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A Human Monoclonal Antibody Recognizing a Surface Antigen on Stomach Cancer Cells

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Lymph-node lymphocytes of a patient with stomach cancer were fused with the mouse-human heterohybridoma, HM-5. A clone (2F9) was isolated that showed stable production of an IgM antibody reactive with NUGC-4 stomach cancer cell line. This antibody reacted predominantly with a cell surface antigen on cell lines originating from gastro-intestinal cancer and adenocarcinoma of lung, whereas it was not generally reactive with other types of cancers, or with normal kidney cells or fibroblasts. Biotin-labeled 2F9 antibody clearly stained cell smears and the nude mouse tumor of NUGC-4, but it did not show a positive reaction with stomach cancer tissues obtained from more than 10 patients, indicating that the antigen detected is very weakly expressed on tumor cells or on a limited number of stomach cancers. The antigen shed from NUGC-4 cell line was detected in the culture supernatant. 2F9 antibody precipitated a glycoprotein with a molecular weight of over 200 kilodaltons as well as a possible glycolipid, from NUGC-4 cells labeled with [^{3}H]glucosamine or [^{35}S]-H_2SO_4. Periodic acid treatment of the tissue section decreased reactivity with 2F9 antibody, but heat, neuraminidase or protease treatment did not. These results suggested that the epitope is present on a carbohydrate moiety not containing sialic acid, and that a part of the antigen molecule is sulfated.

Key words: Human monoclonal antibody — Stomach cancer — Cell surface antigen — Carbohydrate antigen

The production of human monoclonal antibodies for possible use in the treatment of cancer patients has been attempted by various investigators,[1,2] because mouse monoclonal antibodies may induce adverse effects[3,4] when injected into patients. A recent report by Irie *et al.* demonstrated the possible clinical utility of human monoclonal antibodies and showed that the local injection of the human monoclonal antibody to GD2 ganglioside into melanoma lesion induced tumor regression in a significant number of cases.[5]

We previously reported the production of human monoclonal antibodies reactive to stomach carcer cells by a mouse-human hybridoma technique.[6] All of these antibodies, however, recognized cytoplasmic antigens. Thus, in this study, we attempted to produce human monoclonal antibody reactive to surface antigens of stomach cancer cells by a (mouse-human)-human hybridoma technique. We used the HM-5 heterohybridoma cell line that has been reported to provide a better stability of the hybridoma produced than do conventional mouse myeloma lines.[7] We report here a human monoclonal antibody produced using this approach that recognizes a cell-surface carbohydrate epitope carried on a high-molecular-weight glycoprotein and possibly a glycolipid also.

Abbreviations used: Mr, molecular weight; kD, kilodaltons; Ig, immunoglobulin; FBS, fetal bovine serum; HAT, hypoxanthine, aminopterin, thymidine; IA, immune adherence; IF, immunofluorescence; MHA, mixed hemadsorption; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; As, ascites; SN, culture supernatant.

MATERIALS AND METHODS

Tumor cell lines and nude mouse tumor NUGC-4 stomach cancer cell line[8] and fibroblast culture from the patient from whom NUGC-4 line had been derived as well as NUGC-4 nude mouse tumor originated from a surgical specimen were kindly provided by Dr. T. Watanabe, Nagoya University School of Medicine, Nagoya. Other nude mouse tumors were kindly given by Dr. Saito, Central Institute for Experimental Animals, Kanagawa.

Production of human monoclonal antibody Mouse-human heterohybridoma, HM-5, was used as the parental fusion cell line.[7] This cell line, which did not secrete human immunoglobulin (Ig), was maintained in RPMI-1640 medium with 10% fetal bovine serum (FBS) and 100 μM 8-azaguanine.

Lymph-node lymphocytes were obtained from a patient (59 years old, female) with stomach cancer (scirrhus type carcinoma with signet ring cell) of stage III. Preparation and treatment of lymphocytes with pokeweed mitogen, and fusion with the parent cells were carried out according to the method described previously.[6] The cells were plated (3072 plate, Falcon, Oxnard, CA) in a total volume of 200 μl containing 5×10^4 cells/well. Feeding medium was changed from hypoxanthine, aminopterin, and thymidine (HAT) to HT two weeks after the fusion.

Ascites fluids were obtained from nude mice injected intraperitoneally with hybridoma cells and stored at -80°C. The Ig concentration in ascites and culture supernatant was measured by laser nepherometry.

Purification and biotin-labeling of a human monoclonal antibody Human monoclonal antibody (IgM) was purified from ascites by 60% ammonium sulfate salt fractionation, followed by gel filtration using Sephacryl S-200 (Pharmacia, Uppsala, Sweden). The purified antibody was conjugated with biotin hydrazide by the method of O'Shannessy *et al.*[9]

Serological assays Immune adherence (IA) and indirect immunofluorescence (IF) assays for detecting cell surface antigens have been described.[6] Human mixed hemadsorption (MHA) assay as described by Fagraeus *et al.*[10] was also used. The immunoperoxidase method was performed as described previously[11] with a slight modification involving biotin-labeled human monoclonal antibody. For indirect IF and immunoperoxidase staining, human tumors transplantable into nude mouse, fetal and adult tissues, and patients' tumors embedded in OCT compound (Miles Scientific, Naperville, IL) and stored at -80°C were used.

The antigen in culture supernatant of cell lines was measured by an enzyme-linked immunosorbent assay (ELISA) by the method of Castro *et al.*[12] Microplates (Immunoplate II, NUNC, Denmark) were coated with the purified human monoclonal antibody at 4°C overnight and then the residual protein binding sites were blocked with FBS, dried milk and gelatin. Serial two-fold-diluted supernatants were incubated in the plates and then biotin-conjugated human monoclonal antibody and avidin-biotinylated alkaline phosphatase complex (Vector Lab., Burlingame, CA) were added. The bound alkaline phosphatase activity was determined with *p*-nitrophenyl phosphate by reading A_{405} with a microplate reader (MTP-32, Corona Electric, Tokyo).

Biochemical characterization of antigen The epitope and the antigen molecule detected by the human monoclonal antibody were characterized as follows;

1) Heat stability: NUGC-4 stomach cancer cell line was heated at 100°C for 10 min and the remaining ability to absorb monoclonal antibody was tested by flow cytometry (FACS 440, Becton Dickinson, Sunnyvale, CA).

2) Periodic acid treatment: NUGC-4 cells were treated with 1% $NaIO_4$ at room temperature for 10 min[13] and then the antigen was tested by flow cytometry.

3) Enzymatic treatment: NUGC-4 cells were resuspended in 0.25% trypsin (Difco Lab., Detroit, MI), 0.5 mg/ml protease V8 (ICN ImmunoBiologicals, Lisle, IL) or 0.1 U/ml neuraminidase (Sigma Chemical Co., St. Louis, MO) in serum-free medium (RPMI-1640), and were incubated at 37°C for 1 h.[14] Thereafter, the antigen was tested by flow cytometry.

4) Immunoprecipitation: NUGC-4 cell line and SK-MEL-31 melanoma cell line (control) in a 75 cm^2 T-flask (Corning) were labeled for 48 h with 0.2 mCi of D-[6-^{3}H(N)]glucosamine hydrochloride (29.0 Ci/mmol, New England Nuclear, Boston, MA) in Eagle's minimum essential medium containing 10% FBS or with 1 mCi of [^{35}S]H_2SO_4 (110 mCi/mmol of SO_4^{-2}, New England Nuclear) in sulfate-free medium 199.[15] The cells thus labeled were extracted with lysis buffer, which consisted of 10 m*M* Tris-HCl, pH 7.2, 0.15 *M* NaCl, 1 m*M* $MgCl_2$, 0.5% Nonidet P-40, 0.02% NaN_3, 1 m*M* phenylmethylsulfonyl fluoride, and 0.023 U/ml aprotinin (Sigma Chemical Co.). Cell lysates were cleared by centrifugation and passed through 0.45 μm filter. Aliquots were incubated with human monoclonal antibody at 4°C overnight and then with a rabbit anti-human IgM serum (Cappel Lab., West Chester, PA) and Protein A agarose (Repligen, Cambridge, MA) to adsorb the antigen-antibody complex. The antigen precipitated was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on slab gels[16] and visualized by fluorography.

RESULTS

Production of HM-5 (mouse-human)-human hybridoma The lymph-node lymphocytes from a patient with stomach cancer were fused with HM-5 heterohybridoma parent line. Antibody reactivity in the hybridoma culture fluid was tested against cell-surface antigens of three stomach cancer cell lines (NUGC-2, -3 and -4) by the IA and MHA assays. One positive well, 71-5.2F9 (2F9), was selected and cloned three times, resulting in a clone stably secreting a human monoclonal antibody (IgM) reactive with NUGC-3 and -4, but not NUGC-2 by the IA assay.

2F9 hybridoma was transplanted into nude mice and 2F9 antibody was purified from the ascites collected containing 101–3,900 μg IgM/ml and used for the following serological analysis.

Reactivity of 2F9 antibody with cell-surface antigens of various cell lines The antibody reacted with 4 of 7

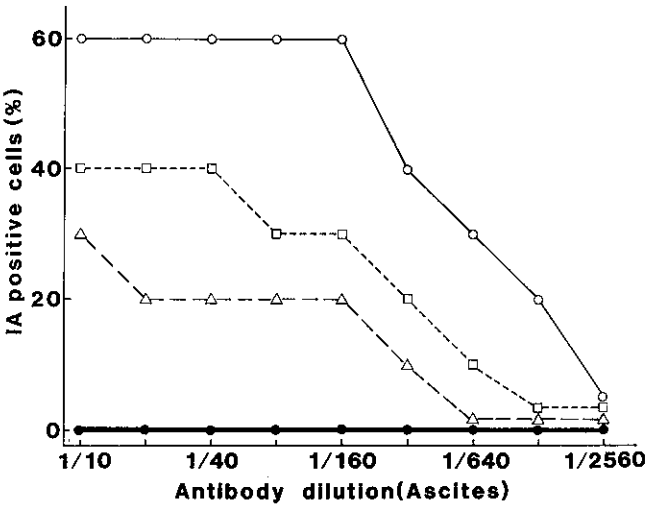

Fig. 1. Reactivity of 2F9 antibody with various cultured cell lines by IA. Target cells used were NUGC-4 stomach cancer line (○), HCT-15 colon cancer line (□), SW-480 colon cancer line (△), NUGC-2 stomach cancer line, SW-1083 colon cancer line, SCLC-1 lung cancer line and a fibroblast culture from the patient from whom NUGC-4 line was derived (●).

Table I. The Reactivity of 2F9 Antibody Tested against Various Cancer Cell Lines by IA

Cell lines, Tumor type	No. tested	No. of IA-positive cell lines[a]
Tumor		
Stomach ca.	7	4
Colon ca.	15	5
Lung ca.		
Adeno	5	2
Squamous	4	0
Large	1	0
Small	8	0
Other tumors		
Epithelial	21	0
Hematopoietic	16	1
Melanoma	5	0
Neural	5	0
Sarcoma	2	1
Normal		
Fibroblast[b]	3	0
Kidney[c]	2	0

a) They were scored as positive, when more than 20% of the cells of the target cell lines were positive at the dilution of 1/80. (As shown in Fig. 1, 2F9 antibody was positive for NUGC-4 line up to the dilution of 1/1280.) Positive cell lines (antibody titer): stomach ca., NUGC-3 (1/320), −4 (1/1280), MKN-28 (1/320), KATO-III (1/640); colon ca., HCT-15 (1/320), SK-CO-17 (1/640), SW-403 (1/640), −480 (1/160), −1222 (1/320); lung ca., ADLC-DA (1/320), −MH (1/160); sarcoma, SAOS-2 (1/160); myelogenous leukemia, HL-60 (1/60).
b) One of these had originated from the patient from whom NUGC-4 line had been derived.
c) Early culture from kidney tissue with appearance of epithelial cells.

stomach cancer lines, and 5 of 15 colon cancer lines using the IA assay. Two of 5 adenocarcinomas of lung were also positive, whereas other types of lung cancer were not. Thus, 2F9 antibody reacted predominantly with adenocarcinoma of gastrointestinal tract and lung (Table I). It was, however, also positive with HL-60 promyelocytic leukemia and SAOS-2 sarcoma cell lines. Among these positive lines, NUGC-4 showed the strongest reactivity (Fig. 1).

Reactivity of 2F9 antibody with human tumors transplantable in nude mice and fetal tissues Frozen sections of 10 human tumors in nude mice including 4 stomach cancers (NUGC-4, PTGC-1, PTGC-2 and SC6-JCK) were stained by indirect IF. Only NUGC-4 tumor showed distinct staining. Fetal tissues (the 17th gestational week) were also tested by indirect IF. All the tissues tested (stomach, intestine, lung, liver and kidney) were unreactive with 2F9 antibody.

Immunoperoxidase staining of patients' tumors and normal adult tissues with biotin-labeled 2F9 antibody Due to the background staining caused by the endogenous Ig in patients' tumor tissues and normal adult tissues, indirect IF was not appropriate for staining these tissues. Thus, 2F9 antibody was labeled with biotin, and combined with avidin-biotinylated peroxidase complex. In order to determine whether the biotin-labeling was conducted appropriately, smears of NUGC-4 line and frozen sections of NUGC-4 nude tumor were stained. The cell surface of smeared cells and tumor cells in frozen section was clearly stained (Fig. 2). Ten stomach cancer tissues including normal part of stomach were stained with the biotin-labeled 2F9 antibody, but neither tumor nor normal tissues were positive. Three colon cancers were also tested, but were negative.

Detection of 2F9 antigen in culture supernatant by sandwich ELISA The antigen shed in culture fluid was detected by sandwich ELISA using biotin-labeled 2F9 antibody (Fig. 3). The antigen was demonstrated in culture supernatant of NUGC-4, but not that of several other cell lines which are positive with 2F9 antibody by the IA assay.

Characterization of the antigen recognized by 2F9 antibody The epitope detected by 2F9 antibody on NUGC-4 line was resistant to treatment with 0.25% trypsin (Fig. 4B), 0.5 mg/ml protease V8 (Fig. 4C) or 0.1 U/ml

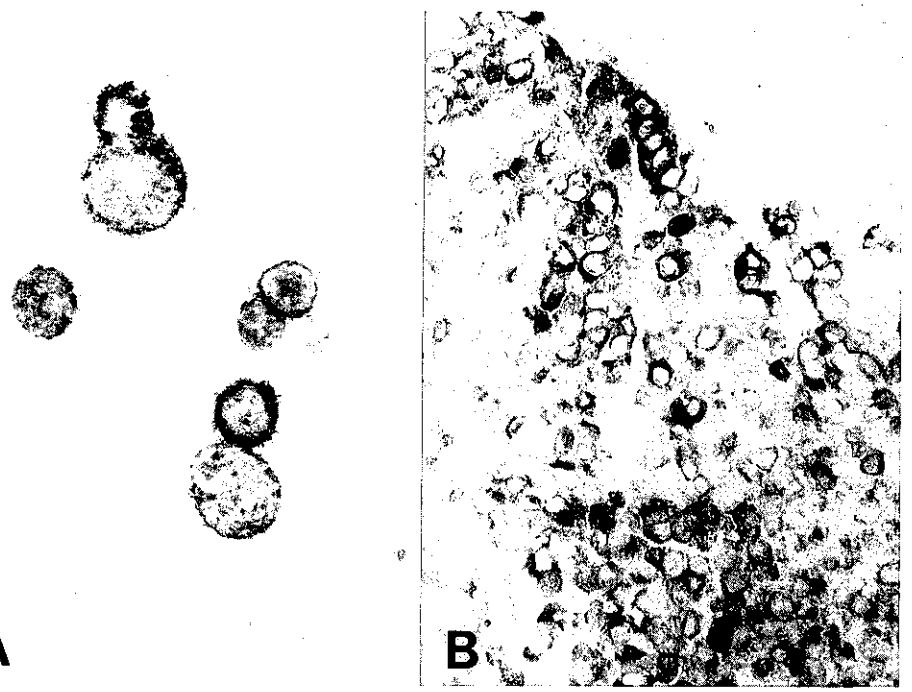

Fig. 2. Immunoperoxidase staining of smears of NUGC-4 cell line and frozen section of NUGC-4 nude mouse tumor. The acetone-fixed smears (A) and the frozen section (B) were stained with biotin-conjugated 2F9 antibody. More than 50% of the smeared cells showed distinct membrane staining (A). Most of the tumor cells in the section were clearly stained, while the stroma cells were negative (B). Magnification: (A), ×400; (B), ×200.

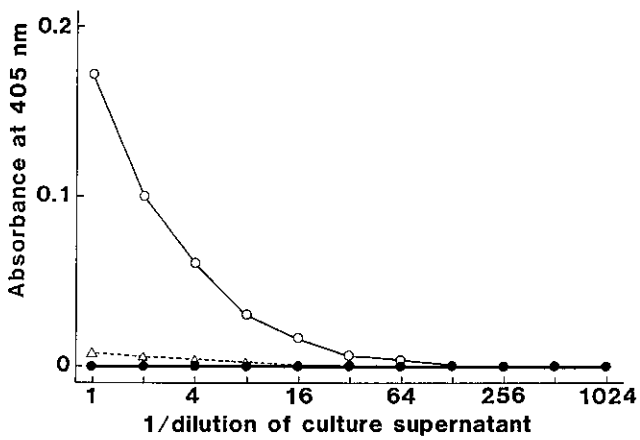

Fig. 3. Detection of the antigen recognized by 2F9 antibody in culture supernatant of NUGC-4 cell line by sandwich ELISA. Culture supernatants were collected from the cell lines which showed a positive reaction to 2F9 antibody by the IA (Table I). The antigen shed was detected with 2F9 antibody coated on the microplate and biotin-conjugated 2F9 antibody. NUGC-4 culture supernatant (○) showed a definite positive reaction. The other supernatants (●; NUGC-3 and SW-403) were negative, although KATO- III (△) may be very weakly positive. Derivation of cell lines: NUGC-3 and NUGC-4 and KATO-III, stomach cancer; SW-403, colon cancer.

neuraminidase (Fig. 4D), and also to heating (Fig. 4H). In the case of the former three enzymes, the reactivity to 2F9 antibody was enhanced by the treatments. Treatment with 1% $NaIO_4$, however, reduced the reactivity to 2F9 antibody (Fig. 4E). The results altogether suggested that the epitope was present on a carbohydrate moiety.

2F9 antibody immunoprecipitated as diffuse bands with Mr of over 200 kilodaltons (kD), and a band on the dye front, from the lysates of NUGC-4 cells labeled with [^{3}H]glucosamine (Fig. 5A). Two weak bands of 48 kD and 22 kD were also recognized. Immunoprecipitates obtained from lysates labeled with [^{35}S]H_2SO_4, also contained diffuse bands with Mr of over 200 kD and a band on the dye front (Fig. 5B), as did samples from cells labeled at the cell surface with ^{125}I (data not shown). Thus, the antigen molecule is a high-molecular-weight glycoprotein, a part of which is sulfated. It is possible that the monoclonal antibody also recognizes a glycolipid, corresponding to the component migrating on the dye front, although we could not detect any specific band in thin-layer chromatography-immunostaining of glycolipids extracted from NUGC-4 (data not shown).

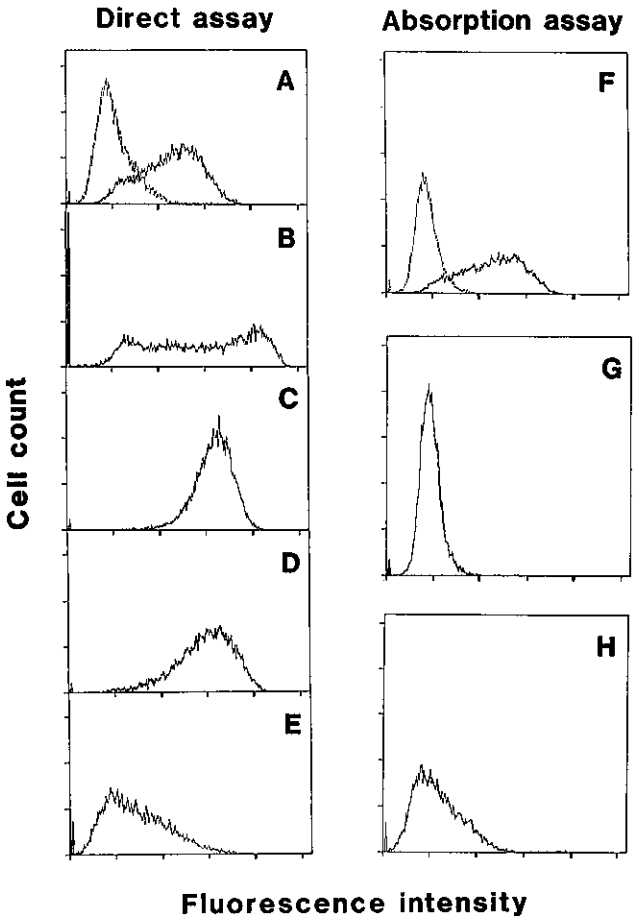

Fig. 4. Characterization of the epitope detected by 2F9 antibody. The left row (A, B, C, D and E) shows the results of direct assay, while the right one (F, G and H) shows those of absorption assay. Figures A and F show control experiments (left peak, negative-staining by ×100 diluted nude mouse ascites containing control human monoclonal antibody, 3E12[6]; right peak, positive staining by ×200 diluted nude mouse ascites containing 2F9 antibody). NUGC-4 stomach cancer cells were resuspended at 2×10^6/ml in serum-free RPMI-1640 medium containing 0.25% trypsin (B), 0.5 mg/ml protease V8 (C), 0.1 U/ml neuraminidase (D) or in PBS with 1% $NaIO_4$ (E). The mixtures with these enzymes were incubated in a 37°C water bath for 1 h, while that with $NaIO_4$ was incubated at room temperature for 10 min. Thereafter, the cells were washed once with RPMI-1640 with 10% FBS, and then twice with PBS. The cells thus treated were stained by indirect IF and the fluorescence intensity was measured by the FACS 440. Treatments of NUGC-4 cells with either trypsin, protease V8 or neuraminidase resulted in the significant enhancement of the reactivity to 2F9 antibody. $NaIO_4$ treatment destroyed the antigen. NUGC-4 cells (5×10^6) in PBS were heated in a 100°C water bath for 10 min and were washed three times with PBS. The heated cells (H) and non-heated cells (G) were incubated with 1/200 2F9 antibody in an ice bath for 1 h. The residual reactivity of 2F9 antibody was assayed by flow cytometry. Figure F shows the results of 2F9 antibody activity before absorption. Both heated and non-heated NUGC-4 cells absorbed 2F9 antibody activity.

DISCUSSION

In this study, we established a hybridoma (2F9) producing a human monoclonal antibody reactive with a surface antigen of gastro-intestinal cancer cells. The procedure used a new parental cell line, named HM-5, which was derived from a hetero-hybridoma between P3U1 mouse myeloma line and human peripheral blood lymphocytes.[7] When 5×10^4 lymphocytes were fused with HM-5 and seeded into the wells of 96-well microplates, hybridomas grew in more than 90% of the wells. Hybridomas secreting human monoclonal antibody reactive to surface antigens of stomach cancer cell line comprised 2.6% (1.5% by MHA assay and 1.1% by IA assay) of the wells with hybridomas growing. In our previous study, cell hybridization was carried out predominantly with NS-1 mouse myeloma line as a parent line.[6] Under experimental conditions which were quite similar to those of the present study, outgrowth of hybridomas was observed in 18% of the wells, even when 10^5 lymphocytes were fused and plated. No hybridoma producing an antibody reactive to surface antigen was established, although four antibodies showing a selective specificity to cytoplasmic antigen of stomach cancer cells were obtained. Thus, HM-5 line seems to be superior to NS-1 line as a fusion partner, confirming the usefulness of a heterohybridoma as a parental line, as reported previously by three groups of investigators.[17-19]

2F9 antibody showed a selective reactivity to stomach and colon cancer cell lines, although the reactivity with lines other than NUGC-4 is weak. Immunostaining of ten stomach cancers from patients and four stomach cancers transplantable in nude mice, however, did not show a positive reaction except for NUGC-4 nude tumor, suggesting that this antigen is expressed weakly on tumor cells or on a very limited number of stomach cancers. Immunoprecipitation experiments showed that the major fraction of 2F9 antigen molecule has a broad band of Mr of over 200 kD, which is glycosylated and sulfated, showing that this antigen has the characteristics of a high-molecular-weight glycoprotein, possibly a mucin. The epitope is present at least in part on a carbohydrate moiety not containing sialic acid, because it is sensitive to $NaIO_4$ treatment, but resistant to neuraminidase, protease and heat treatments. The epitope may also be possibly carried by a glycolipid component. Interestingly, this

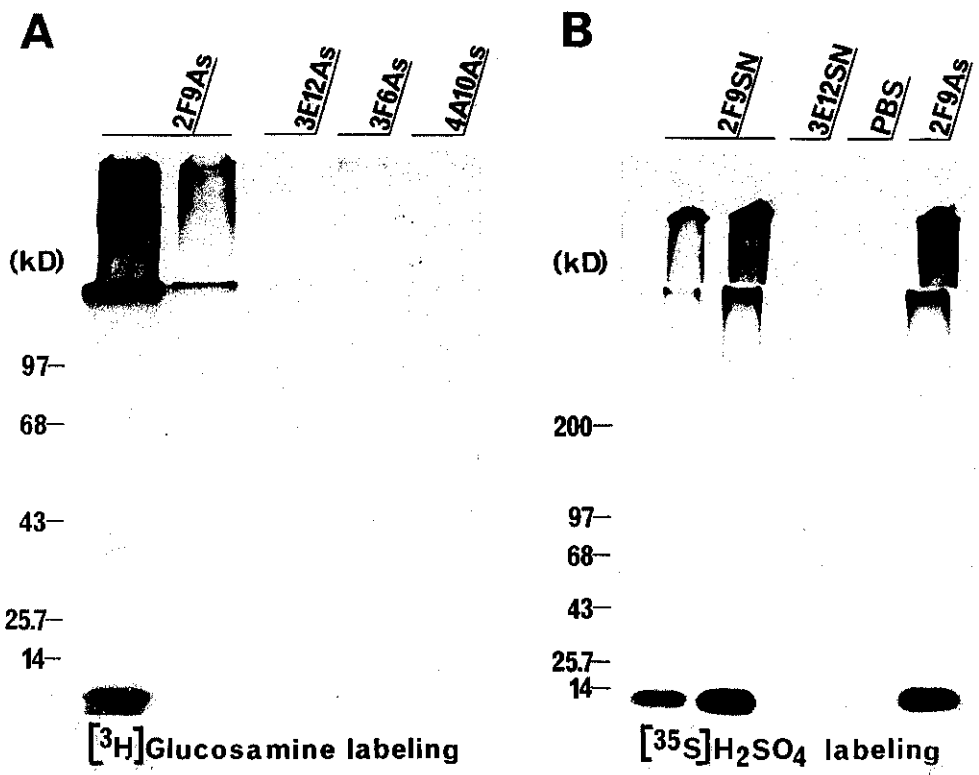

Fig. 5. SDS-PAGE analysis of cell-surface antigen after immunoprecipitation of radio-labeled NUGC-4 cell lysates with 2F9 antibody. Autoradiogram of [^{3}H]glucosamine-labeled (A) and [^{35}S]H_2SO_4-labeled (B) proteins from lysates of NUGC-4 immunoprecipitated by monoclonal antibodies and analyzed by SDS-10% PAGE (A) and 3–10% PAGE (B) under reducing conditions. To evaluate the specificity of the reactivity of antibody 2F9, antibodies from different sources, ascites (As) or culture supernatants (SN), and different volumes of the antibodies, were used. 3E12, 3F6 and 4A10 antibodies (As and SN), which were reported to recognize cytoplasmic antigens,[6] were used as controls. Phosphate-buffered saline (PBS) was also used as a control. Ig concentrations of As and SN were in the ranges of 2,000–4,000 μg/ml and 40–100 μg/ml, respectively. Volumes of 25 μl of As and 100 μl of SN were used for immunoprecipitation, except for two experiments, the first lane in A and the second lane in B, where 100 μl of As and 400 μl SN were used. The Mr markers are shown on the left. kD, kilodaltons.

antigen was detected in the culture supernatant of NUGC-4 cells. A preliminary study, however, did not show the presence of this antigen in the serum of the patient from whom NUGC-4 line had been derived.

Many groups of investigators have already succeeded in producing human monoclonal antibodies reactive to surface antigens of various cancers. In the case of melanomas, in which analysis has progressed the furthest, antibodies showing specificity for gangliosides such as GM3, GM2, GD3 or GD2 have been reported.[20–22] In the case of epithelial cancers, however, the nature of the antigens detected has not been fully analyzed. Cote *et al.* in 1986 characterized five surface antigens present on lung and other types of cancer.[23] Three of them (Ma4, Gr169 and Sp909) were stable to heat and protease treatment. NCC-1004 antibody generated by Hirohashi *et al.* had specificity for i antigen, a blood group precursor structure.[24] A recent report by Schrump *et al.* revealed that the antigen recognized by two antibodies, J309 and D579, is galactosylgloboside.[25] MAC 40/43 antibody was reported by Kjeldsen *et al.* to detect the carbohydrate part of a 47 kD glycoprotein, although the antigen may or may not be expressed on the surface membrane.[26] A glycoprotein antigen with Mr of 65 kD detected by 4G12 antibody was reported by Saito *et al.* in 1988.[27] Thus, many of the antibodies were found to detect an epitope present on a carbohydrate moiety, which is carried on protein and/or lipid moieties. The serological specificity of 2F9 antibody and the nature of the antigen molecule seem to be different from those reported previously as described above, but further analysis is needed to allow a final conclusion.

Recent advances in recombinant DNA technology have made it possible to construct chimeric Ig genes consisting of the constant regions of human Ig gene and the variable regions of mouse Ig gene isolated from mouse hybridoma (which is producing an antibody reactive to human tumor-associated antigens). By this approach, it is possible to produce human-mouse chimeric antibody with the same specificity as the original mouse monoclonal antibody.[28, 29] Such antibodies may be less immunogenic than mouse antibodies, when injected into patients. Patients may, however, respond to the variable part of the chimeric antibody and produce anti-idiotype antibody, which may neutralize the activity of the chimeric antibody injected. Thus, it may still be preferable to use human monoclonal antibody for the treatment of patients. Accordingly, efforts should be continued to improve the techniques for producing human monoclonal antibody.

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