Freeze-stable sialidase activity in human leucocytes: substrate specificity, inhibitor susceptibility, detergent requirements and subcellular localization

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Human leucocytes contain a freeze-stable sialidase (neuraminidase; EC 3.2.1.18) activity in addition to the better-characterized lysosomal freeze-labile enzyme. In order to discriminate between the sialidase activities detected with the synthetic fluorimetric substrate 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (MU-Neu5Ac), different tritiated sialoglycoconjugate substrates were prepared. Using this sensitive radioactive assay system, leucocyte sialidase activity towards glycoproteins was shown to be labile to repeated freeze–thawing, but a Triton-stimulated activity towards gangliosides was entirely freeze-stable. Assay conditions were optimized for this freeze-stable ganglioside sialidase activity. Subcellular fractionation of mononuclear leucocytes (MNLs) on Percoll-density gradients showed that this ganglioside sialidase activity was entirely associated with the plasma membrane. Study of the detergent requirements showed that MNLs also demonstrated ganglioside sialidase activity when sodium cholate was present in place of Triton. Cholate-stimulated ganglioside sialidase activity was found to be entirely freeze-stable and localized at the plasma membrane. Studies on whole homogenates of MNLs demonstrated that the Triton-stimulated and cholate-stimulated activities showed similar acidic pH optima at ⩽ 3.9 and were both strongly inhibited by 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid and Cu^{2+}, but not by free *N*-acetylneuraminic acid, *N*-(4-nitrophenyl)oxamic acid or heparan sulphate. These results suggest that human MNLs contain, in addition to the lysosomal freeze-labile sialidase, a single sialidase activity which is freeze-stable, ganglioside-specific, plasma membrane-associated and stimulated both by Triton and by cholate.

INTRODUCTION

Human leucocytes contain at least two sialidase (neuraminidase) isoenzymes [1,2]. Assays using the synthetic fluorimetric substrate 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (MU-Neu5Ac) detect a freeze-labile activity corresponding to the classic lysosomal enzyme which is absent in certain inherited disorders [3,4]. Although detailed characterization of this lysosomal sialidase in leucocytes was severely limited by its low abundance [5] and membrane- or particle-bound nature [6], a soluble analogue with high activity in human placenta has been identified [7]. This enzyme, existing in a complex with β-galactosidase, a 'protective protein' and several other proteins, has been extensively studied [7–10]. In addition to the labile activity, low levels of a freeze-stable MU-Neu5Ac sialidase activity have been observed in human leucocytes. Because of its presence in the cells both of healthy subjects and of patients with the inherited disorders sialidosis and galactosialidosis [1,4] it has been regarded as genetically distinct from the labile lysosomal sialidase [1]. The stable isoenzyme has, however, remained poorly understood in terms of its natural substrates, subcellular localization and pathophysiological roles. In particular, previous attempts to define the subcellular localization of this enzyme have produced conflicting results, and it has variously been proposed to be lysosomal [1,6] or plasma membrane-associated [11].

Much of the recent evidence implicates altered sialic acid metabolism in leucocyte activation, differentiation and adhesion phenomena, and therefore in regulation of the immune system [12–16]. In particular, altered patterns of sialylation on cell-surface glycoconjugates affect specific interactions with vascular endothelium [15], as well as affecting numerous other interactions between haematopoietic cells which modify immune function [16]. Therefore, to understand fully many of the processes involved in leucocyte function, a detailed knowledge of the properties of the various leucocyte sialidase isoenzymes, especially their sites of action within cells and their specificities towards different classes of glycoconjugates, is essential.

We report here the characterization of the freeze-stable sialidase in human leucocytes as a predominantly ganglioside-specific activity, and demonstrate in mononuclear cells its association solely with plasma membranes. We identify potential reasons for discrepancies between this report and previous studies [1,6] regarding the subcellular localization of this activity. We show that this activity may be stimulated either by non-ionic Triton detergents or by anionic cholate-based detergents. The pH-dependency and inhibition patterns of the detergent-stimulated sialidase activity suggest that a single enzyme is involved.

MATERIALS AND METHODS

Reagents

Gangliosides [type-III mixture, from bovine brain; chief constituents disialogangliosides GD1a and GD1b (50%), monosialoganglioside GM1 (30%) and trisialogangliosides GT1a and GT1b (5%)], fetuin, MU-Neu5Ac, Dowex 50X8-100, Dowex 1X8-100, Sephadex G-50, ovalbumin, trichloroacetic acid, Triton X-100, Triton CF-54, Percoll and density marker beads were

Abbreviations used: sialidase (neuraminidase), *N*-acylneuraminyl hydrolase (EC 3.2.1.18); Neu5Ac, *N*-acetylneuraminic acid; MU-Neu5Ac, 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid; MU, 4-methylumbelliferone; Neu5Ac2en, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid; MNLs, mononuclear leucocytes; PMNLs, polymorphonuclear leucocytes; PTA/TCA solution, [5% (w/v) phosphotungstic acid/15% (w/v) trichloroacetic acid].

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obtained from Sigma (Poole, Dorset, U.K.). Sodium cholate and sodium glycodeoxycholate were obtained from the same company and were more than 98% pure according to manufacturer's specifications. α_1-Acid glycoprotein was obtained from the Scottish National Blood Transfusion Service, Edinburgh, U.K. Bovine submandibular gland mucin was prepared according to Corfield et al. [17] and de-O-acetylated by saponification [17]. Sodium boro[^{3}H]hydride was supplied by Amersham Life Science (Aylesbury, Bucks., U.K.). HiSafe 3 scintillation cocktail was supplied by LKB–Wallac, Milton Keynes, U.K. *N*-(4-Nitrophenyl)oxamic acid was a gift from Dr. R. W. Veh, Hamburg, Germany. 2-Deoxy-2,3-didehydro-*N*-acetylneuraminic acid (Neu5Ac2en) was supplied by Boehringer–Mannheim (Mannheim, Germany). All other reagents were obtained from BDH (Poole, Dorset, U.K.) and were of AnalaR grade.

Preparation of isotopically labelled substrates

Glycoproteins and gangliosides were labelled in the glycosidically bound *N*-acetylneuraminic acid (Neu5Ac) moiety by mild periodate oxidation followed by boro[^{3}H]hydride reduction under conditions maximizing ^{3}H incorporation into the C-8 position, and were then purified by dialysis, passage through Dowex 50 (H^+ form) and chromatography on Sephadex G-50, as described in [18].

Preparation of leucocytes

Mononuclear leucocytes (MNLs) and polymorphonuclear leucocytes (PMNLs) were prepared by Dextran sedimentation and Isopaque–Ficoll centrifugation [19], with all washing and erythrocyte-lysis stages carried out in the cold. Leucocytes were routinely homogenized by sonication on ice for 6 s using a Microson model MS-50 with a P_3 microprobe (Heat Systems Ultrasonics) on output setting 0.4 (scale 0–20), and whole sonicate was used immediately for sialidase assays.

Sialidase assays

Radiometric assays included ^{3}H-labelled substrate such that incubations contained $(1–2) \times 10^5$ d.p.m. (1.7–3.4 kBq). Substrate concentrations were adjusted relative to the amount of Neu5Ac in radioactive and additional unlabelled substrate. The final substrate concentration was adjusted using non-radioactive substrate to give a final concentration of 1 mM in initial assays with all substrates, and of 5 μM in routine ganglioside assays. In experiments on the dependence of sialidase activity on substrate concentration, non-radiolabelled ganglioside was added to achieve the final concentrations.

In initial studies to screen for and compare enzyme activity toward different substrates, all substrate concentrations were fixed at 1 mM. In assays of glycoprotein sialidase activities incubations contained 100 nmol of substrate (fetuin, α_1-acid glycoprotein, or bovine submandibular gland mucin), leucocyte sonicate and a final concentration of 0.15 M sodium acetate buffer, pH 4.2, in a total volume of 100 μl. After incubation at 37 °C for 60 min, the reaction was stopped by adding 100 μl of ice-cold ovalbumin solution (80 mg/ml in 0.1 M sodium phosphate buffer, pH 7.0) and mixing, followed by 500 μl of a PTA/TCA solution [5% (w/v) phosphotungstic acid/15% (w/v) trichloroacetic acid] to precipitate unreacted substrate. After mixing and leaving at 4 °C for at least 20 min tubes were microfuged for 3 min at 14000 *g*, 500 μl of supernatant was removed and radioactivity determined by adding 4 ml of Hi-Safe 3 scintillation cocktail and counting in an LKB–Wallac LS 1410 scintillation counter. Sialidase activity towards gangliosides was assayed using a similar protocol, except that Triton–CF-54, at a final concentration of 0.2% (w/v), was also included and incubations were stopped with 500 μl of ovalbumin solution and 200 μl of PTA/TCA. The lower detection limit for activity in these assays was 0.1 nmol of Neu5Ac released per incubation.

Subsequent ganglioside sialidase assays were modified to enhance sensitivity. Except where otherwise stated, Triton-stimulated activity was assayed using 45 min incubations containing 500 pmol of substrate (based on [20]), 110–150 μg of MNL protein, sodium acetate buffer, pH 4.5, at a final concentration of 0.15 M, and Triton CF-54 at a final concentration of 0.07% (w/v), in a total volume of 100 μl. The lower detection limit for activity was 0.5 pmol of Neu5Ac released per incubation.

Ganglioside sialidase activity was also determined with sodium cholate or glycodeoxycholate in place of Triton, as they were reported to selectively activate a lysosomal ganglioside sialidase activity [20,21]. Except where otherwise stated these incubations contained a final concentration of 0.15% (w/v) cholate.

Assays of sialidase activity towards MU-Neu5Ac routinely contained 70 nmol of substrate, leucocyte sonicate and a final concentration of 0.14 M sodium acetate buffer, pH 4.2, in a total volume of 100 μl. After incubation for 60 min at 37 °C the reaction was stopped by addition of 1 ml of glycine buffer (prepared by mixing 0.085 M glycine and 0.085 M sodium carbonate to attain pH 10.0). Fluorescence was measured using an Hitachi F-2000 fluorimeter, at an excitation wavelength of 365 nm, and an emission wavelength of 448 nm, and related to that of standard solutions of free 4-methylumbelliferone (MU). The lower limit for detection of activity was 0.02 nmol of MU released per incubation. MU-Neu5Ac sialidase activity was found to be unaffected by 0.15% cholate or 0.07% Triton CF-54.

All enzyme assays were performed in duplicate. Appropriate blank incubations were carried out for all assays and activities were calculated by subtraction of blank values from test values. All blank incubations contained water in place of MNL protein, except in studies to determine the optimal substrate concentration for cholate-stimulated ganglioside sialidase assays, where heat-inactivated MNL protein was included. Protein concentrations were measured using the Lowry method [22] with BSA as standard.

Subcellular fractionation

MNLs were isolated from 350 ml of freshly collected blood obtained from a healthy donor and suspended in 650 μl of 0.12 M sucrose in phosphate buffer (10 mM sodium phosphate buffer, pH 6.8). Cells were pelleted in a 1-ml tapered glass mortar by centrifugation at 600 *g* for 10 min, then were disrupted by 40 strokes with a Teflon pestle (clearance 0.06 mm). Nuclei and intact cells were removed by centrifuging for 10 min at 600 *g*, transferring the supernatant to a clean tube and repeating the centrifugation. To 350 μl of final supernatant was added 150 μl of 0.55 M sucrose in phosphate buffer to restore the isotonicity and the whole of the resulting preparation was immediately loaded on a Percoll gradient.

Stock ('100%') Percoll was prepared by diluting Percoll (9:1, v/v) with phosphate-buffered 2.5 M sucrose. This solution was diluted to a final Percoll concentration of 50% with phosphate-buffered 0.25 M sucrose. Aliquots (8 ml) of 50% Percoll were placed in polycarbonate tubes, overlaid with 500 μl of leucocyte preparation or a suspension of density-marker beads in phosphate-buffered 0.25 M sucrose, and centrifuged in a Beckman 70.1 Ti fixed-angle rotor for 45 min at 4 °C and 50000 *g* ($r_{av.}$ 61.2 mm). The gradient formed was removed from the top

through a glass capillary connected to a peristaltic pump and 300 μl fractions were collected at 4 °C directly into tubes containing 50 μl of a cold solution of 10 mg/ml BSA in phosphate-buffered 0.25 M sucrose. The BSA used did not contain any contaminating sialidase activity when tested with MU-Neu5Ac or gangliosides. Fractionation was also carried out without collection into BSA.

Marker enzymes were assayed in fractions. Hexosaminidase (β-D-N-acetylglucosaminidase), a lysosomal marker, was assayed fluorimetrically as in [23] with the inclusion of 0.1 % Triton X-100. 5′-Nucleotidase (plasma membrane marker) was assayed using [^{3}H]AMP in the presence of 0.1 % Triton X-100, by a modification of [24]. Lactate dehydrogenase (cytosolic) was assayed according to [25] with inclusion of 0.01 % Triton X-100. Sialidases were immediately assayed as described above on 30-μl aliquots. MU-Neu5Ac sialidase assays included 0.15 % cholate. All enzyme assays were performed in duplicate.

The percentage recovery of hexosaminidase from the initial leucocyte suspension into the postnuclear supernatant was used as an index of the percentage cell disruption; percentage recoveries of all three marker enzymes into the supernatant were similar. Lysosomal latency in the supernatant was assessed by hexosaminidase assays under isotonic conditions in the presence and absence of 0.1 % Triton X-100. In determining recoveries of marker enzyme and sialidase activities after Percoll fractionation, BSA was added to the postnuclear supernatant at a concentration equivalent to that present in the fractions.

Evaluation of Percoll effects on enzyme activities

The presence of Percoll concentrations exceeding those present in enzyme assays had no effect on marker-enzyme activities. Inhibition of sialidase activity by Percoll was tested by the inclusion of 30 μl of 0.4–45 % Percoll solutions, with various amounts of leucocyte-postnuclear-supernatant protein, in a final assay volume of 100 μl.

RESULTS

Freeze-lability of sialidase activities toward different substrates

Repeated freeze–thawing using liquid nitrogen was shown to cause a progressive decrease of MU-Neu5Ac sialidase activity in MNLs and PMNLs pooled from healthy blood donors. After 16 freeze–thaw cycles a final residual activity of 22 % was found in PMNLs, while 9 % remained in MNLs (Table 1). Assay with other sialidase substrates showed that this treatment caused a complete loss of activity towards sialoglycoproteins, while the ganglioside sialidase activity stimulated by Triton was freeze-stable, with 93 % remaining in MNLs and 87 % in PMNLs (Table 1). All subsequent studies were carried out on MNLs only.

Table 1 Effects of freeze–thawing on sialidase activities towards various substrates

Assays were carried out as described in the Materials and methods section; on fresh cell sonicates and after 16 freeze–thaw cycles using liquid nitrogen. Incubations were of 60 min duration and mixtures contained 291 μg of MNL protein, or 694 μg of PMNL protein, and 100 nmol of substrate (with respect to Neu5Ac), in a volume of 100 μl. ND, not detectable.

	Activity (nmol of Neu5Ac released/h)			
	MNLs		PMNLs	
Substrate	Fresh	After freezing	Fresh	After freezing
MU-Neu5Ac	2.76	0.26	0.84	0.18
Fetuin	0.57	ND	0.63	ND
α_1-Acid glycoprotein	0.24	ND	0.19	ND
Mucin*	ND	ND	0.29	ND
Gangliosides	0.27	0.25	0.63	0.55

* Bovine submandibular gland mucin.

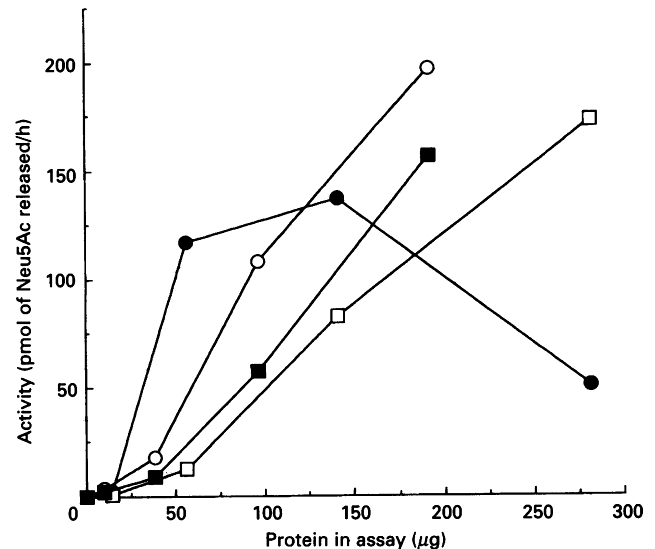

Figure 1 Variation of ganglioside sialidase activity with Triton CF-54 and MNL protein

Different amounts of MNL-sonicate protein were incubated for 60 min in a total volume of 100 μl with 500 pmol of gangliosides, in the presence of various Triton concentrations (●, 0.04%; ○, 0.07%; ■, 0.10%; □, 0.15%). Assay protocol was as described in the Materials and methods section.

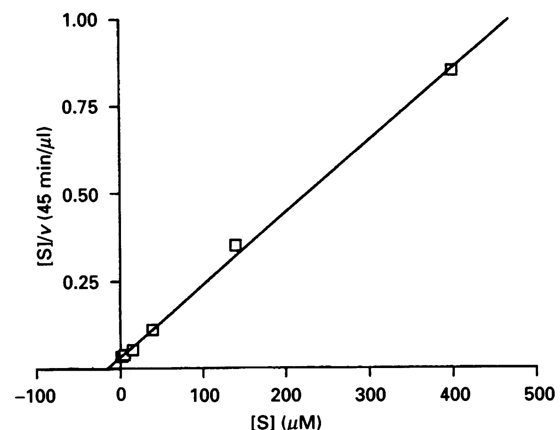

Figure 2 Hanes–Woolf plot displaying the dependence of Triton-stimulated ganglioside sialidase activity upon substrate concentration

Assay protocol was as described in the Materials and methods section. Incubations each contained 135 μg of MNL-sonicate protein in a volume of 100 μl containing isotopically labelled substrate (1.7 × 10^5 d.p.m.) and various amounts of non-labelled substrate to attain the final concentrations indicated. v is expressed as pmol of Neu5Ac released/45 min.

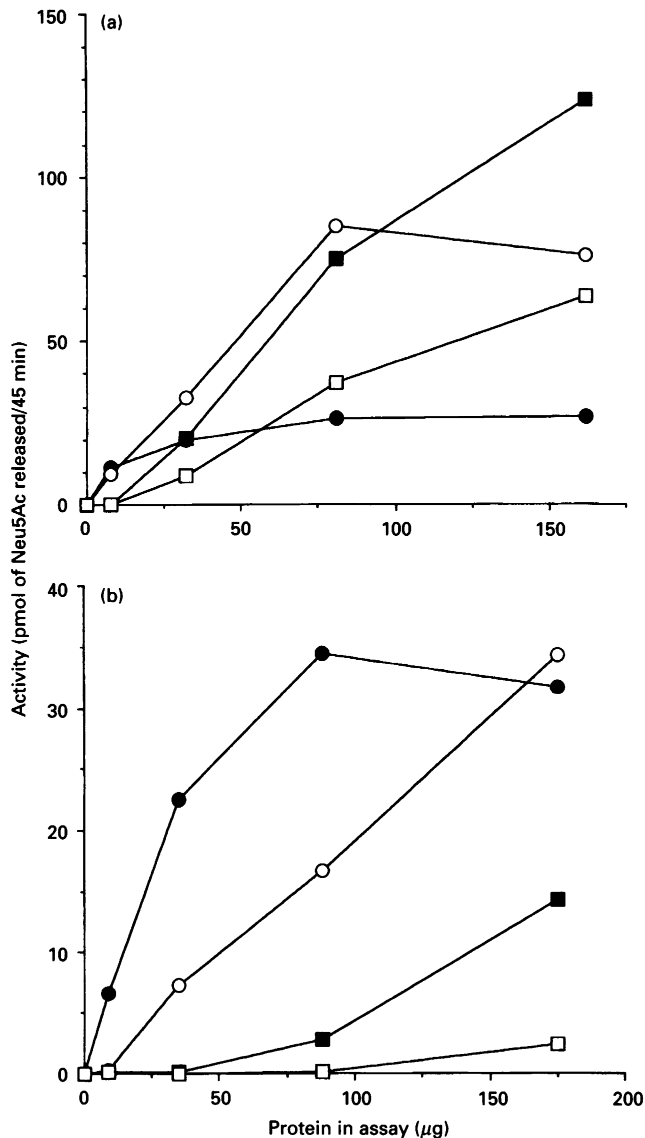

Figure 3 Effects of cholate-based detergents upon ganglioside sialidase activity

Various amounts of MNL-sonicate protein were incubated in a total volume of 100 μl containing 500 pmol of gangliosides, in the presence of various concentrations of: (a) cholate (●, 0.04%; ○, 0.08%; ■, 0.15%; □, 0.25%) and (b) glycodeoxycholate (●, 0.04%; ○, 0.08%; ■, 0.15%; □, 0.25%). Assay protocol was as described in the Materials and methods section.

Assay of Triton-stimulated ganglioside sialidase

A more sensitive assay was developed for characterization of the Triton-stimulated ganglioside sialidase activity. Stimulation of ganglioside sialidase activity by Triton CF-54 was shown to depend both on the concentration of detergent and of leucocyte homogenate protein. Higher protein concentrations required higher Triton concentrations for sialidase activation (Figure 1), but above an optimal detergent:protein ratio activity decreased. Activity was not linear over a wide range of protein concentrations, therefore the inclusion of 110–150 μg of MNL protein per incubation (100 μl total volume), with a fixed final Triton CF-54 concentration of 0.07 % (w/v), was chosen as it gave substantial enzymic release of Neu5Ac within the linear portion of the

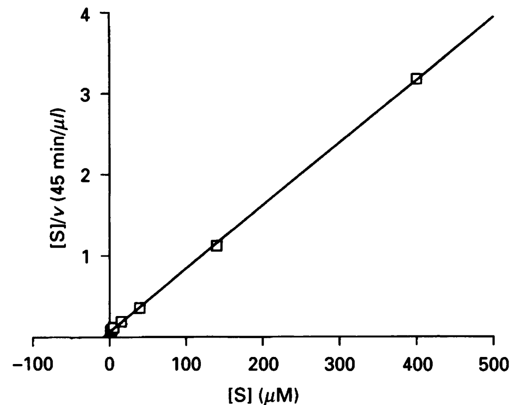

Figure 4 Hanes–Woolf plot displaying the dependence of cholate-stimulated ganglioside sialidase activity upon substrate concentration

Assay protocol was as described in the Materials and methods section. Incubations each contained 112 μg of MNL-sonicate protein in a volume of 100 μl containing isotopically labelled substrate (1.7×10^5 d.p.m.) and various amounts of non-labelled substrate to attain the final concentration indicated. v is expressed as pmol of Neu5Ac released/45 min.

activity–protein concentration curve (Figure 1). These conditions were therefore adopted in all subsequent experiments.

Release of Neu5Ac was linear with incubation times up to 60 min, after which a decrease in the rate was observed. All incubations in subsequent experiments were of 45 min duration.

The dependence of Triton-stimulated ganglioside sialidase activity upon substrate concentration is illustrated in a Hanes–Woolf plot (Figure 2). K_m and $V_{max.}$ values, calculated using the direct linear plot [26], were respectively 15 μM and 80.2 pmol/min per mg of protein. Substrate inhibition apparently occurred at concentrations above 400 μM. A substrate concentration of 5 μM was adopted for subsequent assays; this concentration yielded a near maximal percentage release of radiolabelled NeuAc, resulting in a highly sensitive ganglioside sialidase assay.

Assay of cholate-stimulated ganglioside sialidase

Optimal protein and detergent concentrations for cholate-based assays were established in a similar fashion to those required for the Triton-stimulated sialidase assay. Activity was affected by variation of cholate and protein concentration as shown in Figure 3(a). Using this data, the inclusion of 110–150 μg of MNL homogenate protein in an incubation volume of 100 μl, with a final sodium cholate concentration of 0.15 % (w/v), was chosen for routine use. Since studies on fibroblasts had indicated that glycodeoxycholate stimulated ganglioside sialidase considerably more effectively than did cholate [21], we also investigated the potential use of this detergent. However, relatively low MNL sialidase activities were achieved (Figure 3b); therefore the use of glycodeoxycholate was not pursued further. The potential influence of minor contaminants in the cholate and glycodeoxycholate batches used in these studies was not determined.

Linear release of Neu5Ac was found with cholate-stimulated ganglioside sialidase activity after 60 min and an incubation time of 45 min was used in all subsequent experiments.

The dependence of cholate-stimulated ganglioside sialidase activity upon substrate concentration is illustrated in a Hanes–Woolf plot (Figure 4). K_m and $V_{max.}$ values, calculated using the direct linear plot, were respectively 10 μM and 25.6 pmol/min per mg of protein. A substrate concentration of

Table 2 Inhibition profiles of ganglioside sialidase activities

Assay protocol was as described in the Materials and methods section. Substrate concentration was 5 μM with respect to Neu5Ac. 100% activity values (pmol/mg of protein per h) were 1179 (with cholate) and 1869 (with Triton) in experiments on Neu5Ac, Neu5Ac2en and Cu^{2+}; and were 853 (with cholate) and 1859 (with Triton) in experiments on *N*-(4-nitrophenyl)oxamic acid (NNPOA) and heparan sulphate.

		Activity (% of activity in absence of inhibitor)	
Inhibitor	Concn.	+ Cholate	+ Triton
Neu5Ac	0.1 mM	96	100
	2.0 mM	94	96
Neu5Ac2en	0.1 mM	15	15
	2.0 mM	4	2
Cu^{2+}	0.1 mM	16	13
	2.0 mM	1	0
NNPOA	0.1 mM	100	88
	2.0 mM	103	79
Heparan sulphate	20 μg/ml	100	95
	50 μg/ml	101	88
	150 μg/ml	98	75

5 μM was adopted as the optimal concentration for maximum assay sensitivity.

Comparison of the properties of Triton- and cholate-stimulated ganglioside sialidase activities

Re-examination of the freeze-stability of the Triton-stimulated ganglioside sialidase activity was carried out under optimal assay conditions. In two separate MNL preparations the activities remaining after 16 freeze–thaw cycles were 116% and 104% of the original. In a corresponding study on cholate-stimulated activity, the final activities were 104% and 111% of original. Control experiments showed that freeze-lability of MU-Neu5Ac sialidase activity was unaffected by the presence of corresponding concentrations of Triton CF-54 or cholate in the assays. These results demonstrated conclusively that ganglioside sialidase activity, whether stimulated by cholate or by Triton CF-54, was entirely freeze-stable.

Ganglioside sialidase activity, in the presence of Triton and of cholate, was assayed at pH values from 5.4 to 3.9, and in both cases was found to increase monotonically over this pH range.

The results of inhibitor studies are shown in Table 2. Neither cholate-stimulated nor Triton-stimulated ganglioside sialidase activity showed product inhibition by free Neu5Ac, but the

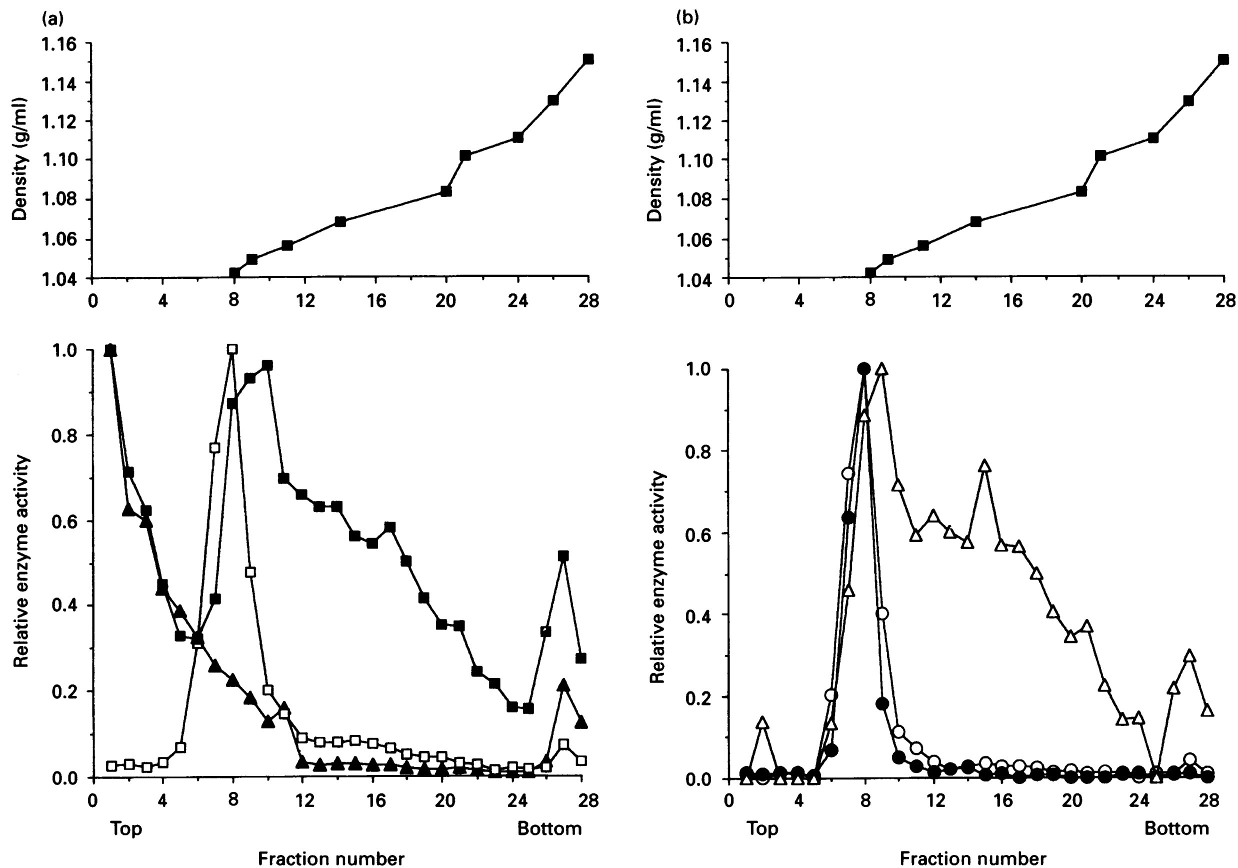

Figure 5 Subcellular fractionation of MNLs

Postnuclear supernatant of MNL homogenate (containing 3.6 mg of protein) was loaded on a Percoll gradient, fractionated and analysed as described in the Materials and methods section. Fractions each contained 500 μg of BSA in a final volume of 350 μl. The density profile was assessed by distribution of density-marker beads. (**a**) Distribution of marker enzymes: ■, hexosaminidase (lysosomal); □, 5′nucleotidase (plasma membrane-associated); ▲, lactate dehydrogenase (cytosolic). (**b**) Distribution of sialidases. Enzyme activities in the most active fractions were: MU-Neu5Ac sialidase (△), 171 pmol/h per 30 μl (fraction 9); cholate-stimulated ganglioside sialidase (○), 101 pmol/45 min per 30 μl (fraction 8); Triton-stimulated ganglioside sialidase (●), 150 pmol/45 min per 30 μl (fraction 8).

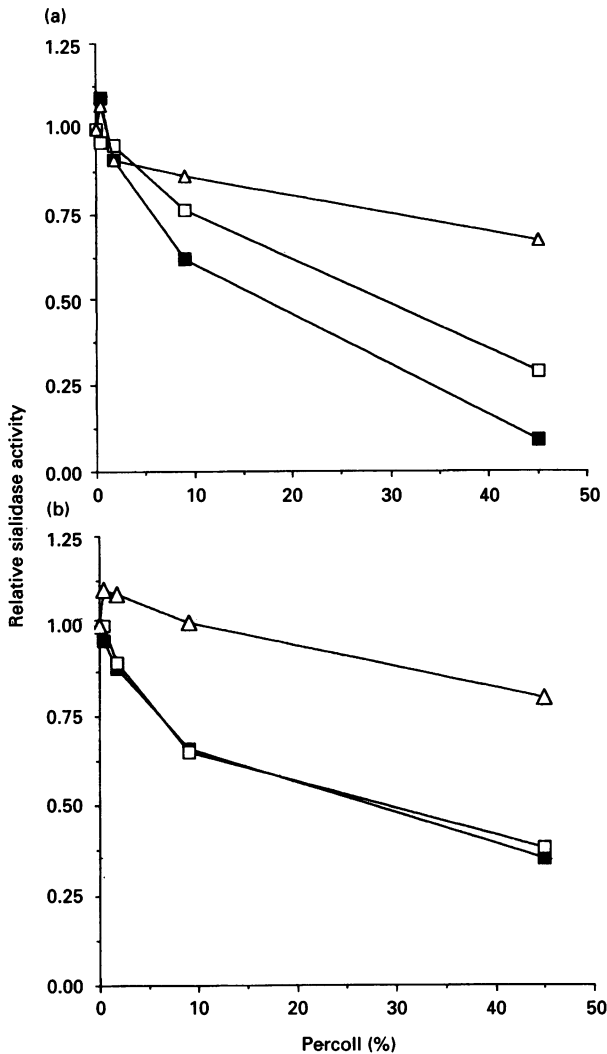

Figure 6 Percoll inhibition of ganglioside sialidase activities stimulated by (a) Triton CF-54 and (b) cholate

Assay protocol was as described in the Materials and methods section. Activities are expressed relative to the activity observed in the absence of Percoll with a given protein concentration (■, 15 μg; □, 30 μg; △, 60 μg) and a given detergent. Values quoted are for the percentage of Percoll in solutions, 30 μl of which were included in a final assay volume of 100 μl.

synthetic unsaturated analogue Neu5Ac2en had a marked inhibitory effect, similar towards both activities. Cu^{2+} also inhibited both activities to a similar extent. *N*-(4-Nitrophenyl)oxamic acid and heparan sulphate both had a minor inhibitory effect on the Triton-stimulated activity.

Subcellular fractionation by Percoll-density-gradient centrifugation

The results of subcellular fractionation, collected into BSA, are shown in Figure 5. Hexosaminidase recovery from the initial cell suspension into the homogenate postnuclear supernatant was 99 %, with a lysosomal latency of 68 %. Recoveries of marker enzymes after fractionation were 78 % (hexosaminidase; lysosomal marker), 102 % (5′ nucleotidase; plasma membrane marker) and 95 % (lactate dehydrogenase; cytosolic). The profile demonstrates that plasma membranes formed a sharp band at a density of 1.04 g/ml: lysosomes, however, were diffusely spread, partially overlapping with the plasma membrane band, indicating considerable heterogeneity in density. This marker profile is consistent with that reported earlier in resting lymphocytes [27]; the presence of a discrete band of dense secondary lysosomes would only be expected after cell activation.

Distribution of MU-Neu5Ac sialidase activity largely paralleled that of lysosomes. Although a minor portion of hexosaminidase activity was located in cytosolic fractions (representing the non-latent enzyme released by lysosomal disruption) no MU-Neu5Ac sialidase was found in these fractions, implying that the latter enzyme is not released in an active soluble form by lysosome disruption.

Ganglioside sialidase activity, whether stimulated by Triton or by cholate, formed a sharp band co-distributing entirely with the plasma membrane marker. No evidence of any lysosomal ganglioside activity was seen. Sialidase recoveries after fractionation were 73 % (MU-Neu5Ac activity), 80 % (cholate-stimulated ganglioside activity) and 76 % (Triton-stimulated ganglioside activity).

Fractionation carried out omitting BSA (results not shown) yielded similar profiles and recoveries of marker enzymes. However, no lysosomal MU-Neu5Ac sialidase was observed in this case. A band of MU-Neu5Ac sialidase activity co-distributed with the plasma membranes, but represented only an 18 % enzyme recovery. Ganglioside sialidase activities, stimulated by cholate or Triton, were both present in a sharp band co-distributing with plasma membranes. Apparent recoveries were 13 % and 19 % respectively.

Percoll effects on observed sialidase activities

Figure 6 indicates that Percoll caused a decrease in the measured ganglioside sialidase activity in MNL postnuclear supernatant, although the percentage decrease was markedly reduced by the presence of higher concentrations of MNL supernatant protein in the assays. The apparent inhibition was not due to interaction of Percoll with the reaction product, free [^{3}H]Neu5Ac. Decreases in observed activity could therefore be ascribed either to a true inhibition of the enzyme, or to Percoll interaction with gangliosides reducing the availability of substrate to the enzyme. In contrast, Percoll did not significantly affect the assay of MU-Neu5Ac sialidase activity.

DISCUSSION

The work presented here demonstrates the predominantly ganglioside-specific nature of human leucocyte freeze-stable sialidase and its localization in the plasma membrane. Under the optimal conditions established for measurement of this activity in leucocytes using ganglioside substrates, the assay is considerably more sensitive than when using the artificial fluorimetric substrate MU-Neu5Ac.

Determination of the optimal assay conditions has indicated potential reasons for discrepancies between this report and previous studies [1,6], particularly with respect to the subcellular localization of the freeze-stable sialidase.

Verheijen et al. [1] reported that the human leucocyte freeze-stable sialidase was, like the labile enzyme, lysosomal because only a single peak of MU-Neu5Ac sialidase activity was observed on subcellular fractionation and it co-distributed with β-galactosidase. However, the freeze-stable MU-Neu5Ac activity represents only a small proportion of the total (Table 1), smaller indeed than that implied by studies which have not used such extensive freezing or sonication as to inactivate totally the labile enzyme [1,2]. Inspection of the fractionation data from [1] shows that the total MU-Neu5Ac sialidase activity on the Percoll

gradient was relatively low and a second smaller peak of activity might well not have been discernible, particularly against the high substrate blanks which arise in MU-Neu5Ac assays due to substrate instability. The same study did not examine other substrates; however, its conclusion that all sialidase activity in leucocytes is lysosomal was endorsed by a more recent report on rat leucocytes [6] which used gangliosides, as well as MU-Neu5Ac, as substrates. The latter workers observed low levels of ganglioside sialidase activity in whole homogenates of leucocytes, but none in fractions from a Percoll gradient, while a single lysosomal peak of MU-Neu5Ac sialidase was observed. We have identified two factors which may have contributed to this failure to detect ganglioside activity in isolated fractions. First, non-linearity of ganglioside sialidase activity is observed at low protein concentrations (Figures 1 and 3). Secondly, Percoll may reduce the observed ganglioside sialidase activities in fractions, a problem which is more pronounced at low protein concentrations (Figure 6). It is surprising that this effect has not been reported previously, as Percoll has been used in several studies on ganglioside sialidase localization in various cell types [6,28,29]. The inclusion of BSA in fractions, which we and one previous author [28] have found to be essential to stabilize labile lysosomal sialidase activity, evidently was also sufficient to eliminate effectively problems in the ganglioside sialidase assay of fractions; in the presence of BSA we achieved good recoveries of all the sialidase activities.

Our results concur with those of Yamada et al. [11], who reported the presence of ganglioside and MU-Neu5Ac sialidase activity in a plasma membrane fraction obtained by sucrose-density-gradient centrifugation of lymphocytes both from healthy subjects and from patients with galactosialidosis. These authors also reported the presence of [^{3}H]α-2,3-sialyl-lactitol sialidase activity in plasma membranes. Isoenzyme resolution on the basis of enzyme stability was not clear-cut [11], lysosomes and plasma membranes appearing to possess activities which were respectively more or less labile to sonication, but that may be ascribed to incomplete separation of lysosomes from plasma membranes on the density gradient.

The plasma membrane localization of this sialidase implies a role in the modulation of cell-surface properties and hence of cell–cell interactions [12–16]. Of particular relevance here are observations of altered cell-surface gangliosides during the differentiation of haematopoietic cells [14].

The ganglioside sialidase activities which we observed in the presence of Triton CF-54 and of sodium cholate appear to correspond to a single enzyme, as both activities showed identical freeze-stability and an exclusively plasma membrane-associated localization. Also studies on whole homogenates provided no evidence for a distinction between the two activities. Inhibition profiles, which may be of use in distinguishing certain classes of sialidases [30,31], were similar (Table 2). High concentrations of oxamic acid and heparan sulphate did not inhibit the cholate-stimulated activity and gave only slight inhibition of the Triton-stimulated activity. This probably reflects non-specific ionic effects [21]. In addition pH profiles were similar in the presence of Triton or cholate.

Stimulation of plasma membrane ganglioside sialidases by non-ionic Triton detergents has been observed in various cell types [6,20,29]. This is thought to result from increased membrane fluidity, promoting interaction of the integral membrane enzyme and its ganglioside substrates via lateral diffusion within the membrane. Earlier work suggested that lysosomal sialidase hydrolysed only water-soluble substrates (oligosaccharides and glycoproteins); however, studies on fibroblasts have indicated that the use of an anionic cholate-type detergent, instead of Triton, revealed both lysosomal and plasma membrane ganglioside sialidase activities [28]. It was proposed that this detergent mimicked the action of a putative activator protein found intralysosomally *in vivo*. Cantz and co-workers [20,21] have reported the use of cholate or glycodeoxycholate, as opposed to Triton, to permit an absolute discrimination between the lysosomal and plasma membrane ganglioside sialidases assayed in whole fibroblast homogenates. However, subcellular fractionation of the cholate-stimulated activity was not carried out, and this conclusion derived entirely from studies on intact or homogenized fibroblasts from healthy subjects and sialidosis patients. A recent study [29] on lysosomal sialidase purified from placenta identified the putative activator protein as saposin B ('sulphatide activator protein'), a broad specificity 'physiological detergent' which appears to act by interacting with substrates rather than with the various lysosomal enzymes responsible for their degradation [32,33]. In contrast with the findings by Cantz and co-workers [20,21] these studies showed that both taurodeoxycholate and the purified activator protein stimulated not only the lysosomal ganglioside sialidase but also the plasma membrane-associated enzyme in fibroblasts.

Our results do not rule out the existence of a leucocyte lysosomal ganglioside sialidase but show that under optimal assay conditions for the freeze-stable ganglioside sialidase the observed activity is entirely plasma membrane-associated. Yamada et al. [11] reported the presence in lymphocytes of low levels of lysosomal GM3 ganglioside sialidase activity. The ganglioside preparation used in the present study comprised chiefly the disialogangliosides GD1a and GD1b, in contrast with the use of the monosialoganglioside GM3 in some other studies [11,20,21]. It has, however, been shown that purified lysosomal sialidase from human placenta acts both on GM3 and on more highly sialylated gangliosides [29]. The complexities involved in assaying a lysosomal ganglioside sialidase have been extensively discussed elsewhere [29]. The enzyme may behave in certain respects as a membrane-associated enzyme and in others as a water-soluble enzyme. Difficulties may also arise from the need to use an ionic detergent which presents the substrate in a form available to the enzyme, but which simultaneously tends to cause enzyme inactivation. Conclusive identification of a lysosomal ganglioside sialidase in leucocytes may require the use of purified activator protein. It would also be of interest to examine activated lymphocytes for the presence of lysosomal ganglioside sialidase activity, as the activities of various lymphocyte lysosomal enzymes increase dramatically on activation [27].

The relationship between the freeze-labile lysosomal sialidase and the freeze-stable enzyme, which we have identified as plasma membrane-associated, still requires elucidation. It has been assumed that since the stable enzyme is present in patients with inherited lysosomal disorders the two isoenzymes are genetically distinct [1]. Alternative explanations should, however, be considered. The two isoenzymes may derive from a single gene, but inherited defects may prevent the correct trafficking to the lysosome and/or incorporation into the complex with β-galactosidase and 'protective protein'. Improved understanding of the interrelationship between different forms of sialidase will require more detailed characterization and complete protein purification. A partial purification of a leucocyte sialidase was reported in 1984 [34], but the purification factor was low, apparently due to lability. This enzyme, oligosaccharide- and glycoprotein-specific and freeze-labile, was proposed to be the lysosomal sialidase. However, because of the apparent lack of β-galactosidase association with this enzyme, it is not now considered to be the classic lysosomal enzyme.

Purification of the leucocyte freeze-stable sialidase, to obtain

terminal protein sequence data and thus information on mammalian sialidase genes, may raise fewer problems than have been encountered with the lysosomal enzyme. There is no indication that the stable isoenzyme requires the presence of additional proteins for its activity or stability. The sensitive ganglioside sialidase assay which we have established, specifically identifying the freeze-stable enzyme activity, may prove to be a useful tool in its purification.

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