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Immunologic Heterogeneity of Dihydrofolate Reductase from Methotrexate Sensitive and Resistant L1210 Leukemia Cells (42149)

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Abstract. Dihydrofolate reductase from L1210 leukemia cells which are sensitive and resistant to methotrexate has the same physical and kinetic properties and immunoreactivity with a guinea pig antiserum raised to the enzyme purified from the methotrexate resistant strain. However, a chicken antiserum to dihydrofolate reductase from methotrexate sensitive L1210 cells has greater affinity for the homologous enzyme than for the enzyme from the MTX resistant cells indicating that there is some antigenic difference in these molecules.

The physical and kinetic properties of dihydrofolate reductase (DHFR) from bacteria which have become resistant to the antifolate drug, methotrexate (MTX), are distinctly different than the enzyme from the MTX sensitive strain indicating that a structural alteration of the enzyme occurs as MTX resistance is acquired in these procaryotes (1). In contrast, studies of the properties of DHFR from MTX sensitive and resistant mammalian cells have been variable. Kashket *et al.* (2) and Perkins *et al.* (3) found no difference in the properties of DHFR from MTX sensitive (MTX/S) and resistant (MTX/R) L1210 cells. However, Dedhar and Goldie (4) observed that L5178Y mouse leukemia cells which developed resistance to MTX expressed two forms of DHFR, each form having similar catalytic properties but differing in sensitivity to MTX inhibition and immunologic cross-reactivity.

In this study we can demonstrate that a chicken antiserum raised to DHFR purified to homogeneity from MTX/S L1210 cells has higher affinity for the homologous enzyme than for DHFR from MTX/R cells, yet the enzyme from both strains of leukemia cells has similar physical and kinetic properties and reacts similarly with a guinea pig antiserum to DHFR purified to homogeneity from MTX/R cells.

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Materials and Methods. Tritiated methotrexate ([^{3}H]MTX), specific activity greater than 5 Ci/mmole and [^{125}I]iodide were purchased from Amersham, Arlington Heights, Illinois. NADPH was purchased from Sigma Chemical Company. MTX was obtained through the courtesy of Lederle Laboratories, Inc., Pearl River, New York, and was purified by the method of Gallelli and Yokoyama (5). Sephadex G-75 and Sepharose 4B were obtained from Pharmacia Fine Chemicals, Piscataway, New Jersey. Trasylol was obtained from the Mobay Chemical Corporation, New York. Rabbit anti-chicken γ-globulin and rabbit anti-guinea pig γ-globulin were purchased from Miles Laboratories, Inc., Elkhart, Indiana. All other chemicals were of reagent grade.

DHFR from MTX/S L1210 murine leukemia cells was purified to homogeneity by affinity chromatography as previously described (6). A stable MTX/R L1210 cell line was obtained through the courtesy of Dr. F. Sirotnick, Sloan-Kettering Research Institute, New York. The ascitic form of these leukemia cells was maintained in BDF_1 mice (C57B1 $\times$ $DBA/2F_1$) and the DHFR concentration in the MTX/R strain was approximately 16-fold greater than the concentration in the MTX/S strain. This strain also had more than 16-fold greater *in vivo* resistance (7). DHFR from these MTX/R cells was similarly purified by affinity chromatography to homogeneity by disc gel electrophoresis (6).

Antiserum was raised in a chicken to purified DHFR from the MTX/S cells by

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two subcutaneous injections in the breast area of 10 μg of the enzyme emulsified with complete Freund's adjuvant administered at 7- to 10-day intervals, followed by a third injection of similarly emulsified 25 μg of enzyme.

Antiserum was raised to the purified DHFR from MTX/R cells by four weekly subcutaneous injections of 50 μg of the enzyme emulsified with complete Freund's adjuvant. We fortuitously selected the guinea pig instead of the chicken because in the interval between purifying the enzyme from MTX/S cells and the enzyme from MTX/R cells our laboratory moved to another institution which had no facility for housing chickens.

The radioimmunoassay (RIA) for DHFR using ^{125}I-labeled purified DHFR from calf liver as the tracer has been previously described (8). The sensitivity of the RIA is approximately 1 ng of DHFR.

Preparation of partially purified DHFR from MTX/S and MTX/R cells. The L1210 cells in the ascitic fluid from the BDF_1 mice, inoculated into separate groups 6 to 7 days previously with MTX/S and MTX/R L1210 leukemia cells, were washed three times with cold 0.15 *M* sodium chloride. The contaminating erythrocytes were lysed by dilution of 1 vol of the cell suspension with 3 vol of cold distilled water, followed within 30 sec by the addition of 1 vol of 0.6 *M* sodium chloride. The cells were then washed with 0.15 *M* sodium chloride to remove the free hemoglobin, packed by centrifugation at 1400*g* for 15 min, and then suspended in 3 vol of 0.05 *M* sodium citrate, pH 7.2, and frozen.

The cytosol of these cells was obtained by centrifugation of the thawed cells at 100,000*g* for 60 min. DHFR was partially purified from the cytosol by filtration (2 ml) through a Sephadex G-75 column (1.5 × 75 cm) equilibrated and packed with 0.05 *M* Tris–HCl buffer, pH 7.4, at a constant flow rate of 12 ml/hr. Each fraction was assayed for DHFR by the binding of [^{3}H]MTX (8) and the fractions containing the highest DHFR concentration were pooled.

Immunoinhibition of MTX binding. An aliquot of the partially purified DHFR from MTX/S and MTX/R cells was diluted in 0.02 *M* Tris–HCl buffer, pH 7.4, to bind approximately 250–350 pg of [^{3}H]MTX and then incubated for 16 hr at 4°C with 0–10 μl of chicken antiserum to the purified DHFR from MTX/S cells. [^{3}H]MTX (520 pg) and 0.024 μmole of NADPH were then added to each incubation mixture and the reaction volume was brought to 0.5 ml with 0.06 *M* citrate buffer, pH 4.8, containing bovine serum albumin (1 mg/ml). After 30 min incubation at room temperature, the bound and free [^{3}H]MTX were separated by the addition of 0.4 ml of a 1% suspension of dextran-coated charcoal (9). After centrifugation of the pellet, the radioactivity in the supernatant solution, which represented the bound [^{3}H]MTX, was determined using a liquid scintillation counter as previously described (9).

Results. The MTX binding activity of the cytosol from the MTX/S and MTX/R L1210 cells elute from the Sephadex G-75 column in exactly the same fractions (Fig. 1) which correspond to an apparent molecular weight of 20,000, the established molecular weight of DHFR (10).

The affinity for the binding of MTX by the partially purified DHFR from the MTX/S and MTX/R cells also appears to be similar by Scatchard plot analysis (Fig. 2). The ap-

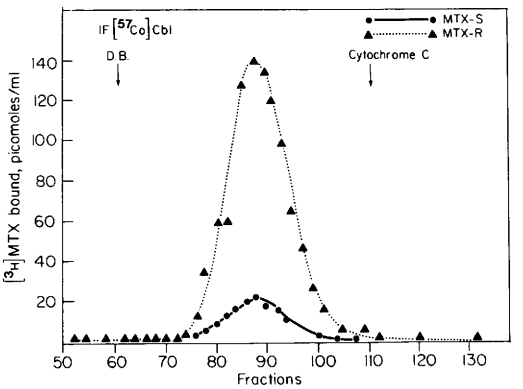

FIG. 1. Gel filtration elution profile of the cytosol from MTX/S and MTX/R L1210 cells through a Sephadex G-75 column. The column markers were Dextran blue (D.B.), cyano[^{57}Co]cobalamin bound to human intrinsic factor (IF, M_r = 50,000), and cytochrome *c* (M_r = 13,000). Each fraction was assayed for the binding of [^{3}H]MTX.

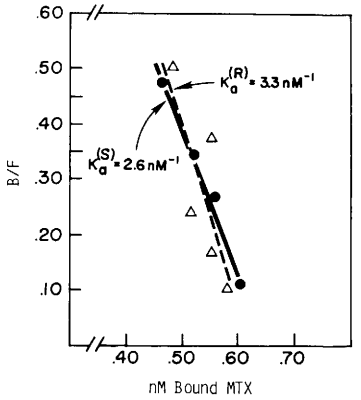

FIG. 2. Scatchard plot of the binding of MTX by DHFR from MTX/S and MTX/R cells. $K_a^{(S)}$ and $K_a^{(R)}$ are the apparent affinity constants for the binding of MTX by the cytosol from the sensitive and resistant cells, respectively.

parent affinity constant (K_a) for the MTX/S and MTX/R enzyme are 2.6 and 3.3 mM^{-1}, respectively.

In a competitive RIA, where purified DHFR from MTX/S and MTX/R cells competed for antibody binding sites in an antiserum raised in a guinea pig to the purified MTX/R DHFR, both enzymes competed equally (Fig. 3). However, when the same experiment was carried out using the chicken antiserum to the purified MTX/S enzyme, there is clear evidence of different antigenic properties (Fig. 4). Whereas 0.35 pmole of

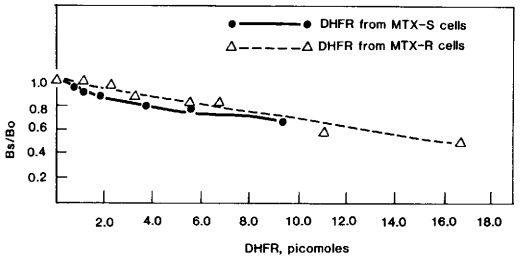

FIG. 3. Dose-response curves for the RIA for DHFR from MTX/S and MTX/R L1210 cells using a guinea pig antiserum raised to DHFR purified from MTX/R cells. B_s/B_0 is the ratio of ^{125}I-labeled DHFR bound to the antibody in the presence of competing unlabeled antigen (B_s) to the tracer antigen bound to the antibody in the absence of competing unlabeled antigen (B_0).

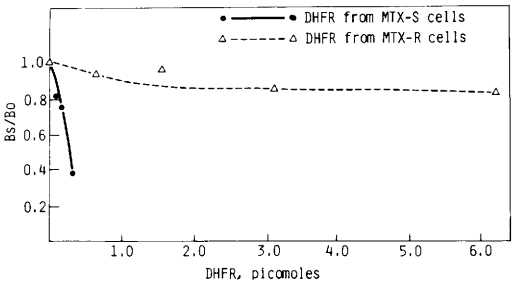

FIG. 4. Dose-response curves for the RIA for DHFR from MTX/S and MTX/R L1210 cells using chicken antiserum raised to DHFR purified from MTX/S cells.

DHFR from the MTX/S cells inhibits the immune binding of the ^{125}I-DHFR tracer by approximately 60%, 6 pmole of DHFR from the MTX/R cells inhibits the reaction by only 20%.

This experiment by itself could have also suggested that the preparation of DHFR from the MTX/S cells, which was used for the dose-response curve, might also contain an immunoreactive protein which did not bind MTX (6, 8). However, the results of the experiment shown in Fig. 5 demonstrate that equivalent amounts of the chicken antiserum (raised to DHFR from MTX/S cells) inhibit more effectively the binding of [^{3}H]MTX to the enzyme from the MTX/S cells than to the enzyme from the MTX/R cells. This experiment indicates that those antibodies binding to or near the functional site of DHFR have higher affinity for the homologous MTX/S enzyme.

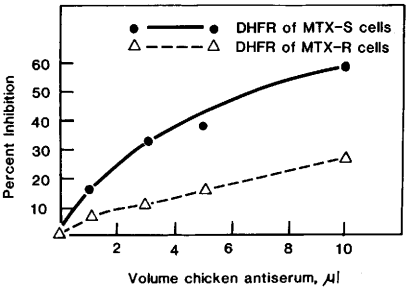

FIG. 5. The inhibition by the chicken antiserum of the binding of [^{3}H]MTX by the cytosol from MTX/S and MTX/R cells.

Initially, we tested each antiserum against the purified and partially purified forms of the homologous enzyme and observed no difference in the immunoinhibition. Accordingly, for these studies, which were repeated several times for confirmation, we used the partially purified DHFR because it was readily prepared fresh by gel filtration chromatography from the crude cell lysate.

It has been previously demonstrated that DHFR undergoes conformational changes in the presence of dihydrofolate, NADPH, or MTX which can be detected by proton magnetic resonance spectroscopy (11). Again, in preliminary studies of the property of these antiserums, such conformational changes which were induced by NADPH or the binding of [^{3}H]MTX to the enzyme did not block the immunoinhibition of the catalytic reduction of dihydrofolate or the immunoprecipitation of the DHFR–inhibitor complex (data not shown).

Discussion. It has been presumed from the studies of the physical properties, catalytic activity, inhibition by MTX, and immunologic cross-reactivity that DHFR from MTX/S and MTX/R L1210 cells have the same structure (2, 3). In fact the purpose of our initial experiment was to prepare an antiserum equally reactive with both enzymes using DHFR from MTX/R cells because greater amounts of purified enzyme are more readily prepared from the resistant cell line. The studies using the guinea pig antiserum to the purified MTX/R DHFR initially provided supporting evidence that DHFR from both strains of L1210 cells was antigenically homologous. It is clear, however, that the immune system of the chicken was able to detect some antigenic site(s) on DHFR from the MTX/S cells which is different than the DHFR from the MTX/R cells. It should be emphasized that the guinea pig and the chicken were immunized by DHFR purified by affinity chromatography and proven to be homogenous by PAGE (6).

The studies of Perkins *et al.* (3) which demonstrated similar immunochemical properties of DHFR from MTX/S and MTX/R L1210 cells were carried out using a rabbit antiserum to the enzyme. The rabbit, however, like the guinea pig, may not be able to detect a subtle structural difference in the molecule that an avian species could. For example, Simonsen and Levinson (12), on the basis of a difference in the cDNA clone for the wild-type and MTX induced mutant DHFR, predicted a single amino acid substitution between the two enzymes from a murine cell line. The exression of a single amino acid difference in these proteins could alter the tertiary structure of the molecules and hence their antigenic properties. These findings also indicate that gene amplification, which is believed to be the mechanism for the induction of DHFR in mutant MTX resistant cells (13), may also be accompanied by a change in the nucleotide sequence of the amplified gene.

Although it is an unusual phenomenon, a change in the tertiary antigenic structure of a protein may also be a consequence of a conformational alteration without a change in the primary amino acid sequences. For example, a human antiserum to insulin from the dog and the pig can detect an antigenic difference in these hormones even though their amino acid sequences are identical (14).

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