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Production of a monoclonal antibody-mitomycin C conjugate, utilizing dextran T-40, and its biological activity

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Attempts to bind anticancer agents to antibodies have increased because of the hydbridoma technique developed by Köhler and Milstein [1] which permits the production of large amounts of monoclonal antibodies. 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 drugs covalently to antibodies have been investigated [2, 3]. 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 [4].

Mitomycin (MMC), an antibiotic isolated from *Streptomyces caespitosus* by Wakaki *et al.* [5], has potent anticancer activity; however, its clinical use is limited by its detrimental effects on normal tissues. MMC-antibody conjugates were first prepared by Suzuki *et al.* [6], employing the cyanogen bromide method.

In this report, we conjugated MMC to H-1 IgG_1 antibody with the use of dextran T-40 as a multivalent carrier, and studied the cytotoxic activity of the conjugate against cells bearing or lacking HLA *in vitro*.

Materials and methods. MMC was obtained from the Kyowa Hakko Co., Ltd., Tokyo. Dextran T-40 was purchased from Pharmacia Fine Chemicals, Sweden, and sodium periodate and sodium borohydride were from the Sigma Chemical Co., U.S.A. A null cell line (NALL-1) [7], which has HLA, was derived from human acute lymphoblastic leukemia cells, and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. A mouse myeloma cell line, P3-NS1/1-Ag4-1 (NS-1) [8], which does not have HLA, was used as a control. The hybridoma cells secreting H-1 antibody [4] were grown in the peritoneal cavity of BALB/c mice, and the ascitic fluid from several mice was pooled. Control IgG was obtained similarly using NS-1 cells. H-1 and control IgG were purified from the ascitic fluid pool by affinity chromatography on Protein-A Sepharose CL-4B [9, 10].

Dextran T-40 (molecular weight: 4×10^4) was oxidized to polyaldehyde-dextran (PAD) by the following Malaprade reaction [11, 12]. Dextran T-40 (1.0 g) was mixed with sodium periodate (0.33 g) in 200 ml of distilled water, and stirred for 1 hr at room temperature. The resultant mixture was concentrated using a Collodion bag (cut-off level: 2.5×10^4; Sartorius, Germany), applied to a Sephadex G-25 column equilibrated with distilled water, and lyophilized. Sixty milligrams PAD was incubated with 20 mg H-1 in 10 ml of 0.15 M phosphate-buffered saline (PBS), pH 7.2, for 24 hr at 4°, after which 12 mg MMC in 5 ml PBS was added. The solution was stirred for another 24 hr. The Schiff bases thus formed were reduced by 2 hr of incubation with 0.3 ml of sodium borohydride solution (10 mg sodium borohydride in 10 ml PBS) [13]. The mixture was then applied to a Sephadex G-200 column (2.6×100 cm) equilibrated with PBS. The optical density of the fraction was measured at 280 nm, and the two major peaks were lyophilized.

The antimicrobial activity [MMC equivalent (μg/ml)] of the conjugate and of free MMC was assayed by a cup method using *Escherichia coli* ATCC 11303 agar plates and measuring the zone of growth inhibition [14]. The degree of MMC substitution of the conjugate was estimated by the absorbance at 363 nm, assuming an $E^{1\%}_{1cm,623}$. The IgG concentration was measured by the Bio-Rad Protein Assay (Bio-Rad Laboratories, U.S.A.) [15]. MMC activity (%) of the conjugate was calculated as follows: MMC activity (%) = MMC equivalent determined by the cup method/MMC substitution determined spectrophotometrically $\times$ 100.

NALL-1 cells were incubated with H-1 or MMC-(H-1) for 30 min at 4° and then stained with fluorescein isothiocyanate (FITC)-conjugated goat antiserum to mouse IgG (E. Y Laboratories, U.S.A.). Membrane staining of the cells was examined by fluorescence microscopy, as was the reactivity of the MMC-(H-1) conjugate to NS-1 cells.

The cytotoxic activity of the conjugate was measured against the HLA-bearing cell line, NALL-1, and the HLA-lacking cell line, NS-1. Cells (3×10^6), grown to the logarithmic phase, were incubated in 1 ml medium containing the test substances for 30 min at 37% and washed two times with fresh medium and cultured further for 3 days in 5 ml medium. Viable cells were measured by the trypan blue dye exclusion test. Data were analyzed for statistical significance according to Student's *t*-test.

Whether MMC was released from MMC-(H-1) conjugate was examined. One milligram of MMC-(H-1) was sus-

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

pended in 2.5 ml PBS maintained at 37°. The MMC remaining in the conjugate was determined after separation by Sephadex G-25 gel chromatography. H-1 IgG content in the conjugate after gel filtration was also determined.

Results and discussion. The reaction mixture of MMC and H-1 monoclonal IgG_1 antibody with oxidized dextran T-40 (PAD) was applied to a Sephadex G-200 column and eluted with PBS, yielding the elution profile shown in Fig. 1. Fractions 1 and 2 contained substances inhibitory to *E. coli* ATCC 11303 growth. When mouse IgG was applied to the column, a single peak appeared in the same position as the Fraction 1 peak. A free MMC peak was eluted in approximately the same position as the Fraction 2 peak. Figure 2 shows the absorption spectrum of Fraction 1 that differs from that of free MMC or H-1. When MMC and H-1 were simply mixed and applied to a Sephadex G-25 column, the absorption spectrum of the eluted material showed the same pattern as that of H-1, indicating that no MMC-(H-1) conjugate was formed. These results indicate that MMC bound covalently to H-1 by a dextran bridge.

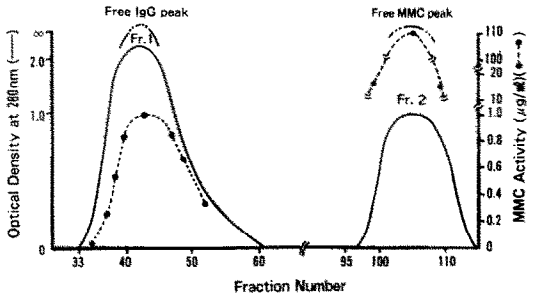

Fig. 1. Elution profile of the dextran T-40-treated mixture of H-1 and MMC (2.6 × 100 cm Sephadex G-200 column). Five-ml fractions were used. Key: (——) O.D. at 280 nm; (- - -) MMC activity assayed by *E. coli* ATCC 11303 growth inhibition (μg/ml).

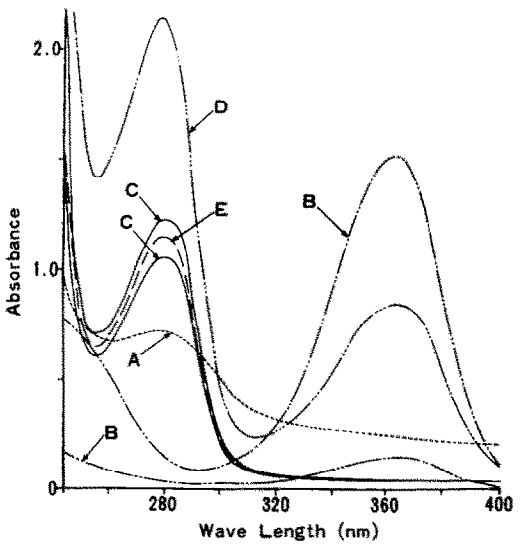

Fig. 2. Absorption spectra. (A) MMC-(H-1); (B) MMC (upper curve: 20 μg/ml; lower one: 2 μg/ml); (C) H-1 (upper curve: 0.6 mg/ml; lower one: 0.5 mg/ml); (D) an equivolume mixture of MMC (20 μg/ml) and H-1 (1.1 mg/ml); and (E) absorption spectrum of the first eluted material after the mixture (D) was applied to a Sephadex G-25 column.

The extent of substitution of the conjugate was 87.6 moles of MMC per mole of H-1 IgG_1, and the MMC activity was calculated to be 2.42%. The IgG recovery rate was 69%.

Figure 3 shows the antibody activity of the conjugate, as measured by an indirect membrane immunofluorescence assay when NALL-1 cells were incubated with MMC-(H-1) 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. MMC-(H-1) did not react with NS-1 cells.

Figure 4 shows the cytotoxic activity of MMC-(H-1) conjugate against HLA-bearing NALL-1 and HLA-lacking NS-1 cells after a 30-min exposure to the conjugate. H-1 did not inhibit NALL-1 cell growth. The IC_{50} (drug concentration required for 50% cell growth inhibition) of MMC-(H-1) to NALL-1 cells was $7.00 \pm 0.10 \times 10^{-2}$ μg/ml, while that of free MMC was $7.10 \pm 0.25 \times 10^{-1}$ μg/ml.

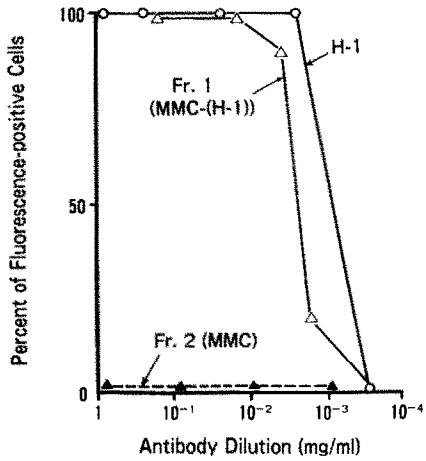

Fig. 3. Comparison of antibody titer before and after conjugation. The antibody activity against NALL-1 cells was measured by the indirect membrane immunofluorescence assay. Key: (○—○) H-1; (△—△) MMC-(H-1) (Fr. 1); and (▲—▲) MMC (Fr. 2).

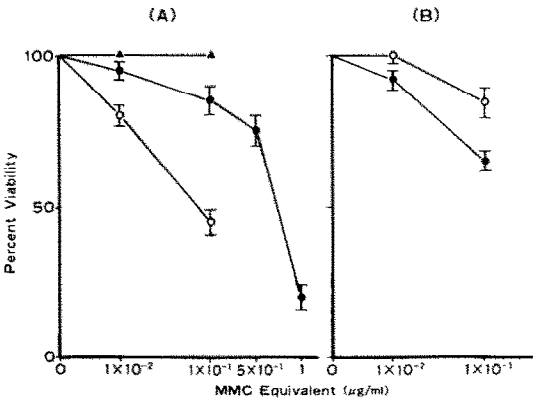

Fig. 4. Cytotoxic activities of MMC-(H-1), MMC, and H-1 to NALL-1 (A) and NS-1 (B) cells. Cells (3×10^6) were cultured for 3 days after a 30-min drug exposure. Key: (▲—▲) H-1; (○—○) MMC-(H-1); and (●—●) MMC. Each point and bar indicates the mean and S.E. of three determinations.

The difference was statistically significant ($P < 0.001$). This result indicates that MMC-(H-1) is ten times more toxic to NALL-1 cells than MMC. On the other hand, the conjugate showed statistically less toxicity than MMC against HLA-lacking NS-1 cells ($P < 0.02$).

Figure 5 shows the time course for MMC remaining in MMC-(H-1) conjugate. During the period of 3 days, nearly all amounts of MMC remained as MMC-(H-1) conjugate. IgG content in the conjugate was not changed, indicating that MMC-(H-1) coupled by dextran bridge was stable. These results indicate that the conjugate bound to the cell surface antigen does exert the cytotoxic activity of MMC.

Suzuki *et al.* [6] reported that the degree of substitution by the cyanogen bromide method was 1 mole of MMC per mole of antibody, and that the conjugate was composed of 0.535 mole of MMC with antimicrobial activity per mole of antibody. These results indicate that our dextran method could bind four times more moles of pharmacologically active MMC to one mole antibody than the cyanogen bromide method. MMC was released from MMC-antibody conjugate coupled by the cyanogen bromide method, while MMC was not released from MMC-(H-1) conjugate coupled by the dextran method.

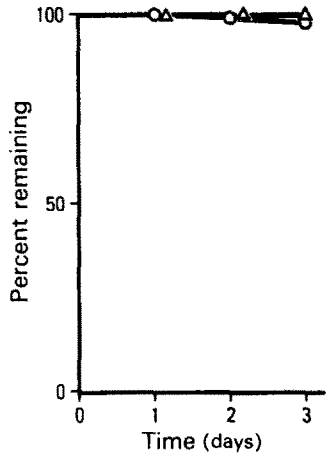

Fig. 5. Time course of MMC in MMC-(H-1) conjugate. MMC-(H-1) (1 mg) was suspended in 2.5 ml PBS maintained at 37°. The MMC remaining in the conjugate was determined after separation by Sephadex G-25 gel filtration. Key: (○—○) MMC in MMC-(H-1); and (△—△) H-1 IgG in MMC-(H-1).

MMC was linked covalently to monoclonal anti-HLA IgG_1 antibody (H-1) with the use of dextran T-40. The MMC-(H-1) conjugate showed stronger cytotoxicity than MMC alone against HLA-bearing cells in cultivation after a 30-min exposure to the drugs. In the same experiment, the conjugate was less toxic than MMC against cells lacking HLA. MMC was not released from MMC-(H-1) *in vitro*. These results indicate that the MMC-(H-1) conjugate attached by antigen-antibody action to the cell surface exerts MMC cytotoxicity.

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