HUMAN BLOOD GROUP B GENE-SPECIFIED α-3-GALACTOSYLTRANSFERASE : PURIFICATION OF THE ENZYME IN SERUM BY BIOSPECIFIC ADSORPTION ONTO BLOOD GROUP O ERYTHROCYTE MEMBRANES

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Received June 14,1977

SUMMARY: The α-3-galactosyltransferase present only in the serum of group B and AB individuals binds to human erythrocytes in the presence of UDP-D-galactose and Mn^{2+} ions. The degree of binding corresponds to the relative numbers of H-active sites on the erythrocytes, that is, group $O > B > A_1 \geq A_1B$. Bombay O_h erythrocytes which lack H serological activity do not bind the transferase. By means of this affinity binding the α-3-galactosyltransferase may be purified over 100,000 fold by a one-step procedure involving adsorption onto group O erythrocyte membranes followed by elution with the low molecular weight H-active trisaccharide 2'-fucosyllactose. The purified enzyme transfers D-galactose in α-linkage to oligosaccharides, glycolipids and glycoproteins with terminal non-reducing H-active structures and confers blood group B activity on group O erythrocytes.

INTRODUCTION. The UDP-galactose : O-α-L-fucosyl(1→2)-D-galactose α-3-D-galactosyltransferase (EC 2.4.1.37) specified by the human blood group B gene converts group O erythrocytes into B-active cells by the transfer of galactose in α(1→3) linkage to H-active structures on the erythrocyte surface (1,2). Binding of this enzyme in serum to H sites on red cell membranes, followed by elution with a low molecular weight compound containing the H determinant structure was therefore investigated as a specific affinity method for the isolation of the B gene-specified transferase. The purification procedure and some properties of the enzyme isolated from group B serum are described in this paper.

MATERIALS AND METHODS. UDP-D-[^{14}C]galactose (238 Ci/mol), UDP-N-acetyl-D-[^{14}C] galactosamine (61 Ci/mol), GDP-L-[^{14}C]fucose (195 Ci/mol) and CMP [^{14}C]sialic acid (195 Ci/mol) were purchased from the Radiochemical Centre (Amersham, U.K.). Unlabelled UDP-D-galactose was obtained from Sigma (Lond. Ltd., U.K.). 2'-Fucosyllactose and lacto-N-fucopentaose I were isolated from human milk by the method of Kuhn et al (3,4). 3-Fucosyllactose was a gift from Dr. V. Ginsburg; other milk oligosaccharides were kindly supplied by Dr. A. Gauhe, O-α-L-fucosyl(1→6) N-acetyl-D-glucosamine was a gift of Dr. H.M. Flowers. H-active glycoprotein was isolated from an ovarian cyst fluid as described (5) and H-active macroglycolipid isolated from red cells (6) was a gift from Dr. A. Gardas. Neuraminidase-treated bovine cervical mucin (7) was a gift from Dr. R.A. Gibbons.

ISSN 0006-291X

Enzyme source. Serum from clotted blood samples was separated at 4°C and stored frozen at -18°C. Immediately before use the serum was thawed out and centrifuged at 4°C for 30 min. at 30,000g.

Red cell membranes. Group O red cell stroma were prepared by the method of Dodge et al (8) and freeze dried. To prepare the stroma as an adsorbent for the B transferase 170 mg were suspended in 10 ml of distilled water, centrifuged at 30,000g, washed three times with 0.1M sodium cacodylate buffer pH 6.5 containing 0.1% 2-mercaptoethanol (buffer A) and left suspended in this buffer overnight. The membranes were washed a further three times with buffer A and then treated for the selective solubilisation of proteins as described by Steck and Yu (9). The membranes (1.2g wet weight), were suspended finely in 6 ml of distilled water, mixed with 60 ml of ice-cold 0.1M sodium hydroxide and centrifuged immediately at 4°C for 20 min. at 30,000g. The resulting "stripped" membranes were washed six times in buffer A and stored at 4°C until required. The treated membranes specifically absorbed H antibodies from immune rabbit anti-H serum (10); demonstrating that they retained their H-active sites after the treatment with sodium hydroxide.

Transferase assays. The blood group B and A gene associated transferases were assayed by transfer of labelled sugars from UDP-[^{14}C]galactose or UDP-N-acetyl [^{14}C]galactosamine, respectively, to the appropriate acceptors. Details of the reaction mixtures are given in the tables. When macromolecular acceptors were used the reaction mixtures were subjected to paper electrophoresis in 0.1M ammonium formate buffer pH 3.7 and the neutral compounds were eluted and chromatographed on Whatman No. 40 paper for 20 hours in ethyl acetate-pyridine-water (2:1:2 by vol., upper phase, Solvent a). The radioactive peaks were detected with a 7201 Packard Radiochromatogram Scanner and counted in a Nuclear Chicago Scintillation Counter, Mark II. With low molecular weight acceptors the incubation mixtures were first applied to ion exchange papers (11) and the eluted material was then chromatographed in Solvent a as described above. The B transferase was also assayed by the conversion of normal group O cells to B-active cells by the transfer of D-galactose from unlabelled UDP-galactose as described (2).

β-Galactosyltransferase activity was assayed by the transfer of [^{14}C]-galactose from UDP-[^{14}C]galactose to N-acetylglucosamine (12) and the peptidyl : N-acetylgalactosaminyltransferase activity by the transfer of N-acetyl[^{14}C] galactosamine from UDP-N-acetyl[^{14}C]galactosamine to a glycopeptide prepared from human blood group substance (13). The anomeric linkages of the transferred N-acetyl[^{14}C]galactosamine and [^{14}C]galactose were determined by means of purified glycosidases as described previously (11,12). H-gene specified α-2-fucosyltransferase activity was assayed by the transfer of [^{14}C]fucose to phenyl β-D-galactoside (14). Sialyltransferase activity was measured by the transfer of [^{14}C]sialic acid from CMP-[^{14}C]sialic acid to neuraminidase-treated bovine cervical mucin essentially by the method of Bartholomew et al (15).

Protein analysis. Protein was estimated by the method of Bradford (16) with bovine serum albumin as a standard. Disc gel electrophoresis was carried out by the method of Clarke (17).

RESULTS AND DISCUSSION. Preliminary experiments demonstrated that, with the addition of the appropriate co-factors, the α-3-galactosyltransferase in serum from group B persons was adsorbed onto human erythrocytes and that the degree of binding was proportional to the relative number of H active sites on the

Table 1. Co-factor requirements for adsorption of α-3-galactosyltransferase to group O erythrocyte membranes

	Transferase bound to membranes (% of total)
Complete system	48.4
Minus UDP-galactose	0
Minus $MnCl_2$	15.8

The complete system for binding the transferase contained : group B serum, 1 ml; UDP-galactose 1.5 mM, 25 µl; $MnCl_2$, 0.4M, 50 µl; sodium cacodylate buffer 2M, pH 6.5, 50 µl; "Stripped" erythrocyte membranes (300 mg wet weight per ml), 10 µl. The reactants were mixed, the tubes left to stand at 4°C for 15 minutes and then centrifuged at 16,000g for 10 minutes. The supernatant (50 µl) was assayed for α-galactosyltransferase by the addition of : UDP-[^{14}C]-galactose, 0.27 nmol (128,000 c.p.m.); 2'-fucosyllactose 0.25 µmol; $MnCl_2$, 2 µmol; buffer A, 5 µmol, NaN_3, 0.8 µmol, ATP, 0.5 µmol. The mixtures (total volume 100 µl) were incubated 14 hours at 37°C. Serum controls were treated with all the components of the binding system except the erythrocyte membranes. The estimate of the α-galactosyltransferase bound to the membranes was taken as the difference between the activities of the control and adsorbed serum samples.

cells (18), that is, the order of binding was group $O > B > A_1 \geq A_1B$. Cells from persons of the Bombay O_h phenotype, which do not react with anti-H reagents (19), failed to bind the α-galactosyltransferase. These results therefore strongly indicated that the transferase was specifically combining with the H acceptor substrate on the red cell membranes. In order to exploit this property for the purification of the transferase, group O red cell membranes (8) treated for the selective removal of proteins (9) were used as the adsorbent. The co-factors required for binding the transferase are shown in Table 1. The transferase was not adsorbed unless UDP-galactose was added to the serum and binding was greatly enhanced by the addition of Mn^{2+} ions. Binding could be demonstrated by assaying the red cell membranes directly for α-galactosyltransferase activity or by testing the residual serum for loss of activity; the latter method was considered to give a more accurate estimate of the amount of enzyme adsorbed. In order to retain maximal transferase

Table 2. Purification of the B gene specified α-3-galactosyltransferase

Enzyme source	Total volume (ml)	Total units*	Total protein (μg)	Specific activity (Units/mg protein)	Purification (fold)	Percentage of total enzyme activity
Untreated serum	400	31,500	27×10^6	1.2	-	100
Enzyme bound to membranes	-	16,006	-	-	-	51
1st eluate from membranes	4.0	12,256	40	306,400	255,000	39
2nd eluate from membranes	4.0	3,672	32	114,750	96,000	12

* One unit is defined as the amount of enzyme that incorporates 1 pmol of galactose into acceptor per hour.

To bind the transferase 450 ml of solution containing 400 ml of group B serum, 10 ml of 1.5 mM UDP-galactose, 20 ml of 0.4M $MnCl_2$ and 20 ml 2M sodium cacodylate buffer pH 6.5 were added to 1.2g (wet weight) of "stripped" erythrocyte membranes suspended in 3 ml of buffer A. The mixture was left 15 minutes at 4°C and then centrifuged at 30,000g for 20 minutes. The pellets were pooled and washed 5 times in buffer A made 34 μM with respect to UDP-galactose and 18 mM with respect to $MnCl_2$. The 5th wash was completely free of α-galactosyltransferase activity. To elute the transferase 400 μl 0.05M 2'-fucosyllactose, 200 μl 0.4M $MnCl_2$, 200 μl 1.5 mM UDP-galactose and 3.2 ml buffer A were added to the erythrocyte membranes. The contents of the tube were mixed, left 2 hours at 4°C, and then centrifuged at 4°C for 20 minutes at 30,000g. The supernatant was removed and the sedimented membranes were eluted a second time as described above. The two supernatants were each centrifuged once more at 30,000g for 30 minutes to ensure complete removal of membrane fragments and then dialysed against buffer A for 24 hours. Finally the dialysed enzyme solution was centrifuged for 1 hour at 100,000g.

The reaction mixtures for the enzyme assays contained in a total volume of 100 μl : UDP-[^{14}C]galactose, 0.27 nmol (128,000 c.p.m.); 2'-fucosyllactose 0.25 μmol, buffer A, 5 μmol; $MnCl_2$, 2.0 μmol; NaN_3 0.8 μmol; enzyme solution 10 μl. The mixture with untreated serum also contained 0.5 μmol ATP. Bovine serum albumin (1%, 10 μl) was added to the tubes containing the eluted enzymes. The mixtures were incubated at 37°C (12 hours for the untreated serum and 2 hours for the eluted enzymes) and assayed as described for low molecular weight acceptors.

activity, UDP-galactose and Mn^{2+} ions were required to be present in the buffer used for washing the membranes after adsorption of the enzyme.

Attempts to elute the B transferase from the membranes showed, in small scale experiments, that either of the low molecular weight H-active milk oligosaccharides, 2'-fucosyllactose (3) or lacto-N-fucopentaose I (4) would elute the enzyme but 2'-fucosyllactose was more effective on a molar basis. This trisaccharide was therefore used in a larger scale experiment in which the starting material was 400 ml of serum from a group B donor (Table 2). In this experiment about half the α-3-galactosyltransferase in the serum was bound to the membranes, and, on elution, recovery of enzyme activity was almost quantitative. In similar experiments the overall purification has varied from 50,000 to 250,000-fold. The eluted transferase is stable to dialysis against buffer A but rapidly loses activity under the assay conditions at 37°C unless bovine serum albumin is added to the incubation mixtures. The purified enzyme transferred [^{14}C]galactose from UDP[^{14}C]galactose to H-active glycoprotein, H-active glycolipid and to the low molecular weight acceptors, O-α-L-fucosyl(1→2)galactose, 2'-fucosyllactose, and lacto-N-fucopentaose I. The apparent Km values for the last three substrates are given in Table 3. Treatment with α- and β-galactosidases confirmed that in each of the acceptor substrates the transferred [^{14}C]galactose was in α-anomeric linkage. Lacto-N-fucopentaose II, lacto-difucohexaoses I and II, 3-fucosyllactose and O-α-L-fucosyl(1→6)N-acetylglucosamine were not acceptors for the purified transferase. Incubation of the enzyme (100 µl) with H-active glycoprotein (500 µg), unlabelled UDP-galactose (200 µg), and $MnCl_2$ (40 µmol) converted the H into a B-active substance as demonstrated by inhibition of haemagglutination of B cells by human anti-B serum. Such a change could not be demonstrated when the untreated group B serum was tested as the transferase source under similar conditions although both the original serum and the eluted transferase readily conferred B activity on group O red cells when the enzymes were incubated with the cells and the appropriate additives.

Table 3. Apparent Km values for substrates, and Ki values for inhibitors, of the purified α-3-galactosyltransferase

Substrate	Km (M)	Inhibitor	Ki* (M)
α-Fuc(1→2)Gal	2.2×10^{-3}	UDP	2.3×10^{-6}
2'-Fucosyllactose	0.5×10^{-3}	UDP-N-acetyl-galactosamine	102×10^{-6}
Lacto-N-fucopentaose I	2.5×10^{-3}		
UDP-galactose	10×10^{-6}		

* With respect to UDP-galactose.

Reaction mixtures were the same as for the purified enzyme in Table 2 except that for the Km determinations α-Fuc(1→2)Gal, 2'-fucosyllactose and lacto-N-fucopentaose I were assayed over the range 5-500 nmol and UDP-galactose over the range 0.27-100 nmol. For the Ki determinations a series of concentrations of UDP or UDP-N-acetylgalactosamine (0.2-50 nmol) were added to either 0.27 nmol or 2.7 nmol of UDP-galactose. Apparent Km values were determined from Lineweaver-Burk plots (22) and apparent Ki values from Dixon plots (23).

The serum samples from which the B transferase was isolated all contained a β-galactosyltransferase which used N-acetylglucosamine as an acceptor (12); this activity could not be detected either bound to the red cell membranes or in the eluted enzyme preparations. Similarly tests on the purified enzyme preparations were negative for the peptidyl α-N-acetylgalactosaminyl-, α-2-fucosyl-, and sialyltransferase activities which were also present in the starting material. When serum from a group A donor was treated with group O erythrocyte membranes, under the same conditions as the group B serum, some A transferase activity was bound to the membranes and could be eluted with 2'-fucosyllactose. The amount of adsorbed enzyme was about 10% of the total amount of B transferase bound to an equivalent volume of erythrocyte membranes. The adsorption of the A transferase is probably explained by the fact that this enzyme also requires H-active sites as an acceptor substrate and that it combines with UDP-galactose although this nucleotide sugar is not a donor substrate

for the transferase (11).

The purified B transferase has a pH optimum of 6.5. In contrast to the enzyme in crude serum, which is stable to heat for 20 minutes at 55°, the purified transferase loses 20% of its activity after 5 minutes at 55° and 60% after 20 minutes at this temperature. The enzyme has a requirement for divalent metal ions, Mn^{2+} being the most effective (optimum concentration 20 mM). Slight activation was observed in the presence of Co^{2+} ions, but Mg^{2+} and Zn^{2+} gave no activation over the range 5-50 mM. UDP is a strong competitive inhibitor with respect to UDP-galactose and UDP-N-acetylgalactosamine is a weak competitive inhibitor (Table 3).

A total of 72 µg of protein was eluted from the erythrocyte membranes that had adsorbed about 50% of the B transferase present in 400 ml of serum (Table 2). Exposure of an equivalent amount of erythrocyte membranes to the same volume of serum from a group O person, followed by elution with 2'-fucosyllactose, yielded approximately the same amount of protein in the eluate. Whilst it cannot be excluded that the group O serum contains a protein product of the O gene that, although lacking transferase activity, binds to H receptors in the presence of UDP-galactose, the result with O serum casts doubts on how much of the protein in the eluate containing α-3-galactosyltransferase activity is in fact enzyme protein. Disc electrophoresis (100 µl samples) failed to show any bands which stained with naphthalene black and the amino acid analyses of the proteins eluted from erythrocyte membranes exposed to either group B or O sera were very similar. The precise origin of all the blood group A and B gene-specified transferase activities in serum is not known; transferase assays on haemopoietic twin chimaeras suggest that only 20-30% is derived from the blood forming tissues (20,21) and the source of the remainder is not established. Further large scale isolation experiments are needed to determine the quantity of B gene-specified transferase protein in serum but the results presented in this paper emphasise the fact that the total amount is probably extremely small.

ACKNOWLEDGEMENTS. We are grateful to Dr. A.S.R. Donald for carrying out the amino acid analyses. L.R.C. thanks the Governing Body of the Lister Institute of Preventive Medicine, London, U.K. for the award of a studentship (1973-76).

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