Developmental Regulation of a Mucinlike Glycoprotein Selectively Expressed on Natural Killer Cells

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Summary

Natural killer (NK) cells are CD3:TCR$^-$, CD16$^+$, CD56$^+$ large granular lymphocytes capable of recognizing and eliminating a variety of virus-infected, malignant, and antibody-coated target cells. Two functionally distinct populations of peripheral blood NK cells can be differentiated by their surface expression of an isoform of the neural cell adhesion molecule (CD56). CD56^{bright} NK cells have the attributes of an undifferentiated cell, in that they proliferate in response to exogenous cytokines, but exert poor cytolytic activity. CD56^{dim} NK cells have the attributes of a more differentiated cell, in that they proliferate poorly in response to exogenous cytokines, but are potent cytolytic effector cells. Here we describe the molecular characterization of a NK cell restricted epitope (PEN5) that is selectively expressed on the functionally differentiated CD56^{dim} NK cells. PEN5$^+$ NK cells proliferate poorly in response to interleukin 2 (IL-2), but are potent cytolytic effectors, whereas PEN5$^-$ NK cells proliferate in response to IL-2, but are poor cytolytic effectors. Biochemical and immunochemical analyses reveal the PEN5 epitope to be an unusual sulfated poly-*N*-lactosamine carbohydrate related to keratan sulfate glycosaminoglycans. Immunoprecipitates prepared using a monoclonal antibody reactive with PEN5 include two polydisperse membrane-bound glycoproteins, PEN5α (120–170 kD) and PEN5β (210–245 kD). Enzymatic deglycosylation reduces the apparent molecular weight of both PEN5 isoforms by 80–90%, and classifies PEN5β as a mucinlike glycoprotein. The surface expression of the PEN5 epitope is downmodulated by stimuli that induce NK cell proliferation, and it is absent from leukemic NK cells of patients with granular lymphocyte proliferative disorder. Taken together, these results indicate that PEN5 is a developmentally regulated poly-*N*-lactosamine epitope associated with a mucin-type glycoprotein, whose expression is restricted to the population of nonproliferative NK cells fully committed to cytolytic effector function.

NK cells are large granular lymphocytes (LGLs)[1] comprising 2–15% of PBMC in healthy individuals (1). Although NK cells do not rearrange or express either of the known antigen receptor complexes, they can recognize and kill a specific repertoire of virus-infected and transformed cells in a non-MHC–restricted fashion (2). With the exception of CD16, an Fc receptor for Ig that recognizes Ab-coated target cells (3), the NK cell surface receptors responsible for target cell recognition have not been identified. The lack of a defining surface receptor requires NK cells to be identified by a combination of phenotypic and functional characteristics. Although most peripheral blood NK cells are CD3:TCR$^-$, CD16$^+$, CD56$^+$ (neural cell adhesion molecule [N-CAM]) LGL, there is substantial phenotypic and functional heterogeneity within this population (1). For example, the surface density of CD56 has been shown to define functionally distinct NK cell populations. CD56^{bright} NK cells are largely CD16$^-$, agranular lymphocytes deficient in cytolytic effector function, which proliferate vigorously in response to IL-2 and resemble embryonic/fetal NK cells (4–6). By contrast,

[1] *Abbreviations used in this paper:* ADCC, antibody-dependent cell cytotoxicity; APC, allophycocyanine; BC, bovine cornea keratan sulfate; BNC, bovine nasal cartilage aggrecan; CD1, embryonic chick cartilage aggrecan; GlcNac, *N*-acetyl glucosamine; GLPD, granular lymphocyte proliferative disorder; LCM, leucocyte-conditioned medium; LGL, large granular lymphocyte; N-CAM, neural cell adhesion molecule; PNgase, peptide-*N* glycosidase.

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$CD56^{dim}$ NK cells are $CD16^+$ LGL possessing potent cytolytic effector function, which proliferate very poorly in response to IL-2 (4, 5). Because CD16 and CD56 are not restricted to the lymphocyte population (1), and a T cell subset also expresses both CD16 and CD56 (1), these molecules cannot define, by themselves, the NK cell population.

To identify cell surface structures more selectively expressed on NK cells, we generated a panel of mouse mAb reactive with freshly isolated human NK cells. In this article, we report the molecular characterization of PEN5, a sulfated poly-*N*-lactosamine epitope whose expression is restricted, within hematopoietic cells, to the functionally differentiated population of LGL previously characterized as $CD3^-TCR^-$, $CD56^{dim}$, $CD16^+$ cytolytic effectors. Because PEN5 is not expressed on activated cytotoxic T cells, it can be used to directly identify this important NK cell subset. We show that $PEN5^+$ NK cells are potent cytolytic effectors that proliferate poorly in response to IL-2, whereas $PEN5^-$ NK cells are less potent cytolytic effectors that proliferate strongly in response to IL-2. The developmental regulation of PEN5 expression suggests that its presence may be required for some aspect of NK cell function.

Materials and Methods

Reagents. Peptide-*N*-glycosidase (PNgase F) and Endo-α-*N*-acetylgalactosaminidase (*O*-glycanase) were used in the buffer provided by the manufacturer (Oxford Glycosystems). Keratanase I (keratan sulfate 1,4 β-D-galactanohydrolase; ICN Biomedicals, Cleveland, OH), keratanase II which attacks oversulfated forms of keratan sulfate resistant to keratanase I (Seikagaku America, Inc., Rockville, MD) and neuraminidase (Calbiochem-Novabiochem Corp., La Jolla, CA) were used in either PBS, PNgase F or *O*-glycanase buffers. Chondroitinase ABC (ICN Biomedicals) was used in sodium acetate 0.05 M, pH 7.4. Bovine cornea keratan sulfate (BC), as well as other glycoaminoglycans and carbohydrates, were purchased from Sigma. Trypsin, chymotrypsin, pronase E and streptavidin were also obtained from Sigma Chemical Co. (St. Louis, MO). Allophycocyanine (APC) was obtained from Molecular Probes, Inc. (Eugene, OR).

Antibodies. Mouse mAb reactive with CD2 (SFCI3Pt2H9, IgG1), CD3 (SFCIRW2–8C8, IgG1), CD56 (N901, IgG1), and CD20 (H299, IgG2a), as well as isotype-matched control mouse mAb (IgG and IgM) were obtained from Coulter Electronics (Hialeah, FL). The anti-PEN5 mAb (5H10, IgM) was produced by immunizing BALB/c mice with digitonin-permeabilized peripheral blood NK cells (7). Radioiodination of PEN5 mAb was performed using Iodobeads (Pierce, Rockford, IL) as previously described (8). The characterization of the anti-CD16 mAb (3G8, IgG1), and the anti-keratan sulfate mAb 5D4 (IgM) was reported elsewhere (9, 10). The following mAb recognize distinct epitopes on most keratin sulfate chains: 1B4 (IgG), 2D3 (IgG), 3D2 (IgM), 4D1 (IgM), and 8C2 (IgM) (11). FITC-labeled goat anti-mouse Ig (G+M) was purchased from Tago Inc. (Burlingame, CA).

Cells. All cells were cultured in final medium consisting of RPMI 1640 (Whittaker Bioproducts, Walkersville, MD) supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM glutamine, and 50 μg/ml gentamycin, all obtained from GIBCO BRL (Gaithersburg, MD). Peripheral blood samples were obtained from healthy volunteers. Purified NK and T cells were isolated from PBMC by negative selection using immunomagnetic bead depletion (12). In some experiments, NK cells and cell subsets ($CD56^{bright}$ and $CD56^{dim}$) were further purified by cell sorting on an Epics V flow cytometer (Coulter Electronics) after staining with anti-CD56 mAb. Activation of NK cells was as described previously (13). PBMC from three patients with a $CD3^-TCR^-$, $CD16^+$, $CD56^+$ granular lymphocyte proliferative disorder (GLPD) (14), were isolated by Ficoll-Hypaque gradient centrifugation. The NK cell line NKL was derived from one of these patients (Robertson, M. J., unpublished results). Splenic B cells were isolated and activated as described (15). The isolation of peripheral B cells, monocytes, and thymocytes was performed using standard methods (16–18). Peripheral blood platelets were kindly obtained from Dr. E. Weinman (Beth Israel Hospital, New York). RBC were obtained from peripheral blood, and granulocytes were purified by Ficoll-Hypaque gradient centrifugation and RBC lysis. T4C1 and T4T8C1 CTL clones, as well as YT.N17, 3.3 NK cell lines, have been described elsewhere (19–22). Other allogenic CTL clones were generated by limiting dilutions in rIL-2–containing final medium, after 8 d of MLR between PBL (5×10^6/ml) and the EBV-transformed lymphoblastoid cell line JY (10^6/ml).

Immunoprecipitations. Cells were resuspended in PBS and subjected to radioiodination using ^{125}I by the lactoperoxidase method (23). Sepharose-bound immune complexes were washed four times in lysis buffer, and eluted either directly into sample buffer (2% SDS, 10% glycerol, 0.1 M Tris-HCl, pH 6.8, 0.02% bromophenol blue) before electrophoretic separation, or in elution buffer (0.15 M NH_4OH, pH 10.5) before deglycosylation experiments.

Deglycosylation of Radioiodinated PEN5 Glycoproteins. Radioiodinated PEN5 samples eluted from 5H10-coated Sepharose beads were dried under vacuum and resuspended in appropriate deglycosylated enzyme buffers. The following enzymes were used alone or in combination: PNgase F (310 U/ml), *O*-glycanase (0.06 U/ml), keratanase I (0.25 U/ml), and neuraminidase (0.2 U/ml). Deglycosylation was for 24 h at 37°C.

ELISA for Aggrecan-type Proteoglycans. Wells of microtiter plates were incubated with 10 μg/ml solutions of the indicated aggrecan-type proteoglycans overnight at 4°C. After washing, wells were incubated with 0.1 M Tris, pH 7.6, containing 1% BSA, or with the indicated enzymes in this buffer. After enzymatic digestion, a standard ELISA was performed using 1:1,000 dilution of 5H10 (anti-PEN5) and 5D4 (anti-keratan sulfate) mAb, and 1:500 dilution of anti–mouse Ig(G+M) conjugated with alkaline phosphatase. Color was developed using *p*-nitrophenyl phosphate substrate in 0.86 M diethanolamine, pH 9.8. All absorbance values are the mean of 4 wells (SD <10%) and have been corrected for nonspecific binding of the second Ab.

NK Cell Cytotoxicity and Proliferation Assays. NK cells were prepared from PBMC by negative selection using immunomagnetic beads (12). These cells were double labeled with rhodamine-conjugated 5H10 (anti-PEN5) and FITC-conjugated anti-CD3 and sorted on an Epics V flow cytometer (Coulter Electronics) to isolate $CD3^-PEN5^+$ and $CD3^-PEN5^-$ subsets. Post-sort analysis confirmed that the selected markers were >98% present or absent from the indicated population. These cells were then assayed for target cell killing and IL-2–mediated proliferation using standard methods (5). The reported stimulation index was calculated as: [average cpm (IL-2 cultures)/average cpm (media cultures)]. An identical analysis was performed using NK cells labeled first with anti-CD56 (NKH1, IgG1) followed by FITC-labeled goat anti–mouse IgG, and second with rhodamine-conjugated 5H10 (anti-PEN5). These cells were sorted for $CD56^+PEN5^+$ and $CD56^+PEN5^-$ populations, and assayed for cytotoxicity and proliferation as described above.

Results

Expression of PEN5 on Hematopoetic Cells. To identify novel cell surface structures selectively expressed on NK cells, we generated a panel of mouse mAb (anti-PEN mAb) that recognized NK cells but not T cells. The hematopoietic expression of one of these mAb (5H10, anti-PEN5) was found to be restricted to NK cells. As shown in Fig. 1, two-color flow cytometric analysis of PBL revealed PEN5 to be expressed on the majority of $CD56^+$ (Fig. 1, *upper left*) and $CD16^+$ (Fig. 1, *upper right*) PBL. In contrast, PEN5 was not expressed on $CD3^+$ T cells (Fig. 1, *lower left*) or on $CD20^+$ B cells (Fig. 1, *lower right*). Neither T cell activation induced by mitogenic concentrations of PHA nor Con A, nor B cell activation induced by *Staphylococcus aureus* Cowan strain I, for 1–6 d, induced the cell surface expression of the PEN5 epitope (Table 1). Similarly, a panel of allogeneic cytotoxic T cell clones ($CD3^+CD4^+CD56^-$, $CD3^+CD8^+CD56^-$ as well as $CD3^+CD4^+CD56^+$) did not express the PEN5 epitope (Table 2). Cell surface staining of monocytes, granulocytes, platelets, and erythrocytes also failed to reveal the PEN5 epitope (Table 1), confirming that PEN5 is an NK cell–restricted molecule. To more precisely analyze the expression of PEN5 on NK cells, flow cytometric analysis of PEN5 expression was performed on freshly isolated peripheral blood NK cells purified by negative selection using immunomagnetic bead depletion (see Materials and Methods). PEN5 was brightly expressed on 71.7 ± 3.5% (mean ± SEM, n = 16) of these NK cell preparations whose average phenotype was 75.6 ± 3.3% $CD56^+$, 74.2 ± 4.0% $CD16^+$, and 8.3 ± 3.5% $CD3^+$.

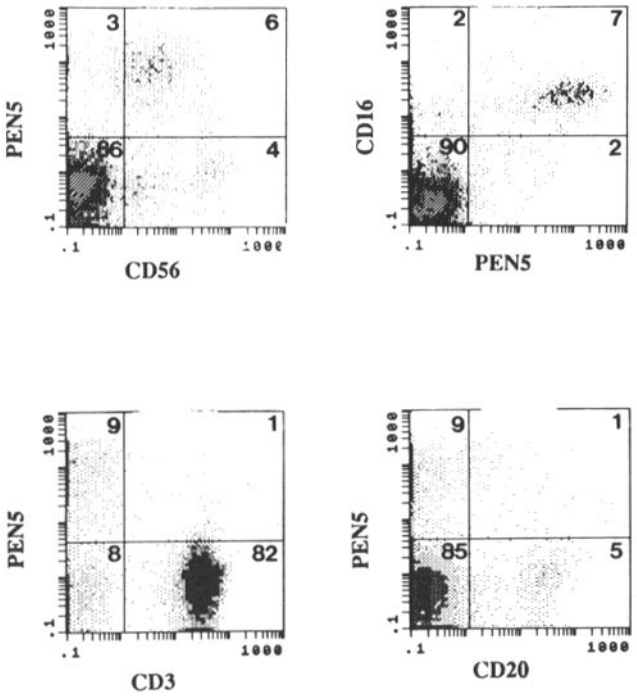

Figure 1. PEN5 expression on PBL. PBL were stained by two-color flow cytometry using the indicated mAbs. Numbers in each quadrant indicate the percentage of positively stained cells.

Table 1. *Cell Surface Expression of PEN5 on Hematopoietic Cells*

Cell type	Relative expression
Peripheral blood T cells	−*
Activated T cells‡	−
Thymocytes	−
Peripheral blood NK cells	+ +
NK cell lines: YT.N17	−
3.3	±
NKL	−
Peripheal blood B cells	−
Splenic B cells	−
Activated B cells§	−
Monocytes	−
Granulocytes	−
Platelets	−
RBC	−

* The cell surface expression of 5H10 was assessed by indirect immunofluorescence and flow cytometry; (−) <5% positive stained cells; (±) between 5 and 35% positive-stained cells; (+ +) >60% positive stained cells.

‡ Peripheral blood T cells were activated with optimal mitogenic concentrations of PHA and Con A, and immunofluorescence staining was performed at days 2, 4, and 6 after activation.

§ Splenic B cells were activated with optimal mitogenic concentrations of *Staphylococcus aureus* Cowan strain I, and immunofluorescence staining was performed at days 2, 4, and 6 after activation.

The phenotypic heterogeneity of peripheral blood NK cells required a more careful comparison of the relative expression of PEN5 and CD56. The two-color flow cytometric comparison shown in Fig. 1, suggested that PEN5 was preferentially expressed on the $CD56^{dim}$ population. This was confirmed by comparing the expression of PEN5 on sorted populations of $CD56^{dim}$ and $CD56^{bright}$ NK cells (Fig. 2). PEN5 was expressed at a high density on 85.9 ± 2.2% of $CD56^{dim}$ NK cells (n = 4), and at low density on 31.1 ± 5.3% of $CD56^{bright}$ NK cells. These results indicate that high density cell surface expression of the PEN5 epitope is restricted to the functionally differentiated $CD56^{dim}$ NK cells. These results also indicate that the cell surface expression of PEN5 defines two distinct subsets of NK cells, $PEN5^+$ and $PEN5^{dim/-}$ which overlap with the $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets, respectively. Because the relative expression of CD56 on the surface of NK cells has been shown to correlate with their proliferative capacity and cytolytic effector function (4), we compared the ability of highly purified populations of $PEN5^+$ and $PEN5^-$ NK cells to proliferate in response to IL-2 and effect natural cytotoxicity. Mononuclear cells enriched for NK cells by magnetic bead

Table 2. *Absence of Surface Expression of PEN5 on Cytotoxic T Cell Clones*

Clone	Cell surface expression					
	CD3	CD2	CD4	CD8	CD56	PEN5
T4Cl	+*	+	+	−	−	−
6.5 B4	+	+	+	−	−	−
6.5 C1	+	+	+	−	−	−
20.1 A2	+	+	+	−	−	−
8.17 A	+	+	+	−	+	−
20.1 D8	+	+	−	+	−	−
T4T8Cl	+	+	+	+	−	−

* The cell surface phenotype of the indicated T cell clones was performed by immunofluorescence and flow cytometry. (−) <5% positive stained cells; (+) >60% positive-stained cells.

depletion (see Materials and Methods) were sorted into PEN5$^+$ and PEN5$^-$ cell populations that were either CD3$^-$ (Fig. 3, *top*) or CD56$^+$ (Fig. 3, *bottom*). In both cases, PEN5$^+$ NK cells killed K562 and Molt-4 targets more effectively than PEN5$^-$ NK cells. Conversely, the IL-2 induced proliferation of PEN5$^-$ cells (stimulation index = 14 ± 6 [n = 3] in CD3$^-$ sorted cells and 14 ± 8 in CD56$^+$ sorted cells) was significantly greater than that of PEN5$^+$ cells (Stimulation index = 2.3 ± 1.7 [n = 3] in CD3$^-$ cells and 3.1 ± 1.7 in CD56$^+$ cells). These results establish the phenotypic and functional similarities between PEN5$^+$ cells and CD56^{dim} cells, and PEN5$^-$ cells and CD56^{bright} cells, respectively.

PEN5 Expression Is Downregulated by NK Cell Activation. CD56^{dim} and CD56^{bright} NK cells strongly differ in their response to proliferative stimuli (4, 5, 24): CD56^{dim}

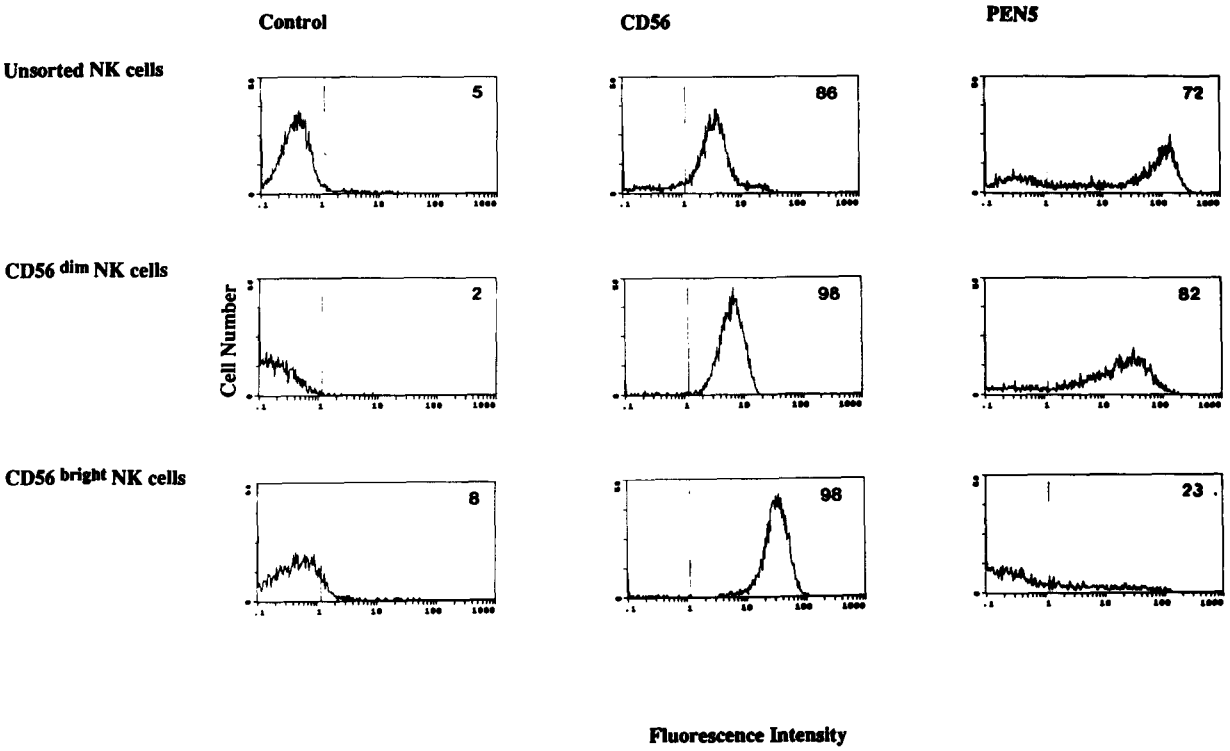

Figure 2. Expression of PEN5 on distinct NK cell subsets. Unsorted NK cells, or sorted CD56^{dim} and CD56^{bright} NK cells were analyzed for the expression of 5H10 using biotinylated anti-PEN5 mAb and APC-conjugated streptavidin. Controls were performed using mouse isotype-matched control mAb. The numbers in each histogram indicate the percentage of positively stained cells.

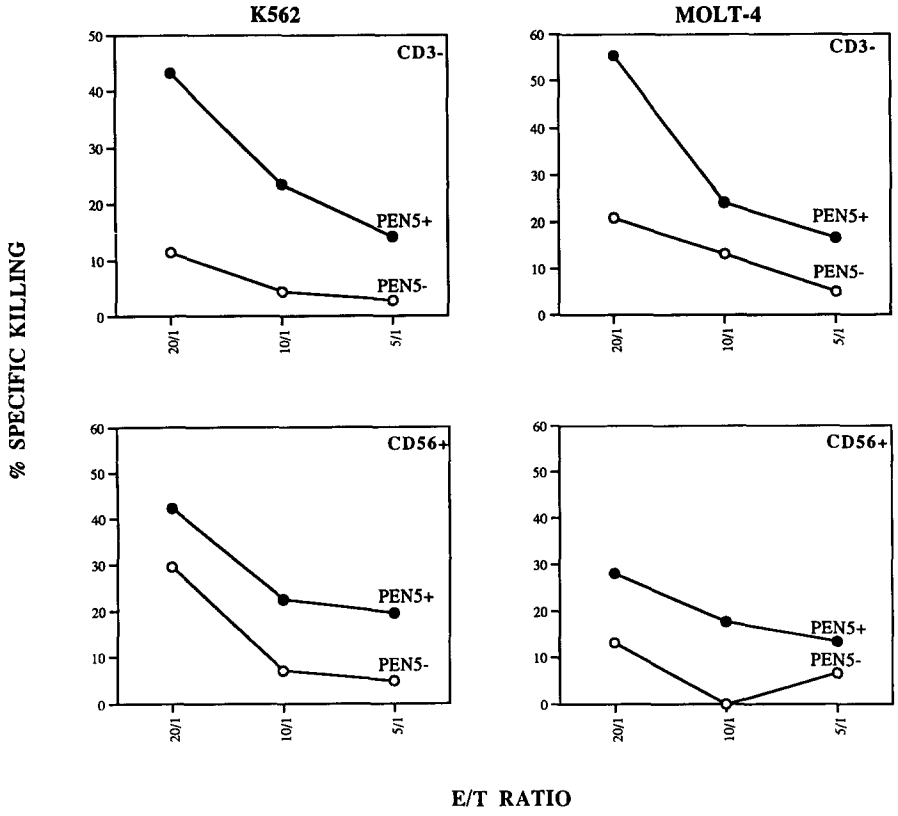

Figure 3. Natural cytotoxicity mediated by purified populations of PEN5$^+$ and PEN5$^-$ NK cells. (*Top*) NK cells were sorted for CD3$^-$PEN5$^-$ (O) and CD3$^-$PEN5$^+$ (●) populations. (*Bottom*) NK cells were sorted for CD56$^+$PEN5$^-$ (O) and CD56$^+$PEN5$^+$ (●) populations. Results are representative of three independent experiments using different donors.

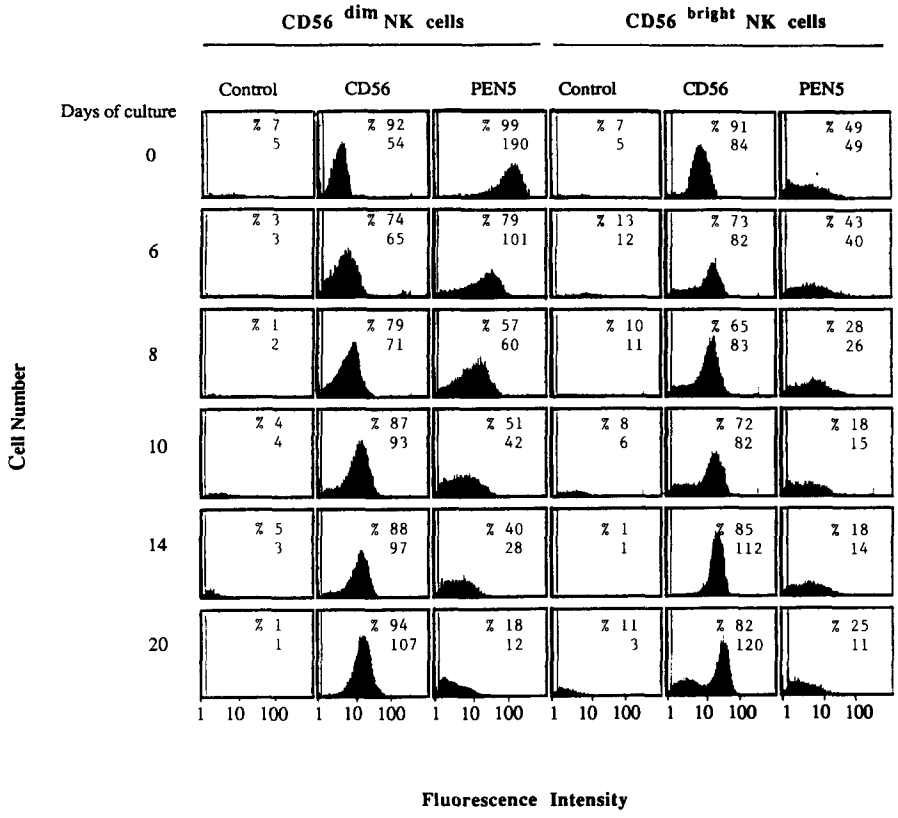

Figure 4. Kinetics of PEN5 expression on activated NK cells. Sorted CD56^{dim} and CD56^{bright} NK cells were activated for 20 d with ionomycin and LCM as described (13). At the indicated times, aliquots of the activated NK cell populations were analyzed for their cell surface phenotype by flow cytometry using isotype-matched control mAb, anti-CD56 and 5H10 mAb. Results indicate the percentage of positively stained cells (%); the total mean fluorescence intensity is indicated below.

NK cells proliferate very poorly in response to IL-2, whereas CD56^{bright} NK cells proliferate in response to this stimulus. However, CD56^{dim} NK cells can be induced to proliferate in response to a combination of LCM and ionomycin (24). We took advantage of this observation to correlate PEN5 expression with the proliferative state of sorted CD56^{dim} and CD56^{bright} NK cells. The cell surface expression of CD56 and PEN5 was determined on both NK cell subsets by flow cytometic analysis at the indicated time intervals after activation (Fig. 4). Activation of CD56^{dim} NK cells resulted in the temporal reduction of PEN5 expression. In parallel, the cell surface expression of CD56 was temporally increased, and after 20 d of activation, the cell surface expression of PEN5 and CD56 on the CD56^{dim} NK cells was largely similar to that of unactivated CD56^{bright} NK cells (i.e., PEN5$^{dim/-}$ and CD56^{bright}). These results are consistent with the absence of PEN5 from the cell surface of long-term human NK cell clones (Moretta, A., personal communication). In addition, PEN5 was not expressed on leukemic NK cells (CD3$^-$TCR$^-$, CD16$^+$, CD56$^+$) isolated from patients with GLPD (Fig. 5). Finally, PEN5 was absent or dimly expressed on three long-term human NK cell lines, YT.N17, 3.3, NKL (Table 1). These results indicate that PEN5 expression inversely correlates with the proliferative capacity of NK cells.

Biochemical Characterization of the PEN5 Epitope. Radioiodinated lysates prepared from resting NK cells were immunoprecipitated using the 5H10 (anti-PEN5) mAb or an isotype-matched control mAb. Immunoprecipitates were then separated under nonreducing conditions on SDS-polyacrylamide gels. As shown in Fig. 6, two diffuse bands were selectively immunoprecipitated by the 5H10 mAb. The average molecular weight of the larger species, PEN5α, was 227 ± 4 kD (n = 12 different donors). The molecular weight range of the polydispersed PEN5α species was 210 ± 3–245 ± 5 kD. The average molecular weight of the smaller species, PEN5β was 140 ± 3 kD, with a range of 123 ± 3 to 170 ± 4 kD. The migration of both PEN5α and β molecules as polydisperse bands suggested that they were highly glycosylated. This was confirmed in the deglycosylation experiments shown in Fig. 7. PEN5α and β were affinity purified from radioiodinated NK cell lysates using anti-PEN5 mAb and eluted as described in Materials and Methods. The eluted glycoproteins were treated with the indicated enzymes before electrophoretic separation on an SDS-polyacrylamide gel. Compared with the migration of untreated PEN5 glycoproteins (Fig. 7, lane *1*), PNgase F treatment induced the disappearance of PEN5α from the 210–245-kD range, and the appearance of a deglycosylated form of PEN5α migrating at 20–25 kD (Fig. 7, lane *6*, *c2*). In contrast, the apparent mobility of PEN5β was reduced by only ~20 kD after PNgase F incubation. Treatment of PEN5 glycoproteins with *O*-glycanase (Fig. 7, lane *3*) did not significantly affect their SDS-PAGE migration pattern. These results indicate that the PEN5α and PEN5β differ markedly in their carbohydrate attachment sites, and that 80–90% of the apparent molecular weight of PEN5α is due to N-linked carbohydrates. Although the recognition of PEN5 in Western blots was weak, the epitope was expressed on both PEN5α and PEN5β (data not shown).

The extensive N-linked glycosylation of PEN5α suggested that it might be a member of one of the classes of glycoproteins characterized by such high carbohydrate content (50–90%), such as proteoglycans. Affinity purified radioiodinated PEN5α and β molecules were digested by enzymes that cleave the

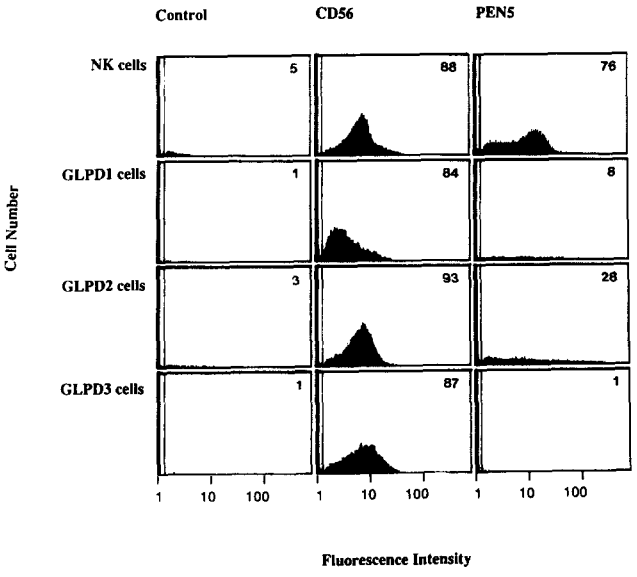

Figure 5. Cell surface expression of PEN5 epitope on leukemic NK cells. Peripheral blood NK cells, as well as PBMC isolated from three patients undergoing GLPD blast crisis (GLPD1–3) were analyzed for the cell surface expression of CD56 and PEN5 using indirect immunofluorescence and flow cytometry. The numbers in each histogram indicate the percentage of positively stained cells.

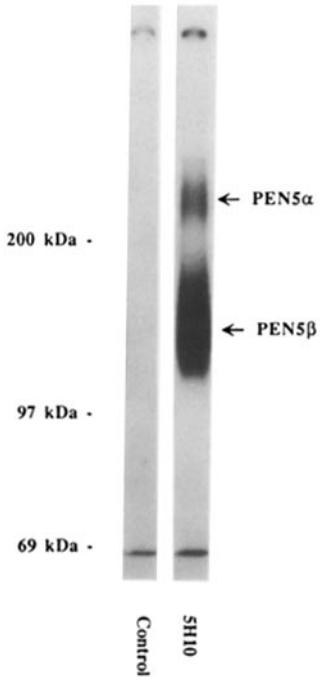

Figure 6. Immunoprecipitation of PEN5 glycoproteins. Detergent lysates prepared from radioiodinated NK cells were immunoprecipitated using 5H10 mAb or mouse IgM control. Samples were then separated under nonreducing conditions on a 6% SDS-polyacrylamide gel.

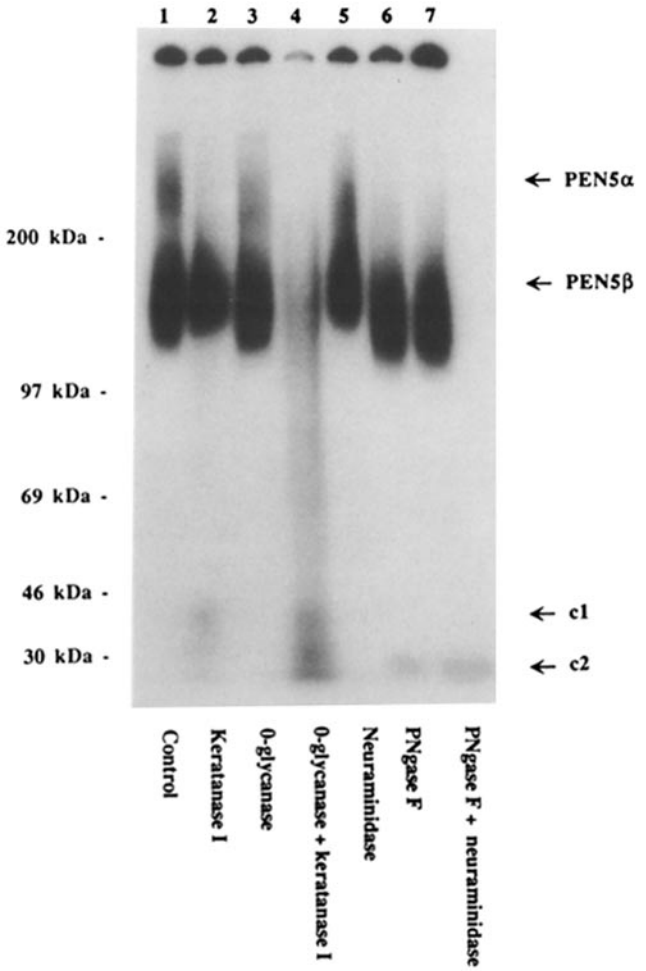

Figure 7. Enzymatic deglycosylation of PEN5 glycoproteins. Affinity-purified PEN5α and β glycoproteins were eluted from antibody-coated Sepharose beads, then subjected to deglycosylation for 24 h at 37°C using PNgase F (lane *6*), O-glycanase (lane *3*), keratanase I (lane *2*), O-glycanase and keratanase (lane *4*), neuraminidase (lane *5*), and PNgase F and neuraminidase (lane 7) and appropriate buffers. Control eluates incubated in PBS without any enzymes were separated in lane *1*. Samples were separated under nonreducing conditions on a 6–12% SDS-polyacrylamide gradient gel.

glycosaminoglycan side chains from proteoglycan core proteins. Chondroitinase ABC, heparitinase and heparinase did not affect the migration pattern of PEN5α or PEN5β (data not shown). By contrast, incubation of PEN5 molecules with keratanase I reduced the apparent molecular weight of PEN5α from 210–245 to 35–40 kD (Fig. 7, lane *2*, *c1*). It is likely that the difference between the PNgase F-digested (35–40 kD) c1 core protein (Fig. 7, lane *2*) and the keratinase-digested (25–30 kD) c2 core protein (Fig. 7, lane *4*), results from a more complete deglycosylation of the PEN5α glycoprotein. Whereas keratanase treatment only slightly reduced the polydispersity of PEN5β, the combination of O-glycanase and keratanase I treatment reduced the apparent molecular weight of PEN5β from 120–170 to 25–30 kD (Fig. 7, lane *4*). Taken together, these results indicate that PEN5α contains 80–90% N-linked keratanase I–sensitive carbohydrates, whereas PEN5β contains 80–90% O-linked carbohydrates and keratanase I–sensitive chains. This extensive O-linked glycosylation is consistent with PEN5β being a mucinlike glycoprotein. In addition, treatment with neuraminidase induced a slight reduction in the polydispersity, as well as a shift in the apparent molecular weight of both PEN5α and β, indicating that sialic acid residues are also present on both glycoproteins (Fig. 7, lane *5*). Treatment of PEN5 glycoproteins with a combination of PNGase F and neuraminidase (Fig. 7, lane 7), resulted in the same effect that PNGase F had alone, confirming the presence of terminal sialic acid residues on N-linked carbohydrates present on PEN5α. The c1 and c2 deglycosylated forms of PEN5α and β proteins were not immunoprecipitable by the 5H10 mAb (data not shown), indicating that the epitope recognized by the anti-PEN5 mAb contains keratanase I–sensitive carbohydrate chains.

Reactivity of Anti-PEN5 mAb with Keratan Sulfate Glycosaminoglycans. To test whether the anti-PEN5 mAb was directly raised against keratan sulfate carbohydrates, we examined the effect of exogenous keratan sulfate carbohydrates on the binding of PEN5 mAb to NK cells. Radioiodinated 5H10 (anti-PEN5) mAb was combined with various concentrations of BC, and the mixture was then incubated with NK cells. As shown in Fig. 8 *A*, the binding of radiolabeled 5H10 mAb to NK cells was inhibited by BC keratan sulfate in a dose-dependent manner. Preincubation of NK cells with the same concentrations of BC keratan sulfate followed by washes, did not affect the binding of 5H10 mAb (data not shown), indicating that the anti-PEN5 mAb reacted with carbohydrate determinants present on keratan sulfate glycosaminoglycans. Incubation of anti-PEN5 mAb with simple sugars or other glycosaminoglycans was without any effect (see legend to Fig. 8). Furthermore, treatment of NK cells with keratanase I induced a 58.5% ± 8.4 (n = 4) decrease in the reactivity of 5H10 mAb with NK cells (Fig. 8 *B*). Parallel treatment of NK cells with chondroitinase ABC or neuraminidase did not have any effect on 5H10 reactivity. Interestingly, the 5H10 epitope was totally insensitive to trypsin and chymotrypsin but was removed by pronase E treatment. Finally, an ELISA was used to compare the binding of 5H10 (anti-PEN5) and 5D4 (anti-keratan sulfate) to the keratan sulfate/chondroitin sulfate proteoglycans expressed in cartilage from different species. As shown in Fig. 8 *C* (*top*), the 5H10 mAb (*solid bars*) recognized aggrecan-type proteoglycans derived from embryonic chick cartilage (*CD1*, *top*) and from bovine nasal cartilage (*BNC*, *middle*). As a positive control, the anti-keratan sulfate mAb 5D4 (Fig. 8, *open bars*) also reacted with untreated CD1 and BNC, whereas its reactivity with keratanase-treated samples was reduced. Treatment of CD1 and BNC with either keratanase I or II, reduced 5H10 reactivity. Treatment of CD1 and BNC with chondroitinase ABC is known to increase the expression of keratan sulfate epitopes (11). Consequently, digestion of CD1 and BNC with chondroitinase ABC increased the binding of both 5H10 and 5D4. As a negative control, neither mAb recognized the Swarm rat chondrosarcoma aggrecan (RC), which does not contain keratan sulfate (Fig. 8 *C*, *bottom*). Although 5D4 also

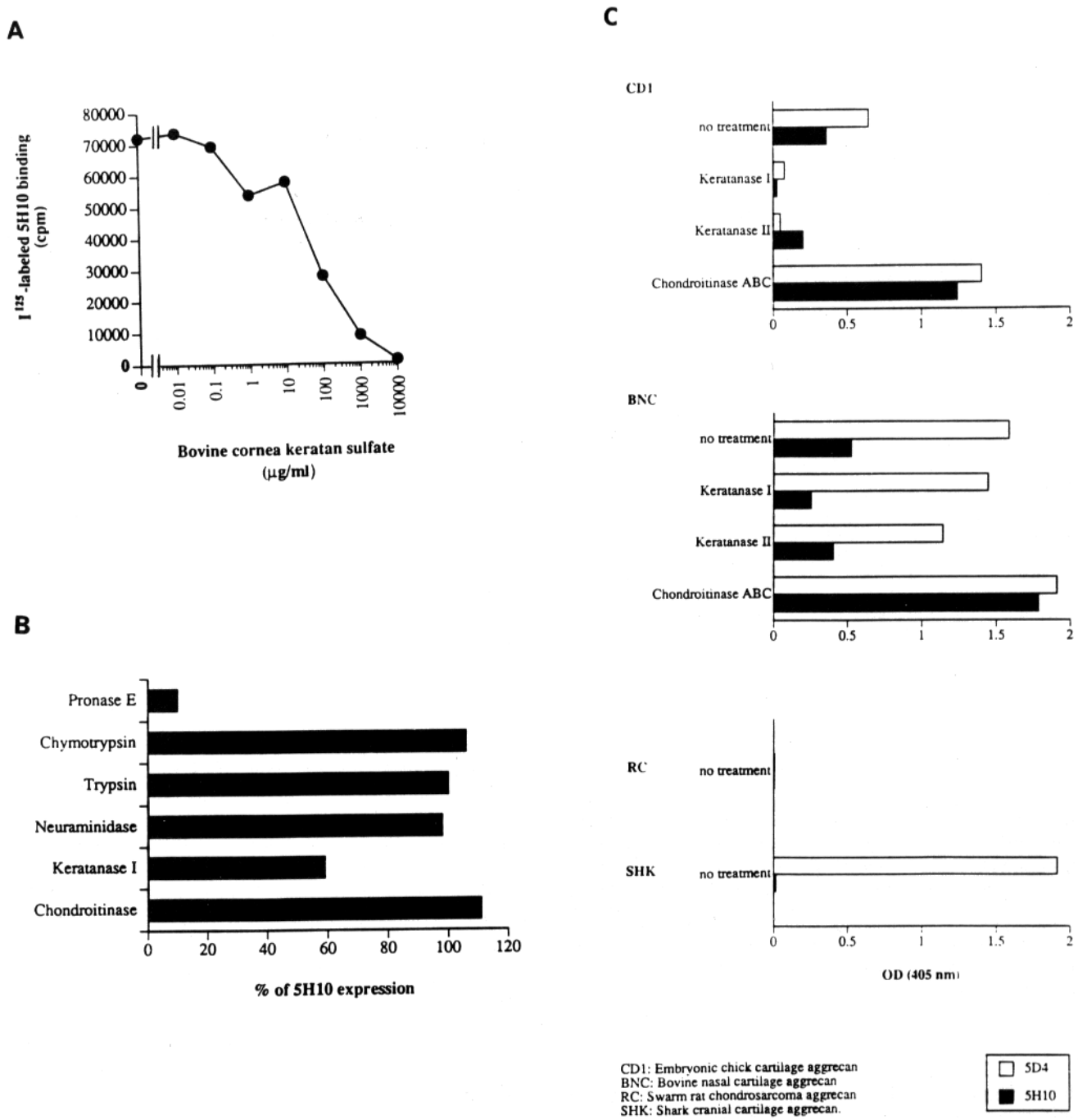

Figure 8. Reactivity of anti-PEN5 mAb with keratan sulfate glycosaminoglycans. (*A*) I^{125}-labeled 5H10 mAb (10^6 cpm/sample) was preincubated for 20 min at 4°C in PBS in the presence of the indicated concentrations of BC. The mixture was then added to NK cells for another 20-min incubation at 4°C, before three washes in PBS-1% BSA. Samples were counted in a γ-counter, and results are expressed as mean cpm of duplicate samples (SD <10%). When used in incubation with NK cells or anti-PEN5 mAb, the following carbohydrates used at 10 mg/ml were without any effect on 5H10 binding to NK cell surface: chondroitin sulfate B, heparin, heparan sulfate, dextran sulfate, GlcNAc, mannose 6-phosphate, lactose, galactose-6-phosphate, fucose, glucose 6-phosphate, glucose, and galactose. (*B*) Peripheral blood NK cells were incubated in PBS-1% BSA for 3 h or 45 min at 37°C with glycosidases (0.025 U/ml) or proteases (5 μg/ml), respectively. Cell surface expression of PEN5 epitope was then analyzed by flow cytometry using 5H10 mAb. Percent modulation was calculated as the ratio of the total linear mean fluorescence intensity of the treated cells over that of untreated control cells. (*C*) The antigenicity of 5H10 mAb for aggrecan proteoglycans was analyzed by ELISA as described in Materials and Methods. The anti-keratan sulfate mAb 5D4 was used as a positive control. Chondroitinase ABC was used at 0.04 U/ml, keratanase I was used at 0.05 U/ml, and keratanase II was used at 0.004 U/ml, for 1 h at 37°C.

reacted with the keratan sulfate proteoglycan isolated from shark cranial cartilage (SHK), 5H10 did not. These results indicate that the PEN5 epitope is present in some, but not all, keratan sulfate chains, and is therefore distinct from the standard sulfated poly-*N*-lactosamine repeat sequence, Galβ1–4(sulfated) G1cNAc. This result is consistent with the inability of six distinct anti-keratan sulfate mAbs (1B4, 2D3, 3D2, 4D1, 8C2, and 5D4, data not shown) to bind to NK cells. Preliminary results (Vivier, E., and P. Anderson, manuscript in preparation) using immunoelectron microscopy show that the PEN5 epitope is located some distance from the plasma membrane (43.4 + 12.8 nm [n = 50]), suggesting that, like other cell surface mucins, the membrane-bound glycoproteins carrying the PEN5 epitope are extended threadlike proteins.

Discussion

We describe the identification and molecular characterization of PEN5, a novel cell surface epitope selectively expressed on peripheral blood NK cells. Several lines of evidence indicate that the PEN5 epitope is an unusual sulfated poly-*N*-lactosamine carbohydrate that can be expressed on keratan sulfate glycosaminoglycans. First, keratan sulfate glycosaminoglycans selectively compete with PEN5 molecules for binding to the 5H10 (anti-PEN5) mAb. Second, treatment of NK cells with keratanase I reduces the cell surface expression of the PEN5 epitope. This enzyme attacks sites where there is an unsulfated Gal unit, which may be adjacent to a 6-sulfated G1cNAc unit; completely unsulfated disaccharides cannot serve as substrates for this enzyme (25). Third, the 5H10 mAb recognizes epitopes located on keratan sulfate chains from aggrecan-type proteoglycans obtained from embryonic chick and bovine nasal cartilages. These keratan sulfate glycosaminoglycans contain, as their prominent structure, a repeating Galβ1-4GlcNac, disaccharides that can be sulfated on either, or both saccharide units. Failure of the anti-PEN5 mAb to recognize an epitope on a third aggrecan-type proteoglycan, from shark cranial cartilage, suggests that this mAb does not recognize a structure common to all keratan sulfate chains. Further modification of keratan sulfate chains can result from differential fucoslyation, sialylation, or perhaps addition of other carbohydrates, such as mannose to the basic repeating structure (26). It is possible that the PEN5 epitope might contain one, or more, unusual structures, which could account for its presence in some, but not all, keratan sulfate chains.

Immunoprecipitation of NK cell detergent lysates with the anti-PEN5 mAb, revealed two distinct glycoproteins, PEN5α and PEN5β. Enzymatic deglycosylation indicated that both species were 80–90% carbohydrate by weight. This result raises the possibility that these proteins are either proteoglycans or mucinlike glycoproteins. Proteoglycans are high molecular weight glycoproteins in which specific glycosaminoglycans are bound to proteins via Gal-xylose-Ser linkages, with the exception of keratan sulfate as discussed below (27). PEN5 molecules are free of xylose-linked carbohydrates, since the cell surface expression of PEN5, and the SDS-PAGE migration of both PEN5α and β molecules are not affected by treatments with chondroitinase ABC (Fig. 8 *B*), heparitinase, heparinase, or *p*-nitrophenyl β-D-xylopyranoside (data not shown). However, the reactivity of anti-PEN5 mAbs with sulfated poly-*N*-lactosamine carbohydrates present on keratan sulfate glycosaminoglycans raises the possibility that PEN5α and/or PEN5β glycoproteins may be cell surface–associated keratan sulfate proteoglycans. Keratan sulfate may be N-linked or O-linked to core proteoglycan proteins (26). To date, O-linked keratan sulfate chains have been identified only in cartilage, bone, and brain (26). In the cornea, keratan sulfate chains are N-linked to Asn residues on 37- and 25-kD core proteins (26). The linkage oligosaccharide is related to biantiennary oligosaccharides found in complex-type glycoproteins, with the keratan sulfate chain extending from one branch and sialic acid terminating the second branch (28). Antigenically similar core proteins, with similar molecular weight, have been identified in a variety of tissues (29). However, there appears to be considerable variability in the glycosylation of these keratan sulfatelike molecules. The PEN5α glycoprotein shares many of the same characteristics as these keratan sulfatelike molecules. By contrast, PEN5β is an O-linked glycoprotein containing keratanase I–sensitive carbohydrates. It has been reported that mucin-type glycoproteins secreted by cultured hamster tracheal epithelial cells contain keratanase I sensitive poly-*N*-lactosamine carbohydrates (30). Mucinlike glycoproteins are highly glycosylated proteins containing a majority of O-linked oligosaccharides, and are associated with the cell membrane in a number of cell types (31). Classification of PEN5β as a NK cell–specific membrane-bound mucinlike glycoprotein is most consistent with our data. Therefore, if PEN5α and PEN5β are associated, the PEN5α–Pen5β complex may be analogous to the ASGP-1–ASGP-2 complex derived from ascitic mammary adenocarcinoma cells (32), in which only one component (ASGP-1) of the complex is a mucin-like glycoprotein. The precise biochemical identification of the carbohydrates present on PEN5α and PEN5β glycoproteins, as well as the amino acid sequence of the core proteins, will allow definitive classification.

Our results clearly show that the cell surface expression of this unique sulfated poly-*N*-lactosamine carbohydrate is restricted, within the hematopoetic compartment, to NK cells. It is intriguing that other cell surface carbohydrates, such as the acidic polylactosamine carbohydrate determinants CD57 (HNK-1) and sialyl-Lewis^x-i in humans, and asialo-GM1 ganglioside in mice, have previously been identified on the surface of NK cells (1). Taken together, these results suggest that a general characteristic of NK cells is their expression of several membrane-bound poly-*N*-lactosamine carbohydrates. The selective expression of the PEN5 epitope on resting CD56^{dim} NK cells, and its downregulation in response to NK cell proliferation, strongly suggest that PEN5 is a marker of functionally differentiated NK cells that are specialized for cytolytic effector functions. It is possible that the absence of PEN5 expression on CD56^{bright}, NK cells and GLPD cells reflects a developmentally regulated shift in the activities of

glycosyltransferases involved in keratan sulfate metabolism. Modulation of glycosyltransferase activities induced by cell differentiation and activation has been described in several cell types including T lymphocytes (33, 34).

Some of the biochemical features of PEN5 glycoproteins point the way for the study of their function. First, carbohydrates are major mediators of cell–cell interactions (35). Second, in their protease-resistance as well as their extended rodlike structure, in addition to the high extent of PEN5β O-glycosylation, the PEN5 glycoproteins resemble epithelial cell mucins. Since the mucin-type glycoproteins serve a protective role on the epithelial cell surface, it is tempting to speculate that PEN5 glycoproteins protect NK cells from their own cytolytic machinery. The selective expression of such shielding proteins on the terminally differentiated subset of NK cells would be consistent with the concomitant acquisition of fully competent cytotoxic functions. Exogenous mucins have been shown to inhibit NK cell killing (36), as well as ADCC mediated by eosinophils (37) supporting their potential involvement in resistance to NK cell cytolytic functions (36). Finally, glycosaminoglycans expressed on cell surface proteoglycans have been shown to capture growth factors (i.e., TGF-β and basic fibroblast growth factor), as well as proadhesive cytokines (i.e., macrophage inflammatory protein 1β), and it remains to be investigated whether, on NK cells, PEN5 glycoproteins could also exert such functions (38, 39).

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