A Monoclonal Antibody, KM10 Reactive with Human Gastrointestinal Cancer and Its Application for Immunotherapy

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A monoclonal antibody, KM10 (IgG₁) was produced by fusing spleen cells from a human gastric cancer cell (MKN45)-primed BALB/c mouse with the murine myeloma cell line X63-Ag8-653. The antibody reacted strongly with the plasma membrane of human gastrointestinal carcinoma. Sections of the malignant and benign tissues were tested with immunoperoxidase. All of 10 (100%) large intestinal cancers, 26 of 31 (84%) gastric cancers, 5 of 7 (71%) pancreatic cancers and all of 3 (100%) ampullary cancers reacted positively. Moderate or weak reactivity was observed with normal human tissues, hepatoma and carcinomas of mammary, thyroid and adrenal glands. According to a study of the distribution of ¹²⁵I-labeled KM10 in nude mice bearing human gastric cancer, KM10 selectively localized in tumor tissue rather than normal tissue. Whole body autoradiography also supported such a selective distribution. Destruction of antigenic properties by pronase digestion demonstrated its protein nature and by Western blot analysis, it was identified as a protein with an Mr of 180-200 kd. KM10-adriamycin (ADM) conjugate was prepared via an oxidized dextran bridge and this immunoconjugate retained the binding activity against human gastric cancer. MKN45 cells were inoculated subcutaneously into athymic mice and intravenous treatment was begun when the tumor became measurable. A dose-dependent antitumor activity was observed *in vivo* with KM10-ADM conjugate, while this conjugate was less toxic than free ADM.

Key words: Monoclonal antibody, KM10 — Gastrointestinal cancer — Immunotherapy — Targeting therapy

Monoclonal antibodies having specificity for human cancer tissues are under development by many investigators for diagnostic and therapeutic use.⁽¹⁻⁵⁾ In the future it may be feasible to use monoclonal antibodies in targeting therapy by coupling an anti-tumor agent with a monoclonal antibody specific for the cancer. In this sense, the histological and cytochemical characterization of monoclonal antibodies to human normal and malignant tissues is fundamental in determining the specificity of monoclonal antibodies. The effect of and absence of untoward reactions to the conjugates of antibody to antitumor

agents are other major problems in developing facilitated immunotherapy.^(6,7)

The present study was performed to examine our newly produced monoclonal antibody, KM10, and the application of KM10-conjugated ADM^{*4} for targeting chemotherapy to human gastrointestinal cancer in athymic (*nu/nu*) mice.

MATERIALS AND METHODS

Production of Monoclonal Antibodies We produced hybridomas secreting monoclonal antibodies to the cancer cell line MKN45 which was derived from a human gastric adenocarcinoma using standard hybridoma technology. A BALB/c mouse was immunized subcutaneously with 0.1 mg of membrane protein from stomach adenocarcinoma tissue (MKN45) emulsified in complete Freund's adjuvant. The immunized spleen cells were fused with mouse myeloma cells X63-Ag8-653 at a 4:1 ratio using 45% polyethyleneglycol (Mr 4000) ac-

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^{*4} Abbreviations: ADM, adriamycin; ELISA, enzyme-linked immunosorbent assay; NC, nitrocellulose; TCA, trichloroacetic acid; PBS, phosphate-buffered saline; HBs, hepatitis B surface antigen.

cording to the method of Galfre and Milstein.⁸⁾ The hybrid cells were then seeded in 96-well microplates.

Screening of Monoclonal Antibodies Supernatants from growing hybridomas were screened for specific antibodies using an enzyme-linked immunosorbent assay (ELISA). The hybridoma culture supernatant was added to microplates coated with antigen (cultured human gastric cancer cell line MKN45 fixed by 0.1% glutaraldehyde solution or partially purified cancer-associated antigens). These plates were incubated for 1 hr at 37°, rinsed with phosphate-buffered saline (PBS), converted with peroxidase-labeled rabbit anti-mouse immunoglobulin (IgA + IgG + IgM) antibody (Zymed Lab., Inc.) and then incubated for 1 hr at 37°. The plates were washed with PBS and incubated with O-phenylenediamine-H₂O₂ substrate for 30 min. The enzyme reaction was stopped with 2M H₂SO₄ and the absorbance was read at 490 nm by an autoreader (Type MR 580, Dynatech).

Determination of Isotype of Monoclonal Antibodies The isotype of monoclonal antibodies was determined by Ouchterlony's method.⁹⁾

Immunoperoxidase Procedure Fresh normal and malignant tissues were obtained at the time of operation from patients at the First Department of Surgery, Kobe University, School of Medicine. They were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and embedded in optimum cutting temperature (OCT) compound (Miles Lab., Inc.). Frozen sections were stained using an indirect immunoperoxidase method described by Nakane.¹⁰⁾ As control, sections from the same block were incubated with murine myeloma protein at an immunoglobulin concentration equivalent to that of monoclonal antibody, KM10.

Grading of Immunoperoxidase Staining Tissue sections were graded according to staining intensity: that is, strongly positive, staining more than 50% of the cells in the specimen; moderately positive, staining more than 10% of the cells in the specimen; weakly positive, very slight staining or negative.¹¹⁾

Biochemical Characterization of the Antigen The antigenic determinant defined by the reaction of monoclonal antibody KM10 was characterized by using the following procedure.

Enzyme Treatment: The antigen was partially purified from MKN45 cell lysates by affinity chromatography using both KM10 and anti-mouse immunoglobulin coupled to a Sepharose 4B column. The antigen was treated with pronase (Kaken Pharmaceutical Co., Ltd., Tokyo, 10 µg/ml, 25° for 17 hr), trypsin (Sigma Chemical Co., St. Louis, Mo.; 4 mg/ml, 22° for 1 hr), neuraminidase (Sigma; 20 units/ml, 37° for 1 hr) and

sodium periodate (4mM, 4° for 45 min). The treated antigens were each added to KM10 (2 µg/ml) and incubated at 37° for 1 hr. These solutions were added to the microplate coated with MKN45 and ELISA was performed to evaluate the inhibition of the antigen with respect to KM10. Sodium periodate (10mM) was added to the microplates coated with MKN45 cells. After the treatments, 50 µl of diluted KM10 and carcinoembryonic antigen (CEA) antibody (2 µg/ml) were added to separate wells and incubated at 37° for 1 hr. Then ELISA was performed.^{12, 13)}

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting: SDS-PAG plates were purchased from Daiichi Kagaku, Japan, and nitrocellulose (NC) membrane filters (Type TM-2) from Toyo Roshi Co., Ltd., Tokyo. The samples (purified antigen) were dissolved in buffer (2.5% SDS, 5% 2-mercaptoethanol and 0.01M Tris-HCl, pH 8.0) and boiled for 5 min. As a control, CEA-CT6 was purchased from Morinaga Institute of Biological Science, Yokohama. CEA-CT6 was dissolved in the same buffer and boiled for 5 min. Electrophoresis was carried out with a current of 300 V for 10 min and 150 V for the subsequent 60 min. The samples were fixed overnight with 5% trichloroacetic acid (TCA)¹⁴⁾ and then stained with silver.¹⁵⁾ The molecular weight was estimated using protein standards (Pharmacia, Tokyo).

Upon completion of electrophoresis, the gel was placed on a wet Scotch-Brite pad (3M, St. Paul, Minn.) and its surface was rinsed with cool (4°) transfer buffer (25mM Tris-192mM glycine, pH 8.3 in 20% methanol) and an NC filter was placed on the gel. Electrophoretic transfer was carried out with a current of 12 V for 1 hr and subsequently at 36 V overnight.^{16, 17)} Upon completion of electrophoretic transfer, the NC filter was stained with a Biorad gold staining kit, (Biorad, Tokyo) using the indirect immunoperoxidase method as described above, making certain that the antigen was transferred.

Distribution Study of ¹²⁵I-labeled Monoclonal Antibody, KM10 Congenital athymic (nude), 4-week-old female BALB/c mice were inoculated subcutaneously with 1 × 10⁶ MKN45 cells derived from human gastric cancer cells. ¹²⁵I-labeled monoclonal antibodies were prepared according to the solid-state lactoperoxidase method.¹⁸⁾ Seven days before the injection, they received 10mM KI solution to block uptake of free iodine by the thyroid. Tumors appeared in 3 weeks. The nude mice were injected with 165 µg of ¹²⁵I (5.07 × 10⁶ cpm)-labeled KM10 or 156 µg of ¹²⁵I (5.00 × 10⁶ cpm)-labeled anti-HBs monoclonal antibody (IgG₁).¹⁹⁾ The negative control antibody, via the tail vein. At 3, 24 and 72 hr post-injection, the tumor-bearing mice were sacrificed by exsanguination and dis-

sected. The tumors, heart, lung, liver, spleen and kidney were individually weighed and assayed for radioactivity. The results were expressed as specific radioactivity (cpm/g). The statistical significance of differences between experimental and control values was determined with Student's *t* test.

At 72 hr after administration of ^{125}I -labeled monoclonal antibodies the nude mice were killed with chloroform, frozen with dry ice-acetone and embedded in 7% carboxymethylcellulose paste as a block. The whole body was cut into $7\text{ }\mu\text{m}$ sections with a cryomicrotome (CMT-400-NM, Medical Technology Equipment Service Corp., Tokyo). The resulting slices were exposed on a sheet of X-ray film (Fuji Photo Corp., Tokyo) for 12 days.

Preparation of an Antibody-ADM Conjugate An antibody-ADM conjugate was prepared via a dextran bridge according to the methods described by Hurwitz *et al.*,²⁰ but reduction of Schiff's base was not done. Namely, polyaldehyde-dextran prepared by oxidation of dextran with sodium periodate was reacted first with ADM at 20° for 18 hr and then antibody was added to the reaction mixture. After 18 hr the solution was applied to a Sephacryl S300 column ($3.5 \times 40\text{ cm}$) and the conjugate fractions

were collected. The antibody titer of the conjugate was assayed by ELISA as described above. Combined molar ratio between KM10 and ADM was measured by gel permeation chromatography.

Antitumor Activity *in vitro* MKN45 cells were seeded in a 96-well tissue culture plate with 10^5 cells per well and cultured at 37° for 18 hr. Quadruplicate sets of cell cultures were incubated with KM10-ADM conjugate, non-specific conjugate (anti-HBs monoclonal antibody-ADM conjugate) or free ADM at 37° for 1 hr. The concentration of ADM in the well of each series was varied in 5 steps from 0.9 to $15\text{ }\mu\text{g/ml}$. The cells were pulsed with [^3H]uridine ($0.2\text{ }\mu\text{Ci}$ per well; specific activity, 25.8 Ci/mmol , New England Nuclear) at 37° for 3.5 hr, collected on a Millipore filter ($0.45\text{ }\mu\text{m}$) and treated with 5% TCA. Radioactivity in the 5% TCA-insoluble fraction was measured with a liquid scintillation counter (Packard Tri-Carb 4640).

Antitumor Activity *in vivo* MKN45 cells (10^7 cells/mouse) were inoculated subcutaneously into athymic mice and intravenous treatment was begun when the tumor became measurable (about 8 mm in diameter). Cross-sectional diameters were measured using a caliper at 2- or 3-day intervals. Tumor

Fig. 1. Light micrographs of malignant tissues. A: Colon cancer stained by KM10 monoclonal antibody, peroxidase-antiperoxidase method. $\times 150$. B: Serial section of Fig. 1-A stained with hematoxylin and eosin. $\times 150$. C: Gastric cancer stained by KM10 monoclonal antibody, peroxidase-antiperoxidase method. $\times 150$. D: Serial section of Fig. 1-C stained with hematoxylin and eosin. $\times 150$.

weight was calculated according to the following equation: Tumor volume (mm^3) = length (mm) $\times$ [width (mm)]² $\times$ 1/2.²¹⁾ KM10-ADM conjugate, anti-HBs monoclonal antibody-ADM conjugate, free ADM and saline were administered via the tail vein at the doses of 7.5, 15 and 30 mg/kg respectively. These agents were administered once or three times at 3-day intervals.

RESULTS

The Isotype of Monoclonal Antibody KM10
It was determined to be IgG₁ using Ouchterlony's method.

Immunohisto-cytochemical Reaction of KM10 with Malignant and Normal Tissues Various tumorous and normal tissues were examined immunohisto-cytochemically with KM10 using an indirect immunoperoxidase method. The positive reaction sometimes occurred unevenly in a specimen. In spite of this fact, these positive findings in specimens could be broadly divided into two types, that is to say, apical

type and cytoplasmic type. The apical type showed the positive reaction localized as a thin line along the luminal surface of the cell and the cytoplasmic type showed the positive reaction in the cytoplasm as a diffuse pattern.

In the specimens of malignant tumors, 47 of 69 (68%) specimens showed immunoperoxidase staining. All cases of 10 carcinomas of the large intestine and 3 ampullary cancers, 26 cases of 31 (84%) gastric cancers and 5 cases of 7 (71%) pancreas cancers were positive (Fig. 1). Hepatocellular carcinoma and squamous cell carcinoma of the esophagus showed positive staining in 1 of 11 (9%) and 2 of 7 (29%), respectively. Malignant tumors of mammary gland, thyroid gland and gall bladder were negative for KM10.

In normal tissues, 12 of 16 (75%) large intestine, 1 of 3 (33%) small intestine, and 13 of 34 (38%) stomach were weakly or moderately positive for KM10 as apical type. Pan-

Table I. Immunoperoxidase Staining of Specimens of Human Tissue Using KM10 Monoclonal Antibody

Tissues	Negative	Positive		
		Weak	Moderate	Strong
Malignant				
Colon	0	1	1	8
Stomach	5	4	4	18
Ampulla of Vater	0	0	0	3
Pancreas	2	1	3	1
Liver	10	0	1	0
Esophagus	5	1	1	0
Breast	1	0	0	0
Thyroid gland	1	0	0	0
Gall bladder	2	0	0	0
Normal				
Colon	4	10	2	0
Stomach	21	10	3	0
Small intestine	2	1	3	0
Pancreas	7	0	0	0
Liver	4	0	0	0
Lung	2	0	0	0
Kidney	1	0	0	0
Esophagus	0	3	1	0
Mammary gland	2	0	0	0
Thyroid gland	1	0	0	0
Adrenal gland	1	0	0	0
Gall bladder	2	0	0	0
Lymph node	3	0	0	0

creas, liver, lung, kidney, mammary gland, thyroid gland, adrenal gland, lymph node and gall bladder were negative (Table I).

Analysis of the Antigen by Enzyme Treatment, SDS-Polyacrylamide Gel Electrophoresis and Western Blotting In ELISA with pronase treatment the value of absorbance at 490 nm was high, but in the case of no treatment or sialidase treatment it was low. The pronase treatment of the antigen refined from

Table II. Sensitivity of KM10 Antigen to Several Enzymes

Enzyme	Absorbance at 490 nm
None	0.362 ± 0.01 ^{b)}
Sialidase	0.360 ± 0.01
Pronase	0.606 ± 0.02
Trypsin	0.353 ± 0.03
Control ^{a)}	0.873 ± 0.03

a) Reaction without KM10 antigen.

b) Mean ± SD.

Fig. 2. Western blot analysis of KM10 antigen. 1: Prestained marker. 2: KM10 antigen. 3: CEA-CT6.

Fig. 3. Distribution of ¹²⁵I-labeled KM10 (●) and anti-HBs (○) monoclonal antibody in nude mice bearing MKN45 human gastric cancer.

the cell line MKN45 markedly reduced the binding activity of the antigen to KM10, but trypsin or sialidase did not affect the activity (Table II). In SDS-polyacrylamide gel electrophoresis, the sample was near the CEA-CT6 band. Therefore, the molecular weight of the antigen was estimated to be about 180–200 kD, and KM10 seemed to be similar to CEA-CT6. The sample transferred to the NC filter by Western blotting reacted with KM10, confirming that protein was a component of the antigen (Fig. 2).

In vivo Distribution of ^{125}I -labeled Monoclonal Antibodies The time courses of localization of radioactivities in the tumor, blood, liver, lung, heart, spleen and kidney are shown in Fig. 3. In the case of KM10, the specific radioactivity of the tumor remained higher than those of other tissues except blood during 72 hr after administration. In other tissues (except blood), the radioactivity was

highest (4.29×10^5 cpm/g) at 3 hr and then decreased over time. In the case of anti-HBs monoclonal antibody, there were no remarkable differences in the distribution of radioactivity in tissues except blood at 24 and 72 hr. Thus the differences between KM10 accumulation and anti-HBs monoclonal antibody accumulation in the tumor at 24 and 72 hr were statistically significant ($P < 0.01$). Also, the accumulation of KM10 in tumor tissue differed significantly ($P < 0.01$) from that in other tissues at both 24 and 72 hr. Whole body autoradiography also supported the selective distribution of KM10 to tumor tissues. Radioactivity was localized only in the tumor and was scarcely detected in other tissues (Fig. 4A). However, such selective distribution of radioactivity was not observed in the mouse administered anti-HBs monoclonal antibody (Fig. 4B).

Nature of KM10-ADM Conjugate In ELISA, the antibody titer of KM10-ADM conjugate was about 75% of that of KM10 alone. By gel permeation chromatography, 1 mol of KM10 was found to be combined with 7 mol of ADM.

Antitumor Effect of KM10-ADM Conjugate *in vitro* In the *in vitro* experiment, the inhibitory effect of ADM on the incorporation of [^3H]uridine into MKN45 cells was reduced moderately when ADM was conjugated to either KM10 or anti-HBs monoclonal antibody (Fig. 5). The IC₅₀s (the concentration

Fig. 4. Whole-body autoradiographs of MKN45 human gastric cancer-bearing mice at 72 hr after administration of ^{125}I -labeled monoclonal antibodies. A: Mouse administered ^{125}I -labeled KM10. B: Mouse administered ^{125}I -labeled anti-HBs monoclonal antibody.

Fig. 5. [^3H]Uridine incorporation of MKN45 cells with KM10-ADM (○), anti-HBs antibody-ADM conjugate (△) and free ADM (●). Mean $\pm$ SD.

Fig. 6. Tumor growth curves after single intravenous administration of KM10 conjugate (■), anti-HBs antibody conjugate (●), free ADM (▲) and saline (×) (ADM 7.5 mg/kg, n=7). Mean $\pm$ SD. *: $P < 0.05$.

Fig. 7. Tumor growth curve after single intravenous administration of KM10 conjugate (■), anti-HBs antibody conjugate (●), free ADM (▲) and saline (×) (ADM 30 mg/kg, n=7). Mean $\pm$ SD. †: Dead. *: $P < 0.05$.

inhibiting uptake of [3 H]uridine to 50% of the control) of KM10-ADM conjugate, free ADM and non-specific conjugate were estimated to be 5.8, 2.8 and 12.2 μ g/ml, respectively.

Antitumor Effect of KM10-ADM Conjugate *in vivo* Figures 6 and 7 show the tumor growth curve after single intravenous administration of KM10-ADM conjugate and two control agents, free ADM and non-specific conjugate. A dose-dependent antitumor activity was observed in mice treated with KM10-ADM conjugate. On the other hand, free ADM and non-specific conjugate were not effective at low dose levels (7.5 and 15 mg/

kg). When the dose was increased to 30 mg/kg, all mice given free ADM were dead by 7 days after the administration, but no mice died in the conjugate groups. KM10-ADM conjugate was the most effective in this series. Relative tumor volume of the mice given non-specific conjugate at 10 days after treatment seemed to be smaller than that at 7 days after treatment, but the difference was not statistically significant. The effect of KM10-ADM conjugate on MKN45-bearing mice was seen more clearly in the multiple treatment schedule (Fig. 8). KM10-ADM conjugate showed not only higher antitumor activity but also markedly less toxicity than free ADM.

Fig. 8. Tumor growth curves after the multiple administration of KM10-ADM conjugate (■, 15 mg/kg/day; ▲, 5 mg/kg/day), free ADM (□, 15 mg/kg/day; △, 5 mg/kg/day) and saline (×). Mean $\pm$ SD. †: Dead.

DISCUSSION

High incidence of positive staining by immunoperoxidase in carcinoma of the stomach, large intestine, pancreas and ampulla of Vater reveals that KM10 has an affinity for adenocarcinoma of the digestive organs. However, the weak and localized positive staining of KM10 on the luminal surface of normal mucosa such as large intestine suggests that KM10 is not absolutely specific for carcinoma and that the distribution of KM10 is similar to that of CEA, which is detected in both normal and malignant tissue.^{22, 23)} In normal colonic epithelial cells, CEA is localized at a thin line along the luminal surface of the columnar cells and within the mucus of goblet cells by polarity. But in malignant cells, because of the lack of polarity, CEA is observed in a diffuse pattern throughout the cytoplasm without the apical concentration seen in normal cells. Our study suggests that the distribution of KM10 depends on the polarity of cells, as in the case of CEA.

The epitope of the antigen is considered not to be carbohydrate, since it was not destroyed

by neuraminidase or sodium periodate. Reduced activity of the antigen after pronase treatment supports the hypothesis that the epitope of the antigen is associated with a protein. The molecular weight of the antigen is estimated to be about 180–200 kD. Because KM10 and CEA showed immunological cross reactivity, they have a common epitope, but the solid structure of KM10 has not yet been fully analyzed.

With regard to the distribution of KM10 in tissues of nude mice bearing MKN45 human gastric cancer, high selectivity of KM10 for adenocarcinoma was observed. The selective distribution observed in whole body autoradiography also confirms that KM10 is highly targeted to gastrointestinal carcinoma.

In summary, even though it reacts slightly with some normal tissues, the reactivity of KM10 with malignant tissues is stronger and more frequent, suggesting its application to targeting therapy.

It has been reported that several monoclonal antibody-toxin conjugates directed against human and animal tumor cells surface antigens had specific toxicity toward the

target cells *in vitro* and *in vivo*. Our monoclonal antibody, KM10, was conjugated to an anticancer agent, ADM, with a 1 to 7 molar ratio; the antibody titer remained at about 75% after conjugation. Maintenance of 75% binding activity against target cells is adequate, suggesting KM10-ADM conjugate is available for immunotherapy.

Compared with conjugate groups, higher inhibition of [^3H]uridine uptake (lower IC $_{50}$ s) of MKN45 cells in the free ADM group reveals that ADM activity is depressed by conjugation. Thus, KM10-ADM conjugate should be administered at twice the amount of free ADM on the basis of ADM activity alone. Between conjugate groups, however, greater inhibition of [^3H]uridine uptake in the KM10-ADM conjugate group than in the non-specific conjugate group supports the view that ADM combined to KM10 is transferred into tumor cells more effectively due to specific binding activity of KM10 to cancer cells.

In the *in vivo* experiment, dose-dependent antitumor activity was observed with KM10-ADM conjugate. KM10-ADM conjugate, non-specific conjugate and free ADM showed antitumor activity owing to ADM. But, KM10-ADM conjugate has greater antitumor activity than non-specific conjugate or free ADM in spite of loss of ADM activity by conjugation. This fact reveals that KM10 carries the antitumor agent toward target cells selectively. The effect of KM10-ADM conjugate on MKN45-bearing mice is shown more clearly in multiple administration, as would be used in a clinical schedule.

The marked difference in incidence of death due to adverse effect of ADM between free ADM and conjugates suggests that conjugation of ADM to antibody decreases the side effects of free ADM. In conclusion, therefore, KM10-ADM conjugate not only retains higher antitumor activity but also shows markedly less toxicity than free ADM.

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REFERENCES

- 1) Koprowski, H., Steplewski, Z., Mitchell, K., Herlyn, M., Herlyn, D. and Fuhrer, P. Colorectal carcinoma antigens detected by hybridoma antibodies. *Somatic Cell Genet.*, **5**, 957-972 (1979).
- 2) Houston, L. L. and Nowinski, R. C. Cell specific cytotoxicity expressed by a conjugate of ricin and murine monoclonal antibody directed against Thy1.1 antigen. *Cancer Res.*, **41**, 3913-3917 (1981).
- 3) Thurlow, P. J. and McKenzie, I. F. C. Monoclonal antibodies in clinical medicine. A review. *Aust. N.Z. J. Med.*, **13**, 91-100 (1983).
- 4) Baldwin, R. W., Embleton, M. J. and Pimm, M. V. Monoclonal antitumor antibodies for detection and therapy. *Behring Inst. Mitt.*, **74**, 3-13 (1984).
- 5) Sears, H. F., Herlyn, D., Steplewski, Z. and Koprowski, H. Initial trial use of murine monoclonal antibodies as immunotherapeutic agents for gastrointestinal adenocarcinoma. *Hybridoma*, **5**, 109-115 (1986).
- 6) Bumol, T. F., Wang, Q. C., Reisfield, R. A. and Kaplan, N. O. Monoclonal antibody and an antibody-toxin conjugate to a cell surface proteoglycan of melanoma cells suppress *in vivo* tumor growth. *Proc. Natl. Acad. Sci. USA*, **80**, 529-533 (1983).
- 7) Uadia, P., Blair, A. H. and Ghose, T. Tumor and tissue distribution of a methotrexate-anti-EL4 immunoglobulin conjugate in EL4 lymphoma-bearing mice. *Cancer Res.*, **44**, 4263-4266 (1984).
- 8) Galfre, G. and Milstein, C. Antibodies to major histocompatibility antigens produced by hybrid cell lines. *Nature*, **266**, 550-552 (1977).
- 9) Ouchterlony, Ö. Diffusion-in-gel methods for immunological analysis. *Prog. Allergy*, **5**, 1-78 (1958).
- 10) Nakane, P. K. Recent progress in the peroxidase-labeled antibody method. *Ann. N.Y. Acad. Sci.*, **234**, 203-211 (1975).
- 11) Iacobelli, S., Natoli, V., Scambia, G., Santeusano, G., Negri, R. and Natoli, C. Monoclonal antibody (AB/3) reactive with human breast cancer. *Cancer Res.*, **45**, 4334-4338 (1985).
- 12) Cheresch, D. A. and Reisfeld, R. O-Acetylation of disialoganglioside GD by human melanoma cells creates a unique antigenic determinant. *Science*, **225**, 844-845 (1984).
- 13) Edwards, D. P., Grzyb, K. T., Dressler, L. G., Mansel, R. E., Zava, D. T., Sledge, G. W., Jr. and McGuire, W. L. Monoclonal

- antibody identification and characterization of Mr 43,000 membrane glycoprotein associated with human breast cancer. *Cancer Res.*, **46**, 1306-1317 (1986).
- 14) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685 (1970).
 - 15) Oakley, B. R. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.*, **105**, 361-363 (1980).
 - 16) Gershoni, J. M. and Palade, G. E. Electrophoretic transfer of protein from sodium dodecyl sulfate-polyacrylamide gels to a positively charged membrane filter. *Anal. Biochem.*, **124**, 396-405 (1982).
 - 17) Bittner, M., Kupferer, P. and Morris, C. Electrophoretic transfer of proteins and nucleic acids from slab gels to diazobenzyl oxymethyl cellulose or nitrocellulose sheets. *Anal. Biochem.*, **102**, 459-471 (1980).
 - 18) David, G. S. Solid state lactoperoxidase: a highly stable enzyme for simple, gentle iodination of proteins. *Biochem. Biophys. Res. Commun.*, **48**, 464-471 (1972).
 - 19) Uemura, Y., Fukuyama, K. and Suyama, T. Establishment of passive hemagglutination assay (PHA) system for anti-HBc in plasma. *Tohoku J. Exp. Med.*, **149**, 11-20 (1986).
 - 20) Hurwitz, E., Maron, R., Berstein, A., Wilchek, M., Sela, M. and Armon, R. The effect *in vivo* of chemotherapeutic drug antibody conjugates in two murine experimental tumor systems. *Int. J. Cancer*, **21**, 747-755 (1978).
 - 21) Ovejera, A. A., Houchens, D. P. and Barker, A. D. Chemotherapy of human tumor xenografts in genetically athymic mice. *Anal. Clin. Lab. Sci.*, **8**, 50-56 (1978).
 - 22) Ahnen, D. J., Nakane, P. K. and Brown, W. R. Ultrastructural localization of carcinoembryonic antigen in normal intestine and colon cancer. *Cancer*, **49**, 2077-2090 (1982).
 - 23) Nakane, P. K. and Watanabe, T. Distribution of oncodevelopmental markers in neoplastic cells. *J. Histochem. Cytochem.*, **32**, 894-898 (1984).
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