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Monoclonal Antibody Recognizing a Determinant on Type 2 Chain Blood Group A and B Oligosaccharides Detects Oncodevelopmental Changes in Azoxymethane-Induced Rat Colon Tumors and Human Colon Cancer Cell Lines

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Abstract Altered expression of ABH blood group substances is a common feature of human colorectal carcinoma, yet it remains unclear how these structural changes influence the biological properties of tumor cells. Azoxymethane-induced rat colon tumors display many features of the human disease, thereby providing a potentially useful model to study the role of blood group substances in colon cancer progression. We have prepared monoclonal antibodies to a microsomal fraction isolated from an azoxymethane-induced rat colon tumor and selected an antibody that detects cancer-associated changes. Monoclonal antibody (mAb) 3A7 recognizes a determinant on type 2 chain blood group A (GalNAcα1-3[Fucα1-2]Galβ1-4GlcNAc-R) and B (Galα1–3[Fucα1-2]Galβ1-4GlcNAc-R) oligosaccharides. Expression of the epitope detected by this antibody was developmentally regulated in rat colon, with maximal expression from day 4–21 after birth. Immunohistochemical staining and Western blotting analyses of azoxymethane-induced colon tumors revealed increased expression of the epitope in all of the 21 colonic tumors examined, including preneoplastic glands within transitional mucosa. Conventional and signet-ring adenocarcinomas that had invaded through the muscularis propria (Duke's B2) consistently showed the most intense staining with mAb 3A7, including regions depicting angioinvasion. Some of the lymph node metastases (Duke's C2) stained poorly with the antibody. The epitope was also expressed in blood group A positive human colon carcinoma cell lines, including HT29 and SW480 but not by SW620, a cell line derived from a lymph node metastasis isolated in vivo from the SW480 primary tumor, or in the blood group B cell line SW1417. The glycoproteins detected by mAb 3A7 in rat colon tumors and HT29 cells ranged in size between 50 and 200 kd, including a major species of 140 kd. Affinity chromatography of detergent lysates of normal rat colon on the blood group A specific lectin *Dolichos biflorus* (DBA)-agarose resulted in nearly quantitative binding of glycoprotein species detected by the antibody. By contrast, immunoreactive glycoproteins from rat colon tumors or HT29 cells bound poorly to DBA-agarose but were retained by another blood group A-binding lectin, *Helix-pomatia* (HPA)-agarose. These results indicate that colon carcinogenesis results in quantitative as well as qualitative changes in oligosaccharides detected by mAb 3A7 and suggest that the combined use of mAb 3A7 and blood group A-specific lectins may provide a useful tool for early detection of colon cancer.

Key words: oncodevelopmental antigen, type 2 chain blood group A and B determinants, azoxymethane-induced rat colon tumors, lectin-affinity chromatography, human colon cancer cell lines

Abbreviations used: mAb, monoclonal antibody; ABH, serologically defined carbohydrate epitopes having the following structures: A = GalNAcα1-3[Fucα1-2]Gal-R, B = Galα1-3[Fucα1-2]Gal-R and H = Fucα1-2Gal-R; Le, Lewis blood group; Asn-linked, asparagine-linked; Gal, D-galactose; Fuc, L-fucose; GlcNAc, N-acetyl-D-glucosamine; GalNAc, N-acetyl-D-galactosamine; pmole, picomole; DMEM, Dulbecco's minimum essential medium; BSA, bovine serum albumin; NMS, normal mouse serum; NGS, normal goat serum; TBT, 50 mM Tris HCl pH 8.0, 0.1% bovine serum albumin, 0.025% Tween-20, 0.02% sodium azide; TBS, Tris-buffered saline (50 mM Tris HCl pH 8.0, 0.02% sodium azide); PBS, phosphate-buffered saline (2.5 mM NaH_2PO_4, 7.3 mM Na_2HPO_4, 144 mM NaCl, pH 7.2); BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitroblue tetrazolium; PMSF, phenylmethanesulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; L-PHA, leukophytohemagglutinin; DBA, *Dolichos biflorus;* HPA, *Helix pomatia.*

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Colorectal cancer remains one of the most prevalent epithelial cancers, resulting in nearly 70,000 deaths annually in North America [1,2]. These statistics emphasize the need for improved methods of early detection as well as rationalized treatments for disseminated disease, i.e., metastasis. During the past decade, significant advances have been made in identifying genetic alterations associated with various stages in the carcinogenic process, including activation of dominant acting oncogenes (e.g., *ras*) and loss of tumor suppressor genes (e.g., p53,DCC[deleted in colon carcinoma]) [3,4]. In addition, colorectal cancer cells exhibit a number of epigenetic changes affecting the activity or level of expression of specific gene products, some of which have been shown to contribute to the malignant behavior of colon tumor cells [4].

One of the most consistent biochemical change associated with colon tumorigenesis and acquisition of the metastatic phenotype is altered expression and/or structure of cell-associated complex carbohydrates [5–8]. Some of the most notable carbohydrate changes detected on colon cancer cells include altered expression and structure of oligosaccharides bearing ABH and Lewis (Le) blood group antigens [5,9,10]. Although the biological significance of altered glycosylation on colon cancer cells is not completely understood, complex carbohydrates have been shown to serve as ligands for mammalian lectins [11], modulate tumor cell adhesion [8], motility [12], and influence antitumor immunity [13,14], thereby affecting their malignant behavior. The recent demonstration that Le^x and sialylated Le^x determinants constitute the ligands for recognition by selectins, a family of glycoproteins involved in cell adhesion [15–17], as well as recent reports documenting increased expression of these glycans on metastatic human colon cancer cells [18], have provided new insights about the functional role of complex carbohydrates in cancer cells.

Much less is known about the biological function of ABH determinants on colon cancer cells [5,19]. In contrast to normal adult large intestine, where expression of ABH determinants is restricted to the proximal region of the colon [20], colonic tumors display deregulated expression of ABH determinants [5,10]. This results in either loss of expression of blood group substances in tumors of the proximal colon [21], re-expression of the fetal pattern of ABH determinants in tumors of distal colon [22,23] or neo-expression of blood group substances not compatible with the patient's blood type [24,25]. Although altered expression of ABH glycosyltransferases has been implicated as a possible mechanism responsible for aberrant expression of ABH determinants [26,27], regulation of core structure synthesis [28] and branching of oligosaccharides may also be important [8,29–31]. Thus, elucidation of the mechanisms regulating expression of ABH determinants during colon carcinogenesis as well as their biological role in tumorigenesis and metastasis is likely to contribute significantly to our understanding of colon cancer progression. In addition, this knowledge may form the basis of improved detection methods for preneoplastic colonic lesions and lead to rationalized treatments for metastatic disease.

Induction of experimental tumors in rodents provides a powerful approach to examine the biological role of ABH determinants in multistage carcinogenesis. The carcinogens dimethylhydrazine and azoxymethane have been used extensively to induce colorectal tumors in rodents and studies have shown that these tumors display many features of human colonic tumors including hyperplasia, dysplasia, carcinomas and less frequently, liver metastases [32,33]. In addition, many of the rodent strains used in these studies, including Sprague-Dawley, express glycoconjugates bearing ABH determinants [34,35]. Finally, individual animals usually harbor multiple colonic lesions differing in their malignant potential, thereby providing a unique opportunity to identify biochemical and genetic alterations directly associated with colon tumorigenesis and progression to malignancy [36]. For these reasons, the azoxymethane model of colon carcinogenesis may provide a very good system to examine the underlying mechanisms responsible for altered ABH expression during colon cancer progression as well as their functional significance in this process.

As part of ongoing studies aimed at understanding the contribution of glycoconjugates to colon cancer progression [37], we have prepared monoclonal antibodies (mAb) to a microsomal fraction prepared from an azoxymethane-induced rat colon tumor. Antibodies were screened for their ability to detect glycoproteins which are differentially expressed in normal and neoplastic colon. mAb 3A7 was selected for further study because it detected an epitope expressed at elevated levels in azoxymethane-induced rat colonic tumors and some human colon cancer cell lines. In this report, we demonstrate that

mAb 3A7 detects an epitope on type 2 oligosaccharide chains (Galβ1–4GlcNAc-R) bearing either blood group A (GalNAcα1–3[Fucα1–2]Gal) or B (Galα1–3[Fucα1–2]Gal) determinants whose expression is oncodevelopmentally regulated. Moreover, we demonstrate, using a combination of mAb 3A7 and blood group A-specific lectins, that induction of rat colon tumors and progression towards a more malignant phenotype result in quantitative as well as qualitative changes in oligosaccharides detected by mAb 3A7. Finally, we compare the glycoproteins detected by mAb 3A7 in rat colon tumors and human colon carcinoma cells in order to assess the relevance of the rat model of colon carcinogenesis to corresponding studies of human colon tumors.

EXPERIMENTAL

Cell Lines

The human colon carcinoma cell lines HT29 (HTB 38), SW480 (CCL 228), SW620 (CCL 227), and SW1417 (CCL 238), as well as the nonsecreting mouse myeloma cell line FO (CRL 1646), were obtained from the American Type Culture Collection (A.T.C.C., Rockville, MD) and cultured in Dulbecco's minimum essential medium (DMEM, high glucose) containing 10% fetal bovine serum. In routine testing, all of the cell lines used were found to be free of mycoplasma contamination.

Induction of Rat Colon Tumors With Azoxymethane

Female Sprague-Dawley rats (6–8 weeks old) were administered 8 weekly intraperitoneal injections of azoxymethane (15 mg/kg) (Ash Stevens, Detroit, MI) [33]. Four to 6 months later, animals were sacrificed, the intestine removed and washed in ice-cold PBS. Tumors were detected in the proximal small intestine as well as the descending colon. Tumors from the descending colon were excised and divided into two representative sections. One-half of the sample was fixed in 10% neutral buffered formaldehyde and routinely embedded in paraffin, while the other half was frozen immediately in liquid nitrogen and stored at −70°C until further analysis; 5-μm-thick sections of rat colon tumors were examined histologically following staining with hematoxylin and eosin (H & E) and classified according to the Astler-Coller modification of Duke's staging system for human colorectal cancer [38] (see footnote of Table III for summary of staging criteria).

Preparation of Rat Colon Tumor Microsomal Membranes (RTMM)

Tumor tissue was homogenized in 4 vol of 10 mM HEPES pH 7.4, 0.25 M sucrose, 1 mM EDTA, 0.5 mM dithiothreitol, 2 mM PMSF, 10 μg/ml aprotinin using a Polytron homogenizer (Brinkman Instruments, Edmonton, Alberta, Canada) and centrifuged at 300*g* for 10 min. The supernatant was collected and centrifuged at 25,000*g* for 60 min. The resultant microsomal pellet was solubilized with 50 mM Tris–HCl, pH 7.4, 1% Triton-X100 and centrifuged at 20,000*g* for 30 min. The supernatant was assayed for protein content using the Bio-Rad protein assay kit with gamma globulin as standard (Bio-Rad, Mississauga, Ontario, Canada), according to manufacturer's instructions. This preparation of microsomal membranes was used for immunizing mice and coating 96-well immunoassay plates (Immulon 2, Fisher Scientific, Edmonton, Alberta), as described below.

Monoclonal Antibodies

Monoclonal antibodies were prepared as described previously [37]. Briefly, female Balb/c mice were injected intraperitoneally with 100 μg of rat tumor microsomal membranes (RTMM) emulsified with Freund's complete adjuvant. Three, six and nine weeks later, mice were boosted intraperitoneally with 50 μg of RTMM. Serum was taken from each mouse and tested by solid-phase immunosorbent assay (ELISA) with RTMM, as described below. One month after the final boost, the mouse producing serum with the highest titer against RTMM was given three daily intraperitoneal injections of 25 μg RTMM. On the fourth day, spleen cells and F0 myeloma cells were fused in the presence of 40% polyethylene glycol 1540 by standard protocols [39] and 2–3 weeks later supernatants were tested by standard ELISA protocols, using 10 μg RTMM/well [39]. Positive hybridoma lines were subcloned twice by limiting dilution and stored frozen in liquid nitrogen. Hybridoma subclones were injected intraperitoneally into Balb/c mice for the production of ascitic fluid which was subsequently used for immunohistochemical staining, ELISA, immunoprecipitation and Western blotting analyses. Isotyping of hybridomas was carried out using the Isotyping Kit (Bio-Rad Laboratories, Mississauga, Ontario, Canada) ac-

cording to manufacturer's instructions. mAb 3A7 was found to be of the IgM subclass.

The mAb specific for blood group H determinant (Fucα1-2Gal-R) was generously provided by Dr. Monica Palcic (University of Alberta, Edmonton, Alberta, Canada) [40].

Hemagglutination Assay

Assays were carried out as described [41] using a 4% suspension of human A_1, A_2, B, or O erythrocytes.

Preparation of BSA–Glycan Conjugates

H type 2-BSA and A type 2-BSA conjugates were a generous gift from Dr. Monica Palcic (University of Alberta, Edmonton, Alberta, Canada). H type 2 oligosaccharide with an 8-methoxycarbonyl aglycone was chemically synthesized following a procedure described by Hindsgaul et al. [42], enzymatically converted to the A type 2 structure [43] and coupled to bovine serum albumin (BSA) via its acyl azide, as described by Pinto and Bundle [44]. The carbohydrate content of the conjugate was determined using a phenol sulfuric acid assay [45] with A type 2 as a reference standard. An incorporation of 57 moles of oligosaccharide per mole BSA was achieved (denoted as n = 57). All other BSA–glycan conjugates listed in Table 1 were generous gifts from the Alberta Research Council (Edmonton, Alberta, Canada). The incorporations achieved for the remaining compounds were as follows (where n = number of moles of oligosaccharide incorporated per mole BSA): H type 2-BSA (n = 19); A disaccharide–BSA (n = 17); A type 1-BSA (n = 13); A type 4-BSA (n = 17); A type 6-BSA (n = 13); B disaccharide–BSA (n = 19); B type 2-BSA (n = 18); B type 4-BSA (n = 14); B type 5-BSA (n = 15); B type 6-BSA (n = 16). Because of the different level of incorporation of oligosaccharide chains per BSA molecule, all BSA–glycan conjugates were diluted to a standard concentration of 50 nmol oligosaccharide/ml. Suitable dilutions of this stock solution were used in ELISA and Western blotting analyses, as described in the Results section.

Enzyme-Linked Immunosorbent Assay

RTMM (10 μg/well) or BSA–glycan conjugates were applied to 96-well microtiter plates (Immunolon-2) and incubated overnight at 4°C. Plates were washed with TBT (50 mM Tris–HCl pH 8.0, 0.1% BSA, 0.025% Tween-20, 0.02% sodium azide) and blocked at room temperature for 1 hr with 4% BSA. After washing with TBT, plates were incubated for 2 hr with 100 μl of mAb 3A7 (1:3,000 dilution in TBT), washed in TBT and subsequently incubated for 1 hr with 100 μl of alkaline phosphatase-coupled affinity-purified goat antimouse Ig (1:3,000 dilution in TBT) (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Plates were washed three times with TBT, once with TBS (50 mM Tris HCl, pH 8.0, 0.02% sodium azide) supplemented with 0.05% Tween-20, and twice with TBS. Immunoreactive material was detected colorimetrically at 405 nm following addition of the substrate *p*-nitrophenyl phosphate (Bio-Rad EIA kit).

TABLE I. Structure of Oligosaccharide Moiety of BSA–Glycan Conjugates

Name	Structure
H type 2	Fucα1-2Galβ1–4GlcNAc-BSA
A disaccharide	GalNAcα1–3Gal-BSA
A type 1	GalNAcα1–3[Fucα1–2]Gal β1–3GlcNAc-BSA
A type 2	GalNAcα1–3[Fucα1–2]Gal β1–4GlcNAc-BSA
A type 4	GalNAcα1–3[Fucα1–2]Gal β1–3GalNAc-BSA
A type 6	GalNAcα1–3[Fucα1–2]Gal β1–4Glc-BSA
B disaccharide	Galα1–3Gal-BSA
B type 2	Galα1–3[Fucα1–2]Gal β1–4GlcNAc-BSA
B type 4	Galα1–3[Fucα1–2]Gal β1–3GalNAc-BSA
B type 5	Galα1–3[Fucα1–2]Gal β1–3Gal-BSA
B type 6	Galα1–3[Fucα1–2]Gal β1–4Glc-BSA

Glycosidase Digestion

BSA–glycan conjugates containing 5 pmole oligosaccharide were digested for 24 hr at 37°C with either α-N-acetyl-D-galactosaminidase from *Acremonium* sp. (2 U/ml, Seikagaku, PDI BioScience, Calgary, Alberta, Canada) in 0.1 M sodium citrate buffer, pH 4.5 or α-D-galactosidase from green coffee beans (5 units/ml, Oxford GlycoSystems, Rosedale, New York) in 0.1 M sodium phosphate citrate, pH 6.5. Following enzyme digestion, samples were adjusted to a final volume of 100 μl with TBS pH 8.0, added to indi-

vidual wells of a 96-well Immulon-2 plate, and assayed by ELISA.

Immunohistochemical Staining of Normal and Neoplastic Rat Tissues

Adult rat large intestine as well as azoxymethane-induced colon tumors were harvested, washed in ice-cold phosphate-buffered saline (PBS), fixed in 10% neutral buffered formaldehyde and embedded in paraffin; 5-μm-thick sections were deparaffinized and prepared for immunohistochemical staining, as previously described [46]. Sections were then blocked for 30 min at room temperature with PBS containing 4% heat-inactivated normal goat serum (NGS) and subsequently incubated with either normal mouse serum or monoclonal antibody 3A7 (1:500 dilution in PBS/4% NGS). Sections were washed three times with PBS, incubated for 30 min with biotinylated goat antimouse IgG (1:400 dilution in 4% NGS/PBS) (Jackson Laboratories, Bio/Can Scientific, Mississauga, Ontario, Canada) followed by incubation with the avidin–biotin–complex (ABC reagent), according to manufacturer's instructions (Vector, Dimension Labs, Mississauga, Ontario, Canada). Following three washes in PBS, sections were dehydrated and mounted in Entellan (BDH, Saskatoon, Saskatchewan, Canada).

Preparation of Detergent Lysates of Tissues and Cells

Normal rat tissues, azoxymethane-induced colon tumors or HT29 cells were homogenized using a Polytron (Brinkmann Instruments, Edmonton, Alberta) in 4 vol of 50 mM Tris–HCl pH 7.4, 0.15 M NaCl, 1 mM PMSF, 1 mM benzamidine, 10 μg/ml aprotinin, 0.5 μM leupeptin, 0.7 μM pepstatin (buffer A). The sample was adjusted to 1% Triton X-100, incubated on ice for 1 hr and centrifuged at 20,000*g* for 30 min. The supernatant was assayed for protein using the Bio-Rad assay. For experiments involving lectin affinity chromatography of tissue or cell lysates on DBA- and HPA-agarose, which require dialysis and concentration of samples to small volumes (see below), tissue and cell lysates were prepared using the dialysable detergent CHAPS (0.5%).

SDS–PAGE

SDS–PAGE was performed as described by Laemmli [47]. [^{35}S]-methionine-labeled proteins separated by 7.5% SDS–PAGE were visualized by fluorography [48], using Enlightning (NEN, DuPont, Mississauga, Ontario, Canada).

Western Blotting Analysis

Proteins separated by SDS–PAGE were transferred electrophoretically onto nitrocellulose (Schleicher and Schull, Mandel Scientific, Guelph, Ontario, Canada) for 1 hr at 100 V. Nitrocellulose blots were blocked for 1 hr at room temperature or overnight at 4°C in TBS/4% BSA. Following three 5-min washes in TBT buffer, blots were incubated for 2 hr with NMS or mAb 3A7 diluted 1:3,000 in TBT. Following three 5-min washes in TBT, blots were incubated for 1 hr with alkaline phosphatase-coupled affinity purified goat antimouse Ig (Bio-Rad Laboratories, Mississauga, Ontario, Canada). The substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) were used for color development.

Affinity Chromatography of Normal Rat Colon, Azoxymethane-Induced Rat Colon Tumors and HT29 Cells on DBA- and HPA-Agarose

Detergent lysates of normal adult rat colon (2 g protein), rat colon tumors (2 mg protein), or HT29 cells (2 mg protein) were applied to a 5-ml column of *Dolichos biflorus* (DBA)-agarose (4 mg lectin/ml gel, E-Y Labs, Intermedico, Markham, Ontario, Canada) equilibrated in TBS pH 7.4, 0.5% CHAPS, 0.02% sodium azide and allowed to bind to the resin overnight at 4°C. The column was washed with 10 vol of 50 mM Tris–HCl pH 7.4, 0.5 M NaCl, 0.1% CHAPS, 0.02% sodium azide (DBA buffer), and subsequently eluted with 5 column volumes of DBA buffer containing 100 mM N-acetyl-D-galactosamine (GalNAc). Fractions of 1 ml were collected and assayed by Western blotting with mAb 3A7 (1:3,000 dilution). mAb 3A7-reactive glycoproteins that failed to bind to DBA-agarose following repeated application of the sample to the column were concentrated to 0.5 ml, using a Centricon 10 microconcentrator (Amicon, Beverly, MA) and applied to a 5-ml column of *Helix pomatia* (HPA)-agarose (1.9 mg lectin/ml gel, Sigma, St. Louis, MO). Elution of glycoproteins from HPA-agarose was carried out as described for DBA-agarose affinity chromatography. The combined recovery of protein from HPA-unbound and eluted fractions obtained from each

sample was 75–85%, as measured with the Bio-Rad protein assay.

Metabolic Labeling of Cells

Cell monolayers grown to 70–80% confluence were washed twice with D-PBS and radiolabeled for 24 hr in methionine-free DMEM supplemented with one-tenth the normal concentration of methionine, 10% FCS, and 50 μCi of [^{35}S]methionine/ml (800 Ci/mmol; NEN, Calgary, Alberta, Canada). The plates were washed three times with ice-cold PBS, and the cells were harvested with a rubber policeman and pelleted. Cell pellets were solubilized on ice for 1 hr in buffer A containing 1% Triton X-100 and cleared by centrifugation at 22,500*g* for 30 min.

Immunoprecipitation

[^{35}S]Methionine-labeled cell lysates (2×10^6 cpm) were incubated overnight at 4°C with 3 μl of normal mouse serum or mAb 3A7 ascitic fluid. Then, 100 μl of a 1:1 suspension of protein A Sepharose-4B beads (Pharmacia, Montreal, Canada) was added and the suspension gently rocked at 4°C for 1 hr. After washing the beads once with immunoprecipitation (IP) buffer (50 mM Tris–HCl pH 8.0, 1 mM PMSF, 1 mM benzamidine, 1% deoxycholate, 1% Triton X-100, 0.02% sodium azide), twice with IP buffer containing 0.5M NaCl, twice with IP buffer containing 0.1% SDS and once with IP buffer, the immunoprecipitated proteins were eluted by boiling for 5 min in SDS–PAGE sample buffer (0.125 M Tris–HCl pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol).

RESULTS

Characterization of the Epitope Detected by mAb 3A7

Recent studies in this laboratory have focused on identifying and characterizing glycoproteins that may contribute directly to colon carcinogenesis and metastasis [37]. Our approach has consisted of preparing monoclonal antibodies to membrane fractions of human colon cancer cell lines [37] or, as described in this report, a chemically induced rat colon tumor. mAb 3A7 was selected for further study because it detected an epitope expressed at higher level in azoxymethane-induced colon tumors compared to normal colon (cf. Figs 4B and 6), suggesting that this epitope may constitute a useful marker of colon cancer progression.

Several approaches were used to elucidate the structure of the epitope detected by mAb 3A7. mAb 3A7 was found to agglutinate both A_1 and A_2 erythrocytes at titers of 1:51,000 and 1:12,800, respectively. Significant reactivity of the antibody towards blood group B red blood cells was also observed (titer of 1:12,800). In contrast, no hemagglutination was detected using type O cells, even at 1:100 dilution of the antibody.

The specificity of the antibody was characterized further by Western blotting, using a panel of blood group A (GalNAcα1–3[Fucα1–2]Gal-R) and B (Galα1–3[Fucα1–2]Gal-R) variant oligosaccharides chemically coupled to bovine serum albumin (BSA) (see Table I for structures). Analysis of the reactivities of the available BSA-glycan conjugates with mAb 3A7 revealed that the antibody specifically recognizes A type 2-BSA (Fig. 1, lane 4), A type 6-BSA (Fig. 1, lane 6) and B type 2-BSA (Fig. 1, lane 8). In contrast, no binding was detected with H type 2-BSA (Fig. 1, lane 1), A and B disaccharides (Fig. 1, lanes 2 and 7, respectively) or with any of the BSA-glycans containing A and B determinants present on a type 1 chain (Galβ1–3GlcNAc) (Fig. 1, lanes 3, 5, 9, 10). The different sizes of BSA-glycans detected in Western blots most likely result from the different numbers of glycan chains per BSA molecule (see Experimental section) as well as the different sizes of glycan chains in the various BSA conjugates (see Table I).

While Western blotting analysis identified which of the BSA-glycans were recognized by mAb 3A7, it did not provide any information about the relative affinity of binding of the antibody to these glycoconjugates. Using a solid-phase immunosorbent assay (ELISA), we examined binding of mAb 3A7 to serial dilutions of each of the BSA-glycans listed in Table I. As can be seen in Figure 2, the strongest reactivity of the antibody was observed with A type 2-BSA (GalNAcα1–3[Fucα1–2]Galβ1–4GlcNAc-BSA, closed squares) and B type 2-BSA (Galα1–3[Fucα1–2]Galβ1–4GlcNAc-BSA, closed triangles). By contrast, the relative affinity of mAb 3A7 for A type 6-BSA (closed circles) (GalNAcα1–3[Fucα1–2]Galβ1–4Glc-BSA) was much lower than A- and B-type 2-BSA, as shown by the decreased slope of the curve [49]. In fact, little binding was detected with B type 6-BSA (open diamonds).

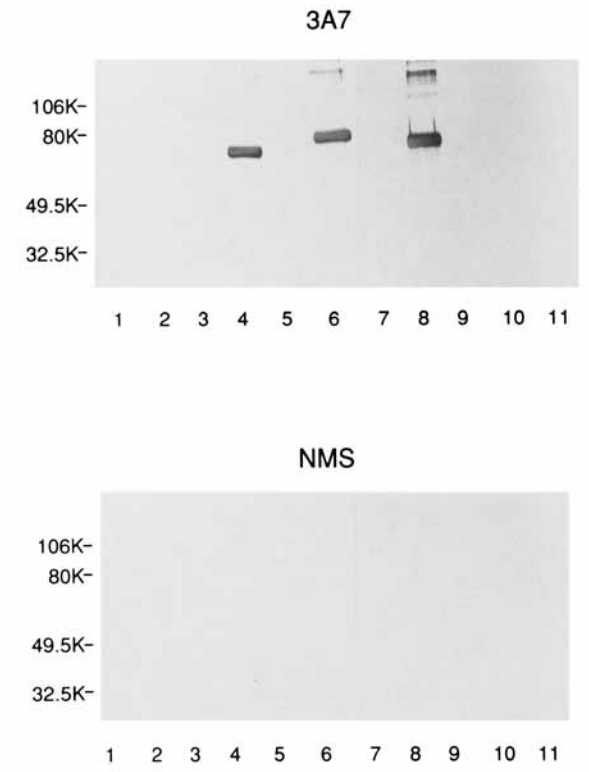


Fig. 1. Western blotting analysis of BSA–glycan conjugates. BSA-glycan conjugates (1 nmole oligosaccharide), depicted in Table I were separated by 7.5% SDS-PAGE, transferred to nitrocellulose and blotted with mAb 3A7 or normal mouse serum (NMS) (1:3,000, 2 hr) followed by alkaline-phosphatase coupled goat antimouse Ig (1:3,000, 1 hr). Lane 1, H-type 2-BSA; lane 2, A disaccharide-BSA; lane 3, A type 1-BSA; lane 4, A type 2-BSA; lane 5, A type-4-BSA; lane 6, A type 6-BSA; lane 7, B disaccharide-BSA; lane 8, B type 2-BSA; lane 9, B type 4-BSA; lane 10, B type 5-BSA; lane 11, B type 6-BSA. The numbers in the left margin represent sizes of prestained molecular weight standards.

In order to confirm that the terminal α1–3 linked sugar in A and B determinants (i.e., GalNAc or Gal, respectively) is an integral part of the epitope recognized by mAb 3A7, A type 2-BSA and B type 2-BSA were digested with α-N-acetyl-D-galactosaminase from *Acremonium* sp. or α-D-galactosidase from green coffee beans, respectively, which specifically remove the α1–3-linked terminal sugars. As shown in Table 2, removal of either N-acetyl-D-galactosamine or D-galactose from the BSA-glycan samples greatly reduced binding of mAb 3A7 to these glycoconjugates. To rule out the possibility that the decrease in antibody binding observed in the enzyme-treated BSA conjugates resulted from destruction of the epitope by possible contaminating glycosidases present in the enzyme preparations, enzyme-treated and untreated A type 2 and B type 2-BSA were tested for their ability to bind to a monoclonal antibody specific for blood group H determinant, the expected product of glycosidase treatment (see structures in Table I). As can be seen from Table II, removal of terminal α1,3-linked N-acetyl-D-galactosamine or D-galactose from A type 2-BSA or B type 2 BSA, respectively, generated glycans that were detectable by anti-H antibody, whereas untreated samples were only recognized by mAb 3A7.

Immunohistochemical Analysis of Normal Rat Colon and Azoxymethane-Induced Colon Tumors Using mAb 3A7

To investigate the potential value of mAb 3A7 for studies of colon cancer progression, we compared normal rat colon with a panel of azoxymethane-induced colon tumors by immunohistochemical staining. Figure 3 depicts the immunohistochemical staining pattern of normal adult large intestine as well as representative azoxymethane-induced colon tumors obtained with mAb 3A7. In normal colon, expression of the epitope was highest in the cecum (Fig. 3A), followed by ascending (data not shown) and descending colon (Fig. 3B). In the cecum, antibody staining was detected in the entire epithelium with the strongest staining localized within the crypts. In contrast, staining in the ascending and descending colon was restricted to the lower half of the crypts, which is within the proliferative zone of the colonic epithelium [50]. Furthermore, antibody staining predominantly involved the columnar absorptive cells with lesser staining of the goblet cells.

Analysis of azoxymethane-induced rat colon tumors revealed several marked changes in the pattern of antibody staining. As shown in Table III, colonic tumors were assessed histologically with respect to their degree of differentiation and invasiveness, using as criteria the Astler-Coller modification of Duke's staging system for human colorectal carcinomas (see figure legend of Table III for summary of staging criteria) [38]. Twenty-one colon tumors were examined, which included intramucosal adenocarcinomas (Duke's A), invasive conventional adenocarcinomas (Duke's B1 and B2), and tumors that had metastasized to the lymph nodes (Duke's C). In addition to conventional adenocarcinomas, some of the tumors were of signet-ring cell type. Liver metastases were not detected in any of the tumor-bearing animals. All the colonic neoplasms

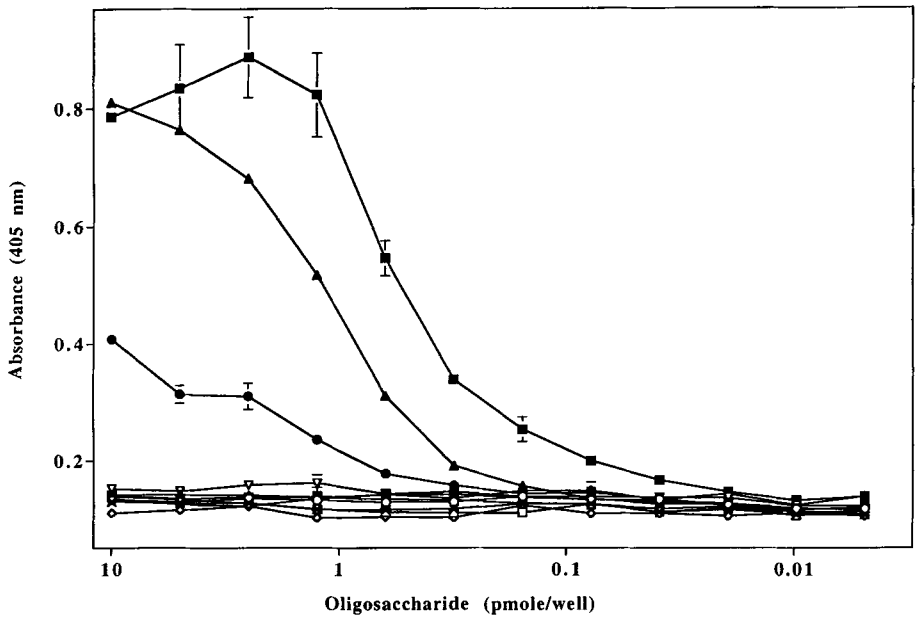


Fig. 2. Solid-phase immunosorbent assay of BSA–glycan conjugates with mAb 3A7. Serial dilutions of BSA-glycan conjugates (10–0.01 pmole oligosaccharide/well), depicted in Table I, were added in triplicate to 96-well Immulon-2 plates and incubated overnight at 4°C. An ELISA was carried out using a 1:3,000 dilution of mAb 3A7. Color development was monitored at 405 nm following addition of the substrate p-nitrophenyl phosphate. Each point on the graph represents the mean and standard deviation (note that for some of the data points, the standard deviation does not appear because values were lower than graphic resolution). H type 2-BSA (◆); A disaccharide-BSA (×); A type 1-BSA (○); A type 2-BSA (■); A type 4-BSA (◩); A type 6-BSA (●); B disaccharide (□); B type 2-BSA (▲); B type 4-BSA (△); B type 5-BSA (▽); B type 6-BSA (◇).

TABLE II. Effect of Partial Deglycosylation of A Type 2 and B Type 2-BSA Conjugates on mAb 3A7 and Anti-H Binding*

	MAb 3A7	Anti-H	NMS
A type 2-BSA			
Untreated	1.079 ± 0.030[a,b]	0.169 ± 0.004	0.161 ± 0.004
20 mU α-N-acetylgalactosaminidase	0.189 ± 0.047	0.463 ± 0.055	0.148 ± 0.002
B type 2-BSA			
Untreated	1.023 ± 0.013	0.168 ± 0.007	0.154 ± 0.005
20 mU α-galactosidase	0.295 ± 0.028	0.934 ± 0.005	0.177 ± 0.001

*A type 2-BSA or B type 2-BSA conjugates (5 pmol oligosaccharide) were digested with α-N-acetyl-D-galactosaminidase from *Acremonium* sp. or α-D-galactosidase from green coffee beans, respectively, and assayed by ELISA with normal mouse serum (NMS) and mAb 3A7 at 1:3000 dilution or affinity-purified anti-H immunoglobulin (300 μg/ml), as described in the Experimental section.

[a]Optical density at 405 nm was recorded 3 hr after addition of substrate. Numbers indicate the mean optical density of triplicate samples and the standard deviation.

[b]Optical density at 405 nm of control wells containing only TBS pH 8.0 was 0.120 ± 0.010.

examined showed variable slight to marked immunoreactivity with the antibody. In general, there was a gradation in the degree of immunoreactivity. Most areas in the invasive cancers were strongly positive (Duke's B2, Fig. 3D) whereas with the early lesions (Duke's A, Fig. 3C), there were some sizeable areas that were only slightly positive. However, lymph node metastases (Duke's C, see Table III) tended to stain less intensely than invasive primary tumors (Duke's B2). When compared to conventional colonic adenocarcinomas (Fig. 3D), signet-ring adenocarcinomas stained with equal intensity (Fig. 3E, arrow). However, the neoplastic cells in the latter were associated with relatively little stroma (Fig. 3F); hence their staining intensity tended to be more diffuse. As is the case in humans, the signet-ring tumors tended to be

biologically more aggressive than conventional adenocarcinomas as shown by the greater propensity of the former for vascular invasion (Fig. 3G, arrow).

The transitional mucosa adjacent to colonic neoplasms stained differently from normal colonic mucosa in a number of aspects. First, unlike normal mucosa, the epithelial staining was not restricted to the crypts but tended to extend further up toward the surface epithelium (Fig. 3G). Second, there appeared to be relatively more staining on the luminal aspects of enterocytes in transitional epithelium than in normal enterocytes (Fig. 3G). Third, there was no regularity in the areas that stained. For example, there were areas of strong positive staining in the vicinity of areas that were otherwise negative (Fig. 3G). On routine hematoxylin and eosin staining, some of these areas of positivity corresponded to intramucosal dysplastic changes whereas other areas of positivity appeared unremarkable (Fig. 3H). This suggests that the antibody may be detecting preneoplastic changes that are not apparent on the routine hematoxylin and eosin stain.

Expression of Glycoproteins Detected by mAb 3A7 in Normal Rat Tissues

While imunohistochemical analysis of normal and neoplastic colon provided valuable information about the morphological distribution of epitopes detected by mAb 3A7, it did not provide any information about the biochemical nature of glycoconjugates bearing epitopes detected by this antibody. Preliminary evidence indicated that the epitope was undetectable in the neutral and acidic glycolipid fractions extracted from normal and neoplastic rat colon (data not shown). By contrast, Western blotting analysis of detergent-solubilized adult rat tissues revealed that the epitope detected by mAb 3A7 is found on glycoproteins which are expressed primarily in gastrointestinal tissues, including stomach, small intestine, and to a lesser extent large intestine (Fig. 4). Several glycoproteins, including species of 130–140 kDa, 90 kDa, and 60 kDa, were detected in proximal small intestine (Fig. 4B, lane 1). Glycoprotein species of 130–140 kDa were also detected in the middle and distal portion of the small intestine (Fig. 4B, lanes 2 and 3), however, the structural relationship of this glycoprotein species to that detected in proximal small intestine has not yet been determined. In the large intestine, mAb 3A7 detected primarily a glycoprotein species of 140 kDa that was expressed at higher level in the ascending and descending colon than in the cecum (Fig. 4, panel B, lanes 4, 5, 6).

Similar analysis of fetal and neonatal rat intestine revealed that expression of the epitope detected by mAb 3A7 is developmentally regulated (Fig. 5). The epitope was first expressed at low level on day 19 of gestation, coincident with emergence of simple columnar epithelium, appearance of globlet cells and expression of enzymes such as lactase and aminopeptidase N [50,51]. From postnatal days 1–3, during which time the distal intestine develops a typical colon morphology [50,51], the level of expression remained low in the proximal (Fig. 5, upper panel) as well as distal (Fig. 5, lower panel) half of the intestine. On day 6, which coincided with the enlargement of the cecum resulting in a clear demarcation between small and large intestines, there was a significant increase in the level of staining of the large intestine by mAb 3A7. This elevated level of staining persisted until weaning occurred on day 21 (Fig. 5, upper panel). By day 31, the level of staining in the colon had dramatically decreased to that found in adult colon. By contrast, staining of the small intestine (Fig. 5, lower panel) increased gradually from postnatal days 6–31, where the level reached that detected in adult small intestine.

Western Blotting Analysis of Azoxymethane-Induced Rat Colon Tumors

Western blotting analysis of detergent-solubilized colon tumors using mAb 3A7 revealed a quantitative increase in staining of tumor extracts compared to normal colon (cf. Figs. 4B and 6). Glycoproteins ranging in size from 50 kDa to 200 kDa were detected, including a major species at 140 kDa. In all samples examined, the intensity of staining of tumors was higher than normal colon analyzed. However, examination of colon tumors of a given histopathological stage showed variation in the intensity of staining which may result from heterogeneity in antigen expression within the tumor, as was observed by immunohistochemical staining (Fig. 3). In addition, individual tumors contained variable amounts of residual "normal" mucosa that would result in an apparent decrease in signal intensity detected by Western blotting. As can

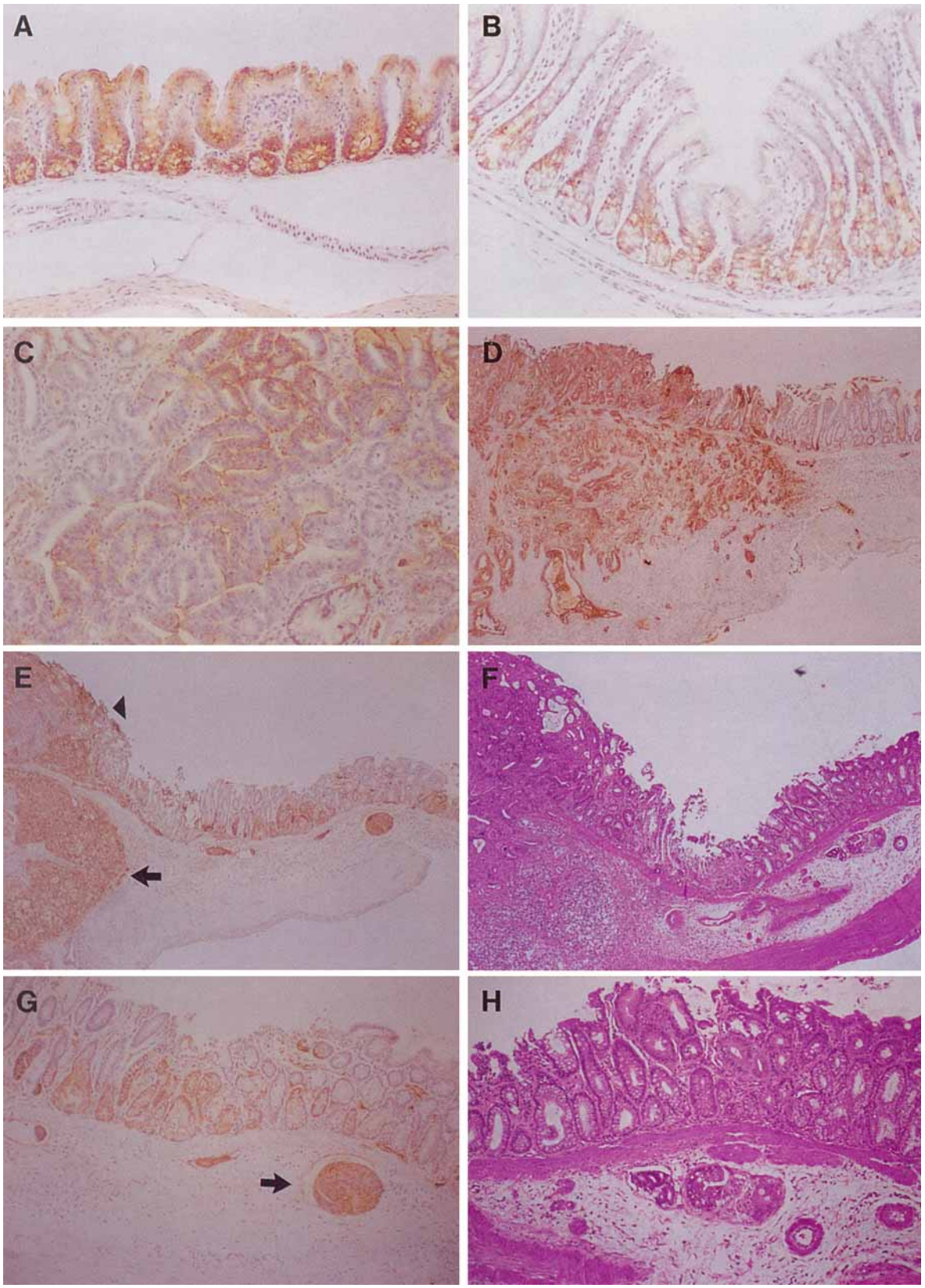


Figure 3

be seen from Table III, there was generally good agreement between immunohistochemical staining and Western blotting data. In confirmation of immunohistochemical staining data presented above (Fig. 3 and summary in Table III), increased expression of mAb 3A7-immunoreactive glycoproteins was an early event in colon carcinogenesis, since many of the early-stage carcinomas (Duke's A and B1) stained strongly with the antibody. Well-differentiated A and B1 tumors stained more intensely with the antibody than moderately and poorly differentiated tumors. Moderate to high level of expression of mAb 3A7 immunoreactive glycoconjugates was consistently detected in the more advanced invasive tumors (Duke's B2), with the poorly differentiated signet-ring type tumors showing the most intense staining. Examination of two lymph node metastases (Duke's C2, see Table III) showed very different staining patterns with the antibody. One of the samples stained intensely by Western blotting (Fig. 6, lane 20) but not by immunohistochemical staining (Fig. 6, lane 20), whereas the other showed little staining using either method (Fig. 6, lane 21 and Table III).

TABLE III. Comparative Analysis of Histopathological Staging of Azoxymethane-Induced Colon Tumors and mAb 3A7 Reactivity Measured by Western Blotting and Immunohistochemical Staining

Tumor[a]	Stage[b]	Tumor type and degree of differentiation[c]	Western blotting[d]	IHC[e]
Azo1.6	A	WD (cv)	+++	+++
Azo3.11	A	WD (mc)	+	+++
Azo6.9	A	WD (cv)	++	++
Azo7.8	A	WD (cv)	+++	++
Azo2.8	B1	WD (cv)	+++	+++
Azo5.11	B1	WD (cv)	++	++
Azo6.5	B1	WD (mc)	+	+
Azo6.6	B1	MD (cv)	+	+
Azo6.8	B1	MD (cv)	+	++
Azo4.1	B1	PD (sg)	+/−	+/−
Azo2.7	B2	WD (cv)	++	++
Azo5.10	B2	WD (cv)	++	+++
Azo1.7	B2	MD (cv)	++	+++
Azo1.10	B2	MD (cv)	++	++
Azo2.9	B2	MD (cv)	++	++
Azo3.9	B2	MD (cv)	++	+++
Azo1.8	B2	PD (sg)	+++	+++
Azo1.9	B2	PD (cl)	+++	+++
Azo7.11	B2	PD (mc)	+++	+++
Azo4.13	C2	PD (sg)	+++	+
Azo4.15	C2	PD (sg)	+/−	+

[a]The first digit refers to the rat from which the tumor was removed and the number following the decimal point refers to the individual tumor removed from that animal.
[b]Azoxymethane-induced tumors were assessed histologically according to the Astler-Coller-modified Duke's staging of colorectal carcinoma (37) (A, mucosal involvement; B1, extending into, but not penetrating, the muscularis propria; B2, invasion into the entire wall with uninvolved nodes; C1, limited to wall with involved nodes; C2, through all layers of wall with involved nodes. Tumor types included conventional (cv), mucinous (mc), signet-ring type (sg), or collision (cl) (made up of both conventional and signet-ring types).
[c]WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.
[d]Summary of Western blotting data shown in Figure 6. The plus signs refer to the relative staining intensity for each tumor sample: +++, marked; ++, moderate; +, slight; +/−, weak/minimal.
[e]Summary of immunohistochemical (IHC) staining intensity of paraffin-embedded tumor sections obtained with mAb 3A7 (1:500 dilution). Representative examples are shown in Figure 3. The plus signs refer to the relative staining intensity of the neoplastic region within a given tumor: +++, marked; ++, moderate; +, slight; +/−, weak/minimal.

Fig. 3. Immunohistochemical staining of normal rat intestine and rat colon tumors with mAb 3A7. Segments of small and large intestine as well as representative azoxymethane-induced rat colon tumors were examined by immunohistochemical staining using either normal mouse serum or mAb 3A7 (1:500 dilution), as described in the Experimental section. Only staining patterns obtained with mAb 3A7 are shown, since parallel staining of sections with normal mouse serum yielded negative results. **A:** Normal cecum depicting generalized staining of the epithelium with the strongest staining present in the crypt zones ×200. **B:** Normal mucosa from the descending colon illustrating immunopositivity for the antibody predominantly in the crypt zones (original magnification, ×150). **C:** Duke's stage A colonic adenocarcinoma (tumor specimen Azo 1.6 depicted in Table IV) showing areas of weak positivity, as well as other areas of marked positivity. ×200. **D:** Conventional Duke's B2 adenocarcinoma (tumor specimen Azo 1.7) depicting a marked degree of positivity for the antibody. ×40. **E:** Collision colonic adenocarcinoma with both a conventional adenocarcinoma (arrowhead) and a signet-ring carcinoma pattern (arrow). Note the more diffuse staining in the signet-ring carcinoma. ×30. **F:** Hematoxylin and eosin-stained micrograph from an area of a step section roughly equivalent to Figure 3E. ×30. **G:** High-power view of the mucosa adjacent to the tumor in Figure 3E. This highlights the irregularity in the staining of this transitional mucosa, with some focal areas close to the lumen markedly positive (arrow). Note the submucosal vessels depicting vascular invasion. ×200. **H:** Hematoxylin and eosin-stained micrograph is from an area of a step-section roughly equivalent to Figure 3G. In contrast to Figure 3G, no overtly abnormal areas are seen in the transitional mucosa. ×200.

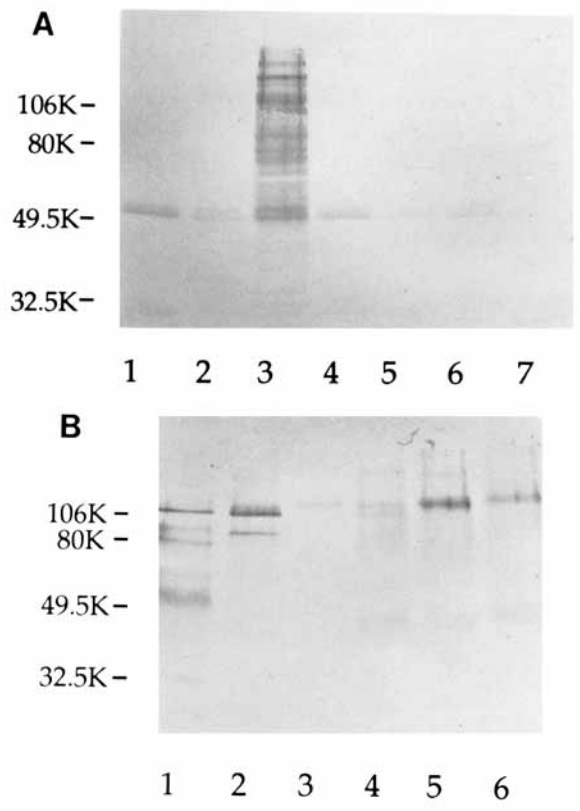


Fig. 4. Expression of glycoproteins detected by mAb 3A7 in *normal adult rat tissues.* **A:** Detergent-solubilized rat tissues containing 20 μg protein separated by SDS–PAGE (7.5%), transferred to nitrocellulose, and blotted with mAb 3A7 (1:3,000 dilution). Lane 1, lung; lane 2, liver; lane 3, stomach; lane 4, kidney; lane 5, spleen; lane 6, heart; lane 7, brain. **B:** Detergent-solubilized rat intestine containing 20 μg protein separated by SDS–PAGE (7.5%), transferred to nitrocellulose, and blotted with mAb 3A7 (1:3,000). Proximal (lane 1), medial (lane 2), and distal (lane 3) segments of rat small intestine as well as the cecum (lane 4), ascending (lane 5), and descending (lane 6) regions of the large intestine were analyzed.

Characterization of mAb 3A7-Immunoreactive Glycoproteins from Normal Colon and Azoxymethane-Induced Rat Colon Tumors

To investigate further the possible differences in carbohydrate structure associated with mAb 3A7-immunoreactive glycoproteins in normal and neoplastic colon, detergent lysates of normal rat colon and azoxymethane-induced rat colon tumors representative of different histopathological type were subjected to affinity chromatography on agarose columns containing the blood group A-specific lectins *Dolichos biflorus* (DBA) and *Helix pomatia* (HPA). In contrast to *Helix pomatia*, which recognizes blood group A determinants irrespective of the core oligosaccharide structure [52], *Dolichos biflorus* lectin exhibits more restricted specificity [53]. DBA has been shown to bind A_1 erythrocytes with higher affinity than A_2 erythrocytes [54], although the structural basis of this difference is unclear. In addition, studies have shown that the best inhibitor of DBA binding is a type 2 chain blood group A oligosaccharide having the structure GalNAcα1–3[Fucα1–2]Galβ1–4GlcNAcβ1-6–R) [53]. As shown in Figure 7A, most mAb 3A7-immunoreactive glycoproteins from normal colon (lanes 1 and 2) were adsorbed to DBA-agarose (note that 100-fold more normal colon protein was applied to the column compared to tumor extracts because of the low level of mAb 3A7 immunoreactive glycoproteins in normal rat colon). Glycoproteins ranging in size between 50 and 200 kDa were specifically eluted from the column, including a major species of 140 kDa (Fig. 7A, lane 2). Similar analyses were carried out on a panel of azoxymethane-induced rat colon tumors which included a Duke's A intramucosal carcinoma (Fig. 7A, lanes 3 and 4), a Duke's B1 carcinoma (Fig. 7A, lanes 5 and 6), a B2 carcinoma (Fig. 7A, lanes 11 and 12), a B2 collision tumor (conventional adenocarcinoma and signet-ring cell carcinoma, Fig. 7A, lanes 7 and 8), the original rat tumor used for the production of mAb 3A7 (Fig. 7A, lanes 9 and 10) and a lymph node metastasis (Duke's C2) (Fig. 7A, lanes 13 and 14). Western blotting analysis of DBA-agarose unbound (U) and eluted (E) fractions from each sample revealed an apparent cancer-associated decrease in the ability of mAb 3A7-immunoreactive glycoproteins to bind to DBA-agarose. In contrast to normal colon, glycoproteins from Duke's A, B1, B2, and C2 tumors were distributed in both DBA-unbound and eluted fractions. In fact, the Duke's A tumor (Fig. 7A, lane 4), one of the B2 tumors (Fig. 7A, lane 12) and the C2 tumor (Fig. 7A, lane 14) contained very little material in the DBA-eluted fraction. When mAb 3A7-immunoreactive glycoproteins from the DBA-unbound fractions were subsequently applied to HPA-agarose, nearly all of the immunoreactive material was recovered in the eluted fractions, confirming the presence of blood group A determinants on these glycoproteins (Fig. 7B). Comparison of the glycoprotein profiles of HPA-eluted fractions from various tumors revealed an apparent reduction in the number of glycoprotein species detected by mAb 3A7 in the more aggressive tumors, concomi-

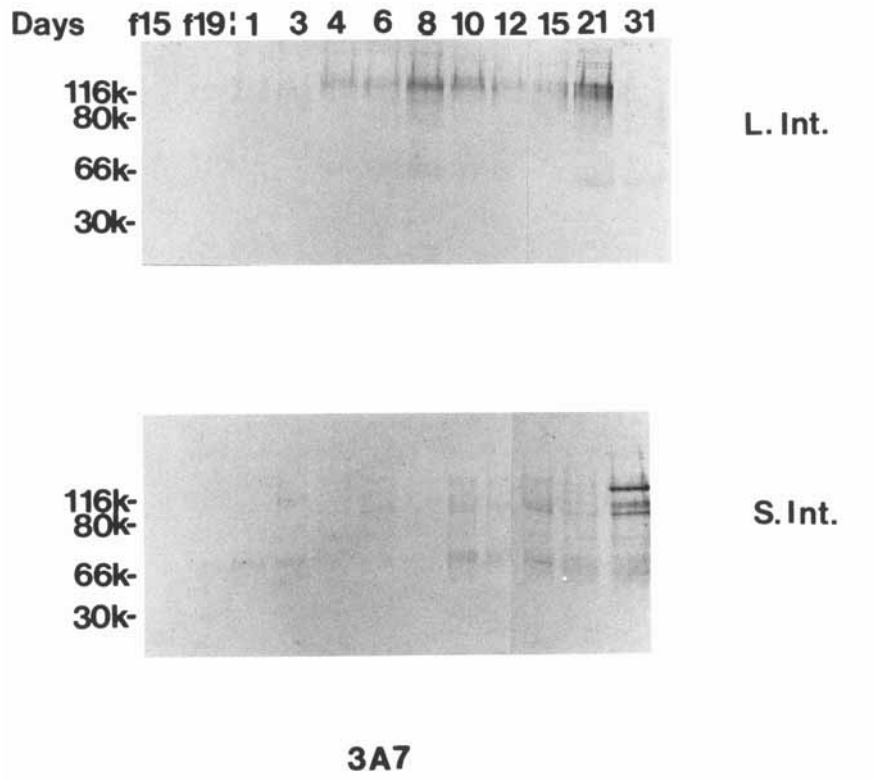


Fig. 5. Western blotting analysis of fetal and neonatal rat intestine with mAb 3A7. Segments of intestine were isolated from fetal (day 15 and 19 of gestation) and neonatal rats (day 1–31 after birth). Detergent-solubilized extracts containing 20 μg protein were separated by SDS–PAGE (7.5%), transferred to nitrocellulose, and blotted with mAb 3A7 (1:3,000). **Upper:** large intestine (L. int.); **lower:** small intestine (S. int.). Note that the fetal intestine samples removed at day 15 (f15) and 19 (f19) of gestation are shown in both upper and lower panels. Also, neonatal intestine removed on day 1 to 5 was divided into a proximal segment (upper) and distal segment (lower) because of the lack of clear demarcation between small and large intestines. A well-defined cecum appears on postnatal day 6.

tant with an increased expression of a major glycoprotein species of 140 kDa.

Differential binding of mAb 3A7 immunoreactive glycoproteins to DBA lectin was not unique to rat colon tumors. Affinity chromatography of a detergent lysate of the human colon carcinoma cell line HT29 on DBA-agarose revealed that the 140-kDa glycoprotein, which is the major mAb 3A7-immunoreactive species, was present in the unbound fraction (Fig. 7A, lane 15). By contrast, a less abundant 120-kDa species was specifically eluted from DBA-agarose (Fig. 7A, lane 16). Subsequent chromatography of the DBA-unbound fraction on HPA-agarose resulted in quantitative recovery of the 140-kDa glycoprotein in the eluted fraction (Fig. 7B, lane 14). The structural relationship between the 140-kDa glycoproteins present in HPA-agarose eluted fractions of rat colon tumors and HT29 cells has not yet been determined.

Expression of Glycoproteins Detected by mAb 3A7 in Human Colon Carcinoma Cell Lines

To determine whether glycosylation changes observed in the azoxymethane model of colon carcinogenesis are relevant to the study of human colon cancer, expression of glycoproteins detected by mAb 3A7 was also analyzed in four human colon cancer cell lines established from tumors differing in histopathological stage. These included the moderately differentiated cell line HT29 (blood group A) established from a grade II primary tumor [55], the poorly differentiated cell line SW1417 (blood group B) established from a grade III primary tumor [56], the poorly differentiated cell lines SW480 (blood group A) and SW620 (blood group A) [56] established from a grade III/IV tumor and lymph node metastasis, respectively, isolated from the same patient. A major glycoprotein species with apparent molecular weights of 140 kDa and 132

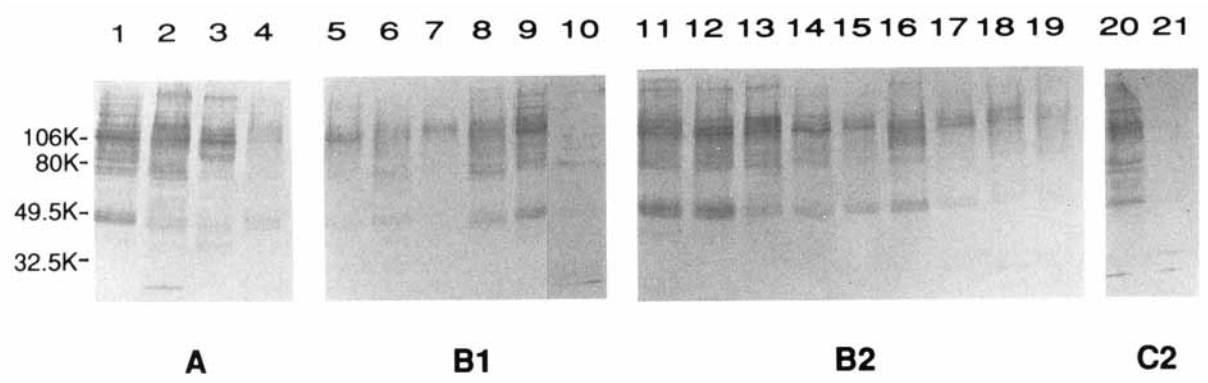
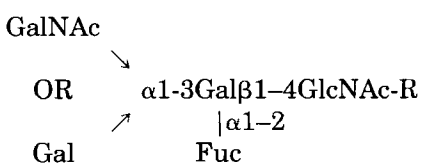


Fig. 6. Western blotting analysis of azoxymethane-induced rat colon tumors with mAb 3A7. Rat colon tumors (listed in Table 3) were homogenized and solubilized in the presence of Triton-X-100. Aliquots containing 20 μg protein were separated by SDS–PAGE (7.5%), transferred to nitrocellulose and blotted with mAb 3A7 (1:5,000 dilution). Samples included Duke's A adenocarcinoma (A), Duke's B1 and B2 adenocarcinomas and Duke's C2 tumors (lymph node metastasis). Lane 1, Azo 1.6; lane 2, Azo 7.8; lane 3, Azo 6.9; lane 4, Azo 3.11; lane 5, Azo 6.8; lane 6, Azo 6.6; lane 7, Azo 6.5; lane 8, Azo 5.11; lane 9, Azo 2.8; lane 10, Azo 4.1; lane 11, Azo 1.8; lane 12, Azo 1.9; lane 13, Azo 7.11; lane 14, Azo 2.9; lane 15, Azo 2.7; lane 16, Azo 1.10; lane 17, Azo 1.7; lane 18, Azo 3.9; lane 19, Azo 5.10; lane 20, Azo 4.13; lane 21, Azo 4.15.

kDa was immunoprecipitated by mAb 3A7 from lysates of [^{35}S]methionine labeled HT29 and SW480 cells, respectively (Fig. 8, lanes 2 and 6, respectively). In contrast, the lymph node metastasis cell line SW620 (Fig. 8, lane 8) failed to synthesize appreciable levels of the 140 kDa glycoprotein recognized by the antibody, suggesting that there was a block in the synthesis of the polypeptide chain or synthesis of the determinant recognized by the antibody. The absence of detectable mAb 3A7 immunoreactive glycoconjugates in the blood group B cell line SW1417 suggested that these cells do not synthesize blood group B epitopes on a type 2 core. In fact, Dahiya et al. [57] have shown that SW1417 cells fail to express blood group B determinants, despite sufficient levels of B transferase.

DISCUSSION

This report describes the isolation and characterization of a monoclonal antibody prepared against an azoxymethane-induced rat colon tumor. mAb 3A7 detects an oncodevelopmentally expressed carbohydrate epitope in rat colon that is also present in some human colon cancer cells. Hemagglutination of human erythrocytes, Western blotting (Fig. 1), and solid-phase immunosorbent assays of BSA-glycan conjugates carrying variant A and B blood group determinants (Fig. 2), have established that mAb 3A7 recognizes a determinant found on type 2 chain blood group A (GalNAcα1–3[Fucα1-2]Galβ1–4GlcNAc-R) and B (Galα1–3[Fucα1–2]Galβ1–4GlcNAc-R) oligosaccharides. Loss of antibody reactivity following enzymatic removal of the terminal α1–3 linked N-acetyl-D-galactosamine or D-galactose from A type 2-BSA or B type 2-BSA, respectively, confirmed the requirement for an α1–3 linked sugar for optimal antibody binding. The fact that both N-acetyl-D-galactosamine and D-galactose are recognized equally well by the antibody in ELISA (Fig. 2), suggested that the N-acetyl group on N-acetyl-D-galactosamine plays a lesser role in proper positioning of the epitope within the antibody combining site. By contrast, replacement of the N-acetylglucosamine in the N-acetyllactosamine unit (Galβ1–4GlcNAc-R) by a glucose residue, as is found in A type 6-BSA (GalNAcα1–3[Fucα1–2]Galβ1–4Glc-R), resulted in decreased antibody binding. Collectively, these data suggest that the antigen-binding site on mAb 3A7 can accomodate a tetrasaccharide made up of at least three essential components: an α1–3 linked D-galactose or N-acetyl-D-galactosamine, an α1–2 linked L-fucose and an N-acetyllactosamine unit (i.e., type 2 chain, Galβ1–4GlcNAc-R), as shown below:



The specificity of mAb 3A7 for type 2 chains bearing either blood group A or B determinants as well as its ability to distinguish A type 2 and A type 6 structures are not unique, since Oriol et al. [40] have previously described a subset of anti-AB monoclonal antibodies having a similar

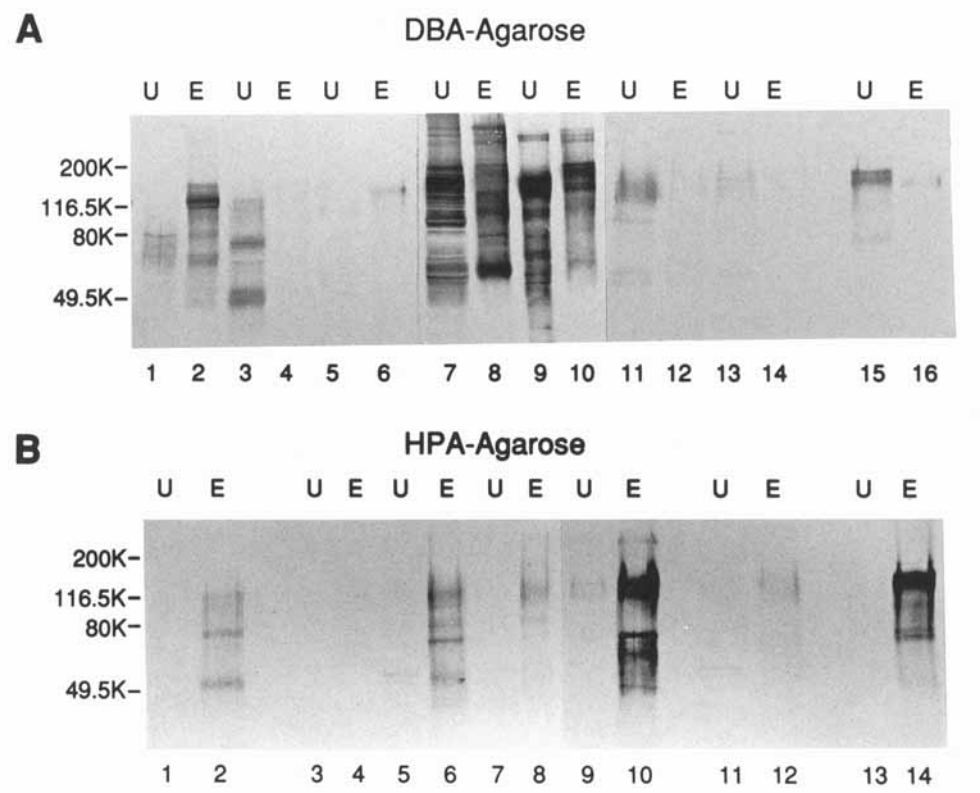


Fig. 7. Serial lectin-affinity chromatography of detergent solubilized normal rat colon, rat colon tumors, and HT29 cells on *Dolichos biflorus*- and *Helix pomatia*-agarose. **A:** Detergent lysates of normal colon (2 g protein), azoxymethane-induced tumor extracts of different histopathological types (2 mg protein) or HT29 cells (2 mg protein) were applied to *Dolichos biflorus* (DBA)-agarose and the column washed extensively prior to elution with 5 mM N-acetylgalactosamine. Unbound (U) and eluted (E) fractions were collected, pooled separately, and concentrated to 0.5 ml; 20-μl aliquots were analyzed by Western blotting with mAb 3A7 (1:3,000 dilution). Lanes 1, 2, normal colon; lanes 3, 4, Duke's A (Azo 1.6); lanes 5, 6, Duke's B1 (Azo 4.1); lanes 7, 8, Duke's B2 collision tumor (conventional adenocarcinoma and signet ring type carcinoma; Azo 1.9); lanes 9, 10, original rat tumor used for preparation of monoclonal antibodies; lanes 11, 12, Duke's B2 (Azo 1.10); lanes 13, 14, Duke's C2 (Azo 4.15); lanes 15, 16, HT29 cells. **B:** DBA-unbound fractions depicted in **A** (with the exception of normal colon) were subsequently applied to *Helix pomatia* (HPA)-agarose and the column eluted with N-acetylgalactosamine. HPA-unbound (U), and eluted (E) fractions were concentrated to 0.5 ml; 20-μl aliquots were analyzed by Western blotting with mAb 3A7 (1:3,000 dilution). Lanes 1, 2, Duke's A (Azo 1.6); lanes 3, 4, Duke's B1 (Azo 4.1); lanes 5, 6, Duke's B2 collision tumor (Azo 1.9); lanes 7, 8, Duke's B2 (Azo 1.10); lanes 9, 10, original rat tumor; lanes 11, 12, Duke's C2 (Azo 4.15); lanes 13, 14, HT29 cells.

specificity. However, the ability of mAb 3A7 to detect an oncodevelopmentally regulated carbohydrate epitope in rat colon provided a unique opportunity to examine the expression and structure of type 2 oligosaccharides bearing either blood group A or B in developing colon and azoxymethane-induced colon tumors.

Analysis of fetal and developing rat colon revealed that the 3A7 epitope is expressed maximally between days 4 and 21 after birth. Although the mechanisms regulating expression of this epitope are unclear, recent studies have demonstrated that maturation of the rat proximal colon during the first 10 days after birth is accompanied by the transient appearance of villi and coincident expression of a number of glycoprotein enzymes normally associated with small intestine brush-border membranes, including lactase and alkaline phosphatase [58,59]. It is therefore possible that the developmental expression of the 3A7 epitope may be due in part to expression of specific glycoproteins at distinct stages of colon maturation which are targets for addition of the 3A7 epitope. Additional factors regulating expression of the 3A7 epitope may include the level of A, B and H glycosyltransferases, which have been shown to be expressed in a developmentally regulated manner [5,9,10,20], as well as synthesis of type 2 chains [5,10]. In this regard, Dabelstein et al. [60] have demonstrated, using a panel of monoclonal antibodies that detect blood group A variants on either type

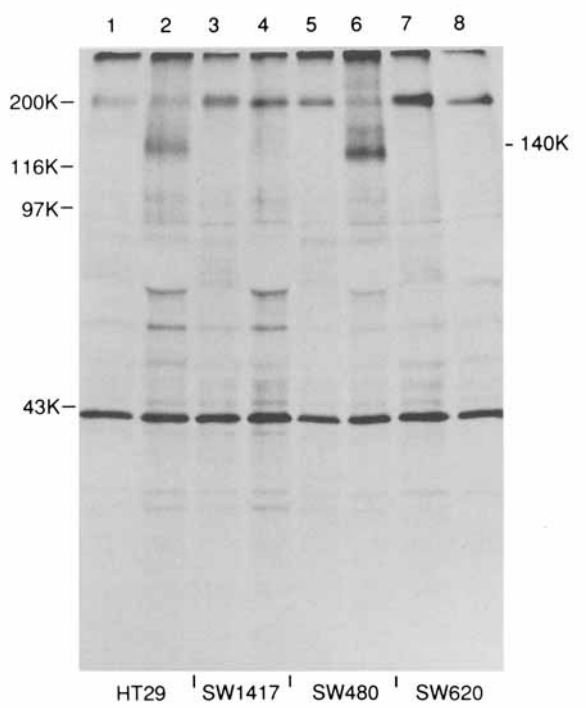


Fig. 8. Expression of glycoproteins detected by MAb 3A7 in human colon cancer cell lines. The human colon cancer cell line HT29 (grade II, moderately differentiated), SW480 (grade III, poorly differentiated), SW620 (lymph node metastasis isolated from SW480 primary tumor) and SW1417 (grade III/IV) were radiolabeled for 24 hr with [^{35}S]methionine (100 μCi/ml). Radiolabeled proteins from cell lysates (2 × 10^6 cpm) were immunoprecipitated with normal mouse serum (lanes 1,3,5,7) or MAb 3A7 (lanes 2,4,6,8) and separated by 7.5% SDS–PAGE. Bands were visualized following fluorography and autoradiography.

1 (Galβ1–3GlcNAc-R), type 2 (Galβ1–4GlcNAc-R), or type 3 (Galβ1–3GalNAc-R) core oligosaccharide chains, that normal human colon expresses exclusively type 1 chain A antigens, whereas fetal intestinal mucosa and adenocarcinomas of distal colon express blood group A antigens on type 1, 2, and 3 chains.

In contrast to normal adult colon that expressed low but detectable levels of mAb 3A7–immunoreactive glycoconjugates, azoxymethane-induced colon tumors expressed elevated levels of these glycans. This appeared to be an early event in colon carcinogenesis since the epitope was expressed at high level in some early-stage tumors (i.e., Duke's A). Even more significant was the fact that some glands within the transitional mucosa, which appeared histologically normal, as well as others depicting cytologic atypia stained with the antibody. This suggested that mAb 3A7 may provide a valuable tool for the detection of preneoplastic colonic lesions, a key to decreased incidence of human colon cancer. In addition, mAb 3A7 detected apparent glycosylation differences between blood-borne (i.e., angioinvasion in Fig. 3G) and lymph node metastatic cells which may point to biochemical alterations required for organ-specific metastasis.

The biochemical mechanism responsible for increased synthesis of mAb 3A7 immunoreactive determinants in colon tumors is not yet known. However, recent studies by Ørntoft et al. [26] have revealed that increased activity of α-2-L-fucosyltransferase is a consistent feature of cancers of the distal colon. By contrast, no significant change of A and B transferase levels was observed in these studies, suggesting a key role for α-2-L-fucosyltransferase in regulating expression of ABH determinants in the distal colon [26]. Indeed, we have provided evidence that α-2-L-fucose is a necessary component of the epitope detected by mAb 3A7, suggesting that this enzyme may play an important role in regulating expression of this determinant in normal intestinal development as well as during colon carcinogenesis. However, it is likely that regulation of mAb 3A7 immunoreactive determinant expression, like other type 2 chain oligosaccharides, is more complex, involving the concerted action of enzymes involved in synthesis and extension of type 2 chains as well as enzymes regulating terminal glycosylation, including fucosylation [8,16–19,28,30,61]. In this regard, we have obtained evidence that in HT29 cells, the 3A7 epitope is added primarily to GlcNAcβ1–6Manα1-6Manβ- branched Asn-linked oligosaccharides (data not shown). Thus, increased expression of β-N-acetylglucosaminyltransferase V, which initiates the β1–6 antenna [30], and subsequent addition and extension of type 2 chains [28] may play key roles in regulating the extent to which Asn-linked oligosaccharides are modified with A and B determinants. In fact Fernandes et al. [62] have shown that human colon tumors express elevated levels of β1–6 branched Asn-linked oligosaccharides compared with normal colonic mucosa, as detected by leukoagglutin (L-PHA) binding.

In addition to the cancer-associated increase in expression of mAb 3A7 immunoreactive type 2 chains, azoxymethane-induced tumors displayed qualitative glycosylation changes. Affinity chromatography of detergent extracts of normal and neoplastic colon on the blood group A-specific lectin *Dolichos biflorus*-agarose revealed a cancer-associated decrease in binding of

mAb 3A7 immunoreactive glycoproteins to this lectin. However, the tumor-derived immunoreactive glycoproteins in the unbound fraction of DBA-agarose were subsequently bound by *Helix pomatia*-agarose. The cancer-associated decrease in DBA binding seen with rat colon tumors as well as HT29 cells (Fig. 7A, lane 15) described herein has also been detected in human colorectal polyps [63] and colon tumors [22] by immunohistochemical staining. In this regard, Bresalier et al. [64] demonstrated that only 9% of tumors from the distal colon bound to the lectin whereas 58% of tumors in the proximal colon bound to the lectin. In contrast, DBA binding was detected throughout normal colon, associated primarily with goblet cell mucin. Collectively, these results indicate that there are structural differences on glycoproteins detected by mAb 3A7 in normal and neoplastic colon and suggest that these differences may be due in part to alterations of blood group A oligosaccharides.

Although the biochemical basis for differential binding of mAb 3A7 immunoreactive glycoproteins from normal and neoplastic colon to *Dolichos biflorus* is unclear, Nakayama et al. [65] have shown that prior treatment of normal colon with neuraminase results in increased DBA-binding, suggesting that modification of blood group A oligosaccharides by sialylation may influence lectin binding. The core structure of blood group A oligosaccharides may also modulate their binding to *Dolichos biflorus*. In fact, Clausen et al. [66] have demonstrated that A_1 erythrocytes, which are preferentially agglutinated by DBA lectin [54], express A type 3 chains, GalNAcα1–3[Fucα1–2]GalNAcα1–3[Fucα1–2]Galβ1–4GlcNAc-R, that are not detectable on A_2 erythrocytes. Whether the differential binding of mAb 3A7 immunoreactive glycoproteins to DBA-agarose results from modification of oligosaccharides specifically recognized by the antibody or whether other carbohydrate modifications on the glycoproteins influence lectin binding is not known at this time. Detailed analyses of mAb 3A7 immunoreactive glycopeptides or oligosaccharides derived from normal and neoplastic colon will be required to provide a structural basis for differential lectin binding. This in turn should provide valuable information about the biochemical changes responsible for the cancer-associated glycosylation changes detected by mAb 3A7 in normal and neoplastic colon (e.g., alteration in size, core structure and/or branching of oligosaccharides).

In conclusion, we have demonstrated that mAb 3A7 detects an oncodevelopmentally expressed carbohydrate epitope in rat colon tumors and some human colon cancer cell lines. In addition, we have illustrated the potential usefulness of the azoxymethane model of colon carcinogenesis for studying mechanisms regulating blood group antigen expression. Finally, we have shown that the combined use of mAb 3A7 and the blood group A-specific lectin *Dolichos biflorus* may provide a useful tool for the early detection of colon cancer. Although the biological role of mAb 3A7 immunoreactive glycoconjugates in health and disease is not clear, one can speculate that like other N-acetyllactosamine-containing oligosaccharides, including extended type 2 chains (i.e., polylactosamine units), oligosaccharides detected by mAb 3A7 may play a role in cell–cell adhesion [8], adhesion of tumor cells to extracellular matrix proteins [8,67,68] or influence release of lysosomal enzymes capable of degrading the extracellular matrix [18], affecting the metastatic potential of colon cancer cells.

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