Induction of lysosomal and plasma membrane-bound sialidases in human T-cells via T-cell receptor

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Among the three isoenzymes of neuraminidase (Neu) or sialidase, Neu-1 has been suggested to be induced by cell activation and to be involved in IL (interleukin)-4 biosynthesis in murine T-cells. In the present study, we found that antigen-induced airway eosinophilia, a typical response dependent on Th2 (T-helper cell type 2) cytokines, as well as mRNA expression of Th2 cytokines, including IL-4, are suppressed in Neu-1-deficient mice, thereby demonstrating the *in vivo* role of murine Neu-1 in regulation of Th2 cytokines. To elucidate the roles of various sialidases in human T-cell activation, we investigated their tissue distribution, gene induction and function. Neu-1 is the predominant isoenzyme at the mRNA level in most tissues and cells in both mice and humans, including T-cells. T-cells also have significant levels of Neu-3 mRNAs, albeit much lower than those of Neu-1, whereas the levels of Neu-2 mRNAs are minimal. In human T-cells, both Neu-1 and Neu-3 mRNAs are significantly induced by T-cell-receptor stimulation, as is sialidase activity against 4-methylumbelliferyl-*N*-acetylneuramic acid (a substrate for both Neu-1 and Neu-3) and the ganglioside G_{D1a} [NeuAcα2-3Galβ1-

3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-cer] (a substrate for Neu-3, but not for Neu-1). The expression of the two sialidase genes may be under differential regulation. Western blot analysis and enzymic comparison with recombinant sialidases have revealed that Neu-3 is induced as a major isoform in activated cells. The induction of Neu-1 and Neu-3 in T-cells is unique. In human monocytes and neutrophils stimulated with various agents, the only observation of sialidase induction has been by IL-1 in neutrophils. Functionally, a major difference has been observed in Jurkat T-cell lines over-expressing Neu-1- and Neu-3. Upon T-cell receptor stimulation, IL-2, interferon-γ, IL-4 and IL-13 are induced in the Neu-1 line, whereas in the Neu-3 line the same cytokines are induced, with the exception of IL-4. Taken together, these results suggest an important immunoregulatory role for both Neu-1 and Neu-3 in humans.

Key words: cytokine, gene regulation, T-lymphocyte, neuraminidase, sialidase.

INTRODUCTION

Sialic acids exist mostly in the terminal positions of biomolecules (such as glycoproteins, glycolipids and gangliosides) and cell membranes, and are involved in a wide variety of physiological processes, including immune functions [1,2]. Neuraminidases (Neu or sialidases, EC 3.2.1.18) are a family of exoglycosidases that hydrolyse the terminal sialic acid linkage in these biomolecules and hence play important physiological roles. To date, three mammalian sialidases have been identified at the molecular level in both mice and humans: Neu-1, Neu-2 and Neu-3 [3,4]. They share high degrees of similarity and amino acid blocks of highly conserved residues. However, the three sialidases are different from one another in terms of subcellular localization, substrate preference *in vitro* and tissue distribution. Neu-1 is a lysosomal sialidase which is presumed to have a narrow substrate specificity. The natural substrate for this enzyme is unknown and activity has thus only been reported on artificial substrates such as 4-MU-NANA (4-methylumbelliferyl-*N*-acetylneuramic acid), and nitrophenyl-NANA, but not gangliosides, fetuin or sialyllactose [5]. Neu-1 mRNA is widely expressed in various tissues [4]. Neu-2 is a cytosolic enzyme with wide substrate specificity [6,7]. Neu-2 mRNA is expressed at relatively lower levels compared with the other sialidases, detectable only in skeletal muscle among various human tissues by Northern blot analysis [8]. Neu-3 is a

plasma membrane-bound sialidase, originally described as ganglioside sialidase [9–11]. Neu-3 preferentially hydrolyses gangliosides, although glycoproteins, 4-MU-NANA, sialyllactose, etc. are also hydrolysed [12–14]. Brain expresses the highest level of ganglioside sialidase enzyme activity [10], and this tissue was used for purification of the enzyme [9–11]. However, recent Northern blot analysis shows that Neu-3 mRNA is expressed in multiple human tissues, including adrenal gland, skeletal muscle, testis and thymus [8].

In mouse T-cells, stimulation by concanavalin A [15], lyso-phosphatidylcholine [16], allogeneic splenic adherent cells [17] or anti-CD3 antibody [18] resulted in an increase in Neu-1 enzyme activity. Treatment of the T-cells with a bacterial [18] or viral [19] sialidase increased production of IL (interleukin)-4, but not of the Th1 (T-helper cell type 1) cytokines IL-2 and IFN (interferon)-γ. Moreover, in Neu-1-deficient mouse strains there was little IL-4, but normal levels of the Th1 cytokines were produced [18]. These results suggest a specific role of a sialidase, particularly Neu-1, in IL-4 biosynthesis in mice. In human T-cells, on the other hand, concanavalin A stimulation did not lead to an increase in sialidase enzyme activity, but instead to translocation of a sialidase (presumably Neu-1) from lysosomes to cell surface [20]. However, the physiological role of the translocation and whether or not Neu-1 is involved in cytokine gene regulation in human T-cells remain unknown. It has been suggested that gangliosides

Abbreviations used: BAL, bronchoalveolar lavage; G_{D1a}, NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-cer; G_{D1b}, Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-cer; G_{M3}, NeuAcα2-3Galβ1-4Glcβ1-cer; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; G_{T1b}, NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-cer; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; 4-MU-NANA, 4-methylumbelliferyl-*N*-acetylneuramic acid; Neu, neuraminidase; Th, T-helper cell; TNF, tumour necrosis factor.

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may be involved in cytokine gene regulation [21,22], but nothing is known about any ganglioside-hydrolysing sialidase (Neu-2 or Neu-3) in T-cells in humans or mice.

To elucidate the roles of the various sialidases in T-cell activation, we have investigated the tissue distribution profile and gene induction of Neu-1, Neu-2 and Neu-3 in T-cells, as well as their functions in T-cell activation. In the present study, we found that in activated human CD4⁺ T-cells both Neu-1 and Neu-3 are induced. Neu-3 is induced as a major sialidase isoenzyme at both protein and enzyme activity levels, despite the fact that Neu-1 is overall the major isoform at the mRNA level in various tissues and cells, including T-cells. Furthermore, we have found that sialidase induction is highly T-cell specific. Finally, using recombinant over-expression T-cell lines, we have found that Neu-1 and Neu-3 affect differentially cytokine expression.

EXPERIMENTAL

Isolation of total RNA and synthesis of cDNA

Total RNA was prepared using TRIzol Reagent (Invitrogen, Carlsbad, CA, U.S.A.). cDNA was synthesized as described previously [23].

Isolation and stimulation of human blood CD4⁺ T-cells, monocytes and neutrophils

Human leucocytes were isolated from peripheral venous blood of healthy donors. T-cells were prepared using a CD4⁺ T-cell Isolation Kit from Miltenyi Biotec (Auburn, CA, U.S.A.), and neutrophils and monocytes were isolated as described previously [24].

T-cells were grown for a total of 6 or 12 days on one or two alternating 3-day stimulation and rest cycles. The cells were suspended at 4×10^6 cells/ml in RPMI 1640 medium supplemented with 1 % each of penicillin/streptomycin and glutamine and with 10 % fetal bovine serum (all from Invitrogen), and stimulated with immobilized anti-CD3 antibody (10 µg/ml; R & D Systems, Minneapolis, MN, U.S.A.). During the rest phase, the cells were cultured with IL-2 (10 ng/ml, R & D Systems). After cell growth, cells were stimulated at 1×10^6 cells/ml with immobilized anti-CD3 and soluble anti-CD28 (1 µg/ml, R & D Systems) antibodies for a time period as indicated to investigate sialidase induction. Cells were cultured at 37 °C in a humidified atmosphere of 5 % CO₂/95 % air.

After stimulation, whole cell lysate for the sialidase activity assay was prepared by sonication for 10 s in Branson's Sonifier 250 (Danbury, CT, U.S.A.), with output set at 4, in 12 mM CaCl₂ on ice (when using 4-MU-NANA as substrate) or 100 mM sodium acetate buffer (pH 4.6) on ice (when using a ganglioside as substrate).

Neutrophils and monocytes were stimulated as described previously [24]. The concentrations of stimuli used were as follows: LPS (lipopolysaccharide), 100 ng/ml; and TNF (tumour necrosis factor), granulocyte/macrophage colony-stimulating factor, IL-1β and IFN-γ, 10 ng/ml. LPS was obtained from Sigma (St. Louis, MO, U.S.A.), whereas all cytokines were from R & D Systems.

Quantification of Neu-1, Neu-2 and Neu-3 mRNAs by real-time PCR

Mouse CD4⁺ T-cells were isolated from spleens using a CD4⁺ T-cell Isolation Kit from Miltenyi Biotec. Mouse T-cells, similar to human T-cells, were grown on one alternating 3-day stimulation and rest cycle (see above). Anti-mouse CD3 and IL-2 antibodies were obtained from R & D Systems. cDNAs from human T-cells, monocytes and neutrophils, and from mouse T-cells, lymph node and thymus, were synthesized as described above, whereas those

Table 1 Positions and sequences of primers used, and sizes of resultant PCR products, in mRNA quantification by real-time PCR

For each gene, the upper and lower sequences are forward and reverse primers respectively. All sequences are from 5' to 3'. Primers for mouse G3PDH were obtained from ClonTech. m, mouse; h, human.

Gene	Primer sequence	Position	Product size (bp)
hG3PDH	CCTCTGACTTCAACAGCGACA CATGACAAGGTGCGGCTCCC	923–1253	331
hNeu-1	ACTGCCACTGCCGAATTG GGTTGCCAGGGATGAATAGCC	857–1105	249
hNeu-2	CCAGGGGAGCGTCATCAG CCTCGTAATCATTGGCTTCGT	816–1061	246
hNeu-3	GAGACTGGCCCTGAGTCGACA AACAGGCGGAAGGCAATC	891–1287	397
mNeu-1	GCCGCTGCAGGATCGTC CCGGTTCAGGCCCTTCTCGTA	875–1192	318
mNeu-2	CACCCCTGAAGCAAGCTTTC AACAGGCGAGTTAACTAGCA	1179–1500	322
mNeu-3	ATTCAGGTTAGTCATGATGC TGAGTCCAGGATAGCTAGG	2449–2734	286
mIL-2	GCAGCTGTGATGGACCTACA GGGCTTGTGAGATGATGCTT	193–529	337
mIFN-γ	TGATTGCGGGTGTATCTGG TGTCATTGCGGTGTAGTCACA	804–1079	276
mIL-4	TGACGGCAGACAGCTATTGAT GAAGCACCTTGAAGCCCTAC	69–293	225
mIL-5	ATCACATACCCAACCTTAGCA CTGAATAACATCCCAACATT	935–1352	418
mIL-9	TTGTGTCTCTCCGTCCCAACT TCTGTCTTCATGGTCGGCTTT	178–427	250
mIL-13	AAACTGCAGCAAGACCGTGAG TGAGACCTGGAGGCTACAGTG	680–920	241

for other tissues were obtained from ClonTech (Palo Alto, CA, U.S.A.).

Real-time quantitative PCR was performed as described previously [23]. The positions and sequences of gene-specific primers and G3PDH (glyceraldehyde-3-phosphate dehydrogenase; as control for normalization) primers used and the sizes of their corresponding PCR products are shown in Table 1. The primers were designed using Primer Express software from Applied Biosystems (Foster City, CA, U.S.A.). The specificity of each primer set was confirmed by PCR (only one PCR product with the expected molecular size was detected), and the similarities of PCR efficiencies of all the primer sets were confirmed.

Quantification of cytokine mRNAs by real-time PCR

For mouse cytokine mRNA quantification, the positions and sequences of cytokine gene-specific primers were used; the sizes of their corresponding PCR products are shown in Table 1. Other details are as described above.

For human cytokine mRNA quantification, all primer sets and probes (including those for G3PDH) were obtained from Applied Biosystems, and the quantification was performed by the TaqMan method as instructed by the manufacturer.

Assay for protein concentration

Protein concentrations were determined using Protein Assay solution from Bio-Rad (Hercules, CA, U.S.A.), with BSA as the standard.

Assays for sialidase activity

Sialidase activity for 4-MU-NANA (Sigma) was assayed by spectrofluorometrical measurement of 4-MU (4-methylumbelliferyl)

as described previously [18], whereas that for gangliosides (Sigma) was assayed by measuring released sialic acid by the thiobarbituric acid method [25].

For the mouse liver sialidase activity assay, liver was homogenized in 20 mM Tris/HCl (pH 7.4) containing 12 mM CaCl₂ and Complete Protease Inhibitor Cocktail (Boehringer Mannheim, Indianapolis, IN, U.S.A.) by sonication as described above. The homogenate was centrifuged at 350 g for 5 min at 4 °C, and the supernatant was used as enzyme sample.

Sialidase activity in stable Jurkat cell lines (see below) was assayed as described previously [26].

Expression of recombinant human Neu-1 and Neu-3

A cDNA encoding full-length human Neu-1 was generated by PCR using a primer set designed from the published sequence information [5]. The primers had the following sequences: 5'-CTGTGGAGTCTAGCTGCCAGG-3' (forward) and 5'-GAAT-ACCCCTGTGGCAGTGGC-3' (reverse). Human liver cDNA (Clontech) was used as a template. A cDNA encoding full-length human Neu-3 [12], starting at -19 bp upstream of the translation start site, was generated by chemically synthesizing then combining together smaller DNA fragments by standard method. The Neu-1 and Neu-3 cDNAs were subcloned into pcDNA1.1/AMP vector (Invitrogen), followed by double-strand DNA sequencing for confirmation. For enzymic property analysis, recombinant Neu-1 and Neu-3 were highly expressed in COS-7 cells as described in [27], so that the endogenous sialidase activity was negligible. Whole cell lysates were prepared as above and used in the analysis.

Recombinant Neu-1 and Neu-3 were also expressed in SF9 insect cells as described [28], with a 6xHis tag for normalization using anti-His antibody in Western blot analysis (see below).

Western blot analysis of human Neu-1 and Neu-3 proteins

Rabbit anti-human Neu-1 and Neu-3 polyclonal antibodies were raised against the peptides KARKMSSSDEGAKFIALRR and EKRSTRRDEDALHLVLRRL respectively. The peptides were conjugated to KLH for immunization. Each resultant anti-serum was confirmed by ELISA and Western blot analysis to contain an appropriate isoenzyme-specific anti-sialidase antibody. Using ELISA, each anti-serum showed a highly increased titre against relevant immunogen, compared with pre-immunization serum (results not shown). Using Western blot analysis, each anti-serum demonstrated a single band with the expected apparent molecular mass in cell lysate of SF9 cells expressing the appropriate sialidase, but not in cell lysate of uninfected cells. Moreover, the recognition of sialidase by each anti-serum was blocked by treatment with the relevant immunogen (results not shown).

T-cells or recombinant SF9 cells were mixed with SDS gel loading buffer and heated at 100 °C for 3 min. After SDS/PAGE (10% gel) and subsequent Western blotting, the blot was blocked with 1% BSA in PBS. The blot was then probed with an anti-sialidase (1:1000) or anti-His antibody (1:5000, Invitrogen). Signals were detected using a SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, U.S.A.). Anti-(human IL-2 receptor) antibody, used as a positive control, was obtained from R & D Systems.

Development of stable Jurkat cell lines over-expressing human Neu-1 and Neu-3

The Neu-1 and Neu-3 cDNAs were subcloned into pcDNA3.1/V5-HisA vector (Invitrogen), followed by double-strand DNA se-

quencing for confirmation. Jurkat cells (A.T.C.C.) were cultured as instructed by the provider. The cells were transfected with either the Neu-1 plasmid, Neu-3 plasmid or control vector using Lipofectamine 2000™ and Opti-MEMI (Invitrogen). The three stable cell lines were developed by selection in the presence of 0.5 mg/ml G418 (Invitrogen) and by limiting dilution.

Quantification of IL-2 protein

IL-2 protein in culture supernatant was quantified using a specific ELISA kit from Pierce.

Antigen-induced airway eosinophilia in mice

Ovalbumin sensitization and challenge of mice, BAL (bronchoalveolar lavage) and determination of total and differential cell counts were performed as described previously [29].

RESULTS

Suppression of antigen-induced airway eosinophilia in the Neu-1-deficient mouse strain SM/J

It was reported that production of IL-4, but not Th1 cytokines, was suppressed in the Neu-1-deficient mouse strain SM/J [18]. To confirm that Neu-1 is involved in Th2 development *in vivo*, we evaluated ovalbumin-induced airway eosinophilia, a typical Th2 phenotype, in SM/J in comparison with the mouse strain DBA/1J whose genetic background is the closest to that of SM/J [30]. As shown in Figure 1(A), the number of eosinophils and the percentage of eosinophils in total cells in BAL fluid were both significantly lower in the SM/J strain than in the DBA/1J strain. Because eosinophils are a major cell type in this model, the total cell number in BAL fluid was also significantly lower in SM/J than in DBA/1J mice. The DBA/1J strain has not previously been evaluated for Neu-1 activity. Therefore, liver sialidase activity, a standard assay for the Neu-1 deficiency in SM/J mice, was measured in all animals used. As shown in Figure 1(B), the enzyme activity in the SM/J strain was very low, as reported previously [31], whereas DBA/1J exhibited a high level of activity, confirming that it was an appropriate control strain. The enzyme activity level in sensitized DBA/1J animals was slightly lower than that in non-sensitized animals, but the mechanism for this reduction or implication of this observation is not known.

We also measured mRNAs for various cytokines in the lungs of these mice. As shown in Figure 1(C), by allergen sensitization followed by challenge, the levels of all the Th2 cytokines were lower in SM/J than in DBA/1J mice. As for Th1 cytokines, there was no significant difference in IL-2 between the two strains, with the sensitization and challenge causing a slight increase in both strains. With regard to INF- γ , the level in the SM/J strain was lower than that in the DBA/1J strain following sensitization and challenge. However, whether the lower level was due to the Neu-1 deficiency is not clear, since the DBA/1J strain has been known to highly express INF- γ [32]. Thus it is suggested that Neu-1 may be specifically involved in Th2 cytokine expression.

Tissue distribution profiles of Neu-1, Neu-2 and Neu-3 mRNAs in mice and humans

To evaluate the roles of various sialidase isoforms in T-cells, we first investigated the expression profile of all of Neu-1, Neu-2 and Neu-3 mRNAs in T-cells in comparison with various tissues and cells in mice and humans. The main aim was to compare T-cells with T-cell-rich tissues (spleen, thymus and lymph node in mice) and other routinely available blood leucocytes (monocytes and neutrophils in humans). As shown in Figure 2, Neu-1

Figure 1 Suppressed allergic response in the Neu-1-deficient mouse strain SM/J

(A) Suppressed ovalbumin sensitization-induced airway eosinophilia. The DBA/1J mouse strain, which has the closest genetic background to SM/J, was used as control. The animals were non-sensitized or sensitized with ovalbumin twice over a period of 12 days then challenged with ovalbumin. Total cells and eosinophils in BAL were counted, and percentages of eosinophils were calculated. The results are expressed as means $\pm$ S.E.M. *Statistically significant ($P < 0.05$, $n = 5$ animals per group). (B) Confirmation of Neu-1 deficiency in the SM/J strain. Livers were collected and sialidase activity was assayed using 4MU-NANA as substrate to confirm the Neu-1 deficiency in the SM/J strain, as reported previously [31]. The assay was performed in duplicate in each animal. The results shown represent the means $\pm$ S.E.M. of each group as nmol of substrate/h per mg of protein at 37 °C. (–), Without sensitization; (+), with sensitization. (C) Cytokine expression in lung. Lungs were collected and mRNAs for the cytokines were quantified by real-time PCR. The determinations were performed in duplicate in each animal. The results represent the means $\pm$ S.E.M. for each group of the relative expression levels of each cytokine compared with G3PDH.

was highly expressed in all the tissues and cells in both species, whereas Neu-2 and Neu-3 exhibited certain differential expression patterns. Notably, Neu-2 was poorly expressed in heart, spleen, lymph node and T-cells in mice (Figure 2A) and in skeletal muscle, kidney, T-cells and monocytes in humans. Neu-3 expression was particularly low in human monocytes (Figure 2B). Thus among the three sialidase isoforms, Neu-1 expression level was the highest in all the tissues and cells, except for skeletal muscle in mice and neutrophils in humans. Between humans and mice, the expression profile of the three sialidase isoforms was similar across various tissues except for skeletal muscle.

Induction of Neu-1 and Neu-3 in human T-cells by CD3/CD28 co-stimulation

Subsequently, we investigated whether sialidases are induced in human T-cells by stimulation via T-cell receptor. As shown in Figure 3(A), in blood CD4⁺ cells, CD3/CD28 co-stimulation induced both Neu-1 and Neu-3 mRNAs. For both sialidases, the induction occurred rapidly with the mRNA levels peaking at 2 h. For Neu-1, there appeared to be a second phase of induction

between 6 and 24 h, which might represent a secondary effect. For Neu-3, the mRNA level gradually decreased between 2 and 24 h, but after 24 h there was still a significantly elevated mRNA level in most donors. The significant variations were due to use of different donors. Neu-2 mRNA levels before and after stimulation were both too low to draw a reliable conclusion.

At enzyme activity level, assays were performed using two different substrates: 4-MU-NANA and ganglioside G_{D1a} [NeuAc α 2-3Gal β 1-3GalNAc β 1-4(NeuAc α 2-3)Gal β 1-4Glc β 1-cer]. 4-MU-NANA can be hydrolysed by both Neu-1 and Neu-3, whereas G_{D1a} can be hydrolysed by Neu-3 but not Neu-1 (Table 2 and [5,12–14]). Accordingly, the G_{D1a}-hydrolysing activity observed was presumably due to Neu-3 (Figure 3B). The G_{D1a}-hydrolysing activity profile was very similar to the above Neu-3 mRNA time course: peaking at 2 h then sustaining an elevated level up to 24 h. 4-MN-NANA-hydrolysing activity increased up to 24 h, probably due to the fact that all sialidase isoforms were measured when this substrate was used.

To clarify if CD3/CD28 co-stimulation is required for the induction, the co-stimulation was compared with stimulation via CD3 or CD28 alone (Figure 4). For both Neu-1 and Neu-3,

Figure 2 Tissue distribution of Neu-1 and Neu-3 mRNAs in mice (A) and humans (B)

mRNAs for Neu-1 and Neu-3 were quantified by real-time PCR. All mouse cDNAs used as PCR template were from BALB/c, except for lung cDNA which was from equal numbers of SM/J and DBA/1J mice (the two strains exhibited similar mRNA levels for each sialidase). The determinations were performed in duplicate, and each set of experiments was performed at least twice. The results represent the means $\pm$ S.D. of the relative expression levels of Neu-1 or Neu-3 compared with G3PDH. SM, skeletal muscle; LN, lymph node; F-liver, fetal liver; Mono, monocyte; PMN, neutrophil.

Table 2 Significant induction of Neu-3 (ganglioside-hydrolysing) activity in activated T-cells

All assays were performed in duplicate, and each set of experiments was performed three times. The results are expressed as the means $\pm$ S.E.M. The concentration of Triton X-100 was 0.1 %. r, Recombinant; –, without; +, with; N.D., not determined.

Substrate	Triton X-100	Sialidase activity (nmol of substrate/h per mg of protein)			
		rNeu-1	rNeu-3	Resting T-cells	Activated T-cells
4-MU-NANA	–	32.80 $\pm$ 4.33	29.67 $\pm$ 1.79	N.D.	N.D.
	+	37.88 $\pm$ 8.75	40.02 $\pm$ 4.28	1.87 $\pm$ 0.02	10.60 $\pm$ 1.19
G _{M3}	–	0.36 $\pm$ 0.16	0.69 $\pm$ 0.21	N.D.	N.D.
	+	0.05 $\pm$ 0.03	3.03 $\pm$ 0.06	N.D.	N.D.
G _{D1a}	–	0.18 $\pm$ 0.05	0.84 $\pm$ 0.24	0.55 $\pm$ 0.14	0.30 $\pm$ 0.05
	+	0.50 $\pm$ 0.17	5.47 $\pm$ 1.01	0.46 $\pm$ 0.09	1.59 $\pm$ 0.09
G _{D1b}	–	0.08 $\pm$ 0.05	0.49 $\pm$ 0.11	N.D.	N.D.
	+	0.29 $\pm$ 0.14	3.74 $\pm$ 0.82	N.D.	N.D.
G _{T1b}	–	0.24 $\pm$ 0.08	0.68 $\pm$ 0.24	0.50 $\pm$ 0.08	0.43 $\pm$ 0.14
	+	0.14 $\pm$ 0.12	5.17 $\pm$ 0.77	0.50 $\pm$ 0.09	1.77 $\pm$ 0.03

CD28 stimulation caused a smaller induction, whereas CD3 stimulation caused a larger induction. For Neu-1, though not statistically significant, the result suggests that only the co-stimulation caused maximal induction. For Neu-3, on the other hand, maximal induction was achieved by CD3 stimulation alone ($P < 0.05$ between CD28 and CD3 or CD3/CD28, but $P > 0.05$ between CD3 and CD3/CD28 groups). Thus it is suggested that these two sialidase isoforms may be expressed under differential regulation.

Identification of Neu-3 as a major inducible isoform in stimulated T-cells

At the protein level, Western blot analysis suggests that there was induction of Neu-3 in T-cells activated via CD3/CD28 co-stimulation (Figure 5). There was no Neu-1-like band detected

Figure 3 Induction of Neu-1 and Neu-3 in CD3/CD28-co-stimulated human T-cells at mRNA (A) and enzyme activity (B) levels

Human CD4⁺ cells were co-stimulated with CD3/CD28 for various periods of time. Neu-1 and Neu-3 mRNAs were quantified by real-time PCR, and enzyme assays were performed using 4-MU-NANA and ganglioside G_{D1a}. All determinations were performed in duplicate, and the results are expressed as the means $\pm$ S.E.M. ($n = 9$ for mRNAs and $n = 12$ for enzyme activity). Enzyme activity is expressed as nmol of substrate/h per mg of protein at 37 °C, and mRNA is expressed as the relative expression levels of Neu-1 or Neu-3 compared with G3PDH.

Figure 4 Comparison of Neu-1 and Neu-3 induction after stimulation via CD3, CD28 or CD3/CD28 co-stimulation in human T-cells

Human CD4⁺ cells were stimulated via CD3, CD28 or both for 24 h, then Neu-1 and Neu-3 mRNAs were quantified by real-time PCR. All determinations were performed in duplicate, and the results represent the means $\pm$ S.E.M. ($n = 9$) of the relative expression levels of Neu-1 or Neu-3 compared with G3PDH.

(results not shown), despite the anti-Neu-1 antibody being confirmed to be at least as efficient as the anti-Neu-3 antibody for Western blot analysis (Figure 5). Multiple bands were detected for the recombinant Neu-1, presumably due to its N-linked glycosylation in insect cells [5]. As a positive control to validate the experiments, IL-2 receptor was greatly induced upon T-cell activation (Figure 5). This result demonstrates that in activated T-cells Neu-3 becomes the predominant sialidase isoenzyme over Neu-1 at the protein level. This result also suggests that both Neu-1 and Neu-3 proteins are expressed at very low levels in these cells, since only Neu-3 became visible as a minor band in the activated cells after intensive exposure.

To further confirm the significant induction of Neu-3 in activated T-cells at the enzyme activity level, the sialidase activity of activated T-cells was compared with recombinant human Neu-1 and Neu-3. As reported previously [5,12–14], both recombinant Neu-1 and Neu-3 hydrolysed 4-MU-NANA, with the enzyme activity slightly activated by Triton X-100 (Table 2). For the gangliosides G_{M3} (NeuAc α 2-3Gal β 1-4Glc β 1-cer), G_{D1a} , G_{D1b} [Gal β 1-3GalNAc β 1-4(NeuAc α 2-8NeuAc α 2-3)Gal β 1-4Glc β 1-cer] and G_{T1b} [NeuAc α 2-3Gal β 1-3GalNAc β 1-4(NeuAc α 2-8NeuAc α 2-3)Gal β 1-4Glc β 1-cer], Neu-3 exhibited significant activity in the presence of Triton X-100, as reported previously [5,12–14], but Neu-1 did not (Table 2). In resting T-cells, there was a low level of sialidase enzyme activity. Since there was little Triton X-100-dependent activity against ganglioside, Neu-1 probably accounted for the majority of the enzyme activity in the resting cells. In activated T-cells, there was a large increase

Figure 5 Identification of Neu-3 as the major sialidase isoform at protein level in activated human T-cells

(A) Human CD4⁺ cells were co-stimulated with CD3/CD28 for 24 h, then equal amounts of whole cell lysates from resting (RT) and activated (AT) T-cells were subjected to Western blot analysis using anti-Neu-3 antibody. Recombinant human Neu-3 protein (rN) was used as a positive control to identify the Neu-3 band. The apparent molecular mass of Neu-3 is 50 kDa. Note that this blot was exposed intensively to detect the Neu-3 band. No Neu-1-like protein band was observed using anti-Neu-1 antibody (results not shown). IL-2 receptor (IL-2R) was used as positive control to validate the T-cell activation. (B) Demonstration of anti-Neu-1 antibody being at least as good as Neu-3 antibody for Western blot analysis. Various amounts of whole cell lysates from Sf9 cells expressing recombinant human Neu-3 (N-3) or Neu-1 (N-1), both with a His-tag, were subjected to Western blot analysis using an anti-(His-tag) antibody. For each recombinant sialidase sample a particular amount of cell lysate was selected, so that the Neu-1 protein amount was not larger than the Neu-3 amount (left-hand panel). The selected amounts of recombinant Neu-3- and Neu-1-containing cell lysates were then subjected to Western blot analysis separately using the anti-Neu-3 and anti-Neu-1 antibodies. The fact that an amount of Neu-1 protein smaller than that of Neu-3 gave rise to a band at least as intense as Neu-3 (right-hand panel) demonstrated that the anti-Neu-1 antibody was at least as efficient as the anti-Neu-3 antibody for Western blot analysis.

in 4-MU-NANA-hydrolysing activity, which could be due to all sialidase isoenzymes. However, there were also significant levels of Triton X-100-activated ganglioside-hydrolysing activity, which is exhibited only by Neu-3. Since the two sialidase assays using 4-MU-NANA and ganglioside were performed using different methods under different conditions, it is difficult to quantitatively compare Neu-1 and Neu-3 activity levels. Nevertheless, it appears clear that Neu-3 was induced as a major sialidase isoform in activated T-cells.

High specificity of sialidase induction for T-cells

To investigate whether or not the sialidase induction is T-cell-specific, purified human blood monocytes and neutrophils were stimulated with various potent stimuli, then Neu-1, Neu-2 and Neu-3 mRNAs were measured. As shown in Figure 6, the only observation was IL-1-induced induction of Neu-1 and Neu-2 in neutrophils. As a positive control to validate the experiments, TNF was greatly induced in these cell preparations. Moreover,

Figure 6 Specificity of sialidase induction

Human monocytes (**A**) and neutrophils (**B**) were stimulated with various agents for 2 h, then Neu-1, Neu-2 and Neu-3 mRNAs were quantified by real-time PCR. All determinations were performed in duplicate, and each set of experiments was performed at least twice. The results represent the means $\pm$ S.D. of the relative expression levels of each sialidase compared with G3PDH. GMCSF, granulocyte/macrophage colony-stimulating factor. TNF induction by LPS in both cell types was used as positive control to validate the cell stimulation (**C**). IFN-g, IFN- γ ; Mono, monocytes; PMN, neutrophil.

all the concentrations of the stimuli used have been shown to cause optimal functional responses in these cells. Thus sialidase induction seems to be highly specific for T-cell activation.

Differential involvement of Neu-1 and Neu-3 in T-cell cytokine expression

To investigate possible roles of Neu-1 and Neu-3 in T-cell activation, we developed Neu-1- and Neu-3-over-expressing recombi-

Table 3 Establishment of Neu-1- and Neu-3-over-expressing Jurkat cell lines and effects of sialidase over-expression on cytokine production

All determinations were performed in duplicate, and each set of experiments was performed twice. The results are expressed as the means $\pm$ S.D. The absolute values for IL-2 protein (ng/ml) were 91.6 ± 0.3 (experiment 1) and 61.8 ± 0.4 (experiment 2).

Cell line ...	Relative level compared with vector control	
	Neu-1	Neu-3
Neu-1 mRNA	6.5 ± 0.6	1.7 ± 0.1
Neu-3 mRNA	1.1 ± 0.4	4.1 ± 0.6
Sialidase activity	2.2 ± 0.6	1.6 ± 0.2
Cytokine mRNA*		
IL-2	4.7 ± 0.5	3.2 ± 0.1
IFN- γ	22.1 ± 3.1	16.0 ± 5.1
IL-4	13.0 ± 1.2	1.0 ± 0.4
IL-13	20.6 ± 0.7	8.7 ± 1.6
IL-2 protein†		
Experiment 1	2.6 ± 0.0	2.5 ± 0.0
Experiment 2	2.6 ± 0.0	1.6 ± 0.0

* After 5-h CD3/CD28 co-stimulation.

† After 24-h co-stimulation.

nant Jurkat cell lines, and evaluated cytokine expression in these cells. As shown in Table 3, compared with vector control cell line, the Neu-1 cell line expressed a significantly (6.5-fold) higher level of Neu-1 mRNA, but not Neu-3 mRNA. Similarly, the Neu-3 cell line expressed a higher level of Neu-3 mRNA, but not Neu-1 mRNA. Moreover, at the enzyme activity level, both of the over-expression cell lines exhibited a level approx. 2-fold higher than that of the vector control cell line. Thus the recombinant over-expression cell lines were validated.

When co-stimulated via CD3/CD28, the induction of all the mRNAs for IL-2, IFN- γ , IL-4 and IL-13 was significantly enhanced in the Neu-1 cell line. In the Neu-3 cell line, the induction of IL-2, IFN- γ and IL-13, but not of IL-4, was significantly enhanced. Thus there was a major difference in cytokine expression between Neu-1 and Neu-3 in that only Neu-1 was able to enhance the expression of IL-4, the driving force in Th2 phenotype development. The enhancement of cytokine expression by sialidase over-expression at the mRNA level was confirmed at the protein level by IL-2 protein measurement by ELISA (Table 3).

DISCUSSION

In the present study, we have confirmed that the Neu-1 induction in T-cells stimulated via T-cell receptor, which has been reported in mice, also occurs in human blood CD4⁺ T-cells. Furthermore, we have, for the first time, found that Neu-3 is also induced in human T-cells (Figure 3). The expression level of Neu-2 in T-cells was too low to conclude whether or not Neu-2 is also induced. Interestingly, although in most (if not all) tissues and cells Neu-1 is the major form of sialidase at the level of mRNA (Figure 2), as well as of enzyme activity (Table 2 and [33]), Neu-3 becomes a major isoform of sialidase in T-cells at both protein and enzyme activity levels upon CD3/CD28 co-stimulation (Figure 5 and Table 2).

Although both Neu-1 and Neu-3 are induced, it seems that their gene expression may undergo differential regulation. Thus maximal induction of Neu-1 gene requires signals from both CD3 and CD28 pathways, whereas for Neu-3 maximal induction is

achieved by stimulation via CD3 alone (Figure 4). Moreover, the time courses of gene induction seem to be different in that there may be a second phase of induction between 6 and 24 h for Neu-1 only (Figure 3). However, at present the second phase of induction is speculative and would require more time points to be conclusive. On the other hand, future isolation and analysis of Neu-1 and Neu-3 gene promoters may shed light on how their gene expression is differentially regulated.

The sialidase induction is highly specific for T-cells. To date, our only other observation has been the induction of Neu-1 and Neu-2 genes in human blood neutrophils by IL-1 (Figure 6). In previous studies, LPS stimulation increased sialidase activity in monocytes, but it is not known whether it was due to gene induction or post-transcriptional activation [34,35]. It has been reported that a sialidase is involved in tissue recruitment of blood neutrophils [36]. IL-1 is known to have a specific stimulatory effect on neutrophil recruitment [37,38]. Whether or not the sialidase induction plays a role in the IL-1-induced neutrophil recruitment remains to be elucidated.

In Neu-1-deficient mice, Neu-1 deficiency is specifically associated with the suppressed expression of Th2, but not Th1, cytokines (Figure 1C and [18]). However, in human T-cells, over-expression of Neu-1 resulted in enhanced production of both Th1 and Th2 cytokines (Table 3). There are two possible explanations for this apparent discrepancy. One possibility relates to species difference, as suggested by the differential T-cell sialidase regulation between mice and humans [15,20]. Alternatively, and more plausibly, the specific association of Neu-1 with Th2 cytokine expression may be a unique phenomenon under the condition of Neu-1 deficiency *in vivo*. In fact, it is reported that in mouse T-cells and dendritic cells treatment with a viral sialidase resulted in enhanced production of all cytokines measured, including both Th2 and Th1 cytokines [39]. Taken together with our results, this suggests that Neu-1 may indeed be involved in the expression of both Th1 and Th2 cytokines in both humans and mice.

Neu-3 over-expression in T-cells resulted in enhanced production of several cytokines of both Th1 and Th2 types, but not of IL-4 (Table 3). Our result agrees with the study by Irani et al. [40] in which gangliosides blocked Th1 cytokine expression without affecting IL-4. The differential effects of Neu-3 on IL-4 and the other cytokines may be derived from the unique mechanisms for IL-4 gene expression regulation [41]. On the other hand, the differential effects of Neu-1 and Neu-3 on IL-4 expression may be due to the fact that the two sialidases have differential substrate specificities (Table 2 and [5,12–14]).

Neu-1 and Neu-3 may play a role in the cytokine gene expression via their degradation of gangliosides. Gangliosides are known to be involved in a variety of biological functions in most (if not all) cells [1,2], including cytokine gene regulation in T-cells [21,22,40] and antigen-presenting cells [42,43]. Although Neu-1 is able to hydrolyse only the artificial substrates 4-MU-NANA and nitrophenyl-NANA *in vitro* [5], there have been several lines of evidence for its degradation of gangliosides *in vivo* [18,44,45]. It is possible that Neu-1 and Neu-3 may hydrolyse different gangliosides *in vivo*, which may explain why they have differential effects on cytokine gene expression.

In conclusion, in the present study it has been demonstrated that both Neu-1 and Neu-3 sialidases are specifically induced in human T-cells stimulated via T-cell receptor. It was further demonstrated that Neu-3 becomes a major sialidase isoform upon T-cell receptor stimulation, despite the fact that in most (if not all) tissues and cells, including resting T-cells, Neu-1, but not Neu-3, is the predominant isoform. In addition, using stable over-expression T-cell lines, it is suggested that Neu-1 may be involved

in gene expression of various cytokines of both Th1 and Th2 types. It is further suggested that Neu-3 may also be involved in the expression of various cytokines, except for IL-4. Whether Neu-3 has some other unique role(s) in T-cell activation remains to be elucidated.

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