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CHARACTERIZATION AND ISOLATION OF A TRYPSIN-LIKE SERINE PROTEASE FROM A LONG-TERM CULTURE CYTOLYTIC T CELL LINE AND ITS EXPRESSION BY FUNCTIONALLY DISTINCT T CELLS

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We describe the characterization and purification of a trypsin-like serine protease isolated from cloned long-term culture cytolytic T cell line (CTLL AK). High amounts of proteolytic activity were isolated from extracts of CTLL AK after either nitrogen cavitation or detergent lysis. Trypsin-like protease was detected by using either the ester compound N$^{\alpha}$-benzyloxycarbonyl-L-lysine thiobenzyl ester or a panel of low molecular amide substrates. The latter compounds were preferentially cleaved at the carboxyl termini of lysine and arginine residues. The enzyme activity was completely inhibited by two serine esterase inhibitors, diisopropylfluorophosphate and phenylmethanesulfonyl fluoride, and by aprotinin and meta-aminobenzamidine, which are known to block trypsin-like proteases. The pH optimum for CTLL AK-derived protease activity is 8 to 9. Analysis of the enzyme by gel filtration revealed that the cell-bound proteolytic activity was associated with a complex that could not be dissociated by treatment with Triton X-100. The CTLL AK-derived protease activity was found to reside in two proteins with relative molecular masses (M_r) of 32,000 and 40,000 daltons as determined by affinity labeling with [^{3}H]diisopropylfluorophosphate and sodium dodecyl sulfate gel electrophoresis. High levels of enzyme activity were found in a panel of H-Y-specific cloned T cell lines with either cytolytic/suppressor (CTLL) or helper potential (THL), indicating a lack of correlation between trypsin-like protease activity and a particular T cell function. High enzyme activity was also detected in tumorigenic variants of CTLL. Furthermore, it was excluded that the trypsin-like activity detected was attributable to plasminogen activator activity. In contrast to cloned T effector cells and their in vitro or in vivo derived variants, considerably less activity was found in normal nonactivated or activated lymphocyte populations. The possible role of the trypsin-like serine protease in the function of T effector cells is discussed.

Hydrolytic enzymes attacking amide bonds of polypeptides (so-called peptidases or, synonymously, proteases) have been detected in a large number of cell types, including lymphocytes, monocytes, basophils, and cultured cell lines from several tissue sources (1–4). These enzymes are produced constitutively and seem to participate in basic metabolic and regulatory processes within a cell, at the cellular surface, or in the close vicinity of the cell (4). In lymphoid cells, such proteases in particular have been implicated in cytolytic functions mediated by both cytolytic T cells (CTL) and natural killer (NK) cells (5, 6) or in the hematogenous traffic of metastasizing lymphoma cells (7). Moreover it was shown that cloned murine CTL possess high trypsin-like esterase activity (2). However, because most previous studies explored heterogeneous cell populations expressing numerous protease activities, a clear picture of the association of these enzymes with individual lymphocyte subsets has not yet emerged. Recent progress in cell culture technique now allows the establishment and expansion of functionally distinct cloned T cell lines as suitable tools for the characterization of T lymphocyte-associated proteases.

In an attempt to explore the biochemical properties of defined T cell lines, we analyzed a long-term culture murine CTL like (CTLL AK)[2] derived from cloned H-Y-specific CTL (CTLL) for cell-associated and secreted protease activities. We found that the interleukin 2 (IL 2)-dependent, antigen independent CTLL AK 1.3E6, which has lost its original specificity but exerts so called "aged killer activity" on P815 tumor target cells (8) expresses high levels of a trypsin-like serine protease. The enzyme activity was characterized by using a panel of appropriate substrates, as well as inhibitors, and molecular identification of the enzyme was achieved by using affinity chromatography in combination with esterase-specific radiolabeling and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In addition, it was found that a series of H-Y-specific cloned T cell lines express comparable levels of cell-associated and extracellular protease activity irrespective of their cytolytic or helper function. The possibility to grow long-term culture CTLL to large numbers in the absence of contaminating stimulator cells will allow the production of highly pure enzyme preparations for studies on the physiologic role of

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[2] Abbreviations used in this paper: BLT, N$^{\alpha}$-benzyloxycarbonyl-L-lysine thiobenzyl ester; DFP, diisopropylfluorophosphate; PNSN, postnuclear supernatant; pNA, Para-nitroanilide; SN, supernatant; CTLL AK, CTLL variant with aged killer activity; CTLL TU, tumorigenic variant of CTLL; THL, cloned helper T cell line.

the described amido-/proteolytic activity in T cell populations.

MATERIALS AND METHODS

Generation and maintenance of long-term culture CTL lines. Cloned H-Y-specific cytolytic T cells (CTLL) were derived from long-term mixed lymphocyte culture containing female B6 responder T cells (prepared by nylon wool passage of spleen cells derived from mice previously primed to male B6 cells) and male B6 stimulator cells, as described (8). Briefly, the H-Y-specific T cell line was restimulated in vitro in 50 ml tissue culture flasks (No. 16337, Nunc, Wiesbaden, FRG) at weekly intervals with 2.5×10^7 irradiated male B6 stimulator cells in RPMI 1640 medium in 5% CO_2 atmosphere, and after 6 wk, was maintained on antigen in the presence of supernatant (SN) from concanavalin A (Con A)-activated rat spleen cells (Con A SN 10%). H-Y-specific CTL clones were obtained by limiting dilution procedures seeding 10 to 0.3 long-term culture responder cells/well on 5×10^5 irradiated (2200 rad) male B6 stimulator cells in Con A SN-conditioned medium (10 to 20%) in round-bottomed microtiter plates (No. 163320; Nunc). After 2 to 3 wk, cytotoxic activities were tested in individual wells on ^{51}Cr-labeled Con A-stimulated male B6 target cells. Positive cultures from wells which had received either 1 or 0.3 cell/well were picked, were expanded on antigen in the presence of Con A SN, and were recloned twice at dilutions of 0.3 cell/well under similar conditions. After 3 mo in culture, one line, 1.3E6, was adjusted to grow in Con A SN-conditioned medium in the absence of antigen and subsequently was recloned by limiting dilution procedures as described above. Lymphocytes of this line have lost their specific cytolytic activity on male B6 target cells and have acquired the ability to specifically lyse P815 mastocytoma target cells. These cells have been termed aged killer cells (CTLL AK) (8). After 9 mo in culture with Con A SN, a variant was isolated from CTLL AK that grew as solid tumor or as metastases when injected into female C57BL/6 mice (CTLL TU; M. M. Simon et al., manuscript in preparation). Cloned H-Y-specific T helper lines (THL) were derived from long-term culture cell lines after selection for Lyt-2$^-$ lymphocytes as described in detail (9).

Enrichment for T cells and removal of adherent cells. T cells were enriched from spleen cells by passage over a nylon-wool column as described by Julius et al. (10). Macrophages were removed from spleen cells by passage over a Sephadex G-10 column (11).

Generation of activated lymphocyte blasts and cell-mediated lympholysis. Polyclonally activated T and B cell blasts were obtained from C57BL/6 spleen cells incubated for 2 days at 1.2×10^7 cells/6 ml with either Con A (5 μg/ml), or lipopolysaccharide (LPS) (12 μg/ml). The culture medium was RPMI 1640 medium (GIBCO, Bonn, FRG) supplemented with L-glutamine (2 mM), kanamycin (100 μg/ml), tylosine (10 μg/ml), HEPES buffer (25 mM), 2-mercaptoethanol (2×10^{-5} M), and 10% selected fetal calf serum. Lymphoblasts were freed from small cells by centrifugation on a Ficoll gradient (Ficoll/metrizoate; 1.077; Pharmacia Fine Chemicals, Freiburg, FRG) for 15 min at 2500 rpm. The interphase containing the blasts was recovered, and the cells were washed three times in medium.

Specific ^{51}Cr release was determined by incubating serial dilutions of effector cells with a fixed number (2×10^3/well) of ^{51}Cr-labeled day 2 Con A-activated blasts from male C57BL/6 mice or with ^{51}Cr-labeled P815 tumor cells in the absence or presence of phytohemagglutinin (PHA; 4% of stock solution, final concentration; GIBCO, Bonn, FRG) in a final vol of 0.2 ml for 4 hr. Afterwards, plates were centrifuged, and 100 μl of SN were removed for counting. The percentage specific lysis was calculated by using the equation: % ^{51}Cr release $= (x - y)/(z - y) \times 100$ in which x is cpm in the SN of target cells mixed with effector cells, y is cpm in the SN of target cells incubated alone, and z is cpm after lysis of target cells in 1 N HCl.

Preparation of cell lysates and culture SN from cloned T cell lines and normal lymphocyte populations. Lymphocytes of CTLL AK 1.3E6 were expanded in Con A SN in the absence of stimulator or feeder cells, taken from exponentially growing cultures and were washed twice in phosphate-buffered saline (PBS). Cells (1×10^8 to 2×10^9) were then suspended in 20 ml of ice cold relaxation buffer (100 mM KCl, 3.5 mM $MgCl_2$, 1 mM ATP, 10 mM piperazine-N,N'-bis-2-ethane-sulfonic acid), pH 6.8, containing 1.25 mM ethyleneglycol-bis(β-amino-ethyl-ether)N,N'-tetraacetic acid (EGTA) (12). Cellular plasma membranes were disrupted by nitrogen cavitation after equilibration in nitrogen for 30 min at 4°C at 30 kg/cm^2, and the lysate was collected. The lysate was afterwards freed from nuclei by centrifugation in a Heraeus minifuge at 1500 rpm for 5 min resulting in the so called "postnuclear supernatant" (PNSN).

To study different functional T cell clones or natural T lymphocyte populations, we prepared cell lysates by nitrogen cavitation and for comparison by detergent lysis. Furthermore we analyzed the cloned T cell lines for the constitutive and lectin-induced secretion of amidolytic activity into their culture media. The cell concentration used was 1×10^7/ml throughout. Nitrogen cavitation was performed as described above. Lysis by Triton X-100 was achieved by incubation of cells in 0.01 M Tris-(hydroxymethyl)-amino methane (Tris; Roth, Karlsruhe, FRG), pH 8.0, containing 0.1% Triton X-100 at 4°C for 1 hr. Afterwards the lysate was freed from particulate material by centrifugation at 10,000 × G in an Eppendorf tabletop centrifuge. Culture supernatants were obtained from 1×10^7 cells/ml incubated in Eagle's medium (GIBCO, Bonn, FRG) without phenol red in the absence or presence of Con A (5 μg/ml). The cell-free supernatant was removed after 8 hr of incubation at 37°C.

Assay for amidolytic activity. The detailed performance of amidolysis assays for the determination of substrate specificity and sensitivity to inhibitors is given in the legend to Table I or below, respectively. After the initial identification and characterization of the enzyme activity, the assay for amidolysis by cell lysates or culture SN has been standardized as follows: 100 μl of cell lysates or SN were mixed with 100 μl (3.5×10^{-4} M) of the commercially available chromogenic substrate Ile-Pro-Arg-para-nitroanilide (-pNA; AB KABI, Peptide Research, Moindal, Sweden). The absorbance was tested at 405 nm after 1 hr at 37°C, and the OD of 0.01 was defined as 1 U of amidolytic activity. The molar extinction coefficient for the resulting para-Nitroaniline at 405 nm is 10.4 $mMol^{-1} \times 1 \times cm^{-1}$ (product information; AB KABI Peptide Research, Moindal, Sweden).

Assay for esterolytic activity. Trypsin-like esterase activity was tested on N$^\alpha$-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) as described by Green and Shaw (13). Briefly, 100 μl of appropriate dilutions of cell extracts in 0.1% Triton X-100/Tris buffer (pH 8.0) were mixed with 900 μl of a reaction mixture containing 2×10^{-4} M BLT (Sigma, München, FRG) and 2.2×10^{-4} dithiobis (nitrobenzoic acid) (Sigma, München, FRG) in 0.2 M Tris-HCl, pH 8.1. After 30 min at room temperature, the absorbance was measured at 412 nm by using 100 μl 0.1% Triton X-100/Tris buffer (pH 8.1) plus 900 μl of the reaction mixture as a blank. The absorbance of 1.0 was defined as 1 U of esterase activity.

Assay for plasminogen activator. Plasminogen activator activity was detected in an indirect chromogenic assay by using highly pure, inhibitor-free human plasminogen (P-7397; Sigma) and the chromogenic plasmin substrate Val-Leu-Lys-pNA (No. 2251; AB KABI Peptide Research, Moindal, Sweden). The assay was performed with slight modifications as described by Overwien et al. (14). In short, 100 μl of plasminogen (5 μM) and 100 μl of the chromogenic plasmin substrate Val-Leu-Lys-pNA (3.5×10^{-4} M) in reaction buffer (30 mM Tris-HCl, 60 mM NaCl, and 3 mM NaN_3, pH 7.4) were mixed with appropriate dilutions in reaction buffer of either human low m.w. urokinase (U-8627; Sigma) or of PNSN prepared from CTLL TU 1.3E6 K1. The total volume of the reaction mixture was finally reached by addition of reaction buffer. The reaction was run for 4 hr at 37°C and was then terminated by addition of 100 μl of 2% acetic acid. Enzyme activity was then measured by reading the OD at 405 nm. The low control was a mixture of plasminogen with chromogenic substrate alone in reaction buffer. This set up did not yield a measurable color reaction within up to 10 hr of incubation at 37°C.

Inhibitor studies and labeling with [^{3}H]diisopropylfluorophosphate. Triton X-100 (25 μl) lysed PNSN or of a solution of bovine pancreatic trypsin (Sigma No. T-2395; 1 μg/ml in PBS) were mixed with 30 μl of 0.1 M Tris (pH 8.0) and 75 μl of the appropriate inhibitor dilution or as a control with the respective organic solvent diluted in double distilled water. Substrate (25 μl) Ile-Pro-Arg-pNA (0.266 mg/ml) in double distilled water was added after 30 min of preincubation. The enzyme reaction was run for 3 hr at 37°C and the OD at 405 nm was then read directly from the 96-well flat bottomed plates. The appropriate dilutions of the inhibitors were prepared in double distilled water from the following stock solutions: phenylmethanesulfonylfluoride (PMSF) (Sigma Biochemicals, Munich, FRG; P-7626) 10^{-1} M in dimethylsulfoxide (DMSO) (Merck, Darmstadt, FRG); PMSF 10^{-1} M in isopropanol (Merck, Darmstadt, FRG); aprotinin (Sigma; A-1153), and meta-aminobenzamidine (Sigma; A-6391), 2 mg/ml in double distilled water.

Enzyme labeling was performed by adjusting 200 μl of PNSN or of affinity-purified protease to 0.1 M Tris buffer (pH 8.0) and incubation for 18 hr at room temperature with 30 μCi of [^{3}H]diisopropylfluorophosphate ([^{3}H-DFP), (NEN-605; New England Nuclear, Boston, MA) under permanent shaking. For gel filtration, unbound radioactivity was separated by passage over a small column of Sephadex G 50 (1 ml column vol). For SDS-PAGE, the mixture was adjusted to 20% trichloroacetic acid (TCA; using a 100% w/v solution of TCA) and was kept at 4°C overnight. The precipitate was pelleted by using an Eppendorf tabletop centrifuge and was washed once in acetone:1 M HCl (9:1) and once in pure acetone. The dried pellet was dissolved in sample buffer containing the reducing agent dithiothreitol and

was electrophoresed using a 10% polyacrylamide slab gel. Separated gel lanes were cut into 5 mm slices, and radioactivity was determined by liquid scintillation counting after solubilization in protosol/water (9/1; v/v).

Column chromatography and affinity purification of CTLL AK 1.3E6-associated protease. For molecular sieving of PNSN, we used a Pharmacia Fast Protein Liquid Chromatography (FPLC) unit equipped with a prepacked Sepharose 6 gel filtration column. Chromatography was performed by using an isocratic buffer system of 0.1 M Tris (pH 8.0) at a flow rate of 1 ml/min and collecting 2 ml fractions. The protein contents of the eluate was continuously monitored by absorption measurement at 280 nm by an integrated spectrophotometer. Aliquots of the resulting fractions were tested for amidolytic activity by using the chromogenic substrate Ile-Pro-Arg-pNA or for radioactivity, by liquid scintillation counting.

For enrichment, the protease activity in Triton X-100-treated PNSN of CTLL AK 1.3E6 was bound to and was eluted from immobilized arginine (Arginine-Sepharose 4B, Pharmacia; 17-0524-01). The column (4 ml column vol) was equilibrated with 0.1 M Tris buffer (pH 8.0) containing 0.1% Triton X-100. Five milliliters of Triton X-100 lysed PNSN were applied to the column, and the column was sequentially washed with 10 ml of 0.1 M Tris buffer (pH 8.0) containing 0.1% Triton X-100, then with 10 ml of 0.1 Tris buffer (pH 8.0), and finally the bound material was eluted with 0.1 M Tris buffer (pH 8.0) containing 0.5 M arginine hydrochloride (Sigma; A-5131). Aliquots of the resulting fractions were analyzed for amidolytic activity by using the substrate Ile-Pro-Arg-pNA.

RESULTS

Substrate specificity and pH optimum of CTLL AK 1.3E6-associated amidolytic activity. CTLL AK 1.3E6 was derived from a long-term culture H-Y-specific T cell clone and is maintained in culture medium supplemented with Con A SN without stimulator cells. As detailed (8), 1.3E6 has lost its original specificity but not its cytolytic potential and acquired new specificity for P815 mastocytoma cells. For determination of enzyme activity, 1.3E6 cells were lysed by nitrogen cavitation, and PNSN was derived after removal of cellular nuclei by centrifugation. PNSN was additionally treated with the nonionic detergent Triton X-100 for complete solubilization of cellular constituents, and enzyme activity was then tested on a panel of 11 low m.w. chromogenic peptide substrates. As can be seen in Table I, the lysate was highly active on substrates Pro-Phe-Arg-pNA, Ile-Pro-Arg-pNA, and Val-Leu-Lys-pNA. Considerably lower or no activity was found against substrates carrying the chromogenic group coupled to amino acids other than L-arginine or L-leucine. These observations indicate that the primary specificity of the enzyme(s) as determined by the amino acid after which cleavage of the chromogenic substrates occurs depends on the amino acids L-arginine or L-lysine. Maximum enzyme activity on the appropriate chromogenic substrates was obtained at pH 8 to 9 (data not shown).

TABLE I

Relative amidolytic activity of CTLL AK 1.3E6 lysate on chromogenic substrates at pH 8.0[a]

Amino Acid Composition of Chromogenic Substrate	Absorbance at 405 nm	Percent Relative Activity
Pro-pNA	0.059	9.8
Trp-pNA	0.010	1.6
Glu-Pro-Val-pNA	0.002	0.4
Phe-pNA	0.002	0.4
Ala-pNA	0.014	2.4
Met-pNA	0.004	0.6
Arg-Pro-Tyr-pNA	0.010	1.7
Val-Leu-Lys-pNA	0.147	24.5
Gly-Arg-pNA	0.050	8.3
Ile-Pro-Arg-pNA	0.314	52.4
Pro-Phe-Arg-pNA	0.598	100.0

[a] Substrate specificity of CTLL AK 1.3E6 associated amidolytic activity. The original PNSN of 1.3E6 cells was diluted 1/6 in 0.1 M Tris-HCl, pH 8.0, 0.1% Triton X-100 and was kept on ice for 20 min. The superantant after centrifugation at 10,000 × G at 4°C for 10 min was taken for determination of amidolytic activity; 20 μl of the respective substrates (2 mg/ml in double-distilled water, 0.2 μm filtered) were mixed with 200 μl of the final detergent lysate of 1.3E6 PNSN in individual wells of a 96-well, flat-bottomed microtiter plate. The optical density at 405 nm of six replicates was measured after 18 hr at room temperature. The data represent the mean value of these replicate determinations, and the standard deviation never exceeded 10% of the mean value. Substrates were kindly provided by Dr. Petter Friberger (AB KABI Peptide Research, Sweden).

Inhibition of amidolytic activity by PMSF, aprotinin, and m-amino-benzamidine. The finding that the CTLL AK 1.3E6-derived amidolytic activity on substrate Ile-Pro-Arg-pNA was completely inhibited by the irreversible protease inhibitors DFP (not shown) and PMSF (Table II) indicates that this activity is due to a serine esterase. Moreover, Table II shows that the amidolytic activity was completely blocked by aprotinin and meta-amino-benzamidine (mABA), both of which are inhibitors for trypsin-like proteases, thus providing additional evidence for the trypsin-like nature of the enzyme activity tested (15). In control experiments it was found that the organic solvents isopropanol and DMSO used to solubilize PMSF caused a massive increase in protease activity (Table II). Because neither solvent influenced amidolysis by trypsin or destabilized the chromogenic substrate alone (shown only for isopropanol, Table II), we conclude that their enhancing effect on the enzyme activity in cell lysates is mediated indirectly, possibly via inactivation of suppressive molecules.

TABLE II

Inhibition of CTLL AK 1.3E6-derived amidolytic activity[a]

Addition to 1.3E6 Lysate	Absorbance at 405 nm	Percent of Positive Control
—	0.406	100
PMSF (IP) 10^{-2} M	0.045	11
PMSF (IP) 10^{-3} M	0.045	11
PMSF (IP) 10^{-4} M	0.266	65.5
Isopropanol 10%	1.399	344
Isopropanol 1%	0.826	203.4
Isopropanol 0.1%	0.529	130
PMSF (DMSO) 10^{-2} M	0.056	13.7
PMSF (DMSO) 10^{-3} M	0.136	33.4
DMSO 10%	0.867	213.5
DMSO 1%	1.078	265.5
Aprotinin 1 mg/ml	0.022	5.4
Aprotinin 0.5 mg/ml	0.016	3.9
Aprotinin 0.1 mg/ml	0.046	11.3
mABA 1 mg/ml	0.022	5.4
mABA 0.1 mg/ml	0.044	10.8
Trypsin	0.504	100
Trypsin + isopropanol 10%	0.501	99.4
Isopropanol 10%	0.025	4.9

[a] Inhibition of CTLL AK 1.3E6 associated amidolytic activity. Triton X-100 lysates of 1.3E6 PNSN (prepared as described in the legend to Table I) or a trypsin solution (1 μg/ml in PBS) were reacted with various inhibitors as described in *Materials and Methods*. The amidolysis assay was run for 3 hr at 37°C, and the absorbance at 405 nm was read directly from the plates; the data are depicted as the mean value of four replicate determinations. The standard deviation never exceeded 10% of the mean value. In addition, the data have been calculated by referring to the high control value obtained in the presence of double-distilled water instead of inhibitor (O.D., 0.406) and are expressed as "percent of positive control". Trypsin (1 μg/ml in PBS) instead of cell lysates was tested in parallel. In this case, the absorbance at 405 nm was determined after 30 min at 37°C.

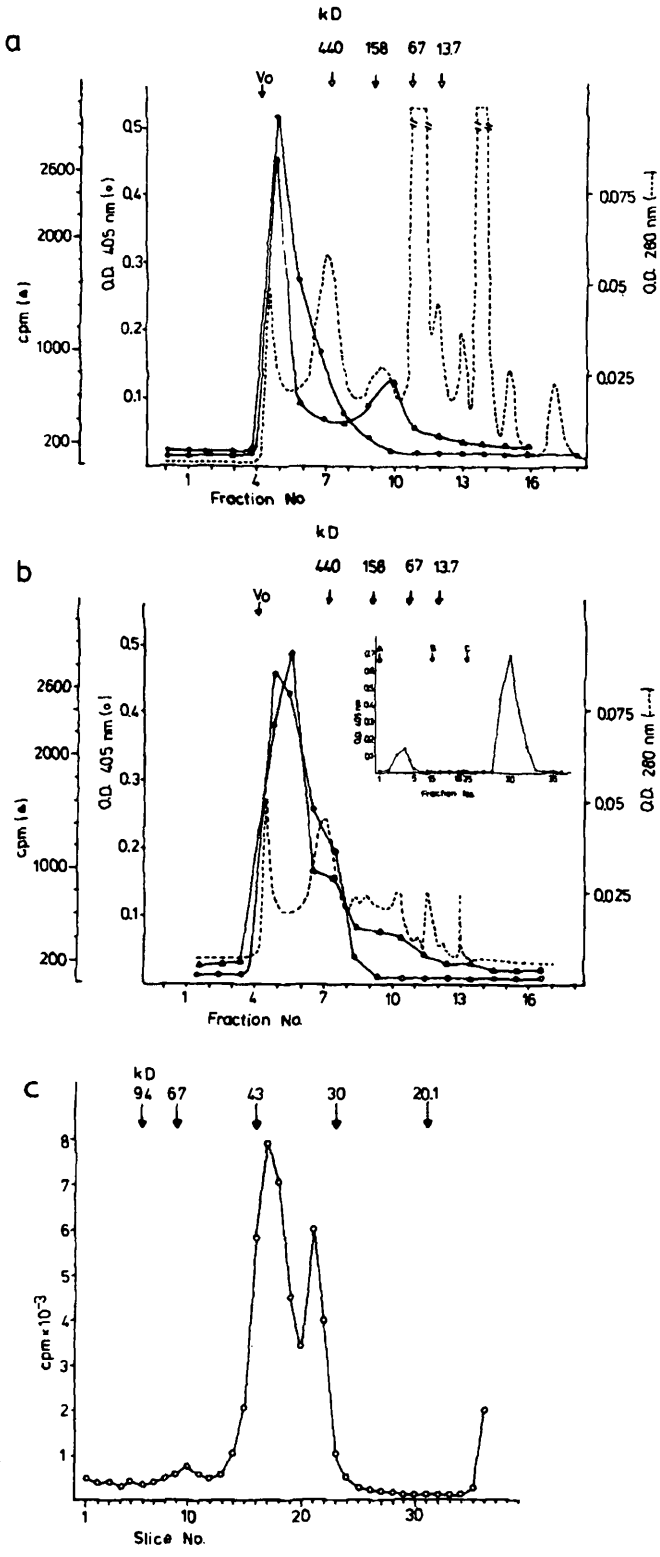

Figure 1. Gel chromatography and SDS gel electrophoresis of untreated and affinity labeled CTLL AK 1.3E6 PNSN. In *Panel a*, PNSN of 1.3E6 cells (200 μl; corresponding to 2×10^6 cell equivalents) was cleared from particulate material by centrifugation at 10 000 × G for 10 min and then was applied directly or after reaction with ^{3}H-DFP to a superose 6 column and was chromatographed as described in *Materials and Methods*. The fractions were tested for protein content at 280 nm (---), amidolytic activity at 405 nm (○), and radioactivity (▲). The column was standardized with the following proteins: ferritin (440,000 daltons), aldolase (158,000 daltons), bovine serum albumin (67,000 daltons), and ribonuclease (13,700 daltons). In *Panel b insert*, 5 ml (corresponding to 5×10^7 cell equivalents) of Triton X-100 treated 1.3E6 PNSN were applied to a 4 ml column of immobilized arginine. The column was washed with 1 ml fractions of (*A*) 0.1 M Tris buffer (pH 8.0) containing 0.1% Triton X-100 and (*B*) 0.1 M Tris buffer (pH 8.0). Elution (*C*) was achieved with 0.1 M Tris (pH 8.0) containing 0.5 M arginine. In *Panel b*, 200 μl of the fraction with highest amidolytic activity (No. 30) either without treatment or after reaction with ^{3}H-DFP were applied to the Superose 6 FPLC column and were eluted as described above. The resulting fractions were monitored for protein content at 280 nm (---), amidolytic activity on the substrate Ile-Pro-Arg-pNA at 405 nm (○), and radioactivity (▲). In *Panel c*, PNSN of 1.3E6 cell (200 μl; corresponding to 2×10^6 cell equivalents) was reacted with ^{3}H-DFP and was processed for SDS-PAGE as described in *Materials and Methods*. The chromatography was performed overnight at 22 mA by using a 10% polyacrylamide slab gel. Molecular weight standards were phosphorylase-b (94,000 daltons), bovine serum albumin (67,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (30,000 daltons), and soybean trypsin inhibitor (20,000 daltons). After separation, the gel was cut into individual lanes that were then cut into 5 mm slices and were processed for radioactivity determination.

Biochemical characterization of CTLL AK 1.3E6-associated amidolytic activity. Untreated 1.3E6 cell lysates or lysates treated with the radioactive affinity ligand ^{3}H-DFP were fractionated by molecular sieve chromatography under FPLC conditions. The analysis of the fractions for protein content, enzymatic activity, and radioactivity revealed that both amidolytic activity and specific radioactivity were excluded from the gel matrix and were eluted in the void volume (Fig. 1*a*). Pretreatment of lysates with Triton X-100 and subsequent molecular sieving in the presence of this detergent did not alter the migration behavior of the enzyme activity (not shown). In an attempt to additionally enrich the enzyme, lysates were subjected to chromatography on arginine coupled to Sepharose 4B. As illustrated (Fig. 1*b*, insert), enzyme activity could be bound onto the gel matrix and could be competitively eluted with free arginine. Untreated and ^{3}H-DFP-treated affinity-purified enzyme activity was reanalyzed by gel filtration. Again, as shown before for the crude lysate, the enzymatic activity and radioactivity eluted in the void volume (Fig. 1*b*), indicating that the amidolytic activity in 1.3E6 lysates corresponds to a compound with a high m.w. Therefore, we tried to determine the m.w. of the enzyme under denaturing and reducing conditions by SDS-PAGE. For this purpose, 1.3E6 lysate was treated with ^{3}H-DFP and was separated via SDS-PAGE under reducing conditions. As shown in Figure 1c, two peaks of radioactivity were observed, with a relative molecular mass of approximately 32,000 and 40,000 daltons, respectively.

Expression of amidolytic activity by functionally distinct cloned T cell lines and by normal lymphocyte populations. Protease activity was determined in lysates (PNSN, nitrogen cavitation; Triton X-100) and in culture SN of several cloned H-Y-specific T cell lines with cytolytic (CTLL 2.A4.1, 2.A4.2, 2.C10.1) or helper (THL G8, H3, H12.H2, B5) potential. In addition, in vitro and in vivo variants of specific CTLL that lost their original specificity for H-Y antigen but specifically lyse P815 tumor target cells (1.3E6, CTLL AK; 8), or lost both their specificity and function and are tumorigenic (1.3E6 K1, 1.3E6 U, CTLL TU) were tested. The etiology and the characteristics of CTLL TU will be described elsewhere (M. M. Simon et al., manuscript in preparation). Furthermore, the expression of amidolytic activity in the C57BL/6-derived T cell lymphoma line EL-4 and in normal lymphocyte populations has been tested. In Table III, enzyme activity as determined at a single time point (1 hr, 37°C) is given. Figure 2 illustrates the time dependence of

TABLE III

Cytolytic and amidolytic activity expressed by cloned H-Y specific T cell lines, their in vitro and in vivo derived variants and normal lymphocyte populations[a]

T Cell Clone or Lymphocyte Population	Type	Cytolytic Activity[b] on			Relative Amidolytic Activity (U)[c]			
					Lysates		SN	
		B6 δ	P815	P815 + PHA	Triton X-100	PNSN	ϕ	Con A
2.A41	CTLL	32.4	0	63.6	38.7	ND	8.0	15.4
2.A4.2	CTLL	35.6	0.7	81.0	31.5	32.7	3.5	16.4
2.C10.1	CTLL	33.0	1.3	51.5	41.8	34.9	4.6	14.0
G8	TH	0	0	23.2	18.7	ND	4.8	6.0
H3	TH	0	0.3	31.9	35.2	27.5	3.1	21.9
H12.H2	TH	0.9	0	32.6	34.5	ND	2.6	22.0
B5	TH	0	0	0	43.5	ND	ND	ND
1.3E6	CTLL AK	2.2	54.7	19.1	51.8	ND	ND	ND
1.3E6 K1	CTLL TU	0	0	0	25.2	ND	ND	ND
1.3E6.U	CTLL TU	0	0	0	42.3	ND	ND	ND
EL4.F15	Thymoma	0	0	0	0.8	0.5	ND	ND
Thymocytes					0.3	0.3		
Resting spleen cells			ND		12.4	12.1		
Nylon-purified T cells					7.8	ND	ND	
Con A-activated spleen cells		ND	20.9	53.9	15.4	ND		
LPS-activated spleen cells		ND	2.0	7.7	12.8	ND	ND	

[a] Individual T cell clones and activated (2 days Con A, LPS) spleen lymphocytes were tested for cytotoxic activity on ^{51}Cr-labeled target cells that were either male C57BL/6 Con A-induced blasts or P815 tumor target cells in the absence or presence of PHA. The effector to target cell ratio was 25:1. Cell-associated enzyme activity in 100 μl aliquots of either Triton X-100 extracts or in PNSN obtained after nitrogen cavitation of cells was tested with chromogenic substrate Ile-Pro-Arg-pNA at 405 nm after 1 hr at 37°C as described in *Materials and Methods*. Culture supernatants from lymphocytes incubated in medium alone or together with Con A for 8 hr were tested in the same way for protease activity. The cell concentration was always 1×10^7 lymphocytes/ml. The absorbance of 0.01 at 405 nm after 1 hr of incubation was defined as 1 U of amidolytic activity.

[b] Effector:Target cells ratio = 25:1.

[c] Units per 10^6 cells; the absorbance of 0.01 at 405 was defined as 1 unit of amidolytic activity. Test was performed after 1 hr of incubation.

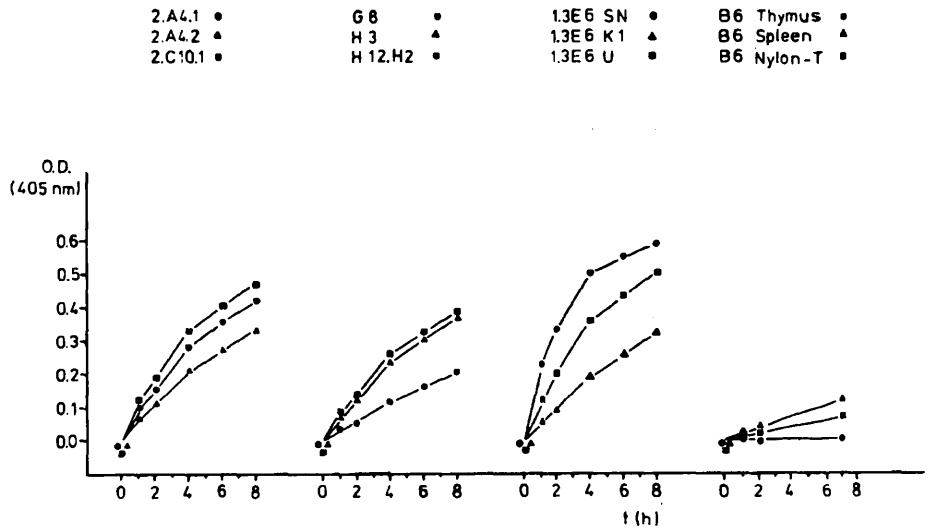

Figure 2. Time dependence of amidolysis by different cell lysates. Triton X-100 lysates were prepared as described in *Materials and Methods*. Appropriate dilutions (corresponding to 1.25×10^5 cell equivalents) of these cell extracts (100 μl) were incubated at 37°C with 100 μl of chromogenic substrate Ile-Pro-Arg-pNA in Tris-HCl (pH 8.0) at 37°C, and the OD at 405 nm was measured as indicated.

amidolysis by individual cell lysates and proves linearity of the resultant slopes in the absorbance range from 0.0 to 0.3 OD. As seen in Figure 1 and in Table III, lysates of all specific T cell clones except one (THL G8) expressed similarly high activities independent of whether they belong to the cytolytic or helper cell lineages. The THL G8 expressed less protease activity. When tested for cytolytic function, all CTLL and three out of four THL (G8, H3, H12.H2) were positive in the lectin-facilitated lympholysis assay (P815 + PHA), but only CTLL specifically lysed male B6 target cells. The fourth THL B5 with helper potential for the generation of cytotoxic lymphocytes and antibody-secreting cells (9) did not show any cytolytic activity on all three target cell populations. The EL-4 tumor line which is of the T helper phenotype and secretes IL 2 either constitutively (data not shown) or upon stimulation with phorbol myristate acetate (16) had no cytotoxic activity and minimal enzyme activity. Furthermore, lysates of three CTLL derived variants, 1.3E6, IL 2 dependent, nontumorigenic; 1.3E6 K1, IL 2 independent, tumorigenic; and 1.3E6 U, IL 2 independent, tumorigenic metastatic (M. M. Simon, manuscript in preparation), also contained high enzyme activity. Note that from the three variants, only 1.3E6 showed cytolytic potential on P815 tumor target cells (±PHA), whereas the two other tumor variants were negative on all three target cells tested.

In contrast with the cloned T cell lines, only marginal protease activity was found in nonactivated thymocytes (2%). In lysates of resting or polyclonally activated (Con A, LPS) spleen cells or of nylon-enriched T cells, the enzyme activities were 20 to 30% compared with those expressed by cloned T cells. However, additional experiments suggested that it is the contaminating adherent cells that mainly contribute to protease activity detected in unselected or nylon wool-purified spleen cells. It is shown in Table IV (right column) that the amidolytic activity present in unselected spleen cells was drastically reduced after their separation on Sephadex G-10 column,

TABLE IV

Protease activity in unseparated and separated spleen cell populations[a]

Cell Source	Separation Procedure	Protease Activity on: BLT substrate U/10^6 cell equivalents	Protease Activity on: para-nitroanilide substrate U/10^6 cell equivalents
Spleen	—	3.6	19.1
Spleen	Nylon	4.4	9.7
Spleen	Sephadex G-10	0.15	1.2
Spleen	Sephadex G-10/nylon	0.90	1.9

[a] Triton X-100 lysates were prepared as described in Table III, and 100 μl of appropriate dilutions were mixed with either of the two substrates as described in *Materials and Methods*. Enzyme activity on BLT was assessed after 30 min of incubation at room temperature by measuring absorbance at 412 nm. Absorbance of 1.0 was defined as 1 U of esterolytic activity. The cleavage of the Ile-Pro-Arg-pNA was measured after 1 hr of incubation at 37°C. Absorbance of 0.01 at 405 nm corresponds to 1 U of amidolytic activity.

TABLE V

Comparison of trypsin-like enzyme activity as determined by means of two different substrates[a]

Cell Population	Type	Protease Activity on: BLT substrate U/10^6 cell equivalents	Protease Activity on: para-nitroanilide substrate U/10^6 cell equivalents
2.A4.1	CTLL	50.0	23.2
2.A4.2	CTLL	42.0	16.7
2.C10.1	CTLL	69.0	24.6
G8	THL	16.0	18.5
H3	THL	57.0	28.6
H12.H2	THL	50.0	32.3
1.3E6	CTLL AK	78.0	36.9
1.3E6 K1	CTLL TU	25.0	24.0
EL4.F15	Thymoma	0.8	0.25
P815	Mastocytoma	1.3	1.5
L929	Fibroblast tumor	0.9	1.5
IC-21	Macrophage tumor	0.8	1.5
B6 spleen cells	Normal lymphocyte	3.6	15.5
B6 nylon T	Normal lymphocyte	4.4	9.7

[a] Triton X-100 lysates were prepared from unseparated and separated spleen cells, and 100 μl (corresponding to 10^6 cell equivalents) or dilutions thereof were incubated with either of the two substrates as described in *Materials and Methods*. For the calculation of units of enzyme activity see legend to Table IV.

which is known to efficiently remove macrophages (11). When tested for extracellular enzyme activity low but significant amounts were detected in all SN of CTLL and THL. Lectin activation of individual CTLL and THL led to an approximate twofold increase of protease activity in SN of five out of six clones as compared with controls. In contrast, activation of THL G8 by Con A resulted only in minimal increase of secreted activity.

Comparison of esterolytic and amidolytic activity and test for plasminogen activator in cell lysates. The following experiments were performed to compare the distribution of amidolytic activity with that of an esterolytic activity that has recently been described by Pasternak et al. (2) to be present in T cell populations. Lysates of various cell populations have been tested in parallel for amidolysis on Ile-Pro-Arg-pNA and for esterolysis of BLT. The relative enzyme activities in the tested cell populations were found to be comparable in both assay systems (Tables IV and V). However, the data reveal that the esterolytic assay with the use of BLT as the substrate is approximately 100-fold more sensitive than the amidolysis assay. This appears by comparison of the definition of 1 U of enzyme activity for the respective assay system (see the definition of 1 U of enzyme activity in the legend to Table V). Furthermore, we found that the affinity-purified protease activity also exerts strong activity on the ester substrate BLT (data not shown). Taken together these correlation studies and the preliminary biochemical data suggest that the activity in both assay systems may be attributed to an identical trypsin-like enzyme.

From the literature, it is known that transformed cells of different origin (17) and lymphoid cell populations (18) express a trypsin-like protease called plasminogen activator. However, purified normal murine T cells do not express detectable levels of plasminogen activator (17). Nonetheless we analyzed a panel of cell lysates for plasminogen activator, and a representative experiment that made use of the in vitro-derived transformed variant CTLL TU 1.3E6 K1 is depicted in Figure 3. In contrast to urokinase, which displays strong plasminogen-dependent lysis of the plasmin-specific amide substrate Val-Leu-Lys-pNA, the CTLL TU 1.3E6 K1 lysate exerts no plasminogen-dependent amidolysis. However, low but significant plasminogen independent lysis of the amide substrate was observed. Thus it appears that the CTLL TU 1.3E6 K1 lysates do not contain detectable levels of

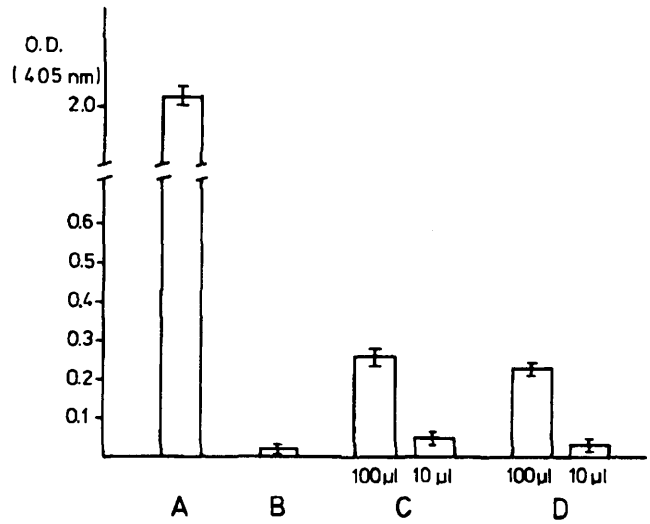

Figure 3. Test for plasminogen activator activity. Triton X-100 lysates of CTLL TU 1.3E6 K1 were prepared as described above. Cell lysates (corresponding to 1 × 10^6 cell equivalents) were mixed with the plasmin-specific chromogenic substrate Val-Leu-Lys-pNA in the presence (*Column C*) or absence (*Column D*) of the proenzyme plasminogen. Enzyme activity was assessed by measuring OD at 405 nm. *Column A*, urokinase (10 ng/ml) as control plasminogen activator, chromogenic substrate, and plasminogen. *Column B*, urokinase and chromogenic substrate alone. *Column C*, cell lysate (100 μl and 10 μl) in the presence of plasminogen and chromogenic substrate. *Column D*, cell lysate (100 μl and 10 μl) in the presence of chromogenic substrate only.

plasminogen activator, but the chromogenic substrate is directly attacked by the trypsin-like protease (note that this is in accordance with the data presented in Table I). Similar results were obtained when radioactive labeled casein was taken as the plasmin/protease substrate (data not shown). Taken together, no plasminogen-dependent protease activity could be detected in lysates of long term culture H-Y-specific CTLL and their variants CTLL AK and CTLL TU or in purified normal T cell populations (data not shown) (18).

DISCUSSION

In this report, we describe the characterization and isolation of a trypsin-like serine protease from the long-term culture CTLL AK 1.3E6. High enzymatic activity was detected in lysates of 1.3E6 cells on either BLT or low m.w. amide substrates carrying a nitroaniline group at the carboxy-terminus of either lysine or arginine. No or only marginal effects were seen with para-nitroanilide substrates carrying other aminoacids at the same position (Table I). Linearity of enzyme activity in lysates of CTLL AK 1.3E6 and other cloned or heterogeneous lymphocyte population was established in detailed kinetic experiments (Fig. 2). The amidolytic activity was blocked by the two serine esterase inhibitors DFP and PMSF, and by the two trypsin inhibitors aprotinin and meta-aminobenzamidine. Analysis of CTLL AK 1.3E6 lysates by gel filtration showed that enzyme activity and specific radiolabel after pretreatment with ^{3}H-DFP was eluted in the void volume of the gel filtration column, thus indicating a m.w. >5000 daltons. A similar elution profile was obtained in the presence of 0.1% Triton X-100, thus indicating that the protease containing complex is not formed by plasma membrane particles. Preliminary experiments have shown that this protease complex can be dissociated in the presence of high salt concentrations (unpublished results). Isolation of proteolytic activities in complex form has been shown in other systems (7, 19), and may represent a way of anchoring the enzyme to a matrix, such as the plasma membrane. Future studies will have to elucidate the significance of this phenomenon in the case of the CTLL AK-derived hydrolytic enzyme.

We found that the organic solvents isopropanol and DMSO cause a massive increase in apparent enzymatic activity (Table II). Recently, Vassalli et al. (20) reported that human mononuclear cells contain an inhibitor(s) for the so-called urokinase-type plasminogen-activator (a trypsin-like endopeptidase) and showed that the inhibitor but not the endopeptidase was sensitive to treatment with isopropanol. Because isopropanol and DMSO influenced neither substrate alone nor the activity of pancreatic trypsin on the chromogenic substrate, the existence of a similar inhibitor(s) in murine CTLL is probable. However, this conclusion is premature, and a final explanation of this phenomenon has to await additional investigation.

Labeling with ^{3}H-DFP and separation of cell lysates by SDS-PAGE under reducing conditions demonstrates that protease activity resides in two proteins of approximate relative molecular mass 32,000 and 40,000, respectively. By using a similar approach, it was found recently by Pasternack and Eisen (2) that ^{3}H-DFP labels a protein of 28,000 daltons in Nonidet P-40 lysates of cloned CTLL. In their study, the enzyme was also shown to be trypsin-like as assessed by its specificity for the ester-substrate BLT and by experiments with appropriate inhibitors. By comparing trypsin-like protease activity by using both Ile-Pro-Arg-pNA and BLT substrates, we found that the relative activities detected in various lysate preparations were similar in both assays (Table IV and V). In addition the BLT assay was found to be much more sensitive (approximately 100-fold) as compared with that using the para-nitroanilide substrate. In spite of the divergence in the determination of molecular mass of the enzyme in both studies, the results suggest that esterolysis and amidolysis are caused by an identical trypsin-like enzyme molecule(s). However, future experiments will have to explore the validity of this assumption.

It is known that transformation of normal cells, for instance by oncogenic viruses, may be accompanied by the appearance of a cell-associated endopeptidase called plasminogen activator and in particular of the urokinase-type plasminogen activator (17). We asked whether this type of protease was responsible for the enzyme activity either observed as esterolysis or amidolysis activity in CTLL AK 1.3E6 cells or in the tumorigenic variants (CTLL TU) thereof. When testing cell lysates for plasminogen activator, we found that neither those of purified normal T cells, which is in accordance with previous findings of Maillard et al. (18), nor those of the CTLL AK or CTLL TU contain detectable levels of plasminogen activator. Taken together these data propose that the amidolytic or esterolytic activity in these T cell populations is not due to plasminogen activator activity. Cell-associated peptide hydrolases and especially endopeptidases (proteinases) have been implied in cell-mediated cytolysis ("killing") (2, 5, 6, 21) and in the degradation of connective tissue barriers during the migration of normal or transformed cells (7, 22). In particular, it has been demonstrated that inhibitors of proteases interfere with the lytic process elicited by either T lymphocytes or NK cells (6). Moreover, in a recent study on functionally distinct T cell lines, protease activity was correlated with CTL and their effector function (2). However, the results described in this study demonstrate that lysates of a panel of H-Y-specific T cell clones with either cytolytic/suppressor (23, 24) or helper function (11) express comparable amounts of trypsin-like amidolytic/esterolytic activity, which were higher than those expressed in resting or polyclonally activated normal lymphocyte populations. This was also true for CTLL AK and tumorigenic variants CTLL TU that have lost the original specificity (8) or both specificity and cytolytic function. The finding that one THL (B5) did not express any cytolytic potential, as tested on P815 tumor target cells in the presence of PHA, but had high amounts of enzyme activity in its lysates also argues against a strict correlation between the expression of cell-associated protease activity and cytolytic function. This is supported by the fact that both H-Y-specific CTLL and THL secreted significant amounts of enzyme activities either constitutively or after lectin activation. The discrepancy between our findings and the study by Pasternack and Eisen (2) cannot be due to the use of the two different substrates, Ile-Pro-Arg-pNA and BLT, for the reasons given above. Thus our findings do not support the conclusion that cell-associated trypsin-like protease is a specific marker for CTL. Rather the data indicate that protease activity may be associated with all functionally active T cell subsets, but expression and/or secretion of the enzyme in individual lymphocytes is regulated differentially via distinct activation signals.

Our data obtained with nonactivated or polyclonally activated unselected or selected spleen cells suggest that nonlymphoid cells, probably macrophages, mainly contribute to protease activity detected in these populations, and resting T lymphocytes only express marginal trypsin-like serine esterase activity. It has been shown by others that in macrophages, the expression of neutral proteases

are differentially regulated during activation (25). Therefore, additional studies with highly purified T lymphocyte populations are required to follow-up the expression of proteases during T cell maturation. The involvement of hydrolytic enzymes and especially of proteases in the hematogenous traffic of nonlymphoid and lymphoid tumor cells has been evinced by the blocking of metastatogenic behavior by protease inhibitors (26) or specifically blocking antisera against proteases (27). We could recently show that highly metastatic T lymphoma cells contain a trypsin-like serine protease that synergizes with an endoglycosidase in the degradation of extracellular matrix components (28). Preliminary results indicate that the CTLL 1.3E6-associated protease is also able to degrade certain components of extracellular matrix (unpublished observations). In future experiments, the purified enzyme preparation derived from CTLL AK 1.3E6 or from the tumorigenic variants (CTLL TU) will be used to generate specific antisera and monoclonal antibodies for immunohistochemical and functional studies, which should help to clarify the role of this protease in the T cell economy and the functional significance of its upregulation in distinct effector cells of the immune system.

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