Clinical Application of Monoclonal Antibody–Drug Conjugates for Immunotargeting Chemotherapy of Colorectal Carcinoma

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Monoclonal antibody–drug conjugates were applied as a clinical trial for patients who, based on the experimental study, had colorectal cancer. Monoclonal antibody A7, from a mouse splenocyte immunized against human colon cancer, was used as a drug carrier for colon cancer. The anti-cancer drugs mitomycin C (MMC) and neocarzinostatin (NCS) were bound covalently to A7 to form the conjugates A7–MMC and A7–NCS. The *in vitro* cytotoxic effects of the conjugates on SW1116 cells were stronger than those on free MMC or NCS. The conjugate A7–NCS, when administered to nude mice, brought about the highest NCS tumor concentration, whereas normal immunoglobulin G (IgG)–NCS distributed evenly in all tissues. The conjugates showed a strong antitumor effect on colon cancer transplanted into nude mice. Forty-one patients with colorectal cancer, including ten patients with postoperative metastasis, were given A7–NCS. The immunoperoxidase and drug concentration studies of the resected specimens showed that NCS was localized specifically in cancer. Patients receiving the conjugate did not experience serious adverse effects. Of the eight patients with postoperative liver metastasis, three showed evidence of tumor reduction on computed tomography (CT) scan and three claimed pain relief. The conjugate did not benefit patients with multiple lung metastasis or peritoneal metastasis.

***Cancer* 61:881–888, 1988.**

ONE OF THE MAJOR PROBLEMS with cancer chemotherapy is the detrimental effect of an anti-cancer drug on normal cells. Targeting chemotherapy is one way to overcome such adverse effects. We have applied clinically targeting chemotherapy using various drug carrier systems.[1–3] Of the various drug carriers, the monoclonal antibody against cancer cells seems to be the most suitable for drug targeting to cancer cells. Thus, we have prepared the monoclonal antibody A7,[4] which is specific highly to colon and rectum carcinoma, and its antibody–drug conjugates. This study describes these antibody–drug conjugates as drug carrier systems and their clinical application for colorectal carcinoma patients.

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Supported by a Grant-in-aid for Cancer Research from the Ministry of Health and Welfare of Japan.

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Accepted for publication September 9, 1987.

Materials and Methods

Monoclonal Antibody

The monoclonal antibody A7 was produced by a hybridoma obtained after the fusion of splenocytes from a mouse immunized against human colon carcinoma cells and murine myeloma P3.X63.Ag8.653 cells as described previously.[4] The monoclonal antibody, which belonged to the immunoglobulins (Ig) and G1 K, was purified from ascites fluid in BALB/c mice by chromatography on Affi-Gel Protein A (Bio-Rad Laboratory, Richmond, CA).

Mitomycin C and Neocarzinostatin Conjugation to the Monoclonal Antibody

A7–mitomycin C: Mitomycin C ([MMC] Kyowa Hakko, Tokyo, Japan) was bound covalently to A7 (IGg1) by cyanogen bromide as Suzuki described.[5] Briefly, purified A7 (IgG1) was mixed with cyanogen bromide for 10 minutes at room temperature. The pH of the mixture was maintained at 11 by adding 0.5 mol/l of sodium hydroxide. The pH was then adjusted to 7.5 by adding 5% of acetic acid after adding MMC. The mixture was stirred for an additional 24 hours under

TABLE 1. Clinical Cases of A7–Neocarzinostatin Conjugate

Target lesion	No. of patients	Administration route	Doses [A7 (mg)/NCS (U)]			
			15/1000	30/2000	45/4000	90/6000
Colon and rectum carcinoma (primary)	31	IA*	3	18	3	7
Liver metastasis (postoperative)	8	IA	2	3		3
Lung metastasis (postoperative)	1	IV			1	
Peritoneal metastasis (postoperative)	1	IP		1		

A7–NCS: A7–neocarzinostatin; IA: intraarterially; IV: intravenously; IP: intraperitoneally.

* Fourteen patients had intraoperative administration.

nitrogen at room temperature in the dark. The products were fractionated on a Sepharose 6B (Pharmacia, Uppsala, Sweden) column. The extent of substitution in this conjugate (A7–MMC) was about 2 mol of drug per mol of A7.

A7–neocarzinostatin: The A7 was conjugated with neocarzinostatin ([NCS] Kayaku, Tokyo, Japan) as Fukuda described.[6] The NCS was incubated first with a four-fold molar excess of N-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) in a 0.1 mol/l phosphate buffer (pH, 6.5) at 25°C for 30 minutes. The 3-(2-pyridyldithio)-propionated (PDP) NCS was reduced with 10 mmol of dithiothreitol (DTT) in a 0.1 mol/l acetete buffer (pH, 4.5) at 25°C for 30 minutes. The resulting thiol group-introduced NCS(HS–NCS) was passed immediately through a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column. The A7 was incubated with a ten-fold molar excess of SPDP in a 0.1 mol/l phosphate buffer (pH, 6.5) at 25° C for 30 minutes. The PDP was purified by gel filtration on a Sephadex G-25 column. Finally, the PDP was mixed with a six-fold molar excess of HS–NCS and allowed to stand at 25°C overnight in the dark. Then, the mixture was applied to a Sephacryl S-200 (Pharmacia, Uppsala, Sweden) column equilibrated with phosphate-buffered saline (PBS) (pH, 6.0) and eluted with the same buffer. The peak fractions were pooled, concentrated, sterilized, and stored at −20° C in the dark. The conjugation ratio was 2 to 3 mol of NCS per mol of A7.

Experimental Studies

In vitro *study:* The *in vitro* cytotoxicity of A7–MMC and A7–NCS was measured by incubation target cells (SW1116) and in a range of conjugate concentrations in microtiter plates at 45 minutes after washing them for 48 hours and measuring tumor cell survival by trypan blue exclusion.

In vivo *study:* The localization of A7 was investigated using iodine 125 (^{125}I)-radiolabeled A7 or ^{125}I-radiolabeled normal mouse IgG in BALB/c (nu/nu) athymic nude mice transplanting human colon cancer C-6 that was used as the immunogen of A7. The mice were killed 4 days after intraperitoneal injection of ^{125}I–A7 or ^{125}I–normal–IgG. Blood, tumor, and visceral organs were counted for radioactivity. Whole body sections of the mice were prepared for autoradiograph.

The NCS concentration in various tissues of the nude mice was measured to demonstrate the localization of NCS by the passive hemagglutination inhibition (PHAI) method.[7] The nude mice transplanting C-6 were killed on days 4 and 8 after intraperitoneal injection of A7–NCS (A7: 1 mg; NCS: 150 units), normal IgG–NCS (IgG: 1 mg; NCS: 150 units), or free NCS (150 units).

To investigate antitumor effect, nude mice were injected with A7 or one of its conjugates or free drugs 7 days after C-6 transplantation. Tumor volume was evaluated by multiplying one half of the length of the long axis by double the length of the short axis after injection of the drugs.

Clinical Applications

Forty-one patients with colon and rectum carcinoma, including eight patients with postoperative liver metastasis, one with postoperative lung metastasis, and one with postoperative peritoneal metastasis, were given A7–NCS. The conjugate was administered intraarterially to 39 patients, intravenously to one patient with lung metastasis, and intraperitoneally to one patient with peritoneal metastasis. Of the patients given the conjugate intraarterially, 14 were given the conjugate intraoperatively from the artery proximal to the tumor, and others were given the conjugate by introducing the catheter inserted from the femoral artery to the tumor artery. The conjugate dose was 15 to 90 mg of antibody and 1000 to 6000 units of NCS. Thirty-eight patients received the conjugate once and three patients received it twice (Table 1).

All patients were given A7–NCS first. Then, 30 patients with primary carcinoma of the colon or rectum

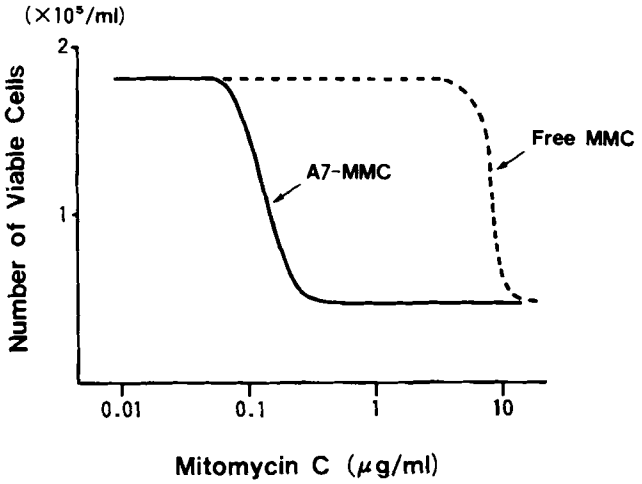


FIG 1. Cytotoxic effect of A7–MMC on SW1116 cells. The dose of MMC that killed 50% of the SW1116 cells was 0.14 μg/ml for A7–MMC and 10.8 μg/ml for free MMC.

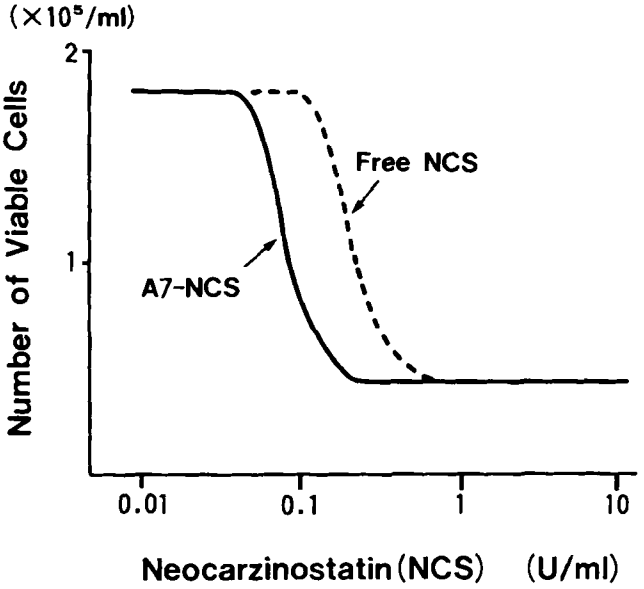


FIG. 2. Cytotoxic effect of A7–NCS on SW1116 cells. The dose of NCS that killed 50% of SW1116 cells was 0.059 U/ml for A7–NCS and 0.22 U/ml for free NCS.

underwent surgery for resection of the carcinoma. One patient with postoperative liver metastasis underwent right hepatectomy of the liver. The remaining ten patients with recurrent carcinoma were observed without surgery or any other chemotherapy.

The resected specimens were investigated with immunoperoxidase staining using antibody against NCS for evaluation of NCS localization in carcinoma and normal tissues. The NCS concentration of the resected specimens was measured in six colon patients who were given A7–NCS (A7: 60 mg; NCS: 4,000 units) intraarterially.

Patients given A7–NCS were followed 5 to 36 months after the treatments with serial checks of laboratory data and computed tomography (CT) scans.

Results

Experimental Study

In vitro *study:* The cytotoxic effect of A7–MMC on SW1116 was much stronger than that of free MMC (Fig. 1). The dose that killed 50% of SW1116 was 0.14 μg/ml and 10.8 μg/ml, respectively, for A7–MMC and free MMC (*i.e.*, A7–MMC showed a 77 times stronger cytotoxic effect than free MMC).

A7–NCS also was more effective than free NCS (Fig. 2). The dose that killed 50% of the target cells was 0.059 U/ml and 0.22 U/ml, respectively, for A7–NCS and free NCS.

In vivo *study*

Monoclonal antibody A7 localization in athymic nude mice: There was accumulation of ^{125}I–A7 in colon cancer transplanted into nude mice, compared with ^{125}I–normal mouse IgG and other organs (Fig. 3). The tissue–blood ratio of ^{125}I in cancer was 2.03:1 for A7 compared with 0.57:1 for normal IgG. The ^{125}I–A7 levels in other normal organs were comparable with those of ^{125}I–normal IgG.

Whole body sections of the nude mice bearing colon cancer C-6 were prepared for autoradiograph 4 days after intraperitoneal injection of ^{125}I–A7. As shown in Figure 4, ^{125}I–A7 was localized specifically in cancer, whereas there was little localization in normal organs.

Concentration of NCS in the tissues of nude mice: NCS was not detected in all tissues at the fourth and eighth days after free NCS injection. The A7–NCS generated the highest concentration of NCS in the tumor on

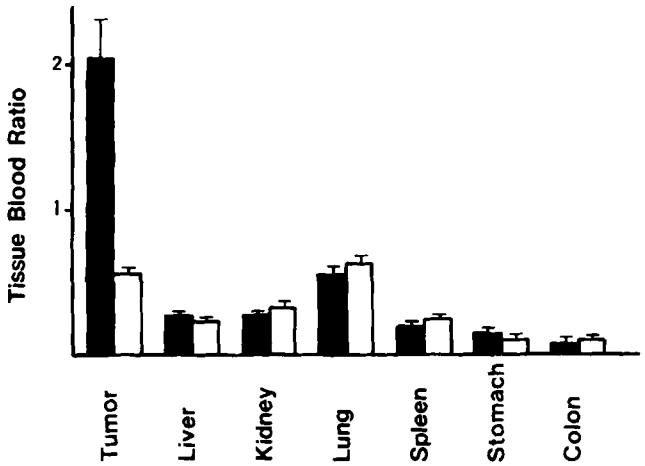
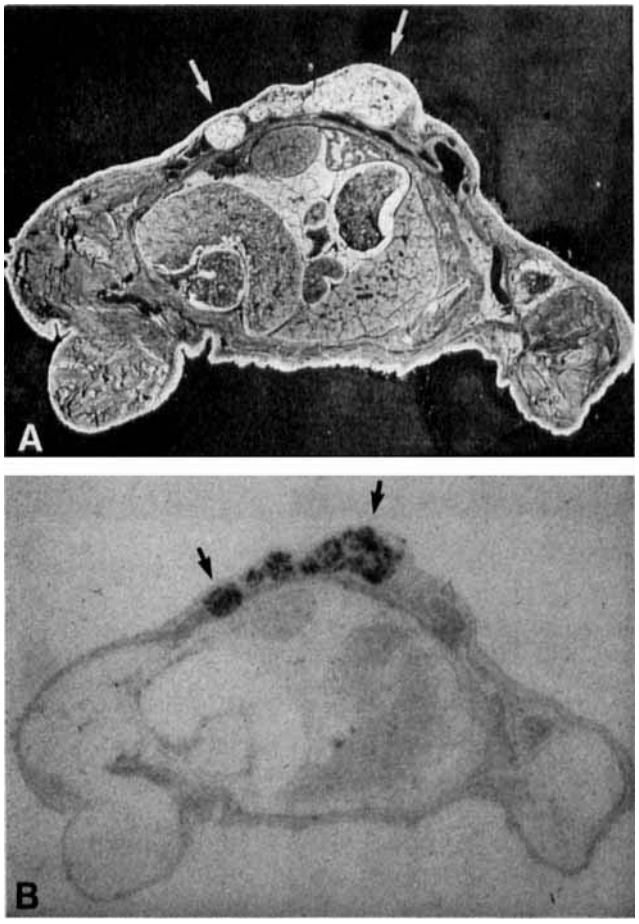


FIG. 3. Tissue blood ratio of ^{125}I 4 days after intraperitoneal injection of ^{125}I–A7 or normal ^{125}I–IgG into nude mice bearing the colon cancer C-6. ■ ^{125}I–A7; □ ^{125}I–normal mouse IgG (mean ± SE)



FIGS. 4A AND 4B. Autoradiograph of nude mouse bearing C-6 4 days after intraperitoneal injection of ^{125}I-A7. (A) Arrows indicate the subcutaneously transplanted C-6 in the nude mouse (B) Black grains seen at the same site indicate the accumulation of ^{125}I-A7 at the C-6.

both the fourth and eighth days after injection, whereas normal IgG-NCS distributed evenly in the tissues of nude mice (Fig. 5).

Effect of conjugates on tumors transplanted into nude mice: Figure 6 shows the tumor volume after intraperitoneal injection of A7 (6mg/kg), A7-MMC (A7: 6mg/kg; MMC: 30 μg/kg), and free MMC (30 μg/kg) twice a week with untreated control. The A7 alone had no effect on tumor growth. The A7-MMC had the most favorable effect with a T/C (treated/control) of 0.35, compared with free MMC and free A7 that had a T/C of 0.83 and 0.8, respectively.

Figure 7 shows the tumor volume after intravenous injection of A7 (3.75 mg/kg), A7-NCS (A7: 3.75 mg/kg; NCS: 500 units), and NCS (500 U/kg) twice a week with untreated control. The A7-NCS exhibited an inhibitory effect on tumor growth with a T/C of 0.22, whereas NCS alone was toxic but had no effect.

Clinical application of A7-NCS for patients with colon and rectum carcinoma: An immunoperoxidase study of resected specimens showed that NCS was localized specifically in cancer cells (Fig. 8).

The NCS concentration in the tumor was higher than that of normal mucosa of the colon in all the patients given A7-NCS 1 to 72 hours before tumor removal (Table 2).

Of the eight patients with postoperative liver metastasis who received A7-NCS through the hepatic artery, four responded favorably to the conjugate (Table 3). Three patients showed evidence of tumor reduction on CT scan. Three patients claimed pain relief. Four patients died of cancer progression 8 to 25 months after the treatment. Three patients are alive for 12 to 26 months after treatment with liver metastasis, and two patients have had stable liver tumors without regrowth for 12 and 26 months after treatment, respectively. One patient, subjected to right hepatectomy, is alive without liver metastasis 10 months after treatment.

Figure 9A shows the CT scan of a patient with liver metastasis 5 years after surgery for colon carcinoma. This patient was given A7-NCS (A7: 90 mg; NCS: 6000 units) once through the hepatic artery. The patient had had a temperature of 38.0°C for 3 days after injection. The CT scan showed a 67% tumor reduction 3 weeks later (Fig. 9B). The patient is alive and in good condition 26 months after the treatment, without progress of the liver metastasis.

A patient with multiple lung metastasis died of the metastasis 1 month after treatment. A patient with peritoneal metastasis died of the metastasis 3 months after treatment. Therefore, the conjugate was of no benefit to these patients.

The clinical course of 31 patients with primary carcinoma of the colon and rectum resected surgically was uneventful. The follow-up study is now carried out in serial observations.

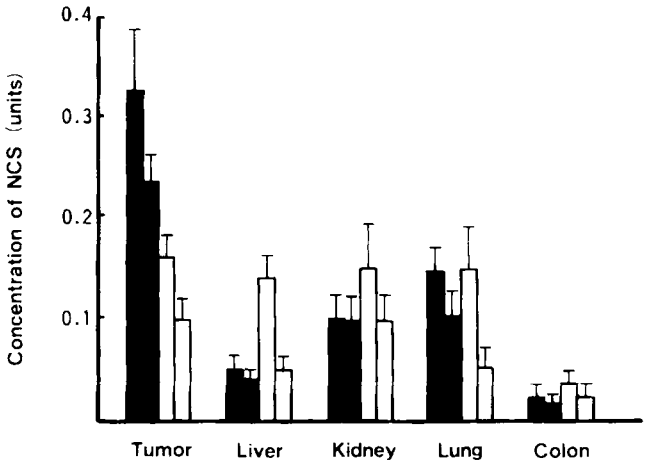


FIG. 5. Drug concentration (U/g) of NCS in the tissues after injection of A7-NCS and normal mouse IgG-NCS. ■ Left on fourth day, right on eighth day; □ left on fourth day, right on eighth day.

There were no serious adverse effects on the patients who received the conjugate, regardless of the administration route. Of the 41 patients, a fever greater than 38.0°C was the most common adverse effect and was evident in 20 patients. Other side effects included leukocytosis (leukocyte count of greater than 10,000/μl) in five patients, slight pain at the portion of tumor region and eruption at the site of intracutaneous test injection in two patients, and slight hypotension with a systolic pressure of 100 in one patient.

Discussion

There have been reports[8–12] on the successful application of the murine monoclonal antibody alone in the immunotherapy of patients with tumors. However, there is little information on the clinical application of monoclonal antibody–drug conjugates. Our investigations with the monoclonal antibody and its drug conjugates demonstrated that A7, which we prepared for human colon carcinoma, can be used effectively to deliver drugs to human colon and rectum carcinomas in both nude mice and patients.

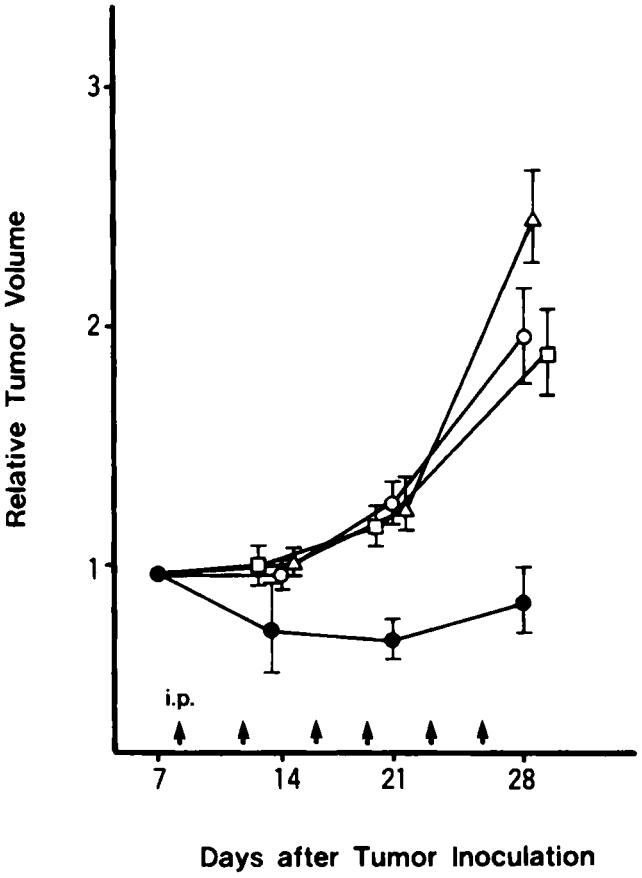


FIG. 6. Tumor volume after intraperitoneal injection. ⊟ A7 (6 mg/kg); ⊖ MMC (30 μg/kg); ⧗ A7–MMC (A7: 6 mg/kg; MMC: 30 μg/kg); and ⊻ saline twice a week (mean ± SE [n = 5]).

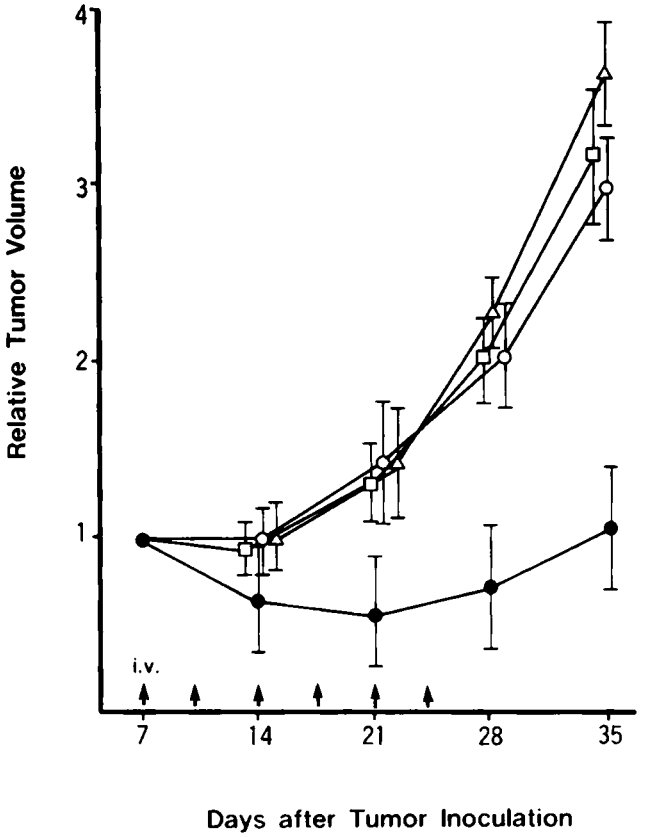


FIG. 7. Tumor volume after intravenous injection. ⊟ A7 (3.75 mg/kg); ⊖ NCS (500 U/kg); ⧗ A7–NCS (A7; 3.75 mg/kg; NCS: 500 U/kg); and ⊻ saline twice a week (mean ± SE [n = 5]).

The A7 was localized specifically to human colon carcinoma transplanted into nude mice. This suggested that A7 was a suitable drug carrier to colon cancer. In our previous study,[13–14] A7 alone had no cytotoxic effect on SW1116 cells *in vitro*. Herlyn *et al.*[15] reported that IgG1, IgG2b, IgM, and IgA isotypes were ineffective against colorectal carcinomas when used as monoclonal antibodies alone, compared with IgG2a and IgG3. Because A7 belongs to the IgGl subclass that has no effect on cancer, we prepared antibody–drug conjugates for targeting chemotherapy of colorectal carcinomas.

The MMC and NCS were bound covalently to the A7. For targeting immunochemotherapy, it is essential that the conjugate retains the activities of both the antibody and the drug. In our previous study,[6] A7–MMC retained antibody activity but reduced the antimicrobial activity of MMC to only 5% of its spectrophotometrical value. This was due presumably to binding with one of the three active amino radicals of MMC for cancer[16–17] to the Fc portion of the antibody.[5] Despite such drug activity reduction, the cytotoxic effect of A7–MMC on

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