Selection of Monoclonal Antibodies Detecting KM01 Antigen

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KANO, Y., TANIGUCHI, T., UEMURA, Y., YOKOYAMA, K., OHYANAGI, H. and SAITOH, Y. *Selection of Monoclonal Antibodies Detecting KM01 Antigen*. Tohoku J. Exp. Med., 1990, **162** (2), 127-136 — Monoclonal antibodies (MoAbs) were selected for specific binding inhibition of KM01 to the KM01 antigen, which was detected in the serum of colorectal and other gastrointestinal carcinoma patients. Out of 91 MoAbs tested, 8 were found to have the inhibiting activity. The reactivity of these MoAbs with glycolipid antigen, which was isolated from cancer cell line, corresponded well with that of cancer patients sera. These monoclonal antibodies are thought to be useful in combination with KM01 for the construction of the second generation of the KM01 antigen-detecting system. ——— mono-clonal antibody ; KM01 ; tumor marker

The KM01 antigen, which is recognized by MoAb KM01, is a new tumor marker for pancreatic carcinoma. The antigen is detected in the serum of approximately 80% of pancreatic carcinoma patients, 50% of bile duct carcinoma patients, 50% of hepatoma patients and 30% of colorectal carcinoma patients as a high-molecular-weight glycoprotein (Ohyanagi et al. 1987 ; Kano et al. 1990b). The antigen also exists as a glycolipid on the surface of some cancer cell lines (Kano et al. 1990b). Though the precise sequence of the antigen has not been identified yet, the antigenic determinant is shown to be a carbohydrate ; its whole structure is a long carbohydrate, the terminal structure of which is thought to be sialylated Lewis^a (Kano et al. 1990b). The antigenic determinant of KM01 includes that of 19-9 antibody and this results in the wider reactivity of KM01 as

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Abbreviations used are : MoAb, monoclonal antibody ; HPLC, high performance liquid chromatography ; DMEM, Dulbecco's modified MEM ; TLC, thin layer chromatography ; HPTLC, high performance thin layer chromatography ; SRBC, sheep red blood cell ; CELISA, cellular enzyme-linked immunosorbent assay ; BSA, bovine serum albumin ; PBS, phosphate-buffered saline ; RPHA, reversed passive hemagglutination assay.

compared with 19-9. In the present report, we describe the selection of MoAbs detecting KM01 antigen based on their specific inhibition of the binding of KM01 to the antigen and the characterization of selected MoAbs as compared with KM01.

MATERIALS AND METHODS

Monoclonal antibodies. Murine MoAb KM01, AF6, BF2, BG3, CE5, FD5, HH9, IE3, and JG2 were developed in our laboratories using cancer cell lines as the immunogen (Ohyanagi et al. 1987; Kano et al. 1990a). Anti-hepatitis B surface (HBs) antigen monoclonal antibody 2H7 (IgM), the negative control antibody was also developed in our laboratories. Purification of these MoAbs was performed by precipitation with 50% ammonium sulfate and HPLC on a TSK G3000SW column (Toso, Tokyo). Antibody 19-9 used in this study was a component of HISTO CA19-9™ kit (The Green Cross Corp., Osaka). Antibody C-50 used was a component of CanAg, CA-50 immunohistochemistry kit (Stena Diagnostics, Gothenburg, Sweden). The immunogens and isotypes of these MoAbs were summarized in Table 1.

Cell lines. Lung cancer cell lines (PC-1, PC-3, PC-9, PC-10), stomach cancer cell lines (MKN-28, MKN-45, KATO-III), renal cancer cell line (NRC-12), and bladder cancer cell line (NBT-2) were purchased from Immunological and Biological Laboratory, Takasaki. Esophagus cancer cell lines (TE-1, TE-2, TE-3, TE-4, TE-5, TE-6, TE-7, TE-8, TE-9, TE-10, TE-11) were kindly provided by Dr. T. Nishihira (Tohoku University, Sendai). The cell line HEK was established in our laboratory from the specimens of the patient bearing biliary duct carcinoma. Colorectal cancer cell lines (COLO-201, COLO-320DM), breast cancer cell line (ZR75-1), ovarian cancer cell line (PA-1), laryngeal cancer cell line (HEp2), oral cancer cell line (KB) and pancreatic cancer cell lines (PANC-1, MIA-PaCa-2) were purchased from Dainihon Pharmaceutical Company, Osaka. All cancer cell lines were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum. Mouse myeloma and hybridomas were cultured in DMEM medium supplemented with 10% heat inactivated horse serum.

CELISA. Reactivity of MoAbs with cancer cell lines was determined by CELISA as previously described (Kano et al. 1990b). In brief, cancer cell lines were cultured overnight in 96-well microplate and then fixed with 0.1% glutaraldehyde at room temperature for 10 min. After blocking with 1% BSA in PBS, MoAb solution was added and incubated at 37°C for 1 hr. After washing, horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (IgA + IgG + IgM) (Zymed Ltd. Inc., San Francisco, CA, USA) was added and incubated at 37°C for 1 hr, followed by washing. Substrate solution was added and the peroxidase reaction was carried out at room temperature for 30 min. The reaction was stopped by adding 4N-H₂SO₄ and the absorbance at 492 nm was read on a Titertek Multiskan spectrophotometer (Flow Lab. Inc., McLean, CA, USA).

Hemagglutination inhibition. For the screening of KM01-related antibodies, MoAbs were examined for their inhibitory effect on KM01™ RPHA (The Green Cross Corp., Osaka) in which KM01 was coupled to SRBC according to the method of Uemura et al. (1986). The spent medium of COLO-201 was used for the KM01 antigen solution. Serial doubling dilutions of the MoAb solutions (1 µg/ml) were prepared using the dilution buffer provided in KM01™ RPHA kit. Each dilution was mixed with an equal volume of KM01 antigen solution and left at room temperature for 1 hr before the remaining KM01 antigen was determined by KM01™ RPHA. The inhibitory titer of each antibody solution was expressed as the lowest dilution ratio at which hemagglutination was observed in RPHA.

Absorption by sugar determinant. The reactivity of MoAb with Lewis^a and its related determinants was investigated by the absorption test using Synsorb immunoabsorbent (Chembiomed Ltd, Edmonton, Alberta, Canada). One milliliter of MoAb solution was incubated with 50 mg of Synsorb Lewis-A, Lewis-B, H (type I), or H (type II) at room

temperature for 2 hr with continuous shaking. After removal of Synsorb by centrifugation, the remaining MoAb was determined by CELISA.

Sialidase treatment. The glutaraldehyde-fixed COLO-201 cells in 96-well microplate were treated with 0.1 $\mu\text{g/ml}$ of sialidase (Sigma Chemical Co., St. Louis, MO, USA) at 37°C for 1 hr. After incubation, the plate was washed and MoAb was added. The rest of the procedure was the same as that used in CELISA.

Preparation of ganglioside fraction. The glycolipid antigen was isolated according to a slight modification of the method of Magnani et al. (1982). COLO-201 cells were homogenized in 3 volumes (v/v) of distilled water with a homogenizer on ice. Glycolipids in the homogenate were extracted with chloroform/methanol/water (4:8:3, v/v/v) for 30 min at room temperature and centrifuged at 1,500 rpm for 10 min. The pellets were rehomogenized in distilled water, extracted, and centrifuged as before. The supernatant solutions were combined and phase-separated in chloroform/methanol/water (1:2:1.4, v/v/v). The lower phase was re-extracted with the addition of methanol and 10 mM KCl solution. The combined upper phase was evaporated under a N_2 stream. The residue was dissolved in 0.2 N NaOH and incubated for 2 hr at 37°C. After neutralization with 0.2 N HCl in methanol and evaporation, the dry residue was suspended in distilled water, dialyzed against cold distilled water, and lyophilized. The residue was dissolved in chloroform/methanol (1:1) and filtered to remove insoluble materials from the crude ganglioside fraction.

TLC-immunostaining. TLC-immunostaining was performed by a modified method of Magnani et al. (1982). Glycolipid were developed with chloroform/methanol/0.2% CaCl_2 (60:40:9, v/v/v) on HPTLC plate (Silica Gel 60, Merk, Darmstadt, Germany). The dried plate was soaked in 0.4% polyisobutylmethacrylate in *n*-hexane for 1 min. After drying, the plate was soaked in PBS containing 1% BSA and 1% polyvinylpyrrolidone (immunostaining buffer) for 1 hr at room temperature. Monoclonal antibody was diluted with the immunostaining buffer and was incubated with the plate for 1 hr at room temperature. After washing out excess antibody with PBS, 0.5 $\mu\text{Ci/ml}$ of ^{125}I -labeled F(ab')₂ sheep anti-mouse Ig (40 $\mu\text{Ci}/\mu\text{g}$, Amersham Japan Co., Tokyo) in the immunostaining buffer was overlaid on the plate and incubated for 1 hr at room temperature. The plate was washed, dried, and exposed to XR-5 x-ray film (Eastman Kodak, Rochester, NY, USA) for 20 hr. Gangliosides were also detected with resorcinol (Svennerholm 1957). Gangliosides GM1, GM2, GD1a, GD1b, and GT1b were purchased from Funakoshi Pharmaceutical Company, Tokyo.

Detection of antigen in human serum. The serum of cancer patients was obtained from the First Department of Surgery, Kobe University School of Medicine. Normal human serum was collected from healthy donors. The antigen in serum sample was determined by RPHA reagent, in which each purified MoAb was coupled to SRBC according to the method of Uemura et al. (1986). The specimen under examination was judged as positive, if a 2 well or greater difference in the endpoint of hemagglutination exists between MoAb-coated SRBC and control SRBC annexed to KM01™ RPHA kit.

RESULTS

Selection of MoAbs. In Table 1 are presented data on the selection of MoAbs inhibiting hemagglutination of KM01™ RPHA. In the 14 fusions with spleen cells of mice immunized with various cancer cell lines, approximately 10,000 hybridomas were obtained and 91 of them secreted antibodies reactive to cancer cells used for the immunization of mice but not to normal fibroblast cells. These 91 MoAbs were tested for the hemagglutination inhibition of KM01™ RPHA, and 8 were found to be inhibitory. Seven of them were obtained from the fusion with

TABLE 1. *Monoclonal antibodies inhibiting binding of KM01*

Monoclonal antibody	Isotype	Immunogen	HAI-titer
KM01	IgG1	COLO-201	1 : 128
19-9	IgG1	SW1116	ND
C-50	IgM	COLO-205	1 : 64
AF6	IgM	HEK	1 : 128
BF2	IgA	HEK	1 : 32
BG3	IgM	HEK	1 : 32
CE5	IgG1	HEK	1 : 2
FD5	IgM	HEK	1 : 128
HH9	IgM	HEK	1 : 64
IE3	IgG1	HEK	1 : 8
JG2	IgG3	COLO-201	1 : 64

HAI, hemagglutination inhibition.

ND, not detected.

spleen cells immunized with HEK and one was from the fusion with spleen cells immunized with COLO-201. The results with MoAbs 19-9 and C-50, of which were considered to recognize antigens similar to KM01 antigen, were also shown in Table 1.

Reactivity of MoAbs with cancer cell lines. The reactivity of MoAbs listed in Table 1 with various cancer cell lines was examined. As shown in Table 2, MoAbs were divided into 4 groups on the basis of the reaction pattern with cancer cell lines: group 1, BF2 and 19-9, which reacted with PC-3, KATO-III, COLO-201 and HEK; group 2, CE5 and IE3, which reacted with TE-2 and TE-7 in addition to those cell lines detected by group 1 MoAbs; group 3, KM01, AF6, BG3, JG2 and C-50, which reacted with TE-1, TE-9 and TE-10 in addition to those cell lines detected by MoAbs of the former two groups; group 4, FD5 and HH9, which reacted with MKN-45 in addition to all cell lines detected by MoAbs of the former three groups.

Biochemical studies. The reactivity of all MoAbs except for JG2 to COLO-201 cells was markedly diminished, when the cells were treated with sialidase (Fig. 1). The absorption of MoAbs with Synsorb Lewis-A diminished the binding activity of AF6 and JG2 to COLO-201 cells (Fig. 2). Other immunoabsorbents including Synsorb Lewis-B, H (type I) and H (type II) had no effects (data not shown). The reactivity of MoAbs with ganglioside antigens from COLO-201 cells was examined by TLC-immunostaining. The major component of the ganglioside recognized by KM01 showed almost the same mobility as that recognized by 19-9, that is, between GD1a and GM1. Antibody C-50 also recognized the same ganglioside, but the intensity of the immunostaining was far weaker than that of KM01 (Fig. 3). MoAbs selected as shown in Table 1 were divided into 3 groups based on their reactivity pattern with the ganglioside fraction (Fig. 4).

TABLE 2. *Reactivity of monoclonal antibodies with cancer cell lines*

Cell line	Monoclonal antibody (20 ng/ml)										
	KM01	AF6	BF2	BG3	CE5	FD5	HH9	IE3	JG2	19-9	C-50
TE-1	+	+	—	+	—	+	+	—	+	—	+
TE-2	#	#	—	#	+	#	#	#	#	—	+
TE-3	—	—	—	—	—	—	—	—	—	—	—
TE-4	—	—	—	—	—	—	—	—	—	—	—
TE-5	—	—	—	—	—	—	—	—	—	—	—
TE-6	—	—	—	—	—	—	—	—	—	—	—
TE-7	#	#	—	#	+	#	#	#	#	—	+
TE-8	—	—	—	—	—	—	—	—	—	—	—
TE-9	+	+	—	+	—	+	+	—	+	—	+
TE-10	#	#	—	#	—	#	#	—	#	+	#
TE-11	—	—	—	—	—	—	—	—	—	—	—
PC-1	—	—	—	—	—	—	—	—	—	—	—
PC-3	#	#	#	#	#	#	#	#	#	#	#
PC-9	—	—	—	—	—	—	—	—	—	—	—
PC-10	—	—	—	—	—	—	—	—	—	—	—
MKN-28	—	—	—	—	—	—	—	—	—	—	—
MKN-45	—	—	—	—	—	#	+	—	—	—	—
KATO-III	#	#	#	#	#	#	#	#	#	#	+
COLO-201	+	+	#	#	#	#	#	#	#	+	+
COLO-320DM	—	—	—	—	—	—	—	—	—	—	—
ZR75-1	—	—	—	—	—	—	—	—	—	—	—
NRC-12	—	—	—	—	—	—	—	—	—	—	—
PA-1	—	—	—	—	—	—	—	—	—	—	—
HEp-2	—	—	—	—	—	—	—	—	—	—	—
KB	—	—	—	—	—	—	—	—	—	—	—
NBT-2	—	—	—	—	—	—	—	—	—	—	—
HEK	#	#	#	#	#	#	#	#	#	#	+
PANC-1	—	—	—	—	—	—	—	—	—	—	—
MIA-PaCa-2	—	—	—	—	—	—	—	—	—	—	—

The intensity of the reaction was scored according to the absorbance at 492 nm :
that is, — ; less than 0.099, + ; 0.100–0.399, # ; 0.400–0.699, ## ; more than 0.700.

Group I MoAbs (19-9, CE5, IE3) recognized a single major ganglioside component with a mobility between GD1a and GM1. Group II MoAbs (KM01, BG3, BF2, HH9, FD5) recognized not only the same ganglioside as detected by group I but also other ganglioside components with a slower mobilities. Group III MoAbs (AF6, JG2) recognized a ganglioside component with a higher mobility in addition to those recognized by group II.

Serum detection. Table 3 shows the positive ratio of antigens in human

Fig. 1. Effect of sialidase treatment on the reactivity of monoclonal antibodies. The COLO-201 cells were treated with 0.1 u/ml of sialidase at 37°C for 1 hr. Reactivities of MoAbs with the sialidase treated cells and untreated control cells were determined by CELISA. The concentration of MoAb was 0.1 μ g/ml. For details of the procedure, see "Materials and Methods." ■, sialidase-treated; □, untreated control.

Fig. 2. Effect of absorption by Synsorb Lewis-A on the reactivity of monoclonal antibodies. Each MoAb (0.1 μ g/ml) was incubated with Synsorb Lewis-A at room temperature for 2 hr with continuous shaking. Reactivity of MoAb with COLO-201 cells was determined by CELISA. For details of the procedure, see "Materials and Methods." ■, absorbed with Synsorb Lewis-A; □, untreated control.

Fig. 3. TLC-immunostaining of ganglioside from COLO-201 cells by KM01 and C-50 monoclonal antibodies. The concentrations of MoAbs were as follows; lane 1, 25 $\mu\text{g}/\text{ml}$ of anti-HBs MoAb; lane 2, 100 $\mu\text{g}/\text{ml}$ of anti-HBs MoAb; lane 3, 25 $\mu\text{g}/\text{ml}$ of C-50; lane 4, 100 $\mu\text{g}/\text{ml}$ of C-50; lane 5, 5 $\mu\text{g}/\text{ml}$ KM01. For details of the procedure, see "Materials and Methods."

Fig. 4. TLC-immunostaining of ganglioside from COLO-201 cells by monoclonal antibodies inhibiting binding of KM01. The concentration of MoAb was 1 $\mu\text{g}/\text{ml}$. Lane 1, BG3; lane 2, BF2; lane 3, AF6; lane 4, 19-9; lane 5, KM01; lane 6, HH9; lane 7, CE5; lane 8, IE3; lane 9, FD5; lane 10, JG2. For details of the procedure, see "Materials and Methods."

TABLE 3. *Reactivity of monoclonal antibodies with sera from cancer patients*

Disease	Monoclonal antibodies					
	KM01	BG3	CE5	IE3	AF6	JG2
Pancreatic cancer	11/12	10/12	8/12	5/12	6/6	6/6
Bile duct cancer	4/6	4/6	3/6	1/6	6/6	6/6
Liver cancer	7/12	4/12	0/12	0/12	3/6	5/6
Stomach cancer	6/12	4/12	2/12	0/12	4/6	5/6
Colorectal cancer	8/12	4/12	2/12	0/12	5/6	5/6
Normal	0/12	0/12	0/12	0/12	4/6	2/6

serum detected by MoAbs coupled to SRBC. Each group of MoAbs in Fig. 4 showed different reactivity with cancer patients sera. KM01 and BG3 reacted with sera from the patients with bile duct, liver, stomach and colorectal carcinoma besides sera from pancreatic carcinoma patients. CE5 and IE3 reacted preferentially with sera from pancreatic carcinoma patients. AF6 and JG2 showed broad reactivity spectra including normal human sera.

DISCUSSION

We have recently developed a MoAb designated KM01, which detects serum antigens for pancreatic carcinoma and other several gastrointestinal carcinomas (Ohyanagi et al. 1987). We also have been developing new MoAbs useful for the diagnosis and therapy of cancer (Ohyanagi et al. 1988; Kano et al. 1990a), some of which were found to be cross-reactive with KM01. They were divided into three groups depending on their reactivity with glycolipid antigens isolated from COLO-201. The reactivity of MoAbs with sera of cancer patients appeared to be correlated to that with the glycolipid antigens. MoAbs in group I, represented by 19-9, had a weak inhibitory activity toward the binding of KM01 to KM01 antigen. Each MoAb in group II, represented by KM01, showed almost the same inhibitory activity as KM01. MoAbs in group III had the same inhibitory activity as group II. The glycolipid antigen of group I migrates between GD1a and GM1. This glycolipid is thought to be lacto-N-fucopentaose II as reported by Magnani et al. (1982). MoAbs in groups II and III detected slowly migrating glycolipids in addition to the antigen detected by group I. This suggests that the antigenic determinants for groups II and III have longer sugar chains than that for group I. The weaker ability of group I to inhibit the binding of KM01 to KM01 antigen as compared with that of groups II and III shows the importance of the long-chain structure as the determinant of KM01. The terminal structure of the KM01 antigen is assumed to be sialylated Lewis^a, which is composed of a type 1 chain (Kano et al. 1990b). It is reported that the type 1 chain is not elongated by itself and that, in the case of long-chain glycolipids, the determinants are carried by type 2 chain (Kannagi et al. 1985). Such a case may occur

in KM01 antigen. The antigen detected by group III might be a long-chain Lewis^a, because the reactivity of MoAbs in group III was diminished by the absorption with Lewis^a determinant. The reactivity of MoAb AF6 to COLO-201 cells was markedly reduced when the cells were treated with sialidase, but the reactivity of JG2, the another group III MoAb, was not. This result indicates that these two MoAbs recognize different epitopes on long-chain Lewis^a. Kitagawa et al. (1988) have reported similar observations with MoAbs to sialylated Lewis^a. They developed MoAb MSW 113 detecting sialylated Lewis^a. The antigen of MSW 113 was more sensitive to sialidase treatment than the NS 19-9 antigen. From the results of inhibition studies using oligosaccharides, they showed that NS 19-9 was directed toward a fucose-containing terminal structure, while MSW 113 was directed toward a sialic acid-containing terminal structure. From the result of the reactivity with cancer cell lines, MoAbs were divided into 4 groups. It appeared that MoAbs of groups 1 and 2 corresponded to group I, and MoAbs of groups 3 and 4 corresponded to groups II and III. This result is thought to reflect both the expression level of the antigen in each cancer cell line and the avidity of each MoAb to the antigen. MoAbs of groups 1 and 2 recognized the same antigen, but the avidity of group 2 MoAbs may be stronger than that of group 1 MoAbs. The avidity of group 4 may be stronger than all other MoAbs in groups II and III. The reason for the poor reactivity of BF2 to cancer cell lines remains to be solved, but it may partly because of the poor reactivity of the peroxidase-conjugated anti-mouse immunoglobulin with IgA subclass. In those cancer cell lines with which groups 1 and 2 MoAbs reacted, PC-3, KATO-III, COLO-201, HEK, TE-2 and TE-7, the expression level of KM01-specific long-chain sialylated Lewis^a might be very low when compared with that of sialylated Lewis^a. On the other hand, in cancer cell lines TE-1, TE-9, TE-10 and MKN-45, significant amounts of long-chain carbohydrate may be expressed. These cancer cell lines may be the better source than COLO-201 to analyze the long-chain structure of KM01 antigen. MoAbs obtained here will be useful for this purpose. The investigation of the sandwich immunoassay using these MoAbs in combination with KM01 is of another interest. Mansson et al. (1985) have found that MoAb C-50 could react with both sialylated Lewis^a and its defucosylated derivative. In this study, we showed that MoAb C-50 has strong inhibitory activity toward the binding of KM01 to KM01 antigen and reacts with the same antigens, which are thought to be sialylated Lewis^a and its long-chain derivative, as those detected by KM01. Mansson et al. (1985) have also reported that C-50 could react with octasaccharide fraction of the monosialoganglioside in addition to hexasaccharide fraction, which corresponded to sialylated Lewis^a. Holmgren et al. (1984) have shown the broad reactivity of C-50 with sera of patients with different carcinomas including hepatocellular carcinoma when compared with that of 19-9 antibody. These results suggest the close relationship between C-50 and KM01. Further studies are needed to identify the structural differences of the antigens

recognized by these two MoAbs.

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