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Conjugation of methotrexate to IgG antibodies and their $F(ab)_2$ fragments and the effect of conjugated methotrexate on tumor growth in vivo

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Summary. *Methotrexate (MTX) was first conjugated to antibovine serum albumin IgG (antiBSA) or its $F(ab)_2$ fragment to define conditions for retention of drug and antibody activity. With identical drug: protein molar ratios, incorporation in the $F(ab)_2$ fragment was lower than in intact antiBSA, an observation consistent with analysis of the number of lysine residues (22 in $F(ab)_2$ compared to 40 in antiBSA). In either case, up to approximately 10 mol MTX could be incorporated per mol protein, with recovery of 70% of the protein. At an incorporation ratio of 6 mol MTX per mol protein, MTX-antiBSA retained 100% of antibody activity and MTX-$F(ab)_2$antiBSA retained 75%. MTX-antiBSA and MTX-$F(ab)_2$antiBSA were equally potent in vitro inhibitors of dihydrofolate reductase. Conjugates prepared from antiEL4 IgG (AELG) and from $F(ab)_2$AELG significantly increased survival in EL4 lymphoma-bearing mice compared with mice receiving equal amounts (5 mg MTX/kg) of free MTX, MTX linked to the $F(ab)_2$ fragment of normal rabbit IgG, or a simple mixture of MTX and $F(ab)_2$AELG. MTX-AELG at this dose level produced longer survival than MTX-$F(ab)_2$AELG ($0.005 < P < 0.01$).*

Introduction

Currently available cancer chemotherapeutic agents (such as the folic acid antagonist methotrexate) are not tumor-specific; they damage all proliferating cells. One way to render them tumor-specific is to link them to antibodies that bind to cell-surface TAA [2]. Indeed, linking MTX to AELG, for example, yields a conjugate that inhibits EL4 lymphoma growth more effectively in vivo than does free MTX or MTX linked to NRG [8]. The $F(ab)_2$ fragment of antiTAA antibodies may be better suited as a carrier for therapy, because (i) it will more easily cross the capillaries and diffuse in the tissue space, qualities that are of considerable importance in determining the accessibility of systemically administered agents to target cells [2]; (ii) the faster plasma clearance of $F(ab)_2$ (compared with the parent IgG) not bound by the tumor may increase the tumor: plasma (or tumor: other normal tissues) ratio of localization and thus improve the conjugate's chemotherapeutic index [5, 7]; and (iii) the removal of the Fc fragment of carrier immunoglobulins is likely to reduce their antigenicity and render them less susceptible to binding to and subsequent catabolism by normal cells that have Fc receptors, e.g., phagocytes. Furthermore, effective tumor suppression by MTX-$F(ab)_2$antiTAA would exclude the involvement of Fc-mediated complement or effector-cell activation in the process of tumor inhibition by MTX-IgG conjugates in vivo [3].

Using the $F(ab)_2$ fragment of rabbit antiBSA IgG, we first assessed retention of antibody activity as a function of molar incorporation of MTX in the protein, coupling being carried out by an active ester method [6]. We compared the potency of MTX-AELG and MTX-$F(ab)_2$AELG conjugates (prepared under optimal conditions defined with the BSA-antiBSA system) with regard to retention of drug and antibody activities in vitro, and systemic toxicity and tumor inhibition in vivo.

Materials and methods

Chmicals. Biogel P6 and the Bio-Rad protein assay kit were from Bio-Rad Laboratories, Richmond, Calif; BSA, DHFR, NADPH, MTX and pepsin from Sigma Chemicals Co., St. Louis, Mo., DCC from Eastman Organic Chemicals, Rochester, NY; NHS from Aldrich Chemical Co., Milwaukee, Wis.; and Protein A Sepharose and Sephadex G150 from Pharmacia, Uppsala, Sweden.

Mice and tumors. We used 12- to 16-week-old inbred female C57BL/6J mice (Jackson Laboratories, Bar Harbor, M) in all experiments. The EL4 lymphoma was obtained from the Chester Beatty Research Institute (London, England) in 1969 and has been maintained by serial IP passage in C57BL/6J mice. We have described its pattern of growth and lethality elsewhere [4].

Production and assay of antiBSA and antiEL4 antibodies. We produced antibodies in rabbits, using (as appropriate)

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Abbreviations: AELG, antiEL4 IgG; antiBSA, antibovine serum albumin IgG; BSA, bovine serum albumin; DCC, dicyclohexylcarbodiimide; DFA, dihydrofolic acid; DHFR, dihydrofolate reductase; DMF, dimethylformamide; IP, intraperitoneal; MTX, methotrexate; MTX-AELG, MTX linked to AELG; MTX-antiBSA, MTX linked to antiBSA; MTX-$F(ab)_2$AELG, MTX linked to the $F(ab)_2$ fragment of AELG; MTX-$F(ab)_2$antiBSA, MTX linked to the $F(ab)_2$ fragment of antiBSA; MTX-$F(ab)_2$NRG, MTX linked to the $F(ab)_2$ fragment of NRG; MTX-NRG, MTX linked to NRG; NHS, N-hydroxysuccinimide; NRG, normal rabbit IgG; PBS, 0.01 *M* sodium phosphate (pH 7.1) containing 0.45 *M* sodium chloride; TAA, tumor-associated antigen; $t_{1/2}$, half-life

either BSA or EL4 lymphoma cells as immunogens. The details of the immunization schedule and the purification and assay conditions for these two antibodies have been reported [8].

Preparation of the $F(ab)_2$ fragments. We obtained 40 mg rabbit IgG by column chromatography on Protein A Sepharose, dialysed it against 0.1 *M* sodium acetate buffer (pH 4.5) for 24 h, then digested it with 1 mg of pepsin at 37 °C for 18 h. We centrifuged the reaction product to remove precipitates and passed the clear supernatant through a Sephadex G150 column using 0.1 *M* $NaPO_4$ buffer (Ph 7.0). The high-molecular-weight fractions, containing both the $F(ab)_2$ and undigested IgG, were pooled and were passed through Protein A Sepharose to remove any contaminating whole IgG. The $F(ab)_2$ fraction thus purified was found to be homogeneous by disk electrophoresis [14]. Protein determination was achieved either by the Lowry method [9] or by using the Bio-Rad protein assay kit. Antibody activity of $F(ab)_2$antiBSA was estimated by a modification of Mancini's single radial immunodiffusion technique, using 25 µg BSA/ml agar [8, 10]. Antibody activity of AELG and $F(ab)_2$AELG was assayed by membrane immunofluorescence [8].

Coupling of MTX to IgG or $F(ab)_2$. We conjugated IgG or $F(ab)_2$ with MTX, using an active ester intermediate of MTX [6, 8]. Briefly, equimolar MTX, NHS, and DCC were reacted in DMF for 18 h at 4 °C. Then the urea, which precipitated during the reaction, was separated by decantation, and the supernatant, which contained the active ester derivative of MTX, was stirred with IgG or $F(ab)_2$ in 0.01 *M* PBS (pH 7.0) for 4 h at 4 °C. Control experiments showed that the amounts of DMF used in coupling did not decrease the antibody activity of antiBSA IgG or its $F(ab)_2$ fragment. Free unreacted MTX and reaction side products were removed by gel filtration on a Biogel P6 column, followed by 24 h dialysis. To determine the amount of MTX incorporated per mole of IgG or $F(ab)_2$ we measured absorbance at 370 nm, using the previously established molar absorbance coefficient of 6.5×10^3 $1\ mol^{-1}\ cm^{-1}$ for bound MTX [8].

Assay of MTX activity. The capacity of conjugated MTX to inhibit DHFR was determined spectrophotometrically, using the assay procedure of Peterson et al. [12].

Assay of lysine residues in the whole IgG and its $F(ab)_2$ fragment.

Samples were digested with 6 *N* HCl in evacuated sealed ampules at 100 °C for 22 h, after which the acid was removed by vacuum evaporation. The residue, which contained liberated amino acids, was suitably diluted in lithium citrate buffer (Beckman Instruments, Inc., Palo Alto, Calif, USA) and was analyzed using a Beckman 6300 amino acid analyzer.

Tumor inhibition. Tumor inhibition was assessed in groups of mice (at least 5 per group), each mouse being inoculated IP with 10^4 EL4 cells and then, starting 24 h thereafter, injected with a test material on days 1, 4, and 7. The significance of differences between the groups' mean survival was determined by the Mantel-Haenszel summary chi-square procedure [11].

Results

Incorporation of MTX in $F(ab)_2$ and IgG and retention of antibody and drug activity

The lysine content of antiBSA and its $F(ab)_2$ fragment used in this study was assayed since amino groups are likely linkage sites for MTX [6]. The antiBSA contained approximately 40 lysine residues, and its $F(ab)_2$ fragment had about 22. For each concentration of MTX in the reaction mixture, incorporation was always lower in antiBSA's $F(ab)_2$ fragment than in whole antiBSA (Fig. 1a). Up to 10 mol MTX could be incorporated in either antiBSA or $F(ab)_2$antiBSA, with about 70% of the original protein be-

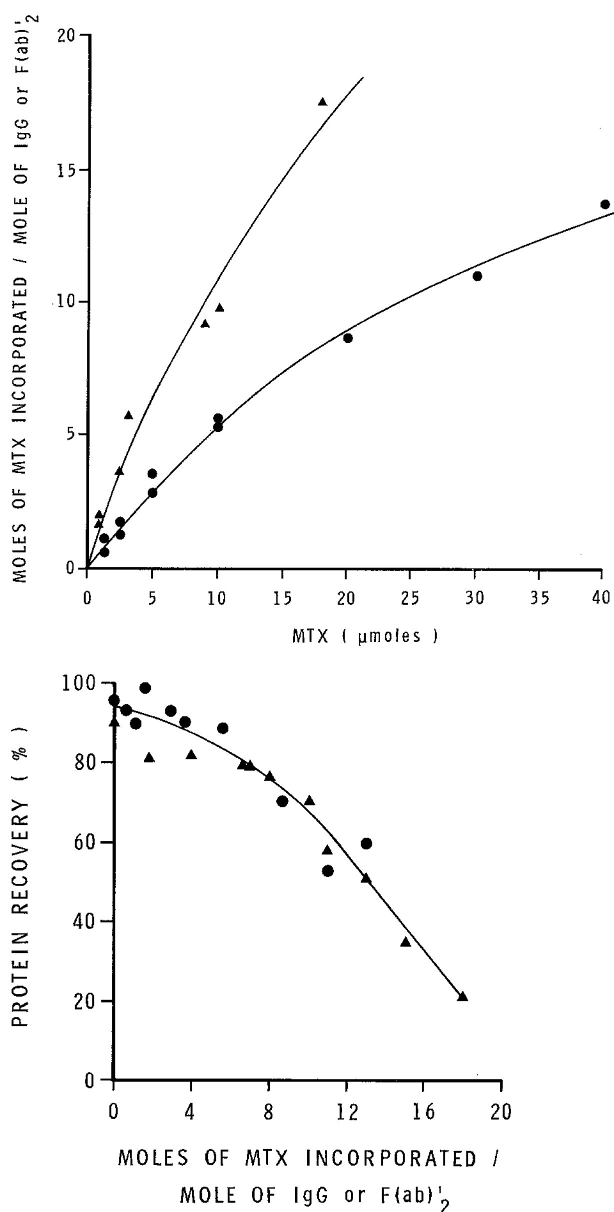

Fig. 1a, b. Coupling of MTX to rabbit antiBSA IgG (▲) or its $F(ab)_2$ fragment (●). **a** Number of mol of MTX incorporated per mol of IgG or $F(ab)_2$ versus the amount of MTX in the reaction mixture; **b** protein recovery as a function of MTX incorporation. Reaction mixtures contained 16 mg IgG or $F(ab)_2$ in 4 ml PBS, MTX active ester as indicated, and 0.8 ml of DMF; reaction time was 4 h at 4 °C

ing recovered in the form of soluble conjugates. Conjugates with higher incorporation of MTX (produced by higher concentrations of active ester in the reaction mixture), were progressively less soluble (Fig. 1 b), and MTX-$F(ab)_2$antiBSA in which 14 or more mol MTX were incorporated per mol $F(ab)_2$antiBSA completely precipitated out.

Relative to equal amounts of unreacted IgG or $F(ab)_2$, respectively, the MTX-$F(ab)_2$antiBSA lost its antibody activity at lower incorporation levels than did MTX-antiBSA (Fig. 2). For example, 10 mol MTX could be incorporated in antiBSA with retention of 90% of its antibody activity.

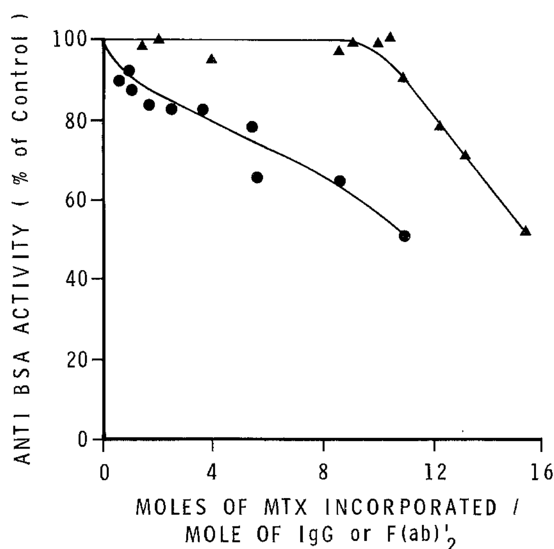

Fig. 2. AntiBSA activity retained versus mol of MTX incorporated per mol of IgG (▲) or $F(ab)_2$ (●). AntiBSA activity was determined by radial immunodiffusion and is expressed as a percentage of the square of the diameter of the precipitin ring given by equimolar amounts of unreacted antiBSA IgG or its $F(ab)_2$ fragment. Experimental conditions as in Fig. 1

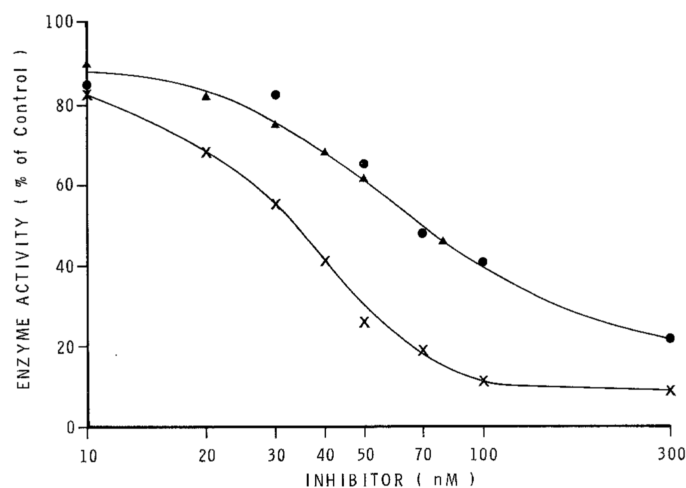

Fig. 3. Inhibition of DHFR activity by MTX, free (×) or conjugated to rabbit antiBSA IgG (▲), or its $F(ab)_2$ fragment (●). Enzyme activity is plotted as a function of the concentration of MTX in the assay system. Free antiBSA IgG or its $F(ab)_2$ fragment in amounts present in conjugates did not inhibit DHFR

Even at an incorporation level of 15 mol, the recovered soluble MTX-antiBSA retained 50% of its original activity, whereas MTX-$F(ab)_2$antiBSA started to lose antibody activity with the incorporation of as little as 2 mol MTX per mol $F(ab)_2$antiBSA. Incorporation of 25 mol MTX per mol intact antiBSA was possible, although this was associated with considerable precipitation and complete loss of antibody activity. When IgG and $F(ab)_2$ conjugates with equimolar incorporations of MTX were compared for their capacity to inhibit DHFR in vitro, both conjugates inhibited the enzyme to the same extent (Fig. 3). The conjugates used for studying tumor inhibition in vivo contained 5 – 7.5 mol MTX per mol $F(ab)_2$AELG and 10 – 12 mol MTX per mol AELG. Indirect immunofluorescent staining of 2×10^7 EL4 cells/ml with serial dilutions of AELG or its $F(ab)_2$ fragment, starting at 1.0 mg/ml, yielded titers (i.e., detectable staining of 50% of cells) of 3 μg/ml for both AELG and $F(ab)_2$AELG. In contrast to the partial loss of antibody activity in MTX-$F(ab)_2$antiBSA at 6 mol drug per mol protein, no decrease in titer was observed in either conjugate at these incorporation levels.

Toxicity and tumor-inhibitory effect of MTX conjugates

Intraperitoneal injections of 5 mg/kg MTX linked to NRG or to its $F(ab)_2$ fragment on days 1, 4, and 7 produced no observable toxicity in the mice. When the dose of conjugated MTX in each injection was 7.5 mg/kg and injections were given on days 1, 4, 7, and 10, two of six mice given the MTX/NRG conjugate died of toxicity but none of six mice given MTX-$F(ab)_2$NRG did.

Compared with untreated tumor-inoculated mice, which survived 16.9 ± 0.3 days ($n=28$), survival was significantly prolonged in groups given (at a dosage of 5 mg MTX/kg) MTX-NRG (24.6 ± 1.7 days; $P<0.005$; $n=6$) or MTX-$F(ab)_2$NRG (21.4 ± 0.7 days; $P<0.005$; $n=7$). Mice given 200 mg/kg AELG alone survived 23.0 ± 1.1 days ($P<0.001$; $n=8$). In contrast, 200 mg/kg $F(ab)_2$AELG alone (19.3 ± 0.8 days; $n=5$), 5 mg/kg free MTX (20.3 ± 0.7 days; $n=6$), or a mixture of $F(ab)_2$AELG and MTX (17.2 ± 1.2 days; $n=5$) had no significant effect on survival. Tumor-free long-term (>200 days) survivors were observed only in the groups given 5 mg/kg MTX linked to AELG or to $F(ab)_2$AELG. In the case of MTX-AELG, 9/15 mice survived tumor-free and the remaining 6 mice died on days 22, 26, 27, 30 (2), and 32 after tumor inoculation. In the case of MTX-$F(ab)_2$AELG, 4/16 mice survived tumor-free and the remaining 12 mice died on days 21 (2), 22 (4), 23 (2), 28 (3), and 44 after tumor inoculation. MTX-AELG at this dose level produced longer survival than MTX-$F(ab)_2$AELG ($0.005 < P < 0.01$).

Discussion

Side-chain amino groups of lysine are the most likely sites for reaction of the active ester derivative of MTX, leading to the formation of stable amide linkages [6]. The lower incorporation of MTX in the $F(ab)_2$ moiety than in the parent IgG after reaction of MTX with equimolar amounts of the $F(ab)_2$ fragment or the parent IgG is consistent with the lower number of lysine residues in the $F(ab)_2$ fragment. It is also possible that these residues in the $F(ab)_2$ fragment were less accessible than they are in the parent IgG. The loss of antibody activity in MTX-$F(ab)_2$antiBSA conju-

gates after the incorporation of as little as 2 mol MTX indicates possible substitution in the antigen-binding site of the $F(ab)_2$ fragment of antiBSA. However, the observed precipitation of the MTX-$F(ab)_2$ conjugates at low levels of substitution suggests changes in the tertiary structure and charge of the $F(ab)_2$ fragments after conjugation. These changes could also contribute to the loss of antibody activity in the MTX-$F(ab)_2$ conjugates.

MTX, when linked to non-tumor-specific macromolecular carriers such as serum albumin [1] or NRG [8], has been shown to be a more effective tumor inhibitor in vivo than equivalent amounts of free MTX. This has been attributed to the slower clearance of macromolecule-linked MTX than of free MTX [1]. In the present study also, MTX linked to NRG or $F(ab)_2$NRG was more effective in tumor inhibition than unlinked MTX. However, MTX-AELG and MTX-$F(ab)_2$AELG were, respectively, more effective in prolonging survival of tumor inoculated mice than MTX-NRG ($P < 0.001$) and MTX-$F(ab)_2$NRG ($P = 0.01$). It is interesting that MTX-$F(ab)_2$AELG could effectively inhibit tumor in vivo in the absence of Fc-dependent complement or killer cell activation.

The serum $t_{1/2}$ value of ^{131}I-labeled rabbit antitumor IgG preparations in murine neuroblastoma or EL4-lymphoma-bearing mice was found to be 24 ± 2 h [5; unpublished observations], i.e., approximately the same as the $t_{1/2}$ value of MTX-ALEG in the ascites fluid of intraperitoneal EL4 lymphoma-bearing C57BL/6J mice [13]. The serum $t_{1/2}$ value of the $F(ab)_2$ fragments of these antibodies was 8 ± 2 h. The serum $t_{1/2}$ values of a mouse hybridoma-derived antihuman melanoma IgG and its $F(ab)_2$ fragment in human melanoma xenograft-bearing nude BALB/c mice (i.e., an IgG preparation from the same species) were somewhat longer, with 36 h and 14 h, respectively [7]. Thus all these $F(ab)_2$ fragments had much shorter serum $t_{1/2}$ values than their parent antitumor IgG preparations. The faster clearance of the $F(ab)_2$ fragment from serum and other tissues was able to produce a very high tumor: normal tissue ratio of localization but a much lower percentage of the administered dose of $F(ab)_2$ than of the parent antitumor IgG actually localized in the tumor [5, 7]. The lower antitumor effect found in this study for MTX-$F(ab)_2$AELG than for MTX-AELG may thus be due to a decrease in the net amount of tumor localization of MTX linked to $F(ab)_2$. The lower systemic toxicity of MTX-$F(ab)_2$NRG than of MTX-NRG is also consistent with faster clearance of the $F(ab)_2$ fragment of NRG.

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