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Occurrence of Two Fucosyltransferase Activities at the Outer Surface of Rat Lymphocytes

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To demonstrate the existence of ectofucosyltransferase activities on the outer surface of rat lymphocytes, we measured fucosyltransferase activities on whole cells using procedures enabling us to exclude the possibility of misleading results due to precursor hydrolysis and intracellular utilization of the free fucose, and to take into account the contamination by intracellular enzymes freed by the small percentage of broken cells. The described ectofucosyltransferases are able to catalyze the transfer of fucosyl residues from GDP-fucose to the endogenous membrane acceptors but the transfer activity towards exogenous acceptors is restricted to low molecular weight compounds. Use of galactose and di-*N*-acetylchitobiose as exogenous acceptors and concomitant study of the specific inhibition by *N*-ethylmaleimide enabled us to detect both types of ectofucosyltransferases: a GDP-fucose : galactoside ectofucosyltransferase and a GDP-fucose : *N*-acetylglucosaminide ectofucosyltransferase.

The presence of fucosyl residues has been demonstrated in various types of membrane glycoconjugates of biological importance. For example, fucose has been found as a terminal residue attached to β-galactosyl residues of glycoconjugates confering blood-group H-specificity [1], and as a branched residue attached to the asparagine-linked *N*-acetylglucosamine residue of asparagine – *N*-acetylglucosamine-type glycoproteins as in the mouse histocompatibility-2 alloantigen [2] and immunoglobulins [3]. Recently, it has been proved that two different fucosyltransferases were involved in the transfer of fucose from GDP-fucose to each type of acceptor, either to a β-*N*-acetylglucosaminyl residue or to a terminal galactosyl residue [4]. Although during the past few years biochemical evidence for ectoglycosyltransferases has been accumulating (for review see [5]), few reports concerned ectofucosyltransferases and their possible involvement in the fucosylation of the membrane components [6, 7].

The methodology we developed to prove the presence of ectoglycosyltransferases [8 – 10] has been applied, in this work, to detect ectofucosyltransferases on whole lymphocytes avoiding the causes of errors pointed out by Keenan and Morré [11]. These ectofucosyltransferases are able to transfer fucose to low molecular weight acceptors and to their nearby membranous acceptors. However, this transfer reaction is not possible with macromolecular acceptors contrary to observations with intracellular enzymes. In addition, using different types of low molecular weight acceptors and the selective inhibition of the galactoside fucosyltransferase by *N*-ethylmaleimide [12] we demonstrated the presence of two ectofucosyltransferase activities: a GDP-fucose : galactoside fucosyltransferase and a GDP-fucose : *N*-acetylglucosaminide fucosyltransferase.

Abbreviation. GDP-Fuc, guanosine diphospho-L-fucose.
Enzyme. β-Galactosidase (EC 3.2.1.23).

MATERIALS AND METHODS

Chemicals

All reagents were of analytical grade. All sugars were of the D configuration except the L-fucose. GDP-[^{14}C]fucose (specific activity 89 Ci/mol) was obtained from the Radiochemical Centre (Amersham, England). L-Fucose, D-galactose, GMP, GDP, UMP, *N*-ethylmaleimide and fetuin were purchased from Sigma (St Louis, U.S.A.). β-Galactosidase from Jack bean was a gift from Dr G. Spik. Di-*N*-acetylchitobiose was obtained from l'Industrie Biologique Française (Clichy, France).

Preparation of Cells and Homogenate

Spleen lymphocytes were prepared from six-week-old Wistar rats as previously described [8]. Homo-

genates were prepared from a lymphocyte suspension (6×10^8 cells/ml) in a cooled Potter-Elvejhem apparatus (3×10 strokes at 1250 rev./min).

Preparation of Macromolecular Acceptors

Fetuin was desialylated by mild acid hydrolysis (0.005 M H_2SO_4, 100 °C, 30 min). The asialofetuin was further treated with β-galactosidase purified from Jack bean and the enzyme was removed by passing through a column of Ultrogel AcA-44. Effective desialylation (complete) and degalactosylation (about 50% galactosyl residues removed) were checked by gas-liquid chromatography.

Standard Fucosyltransferase Assays

The incubation mixture contained 0.1 M sodium cacodylate pH 7.4, 0.154 M NaCl, 2 mM $MgCl_2$, 1 mM fucose, 5 mM UMP, and 5 μM GDP-[^{14}C]-fucose i.e. an average value of 200 counts × min^{-1} × $pmol^{-1}$. For a final volume of 100 μl, a standard assay solution contained 3×10^7 cells and 1 mg of acceptors. Incubation was at 30 °C for 10 min.

Analysis of [^{14}C]Fucose Incorporated on Cells and on Macromolecular Acceptors

After incubation, cells were separated from exogenous macromolecular acceptors by low-speed centrifugation before acid precipitation. As we had observed adsorption of labelled GDP-fucose on cells and macromolecular acceptors (about one-third of the enzymatically incorporated radioactivity) when direct addition of trichloroacetic acid or phosphotungstic acid had been used, we developed a procedure to eliminate this background radioactivity. After separation, cells or macromolecular acceptors were first diluted 25 times with cold 0.154 M NaCl, precipitated with trichloroacetic acid (10% final) and desorption was completed by increasing the ionic strength using NaCl to give a final concentration of 2 M. The acid-precipitable material was collected on a glass fibre filter (Whatman GF 83) and extensively washed with 5% trichloroacetic acid and then with cold ethanol. The radioactivity was determined by counting in scintillation liquid. In these conditions, adsorption was reduced to less than 1% of the enzymatically incorporated label as illustrated in Fig. 1.

Analysis of [^{14}C]Fucose Incorporated into Glycoproteins or Glycolipids

Glycolipids were extracted from the acid-precipitable material by solubilization in chloroform/methanol mixture as described by Patt and Grimes [13] for cell surface glycolipids and glycoproteins. Following

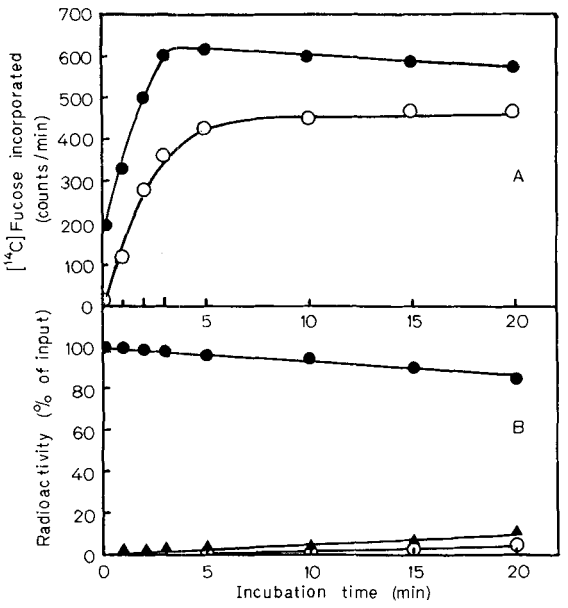

Fig. 1. *Kinetic studies of the transfer of [^{14}C]fucose to the cells from GDP-[^{14}C]fucose and concomitant analysis of nucleotide sugar degradation.* Incubations were performed in the standard conditions at 30 °C with 3×10^7 cells in a final volume of 100 μl, in the cacodylate/NaCl/$MgCl_2$ buffer pH 7.4 containing 5 μM GDP-[^{14}C]-fucose, 5 mM UMP and 1 mM fucose. (A) Total radioactivity bound to the cells after trichloroacetic acid precipitation (●——●). (B) Radioactivity bound to the cells after desorption of radioactive precursors by high saline treatment and washings as indicated under Materials and Methods (○——○). (B) GDP-Fuc integrity (●——●), fucose 1-phosphate (▲——▲) and appearance of free fucose (○——○)

incubations, cells were precipitated as described above. The precipitated material was collected by centrifugation and the pellet was washed twice with 5% trichloroacetic acid and then by successive addition of water until the supernatant contained less than 100 counts × min^{-1}. The washed pellet was extracted three times with 2 ml of chloroform/methanol (2/1) and the extractions were pooled and dried in scintillation vials for counting. The extracted pellet was suspended in 1 ml of 1 M NH_4OH and heated to 70 °C overnight. Proteins solubilized by this procedure were transferred to scintillation vials and counted.

Analysis of [^{14}C]Fucose Incorporated in Low Molecular Weight Acceptors and of the Precursor Degradation Products

When low molecular weight acceptors were used, fucosylated products were separated from precursor and precursor degradation products (GDP-[^{14}C]fucose, [^{14}C]fucose 1-phosphate and [^{14}C]fucose) by paper chromatography in the following solvent [14]: ethyl acetate/pyridine/glacial acetic acid/water (5/5/1/3). Fucosylated acceptors were further eluted free of degradation products and chromatographed together with unlabelled sugars.

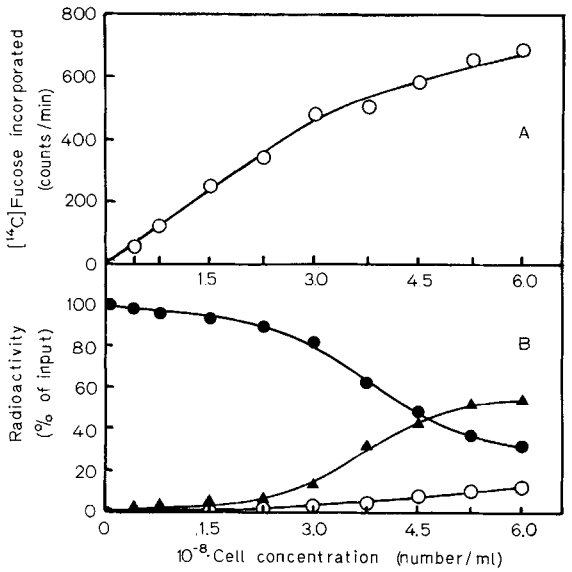

Fig. 2. *Effect of the cell concentration on the transfer of [^{14}C]fucose to the cells from GDP-[^{14}C]fucose and concomitant analysis of nucleotide sugar degradation.* Incubations were performed at 30 °C for 10 min in the cacodylate/NaCl/$MgCl_2$ buffer pH 7.4, containing 5 μM GDP-[^{14}C]fucose, 5 mM UMP and 1 mM fucose in a final volume of 100 μl. (A) Transfer of radioactive fucose to the cells (O——O). (B) GDP-fucose integrity (●——●), fucose 1-phosphate (▲——▲) and appearance of free fucose (O——O)

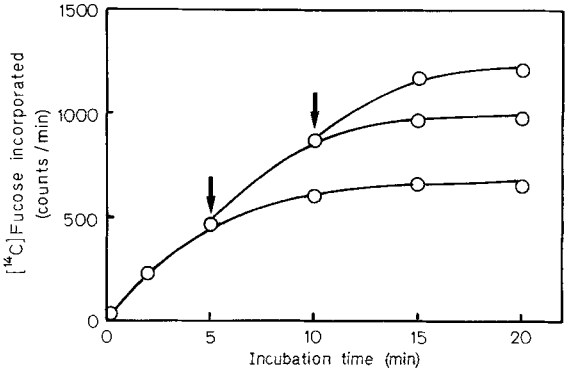

Fig. 3. *Kinetic studies of the transfer of [^{14}C]fucose to the cells from GDP-[^{14}C]fucose.* Incubations were performed in the standard conditions at 30 °C with a final concentration of 3×10^8 cells/ml in the cacodylate/NaCl/$MgCl_2$ buffer pH 7.4 with 5 mM UMP and 1 mM fucose, in a final volume of 100 μl containing 0.5 nM of GDP-[^{14}C]fucose. Arrows indicate new additions of 0.5 nM of GDP-[^{14}C]Fuc after 5 and 10 min

RESULTS AND DISCUSSION

Determination of the Conditions for Measurement of Ectofucosyltransferase Activities

In our previous studies on ectoglycosyltransferases [8–10], we pointed out that precursor degradation by plasma membrane nucleotide-pyrophosphatase(s) and phosphatase(s) leads to the formation of labelled free sugar which may enter the cells and contribute to a non-enzymatic label of these cells. To measure a real enzymatic glycosylation of the plasma membrane acceptors from nucleotide sugars, the formation and entry of free radioactive sugar have to be reduced to a negligible level. For incubation with whole cells this can be achieved by addition of nucleoside monophosphates [15], phloridzin and an excess of unlabelled sugar. In the case of fucosyltransferase, we determined that the UMP concentration had to be raised to 5 mM to inhibit the degradation of GDP-fucose without affecting the transfer activity. Furthermore, the addition of UMP lowered the amount of free fucose generated during the incubation to less than 10 % of the input radioactivity in standard conditions. In addition, a 1000-fold excess of unlabelled fucose (1 mM) was sufficient, without any need for phloridzin, to reduce the entry of labelled fucose into the cells to less than 0.1 % of the extracellular radioactive fucose.

Taking these conditions into account, we studied the optimal cell concentration for the measurement of the ectofucosyltransferase activity with suspensions of intact lymphocytes. Fig. 2 shows that, up to 3.10^8 cells/ml, the radioactivity bound to the cells was proportional to the number of cells i.e. the enzyme concentration. As, in this concentration range, no significant amount of free radioactive fucose was formed during the 10-min reaction, the GDP-[^{14}C]fucose was the only donor of fucose indicating a fucosyltransferase activity exhibited by whole cells. At cell concentrations higher than 3.10^8 cells/ml, the transfer reaction was still observed but the degradation of GDP-[^{14}C]fucose led to a decrease of the slope. In the further assays, cell concentration was adjusted to 3.10^8 cells/ml.

Kinetic Studies of the Ectofucosyltransferase Reaction

Fig. 1 A shows the time dependence of the ectofucosyltransferase activity. The transfer reaction reached a plateau after 5 min of incubation although the precursor integrity was preserved far over this time (Fig. 1 B). This result could be explained if either the enzyme was no longer active or if there were no more membranous acceptors available after this reaction period. However, experiments described in Fig. 3 indicate that when further precursor was added after 5 min, the reaction started again with the same initial velocity to reach a plateau which could be again overcome by a new addition of precursor. Consequently, neither the inactivation of the enzyme nor the lack of acceptors can explain the plateau of the reaction after 5 min of incubation. The kinetics of the reaction rather indicate that an inhibitor was produced during the reaction and that it could be displaced by the substrate. Fig. 4 shows that GDP, a product of the transfer reaction, exhibited an inhibitory effect and is 1000-times more effective than GMP. This fact is in a good agreement with studies of Bella and Kim [16] who proved that GDP is a competitive inhibitor of solu-

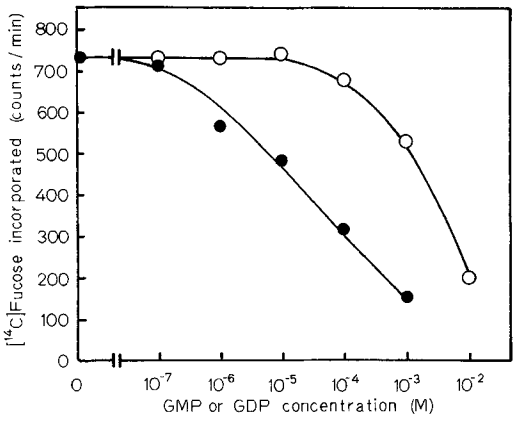

Fig. 4. *Effect of various concentrations of GDP or GMP on the transfer activity.* Incubations were performed in the standard conditions at 30 °C for 10 min with a final concentration of cells of 3×10^8 cells/ml in a final volume of 100 µl in the cacodylate/NaCl/$MgCl_2$ buffer pH 7.4 with 10 µM GDP-[^{14}C]fucose, 5 mM UMP and 1 mM fucose. A logarithmic scale is used for the GDP (●——●) and GMP (○——○) concentrations

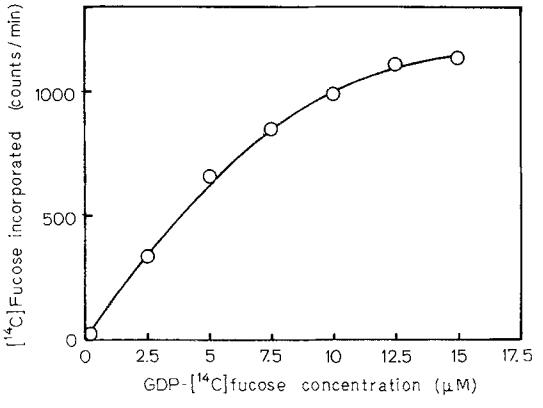

Fig. 5. *Effect of the GDP-[^{14}C]fucose concentration on the transfer of [^{14}C]fucose to the cells.* Incubations were performed at 30 °C for 10 min with 3×10^7 cells in a final volume of 100 µl, in the cacodylate/NaCl/$MgCl_2$ buffer pH 7.4 containing 5 mM UMP, 1 mM fucose and increasing concentrations of GDP-[^{14}C]fucose

bilized intestinal fucosyltransferase. Consequently, the plateau we obtained in kinetic studies of ecto-fucosyltransferase could be explained by a retro-inhibition by the GDP produced during the reaction.

Maximal incorporation (around 1000 counts $\times$ min^{-1}) of fucosyl residues in the membranous acceptors could be obtained for an input of 1.5 nM of GDP-[^{14}C]fucose per 3×10^7 cells (Fig. 5). These conditions led to an estimate of 100000 newly bound fucosyl residues per cell. These fucosyl residues were bound to glycolipids and glycoproteins in an average proportion of 40% and 60% respectively as measured after chloroform/methanol extraction of the total acid-precipitable material.

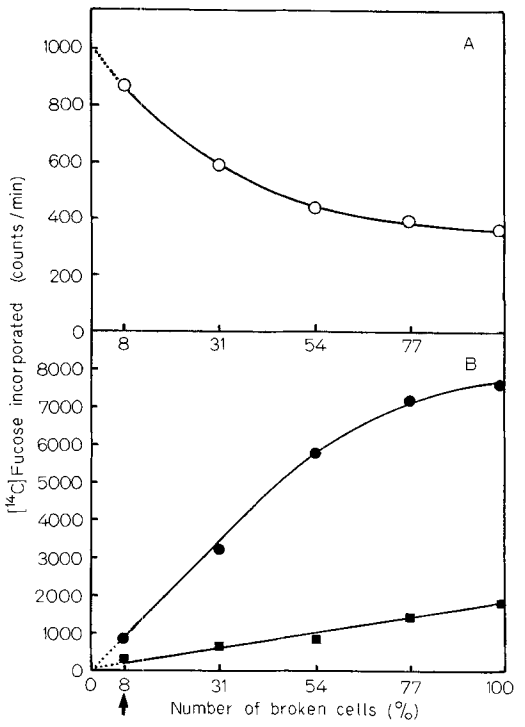

Fig. 6. *Fucosyltransferase activity as a function of the percentage of broken cells present in an incubation.* Incubations were performed at 30 °C for 10 min in the cacodylate/NaCl/$MgCl_2$ buffer pH 7.4 containing 10 µM GDP-[^{14}C]fucose, 5 mM UMP and 1 mM fucose. Mixtures of known proportion of intact and broken cells were added to the incubation medium. In the experiment 92% of the stock suspension of cells excluded trypan blue (arrow). (A) Incorporation of radioactive fucose on endogenous acceptors (○——○). (B) Incoporation of radioactive fucose on asialofetuin (■——■) and asialo-agalactofetuin (●——●). Each assay contained 1 mg of exogenous macromolecular acceptor

Evidence for Ectofucosyltransferase Activities

By avoiding errors due to precursor hydrolysis, the above experiments define the optimal conditions for measuring fucosyltransferase activities in incubations with whole cells. However, the assignment of the fucosyltransferase activity to ectoenzyme is complicated by the presence of a certain percentage of broken cells. To determine whether the measured activities were due to broken cells, mixtures containing definite proportions of intact and broken cells were incubated with GDP-[^{14}C]fucose as recommended by Struck and Lennarz [17]. Fig. 6A shows that the radioactivity bound to endogenous acceptors decreased while the proportion of broken cells increased. This phenomenon cannot be accounted for by an increased degradation of precursor by broken cells as was revealed by the GDP-fucose integrity checked after incubation. This reduced incorporation of fucosyl residues by broken cells is under investigation and appears, up to now, to be a similar phenomenon to the one we observed in the case of ectosialyltransferase [18]. Whatever the case may be, it is important that

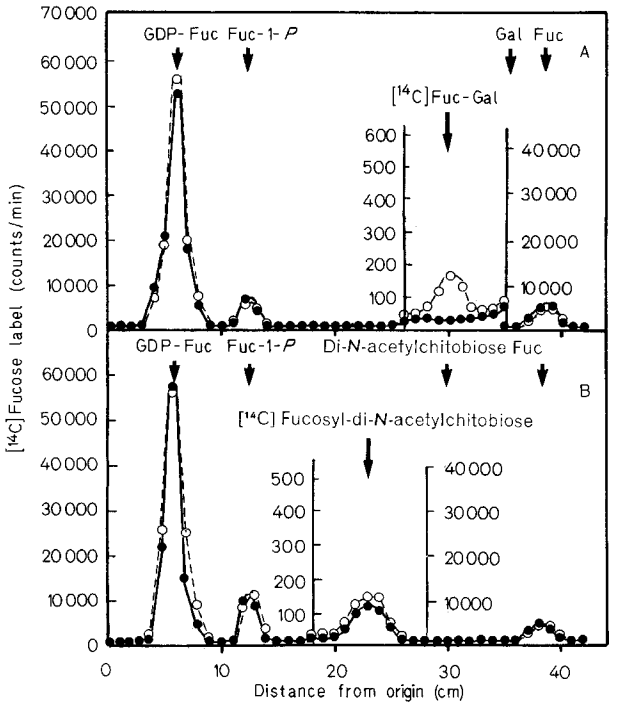

Fig. 7. *Chromatography of the products of a transfer reaction of radioactive fucose from GDP-[^{14}C]fucose to low molecular weight acceptors.* Whole cells incubations were performed at 30 °C for 10 min in the cacodylate/NaCl/$MgCl_2$ buffer pH 7.4 containing 10 μM GDP-[^{14}C]fucose and 5 mM UMP. After incubation, cells and acceptors were separated by low-speed centrifugation and the supernatant was submitted to paper chromatography in the following solvent: pyridine/ethyl acetate/acetic acid/ water (5/5/1/3). (A) 1 mg of galactose was added to the incubation medium without (O– O) or with 5 mM *N*-ethylmaleimide (●——●). (B) 1 mg of di-*N*-acetylchitobiose was added to the incubation medium without (O– O) or with 5 mM *N*-ethylmaleimide (●——●). Gal, galactose; Fuc, fucose; Fuc-1-*P*, fucose 1-phosphate; Fuc-Gal, fucosyl galactose

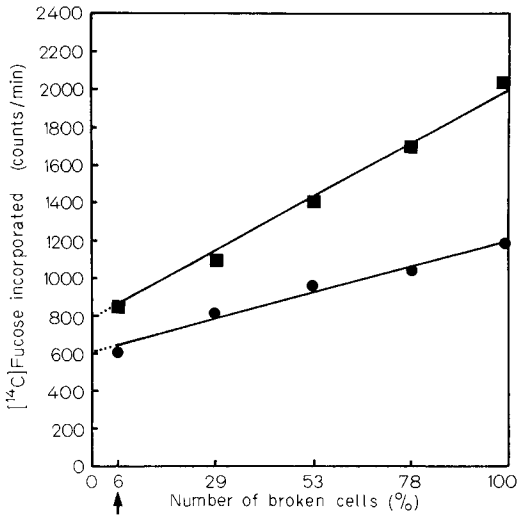

Fig. 8. *Fucosyltransferase activity measured with low-molecular-weight acceptors as a function of the percentage of broken cells present in an incubation.* Incubations were performed at 30 °C for 10 min in the cacodylate/NaCl/$MgCl_2$ buffer pH 7.4 containing 10 μM GDP-[^{14}C]fucose and 5 mM UMP. Mixtures of known proportions of intact and broken cells were added to the incubation medium. In this experiment 94% of the stock suspension of cells excluded trypan blue (arrow). Each assay contained 1 mg of galactose (■– ■) or di-*N*-acetylchitobiose (● – ●)

extrapolation to 100% intact cells reveals a significant ectofucosyltransferase activity.

Study of the Fucosylation of Macromolecular Acceptors

To distinguish between GDP-fucose : galactoside fucosyltransferase and GDP-fucose : *N*-acetylglucosaminide fucosyltransferase activities, the use of exogenous acceptors is required. Asialofetuin and asialoagalactofetuin are useful to demonstrate these two activities as were shown by Jabbal and Schachter [19]. These two macromolecules are good acceptors for intracellular enzymes as shown by the transfer of [^{14}C]fucosyl residues catalyzed by cell homogenate (Fig. 6 B; 100% broken cells). However, extrapolation to zero percent broken cells indicates no detectable ectofucosyltransferase activities measured with these macromolecular acceptors. As has been postulated for ectosialyltransferase [10], ectofucosyltransferase could be rather buried in the membrane and its accessibility to exogenous acceptors restricted to those of low molecular weight.

Fucosylation of Low-Molecular-Weight Acceptors to Reveal Two Ectofucosyltransferase Activities

A comparative study of the incorporation of [^{14}C]-fucosyl residues in low-molecular-weight acceptors required a simple separation procedure to avoid any loss of material. Galactose and di-*N*-acetylchitobiose were chosen as acceptors since their fucosylated derivatives separate well from precursor degradation products in the one-dimensional paper chromatography system described above (see Fig. 7). By this procedure, such a comparative study of the transfer activity in incubation mixtures containing various proportions of intact and broken cells (Fig. 8) indicates that whole cells (extrapolation to zero percent broken cells) exhibit ectofucosyltransferase activities toward low molecular weight acceptors. In addition, this data shows that lymphocytes possess both fucosyltransferase activities on their outer surface: galactoside fucosyltransferase and *N*-acetylglucosaminide fucosyltransferase activities. It has been recently shown, in the case of plasma fucosyltransferases [12] that inhibition by *N*-ethylmaleimide could be used to distinguish between these two activities since the galactoside fucosyltransferase is the only one affected by *N*-ethylmaleimide. Preliminary studies indicate that, in our case, optimal inhibition was obtained in the presence of 5 mM *N*-ethylmaleimide in the incubation medium. Fig. 7 shows the

chromatographic pattern of [^{14}C]fucose-labelled products obtained after whole cell incubation with GDP-[^{14}C]fucose in standard conditions with either galactose (Fig. 7A) or di-*N*-acetylchitobiose (Fig. 7B) as acceptors and measured with or without 5 mM *N*-ethylmaleimide. It reveals that *N*-ethylmaleimide completely abolishes the transfer of fucosyl residues to galactose but it only partially affects the transfer on *N*-acetylglucosaminyl residues (20% inhibition). These results confirm for ectofucosyltransferases the observations of Chou et al. [12] with plasma fucosyltransferases, and, moreover, enable us to conclude that the rat spleen lymphocytes possess the two fucosyltransferase activities on their outer surface.

CONCLUSION

Taking into account the misleading effects of precursor hydrolysis, of free sugar entry into the cell and of contamination by broken cells, our results clearly prove that ectofucosyltransferase activities are present on intact lymphocytes. These ectofucosyltransferases are able to transfer fucose from GDP-fucose to endogenous membranous glycolipid and glycoprotein acceptors to the extent of 100000 fucosyl residues per cell as estimated by the specific radioactivity of [^{14}C]fucose. This number is in the range that has been observed for ectogalactosyltransferase [9] and for ectosialyltransferase [18]. However, these ectofucosyltransferases are not able to transfer fucosyl residues to macromolecular exogenous acceptors although the lymphocyte possesses intracellular enzymes which are capable of doing so. This property may indicate that enzymes of the cell surface and of the intracellular membranes are different, but it may also be the case that the same enzyme is in different environments on cytomembranes and on plasma membranes. This peculiar property has already been observed for ectosialyltransferase [10] but not for ectogalactosyltransferase [8].

As the occurrence of two fucosyltransferases in microsomal membranes has been well defined [4, 19], it was worthwhile examining whether these two activities could be recovered on the plasma membrane. As the use of different macromolecular acceptors was not possible in our case, our investigation was restricted to low molecular weight acceptors. We chose galactose and di-*N*-acetylchitobiose to demonstrate the GDP-fucose:galactoside fucosyltransferase and GPD-fucose : *N*-acetylglucosaminide fucosyltransferase activities respectively. To distinguish these two activities we used the selective inhibition of the galactoside fucosyltransferase observed with *N*-ethylmaleimide. The results prove the presence of the two ectofucosyltransferase activities on the outer surface of rat lymphocytes.

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