Blood-group-related carbohydrate antigens are expressed on human milk galactosyltransferase and are immunogenic in rabbits

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Immunochemical evidence is presented for the presence of blood-group-related carbohydrate structures on human milk galactosyltransferase and for the occurrence of the corresponding specificities among rabbit antibodies to this enzyme. Although these carbohydrate specificities constitute minor populations among antisera and affinity-purified antibodies to galactosyltransferase, their presence is important in the immunohistochemical approach to enzyme localization, since they give rise to strong reactivities with epithelial cells of the gastrointestinal tract.

INTRODUCTION

Galactosyltransferase of human milk is a sialoglycoprotein of M_r approx. 55000 that, on isoelectric focusing, resolves into multiple bands (Gerber *et al.*, 1979). Although the pI of the bands is in part sialic acid-dependent, the heterogeneity is only slightly decreased by neuraminidase treatment. The structural basis of the isoforms remains to be elucidated. One possibility addressed in the present study is that they represent glycosylation variants of the enzyme glycoprotein. Monoclonal antibodies are available that recognize peripheral and backbone structures of glycoprotein oligosaccharides of *N*- or *O*-glycosidic type (Feizi *et al.*, 1984) and they can be applied with ease for deriving structural information on glycoprotein oligosaccharides (Childs *et al.*, 1978; Childs & Feizi, 1981; Childs *et al.*, 1984). In the present study we have carried out immunoblotting of human milk galactosyltransferase after isoelectric focusing. We detected several blood-group-related antigens on the enzyme glycoprotein, but no major differences in their expressions on the different isoforms. Solid-phase radiobinding and inhibition of binding assays using reference blood-group substances and structurally defined oligosaccharides revealed that rabbit antisera to this enzyme contain variable amounts of antibodies directed at the blood-group-related carbohydrate antigens. Immunofluorescence microscopy with human gastrointestinal tissues revealed that these blood-group-related specificities give rise to strong immunofluorescence of epithelial cells.

MATERIALS AND METHODS

Purification of galactosyltransferase and immunization of rabbits

Pooled human milk, or milk from individual donors, was stored until use at -20°C . Galactosyltransferase was isolated as described previously (Gerber *et al.*, 1979)

and stored at -70°C . Portions were passed over an insolubilized-protein A column to remove trace contaminants of IgG (Wilson *et al.*, 1982) and used to immunize rabbits with five to six subcutaneous injections of 200 μg of enzyme per injection, of which the first preparation was emulsified in complete Freund's adjuvant. Injections were fortnightly and the immune response was monitored by enzyme-linked immunoadsorbent assay (Berger *et al.*, 1981).

Antisera

Three rabbit antisera against purified galactosyltransferase were studied. One of these, designated X, was raised against the enzyme from a blood-group-O individual (secretor status unknown); antisera P and Z were against enzyme from milk pools from individuals of unknown blood groups. Affinity-purified antibodies were available from sera X and P and had been isolated by using immunoadsorbents of galactosyltransferase from pooled milk as described by Berger *et al.* (1981).

Monoclonal anti-carbohydrate antibodies

The monoclonal antibodies were those used in previous studies (Childs *et al.*, 1983, 1984) unless otherwise stated. These consisted of human autoantibodies or mouse hybridoma-derived antibodies to epitopes on accessible backbone structures: IgM^{WOO} (human) anti-Type 1 backbone; anti-*i* Den, anti-I Ma and anti-I Step (human) anti-Type 2 backbones; M18 (mouse) anti-Type 2 backbones; mouse hybridoma-derived antibodies to antigens on fuco-oligosaccharide sequences: CF4 and 115C2 anti-(blood-group-Lewis^a)-related (Gooi *et al.*, 1983a), 64/5B93 anti-(blood-group Lewis^b) (Messeter *et al.*, 1984); H11 anti-(Type 2 blood group H) (Knowles *et al.*, 1982); HF33 anti-(Types 1 and 2 blood-group H) (J. Picard & T. Feizi, unpublished work); anti-(SSEA-1) anti-(α 1–3-fucosylated Type 2 chain); C14 anti-(2',3-difucosyl Type 2 chain); G49 anti-(blood-group ALe^{b/y}). The human antibodies were used as sera (1:300

Abbreviations used: affi-P and affi-X, affinity-purified antibodies from serum P and serum X respectively; LNDF I, lacto-*N*-difucohexaose I; LNF II, lacto-*N*-fucopentaose II; LNF III, lacto-*N*-fucopentaose III; 3FL, 3-fucosyl-*N*-acetyl-lactosamine; PBS/BSA, 20 mM-sodium phosphate-buffered saline, pH 7.2, containing 1 mg of bovine serum albumin/ml.

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Fig. 1. Immunoblotting after isoelectric focusing of galactosyltransferase from a presumed blood-group-A donor (panel A) and a blood-group-O non-secretor (panel O) using monoclonal anti-carbohydrate antibodies

Lanes R are reference lanes showing immunostaining of the two preparations with anti-galactosyltransferase serum, and lanes ALe^{b/y}, Le^a, SS and H show autoradiography using antibodies G49, CF4, anti-SSEA-1 and H11 respectively. H11 antibody in panel A and G49 antibody in panel O gave no reactions with the group-A and -O enzymes respectively. The six isoforms of the enzyme glycoprotein are designated 1–6; additional bands showing Le^a reactivity are designated 2', 3' and 6'.

group-A secretor, difucosylated blood group A (ALe^b or *y*):

recognized by monoclonal antibody G49, was detected in addition to Le^a and SSEA-1. All six isoforms of the enzyme separated by isoelectric focusing reacted with these antibodies. The blood-group-A-active enzyme contained two additional bands (designated 2' and 3'), reacting with CF4 antibody and weakly with G49. By contrast, the 2' and 3' bands were absent in the blood-group-O non-secretor enzyme, which showed an additional weak band, 6', revealed by antibody CF4. The expression of Le^a on galactosyltransferase was confirmed by a solid-phase radiobinding assay using the second monoclonal antibody, 115C2, with Le^a-related specificity (results not shown). No immunostaining was observed with the other monoclonal antibodies used and no reactivities were revealed with the anti-I and anti-i antibodies and IgM^{wo} after neuraminidase treatment of the enzyme glycoprotein.

Blood-group-related specificities occur in rabbit antisera to galactosyltransferase

In view of the finding of blood-group-related antigen activities on galactosyltransferase, a search was made for the corresponding specificities in rabbit anti-galactosyltransferase sera. Binding assays with the reference blood-group-A-active ovarian-cyst glycoprotein (MSS) and meconium glycoproteins of non-secretor type showed that the galactosyltransferase antisera contained antibodies reactive with antigenic determinants on one or both glycoproteins, although their titres were lower than those obtained using galactosyltransferase as the immobilized antigen (Fig. 2). The reactivities of the

Fig. 2. Binding of rabbit anti-galactosyltransferase sera and affinity-purified antibodies (insets) to galactosyltransferase isolated from a blood-group-O non-secretor (a) and to a blood-group-A-active reference glycoprotein (MSS) (b) and meconium glycoproteins of non-secretor types (c)

Symbols: ▼, antiserum and eluate P; △, pre-immune serum X; ▲, antiserum and eluate X; ◆, antiserum Z; □, ○, normal rabbit sera.

affinity-purified antibodies, affi-P and affi-X, reflected those of the original antisera (Fig. 2, insets); affi-P showed binding with glycoprotein MSS and affi-X with the meconium glycoproteins.

Fig. 3. Inhibition of binding of antiserum Z, antiserum P and affi-P to the blood-group-A-active glycoprotein MSS and of antiserum X and affi-X to meconium glycoproteins of non-secretor type

Abbreviations for inhibitors: Mec(s) and Mec(ns), meconium glycoproteins of secretor and non-secretor type respectively; NI, MSS, Beach, JS, FL, reference blood-group substances as described in the Materials and methods section; Mec(s)^a, meconium glycoproteins subjected to mild acid hydrolysis in order to remove fucose residues. LNDI, LNF II, LNF III and 3FL are fucose-containing oligosaccharides described in the Materials and methods section. The concentrations of the glycoprotein inhibitors are given as $\mu\text{g/ml}$ and, of the oligosaccharides, nmol in a total reaction volume of 110 μl .

Inhibition-of-binding studies (Fig. 3) showed that the reactivity of serum Z with glycoprotein MSS was predominantly blood-group-A-related; only glycoprotein MSS and the meconium glycoproteins of secretor type with blood-group-A and -H activities were inhibitory, whereas the reactivities with antiserum P and affi-P involved specificities towards structures shared by MSS and the several other glycoproteins, particularly Beach, JS and meconium glycoproteins derived from secretors. The inhibitory activity of the meconium glycoproteins of secretor type was abolished (with affi-P) or almost completely abolished (with serum P) after mild acid hydrolysis, under conditions which release most of the fucose residues. This is consistent with specificities related to H and Le^b antigens or with the Le^y antigen structures being predominant in serum P and affi-P.

Inhibition studies with serum X and affi-X (Fig. 3) indicated that binding of meconium glycoproteins is mediated by structures that are most strongly expressed on the two meconium glycoproteins and the ovarian-cyst

glycoprotein, N-1, all of which are rich in Le^a and SSEA-1 antigen structures. This pattern was more clearly seen with affi-X, and here also the antigenic determinant was susceptible to mild acid hydrolysis, indicating the importance of fucose residues for reactivity. The results of the oligosaccharide inhibition assays with affi-X (Fig. 3) confirmed the presence of antibody populations related to Le^a and SSEA-1. There was a preferential reaction with the Type 1-based Le^a structure, since LNDI and LNF II were more active inhibitors than the Type 2-based oligosaccharides LNF III and 3FL.

These antibodies with blood-group-related specificities were minor populations among the antibodies to galactosyltransferase, for the binding of the antisera and affinity-purified antibodies to the immobilized enzyme could not be inhibited by the ovarian-cyst and meconium-derived glycoproteins individually or as a mixture at the highest concentrations tested (50 μg of glycoprotein/well), whereas galactosyltransferase gave complete inhibition at 3 μg /well (results not shown).

Fig. 4. Immunofluorescence of sections of epithelial cells and of cultured HeLa cells with antibodies to galactosyltransferase

Panel A shows immunofluorescence of the surface epithelium of the stomach of a blood-group-A secretor with anti-galactosyltransferase serum Z, and panel B shows the lack of immunofluorescence after absorption with blood-group-A substance, as described in the Materials and methods section. Panel C shows immunofluorescence of the surface epithelium of small intestine from a blood-group-O secretor with affinity-purified antibodies to galactosyltransferase, affi-P, and panel D shows lack of immunofluorescence after absorption with blood-group-H substance. Panels E and F shows similar immunofluorescence of HeLa cells with antiserum Z before and after absorption with blood-group-A substance. Panels B^I, D^I, E^I and F^I are phase-contrast micrographs corresponding to panels B, D, E and F respectively. Magnification $\times 350$ (panels A, B, C and D) and $\times 560$ (panels E and F).

Blood-group-related specificities are involved in surface immunofluorescence of epithelial cells

The blood-group-related specificities, although minor components, were clearly predominant in the reactivities of the anti-galactosyltransferase sera and affinity-purified

antibodies with human gastric and intestinal epithelia derived from individuals of known blood-group and secretor status. Immunohistochemical studies (not described here in detail) have shown that serum Z resembles anti-(blood-group A) serum in reacting with

epithelia from blood-group-A individuals but not with those of blood-group O; serum P and affi-P resemble anti-(blood-group H) serum in reacting more strongly with epithelia from secretors than those from non-secretors, and serum X and affi-X resemble anti-(blood group Le^a) and anti-(SSEA-1) antibodies in reacting more strongly with epithelia from non-secretors than those from secretors. Absorption of the antisera and the affinity-purified antibodies with appropriate blood-group substances abolished this epithelial immunofluorescence. Illustrated in Fig. 4, panel A, is immunofluorescence of the gastric surface epithelium of a blood-group-A secretor using antiserum Z, and in Fig. 4, panel B, the abolition of immunofluorescence after absorption with blood-group-A substance (MSS). Fig. 4, panel C, shows immunofluorescence of small-intestinal epithelium from a blood-group-O secretor with affi-P, and Fig. 4, panel D, shows the loss of reactivity after absorption with blood-group-H substance (JS). In contrast with the findings with epithelial cells, absorption of the antisera and the affinity-purified antibodies with blood-group substances had no perceptible effect on the Golgi-region immunofluorescence of HeLa cells, as shown for serum Z in Fig. 4 (panel E and F). These results indicate that immunofluorescence of the surface epithelial cells, but not that of HeLa cells, is mediated by antibodies with blood-group-related specificities.

DISCUSSION

The conclusions that can be drawn from the present study are, firstly, that the six charge isoforms of human milk galactosyltransferase exhibit blood-group-related carbohydrate determinants to comparable extents. Thus the structural features that determine the differences in isoelectric point of the isoforms are neither related to these epitopes nor to sialic acid, as shown previously (Gerber *et al.*, 1979). Secondly, the carbohydrate antigens on galactosyltransferase are in part influenced by the blood-group and secretor genes, and antibodies with corresponding specificities occur among rabbit antisera to this enzyme. Thirdly, these blood-group specificities give rise to immunofluorescence of surface epithelial cells.

The finding of blood-group-related carbohydrate specificities in antisera and affinity-purified antibodies to galactosyltransferase has important implications, since these carbohydrate antigens occur on a variety of other glycoproteins. In a previous study (Roth *et al.*, 1985), immunoblotting with affi-X of partially purified intestinal galactosyltransferase and a crude intestinal homogenate after electrophoresis in 10%-(w/v)-polyacrylamide gel, showed in each case an immunoreactivity in the M_r range of galactosyltransferase of human milk. The absence of other major cross-reactive components suggested that the antibodies were monospecific for galactosyltransferase. However, experience with monoclonal antibodies has shown that, even when they react with discrete glycoproteins, the epitopes they recognize may be carbohydrate. Examples are a monoclonal antibody to an antigenic marker of human fetal endoderm (Gooi *et al.*, 1983c) and several antibodies to the receptor for epidermal growth factor in A431 cells (reviewed by Gooi *et al.*, 1985). These antibodies, which appeared monospecific and reacted with single immunoreactive components in the cells used as immunogens, were directed

against blood-group chains. In each case, the glycoprotein in question (a 200 kDa fetal glycoprotein or the 170 kDa receptor for epidermal growth factor) happened to be the main immunoreactive component among the total solubilized glycoproteins of these cells, but in other cell types the antigenic determinants are shared by a variety of glycoproteins and glycolipids (Feizi *et al.*, 1984). A major class of epithelial glycoproteins, mucins, which express carbohydrate antigens of the blood-group family, do not penetrate 10% polyacrylamide gels and would be overlooked unless electrophoresis with low concentrations of polyacrylamide gel is performed (Feizi, 1983). In addition they may fail to be electrotransferred on to nitrocellulose and thus fail to immunostain.

Although the carbohydrate antibodies are minor components among the antibodies to galactosyltransferase, they are detectable at the dilutions used for immunohistochemistry of the enzyme glycoprotein. The cell-surface immunoreactivity of epithelial cells (Pestalozzi *et al.*, 1982; Davis *et al.*, 1984; Roth *et al.*, 1985) and of pre-implantation embryos (Sato *et al.*, 1984) with anti-galactosyltransferase sera or with affinity-enriched antibodies has previously been taken as evidence for the occurrence of this enzyme on cell surfaces. In view of the occurrence of SSEA-1 on pre-implantation embryos (Gooi *et al.*, 1981) and blood-group A, Le^a, SSEA-1 and related carbohydrate antigens on epithelial surfaces [reviewed by Gooi *et al.* (1983b) and Feizi *et al.* (1984)], it will be important to re-evaluate these previous immunohistochemical observations after absorptions to deplete any carbohydrate specificities in the anti-galactosyltransferase reagents and by raising antibodies that react exclusively with the protein moiety of the enzyme glycoprotein.

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