Production of Monoclonal Antibodies Directed against Carbohydrate Moieties of Cell Surface Glycoproteins

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Through the use of a technique for raising monoclonal antibodies, coupled with a solid-phase radioimmunoassay utilizing immobilized glycopeptides prepared from the surface membranes of the colorectal cancer cells (LS 180) used for the immunization, carbohydrate-directed monoclonal antibodies were obtained. One of the monoclonal antibodies, MLS 102, reacted immunohistochemically intensely with the colorectal cancer cell surface and the mucinous glycoproteins secreted by the cancer cells, but only weakly with normal colon tissue. The antigenic determinant recognized by MLS 102 was the carbohydrate moiety of glycoproteins with terminal sialic acid. The antigens defined by other monoclonal antibodies, MLS 103 and 104, were immunohistochemically detected in both normal colonic epithelial and cancer cells. These antibodies seemed to recognize the carbohydrate moieties of both glycoproteins and glycolipids. The method described in this report can be generally applied to raise cell surface carbohydrate-directed antibodies.

Key words: Monoclonal antibody — Colorectal cancer — Tumor-associated antigen — Mucin-type glycoprotein — Mucin

Carbohydrate-directed monoclonal antibodies are regarded as being useful for analyzing changes in the glycosylation of glycoconjugates accompanying the malignant transformation of cells.[1, 2] These antibodies recognize small changes in carbohydrate structure, such as the addition or removal of a single sugar residue or a change in anomeric structure. The use of carbohydrate-directed monoclonal antibodies has revealed, for example, the occurrence of carbohydrate chains with sialyl Lewis[a] and sialy Lewis[x] structures at the non-reducing ends,[2, 3] and the presence of a heterophile antigen, Hanganutziu-Deicher (H-D) antigen (N-glycolylneuraminyl residue), in some cancer cells.[4, 5]

To raise carbohydrate-directed monoclonal antibodies, two methods have been used with some success. One involves the use of intact cancer cells as immunogens and the screening of hybridomas based on their ability to produce antibodies that bind selectively to cancer cells. Antibodies produced by such selected hybridomas usually bind to cell-surface glycoproteins or glycolipids. The drawback of this method is that it is tedious to select hybridomas that produce cancer cell-related antibodies out of a huge number of hybridomas, most of which produce antibodies reacting with both cancer and corresponding normal cells.[2] The other involves the use of isolated glycopeptides as immunogens, together with special adjuvants.[1, 6] However, carbohydrate moieties of glycoproteins have been shown to be poorly antigenic.[1]

We have developed a new procedure for specifically assaying carbohydrate-directed antibodies, involving a solid-phase radioimmunoassay using glycopeptide-coated plastic assay plates. In this report, we describe the production and properties of monoclonal antibodies reactive with carbohydrate chains of glycoproteins.

MATERIALS AND METHODS

Materials   [^{125}I]NaI, carrier-free, and [1-^{14}C]-glucosamine-HCl, 56.8 mCi/mmol, were purchased from Amersham.

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FITC[*5]-Conjugated antimouse IgG rabbit IgG and endo-β-galactosidase (*Escherichia freundii*) were obtained from Seikagaku Kogyo, Tokyo. Sialidase (*Arthrobacter ureafaciens*) and Protein A (*Staphylococcus aureus*) were from Nakarai Chemicals Ltd., Kyoto. Pronase P (75,000 units/g) was from Kaken Kagaku Co., Tokyo, and fetal calf serum (FCS) from Boehringer-Mannheim, Mannheim. Preparation of ^{125}I-labeled Protein A was carried out by the chloramine T method[7] except that the reaction was terminated with tyrosine.

Cell Culture and Immunization Human colorectal cancer cells, LS 180, obtained from the American Type Culture Collection, (ATCC), Rockville, were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% FCS. For immunization, male Balb/c mice were intraperitoneally injected 9 times with 2×10^6 cells, spread over a period of 3 months. Three days before cell fusion, LS 180 cells were administered intravenously as a booster immunization.

Cell Fusion and Cloning The fusion of immune splenic lymphocytes (1.5×10^8 cells) with mouse myeloma cells, SP 2/0-Ag 14 (2.0×10^7 cells), was performed using polyethylene glycol 1000 according to Kohler and Milstein[8] with the modification described by Kennett et al.[9] Fused cells were seeded in seven 96-well tissue culture plates, and hybridomas were selected by adding the hybridoma medium (72% Dulbecco's modified Eagle medium, 8% NCTC 109 medium, 20% fetal calf serum) containing hypoxanthine, aminopterin and thymidine (HAT). After ten days, the culture fluids were assayed for antiglycopeptide antibody activities by means of a solid-phase radioimmunoassay (RIA) using ^{125}I-labeled Protein A, as described below. Hybridomas producing such antibodies were cloned twice by the limiting dilution method in 96-well tissue culture plates at an average cell density of 0.3 cell/well, using mouse splenic lymphocytes as a feeder layer.

Preparation of Glycopeptides Cell membrane glycoproteins of LS 180 cells were isolated accord-

ing to Funakoshi and Yamashina.[10] In brief, cell membranes were solubilized in 10mM phosphate buffer, pH 7.2, containing 1% Triton X-100 and 0.15M NaCl, and the extract was heated at 100° for 7 min to denature and inactivate glycosidases that might have been present in the extract. The heated extract was lyophilized, and the dried material was treated with 80% ethanol and then chloroform/methanol (2:1, v/v), and finally the residue was suspended in 0.1M borate buffer, pH 8.0, containing 0.1% calcium acetate. Pronase P was added to the suspension at the ratio of one part to 50 parts of protein. Exhaustive digestion was performed, and the resulting glycopeptides were fractionated into mucin-type glycopeptides, G-50I, and serum-type glycopeptides, G-50 II, on a column of Sephadex G-50 according to Nakada et al.[11]

Solid-phase Radioimmunoassay (RIA) One μg of G-50I was adsorbed on each well of 96-well microtiter plates (Costar, Cambridge) according to Hawrot and Patterson[12] with some modifications. G-50II was acylated with palmitic anhydride, and then the acylated G-50II (2 μg) dissolved in pyridine was adsorbed on each well of 96-well microtiter plates according to Brockhaus et al.[13] The antibody binding assay was performed as follows. The microtiter plates were treated with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) to prevent nonspecific binding of antibodies. Twenty μl of culture fluid was added to each of the glycopeptide-coated wells, followed by standing at 4° overnight. Unbound immunoglobulin was removed from the wells by washing them three times with PBS containing 1% BSA. Then, ^{125}I-labeled Protein A (12.6 ng, about 100,000 cpm) in 50 μl of PBS containing 0.1% BSA was added to each well, followed by incubation at room temperature for 2 hr. Unbound Protein A was removed from the wells by washing them with PBS. The wells were then cut out, and the bound radioactivity was determined with a gamma counter (Beckman Gamma-5500).

Metabolic Labeling of Glycoproteins with [1-14C]-Glucosamine LS 180 cells were grown on 100 mm Falcon dishes in a medium comprising Eagle's MEM supplemented with 10% FCS plus 5 μCi/ml of [1-14C]glucosamine-HCl. After a 24 hr incubation, the cells were scraped from the dishes with a rubber policeman and then washed with 0.15 M NaCl three times. The cell membranes were solubilized by homogenizing the cells with a Dounce-type homogenizer in 20mM phosphate buffer, pH 7.1, containing 1mM 2-mercaptoethanol, 0.5% DOC, 1% NP-40, 1mM EDTA, 1mM phenylmethylsulfonyl fluoride and 100 units/ml of aprotinin (Ohkura Pharm. Co., Kyoto). After centrifugation of the homogenates at 100,000g for 60 min, the supernatants were recovered and

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[*5] The abbreviations used are: FITC, fluorescein isothiocyanate; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; RIA, radioimmunoassay; HAT, hypoxanthine, aminopterin and thymidine; PBS, phosphate-buffered saline; NET, NaCl-EDTA-Tris buffer; DOC, sodium deoxycholate; NP-40, Nonidet P-40; G_{M2}, GalNAcβ1 → 4 [NeuAcα 2 → 3] Galβ 1 → 4 Glcβ 1 → 1 ceramide; G_{M3}, NeuAcα2 → 3 Galβ1 → 4 Glcβ 1 → 1 ceramide; G_{D1b}, Galβ 1 → 3 GalNAcβ 1 → 4 [NeuAcα 2 → 8NeuAcα2 → 3] Galβ1 → 4Glcβ1 → 1ceramide; G_{T1b}, NeuAcα2 → 3Ga 1β1 → 3GalNAcβ1 → 4 [NeuAcα2 → 8NeuAcα2 → 3]Galβ1 → 4Glcβ1 → 1ceramide.

then subjected to immunoprecipitation with antibody and formalin-fixed *Staphylococcus aureus* cells (Immusorbin, Wako Chemicals Ltd., Osaka). The ^{14}C-labeled glycoproteins were released from the immunoprecipitate with a buffer containing SDS.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) SDS-PAGE was performed in 7.5% polyacrylamide gels by the procedure described by Laemmli.[14] Fluorography to detect ^{14}C-labeled glycoproteins was carried out according to Bonner and Laskey.[15]

Preparation of Glycolipids Glycolipids were extracted from LS 180 cells by the method of Svennerholm and Fredman.[16] High-performance thin layer chromatography (HPTLC) was carried out on aluminum plates coated with silica gel 60 (Merck, 0.25 mm thick). The solvent system used was chloroform/methanol/0.25% KCl (5:4:1, v/v). The reference gangliosides (G_{M2}, G_{M3}, G_{D1b} and G_{T1b}) were gifts from Dr. A. Suzuki, Tokyo Metropolitan Institute of Medical Science, Tokyo. Glycolipids were visualized by resorcinol/HCl staining.[17]

Immunostaining of Glycolipids After separation of glycolipids on an HPTLC plate, they were stained with monoclonal antibodies according to Magnani *et al.*[18]; ^{125}I-labeled Protein A was used to detect the bound antibody.

Immunohistochemical Analysis Tissues obtained from surgically resected materials or from autopsy materials were fixed in 10% formalin and embedded in paraffin. They were cut into sections, 4 μm thick. Indirect immunostaining was performed with monoclonal antibodies and FITC-conjugated antimouse IgG rabbit IgG. The staining was also carried out using the indirect immunoperoxidase method according to Heyderman.[19]

RESULTS

Titers of Carbohydrate-directed Antibodies in the Immune System Figure 1 shows typical immune response of mice immunized with LS 180 cells to two types of glycopeptides. The binding activities of the antiserum, to G-50I and G-50II, became maximun after nine immunizations spread over a period of 3 months, but the titers of the antibodies were not very high, being marginal after 100-fold dilution of the serum.

Cell Fusion and Screening of Hybridomas Splenic lymphocytes from the Balb/c mice with the antibody levels shown in Fig. 1 were fused with SP 2/0 myeloma cells. Visible colonies of hybrid cells were present in all the wells at 10 days after the cell fusion. The culture fluids from the wells were tested for antiglycopeptide antibody activities by means of the solid-phase RIA, using wells coated with G-50I and G-50II glycopeptides. The culture fluids produced by hybridomas in 12 out of 7×96 wells showed significant binding activities to G-50I and G-50II plates, and one of them showed binding activity toward only the G-50I plate. However, cloning of the hybridomas by the limiting dilution method led to the establishment of five clones. Monoclonal antibodies produced by these clones were designated as MLS 102–106. The binding activities of these antibodies toward G-50I and G-50II glycopeptides are shown in Fig. 2. MLS 102 bound only to G-50I whereas the others bound to both G-50I and G-50II.
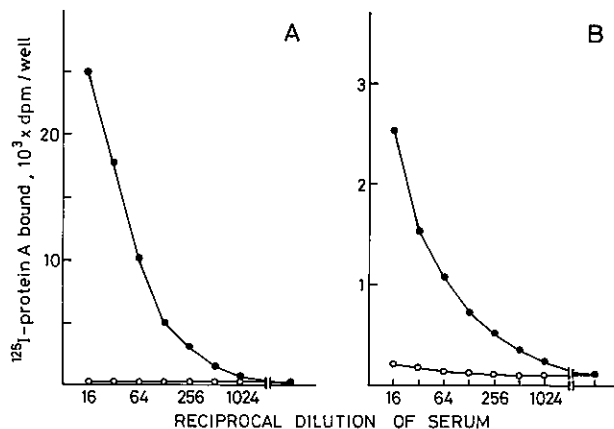

Fig. 1. Solid-phase radioimmunoassay (RIA) for serum antiglycopeptide activity. Colorectal carcinoma cells (LS 180) were injected intraperitoneally into Balb/c mice. Mucin-type and serum-type glycopeptides (G-50I and G-59II, respectively) prepared from LS 180 cells were coated on microtiter plates, and the antibody assay was carried out with 20 μl of serially diluted serum. The amounts of antibody bound to the plates were determined with ^{125}I-labeled Protein A. (A) Activity towards G-50I and (B) activity towards G-50II. (○) Preimmune serum, (●) immunized serum.
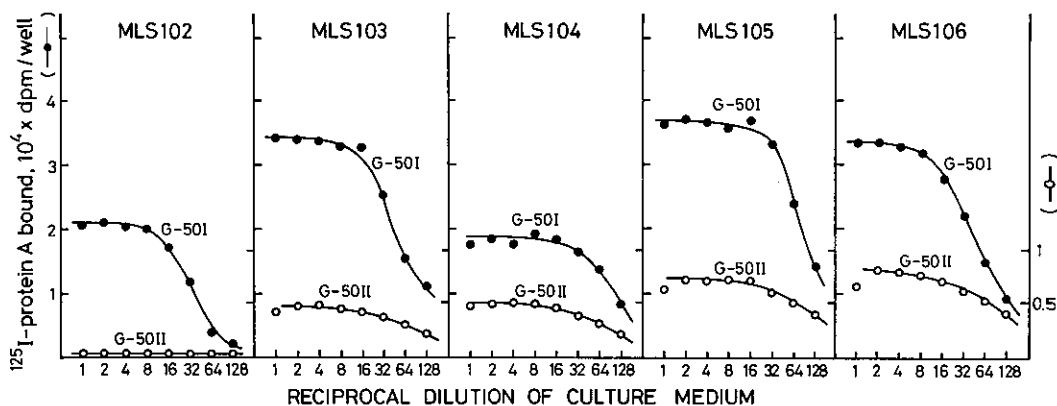
Fig. 2. Reactivity of monoclonal antibodies with G-50I and G-50II glycopeptides. (●) G-50I coated plates and (○) G-50II coated plates.

However, the antigenic specificities of MLS 103, 105 and 106 seemed to be similar, if not identical, as judged from the amounts of the antibodies that bound to the glycopeptide-coated plates. In fact, each of these three antibodies inhibited the binding to the plates of the other two (Fig. 5).

Immunohistochemical Studies on the Antibodies These monoclonal antibodies, MLS 102–106, were studied immunohistochemically. Individual sections of a colon specimen were exposed to 0.2 ml of monoclonal antibody solutions (culture fluids) at 4° for 16–20 hr with mild agitation. The sections were then treated with FITC-conjugated antimouse IgG rabbit IgG for one hour at room temperature in the dark and examined under a Nikon fluorescence photomicroscope. As shown in Fig. 3, MLS 102 intensely stained cancerous colon tissue whereas the other antibodies stained equally both cancerous and adjacent normal colon tissues. MLS 102 seemed to stain not only the cancer cells but also the mucin-like glycoproteins secreted by them. The other antibodies (MLS 103 to 106) strongly stained the normal mucosal epithelium, especially the goblet cells. To confirm the cancer-associated staining property of MLS 102, we stained various normal tissues with this antibody using the indirect immunoperoxidase method. The results are shown in Fig. 4 in comparison with the staining property of MLS 103.

Figure 4-1 shows that MLS 102 did not stain normal colonic mucosa, whereas MLS 103 stained the cytoplasm and luminal surface of the tissues. Note that granular deposits correspond to Golgi apparatus and endoplasmic reticulum. Figure 4-2 shows that both MLS 102 and MLS 103 stained colonic cancer cells and their secretions. This cancer was classified as well differentiated adenocarcinoma. Figures 4-3 and 4-4 show the staining of normal gastric mucosa and bronchial glands, respectively, with MLS 102 and MLS 103. Gastric mucosa was negatively stained with both of the antibodies, whereas mucinous epithelia of bronchial glands were stained with MLS 103, but not with MLS 102. Thus, it seems that MLS 102 is directed more to cancer-associated antigen compared to other antibodies represented by MLS 103.

Reactions of the Antibodies with Glycoproteins and Glycolipids from LS 180 Cells Glycoproteins were extracted with a solvent containing NP-40 plus sodium DOC from LS 180 cells which had been metabolically labeled with [1-^{14}C]glucosamine. The glycoprotein fraction was reacted with the monoclonal antibodies, and then the immunocomplexes were precipitated with Immusorbin. After washing of the precipitates with NET as described by Kessler,[20] the antigens were solubilized with the sample buffer used for SDS-PAGE. Figure 6 shows an electrophoretic profile of immunoprecipitated glyco-
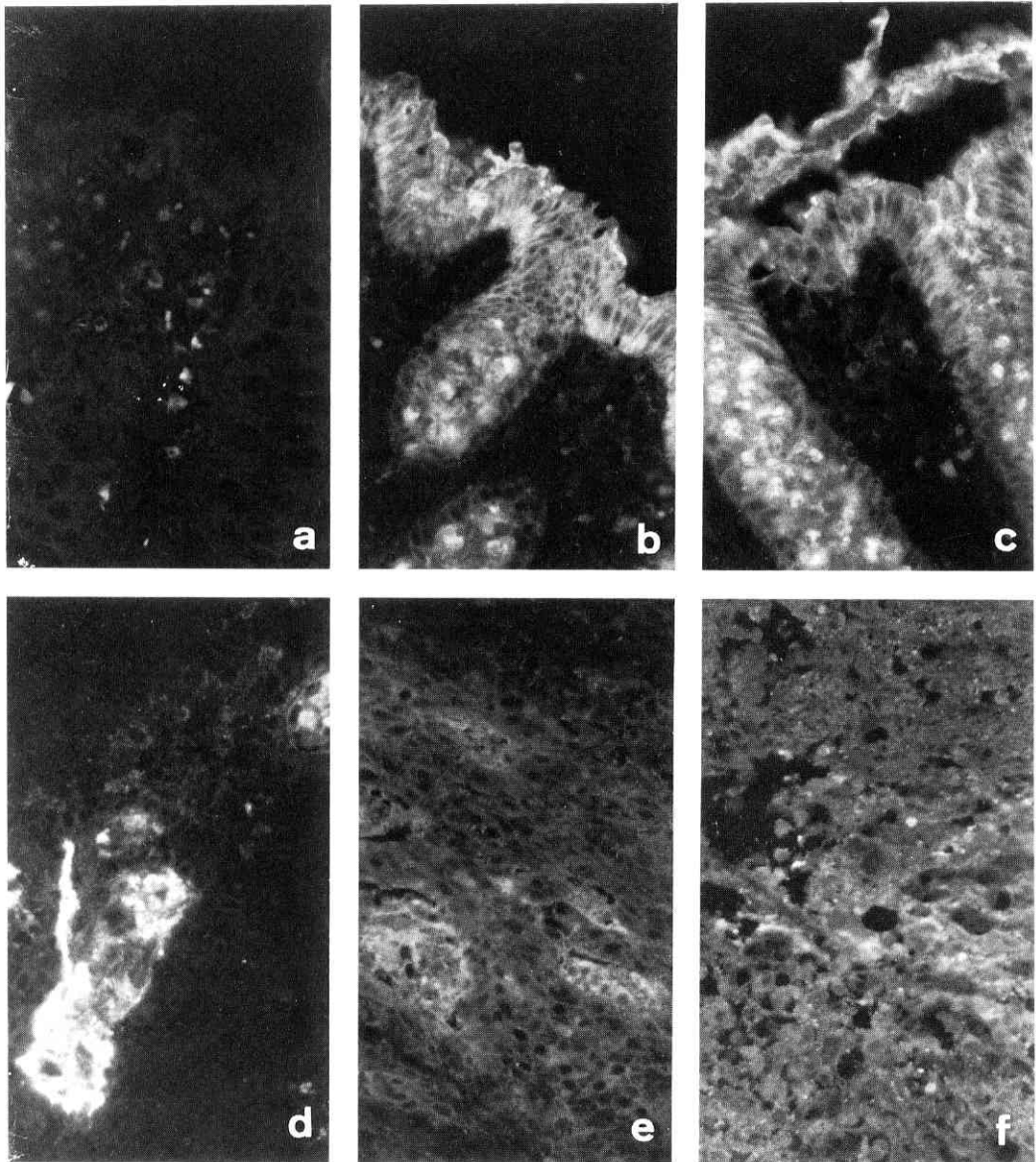
Fig. 3. Histochemical detection of antigens defined by monoclonal antibodies on colorectal cancer cells and adjacent normal colon. Individual sections of a colon specimen which had been fixed with 10% formaldehyde were exposed to monoclonal antibodies (culture fluids), washed with PBS and then treated with FITC-conjugated antimouse IgG rabbit IgG (Miles, Chicago). (a–c) Normal colon; (d–f) colorectal cancer; (a, d) MLS 102 staining; (b, e) MLS 103 staining; (c, f) MLS 104 staining.

proteins from LS 180 cells. All the antigenic glycoproteins were of high molecular weight, migrating only slightly in the separation gel with considerable portions remaining in the stacking gel, although the amount of radioac-

tivity and the electrophoretic profile differed among the MLS 102, 103 (as well as 105 and 106) and 104 antigens.

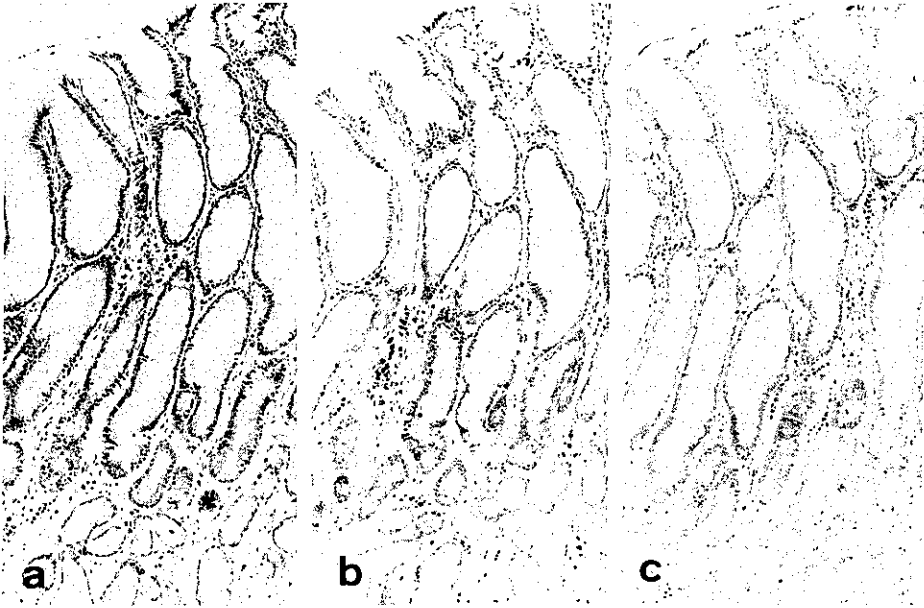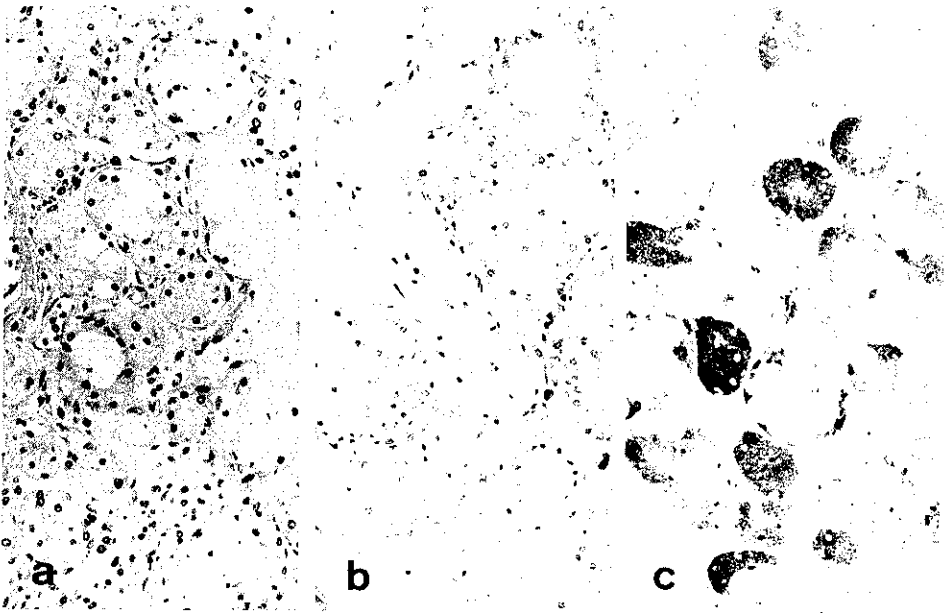For the glycolipids, solvent extracts prepared according to Svennerholm and
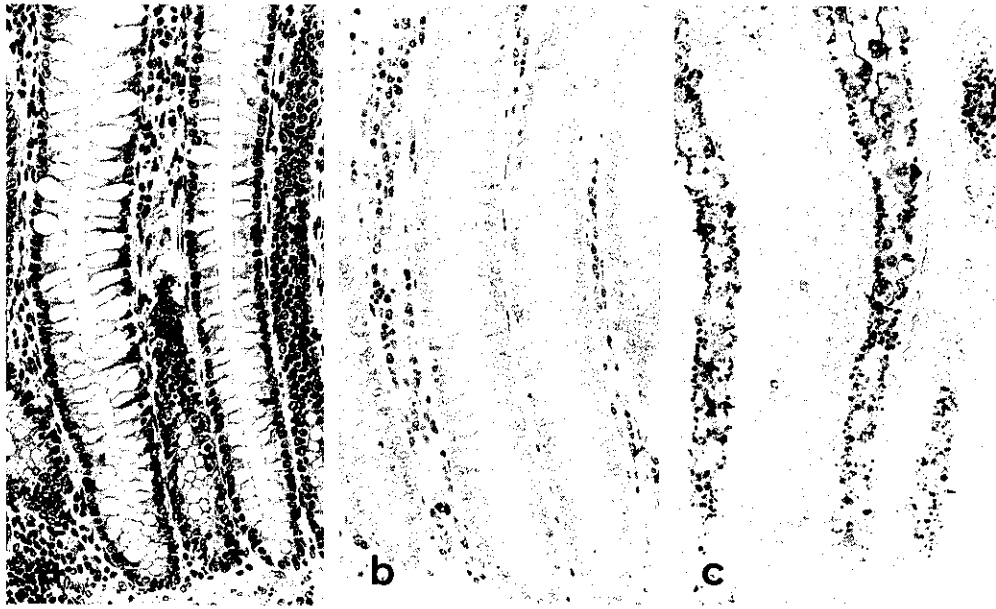
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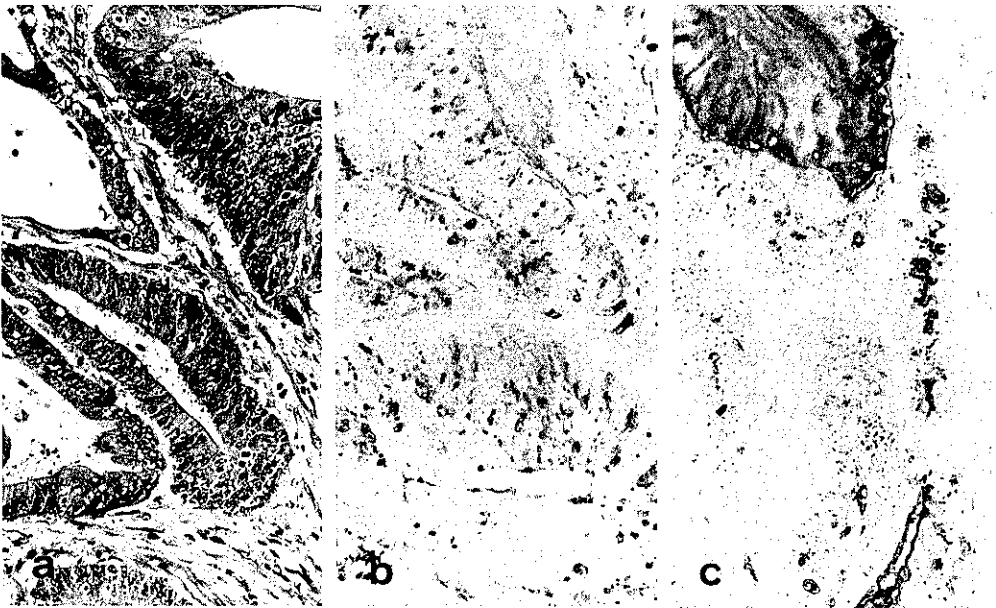
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Fig. 4. Detection of MLS 102 and MLS 103 antigens on sections of intestinal colon (normal and cancer), gastric mucosa, and bronchial glands. The staining was carried out using the indirect immunoperoxidase method of Heyderman.[19] 4-1, Normal colon; 4-2, colonic cancer cells; 4-3 normal →

4-3

4-4

→gastric pyloric mucosa; 4-4 bronchial glands. In every staining, a, b, and c show staining with hematoxylin-eosin (for 4-1 and 4-2) or Giemsa (for 4-3 and 4-4), staining with MLS 102 and staining with MLS 103, respectively.
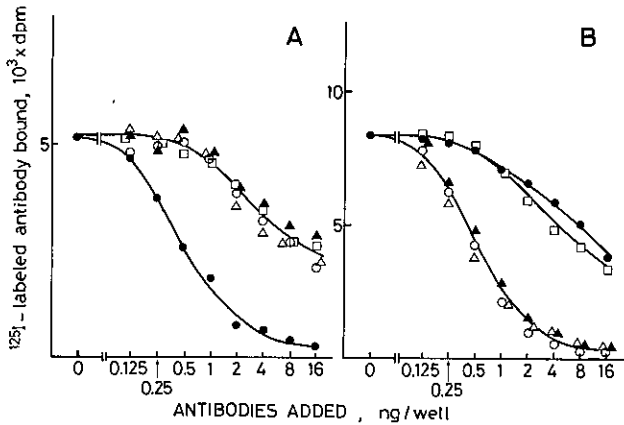
Fig. 5. Mutual inhibition of the antigen-binding activities of the MLS 102–106 antibodies. ^{125}I-Labeled MLS 102 (A) or MLS 103 (B) was used as the reference antibody and its ability to bind to G-50I coated plates was determined in the presence of other antibodies. Equivalent amounts of all the antibodies were used. ●, MLS 102; △, MLS 103; □, MLS 104; ▲, MLS 105; ○, MLS 106.
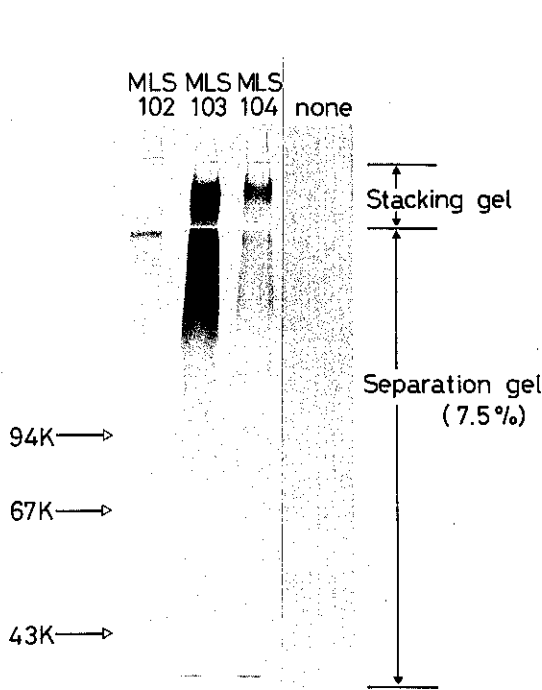

Fig. 6. SDS-PAGE of antigens defined by monoclonal antibodies in a cell membrane extract of LS 180 cells. Detergent extracts of LS 180 cells were immunoprecipitated with the monoclonal antibodies using formalin-fixed *Staphylococcus aureus* cells, and then the antigens were solubilized with the sample buffer used for SDS-PAGE. Samples were analyzed in 7.5% polyacrylamide gels. Immunoprecipitates obtained with MLS 102, MLS 103 and MLS 104, and without an antibody are indicated at the tops of the lanes.
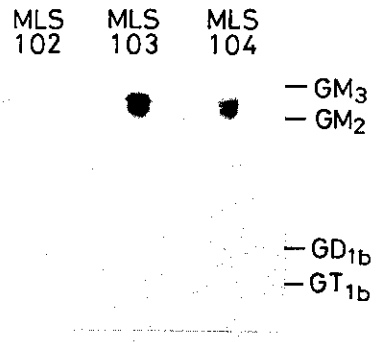

Fig. 7. Thin layer chromatogram of glycolipids from LS 180 cells defined by monoclonal antibodies. Glycolipids from LS 180 cells and reference glycolipids were separated by thin layer chromatography on an aluminum-backed HPTLC plate (Merck, Darmstadt) in chloroform/methanol/0.25% KCl (5:4:1 v/v). After development, the reference lane was cut out and stained with resorcinol/HCl reagent, and the lanes for the cellular glycolipids were treated with the monoclonal antibodies (MLS 102–104) followed by staining with ^{125}I-labeled Protein A as described under "Materials and Methods." No spot was detected in the case of MLS 102.

Fredman[16] were developed on HPTLC plates and then immunostained with the antibodies. As shown in Fig. 7, MLS 103 (as well as 105 and 106) and 104 bound to at least two apparently different glycolipids which migrated to positions close to G_{M2}, whereas no radioactive band was detected with MLS 102.

Table I. Effects of Glycosidase and Acid Treatments on G-50I Glycopeptides that React with the Monoclonal Antibodies

Treatment of G-50I	Monoclonal antibodies		
	MLS 102	MLS 103	MLS 104
None	0.053	0.088	0.047
Neuraminidase	>10	0.015	0.022
Neuraminidase + endo-β-galactosidase	>10	0.038	0.022
0.05 M H₂SO₄, 100°, 90 min	>10	>10	>10

The reactivity was expressed as inhibitory activity towards the reaction of the antibodies with a G-50I coated plate. The values are the amounts (μg/well) of the modified G-50I giving 50% inhibition.

Characterization of Glycoprotein Antigens

To characterize the carbohydrate structures of the antigenic glycoproteins, glycopeptides (G-50I) were treated with glycosidases or with acid, and then the modified glycopeptides were tested for the ability to react with the antibodies by determining the inhibition of the reaction of the antibodies with G-50I. The results are summarized in Table I. Sialidase from *Arthrobacter ureafaciens* abolished the activity of the MLS 102 antigen completely, as described in papers published recently[21, 22]; in contrast, asialoglycopeptides were more reactive toward MLS 103 and 104 than the original glycopeptides. Sequential treatment with sialidase and endo-β-galactosidase depressed the activity of the MLS 103 antigen slightly, but had no effect on that of the MLS 104 antigen. Mild acid treatment abolished the activities of both the MLS 103 and 104 antigens, suggesting that fucose residues may be involved in these antigens.

DISCUSSION

It is believed that the carbohydrate moieties of glycoproteins are poorly antigenic, except for those exhibiting blood group activities.[4] Immunization with isolated glycoproteins emulsified with appropriate adjuvants usually results in the production of antibodies directed against their polypeptide moieties. Glycoproteins embedded in cell surface membrane matrices are often potent antigens. Thus, mice (Balb/c) have been immunized with human cancer cells, which led to the production of cancer-associated monoclonal antibodies that recognize glycoproteins. However, it is tedious to select hybridoma clones that produce carbohydrate-directed and cancer-associated antibodies from among large numbers of hybridomas by means of cell-binding assay of the antibodies, since many antigenic groups of a peptide nature occur on the cell surface and they are usually not cancer-associated. In fact, Koprowski *et al.*[23, 24] established a hybridoma that produces a monoclonal antibody that recognizes a cancer-associated antigen, CA 19-9, after screening of thousands of hybridomas, most of which were not cancer-associated. Similar efforts have been made to establish hybridomas producing cancer-associated antibodies, and most of them turned out to be directed against the carbohydrate moieties of glycoconjugates. Hakomori[1] and others[2] have succeeded in producing monoclonal antibodies by immunizing mice with glycolipids isolated from cancer cells. Some of these antibodies reacted with glycoproteins.

We have been interested in raising antibodies directed specifically against the carbohydrate moieties of glycoproteins, especially those on cell surfaces. We used whole cells (human colorectal cancer cell lines) as immunogens, and developed a system specifically assaying carbohydrate-specific antibodies. For this, we coated immunoassay plates with glycopeptides. Through a single fusion of splenic lymphocytes from a Balb/c mouse immunized with LS 180 cells with myeloma cells, we were able to establish five hybridomas that produced carbohydrate-directed antibodies.

Our method for the preparation of carbohydrate-coated assay plates differs from that developed by Tang *et al.*[25] in that they converted oligosaccharides released from the polypeptide chains into neoglycolipids, whereas we used glycopeptides with the peptide moieties modified chemically.

One of the monoclonal antibodies, MLS 102, reacted immunohistochemically intensely with colonic cancer cells and their secretions, but only weakly with normal colon, gastric mucosa and mucinous epithelia of bronchial glands, so far investigated. MLS 102 also reacted with the intestinal metaplasia of gas-

tric pyloric glands and the normal esophageal mucosa in some of the specimens tested (data not shown). However, further immunohistochemical studies are needed to characterize more precisely the staining properties of MLS 102. Other antibodies, MLS 103 (as well as 105 and 106) and MLS 104, immunohistochemically reacted with both normal mucosal epithelial and colon cancer cells.

MLS 102 was directed against the carbohydrate moiety of a mucin-type glycoprotein with terminal sialic acid. The epitope for MLS 102 has been shown to be shared by ovine and bovine submaxillary mucins.[21, 22] The epitopes for MLS 103 (as well as 105 and 106) and MLS 104 were present in both glycoproteins and glycolipids, probably involving terminal fucose. The glycolipid antigens detected by MLS 103 and MLS 104 are probably ceramides with neutral pentasaccharides, as judged from their behavior on HPTLC and the absence of sialic acid. Each of them gave two spots on HPTLC, due probably to different ceramide moieties. The mobilities of the MLS 103 and 104 antigens on HPTLC were similar, but the amount of antigen detected with MLS 103 was apparently greater than that detected with MLS 104. This was also obvious in the binding experiments with the G-50I plate for the antibodies, as shown in Fig. 2, since each plate contained the same amount of G-50I. Structural differences between these two antigens are currently under investigation.

ACKNOWLEDGMENTS

REFERENCES

1) Hakomori, S. Tumor-associated carbohydrate antigen. *Ann. Rev. Immunol.,* **2,** 103–126 (1984).
2) Magnani, J. L., Nilsson, B., Brockhaus, M., Zopf, D., Steplewski, Z., Koprowski, H. and Ginsberg, V. A monoclonal antibody-defined antigen associated with gastrointestinal cancer is a ganglioside containing sialylated lacto-N-fucopentaose II. *J. Biol. Chem.,* **257,** 14365–14369 (1982).
3) Fukushima, J., Hirota, M., Terasaki, P. I., Wakisaka, A., Togashi, H., Chia, D., Suyama, N., Fukushi, Y., Nudelman, E. and Hakomori, S. Characterization of sialosylated Lewis^x as a new tumor-associated antigen. *Cancer Res.,* **44,** 5279–5285 (1982).
4) Ikuta, K., Nishi, Y., Shimizu, Y., Higashi, H., Kitamoto, N., Kato, S., Fujita, M., Nakano, Y., Taguchi, T. and Naiki, M. Hanganutziu-Deicher type-heterophile antigen-positive cells in human cancer tissues demonstrated by membrane immunofluorescence. *Biochem. J.,* **25,** 47–50 (1982).
5) Higashi, H., Nishi, Y., Fukui, Y., Ikuta, K., Ueda, S., Kato, S., Fujita, M., Nakano, Y., Taguchi, T., Sakai, S., Sako, M. and Naiki, M. Tumor-associated expression of glycosphingolipid Hanganutziu-Deicher antigen in human cancers. *Gann,* **75,** 1025–1029 (1984).
6) Hakomori, S. Aberrant glycosylation in cancer cell membranes as focused on glycolipids: overview and perspectives. *Cancer Res.,* **45,** 2405–2414 (1985).
7) Langone, J. J. ^{125}I-Labeled Protein A: reactivity with IgG and use as a tracer in radioimmunoassay. *Methods Enzymol.,* **70,** 356–375 (1980).
8) Köhler, G. and Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature,* **256,** 495–497 (1975).
9) Kennett, R. H., Denis, K. A., Tung, A. S. and Kilnman, N. R. Hybridoma plasmacytoma production: fusion with adult spleen cells, and human spleen cells. *Curr. Top. Microbiol. Immunol.,* **81,** 77–91 (1978).
10) Funakoshi, I. and Yamashina, I. Structure of O-glycosidically linked sugar units from plasma membranes of an ascites hepatoma, AH 66. *J. Biol. Chem.,* **257,** 3782–3787 (1982).
11) Nakada, H., Funakoshi, I. and Yamashina, I. The isolation and characterization of glycopeptides and mucopolysaccharides from plasma membranes of an ascites hepatoma, AH 130. *J. Biochem.,* **78,** 863–872 (1975).
12) Hawrot, E. and Patterson, P. H. Long-term culture of dissociated sympathetic neurons. *Methods Enzymol.,* **58,** 574–584 (1979).
13) Brockhaus, M., Magnani, J. L., Blaszczyk, M., Steplewski, Z., Koprowski, H., Karlsson, K. A., Larson, G. and Ginsburg, V. Mono-

clonal antibodies directed against the human Le blood group antigen. *J. Biol. Chem.,* **256**, 13223–13225 (1981).

14) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* **227**, 680–685 (1970).

15) Bonner, W. M. and Laskey, P. A. A film detection method for tritium labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.,* **46**, 83–88 (1974).

16) Svennerholm, L. and Fredman, P. A procedure for the quantitative isolation of brain gangliosides. *Biochim. Biophys. Acta,* **617**, 97–109 (1980).

17) Svennerholm, L. Quantitative estimation of sialic acids. *Biochim. Biophys. Acta,* **24**, 604–611 (1957).

18) Magnani, J. L., Smith, D. F. and Ginsberg, V. Detection of gangliosides that bind cholera toxin: direct binding of ^{125}I-labeled toxin to thin layer chromatograms. *Anal. Biochem.,* **109**, 399–402 (1980).

19) Heyderman, E. Immunoperoxidase technique in histopathology: applications, methods and controls. *J. Clin. Pathol.,* **32**, 971–978 (1979).

20) Kessler, S. W. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. *J. Biol. Chem.,* **115**, 1617–1624 (1975).

21) Kurosaka, A., Fukui, S., Kitagawa, H., Nakada, H., Numata, Y., Funakoshi, I., Kawasaki, T. and Yamashina, I. Mucin-carbohydrate directed monoclonal antibody. *FEBS Lett.,* **215**, 137–139 (1987).

22) Kurosaka, A., Kitagawa, H., Fukui, S., Numata, Y., Nakada, H., Funakoshi, I., Kawasaki, T., Ogawa, T., Iijima, H. and Yamashina, I. A monoclonal antibody that recognizes a cluster of a disaccharide, NeuAcα2→6GalNAc, in mucin-type glycoproteins. *J. Biol. Chem.,* **263**, 8724–8726 (1988).

23) Koprowski, H., Steplewski, Z., Mitchell, K., Herlyn, M., Herlyn, P. and Fugrer, P. Colorectal carcinoma antigens detected by hybridoma antibodies. *Somatic Cell Genet.,* **5**, 957–972 (1979).

24) Koprowski, H., Herlyn, M. and Steplewski, Z. Specific antigen in serum of patients with colon carcinoma. *Science,* **212**, 53–55 (1981).

25) Tang, P. W., Goul, H. C., Hardy, M., Lee, Y. C. and Feizi, T. Novel approach to the study of the antigenicities and receptor functions of carbohydrate chains of glycoproteins. *Biochem. Biophys. Res. Commun.,* **132**, 474–480 (1985).