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Abstract

Macromomycin (MCR), an unique membrane-reactive anticancer antibiotic, was incubated with murine monoclonal anti-HLA IgG1 antibody (H-1) in the presence of carbodiimide. The resulting mixture was fractionated with a Sephadex G-200 column. The first and second fractions were shown to contain MCR-(H-1) conjugate by the elution profile, as well as by the Sarcina lutea growth inhibition assay and Ouchterlony double-diffusion method. A membrane immunofluorescence test with anti-MCR and anti-mouse IgG antibodies demonstrated specific localization of MCR-(H-1) on the surface of HLA-bearing NALL -1 cells. MCR-(H-1) inhibited the growth of HLA-lacking NS-1 cells statistically less effectively than MCR alone (p less than 0.01). On the other hand, the conjugate and free MCR equally inhibited the growth and 3H-TdR incorporation of HLA-bearing NALL -1 cells. These results indicate that the antibody-bound MCR retained both MCR and antibody activities, and thus exerted antibody-targeting MCR cytotoxicity in vitro.

KEYWORDS: macromomycin(MCR), anti-HLA monoclonal antibody, carbodiimide

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PRODUCTION OF A MACROMOMYCIN (MCR)-MONOCLONAL ANTIBODY CONJUGATE AND ITS BIOLOGICAL ACTIVITY

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Abstract. Macromomycin (MCR), an unique membrane-reactive anticancer antibiotic, was incubated with murine monoclonal anti-HLA IgG_1 antibody (H-1) in the presence of carbodiimide. The resulting mixture was fractionated with a Sephadex G-200 column. The first and second fractions were shown to contain MCR-(H-1) conjugate by the elution profile, as well as by the *Sarcina lutea* growth inhibition assay and Ouchterlony double-diffusion method. A membrane immunofluorescence test with anti-MCR and anti-mouse IgG antibodies demonstrated specific localization of MCR-(H-1) on the surface of HLA-bearing NALL-1 cells. MCR-(H-1) inhibited the growth of HLA-lacking NS-1 cells statistically less effectively than MCR alone ($p < 0.01$). On the other hand, the conjugate and free MCR equally inhibited the growth and ^{3}H-TdR incorporation of HLA-bearing NALL-1 cells. These results indicate that the antibody-bound MCR retained both MCR and antibody activities, and thus exerted antibody-targeting MCR cytotoxicity *in vitro.*

Key words : macromomycin (MCR), anti-HLA monoclonal antibody, carbodiimide.

Attempts to bind anticancer drugs or toxins to antibodies have increased since the hybridoma technique, which permits the production of large amounts of monoclonal antibodies, was developed by Köhler and Milstein (1). Cancer chemotherapeutic agents are not selective in their action, and the need to preserve vital tissues reduces the maximum potential dose of cytotoxic drugs. Theoretically, selective cytotoxicity of anticancer agents should be enhanced by conjugation to antibodies raised against antigens on the surface of tumor cells. For this reason, methods of linking anticancer drugs or toxins covalently to antibodies have been investigated (2, 3, 15). In our laboratory, a hybridoma cell line which produces an anti-HLA IgG_1 monoclonal antibody (H-1) was established ,and H-1 was shown not to exhibit cytotoxic activity *in vitro.* H-1 seemed to be ideal for assessing the activity of linked drugs and the antigen-targeting potential of anticancer drug-(H-1) conjugates (4).

Macromomycin (MCR), a proteinaceous anticancer antibiotic, was isolated by Chimura *et al.* (5) from the culture filtrate of *Streptomyces macromomyceticus.* MCR has a molecular weight of 12,500 and inhibits mouse leukemia L1210, B 16 mela-

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noma, Lewis lung carcinoma and other tumor cells (6, 7). It has been demonstated that MCR binds to the membrane of tumor cells and inhibits DNA synthesis preferentially (8, 9), which is why we chose MCR for this experiment.

In this study, we conjugated MCR to H-1 IgG_1 monoclonal antibody, and examined the cytotoxic activity of the conjugate against cells bearing or lacking HLA *in vitro.*

MATERIALS AND METHODS

Chemicals and drugs. Purified MCR was obtained from Kanegafuchi Co., Ltd., Osaka, Japan. 1-Hydroxybenztriazole (HOBt) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (WSCD-HCL) were purchased from SIGMA, U.S.A..

Cells. A null cell line (NALL-1) (10), which has HLA, was derived from acute lymphoblastic leukemia cells, and maintained in RPMI 1640 medium supplemented with 10 % fetal calf serum. A mouse myeloma cell line, P3-NSl/1-Ag4-1 (NS-1) (11), which dose not have HLA, was used as a control.

Monoclonal antibody. A hybridoma cell line which secreted an antibody specific against an HLA framework determinant was established. The antibody (H-1) reacted with human nucleated cells but not with HLA-lacking Daudi cells (12). H-1 was confirmed to react with NALL-1 cells but not with NS-1 cells. The hybridoma cells were grown in the peritoneal cavity of BALB/c mice, and the ascitic fluid was pooled. H-1 was purified from the ascitic fluid pool by affinity chromatography on Protein A-Sepharose CL-4B (13, 14).

Binding of MCR to H-1 monoclonal antibody. The following were mixed together : 40 mg

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Abstract. Basophil histamine release induced by allergens (house dust and *Candida*

100 cm) column equilibrated with ... resultant mixture was applied to a Sephadex G-200 (2.6 × PBS. The optical density of each fraction was measured at 280 nm, and the fractions corresponding to the first two major peaks were lyophilized.

Immunological analysis. Double-diffusion tests were carried out in agarose gels according to the procedure of Ouchterlony.

NALL-1 and NS-1 cells were incubated with H-1 or HCR-(H-1) conjugates for 30 min at 4 ℃ and then stained with fluorescein isothiocyanate (FITC)-conjugated goat antiserum to mouse IgG (E.Y Laboratories, U.S.A.) for a further 30 min at 4 ℃. NALL-1 cells were also incubated with MCR-(H-1) for 30 min at 4 ℃ and then with rabbit anti-MCR IgG (gift from Kanegafuchi Co., Osaka, Japan) for 30 min at 4 ℃, followed by staining with FITC-conjugated goat antiserum to rabbit IgG. Membrane staining of the cells was examined by fluorescence microscopy.

Biological activity. The inhibitory activity of MCR and of MCR-(H-1) conjugate against *Sarcina lutea PCI 1001* was determined with paper disks by measurement of the zone of inhibition. The MCR concentration was adjusted according to the MCR activity equivalent determined by this procedure. The protein concentration was measured by the Bio-Rad Protein Assay (Bio-Rad Laboratories, California) (16).

The cytotoxic activity of the conjugate was measured against the HLA-bearing cell line, NALL-1, and the HLA-lacking cell line, NS-1. After 3×10^6 cells in the logarithmic phase, were incubated in 1 ml medium containing the test substances, MCR, MCR-(H-1) and H-1,

for 30 min at 37 ℃ and washed 2 times with fresh medium, the cells were cultured further for 3 days in fresh medium. In another experiment, 3×10^6 cells were cultured for 3 days in 5 ml culture medium containing the test substances. In both experiments, viable cells were counted by the trypan blue dye exclusion test. Data were analyzed for statistical significance with Student's t test.

Inhibition of ^{3}H-TdR incorporation into NALL-1 cells was assayed as follows : 2×10^6 NALL-1 cells were mixed with 1 ml culture medium containing the test substances for 30 or 60 min at 37 ℃ and washed 2 times with fresh medium. Aliquots (100 ul) of each cell culture were dispensed into a microplate (Nunc, Denmark). ^{3}H-TdR (specific activity : 2 Ci/mmol) was added to each well (1 μCi/well). After 1 h incubation, cells were harvested on millipore filters, and the radioactivity was counted in a liquid scintillation counter.

RESULTS

MCR binding to H-1 antibody. The reaction mixture of MCR and H-1 monoclonal antibody IgG_1 with WSCD-HCL was applied to a Sephadex G-200 column and eluted with PBS (Fig. 1). The elution profile showed four major peaks (Fractions 1, 2, 3 and 4). Fractions 1 and 2 were eluted continuously and completely separated from Fraction 3. When purified mouse IgG was applied to the column, a single peak appeared in approximately the same position as Fraction 2. A free MCR peak was eluted in the same position as Fraction 3. Fractions 1, 2 and 3 showed inhibitory activity against *Sarcina lutea*, but Fraction 4 did not show any antimicrobial activity.

Immunological analysis of the conjugate. A single precipitation line was observed

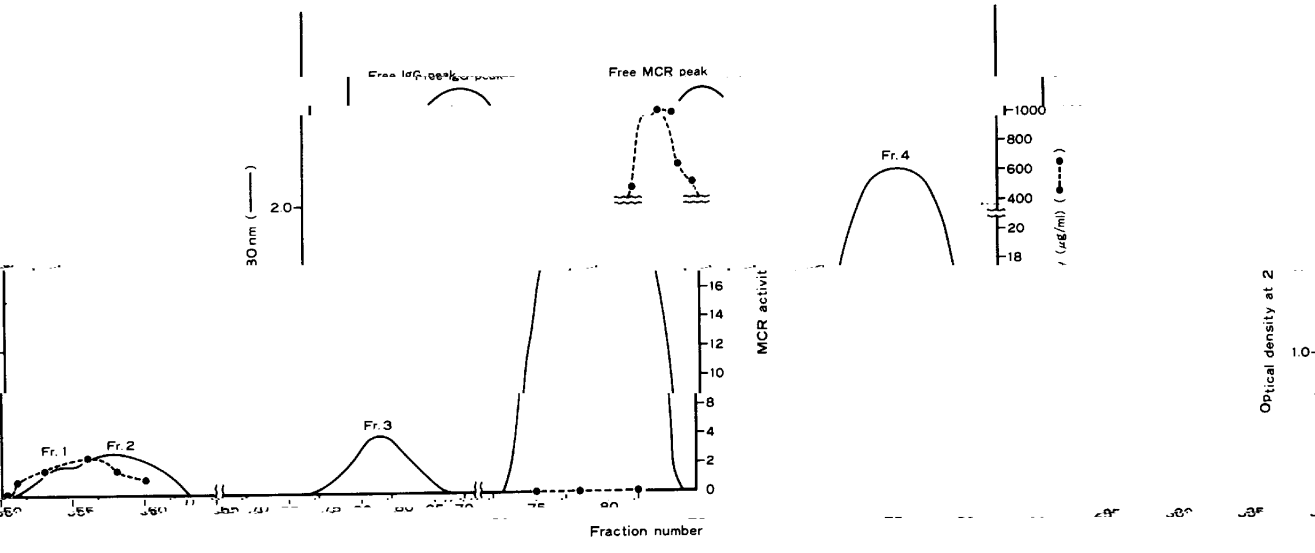

Fig. 1. Elution profile of the carbodiimide-treated mixture of H-1 and MCR (2.6×100 cm Sephadex G-200 column). 5.5 ml fractions. ——, OD at 280 nm ; -----, MCR activity assayed by *Sarcina lutea* growth inhibition (ug/ml).

TABLE 1. REACTIVITY OF MCR-(H-1), H-1 AND MCR TO NALL-1 CELLS TESTED BY THE INDIRECT MEMBRANE IMMUNOFLUORESCENCE TEST

1st antibody		2nd antibody	3rd antibody	Full surface fluorescence
MCR-(H-1)	Fraction 1	Rabbit anti-MCR	FITC-anti-rabbit 7S	+[a]
	Fraction 2	Rabbit anti-MCR	FITC-anti-rabbit 7S	+[a]
	Fraction 3	Rabbit anti-MCR	FITC-anti-rabbit 7S	−
MCR	100 ug/mll	Rabbit anti-MCR	FITC-anti-rabbit 7S	−
	1 ug/ml	Rabbit anti-MCR	FITC-anti-rabbit 7S	−
H-1		Rabbit anti-MCR	FITC-anti-rabbit 7S	−
HCR-(H-1)	Fraction 1	FITC-anti-mouse 7S		++[b]
	Fraction 2	FITC-anti-mouse 7S		++[b]
	Fraction 3	FITC-anti-mouse 7S		−
MCR		FITC-anti-mouse 7S		−
H-1		FITC-anti-mouse 7S		++[b]

[a]Approximately 100 % of cells were positive, but fluorescence was weak.
[b]100 % of cells were strongly positive.

in Ouchterlony double-diffusion plates when Fractions 1 and 2 were incubated with anti-MCR antiserum, and this precipitation line fused with the line formed between MCR and anti-MCR. Fractions 1 and 2 and mouse IgG formed a single precipitation line to rat anti-mouse IgG, and fused completely. These results indicate that Fractions 1 and 2 contained both IgG and MCR.

Table 1 shows the reactivity of the conjugate to NALL-1, as measured by an indirect membrane immunofluorescence assay. Fractions 1 and 2 gave weak

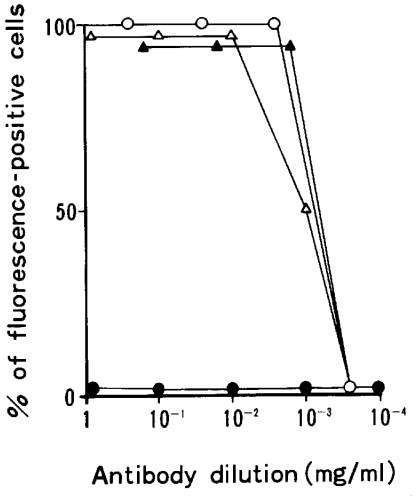

Fig. 2. Comparison of antibody titer before and after conjugation. The antibody activity against NALL-1 cells was measured by an indirect membrane immunofluorescence assay. ○—○, H-1; △—△, Fraction 1 [MCR-(H-1)]; ▲—▲, Fraction 2 [MCR-(H-1)]; ●—●, Fraction 3 (MCR).

but definite full membrane fluorescence to NALL-1 cells when rabbit anti-MCR as the second antibody, and FITC-conjugated goat anti-rabbit IgG as the third antibody, were used. Strong full membrane fluorescence was observed when NALL-1 cells were reacted with Fractions 1 or 2, and then stained with FITC-conjugated goat anti-mouse IgG. These results indicate that Fractions 1 and 2 were composed of IgG and MCR, which had both antibody and antimicrobial activities.

Fig. 2 shows the antibody activity of the conjugate, as measured by an indirect membrane immunofluorescence assay when NALL-1 cells were incubated with MCR-(H-1) conjugate and stained with FITC-conjugated goat anti-mouse IgG. There was hardly any decrease in the antibody activity after conjugation, suggesting that the conjugation method did not result in a significant loss of antibody activity. MCR-(H-1) did not react with NS-1 cells.

Cytotoxicity assay. Fractions 1 and 2 were mixed, and the cytotoxicity assay was performed. The protein concentration of MCR-(H-1) was 8 ug/ml when the conjugate had 4×10^{-2} ug/ml of MCR activity. Fig. 3 shows the cytotoxic activity of MCR-(H-1) conjugate against HLA-bearing NALL-1 (Fig. 3-A) and HLA-lacking NS-1 (Fig. 3-B) cells when the cells were exposed to the test solutions for 30 min and cultivated further for 3 days in fresh medium. H-1 alone did not inhibit cell growth. MCR-(H-1) conjugate showed the same toxicity to the HLA-bearing NALL-1 cells as free MCR did, while MCR-(H-1) was statistically less toxic to the HLA-lacking NS-1 cells than free MCR ($p < 0.01$).

Fig. 4 shows the cytotoxic activity of MCR-(H-1) conjugate against HLA-

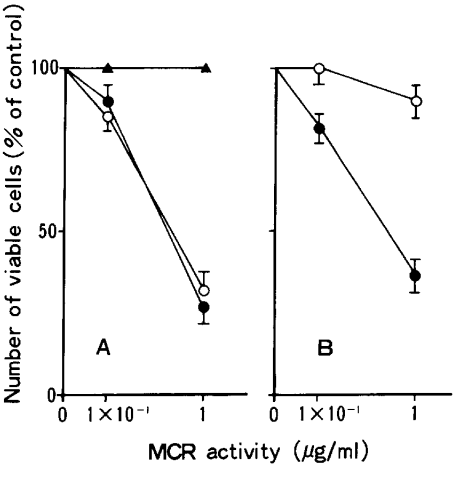

Fig. 3. Cytotoxic activities of MCR-(H-1), MCR and H-1 to NALL-1 (A) and NS-1 (B) cells. Three $\times 10^6$ cells were cultured for 3 days after a 30-min drug exposure. ▲—▲, H-1 ; ○—○, MCR-(H-1) ; ●—●, MCR. Each point and bar indicates the mean and S.E. of three determinations. H-1 concentration was abjusted to the IgG content of the conjugate.

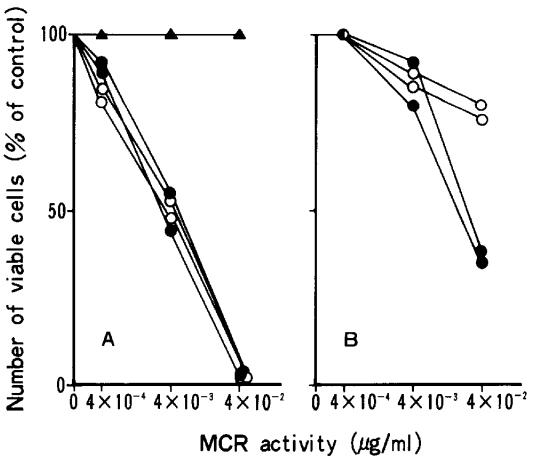

Fig. 4. Cytotoxic activities of MCR-(H-1), MCR and H-1 to NALL-1 (A) and NS-1 (B) cells. Three × 10^6 cells were cultured with the test drugs for 3 days. ▲—▲, H-1 ; ○—○, MCR-(H-1) ; ●—●, MCR. Each point indicates the mean of two determinations. H-1 concentration was adjusted to the IgG content of the conjugate.

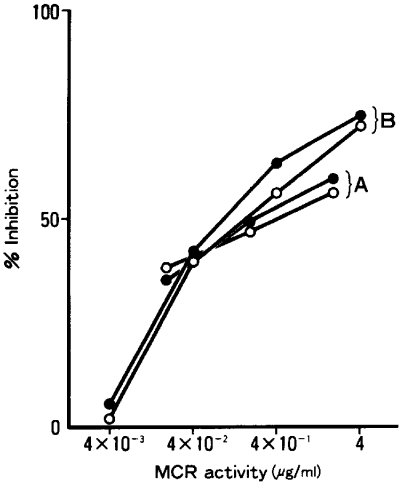

Fig. 5. Effect of MCR-(H-1) and MCR on the ^{3}H-TdR incorporation into NALL-1 cells after a 30 (A) or 60 (B)-min exposure to the test drugs. ○—○, MCR-(H-1) ; ●—●, MCR.

bearing NALL-1 and HLA-lacking NS-1 cells after 3 days exposure to the conjugate in culture. H-1 did not inhibit cell growth, while the cytotoxic activities of MCR-(H-1) and MCR to NALL-1 were almost the same. The MCR-(H-1) conjugate was less toxic to NS-1 cells than MCR.

Fig. 5 shows the percent inhibition of ^{3}H-TdR incorporation into NALL-1 cells after the cells were exposed to the test substances for 30 or 60 min. The MCR-(H-1) conjugate inhibited ^{3}H-TdR incorporation into NALL-1 cells as much as free MCR did.

DISCUSSION

MCR binds to the membrane of tumor cells and inhibits DNA synthesis (8, 9). Neocarzinostatin (NCS), a antibiotic protein with similar antitumor properties, is another agent that has been reported to be active at the cell surface (17). If antitumor agents induce cytotoxicity by interaction with cell surface receptors, such antitumor agents covalently coupled to a tumor-specific antibody might be efficiently transported to target sites on the tumor cells, and kill the tumor cells selectively. Thus, NCS and MCR appear to be ideal agents for this novel approach to selective cancer chemotherapy.

We have reported that NCS can be covalently bound to rabbit IgG and that the conjugate retained both pharmacological and antibody activities (15). In the present study, an MCR-(H-1) conjugate was produced using WSCD-HCL with little loss of antibody activity. The conjugate also retained pharmacological capacity.

H-1 alone did not inhibit cell growth. MCR-(H-1) and free MCR had the same cytotoxicity to HLA-bearing cells, while the conjugate was significantly less toxic than free MCR against cells lacking HLA. The equivalent cytotoxicity of MCR-(H-1) and MCR against HLA-bearing cells was confirmed by a ^{3}H-TdR incorporation study. Moreover, we observed that H-1 antibody-bound MCR was localized on the surface of HLA-bearing NALL-1 cells, but not on the surface of HLA-lacking cells, in the membrane immunofluorescence test. The reason why MCR activity of the conjugate against HLA-lacking cells was reduced is not clear, but when the conjugate was reacted with HLA-bearing cells, there was no reduction in MCR activity. These results indicate that MCR-(H-1) exerted antigen-targeting MCR cytotoxicity, and clearly show that MCR-IgG conjugate induced weaker cytotoxicity than free MCR when antigen-antibody action was not involved. MCR-bound non-immune IgG may weaken the capacity of MCR to attach to the cell membrane, and thus result in weaker cytotoxicity than free MCR.

If a monoclonal antibody directed against tumor-specific antigens is obtained, the MCR-antitumor antibody conjugate may be produced to assess its therapeutic usefulness.

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