Ultrastructural and Biochemical Characterization of Glycosaminoglycans in HNK-1-Positive Large Granular Lymphocytes

By Richard T. Parmley, Firoz Rahemtulla, Max D. Cooper, and Lennart Rodén

Natural killer (NK) cells are large granular lymphocytes (LGLs) that contain distinct lysosomal granules. The present study was undertaken to determine if these lysosomes contain glycosaminoglycans (GAGs) similar to those previously described in myeloid cells. Mononuclear cells from human blood were stained with HNK-1 fluoresceinated monoclonal antibody, and the NK cell population reactive with this antibody were isolated with a fluorescence-activated cell sorter (FACS). Specific staining of sulfated macromolecules with the cationic reagent, high iron diamine, was observed in the lysosomal granules of 90% of the HNK-1-positive cells. Staining in the same location was also observed in the unsorted LGLs, presumed to be NK cells, and intense staining of the cell surface was also a prominent feature of these cells. Surface staining was not evident in the majority of the FACS-separated NK cells. Digestion with chondroitinase ABC or treatment with nitrous acid reduced the staining in both locations; after sequential treatment with both chondroitinase and nitrous acid, little or no staining was seen. The presence of chondroitin sulfate (and/or dermatan sulfate) and heparan sulfate was also shown by the finding that incubation of the isolated NK cells with $^{35}$S-sulfate yielded cell-associated radiolabeled macromolecules with the characteristics of these two groups of GAGs. Of the labeled GAG pool, 60% was degraded by chondroitinase and 40% was susceptible to nitrous acid treatment. LGLs of a patient with Chediak-Higashi syndrome was also stained, and intracellular sulfation was clearly localized to the enlarged granules, supporting the conclusion that the lysosomes are the major site of intracellular accumulation of GAGs in normal NK cells.

© 1985 by Grune & Stratton, Inc.

MATERIALS AND METHODS

Blood samples. Human blood samples were obtained by venipuncture from normal volunteers after informed consent, as established by The Helsinki Declaration. The samples were collected in heparinized or citrated glass tubes. Mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation. The cells were plated on plastic dishes (Falcon, Oxnard, Calif) for depletion of adherent cells. NK cells were isolated with a FACS IV (Becton Dickinson, Mountain View, Calif) after staining of cells with the fluoresceinated HNK-1 monoclonal antibody (Leu-7, Becton Dickinson) as described previously. Preparations containing at least 96% HNK-1-positive cells were obtained with this methodology.

Ultrastructural cytochemistry. Suspensions of $10^4$ to $10^5$ lymphocytes in phosphate buffered saline (PBS) were pelleted by centrifugation in 1-mL glass tubes at 1,700 g for three minutes at 4°C. The cells were resuspended in H2O and adjusting the pH to 1.4 with 0.1 mol/L HC1. Some specimens were postfixed in 1% OsO4 in 0.1 mol/L cacodylate, pH 7.35. The samples were then rinsed three times in 0.1 mol/L cacodylate, 7% (wt/vol) sucrose, pH 7.35. In addition to normal lymphocytes, blood buffy coat samples from a child with Chediak-Higashi syndrome were similarly prepared.

Staining of sulfated macromolecules was carried out as a preembedding procedure using the high iron diamine (HID) method. The cells were stained for 18 hours at 22°C in HID solution (pH 1.4) prepared as described previously. Control specimens for evaluation of intrinsic density were incubated for 18 hours in an acid MgCl2 solution prepared by adding 1.4 mL of 40% MgCl2 to 50 mL H2O and adjusting the pH to 1.4 with 0.1 mol/L HC1. Some specimens were postfixed in 1% OsO4, samples were routinely dehydrated in graded alcohols, and embedded in Spurr low-viscosity medium (Ladd Research Industries, Burlington, Vt). HID staining was enhanced in unosmicated specimens by treating thin sections (50 to 70 μm) on stainless steel grids with thiocarbohydrazide and silver proteinate, as described previously. All sections were examined with a Phillips 300 or 301 electron microscope at an accelerating voltage of 60 kV.


From www.bloodjournal.org by guest on October 21, 2017. For personal use only.
LYMPHOCYTE GLYCOSAMINOGLYCANS

Prior to HID staining, some specimens were digested with chondroitinase ABC (Miles Laboratories, Elkhart, Ind) or treated with nitrous acid, according to procedures which have been described previously. In brief, digestion with chondroitinase ABC was carried out for 4 to 18 hours at 37 °C in 0.1 mol/L Tris-HCl/0.1 mol/L sodium acetate buffer, pH 7.35, at an enzyme concentration of 1 U/mL. For treatment with nitrous acid, approximately 10^6 cells were suspended in 1 mL of the pH 1.5 reagent of Shively and Conrad at 4 °C; after the sample had been kept at room temperature for three hours, the reagent was removed and replaced with cacodylate buffer. Control samples were incubated in buffer without enzyme or in 1 mol/L HCl. Some specimens were treated sequentially with chondroitinase ABC and nitrous acid.

Incorporation of [35S]-sulfate into proteoglycans. FACS-separated HNK-1-positive lymphocytes (10^6) were incubated for 12 hours at 37 °C in 1 mL of RPMI 1640 medium (GIBCO, Grand Island, NY), supplemented with 10% heat-inactivated donor serum, and were digested with 0.2 U of enzyme at 37 °C for four to eight hours. The cells were extracted as described above, while the medium was mixed with an equal volume of 8 mol/L guanidinium chloride, pH 7.2, and the void volume material was used directly for enzymatic digestions after addition of bovine serum albumin (BSA) to a final concentration of 0.1%. Radioactivity was measured by liquid scintillation counting with Scintiverse I (Fisher Scientific, Atlanta) as scintillant.

Chemical and enzymatic degradation of proteoglycans. Conditions for digestion with chondroitinase ABC or AC II were similar to those described by Saito et al. [14]. [35S]-labeled samples (2,000 to 5,000 cpm) were dissolved in 1 to 2 mL of 0.05 mol/L Tris-HCl, pH 7.2, containing 0.1% BSA, and were digested with 0.2 U of enzyme at 37 °C for four to eight hours.

Deaminative cleavage of N-sulfated glucosamine residues was carried out essentially as described by Shively and Conrad. To samples (2,000 to 3,000 cpm) of [35S]-labeled proteoglycan in 1 mL of distilled water was added nitrous acid, pH 1.5, until the pH of the mixture was 1.5 to 1.6; this usually required between 100 and 150 μL of reagent. The mixture was incubated at room temperature for one hour.

Product formation after enzymatic or chemical degradation was assessed by chromatography of the reaction mixtures on a column (2 × 20 cm) of Bio-Gel P-10, which was eluted with 0.2 mol/L ammonium bicarbonate.

RESULTS

Ultrastructural cytochemistry. Most cytoplasmic granules of FACS-separated NK cells were moderately to intensely stained with the HID-TCH-SP method, which allows visualization of sulfated macromolecules (Fig 1). A few granules lacked staining despite the presence of positive granules in the same cell profile. Some cytoplasmic vesicles near the Golgi region contained a few stain deposits, but Golgi lamellae lacked staining. Cell-surface staining was minimal or absent. HNK-1 negative lymphocytes lacked significant staining in the majority of cell profiles; however, these preparations contained numerous basophils that stained intensely. After treatment of NK cells with either nitrous acid or chondroitinase ABC, the staining intensity

Fig 1. Portions of two NK cells in a FACS-separated specimen are illustrated. HID-TCH-SP intensely stains sulfate, which is diffusely distributed in two lysosomal granules (large arrows). Some granules stain weakly or not at all (small arrows). Unosmicated specimen. Original magnification ×31,250; current magnification ×23,438.
was moderately reduced. However, after sequential treatment with both agents, little or no staining was seen (Fig 2). These results suggested that the granules of the NK cells contained heparan sulfate and galactosaminoglycans (chondroitin sulfates and/or dermatan sulfate).

Both granule and surface staining was present in morphologically identified LGLs in separated cell preparations not exposed to the HNK-1 antibody (Figs 3 and 4). The surface staining present on the majority of LGLs varied from weak to intense in both citrated and heparinized samples. Sequential chondroitinase ABC and nitrous acid treatment markedly reduced staining of the cytoplasmic granules and of the cell surfaces. A similar reduction of staining was not observed in control specimens that had been kept in buffer or HCl.

LGLs from a patient with Chediak-Higashi syndrome contained giant lysosomes that stained intensely with the HID-TCH-SP method (Fig 5A). This intense staining was also evident without TCH-SP enhancement in HID-stained preparations that had been postfixed in 1% OsO₄ (Fig 5B). HID-TCH-SP staining was performed on unosmicated specimens because some TCH-SP staining was present in osmiophilic material in granules of control preparations incubated in MgCl₂. TCH-SP staining was not observed in unosmicated control samples.

Incorporation of ³⁵S-sulfate into glycosaminoglycans. The ability of mature NK cells to incorporate ³⁵S-sulfate into glycosaminoglycans was examined in two experiments that are summarized in Table 1 and Fig 6. After
incubation of $10^6$ HNK-1-reactive cells with 0.5 mCi $^{35}$S-sulfate for 12 hours, a total of 7,000 to 9,000 cpm was found in macromolecular products separated by chromatography on Bio-Gel P-10. Under the conditions chosen, 70% to 80% of the products was secreted into the culture medium.

Characterization of the $^{35}$S-labeled products was carried out by digestion with chondroitinase ABC and deaminative cleavage by nitrous acid. As shown in Table 1, more than half of both medium-derived and cell-derived products was susceptible to digestion with chondroitinase ABC, and the remainder was degraded by HNO$_3$. (In one experiment, the total recovery of chondroitinase-susceptible and HNO$_3$-susceptible material in the cell-associated fraction was 131%, of which one third was degraded by HNO$_3$ and two thirds was degraded by chondroitinase.)

Rigorous identification of the chondroitinase ABC-susceptible GAG was not undertaken in view of the previous investigation, which has shown that unsorted human lymphocytes produce chondroitin-4-sulfate proteoglycans. However, analysis of the proteoglycans synthesized by unsorted
Table 1. Incorporation of 35S-Sulfate Into Glycosaminoglycans of NK Cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Radioactivity in Cells (cpm/10^6 Cells)</th>
<th>Radioactivity in Medium (cpm/10^6 Cells)</th>
<th>Total Radioactivity (cpm)</th>
<th>Chondroitinase ABC-Susceptible Cells (%)</th>
<th>Medium (%)</th>
<th>Cells (%)</th>
<th>Medium (%)</th>
<th>Nitrous Acid-Degradable Cells (%)</th>
<th>Medium (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,342</td>
<td>4,949</td>
<td>7,291</td>
<td>91</td>
<td>63</td>
<td>40</td>
<td>52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1,969</td>
<td>7,023</td>
<td>8,992</td>
<td>53</td>
<td>59</td>
<td>47</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

lymphocytes over a 48-hour period showed that 97% of the incorporated radioactivity was present in the medium, and that 81% and 84% of this fraction was degraded by chondroitinase AC and ABC, respectively. These findings confirm the chondroitin sulfate nature of the glycosaminoglycans and showed that little if any dermatan sulfate was present.

**DISCUSSION**

The present study has localized sulfated GAG in human NK cells using ultrastructural cytochemical and biochemical methods. Previous studies have demonstrated the presence of chondroitin sulfate in cloned lines of mouse lymphocytes with NK activity, using biochemical and radioautographic methods. Our results extend these observations to directly sampled normal human NK cells and localize both chondroitin sulfate and an N-sulfated GAG in the cytoplasmic granules and on the cell surface of NK cells. The identification of intense sulfate staining in the giant granules of LGLs from a patient with Chediak-Higashi syndrome in the present study further confirms the lysosomal nature of the GAG-stained granule and is consistent with previous observations of giant lysosomes in Chediak-Higashi NK cells. The intense staining of many NK cell granules appears to separate NK cells from other lymphoid cells, which usually lack intense lysosomal staining. Previous studies have similarly localized sulfated GAG in human granulocyte lysosomes but not in human monocytes. These myeloid cell lysosomes further resemble NK cell lysosomes in that at least some of the GAG staining appears to result from chondroitin sulfate and to a lesser extent from heparan sulfate, cytochemically and biochemically identified in these cells. In addition to these lysosomal similarities, NK cells and granulocytes also share specific surface antigens not found on other leukocytes. The shared features of these cells presumably reflect similar functions for these components within the cell.

The intensity of the cell-surface staining in many LGLs appears to be unique when compared with other leukocytes including myeloid cells. The loss of GAG staining after treatment with the HNK-1 antibody suggests either internalization of the cell surface GAG or masking of its reactivity by the antibody. The identification of the cell-surface-reactive LGL as NK cells is inferred since NK negative cells generally lack staining and the surface staining was associated with the intense LGL granule staining observed for FACS-separated NK cells. The ability to remove this staining with chondroitinase ABC and HNO₂ indicates that it is similar to the granule GAG and is probably endogenous in origin. Hid-TCH-SP staining has previously demonstrated heparan sulfate on the surface of hepatoma cells that appears to be covalently bound to the cell surface. Further studies of NK cells are required to determine whether the cell-surface GAG is covalently or ionically attached. The presence of highly anionic cell surface GAGs could conceivably influence the function of NK cells, especially their interaction with other cell types that lack similar sulfate staining.

The incorporation of 35SO₄ into isolated NK cells indicated that NK cells have the ability to synthesize at least some of the chondroitin sulfate and heparan sulfate cytologically identified in this study. Ultrastructural staining of sulfate in vesicles presumed to be Golgi-derived would support this observation. Release of label into the NK cell culture media in this study further suggests an extracellular role for at least some of the chondroitin sulfate and heparan sulfate, which could function as mediators in yet-to-be-identified immunologic reactions.

The ability to incorporate 35SO₄ into GAG is not unique to NK lymphocytes but has been reported for other mononuclear cells, including T and B lymphocytes. In monocytes,
the in vitro incorporation of $^{35}$SO$_4$ into GAG appears to increase with time. In contrast, in vivo studies have shown relatively little incorporation of $^{35}$SO$_4$ into lymphocytes and monocytes, in keeping with their general lack of sulfate staining. The failure to demonstrate sulfate staining in the majority of NK-negative cells in this study, despite the previously observed in vitro incorporation of $^{35}$SO$_4$ into T and B lymphocytes may indicate the accumulation of only a small amount of chondroitin sulfate that cannot be resolved with this staining method.

REFERENCES

19. Abo T, Roder JC, Abo W, Cooper MD, Balch CM: Natural killer (HNK-1)" cells in Chediak-Higashi patients are present in normal numbers but are abnormal in function and morphology. J Clin Invest 70:193, 1982
Ultrastructural and biochemical characterization of glycosaminoglycans in HNK-1-positive large granular lymphocytes

RT Parmley, F Rahemtulla, MD Cooper and L Roden