed upon exposure of the NK cells to equal or greater numbers of specific target cells. When the resistant target KG1 was used, no more than 3% ($n = 3$) of the ^{35}S-labeled proteoglycan was specifically released at any of the effector/target cell ratios examined (data not shown).

The JTB18 clone is cytolytic for certain targets, such as K562, Molt4, REX, and CEM, but does not kill KG1 or Laz156 (22). The net percent cytotoxicity mediated by the JTB18 cloned NK cells against various targets was compared with the net percent proteoglycan release induced by these same targets in parallel assays. The induction of exocytosis of proteoglycans from the NK cell by specific targets was related to the degree of cytotoxicity of the NK cell for the same targets (Table I). In this experiment, the K562 target induced a mean 49% net release of ^{35}S-labeled proteoglycan from the NK cell.

Discussion

These studies demonstrate the presence of a chondroitin sulfate A proteoglycan in the granules of a cloned human NK cell and induction of its specific exocytosis by target cells sensitive to lysis. For these studies, a clone (JTB18) was used with a phenotype typical of peripheral blood NK cells. These cells are similar in ultrastructure to peripheral blood human LGL isolated by fluorescence-activated cell sorting (Figs. 1 and 2). The cloned cells also resembled published descriptions of LGL isolated by a combination of gradient and rosetting techniques (31-36), with the exception that parallel tubular arrays were not seen. Although some authors have considered these structures to be characteristic of LGL (33, 34, 36), they have not been observed in all studies (31, 32, 35). When the human cloned NK cell JTB18 was cultured for 24 h in medium containing [^{35}S]sulfate, radioactivity was incorporated into macromolecules in a linear fashion (Fig. 3). Because even after 24 h of culture, most of the newly synthesized ^{35}S-labeled macromolecules were not released, the NK cell–produced ^{35}S-labeled macromolecules were destined to remain cell associated.

The first indication that these ^{35}S-labeled macromolecules were proteoglycans was their possession of a buoyant density in CsCl typical of proteoglycans in secretory granules (17). Upon filtration of the density gradient–purified [35]S-

TABLE I

Net Cytotoxicity and Net Proteoglycan Release with JT_B18 Cloned NK
Cells and Either Sensitive or Resistant Targets

Targets	Net cytotoxicity	Net proteoglycan release
	%	
Sensitive		
REX	82	36
K562	77	49
Molt4	73	39
CEM	65	27
Resistant		
KG1	15	2
Laz156	8	0

Cells were incubated for 4 h at an effector/target ratio of 0.5:1 for the net percent release of proteoglycan and 10:1 for the net percent cytotoxicity. Numbers are means for three experiments performed simultaneously, in which both cytotoxicity and proteoglycan release were analyzed. The cytotoxicity experiments were performed in quadruplicate, and the proteoglycan release experiments were performed in duplicate.

labeled proteoglycans on Sepharose CL-4 B (Fig. 4 A), a polydispersed peak of radioactivity with a K_{av} of 0.42–0.47 was obtained, indicating ~200,000 M_r. When these ^{35}S-labeled NK cell proteoglycans were incubated with pronase or V8 protease and again chromatographed, their filtration was unchanged (Fig. 4 A). Because protease-resistant proteoglycans have been found in the granules of rat serosal mast cells (15), mouse bone marrow–derived mast cells (37), and rat basophilic leukemia cells (16), it seemed possible that these NK cell–associated proteoglycans belonged to a unique family of proteoglycans that reside intracellularly in granules, rather than on the plasma membrane or in the extracellular matrix. Glycosaminoglycan analysis of the purified NK cell proteoglycan revealed the presence of chains with ~50,000 M_r (Fig. 4 B). Because these glycosaminoglycans were equally sensitive to digestion with chondroitinase ABC and chondroitinase AC, it was concluded that chondroitin sulfate chains containing glucuronic acid rather than iduronic acid were attached to the peptide core of this proteoglycan. HPLC analyses of the chondroitinase digests revealed only the disaccharide ΔDi-4S (Fig. 5), indicating that these glycosaminoglycans were exclusively chondroitin sulfate A. Thus, the chondroitin sulfate glycosaminoglycans on the proteoglycans from this human cloned NK cell were different from those on the proteoglycans of the mouse bone marrow–derived mast cell (17) and the rat basophilic leukemia cell (16), in that they were larger (M_r) and did not contain substantial amounts of disulfated disaccharides.

Mouse cloned NK cells (38, 39), human peripheral blood mononuclear cells (40), and human T cell lines (40) possess cell-associated proteoglycans bearing chondroitin sulfate glycosaminoglycan side chains. Transmission electron microscopy and autoradiography localized ^{35}S-labeled molecules to the cytoplasmic granules of one clone of ^{35}S-labeled mouse NK cells, suggesting that these proteoglycans are present in the secretory granules (39). In this study, sulfur was

detected in the granule of the JT_B18 cells by x-ray energy-dispersive spectroscopy (Fig. 6). This sulfur is most likely present as sulfate bound to the proteoglycan molecule, because ionic sulfate is normally lost during the processing procedure, and because it is highly unlikely that sulfur-containing amino acids are present in sufficient concentration to provide detectable signals. The failure to detect sulfur in 17% of the granules is most likely due to technical difficulties in identifying and measuring poorly visualized granules, although a lack of proteoglycan in a small population of the granules cannot be ruled out. However, the presence of these sulfur-containing molecules in most of the granules (Fig. 6), coupled with the absence of chondroitinase ABC–susceptible ^{35}S-labeled proteoglycans on the surface of the NK cell, indicates that most of the chondroitin sulfate A proteoglycans of the JT_B18 cell are located within the secretory granules of the cell.

Specific exocytosis of chondroitin sulfate A proteoglycans from the granules of the ^{35}S-labeled cloned JT_B18 cell occurred during exposure of the cells to JT_B18-susceptible target cells such as K562, REX, Molt4, and CEM, but not to JT_B18-resistant targets such as KG1 or Laz156 (Table I). The stimulation of proteoglycan release by K562 targets was time dependent, reaching a plateau at ~60 min (Fig. 7), and was optimal at an effector/target cell ratio of 0.5:1, with progressively less release being obtained at increasing ratios (Fig. 8). A cell-associated proteoglycan of 200,000 M_r containing only chondroitin sulfate A glycosaminoglycans was also produced by a human NK clone, CNK8 (T3$^+$, T11$^+$, NKH1$^+$, NKH2$^+$). Exocytosis of the ^{35}S-labeled proteoglycan from the CNK8 cell was also stimulus specific, being 53% upon incubation with the K562 sensitive target, and 3% for the CEM-resistant target. The correlation of proteoglycan release with target cell susceptibility, coupled with the inhibition of proteoglycan release by monoclonal antibodies that block JT_B18 and CNK8 cytotoxicity indicates that specific NK effector cell receptors and target cell recognition structures are involved in triggering proteoglycan exocytosis (42). The ability to define one of the granule constituents, proteoglycan, exocytosed from the NK cell via receptor-mediated activation should enable the identification of other granule-associated molecules and their possible association with this proteoglycan molecule.

Because of stimulation of their specific release by NK-susceptible targets, there are several possible roles for chondroitin sulfate A proteoglycans in cell-mediated cytotoxicity. Due to its acidic nature, chondroitin sulfate A proteoglycan may act as a packaging and/or carrier molecule that forms complexes with cationic cytolysins or perforins (9–13) and/or other proteins (5, 6). A serine esterase has been recently identified in mouse cytotoxic T cell clones (41), and the chondroitin sulfate A proteoglycan could function as a carrier for analogous human proteases. Such complexes could reduce granule osmotic pressure before secretion, thereby condensing the size of the granule and making storage more efficient.

Alternatively, the chondroitin sulfate A proteoglycans may protect the NK cell from being lysed by the cytotoxic molecules in the granules. Before secretion, the proteoglycan may orient the lytic proteins and prevent lysis of the granule membrane. After secretion, the complex of proteoglycan and cytotoxic molecules may persist and permit the transfer of the cytotoxic molecules to the membrane

of the target cell. Thus, the proteoglycan molecule may allow the cytotoxic effector cell to deliver a series of lethal hits to target cells without incurring damage.

On the target cell membrane, chondroitin sulfate A proteoglycans may either participate in the assembly of the lytic subunits or act as insertion molecules in the final ring-forming structure. Their large size, with multiple glycosaminogly-can side chains, could bind and orient multiple lytic subunits in a ring formation. Alternatively, a terminal hydrophobic region of the peptide core could promote lytic complex insertion into the target cell membrane.

Summary

A clone of natural killer (NK) cells (JT_B18) was found to be ultrastructurally similar to peripheral blood large granular lymphocytes (LGL). These cells incorporated [^{35}S]sulfate into cell-associated proteoglycan molecules, which were then isolated by CsCl density gradient centrifugation. As assessed by gel filtration chromatography, the native ^{35}S-labeled proteoglycan and its β-eliminated ^{35}S-labeled glycosaminoglycans were of M_r ~200,000 and 50,000, respectively. The ^{35}S-labeled proteoglycans were resistant to proteolysis, since their M_r were apparently not altered by incubation with either pronase or S. aureus V8 protease. The purified NK cell ^{35}S-labeled proteoglycans were degraded by ~90% to ^{35}S-labeled disaccharides with either chondroitinase ABC or AC. High performance liquid chromatographic analysis of the digests revealed these disaccharides to be composed entirely of chondroitin sulfate A (glucuronic acid → N-acetylgalacto-samine-$4SO_4$). Whole ^{35}S-labeled cells incubated with chondroitinase ABC failed to release ^{35}S-labeled disaccharides into the supernatant, and x-ray energy-dispersive analysis revealed that sulfur-containing molecules were present in the intracellular granules, thereby localizing the NK cell–associated proteoglycan primarily in the granules of the cell, rather than on the plasma membrane.

The ^{35}S-labeled cloned NK cells incubated for 30 min to 4 h with K562 tumor cell targets at a 0.5:1 ratio exocytosed a mean of 49% of the granular ^{35}S-labeled proteoglycans during the first 60 min of the culture. Proteoglycan release was maximal with an effector/target cell ratio of 0.5:1 for JT_B18:K562. Significant proteoglycan release from JT_B18 NK cells was also obtained with other sensitive target cells such as REX, Molt4, and CEM, but not with cells such as KG1 and Laz156, which have been shown previously (22) to be resistant to killing by this NK cell. Thus, protease-resistant intracellular proteoglycans with chondroitin sulfate A side chains are specifically exocytosed from the granules of human NK effector cells upon contact with sensitive targets, suggesting that these proteogly-cans may be involved in the mechanism of cytotoxicity.

References

1. Herberman, R. B., and J. R. Ortaldo. 1981. Natural killer cells: their role in defenses against disease. *Science (Wash. DC)*. 214:24.

2. Trinchieri, G., and B. Perussia. 1984. Human natural killer cells: biologic and pathologic aspects. *Lab. Invest.* 50:489.

3. Goldfarb, R. H., and R. B. Herberman. 1982. Characteristics of natural killer cells and possible mechanisms for their cytotoxic activity. *Adv. Inflammation Res.* 4:45.

4. Targan, S. R., and W. Newman. 1983. Definition of a "trigger" stage in the NK cytolytic reaction sequence by a monoclonal antibody to the glycoprotein T-200. *J. Immunol.* 131:1149.

5. Wright, S. C., and B. Bonavida. 1982. Studies on the mechanism of natural killer (NK) cell-mediated cytotoxicity (CMC). I. Release of cytotoxic factors specific for NK-sensitive target cells (NKCF) during co-culture of NK effector cells with NK target cells. *J. Immunol.* 129:433.

6. Farram, E., and S. R. Targan. 1983. Identification of human natural killer soluble cytotoxic factor(s) (NKCF) derived from NK-enriched lymphocyte populations: specificity of generation and killing. *J. Immunol.* 130:1252.

7. Hiserodt, J. C., L. Britvan, and S. R. Targan. 1983. Studies on the mechanism of the human natural killer cell lethal hit: analysis of the mechanism of protease inhibition of the lethal hit. *J. Immunol.* 131:2705.

8. Dourmashkin, R. R., P. Deteix, C. B. Simone, and P. Henkart. 1980. Electron microscopic demonstration of lesions in target cell membranes associated with antibody-dependent cellular cytotoxicity. *Clin. Exp. Immunol.* 42:554.

9. Henkart, M. P., and P. A. Henkart. 1982. Lymphocyte-mediated cytolysis as a secretory phenomenon. *Adv. Exp. Med. Biol.* 146:227.

10. Henkart, P. A., P. J. Millard, C. W. Reynolds, and M. P. Henkart. 1984. Cytolytic activity of purified cytoplasmic granules from cytotoxic rat large granular lymphocyte tumors. *J. Exp. Med.* 160:75.

11. Millard, P. J., M. P. Henkart, C. W. Reynolds, and P. A. Henkart. 1984. Purification and properties of cytoplasmic granules from cytotoxic rat LGL tumors. *J. Immunol.* 132:3197.

12. Podack, E. R., and G. Dennert. 1983. Assembly of two types of tubules with putative cytolytic function by cloned natural killer cells. *Nature (Lond.).* 302:442.

13. Dennert, G., and E. R. Podack. 1983. Cytolysis by H-2-specific T killer cells. Assembly of tubular complexes on target membranes. *J. Exp. Med.* 157:1483.

14. Rodén, L. 1980. Structure and metabolism of connective tissue proteoglycans. *In* The Biochemistry of Glycoproteins and Proteoglycans. W. J. Lennarz, editor. Plenum Press, New York. p. 267.

15. Yurt, R. W., R. W. Leid, K. F Austen, and J. E. Silbert. 1977. Native heparin from rat peritoneal mast cells. *J. Biol. Chem.* 252:518.

16. Seldin, D. C., K. F. Austen, and R. L. Stevens. 1985. Purification and characterization of protease-resistant secretory granule proteoglycans containing chondroitin sulfate di-B and heparin-like glycosaminoglycans from rat basophilic leukemia cells. *J. Biol. Chem.* 260:11131.

17. Razin, E., R. L. Stevens, F. Akiyama, K. Schmid, and K. F. Austen. 1982. Culture from mouse bone marrow of a subclass of mast cells possessing a distinct chondroitin sulfate proteoglycan with glycosaminoglycans rich in N-acetylgalactosamine-4,6-disulfate. *J. Biol. Chem.* 257:7229.

18. Metcalfe, D. D., R. A. Lewis, J. E. Silbert, R. D. Rosenberg, S. I. Wasserman, and K. F. Austen. 1979. Isolation and characterization of heparin from human lung. *J. Clin. Invest.* 64:1537.

19. Metcalfe, D. D., N. A. Soter, S. I. Wasserman, and K. F. Austen. 1980. Identification of sulfated mucopolysaccharides including heparin in the lesional skin of a patient with mastocytosis. *J. Invest. Dermatol.* 74:210.

20. Razin, E., J.-M. Mencia-Huerta, R. L. Stevens, R. A. Lewis, F.-T. Liu, E. J. Corey, and K. F. Austen. 1983. IgE-mediated release of leukotriene C₄, chondroitin sulfate E proteoglycan, β-hexosaminidase, and histamine from cultured bone marrow–derived mouse mast cells. *J. Exp. Med.* 157:189.

21. Hercend, T., J. D. Griffin, A. Bensussan, R. E. Schmidt, M. A. Edson, A. Brennan, C. Murray, J. F. Daley, S. F. Schlossman, and J. Ritz. 1985. Generation of monoclonal antibodies to a human natural killer clone: characterization of two natural killer-associated antigens, NKH1ₐ and NKH2, expressed on subsets of large granular lymphocytes. *J. Clin. Invest.* 75:932.

22. Hercend, T., E. L. Reinherz, S. Meuer, S. F. Schlossman, and J. Ritz. 1983. Phenotypic and functional heterogeneity of human cloned natural killer cell lines. *Nature (Lond.).* 301:158.

23. Hercend, T., S. Meuer, E. L. Reinherz, S. F. Schlossman, and J. Ritz. 1982. Generation of a cloned NK cell line derived from the "null cell" fraction of human peripheral blood. *J. Immunol.* 129:1299.

24. Hercend, T., S. Meuer, A. Brennan, M. A. Edson, O. Acuto, E. L. Reinherz, S. F. Schlossman, and J. Ritz. 1983. Identification of a clonally restricted 90 kD heterodimer on two human cloned natural killer cell lines. *J. Exp. Med.* 158:1547.

25. Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell. Biol.* 27:137a (Abstr.).

26. Stevens, R. L., E. Razin, K. F. Austen, A. Hein, J. P. Caulfield, N. Seno, K. Schmid, and F. Akiyama. 1983. Synthesis of chondroitin sulfate E glycosaminoglycan onto p-nitrophenyl-β-D-xyloside and its localization to the secretory granules of rat serosal mast cells and mouse bone marrow–derived mast cells. *J. Biol. Chem.* 258:5977.

27. Goldstein, J. I., and J. W. Colby. 1975. Special techniques in the x-ray analysis of samples. *In* Practical Scanning Electron Microscopy: Electron and Ion Microprobe Analysis. J. I. Goldstein and H. Yakowitz, editors. Plenum Press, New York, p. 435.

28. Wasteson, Å. 1971. A method for the determination of the molecular weight and molecular-weight distribution of chondroitin sulfate. *J. Chromatogr.* 59:87.

29. Saito, H., T. Yamagata, and S. Suzuki. 1968. Enzymatic methods for the determination of small quantities of isomeric chondroitin sulfates. *J. Biol. Chem.* 243:1536.

30. Seldin, D. C., N. Seno, K. F. Austen, and R. L. Stevens. 1984. Analysis of polysulfated chondroitin disaccharides by high-performance liquid chromatography. *Anal. Biochem.* 141:291.

31. Grossi, C. E., S. R. Webb, A. Zicca, P. M. Lydyard, L. Moretta, M. C. Mingari, and M. D. Copper. 1978. Morphological and histochemical analyses of two human T-cell subpopulations bearing receptors for IgM or IgG. *J. Exp. Med.* 147:1405.

32. Grossi, C. E., A. Cadoni, A. Zicca, A. Leprini, M. Ferrarini. 1982. Large granular lymphocytes in human peripheral blood: ultrastructural and cytochemical characterization of the granules. *Blood.* 59:277.

33. Payne, C. M., L. Glasser, R. Fiederlein, and R. Lindberg. 1983. New ultrastructural observations: parallel tubular arrays in human Tᵧ lymphoid cells. *J. Immunol. Methods.* 65:307.

34. Smit, J. W., N. R. Blom, M. van Luyn, and M. R. Halie. 1983. Lymphocytes with parallel tubular structures: morphologically a distinctive subpopulation. *Blut.* 46:311.

35. Ferrarini, M., A. Cadoni, A. T. Franzi, C. Ghigliotti, A. Leprini, A. Zicca, and C. E. Grossi. 1980. Ultrastructure and cytochemistry of human peripheral blood lymphocytes. Similarities between the cells of the third population and T_G lymphocytes. *Eur. J. Immunol.* 10:562.

36. Zucker-Franklin, D., G. Grusky, and J.-S. Yang. 1983. Arylsulfatase in natural killer cells: its possible role in cytotoxicity. *Proc. Natl. Acad. Sci. (USA).* 80:6977.

37. Stevens, R. L., K. Otsu, and K. F. Austen. 1985. Purification and analysis of the core glycopeptide of the protease-resistant intracellular chondroitin sulfate E proteoglycan from the interleukin 3–dependent mouse mast cell. *J. Biol. Chem.* In press.

38. Bland, C. E., K. L. Rosenthal, D. H. Pluznik, G. Dennert, H. Hengartner, J. Bienenstock, and D. D. Metcalfe. 1984. Glycosaminoglycan profiles in cloned granulated lymphocytes with natural killer function and in cultured mast cells: their potential use as biochemical markers. *J. Immunol.* 132:1937.

39. Dvorak, A. M., S. J. Galli, J. A. Marcum, G. Nabel, H. Der Simonian, J. Goldin, R. A. Monahan, K. Pyne, H. Cantor, R. D. Rosenberg, and H. F. Dvorak. 1983. Cloned mouse cells with natural killer function and cloned suppressor T cells express ultrastructural and biochemical features not shared by cloned inducer T cells. *J. Exp. Med.* 157:843.

40. Levitt, D., and P.-L. Ho. 1983. Induction of chondroitin sulfate proteoglycan synthesis and secretion in lymphocytes and monocytes. *J. Cell Biol.* 97:351.

41. Pasternak, M. S., and H. N. Eisen. 1985. A novel serine esterase expressed by cytotoxic T lymphocytes. *Nature (Lond.).* 314:743.

42. Schmidt, R. E., R. P. MacDermott, G. Bartley, M. Bertovich, D. A. Amato, K. F. Austen, S. F. Schlossman, R. L. Stevens, and J. Ritz. Specific release of proteoglycans from human natural killer cells during target lysis. *Nature (Lond.).* In press.