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DETECTION OF ECTOSIALYLTRANSFERASE ACTIVITY USING WHOLE CELLS

CORRECTION OF MISLEADING RESULTS DUE TO THE RELEASE OF INTRACELLULAR CMP-*N*-ACETYLNEURAMINIC ACID

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Summary

An inhibitory effect due to broken cells is observed when sialyltransferase (CMP-*N*-acetylneuraminate:D-galactosyl-glycoprotein *N*-acetylneuraminyltransferase, EC 2.4.99.1) is measured with mixture of intact and homogenized lymphocytes. This intracellular inhibitory factor is purified and characterized as CMP-*N*-acetylneuraminic acid (CMP-NeuNAc) by its behavior in various chromatographic and electrophoretic systems and by its susceptibility to CMP-NeuNAc hydrolase. This endogenous CMP-NeuNAc leads to an isotopic dilution of the exogenous labelled CMP-NeuNAc explaining the apparently lower activity of homogenate when compared to whole cells. Consequently, the radioactivity bound to acceptors may not be related to a known number of sialyl residues transferred, calling into question the validity of comparing the incorporation of [^{14}C]NeuNAc by homogenate and whole cells in order to assign sialyltransferase activity to ectoenzyme. A new approach is developed to detect ectoglycosyltransferases with whole cells, taking into account that both intracellular enzymes and endogenous precursor may be introduced by the small percentage of broken cells.

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Abbreviation: CMP-NeuNAc, CMP-*N*-acetylneuraminic acid.

Introduction

Ectoglycosyltransferases are defined as membranebound enzymes whose active sites are accessible from the outside of the cell [1]. During the past few years, ultrastructural [2] and biochemical evidence [3—7] for ectosialyltransferase activity has accumulated (for review see Refs. 8 and 9). In most experiments the biochemical proofs for the surface localisation of such ectoenzymes are obtained by incubating whole cells with labelled nucleotide-sugar, assuming that the resulting incorporation into acid precipitable acceptor molecules is due to ectoglycosyltransferase activity. As pointed out by Keenan and Morré [10], this methodology is not without faults and in previous studies [11,12] we developed procedures to avoid the main causes of error. Particularly, attention has been focused on determining whether or not the detected sialyltransferase activity is due to the small percentage of broken cells in the assay. For this, we used the method recommended by Struck and Lennarz [13]: sialyltransferase activity of mixtures containing definite proportions of intact and broken cells are measured and extrapolation to zero percent broken cells gives the measurement of the ectoenzyme activity alone. In our hands, using lymphocytes, we were surprised to observe that the radioactivity bound to endogenous acceptors decreases while the proportion of broken cells increases. Further investigations [12] lead us to postulate the presence of a heat-labile, cytosoluble inhibitor of ectosialyltransferase activity.

In this paper, we demonstrate that the inhibitory effect is due to the release of intracellular CMP-*N*-acetylneuraminic acid (CMP-NeuNAc) by broken cells leading to a decrease of the transferred radioactive sialyl residues caused by isotopic dilution of the labelled precursor. This result brings the experimental procedure proposed by Struck and Lennarz [13] into question again: the radioactivity bound to endogenous acceptors does not reflect the real amount of incorporated NeuNAc residues and consequently extrapolation to 100% intact cells is no longer valid. Thus we developed an original procedure to detect ectosialyltransferase activity at the outer surface of cells taking into account the fact that both intracellular sialyltransferases and endogenous CMP-NeuNAc may be introduced by the small percentage of broken cells in the assay.

Experimental

Materials. All reagents were of analytical grade. CMP-[^{14}C]NeuNAc was purchased from Radiochemical Centre Amersham, U.K. (specific activity 214 Ci/mol). 4 U/mg pronase and insolubilized papain (EC 3.4.22.2, 80 U/g carrier-fixed enzyme) was obtained from Sigma Chemical Co (St. Louis, MO, U.S.A.) and from Boehringer (Mannheim, F.R.G.), respectively. CMP-NeuNAc hydrolase was partially purified from rat serum and was a gift from Dr. S. Bouquelet (unpublished results).

Preparation of cells and homogenate. Spleen lymphocytes were prepared from 6-week-old Wistar rats as previously described [14]. Microscopic examination was routinely used to control the viability of cells by the trypan blue exclusion test. Homogenates were prepared from lymphocytes suspensions

(10^9 cells/ml) in a cooled Potter-Elvejhem apparatus (three times ten strokes at 1250 rev./min.)

Standard sialyltransferase assay. The incubation mixture contained 0.1 M sodium cacodylate (pH 7.4), 0.154 M NaCl and 5 mM $MnCl_2$. Unless otherwise stated, standard assays (120 µl) contained 0.02 µCi CMP-[^{14}C]NeuNAc and $2 \cdot 10^7$ cells. Incubation was at 30°C with slow rotation for 1 h. At the end of each incubation, the integrity of CMP-[^{14}C]NeuNAc was controlled by submitting an aliquot fraction to descending paper chromatography in solvent: pyridine/ethyl acetate/glacial acetic acid/water (5 : 5 : 1 : 3, v/v) [15]. Radioactivity bound to endogenous acceptors was measured after phosphotungstic acid precipitation as previously described [14].

Purification of the inhibitor. After homogenization of the cells, the 150 000 × *g* supernatant [12] was successively passed through Sephadex G-50, G-25 and G-10 columns equilibrated with 0.154 M NaCl in 0.1 M sodium cacodylate (pH 7.4). A 60 µl aliquot of each fraction was tested for inhibitory effect in the standard sialyltransferase assay. Further identification of the inhibitor was achieved by paper chromatography using two systems: system A [15] and system B, isobutyric acid/concentrated ammonia/water (66 : 1 : 33, v/v) [16] and by high voltage paper electrophoresis in pyridine/glacial acetic acid/water (3 : 1 : 387, v/v, pH 5.4, 100 V/cm, 1 h). After chromatography or electrophoresis, paper strips were cut perpendicular to migration direction and the products eluted with 0.154 M NaCl, 0.1 M sodium cacodylate (pH 7.4) and again used to check their inhibitory effect.

Proteolytic treatment of the inhibitor. The susceptibility to proteolytic enzymes was tested in two ways on fractions of the inhibitor after the Sephadex G-10 purification step. In one procedure, digestion was performed with 1 unit insolubilized papain at 37°C for 1, 4, 8 and 18 h in the cacodylate/NaCl buffer (pH 7.4). Insolubilized enzyme was discarded by low speed centrifugation and supernatant was tested for its inhibitory effect. In the second procedure, 2 units pronase were used and incubation was performed at 37°C in 0.02 M calcium acetate (pH 7.5). After 24 h and 48 h digestion, the mixture was passed through a Sephadex G-25 column and each fraction was tested for the inhibitory effect.

CMP-NeuNAc hydrolase treatment of the inhibitor. The inhibitory fraction obtained after the Sephadex G-10 purification step was treated with CMP-NeuNAc hydrolase purified from rat serum, in the sodium cacodylate buffer. The incubation was at 37°C for 16 h to obtain a complete degradation of the CMP-NeuNAc as monitored by the degradation of CMP-[^{14}C]NeuNAc added to the medium as internal marker in an aliquot fraction. The enzyme was further removed from the medium by passing through a Sephadex G-25 column. Fractions were tested for their inhibitory effect, using as control a similar Sephadex G-25 chromatography of the inhibitor which has been incubated in the same conditions but without CMP-NeuNAc hydrolase.

Results and Discussion

Purification and identification of the inhibitor

In sialyltransferase assays using varying proportions of intact and broken

cells, the quantity of radioactive sialyl residues transferred to endogenous acceptors decreases as the proportion of broken cells increases (Fig. 1A). Compared to whole cells, the lower sialyltransferase activity observed with broken cells had also been mentioned by other authors [7,17] but no explanation was given. This inhibitory effect is recovered in the 150 000 $\times g$ supernatant of lymphocyte homogenate and appears to be heat labile [12]. This crude inhibitory fraction was purified by the gel filtration technique. Fig. 2 shows a typical pattern observed on passing the supernatant through a Sephadex G-50 column. The active fraction was recovered at the $V_o + V_i$ volume. This was also the case for the further Sephadex G-25 and G-10 chromatographies allowing us to conclude that the inhibitory effect is due to a small molecular weight compound. This latter purified fraction was tested for its susceptibility to proteolytic enzymes using either papain or pronase as described in the experimental section. No difference was observed after either proteolytic treatment. This fraction was also submitted to paper chromatography in two different solvent systems (A and B) and to high voltage paper electrophoresis. Fig. 3 shows that in each case, the inhibitory effect is recovered in the fraction comigrating with the CMP-[^{14}C]NeuNAc used as a marker. As it was checked that CMP-NeuNAc was resistant to the commercialized proteases used, was completely degraded by heat treatment at 100°C, for 3 min and has the same

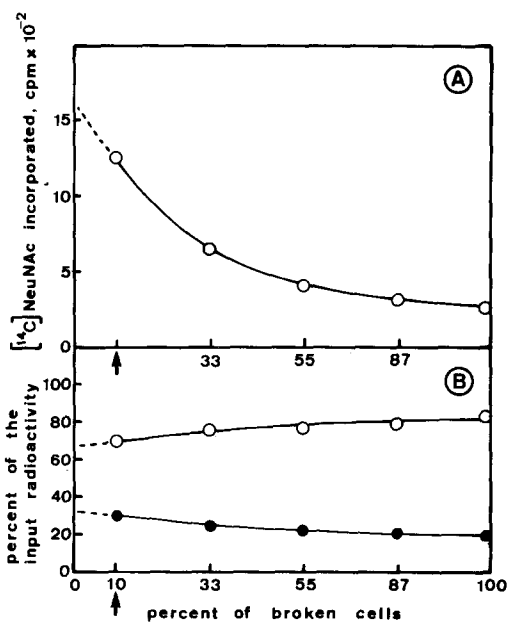

Fig. 1. Simultaneous studies of the incorporation of [^{14}C]NeuNAc into endogenous acceptors and of the CMP-[^{14}C]NeuNAc degradation as a function of the percentage of broken cells. Mixture of known proportions of intact and broken cells were incubated with CMP-[^{14}C]NeuNAc in the standard conditions. Arrow indicates the percentage of broken cells in the stock suspension of whole cells as judged by the trypan blue exclusion test. (A) ○———○, incorporation of [^{14}C]NeuNAc into endogenous acceptors (B) ○———○, CMP-[^{14}C]NeuNAc integrity, and ●———●, free [^{14}C]NeuNAc appearance as checked after separation by paper chromatography in the solvent system A.

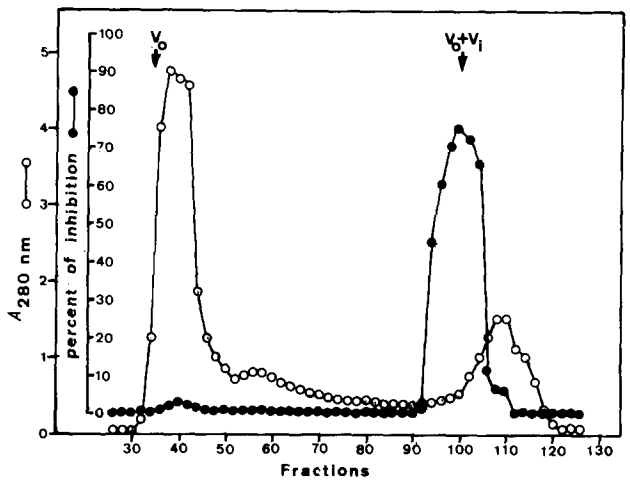

Fig. 2. Gel filtration behavior of the sialyltransferase inhibitor extracted from lymphocyte homogenate. 10 ml of the 150 000 X *g* supernatant were put on a 5 X 110 cm Sephadex G-50 column; flow rate: 80 ml/h; volume of each fraction: 20 ml; absorbance was measured at 280 nm (○———○). The inhibitory effect was assayed for each fraction and expressed as a percent of the activity measured without inhibitor (●———●).

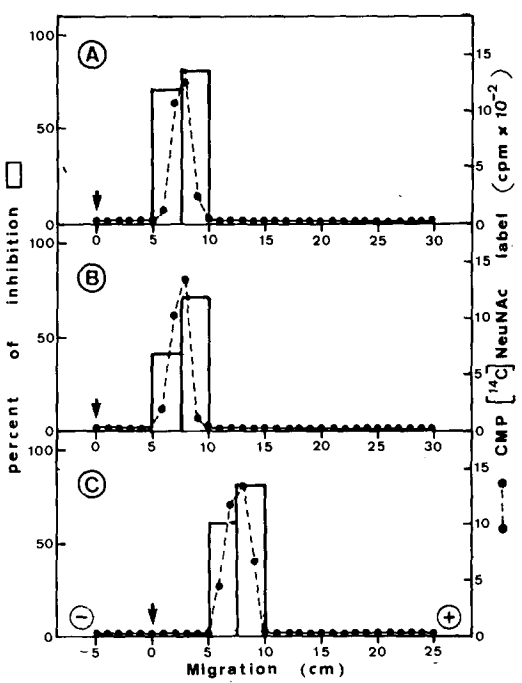

Fig. 3. Characterization of the inhibitor of the sialyltransferase activity found in lymphocyte homogenate. After purification, the inhibitor is spotted on Whatman 3 paper and chromatographed using (A) solvent A or (B) solvent B or (C) submitted to electrophoresis for 1 h at 100 V/cm in pH 5.4 buffer. In each case the CMP-[^{14}C]NeuNAc was used as internal marker. (●------●). The inhibitor effect in the sialyltransferase assay was tested after elution of 2.5 X 5 cm paper strip cut perpendicular to the migration direction. Results are expressed as a percent of the control activity without inhibitor (rectangles).

behaviour on Sephadex column chromatography as the 'so-called' inhibitor, we though that the lower transfer activity observed in the presence of broken cells could be due to the release of endogenous CMP-NeuNAc causing an isotopic dilution of the CMP-[^{14}C]NeuNAc used as precursor in the sialyltransferase assay. In addition, this was confirmed by the fact that a treatment of the inhibitory fraction with CMP-NeuNAc hydrolase leads to a complete loss of the inhibitory effect. Considering Fig. 1 again, this unexpected isotopic dilution of radioactive precursor explains both the observed apparent inhibition of the sialyltransferase (Fig. 1A) and of the CMP-NeuNAc hydrolase (Fig. 1B). In addition, more important is the fact that the real sialyltransferase activity (expressed in pM of sialyl residue transferred) is different from the amount of [^{14}C]NeuNAc incorporated as each point should be corrected according to the level of unlabelled CMP-NeuNAc released. As this correcting factor increases as the proportion of broken cells increases, the corrected points could lead to a curve which extrapolates to zero, calling into question the presence of ecto-enzyme activity.

As current methods for CMP-NeuNAc determination [18] are not sensitive enough to allow precise evaluation of the CMP-NeuNAc content in such assays, we developed a different approach to detect ectosialyltransferase activity using whole cell assays but taking into account that broken cells add both intracellular sialyltransferase and endogenous precursor.

New evidence for ectosialyltransferase activity using whole cells

To avoid a significant variation of the specific radioactivity of the precursor in the sialyltransferase assay, the transfer activity was measured in the presence of a quasi-constant and known amount of broken cells. Fig. 4 represents the [^{14}C]NeuNAc incorporated by mixtures of increasing amounts of whole cells (0—45 μl of a 10^9 cells/ml of stock solution containing 10% of broken cells) in the presence of 20, 35, 50 or 65 μl of broken cells (stock solution: 10^9 broken cells/ml, Fig. 4A—D, respectively). The presence of increasing amounts of whole cells leads to an increased transfer of radioactive sialyl residues on endogenous acceptors, as illustrated by the difference between total incorporation and incorporation uniquely due to broken cells (homogenate plus broken cells brought by whole cells suspension, Fig. 4, dotted lines). Since for each set of experiments, the additional bound [^{14}C]NeuNAc was measured for different amounts of broken cells, i.e. for different specific radioactivities of the CMP-[^{14}C]NeuNAc, valid comparison of the increased transfer due to 45 μl of stock solution of cells (i.e. 40.5 μl of intact cells, values a—d on Fig. 4) requires corrections for identical isotopic dilution. Assuming that this correcting factor is the same for homogenate alone and for mixtures of whole cells and an identical amount of homogenate, this correction is illustrated in Table I. It gives comparative values of the enhancement of the transfer of sialyl residues due to the presence of whole cells for various amounts of homogenate. This enhancement may be due to ectoenzyme activities exhibited by whole cells and/or to the presence of additional cell surface acceptors for the intracellular sialyltransferases. To distinguish between these two possibilities, it is necessary to plot the enhancement of incorporation given by whole cells versus the various amounts of homogenate: either there is no extoenzymatic activity, and

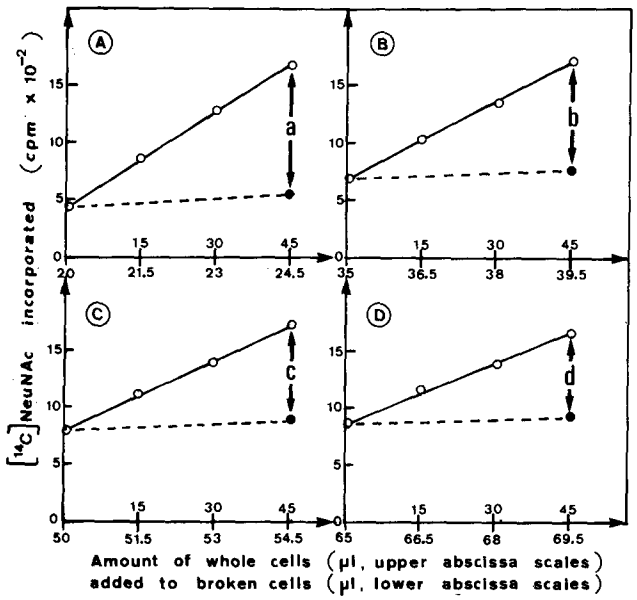

Fig. 4. Incorporation of [^{14}C]NeuNAc into endogenous acceptors using mixture of broken cells with increasing amount of whole cells. The upper abscissa scales indicate the amount of whole cells (from 0 to 45 µl of a stock suspension of 10^9 cells/ml containing 10% of broken cells as checked by the trypan blue exclusion test) which were incubated with the various amount of broken cells (20 µl, 35 µl, 50 µl and 65 µl of a stock suspension of 10^9 broken cells/ml for A—D, respectively). The lower abscissa scales indicate the total amount of broken cells present in the final 120 µl assay, i.e. the amount of homogenate added plus the amount of broken cells brought with the whole cell suspension. The additional [^{14}C]-NeuNAc incorporated when intact cells (40.5 µl) are incubated with either 24.5, 39.5, 54.5 or 69.5 µl of homogenate, - - - - -, the incorporation due to broken cells alone.

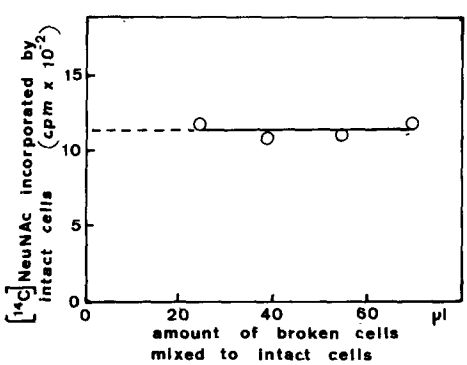

Fig. 5. Variation of the incorporation of [^{14}C]NeuNAc by constant amount of whole cells when assayed in the presence of various amount of broken cells. The amount of [^{14}C]NeuNAc incorporated into endogenous acceptors by 40.5 µl of intact cells measured in the presence of 24.5, 39.5, 54.5 and 69.5 µl of homogenate (values a—d of Fig. 4) are corrected for identical isotopic dilution as indicated in Table I and then are plotted versus the amount of broken cells. Extrapolation to zero give the intrinsic activity due to intact cells alone.

TABLE I

CORRECTION FOR IDENTICAL ISOTOPIC DILUTION OF THE [^{14}C]NeuNAc TRANSFERRED ON WHOLE CELLS

The sialyltransferase activity was assayed for mixture of intact cells (40.5 μl) with various amount of homogenate (24.5 μl, 39.5 μl, 54.5 μl and 69.5 μl) as indicated in the legend of Fig. 4. The corrective factor for identical isotopic dilution is obtained by assuming that a linear relationship should be observed between amount of homogenate and amount of [^{14}C] NeuNAc transferred.

Amount of homogenate in the incubation (μl)	A: [^{14}C]-NeuNAc incorporated on endogenous acceptors by homogenate alone (cpm)	B: Corrected values for identical isotopic dilution (cpm)	Corrective factor B/A	Enhancement of the transfer of [^{14}C]-NeuNAc residues by by whole cells (40.5 μl) in the presence of homogenate * (cpm)	Corrected values for identical isotopic dilution using factor B/A (cpm)
24.5	539	539	1.00	1160	1160
39.5	770	869	1.13	950	1070
54.5	897	1199	1.34	826	1106
69.5	944	1529	1.62	720	1166

* These values are obtained from Fig. 4 as indicated by a, b, c, d.

thus the transfer activity is strictly proportional to the homogenate enzyme content (i.e. extrapolation of the curve to origin) or there is ectoenzyme activity and extrapolation to no broken cells reveals a significant transfer activity. This final extrapolation is illustrated in Fig. 5 which shows that intact lymphocytes alone exhibit a sialyltransferase activity. That is to say that the outer surfaces of these cells possess ectosialyltransferases. Furthermore, as the additional transfer of sialyl residues given by intact cells is constant whatever the amount of broken cells in the incubation medium, this suggests that the plasma membrane glycoconjugates are not acceptors for intracellular sialyltransferases.

Conclusions

Among the various causes of error in the detection of ectoenzyme activity using incubations with whole cells, one of the most difficult to avoid is the presence of a small percentage of broken cells which could be responsible for all the transfer activity detected. To take into account the inevitable presence of broken cells, the easiest way is to use the method described by Struck and Lennarz [13] plotting the transfer activity versus various mixtures of whole and broken cells and using extrapolation to 0% broken cells for assigning transfer activity to ectoenzymes. However, this method may be faulty if broken cells release endogenous precursor causing an isotopic dilution of the labelled precursor used in the assay. In this case, the radioactivity bound to acceptors does not reflect the real amount of transferred sialyl residues and furthermore the profile of the curve may be so modified that extrapolation to zero percent broken cells is no longer valid. We made this observation when testing ectosialyltransferase [12] and ectofucosyltransferase [19] activities

with whole rat spleen lymphocytes. The decreasing radioactivity bound to endogenous acceptor with an increasing percentage of broken cells lead us to the detection of a heat-labile cytosoluble factor producing an inhibitory effect in the sialyltransferase assay. In this paper, we assign the inhibition to the release of intracellular CMP-NeuNAc by broken cells causing an isotopic dilution of the labelled precursor. Thus, to have a valid test for ectosialyltransferase, we developed a new procedure based on the measurement of whole cell sialyltransferease activity in the presence of constant amount of broken cells, i.e. a constant, thus comparative isotopic dilution. This technique permitted us to ascertain whether the sialyltransferase activity measured with whole cells was really due to ectoenzyme. Moreover, since first, a constant level of sialylation of the whole cells is observed whathever the amount of homogenate present in the incubate and second that ectosialyltransferase is not able to sialylate high molecular weight exogenous acceptors [11], it is suggested that in these in vitro assays, there is a compartmentation of sialyltransferase activity: the ectoenzyme and the intracellular enzyme endogenous activities being restricted to their own membrane acceptors.

Acknowledgements

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