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Identification of Tissue-Infiltrating Lymphocytes Expressing PEN5, a Mucin-Like Glycoprotein Selectively Expressed on Natural Killer Cells

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PEN5 is a carbohydrate epitope selectively expressed on peripheral blood natural killer cells. We have used a monoclonal antibody reactive with PEN5 to survey the expression of PEN5$^+$ large granule lymphocytes in a variety of human tissues. PEN5$^+$ cells are scattered throughout lymphoid (eg, lymph node, tonsil, thymus, spleen, and intestine) and nonlymphoid (eg, liver, esophagus, lung, and uterus) organs. Due to their relatively abundant cytoplasm, these cells are somewhat larger than resting tissue lymphocytes. The majority of splenic (87 ± 13%, n = 5) and hepatic (92 ± 6%, n = 5) PEN5$^+$ lymphocytes coexpress TIA-1, a cytotoxic granule protein found in natural killer cells. In some tissues (eg, tonsil and Peyer's patch), however, relatively few PEN5$^+$ lymphocytes coexpress TIA-1, possibly reflecting different stages of activation or differentiation. Our results indicate that PEN5 may be a useful marker of tissue-infiltrating natural killer cells and reveal these cells to be surprisingly abundant in lymphoid tissues. (Am J Pathol 1995, 146:409–418)

We recently described a monoclonal antibody (anti-5H10) reactive with a sulfated poly-*N*-lactosamine-related epitope found on two natural killer cell glycoproteins designated PEN5-α and PEN5-β.[1] Several natural killer (NK) cell-restricted cell surface glycoproteins have been shown to express poly-*N*-lactosamine epitopes. These include the NK cell-restricted CD57 (HNK-1) glycoprotein[2] and the sialyl-Lewis^{x-i} in man[3] and the GM1 ganglioside in mouse.[4] Thus, PEN5-α and PEN5-β are new members of the family of surface glycoproteins expressing the poly-*N*-lactosamine epitope. The two related isoforms of PEN5 differ in their carbohydrate branching: PEN5-α is a 210 to 245-kd glycoprotein modified primarily with *N*-linked sugars, whereas PEN5-β is a 120- to 170-kd glycoprotein modified primarily with *O*-linked sugars. Deglycosylation of PEN5-α with peptide-*N*-glycanase produces a core polypeptide of 20 to 25 kd. Deglycosylation of PEN5-β with a combination of *O*-glycanase and keratanase I produces a core polypeptide of 25 to 30 kd. Thus, both PEN5 isoforms are highly glycosylated species, with PEN5-α resembling a keratan sulfate proteoglycan and PEN5-β resembling a keratan sulfate-derivatized mucin-like glycoprotein. Among hematopoietic cells, PEN5 expression is restricted to the CD56^{dim} subset of NK cells.[1] Whereas CD56 is expressed on a subset of cytotoxic T cells, the poly-*N*-lactosamine epitope comprising PEN5 is not.[1] This characteristic makes PEN5 a highly specific marker for peripheral blood NK cells. Here we show that a monoclonal antibody (MAb) reactive with PEN5 also recognizes tissue-infiltrating lymphocytes that are likely to be NK cells.

Materials and Methods

Source of Tissues

Histologically normal fetal (20 weeks gestation) and adult human tissues were obtained from surgical and autopsy specimens. Frozen tissues embedded in OCT compound (Baxter Corp., McGaw Park, IL) were

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stored at −70 C until needed. All tissues were used as frozen tissue sections and were adequately preserved histologically. The panel of normal tissues that were screened included the following (in parentheses is the number of individuals screened for each tissue examined): adrenal (3), brain (2), breast (2), cervix (3), colon (3), esophagus (3), heart (2), kidney (3), liver (3), lung (3), lymph node (3), ovary (2), peripheral nerve (3), pancreas (3), skeletal muscle (3), skin (3), small intestine (3), spleen (3), stomach (3), testis (3), thyroid (3), tonsils (3), thymus (3), fetal thymus (1), fetal liver (1), and uterus (2). Representative sections from each tissue were selected for the preparation of photomicrographs.

Reagents

Anti-5H10, a murine mAb of the IgM subclass, was used at a concentration of 2.5 μg/ml in phosphate-buffered saline (PBS) containing 0.06% crystalline bovine serum albumin and 0.1% sodium azide. Purified mouse immunoglobulin M (IgM; Coulter Immunology, Hialeah, FL) served as the negative control. For use it was diluted to the same concentration with the same buffer solution as the test antibody. N901, a murine mAb of the IgG1 subclass, binds to the NKH1 antigen (CD56) expressed on NK cells. The antibody was used at a concentration of 2.5 μg/ml in PBS containing 0.06% crystalline bovine serum albumin and 0.1% sodium azide. Biotinylated affinity-purified goat anti-mouse IgM (μ-chain specific) and horse anti-mouse IgG (heavy and light chain specific) antibodies (Vector Laboratories, Burlingame, CA) were used as secondary antibodies at a dilution of 1:150 in PBS containing 2% human AB^+ serum and 0.1% sodium azide. Avidin-biotin-peroxidase complexes (Vector) were used as the labeling reagent at a dilution of 1:1:80 in PBS.

Immunohistochemistry

Immunohistochemical studies were performed by the avidin-biotin immunoperoxidase technique.[5] To assure that tissue sections adhered, slides were coated with poly-*L*-lysine (Sigma Chemical Co., St. Louis, MO) reconstituted in purified water. Frozen sections were cryostat cut (6 to 8 μ thick), collected onto coated slides, air dried, and fixed in 2% neutral buffered paraformaldehyde at 4 C for 20 minutes followed by several washes with PBS. To block endogenous biotin content and reduce cross-reactivity of the biotinylated antibody, all tissues were incubated with a solution of avidin (Vector) and 10% normal horse serum (Vector) in bovine serum albumin dilution buffer, at room temperature for 15 minutes. Tissue sections were drained of avidin/horse serum buffer and incubated with the antibody at 4 C, overnight. After washing in PBS, slides were incubated for 30 minutes in 0.3% hydrogen peroxide and biotin blocking solution to quench endogenous peroxidase activity and to block remaining avidin. Sections were then washed with PBS, incubated with either biotinylated goat anti-mouse IgM or horse anti-mouse IgG antibodies for 30 minutes, washed in PBS, incubated with avidin-biotin-peroxidase complexes for 45 minutes, and then washed again with PBS. After incubating the slides for 5 minutes in Tris-imidazole/HCL buffer, the peroxidase reaction was initiated by incubating for 5 minutes with 3,3-diaminobenzidine (Sigma) dissolved in Tris-imidazole/HCL buffer containing 0.11% hydrogen peroxide. Tissue sections were washed in water, counterstained with Harris hematoxylin, and dehydrated through graded alcohols and xylenes. Coverslips were then mounted on slides with E-Z-Mount mounting media (Shandon, Pittsburgh, PA).

Immunofluorescence Microscopy

Immunofluorescence microscopy was performed on cryostat sections of human tissues with optimal concentrations of anti-PEN5 (5H10, IgM), anti-TIA-1 (2G9, IgG1), and isotype-matched control antibodies. Tissues examined by double immunofluorescence were (number of sections in parentheses): liver (5), spleen (5), tonsil (4), and Peyer's patches or appendiceal lymphoid structures (4). After 20 minutes fixation in paraformaldehyde (2% at 4 C), sections were washed in PBS, preincubated in 10% goat serum, and exposed to both primary antibodies at 4 C for 16 to 18 hours. The sections were then washed again and incubated with rhodamine-labeled goat anti-IgG1 and fluorescein-labeled goat anti-IgM secondary reagents (Southern Biotechnology Associates, Birmingham, AL) each at 25 C for 1 hour, washed, coverslipped, and viewed under a fluorescence microscope with appropriate filters. Twenty-five $PEN5^+$ cells were identified and assessed for potential simultaneous binding of anti-TIA-1. Controls included incubating sections with single primary

antibodies and then exposing them to a single mismatched secondary reagent, which did not yield staining.

Transmission Electron Microscopy

Peripheral blood NK cells purified as previously reported[6] were first stained with 5H10 (anti-PEN5) and colloidal gold-labeled goat anti-mouse IgM (Amersham Arlington Heights, IL). After fixation for 1 hour in 0.1% glutaraldehyde and 2% paraformaldehyde, the stained cells were examined by transmission electron microscopy as described.[7]

Results

Comparative Expression of PEN5+ and CD56+ Lymphocytes Infiltrating Lymphoid Tissues

Although CD56 is expressed on the surface of most peripheral blood NK cells, its density of expression on most NK cells is quite low. Because of this, antibodies reactive with CD56 may not be ideal reagents for the identification of tissue-infiltrating NK cells. The relative inability of anti-CD56 to detect NK cells in lymphoid tissues is demonstrated in Figures 1 and 2, in which CD56+ cells are rarely detected in lymph node,

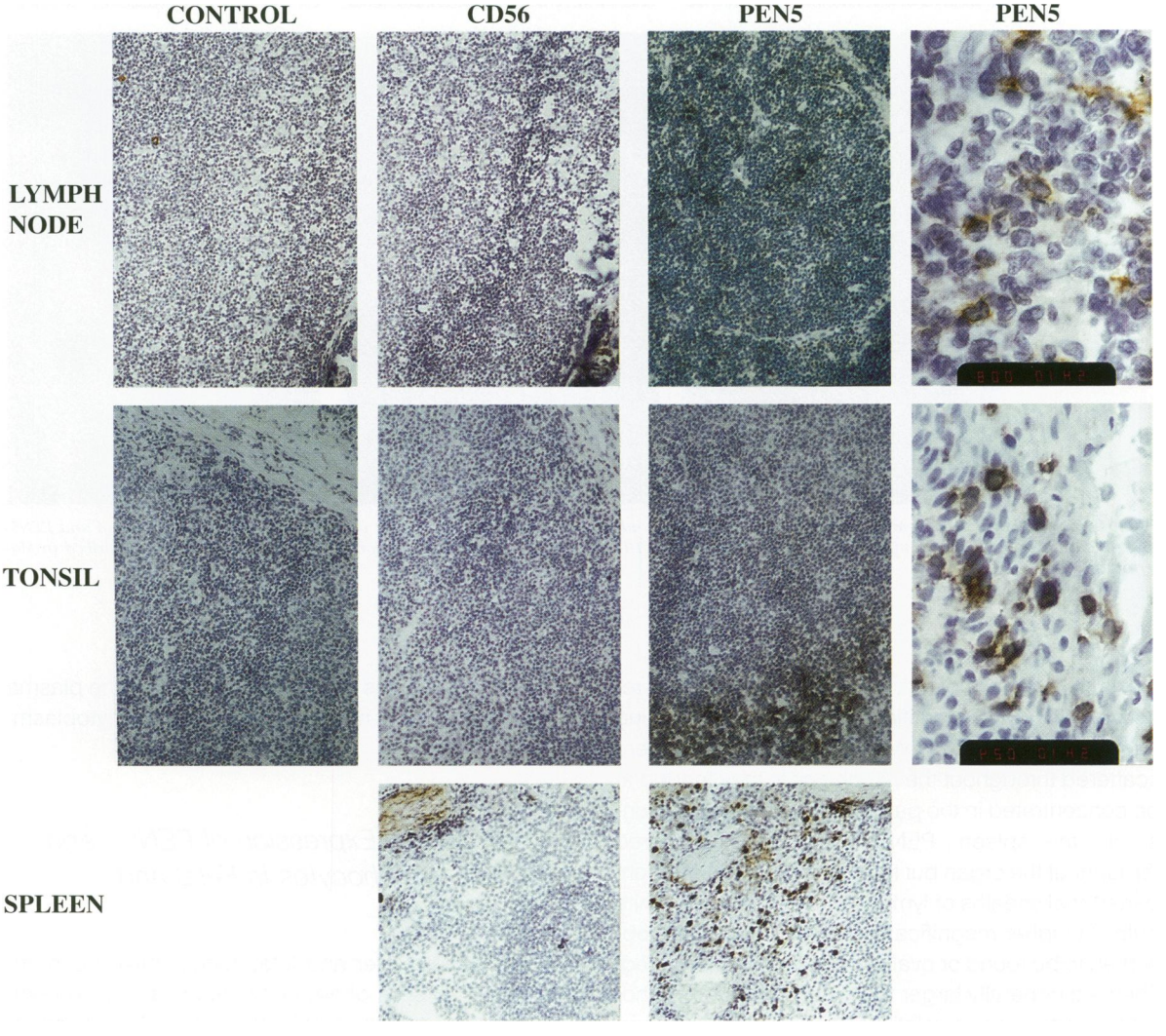

Figure 1. *Comparative histochemical staining of normal adult lymph node, tonsil, and spleen. Magnification of PEN5 staining shown in the far right panels is ×40. Magnification in all other panels is ×10. MAbs used to stain tissue sections and specific methods are described in Materials and Methods.*

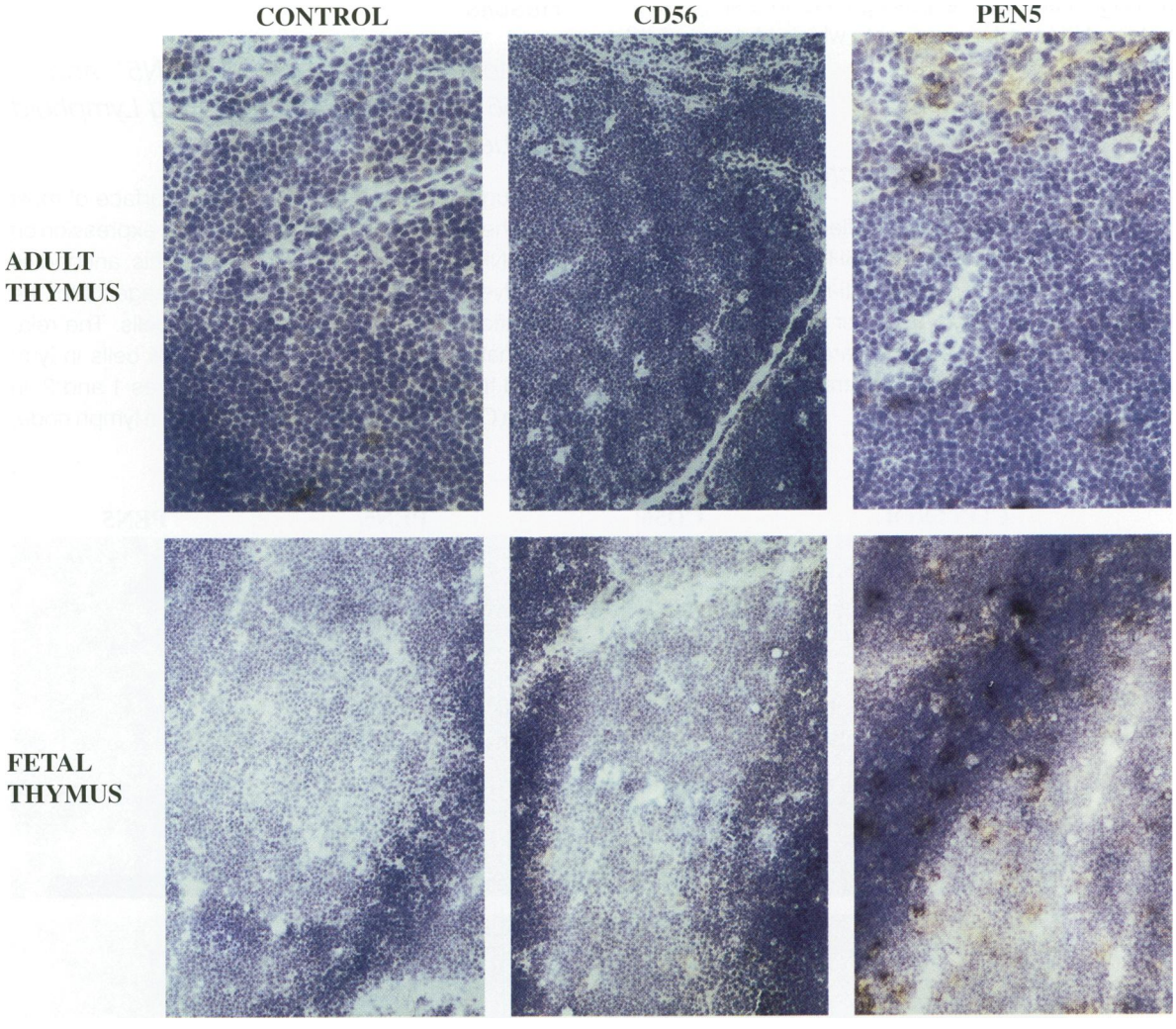

Figure 2. *Comparative histochemical staining of normal adult and fetal thymus. Magnification of adult thymus stained with control and PEN5-specific antibodies is ×20. Magnification of all other panels is ×10. MAbs used to stain tissue sections and specific methods are described in Materials and Methods.*

tonsil, thymus, or spleen. In contrast, antibodies reactive with PEN5 identified lymphocytes infiltrating each of these tissues. Whereas PEN5$^+$ cells were scattered throughout the lymph node, they tended to be concentrated in the parafollicular areas of the tonsil. In the spleen, PEN5$^+$ cells were scattered throughout the organ but tended to be excluded from periarterial sheaths of lymphoid cells within the white pulp. At higher magnification, PEN5$^+$ cells were observed to be round or oval or occasionally elongated. They are generally larger than resting tissue lymphocytes, containing a relatively abundant cytoplasm. The nuclei are set eccentrically within the cells and are slightly larger than those of resting lymphocytes. The nuclear chromatin is dense and homogeneous. Immunostaining is usually in the region of the plasma membrane, but it may also be seen in the cytoplasm.

Comparative Expression of PEN5$^+$ and CD56$^+$ Lymphocytes in Fetal and Adult Tissues

Because fetal liver and fetal thymus have been implicated as sites of NK cell differentiation, we compared the expression of PEN5$^+$ and CD56$^+$ lymphocytes in each of these tissues with that of their adult counterparts. As shown in Figure 2, CD56$^+$ cells were not easily detected in either fetal or adult thymus. In

each of these tissues, scattered lymphocytes expressing low levels of CD56 could be detected at high magnification, suggesting that CD56+ cells are present but difficult to detect by this histochemical method. This might result from lability of the antigen under these fixation conditions or the low level of CD56 expression, as Sanchez et al[8] have shown that CD56+ lymphocytes can be identified in these tissues by flow cytometric analysis. In contrast, PEN5+ cells were easily detected, scattered throughout both adult and fetal thymus. The density of PEN5+ cells was consistently greater in fetal thymus than in adult thymus. Occasional CD56+ cells could be detected in adult liver, but again the intensity of staining was very weak (Figure 3). Scattered PEN5+ cells were easily detected in the adult liver because of their more intense staining. Relatively more PEN5+ cells were observed in fetal liver compared with adult liver. At higher magnification, PEN5 expression in liver-infiltrating lymphocytes appeared, at least in part, cytoplasmic. Previous results have shown that mucin-like glycoproteins can be identified in the trans-Golgi reticulum and in cytoplasmic vesicles that eventually fuse with the plasma membrane.[7] It is possible that liver-infiltrating NK cells express PEN5 primarily in these intracellular compartments.

Expression of PEN5 Antigen on Nonlymphoid Cells

Antibodies reactive with PEN5 also recognized some nonleukocytic cells. These were generally epithelial cells found in the esophagus, cervix, endometrium, trachea, bile ducts, colon, and pancreas. The most dramatic example of this nonlymphoid staining was seen in the lung and colon, where anti-PEN5 strongly stained the mucous layer lining bronchial and colonic epithelial cells (Figure 4). The specificity of this staining was confirmed by the inability of either isotype-matched control antibody or anti-CD56 to stain epithelial mucus.

Coexpression of PEN5 and TIA-1 in Tissue-Infiltrating Lymphocytes

Additional evidence that PEN5+ tissue-infiltrating lymphocytes are NK cells comes from double label-

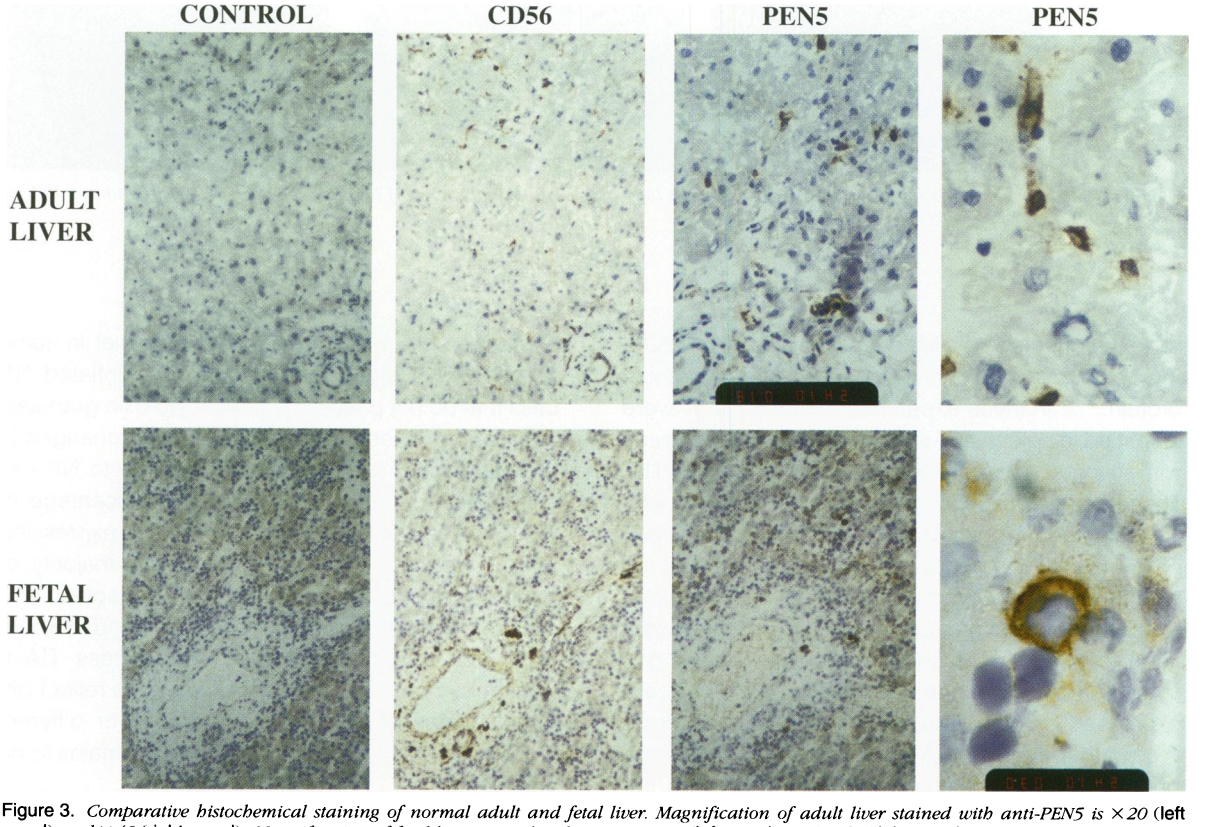

Figure 3. *Comparative histochemical staining of normal adult and fetal liver. Magnification of adult liver stained with anti-PEN5 is ×20* (left panel) *and ×40* (right panel). *Magnification of fetal liver stained with PEN5 is ×10* (left panel) *and ×60* (right panel). *Magnification of all other panels is ×10. MAbs used to stain tissue sections and specific methods are described in Materials and Methods.*

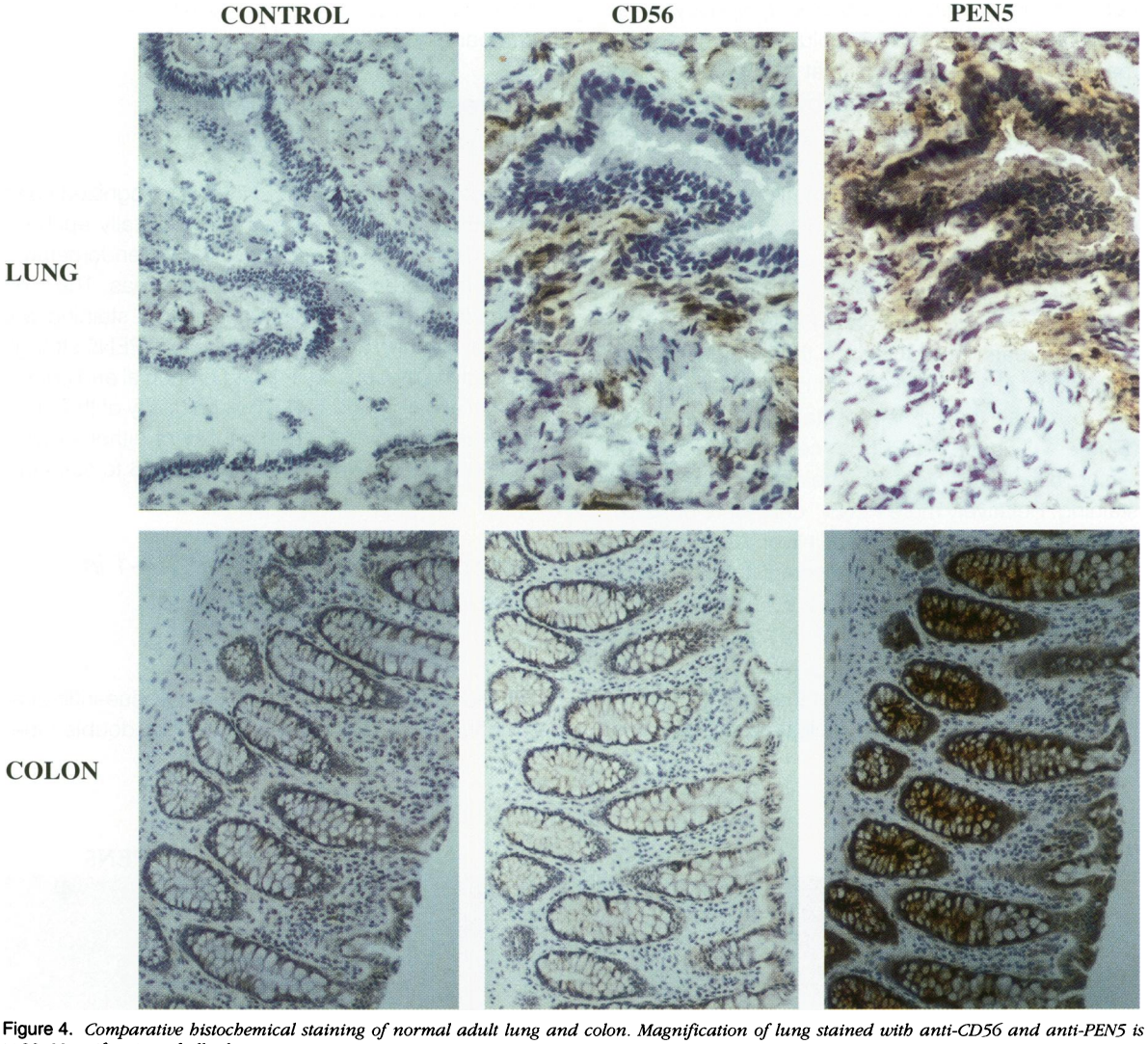

Figure 4. *Comparative histochemical staining of normal adult lung and colon. Magnification of lung stained with anti-CD56 and anti-PEN5 is ×20. Magnification of all other sections is ×10.*

ing experiments with a MAb reactive with TIA-1 (2G9, IgG1), a cytotoxic lymphocyte-restricted granule protein.[9–11] In these experiments, PEN5$^+$ cells were identified in spleen and appendiceal lymphoid tissue with fluorescein isothiocyanate-tagged anti-5H10. These same sections were also labeled with phycoerythrin-tagged anti-2G9. As shown in Figure 5, all four PEN5$^+$ lymphocytes scattered throughout the spleen were also TIA-1$^+$. Consistent with the localization of these antigens, PEN5 staining is largely confined to the cell surface, whereas TIA-1 staining is cytoplasmic and granular. Some PEN5$^-$ cells expressed TIA-1. These cells are likely to be cytotoxic T cells that express TIA-1 but not PEN5. In the appendix, only one of four PEN5$^+$ lymphocytes coexpressed TIA-1. This result suggests that in some tissues PEN5 might identify less differentiated NK cells that do not possess defined cytotoxic granules. Alternatively, these results might reflect changes in the expression of TIA-1 that are related to NK cell differentiation. Table I tabulates the percentage of PEN5$^+$ tissue-infiltrating lymphocytes expressing TIA-1 in several tissues. Whereas the majority of PEN5$^+$ lymphocytes coexpress TIA-1 in spleen and liver, this is not the case in tonsil or appendix, where most PEN5$^+$ lymphocytes do not express TIA-1. Whether these tissue-specific differences reflect different stages of NK cell differentiation or different types of tissue-infiltrating lymphocytes remains to be elucidated.

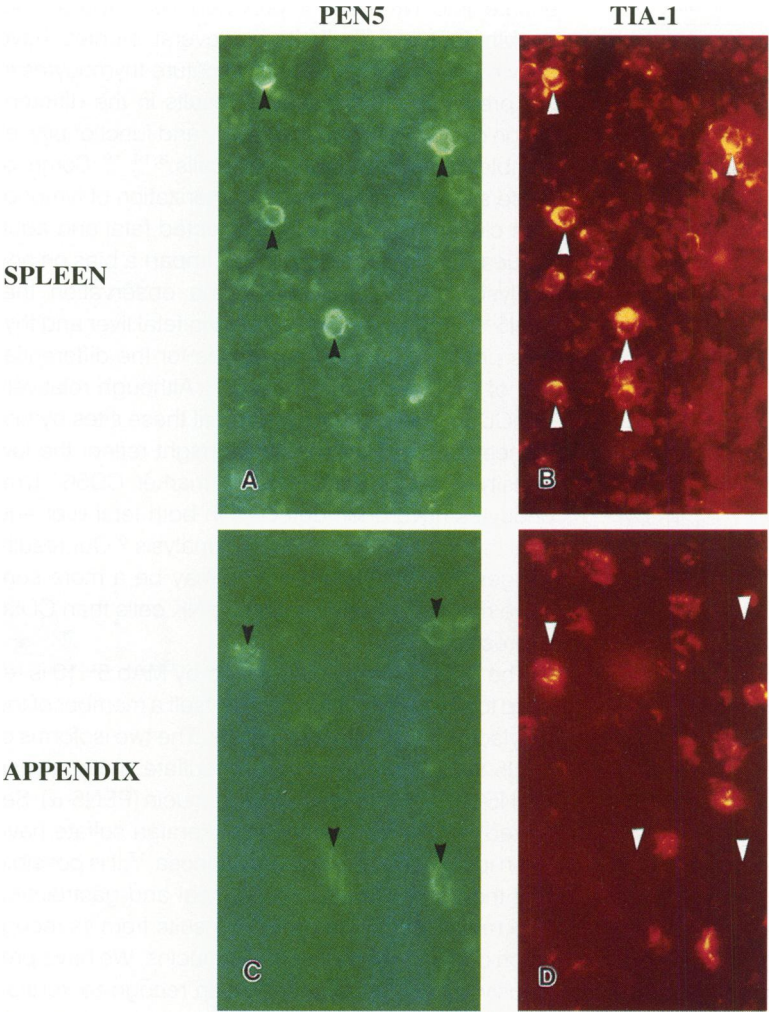

Figure 5. *Dual labeling of tissue-infiltrating lymphocytes. Tissue sections from adult spleen* (**A** *and* **B**) *or adult appendix* (**C** *and* **D**) *were double labeled with fluorescein-tagged anti-PEN5* (**A** *and* **C**) *and rhodamine-tagged anti-TIA-1* (**B** *and* **D**) *before examination by fluorescent microscopy. Black arrowheads show the location of PEN5+ cells in* **A** *and* **C**. *White arrowheads show the location of both PEN5+ cells and TIA-1+ cells in* **B** *and* **D**.

PEN5 Glycoproteins Are Expressed at the NK Cell Surface as Extended Rod-Like Structures

Transmission electron microscopy was performed on NK cells stained by indirect immunogold with the 5H10 MAb and a gold-labeled anti-IgM developing reagent. Ultrathin sections of NK cells showed extensive labeling at the cell surface (Figure 6). Labeling was generally continuous around the entire cell profile, although in some cell preparations there was relatively more labeling over microvilli. More striking was the distance between the plasma membrane and the gold label, which averaged 43.4 ± 12.8 nm (n = 50). This result suggests that, like other cell surface mucins, the membrane-bound glycoproteins carrying the PEN5 epitope are extended thread-like proteins.

Discussion

We have used a MAb reactive with a sulfated poly-*N*-lactosamine epitope expressed on the NK cell-restricted glycoprotein PEN5 to survey the presence of tissue-infiltrating NK cells in lymphoid and nonlymphoid tissues. Whereas antibodies reactive with CD56 were unable to efficiently detect all tissue-infiltrating NK cells, PEN5+ lymphocytes were readily identified in multiple tissues. These results suggest that NK cells can infiltrate multiple lymphoid and non-

Table 1. *Expression of TIA-1 in PEN5$^+$ Tissue-Infiltrating Lymphocytes*

Tissue	Donor No.	TIA-1$^+$	Mean ± SE
Spleen	1	100	
	2	64	
	3	84	
	4	100	
	5	88	87 ± 13
Tonsil	1	16	
	2	40	
	3	36	
	4	28	30 ± 9
Liver	1	96	
	2	88	
	3	100	
	4	84	
	5	92	92 ± 6
Appendix	1	12	
	2	0	
	3	12	
	4	4	7 ± 5

Dual labeling of the indicated tissues was performed as described in Materials and Methods and in the legend to Figure 5. The percentage of PEN5$^+$ cells that expressed TIA-1 is indicated. Tissues from four or five independent donors were evaluated and the mean ± SE is reported.

lymphoid tissues to mediate their immune functions. This conclusion assumes that PEN5 is expressed similarly on both tissue-infiltrating and circulating lymphoid cells. In the periphery, PEN5 is selectively expressed on large granular lymphocytes possessing cytotoxic effector function. These cells express low levels of CD56, which might account for the inability of antibodies reactive with CD56 to recognize these cells in tissues. Double staining with the cytotoxic granule marker TIA-1 supports the conclusion that PEN5$^+$ lymphocytes infiltrating some tissues (eg, spleen and liver) contain cytotoxic granules. Surprisingly, however, many PEN5$^+$ cells infiltrating other tissues (eg, tonsil and appendix) did not coexpress TIA-1. This result suggests that in some tissues PEN5 might be expressed on agranular lymphocytes, reflecting a different stage of activation or differentiation. It is also possible, however, that PEN5 is expressed on tissue-infiltrating lymphocytes that are not classical NK cells. Additional studies will be required to determine the phenotype and function of these tissue-infiltrating lymphocytes.

PEN5$^+$ lymphocytes were particularly prevalent in fetal liver and fetal thymus. Recent studies suggest that NK cells and T cells arise from a common bone marrow-derived progenitor cell.[8,12–14] Homing of these cells to the fetal liver, a major site of prenatal hematopoiesis, fosters the development of CD56$^+$ cells that resemble peripheral NK cells in both phenotype and function. Some evidence suggests that these cells can differentiate into T cells if they leave the liver and home to the thymus.[8] In the absence of the thymic microenvironment, these cells can differentiate into NK cells if provided with appropriate growth factors.[8,14] Similarly, several studies have shown that *in vitro* culture of immature thymocytes in the presence of interleukin-2 results in the differentiation of cells that phenotypically and functionally resemble peripheral blood NK cells.[8,14–16] Some of these studies rely on the characterization of lymphocyte clones that grow out of selected fetal and adult tissues. As clonal selection may impart a bias on any analysis of cell populations, the observation that PEN5$^+$ lymphocytes are present in fetal liver and thymus provides unbiased evidence for the differentiation of NK cells in these tissues. Although relatively few CD56$^+$ cells were identified at these sites by histochemical analysis, this result might reflect the low density of expression of this NK marker. CD56$^+$ lymphocytes have been detected in both fetal liver and fetal thymus by flow cytometric analysis.[8] Our results suggest that PEN5 expression may be a more sensitive marker of tissue-infiltrating NK cells than CD56 expression.

The PEN5 epitope recognized by MAb 5H10 is related to keratan sulfate, which is itself a member of the polylactosamine family of sugars. The two isoforms of PEN5 thus resemble a keratan sulfate proteoglycan (PEN5-β) and a keratan-sulfated mucin (PEN5-α). Secreted mucins derivatized with keratan sulfate have been identified in the tracheal mucosa.[17] It is possible that the recognition of the tracheal and gastrointestinal mucin layer by anti-5H10 results from its recognition of these keratan-sulfated mucins. We have previously shown that anti-5H10 can recognize keratan sulfate-bearing proteoglycans derived from several tissues, including embryonic chick cartilage and bovine nasal aggrecan.[1] In epithelial cells, mucins are secreted to provide protection against environmental toxins.[18] It is possible, by analogy, that PEN5 is expressed on differentiated large granular NK cells to protect them against their own cytotoxic effector molecules. The extended, rod-like structure of PEN5 demonstrated by transmission electron microscopy could facilitate such a functional role. Cell surface mucins have also been identified as ligands for lymphocyte adhesion molecules involved in tissue homing.[19] It is therefore possible that the expression of PEN5 on terminally differentiated NK cells allows its subsequent infiltration into the various tissues in which these cells are found. Additional experiments will be required to determine whether PEN5 performs any of these functions in NK cells.

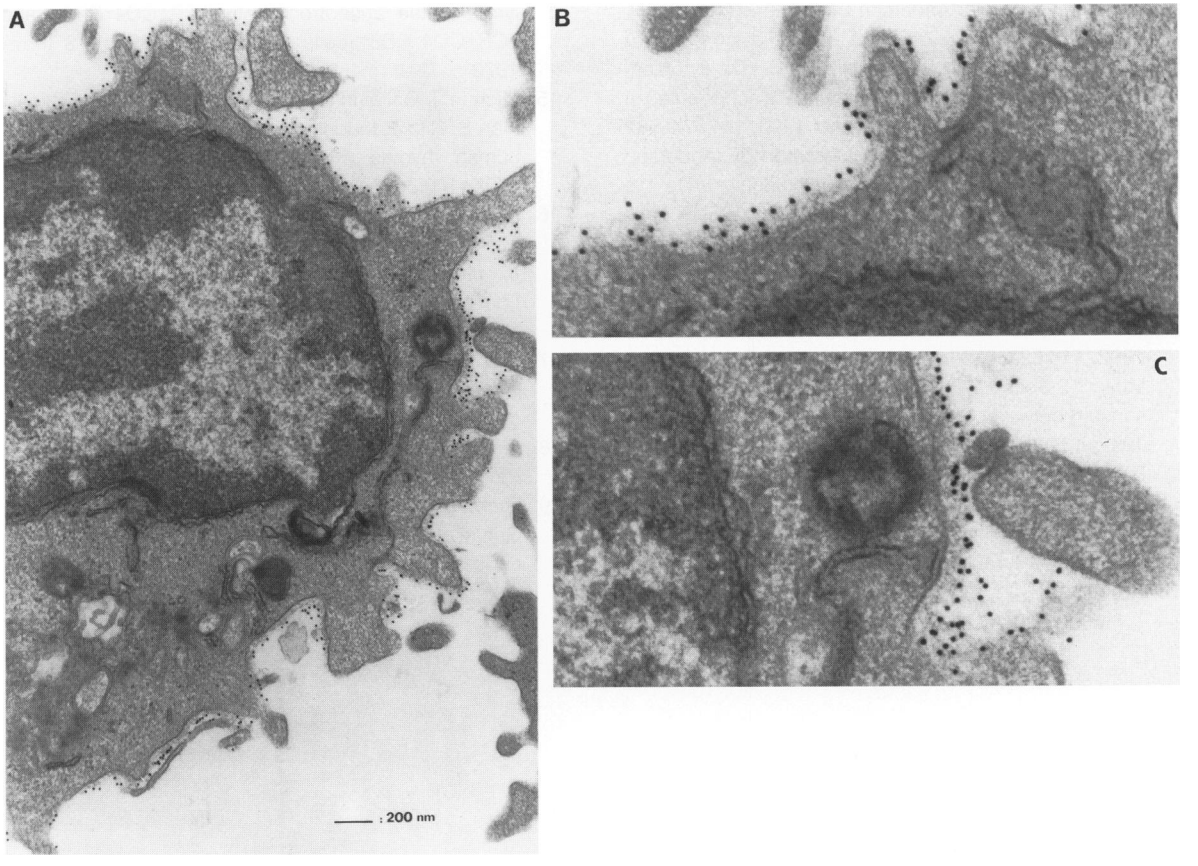

Figure 6. *Immunogold staining of PEN5 epitope on NK cells. Peripheral blood NK cells were stained with 5H10 MAb and then developed with a gold-labeled anti-mouse IgM. Cells were then processed for transmission electron microscopy. Magnification in* A, *×48,500 (0.972 cm = 200 nm). In* B *and* C *two areas of 5H10 labeling are shown at 3 × higher magnification.*

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