Murine Monoclonal Antibodies Directed to the Human Histo-Blood Group A Transferase (UDP-GalNAc:Fucα1→2Gal α1→3-*N*-Acetylgalactosaminyltransferase) and the Presence Therein of N-Linked Histo-Blood Group A Determinant†

T. White,‡ U. Mandel,§ T. F. Ørntoft,∥ E. Dabelsteen,§ J. Karkov,§ M. Kubeja,‡ S. Hakomori,‡ and H. Clausen*,‡,§

The Biomembrane Institute and Department of Pathobiology, University of Washington, Seattle, Washington 98119, The Royal Dental College and University Hospital, Copenhagen, Denmark, and Department of Experimental Clinical Oncology, Danish Cancer Society, and Department of Clinical Immunology, Skejby University Hospital, Aarhus, Denmark

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ABSTRACT: Mouse MAbs (WKH-1 through -3) to the human histo-blood group A glycosyltransferase (Fucα1→2Gal α1→3galactosaminyltransferase) were established by immunization with the purified native A transferase protein. Hybridomas were selected on the basis of solid-phase reactivity with the purified native A transferase, cell immunofluorescence and immunoprecipitation of transferase activity, and absence of reactivity with blood group ABH carbohydrate determinants. Three MAbs, thus selected, were found most likely to react with the protein epitopes unrelated to carbohydrate epitopes of purified A transferase. The MAbs reacted with cells having high A transferase activity and immunoprecipitated the A transferase activity as well as the 40 000 MW iodinated transferase protein. The antibodies were shown, however, to immunoprecipitate and partially inhibit not only A_1 and A_2 but also B transferase activity from plasma and A transferase from human lung, and to react with B cells expressing B transferase, thus indicating a cross-reactivity with B transferase. In contrast, they showed no reactivity with various cells having the O phenotype and did not immunoprecipitate the A transferase from porcine submaxillary glands or the α1→2fucosyltransferase from Colo205 cells. The purified A glycosyltransferase was found to carry blood group A carbohydrate determinants by immunochemical detection with a panel of anti-carbohydrate MAbs. These determinants are believed to be N-linked, since treatment of the purified A transferase with *N*-glycanase removed activity. Immunohistological studies of three epithelial tissues showed that the antibodies stained the Golgi area of cells in epithelia from A and B, but not O, individuals.

Glycosylation of proteins and lipids proceeds through a cooperative multiglycosyltransferase system organized in the rough endoplasmic reticulum and Golgi apparatus (Hirschberg & Snider, 1987). Data on topology and organization of membrane glycosyltransferases are therefore essential for better understanding of the diversity of glycosylation patterns and their changes associated with differentiation and development. For this approach, antibodies directed to glycosyltransferases have been suggested as useful probes (Roth et al., 1985, 1986). Earlier studies utilizing polyclonal antibodies were criticized on the basis of possible presence of antibodies directed to extraneous proteins, in addition to antibodies directed to carbohydrates (Childs et al., 1986). This problem may be overcome by generation of MAbs[1] to glycosyltransferases (Berger et al., 1986; Chatterjee et al., 1984; Podolsky & Isselbacher, 1984). Recently, the problem has also been elegantly addressed through affinity purification of antisera on fusion protein polypeptides (Taatjes et al., 1988).

The histo-blood group[2] A and B gene defined glycosyltransferases offer especially good opportunities to study the glycosylation process in epithelial cells, judging from the well-defined changes found in the expression of the blood group A and B antigens during epithelial differentiation and maturation, as well as oncogenic transformation (Hakomori, 1985; Clausen & Hakomori, 1989). Previous attempts to purify the human blood group A and B transferase most likely did not succeed (Greenwell et al., 1987; Clausen et al., 1990), and polyclonal antibodies raised may not be entirely specific for the peptide portion of the transferase (Cook et al., 1982; Roth et al., 1986). It is thus desirable to have MAbs to the A and B transferases. The present paper describes (i) the production and characterization of MAbs directed to the A transferase and cross-reacting with the B transferase; (ii) evidence that the A and B transferases are homologous proteins and that blood group O individuals do not share such a protein; and

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* Correspondence should be addressed to this author at The Biomembrane Institute, 201 Elliott Ave. W., Seattle, WA 98119.

‡ The Biomembrane Institute and Department of Pathobiology, University of Washington.

§ The Royal Dental College and University Hospital, Copenhagen.

∥ Danish Cancer Society and Skejby University Hospital, Aarhus.

[1] Abbreviations: BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; 2'FL, 2'-fucosyllactose; MAb, monoclonal antibody; MW, molecular weight; PBS, phosphate-buffered saline; PCFI, particle-concentrated fluorescence immunoassay.

[2] The term "histo-blood group ABH antigens" is used in this paper because they constitute the major allogeneic antigens not only on erythrocytes but also in most epithelial tissues. Phylogenetic studies indicate that these antigens appear earlier in evolution in ectodermal and endodermal tissues than in mesenchymal hematopoietic tissues and blood cells. The term "histo-blood group" is therefore more appropriate than "blood group" (Clausen & Hakomori, 1989).

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(iii) evidence that the A transferase carries N-linked A carbohydrate determinants. The combination of MAbs to the primary and secondary gene products, together with gene probes for the ABO blood group system (Yamamoto et al., 1990), should provide valuable tools for further studies.

Materials and Methods

Materials. Human A_1, A_2, O, and B plasma (cryo-product) were obtained from Pacific Northwest Regional Services, American Red Cross, Portland, OR. The histo-blood group A Fucα1→2Gal α1→3galactosaminyltransferase was purified from human lung as described elsewhere (Clausen et al., 1990). Briefly, Triton X-100 extracts of lung tissue were passed over Sepharose 4B, from which the A transferase was eluted with UDP. Subsequently, cation-exchange chromatography (repeated twice) yielded an essentially homogeneous enzyme preparation with specific activity of ≈6 units/mg of protein. Human cell lines were obtained from American Type Culture Collection (ATCC) and kindly provided by Dr. T. Suzuki, Department of Pathology, Niigata University School of Medicine, Niigata, Japan (MKN-45). Porcine submaxillary glands were purchased from Pelfreeze (Rogers, AR). UDP-[^{14}C]GalNAc, UDP-[^{14}C]Gal, and GDP-[^{14}C]fucose (54 mCi/mol) were purchased from Amersham (Arlington Heights, IL). Unlabeled UDP-GalNAc and UDP-Gal were purchased from Sigma Chemical Co. (St. Louis, MO). Labeled and unlabeled sugar nucleotides were mixed to a final specific activity of 22.816 cpm/nmol or 4.00 cpm/nmol (UDP-GalNAc), 18.740 cpm/nmol (UDP-Gal), and 16.108 cpm/nmol (GDP-Fuc). All gel electrophoresis reagents were purchased from Pharmacia (Upsala, Sweden). The substrate 2′-fucosyllactose was either purchased from Sigma or purified from human milk. Fluoricon carboxyl-polystyrene assay particles were purchased from Baxter (Mundeline, IL). Goat anti-mouse antibodies were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). *N*-Glycanase was purchased from Genzyme (Boston, MA). All other reagents were of the highest grade available commercially. MAbs to various isocarrier type variants of blood group A antigen were prepared as previously described (Clausen et al., 1985).

Enzyme activity was determined in 100-μL reaction mixtures containing 50 μL of enzyme source, 50 mM Tris buffer (pH 7.4), 20 mM $MnCl_2$, 0.1% Triton CF-54, 1% BSA, 5 mM CDP choline, and 50 μM UDP-[^{14}C]GalNAc (A enzyme), UDP-[^{14}C]Gal (B enzyme), or GDP-[^{14}C]fucose (fucosyltransferase). The substrate, 2′FL (0–20 mM), was added to the reaction mixture, and incubation was performed at 37 °C for 10 or 30 min and terminated by addition of 250 μL of ice-cold Milli-Q water. The incorporated sugar nucleotide was determined by scintillation counter after passing the reaction mixture over a 300-μL Dowex-1 formic acid cycle column followed by washing with 650 μL of Milli-Q water. In some cases, blood group H glycosphingolipids (H_1 or H_2) were used as substrates in order to use thin-layer chromatography, including immunostaining with MAbs to identify the products [experimental procedure described in Clausen et al. (1990)].

Generation of MAbs. Production of three MAbs, WKH-1, -2, and -3, directed to human blood group A glycosyltransferase, was obtained by immunization of 3-month-old BALB/c mice. Mice were immunized with A transferase emulsified in Ribi's adjuvant (monophosphoryl lipid A + trehalose dimycolate) by intraperitoneal injection 4 times (3-week interval), with ≈30 μg of transferase per injection. Spleen cells were fused with NS-1 myeloma cells 3 days after the last immunization, and hybridomas were cloned by limiting dilution at least three times. Hybridomas were screened by PCFI, fluorescent staining of blood group A cells with high A transferase activity (MKN-45), and immunoprecipitation of transferase activity. Controls included various A glycolipids (Clausen et al., 1986) and cell lines with no A or B transferase activity (Colo205). Isotype and subclass were determined by PCFI using goat anti-mouse FITC-conjugated antibodies (Boehringer Mannheim Biochemicals), as well as by the Ouchterlony method using rabbit anti-mouse antibodies. MAbs were used as tissue culture supernatants unless otherwise indicated. Antibodies were purified on a protein A–Sepharose 4B column (PBS pH 9.0) eluted with 100 mM citrate buffer (pH 4.2), and dialyzed against 20 mM Tris buffer (pH 7.4).

PCFI Screening. Approximately 50 μg of purified transferase (Clausen et al., 1990) was mixed with 1 mL of 0.5% (w/v) Fluoricon carboxyl-polystyrene assay particles (0.86 μm, Pandex) and covalently coupled by adding solid 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide to give a final concentration of 1 mg/mL. Controls for reactivity with carbohydrates included beads similarly coated with salivary or ovarian cyst mucins (a generous gift from Dr. Elvin Kabat), as well as beads coated with A-active glycolipids as described previously (Clausen et al., 1988). After vortexing, the mixture was incubated at room temperature for 1–2 h. The microparticles were then centrifuged (3000*g*, 10 min), washed with PBS, blocked with either BSA/PBS 5% or human serum (1:10 dilution), and brought to a final volume of 0.25% w/v in PBS. Antigen-coated particles were then diluted 1:10 in BSA-coated particles (similar procedure) to give a final particle concentration of 0.225% BSA particles and 0.025% transferase particles. Twenty microliters of BSA transferase or BSA-coated particles was distributed in 96-well Epicon assay plates (Pandex) with 0.2-μm filter. The automated particle concentrated fluorescence immunoassay screen machine (Pandex) (Jolley et al., 1984) performed the following steps sequentially by vacuum suction through the 0.2-μm filter in the bottom of each well and distribution of buffers, through an eight-channel pump: incubation for 10 min with 50 μL of MAb culture supernatant, washing with PBS, incubation for 10 min with 25 μL of affinity-purified goat anti-mouse Ig FITC-conjugated antibody (1:200, Pandex), washing with PBS, and reading at 485 nm/535 nm after final suction centering and concentrating antigen-coated particles in the bottom of the wells.

Immunostaining of Cell Lines and Tissues. Cells were grown in media according to ATCC guidelines, harvested by rubber policeman, and air-dried onto 10-well microslides (Carbon Scientific, Peokone, IL) for 2 h. Slides were "fixed" in ice-cold acetone for 10 min and allowed to dry. Cells were incubated with primary antibody for 45 min at 37 °C, washed with PBS, and incubated with fluorescein-conjugated rabbit anti-mouse antibody (Dakopatts, Denmark) for 30 min at 37 °C. Similarly, human buccal mucosal tissues, salivary glands, and human intestine obtained at surgery were quick-frozen in isopentane precooled with dry ice, sectioned with a cryostat after embedding in Tissue-Tek (Miles Scientific), and processed immediately for immunostaining. Sections were air-dried briefly and "fixed" in acetone and immunostained as described for cell lines, except primary antibodies were incubated for 4 h or overnight at 4 °C.

Slides were examined in a Zeiss fluorescence microscope using epiillumination. The microscope was equipped with FITC interference filters and a 200-W mercury lamp. For control of the staining, primary antibody was replaced with PBS or MAbs of other specificities but with the same isotype as the test antibody. Staining with the MAbs was also per-

Table I: UDP-GalNAc:Fucα1→2Gal α1→3GalNAc Transferase (and UDP-Gal:Fucα1→2Gal α1→3Gal Transferase Where Applicable) Activities in Various Human Cell Lines from Donors with Known Blood Group

cell line Ab	ABO group	origin	specific activity × 10^{-3} (unit/mg)[a]		reactivity with	
			homogenate	detergent soluble	WKH-1	anti-A
MKN-45	A	gastric adenocarcinoma	0.23	0.20	+++	+++
Sw480	A	colonic adenocarcinoma	0.0056	0.0006	-	-/+
A431	A	epidermoid adenocarcinoma	0.15	0.16	+++	+++
Calu-1	A	lung epidermoid carcinoma	0.0000	0.019	-	-/+
Calu-3	A	lung adenocarcinoma	0.0000	0.0000	-	-
Sw48	AB	colonic adenocarcinoma	0.16 (0.10)	0.12 (0.12)[b]	+	+
Sw147	B	colonic adenocarcinoma	0.021 (0.10)	0.032 (0.073)[b]	+	-
Colo205	O	colonic adenocarcinoma	ND[c]	ND	-	-
LS174T	O	colonic adenocarcinoma	ND	ND	-	-
SkLu1	O	lung adenocarcinoma	ND	ND	-	-
SkMes1	O	lung squamous carcinoma	ND	ND	-	-
Ramos	unknown	Burkitt lymphoma	ND	ND	-	-
T cells	unknown	peripheral blood	ND	ND	-	-
B cells	unknown	peripheral blood	ND	ND	-	-

[a] Units defined as 1 μmol/min product formed with 2'FL as substrate in the standard assay described under Materials and Methods. [b] UDP-Gal:Fucα1→2Gal α1→3Gal transferase activity. [c] ND = not determined.

formed after "fixation" with paraformaldehyde or glutaraldehyde on air-dried slides and on cells grown as tumors in nude mice, which were fixed, paraffin-embedded, and sectioned. In the case of colonic tissues, sections were stained by avidin–biotin–peroxidase complex as previously described (Orntoft et al., 1988).

Immunoprecipitation of A Transferase Activity. One milligram of affinity-isolated goat anti-mouse IgG Boehringer Mannheim Biochemicals) was added to 10 mL of 1% Fluoricon polystyrene assay particles (0.85 μm, Pandex) in PBS. After 2 h at room temperature, the suspension was centrifuged (3000*g*) for 10 min, blocked with 3% BSA in PBS, and resuspended to a final concentration of 1% w/v. Goat anti-mouse particles were mixed with MAb hybridoma supernatants in a 1:5 ratio, incubated at 4 °C for 15 min, and centrifuged (3000*g*) for 2 min. The particles were washed with buffer A [50 mM Tris buffer (pH 7.4), 100 mM NaCl, 20 mM $MnCl_2$, 1 mM ethylenediaminetetraacetic acid, 0.1% Triton X-100, and 3% BSA] and resuspended in buffer A to a concentration of 1%. Particles were added to enzyme samples to a concentration capable of binding to twice the amount of A transferase present (≈100 μL of particles for 500 μL of concentrated plasma). After 30 min at 4 °C, the particles were centrifuged at 3000*g* for 2 min and the supernatant was assayed for remaining enzyme. The precipitated particles were washed twice with buffer A, resuspended in 50 μL of wash buffer, and assayed for enzyme activity. The transferase used was either purified or semipurified from human blood group A lungs, or from blood group A_1, A_2, B, or O plasma concentrated 10X by 30–50% ammonium sulfate precipitation followed by concentration in an Amicon stirred-cell membrane concentrator. Fucosyltransferase was from a Triton CF-54 homogenate of Colo205 cells after centrifugation at 100000*g* for 1 h.

Inhibition of Transferase Activity by MAbs. Purified anti-A transferase MAbs, irrelevant MAbs with the same isotype, commercially obtained IgG_1 myeloma standard, or 20 mM Tris buffer (pH 7.4) was added to transferase preparations and incubated at 4 °C for 30 min. The enzyme activity of this mixture was then measured by incubation with reaction mixture at 37 °C for 10 or 30 min.

Results

Production of MAbs to the A Transferase. Three mouse hybridomas, WKH-1, -2, and -3, secreting IgG_1 MAbs, were isolated from one fusion, after immunization with native histo-blood group A transferase. These antibodies were selected on the basis of strong reaction with the purified A transferase coated beads in the PCFI assay (Figure 1) and absence of reactivity with beads coated with BSA, glycolipids from ABO erythrocytes, saliva mucins, or ovarian cyst mucins, all of which carry histo-blood group ABH determinants. The MAbs were also selected for their ability to stain cells with high A transferase activity (MKN-45) and their ability to immunoprecipitate A transferase activity. Thus, the three selected hybridomas secreted MAbs directed to epitopes associated with histo-blood group A transferase, but not immunodominant ABH carbohydrate determinants associated with the A transferase (see "Detection of Histo-Blood Group A Determinant Present on the A Transferase").

In this fusion experiment, numerous antibodies bound the A transferase coated beads (75/500 wells), of which more than 50% also showed reactivity with blood group A glycolipids, as well as membrane staining of MKN-45 similar to the staining pattern of some anti-A carbohydrate MAbs. Of the antibodies (wells) not reacting with A glycolipids, 10 showed granular cytoplasmic staining of MKN-45 (A), but not Colo205 (O). Of these 10, three could immunoprecipitate A transferase activity; from these wells, WKH-1 through -3 were cloned and further characterized.

Immunostaining of Human Cell Lines. A summary of MAb reactivity to various human cell lines is presented in Table I. WKH-1 through -3 stained only cell lines derived from blood group A and B (but not O) indivudals. Only reactivity with cell lines of epithelial origin known to carry ABO blood group antigens was observed. Quantitatively, the staining of cells correlated with the specific transferase activity of detergent-soluble extract of the cell lines. Formaldehyde or glutaraldehyde fixation completely abolished the immunoreactivity of WKH-1 through -3, whereas anti-A carbohydrate MAbs were unaffected. WKH-1 through -3 produced a granular cytoplasmic fluorescence (Figure 8).

Immunoprecipitation of A_1, A_2, and B Transferase Activities. The three MAbs immunoprecipitated A transferase activity quantitatively regardless of whether transferase was homogeneous in buffer A or added to crude plasma (Figure 2). The MAbs were tested for their ability to remove transferase activity from blood group A_1, A_2, and B crude plasma. WKH-1 through -3 were able to remove 90% of A_1 and A_2 transferase and 60% of B transferase activity (Figure 3). Active transferase could be detected in the washed pellet,

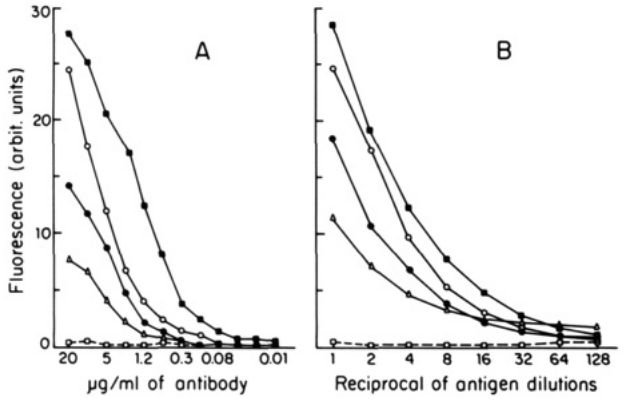

FIGURE 1: Immunoreactivity of MAbs with purified A transferase (PCFI assay). Panel A: Antibody dilution of adjusted culture supernatant at 0.25% antigen bead concentration (see Materials and Methods). Panel B: Antigen dilution with initial concentration of antigen beads of 0.25% using MAb concentration of 20 μg/mL. Symbols: (●) WKH-1; (△) WKH-2; (■) WKH-3; (□) control standard mouse myeloma IgG_1; (○) MAb HH5 directed to blood group A carbohydrate.

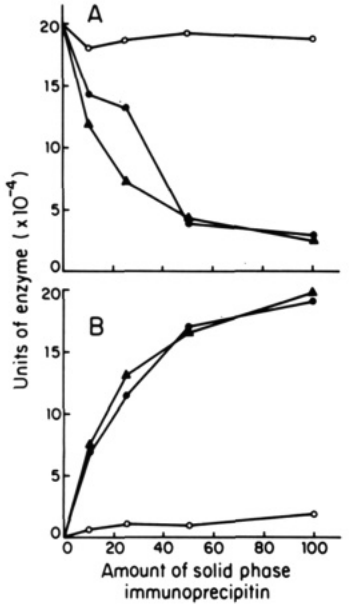

FIGURE 2: Immunoprecipitation of A transferase activity using MAb WKH-1. Assays were carried out with purified A transferase in Tris buffer or purified A transferase added to human plasma ($\approx 2 \times 10^{-3}$ units in 50 μL). The immunoprecipitin [polystyrene beads, 0.86 μm (Pandex), absorbed with affinity-purified rabbit anti-mouse antibodies and MAb] was used at varying concentrations; after centrifugation, transferase activity was measured in supernatant as well as buffer-washed (2×) immunoprecipitin beads. Panel A: Transferase activity remaining soluble. Panel B: Transferase activity recovered in the immunoprecipitin. Symbols: (●) WKH-1 with purified transferase in buffer; (▲) WKH-1 with purified transferase in plasma; (○) IgG_1 myeloma control antibody with purified transferase in buffer.

but the amount of activity depended upon the antibody's ability to inhibit transferase activity (see Figure 5). Standard IgG_1 and other MAbs were unable to remove transferase activity from plasma, and no activity was detectable in the immunoprecipitated pellet. The A transferase MAbs were unable to immunoprecipitate the blood group A detergent-soluble Fucα1→2Gal α1→3-*N*-acetylgalactosaminyltransferase from porcine submaxillary glands or the α-3-fucosyltransferase activity of detergent-soluble Colo205 cell extract (data not shown).

Immunoprecipitation of ^{125}I-Labeled Protein. All three MAbs capable of immunoprecipitating transferase activity were also able to immunoprecipitate an ^{125}I-labeled protein

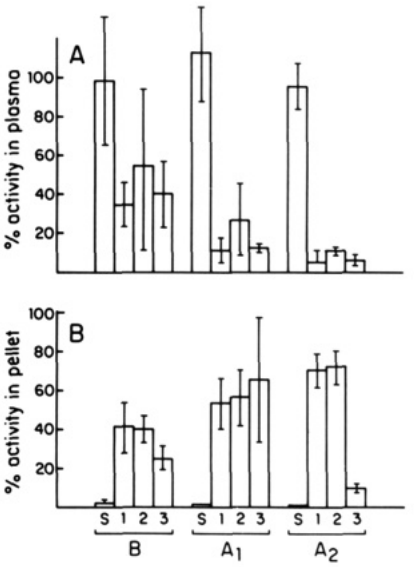

FIGURE 3: Immunoprecipitation of transferase activity from blood group B, A_1, and A_2 plasma by MAbs WKH-1 (1), WKH-2 (2), and WKH-3 (3) and standard IgG_1 (S). Panel A: Percent transferase activity remaining in plasma after immunoprecipitation. Panel B: Percent transferase activity in the washed pellet. Variation shown represents one standard deviation from dual assays of three different sera of each of the various blood groups.

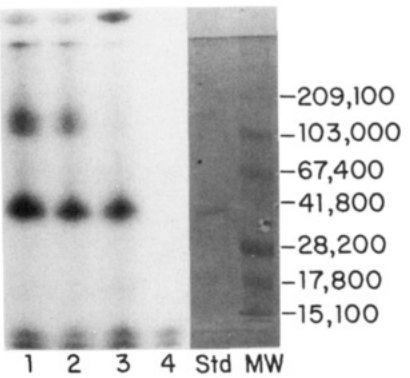

FIGURE 4: Immunoprecipitation of ^{125}I-labeled A transferase protein by MAbs. Lanes 1–4: Immunoprecipitation with WKH-1, WKH-2, WKH-3, and control IgG_1 MAb, respectively. Std: Coomassie Blue strain of A transferase purified to homogeneity. MW: molecular weight standards.

[for iodination see Clausen et al. (1990)] corresponding to the MW (40K) of the purified A transferase (Figure 4), while control MAbs did not. MAb HH5 did immunoprecipitate a small amount of the protein in the purified state (data not shown). WKH-1 and -2 also immunoprecipitated a protein of approximately 100 000 MW, possibly corresponding to a dimer of the A transferase.

Inhibition. Purified A transferase from human lung was significantly inhibited (≈70%) by WKH-3, but not by WKH-1 and -2, nor by various controls (Figure 5). The inhibition by purified WKH-3 was optimal at less than 1 μg/mL MAb, and even at 270 μg/mL inhibition did not increase to more than 70%. When purified MAbs were added to concentrated crude plasma, inhibition of transferase activity was detectable. The degree of inhibition depended on the antibody used, and on blood group type. MAb WKH-3 almost eliminated A transferase activity from blood group A_2 plasma, whereas WKH-1 and -2 only inhibited 30% of the transferase activity (Figure 6). All three anti-A transferase MAbs were able to show an inhibitory effect on B transferase activity in concentrated B plasma but showed no effect on α-3-fucosyl-

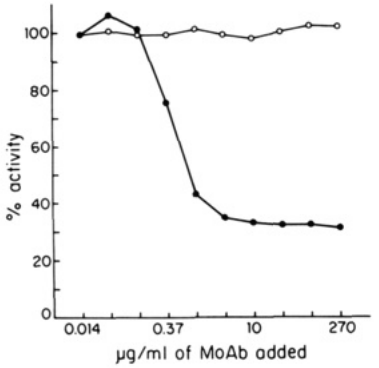

FIGURE 5: Inhibitory effect of WKH-3 on purified A transferase from human lung. Varying concentrations of purified WKH-3 MAb or control mouse myeloma IgG_1 were added to 1×10^{-4} units of purified A transferase. The MAbs were allowed to react at 4 °C for 10 min, after which A transferase activity was measured by addition of the standard reaction mixture and incubation for 20 min at 37 °C. Inhibition is expressed as percent of activity obtained with the addition of buffer alone. (●) WKH-3; (○) control IgG_1.

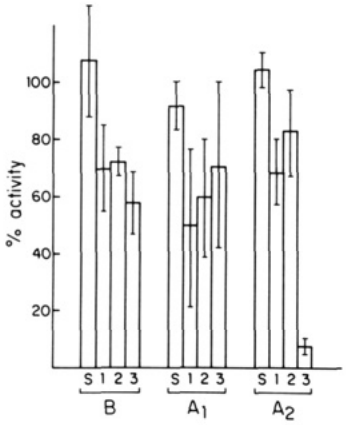

FIGURE 6: Inhibitory effect of MAbs on A_1, A_2 and B plasma transferase activities. Purified MAbs (5 μg each) were added to 100 μL of concentrated (10×) plasma and incubated for 30 min at 4 °C, after which transferase activities were assayed by addition of standard reaction mixture and incubation for 20 min at 37 °C. Inhibition is expressed as percent of activity obtained with the addition of buffer alone. Columns: control IgG_1 (S), WKH-1 (1), WKH-2 (2), and WKH-3 (3). Variation shown represents one standard deviation from dual assays of three different sera of each of the various blood groups.

transferase activity in Colo205 cell extract. Standard IgG_1 and other MAbs of the same isotype had no effect on transferase activity in all assays.

Detection of Histo-Blood Group A Determinant Present on the A Transferase. In order to eliminate the possibility for selection of anti-carbohydrate antibodies when immunizing and screening with intact A glycosyltransferase, a variety of anticarbohydrate MAbs were tested in a PCFI assay on the purified A transferase to determine the presence of carbohydrate. These included antigens related to the ABO, Lewis, T/Tn, and related blood group antigens (data not shown). The only MAb that reacted with transferase-coated beads was HH5, which defines the blood group A carbohydrate determinant found on the type 3 and type 4 chain core (GalNAcα1→3[Fucα1→2]Galβ1→3GalNAcα/β1→R) (Figure 1) (Clausen et al., 1986). HH5 was also able to stain the A transferase in a Western blot (Figure 7). Interestingly, this reactivity could be eliminated by treatment with *N*-glycanase, indicating that the A type 3 or 4 chain determinant is present on N-linked carbohydrate of the protein. HH5 was also able to immunoprecipitate a small amount (≤10%) of active A transferase from a purified transferase preparation

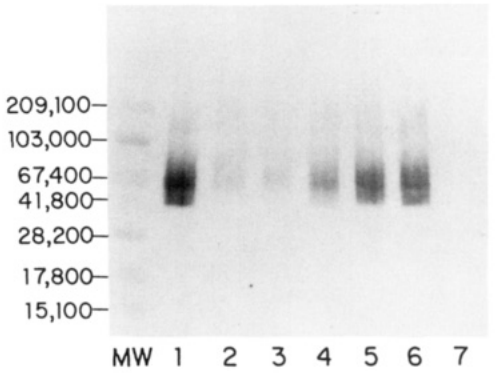

FIGURE 7: Western blot of *N*-glycanase-digested A transferase with MAb HH5. Purified A transferase (≈500 ng) (lanes 1–6) was digested with *N*-glycanase for 4 h at 37 °C according to manufacturer's instructions. Units of *N*-glycanase/mL in lanes 1–7 were 0, 62.5, 15.6, 3.9, 1, 0.25, and 62.5, respectively. Western blot was performed using Pharmacia's Phast system with 12.5% polyacrylamide gel. The gel was released from its plastic backing by using a thin wire, and protein was transferred to Immobilon PVDF membrane by using a semidry electroblotter. The membrane was stained with MAb HH5 according to Janssen's immunogold silver staining procedure (Janssen Biotech, Belgium). MW: molecular weight standards.

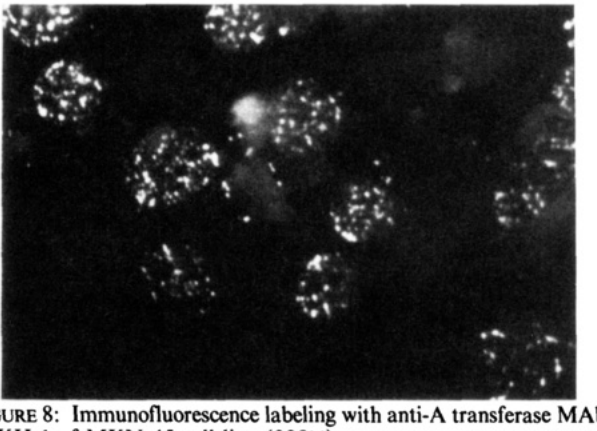

FIGURE 8: Immunofluorescence labeling with anti-A transferase MAb WKH-1 of MKN-45 cell line (320×).

but was unable to immunoprecipitate any activity from plasma. The MAb had no inhibitory effect on the activity of A transferase in an inhibition assay (data not shown).

Immunohistology of Human Tissues. Three different types of epithelia were tested with the anti-A transferase MAbs. In general, the antibodies all stained A_1, A_2, and B epithelial cells, but not O tissues. In normal human colonic intestinal epithelium, as well as in a colonic adenocarcinoma, the antibodies labeled in all cells in the paranuclear region in a granular fashion (Figure 9). Similar staining was found in acinar cells of small salivary glands, where staining was observed in the basolateral area of the cells corresponding to the location of the Golgi complex (Figure 10B,C). In nonkeratinized stratified epithelium, a weak granular staining was observed in the spinous cell layers, in agreement with the appearance of the blood group A carbohydrate phenotype (Dabelsteen et al., 1982) (Figure 10A). Formaldehyde-fixed paraffin-embedded sections did not show immunoreactivity with the anti-A transferase MAbs.

Discussion

Cellular glysoylation patterns showing marked changes associated with differentiation and oncogenesis (Hakomori, 1981, 1985; Clausen & Hakomori, 1989) are believed to be regulated by the quality and quantity of glycosyltransferases, not only through transcriptional and translational control

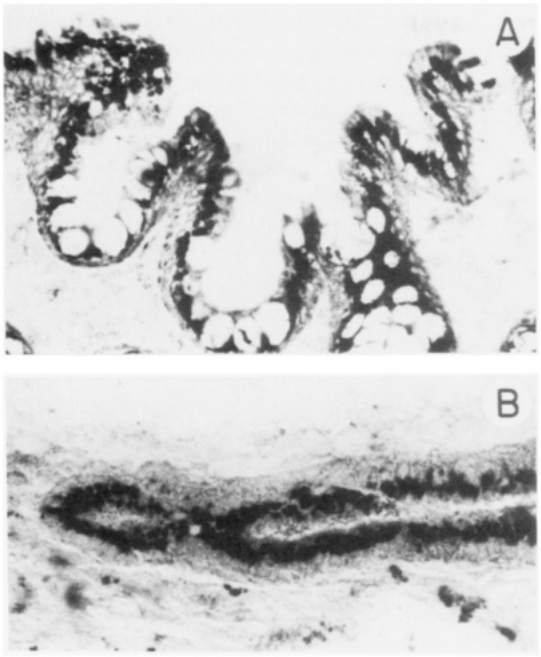

FIGURE 9: Immunoperoxidase staining (black) of normal human colonic epithelium (A) and a colonic adenocarcinoma (B) of a blood group A patient, using MAb WKH-1 (370×).

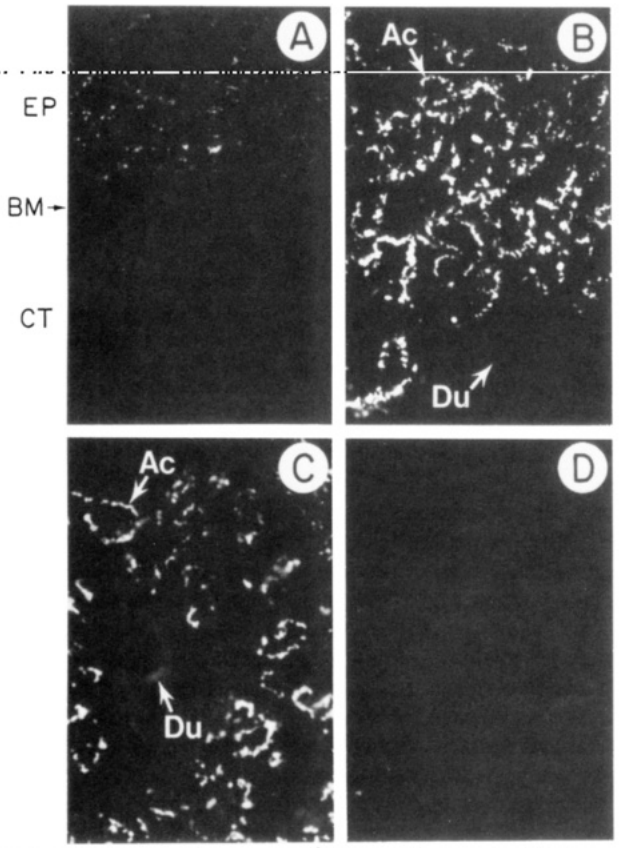

FIGURE 10: Immunofluorescence labeling of human stratified squamous epithelium (A) and glandular epithelium (B–D) using MAb WKH-2 (130×). Specimens were taken from individuals of blood type A_1 (panels A and B), B (panel C), and O (panel D). In panel A is indicated the extent of epithelium (EP) and connective tissue (CT), as well as basement membrane (BM and white stippled line). Immunofluorescence staining in panels B and C is localized to acini (Ac), whereas ducts (Du) are negative.

mechanisms, but also though their organization in membranes (Hubbard & Ivatt, 1981; Ivatt, 1981). Other factors such as substrate availability and competition among transferases for substrates (Schachter et al., 1983), and the ordered processing of sugar chains (Kornfeld & Kornfeld, 1985), have also been shown to contribute to the diversity of carbohydrate chains. As a basis for understanding the diversity of glycosylation processes, the topology and organization of the "multiglycosyltransferase system" in Golgi apparatus and other cell organelles, as originally suggested by Roseman (1970), are of primary importance. In this context, the present study attempted to develop MAbs directed to the A transferase, for use as probes. A and B gene-defined transferases could be located in the late-acting trans region of the Golgi apparatus and plasma membrane, as suggested by Roth et al. (1986).

Three MAbs directed to the histo-blood group A glycosyltransferase, and cross-reacting with the B glycosyltransferase, were generated. The antibodies immunoprecipitated transferase activities as well as the putative A transferase protein and inhibited transferase activities to varying degrees. The MAbs reacted strongly with cells and tissues from histo-blood group A individuals, and with those from group B, but did not react with any group O derived cells or tissues. Activity of fucosyltransferase from Colo205 cells and A transferase from porcine submaxillary glands could not be inhibited or immunoprecipitated by the three MAbs generated, illustrating their specificity for human A and B transferases. The inability of the MAbs to bind to A transferase from porcine submaxillary glands suggests that human A transferase is structurally different from that of porcine A transferase. Previously, polyclonal antibodies have been produced to the blood group A and B transferases. A polyclonal serum raised against the A transferase by Yoshida (1979) was able to neutralize both A and B transferase activities. Takizawa and Iseki (1981) produced rabbit antisera to the B transferase, which neutralized B transferase activity but only partially inhibited A transferase activity. Polyclonal antiserum to the A_1 transferase isolated from plasma has also been produced by Cook et al. (1982). The antiserum was unable to inhibit the A and B transferase activities when using low molecular weight substrate (2′FL) but did prevent the conversion of O cells into A or B cells (Cook et al., 1982). Taken together, these studies with polyclonal antisera and the three MAbs produced in the present study indicate that the A and B glycosyltransferases must exhibit close structural homology.

The MAbs described in this paper are most likely directed to protein epitopes of the A transferase which are shared with the B transferase. Although the purified A transferase has N-linked A carbohydrate determinants (as evidenced by its reactivity with anti-A MAbs and susceptibility to *N*-glycanase) which are very immunogenic in mice, the MAbs generated did not react with histo-blood group ABH determinants. The interesting possibility is suggested that N-linked carbohydrate chains of the A transferase can be modified by addition of A determinant through its own catalytic activity. This could be due to autoglycosylation, or to intermolecular glycosylation resulting from clustering of A transferase. This is the first unequivocal demonstration of the presence of a particular glycosyl residue in a glycosyltransferase which catalyzes the transfer of the same sugar. Whether the same principle may be involved in glycosylation patterns of other glycosyltransferases is of interest.

The strategy used for generating these anti-A transferase MAbs allowed for the selection of "functional" antibodies, i.e., ones capable of immunoprecipitating and inhibiting transferase activity. Since none of the MAbs obtained bound denatured A transferase protein (sodium dodecyl sulfate–polyacrylamide gel electrophoresis Western blot) or fixed (formaldehyde or glutaraldehyde) cells expressing A transferase, the antigenic

epitopes may be conformation-dependent. The observed inhibition, especially by WKH3 on A_2 transferase activity, could suggest that the binding epitope is related to the catalytic site of the transferase. The inhibition by WKH3 of A_2 transferase was, however, not protected by preincubation of the transferase with substrates (data not shown). WKH3 was the only MAb producing inhibition of purified A transferase from human lungs. This inhibition, however, was never total. The nearly complete inhibition of A_2 plasma transferase, but only weak inhibition of A_1 plasma transferase, may explain the result with purified lung transferase, since lung transferase preparations were mixed from several individuals with unknown genotype.

Conflicting data exist as to the presence or absence of a structurally and immunologically related protein associated with the blood group O phenotype. Yoshida (1979) presented data supporting the presence of a "cross-reactive material" in the plasma of O individuals, as detected by a polyclonal antiserum. However, this was not confirmed in subsequent studies with two other polyclonal antisera (Takizawa & Iseki, 1981; Greenwell et al., 1987). Recently, it has been shown that concentrated O plasma contains a transferase activity that can produce the blood group A determinant (Greenwell & Watkins, 1987; Watkins et al., 1988). In the present study, the three MAbs isolated (or any other antibodies not selected for further studies in the same fusion) showed no reactivity with epithelial cell lines of blood group O origin. The three MAbs also stained A and B tissues exclusively in the immunohistological analyses. Preliminary evidence from studies of the ABO genes suggests that the genes are structurally very similar and highly conserved. The O genotype may be a result of structural differences, e.g., leading to a shift in the reading frame rather than expression failure (Yamamoto et al., 1990).

Childs et al. (1986) found that polyclonal antisera raised against the β-4-galactosyltransferase had considerable reactivity with carbohydrates. Initial studies using this antiserum showed immunoreactivity in the Golgi area as well as the cell membrane. The latter reactivity was, however, abolished after preadsorption of the antiserum with carbohydrates. In the present study, it was demonstrated that the A transferase is a glycoprotein carrying at least one N-linked chain, which appears to terminate in blood group A carbohydrate determinants of type 3/4 chain. Interestingly, antibodies to these A determinants (in contrast to "general" anti-A antibodies) show a reactivity pattern in most epithelia very similar to the expected localization of the A transferase, i.e., the Golgi area (Le Pendu et al., 1986; unpublished data).

The blood group ABO(H) carbohydrate antigens are good markers of epithelial differentiation and maturation processes as well as oncogenic transformation (Hakomori, 1985; Clausen & Hakomori, 1989). Using the MAbs WKH-1 through -3, it was possible, for the first time, to provide evidence that these changes in carbohydrate expression may be a direct gene-regulated event. The A or B carbohydrate phenotype in stratified epithelium is acquired in differentiated cell layers above the progenitor region localized in the basal cell layer close to the basement membrane (Dabelsteen et al., 1982). As shown in Figure 10A, immunoreactivity with MAb WKH-1 to the transferase is only localized in the upper differentiated cell layers. The MAb produced a granular cytoplasmic staining which, in colonic adenocarcinoma cells and small salivary acinar cells, localized distinctly to the supranuclear area. No evidence of staining of cell membranes or secretory vesicles was found. Previous immunogold electron microscopy studies using polyclonal anti-A transferase sera (Roth et al., 1986) showed labeling of mucous droplets and plasma membranes. This result could be due to anti-carbohydrate antibodies and requires confirmation with affinity-purified polyclonal antibodies, e.g., on fusion protein, as recently described with polyclonal antisera to the $\alpha 2 \rightarrow 6$sialyltransferase (Taatjes et al., 1988). The blood group ABO system offers a unique model to study glycosylation, and the MAbs produced in the present work will be valuable tools.

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Phospholipids Chiral at Phosphorus. Stereochemical Mechanism of Reactions Catalyzed by Phosphatidylinositide-Specific Phospholipase C from *Bacillus cereus* and Guinea Pig Uterus†

Gialih Lin,‡ C. Frank Bennett,§ and Ming-Daw Tsai*,‡

Department of Chemistry, The Ohio State University, Columbus, Ohio 43210, and Isis Pharmaceuticals Inc., 2280 Faraday Avenue, Carlsbad, California 92008

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ABSTRACT: (R_p)- and (S_p)-1,2-dipalmitoyl-*sn*-glycero-3-thiophosphoinositol (DPPsI) were synthesized as a mixture and their configurations assigned on the basis of the stereospecific hydrolysis catalyzed by phospholipase A_2 (PLA2) from bee venom. PLA2 is known to be stereospecific to the R_p isomer of 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphocholine (DPPsC) and 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphoethanolamine (DPPsE). Since the configurations of (R_p)- and (S_p)-DPPsI correspond to those of (S_p)- and (R_p)-DPPsC, respectively, due to a change in priority, the isomer specifically hydrolyzed by PLA2 was assigned to (S_p)-DPPsI. The DPPsI analogues were then used to probe the mechanism and to elucidate the steric course of the reaction catalyzed by phosphatidylinositide-specific phospholipase C (PI-PLC) from *Bacillus cereus* and for both isozyme I and isozyme II of PI-PLC from guinea pig uterus. It was found that the R_p isomer of DPPsI is the preferred substrate for all three PI-PLCs. Thus PI-PLC shows the same stereospecificity as phosphatidylcholine-specific PLC (PC-PLC), which prefers the S_p isomer of DPPsC. The ratio of the two products inositol 1,2-cyclic phosphorothioate (cIPs) and inositol phosphorothioate (IPs) was not significantly perturbed by the use of phosphorothioate analogue for all three PI-PLCs, which implies that IPs is not produced by enzyme-mediated ring opening of cIPs and supports a parallel pathway for the formation of both products. In order to elucidate the steric course of the cyclization reaction, exo and endo isomers of cIPs were synthesized and their absolute configurations at phosphorus were determined by nuclear magnetic resonance and other techniques. It was found that *exo*-cIPs is the product produced by all three PI-PLCs. Thus the steric course of the conversion DPPsI to cIPs catalyzed by all three PI-PLCs was inversion of configuration at phosphorus. These results taken together suggest that the reaction catalyzed by PI-PLC most likely proceeds via direct attack by the 2-OH group to generate the cyclic product, and parallelly by water to generate the noncyclic inositol phosphates, without involving a covalent enzyme–phosphoinositol intermediate.

Phosphatidylinositides are important phospholipids since their metabolism is highly responsive to various extracellular stimuli acting on the cell (Michell, 1975). A key enzyme for this metabolism is phosphatidylinositide-specific phospholipase C (PI-PLC)[1] [for recent reviews see Majerus et al. (1986) and Shukla (1982)]. PI-PLC has been found in many mammalian tissues and in some bacteria. It uses phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), or phosphatidylinositol 4,5-bisphosphate (PIP_2), and possibly other phosphatidylinositides as a substrate. The relative specificity of these substrates varies with the source of the enzyme, but PI is an acceptable substrate in most cases and is the substrate used in this study. The enzyme is biologically significant since it generates three second messengers from PIP_2: diacylglycerol, inositol 1,4,5-trisphosphate, and inositol 1,2-cyclic 4,5-trisphosphate (Berridge, 1984, 1987). It is mechanistically unique in that it produces both inositol phosphates and inositol cyclic phosphates simultaneously, at a ratio dependent on the source

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‡The Ohio State University.
§Isis Pharmaceuticals Inc.

[1] Abbreviations: cIP, inositol 1,2-cyclic phosphate; cIPs, inositol 1,2-cyclic phosphorothioate; de, diastereomeric excess; COSY, correlated spectroscopy; DEPT, distorsionless enhancement by polarization transfer; DPPsC, 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphocholine; DPPsI, 1,2-dipalmitoyl-*sn*-glycero-3-thiophospho-1-*myo*-inositol; EDTA, ethylenediaminetetraacetate; FAB, fast atom bombardment; GC, gas chromatography; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IP, inositol 1-phosphate; IPs, inositol 1-phosphorothioate; MPPsI, 1-palmitoyl-*sn*-glycero-3-thiophosphoinositol; MOMCl, chloromethyl methyl ether; MPLC, medium-pressure liquid chromatography; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser enhancement spectroscopy; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PI-PLC, phosphatidylinositide-specific phospholipase C; PC-PLC, phosphatidylcholine-specific PLC; PLA2, phospholipase A_2.

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