Glycoconjugate Journal 20, 375–384, 2004

Localization of sialidase-positive cells expressing Mac-1 and immunoglobulin in the mouse thymus

Shigeko Kijimoto-Ochiai, Naoko Doi, Hiroko Matsukawa, Miwako Fujii and Koji Tomobe

Institute for Immunological Science/Genetic Medicine, Hokkaido University, N-15, W-7, Sapporo 060-0815, Japan

We have sought an endogenous membrane bound sialidase acting at neutral pH in immune system, because the removal of sialic acid from cell surfaces will affect the cell-cell interaction directly or indirectly. The levels of activity of unique membrane-bound sialidase at neutral pH and also soluble sialidase are high in the thymus but low in the spleen and lymph nodes. These are thought to be plasma membrane and cytosolic types based on the behavior of inhibition by Cu^{2+} and 2-deoxy-2, 3-dehydro-N-acetylneuraminic acid. Newly synthesized 5-bromo-4-chloro-3-indolyl-N-acetylnueraminic acid was used for histochemical staining of sialidase-positive thymic cells, and the results showed positive cells sparsely distributed in the corticomedullar region or medullary region of the thymus. They expressed immunoglobulin and Mac-1 antigen on their surfaces. These cells must therefore be of a B cell lineage, not a T cell lineage. We also found that some vessels in the thymus were sialidase-positive.
Published in 2004.

Keywords: **active staining of sialidase/Mac-1- and Ig- positive cells/neutral sialidase/thymus sialidase/X-NANA**

Abbreviations: **DAB, diaminobenzidine; Ig, immunoglobulin; mAb, monoclonal antibody; 4MU-Neu5Ac, 4-methylumbelliferyl-N-acetyl-α-D-neuraminic acid; Neu5Ac2en, 2-deoxy-2, 3-dehydro-N-acetylneuraminic acid; PE-R, phycoerythrin-R; PO, peroxidase; X-NANA, 5-bromo-4-chloro-3-indolyl-α-D-N-acetylnueraminic acid cyclohexylamine salt.**

Introduction

Sialic acids play an important role in the regulation of the immune system [1]. The addition of sialic acid to glycans is regulated by sialiyltransferases and the removal of sialic acid from glycans is regulated by sialidases. The effects of these enzymes on immune system have been reported [2–10]. We have also shown that sialidase treatment causes mass aggregation of single cells of an Epstein-Barr virus (EBV)-transformed human B cell line [11]. We concluded that this aggregation is partly due to the low-affinity IgE receptor (CD23) based on our findings that CD23 has galactose-binding lectin activity, acts as a lectin on EBV-transformed B cells, and induces cell aggregation through newly exposed galactose-residues [12,13]. These findings suggest that endogenous sialidase on lymphocytes may play an important role in the interaction or aggregation of lymphocytes. If endogenous sialidase in the immune system plays an important role in the interaction of lymphocytes, it should react under physiologically neutral pH conditions, because the enzyme is probably located on the cell surface. The optimum pH, however, was found to be pH 5.0 or less for plasma membrane [14], although pH 6.5 was found to be optimal for cytosolic sialidase. We have tried to find a membrane bound sialidase acting at neutral pH in immune tissues. We found that this activity was high in the murine thymus, and preliminarily reported that sialidase-positive cells are sparsely located in the thymus-medulla and possess immunoglobulin (Ig) and Mac-1 antigen on their surfaces [15]. As an extension to that study, we have recently cloned the cytosolic sialidase-gene from the thymus [16]. In the present study, we will show that a high level of neutral cytosolic, and membrane sialidase activities in the mouse thymus but not in the spleen, lymph nodes or other organs. We will also show the localization of these sialidase-positive cells in the thymus using 5-bromo-4-chloro-3-indolyl-N-acetylnueraminic acid (X-NANA) as a substrate. The expressions of Mac-1 antigen and IgG on sialidase positive cells are demonstrated by immunostaining.

To whom correspondence should be addressed: Shigeko Kijimoto-Ochiai, PhD, Life Space COSMOS, #602 3-12, N-3 W-13, Chuo-ku, Sapporo 060-0003, Japan. Tel.: +81-11-261-3873; Fax: +81-11-261-3881; E-mail: koshigeko@hkg.odn.ne.jp

Materials and methods

Mice and tissues

C3H/HeSlc and A/J mice were purchased from the Shizuoka Laboratory Animal Center, Hamamatsu, Shizuoka, Japan. After the mice had been sacrificed, the thymus, spleen, peripheral lymph nodes and other tissues were carefully dissected out of each mouse and frozen at −70°C until use or were immediately used for the experiment.

Chemicals and antibodies

4-Methylumbelliferyl-*N*-acetyl-α-D-neuraminic acid (4MU-Neu5Ac) was obtained from Nacalai Tesque (Kyoto, Japan) and X-NANA was obtained from Rose Science (Alberta, Canada).

2-Deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en, Sigma) was a kind gift from Dr. T. Miyagi (Miyagi Cancer Center, Miyagi, Japan). The following antibodies were obtained commercially: phycoerythrin-R (PE-R)-conjugated anti-mouse Mac-1 (CD11b/CD18) monoclonal antibody (mAb) (Cedarlane Laboratories, Ontario, Canada), anti-pan-epithelial cytokeratin mAb as an anti-epithelial cell Ab (mouse IgG1, Progen Biotechnik, Heidelberg, Germany), biotin-labeled anti-mouse IgG (H + L) (horse polyclonal, Vector Laboratories, Burlingame, CA), and horseradish-peroxidase (HR)-labeled streptavidin (Zymed Laboratories, San Francisco, CA). Anti I-A^k mAb (clone 10-2-16) was purified from ascites and biotin-labeled in this laboratory according to the previously reported method [17]. Monoclonal antibody Mac-2 (M3/38, rat IgG2a, culture supernatant) was kindly provided by Prof. T. Ito and Dr. H. Soga (Third Department of Anatomy, School of Medicine, Tohoku University, Sendai).

Preparation of soluble fraction, crude-membrane fraction and NP40 solubilized fraction from frozen thymus

The crude-membrane fraction was prepared by combining two methods used for the isolation of plasma membrane from rat liver with high yield [18,19]. Tissues were homogenized in a homogenizing buffer (1 mM $NaHCO_3$ and 0.5 mM $CaCl_2$) with a Dounce homogenizer using 25 gentle strokes. The homogenate was diluted to 100 times its weight and allowed to stand for 5 min while being slowly stirred. The diluted homogenate was then passed through three layers of nylon mesh. The supernatant was centrifuged at 105,000 × *g* for 1 h at 4°C. The resulting supernatant was taken as the soluble fraction, and the pellet was suspended in phosphate-buffered saline (PBS) and used as the crude-membrane fraction. The residual materials on nylon mesh were solubilized with PN40 solubilization-buffer (10 mM Tris-HCl, pH 7.4/0.15 M NaCl, containing 0.5% NP40/200 μg/ml phenylmethylsulfonyl fluoride), stirred on ice for 30 min, and centrifuged for 30 min at 105,000 × *g*. The supernatant was used as the NP40-solubilized fraction.

Sialidase assay

The assay mixture contained 0.1 mM 4MU-Neu5Ac and 0.1 M acetate buffer (pH 4.5) or PIPES buffer (pH 7.0), with or without an inhibitor, and approximately 50 μg protein of enzyme preparation in a final volume of 0.2 ml. After incubation at 37°C for 3 h (standard method) or the appropriate time, the amount of 4-MU released was determined spectrofluorometrically [20] with 4-MU as a standard, with or without an inhibitor in both buffers. All assays were performed in duplicate, and the mean value was shown as one point without indication of variation unless it exceeded 30%.

Histochemical analysis of sialidase-positive cells

Fresh thymus or other organs at 6 to 8 wks of age were embedded in an O.C.T. compound (Tissue-Tek, Miles Inc., Elkhart, IN), quickly frozen in liquid nitrogen, cryostat-sectioned to 10 or 7 μm in thickness, and applied to poly-L-lysine-coated glass slides. For sialidase staining, a 25-μl reaction buffer containing X-NANA (reaction mixture of 84 mM PIPES buffer, pH 7.0, or 84 mM Na-acetate buffer, pH 4.5, 8.4 mM KCl, 3 mM $K_4Fe(CN)_6$, 3 mM $K_3Fe(CN)_6$, 1 mM $MgCl_2$, 0.1% Triton and 0.05% X-NANA from 0.5% stock solution in dimethyl sulfoxide, with or without 1 mM of Cu^{2+}) was applied to cryostat sections, and the sections were incubated in a humidified chamber at least for 8 h at 37°C. The reaction buffer was then removed, and each section was washed and immersed in stop solution (0.133 M glycine / 83 mM Na_2CO_3 / 60 mM NaCl, pH 10.7), covered with a glass slip, and then photographed using a fluorescent microscope with a UV filter. After that, if necessary, sections were fixed with acetone, and immunochemical staining was performed as follows.

Immunochemical staining

A typical process for diaminobenzidine (DAB) staining is as follows. Cryostat section (10 μm thick) was fixed in acetone (−10°C) for 10 min, treated with 0.3% H_2O_2 in methanol for 30 min to eliminate the endogenous peroxidase (PO) activity. It was reacted with an appropriately diluted primary antibody for 30 min at room temperature, rinsed three times with PBS, incubated with biotin-conjugated secondary antibody, washed three times with PBS and then incubated with streptavidin-PO (1:1000 v/v). The histochemical color was developed in a 0.03% DAB solution (in 15 mM phosphate buffer, pH 6.5, containing 0.05% H_2O_2). A section was also stained with hematoxylin-eosin.

Results

High level of neutral sialidase activity in the thymus crude-membrane fraction

Sialidase activity levels in various tissues so far reported have been investigated using a whole homogenate of tissues at acidic

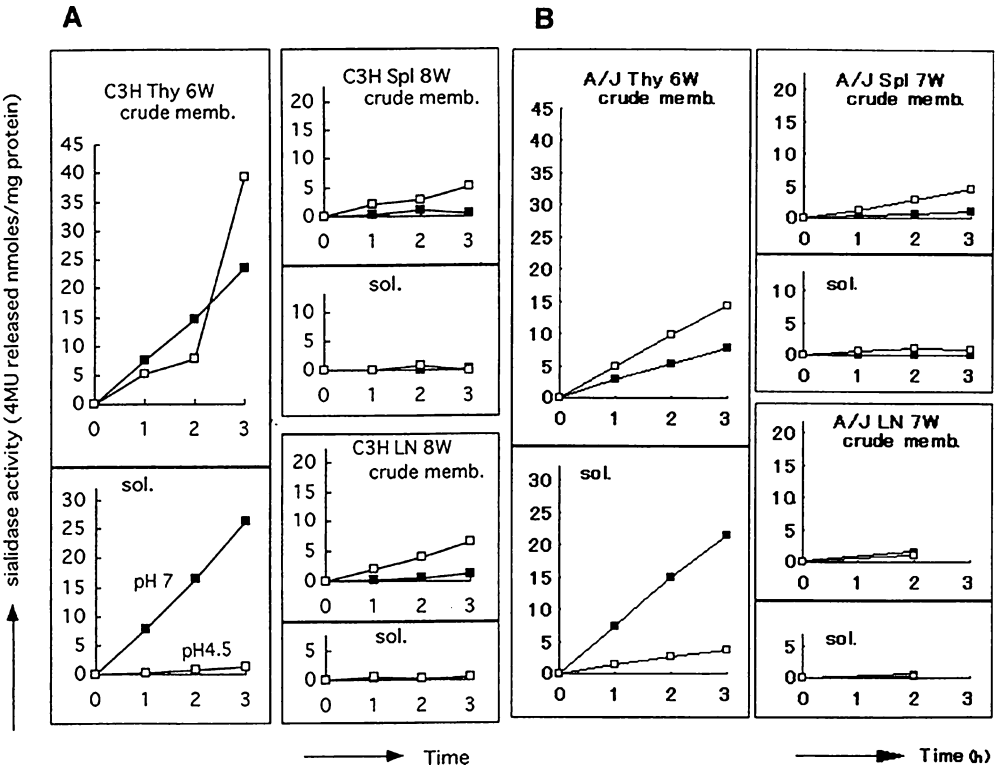

Figure 1. Sialidase activity at pH 7.0 is high in the thymus among immune tissues. Crude-membrane and soluble fractions were prepared from thymus (Thy), spleen (Spl) and lymph nodes (LN) from C3H mice (A) or A/J mice (B) (ages 6–8 wks) as described in *Materials and Methods*. Sialidase was assayed in PIPES buffer at pH 7.0 (■) or in acetate buffer at pH 4.5 (□) for 1, 2 and 3 h with 4MU-Neu5Ac as a substrate. The upper panel of each fraction indicates the activity of the crude-membrane fraction (105,000 × *g* ppt) and the lower panel indicates that of the soluble fraction (105,000 × *g* sup). All assays were performed in duplicate, and the mean value was plotted as one point without indication of variation unless variation exceeded 30%.

pH. In the present study, however, we examined membrane-bound sialidase acting at neutral pH. Four types of sialidase activity were assayed at pH 7.0 and pH 4.5 using soluble and crude-membrane fractions from immune tissues: the thymus, spleen and peripheral lymph nodes. The crude-membrane and soluble fractions from the thymus tissue showed high level of sialidase activity at pH 7.0, while those of the spleen and lymph nodes showed low level of activity at pH 7.0 and also at pH 4.5 both in the C3H and A/J mice (Figure 1). The level of sialidase-activity in the crude membrane fraction of the thymus increased almost linearly with increase in incubation time until 4.5 h in this condition (with approximately 50 μg of protein and 0.1 mM 4MU-Neu5Ac). Such a high level of membrane-bound sialidase activity at pH 7.0 is unusual. Table 1 shows sialidase activities in various tissues and also shows the effect of inhibitors. The mouse thymus clearly showed high sialidase activity level at pH 7.0 in the soluble and membrane fractions, but the liver and bone marrow showed membrane-bound sialidase activities at pH 7.0 that were as low as that of the lymph node. The activity level of the soluble fraction at pH 4.5 was high in the kidney. This enzyme is probably an intralysosomal type as was reported by Miyagi *et al.* [21]. They reported that 1 mM of Cu^{2+} caused complete inhibition of the activities of cytosolic sialidase and plasma membrane sialidase but had no inhibitory effect on the activities of intralysosomal sialidase and lysosomal membrane sialidase. They also reported that Neu5Ac2en was more effective against cytosolic sialidase and intralysosomal sialidase than against membrane-associated sialidases of the plasma membrane and lysosomal membrane. As shown in Table 1, Neu5Ac2en more strongly inhibited the activities of soluble fraction at pH 7.0 than membrane-bound sialidase activities, while Cu^{2+} more strongly inhibited the sialidase activities of soluble fraction and membrane fraction at pH 7.0 than the activity of membrane fraction at pH 4.5. These results suggest that thymus-crude membrane contains at least a sialidase of a plasma membrane-type acting at pH 7.0 and also a sialidase of a lysosomal membrane-type acting at pH 4.5. A plasma membrane-sialidase of rat brain has been reported to have optimal activity at pH 5.0 [14]. We, therefore, examined sialidase activities with 4MU-Neu5Ac at various pH values, ranging from 3.5 to 8.0, with or without Cu^{2+} in the crude-membrane and soluble fractions of the thymus from C3H mice. Two peaks were clearly observed for the crude-membrane fraction; there was complete inhibition of crude-membrane activity with 1 mM Cu^{2+} in the neutral pH range and about 50% inhibition in the acidic pH range (Figure 2A). These results clearly

Table 1. Sialidase activity in various organs from A/J mice

	Silalidase activity (nmoles released 4 MU/mg protein/2 h)							
	Crude membrane fraction				*Soluble fraction*			
	pH 7.0		*pH 4.5*		*pH 7.0*		*pH 4.5*	
	–	$+Cu^{2+}$	–	$+Cu^{2+}$	–	$+Cu^{2+}$	–	$+Cu^{2+}$
Kidney	0.8 ± 0.05	0 ± 0	28.8 ± 1.03	20.6 ± 0.11	0.68 ± 0	0 ± 0	4.5 ± 0.18	5.05 ± 0
Liver	1.45 ± 0	0 ± 0	4.58 ± 0.07	2.44 ± 0.07	1.53 ± 0	0 ± 0	0.6 ± 0.11	0.31 ± 0
Lung	0.67 ± 0.08	0 ± 0	3.87 ± 0.13	1.04 ± 0.08	0.05 ± 0.05	0 ± 0	0.03 ± 0.02	0 ± 0
Heart	0.24 ± 0.03	0 ± 0	1.03 ± 0.13	0.21 ± 0	0.08 ± 0.08	0 ± 0	0.16 ± 0.16	0.29 ± 0.03
Bone marrow	1.29 ± 0	0 ± 0	6.17 ± 0.13	3.75 ± 0.47	1.2 ± 0.13	0.79	0.45 ± 0.04	0.97 ± 0.97
Thymus	5.4 ± 0.21	0 ± 0	9.9 ± 0.13	2.0 ± 0.11	14.9 ± 0.7	0 ± 0	2.7 ± 0.32	1.05 ± 0.07
+0.03 mM (*)	100	39	100	29	100	10		ND
+0.3 mM (*)	100	30	100	25	100	14		ND
Spleen	0.6 ± 0.08	0 ± 0	2.88 ± 0	0.58 ± 0.05	0.09 ± 0	0 ± 0	1.06 ± 0.35	0.26 ± 0
Lymph node	1.4 ± 0.06	0 ± 0	1.1 ± 0.12	0.22 ± 0.04	0.4 ± 0.07	0 ± 0	0.2 ± 0.05	0.05 ± 0.02
Rat thymus (new born)	1.17 ± 0	0.11 ± 0.05	4.8 ± 0.05	3.36 ± 0	1.98 ± 0.14	0.23 ± 0.05	1.06 ± 0.14	0.92 ± 0

A/J mice (6–8 weeks) were used.
*% Activity in the presence of 0.03 mM or 0.3 mM of Neu5Ac2en instead of Cu^{2+}. ND, not done.

show that the activity at pH 6.5–7.0 in the crude membrane fraction is not due to the shoulder or the foot of the acidic activity and suggest that this activity is a plasma membrane type judging from the inhibition with copper ion as reported by Miyagi *et al.* [21]. The sialidase activity at acidic pH in the crude membrane fraction is probably due to the lysosomal membrane enzyme. The optimal activity in the soluble fraction was at pH 6.5 (Figure 2B) and this was completely inhibited by 1 mM of Cu^{2+}. The activities in citrate-phosphate buffer and in PIPES buffer were compared in the neutral region (Figure 3). A higher level of activity was observed in the PIPES buffer than in the

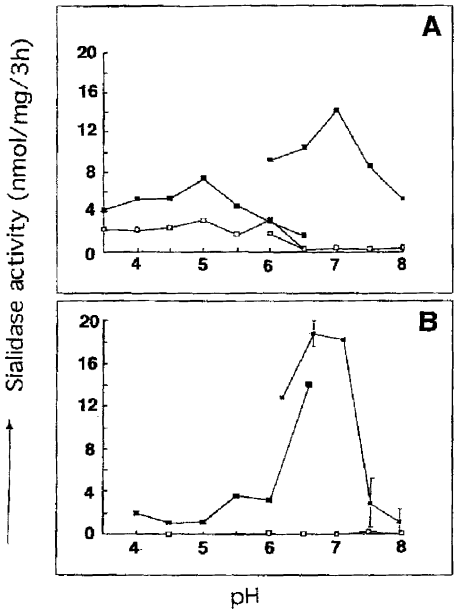

Figure 2. pH curves for sialidase of the thymus from C3H mice (15 wks). Sialidase activity was assayed with the crude-membrane (A) and with soluble fraction (B), at pH 3.5–6.5 using citrate-phosphate buffer and at pH 6–8 using PIPES buffer with (□) or without (■) 1 mM Cu^{2+}.

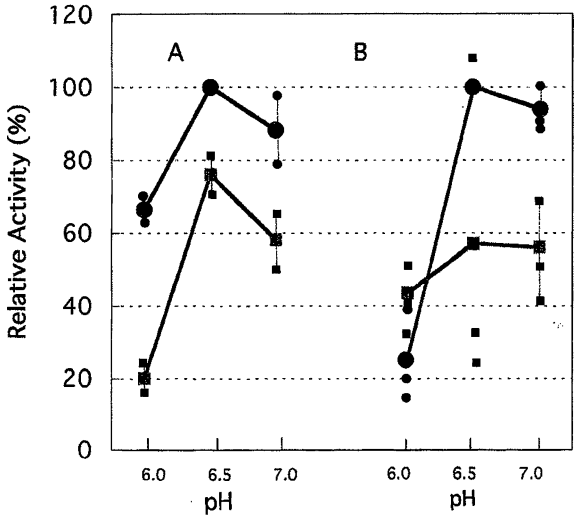

Figure 3. Comparison of relative sialidase activities in PIPES and citrate-phosphate buffers. The relative activity is shown as a percentage of the activity obtained at pH 6.5 in PIPES buffer as a control (100%). Thymi from 8–9-wks-old C3H mice were used. Two experiments for the soluble fraction or three experiments for the crude membrane fraction were performed. The average values from different experiments are shown by line-graphs, and each values were also indicated. A, soluble fraction. B, crude membrane fraction. (—●—) PIPES buffer, (—■—) citrate-phosphate buffer.

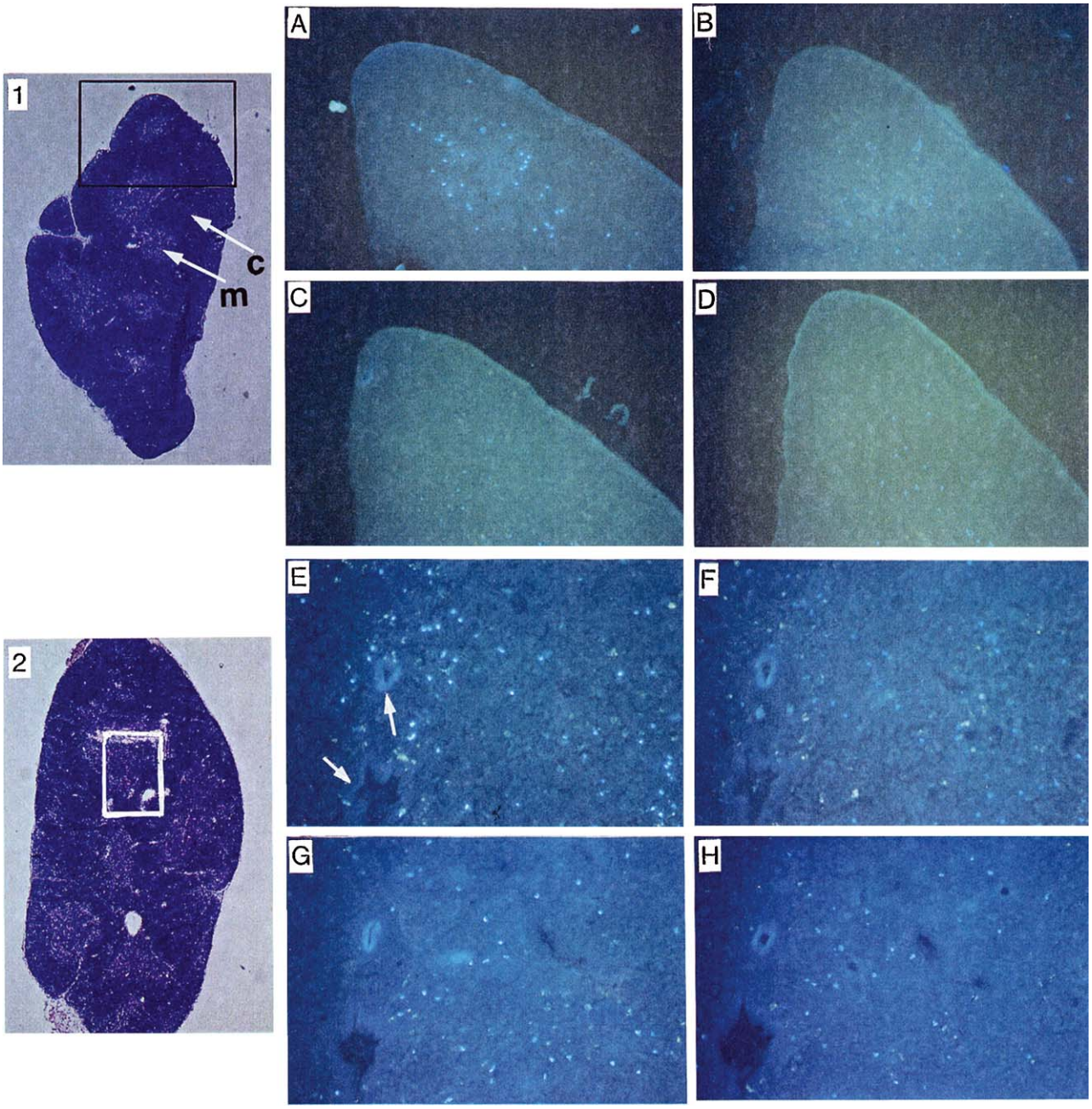

Figure 4. Histochemical active staining of sialidase-positive cells in the thymus. Frozen sections of the thymus from A/J mice (8 wks) (A–D) or C3H mouse (8.5 wks) (E–H) which was reacted with X-NANA solution as described in *Materials and Methods*. The reaction buffers were used at pH 7.0 (A and E), pH 7.0 + 1 mM Cu^{2+} (B and F), pH 4.5 (C and G) and pH 4.5 + 1 mM Cu^{2+} (D and H). These were photographed with ×10 eye lens, and ×10 (A–D) or ×20 (E–H) objective lens. A–D and E–H correspond to the squares in Figure 4-1 and -2, respectively. Figure 4-1 and -2 are stained with hematoxylin-eosin and photographed with ×4 objective and ×10 eye lens. Cortex area (C) and medullar area (M) are indicated.

citrate-phosphate buffer. In the acidic region, the level of activity in the citrate-phosphate buffer and in the sodium-acetate buffer were almost the same and the optimum activity in the acetate buffer was pH 4.5 and higher than that in the citrate-phosphate buffer (data not shown). Thus, at least three kinds of sialidase activity were detected in the murine thymus: soluble enzyme, plasma membrane-type and lysosomal membrane-type activities, and their optimum activities were at pH 6.5, 6.5–7.0 and 4.5–5.0 respectively. This high membrane-bound activity at pH 6.5–7.0 is unique among the activities of enzyme so far reported.

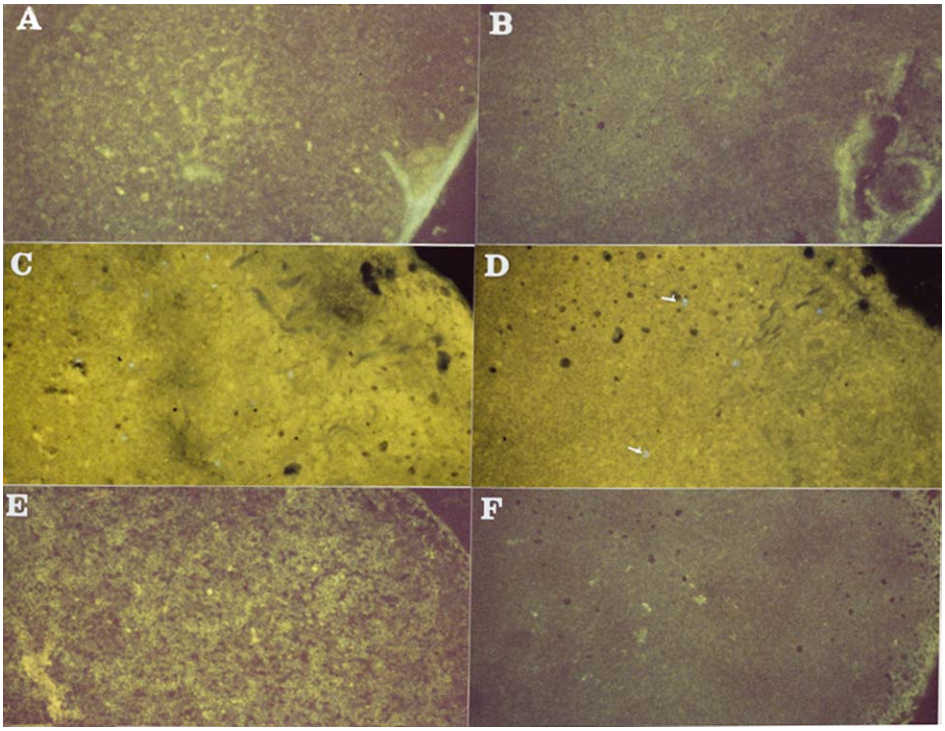

Figure 5. Histochemical active staining of sialidase-positive cells in other organs than the thymus. Frozen sections of the spleen (A and B), lymph node (C and D) and mesenteric lymph node (E and F) from A/J mice (10 wks old) were reacted with X-NANA solution at pH 7.0 (A, C, and E) or at pH 4.5 (B, D, and F) as described in *Materials and Methods* and, were photographed with a $\times$10 eye lens and $\times$20 objective lens. The white arrow indicates positively stained cells.

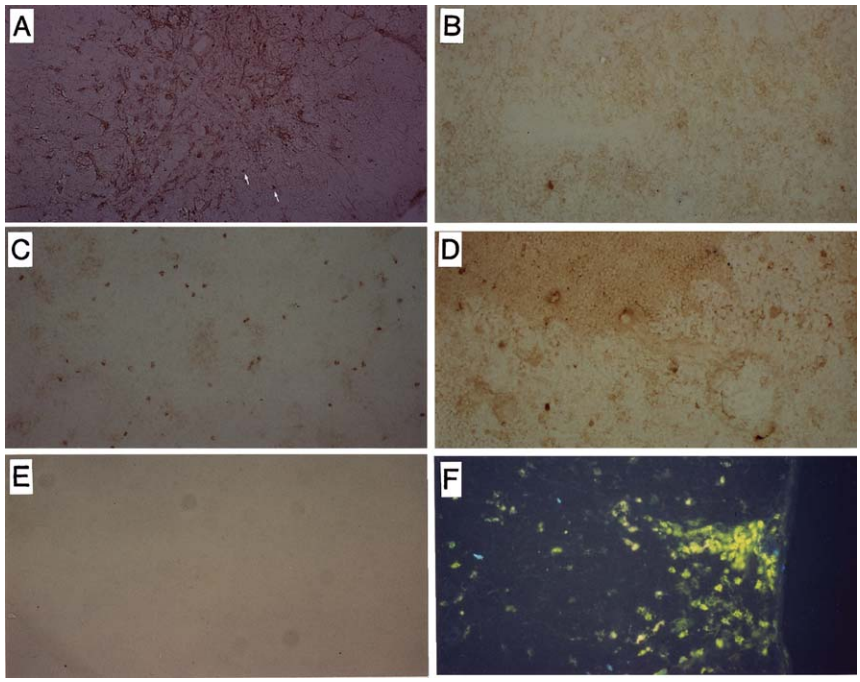

Figure 6. Immunochemical staining of various sections of the thymus. A frozen section of thymus from a C3H mouse (8.5 wks) was reacted with (A) anti-epithelial cell (then biotin-anti-mouse immunoglobulin, avidin-PO), (B) biotin-anti-I-A^k (then avidin-PO), (C) biotin-anti-mouse Ig (then avidin-PO) as a negative control for (A), (D) anti-Mac-2 (then PO-anti-rat Ig), (E) avidin-peroxidase as a negative control for (A)-(D) and (F) PE-R labeled Mac-1. All were photographed with $\times$10 eye lens and $\times$20 objective lens except (A) that was photographed with $\times$10 objective lens.

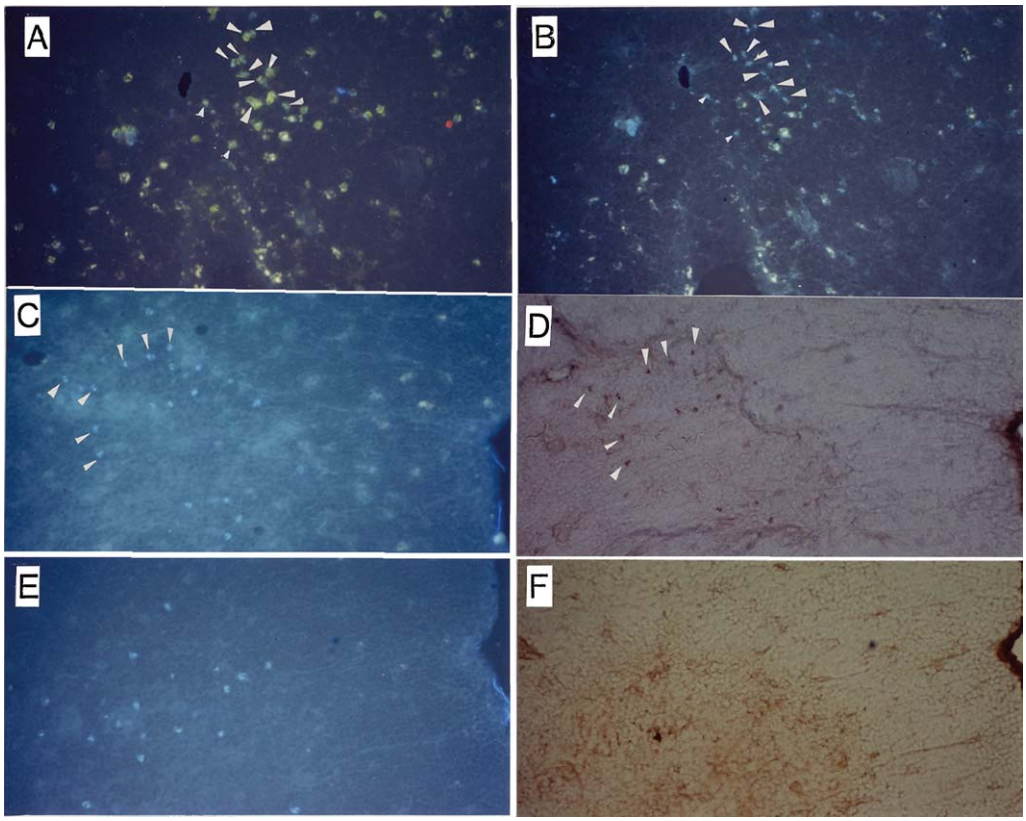

Figure 7. Immunochemical and histochemical active staining of sialidase-positive cells. A frozen section of thymus from a C3H mouse (8.5 wks) was reacted with R-PE labeled Mac-1 (A) and then reactied with X-NANA reagent at pH 7.0 (B). Frozen sections of thymus from a C3H mouse (8.5 wks) were reacted with X-NANA reagent at pH 7.0 (C and E) and then stained with anti-mouse Ig G (D) or anti-Mac-2 (F).

Histochemical staining of sialidase-positive cells

To determine the localization of sialidase positive cells in the thymus, we histochemically stained the thymus tissues using X-NANA as a substrate for sialidase. This reagent fluoresces after hydrolysis by sialidase, and the released molecule (5-bromo-4-chloro-3-indol) becomes insoluble at the position hydrolyzed on the tissue. This reagent was synthesized for this study on the basis of a hint obtained from X-Gal, which is used to select gene-transfected colonies. As shown in Figure 4, strongly white-blue-stained cells were distributed sparsely and unevenly throughout the thymus. Figure 4A–D ($\times$200) and E–H ($\times$400) (thymus tissues from A/J and C3H mice, respectively) correspond to the area enclosed by the black and white squares in Figure 4-1 and -2 ($\times$40), respectively. In the presence of Cu^{2+}, the brightness was reduced at pH 7.0 (Compare Figure 4A and B, or Figure 4E and F.) because the soluble (cytosolic) and membrane-bound enzymes acting at neutral pH were inhibited by Cu^{2+}, while the lysosomal enzymes acting at pH 4.5 were not inhibited by Cu^{2+} (Compare Figure 4C and D, an Figure 4G and H). Hematoxylin-eosin staining distinguished the cortical (C) and medullary (M) areas (Figure 4-1 and -2). X-NANA-stained cells were predominantly seen in the corticomedullar and medullary regions. In addition, a small vessel (indicated by an upper white arrow) showed clearly sialidase-positive, while the larger vessel (indicated by a lower white arrow) was negative. Few cells were stained in the tissues other then the thymus (Figure 5).

Immunochemical characterization of sialidase-positive cells

To study the surface antigens of sialidase-positive cells, thymus sections were stained with several antibodies: anti-epithelial cells (pan-epithelial cytokeratin) (Figure 6A), anti-I-A^k (Figure 6B), anti-Mac-2 (Figure 6D) and anti-Mac-1 (PE-R-conjugated anti-mouse CD11b/CD18 mAb) (Figure 6F). I-A^k, Mac-1 and Mac-2 antigens are MHC class II antigen, β2-integrin and an activated macrophage marker molecule, respectively. As stated in Materials and Methods, thymus section was stained with anti-epitherial cells (first antibody, mouse IgG1), biotinylated horse anti-mouse IgG (H + L) immunoglobulin (second antibody), horse-radish peroxidase-labeled streptavidin and DAB system (Figure 6A). As the negative controls of Figure 6A, thymus sections were stained without the first

antibody and without the first and second antibodies, respectively (Figure 6C and E). Unexpectedly, only with the second antibody (anti-mouse IgG), some cells were stained clearly (Figure 6C). These positive cells can also be seen in Figure 6A (indicated by arrows). The cells stained with anti-mouse IgG (Figure 6C), anti-Mac-2 (Figure 6D), and anti-Mac-1 (Figure 6F, medulla aria) are sparsely distributed similarly as sialidase-positive cells. In order to determine whether those cells are sialidase-positive or not, a PE-R-labeled anti-Mac-1 stained section (Figure 7A) were photographed and then stained with X-NANA to detect the sialidase positive cells (Figure 7B). Some of the PE-R-Mac-1-stained cells were sialidase-positive (shown by arrows), though other larger cells around the large vessel were sialidase-negative. Some Mac-1 positive cells, therefore, are sialidase-positive. Other sections were first stained with X-NANA (Figure 7C and E), photographed, and then stained with an anti-mouse Ig system (Figure 7D) or anti-Mac-2 system (Figure 7F). Mac-2 antigen is a galactose-specific lectin that binds IgE [22] and is expressed on activated macrophages. Unexpectedly, the sialidase-positive cells coincided exactly with the cells stained with anti-mouse Ig (Figure 7C and D) but differed from Mac-2-positive cells (Figure 7E and F). The sialidase-positive cells thus expressed Mac-1 (CD11b/CD18) and Ig molecules on their surfaces, and they were located sparsely in the medulla or in the area bordering the medulla and cortex.

NP40-solublilized sialidase from residual materials, including vessels

As Figure 4E–H shows, a vessel was stained clearly as sialidase-positive. In order to confirm this activity using a 4MU-NANA assay, residual materials, including vessels and thymic capsule, on nylon meshes that had been obtained after filtrating thymus homogenate with nylon meshes were solubilized with NP40. Using the NP40-solubilized fraction, sialidase activity was assayed at various pH values (Figure 8). A high level of sialidase activity was observed at pH 6.5, and this activity was inhibited by 1 mM of Cu^{2+}. A small peak was observed at pH 4.5, and this activity was also inhibited by 1 mM of Cu^{2+}. Figure 4F and H, however, show that the vessel was stained even in the presence of 1 mM of Cu^{2+}. Although further study of this discrepancy is needed, it is clear that a plasma membrane-type sialidase was contained in the NP40 fraction. Miyagi *et al.* [14] reported that a sialidase of rat plasma membrane showed optimal activity at pH 5.0 when gangliosides was used as a substrate. They cloned human ganglioside (plasma membrane) sialidase gene and transfected it in Cos-1 cells, and they observed two optimum peaks for the activity at pH 4.5–4.8 and at pH 6.0–6.5 [23]. Our NP40-solubilized sialidase showing high level of activity at pH 7.0 with 4MU-NANA (Figure 8) probably has substrate specificity other then for gangliosides, *e.g.*, for glycoprotein. Further studies are needed.

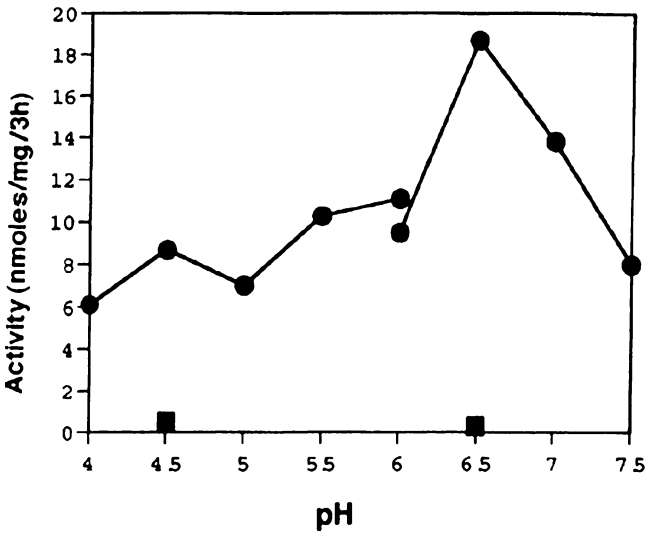

Figure 8. Sialidase activity at various pH with NP40-solublilized sialidase from residual material including vessels. Sialidase activity was assayed with NP40 solubilized fraction at pH 4.0-6.0 using citrate-phosphate buffer, and at pH 6.0–7.5 using PIPES buffer with (■) or without (●) 1 mM Cu^{2+}.

Discussion

We have demonstrated in this study that high sialidase-active cells are present in the thymus. They are sparsely distributed throughout the thymic medulla and express immunoglobulin and Mac-1 antigens. This is the first report of sialidase-positive cells expressing such antigens. Based on the report by Landolfi [24], we expected that T cells would show sialidase activity. Although further study is needed to characterize these sialidase-positive cells, the results of this study have clearly shown that only a small percentage of cells in the thymus have sialidase activity. Mac-1 is a $\beta 2$ integrin consisting of a heterodimer of an α chain (CD11b) and β chain (CD18) [25]. It is found on macrophages, monocytes, granulocytes, large granular lymphocytes, and immature $CD5^+$ B cells [26]. Since the sialidase-positive cells are also IgG-positive, they must be of a B cell lineage. Akashi *et al.* [27] reported that a significant number of pro-B and pre-B cells were found in the thymic cortex, whereas mature B cells that express B220 and IgM were found in the corticomedullar and medullary regions of the thymus, and a significant number (about 3×10^4/day) of the cells are exported from the thymus. It is unclear whether these B cells are the same as the sialidase-positive cells that we identified in the present study. In addition to sialidase activity, the sialidase-positive cells probably have high level of peroxidase activity, because we noticed that these cells in addition to the large number of smaller cells were stained strongly with DAB if we did not pre-treat the section with H_2O_2-methanol. The staining with anti-mouse immunoglobulin with DAB system (Figure 7D), however, is specific to the antigen, because sialidase positive cells in Figure 7E were stained negatively by DAB,

though Figure 7D and F were stained at the same time. The sialidase positive cells are Mac-2 negative (Figure 7F). Soga *et al*. [28] reported that mouse thymic macrophages had phenotypic heterogeneity by immuno-histochemical double staining. All types, except for one located in the subcapsular region of the thymus, expressed Mac-2 antigen. The sialidase-positive cells, therefore, are probably not macrophages. They [28] also showed that some of the heterogeneous thymus macrophages have CD32/16 (Fc receptor for IgG), but our sialidase-positive cells differed from Fc receptor-positive cells (data not shown).

Murine lysosomal sialidase gene from the liver is known as the Neu-1 gene, which is located at the D end of H-2 on chromosome 17 [29,30]. SM mice that lacked the Neu-1 gene showed low level of this sialidase activity at pH 4.4 with the liver whole homogenate [31]. We have cloned the cytosolic sialidase gene from A/J thymus and shown that the gene is localized to the distal part of mouse chromosome 1D [16]. The gene that encodes the membrane-bound sialidase acting at pH 7.0 is also probably different from the Neu-1 gene. Further characterization of these sialidase-positive cells, as well as the enzyme and gene, is needed.

In addition, it should be noted that sialidase was present in a vessel shown in Figure 4E–H. The NP40-solubilized fraction from the residual materials, including vessels, contained a plasma membrane-type sialidase that may mediate cell adhesion. Sialidase on the cell surface has been suggested by a few classic studies to be a mediator of cell adhesion [32,33]. These studies showed that cell adhesion was enhanced on a sialidase-coated plate and was inhibited by sialidase-specific inhibitors and by cytochalasin B. In contrast, enhanced adhesion to a concanavalin A-coated plate was not inhibited by cytochalasin B. This indicates that the process of cell adhesion based on sialidase and sialoglycoconjugate on the cell surface may involve the actin cytoskeletal system. Sialidase on a blood vessel may also bind to mature T cells expressing ganglioside or sialoglycoconjugate such as CD8, CD43 and CD45, whose asialo-forms were reported to be the PNA receptor molecule on immature thymocytes and these were sialylated on matured T cells [34]. Interaction of selectin with sialyl Lewis X ligands is known to play an important role in homing of lymphocytes [35]. In addition, sialidase and other sialoglycoconjugates may also play an important role. The possible functional role of sialidase expressed on venules in recruiting T cells remains to be investigated.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research (C)(2) (07808069) from the Ministry of Education of Japan. S. K.-O. gratefully acknowledges Dr. Yoshino (International Christian University), whom I asked to synthesize X-NANA. He consulted with the proper people, including Professor Dr. Shoure (Kiel, Germany), and I finally have obtained this from Rose Scientific LTD (Edmonton, Alberta, Canada). I also gratefully thank Professor Dr. Hakomori (Seattle, Washington, USA), who has encouraged me for a long time to publish this paper. We also thank Prof. Onoe (Hokkaido University) for permission to use the facilities for microscopic study.

References

1 Pilatte Y, Bignon J, Lambre CR, Sialic acids as important molecules in the regulation of the immune system: Pathophysiological implications of sialidases in immunity, *Glycobiology* **3**, 201–17 (1993).
2 Gascoigne NRJ, T-cell differentiation: MHC class I's sweet tooth lost on maturity, *Current Biology* **12**, R99–101 (2002).
3 Priatel JJ, Chui D, Hiraoka N, Simmons CJ, Richardson KB, Page DM, Fukuda M, Varki NM, Marth JD, The ST3Gal-I sialyltransferase controls $CD8^+$ T lymphocyte homeostasis by modulation 0-glycan biosynthesis, *Immunity* **12**, 273–83 (2000).
4 Daniels MA, Devine L, Miller JD, Moser JM, Lukacher AE, Altman JD, Kavathas P, Hogquist KA, Jameson SC, CD8 binding to MHC class I molecules is influenced by T cell maturation and glycosylation, *Immunity* **15**, 1051–61 (2001).
5 Moody AM, Chui D, Reche PA, Priatel JJ, Marth JD, Reinherz EL, Developmentally regulated glycosylation of the CD8 $\alpha\beta$ coreceptor stalk modulates ligand binding, *Cell* **107**, 501–12 (2001).
6 Hirayama Y, Inaba K, Inaba M, Kato T, Kitamura M, Hosokawa T, Ikehara S, Muramatsu S, Neuraminidase-treated macrophages stimulate allogenic $CD8^+$ T cells in the presence of exogenous Interleukin 2, *J Exp Med* **168**, 1443–56 (1988).
7 Kapsenberg ML, Stiekema FEM, Kallan A, Bos JD, Roozemond RC, The restrictive role of sialic acid in antigen presentation to a subset of human peripheral $CD4^+$ T lymphocytes that requires antigen-presenting dendritic cells, *Eur J Immunol* **19**, 1829–34 (1989).
8 Karasuno T, Kanayama Y, Nishiura T, Nakao H, Yonezawa T, Tarui S, Glycosidase inhibitors (castanospermine and swainsonine) and neuraminidase inhibit pokeweed mitogen-induced B cell maturation, *Eur J Immunol* **22**, 2003–8 (1992).
9 Kearse KP, Cassatt DR, Kaplan AM, Cohen DA, The requirement for surface Ig signaling as a prerequisite for T cell:B cell interactions. A possible role for desialylation , *J Immunol* **140**, 1770–8 (1988).
10 Taira S, Nariuchi H, Possible role of neuraminidase in activated T cells in the recognition of allogeneic Ia, *J Immunol* **141**, 440–6 (1988).
11 Kijimoto-Ochiai S, Horimoto E, Uede T, Demonstration of the interaction between the CD23 molecule and the galactose residue of glycoproteins, *Immunol Lett* **40**, 49–53 (1994).
12 Kijimoto-Ochiai S, Uede T, CD23 molecule acts as a galactose-binding lectin in the cell aggregation of EBV-transformed human B-cell lines, *Glycobiology* **5**, 443–8 (1995).
13 Kijimoto-Ochiai S, CD23 (the low-affinity IgE receptor) as a C-type lectin: A multidomain and multifunctional molecule, *Cell Mol Life Sci* **59**, 648–64 (2002).
14 Miyagi T, Hata K, Hasegawa A, Aoyagi T, Differential effect of various inhibitors on four types of rat sialidase, *Glycoconjugate J* **10**, 45–9 (1993).
15 Kijimoto-Ochiai S, Age-dependence of high sialidase activity in newly discovered cells sparsely located in the thymus medulla , *Glycoconjugate J* **14**(Sup.1.), S138 (1997).
16 Kotani K, Kuroiwa A, Saito T, Matsuda Y, Koda T, Kijimoto-Ochiai S, Cloning, chromosomal mapping, and characteristic

5′-UTR sequence of murine cytosolic sialidase, *Biochem Biophys Res Commun* **286**, 250–8 (2001).
17 Guesdon JL, Ternynck T, Avrameas S, The use of avidin-biotin interaction in immunoenzymatic techniques, *J Histochem Cytochem* **27**, 1131–9 (1979).
18 Ray TK, A modified method for the isolation of the plasma membrane from rat liver, *Biochim Biophys Acta* **279**, 456–74 (1972).
19 David T, Poweel AJ, Lloyd CW, Rees DA, Rapid isolation of plasma membranes in high yield from cultured fibroblasts, *Biochem J* **168**, 187–94 (1977).
20 Potier M, Mameli L, Belisle M, Dallaire L, Melancon SB, Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl-α-D-N- acetylneuraminate) substrate, *Analy Biochem* **94**, 287–96 (1979).
21 Miyagi T, Sagawa J, Konno K, Tsuiki S, Immunological discrimination of intralysosomal, cytosolic, and two membrane sialidases present in rat tissues, *J Biochem* **107**, 794–8 (1990).
22 Cherayil BJ, Weiner SJ, Pilla S, The Mac-2 antigen is a galactose-specific lectin that binds IgE, *J Exp Med* **170**, 1959–72 (1989).
23 Wada T, Yoshikawa Y, Tokuyama S, Kuwabara M, Akita H, Miyagi T, Cloning, expression, and chromosomal mapping of a human ganglioside sialidase, *Biochem Biophys Res Comm* **261**, 21–7 (1999).
24 Landolfi NF, Leone J, Womack JE, Cook RG, Activation of T lymphocytes results in an increase in H-2 encoded neuraminidase, *Immunogenetics* **22**, 159–67 (1985).
25 Kishimoto TK, Larson RS, Corbi AL, Dustin ML, Staunton DE, Springer TA, The Leukocyte Integrins, *Adv Immunol* **46**, 149–82 (1989).
26 De la Hera A, Alvarez-Mon M, Sanchez-Madrid F, Martinez-A C, Durantez A, Co-expression of Mac-1 and p150,95 on CD5+ B cells. Structural and functional characterization in a human chronic lymphocytic leukemia, *Eur J Immunol* **18**, 1131–4 (1988).
27 Akashi K, Richie LI, Miyamoto T, Carr WH, Weissman IL, B lymphopoiesis in the thymus, *J Immunol* **164**, 5221–6 (2000).
28 Soga H, Nakamura M, Yagi H, Kayaba S, Isii T, Gotoh T, Itoh T, Heterogeneity of mouse thymic macrophages: I. Immunohistochemical analysis, *Arch Histol Cytol* **69**, 53–63 (1997).
29 Womack JE, David CS, Mouse gene for neuraminidase activity (Neu-1) maps to the D end of H-2, *Immunogenetics* **16**, 177–80 (1982).
30 Klein J, Klein D, Figueroa F, Should the *Neuraminidase-1* locus be considered as part of the major histocompatibility complex? *Human Immunology* **15**, 396–403 (1986).
31 Potier M, Lu Shun Yan D, Womack JE, Neuraminidase deficiency in the mouse, *FEBS Letters* **108**, 345–8 (1979).
32 Rauvala H, Carter WG, Hakomori S, Studies on cell adhesion and recognition. I. Extent and specificity of cell adhesion triggered by carbohydrate-reactive proteins (glycosidases and lections) and by fibronectin, *J Cell Biol* **88**, 127–37 (1981).
33 Carter WG, Rauvala H, Hakomori S, Studies on cell adhesion and recognition. II. The kinetics of cell adhesion and cell spreading on surfaces coated with carbohydrate-reactive proteins (glycosidases and lectins) and fibronectin, *J Cell Biol* **88**, 138–48 (1981).
34 Wu W, Harley PH, Punt JA, Sharrow SO, Kearse KP, Identification of CD8 as a peanut agglutinin (PNA) receptor molecule on immature thymocytes, *J Exp Med* **184**, 759–64 (1996).
35 Kannagi R, Regulatory roles of carbohydrate ligands for selectins in the homing of lymphocytes, *Current Opinion in Structural Biology* **12**, 599–608 (2002).

Received 11 August 2003; revised 11 February 2004;
accepted 24 February 2004