In Vitro Cytotoxicity of a Human Serum Albumin-mediated Conjugate of Methotrexate with Anti-MM46 Monoclonal Antibody

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ABSTRACT

In studies on antitumor antibody:drug conjugates as potential antitumor agents, methotrexate (MTX) was conjugated with a murine monoclonal antibody (aMM46) to an antigen on ascitic mouse mammary tumor MM46 cells (MM antigen) with human serum albumin (HSA) as an intermediary. MTX was linked to HSA which had been conditioned to have about 1 mol of thiol group per mol of HSA by dithiothreitol treatment followed by oxidation on standing at 4°C. The MTX linking was performed, without protection of the thiol group of HSA, by using MTX N-succinimidyl ester prepared via MTX intramolecular anhydride. The resulting HSA:MTX was reacted with the immunoglobulin with the maleimide group introduced. The aMM46:HSA:MTX obtained retained both antibody binding and drug activities. The cytotoxicity of aMM46:HSA:MTX against MM antigen-positive MM46 cells was greater than that of control 96.5 (anti-human melanoma-associated antigen, p97):HSA:MTX and was inhibited by unconjugated aMM46. No different cytotoxicity of aMM46:HSA:MTX compared with that of 96.5:HSA:MTX was observed against MM antigen-negative mouse mammary tumor MM48 cells. The presence of ammonium chloride or leupeptin abrogated the selective cytotoxicity against MM46 cells of aMM46 conjugate but did not affect the nonspecific cytotoxicity of 96.5:HSA:MTX. These results support the idea that the selective cytotoxicity of aMM46:HSA:MTX is antibody directed and exhibited through lysosomal degradation of the conjugate.

INTRODUCTION

A problem in cancer chemotherapy is that sufficiently potent and selective cytotoxicity to tumor cells is not usually obtained with the cytotoxic agents currently available. One possible way to overcome this problem is to use the drugs in the form of conjugates with antibodies to tumor-associated antigens (1, 2) and thereby to alter the drug distribution so as to deliver more drug to antigen-bearing tumor cells while reducing their delivery to normal tissues.

Previously we developed a method for conjugating mitomycin C with antibody with serum albumin as an intermediate drug carrier (3). For this we used the intrinsic single thiol group of cysteine at amino acid residue 34 in serum albumin as a functional group for the linkage with the antibody without using a method for external introduction of thiol groups, because in this way we could prepare the conjugate by a more controllable reaction and obtain a more homogeneous preparation.

In the present study, we modified our previous method to obtain serum albumin-mediated conjugates of improved quality by a more convenient method. We then used the modified method to prepare an MTX2-conjugate with a murine monoclonal antibody (4-6) (aMM46) to an antigen (MM antigen) on syngeneic, ascitic C3H/He mouse mammary tumor MM46 cells (7, 8). MTX was used after its conversion to an active ester by a new method.

MTX is an interesting candidate drug for being used as conjugates with antitumor antibodies, because the time-dependent nature of its cytotoxic activity may be compatible with the slow kinetics (9, 10) at the tumor site of the i.v. administered antibodies. MTX has been covalently conjugated with antibodies (11-18), but the selective cytotoxicities of the conjugates have not yet been thoroughly studied.

In this work we examined the in vitro cytotoxicity of an MTX conjugate with aMM46 with HSA as an intermediary and verified the antibody-directed cytotoxicity to target MM46 cells. Evidence was also obtained for the involvement of lysosomal enzymes in the mechanism of specific action of the conjugate.

MATERIALS AND METHODS

Tumor Cell Lines. The ascitic tumor cell lines MM46 and MM48 from spontaneous mammary tumor of C3H/He mice were provided by Dr. T. Chihahana, Tohoku University School of Medicine, Sendai, Japan, and were maintained in in vivo passage in C3H/He mice.

Antibodies. The monoclonal IgG1 antibody MM-γ-1 (aMM46) and IgG2a antibody MM-γ-2a-1 against MM antigen were prepared in athymic BALB/c-nu/nu mice with respective hybridoma clones established by Seto et al. (4) and purified from the ascitic fluids on Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The monoclonal antibody 96.5 (19) to a human melanoma-associated antigen p97 was purchased from Hybritech, Inc., San Diego, CA.

Preparation of an Active Ester Derivative of MTX. MTX (545 mg; Fluka AG, Buchs, Switzerland) was treated with N,N′-dicyclohexylcarbodiimide (206 mg) in DMF (15 ml) for 15 h at 4°C. N-Hydroxysuccinimide (115 mg) and pyridine (158 mg) were added to the resulting solution, and the reaction was allowed to proceed for 6 h at 4°C. The precipitate was removed by filtration, and the solution containing the active ester derivative was stored at −20°C.

The content of the active ester was determined by use of Affi-Gel 102 (Bio-Rad Laboratories, Richmond, CA) (11).

Preparation of MTX-linked HSA. HSA (Sigma Chemical Co., St. Louis, MO) (111 mg of protein/ml, 9 ml) was first freed of its dimer by gel filtration on Sephadex G-150, superfine, in PBS, and then reduced with 10 mM DTT and dialyzed against PBS to obtain a preparation with 1.65 mol of thiol group per mol of HSA. The HSA remained at 4°C to allow the thiol group: HSA molar ratio to decrease to 0.72 (for 6 days) (Fig. 1). The thiol group was determined with 5,5′-dithiobis(2-nitrobenzoic acid).

To the resulting solution of HSA with free thiol group (20.9 mg/ml, 1.0 ml) was added dropwise 104.4 mm MTX N-succinimidyl ester in DMF (0.2 ml). The solution was adjusted to pH 8.0 with 0.2 N NaOH, stirred at 4°C for 16 h, and dialyzed against PBS to obtain an HSA:MTX conjugate with free thiol group (HS-HSA:MTX).

Preparation of aMM46:HSA:MTX. To a solution of aMM46 in 10 mM phosphate buffer, pH 7.0, containing 0.14 mM NaCl (23.3 mg/ml, 1 ml) was added dropwise 74.3 mm N-succinimidyl 4-(N-maleimido)butylate in DMF (23 μl). The mixture remained at 25°C for 1 h and was then dialyzed against PBS.

To the resulting solution of the IgG1 with maleimide group (12.9 mg/ml, 1.2 ml) was added to a solution of HS-HSA:MTX in PBS (13.1 mg of HSA equivalent/ml, 1.3 ml). The mixture remained at 4°C for...
18 h. For blocking the remaining unreacted maleimide group, the reaction mixture was treated with 71 mM L-cysteine in PBS (30 μl) at 4°C for 1 h and subjected to gel filtration on a Sephadex G-150, superfine, column (1.5 × 94 cm) in PBS to give aMM46:HSA:MTX. 96.5:HSA:MTX was prepared by the same procedure as for aMM46:HSA:MTX.

Quantitation of the Maleimide Group Introduced to IgG. Quantitation was performed with N-(2,4-dinitrophenyl)cysteine as described previously (3).

Quantitation of MTX Bound to Protein. Quantitation was performed by measuring the absorbance at 372 nm (ε, 7200).

Quantitation of HSA. Quantitation was performed by Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) (20) based on the shift of absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 from 465 to 595 nm when binding to protein occurred.

Quantitation of IgG in IgG:HSA:MTX. Quantitation was performed by measuring the absorbance at 280 nm due to IgG as follows. The UV spectrum of IgG:HSA:MTX was determined, and the absorbance at 280 nm due to HSA:MTX was calculated by multiplying the absorbance at 372 nm of IgG:HSA:MTX by the ratio of the absorbance at 280 nm to that at 372 nm of HSA:MTX. The absorbance at 280 nm due to IgG was then obtained by subtracting the absorbance due to HSA:MTX calculated as above from the absorbance of IgG:HSA:MTX.

In Vitro Cytotoxicity Test. MM46 or MM48 cells were cultured with a test sample in RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) (2.5 × 10^6 cells/ml) supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, NY), 20 μM 2-mercaptoethanol, and 0.1 mg of gentamycin sulfate per ml in a 96-well flat-bottomed microtest plate (Falcon No. 3072) in a humidified atmosphere of 5% CO2 in air at 37°C for 3 days. Viable cells were counted by the trypan blue dye exclusion method. Cells were cultured in duplicate, and mean numbers of viable cells were obtained.

RESULTS

Preparation of Conjugates. HSA was first reduced with DTT and then remained at 4°C until the thiol group:HSA molar ratio decreased to about 1 (Fig. 1). MTX was incorporated into this HSA by use of the MTX N-succinimidyld active ester prepared by a new procedure via MTX intramolecular anhydride to obtain HSA:MTX. In our repeated experiments, the reaction efficiencies in terms of the percentages of MTX incorporated into aMM46 among MTX active ester used and among MTX which had been used for preparation of the ester were 35 to 50 and 25 to 40%, respectively. These are considerably higher, than those described for the active ester method by Garnett and Baldwin (16), and the yield of HSA:MTX based on HSA used was as high as 80 to 95%.

The maleimide group was introduced to aMM46 with N-succinimidyld 4-(N-maleimido)butylate (introduced maleimide group, 5.7 mol per mol of IgG). aMM46:HSA:MTX was then prepared by the addition reaction of the thiol group of HSA:MTX with the maleimide group of aMM46 (Fig. 2).

The binding ratios in aMM46:HSA:MTX and similarly prepared 96.5:HSA:MTX are listed in Table 1.

Antibody Binding Activity in Conjugate. The antibody binding activity of aMM46:HSA:MTX was assessed taking advantage of the fact that complement does not bind to aMM46, a mouse IgG1 antibody, whereas it binds to MM-γ2a-1, a mouse IgG2a antibody directed to the same or a closely related antigenic determinant of MM antigen. Namely, in the assay MM46 cells which had been coated with the conjugate and the IgG2a antibody by successive incubations with various concentrations of the conjugate and a constant concentration of the IgG2a antibody at 0°C were treated with complement (Fig. 3). For comparison, the above procedure was followed with aMM46 instead of the aMM46 conjugate. The IgG1 (aMM46) concentrations required for 50% decrease in the percentage of lysis from the lysis (89% lysis) at an IgG1 concentration of 0 (no

![Fig. 1. Time course of change in the content of thiol group in reduced HSA. HSA in PBS which had been reduced with DTT to contain 1.65 mol of the thiol group per mol of HSA was stored at 4°C. At various times after dialysis to remove DTT, aliquots were withdrawn, and their thiol group contents were determined with 5,5'-dithiobis (2-nitrobenzoic acid).](image-url)
addition of aMM46:HSA:MTX or aMM46) were 45 and 16 
µg/ml for the conjugate and aMM46, respectively.

Cytotoxicity of Conjugates. The cytotoxic activity of MTX conjugated with aMM46 was compared with that of unconjugated MTX (Fig. 4) with MM46 cells as targets. Unconjugated and conjugated MTX showed concentration-dependent cytotoxicity at concentrations above 16 nM. Conjugated MTX was about one-fifth as active as unconjugated MTX. The cytotoxicity of HSA-linked MTX was enhanced when it was coupled with aMM46, but decreased when it was coupled with irrelevant antibody 96.5 (Fig. 5). At the same MTX concentrations of 500 and 1000 nM, more than a 10-fold difference in viable cell numbers was observed with aMM46:HSA:MTX and 96.5: HSA:MTX. The concentrations of the two conjugates required for 90% decrease in the viable cell number compared with the control (no addition of test materials) differed about 4-fold.

MM46 cells were incubated with a constant concentration of the conjugate (2.7 µg/ml by IgG equivalence or 500 nM by MTX equivalence) plus various concentrations of aMM46 or 96.5 (Fig. 6). Increasing concentrations of aMM46 but not of 96.5 inhibited the activity of the conjugate.

Next, the target cell specificity of the aMM46 conjugate was examined. The higher cytotoxic potency of aMM46:HSA:MTX than of 96.5:HSA:MTX on MM46 cells was confirmed (Fig. 7A), but with MM antigen-negative MM48 cells the aMM46 conjugate showed lower cytotoxicity, which was similar to that of the 96.5 conjugate (Fig. 7B).

All these results indicate that the cytotoxicity of aMM46:HSA:MTX is mediated by a specific antibody/antigen interaction on the cell surface and, therefore, is rendered selective.

Effect of Ammonium Chloride on the Cytotoxicity of Conjugates. For examination of the effect of ammonium chloride, a lysosomotropic agent raising the pH in lysosomes (21), on the in vitro cytotoxicities of aMM46:HSA:MTX and 96.5: HSA:MTX, MM46 cells were preincubated with 10 mM ammonium chloride before the culture with the conjugates (Fig. 8). Ammonium chloride decreased the cytotoxicity of aMM46:HSA:MTX, but had no effect on the cytotoxicity of 96.5:HSA:MTX.

Effect of Leupeptin on the Cytotoxicity of Conjugates. For examination of the effect of leupeptin (22), an inhibitor of the lysosomal endopeptidases cathepsin B, H, and L, on the in vitro cytotoxicity of aMM46:HSA:MTX and 96.5:HSA:MTX,
MM46 cells were cultured with the conjugates in the presence or absence of leupeptin (Fig. 9). Leupeptin decreased the cytotoxicity of aMM46:HSA:MTX, but it did not affect that of 96.5:HSA:MTX.

**DISCUSSION**

In order to increase the amount of drug attached to antibody without significantly impairing its antigen-binding activity, indirect conjugation methods with serum albumin as intermediary have been developed (3, 12, 13). Human serum albumin has a large number of amino groups which can be used for drug binding and is relatively stable in aqueous medium even after drug binding. Moreover, it has a single thiol group (23) useful for binding to antibody (3). An intermediate drug carrier which has a single functional group for binding to antibody is desirable to minimize the formation of high-molecular-weight conjugates by avoiding the linking of an intermediary with more than one antibody molecule.

In the present study, we modified our previously reported serum albumin-mediated conjugation method (3). Monomeric HSA contained only 0.2 mol of thiol group per mol of protein, the thiol group probably being in the form of a disulfide bridge with glutathione and cysteine. We generated about 1 mol of thiol group per mol of HSA by DTT treatment followed by oxidation while remaining at 4°C.

We found that even when HSA with an unprotected thiol group was treated with the N-succinimidyl ester of MTX, the HSA:MTX obtained still retained a thiol group that reacted with the maleimide group introduced into IgG. Based on these findings we could avoid use of protection and deprotection processes. An alternative to our method may be the use of HSA unconditioned with respect to the amount of thiol group and generation of an appropriate amount of thiol group after drug binding by DTT treatment (16). However, by binding of drug, various disulfide bonds in HSA become more susceptible to thiol reagents, and the DTT treatment becomes more difficult to control.

Another modification was blocking of the maleimide group that remained in the conjugates after the reaction of HSA:MTX with IgG with maleimide group. This blocking was achieved by treatment with L-cysteine, and this procedure increased the stability of the conjugates by preventing the gradual formation of high-molecular-weight material by the remaining maleimide group (data not shown).

Furthermore, our new method of making the N-succinimidyl ester of MTX, consisting of the conversion of MTX to its intramolecular anhydride followed by its treatment with N-hydroxysuccinimide, afforded an ester preparation with less diester as shown in Table 2.

Contrary to the description by Garaett and Baldwin (16), coupling of MTX with HSA by the N-succinimidyl ester method was found to be as effective as the carbodiimide method, and we could use the ester method with an advantage of eliminating formation of polymeric HSA:MTX encountered by the carbodiimide method.

The method to determine the antibody binding activity in the aMM46 conjugate devised in this study is based on the nature of aMM46 (IgG1) and anti-MM46 IgG2a. Both antibodies recognize the same or a closely related antigenic determinant, and the IgG1 antibody is not active in complement-dependent cytolysis (5). aMM46 caused dose-dependent inhibition of complement-dependent cytolysis by the IgG2a antibody. Thus, the antibody binding activity in aMM46:HSA:MTX was determined by measuring its inhibition of complement-dependent cytolysis with the IgG2a antibody in comparison with that of the unconjugated aMM46.

The antibody binding activity of the aMM46 conjugate determined by this method was 36%. This conjugate was made by introducing 5.7 mol of the maleimide group per mol of aMM46 for binding 1.16 mol of HSA. Even when the molar rate of substitution with the maleimide group was decreased to 3.5, the binding activity of the final conjugate remained the same, while the binding activity of the intermediate maleimide-modified aMM46 was 100%. Therefore, the decrease of the antibody binding activity is not due to the rate of substitution of the antibody molecule.

The present results showed that aMM46:HSA:MTX has antibody-directed, selective cytotoxicity. aMM46:HSA:MTX was more cytotoxic than control 96.5:HSA:MTX to MM46 cells. This greater cytotoxicity of the aMM46 conjugate was not due to greater cytotoxicity of aMM46 itself than of 96.5, because neither 96.5 nor aMM46 was cytotoxic to MM46 cells. The cytotoxicity of aMM46:HSA:MTX to MM46 was not inhibited by 96.5 but was inhibited by unconjugated aMM46, which competed with the conjugate for the MM antigen on the cells. The cytotoxicity of aMM46:HSA:MTX against MM antigen-negative MM48 cells was less than its cytotoxicity against MM46 cells. The fact that control 96.5 conjugate showed similar weak activity against both MM46 and MM48 cells excludes the possibility that aMM46:HSA:MTX simply showed less nonspecific cytotoxicity against MM48 cells than against MM46 cells. When the immunological specificities of the antibody conjugates are examined using antigen-positive and negative cell lines, a nonspecific conjugate that is irrelevant for the two cell lines must be used for comparison, since nonspecific sensitivity itself may differ in different cell lines.

Previous reports concerning the mechanism of action of MTX conjugates (13, 24–26) suggested the involvement of

![Graph](image)

**Fig. 9.** Effect of leupeptin on the cytotoxicity of conjugates. MM46 cells were cultured with a conjugate in the presence (—) or absence (—) of 100 μM leupeptin (Sigma Chemical Co.) at 37°C for 65 h, and then numbers of the viable cells were determined. ■, 96.5:HSA:MTX; ●, aMM46:HSA:MTX.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Diester (%)</th>
<th>Monoester (%)</th>
<th>Remaining MTX (%)</th>
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</thead>
<tbody>
<tr>
<td>Preparation 1*</td>
<td>12.3</td>
<td>58.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Preparation 2*</td>
<td>20.4</td>
<td>38.4</td>
<td>21.7</td>
</tr>
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</table>

* Preparation made via the intramolecular anhydride.

* Preparation made by the method of Kulkarni et al. (11).
lysosomal degradation of the conjugates in their cytotoxic action. The present study demonstrated that the specific cytotoxicity shown by aMM46:HSA:MTX was mostly inhibited by ammonium chloride, which inactivates lysosomal enzymes by raising the pH (21), and by leupeptin, an inhibitor of the lysosomal endopeptidases cathepsin B, H, and L (21, 22). Thus, consistent with previous reports, our results support the idea that lysosomal degradation of the conjugate is involved in the mechanism of its action. The cytotoxicity of unconjugated MTX was only slightly decreased by ammonium chloride, and it was not affected at all by leupeptin (data not shown).

It should be noted that neither ammonium chloride nor leupeptin affected the nonspecific cytotoxicity of 96.5:HSA:MTX. This suggests that nonspecific cytotoxicity of the conjugates outside the cells or escaping from endocytic vesicles in either conjugated or free form before fusion of the vesicles with lysosomes.

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