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THE BINDING OF EXOGENOUSLY ADDED NEURAMINIDASE TO CELLS AND TISSUES IN CULTURE

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SUMMARY

When neuraminidase-treated cells or tissues and untreated cells or tissues were washed thoroughly and incubated with [^{3}H]glucosamine, the culture medium from the neuraminidase-treated cells and tissues contained at least 1.5 times the amount of free sialic acid as that from untreated cells and tissues.

When neuraminidase-treated and control cells were incubated with neuraminlactose, a substrate for neuraminidase, it was cleaved only in the presence of neuraminidase-treated cells.

Increased synthesis of sialic acid after neuraminidase treatment cannot explain both sets of data. It appears that neuraminidase is bound at the cell surface, from which it cannot be removed by washing, and is active there in cleaving newly synthesized sialic acid from polysaccharide and/or glycoprotein.

INTRODUCTION

Current interest in the identification and function of cell surface carbohydrates has generated a need for reagents which can be used to identify and probe the function of specific terminal sugar residues. Two types of reagents lend themselves to studies of this type: plant agglutinins or lectins which combine relatively specifically with carbohydrate residues[1] and terminal or exoglycosidases which are highly specific in their ability to remove the terminal nonreducing sugar residues of oligosaccharide chains. Neither of these techniques extensively damages the cells which can then be studied for changes in functional capabilities. The inherent disadvantage in studies utilizing lectins is that combination of these large molecules with the cell surface may mask adjacent functional groups causing aberrant behavior not related to the specific group under study. This problem should not be encountered in studies utilizing enzymatic cleavage of specific terminal sugar moieties with glycosidases since ideally they would not be bound irreversibly at the cell surface. The specificity and diversity of these enzymes ideally suit them for studies on the position and function of cell surface carbohydrates. Most of these enzymes have not been widely available in pure form. An exception to this is the enzyme neuraminidase. This enzyme has been used extensively to probe the role of sialic acid in specific cell behavior. In a previous publication we reported on the effect of neuraminidase on cell adhesion[2]. In continuing these studies to determine the rate of replacement and

turnover of sialic acid we have found that neuraminidase is bound at the cell surface, and that the enzyme is active in this state. Binding of the enzyme at the cell surface may severely alter the real and apparent rates of replacement and turnover of sialic acid.

MATERIALS AND METHODS

Neural retinas were dissected from 10-day white leghorn chick embryos. The tissues were used whole, mechanically dissociated or dissociated after trypsin treatment (Armour "tryptar" 5000 NF units/ml). The tissues or single cells were washed three times with Tyrode's solution and then incubated with or without neuraminidase (EC 3.2.1.18, 27 units/ml, B grade, Vibrio Cholerae, Cal Biochem). Detailed procedures for tissue dissociation and neuraminidase treatment have been published elsewhere[2]. The cells or tissues (equivalent of 2 retinas) were then washed 4 times with Tyrode's solution and resuspended in a total of 3 ml of Eagle's basal medium containing 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin and either [^{3}H]glucosamine (0.05 μCi/ml, 3.6 mCi/μmole, New England Nuclear), [^{3}H]leucine (0.05 μCi/ml, 39 mCi/μmole, New England Nuclear) or neuramin-lactose (0.2 mg/ml, A grade, Cal Biochem). After incubating at 37 °C for 21 h, the tissue culture medium was collected.

The radioactive supernatants were dialyzed against 10 times their volume of 0.01 M HCl. The dialyzable material from one culture was passed through a Dowex 1 × 8 column (Biorad) in the acetate form and eluted with water, 0.05 M sodium acetate and 0.6 M sodium acetate according to procedures previously published[2]. Fractions of 0.5 ml were counted in Triton–toluene PPO–POPOP cocktail in a Beckman liquid scintillation spectrometer. Aliquots of the non-dialyzable material were counted directly or hydrolyzed in 0.05 M H_2SO_4 at 80 °C for 1 h to release sialic acid, and chromatographed on Dowex as above.

After incubation with neuramin-lactose cell culture supernatants were analyzed directly for free sialic acid by the thiobarbituric acid method of Warren[3].

RESULTS

We have previously observed that during a prolonged period of culture following neuraminidase treatment, surface sialic acid is found not to return to pretreatment levels[2]. This suggests that neuraminidase may be bound at the cell surface in an active state, there cleaving sialic acid from surface carbohydrate as it is synthesized. To investigate this possibility cells or tissues previously treated with neuraminidase were washed and then incubated with [^{3}H]glucosamine, which is incorporated specifically into hexosamine and sialic acid[4]; the free sialic acid content of the culture medium was compared with that from control (non-neuraminidase treated) cells or tissues. If the neuraminidase is bound and active, more free sialic acid should be found in the medium from the enzyme-treated tissue than in the medium from untreated tissue. Typical elution patterns from Dowex for the dialyzable fraction of the culture media are seen in Fig. 1. Peak I coincides with free glucosamine and other positively charged molecules. Peak II coincides with free sialic acid. Peak III has not been identified. Its position is not altered by treatment with hya-

luronidase (EC 3.2.1.35, ovine, grade 1A, Miles) or phosphomonoesterase (EC 3.1.3.1, calf intestine mucosa, P–L Biochemical), indicating that it is not hyaluronic acid, chondroitin sulfate, or glucosamine phosphate. Its position is not altered by hydrolysis in 0.05 M H_2SO_4 at 80 °C for 1 h, 2 M HCl at 100 °C for 2 h, or 6 M HCl at 110 °C for 6 h, indicating that it has no terminal sialic acid and is not *N*-acetylhexosamine or an oligosaccharide. It does not absorb light at 260 nm, suggesting that it does not contain nucleotide components. When Peak III and glucosamine are co-chromatographed on Sephadex G-10 the bulk of peak III migrates coincidently with the glucosamine, although the profile is skewed toward larger molecular weight material. This suggests that peak-III material is heterodisperse. [^{3}H]Leucine added to the culture medium is not incorporated into peak-III material.

Data in Fig. 1, obtained using mechanically dissociated cells, are presented as a representative pattern. These data indicate that there is more free sialic acid in the medium from cells pretreated with neuraminidase than in that from untreated cells. Similar results are obtained using whole tissues and trypsin-dissociated cells. The amount of free sialic acid in the medium from mechanically and trypsin-dissociated cells and whole tissues (two experiments each) are compared in Table I.

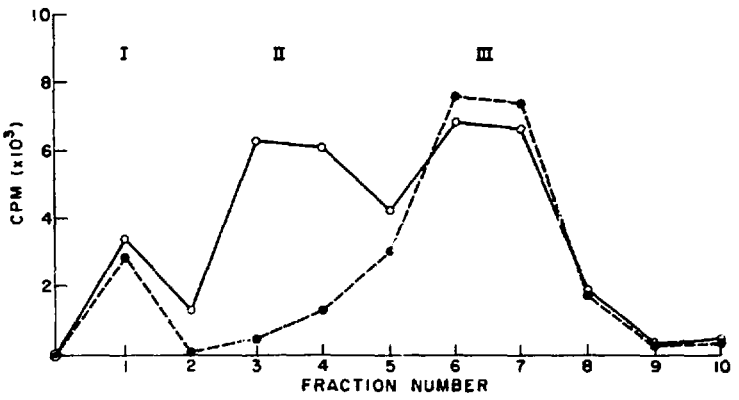

Fig. 1. Ion-exchange chromatography of [^{3}H]glucosamine-labelled dialyzable material from the culture medium from neuraminidase treated (○—○) and untreated (●—●) mechanically dissociated cells.

TABLE I

RADIOACTIVITY IN FREE SIALIC ACID IN CULTURE MEDIUM

Peak II (Fractions 3 and 4) from Dowex chromatography of dialyzable material from one culture vessel.

Tissue or cell type	*Neuraminidase-treated (cpm)*	*Untreated (cpm)*	*Neuraminidase-treated/untreated*
Whole tissue	3275	1630	2.0
Whole tissue	1978	1336	1.5
Mechanically dissociated cells	12600	1962	6.4
Mechanically dissociated cells	5436	671	8.1
Trypsin-dissociated cells	4743	1844	2.5
Trypsin-dissociated cells	3137	2071	1.5

In all cases there is more free sialic acid in the medium from treated cells or tissues. Although this data is consistent with the theory that neuraminidase is bound at the surface, it could also be accounted for if neuraminidase-treated cells synthesize more sialic acid than untreated cells, as has been suggested by Kraemer[5]. If this were the case, all sialic acid containing fractions from neuraminidase-treated cells should incorporate more label than the corresponding fractions from untreated cells. That this is not the case is suggested by the data in Table II. In every case, there has been less incorporation into total non-dialyzable material and into sialic acid hydrolyzable from non-dialyzable material by neuraminidase-treated cells and tissues than by untreated cells and tissues. In addition, medium from neuraminidase-treated cells shows reduced incorporation into peak III of the dialyzable material. Thus, it appears unlikely that increased synthesis accounts for the difference in amount of free sialic acid.

It is interesting to note that both dialyzable and non-dialyzable material containing newly synthesized sialic acid accumulate in the medium when neural retina tissues or cells are cultured. Accumulation of larger molecular weight materials labelled with [^{3}H]leucine has also been observed in this system, indicating that some of the material may be glycoprotein. Similar observations have been reported fo

TABLE II

RADIOACTIVITY IN OTHER FRACTIONS OF THE CULTURE MEDIUM

Culture medium from neuraminidase-treated tissue or cells (N) and from untreated tissues or cells (U)

Tissue or cell type	*Nondialyzable material (cpm)*		*Sialic acid hydrolyzable from nondialyzable material (cpm)*		*Peak III from dialyzable material (cpm)*	
	N	*U*	*N*	*U*	*N*	*U*
Whole tissue	2130	2325	50	60	55760	53455
Whole tissue	3070	3800	no data	no data	43900	44?00
Mechanically dissociated cells	20150	22300	1043	1131	13800	14400
Mechanically dissociated cells	4133	4230	140	230	5922	7111
Trypsin-dissociated cells	7750	10380	623	777	9108	15500
Trypsin-dissociated cells	4000	4500	220	275	26000	28000

TABLE III

SIALIC ACID RELEASED FROM NEURAMIN-LACTOSE

Culture conditions	*μmoles sialic acid per ml culture medium*
Neuramin-lactose alone	0.0125
Untreated cells without neuramin-lactose	0.0017
Neuraminidase-treated cells without neuramin-lactose	0.0043
Untreated cells with neuramin-lactose	0.0140
Neuraminidase-treated cells with neuramin-lactose	0.0270

several cells and tissues, notably for whole rat parathyroid glands[6], ascites tumor cells[7,8], and HeLa cells[4].

The fact that cells do not have the ability to cleave neuramin-lactose (a specific substrate for neuraminidase) unless they have been exposed to neuraminidase lends additional support to the idea the neuraminidase may be bound at the cell surface. Table III gives the results obtained when mechanically dissociated cells, treated with neuraminidase or left untreated, were washed, incubated with neuramin-lactose, and the culture medium analyzed for free sialic acid. The untreated cells release no sialic acid from neuramin-lactose beyond the background levels for the cells and the neuramin-lactose. Neuraminidase-treated cells, however, release 0.01 μmole per ml of culture medium more than would be expected if no residual neuraminidase activity was present. These data does not support the idea that neuraminidase-treated cells synthesize more sialic acid than untreated cells. The only reasonable explanation of the data is that exogenously added neuraminidase is bound at the cell surface, where it is active in cleaving sialic acid from cell surface molecules and from molecules in the medium.

DISCUSSION

These experiments demonstrate that the enzyme neuraminidase when used to remove cell surface sialic acid may be bound at the cell surface where the enzyme is active in cleaving newly synthesized sialic acid residues from polysaccharides and/or glycoproteins.

The fact that more free sialic acid is found in the medium from neuraminidase-treated cells than from control cells could be explained by assuming that there is increased synthesis of sialic acid after neuraminidase treatment. This does not appear to be the case. Two lines of evidence support the contention that the additional free sialic acid found in the medium from neuraminidase-treated cells is due to enzymatic cleavage, not increased synthesis: no increase in sialic acid is found in any other fraction from neuraminidase-treated cells suggesting that the cells do not respond to neuraminidase treatment by increased metabolism directed toward repair of the lesion; secondly, when neuramin-lactose is added to the culture medium, only cells previously treated with neuraminidase are able to cleave it to free sialic acid and lactose.

This last point in addition to verifying the existence of adsorbed enzyme further supports the notion that it is bound at the cell surface.

The implications of this binding for experiments designed to investigate the replacement of sialic acid or the turnover of this component are obvious. Although these experiments have been confined to one tissue type the finding that the enzyme is bound to tissues and cells prepared by both mechanical and enzymatic (trypsin) means suggest that the phenomenon may be more widespread. It is interesting to note that trypsinization of the cells prior to neuraminidase treatment reduces the amount of enzyme bound. This suggests that the binding may depend on surface structures which are at least partially removed by trypsin treatment. Alternatively the previous demonstration[9] that subsequent to trypsin treatment residual trypsin may be bound and active at the cell surface suggests that binding may be reduced or the enzyme may be inactivated by residual trypsin.

The availability of purified glycosidases of varying specificities will undoubtedly provide new tools and insights into surface structure and its relation to cell behavior. The finding that neuraminidase is bound at the cell surface should inject a note of caution in studies utilizing glycosidases as probes for cell surface structure and biochemistry.

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