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SHORT COMMUNICATIONS

Production of a macromomycin (MCR)–monoclonal antibody conjugate and its biological activity

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Attempts to bind anticancer drugs and toxins to antibodies have increased since the development of the hybridoma technique by Köhler and Milstein [1], which permits the production of large amounts of monoclonal antibodies. Cancer chemotherapeutic agents are not selective in their action, and the need to preserve vital tissues reduces the maximum potential dose of such drugs. Theoretically, the 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 agents covalently to antibodies have been investigated [2]. 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*. The antibody (H-1) seemed to be ideal for assessing the activity of linked drugs and the antigen-targeting potential of anticancer drug-(H-1) conjugates [3].

Macromomycin (MCR), a proteinaceous anticancer antibiotic, binds to the membrane of tumor cells and thereby preferentially inhibits DNA synthesis [4]. In the present experiments, we conjugated MCR to H-1 IgG_1 monoclonal antibodies and studied the cytotoxicity of the conjugate to cells that did or did not bear HLA *in vitro*.

Materials and methods. A null cell line (NALL-1) [5], which has HLA, was derived from acute lymphoblastic leukemia cells and was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. A mouse myeloma cell line, P3-NS1/1-Ag4-1 (NS-1) [6], which does not have HLA, was used as a control. The antibody (H-1) reacted with human nucleated cells but not with HLA-lacking Daudi cells [7]. H-1 was confirmed to react with NALL-1 cells but not with NS-1 cells. The hybridoma cells were grown in the peritoneal cavities of BALB/c mice, and the ascitic fluid was pooled. H-1 was purified from the pooled ascitic fluid by affinity chromatography on Protein A-Sepharose CL-4B [8].

Purified MCR was obtained from the Kanegafuchi Co., Osaka, Japan [9, 10]. 1-Hydroxybenztriazole (HOBt) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (WSCD-HCL) were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A.

The following were mixed together: 40 mg MCR dissolved in 4 ml of phosphate-buffered saline (PBS, pH 7.2, 0.15 M), 4.8 mg HOBt in 2 ml PBS and 6.8 mg WSCD-HCL in 1 ml PBS [11, 12]. The mixture was stirred for 1 min at room temperature, and H-1 (20 mg in 2 ml PBS) was added. The reaction was allowed to proceed for 5 hr at room temperature, with continous stirring in complete darkness. The resultant mixture was applied to a Sephadex G-200 column (2.6×100 cm) equilibrated with PBS. The optical density of each fraction was measured at 280 nm, and the first two major peaks were lyophilized.

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 MCR-(H-1) conjugates for 30 min at 4° and then stained with fluorescein isothiocyanate (FITC)-conjugated goat antiserum to mouse IgG 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 (a gift from the Kanegafuchi Co.) for 30 min at 4°, which was followed by staining with FITC-conjugated goat antiserum to rabbit IgG. Membrane staining of the cells was examined by fluorescence microscopy.

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, Richmond, CA) [13].

The cytotoxic activity of the conjugate was measured against the HLA-bearing cell line, NALL-1, and the HLA-lacking cell line NS-1. In 1 ml of medium containing MCR, MCR-(H-1) or H-1, 3×10^6 cells were incubated for 30 min at 37°, washed twice with fresh medium, and cultured further for 3 days in medium. Viable cells were counted by the trypan blue dye exclusion test.

Results and discussion. The reaction mixture of MCR and H-1 monoclonal 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 IgG was applied to the column, a single peak appeared in approximately the same position as Fraction 2. These results suggested that Fr. 1 and Fr. 2 might consist of polymerized and single MCR-IgG conjugate respectively.

A single precipitation line was observed 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, 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 test. Fractions 1 and 2 gave weak but definite full membrane fluorescence to NALL-1 when rabbit anti-MCR as the second antibody and FITC-conjugated goat anti-rabbit IgG as the third antibody were used. After staining with FITC-conjugated goat anti-mouse IgG, strong full membrane fluorescence was observed in the reactions between NALL-1 cells and either Fraction 1 or 2. These results indicate that Fractions 1 and 2 were composed of IgG and MCR. Both fractions had antibody and antimicrobial activities,

Figure 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 goat anti-mouse IgG. There was hardly any decrease in the antibody activity after conjugation, suggesting that the conjugation method did not result in significant loss of antibody activity. The antibody activity of Fraction 1 was weaker than that of Fraction 2. The loss of the antibody activity of Fraction 1 might be due

* HLA, antigens of the major histocompatibility antigen system A, B and C.

to polymerization of MCR-IgG conjugate (Fig. 1). MCR-(H-1) did not react with NS-1 cells.

Fractions 1 and 2 were mixed, and the cytotoxicity assay was performed. The protein concentration of MCR-(H-1) conjugate was 8 μg/ml when the conjugate had 4×10^{-2} μg/ml of MCR activity. Figure 3 shows the cytoxic activity of MCR-(H-1) conjugate against HLA-bearing NALL-1 (Fig. 3A) and HLA-lacking NS-1 (Fig. 3B) cells. 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 MCR ($P < 0.01$).

MCR-(H-1) conjugate, linked by WSCD-HCl, and free MCR showed equivalent cytotoxicity to an HLA-bearing cell line, while MCR-(H-1) was less toxic to an HLA-lacking cell line than MCR, indicating that MCR-(H-1) exerted target-specific cytotoxicity. The reason why MCR activity of the conjugate against HLA-lacking cells was reduced is not clear, but the MCR-IgG conjugate may weaken the capacity of MCR to attach to the cell membrane and thus result in weaker cytotoxicity than free MCR when antigen–antibody action is not involved.

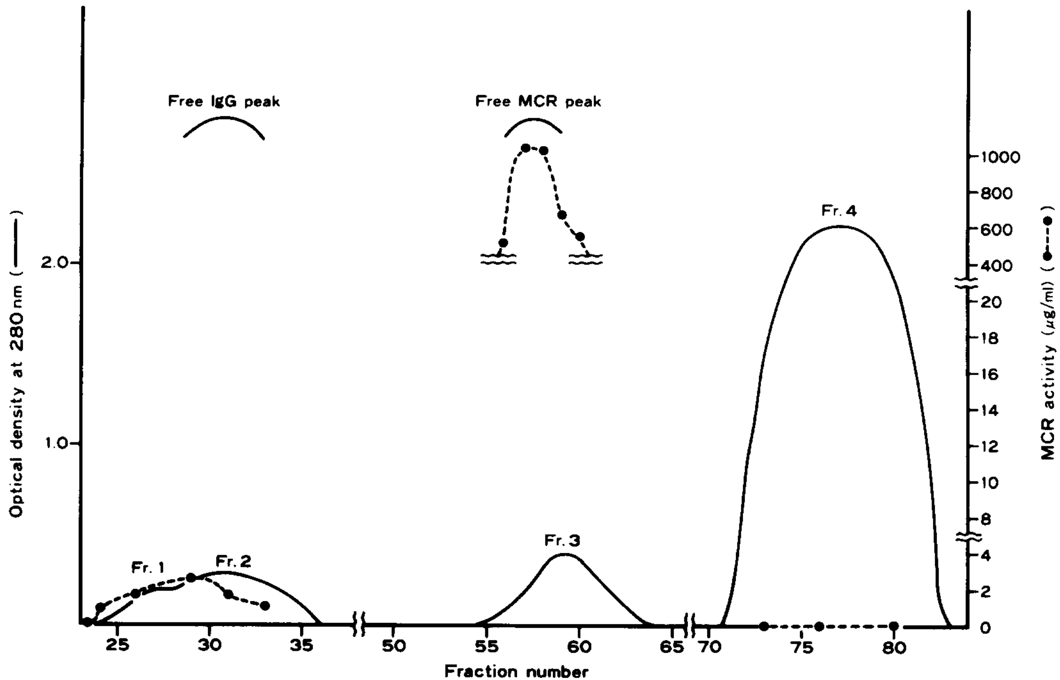

Fig. 1. Elution profile of the carbodiimide-treated mixture of H-1 and MCR (2.6 × 100 cm Sephadex G-200 column). Fractions were 6.5 m. Key (——) O.D. at 280 nm; (- - - - -) MCR activity assayed by *Sarcina lutea* growth inhibition.

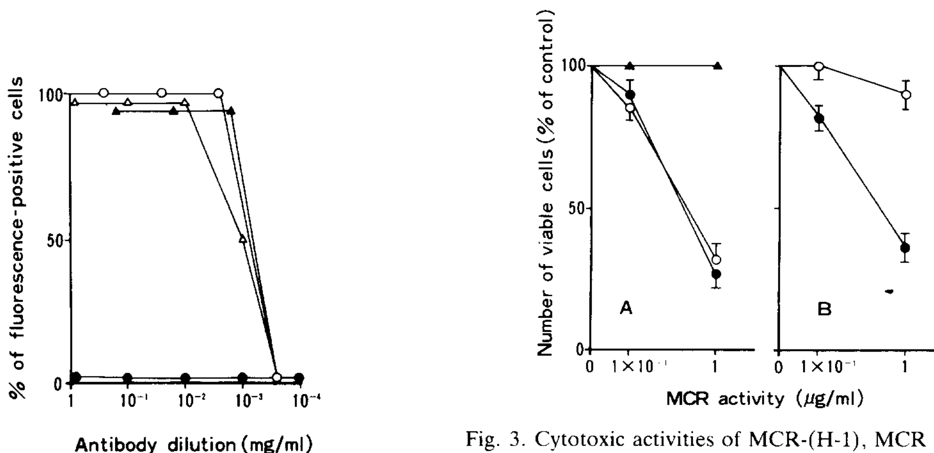

Fig. 2. Comparison of antibody titer before and after conjugation. The antibody activity against NALL-1 cells was measured by an indirect membrane immunofluorescence test. Key: (○) H-1; (△) Fraction 1 [MCR-(H-1)]; (▲) Fraction 2 [MCR-(H-1)]; and (●) Fraction 3 (MCR).

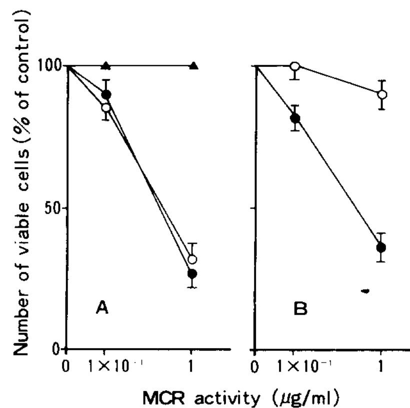

Fig. 3. Cytotoxic activities of MCR-(H-1), MCR and H-1 to NALL-1 (A) and NS-1 (B) cells. After a 30-min drug exposure, 3×10^6 cells were cultured for 3 days. Key: (△) H-1; (○) MCR-(H-1); and (●) MCR. Each point and bar indicates the mean and S.E. of three determinations. H-1 concentration was adjusted to the IgG content of MCR-(H-1) conjugate.

Table 1. Reactivity of MCR-(H-1), H-1 or MCR to NALL-1 cells tested by the indirect membrane immunofluorescent 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 μg/ml	Rabbit anti-MCR	FITC-anti-rabbit 7S	−
1 μg/ml	Rabbit anti-MCR	FITC-anti-rabbit 7S	−
H-1	Rabbit anti-MCR	FITC-anti-rabbit 7S	−
MCR-(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.

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The effect of solvent polarity upon rotational barriers in nikethamide

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Stimulation of central muscarinic receptors, located in the medulla, causes respiratory stimulation [1]. Nikethamide is an analeptic reputed to have specific action on the medulla; however, it is known to cause widespread stimulation of the central nervous system [2]. There are a number of structural features in common between nikethamide and the endogenous neurotransmitter acetylcholine. Many quaternary ammonium compounds have been shown to interact with the acetylcholine receptor [3], and the pyridyl nitrogen of nikethamide and the quaternary nitrogen of acetylcholine may interact with the same anionic site of the receptor. In addition, the carbonyl oxygens of these molecules may participate in hydrogen bonding interactions with a target site on the receptor. We report on a chemical property of nikethamide which could account, in part, for its widespread action on the central nervous system: the hindered internal rotation of the diethylamide group.

Rotational barriers in cholinergic ligands can determine