PECULIAR BEHAVIOR OF ECTOSIALYLTRANSFERASE TOWARD EXOGENOUS ACCEPTORS

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Received 22 December 1976

1. Introduction

The presence of sialic acid in the cell surface glycoconjugates has a crucial importance in a variety of biological cell properties. It has been involved in the electrokinetic potential of the cell [1] and in the permeability of the membrane [2]. More recently, it has been related to the social life of the cell: cell–cell recognition [3], contact inhibition [4] and crypticity of immunogenic loci [5]. Moreover, changes in membrane-bound sialic acid have been reported to occur upon oncogenic transformation [6]. Furthermore, the sialic acid level at the cell surface seems to be correlated to the life-time of the cell [7] and to regulate the activity of some ecto-enzymes [8].

These facts raise the question as to how the surface sialic acid level is maintained or modulated: does it need an intracellular biosynthesis of the complete glycoconjugates and their further integration in the membrane or may it be due to the activity of an ectosialyltransferase as a repair phenomenon? In fact, ectosialyltransferase activity has been widely reported [9–12] and ultrastructural evidence has been obtained [13]. In this paper, we demonstrate that rat spleen lymphocyte possesses an ectosialyltransferase which is able to transfer *N*-acetylneuraminic acid to its own membrane but is not able to transfer it to a macromolecular exogenous acceptor. However, when the size of the same exogenous acceptor is reduced by proteolytic cleavage, it can reach the active site of the ecto-enzyme and is efficiently glycosylated.

2. Materials and methods

2.1. *Preparation of cells, crude homogenate and microsomal fractions*

Spleen lymphocytes were prepared from three month old Sprague-Dawley rats as previously described [14]. Lymphocyte suspension was homogenized in a Potter-Elvejhem apparatus (3 × 10 strokes at 1250 rev./min). The homogenate was centrifuged at 15 000 × *g* for 20 min and the resulting supernatant was used for preparation of microsomal fractions by centrifugation (150 000 × *g* for 90 min). The acceptor capacity of the various asialoglycoproteins was also tested with rat liver microsomal fractions prepared according to the same procedure.

2.2. *Preparation of macromolecular acceptors*

Orosomucoid (α_1-acid glycoprotein) was isolated from pooled human serum by the procedure of Schmid et al. [15]. Glycophorin was extracted from ORh^+ human erythrocyte ghosts as described by Marchesi and Andrews [16]. Orosomucoid and glycophorin were desialylated by mild hydrolysis (0.01 N H_2SO_4, 100°C, 30 min). Effective desialylation was checked by gas–liquid chromatography analysis. Coupling of acceptors to Sepharose 4 B beads was achieved as previously described [14].

2.3. *Preparation of micromolecular acceptor*

Asialoglycophorin was subjected to extensive pronase (Calbiochem) digestion (1 mg for 50 mg of

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asialoglycoprotein, during 72 h at 37°C). A low molecular weight glycopeptide has been purified by chromatography on Biogel P-6 column [17]. Its molecular weight has been estimated to around 1500 according to its distribution coefficient, its carbohydrate molar composition is Gal*NA*c : Gal (1 : 1) as determined by gas–liquid chromatography.

2.4. *Sialyltransferase assays*

The incubation mixture contained 0.1 M sodium cacodylate pH 7.4, 0.154 M NaCl, 5 mM $MnCl_2$ and 0.2 μCi/ml of [^{14}C]NANA–CMP (Amersham, spec. act. 214 mCi/mM). Standard assays (100 μl) contained 2×10^7 cells and 1 mg of acceptors. Incubation was achieved at 30°C, under a constant slow rotation, for 1 h.

2.5. *Separation of cell from acceptors and determination of radioactivity*

Macromolecular or Sepharose-bound acceptors were separated from cells as described previously [14]. When micromolecular acceptor was used, the supernatant after the cell sedimentation was spotted on Whatman 3 paper and submitted to chromatography in the following solvent: ethyl acetate/pyridine/glacial acetic acid/water (5 : 5 : 1 : 3). The acceptor-bound radioactive sialic acid remained near the origin free of precursor ([^{14}C]NANA–CMP) and of degradation products ([^{14}C]NANA) which had migrated. The radioactivity of the material was determined by counting in scintillation liquid.

3. Results and discussion

3.1. *Kinetic studies of ectosialyltransferase activity with macromolecular acceptors*

As we previously demonstrated, the use of an insolubilized acceptor is a good approach to detect ectogalactosyltransferase on intact lymphocytes [14], we devised the same experiments for ectosialyltransferase. Incubation of lymphocytes with asialoorosomucoid coupled beads and [^{14}C]NANA–CMP reveals that incorporation of radioactivity is restricted to the cells. The non-incorporation on an insolubilized acceptor could be due to an alteration of acceptor sites by the chemical treatment for coupling. In fact, fig.1a shows a kinetic study using soluble asialo-

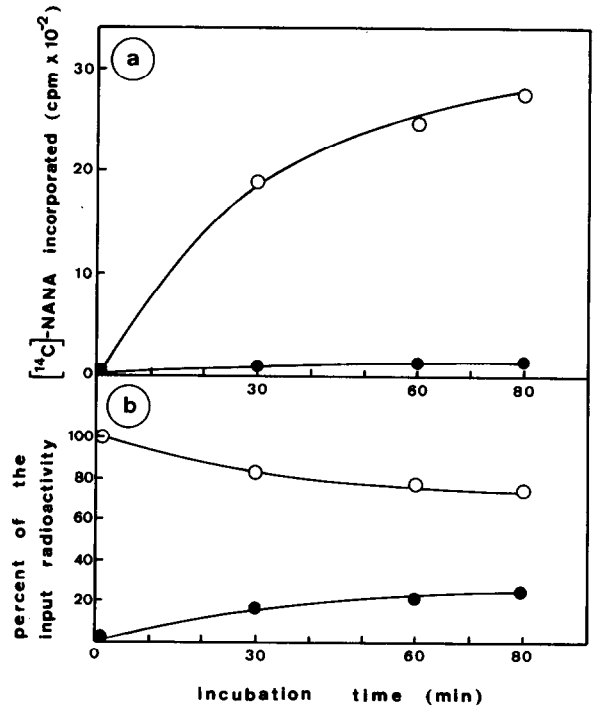

Fig.1.(a) Kinetic study of the incorporation of radioactive sialic acid on lymphocytes (○——○) and on soluble asialoorosomucoid (●——●). (b) Kinetic study of the NANA–CMP integrity (○——○) and free NANA appearance (●——●).

orosomucoid. Again, no significant radioactivity is bound to the acceptor compared to the radioactivity recovered with the cells. This fact has been noticed by Patt and Grimes with fibroblasts [18]. As described for rat liver plasma membrane [19], whole lymphocyte exhibits a NANA–CMP hydrolase activity, which contributes to the formation of free sialic acid in the medium (fig.1b). As sialic acid may enter the cell [20] we checked that a 1000-fold excess of unlabelled sialic acid does not reduce significantly (less than 10%) the radioactivity of the cells. This indicates that entry of [^{14}C]NANA cannot explain the labelling of the cells.

3.2. *Influence of the cell concentration*

As the standard assay contained 2×10^8 cells/ml it was worth examining if higher concentration of cells, i.e., of enzyme, could lead to a sialylation at a higher level of the exogenous acceptor. Figure 2 reveals that,

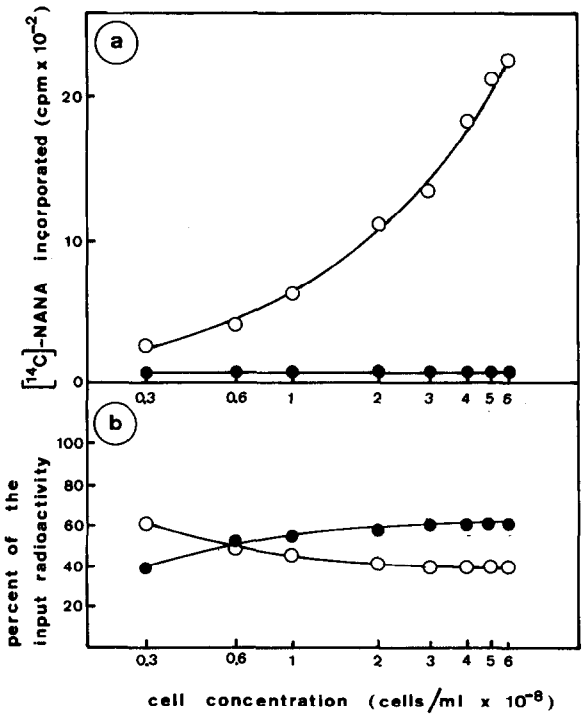

Fig.2.(a) Effect of cell concentration on the incorporation of radioactive sialic acid on the cells (o——o) and on soluble asialo-orosomucoid (•——•). (b) Effect of cell concentration on NANA–CMP integrity (o——o) and free NANA appearance (•——•). Logarithmic scale is used to represent the cell concentration.

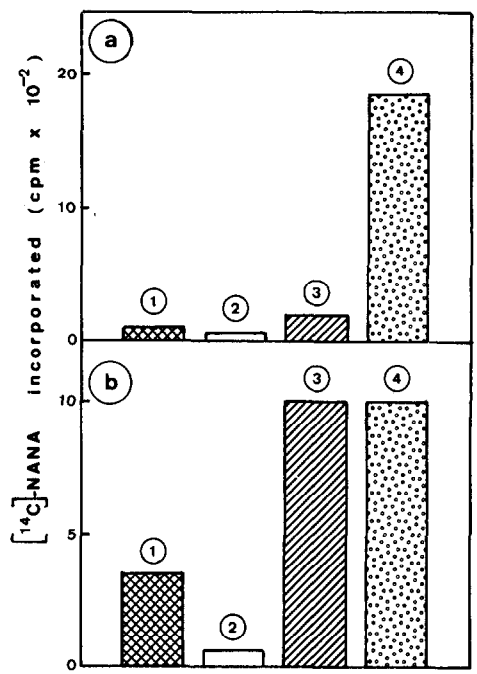

Fig.3. Relative incorporation of various macromolecular and micromolecular acceptors with (a) whole cells and (b) microsomal fraction. The various acceptors are as follows: (1) asialo-orosomucoid, (2) ovomucoid, (3) asialoglycophorin, (4) glycopeptidic acceptor. Acceptors have been used at a final concentration of 10 mg/ml except for the micromolecular (glycopeptidic) acceptor for which the dilution (8 mg/ml) has been done to obtain the 1 : 1 ratio of incorporation as compared to asialoglycophorin when assayed with microsomal fraction.

at any concentration, the cells are not able to catalyze the transfer on a macromolecular acceptor although a cell concentration-dependency is noted for the cell surface sialylation. In addition this shows that, if significant, [^{14}C]NANA incorporation on the exogenous acceptor does not behave as the endogenous membrane acceptors.

3.3. *Comparison between sialylation of macro- and micromolecular acceptors*

Non-incorporation of sialyl residues on asialo-orosomucoid could be due to the inability of this acceptor to form the enzyme–substrate complex with the lymphocyte ectosialyltransferase. To test this possibility we used two other acceptors:

(i) Native ovomucoid which is a poor sialyl acceptor (one terminal galactosyl residue per glycan moiety [21]) but which has been a suitable acceptor in the lymphocyte system [14].

(ii) Asialoglycophorin whose very good acceptor capacity is due to its high content of sialyl residues in the native form, moreover is a membrane glycoprotein.

As asialo-orosomucoid, these two asialoglycoproteins cannot be significantly sialylated by intact lymphocytes (fig.3a) however, they appeared as good acceptors of sialyl residues when assayed with microsomal sialyltransferase (fig.3b).

Thus it raises the question as to whether this peculiar property may be related to the fact that a macromolecular exogenous acceptor cannot reach the active site of the ecto-enzyme. To answer this question, ectosialyltransferase has been tested with a small

glycopeptide obtained after extensive pronase digestion of the asialoglycophorin. Figure 3a shows that this micromolecular acceptor may be highly sialylated by the ectosialyltransferase if compared to the sialylation of the originating macromolecule. When the acceptor capacity is equal for macromolecular and micromolecular acceptor in a microsomal system, the sialylation of the macromolecular acceptor decreases in a large extent (more than 5-times) with intact cell system (95% viability).

3.4. *Additional proof of this peculiar ectosialyltransferase activity*

The peculiar properties of the enzymic activity detected with whole cells favored the existence of an ectosialyltransferase. However, on the one hand, phagocytosis of acceptors and further intracellular glycosylation may not be excluded. On the other hand, whole cell preparations contain from 1–10% broken cells which could be responsible for the detected sialyltransferase activity. To demonstrate clearly the involvement of an ecto-enzyme we devised the experiments recommended by Lennarz [22]. Figure 4 shows that as the proportion of broken cells is increased, incorporation of sialyl residues on asialo-orosomucoid and micromolecular acceptor

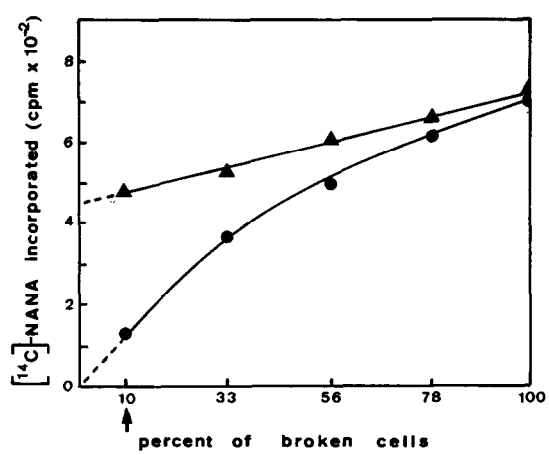

Fig.4. Sialic acid incorporation as a function of the percentage of broken cells present in an incubation, on the soluble asialo-orosomucoid (●——●) and on the glycopeptidic micromolecular acceptor (▲——▲). Mixture of known proportion of intact and broken cells were added to the incubation medium. In this experiment 90% of the stock suspension of cells excluded Trypan Blue (arrow).

increases. More important is the fact that extrapolation to 100% intact cells indicates significant incorporation on the micromolecular acceptor but no incorporation on the macromolecular acceptor. Thus, the intracellular enzyme may be monitored by the sialylation of asialo-orosomucoid and the low level of [^{14}C] NANA transfer on macromolecular acceptors, observed with whole cell incubations, is due to the low proportion of broken cells. Macromolecular acceptor sialylation inability appears to be a peculiar property of the lymphocyte ectosialyltransferase.

4. Conclusion

Demonstration ectoglycosyltransferase occurrence requires three main criteria [23]:

(i) No entry of free carbohydrate into the cell if precursor hydrolysis occurs

(ii) No release or secretion of intracellular enzymes,

(iii) No phagocytosis of acceptors.

Cell-labeling obtained with [^{14}C]NANA–CMP in the presence of excess unlabelled NANA allows us to exclude the first cause of error. Secondly, as transfer on macromolecular acceptor is not observed with intact cells it is rather difficult to consider that phagocytosis and further release of the sialylated product may occur. Finally, experiments with increasing proportion of broken cells allow us to determine that the depicted transferase activity is really due to an ectosialyltransferase. This peculiar property of the ecto-enzyme to transfer sialic acid on micromolecular acceptor or on the nearby membranous acceptors, strongly suggests that it is a rather buried 'intrinsic' membrane enzyme. This enzyme would be more concerned with the sialylation of surrounding membranous acceptors in a '*cis*-glycosylation' process. This role in repairing the cell surface glycoconjugates is in a good accordance with observations of Harms and Reutter [24] showing that membrane sialic acid has a higher turnover rate than the membrane proteins. This sialic acid renewal appears to be crucial for many biological properties of the cell.

Acknowledgements

This research was supported by grants from the CNRS (L.A. No 217: Biologie Physico-Chimique et

Moléculaire des Glucides Libres et Conjugués and ATP du CNRS 'Pharmacodynamie et Chimiothérapie') and by the Commissariat à l'Energie Atomique. We are very thankful to Dr Fournet (Université des Sciences et Techniques de Lille I) for his gift of orosomucoid and for the gas–liquid chromatography analysis.

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