Chemotherapy of Murine Ovarian Carcinoma by Methotrexate-Antibody Conjugates[1]

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Antibodies with specificity for an experimental ovarian carcinoma were coupled to methotrexate by two procedures. Water-soluble carbodiimide gave effective coupling, but a large proportion of the conjugate was rendered insoluble, presumably due to alteration or aggregation of the antibodies. A modification of the mixed anhydride procedure gave rise to products which were completely soluble and allowed a high degree of coupling to be achieved. In vivo testing of the conjugates revealed a significant increase in survival time in treated mice when compared to a variety of control groups; these included groups receiving antiserum or drug alone, mixtures of the two, and conjugates of normal γ-globulin with methotrexate. Our results provide added support for the concept that tumor-associated antibodies coupled to cytotoxic agents are more effective than single agents or noncoupled mixtures of agents.

For some time scattered reports have appeared which suggest that drug-antibody conjugates could be useful agents in cancer therapy; these have been adequately reviewed by Rubens[2] and will not be discussed here. The lack of progress leading to clinical application of this concept is probably due to several factors of a pragmatic nature. First, and most formidable, is the problem of producing purified antibodies to the target tumor cell surface which will have minimal affinity for nontarget cells. Next is the question of which drug to choose as ligand and in what proportion it should be conjugated to give maximum results. Finally, efficient and appropriate chemical procedures are needed to couple the two agents so that they will not dissociate enroute and will be effective upon reaching the tumor cell.

Previous studies from one of our laboratories[3,4] have shown that the first goal, namely, production of a tumor associated antiserum, can be attained in an experimental model. The tumor employed is an embryonal ovarian carcinoma which originated in C3HebFej mice and which is propagated by serial intraperitoneal passage of 10^6 cells biweekly. Cells are harvested from ascitic fluid and a partially purified antigen is injected intradermally with complete Freunds adjuvant into rabbits. The procedures used for production and the characteristics of the antiserum are fully described in previous reports.[3,4]

Preliminary experiments were carried out to determine which drug offered the most promise as a ligand for these antibodies using the ovarian model. Early results suggested that methotrexate might be efficacious in this particular model system (unpublished results). Therefore, this report will describe in some detail the preparation and initial testing of methotrexate-antibody conjugates directed against murine ovarian carcinoma. It should be noted that this model shows several features similar to the clinical disease making it a particularly attractive model.[4]

Results and Discussion

Carbodiimide has been widely used as a reagent for conjugating small molecules to proteins and there are several examples in the literature where this procedure was used to couple methotrexate or other folic acid analogues to proteins.[6-10] In our laboratory this reagent has been used extensively to prepare antigens for radioimmunoassays which involve the coupling of low-molecular-weight haptens to antigenic macromolecules.[11] In most instances, this procedure has led to the production of soluble products with adequate numbers of ligands per macromolecule. However, on several occasions, insoluble materials have been obtained.

Table I. Immunofluorescence of Conjugates[a]

Antibody	+
Antibody-MTX	-
Normal γ-globulin-MTX	

[a] Tested against ovarian tumor cells as described in ref 6.

In attempting to couple methotrexate to either our antibodies or normal γ-globulin, we invariably obtained a high proportion of insoluble conjugate. Moreover, the extent of conjugation was less than we estimated to be necessary for effective action. Nevertheless, we did test the mixture of soluble and insoluble products in our tumor model and the results are presented in Figure 1.

Despite the nature of the preparation, a significant increase in survival time was seen with the drug-antibody conjugate when compared to control groups. In addition to a comparison with untreated tumor-bearing controls, a second control group received an equivalent dose of a conjugate which was identical with the test group except that γ-globulin from normal rabbits was used. Thus the only apparent difference between this control group and the test group was the presence of tumor-binding sites in the latter. A third control group received antiserum alone; none of the control groups showed a significant increase in survival time over the untreated group. These results indicate that the drug-antibody conjugate was effective in delaying the lethal effects of the tumor.

Further experiments were carried out to confirm and possibly improve the above results. A more satisfactory coupling procedure was developed by taking advantage of the fact that methotrexate is a substituted glutaric acid derivative. Under appropriate conditions such compounds would be expected to form six-membered anhydrides which in turn should react readily with the ε-amino groups on the γ-globulin molecule. This represents a variation of the well-known mixed anhydride procedure which has been used in similar applications.[12]

It was found that brief warming of methotrexate in acetic anhydride gave rise to a substance which readily reacted with γ-globulins to yield a soluble conjugate. The extent of conjugation, as monitored by using tritium-labeled drug, was found to be almost quantitative in every case. The radioactivity was stable to prolonged dialysis and could be precipitated by ammonium sulfate treatment indicating that covalent linkage was indeed formed. In a control reaction without globulin, the MTX anhydride was not precipitated by 30% ammonium sulfate. The results in Table I show that the conjugate with antibody gave a

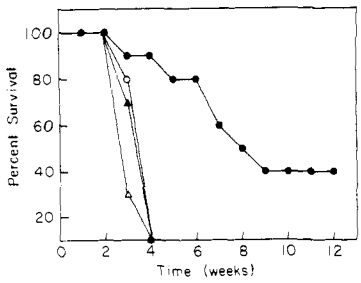

Figure 1. Groups of ten mice received by intraperitoneal route 10^4 tumor cells on day 0. On days 1 and 4 injection of vehicle (▲), antiserum (△), normal γ-globulin–methotrexate conjugate (○), or antibody–methotrexate conjugate (●) was given. The levels of γ-globulin and drug were 200 and 2 mg/kg, respectively, and were comparable.

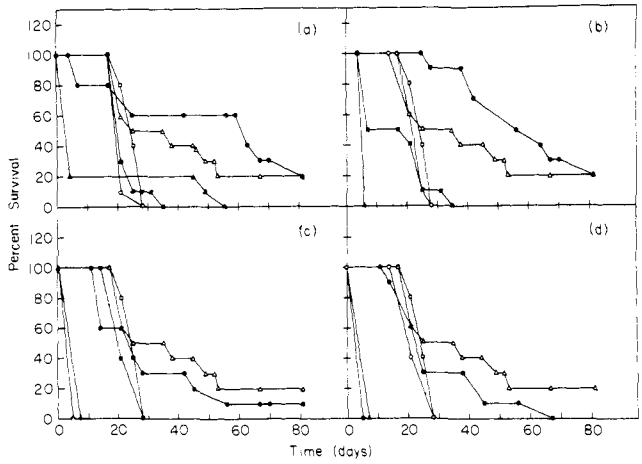

Figure 2. Groups of ten mice received by intraperitoneal injection 10^6 tumor cells on day 0. On days 1 and 2 injections of vehicle (□), antiserum (△), antiserum mixed with drug (▲), drug (■), normal γ-globulin–methotrexate conjugate (○), and antibody–methotrexate conjugate (●) were given. The drug dose levels were (a) 10 mg/kg, (b) 20 mg/kg, (c) 40 mg/kg, and (d) 80 mg/kg while the amounts of γ-globulin were 200 mg/kg in each dose.

positive response in an immunofluorescence test, whereas the normal γ-globulin conjugates did not when assayed against ovarian tumor cells. This indicates that the binding properties of the antibody are retained in the conjugate.

It is possible that the anhydride intermediate is, in fact, a mixed acetic–folic type; however, such a substance would serve the same purpose. We did observe the apparent incorporation of one acetyl group which was still present in the conjugate. This was ascertained by using [^{14}C]acetic anhydride of known specific activity, thereby giving rise to a doubly labeled anhydride. The ^{3}H/^{14}C ratio of the product was consistent with the formation of a monoacetate. The conjugates obtained were tested without further characterization and the results are shown in Table II and Figure 2.

In this experiment once again a pronounced increase in mean survival time was obtained with the drug–antibody conjugate. In terms of methotrexate, four dose levels were studied; the amount of antibody was constant throughout and corresponded to the amount used in all control groups. It is interesting to note that the lower dose levels (10 and 20 mg/kg) gave better results than the higher levels (40 and 80 mg/kg). The explanation for this is not obvious; however, it does point up the existence of optimal drug–antibody ratios.

In addition to the controls used in the previous experiment, two further types of controls were included in the second trial, i.e., tumor-bearing mice receiving drug only and drug mixed with antiserum. In both instances and at every dose level, a decrease in mean survival time was seen which was especially pronounced with the mixtures of drug and antiserum. It appears that these doses of methotrexate are toxic under these conditions, particularly in the 20–80 mg/kg range. The presence of antibodies apparently increases toxicity, an effect which has some precedent in the literature.[13,14]

Table II. Methotrexate–Antibody (MTX–Ab) Treatment of C3H Murine Ovarian Carcinoma[a]

Group no.	Treatment[b]	Dose, mg/kg[c]	Mean survival, days	Survivors/10^d
1	Vehicle		23.6	0
2	Antibody		29.4	2 (81)
3	Ab–MTX conjugate	10	39.4	2 (81)
4	Ab–MTX conjugate	20	50.8	2 (81)
5	Ab–MTX conjugate	40	21.8	1 (81)
6	Ab–MTX conjugate	80	27.9	0
7	NRS–MTX conjugate	10	19.6	0
8	NRS–MTX conjugate	20	21.2	0
9	NRS–MTX conjugate	40	22.3	0
10	NRS–MTX conjugate	80	21.8	0
11	Ab + MTX mixture	10	12.8	0
12	Ab + MTX mixture	20	3.5	0
13	Ab + MTX mixture	40	3.5	0
14	Ab + MTX mixture	80	3.5	0
15	MTX	10	21.2	0
16	MTX	20	21.8	0
17	MTX	40	5.0	0
18	MTX	80	5.0	0
19	Treated MTX[e]	20	21.0	1 (25)

[a] Groups of ten mice received by ip injection 10^6 tumor cells on day 0. [b] Administered ip on days 1 and 2. Conjugates were prepared by the anhydride procedure. NRS refers to normal rabbit serum γ-globulin. Cohn fraction II (Miles Labs). [c] Values represent the amount of MTX per kilogram of body weight; the amount of protein was constant throughout and was 200 mg/kg. [d] Numbers in parentheses refer to the day when the observation was made. [e] This refers to MTX which was allowed to react with acetic anhydride followed by solution in PBS.

In conclusion, we have shown that methotrexate–antibody conjugates are effective in increasing the mean survival time over controls of mice bearing an experimental ovarian carcinoma. The similarity of this experimental model to the clinical disease suggests that this approach may find application in the treatment of human ovarian cancer. The results reported here described effective chemical procedures which could be used toward this goal pending the preparation of human tumor-specific antibodies.

Experimental Section

Drugs and Reagents. Methotrexate was purchased from Nutritional Biochemicals Co., Cleveland, Ohio, and [^{3}H]-methotrexate was obtained from New England Nuclear Corp., Boston, Mass. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (ECDI) came from Sigma Chemical Co., St. Louis, Mo., and normal rabbit γ-globulins (fraction II) from Miles Laboratories, Inc., Elkhart, Ind., were stated to be 95% pure.

Antigens, Antisera, and Therapeutic Trials. The antigens were prepared by chromatography on Sephadex G200 of a soluble extract of tumor cells as described in detail previously.[4] Antisera were obtained and assayed in the same manner as before[3] and the γ-globulin fraction was isolated by precipitation with 30% ammonium sulfate.[5] This fraction was utilized for the coupling procedure. For therapeutic trials, groups of ten C3H mice (Jackson Labs, Bar Harbor, Me.) were used; variations in the published procedure[4] are indicated below.

Coupling Procedures. Carbodiimide Method. Normal rabbit γ-globulin (60 mg) was dissolved in 4 mL of water along

with 6.6 mg of [^{3}H]methotrexate (33 μCi) and adjusted to pH 5.7. ECDI (20 mg) was added with rapid stirring and the reaction allowed to proceed at room temperature for 4 h. The mixture was then dialyzed exhaustively against PBS (phosphate-buffered saline; 0.15 M NaCl, 0.01 M phosphate, pH 8.2). A yellow precipitate which formed during the reaction could not be redissolved. A similar procedure was used for coupling the antibodies to methotrexate.

Mixed Anhydride Procedure. [^{3}H]Methotrexate (28 mg) (10 μCi) was suspended in 2 mL of acetic anhydride and heated at 100 °C for 30 min. The excess anhydride was then removed with a stream of dry nitrogen at 50 °C and the product redissolved immediately in 2 mL of dry dimethylformamide.

A solution of 60 mg of normal rabbit γ-globulin in 10 mL of water at pH 8.5 was treated with appropriate amounts of the above methotrexate "anhydride" at room temperature. After reacting for 18 h the mixture was exhaustively dialyzed against PBS. Tumor-specific anitsera were coupled in the same manner. The molecular ratios of γ-globulin to methotrexate ranged from about 1:15 for lowest dosage (10 mg/kg) to 1:120 for the highest dosage (80 mg/kg). Since there are a limited number of sites on the γ-globulin molecule where coupling could occur, there is some question as to how these high ratios were obtained. One possibility is that the drug underwent self-condensation to give a polymeric methotrexate. Such a derivative would probably be hydrolyzed in vivo to give monomeric drug so that the net effect is a method for coupling high ratios of methotrexate to γ-globulins.

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References and Notes

(1) The abbreviations used are ECDI, 1-ethyl-3-(3-dimethylaminopropy)carbodiimide hydrochloride; PBS, 0.15 M NaCl–0.01 M phosphate, pH 8.2; MTX, methotrexate; Ab, antibody; NRS, normal rabbit serum.
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Synthesis and Central Nervous System Evaluation of Some 5-Alkoxy-3*H*-1,4-benzodiazepin-2(1*H*)-ones

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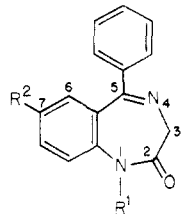
A series of 1-R-5-alkoxy-3*H*-1,4-benzodiazepin-2(1*H*)-ones was prepared and evaluated for central nervous system depressant activity. Several of these compounds, in particular, 7-chloro-5-ethoxy-1-methyl-3*H*-1,4-benzodiazepin-2(1*H*)-one (2), gave a profile and activity level similar to diazepam when measured in mice.

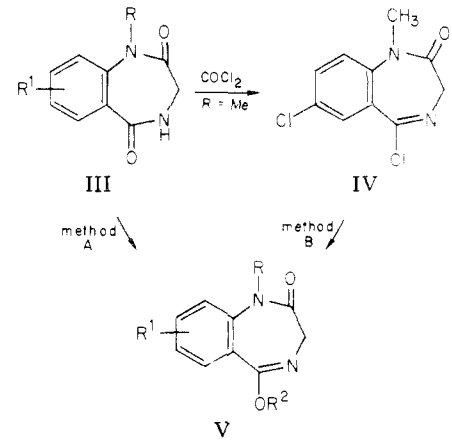
A considerable amount of medicinal chemistry has been carried out on the 1,4-benzodiazepine ring system since the finding that certain 5-aryl derivatives possess useful antianxiety activity in animals and man.[1] Among the more useful compounds developed to date are the 7-chloro-1-methyl (diazepam) and 7-nitro (nitrazepam) derivatives of 5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one (I and II). The present work reports our findings on the central nervous system (CNS) activity of a series of compounds where the phenyl group in I and II has been replaced by an alkoxy or aryloxy group.[2]



I, $R^1 = CH_3$; $R^2 = Cl$
II, $R^1 = H$; $R^2 = NO_2$

Chemistry. The synthesis of the 5-alkoxy- and 5-aryloxy-1-alkyl-3*H*-1,4-benzodiazepin-2(1*H*)-ones (V, Table

Scheme I



IV) was accomplished by the two methods given in Scheme I. Method A consisted of treating a 1-R-3*H*-1,4-benzodiazepine-2,5(1*H*,4*H*)-dione (III) with a trialkyloxonium fluoroborate (Meerwein reagent) in an inert solvent. Method B involved the reaction of 5,7-dichloro-1-methyl-3*H*-1,4-benzodiazepin-2(1*H*)-one[3] (IV) with a so-