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ACCESSIBILITY OF SIALO COMPONENTS IN A MURINE TUMOR CELL TO EXTRACELLULAR *N*-ACETYLNEURAMINATE GLYCOHYDROLASE (SIALIDASE)

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Summary

Lipid-bound sialic acid in the murine melanoma cell is not totally inaccessible to an exogenous macromolecular probe, as formerly believed. Roughly 30% of the sialic acid bound to lipid, and an equal proportion of the sialic acid bound to protein is cleaved by the action of *Clostridium perfringens* *N*-acetylneuraminate glycohydrolase (neuraminidase, sialidase) when the purified enzyme is added to the suspension medium of intact murine melanoma cells freshly derived from the tumor. Cleavage of lipid-bound sialic acid is indifferent to the presence of Ca^{2+} in the medium. However, maximum release from protein requires a physiological concentration of this divalent cation. Variation in ionic strength has no effect on release of sialic acid. These findings show that a restricted portion of the bound sialic acid may be released from the intact murine melanama cell by the extracellularly supplied enzyme acting topographically.

Introduction

Inhibition studies have provided strong indirect evidence that lipid-bound sialic acid in the form of II^3 sialosyl lactosylceramide (hematoside, G_{M3} ganglioside) may act as a potent Pr_2 antigen in erythrocyte surfaces [1]. Other sialolipids that occur in the outer surface of the plasma membrane of mammalian cells are implicated as ligands for interferon [2] and for several bacterial toxins [3—5], and as an immunological receptor, at least on murine thymo-

Abbreviations: GD_3, disialosyl lactosylceramide; G_{M1}, monosialosyl gangliotetraosyl; G_{M2}, triaosylceramide.

cytes [6]. Information on the actual accessibility of cellular sialolipids to macromolecules that arise in the exterior cellular environment is rather scanty. It has been reported that *Vibrio cholerae* *N*-acetylneuraminate glycohydrolase (sialidase, neuraminidase, EC 3.2.1.18), a Ca^{2+}-requiring enzyme of relatively large molecular size [7], cleaves protein-bound but little or no lipid-bound sialic acid from a number of tumorigenic cells [8,9], including the murine B-16 melanoma cell. This finding suggests the possibility of inaccessibility of sialolipids in the cell surface to attack by large exogenous macromolecules, since the isolated lipids alone are found to be sialidase susceptible for the most part. It is claimed that treatment of murine tumor cells with sialidase causes an increase in their immunogenicity [10,11]. The question arises as to whether sialolipids in the murine tumor cell, which may be important antigenic determinants, are in fact accessible to exogenous macromolecules, and under what conditions. We have approached this problem by examining further the susceptibility of the sialo compounds in intact murine melanoma cells freshly derived from the tumor to the degradative action of exogenous microbial sialidase, and we have observed the effect of extracellular Ca^{2+} in regulating this accessibility. Practially all of the sialolipid in this murine tumor cell is II^3 sialosyl lactosylceramide (hematoside) and nearly one third can be degraded to lactosylceramide in the inact cell by exogenously supplied microbial sialidases under specified conditions. These findings may be helpful in attempts at experimental modification of the sialo components of such cells.

Materials and Methods

Clostridium perfringens sialidase (neuraminidase) was purchased from Worthington, Freehold, NJ. The enzyme was purified by a chromatographic procedure utilizing ligand-substituted DEAE-cellulose [12]. A highly purified preparation was obtained (V = 30 μmol sialic acid released/min per mg protein from disialosyl gangliotetraosylceramide substrate) by applying the enzyme preparation to the affinity column in 2 mM calcium chloride and collecting only the final, quantitatively minor, enzymatically active *N*-acetylneuraminiate glycohydrolase fraction eluting from the columns. The enzyme appears to be monomeric with a molecular size in the 60 000 dalton range [13]. We could detect no contaminating proteolytic activity in the purified preparation by incubating 1 mg/ml with 3 mg of diazotized protein (Azocoll, Calbiochem) nor could we detect any trypsin-like activity [14] at this same concentration of the purified enzyme preparation. Tests for phospholipase C activity by the method of Pastan et al. [15] were negative. We could detect no endo-β-*N*-acetyl. glucosaminidase activity [16]. Ca^{2+} up to 10 mM in isotonic sodium chloride solution neither inhibited measurably nor activated the enzyme towards the following substrates: (2 → 3 or 2 → 6) sialosyl lactose, sialosyl lactosylceramide, disialosyl gangliotetraosylceramide, and fetuin.

B-16 murine melanoma cells were obtained from the freshly excised tumor supplied by Dr. J. Kreider of the Department of Pathology. The tumors were minced and suspended in 0.01 M Tris/acetate, pH 7.4, in isotonic sodium chloride solution to which we added 0.25% (w/v) ethylenediaminetetraacetic-acid (EDTA). Brief incubation (15 min) at 37°C dissociated the tumor cells.

The cells were filtered through cheese cloth and collected by centrifugation at 800 × *g* for 10 min at 4°C. They were washed twice with isotonic sodium chloride solution, at pH 7.4, containing 5 mM calcium chloride in order to restore any surface-bound Ca^{2+} removed by EDTA.The washed cells were transferred either to isotonic sodium chloride solution or to iso-osmolar pentaerythritol (300 mM) containing 10 mM Tris/acetate, pH 6.5. The entire preparative operation took 35 min, of which 20 min was in the cold.

Lipids were extracted from the cells [17] and separated by thin-layer chromatography [8]. Enzymatically liberated sialic acid [18], protein-bound sialic acid [19], cell protein [20], and cytoplasmic lactate dehydrogenase [21] were estimated by published procedures.

Results

Sialolipid content of the murine melanoma cell

Fig. 1 shows a thin-layer chromatogram of the total lipid extracted from the cells. Sialosyl lactosylceramide comprises by far the major sialolipid component. Very small amounts of disialosyl lactosylceramide (GD_3) and monosialosyl gangliotetraosyl (G_{M1}) and triaosylceramide (G_{M2}) were also detectable. The latter two are not susceptible to the action of sialidase under the conditions tested. Upon treatment of the lipid fraction extracted from 3 mg of cell protein with 100 μg of *C. perfringens* sialidase in 1 ml of pentaerythritol medium, 95 ± 0.5% ($n = 3$) of the lipid-bound sialic acid was cleaved within 45 min at 37°C. Thin-layer chromatography showed a complete disappearance of sialosyl lactosylceramide and a new spot migrating with lactosylceramide.

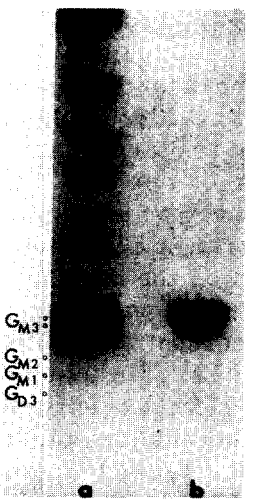

Fig. 1. Sialolipid components in the B-16 murine melanoma cell freshly derived from the tumor. The lipids were extracted from 1 mg of cell protein with chloroform/methanol (2 : 1, v/v), and separated by thin-layer chromatography on silica gel plates with chloroform/methanol/water (70 : 35 : 7, v/v/v). Sialolipids were visualized with resorcinol spray [30]. In addition to the major G_{M3} ganglioside band, faint bands of G_{M2}, G_{M1}, G_{D3} were detected, in order of mobility lower than G_{M3}. (a) Cell lipids. (b) Standard G_{M3}.

Intactness of the cells

We examined the cells freshly prepared from the tumor under the phase-contrast microscope. Their morphology appeared normal. Fewer than 3% of the cells took up trypan blue. Suspensions containing 1 g of cell protein/100 ml of a medium consisting of iso-osmolar pentaerythritol or isotonic sodium chloride stored at 4°C for 24 h did not release any measurable amount of cytoplasmic lactate dehydrogenase into the medium.

Relative content of sialocomponent

The freshly isolated murine melanoma cell was found to contain 7.2 ± 0.4 (n = 6) μg of sialic acid/mg of cell protein. Analysis of the lipid fraction gave 1.2 ± 0.07 μg of lipid-bound sialic acid/mg cell protein. Protein-bound sialic acid estimated by analysis of the lipid-free residue was 6.1 ± 0.5 μg/mg cell protein. There was clonal variability. Some tumor cells contained as much as 1.6 μg of lipid-bound sialic acid/mg protein.

Release of sialic acid

Treatment of cell suspensions, 1 mg of cell protein/ml, with 500 ng of sialidase in calcium-free iso-osmolar pentaerythritol medium at 37°C for 1 h released 1.5 ± 0.03 μg of free sialic acid. Prior to analysis, the released sialic acid from replicate runs were concentrated and purified on micro-columns of Dowex 1 × 10 [22]. The cellular lipid showed a loss of 0.4 ± 0.05 μg of lipid-bound sialic acid/mg cell protein upon treatment with sialidase. This loss was almost entirely from cellular sialosyl lactosylceramide. Incubation of the cells for an additional 90 min caused no addition liberation of sialic acid into the medium. Beyond 90 min, traces of lactate dehydrogenase began to appear in the extracellular medium, and these experiments were discontinued.

Effect of extracellular Ca^{2+}

Upon addition of Ca^{2+} to the extracellular medium, the maximum release of sialic acid from the intact cells increased, at 4 mM Ca^{2+}, to 2.20 ± 0.2 μg of sialic acid/mg cell protein. The amount of lipid-bound sialic acid remaining in the sialidase-treated cells was precisely the same whether or not Ca^{2+} was included in the incubation medium. Therefore, the additional release of sialic acid in the presence of Ca^{2+} presumably was from sialoprotein exclusively. Fig. 2 shows the effect of extracellular Ca^{2+} concentration on the maximum releasability of sialic acid from sialoprotein in the intact cell by *C. perfringens* sialidase. The releasability increased with increasing Ca^{2+} concentration. The optimum was near a physiological, 4 mM, concentration of Ca^{2+}. Higher concentrations gave less of an incrase. The 60 000 dalton *Clostridium* enzyme clearly can readily attack some, but not all, of the enzymatically susceptible sialolipid in the murine melanoma cell. Preincubation of the cells for 1, 2, and 4 h prior to addition of sialidase and Ca^{2+} to the medium had no measurable effect on sialic acid content, or non releasability.

In view of these findings, we reinvestigated accessibility of the sialo compounds of the intact melanoma cell to the larger 90 000 dalton, *V. cholerae* *N*-acetylneuraminate glycohydrolase. This enzyme as supplied commercially (Calbiochem) is preactivated with Ca^{2+}, which it requires, and is ostensibly free

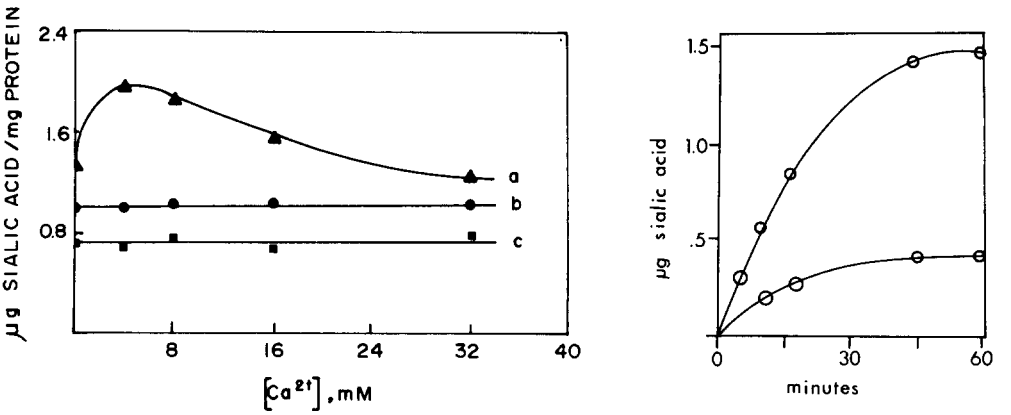

Fig. 2. Effect of extracellular Ca^{2+} concentration on the releasability of bound sialic acid from the surface of the intact B-16 murine melanoma cell by purified *C. perfringens* *N*-acetylneuraminate glycohydrolase. Cells suspended in isotonic sodium chloride, pH 6.5, were incubated at 37°C with 500 ng/ml of purified glycohydrolase until there was no further release of free sialic acid into the medium; maximum time, 90 min. Free sialic acid was measured colorimetrically [18] after concentration and chromatographic purification [22]. Values are for 1 mg of cell protein: (a) maximum release of free sialic acid; (b) lipid-bound sialic acid of control cells incubated without extracellular sialidase and (c) lipid-bound sialic acid of sialidase-treated cells.

Fig. 3. Progress curve for the loss of bound sialic from sialolipid and release of sialoprotein sialic acid of the intact murine melanoma cell exposed to extracellular *C. perfringens* *N*-acetylneuraminate glycohydrolase. Intact cells were exposed to 500 ng/ml of purified glycohydrolase in iso-osmolar pentaerythritol solution, pH 6.5, at 37°C. Free and bound sialic acid were estimated by colorimetric procedures [18, 19]. Upper curve: release from protein; lower curve: loss from lipid.

from other, contaminating, hydrolases. However, we were able to separate away from the active enzyme by affinity chromatography an inert protein fraction comprising as much as half of the total protein in the enzyme preparation. We verified that some 20% of the protein-bound and almost none, less than 4%, of the lipid-bound sialic acid were released from the intact melanoma cell by this enzyme; a plateau was reached after 35 min of treatment of the intact cells with 5 units of enzyme/ml of isotonic sodium chloride, or iso-osmolar pentaerythritol, pH 6.5. However, upon the further addition of Ca^{2+} to the extracellular medium, lipid-bound sialic acid became accessible to the *Vibrio* *N*-acetylneuraminate glycohydrolase and the accessibility of protein-bound sialic acid increased substantially. A plateau of release of sialic acid was reached after 45 min; further treatment released no more sialic acid. Optimum Ca^{2+} concentration was 6 mM which is somewhat higher than the optimum for release of protein-bound sialic acid by the *Clostridium* enzyme. Precisely the same quantity of lipid-bound sialic acid, approximately 30% of the cellular content, was now cleaved by the *Vibrio* enzyme as by the *Clostridium* enzyme. Release from sialoprotein was consistently some 10% less than with the *Clostridium* enzyme under conditions. Preincubation of the cells for 1 h in 5 mM Ca^{2+}, followed by incubation with either enzyme in the absence of added calcium, gave the same results as were obtained in calcium-free medium without such preincubation. Therefore, modulation of accessibility of sialic acid groups is not relatable to high affinity binding of calcium. Incubation of the

cells in the absence of microbial sialidase, at pH 6.5, caused a slow continuous loss of lipid-bound, but not protein-bound sialic acid. This loss amounted to less than 4% of the total cellular lipid-bound sialic acid after 45 min. We attribute it to the action of cellular intrinsic plasma membrane sialidase acting upon endogenous sialolipid substrate [23]. In an attempt to investigate the unlikely possibility of activation of the intrinsic sialidase by unknown contaminants in the bacterial enzyme preparations, we included 3 mg of sialosyl lactose/ml of incubation medium. This small water-soluble substrate is readily attacked by the microbial sialidases but not by the membrane-bound intrinsic cellular enzyme [24]. Under these conditions, we could measure only a very small, roughly 3%, diminution in total bound cellular sialic acid after 45 min incubation with either enzyme, with or without Ca^{2+} in the medium. Removal of the incubation medium, quick rinsing with isotonic sodium chloride solution and reincubation with either of the bacterial enzymes in the absence of the competing exogenous substrate gave the same Ca^{2+}-dependent results within experimental error as were obtained with cells not so pretreated. This finding suggests the absence of non-enzymatic cell surface-modifying substances in the purified *Clostridium* preparation.

The progress curve for the release of sialic acid from the intact murine melanoma cells by *C. perfringens* *N*-acetylneuraminate glycohydrolase in isotonic sodium chloride solution, pH 6.5, containing 4 mM calcium chloride, is shown in Fig. 3. The initial rates of cleavage of sialic acid from sialolipid and sialoprotein are in roughly the same proportion as the total amounts of sialic acid releasable from these two fractions of cellular sialocompounds, (i.e. proportional to the relative amounts of the available substrates in the intact cells). The apparently immediate release of free sialic acid into the medium upon exposure of the cells to the extracellular enzyme suggests a direct interaction of the enzyme with accessible substrate-bearing regions on the cell surface. Nevertheless, we tested the possibility of entry of the enzyme into the cell which could lead to cleavage of putative intracellular sialocompounds. We centrifuged the cells at $500 \times g$ for 5 min and poured off the sialidase-containing medium after 20 min of incubation at 37°C. We then incubated the cells for 4 min with 10% fetal calf serum in isotonic sodium chloride solution to displace any enzyme adhering to the cell surface [25]. After collecting the cells by centrifugation and rinsing them twice with isotonic sodium chloride, we homogenized the cells in a Ten Broeck glass homogenizer. We then added 3 mg of sialosyl lactose to 100 mg of cell homogenate protein in 1 ml of isotonic sodium chloride, pH 6.5, and incubated this mixture for 1 h at 37°C. After correction for a small amount of sialic acid liberated by control preparations lacking sialosyl lactose, we could not detect liberation of sialic acid from the water-soluble substrate. This finding suggests that liberation of sialic acid by the exogenous enzyme is primarily from outer cell surface components since little or no enzyme appears to have entered into the cells.

Discussion

In the current study, we have used the *C. perfringens* *N*-acetylneuraminate glycohydrolase molecules as a topographic probe whose action, apparently, did

not impair the intactness of the plasma membrane of the murine tumor cell. It is clear that some 30% of the sialo components in the B-16 melanoma cell surface are accessible to the enzyme, which constitutes a topical probe roughly 60 000 daltons in size, and extracellular Ca^{2+} partially modulates the accessibility. The findings suggest that a proportion of the sialolipid molecules in the murine tumor cell occur in exposed arrangements that readily accept an extracellular 60 000 dalton entity. Such accessible patches may contain those sialolipids in the cell membrane capable of reacting with exogenous messenger macro-molecules and may consist, conceivably, of self-associated sialolipids [27,28], or else sialolipids associated with intrinsic membrane proteins [29], or a combination of these forms. The experimental partial removal of sialic acid, specifically from the exposed sialosyl lactosylceramide, or from sialoprotein molecules, in the intact cell surface by the techniques described here may in future studies provide useful new information regarding certain normal and oncogenic cellular response mechanisms to exogenous macromolecular messengers. Of particular interest in this regard will be an elucidation of the biological role of the intrinsic plasma membrane sialidase of normal and oncogenic mammalians cells [23] which can liberate sialic acid from the sialolipids of the plasma membrane in situ, exposing β-galactosyl end-groups.

In accordance with accepted concepts concerning cell membrane structure, the lipophilic fatty acyl sphingosine, or ceramide, moiety of the sialosyl lactosylceramide (hematoside) in the plasma membrane of the B-16 murine melanoma cell, and all other mammalian cells, must be associated with the lipid inner layer of the membrane; the short sialosyl lactosyl oligosaccharide moiety should tend to extend out towards the extracellular space. This short trisaccharide unit cannot extend far from the hydrophobic region of the membrane in comparison with the more lengthy intrinsic oligosaccharidyl protein components. The quantity of sialolipid has been reported to vary among cell types. Values of 0.7—1.8 μg of lipid-bound sialic acid/mg of cell protein have been reported for several kinds of tumorigenic cells [8], ranging roughly from 20 to 30% of the total cellular sialic acid. Although a substantial portion of the cell surface sialic acid is bound to lipid, most is protein bound. However, the importance of sialoglycolipids in the cell membrane as determinants in the cells' response to certain environmental signals, e.g. glycoprotein hormones, interferon, serotonin, has been emphasized [26].

Acknowledgement

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