OCCURRENCE OF AN INTRACELLULAR INHIBITOR OF ECTOSIALYLTRANSFERASE IN LYMPHOCYTES

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1. Introduction

One of the final steps in sialoglycoconjugate biosynthesis involves the transfer of sialic acid to glycan moieties by means of sialyltransferases. It has been demonstrated that the presence of sialic acid at the non-reducing end of glycoconjugates has a biological significance either at a molecular level or at a cellular level: sialyl residues appear to be signal for the life time of serum glycoproteins [1] or cells [2,3] and have related to cellular adhesion [4] and crypticity of immunogenic loci [5].

As far as the biogenesis of the membrane is concerned, the question is to know whether the stepwise biosynthesis of glycans follows the pathway of the membrane flow. If such is the case, the final sialylation could occur at the cell surface, thus involving an ectosialyltransferase whose activity has been widely reported [6–9]. In this work we demonstrate the presence of an intracellular inhibitor of the lymphocyte ectosialyltransferase. This finding leads to the hypothesis that sialyltransferases might be 'shut off' inside the cell and would become active, as an ectoenzyme, when it reaches the outer cell surface where the inhibitor is no more present.

2. Materials and methods

2.1. *Preparation of cells, crude homogenate and postmicrosomal supernatant*

Spleen lymphocytes were prepared from two month old Sprague-Dawley rats as previously described [10]. Lymphocyte suspension (5.10^8 cells/ml) was homogenized in a cooled Potter-Elvejhem apparatus (3 × 10 strokes at 1250 rev/min). The homogenate (also called broken cells) was centrifuged at 150 000 × *g* for 1 h. The resulting supernatant was used as source of inhibitor.

2.2. *Preparation of acceptors*

Orosomucoid (α_1-acid glycoprotein) was isolated from pooled human serum by the procedure of Schmid et al. [11] and further desialylated by mild hydrolysis (0.01 N H_2SO_4, 100°C, 30 min). Effective desialylation was checked by gasliquid chromatography analysis.

Ovomucoid was prepared as previously described [10].

2.3. *Glycosyltransferase assays*

For the sialyltransferase assays the incubation mixture contains 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, specific activity 214 mCi/mM, i.e., an average value of 400 cpm/pM). Standard assays (100 μl) contained 2.10^7 cells and 1 mg acceptor. Incubation was achieved at 30°C, under a slow rotation, during 1 h.

The galactosyltransferase assays were performed as previously described [12].

2.4. *Determination of radioactivity of cells and acceptors*

Radioactivity incorporated on the acceptor was measured by difference between two assays, one without exogenous acceptor.

Recovery of acid-precipitable materials, analysis of

precursor degradation and counting were achieved as previously described [10].

3. Results and discussion

3.1. *Galactosyl and sialyltransferase activities as a function of the percentage of broken cells*

Assignment of sialyl- or galactosyltransferase activity to ecto-enzymes by using whole cells incubations may be faulty by the presence of a certain percentage of broken cells. To determine whether the measured activities are due to broken cells, mixtures containing definite propertions of intact and broken cells were incubated with either [^{14}C]Gal–UDP or [^{14}C]NANA–CMP as recommended by Struck and Lennarz [13]. Figure 1a shows that, in the case of galactosyltransferase, extrapolation to zero percent of broken cells leads to a lower but significant incorporation in both endogenous and exogenous (ovomucoid) acceptors. This result is in total agreement with the ectogalactosyltransferase activity we previously depicted on lymphocyte cell surface [10]. Furthermore, as expected, an increasing incorporation is observed with an increasing release of intracellular enzymes. In the case of the sialyltransferase (fig.1B), although an increasing amount of intracellular enzyme is liberated by breakage of the cells as revealed by the sialylation of the asialo-orosomucoid [9], surprisingly, the radioactivity bound to endogenous acceptors decreases while the proportions of broken cells increases.

On one hand the latter phenomenon could be accounted for by an increasing degradation of the precursor by the homogenate. In fact, analysis of the precursor integrity after incubation reveals that NANA–CMP degradation is not meaningfully different between whole cells and broken cells (from 30% to 10% of the input NANA–CMP hydrolysed into free NANA). The availability of NANA–CMP is monitored otherwise by the sialylation of the exogenous acceptor. On the other hand, entry of this newly formed radioactive free NANA into cells [14] could be responsible for the higher labelling of whole cells if compared with broken cells. In fact, a 1000-fold excess of unlabelled sialic acid does not reduce the phenomenon.

Consequently, the reduced incorporation of sialyl residues by broken cells does not reflect an artefactual

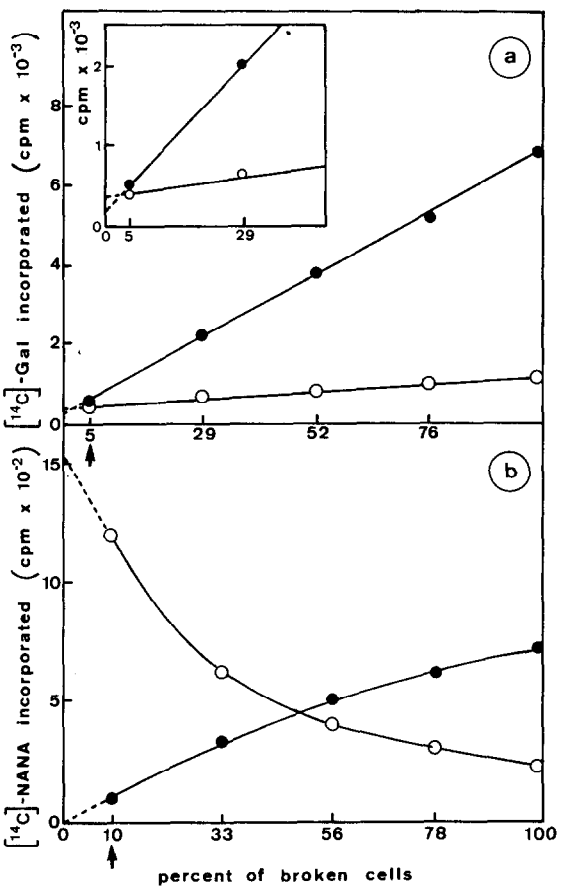

Fig.1. (a) Galactose incorporation as a function of the percentage of broken cells present in an incubation, on the endogenous acceptors (o——o) and on ovomucoid (•——•). (b) Sialic acid incorporation as a function of the percentage of broken cells present in an incubation, on the endogenous acceptors (o——o) and on asialoorosomucoid (•——•). Arrows indicate the percent of broken cells in the stock suspension as judged by trypan blue exclusion test.

event but has to be correlated with either an inhibited transfer reaction or a post reactional removal of sialyl residues such as the action of a neuraminidase [15].

3.2. *Presence of the ectosialyltransferase inhibitor in the postmicrosomal supernatant*

When increasing amounts of post microsomal supernatant are incubated with whole cells in standard ectosialyltransferase assays, the number of radio-

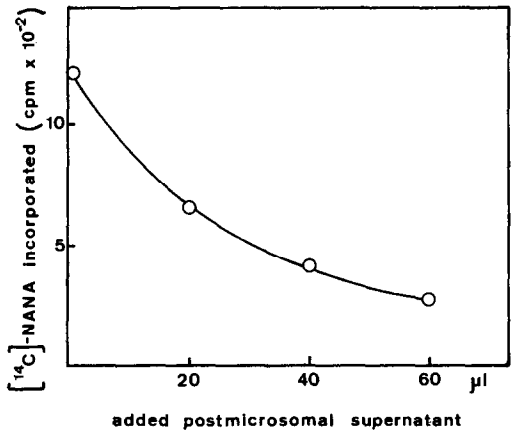

Fig.2. Effect of postmicrosomal supernatant on ectosialyltransferase activity. Increasing amounts of supernatant are added to equal amounts of whole lymphocytes in a standard incubation mixture.

active sialyl residues bound to the cells is decreasing as shown in fig.2. Thus, the effect previously observed with broken cells may be reproduced by using only post microsomal supernatant. This indicates that the effector is present in a subcellular fraction which otherwise does not exhibit any sialyltransferase activity. It was then possible to test whether this effector was an inhibitor or a post reactional destroying factor.

Table 1 shows that the post microsomal supernatant is not able to cleave off the newly bound sialyl residues in a post reactional process (table 1b) but its action is recovered only when it is added during the incubation (table 1a). This fact proves the presence of an inhibitor of the ectosialyltransferase in the post microsomal supernatant. In addition, this inhibitor is a thermolabile factor, which suggests its macromolecular nature.

3.3. *Additional proofs for an intracellular inhibitor of ectosialyltransferase*

If we assume that ectosialyltransferase is active only at the outer cell surface, where no inhibitor occurs, this activity measured in whole cell incubations must be strictly proportional to the number of enzyme, i.e., of cells, leading to a constant specific activity.

On the contrary, in assays with increasing concentration of broken cells where concommitant inhibitor

Table 1

Assay	Radioactivity bound to cells (cpm)
(a) Standard assay	
Complete	1108
Complete plus 50 µl postmicrosomal supernatant	591
Complete plus heat-treated[a] postmicrosomal supernatant	1048
(b) Standard assay[b]	
Complete	1062
Complete plus 1 h postincubation with cacodylate buffer, 30°C	986
Complete plus 1 h postincubation with 50 µl postmicrosomal supernatant, 30°C	993

[a] Heat-treatment has been achieved by maintaining the supernatant at 100°C for 3 min

[b] In this set of experiments the cells were sialylated in standard assays and then the precursor was washed off by low-speed centrifugation before either acid-precipitation or postincubation

concentration increases, the specific activity should fall down. This fact is clearly illustrated by fig.3 for which sialyltransferase specific activity is plotted versus whole cell and broken cell concentrations.

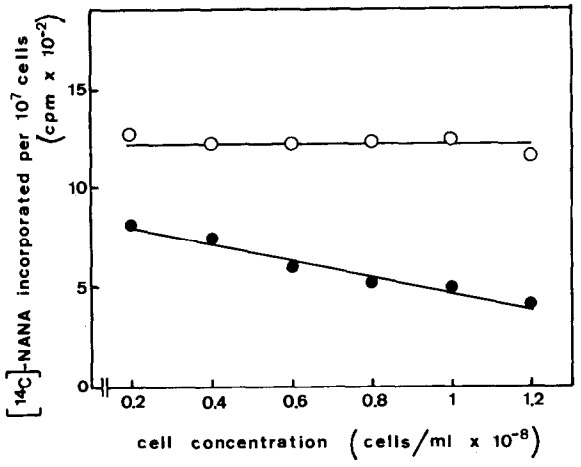

Fig.3. Variations of sialyltransferase specific activity when assays are performed with increasing concentration of whole cells (o——o) and with increasing concentration of broken cells (•——•). The measured activities concerned the transfer of sialyl residues on endogenous acceptors.

4. Conclusion

The starting point of this work has been the observation that the sialyltransferase activity is higher in whole cell incubations than in homogenates. A similar datum has been obtained by Patt and Grimes with SV T2 cells [16]. We demonstrate that this phenomenon is not an artifactual event but is due to release of an intracellular sialyltransferase inhibitor by breakage of the cells.

On one hand, we reported previously [9] that the ectosialyltransferase is ascribed to the sialylation of endogenous membrane acceptors and cannot sialylate macromolecular exogenous acceptors. On the other hand, we demonstrate in this paper that an intracellular inhibitor affects the sialylation of the endogenous acceptors without meaningfully inhibiting the sialylation of exogenous acceptor. These two facts strongly suggest that this inhibitor is specific to ectosialyltransferase.

The finding of such an inhibitor allows us to propose that the final sialylation of the surrounding membrane glycoconjugates could occur only when the ectosialyltransferase reaches the outer cell surface at the end of the membrane flow process.

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References

[1] Ashwell, G. and Morell, A. G. (1974) Adv. Enzymol. Relat. Areas Mol. Biol. 41, 99–128.
[2] Jancik, J. and Schauer, R. (1974) Hoppe Seyler's Z. Physiol. Chem. 355, 395–400.
[3] Woodruff, J. J. and Gessner, B. M. (1969) J. Exp. Med. 129, 551–561.
[4] Deman, J. J., Brugnell, E. A. and Mareel, M. M. (1974) J. Cell Biol. 60, 641–652.
[5] Currie, G. A. and Bagshawe, K. D. (1969) Brit. J. Cancer. 23, 141–149.
[6] Grimes, W. J. (1973) Biochemistry 12, 990–996.
[7] Shur, B. D. and Roth, S. (1975) Biochim. Biophys. Acta 415, 473–512.
[8] Porter, C. W. and Bernacki, R. J. (1975) Nature 256, 648–650.
[9] Verbert, A., Cacan, R., Debeire, Ph. and Montreuil, J. (1977) FEBS Lett. 74, 234–238.
[10] Cacan, R., Verbert, A. and Montreuil, J. (1976) FEBS Lett. 63, 102–106.
[11] Schmid, K., Mac Nair, M. B. and Burgi, A. F. (1958) J. Biol. Chem. 230, 853–864.
[12] Verbert, A., Cacan, R. and Montreuil, J. (1976) Eur. J. Biochem. 70, 49–53.
[13] Struck, D. K. and Lennarz, W. J. (1976) J. Biol. Chem. 251, 2511–2519.
[14] Hischberg, C. B., Goodman, S. R. and Green, C. (1976) Biochemistry 15, 3591–3599.
[15] Carubelli, R. and Yeh, A. K. (1974) Behring Inst. Mitt. 55, 88–90.
[16] Patt, L. M. and Grimes, W. J. (1974) J. Biol. Chem. 249, 4157–4165.