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Interaction of Chondroitin Sulfate with Perforin and Granzymes of Cytolytic T-Cells Is Dependent on pH†

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ABSTRACT: Cytolytic T-lymphocytes (CTL) harbor cytoplasmic granules containing the lytic, pore-forming protein perforin, a family of serine proteases designated granzymes, and proteoglycans as major constituents. Growth of CTL lines in the presence of PNP-xyloside completely inhibited the glycosylation of the granule-associated chrondroitin sulfate A type proteoglycans. Only short glycosaminoglycan molecules were detected. The absence of intact proteoglycans neither altered the sorting of the granule-associated proteins perforin or granzyme A nor influenced their secretion into the extracellular milieu upon T-cell receptor complex stimulation. With a weak base, the pH of the granules was determined to be acidic. At pH 5.2, granzyme A and perforin formed complexes with chondroitin sulfate A. At neutral pH, perforin and only a minor fraction of granzyme A dissociated from the proteoglycan. Upon secretion of the granule contents induced by immobilized anti-CD3 antibodies, most granzyme A molecules remained complexed with the chondroitin sulfate A glycosaminoglycans, even if synthesis of intact proteoglycans was inhibited. We suggest that granule-associated molecules complex with proteoglycans under the acidic conditions prevailing in the trans Golgi and cytolytic granules. A possible pH shift occurring during exocytosis would cause perforin, but only a minor fraction of granzyme A, to dissociate from the proteoglycans.

Cytotoxic T-lymphocytes (CTL) and natural killer (NK) cells contain characteristic electron-dense granules in the cytoplasm. One of the mechanisms of target cell lysis would involve the release of the granule content into the intercellular space between CTL and the target cell upon specific target recognition (Henkart, 1985; Müller-Eberhard, 1988; Podack, 1986; Tschopp & Jongeneel, 1988; Young et al., 1988). Several molecules contained in the granules have been characterized: (1) perforin, a cytolytic protein known to polymerize in the presence of Ca^{2+} into poly(C9)-like tubules on the target cell membrane, thereby rendering it leaky (Masson & Tschopp, 1985; Podack et al., 1985); (2) granzymes, serine esterases with unknown function (Pasternack et al., 1986; Simon et al., 1986; Young et al., 1986a; Masson & Tschopp, 1987); (3) proteoglycans of the chondroitin sulfate A type (MacDermott et al., 1987; Parmley et al., 1985; Stevens et al., 1987). Granzyme A (Pasternack & Eisen, 1985; Takayama & Sitkovsky, 1987; Garcia-Sanz et al., 1987), granzyme B (Jenne & Tschopp, 1988), granzyme D, and proteoglycans (Schmidt et al., 1985) have been detected in the medium during cytolytic assays, indicative of a secretory process.

Other effector cells also participating in the immune response have secretory granules. In particular, mast cells of both the connective tissue and mucosal type harbor proteoglycans and proteases in their intracellular granules (Stevens et al., 1986; Avraham, 1989). Proteoglycans including heparin, heparan sulfate, and chondroitin sulfate consist of long unbranched sulfated polysaccharide chains composed of repeating disaccharide units bound to a core protein. The peptide core contains clusters of alternating serines and glycines (Bourdon et al., 1985). Glycosaminoglycans are attached via an O-

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glycosidic linkage to the serine residue. During biosynthesis, monosaccharides are added to the peptide core, giving rise to the sequence Ser-Xyl-Gal-Gal-GlcUA[1] (Lindahl & Roden, 1964). Depending on the type of proteoglycan, various types of repeating disaccharide units are then added. It is possible to inhibit proteoglycan synthesis by β-D-xylosides (Lohmander et al., 1979), which compete with xylose-substituted protein cores as acceptors for glycosaminoglycan chain synthesis.

The role of proteoglycans in secretory granules is not known. The negatively charged proteoglycans may interact with positively charged secretory proteins (granzymes and mast cell proteases are very basic proteins), thereby leading to the formation of osmotically inactive precipitates. The lytic activity of perforin and the protease activity of the granzymes, which are stored in the granules, may be diminished by complex formation and limited to the environment where exocytosis occurs. Proteoglycans may also be involved in the sorting of proteins into granules (Burgess et al., 1984).

In this report, we have investigated the interaction of chondroitin sulfate with CTL granule-associated proteins in order to evaluate these possible functions.

Materials and Methods

Cell Lines. The murine clone 860/4 is a C57/B16 (H-2^b) anti-DBA/2 (H-2^d) CTL clone kindly provided by Dr. J. C. Cerottini, Ludwig Institute, Lausanne). It is grown in complete RPMI medium supplemented with 5% FCS in the presence of 10% rat con A supernatant and radiated spleen cells (Velotti et al., 1987). It is stimulated weekly by P815 mastocytoma cells.

JS-132 is a human CTL clone specific for a HLA-A2 determinant derived from HLA-A3,3,B7,7, DR2,2 typed normal donor JS. The CTL clone is cultured in Yssel's modification of Iscove's medium (Yssel et al., 1984) supplemented with 2% pooled human serum and weekly stimulated with a feeder cell mixture (Borst et al., 1987).

Radiolabeling of Cells and Granule Isolation. The CTL clone 860/4 was stimulated either in normal RPMI medium or in medium supplemented with 2.5 mM *p*-nitrophenyl β-D-xylopyranoside (Sigma). Three days after stimulation, 40×10^6 cells were labeled for 24 h in 40 mL of sulfate-free RPMI medium containing 5% dialyzed FCS and 1.5 mCi of $Na_2{}^{35}SO_4$ (40 mCi/mL; Amersham) with or without 2.5 mM PNP-xyloside. Cells were collected, washed twice in phosphate-buffered saline (PBS), and suspended in 5 mL of PIPEG [100 mM KCl, 3.5 mM NaCl, 3.5 mM $MgCl_2$, 1 mM ATP, 1.25 mM EGTA, and 10 mM PIPES (pH 6.8)]. Cells were disrupted by nitrogen cavitation, and the organelles were separated on a discontinuous Percoll gradient (Pharmacia) as previously described (Masson et al., 1986a). The gradient consisted of 15.9 mL of 85% (v/v) Percoll and 12.1 mL of 39% Percoll in PIPEG. After 30 min of centrifugation at 19000 rpm in a JA 20 rotor (Beckman) at 4 °C, 1-mL fractions were collected. They were tested for hemolytic activity and BLT [(benzyloxycarbonyl)lysine thiobenzyl ester] esterase (granzyme A) activity as previously described (Masson et al., 1986b). The ^{35}S radioactivity was quantitated by means of a β counter (Beckman). Polyacrylamide gels were prepared according to Laemmli (1970). Labeled molecules were detected by fluorography using XAR-5 X-ray films (Kodak).

Analysis of Proteoglycans. After solubilization of the hemolytic granules in 1.5 M NaCl and subsequent ultracentrifugation at 42000 rpm during 3 h in a SW-55Ti rotor (Beckman) to remove Percoll, the ^{35}S-labeled molecules were analyzed by SDS–PAGE. Glycosaminoglycan side chains were released from proteoglycans by treatment with 50 mM NaOH in 1 M $NaBH_4$ at 37 °C for 48 h (Christmas et al., 1988). Samples were neutralized by adding 10% acetic acid before further analysis.

Degranulation of Granule Proteins. ELISA plates (Dynatech) were coated with 10 μg/mL anti-mouse CD3 monoclonal antibody (17A.2; kindly provided by Dr. R. MacDonald, Ludwig Institute, Epalinges) in PBS overnight at 4 °C. Labeled CTLs were washed in RPMI medium containing 1% bovine serum albumin and resuspended at 10^6 cells/mL in the same medium. The cell suspension was dispensed into the coated ELISA plates at 100 μL/well. After a 5-min centrifugation at 500 rpm, the plates were incubated for different time periods at 37 °C in a 5% CO_2 atmosphere. Per well, 50 μL of supernatant was collected and analyzed for radioactivity and BLT esterase activity (Masson et al., 1986b).

Assay for Cytolytic Activity. CTL lytic activity was determined by a standard ^{51}Cr-release assay (Brunner et al., 1976). Briefly, 10^4 ^{51}Cr-labeled P815 target cells were incubated with varying numbers of effector cells in a final volume of 200 μL or RPMI medium. After a 4-h incubation at 37 °C and 5% CO_2, 100 μL of the supernatant was removed for counting of radioactivity.

Analysis of Proteoglycan–Granzyme Interaction. Granules harvested from the Percoll gradient were disrupted in high salt, and nonsoluble aggregates and membrane debris were removed by high-speed centrifugation (Masson et al., 1986a). The soluble granule protein mixture was dialyzed overnight against 10 mM phosphate buffer, pH 7.4, and 10 mM sodium acetate, pH 5.5, both buffers containing 150 mM NaCl. Samples were centrifuged for 1 h at 10000 rpm in a microfuge, and supernatant and pellet were analyzed for perforin and granzyme A activity. Perforin activity was measured with the hemolytic assay (Masson & Tschopp, 1985) and is expressed in hemolytic units. One hemolytic unit corresponds to the quantity of perforin that lyses 50% of the erythrocytes (Masson & Tschopp, 1985). Since perforin is not hemolytic at low pH (<6.0), acidic samples were diluted at least 10× in the hemolytic test.

Granule release from the ^{35}S-labeled CTL line 860/4 was triggered by anti-CD3 monoclonal antibody during 4 h. Supernatants (50 μL) from wells of a 96-microtiter plate were harvested after exocytosis. A total of 500 μL of the supernatant was loaded onto a Superose 6 gel filtration column (FPLC system, Pharmacia) equilibrated in 10 mM phosphate buffer containing 150 mM NaCl and 1.5 M NaCl. If a detergent was required, 30 mM octyl glucoside (Sigma) was added. Fractions of 500 μL were collected and analyzed for the ^{35}S-labeled proteoglycans and granzyme A activity as described above.

Chondroitin sulfate A (10 mg) from whale cartilage (Sigma) was coupled to 1 mL of CNBr-activated Sepharose (Pharmacia) according to the manufacturer's instructions. Ten micrograms each of purified granzymes A, D, and E (Masson & Tschopp, 1987) was loaded onto the column that had been equilibrated in 10 mM Tris-HCl, pH 7.4, or 10 mM Bis-Tris-HCl, pH 5.5, both buffers containing 150 mM NaCl. The column was washed with 15 mL of the equilibration buffer, and 1-mL fractions were collected. The granzymes were eluted with 15-mL step gradients in which the salt concentration was

[1] Abbreviations: PNP-xyloside, *p*-nitrophenyl β-D-xylopyranoside; BLT, (benzyloxycarbonyl)lysine thiobenzyl ester; PBS, phosphate-buffered saline; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; GlcUA, glucuronic acid.

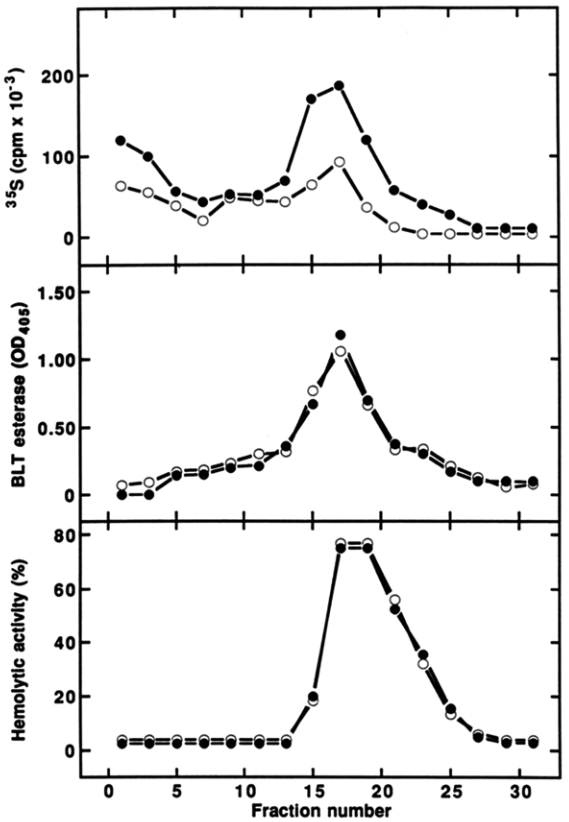

FIGURE 1: Isolation of lytic granules of murine CTL. ^{35}S-Labeled mouse cytolytic T-cells were grown in the presence of PNP-xyloside (open circles) to inhibit de novo synthesis of proteoglycans or in normal medium (closed circles). Cytolytic granules were isolated by Percoll density-gradient fractionation. Each fraction of the gradient was analyzed for ^{35}S-labeled proteoglycans (top), for the esterolytic activity of granzyme A (BLT esterase) (middle), and for the hemolytic activity of perforin (bottom). Fractions of the gradient were collected from the top. The hemolytic activity is given in relative units.

increased (300 mM NaCl, 600 mM NaCl, and 1 M NaCl). Elution of the granzymes was assayed by SDS–PAGE (10%), and the proteins were visualized by silver staining (Harlow & David, 1989).

Accumulation of Weak Bases. CTL clone JS-132 was incubated at 0 °C for 30 min in the weak base 3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropylamine (DAMP, 50 μM). The cells were then washed for 5 min in base-free, ice-cold medium and fixed in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h and processed for immunoelectron microscopy.

Results

Inhibition of Proteoglycan Processing: Effect on Sorting, Exocytosis, and Lytic Activity of Granule Proteins. β-D-Xylosides compete with the xylose-substituted core protein as glycosaminoglycan acceptor molecules and thus inhibit the formation of de novo synthesized proteoglycans. CTL 860/4 were grown during 4 days in the presence or absence of 2.5 mM PNP-xyloside. During the last 24 h, $^{35}SO_4^{2-}$ label was included. An aliquot of the cells was assayed for their ^{35}S content. Cells cultured in the presence of PNP-xyloside incorporated only 60% of the amount of radioactivity incorporated by control cells (data not shown). Cells were disrupted by N_2 cavitation and granules separated from other organelles on Percoll density gradients. Gradients displayed one peak of granule marker activity at the same density independent of the presence of PNP-xyloside during CTL culture. Hemolytic activity of perforin and esterolytic activity of granzyme A were similar in both gradients (Figure 1). Major peaks of ^{35}S radioactivity coincided with the hemolytic and esterolytic activities, while a small fraction of labeled material was present on top of the gradients. This probably reflects sulfated proteins and proteoglycans associated with the plasma membrane or, alternatively, proteoglycans released from damaged granules. Unlike the peaks indicating granzyme A and perforin activity, the glycosaminoglycan-associated ^{35}S radioactivity peak was lower in the cells treated with PNP-xyloside than in the control cells in accord with the decreased ^{35}S incorporation into whole cells. To confirm the efficacy of proteoglycan synthesis inhibition, ^{35}S-labeled molecules were further analyzed by SDS–PAGE and subsequent fluorography (Figure 2). When cells were incubated with [^{35}S]sulfate in the absence of PNP-xyloside and in complete RPMI medium, labeled molecules of M_r approximately 400000 were discerned. In cells that had been treated with PNP-xyloside, the species of high M_r was completely absent and the majority of the ^{35}S-labeled molecules comigrated with the front of the gel, indicating that short glycosaminoglycan species had been attached to PNP-xyloside and that synthesis of high M_r proteoglycans had been prevented. Treatment of the high M_r proteoglycan with alkaline borohydride to release the polysaccharide chains from the core protein revealed that the glycosaminoglycan chains had a molecular mass of approximately 60 kDa (data not shown). The ^{35}S-labeled molecules were chondroitin sulfates, since they migrated with the front of the gel after digestion for chondroitinases A, B, and C (data not shown).

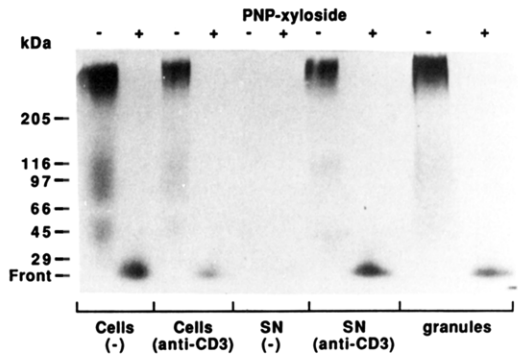

FIGURE 2: Analysis of ^{35}S-labeled proteoglycans released into the supernatant upon anti-CD3 stimulation of CTL. Murine CTLs were stimulated with anti-CD3 during 4 h, and after centrifugation the cells and the supernatant were analyzed by SDS–PAGE and fluorography. Cells and their respective supernatants (SN) that had been treated (anti-CD3) or had not been stimulated (−) are shown. As a control, granules solubilized in 1.5 M NaCl and from which the Percoll had been previously removed are shown. All experiments were carried out with CTLs treated with PNP-xyloside (+) or with untreated cells (−).

Granule exocytosis can be triggered in vitro by incubating CTL in microtiter plates coated with monoclonal antibodies directed against the CD3/T-cell receptor complex, which mimics interaction with target cells. Degranulation can be followed by assaying granzyme A release into the medium with the substrate BLT and/or measuring release of chondroitin sulfate associated [^{35}S]sulfate. The ^{35}S-labeled species secreted into the medium after 4 h of stimulation with anti-CD3 mAb were analyzed by SDS–PAGE and subsequent fluorography (Figure 2). Secreted molecules were identical in M_r with molecules found in whole cell lysates and in isolated cytotoxic granules, indicating that no further processing took place upon exocytosis. Triggering of the CD3/T-cell receptor complex by anti-CD3 mAb induced the release of approximately 40% of the total ^{35}S label incorporated into the cells. Inhibition of proteoglycan synthesis did not alter this value, since short

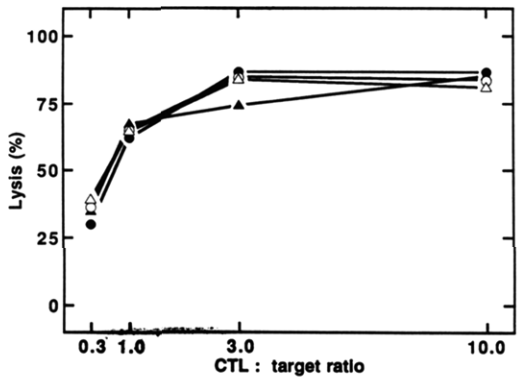

FIGURE 3: Effect of proteoglycan synthesis inhibition on the cytolytic activity of murine CTLs. Effector cells and ^{51}Cr-pulsed target cells (P815) were incubated at various effector to target ratios. Cells were cultured in the presence of PNP-xyloside during 4 days (○) and, in addition, during the 4-h cytotoxicity assay (●). Controls included experiments in which PNP-xyloside was only added during the cytotoxicity assay (▲) or at no time (△). Each value is shown as the percent of maximal chromium released. Spontaneous ^{51}Cr release was below 6%; maximal release amounted to 91%. Data represent mean values of three experiments.

glycosaminoglycan molecules were as efficiently released (Figure 2). Moreover, the kinetics of degranulation of [^{35}S]sulfate-labeled 860/4 cells grown with or without 2.5 mM PNP-xyloside were virtually identical (data not shown).

As it is impossible to follow exocytosis of perforin in vitro using the hemolysis assay system, we performed ^{51}Cr-release assays with intact effector 860/4 cells grown in the presence or absence of 2.5 mM PNP-xyloside using P815 mastocytoma as target cells. Killing of this target cell is strictly calcium dependent. We therefore assumed that perforin is the lytic principle of CTL clone 860/4 mediated cytolysis of P815 target cells [for an extensive discussion on that matter, see Clark et al., (1988)]. To exclude the possibility that PNP-treated cells were synthesizing new proteoglycans during the 4-h cytolytic assay, the assay was carried out in normal RPMI medium, as well as in medium with 2.5 mM PNP-xyloside. In neither case, however, was the cytotoxic activity altered relative to nontreated cells (Figure 3), suggesting that the absence of intact proteoglycans had no influence on the lytic activity of perforin.

Interaction of CTL Granule Proteins with Chondroitin Sulfate. Because intact proteoglycans seemed not to be crucial for the respective activities of perforin and the granzymes, we tested whether the granule components interacted with chondroitin sulfate at all. Earlier studies have already shown that perforin binds to chrondroitin sulfate A and to heparin (Tschopp & Masson, 1987). Since the A type of chondroitin sulfate is present in granules of CTL [see above and Kamada et al. (1989)], chondroitin sulfate A coupled to Sepharose was used to study the proteoglycan–granzyme interaction. Binding was assayed at different pH conditions supposedly prevailing in the granules or in the intercellular cleft upon exocytosis.

Cytotoxic granules are lysosome-like organelles (Peters et al., 1989), and it was postulated that their internal pH might be slightly acidic. Nevertheless, the pH of the cytolytic granules has not been determined to date. Since acidic organelles are capable of accumulating weak bases, the base DAMP was offered to CTL. DAMP contains amino groups that procure their retention by fixing glutaraldehyde and can be conveniently localized by immunocytochemistry (Schwartz et al., 1985; Anderson et al., 1984). As shown in Figure 4, granules of CTL clearly accumulate the bases, which illustrates their acidity.

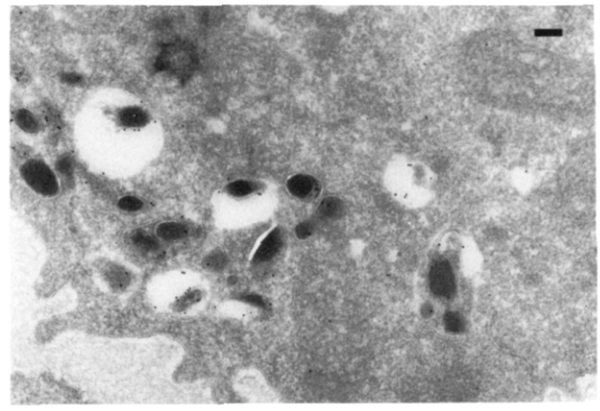

FIGURE 4: Determination of the internal pH of cytolytic granules of CTL. CTL were incubated for 30 min with DAMP and processed for immunolocalization of the weak base. DAMP is accumulated in cytotoxic granules, which illustrates granule acidity. The bar corresponds to 0.2 μm.

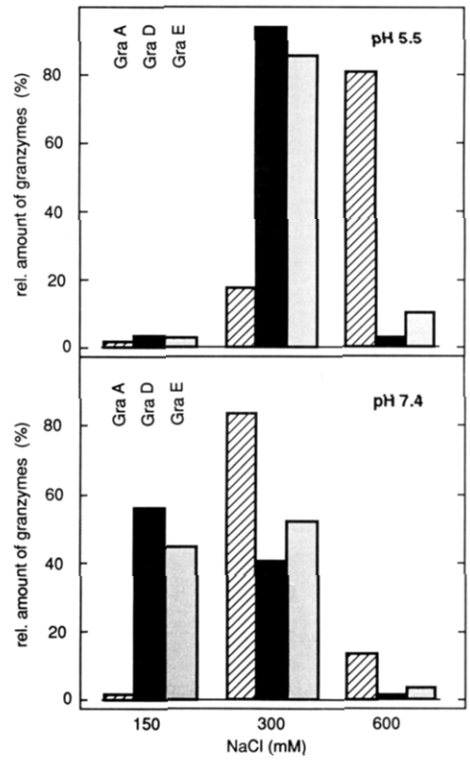

FIGURE 5: Binding of granzymes to chondroitin sulfate A–Sepharose column. Chondroitin A was coupled to CNBr–activated Sepharose. The column was equilibrated in a buffer containing 150 mM NaCl. The granzymes were loaded and eluted with a step salt gradient, 300 mM and 600 mM NaCl, at two different pH levels. The salt concentration with which the granzymes eluted from the column is indicated. Recoveries of granzymes A, D, and E were determined from silver-stained SDS–polyacrylamide gels. The total amount eluted for each granzyme was arbitrarily set to 100%.

At low pH (pH 5.5 was chosen in the experiment), all three granzymes (A, D, and E) tested were retained by the chondroitin sulfate A–Sepharose column at physiological ionic strength (150 mM NaCl). Granzyme D eluted first at increasing ion concentrations, whereas granzyme A eluted last (Figure 5). At neutral pH (pH 7.4), granzyme–proteoglycan interaction was much weaker. The major fraction of granzymes D and E already eluted at normal ionic strength. In contrast, granzyme A still required increased salt concentration for desorption (Figure 5).

We next analyzed the interaction of granzymes and perforin with endogenous T-cell-derived chondroitin sulfate A.

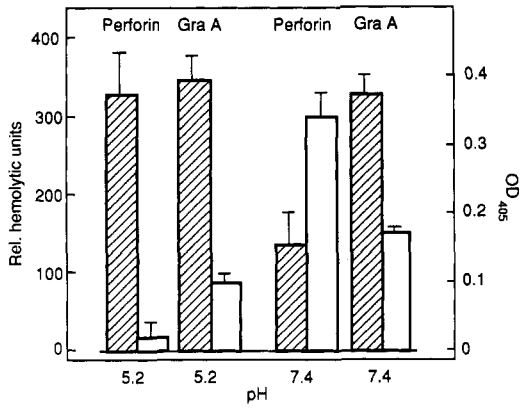

FIGURE 6: pH-dependent association of granule proteins. Soluble granule-associated proteins were equilibrated in a pH 5.2 or 7.4 buffer. The supernatant (50 μL, open bars) or pellet (50 μL, hatched bars) was assayed for relative granzyme A (OD_{405}) and perforin (hemolytic units) activity, respectively. Data are expressed as the mean ± SEM of three separate experiments.

Granules isolated from the 860/4 clone by nitrogen cavitation and subsequent Percoll density centrifugation were solubilized in PBS containing 1.5 M NaCl. This soluble fraction was freed from membranes and Percoll and thus basically contained a mixture of chondroitin sulfate, granzymes, and perforin. When this solubilized material was equilibrated in a buffer containing 150 mM NaCl at pH 5.2, aggregates of chondroitin sulfate, granzyme A, and perforin were formed that were large enough to be pelleted at 10000*g*. In contrast, at 150 mM NaCl, pH 7.4 (Figure 6), more than two-thirds of the perforin activity was detected in the supernatant, indicating the absence of large perforin–chondroitin sulfate complexes, whereas most of the granzyme A esterolytic activity was still found in the pellet. No aggregate formation was observed with purified granzyme A or perforin at 150 mM NaCl, pH 5.2 or 7.4 (data not shown), indicating that aggregation was induced by proteoglycans.

Does dissociation of perforin and granzymes from the proteoglycans occur upon degranulation when the granule content is exposed to a probably neutral environment within the intercellular cleft? To test this notion, CTL 860/4 cells were induced by anti-CD3 mAb to exocytose the granule contents into the supernatant, and the exocytosed material was first filtered on a Superose column in the presence of 1.5 M NaCl (Figure 7C) to induce protein–proteoglycan dissociation. Granzyme A activity eluted in accord with its molecular mass of 60 kDa. Chondroitin sulfate also filtered in the included volume, indicative of a molecule with a molecular mass of approximately 300–600 kDa. When the column was equilibrated in a buffer of physiological ionic strength, more than 80% of the granzyme A activity eluted as a macromolecular complex along with approximately 20% of the proteoglycans (Figure 7A). The remainder of the proteoglycan molecules filtered in the included volume. Thus at 150 mM NaCl, the major part of granzyme A is complexed to chondroitin sulfate, confirming the results obtained with the chondroitin sulfate–Sepharose column. Exocytosed material from PNP-xyloside-treated cells behaved (Figure 7B) similarly to non-treated cells, and most of the granzyme A activity again filtered as a macromolecular complex. $^{35}SO_4$-labeled glycosaminoglycans eluted as two broad peaks close to the total volume of the column. Unfortunately, perforin activity could not be followed after exocytosis, as already noted in earlier reports (Young et al., 1986b). Granzymes were shown to be confined to the electron-dense core of granules (Jenne et al., 1986; Burkhardt et al., 1989). By electron microscopy, this

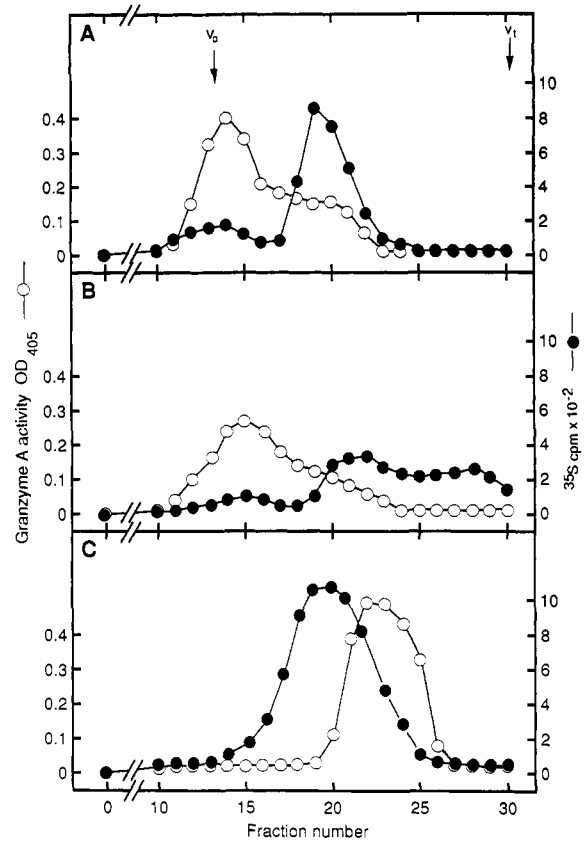

FIGURE 7: Superose 6 chromatography of granule components released onto the supernatant upon CD3 stimulation. A total of 10^6 CTLs were stimulated by cross-linked anti-CD3 antibody, and the exocytosed material was passed over a Superose 6 gel filtration column equilibrated in 150 mM NaCl (A and B) and in 1.5 M NaCl (C). In Figure 6B, CTLs had been treated with PNP-xyloside. Each fraction was analyzed for ^{35}S-labeled proteoglycans and for granzyme A activity. The void volume (v_0) corresponds to fractions 11–14. Purified granzyme A at neutral pH elutes in fractions 22–24. The total volume of the column (v_t) is indicated by an arrow.

core is often perceived with an enveloping membrane (Peters et al., 1989), and the question arose as to whether granzyme A–chondroitin sulfate cofiltration after exocytosis was, at least in part, due to the fact that molecules were contained within membrane vesicles. Incubation of exocytosed material with the nonionic detergent octyl glucoside failed to disrupt the complexes independent of previous PNP-xyloside treatment of the cells, since the elution profiles were identical with the ones shown in Figure 7A,B. We conclude from these results, first, that the proteoglycans form macromolecular complexes due to a direct interaction with granzyme A via ionic forces (we cannot exclude, however, that in addition the complex is stabilized by a surrounding membrane) and, second, that the short glycosaminoglycan molecules generated in the presence of PNP-xyloside suffice to cross-link the protease.

Discussion

We performed [^{35}S]sulfate labeling of the murine CTL clone 860/4 grown in the presence or absence of PNP-xyloside and analyzed the radioactive molecules found in the cytoplasmic granules. The control cells, grown in the absence of PNP-xyloside, contain a chondroitin sulfate A proteoglycan of 400-kDa molecular mass. After β-elimination, glycosaminoglycan chains are observed migrating with a molecular mass of approximately 60 kDa, indicating that the proteoglycan consists of one core protein plus six to eight proteoglycan molecules, assuming a peptide core of 15 kDa (Avraham et al., 1989). Human NK cells have been shown to contain

200-kDa proteoglycans bearing 50-kDa chondroitin sulfate A glycosaminoglycans (MacDermott et al., 1985). Rat large granular lymphocyte tumor cells synthesize 500-kDa proteoglycans containing chondroitin sulfate A glycosaminoglycans of 85 kDa (Stevens et al., 1987).

Although proteoglycan synthesis was completely inhibited in the presence of 2.5 mM PNP-xyloside, no effect on the activities of CTL proteins was observed. First, granzyme A and perforin were still sorted correctly into the granules. Second, granzyme A was normally released into the medium upon T-cell receptor stimulation, and its activity was not impaired. Moreover, intact cells treated with PNP-xyloside were as cytolytic as control cells in accordance with previous results (Christmas et al., 1989). Although PNP-xyloside effectively inhibits new synthesis of proteoglycans, it will not affect the proteoglycans synthesized prior to the addition of the drug. However, since the CTL cultures have gone through five divisions during the experiment, the preexisting chondroitin sulfate would have been diluted approximately 30-fold. If intact proteoglycans were necessary for one of the above mentioned functions, such a decrease in proteoglycan concentration should influence it.

Our results support the experiments reported by Burgess and Kelly (1984), who showed that intact proteoglycans are not required for the sorting of adrenocorticotropin and, consequently, that the core protein does not carry a sorting signal. However, we still consider it possible that chondroitin sulfate may be involved in sorting granule proteins toward the regulated pathway. The aggregation of chondroitin sulfate, perforin, and granzymes induced by the low pH, as shown in this report, may provide a sorting mechanism. The luminal milieu of the trans Golgi network, which is the intracellular site of secretory granule formation, is slightly acidic (Orci et al., 1986). It seems therefore conceivable that proteoglycans, particularly chondroitin sulfate A in the case of CTL, induce low pH dependent aggregation of proteins destined to be sorted into secretory granules. Membrane enwrapping of these aggregates would automatically result in the sorting of chondroitin sulfate A associated molecules from the constitutive pathway. A similar mechanism has been recently proposed for the sorting of secretory proteins in neuroendocrine cells (Gertes et al., 1989). In these cells, secretogranins spontaneously aggregate in a pH- and calcium-dependent manner. Correct sorting in CTLs will not be impaired by preventing proteoglycan synthesis with PNP-xyloside. Our results demonstrate that the short glycosaminoglycan molecules hooked onto the xyloside carrier still efficiently aggregate granzymes and perforin, and thus correct sorting will still ensue as shown in Figure 1.

Complex formation of granule components is highly pH dependent. Although the pH in the cleft between CTL and the target cell is unknown, it is likely to be neutral or only slightly acidic. Thus, if our in vitro results are extrapolated to in vivo conditions, it is conceivable that upon granule exocytosis complexes dissociate due to an increase of pH. Perforin and granzymes D and E may detach immediately from the proteoglycan core as suggested by the experiments shown in Figures 5 and 6, whereas granzyme A may still remain complexed in the extracellular milieu due to its increased affinity for chondroitin sulfate A. This may explain why, first, most of granzyme A is still found in a complexed form upon exocytosis and, second, that after conjugate formation with the target cell and degranulation of the cytotoxic granule in the cleft in addition to small vesicles a dark core corresponding to complexed proteins is found (Peters et al., 1990). Granzyme and perforin dissociation may be crucial for the exertion of their respective activities.

Our results are also fully compatible with the model recently proposed (Peters et al., 1990). In this model for lethal-hit delivery, the dense core is proposed to be released in a membrane-enveloped form into the cleft between killer and target cells, as evidenced by immunoelectron microscopy. As a result, granule components would be delivered in a macromolecular form, separated from the target cell by a membrane. Our results do not allow us to confirm or disprove this model by biochemical methods. Since proteoglycan–granzyme A interaction still occurs under conditions prevailing in the intercellular cleft, the macromolecular complexes seen after exocytosis are maintained independently of the presence of a membrane. Only the examination of the aggregation state of perforin would allow us to test the membrane enwrapping of the core, since the weak affinity of perforin for proteoglycan molecules predicts dissociation in the absence of a membrane. Unfortunately, we are unable to detect exocytosed perforin in a functionally active form. Clearly, further experiments are needed to clarify this issue.

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