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Activation of Methotrexate-α-Alanine by Carboxypeptidase A-Monoclonal Antibody Conjugate†

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ABSTRACT: Carboxypeptidase A (CP-A) and monoclonal antibody KS1/4 directed against an antigen on human lung adenocarcinoma cells (UCLA-P3) were derivatized by treatment with succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate and *N*-succinimidyl 3-(2-pyridyldithio)propionate, respectively. The derivatized proteins were reacted to produce thioether-linked enzyme-antibody conjugates. Sequential HPLC size-exclusion and DEAE chromatography separated the conjugate preparation from unreacted enzyme and antibody. On the basis of SDS-PAGE analysis and measurement of catalytic activity, the preparation contained approximately equal amounts of 1:1 and 2:1 (enzyme:antibody) conjugates; binding activity of the conjugate (1.8×10^5 molecules/cell) was similar to that of unreacted antibody. In vitro cytotoxicity studies with UCLA-P3 cells demonstrated the ability of cell-bound conjugate to convert the prodrug methotrexate-α-alanine (MTX-Ala) to methotrexate (MTX). In the absence of conjugate, ID_{50} values for MTX-Ala and MTX were 8.9×10^{-6} and 5.2×10^{-8} M, respectively. ID_{50} for the prodrug improved to 1.5×10^{-6} M with cells containing bound conjugate. This potentiation of MTX-Ala cytotoxicity by conjugate-bound CP-A, which was at least 30-fold greater than that produced by a comparable amount of free enzyme, is attributed to enhanced effectiveness of MTX generated at the cell surface as opposed to the surrounding medium. Examination of the time course of cytotoxicity over a 96-h period showed that the conjugate-prodrug combination (at 2.5×10^{-6} M) was nearly as effective as MTX in preventing cell replication. These results demonstrate the chemotherapeutic potential of carboxypeptidase-monoclonal antibody conjugates used in conjunction with MTX peptide prodrugs.

Activation of prodrugs by enzyme-monoclonal antibody conjugates, which is a novel and potentially very useful strategy in cancer chemotherapy, provides a mechanism for the *selective* delivery of drugs to tumor sites [reviewed by Bagshawe (1987); Bagshawe, 1989; Senter, 1990]. Methotrexate α-peptides (i.e., derivatives in which amino acids are linked covalently to the α-carboxyl group of MTX[1]) are prodrug forms of the parent antifolate. These compounds are much less toxic to cells than MTX, presumably because of their inability to be internalized by folate transport systems (Sirotnak et al., 1979). MTX peptides can be activated, however, by carboxypeptidase-mediated hydrolysis to yield the parent drug (Kuefner et al., 1989). Regional selectivity of the activation process has been demonstrated in a model system in which L1210 mouse leukemia cells and methotrexate-α-alanine (MTX-Ala) were dispersed in soft agarose and carboxypeptidase A (CP-A) was immobilized on beads in the center of the plate; under these conditions, a zone of cell kill was observed radiating outward from the beads (Vitols et al., 1989).

The present investigation was undertaken to extend the studies with MTX-Ala by determining whether cytotoxic concentrations of MTX could be produced by the amount of CP-A that could be attached to target cells via a monoclonal antibody. For this purpose, CP-A was conjugated to KS1/4, a monoclonal antibody directed toward a cell surface glycoprotein associated with a variety of epithelial carcinomas

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[1] Abbreviations: MTX, methotrexate; MTX-Ala, methotrexate-α-alanine; SMCC, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); CP-A, carboxypeptidase A; PBS, phosphate-buffered saline; DMF, dimethylformamide; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorter; ID_{50}, drug concentration for 50% inhibition of growth; rt, retention time.

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(Varki et al., 1984; Fernsten et al., 1990), and the effectiveness of the conjugate was demonstrated by its ability to enhance the cytotoxicity of MTX-Ala toward human lung adenocarcinoma cells (UCLA-P3).

Experimental Procedures

Materials. The following were obtained from the indicated commercial sources: crystalline bovine pancreas CP-A (type II; 50 units/mg), sulforhodamine B, hippuryl-L-phenylalanine, human plasma fibrinogen (type I), and DTNB (Sigma); goat anti-mouse FITC–IgG conjugate and RPMI 1640 medium (Gibco); fetal bovine serum (Flow/ICN); SMCC (Aldrich); SPDP (Molecular Probes); nylon filters (0.2 μm) (Gelman); prepacked Sephadex G-25 cartridges (PD-10, 9.1-mL bed volume) (Pharmacia LKB); HPLC semipreparative size-exclusion column (Spherogel TSK 3000SW, 21.5 × 300 mm) and HPLC analytical anion-exchange column (DEAE-5PW, 7.5 × 75 mm) (Beckman); ultrafiltration tubes (Centricon 30) (Amicon); $Na^{125}I$ (100 mCi/mL) (Amersham); Iodo-Gen iodination reagent and BCA protein assay reagent (Pierce); precast polyacrylamide gradient gels (3–12%) (iss-enprotech); and high-range SDS–PAGE standards (Bio-Rad). MTX was a gift from Dr. Michael Boyd, National Cancer Institute. Murine monoclonal antibody KS1/4 (95% purity; ca. 10 mg/mL) was obtained from Biotechnetics; SDS–PAGE analysis of the preparations under nonreducing conditions revealed the presence of variable amounts of two components, which may represent protein from two closely related hybridoma clones and/or varying levels of glycosylation. L,L-MTX-Ala was synthesized by the procedure of Kuefner et al. (1990).

Carboxypeptidase A–Monoclonal Antibody (KS1/4) Conjugate. CP-A (20 mg; 0.57 μmol) was dissolved in 1.7 mL of 0.6 M NaCl–0.1 M sodium phosphate buffer, pH 7.0. The solution was passed through a nylon filter to remove a small amount of insoluble material. A 10-fold molar excess (1.82 mg; 5.44 μmol) of SMCC in 70 μL of dry DMF was added, and the mixture was stirred for 1 h at 30 °C. The slightly turbid reaction mixture was centrifuged (10 min; 3100*g*), and the derivatized protein in the supernatant was freed from reagents by two passages through a prepacked column of Sephadex G-25 that had been equilibrated with 25 mL of 0.1 M sodium phosphate buffer, pH 6.0. Yield: 11 mg (58%); 65 units of CP-A/mg; 1.0 mol of maleimide groups/mol of protein.

Monoclonal antibody KS1/4 (8.0 mg) in 1.0 mL of PBS, pH 7.2, was added to 1.5 mL of 0.1 M NaCl–0.1 M sodium phosphate buffer, pH 7.5, and then treated with excess SPDP (167 μg; 533 nmol) in 13.5 μL of ethanol. The solution was stirred gently at room temperature for 30 min, and the buffer was then exchanged for 0.1 M NaCl–0.1 M sodium acetate buffer, pH 4.5, by passage through a prepacked column of Sephadex G-25 that had been equilibrated with the latter buffer. Yield: 7.7 mg in 2.7 mL. Just prior to use in the conjugation reaction (see below), the solution of derivatized antibody was treated with excess DTT (0.77 mg, 5 μmol) in 200 μL of H_2O and stirred gently at room temperature for 30 min. The buffer was exchanged for 0.1 M NaCl–0.1 M sodium phosphate buffer, pH 6.0. Yield: 5.9 mg in 2.6 mL; 33.3 nmol of sulfhydryl groups/mg of protein [5.0 mol/mol of protein, assuming a molecular mass of 150 kDa for the IgG antibody (Parham, 1986)].

For the conjugation reaction, the above solution of derivatized antibody was treated with a 5-fold molar excess (6.8 mg, 196 nmol) of derivatized CP-A in 1.2 mL of 0.1 M sodium phosphate buffer, pH 6.0, and the mixture was stirred gently at room temperature for 18 h. Precipitate was removed by centrifugation (10 min; 3000*g*). Aliquots of the supernatant (1.6 mL; 4.0 mg of protein) were applied with a 2-mL injection loop to an HPLC size-exclusion column that had been equilibrated with 0.1 M NaCl–0.1 M sodium phosphate buffer, pH 7.3. The column was eluted with the same buffer (flow rate, 6 mL/min), and 15-s fractions were collected over a 20-min period; absorbance at 280 nm was monitored continuously. A mixture of unreacted antibody and conjugates eluted first (rt 8.0–12.0 min), followed by CP-A (rt 14.0–18.0 min). Fractions corresponding to rt 9.5–10.5 min were combined and concentrated to 1.2 mL by ultrafiltration. Yield: 4.0 mg. Aliquots (600 μL) of the mixture were applied with a 1-mL injection loop to an HPLC DEAE column that had been equilibrated with 0.1 M NaCl–0.025 M Tris-HCl buffer, pH 7.0. With 0.025 M Tris-HCl buffer, pH 7.0 (pump A) and 0.5 M NaCl–0.025 mM Tris-HCl buffer, pH 7.0 (pump B) as the solvent system, the column was eluted with 20% B for 7.5 min, followed by a linear gradient of 20–100% B over 25.5 min. The flow rate was 1 mL/min, and 30-s fractions were collected over a 40-min period; absorbance at 280 nm was monitored continuously. Unreacted antibody eluted first (rt 2.0–4.5 min), followed by conjugate (rt 15.0–23.0 min). The latter fractions were combined and concentrated to ca. 1.5 mL. Yield: 1.8 mg.

Cells. Human lung adenocarcinoma cell line UCLA-SO-P3 (UCLA-P3) and human melanoma cell line UCLA-SO-M21 (M21) were kindly provided by Dr. D. L. Morton, University of California Los Angeles. UCLA-P3 cells were propagated in RPMI 1640 medium modified to contain 1 μM folate and supplemented with 2 mM glutamine and 5% dialyzed fetal bovine serum; antibiotics were omitted. Twice weekly, cells were released from the plate by trypsinization and subcultured. M21 cells were grown as described previously (Bumol et al., 1982).

Cytotoxicity Measurements. Assays were performed using a 96-well plate microculture technique. Unless otherwise indicated, UCLA-P3 cells were seeded at 20 000 cells per well in 200 μL of the culture medium plus 200 units/mL penicillin and 200 μg/mL streptomycin. After 18 h at 37 °C (for cell attachment), a portion (130 μL) of the culture medium was removed, and (where indicated) cells were incubated with a small volume (5 μL) of conjugate for 1 h at 4 °C. Wells were washed four times with 125-μL portions of sterile culture medium, and the latter was then added to restore the volume to 180 μL. Various amounts of L,L-MTX-Ala or MTX were added (in 20 μL) to obtain the desired final concentrations; saline (20 μL) was added to control cultures. Plates were covered, sealed with semipermeable tape, and incubated at 37 °C under 5% CO_2–95% air. At 48 h, growth was stopped by addition of 50 μL of 50% trichloroacetic acid, and cell protein quantitated by the sulforhodamine B procedure (Skehan et al., 1990) was corrected for the baseline of initial cell inoculum plus growth during the attachment period. Results are expressed as percent growth (100% growth, no drug added). Measurements of the time-dependent growth of cells were conducted in a similar manner, except that growth was stopped at various times and absorbance readings at 570 nm (low cell densities) or 492 nm (high cell densities) were converted to numbers of cells with the use of a standard curve; no corrections were made for baseline growth. To minimize the risk of contamination, separate 96-well plates were used for each incubation period.

Other Methods. CP-A activity was measured spectrophotometrically using the following modification of the procedure

of Folk and Schirmer (1963). The reaction mixture (final volume, 1.0 mL) contained 1.0 mM hippuryl-L-phenylalanine in 0.1 M Tris-HCl buffer–0.2 mM $ZnSO_4$, pH 7.3. The reaction was initiated by addition of CP-A or CP-A-containing conjugate in 5–50 μL of the same buffer, and the absorbance change at 254 nm was monitored with a Gilford spectrophotometer (Model 252) at 37 °C; $\Delta\epsilon_{mM}$ for the reaction is 0.36 (Worthington Enzyme Manual). One unit of activity corresponds to 1 μmol of substrate hydrolyzed/min. Concentrations of CP-A were determined from absorbance at 278 nm [ϵ_{mM} = 64.2 (Bargetzi et al., 1963)]. SDS–PAGE was conducted using precast 3–12% gradient gels under *nonreducing* conditions. Samples (2–5 μg) in 0.0625 M Tris-HCl buffer, pH 6.8, containing 2% SDS, were heated to 100 °C for 5 min and then applied to a 3% stacking gel. Electrophoresis proceeded for ca. 3.5 h at 164 V, using 0.025 M Tris–glycine–0.1% SDS as the running buffer. Proteins were stained with Coomassie brilliant blue. Maleimide groups on SMCC-derivatized CP-A were determined by the following modification of the procedure of Yoshitake et al. (1979). Protein (50 μmol in 680 μL of 0.1 M sodium phosphate buffer, pH 8.0) was incubated at room temperature for 1 h with 20 μL of 10 mM of β-mercaptomethanol; residual sulfhydryl groups were then determined spectrophotometrically by reaction with 300 μL of 0.05 M DTNB [ϵ_{mM} of product = 13.6 at 412 nm (Ellman, 1958)]. Sulfhydryl groups on SPDP-derivatized antibody were determined by the following modification of the procedure of Carlsson et al. (1978). Antibody (2 μmol in 980 μL of 0.1 M acetate buffer, pH 4.5, containing 0.1 M NaCl) was treated with 20 μL of 100 mM DTT, and the pyridine-2-thione released (equal to the sulfhydryl content) was determined spectrophotometrically (ϵ_{mM} = 8.1 at 343 nm). Antigen-binding activity of UCLA-P3 cells was determined by (a) FACS after treatment with conjugate followed by fluorescein-labeled goat anti-mouse IgG or (b) radioactivity after treatment with ^{125}I-labeled conjugate (Mueller et al., 1990); data from the latter determination were analyzed by a Scatchard plot. Concentrations of antibody and conjugates were determined by the BCA microtiter plate colorimetric assay using bovine serum albumin as the standard.

Results

Construction of Enzyme–Monoclonal Antibody Conjugate. As described under Experimental Procedures, CP-A was derivatized by treatment with SMCC, a bifunctional agent containing a maleimide group and an *N*-hydroxysuccinimide ester for attachment to the protein (Yoshitake et al., 1979). The reaction was conducted at 30 °C and in the presence of high salt concentration (0.6 M NaCl) to prevent precipitation of the enzyme. When analyzed by SDS–PAGE, the derivatized enzyme migrated as a single band (molecular mass, 35 kDa) that was indistinguishable from the native protein (data not shown). Even though a 10-fold molar excess SMCC was used, an average of only one maleimide group was incorporated into the enzyme. This result is not unexpected, since derivatization at pH 7.0 favors attachment to the single N-terminal amino acid of CP-A rather than any of the lysine residues (Rana & Meares, 1990). The specific activity of the derivatized protein (65 units/mg of protein), after purification, was higher than that of the commercial starting material (ca. 50 units/mg). Monoclonal antibody KS1/4, an IgG2a isotope (Fernsten et al., 1990), was derivatized with SPDP. This linker also utilizes an *N*-hydroxysuccinimide ester for attachment to the protein, and it contains a disulfide group which, just prior to use in the conjugation reaction, was treated with DTT to generate a thiol group (Carlsson et al., 1978). When analyzed by SDS–PAGE, the derivatized antibody was indistinguishable from the native protein (data not shown). An average of five thiol groups was introduced per molecule of antibody. Reaction of the two derivatized proteins allowed thiol addition to the maleimide double bond to form a stable thioether linkage in the conjugate.

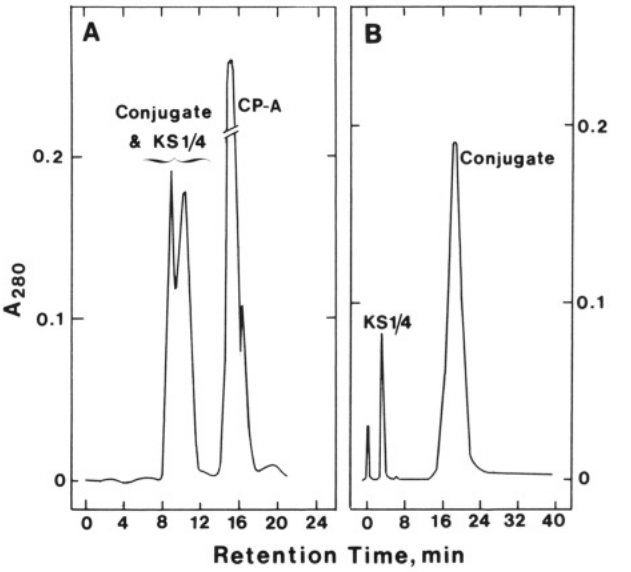

FIGURE 1: Purification of conjugate preparation by sequential HPLC. Panel A: Size-exclusion chromatography. Panel B: DEAE chromatography. For details, see Experimental Procedures. Eluants were monitored continuously for absorbance at 280 nm, and 15-s (A) or 30-s (B) fractions were collected. A_{280} values are plotted as a function of retention time.

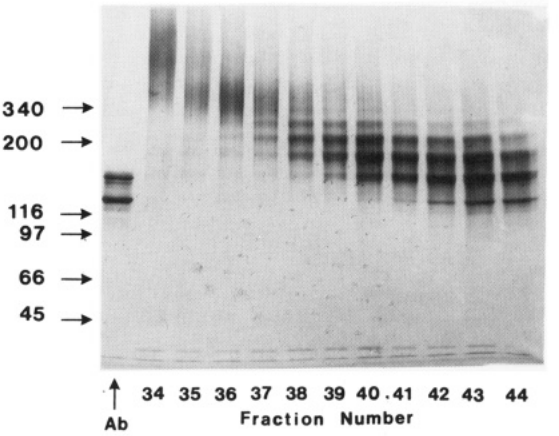

FIGURE 2: SDS–PAGE analysis of conjugate–antibody fractions from size-exclusion column (Figure 1A). Fractions were concentrated by ultrafiltration, and 5-μg aliquots were applied to an SDS–PAGE gradient gel. Left lane: Monoclonal antibody KS1/4 (Ab). Molecular masses (in kilodaltons) of standard proteins (fibrinogen, 340; myosin, 200; β-galactosidase, 116; phosphorylase B, 97; bovine serum albumin, 66; and ovalbumin, 45) are indicated by arrows.

Purification of the conjugate was accomplished by two chromatographic steps. An HPLC size-exclusion column was used first to separate free CP-A (rt 14.0–18.0 min) from unreacted antibody and conjugate; the latter proteins eluted as an incompletely resolved doublet (rt 8.0–12.0 min) (Figure 1A). To obtain further information about the composition of the doublet, fractions from rt 8.5 to 11.0 min were analyzed by SDS–PAGE (Figure 2). Fractions 34–36 (rt 8.5–9.0 min) contained poorly defined, high molecular weight material, but subsequent fractions (37–44; rt 9.25–11.0 min) exhibited a series of well-resolved components with progressively decreasing molecular weight. Accordingly, fractions 43–44

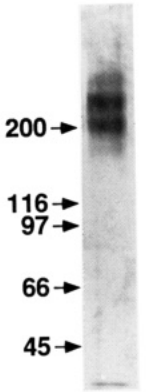

FIGURE 3: SDS–PAGE analysis of purified conjugate preparation.

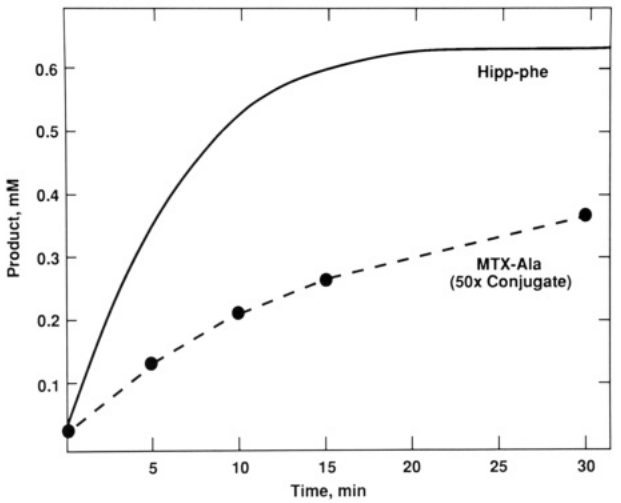

FIGURE 4: Catalytic activity of conjugate-bound CP-A. Upper curve: Hippuryl-L-phenylalanine (Hipp-phe) (1.0 mM) as substrate; conjugate, 0.0046 μg/μL; continuous spectrophotometric assay as described under Experimental Procedures. Lower curve: L,L-MTX-Ala as substrate; conjugate, 0.23 μg/μL; HPLC assay (at the indicated times) as described previously (Kuefner et al., 1989) except that substrate concentration was increased to 1.0 mM and volume of the reaction mixture was decreased to 40 μL.

(containing substantial amounts of unreacted antibody) and 34–37 (high molecular weight material) were discarded; the remaining fractions (38–42; rt 9.5–10.5 min) were combined and chromatographed on an HPLC DEAE column (Figure 1B). Antibody eluted first, followed by a well-separated, symmetrical peak of the more anionic conjugate.

Characterization of Conjugate. SDS–PAGE analysis of the conjugate preparation (Figure 3) revealed the presence of approximately equal amounts of two major components (molecular mass > 200 kDa), along with small amounts of higher and lower molecular weight proteins; unreacted antibody and CP-A were absent. Catalytic activity of the conjugate preparation was measured using hippuryl-L-phenylalanine, a standard substrate for CP-A (Figure 4). From the initial rate of the reaction, the specific activity of conjugate-bound CP-A was calculated to be 18 units/mg of protein. This value was used to estimate the enzyme:antibody ratio. Assuming no change in activity when derivatized enzyme (35 kDa; sp act. = 65 units/mg) is linked to antibody (150 kDa), conjugates ranging from 1:1 to 4:1 in composition should have specific activities of 12, 20, 26, and 31 units/mg, respectively. Thus, the catalytic activity indicated that the preparation was a mixture of 1:1 and 2:1 conjugates, and from SDS–PAGE analysis (Figure 3) these components were present in approximately equal amounts. When the conjugate preparation

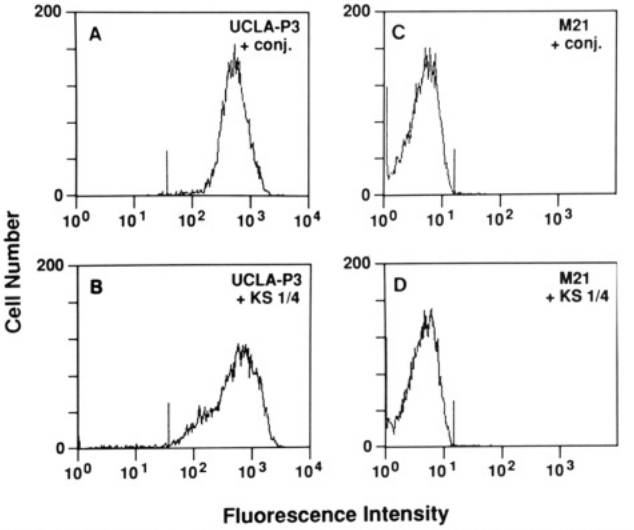

FIGURE 5: FACS analysis of conjugate (CP-A–KS1/4) and antibody (KS1/4) binding to UCLA-P3 cells. Flow cytometric analysis of UCLA-P3 and M21 cells after treatment with conjugate (KS1/4–CP-A) or KS1/4 and goat anti-mouse FITC–IgG as secondary antibody.

was tested with MTX-Ala as the substrate (Figure 4), the specific activity was ca. 1% of that observed with hippuryl-L-phenylalanine; a similar ratio of specific activities was obtained when free CP-A was used to hydrolyze these substrates (Kuefner et al., 1989). Despite its relatively slow hydrolysis by CP-A, the prodrug is able to generate cytotoxic concentrations of MTX in long-term experiments as shown by previous work (Kuefner et al., 1989; Vitols et al., 1989) and by studies in this report (see below).

Antigen-binding activity was retained in the conjugate. When UCLA-P3 cells were exposed to excess conjugate, washed, and then treated with FITC-linked goat anti-mouse IgG, all cells displayed a bright surface fluorescence (data not shown). For quantitative assessment of antigen-binding activity, cells were processed as described above and analyzed with a FACS (Figure 5). Cells treated with conjugate (panel A) were highly fluorescent (10^2–10^3 intensity units), and the distribution curve was narrow and symmetrical. With antibody, however, the distribution curve was skewed toward decreased labeling (panel B). This may be due to heterogeneity in the antibody preparation. When an irrelevant melanoma cell line (M21) devoid of the KS1/4 antigen was tested under the same conditions, binding of both conjugate and antibody was reduced by about 2 orders of magnitude (panels C and D). Conjugate or antibody labeled with ^{125}I was used to determine that UCLA-P3 cells had the capacity to bind 1.8×10^5 molecules of these proteins per cell (data not shown).

Conjugate preparations were stable for at least 6 months when stored at 4 °C. When tested under cell culture conditions (incubation with UCLA-P3 cells, fetal bovine serum, and RPMI 1640 at 37 °C), enzyme activity declined at a rate of approximately 15%/24 h (data not shown).

Cytotoxicity of Conjugate. To evaluate the effectiveness of the conjugate in enhancing the cytotoxicity of the prodrug MTX-Ala, UCLA-P3 cells were exposed to excess conjugate, washed thoroughly, and then treated with varying concentrations of MTX-Ala. As controls, cells that had not been exposed to conjugate were treated with MTX-Ala (or MTX). MTX-Ala was also tested in the presence of 1 milliunit of free CP-A. As shown in Figure 6, ID_{50} for MTX-Ala in the absence of conjugate (8.9×10^{-6} M) was considerably higher than that of the parent drug MTX ($ID_{50} = 5.2 \times 10^{-8}$ M).

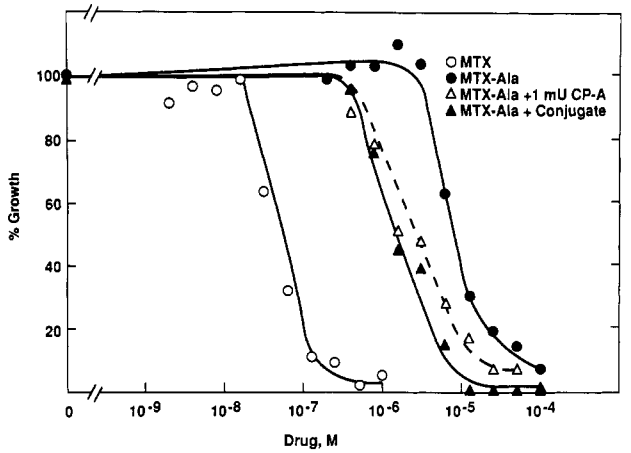

FIGURE 6: Conjugate-mediated enhancement of MTX-Ala cytotoxicity. Cytotoxicity measurements were conducted as described under Experimental Procedures. After being plated, cells were treated with enzyme–antibody conjugate (5 μL; 2.74 milliunits of CP-A/μL), washed, and then exposed to various concentrations of MTX-Ala (▲). Controls: Cells not treated with conjugate but exposed to MTX (○), L,L-MTX-Ala (●), or L,L-MTX-Ala plus 1 milliunit of free CP-A (△).

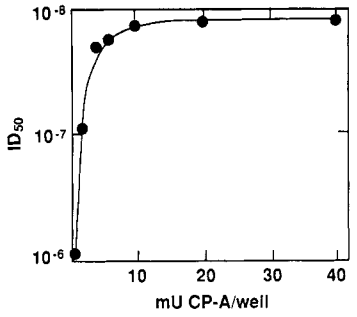

FIGURE 7: Cytotoxicity of MTX-Ala in presence of increasing amounts of CP-A. Cytotoxicity measurements were conducted as in Figure 6, except that cells were seeded at an initial density of 1000/well (ID_{50} values are unchanged over a range of cell densities from 1000 to 20000). After 4 h for attachment, indicated concentrations of L,L-MTX-Ala were added. Amounts of free CP-A: 0, 2, 4, 6, 10, 20, and 40 milliunits. Growth was stopped after 120 h. ID_{50} values are plotted as a function of amount of CP-A.

The ID_{50} decreased approximately 6-fold (1.5×10^{-6} M) when the prodrug was used with conjugate-treated cells. Treatment of cells with conjugate in the absence of MTX-Ala produced no inhibition of growth (data not shown). The enhanced cytotoxicity of MTX-Ala in the presence of conjugate attached to cells was slightly greater than that produced by 1 milliunit of free CP-A ($ID_{50} = 2.7 \times 10^{-6}$ M). In view of the importance of this observation (see Discussion), a more detailed study was made of the concentration dependence of *free* CP-A upon the cytotoxicity of MTX-Ala. As shown in Figure 7, increasing levels of the enzyme produced a progressive increase in the toxicity of MTX-Ala, plateauing at a value ($ID_{50} = 2.4 \times 10^{-8}$ M) comparable to that of MTX. Maximal and half-maximal effects were achieved with ca. 10 and 2 milliunits of enzyme, respectively. Two control experiments (data not shown) demonstrated that the results in Figure 6 were not due to adventitious adsorption of conjugate in the wells: (a) Empty wells treated with conjugate followed by FITC-linked goat anti-mouse IgG showed no fluorescence; and (b) cells exposed to conjugate and washed prior to plating had the same sensitivity to MTX-Ala as those treated with conjugate after attachment in the wells.

Inspection of the data in Figure 6 revealed that the maximal effect of the conjugate–prodrug combination could be achieved when the latter was used at concentrations between 10^{-6} and 10^{-5} M. Under these conditions, MTX-Ala alone had essentially no toxicity, but appreciable growth inhibition was observed with conjugate-treated cells. Accordingly, cytotoxicity was examined as a function of time, using both the prodrug and MTX at a concentration of 2.5×10^{-6} M (Figure 8). Over a 96-h period, control cells (no drug, no conjugate) grew in a typical S-shaped curve plateauing near the end of the period. Cells treated with MTX-Ala alone showed slightly better growth, although the basis for this stimulation has not been determined. Most important, however, cells that had been pretreated with conjugate showed a marked reduction in growth in the presence of MTX-Ala, approaching that obtained by MTX.

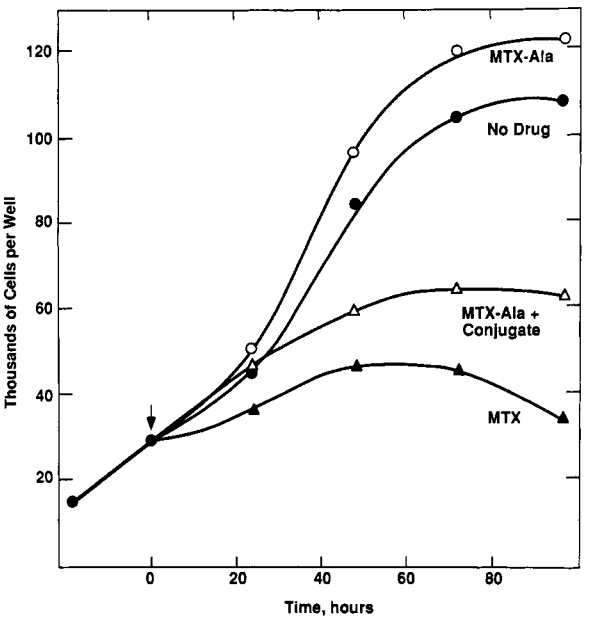

FIGURE 8: Time dependence of MTX-Ala cytotoxicity against cells pretreated with conjugate. Experimental conditions were as in Figure 6 except that cells were seeded at an initial density of 15000 cells/well, activity of the conjugate was 4.1 milliunits/μL, MTX-Ala and MTX were added at concentrations of 2.5×10^{-6} M, and growth was stopped at the indicated times. Absorbance readings at 492 nm were converted to numbers of cells with the use of a standard curve.

Discussion

Conjugates prepared by covalent attachment of drugs to monoclonal antibodies, which in turn are targeted to tumors, have been used extensively in cancer chemotherapy [reviewed by Reisfeld (1989)]. Replacement of bound drugs with enzymes capable of converting inactive derivatives, prodrugs, to their active forms is a modification of this strategy designed to increase the number of drug molecules delivered to the tumor site by each antibody. The feasibility of the enzyme–monoclonal–prodrug approach has been demonstrated, for example, by Bagshawe et al. (1988), who utilized a conjugate of carboxypeptidase G_2 linked to a monoclonal antibody directed against human chorionic gonadotropin to generate the alkylating agent *p*-[*N*,*N*-bis(2-chloroethyl)amino]benzoate from the benzoyl glutamate derivative. The conjugate–prodrug combination showed good cytotoxicity against choriocarcinoma cells in vitro and choriocarcinoma xenografts in nude mice. Similarly, Senter et al. (1988, 1989) coupled alkaline phosphatase to antibodies and found that lung and colon carcinomas pretreated with these conjugates in vitro or as xenografts were highly sensitive to subsequent treatment with phosphorylated derivatives of etoposide, mitomycin alcohol, or doxorubicin. Methotrexate α-peptides can also be utilized as prodrugs. These compounds are relatively nontoxic, pre-

sumably because of their inability to be internalized by folate transport systems (Sirotnak et al., 1979), but they can be hydrolyzed by carboxypeptidases to yield the parent drug (Kuefner et al., 1989). The present investigation demonstrates the ability of the prodrug MTX-Ala, used in conjunction with a CP-A–monoclonal antibody conjugate, to inhibit the in vitro replication of human lung adenocarcinoma cells.

The extensive use of alkaline phosphatase– or peroxidase–antibody conjugates for ELISA determinations and immunocytochemical staining has led to the development of efficient methods for constructing enzyme–antibody conjugates. Although bifunctional agents can be used to join the two proteins, a preferable procedure is to derivatize each protein with "linkers" that can react subsequently to form a stable covalent bond. Accordingly, CP-A was derivatized with SMCC to introduce a single maleimide group, and SPDP was used to thiolate the antibody; in the latter reaction, the protein contained an average of five thiol groups per molecule. Both reactions, which involved use of *N*-hydroxysuccinimide esters of the linkers, were conducted at neutral pH to favor derivatization of the N-terminus of CP-A and the four N-termini of KS1/4 (Rana & Meares, 1990), thereby minimizing the possibility of compromising enzyme activity or antigen-binding activity. Both derivatives were prepared in good yield (ca. 75%). Interaction of the derivatized proteins produced a thioether-linked conjugate. Purification of the conjugate involved passage of the preparation through an HPLC size-exclusion column to remove unreacted CP-A (Figure 1A), followed by chromatography of selected fractions (Figure 2) of the antibody–conjugate mixture on an HPLC DEAE column to provide an antibody-free conjugate preparation (Figure 1B). The overall yield of product was low (ca. 10–15% in various preparations), due largely to the tendency of the proteins to precipitate in the conjugation reaction. Although the two HPLC steps were quite efficient, giving good separation with minimal dilution in a short time (30–60 min), the semipreparative size-exclusion and analytical DEAE columns were able to process only small quantities of conjugate (ca. 10 and 2 mg, respectively). For in vivo evaluation of the conjugate, a scaled-up procedure will be used.

SDS–PAGE analysis of the conjugate preparation (Figure 3) revealed two major components (molecular mass > 200 kDa). Estimates of the enzyme:antibody ratio from the apparent molecular weights of these components were rendered difficult by the heterogeneity of the antibody (Figure 2), the atypical behavior of glycoproteins on SDS–PAGE, and the progressively compressed migration of proteins with molecular masses greater than 200 kDa. Enzymatic activity, however, provided an independent and more reliable parameter for determining the stoichiometry of conjugate preparations. Utilizing values of 35 and 150 kDa for the molecular masses of linker-derivatized CP-A and KS1/4, respectively, and 65 units/mg for the specific activity of the enzyme, the data in Figure 4 indicated that the preparation assayed had an average composition of 1.5 CP-A molecules per antibody molecule. It seems likely, therefore, that the two principal bands in the SDS–PAGE pattern (Figure 3) correspond to the 1:1 and 2:1 (enzyme:antibody) conjugates. Although it was considered desirable a priori to maximize the number of CP-A molecules per antibody, catalytic activity of the enzyme decreased in conjugates when four or more CP-A molecules were present per antibody, and in vivo biodistribution studies indicated that the larger conjugates did not target as well to the tumor and were more readily destroyed and/or excreted (unpublished observations).

The chemotherapeutic effectiveness of the conjugate in mediating the cytotoxicity of MTX-Ala was demonstrated using a human lung adenocarcinoma cell line (UCLA-P3). To aid in interpreting the results, the concentration dependence of *free* CP-A on this process was determined (Figure 7). Increasing levels of enzyme produced a progressive shift of the ID_{50} of MTX-Ala (1.4×10^{-6} M) to a plateau value (2.4×10^{-8} M) comparable to that of MTX. Maximal and half-maximal effects were observed with 10 and 2 milliunits of enzyme, respectively. When the prodrug was used with cells that had been exposed to conjugate, and excess conjugate was removed by extensive washing of the cells, the ID_{50} for MTX-Ala improved approximately 6-fold and was slightly better than that achieved by 1 milliunit of free CP-A (Figure 6). Calculations based upon the number of antigen-binding sites per cell (1.8×10^5), however, indicated that only about 0.03 milliunit of enzyme would be present on cells saturated with conjugate. Thus, it appears that MTX generated at the cell surface, adjacent to transporters for its internalization, is more efficient than drug generated in the surrounding medium. Some confirmation of this hypothesis was obtained by an experiment in which cells exposed to agarose beads containing CP-A (which sink to the bottom of the wells and are in contact with the cell monolayer) produced more cytotoxicity than an equivalent amount of free enzyme (data not shown). Increased effectiveness of drug generated at a cell surface may be one of the advantages to regimens involving enzyme–monoclonal antibody–prodrug combinations.

The basis for the cytotoxicity of MTX-Ala, in the absence of conjugate, is not yet understood. It is not due to serum-mediated hydrolysis of the peptide, since a similar effect was observed when cells were propagated in serum-free medium (unpublished data). Other possible explanations include the following: (a) Existence of an exofacial peptidase on the cells; (b) contamination of MTX-Ala by a small amount of MTX or chemical hydrolysis of the peptide during long-term cytotoxicity experiments; (c) slow uptake of MTX-Ala by cells and subsequent inhibition of dihydrofolate reductase by the prodrug; or (d) inhibition of folate uptake by MTX-Ala leading to folate deprivation. Experiments to test these hypotheses are currently in progress.

The optimum window between cytotoxicity of MTX-Ala in the presence and absence of conjugate occurred at a concentration of about 2.5×10^{-6} M (Figure 6). Using MTX-Ala and MTX at this concentration over an extended time period (96 h), the conjugate–prodrug combination produced a cytotoxicity approaching that of MTX (Figure 8). The inability of MTX-Ala to achieve an ID_{50} identical to that of MTX is not a fatal flaw in the conjugate–prodrug strategy, since only a very small concentration of free MTX (ca. 2×10^{-9} M) is required as a supplement to obtain complete cell kill (unpublished observation). Indeed, prodrug–conjugate regimens will probably be used to decrease the tumor burden while sparing normal cells, followed by sublethal dosages of parent drugs to complete the eradication process.

This investigation has demonstrated the feasibility of using CP-A–KS1/4 monoclonal antibody conjugates in conjunction with MTX-Ala for killing UCLA-P3 human lung adenocarcinoma cells in vitro. The possibility of using this regimen in vivo will be investigated using immunodeficient mice in which the tumor can be propagated. It is likely that carboxypeptidases can be employed to activate the α-peptides of other folate antagonists in current use (e.g., 10-propargyl-5,8-dideazatetrahydrofolate, 10-deazaaminopterin, or 5,8-dideazatetrahydrofolate). Improvements in the substrates (e.g., re-

placement of Ala by other amino acids) and the use of other carboxypeptidases, enzyme–antibody conjugates directed toward more abundant antigens on tumor cells, and combinations of conjugates should enhance the potential of MTX peptides as cancer chemotherapeutic agents.

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