

PRODUCTION OF PLASMINOGEN ACTIVATOR BY HUMAN
NATURAL KILLER CELLS
Large Granular Lymphocytes

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Intense investigation has recently been directed towards defining the exact role, and scope of action, of natural killer (NK)¹ cells in natural cell-mediated immunity against tumors (1, 2). NK cells, nonadherent and nonphagocytic lymphoid cells found in normal individuals, appear to be an important component of host immune defense against primary (1, 3) and metastatic (4, 5) cancer. The mechanism by which NK cells recognize and destroy tumor target cells has remained only poorly understood (6, 7).

Rapid progress in elucidating biochemical aspects of NK cell function has heretofore been precluded by the technical inability to isolate the effector cells in the absence of contaminating cell types. Recent evidence in our laboratory (8) has documented that the lymphoid subpopulation that accounts for human NK activity is comprised of cells that are morphologically defined as large granular lymphocytes (LGL); it also appears that K cells, which mediate antibody-dependent cell-mediated cytotoxicity (ADCC), are LGL (8). Through a combination of purification steps, including discontinuous Percoll gradient centrifugation and elimination of cells that form high affinity rosettes with sheep erythrocytes at 29°C, LGL can be routinely enriched, from their frequency in the peripheral blood of 2–6%, to a purity of 90–95% (8); stringent isolation conditions lead to LGL populations with an average purity of >95% (7). With the availability of highly purified preparations of LGL, studies dealing with the regulation of NK cell reactivity have been performed (9), and studies dealing with the biochemistry

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¹ *Abbreviations used in this paper:* ADCC, antibody-dependent cell-mediated cytotoxicity; A/T, attacker to target ratio; CM, conditioned medium; DFP, diisopropyl fluorophosphate; IFN, interferon; LGL, large granular lymphocytes; LU, lytic units; NADH, reduced nicotinamide-adenine dinucleotide; NK, natural killer; NPGb, *p*-nitrophenyl-*p*-guanidobenzoate; PA, plasminogen activator(s); PHA, phytohemagglutinin; TLCK, *N*- α -*p*-tosyl-lysine-chloromethylketone (TLCK); *N*-*p*-tosyl-lysine-chloromethylketone; TPCK, L-1-tosylamide-2-phenylethyl-chloromethylketone.

of NK cells may now be performed under conditions in which biological properties are directly attributable to a specific lymphoid subpopulation.

We have examined biochemical processes in LGL that have been implicated in tumor cell lysis by activated macrophages or cytolytic T lymphocytes including phospholipase A₂ generation and phospholipid transmethylation (7, 10) and the role of reactive oxygen species in the mechanism of NK cytotoxicity of tumor cells²; we have found that the former, but not the latter, biochemical pathway appears to play a role in the lytic mechanism of NK cells (7). In addition, we have observed that inhibitors of various neutral serine proteases inhibit NK cell-mediated cytotoxicity of tumor cells (7, 12). A number of studies have suggested that neutral serine proteases may play a role in the lysis of tumor cells by NK cells (7, 12–16).

In this report we have examined highly purified LGL for their capacity to produce neutral serine proteolytic activity. We provide, for the first time, direct evidence for production of a neutral serine protease by human NK cells. We report that both freshly isolated, as well as cultured, LGL from normal individuals produce both cell-associated and extracellular forms of the specific neutral serine protease, plasminogen activator (PA).

Materials and Methods

Materials. RPMI-1640 medium and bovine serum were obtained from Biofluids (Rockville, MD). Neuman and Tytell serumless medium, Hepes, and human AB serum were obtained from Grand Island Biological Co. (Grand Island, NY). Conditioned medium (derived from phytohemagglutinin [PHA]-stimulated cultures of human peripheral blood lymphocytes) and nylon/wool were obtained from Associated Biomedical Systems (Buffalo, NY). Ficoll-Hypaque and Percoll were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Lyophilized bovine albumin, and crystallized, lyophilized human albumin, were purchased from Sigma Chemical Co. (St. Louis, MO). Highly purified PA (urokinase), plasminogen, and plasmin were generous gifts of Dr. Genesis Murano, Bureau of Biologics, FDA, Bethesda, MD. Plasminogen-free, highly purified fibrinogen was a gift of Dr. John S. Finlayson, Bureau of Biologics, FDA, Bethesda, MD. Human Type I fibroblast interferon was purchased from HEM Research (Rockville, MD). Heparin was obtained from O'Neal, Jones, and Feldman (St. Louis, MO). Plasticware was from Falcon Labware (Oxnard, CA). Harvesting frames and transfer tubes were purchased from Flow laboratories (McLean, VA). Gentamicin was obtained from the Schering Corp. (Kenilworth, NJ). Carrier-free (¹²⁵I) NaI and (⁵¹Cr) sodium chromate was purchased from New England Nuclear (Boston, MA). A urokinase standard was purchased from Leo Pharmaceuticals, Denmark. All reagents used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were Canalco products and were purchased from Miles Biochemicals, Elkhart, IN).

Culture Medium. Target cells were grown and maintained in RPMI-1640 medium containing 10% fetal bovine serum, 2 mM Hepes buffer, and 100 µg/ml gentamicin (hereafter referred to as complete culture medium).

Assay Medium. The assay for cell-mediated cytotoxicity was performed in Neuman and Tytell serumless medium supplemented with crystalline bovine serum albumin (6 mg/ml), 2 mM Hepes buffer, and 100 µg/ml gentamicin, carefully adjusted to pH 7.4 (hereafter referred to as albumin-supplemented serumless medium).

Cell Counting. Viable cell counts for both effector and target cells were routinely performed both microscopically and with a Cytograf (Model 6300A, Ortho Instruments,

² Goldfarb, R. H., T. Timonen, E. Pick, J. R. Ortaldo, T. Hoffman, and R. B. Herberman. Highly purified natural killer cells (large granular lymphocytes) fail to produce reactive oxygen intermediates. Submitted for publication.

Westwood, MA). Cells were counted before and after preincubation periods, as well as after the cytotoxicity tests.

Culture Conditions. All target cells were cultured at 37°C in a humidified air atmosphere with 5% CO₂ using complete culture medium. Experiments were carried out at 37°C in a humidified air atmosphere with 5% CO₂ using albumin-supplemented medium.

Effector Cells. Human peripheral blood lymphocytes were routinely obtained from male and female adult, healthy volunteers, who had donated their blood for the isolation of platelets in the National Institutes of Health Plateletpheresis Laboratory. Leukocyte-enriched buffy coats were obtained from the plateletpheresis of 300–450 ml of blood, and diluted in complete growth medium containing heparin (20 U/ml). The mononuclear cells were subsequently isolated by Ficoll-Hypaque gradient centrifugation at 400 *g* for 30 min at room temperature. The recovered cells were washed, resuspended, and adsorbed to plastic flasks for 60 min at 37°C to eliminate adherent cells. The recovered cells were then washed, resuspended, and subjected to nylon/wool chromatography (50 × 10⁶/0.6 g nylon wool) (13) in order to further deplete contaminating macrophages and B lymphocytes. The recovered cells were washed by centrifugation and incubated overnight at 4°C. The lymphocyte yield was routinely 0.3–1.0 × 10⁶/ml of blood. Nonlymphocytic contamination, primarily monocytes, varied between 0 and 2%. Under stringent conditions one could further isolate monocytes by G10 Sephadex chromatography.

Alternatively, in experiments with multiple donors, and in experiments with Chediak-Higashi syndrome donors, peripheral blood lymphocytes were obtained by venipuncture of healthy volunteers. The lymphocytes were separated by Ficoll-Hypaque gradient centrifugation, and then further isolated as described below.

Target Cells. K562, a cell line derived from the pleural effusion of a patient with chronic myelocytic leukemia in blast crisis (14) was used as the target cell for measurement of NK cytolytic activity.

Percoll Fractionation. Percoll discontinuous gradient centrifugation was as previously described (8, 17). Culture medium and Percoll were adjusted to 285 mosmol/kg H₂O with sterile distilled water and 10X concentrated phosphate-buffered saline, pH 7.4, respectively. Percoll in medium was prepared at seven different concentrations, ranging from 40 to 57% Percoll, and each varied in increments of 2.5%. Since Percoll varied among batches, refractive indices were used to adjust the concentrations of Percoll to the required density. The refractive index for 57% and 40% Percoll at 25°C, are 1.3454 and 1.3432, respectively. Gradients were carefully and slowly layered into 15 ml conical, plastic test tubes. The gradients were then overlain with 5 × 10⁷ lymphocytes, and centrifuged at 550 *g* for 30 min at room temperature. Cells from the seven layers were then collected from the top with a Pasteur pipette and washed in medium containing 2% fetal bovine serum. Cell recovery was routinely 85–95% and viability was always >95%, as judged by trypan blue exclusion.

Cytotoxicity Assay. Target cells were prepared at a concentration of 10⁷ cells/ml in complete medium. The cells were labeled with ⁵¹chromium at a concentration of 200 μCi/ml for 90 min at 37°C. The cells were then washed three times in albumin-supplemented serumless medium, and resuspended in the same medium at a concentration of 10⁵/ml for use in the chromium release assay. Effector cells (LGL) were also washed three times and resuspended in albumin-supplemented serumless medium. Natural cytotoxicity was assayed by mixing various concentrations of effector cells with 5 × 10³ target cells with resultant 33:1 to 3.7:1 attacker to target (A/T) ratios. The reaction mixtures, in microtiter wells, were centrifuged at 75 *g* for 5 min at room temperature. The reaction mixtures of 0.2 ml were incubated for 4 h at 37°C. All groups were tested in quadruplicate. Autologous controls (unlabeled target cells added to the labeled target cells) and medium controls (labeled target cells in albumin-supplemented serumless medium alone) were used to determine background values in all experiments. Spontaneous release from the target cells was always <10%. The percent of released isotope was calculated as: % release = cpm released from cells during incubation/total cpm incorporated into cells × 100. The percent of specific cytotoxicity was calculated as the percent release in the experimental group minus the percent release in the medium control. The cytotoxic activity of the

effector cell was also expressed as lytic units (LU)/ 10^7 cells, with 1 LU defined as the number of effector cells required to cause 30% lysis of target cells.

Evaluation of Cell Morphology. Morphological analysis of effector cells was performed by Cytospin centrifugation of effector cells onto microscope slides and oil immersion microscopy, as previously described (8, 17). LGL were identified as slightly larger than small-medium sized lymphocytes (10–15 μm). LGL have a relatively high cytoplasmic/nuclear ratio and weakly basophilic cytoplasm, with several azurophilic granules (8). A kidney-shaped nucleus is often detected in these cells. Monocytes were distinguished from LGL on the basis of their size (16–20 μm), vacuolar cytoplasm, and more indented nucleus; in addition, monocytes, but not LGL, were able to ingest fluorescently labeled latex beads. At least 200 cells were analyzed for each morphological determination. LGL from patients with Chediak-Higashi syndrome were found to have only unusually large azurophilic granule(s) in the cytoplasm.

Rosettes. The formation of rosettes between sheep erythrocytes and lymphoid cell populations was performed as previously described (8, 17).

Pretreatment of Lymphocytes with Interferon. Percoll gradient fractions at a concentration of $1 \times 10^6/\text{ml}$ were incubated alone, or in the same medium containing interferon (1,000 IU/ml of human fibroblast IFN, specific activity 2×10^7 IU/mg protein). Following two washes with albumin-supplemented serumless medium, cells were tested for both NK activity, as well as for protease production.

Cultures of Human NK Cells. The preparation and characteristics of purified human LGL cultured in the presence of the interleukin-2-containing conditioned medium (CM) derived from PHA-induced cultures of peripheral blood lymphocytes is described elsewhere, in detail (18).

Assay of Neutral Serine Proteases. Iodinated fibrin, a well-characterized substrate for proteolytic enzymes (19, 20) was used for the assay of neutral serine proteases as previously described (21). ^{125}I -labeled fibrin substrates were prepared as previously reported (22), except that human plasminogen-depleted fibrinogen was used rather than bovine substrate. Where indicated, a plasminogen-containing casein overlay method for the detection of protease production by single cells was used, with a modification of published methods (23).

Electrophoretic Analysis of Proteases. PA (highly purified urokinase) and dialyzed, lyophilized, serumless culture fluids derived from LGL were analyzed by SDS-PAGE, according to the method of Laemmli (24), as described previously (22), with upper stacking gels of 5% and lower resolving gels of 10% acrylamide. Gels were divided into lanes for protein staining or for the measurement of enzymatic activity as previously described (22).

Subcellular Fractionation and Membrane Isolation. Preparation of LGL homogenates, subcellular fractionation of homogenates, and membrane isolation was performed as previously described for the fractionation of Rous sarcoma virus-transformed fibroblasts (25, 26). Overnight cultures of LGL supplemented with serumless media ($5 \times 10^6/75 \text{ cm}^2$ flask) were washed twice with the same media and once with ice-cold phosphate-buffered saline, and twice by centrifugation in isotonic saline-EDTA (1 mM). The washed LGL pellet was resuspended in homogenizing medium (0.25 M sucrose, 10 mM Tris, 1 mM EDTA, pH 7.4), and homogenized by Dounce homogenization in a tight-fitting homogenizer until >90% of the cells were broken, as judged by phase microscopy. Cell fractions were prepared by differential centrifugation as previously described (25, 26); an aliquot of the homogenate was saved, and compared to the resulting crude nuclear, membrane plus granule, and soluble cytoplasmic fractions. Sucrose gradient centrifugation was used to subfractionate the membrane-plus-granule fraction as previously described (25, 26). In brief, the fraction was layered upon a discontinuous sucrose gradient composed of equal volumes of 20%, 40%, and 60% sucrose (wt/vol) and centrifuged at 100,000 g for 3 h at 4°C. Interface fractions comprised of discrete bands of subcellular components were removed from the gradient, diluted with homogenizing medium, and washed by centrifugation at 100,000 g for 1 h at 4°C. The washings were combined and the combination is referred to as the soluble fraction. The individual pellets were resuspended in homogenizing medium and are designated as gradient fractions 1, 2, 3, and 4, respectively. Each

fraction was analyzed for protein, by the method of Lowry, and for enzyme markers by previously described methodology (25, 26). 5'-Nucleotidase and Na^+K^+ ATPase were used as marker enzymes for plasma membrane; B-N-acetylglucosaminidase was used as a lysosomal marker; thiamine pyrophosphatase was used as a marker for Golgi-associated membranes; cytochrome oxidase was used as a marker of mitochondria; NADH diaphorase was used as a marker of the endoplasmic reticulum; and lactic dehydrogenase was used as a marker of soluble cytoplasmic enzymes. All organelle markers were assayed as previously described (25, 26).

Inhibitors of Proteolytic Enzymes. Aprotinin (trasyolol), diisopropyl fluorophosphate (DFP), N- α -p-tosyl-L-lysine-chloromethylketone (TLCK), L-1-tosylamide-2-phenylethyl-chloromethylketone (TPCK), and p-nitrophenyl-p-guanidobenzoate (NPGB), were purchased from Sigma Chemical Co. (St. Louis, MO). Leupeptin, chymostatin, and elastatinal were kind gifts of Dr. Walter Troll, N.Y.U. Medical Center, New York, NY, and the U.S.-Japan Cooperative Cancer Research Program.

Results

Production of Proteolytic Activity by LGL. NK cells (LGL) were freshly isolated from normal volunteers, by stringent conditions to ensure that the LGL purity was 95% or greater. Upon isolation, LGL were washed in albumin-supplemented serumless medium, and incubated overnight in the same medium, under various conditions; the medium, and the albumin used were tested, and shown to be devoid of any neutral serine proteolytic activity, or of any inhibitors of neutral serine proteases, as monitored by the ^{125}I fibrin assay (22). LGL were incubated either in untreated, plastic tissue culture flasks, or in tissue culture wells containing ^{125}I -plasminogen-free fibrin in an insoluble form (22). The culture supernatants of the former cultures served as a source of extracellular proteolytic enzymes, whereas the cells from these cultures, upon harvest and disruption, served as a source of cell-associated proteases. The latter overnight cultures, incubated on ^{125}I -fibrin, utilized intact cells and directly monitored protease production by living cells, and therefore measured both cell-associated and extracellular enzymes.

The results in Fig. 1 (A-C) demonstrate that, under all conditions used, LGL produced only barely detectable levels of fibrinolytic activity following 14 h of culture. However, under all conditions, a striking enhancement of fibrin degradation was observed upon assay in the presence of a source of purified plasminogen. The results therefore demonstrate that LGL produce a plasminogen-dependent proteolytic activity, i.e., a PA (22); this activity was expressed as both a cell-associated enzyme and in an extracellular, soluble form. Panel D of Fig. 1 demonstrates that LGL cultures, grown at first in the presence of interleukin 2-containing conditioned medium, and then washed and cultured in albumin-supplemented serumless medium, also produced a plasminogen-dependent fibrinolytic enzymatic activity.

The inset in panel D (Fig. 1) demonstrates that both the freshly isolated LGL, as well as the cultures of LGL, displayed natural cell-mediated cytotoxicity against K562 cells under serumless conditions. Furthermore, the NK activity of both types of LGL showed a characteristic boost by interferon (1, 6). The results therefore demonstrated that the cells maintained their cytolytic capacity against an NK-susceptible target, under the conditions used for this study.

Production of Plasminogen-dependent Proteases by Distinct Lymphoid Popula-

940 NATURAL KILLER CELLS (LGL) PRODUCE PLASMINOGEN ACTIVATOR

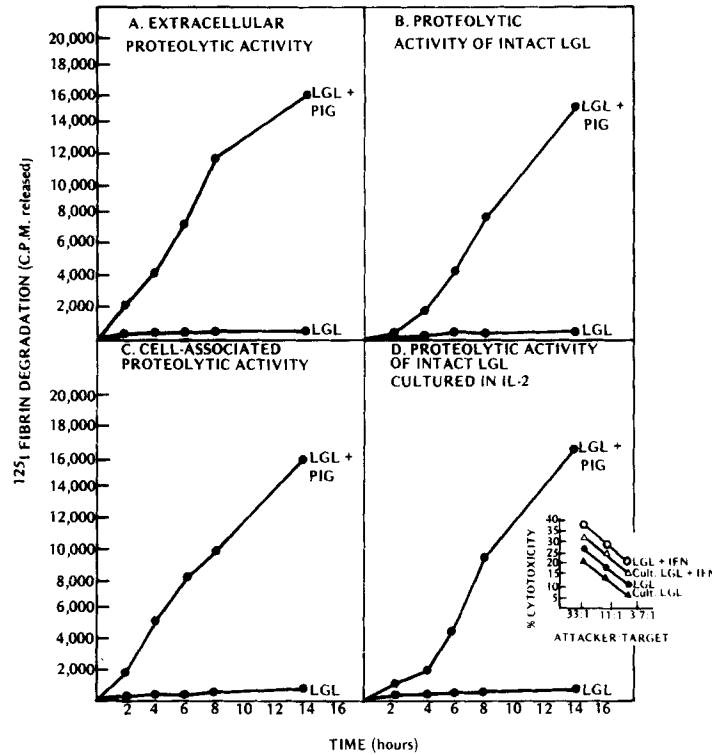


FIGURE 1. Production of plasminogen-dependent proteolytic activity by freshly isolated and cultured LGL. 5×10^6 freshly isolated LGL or IL-2 cultured LGL were washed twice in PBS and resuspended in albumin-supplemented serumless medium. Cells were then incubated in untreated plastic tissue culture flasks, or in tissue culture wells containing plasminogen-free iodinated fibrin. Culture supernatants of the former cultures served as a source of extracellular proteolytic activity and cellular homogenates served as a source of cell-associated enzymatic activity. The latter cultures, incubated on ^{125}I -fibrin, utilized intact cells and directly monitored protease production by living cells. (A) LGL extracellular fibrinolytic activity in the presence or absence of plasminogen (6 μg). (B) Plasminogen-dependent and -independent proteolytic activity of 5×10^6 freshly isolated, intact LGL. (C) LGL cell-associated fibrinolytic activity in the presence or absence of plasminogen. (D) Plasminogen-dependent and -independent proteolytic activity of 5×10^6 IL-2 cultured LGL. The inset in panel D displays spontaneous and interferon-augmented (1,000 U/ml, human fibroblast IFN) natural cell-mediated cytotoxicity against K562 cells by both freshly isolated and cultured LGL under the conditions used in this study.

tions. Fig. 2 compares the production of plasminogen-independent and -dependent proteases by several lymphoid populations: LGL; input nonadherent lymphocytes from which LGL are purified; and plastic adherent monocytes, separated from LGL during the isolation procedure; and small lymphocytes (T cells), separated from LGL by Percoll gradient centrifugation. The results demonstrate that isolated LGL had considerably more plasminogen-dependent activity than input lymphocytes or small T lymphocytes. On a per-cell basis, LGL appeared to produce PA at levels equivalent to those associated with monocytes; monocytes, however, appeared to produce appreciably more plasminogen-independent protease activity than LGL. Although it is well established that macrophages produce PA (27), and it has been reported that B lymphocytes

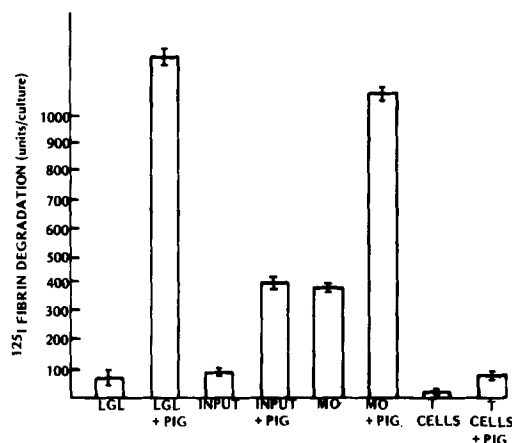


FIGURE 2. Production of plasminogen-dependent proteolytic activity by distinct lymphoid populations. Lymphoid populations derived during LGL purification, LGL; input nonadherent lymphocytes from which LGL are purified; plastic adherent monocytes (Mo); and small lymphocytes (T cells); were tested for plasminogen-dependent and -independent proteolytic activity. 5×10^6 intact, living lymphoid cells of each type were incubated on ^{125}I fibrin as described in Fig. 1. Enzymatic activity is expressed as units/culture. This experiment was performed four times with similar results. Error bars signify SEM. Each determination was run in triplicate.

(28) and thymocytes (29) produce this protease, the findings for NK cells, and T cells, appear to be new and unexpected, respectively (see discussion).

Effect of Interferon on Protease Production by LGL. If neutral proteases play an active role in the lytic mechanism of NK cells, then it is possible that interferon (IFN), the major positive regulator of NK cytolytic activity, might lead to augmentation of enzyme production or release (1, 6). The results depicted in Fig. 3 demonstrate that whereas IFN did not augment extracellular LGL PA levels, it caused a substantial enhancement of cell-associated enzyme (lower panel). When intact LGL were cultured overnight on ^{125}I fibrin, a similar enhancement was also noted (results not shown).

Production of PA by LGL from Patients with Chediak-Higashi Syndrome. Since IFN can enhance PA levels by LGL, it was of interest to examine a converse situation and ascertain whether the LGL of individuals with defects in NK activity (30) showed any deficit in production of this protease. We therefore examined LGL isolated from patients with Chediak-Higashi syndrome, who are known to be defective in NK activity (30), and compared their protease activity, and NK function to LGL isolated from several normal individuals.

The Chediak-Higashi syndrome patients had normal numbers of LGL but upon isolation, their LGL were found to have a distinct morphological appearance; as previously noted (T. Timonen, unpublished observations and reference 31), such LGL have unusually large azurophilic granule(s) in their cytoplasm.

Fig. 4 demonstrates that intact LGL from Chediak-Higashi syndrome donors produced considerably less PA activity than that produced by LGL isolated from a panel of normal donors. The inset (Fig. 4) confirms the previous report (30) that the NK activity of Chediak-Higashi syndrome patients is very low or undetectable.

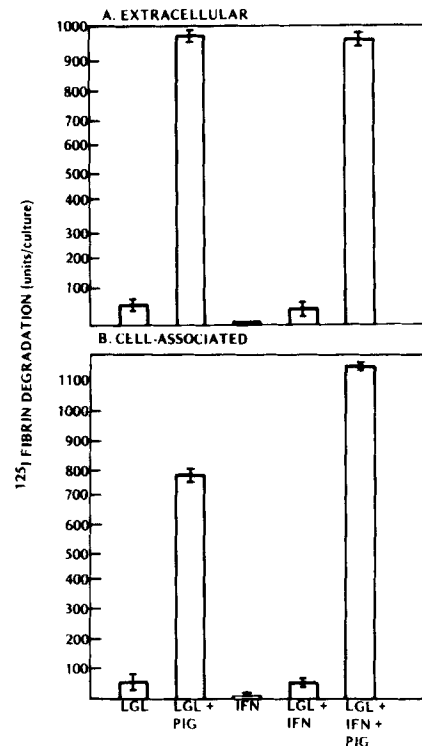


FIGURE 3. Effect of interferon on protease production by LGL. 5×10^6 freshly isolated LGL were prepared and tested for extracellular and cell-associated plasminogen-dependent activity as described in Fig. 1. Human fibroblast interferon (1,000 U/ml) was used as indicated. (A) LGL extracellular plasminogen (PIG)-dependent fibrinolytic activity following incubation of LGL in the presence or absence of human fibroblast interferon (1,000 U/ml) for 14 h at 37°C. (B) LGL cell-associated plasminogen-dependent fibrinolytic activity following incubation in the presence or absence of human fibroblast interferon (1,000 U/ml). This experiment was performed five times with similar results. Each determination was run in triplicate. Error bars signify SEM.

Modulation of LGL Proteolytic Activity by the Exogenous Addition of Protease Inhibitors. In order to further delineate the specificity of the proteolytic activity produced by LGL, various inhibitors were employed. Fig. 5 shows that the neutral serine protease active-site titrants, DFP and NPGb, at the concentrations used, completely abrogated LGL plasminogen-dependent proteolytic activity. The tryptic inhibitors, TLCK, and leupeptin, also dramatically inhibited LGL plasminogen-dependent proteolytic activity; conversely, the chymotryptic inhibitors, TPCK, and chymostatin, had no inhibitory effect in this regard. Similarly, elastatinal, an elastase inhibitor, also showed no effect on inhibition of LGL plasminogen-dependent proteolysis. Trasyolol, a plasmin inhibitor, was able to partially depress NK cell proteolytic activity, at the concentration used, and higher concentrations of this inhibitor led to more pronounced inhibition (data not shown).

Biochemical Characterization of the PA Produced by LGL. In Fig. 6 are shown the PA activity profiles of SDS polyacrylamide gels for both homogeneously pure

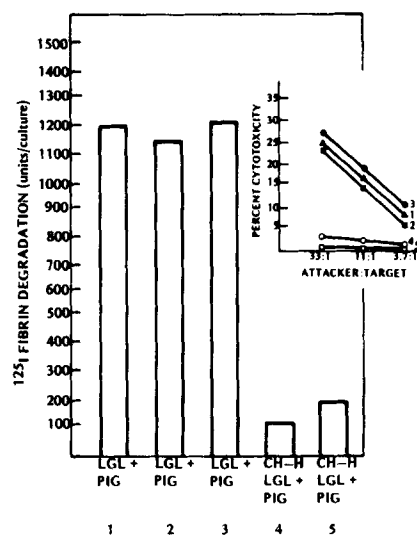


FIGURE 4. Production of PA by LGL from patients with Chediak-Higashi syndrome. Plasminogen (PIG) dependent fibrinolytic activity of 5×10^6 intact LGL from normal donors (#1-3) and CH-H patients (#4-5) were examined. The inset shows natural cell-mediated cytotoxicity against K562 cells by LGL derived from the same normal donors (#1-2) and CH-H patients (#4-5).

urinary PA (urokinase) and concentrated extracellular medium from cultures of freshly isolated LGL. The results show (panel D) that the PA activity produced by human LGL exists in several forms, with molecular weights of 100,000, 78,000, 72,000, 52,000, 45,000, 28,000, and 26,000. In contrast, purified urokinase displayed M_r forms of 53,000 and 34,000, by both enzymatic activity and protein staining, in good agreement with published reports (32). Since LGL produce PA species of both 72,000 and 52,000, one can not state whether the LGL enzyme is a melanoma-like tissue PA (33) or a urokinase-like (32) enzyme, or shares characteristics with each of the previously described human PA.

Subcellular Distribution of PA in LGL. The subcellular distribution of the cell-associated form of LGL PA was investigated by differential centrifugation and sucrose gradient centrifugation (25, 26). A nuclear fraction, a total membrane plus granule fraction, and a cytoplasmic fraction were separated, as shown in Table I. The results demonstrate that >80% of the PA activity was isolated in the total membrane-plus-granule fraction, in excellent agreement with results found upon fractionation of Rous sarcoma virus-transformed chick embryo fibroblasts (25, 26). 30% of the total cellular protein and most of the membrane-associated enzymatic activities were found in this fraction.

Further investigation of the membrane plus granule fraction by sucrose gradient centrifugation indicated that the highest proportion of PA activity (Table II) was associated with a cell-surface membrane-enriched fraction (fraction 2). This fraction contained <10% of the total cellular protein and also showed the highest enrichment of the plasma membrane markers 5'-nucleotidase and Na^+K^+ ATPase, when assayed as previously described (25, 26). Additional enzyme markers (25, 26) for mitochondria, lysosomes, Golgi-associated membranes, and

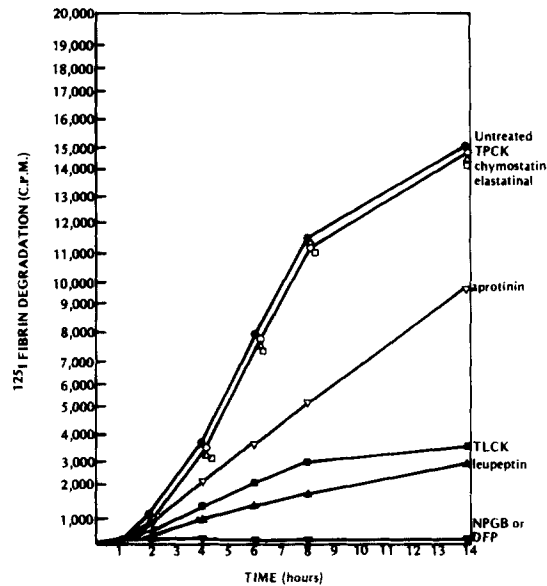


FIGURE 5. Inhibition of LGL proteolytic activity by endogenous inhibitors. LGL were isolated and tested for extracellular fibrinolytic activity as described for Figure 1. 5×10^6 cells were assayed for fibrinolytic activity in the presence of plasminogen ($6 \mu\text{g}$) and in the presence or absence of specific inhibitors, as indicated. The inhibitors were employed at the following concentrations: TPCK, $10 \mu\text{g}/\text{ml}$; chymostatin, $10 \mu\text{g}/\text{ml}$; elastatinal, $10 \mu\text{g}/\text{ml}$; aprotinin, $10 \mu\text{g}/\text{ml}$; TLCK, $10 \mu\text{g}/\text{ml}$; leupeptin, $10 \mu\text{g}/\text{ml}$; NPGb, $10 \mu\text{g}/\text{ml}$; DFP, 100 mM . Enzyme-containing culture fluids were preincubated with inhibitors for 30 min at 4°C before fibrinolytic assay.

rough endoplasmic reticulum were present in only trace amounts in Fraction 2; in addition, little or no cytoplasmic or nuclear material was present (data not shown). Our results therefore indicate that LGL cell-associated PA, is expressed in a subcellular fraction enriched in surface, plasma membrane; this finding is consistent with the subcellular localization of other PA (25, 28, 29).

Discussion

The results of this study demonstrate that human NK cells (LGL) produce a neutral serine protease that proteolytically converts the serum zymogen plasminogen to plasmin, and is therefore a PA.

The proteolytic activity produced by LGL was detectable following overnight culture under serum-free conditions with long-term LGL cultures maintained in IL-2 as well as with freshly isolated LGL, on iodinated fibrin. The LGL PA was associated with cell surface membranes and also was released into culture supernatants.

It therefore appears that NK cells, like macrophages (27), B lymphocytes (28), and thymocytes (29) produce PA. In this study it was critical to ascertain that the cells under study were indeed NK cells and not contaminating monocytes, B lymphocytes, or T lymphocytes; this goal was achieved by the following criteria: high purity of cells with LGL morphology; binding of $\sim 50\%$ of the LGL to, and lysis of, only NK-susceptible target cells (data not shown); ability of LGL to

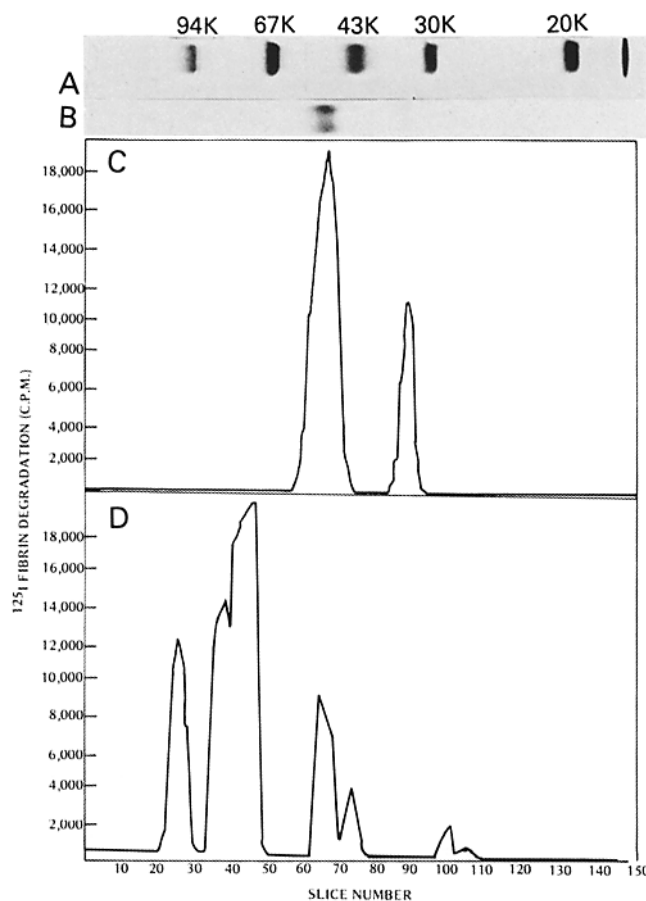


FIGURE 6. SDS-PAGE of extracellular LGL PA activity. 5 U of extracellular plasminogen activator-containing LGL culture fluids and of homogenously purified human PA (urokinase), were subjected to electrophoresis in adjacent lanes of a slab gel. The gels were sliced and assayed for PA activity as previously reported (22). Molecular weight standards were run on the same slab gel, and their positions are indicated at the top of the gel. (A) Molecular weight markers. (B) Coomassie Blue-stained protein: purified urokinase. (C) PA activity contained in 5 U of purified urokinase. (D) PA activity (5 U) from LGL culture supernatants following dialysis and lyophilization by described methods (22).

demonstrate K cell ADCC (8); kinetics and magnitude of IFN-enhanced NK and K cell lysis; inability to detect any cells with the capacity to ingest fluorescently labeled latex beads; inability to detect any superoxide burst in response to phorbol-12-myristate-13-acetate (PMA), F met-leu-phe, digitonin, concanavalin A, A 23187, or phospholipase C²; and the persistence of PA production after depletion of residual T lymphocytes by rosette formation with sheep erythrocytes at 29°C (8). In contrast, monocytes, isolated and tested in parallel, produced a superoxide burst in response to the same stimuli, and also ingested fluorescently labeled latex beads.² Furthermore, purified LGL showed a pattern of reactivity with monoclonal antibodies quite distinct from that expressed by T cells, monocytes, or PMN (34).

In the past, quantitative and reliable assays, including single cell assays, have

TABLE I
Distribution of PA and Marker Enzymes for the Plasma Membrane Following Differential Centrifugation of a Total Cellular Homogenate from LGL

Marker	Differential Centrifugation fractions		
	Nuclear fraction	Membrane + granule fraction	Cytoplasmic fractions
Protein	11 ± 4	29 ± 3	58 ± 5
5'-Nucleotidase	15 ± 2 (0.6)	84 ± 4 (2.5)	1 ± 1 (0.1)
Na ⁺ K ⁺ ATPase	12 ± 2 (0.8)	86 ± 3 (2.4)	2 ± 1 (0.2)
PA	8 ± 2 (1.1)	82 ± 3 (3.3)	9 ± 2 (0.2)

Overnight cultures of LGL were harvested and disrupted as described in Materials and Methods. A cellular homogenate was prepared by Dounce homogenization in 0.25 M sucrose, 10 mM Tris, 0.01 M EDTA, pH 7.4. Cellular fractions were prepared as described in the Materials and Methods. Each fraction was assayed for protein and the indicated enzymes. The values represent the percent distribution in each of the fractions of the total recovered activity. The actual recoveries, based on the original homogenate, ranged from 76% to 118% for all the markers tested. Values in parentheses represent the ratio of the specific activity of the enzyme in the isolated fraction to that in the original homogenate and function as an indication of enzyme enrichment. The specific activities of the original homogenate were: 5'-nucleotidase, 0.35 μ mol/h/mg protein; Na⁺K⁺ ATPase, 0.32 μ mol/h/mg protein; PA, 10.6×10^4 cpm/h/mg protein in previously described units (25). This experiment was performed five times with similar results.

TABLE II
Distribution of PA and Granule-associated Marker Enzymes for the Plasma Membrane Following Sucrose Gradient Centrifugation of the Membrane Plus Granule Fraction

Marker	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Soluble
Protein	10	25	22	9	33
5'-Nucleotidase	14 (3.0)	50 (5.4)	16 (1.2)	2 (0.2)	2 (0.1)
Na ⁺ K ⁺ ATPase	11 (2.2)	52 (5.2)	18 (1.2)	3 (0.4)	2 (0.1)
PA	4 (1.3)	66 (8.3)	8 (2.4)	2 (1.6)	2 (0.2)

Gradient fractions were isolated from a discontinuous sucrose gradient (Table I) and washed by centrifugation as described in the Materials and Methods. Values represent the percent distribution in each of the gradient fractions of the total enzyme activity recovered from the gradient. Actual recoveries are based on the initial membrane + granule fraction (Table I), and ranged from 76% to 105%. Values in parentheses represent the ratio of the specific activity of the enzyme in the isolated fraction to that present in the original homogenate. This experiment was performed three times with similar results.

suggested that lymphocytes do not produce detectable levels of extracellular or cell-associated PA (E. Reich and J. Vassalli, personal communication to R. H. Goldfarb). Nevertheless, with the advent of current lymphoid cell separation technology (8, 17), it has been possible to isolate highly purified NK cell populations, and to demonstrate that LGL produce an enzyme that proteolytically generates plasmin from plasminogen. Through the use of a single cell assay

for PA (23), we have confirmed that individual NK cells within the LGL population produce this enzymatic activity (unpublished observations).

The LGL plasminogen-activating protease appears to exist in multiple molecular weight forms, a common feature for most PA (22, 35). The simultaneous expression of 72,000 and 52,000 *M_r* species suggests that the LGL PA is distinct from typical urokinase-like or tissue-like enzymes (32, 33). Studies with monospecific antibodies will be required to determine the immunologic relationship of the LGL-produced enzyme to other PA. In any event, it is clear that the LGL enzyme is a plasminogen-dependent neutral serine protease, which is completely inhibited upon treatment with the active site inhibitors, DFP and NPGB.

We have previously reported that IFN can augment macrophage PA levels (36). We are now intrigued by the current observation that IFN treatment augments the cell-associated form of LGL PA, but not the extracellular form. This finding suggests that the cell-associated enzyme may play some role in the IFN boost of NK activity. Previous reports have indeed documented an important role for cell-associated PA in the alteration and modulation of cellular morphology (26). A potential role for PA function in the NK lytic mechanism is further supported by the finding that the morphologically atypical LGL isolated from Chediak-Higashi syndrome patients, display both impaired cytolytic activity as well as diminished production of the enzymatic activity. In this regard, it is of interest that polymorphonuclear leukocytes of Chediak-Higashi syndrome patients also have low or undetectable levels of elastase (37). It is therefore possible that multiple proteolytic deficits in various effector cell populations of Chediak-Higashi bearing individuals might contribute to enhanced infection in this patient population.

We have previously observed that inhibitors of both tryptic and chymotryptic enzymes (benzamidine, *p*-aminobenzamidine, TLCK, and leupeptin, and chymostatin and TPCK, respectively) inhibit cytotoxicity mediated by LGL (12). It is of interest to speculate that PA production by LGL might be involved in the control of a regulatory cytolytic cascade of neutral proteases with tryptic and chymotryptic specificity, through limited proteolysis. It has, for example, been reported that a cascade of regulatory DFP-sensitive proteases may regulate the functional capabilities of activated macrophages (38). It is of interest that chymostatin can inhibit the release of PA into extracellular culture medium, suggesting a role for a newly discovered chymotryptic membrane enzyme (39). It is worth noting that several inhibitors that block NK cytolytic activity (benzamidine, *p*-aminobenzamidine, and leupeptin) can directly inhibit PA (26, 40). It remains to be determined whether a highly selective synthetic inhibitor of PA (41) can inhibit NK cytolytic reactivity mediated by LGL. Although the exogenous addition of highly purified urokinase does not augment LGL-mediated cytolysis of K562 cells (unpublished observations), we have not tested LGL-conditioned medium, containing LGL-derived PA, for its cytolytic potential; we await purification of the enzyme to allow for critical testing of LGL PA in the lytic process. A recent report has indicated that urokinase inhibits rather than stimulates murine and chicken natural killing (42); it is difficult to compare studies performed with unpurified cell populations of different species in serum-containing conditions, to studies performed with highly purified human LGL in serum-free

conditions, particularly since endogenous serum-containing protease inhibitors abrogate urokinase activity (32). Although NK cell-mediated cytotoxicity proceeds normally with exogenous protease-inhibitor containing serum, it appears likely that effector cell proteases are activated following contact and binding between NK cells and target cells (15, 43); under these conditions it has been proposed that within the microenvironment of the contact zone between effector and target cell, high molecular weight substances, such as serum antiproteases, would be excluded through diffusion limitation (15). We have used serum-free conditions in this study, and in studies dealing with exogenous addition of protease inhibitors and purified proteases (12) to prevent complications that might arise due to potential enzyme inhibition, or inhibition competition, that would interfere with quantitative analysis of experimental results due to the presence of serum protease inhibitors.

To date, the physiological significance of PA production by highly purified LGL remains obscure. It remains unknown as to whether this protease either directly, or indirectly, plays any role in the NK lytic mechanism. Nevertheless, PA, and the proteolytic activity it can generate through plasminogen activation, plays a role in degradative alterations of extracellular matrix components (44), and also has the ability to modulate both cellular and extracellular protein components (35). It is therefore possible that this protease, with degradative and invasive potential, can contribute in some way to target cell destruction either directly, or indirectly through a sequence involving several interrelated biochemical processes (6). For example, PA, or plasmin, might activate phospholipase zymogens, or produce amphipathic membrane derived fragments which might then be inserted into the target cell phospholipid bilayer, as noted for complement-mediated lysis (P. J. Lachman, personal communication). It remains an open question as to whether the LGL-derived PA has any relationship to a previously described cytotoxic protease, isolated from human lymphocytes, that can mediate lysis of tumor cells (45). It also remains unknown as to whether or not LGL-derived PA bears any relationship to LGL azurophilic granules. Recent studies have shown that LGL requires an intact secretory process to mediate cytotoxicity, and that the carboxylic ionophore, monensin, can irreversibly inhibit the NK lytic mechanism (46). It has also recently been documented that strontium induces degranulation in human LGL, with a concomitant loss of NK reactivity (47); upon *in vitro* culture the strontium-treated LGL recover NK function with the simultaneous reappearance of cytoplasmic granules (48). Judicious experiments with monensin and strontium might illuminate the role, if any, of PA secretion and granule relationship, respectively, in the NK lytic mechanism. An alternative approach awaits the direct examination of isolated LGL granules for their protease content.

Summary

In this report we have used highly purified populations of natural killer (NK) cells: large granular lymphocytes (LGL). This study demonstrates that freshly isolated and interleukin 2-cultured LGL produce the specific neutral serine protease, plasminogen activator (PA). We have found that the enzyme is expressed in both an extracellular form as well as in a cell-associated form. Upon

subcellular distribution the latter form of the enzyme is associated with a cell-surface membrane-enriched fraction. LGL PA exists in multiple molecular weight forms ranging from 100,000 to 26,000. Interferon (IFN), the major positive regulator of NK cytolytic activity, caused a substantial enhancement of cell-associated, but not extracellular, PA. In contrast, LGL isolated from patients with Chediak-Higashi syndrome, who are known to be defective in NK activity, displayed low PA activity, altered morphology, and low NK killing relative to LGL isolated from normal donors. The possible role of LGL PA in the lysis of tumor cells by NK cells, either directly or indirectly, is discussed.

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