Radioimmune Assay and Characteristics of Antibodies to Macromomycin (NSC 170105)

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SUMMARY

A radioimmune assay for the antitumor agent, macromomycin, using purified, radiolabeled macromomycin and antisera raised in rabbits against a carbodiimide-catalyzed macromycin-Limulus polyphemus hemocyanin complex has been developed. Radiolabeled macromomycin was prepared by direct iodination of the polypeptide antibiotic with the use of iodine monochloride or solid-state lactoperoxidase. Antibody-bound drug was isolated from free macromycin with dextran-coated, activated charcoal. The standard curve of the sequential saturation assay was linear on a logit-log plot and indicated a lower limit of sensitivity of approximately 100 pg macromomycin. The radioimmune assay was suitable for measuring macromomycin in the presence of other antitumor drugs, and detection of macromomycin was quantitative when it was added to normal human serum or urine. Drug binding to melanoma and mammary carcinoma cell surfaces could be inhibited by preincubating macromomycin with affinity-purified antiamacromomycin antibodies. However, once the drug was bound to cell surfaces, addition of antiamacromomycin antibodies did not result in removal of the drug from cell surfaces or in reversal of macromomycin-induced inhibition of thymidine incorporation into cellular DNA. Antiamacromycin antibodies and the radioimmune assay should provide useful tools for developing pharmacokinetic and toxicity studies of macromomycin, as well as for analyzing the mechanism(s) of action of the drug.

INTRODUCTION

Macromomycin (NSC 170105), a polypeptide antibiotic isolated from Streptomyces macromyces, is a surface-adsorbing antitumor agent that is active in vivo against L1210 and P388 leukemias, B16 melanoma, and the Lewis lung carcinoma. Although a number of antitumor antibiotics have been isolated, their modes of action have not been studied in any detail. The study of neocamzinostatin is one of the few exceptions. Among the 50 or more drugs currently in clinical use, none is known to exert its effects at the cell surface. MCR is unique in that it apparently inhibits DNA synthesis while binding to the plasma membrane (12, 13). This mechanism of action requires that the binding of MCR to the cell surface freezes or modifies the role of the membrane in mediating cellular metabolism. Kunimoto et al. (13) hypothesize that the interaction between MCR and tumor cells is analogous to the interactions between bacteriocins and bacterial cells.

Since MCR may serve as a valuable tool for elucidating membrane function and because the drug is active against the experimental model tumors that have some, although restricted, predictive value for clinical chemotherapy, development of a sensitive, specific, and rapid method for estimating MCR concentrations in biological materials was warranted. This report describes the development of a radioimmune assay for MCR and use of the assay, as well as purified antiamacromomycin antibodies, in the detection of MCR in body fluids and on tumor cell surfaces.

MATERIALS AND METHODS

Purification and Radiolabeling of MCR

MCR was purified by sequential salt precipitation, ultrafiltration, and ion-exchange and molecular sieve chromatography and stabilized during lyophilization in Lac-PBS as described by Yamashita et al. (34), with the following modifications. DEAE chromatography was performed using DEAE-Sepharose 6B and was followed by a single Sephadex G-50 chromatography in Lac-PBS. Rechromatography on Sephadex G-50 was not necessary, since the Sepharose ion-exchange column also served as a molecular sieve. During purification, fractions were monitored by an agar plate bactericidal technique with Bacillus pumilus as the test organism. This method is sensitive to 0.5 μg MCR. Polyacrylamide slab gel electrophoresis of 300 μg MCR in sodium dodecyl sulfate according to the method of Laemmli (14) resulted in a single band that migrated between RNase (13,700 daltons) and lysozyme (11,400 daltons) standards on 12.5% gels. Since this technique is sensitive to about 10 μg protein, MCR was judged >97% pure. By this criterion and based on the report by Yamashita et al. (34), MCR was assumed to have a molecular weight of 12,500. Drug concentrations were determined spectrophotometrically using a 1% absorbance value of 11.6 at 280 nm. MCR bactericidal activity was purified at least 25-fold over the 30% pure form supplied by Bristol Laboratories, Inc., Syracuse, N. Y. Purified MCR was stored lyophilized in Lac-PBS at −70° with no apparent loss of activity.

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MCR was radiiodinated using cellulose-immobilized lactoperoxidase as previously described in detail (32). γ-Radiation was monitored using a Beckman GM 100-γ counter equipped with a data computer. The specific activity of 125I-labeled MCR was 4 ± 0.5 × 10^6 cpm/mg. The drug could also be radiolabeled using the iodine monochloride method as described by Kunimoto et al. (13), and in neither case was there an apparent loss of bactericidal activity.

**Characterization of MCR**

**Binding of Radiiodinated MCR to Tumor Cells.** To test the activity of the purified MCR in mammalian cell systems, subconfluent monolayer cultures of tumor cells on 60-mm tissue culture dishes were rinsed with PBS and incubated with various concentrations of 125I-labeled MCR for appropriate periods at 37° or at 4°. The cells were then rinsed 3 to 4 times with cold PBS until the final rinse supernatant contained less than 0.1% of the originally added radiolabel. Plates were treated with 0.01 M EDTA in PBS for 10 min, and resulting cell suspensions were monitored for total radioiodine and cell content.

**Effects of MCR Binding on Tumor Cell Macromolecular Biosynthesis.** Subconfluent cultures of tumor cells on 60-mm dishes were preincubated for 2 hr in leucine-free medium prior to incubation with 0.17 μCi/ml [3H]thymidine, 1.0 μCi/ml, and 3.23 μCi/ml [14C]leucine, 1.0 μCi/ml, in leucine-free medium (total volume, 2.0 ml). The uptake of these radioactive precursors into acid-insoluble cellular material over the next 4 hr was determined by a filter disc assay. Briefly, cells were rinsed 3 times with cold PBS and removed from tissue culture dishes with EDTA. After the cells were rinsed twice with PBS, pellets were resuspended to 1.0 ml in PBS, and duplicate 0.1-ml aliquots were applied to 3-cm Whatman No. 3MM filter paper. Discs were then dried under warm air, soaked for 24 hr in cold 10% trichloroacetic acid containing 0.2% L-leucine and 0.2% thymidine, rinsed in 2 changes of 5% trichloroacetic acid containing 0.1% metabolites for 60 min each at 4°, and fixed by incubation in 2 changes of 95% ethanol for 30 min each at 4°. Filter papers were dried under a heat lamp and monitored for 3H and 14C radioactivities in tolune-0.6% PPO scintillation cocktail with a Beckman LS300 liquid scintillation spectrophotometer.

Total cellular protein and DNA were determined using Coomassie blue (2, 29) and diphenylamine (25) reactions on cell aliquots. Both techniques were modified to microassays. For total cell protein, triplicate 0.1-ml cell aliquots were solubilized by adding 50 μl distilled water and 100 μl 1.6% perchloric acid in 0.1% Triton X-100. After incubation at 100° for 10 min, samples were cooled, and 0.5 ml Coomassie reagent (0.06% Coomassie G250 in 1.6% perchloric acid) was added. Absorbance at 620 nm was monitored between 5 and 15 min later using a Beckman DB spectrophotometer. Bovine serum albumin served as the standard for this simple and sensitive protein microassay. The Coomassie reagent is 5- to 10-fold more sensitive than the Folin phenol reagent (20) and has a working range as described here of 1.0 to 50.0 μg protein. For total cell DNA determination, triplicate 0.2-ml cell aliquots were dissolved in 0.3 ml 3.0 N perchloric acid at 100° for 10 min. After incubation, 0.5 ml diphenylamine reagent (4.0 g diphenylamine and 10.0 mg paraldehyde in 100 ml glacial acetic acid, prepared fresh weekly and stored at room temperature protected from the light) was added. The color was allowed to develop for 18 to 24 hr at room temperature, and absorbance was monitored at 600 nm. Salmon testis DNA (0.2 mg/ml solubilized at 100 in 1.5 N perchloric acid) served as the DNA standard, giving this microassay a sensitivity range of 2.5 to 50.0 μg DNA.

**Immunogen Preparation and Immunization**

MCR (5.0 mg/ml) containing 1.0 μg 125I-labeled MCR per ml was mixed with 10.0 mg L. polyphemus hemocyanin per ml at pH 5.5. The carbodiimide, 1-ethyl-3,3-dimethylpropylcarbodiimide HCl, was added to a final concentration of 5.0 mg/ml, and the mixture was stirred at 4° for 90 min. The reaction was terminated by adding 0.1 volume of 0.1 N ethanolamine, and the mixture was passed immediately over a Sephadex G-50 molecular sieve column. On the basis of specific activity, the MCR-carrier conjugate consisted of 4 moles MCR per mole hemocyanin.

New Zealand White rabbits were vaccinated by combined footpad and intrascapular injections of 5 mg immunogen emulsified in complete Freund's adjuvant on Days 1, 14, and 28. Bleedings and booster vaccinations (intrascapular only) followed at biweekly intervals thereafter. Antisera were pooled, inactivated at 56° for 30 min, and stored at −70° until used.

**Antibody Purification and Characterization**

Antibodies were isolated from antisera with MCR immobiolized on bromoacetyl-derivatized cellulose prepared as described by Robbins et al. (26). The resulting immunoabsorbent consisted of 22 mg MCR per ml (packed volume) adsorbent. Antisera (50 ml) was incubated with 5.0 ml (packed volume) immunoabsorbent at 37° for 60 min. After centrifugation, the immunoabsorbent was rinsed with PBS until the absorbance of the rinse supernatant was <0.05, at which point antibodies were eluted with 2 changes of 10.0 ml freshly prepared 2.5 M KI at room temperature for 30 min each (9). Resulting supernatants were dialyzed exhaustively against PBS at 4°. Antibody concentrations were determined spectrophotometrically with a 1% absorbance value of 15.5 at 278 nm (33).

Further treatment of adsorbents with 10% dioxane in 1.0 N acetic acid did not result in measurable protein elution, indicating quantitative removal of adsorbed antibodies during chaotropic salt treatments. The immunoabsorbent could be used repeatedly with no loss of binding capacity, and a 2nd adsorption of once-adsorbed antiserum resulted in no additional antibody recovery. Purified antibodies were 95 to 98% reabsorbed to the affinity matrix. By these criteria, antibody concentration in the pooled rabbit anti-MCR antisera was 452 μg/ml.

Purified antibodies were evaluated for immunoglobulin class and degree of purity with the use of polyacrylamide slab gel electrophoresis in sodium dodecyl sulfate without mercaptoethanol and by conventional immunoelectrophoresis (31). On the basis of these criteria, purified anti-MCR antibodies were of the IgG class with less than 1% detectable IgM.
Radioimmune Assay

The radioimmune assay was performed with whole antiserum and with purified anti-MCR antibodies. A competitive binding assay involving sequential saturation of antibody active sites by unlabelled MCR, and 125I-labeled MCR was used to evaluate drug concentrations in buffer, serum, or urine. Antibody-bound drug was isolated by adsorption of free (unbound) MCR on dextran-coated charcoal. The assay was performed using the following protocol in the order given: 100 μl sample or standard, 100 μl antiserum (1:500 dilution) or purified antibody (10 μg/ml), and 100 μl diluent (2.5% bovine serum albumin in 1% dextran T-40) were mixed thoroughly and incubated for 30 min at 37°. After incubation, 100 μl 125I-labeled MCR (diluted to approximately 400 cpm, generally about 1.0 μg drug) were added, and the mixture was incubated for an additional 30 min at 37° and 30 min at 4°. After the final incubation, 100 μl 2.5% Norit A (prepared by mixing with 0.5% dextran T-40, rinsing by vacuum filtration, and resuspending to 2.5% in PBS) were added for 10 min at 4°. The mixtures were centrifuged at 10,000 rpm for 5 min, and supernatants were monitored for antibody-bound radiolabeled MCR. Controls consisted of preimmunization serum, normal rabbit IgG, and buffer alone.

Effects of Anti-MCR Antibody Binding on MCR Activity

For determination of whether cell surface-adsorbed MCR could be detected with anti-MCR antibodies, unlabelled MCR was added for 15 min to triplicate tissue culture dishes containing subconfluent populations of tumor cells. Normal rabbit IgG (5.0 mg/ml) was added to quench nonspecific surface binding of antibodies. After 15 min, plates were rinsed 3 times with cold PBS containing Mg2+ and Ca2+ (t-cPBS), and 5.0 μg radioiodinated antibodies (anti-MCR) were added at 4° for 15 additional min. Cells were again rinsed 3 times with t-cPBS, and total radiolabel was then evaluated on 0.01 M EDTA-treated samples. Controls for the specificity of anti-MCR antibody binding included normal rabbit IgG with drug and 125I-labeled anti-MCR antibodies without drug.

For measurement of whether surface-bound MCR activity (i.e., inhibition of thymidine incorporation into acid-insoluble material) could be affected by anti-MCR antibody binding to surface-bound drug, cells were incubated for 15 min with 1.0 or 5.0 μg MCR per ml at 37°, rinsed 3 times with t-cPBS, incubated an additional 15 min at 4° with anti-MCR antibodies, and rinsed and finally incubated in fresh tissue culture medium containing 1.0 μCi [3H]thymidine per ml for 60 min at 37°. Incorporation of radioactive thymidine into cellular DNA was evaluated as described above.

Materials

*L. polyphemus* hemocyanin was purified from serum (11) and gave a single band on sodium dodecyl sulfate gel electrophoresis with an apparent molecular weight of 75,000. The 1% extinction coefficient at 280 nm was 11.2. MCR was obtained in approximately 30% pure form from Bristol Laboratories, Inc., through the kind generosity of Dr. W. Bradner. Lactoperoxidase, salmon testis DNA, and the carbodiimide were obtained from Sigma Chemical Co., St. Louis, Mo. Bovine serum albumin was from Reheis Chemical Co., Inc., Chicago, Ill. Radioactive materials were obtained from Schwarz/Mann, Orangeburg, N. Y. ([3H]thymidine, 6 Ci/m mole; L-[14C]leucine, 309 mCi/m mole) and from New England Nuclear, Boston, Mass. (Na125I, 17 Ci/mg). Adriamycin was obtained from the Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, Md. Bleomycin was a gift from Dr. J. Skibba. Actinomycin D was purchased as Cosmegen from Merck Sharp and Dohme, West Point, Pa., and was used directly in mannitol buffer. Methotrexate was obtained from Lederle Laboratories, Pearl River, N. Y., and was purified by ion-exchange chromatography prior to testing in the radioimmune assay (21).

Cloned cell lines were established from the B16 melanoma (The Jackson Laboratory, Bar Harbor, Maine) and from a mammary carcinoma, TNO 3641/75, which was the generous gift of Dr. L. M. van Putten, Radiobiological Institute, TNO, Rijswijk, The Netherlands. All cells were grown in Dulbecco-modified Eagle’s minimal essential medium (30) supplemented with 10% heat-inactivated fetal bovine serum (Grand Island Biological Co., Grand Island, N. Y.), freshly prepared glutamine, and nonessential amino acids. Penicillin (100 units/ml) and gentamicin (10 μg/ml) were present during cell culture.

RESULTS

MCR Activity. The biological activity of purified MCR in eucaryotic cell systems was evaluated by incubating the drug with cloned amelanotic and melanotic B16 melanoma cell lines (B16A and B16M, respectively), as well as with a cloned cell line from the TNO 3641/75 mammary carcinoma. As shown in Chart 1, binding of 125I-labeled MCR to tumor cells occurred rapidly and was essentially complete within 10 min at 37°. Binding to tumor cells was temperature dependent and did not occur to a significant extent at 4°. Data in Chart 1 are representative of experiments performed at MCR concentrations of 5.0 μg/ml. Similar data were obtained at 1.0 and 0.5 μg/ml. Since binding of 125I-labeled MCR at 0.1 μg/ml was not detectable, saturation of surface binding is assumed to occur at concentrations of approximately 1.0 μg/ml under assay conditions.

Incubation of tumor cells with unlabeled MCR in culture medium containing radioactive metabolites ([3H]thymidine and [14C]leucine) revealed that the drug inhibited thymidine uptake but not leucine incorporation into acid-insoluble cellular material (Chart 2). This process was most evident 1 to 2 hr after initial drug contact. The decrease in protein synthesis seen by decreased leucine incorporation after 2 hr (Chart 2) is probably a secondary effect of the drug due to degradation of previously synthesized mRNA in the cells. Inhibition of thymidine incorporation into cellular DNA was not significant at drug concentrations of less than 0.5 μg/ml. The fact that thymidine incorporation was equally inhibited by MCR concentrations of 1.0 and 5.0 μg/ml (total, 2.0 ml per 2 to 4 × 106 cells) further indicates that optimal MCR activity occurs at approximately 1.0 μg/ml.
Chart 1. Kinetics of adsorption of 125I-labeled MCR to tumor cells. 125I-labeled MCR was added at 37° to duplicate 60-mm culture dishes containing subconfluent populations of B16M (■) and B16A (■) melanoma cells and TNO 3641/75 (Δ) mammary carcinoma cells (approximately 3 to 5 x 10^5 cells/dish) at a concentration of 5.0 μg/ml in PBS. Identical sets of cell cultures were incubated at 4°: B16M (○), B16A (○), and TNO 3641/75 (Δ). Radioactivity adsorbed to cells was determined after rinsing at the indicated times. Results are expressed as cpm/10^5 cells (S.E. < 5%).

For verification of membrane adsorption of the antibiotic, B16M melanoma cells were pulsed for 15 min with 5.0 μg MCR per ml, rinsed, and incubated with fresh culture medium containing only [3H]thymidine. At various time intervals after the addition of thymidine (absence of MCR), duplicate cultures were treated with 0.1% trypsin for 5 min at 22°, trypsin was removed by rinsing 3 times with 2.0 ml medium, and cells were again incubated in fresh tissue culture medium containing [3H]thymidine. Chart 3 shows that trypsin treatment 20 min after drug exposure resulted in recovery of normal thymidine uptake. Trypsin treatments after 40 min also partially restored cellular uptake of thymidine into DNA, while trypsin treatments 60 min or more after MCR pulse did not affect MCR cytotoxic activity. These results are analogous to those reported by Kunimoto et al. (12, 13) for Yoshida sarcoma cells.

Antibody Activity and the MCR Radioimmune Assay. After the establishment of the isolation and purification of active MCR, antibodies to the drug were elicited and purified by affinity chromatography. Pooled antisera, as well as purified antibodies, were tested in a dose-response assay with 125I-labeled MCR. Chart 4 shows that optimal antibody concentrations for approximately 50% binding of 125I-labeled MCR added were 3 μg/ml (purified antibody) and a 1:500 dilution of whole antiserum.

A sequential saturation assay between unlabeled MCR and 125I-labeled MCR was used to quantitate drug concentrations in serum, urine, and buffer solutions. The standard

Chart 2. Effects of MCR on DNA and protein synthesis in B16 melanoma (A) and TNO 3641/75 mammary carcinoma cells (B). Subconfluent monolayer cultures of cells were incubated in the presence and absence of MCR with 1.0 μCi [3H]thymidine and [14C]leucine per ml. At the indicated times, cells were harvested and analyzed for thymidine incorporated per μg DNA and leucine incorporated per μg total protein. Two concentrations of drug were used: 1.0 μg/ml (○, ■) and 5.0 μg/ml (Δ, ◇). ○, ■, control cultures.

Chart 3. Effects of trypsin treatment on MCR activity in B16M melanoma cells. Subconfluent monolayer cultures were incubated with 5.0 μg MCR per ml for 15 min. Cells were then rinsed 3 times with tc-PBS and incubated with fresh tissue culture medium containing 1.0 μCi [3H]thymidine per ml. At the indicated time intervals, medium was aspirated, and duplicate cultures were treated with 0.1% trypsin for 5 min at room temperature. After treatment, trypsin was carefully aspirated, and fresh [3H]thymidine-containing medium was added. The incorporation of [3H]thymidine into acid-insoluble cellular material was determined at the indicated times. ○, control cultures (no MCR or trypsin treatment); ◇, treated with MCR but not with trypsin; Δ, treated with MCR followed by trypsin treatment at 20 min; ■, treated with MCR followed by trypsin at 40 min; ●, treated with MCR followed by trypsin at 60 min.
Antibodies to MCR

The affinity of the antibody was determined from binding data plotted according to the method of Scatchard (28), as introduced for radioimmune assays by Berson and Yalow (1). Chart 7 shows the resulting curve. The interrupted line shows the calculated average slope of the binding curve ($m = -2.5$), indicating the average affinity constant of the purified antibody to be $2.5 \times 10^8 \text{ M}^{-1}$.

Effects of Anti-MCR Antibodies on MCR Binding to Cell Surfaces. For determination of whether cell surface-bound MCR could be detected with anti-MCR antibodies, B16M, B16A, and TNO 3641/75 cells were incubated with unla
deleted drug, rinsed well, and incubated with radioiodinated anti-MCR antibodies. Table 1 shows that the antibodies were capable of binding to MCR when the drug was ad-

Chart 4. Dose-response curves for anti-MCR antiserum with MCR and purified anti-MCR antibodies with MCR. The percentage of MCR bound by antibody is plotted as a function of antiserum dilution (O) and as a function of purified anti-MCR antibody concentration (total µg, O). Values represent the average of 6 replicates (S. E. < 3%).

Chart 5. The standard dose-response curve for the radioimmune assay of MCR. The response, $Y$, to dose, $X$, can be defined as a logit function of $Y$ such that $\log Y = \ln(Y/(1 - Y))$. For experimental purposes, $Y$ is further defined as the ratio between antibody-bound radioactivity ($B$) and the amount of radioactivity bound at zero concentration of unlabeled drug ($B_0$). Thus, the response logit $B/B_0$ to dose, log MCR concentration is plotted. Each point represents the mean ± S.D. for 6 replicates using purified antibody. An identical curve is obtained when whole antiserum is used, with a somewhat larger S. D. at 0.1 ng.

Chart 6. Specificity of the MCR radioimmune assay. The proportion of anti-MCR antibody bound to $^{125}$I-labeled MCR is plotted as percentage $^{125}$I-labeled MCR bound in the presence of: MCR (O), methotrexate (MTX) (A), adriamycin (Adria) (O), actinomycin D (Actino D) (A), bleomycin (Bleo) (O).

Chart 7. Scatchard plot for the radioimmune assay of MCR. The ratio of bound to free MCR ($B/F$) is plotted against the concentration of MCR bound. The solid line is curved due to the heterogeneity of the antibody population. Interrupted line, average calculated slope ($m = -2.5$), indicating the average intrinsic association constant of the antibody to be $2.5 \times 10^8 \text{ M}^{-1}$.
Subconfluent monolayer cultures were incubated with 5.0 mg normal rabbit IgG per ml and 1.0 μg MCR per ml in a total volume of 2.0 ml for 15 min. Cultures were rinsed 3 times with cold tc-PBS, and 5.0 μg ¹²⁵I-labeled anti-MCR antibodies (ca. 10,000 cpm) were added at 4° for 15 min. Cells were again rinsed 3 times and removed from plates with 0.01 M EDTA-PBS. Results represent total radioiodine/10⁵ cells.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cells incubated for 15 min with</th>
<th>¹²⁵I-labeled anti-MCR bound (cpm/10⁵ cells)</th>
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<tbody>
<tr>
<td>B16M melanoma</td>
<td>IgG at 37°</td>
<td>127 ± 5.3°</td>
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<td></td>
<td>IgG + MCR at 4°</td>
<td>329 ± 7.5°</td>
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<td></td>
<td>IgG + MCR at 37°</td>
<td>3528 ± 31.2°</td>
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<tr>
<td></td>
<td>IgG + (anti-MCR + MCR) at 37°</td>
<td>539 ± 16.8°</td>
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<tr>
<td>B16A melanoma</td>
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<td></td>
<td>IgG + MCR at 37°</td>
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<td></td>
<td>IgG at 37°</td>
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<tr>
<td>TNO 3641/75 mammary carcino\noma</td>
<td>IgG + MCR at 4°</td>
<td>659 ± 37.0°</td>
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<td></td>
<td>IgG + MCR at 37°</td>
<td>4973 ± 53.6°</td>
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<td>IgG + (anti-MCR + MCR) at 37°</td>
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* Average ± S.E.

* Anti-MCR (purified antibodies) and MCR were preincubated for 15 min at 37° before addition to cell incubations.

Effects of anti-MCR antibody binding to MCR on thymidine incorporation into cellular DNA

Subconfluent monolayer cultures were incubated with 5.0 mg normal rabbit IgG per ml and MCR for 15 min as indicated. Cells were rinsed 3 times with cold tc-PBS, incubated for an additional 15 min at 4° with 5.0 μg anti-MCR antibodies, rinsed 3 times more, and finally placed in culture with 1.0 μCi ¹⁴C]thymidine per ml. Thymidine incorporation into DNA was evaluated after 60 min of incubation at 37° and was compared with controls (normal IgG incubations). Values are presented as percentage of control (±<7%).

<table>
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<tr>
<th>Cell type</th>
<th>Cells incubated for 15 min with</th>
<th>Anti-MCR added (15 min at 37°)</th>
<th>Thymidine incorporated (%)</th>
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<td>B16M melanoma</td>
<td>IgG at 37°</td>
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<td>100</td>
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<td>IgG + MCR (1.0 g/ml) at 37°</td>
<td>+</td>
<td>48</td>
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<td>IgG + MCR (5.0 g/ml) at 37°</td>
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<td>45</td>
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<tr>
<td></td>
<td>IgG at 37°</td>
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<td>100</td>
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<tr>
<td>TNO 3641/75 mammary carcino\noma</td>
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<td>49</td>
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<td>IgG + MCR (5.0 g/ml) at 4°</td>
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<td>&lt;2°</td>
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* Thymidine incorporation was evaluated in these samples after incubation at 4° for 60 min.

DISCUSSION

It is well known that the plasma membrane plays important roles in the regulation of intracellular metabolism. Much of this knowledge has come from studies in which surface-adsorbing proteins are used as biochemical probes. For example, peptide hormones bind to specific receptors on the exterior surface of plasma membranes (8), thereby eliciting a variety of intracellular activities. Toxins also perturb membranes. For example, an enterotoxin secreted by Vibrio cholerae interacts with eucaryotic membranes and stimulates adenylate cyclase (7); the toxin of Corynebacterium diphtheriae binds to specific ganglioside receptors and, after penetration into cells, inhibits protein synthesis by enzymatic modification of Elongation Factor 2 (6); and α-bungarotoxin from Bungarus multicinctus binds specifically to the acetylcholine receptor of muscle cells (3). Mitogens such as phytohemagglutinin bind to cell surfaces and stimulate transport of uridine (24). Lectins such...
as concanavalin A or Robinea pseudocacia lectin bind to plasma membranes and inhibit thymidine incorporation into DNA without affecting its transport into the cells (27). Recently, a plant protein, cesalin, has been shown to bind to the plasma membrane of the human epidermoid carcinoma KB cell line and to inhibit Na-\(^{+}\)-K-\(^{+}\)-ATPase activity (10).

Since the discovery of MCR and of its binding to cell membranes (5, 12, 13), several studies have been performed to verify the antitumor activity of the polypeptide antibiotic (12, 13, 16-19). Lippman et al. (19) have shown that the activity of the drug, when graded according to established methods (4), allows its placement in a category of efficacy with such chemotherapeutic agents as vincristine, actinomycin D, and mitomycin C and in a higher category than methotrexate or 5-fluorouracile. In addition, Lippman et al. (19) feel that the anti-Lewis lung carcinoma activity of MCR alone predicts its potential as a chemotherapeutic agent for solid human tumors. However, much of the work at the whole animal level has been performed using only partially purified drug. The major reason for this has been the problems associated with the apparent storage instability of MCR in highly purified form. With the recent discovery by Yamashita et al. (34) that the drug can be stabilized by disaccharides during lyophilization and storage, it is now possible to proceed with studies of the mechanism(s) of action and biological activities of this potentially useful chemotherapeutic agent.

Several studies have shown that MCR binds to tumor cell surfaces and, as a consequence of this binding, cellular DNA synthesis is terminated (12, 13, 16, 17). Since this activity can be reversed by incubating drug-treated cells with trypsin, Kunimoto et al. (13) have postulated a bacteriologic-like activity for MCR, in which surface binding modifies the role of the plasma membrane in regulating cellular division. However, there is no direct evidence that MCR activity is mediated entirely while the drug is cell surface bound.

The present study confirms the purification and stabilization of MCR as well as the requirement for tumor cell membrane binding in the mechanism of action of the drug (Chart 1). Inhibition of DNA synthesis, but not of protein synthesis (Chart 2), also confirms previously published data (13). However, although binding to cell surfaces is an essential prerequisite and is apparently a saturable phenomenon requiring low concentrations of MCR, optimal inhibition of DNA synthesis takes place 1 to 2 hr after initial contact with the drug (Chart 2). Similar toxicity was measured in the experiments of Kunimoto et al. (13), which show that cell viability, as measured by dye exclusion, drops dramatically 2 hr after exposure to MCR. Cumulatively, these data do not exclude the possibility that MCR enters the cells by endocytosis and may create a primary metabolic lesion(s) inside. Trypsin-mediated rescue of MCR-exposed cells was effective only the 1st 20 to 40 min after drug exposure (Chart 3). We feel the data are, as yet, too indirect to define a precise mode of action.

Since MCR may serve as a valuable probe for further understanding the role(s) plasma membranes play in regulating intracellular metabolism and because the drug is active against many experimental model tumors, a rapid, sensitive assay for MCR was required. The radioimmune assay described in this study fulfills these requirements. With the use of the radioimmune assay, it was possible to quantitate MCR in biological fluids at concentrations below 1.0 ng. In addition, with purified anti-MCR antibodies, it was possible to prove the surface-binding property of MCR (Table 1) and to show that antibody binding to MCR on cell surfaces did not alter the biological activity of the drug (Table 2).

As with all radioimmune assays, sensitive antibody-mediated detection of the drug does not mean quantitative determination of its biological potony. The immunoreactive determinants of MCR may not be the same portions of the molecule that are active in biological assay systems. However, it is probable that the active portion of MCR is also immunogenic, since incubation of the drug with anti-MCR antibodies resulted in apparent inactivation of the ability of the drug to bind to tumor cell surfaces (Table 1). Since MCR is weakly immunogenic in animals even when not covalently attached to a protein carrier (unpublished observation), the ultimate clinical value of this polypeptide antibiotic has yet to be established. Adsorption of MCR to tumor cell surfaces has been hypothesized to increase tumor cell immunogenicity (15, 18), implying that the immunogenic properties of the drug may be beneficial. In the preparation of a relevant therapeutic agent, however, it might be possible that only a small peptide fragment of MCR is required for cytotoxic activity. Further studies are required to determine the precise mechanism(s) of MCR action and to correlate drug detection in the radioimmune assay with the stability and activity of the drug with the use of biological (e.g., bactericidal) assays.

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