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Low expression of Neu2 sialidase in the thymus of SM/J mice—existence of neuraminidase positive cells "Neu-medullocyte" in the murine thymus

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Abstract We have already reported that the homogenate of the A/J mouse thymus shows a high sialidase activity at the neutral pH region and that in both soluble and membrane fractions optimal pH was 6.5–7 (Kijimoto-Ochiai *et al.*, *Glycoconj. J.*, 20:375–384, 2004). In the present study, we investigated the level of sialidase activities in the thymus of the SM/J mouse, a mouse strain that we know to have a Neu1^{a} allele that reveals a low level of sialidase activity in the liver. We found that while in the A/J thymus the soluble sialidase activity at pH 6.5 was high, the SM/J thymus lacked all such activity. A QTL analysis of SMXA recombinant inbred strains showed that soluble sialidase activity correlated well with the D1Mit8/9 marker on chromosome 1. The murine whole DNA-sequence data and the results of our FISH analysis (Kotani *et al.*, *Biochem. Biophys. Res. Comm.*, 286:250–258, 2001) showed that this location is consistent with the position of Neu2 gene. We confirmed that it is hard to detect the Neu2 enzyme of the SM/J mouse thymus by an anti-Neu2 antibody using a Western blot analysis. We also found that

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while the mRNA expression of Neu2 was quite normal in the SM/J mouse liver, it was very low in the SM/J mouse thymus. We therefore conclude that the lack of soluble sialidase activity in the SM/J mouse thymus is due to the thymus-specific low expression level of the Neu2 gene. We have previously shown that the sialidase positive cell which contains the Mac-1 and immunoglobulin, and which is located sparsely in the corticomedullar region or medullary region of the A/J mouse thymus (Kijimoto-Ochiai *et al.*, *Glycoconj. J.*, 20:375–384, 2004). We showed now in this paper that the detection of this cell in the SM/J mouse thymus at pH 7.0 was difficult. We propose, therefore, to name the cell "Neu-medullocyte".

Keywords Sialidase · Neu2-defect · Thymus · SM/J mouse · "Neu-medullocyte"

Introduction

Cell-to-cell interactions are important in the working of the immune system, and in these interactions sialic acids play an important role. The addition and removal of sialic acids from glycans are regulated respectively by sialyltransferases and by sialidases, and we have reported that sialidase-treated EBV-transformed B cells result in large aggregations [1]. We have also reported that the levels of sialidase at neutral pH are high in both soluble and membrane fractions of the mouse thymus and also reported that unique Mac-1- and immunoglobulin-positive cells containing sialidase are distributed in the medulla only sparsely [2].

During the past 10 years, many sialidase genes (about 15 or more) have been cloned from different species. There are four sialidase genes in mammals: they have been designated Neu1, Neu2, Neu3 and Neu4, and their subcellular localizations have been elucidated. Neu1, for example, encodes a lysosomal enzyme [3, 4] and the deletion of this enzyme induces sialidosis or galactosialidosis [5]. This gene is located in the MHC region and in mice has three alleles, a, b and c [6]. The cloning of this gene has shown that replacement of a few amino acids produces the three Neu1 alleles [7] and that this is the reason for the low activity of the $Neu1^a$ enzyme [8]. Neu2 encodes a soluble cytosolic enzyme [9, 10], and Neu3 encodes a plasma membrane enzyme [11, 12], while Neu4 encodes a lysozomal and mitochondrial enzyme [13–16].

Since the SM/J mouse, a small mouse (thus SM), has the $Neu1^a$ allele, its liver shows a low level of sialidase activity [17]. At the same time, it has been reported that the level of Neu1 sialidase activity in T lymphocytes is controlled by the Neu1 locus [18]. We ourselves have reported that the levels of sialidase at neutral pH in the thymus of the A/J mouse were high in both of soluble- and crude membrane-fractions [2]. In this study, we examined the level of sialidase activity in the thymus of the SM/J mouse and found that the soluble sialidase level was very low. We confirmed that this soluble sialidase activity was controlled by Neu2 gene and that it showed a low expression level of Neu2 mRNA.

Materials and methods

Chemicals 4MU-Neu5Ac was obtained from Nacalai Tesque (Kyoto, Japan). X-NANA was purchased from Rose Scientific Ltd. (Edmonton, Alberta, Canada). PNA (peanut agglutinin) was obtained from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan).

Antibodies Anti-Neu2 polyclonal rabbit antibody was a kind gift from Dr. Miyagi (Miyagi Cancer Research Institute, Miyagi, Japan).

Mice and immune tissues $Neu1^b$ mice of A/J, C3H, BALB/c and C57BL/6 strains and B10.S ($Neu1^c$) were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). SM/J ($Neu1^a$) and SMXA recombinant mice were provided by Prof. M. Nishimura while he was working at Hamamatsu University School of Medicine and in Nagoya University, School of Medicine. After his retirement, SM/J mice were purchased from RIKEN BRC (Tsukuba, Ibaraki, Japan). The origins and histories of SM/J and SMXA recombinant mice have been described in Ref [19]. Thymuses, spleens, peripheral lymph nodes, and other organs were taken from the mice and frozen until use.

Preparation of soluble and crude membrane fractions This was done according to the method described in a previous paper [2].

Sialidase assay This was done according to the method described in a previous paper [2].

Western blotting and Neu2 detection Soluble fractions of the thymus homogenate from SM/J and C57BL/6 mice were performed electrophoresis and transferred onto a PVDF membrane. Neu2 antigen was detected by anti-Neu2 rabbit polyclonal antibody, peroxidase-labeled anti-rabbit IgG and ECL solution.

A reverse transcription/polymerase chain reaction (RT-PCR) Total RNA was extracted from tissues by using Isogen (Nippon Gene, Tokyo, Japan). cDNAs were synthesized by reverse transcription of 2 μg of the total RNA at 42°C for 1 h with 100 U of ReverTra Ace (Toyobo, Osaka, Japan) and oligo(dT). Real time PCR was performed with a DyNAmo HS SYBR Green qPCR kit (Finnzymes, Espoo,

Finland) and analysed by Opticon Monitor 2 (MJ Research Inc., St. Bruno, Canada). cDNAs equivalent to 20 ng total RNA were used as templates. Expression levels were calculated as the relative abundance of the template in the cDNA mixture compared to β-actin. The primer pairs used were 5′-AGAGATGTTTGCCCCTGGAC-3′ (forward, F) and 5′-CGTGGTCATCACTGAGGAGA-3′ (reverse, R) for Neu1, 5′-CAGAATCCCTGCTCTGCTCT-3′ (F) and 5′-CTTGGGTCACCACTTCCTCA-3′ (R) for Neu2, 5′-GGAAGAACAGAGTGGGGTGA-3′ (F) and 5′-ATG TGGCCTCCATCAGTAGC-3′ (R) for Neu3, and 5′-CTAAGGCCAACCGTGAAAAG-3′ (F) and 5′-CCATCA CAATGCCTGTGGTA-3′ (R) for β-actin. Neu4 expression was measured by semi-quantitative PCR, since real time PCR did not work for this gene. PCR for Neu4 were performed with cDNA templates equivalent to 0.1 μg total RNA using Taq DNA polymerase (Sigma-Aldrich, St. Louis, MD) for 40 cycles under the condition of 30 s at 94°C, 1 min at 60°C, and 2 min at 72°C. As a standard, β-actin was amplified in separate reactions for 28 cycles. Conventional RT-PCR for Neu2 was carried out by the use of the same program as for Neu4, except for a cycle number of 35 cycles when employing the primers specified above. The products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Bands for Neu4 were quantified by Lane & Spot Analyser (Atto, Tokyo, Japan), and normalized by β-actin. The primers used were 5′-GCTTGGCTCTAGTGGTGGTC-3′ (F) and 5′-TCCTTTCCCTCTGGAAGAGC-3′ (R) for Neu4, and 5′-GTGGGGCGCCCCAGGCACCA-3′ (F), and 5′-CTCC TTAATGTCACGCACGATTTC-3′ (R) for β-actin.

Thymocyte fractionation with PNA Thymocytes were fractionated into PNA-aggregated immature T cells and unaggregated mature T cells [20]. PNA, peanut agglutinin, is a galactose-binding lectin, and Gal-GalNAc is a potent inhibitor of the aggregation [21]. Briefly, thymus tissue was homogenized gently in RPMI medium with a glass homogenizer. The cell suspension was passed through a metal filter, centrifuged, resuspended, and filtered through nylon mesh. The single-cell suspension was incubated with PNA (0.5 mg/ml) for 10 min at room temperature, overlaid on 20% fetal calf serum in a tube, and left for 30 min; the aggregated cells (immature T cells) were at the bottom and single cells (mature T cells) were at the top. Single cells and aggregated cells were harvested, washed with 0.15 M lactose solution in RPMI medium and then washed twice with RPMI medium, and used for FACS analysis.

FACS analysis PNA-separated cells were incubated with PE-labeled anti-CD4, PE-anti-CD8, FITC-anti-CD4 or FITC-anti GA1 in 0.1% BSA—0.1% NaN_3-RPMI for 30 min on ice. The cells were washed twice, and 2 μl (10 μg) of PI (propidium iodide) was added for the detection of dead cells. Fluorescent antibody-labeled cells were analyzed by Becton Dikinson FACSCant using FACS Diva analyzing software.

Histochemical analysis of sialidase-positive cells This was done according to the method reported previously [2].

Confocal microscopic study This was carried out at Nikon Institute at Ohfuna, Tokyo, Japan using a Nikon confocal microscope.

Results

Lack of sialidase activity in the soluble fraction of the thymus of SM/J mice

We have reported that the mouse thymus of the A/J and C3H strains shows high sialidase activity at the neutral pH region with both soluble and crude membrane fractions [2]; yet, on the other hand, it is well known that Neu1 from SM/J ($Neu1^a$) mouse undergoes amino acid mutation at three to four points and that this causes low sialidase activity in the liver or kidney.

We therefore compared the sialidase activity in the thymus of SM/J and A/J mice and found that only the soluble sialidase activity at pH 7.0 of the SM/J mouse showed very low or no activity (Table 1). We next examined the sialidase activities of various $Neu1^b$ strains (A/J, C3H, C57BL/6, MRL/l and MRL/n), and confirmed that only the SM/J mouse ($Neu1^a$) lackes soluble sialidase activity at pH 7.0 when compared with other mice ($Neu1^b$). The crude membrane fraction of the SM/J mouse, on the other hand, showed similar activity to that from $Neu1^b$ mice. (Table 1, Fig. 1).

Our candidate for the gene that controls the sialidase activity at pH 7.0 in the soluble fraction

In order to examine the gene that controls the sialidase activity at pH 7.0 in the soluble fraction, we used SMxA RI mice 22 strains [19]. As for the SM/J mouse, low sialidase

Table 1 Comparison of sialidase activities in thymuses from A/J and SM/J mice

	pH 7.0		pH 4.5	
	A/J	SM/J	A/J	SM/J
Soluble fraction	20.8±0.5	0.2±0.1	3.7±0.1	1.0±0.7
Crude membrane fraction	7.7±0.2	7.6±0.6	14.4±0.3	18.8±5.3

Activity: released 4MU nmol/mg protein per 3 h

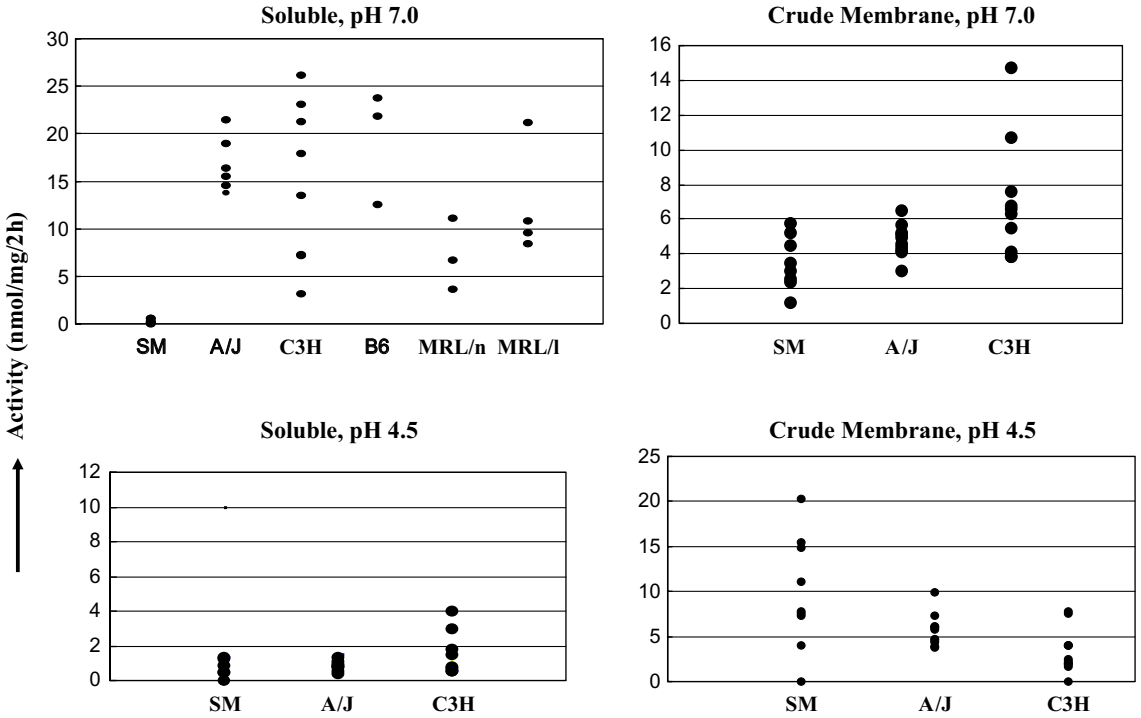

Fig. 1 Lack of sialidase activity in the soluble fraction of the thymus from SM/J mice. Sialidase activities were determined in the soluble fraction at pH 7.0 (**a**) and at pH 4.5 (C), and in the membrane fraction at pH 7.0 (**b**) and at pH 4.5 (**d**) from thymuses of various mice. The number of SM/J mice tested in **a** is 7, though they can not be distinguished

activity has been reported using the whole homogenate of liver and kidney at pH 4.5 [17], and this we confirmed with the curd membrane fraction (Table 2) of A/J and SM/J mice or A group and S group of SMXA RI mice. (A means that the genotype of Neu1 locus of the substrain is the A/J ($Neu1^b$) genotype while S means that the genotype of the substrain is the SM/J ($Neu1^a$) genotype). We therefore conclude that the sialidase activity of the liver and kidney at pH 4.5 is mainly controlled by the Neu1 gene (see "Discussion").

On the other hand, sialidase activities at pH 4.5 with a crude membrane fraction from the spleen, thymus showed very little difference between A/J, SM/J mice, or SMxA RI mice. Moreover, nor, did the level of soluble sialidase activity at pH 7.0 in thymuses taken from 9- to 11-week-old male recombinant inbred (RI) strains (22 substrains) show any difference between A and S group with regard to the Neu1 gene (Table 2). This means that the Neu1 gene does not control the soluble sialidase activity at pH 7.0. In order to examine the gene that controls the sialidase activity at pH 7.0 in the soluble fraction, the activity obtained from the 22 sub-strains was screened for QTL analysis [22], and a candidate was obtained in the region near the marker Mit 8/9 on chromosome 1 (with $p=0.001$). This result accorded

Table 2 Sialidase activities in various organs from SMXA RI mice[a]

Organ	A/J	SMXA (means±S.D.)		SM/J
		A	S	
Liver	6.9	9.1±3.3 (4)	2.5±0.7 (4)	3.6
Kidney	41.1	54.9±7.7 (4)	19.8±9.3 (4)	16.5
Spleen	4.4	3.9±1.1 (4)	3.7±0.7 (4)	4.4
Thymus	14.4	13.4±3.3 (11)	10.1±2.5 (11)	18.8
(pH 7.0)[a]	7.7	5.9±3.9 (11)	5.2±4.1 (11)	7.6
Soluble[b]	21.5	4.7±6.3 (11)	5.2±5.7 (11) (Neu1)	1.0
		9.2±6.5 (10)	1.2±0.6 (12) (D1Mit8/9)	

[a] Activities were assayed using the crude membrane fraction at pH 4.5 except for lane at pH 7.0
[b] Soluble sialidase activity from thymus at pH 7.0 for each substrain of SMxA was shown with average ±S.D. after grouped into A or S concerned with Neu1 or D1Mit8/9

well with our finding by FISH analysis that the Neu2 sialidase gene cloned from the A/J thymus was located on chromosome 1 [9]. We therefore show in Fig. 2 the distribution of soluble sialidase activity of thymuses at pH 7.0 in SMXA RI strains with S or A at the marker Mit 8/9 for each substrain. Consequently, Table 2 reveals that with regard to the marker Mit 8/9 the average value for the soluble sialidase activity of S group was significantly lower than that of the A group. We obtained a similar result when we found that the gene encoding membrane-bound sialidase acting at pH 7.0 also existed on chromosome 1 near the marker D1Rik133, which would correspond to the Neu4 gene described in the DNA sequence database. A murine sialidase Neu4 has been identified by Comelli *et al.* [13]. It therefore became clear that the level of sialidase activity at pH 7.0 in the soluble fraction from the mouse thymus is controlled by the Neu2 gene and that the level of sialidase activity with 4MU-NANA at pH 7.0 in the crude membrane fraction is controlled by the Neu4 gene.

Low expression of Neu2-enzyme and mRNAs in the thymus from SM/J mouse

To explain the low level of soluble sialidase activity in the thymus from the SM/J mouse, we studied the expression levels of Neu2 mRNA in the thymus from five mouse-strains with the Neu1 alleles a (SM/J), b (A/J, C57BL/6 and BALB/c) and c (B10/S). Clearly Neu2 mRNA expression is low in the SM/J thymus (Fig. 3a). Similar quantitative data was obtained by real time PCR experiment (Fig. 3b) Moreover, we were unable to detect a clear band on a Western blot of the soluble fraction from the SM/J mouse thymus with an anti-Neu2 antibody (Fig. 3c). We also compared the mRNA levels for Neu1, Neu3 and Neu4 in the thymuses of SM/J mice with C57BL/6 mice as a control (Fig. 3d,e). The levels of mRNA of these sialidases are rather higher in SM/J mice than in C57BL/6 mice. The mRNA expression of Neu2 in the liver and kidney of SM/J mice was also the same or rather higher than for the control mice (Fig. 3f), and the result for Neu1 were similar to these (Fig. 3g). We have therefore concluded that the low activity level of soluble sialidase in the thymus of SM/J mouse is the result of a low expression level of Neu2 mRNA and that the deletion of Neu2 expression in SM/J mice is specific to the thymus.

FACS analysis of T cells and histochemical study of sialidase positive cell in the mouse thymus—"Neu-medullocyte"

We undertook a FACS analysis, with some markers on the cell surface, to study T cells from the thymus of SM/J and C57BL/6 mice (Fig. 4). T cells from the thymus were separated with PNA into two fractions, aggregated (immature T) and single (mature T) cells. Mature T cells were stained with anti-CD4 and anti-CD8. We found that the populations of both double negative cells and CD4-single positive cells are larger in the SM/J mouse (15.9 and 19.8%) than in the control mouse (8.0 and 13.8%) (Fig. 4). The staining patterns with anti-GA1, which recognizes the NK cell marker of asialo GM1 ganglioside, was the same for both SM/J and A/J mice (data not shown).

Next we performed a histochemical study on the sialidase-positive cells in the mouse thymus. Figure 5 shows the active staining of sialidase-positive cells in thymuses of A/J and SM/J mice with X-NANA as a substrate at pH 7.0 and 4.5. In the A/J mouse thymus stained at pH 7.0, sialidase positive (blue white) cells can be observed mainly in the medullary region (Fig. 5a,b), while they are scarcely observable in the SM/J mouse, although yellow cells were abundant (Fig. 5c). On the other hand, when the sections were stained at pH 4.5, cells similar to those in the A/J mouse were also observed in the SM/J mouse (Fig. 5d,e). The sialidase positive cells stained at pH 7.0 seemed to be larger than those stained at pH 4.5 (Fig. 5b,d).

Sialidase positive cells in Fig. 5a were examined by confocal microscopy (Fig. 5f–h). The image of the sialidase positive cell in the SM/J thymus at pH 7.0 (Fig. 5g)

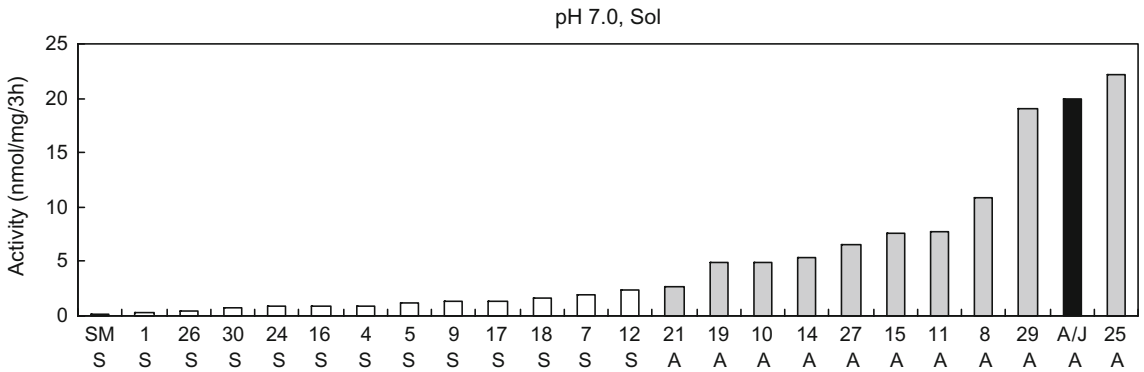

Fig. 2 Distribution of soluble sialidase activity at pH 7.0 in the thymuses from 22 of the SMXA recombinant inbred strains and from two parental strains (SM/J and A/J) at 9–11 weeks of age. A and S under the strain number mean the genotype of each strain at the marker of D1Mit8/9

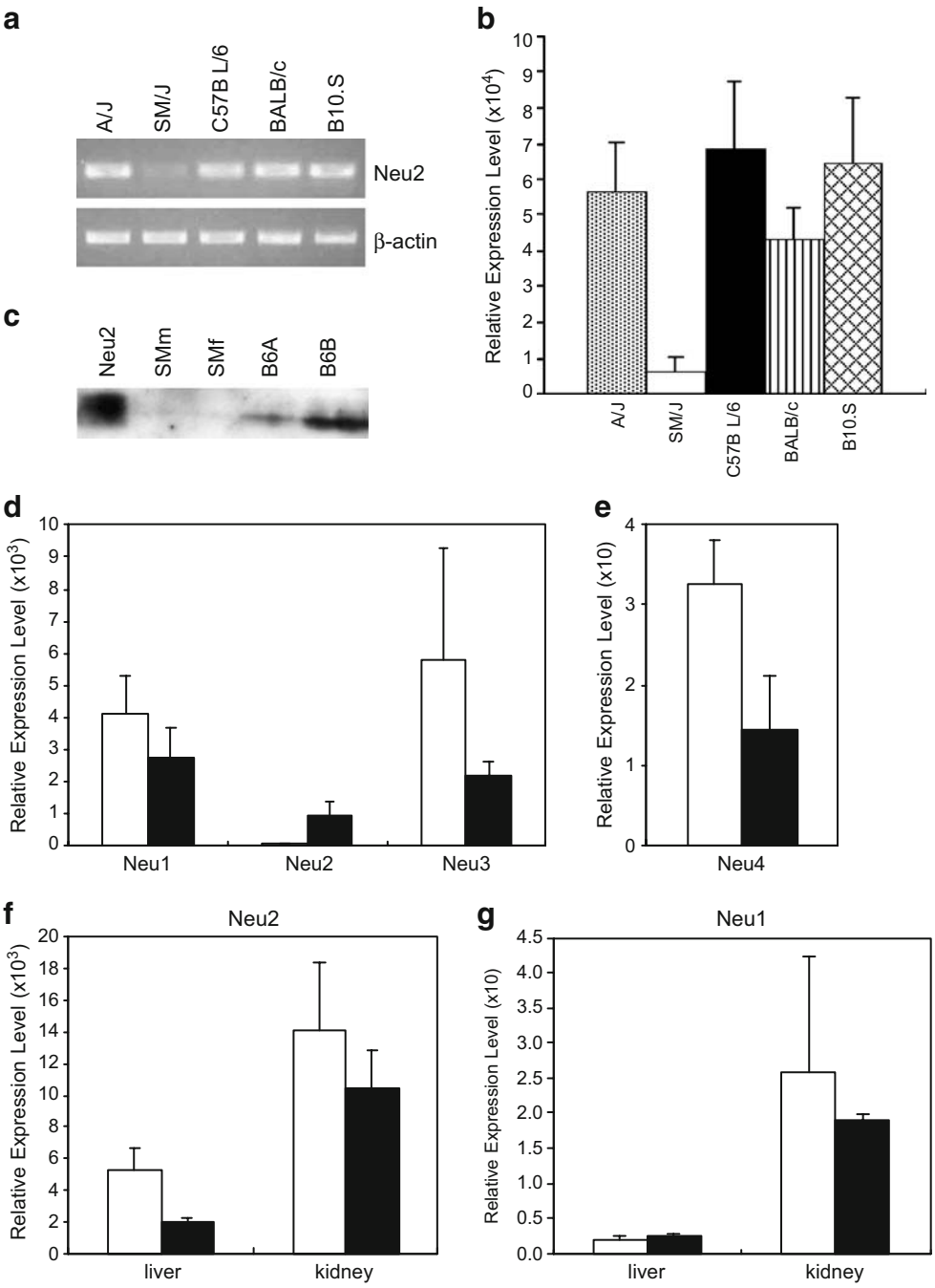

Fig. 3 Low expression of Neu2 mRNA in the thymus of SM/J mice. **a** Expression of Neu2 was examined by RT-PCR of thymus RNA from various mouse strains. **b** Relative expression levels were quantified by real time PCR and normalized to the β-actin level. **c** ECL detection of Neu2 enzyme on a Western blot with anti-Neu2. *Lane Neu2*, positive control of Neu2 enzyme obtained from COS cells transfected with Neu2 expression plasmid. *Lanes SMm and SMf*, soluble fraction obtained from the thymuses of 10-week-old male and female SM/J mice, respectively. *Lanes B6A and B6B*, soluble fraction obtained from the thymuses of 10-week-old male and 7-week-old female C57BL/6 mice, respectively. **d**, **e** Thymic mRNA levels of SM/J (*open bar*) and C57/BL/6 (*closed bar*) mice were measured for Neu1, Neu2, Neu3 (**d**), and Neu4 (**e**). **f**, **g** Relative expression levels of Neu2 (**f**) or Neu1 (**g**) in the liver and kidney of SM/J (*open bar*) or C57BL/6 (*closed bar*) mice

confirmed that the cytoplasm is not stained compared to the cell in A/J thymus (Fig. 5f), although in the SM/J thymus at pH 7.0, a very rare positive blue cell could be seen. When sialidase positive cells at pH 4.5 were also examined, a small number of membrane structures, probably including lysosomal structure but excluding plasma membrane, were stained both in A/J and SM/J cells (the case of SM/J is shown in Fig. 5h). We would like to give the name "Neumedllocyte" to those sialidase positive cells, which were stained at pH 7.0 in the thymus of A/J mouse but lacked in the thymus of SM/J mouse (see "Discussion").

Discussion

Activity and mRNA expression

In this study, we measured the level of mRNA expression of four sialidases in the thymus of the SM/J mouse (Neu1[a]) and compared it with other Neu1[b] or Neu1[c] mice by real-time PCR (Fig. 3). The following points became clear.

1. Not only was the expression level of mRNA of Neu2 in the SM/J mouse thymus was very low, so, too, was its

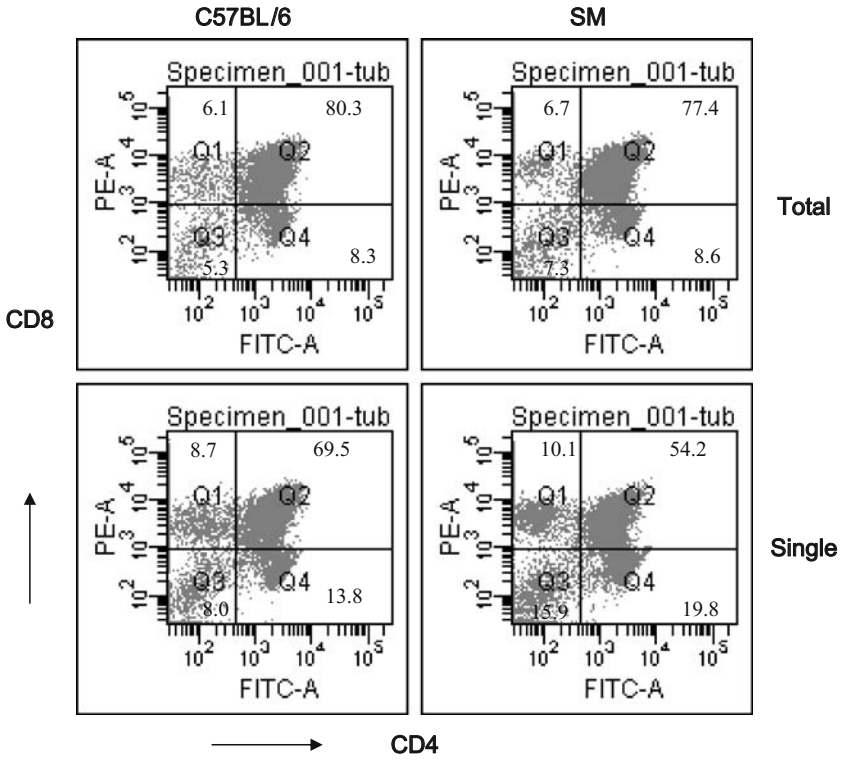

Fig. 4 Fluorescence-activated cell sorting (FACS) analysis of T cells from SM/J and control (C57BL/6) mice. Thymus whole cells and PNA-unaggregated single cells were reacted with FITC-labeled anti-CD4 and phycoerythrin (PE)-labeled anti-CD8. The percent distributions of Q1 (CD8 single positive), Q2 (CD4, CD8 double positive), Q3 (CD4, CD8 double negative) and Q4 (CD4 single positive) for PNA-single cells from C57BL/6 (B6) mouse are 8.7, 69.5, 8.0 and 13.8%, respectively, and those for the single cells from SM/J mouse are 10.1, 54.2, 15.9 and 19.8%, respectively. The percent distribution of Q1–Q4 of thymus whole cells and that of PNA-aggregated cells are almost the same between the SM/J mice and control mice

activity, while other mouse strains showed a high level of both expression and activity.

2. This phenomenon is, however, thymus specific. In the liver or kidney, a similar level or a rather higher level of Neu2 expression was observed in SM/J mouse compared to a control mouse, and the level of Neu1 expression in the two strains was also much the same.
3. The expression levels of Neu1, Neu3 and Neu4 in the thymus were rather higher in the SM/J mouse than in the control mouse.
4. Although it is difficult to compare the relative contribution of the four sialidases in the thymus from our data of quantitative PCR (Neu4 was analyzed by semi-quantitative PCR), the expression level of Neu2 does seems to be lower than that of other sialidases.

The following findings have been reported:

1. Neu1 from Neu1[a] allele (SM/J mouse) undergoes amino acid point mutation. This causes low Neu1 activity although mRNA is expressed [7, 8].
2. Neu3 has high substrate specificity and the artificial substrate 4MU-NANA is hydrolyzed only scarcely [15, 16], but because we used artificial substrates 4MU-NANA and X-NANA, we were unable to obtain useful information about Neu3 activity. Neu4 has double optimal pH peaks at pH 3.5–4.0 and pH 6.5–7.0. The activity at neutral region resisters around 50% of that at the acidic region [15]. The optimal pH of Neu1 and Neu2 is at the acidic and at neutral region, respectively [10, 15].

From the results and the facts described above, we are able to offer a good explanation of the results obtained in this study by the measurement that we made of the activity.

1) The crude membrane activity at pH 7.0 in Table 1 was probably derived from Neu4 activity at the neutral region. The crude membrane activity at pH 4.5 from A/J mouse was dependent upon Neu1 and Neu4 activities, while that from SM/J mouse was dependent upon Neu4 activity. The activity level of the SM/J mouse at pH 4.5 is pretty high, and since the expression level of mRNA of Neu4 is higher in SM/J mouse than in the control, this is reasonable.
2) Because the action is mainly controlled by the Neu1 gene, the high levels of sialidase activity of liver and kidney at pH 4.5 in the A/J mouse and the correspondingly low activity in the SM/J mouse (Table 2) is also reasonable. The rest of the activity of liver and kidney in SM/J mouse is probably due to Neu4 activity at the acidic region.
3) We need to think, however, about another possible candidate for the carrier of the neutral activity in a crude membrane fraction of the mouse thymus. Our microscopic photograph showed a small vessel that is sialidase positive, and had optimal pH at 6.5 with the solubilized sialidase from residual material on a nylon

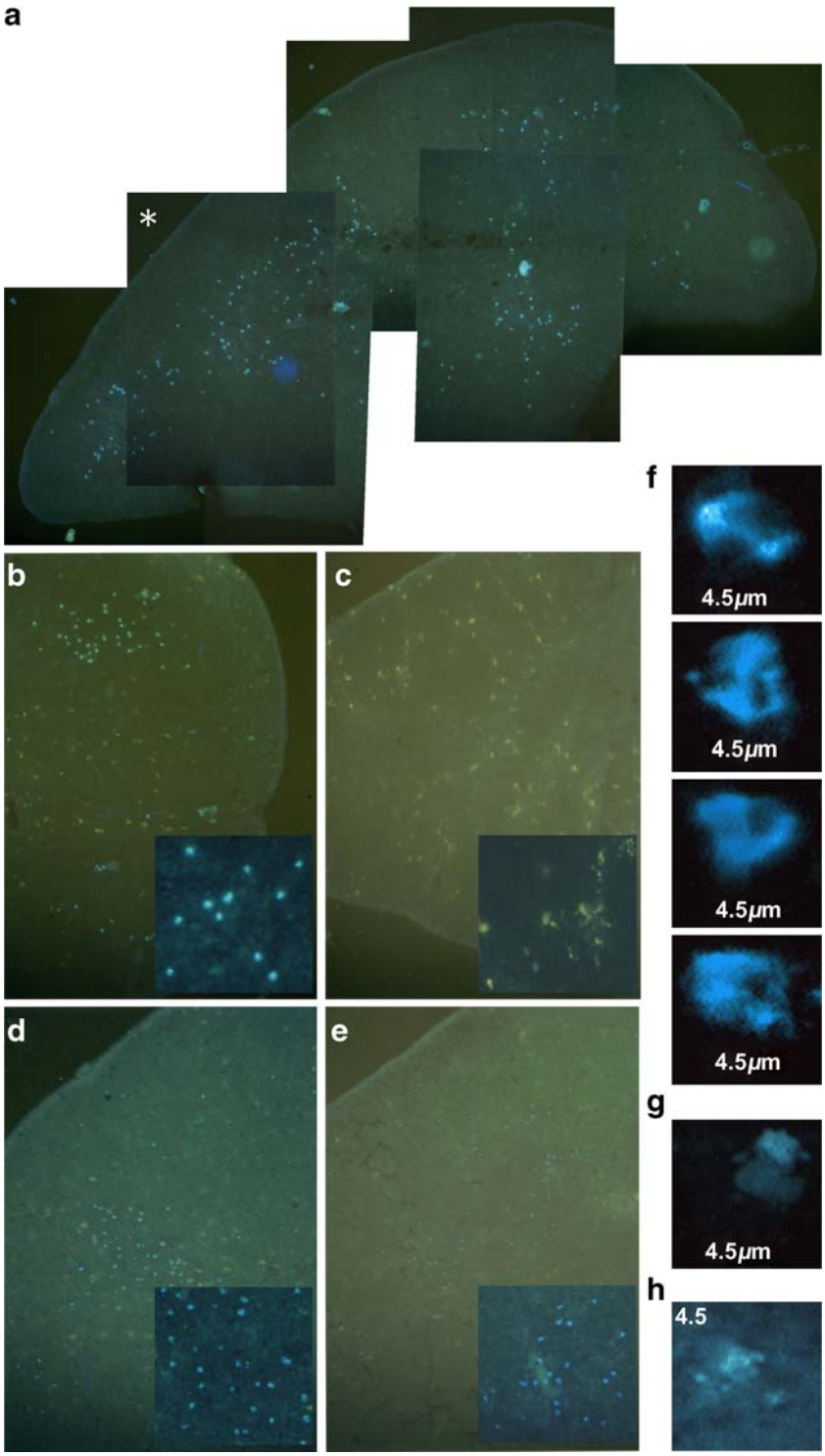

Fig. 5 Sialidase-positive cells in the thymuses from A/J and SM/J mice. Frozen sections (10 μm) of the thymuses were reacted with X-NANA as a sialidase substrate and photographed by a fluorescent microscope with an UV filter (**a**–**e**). **a** A composite of multiple photographs to show a whole section of the A/J mouse thymus stained at pH 7.0. A similar position of photo (*asterisk*) of A/J whole thymus stained at pH 7.0 are shown with A/J (**b**) and SM/J (**c**) stained at pH 7.0, and with A/J (**d**) and SM/J (**e**) stained at pH 4.5. Each small photo was taken with a ×10 eye lens and ×20 objective lens, while each large photo was taken with a ×10 and ×10 lens (**b**–**e**). **f**–**h** Confocal microscopic analysis. Frozen sections of thymuses from A/J and SM/J were reacted with X-NANA at pH 7.0 or at pH 4.5 and sialidase positive cell were analyzed by confocal microscopy. Images at 4.5 μm from the top surfaces of sialidase-positive cells are four cases of A/J at pH 7.0 (**f**), SM/J at pH 7.0 (**g**) and SM/J at pH 4.5 (**h**). The width of each figure is approximately 10 μm

mesh after filtration of loosely disrupted thymus for removing the T cells [2]. Because the whole thymus was homogenized with a Dounce-homogenizer and the homogenate obtained was centrifuged and fractionated to the soluble and crude membrane fractions, our crude membrane fraction in Table 1 contained the above sialidase. Although we have not determined the entity of this enzyme, we need to think about what it might be, should it be in addition to Neu4 for the activity at pH 7.0 with a crude membrane fraction.

Sialidase positive cells observed by microscopic and confocal microscopic study

(1) We observed that the blue-white sialidase-positive cells (Neu2 positive) in the thymus section from A/J mouse stained at pH 7.0 were distributed in the cortico-medullar region or the medullary region (Fig. 5a). We also observed that the smaller positive cells in those of A/J and SM/J mice stained at pH 4.5 were distributed in the similar region (Fig. 5d, e). This will suggest that the positive cells in A/J mouse at pH 4.5 contain Neu1 and Neu4 enzymes, while those in the SM/J mouse contain Neu4 enzyme.
(2) A confocal microscopic image of the A/J mouse stained at pH 7.0 shows what is probably an additive image stained by cytosolic Neu2, as with similar membrane structures observed in the SM/J specimen at pH 7.0 by Neu4. This would be the reason why the positive cells at pH 7.0 looked larger than those at pH 4.5.
(3) It is not clear at this moment however, whether the Neu2 positive cell also contains Neu1 and Neu4, or if the different cells contain different sialidase or that they partially overlap. In order to clarify whether these cells are the same or not, it will be important to consider the physiological role of these sialidases in mouse thymus. It will be possible to do this by a histochemical study using antibodies or by *in situ* hybridization.
(4) Yellow cells were observed in SM/J and also in A/J specimens at pH 7.0. We have reported that the sialidase-positive cells at pH 7.0 are sparsely distributed in the corticomedullar region or medullary region of the thymus, and that they express Mac-1 and immunoglobulin [2]. Mac-1 positive cells probably take up X-NANA, but not all the Mac-1 positive cells contain sialidase, which is why only the sialidase positive cells became blue, and the sialidase-negative Mac-1-positive cells became yellow. Our study has confirmed this sialidase positive cell in A/J mouse with the SM/J mouse as the negative control. Since the Neu2 positive cell observed in A/J thymus at pH 7.0 having Mac-1 and immunoglobulin contain sialidase(s) and are localized mainly in the medullary region, and as "Neu" in German means "new", we would like to name this cell a "Neu-medullocyte".

Physiological roles of sialidases in the murine thymus

Our study has made clear that the thymus of the SM/J strain has lost Neu2 activity. The SM/J mouse is not an autoimmune strain, but produces its own thymocyte-toxic auto-antibody (NTA) [23]. It shows a higher induction of suppressor T cells, higher responsiveness to B cell mitogen, and higher natural killer cell activity, but its lymphocyte response to PHA is lower than that of the A/J mouse [19] and it fails to produce IL-4 [24]. These responses (attributed to Neu1 deficiency) can be related to the lack of Neu2 in the thymus and different distribution of maturated T cells (Fig. 4). Further study will be important.

In the thymus of a young mouse, 5×10^7 cells proliferate day by day. Only 2% are selected for mature T cells, while 98% die by apoptosis and must be cleaned up in the thymus. In the process of the clean up of apoptosis cells, lysosomal enzyme Neu1 and Neu4 have an important role. Recent studies have shown the physiological role of sialidases on apoptosis [25, 26], as well as in the production of IFN-γ [27], and cellular immune response [28], etc. These studies will be important in assisting us to understand the role of sialidases in the thymus. The study on Neu3 in the thymus will also become very important when we make use of the natural substrate. Nakamura *et al.* [29] have reported that disialoganglioside $IV^3\alpha$(NeuGcα2-8NeuGc)-Gg_4Cer is a major ganglioside in the murine thymus. This sialidase-susceptible disialoganglioside may be an important substrate for thymus sialidase during the selection and maturation of T cells.

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