Antibody to Dihydrofolate Reductase from a Methotrexate-Resistant Subline of the L1210 Lymphoma

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SUMMARY

An antiserum has been prepared against dihydrofolate reductase (EC 1.5.1.3) from mouse L1210 R tumor cells by injection of the purified enzyme into rabbits. The antiserum both precipitates and inactivates the enzyme; the antigen-antibody interaction fixes complement. The antibody inactivates dihydrofolate reductase from L5178Y, Ehrlich ascites carcinoma, and Sarcoma 180 murine tumors as well as that from the L1210 lymphoma. It partially inactivates enzyme from human bone marrow, but does not inactivate enzyme from *Escherichia coli* or from mouse, chicken, guinea pig, or rabbit liver. The cofactor NADPH, the substrates dihydrofolate and folate, and the inhibitor methotrexate protect the enzyme from inactivation by the antibody at low concentration.

INTRODUCTION

During recent years the enzyme dihydrofolate reductase (EC 1.5.1.3) has been purified and characterized from bacterial (2–4), avian (5–7), and mammalian sources (8–11), and its function in providing tetrahydrofolate for purine and thymidylate biosynthesis has been elucidated. The ability of the potent antifolate compounds methotrexate (amethopterin) and aminopterin to inhibit cell growth has been shown to be related to their inhibition of this enzyme activity (12, 13).

Recently, the availability of highly purified dihydrofolate reductase from several sources has allowed the determination of some of its physical properties (4, 6, 7, 9–11). We have prepared highly purified reductase from a subline (resistant to methotrexate) of the L1210 leukemia and have used this enzyme as an antigen for the production, in rabbits, of an antiserum. The characterization and specificity of the antigen-antibody reaction are described in this communication.

EXPERIMENTAL PROCEDURE

Materials. Folic acid was purchased from Nutritional Biochemical Corporation. NAD, NADH, NADP, and NADPH were purchased from Sigma Chemical Company. Dihydrofolate was prepared from folate by the modification by Friedkin *et al.* (14) of the dithionite reduction step (15), and was stored frozen in suspension in 0.001 N HCl under nitrogen.

Production of antisera. The preparation and assessment of purity of dihydrofolate reductase from the methotrexate-resistant subline of the L1210 R murine leukemia are described in detail elsewhere (11). In

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essence, the purity was determined from a calculation based on a molecular weight for the enzyme of 20,000, a protein concentration determined by the method of Waddell (16), and an enzyme concentration determined by methotrexate titration. By this criterion, the standard purification procedure produced enzyme at 70–90% purity. "Ammonium sulfate enzyme," "Sephadex enzyme," and "hydroxylapatite enzyme" refer to enzyme obtained from these steps during the purification procedure. The degree of purity of the enzyme protein obtained at these three steps is approximately 1%, 10%, and 80%, respectively (11).

Two rabbits were given different preparations of dihydrofolate reductase. Rabbit I received 0.4 mg of 70% pure enzyme, mixed with Freund's adjuvant; aliquots were injected into all four footpads and also subcutaneously. Eight weeks later a booster dose of the same enzyme preparation (stored at −10°) was administered in the same manner. Rabbit II received 0.4 mg of 90% pure enzyme, injected as described above with the exception that the booster dose (0.4 mg) was administered subcutaneously only 1 month later. The animals were bled 10 days after the second immunization, and the sera were stored frozen.

The antisera were used without further treatment in most experiments. In some studies complement was inactivated by heating the antisera to 55° for 20 min; in addition, partly purified γ_2-globulin fractions were prepared by ammonium sulfate fractionation (17).

Purification of enzyme from sources other than L1210 R. Dihydrofolate reductase was prepared from several transplanted mouse neoplasms (L1210, Sarcoma 180, Ehrlich ascites carcinoma, and L5178Y), livers (chicken, mouse, guinea pig, and rabbit), *Escherichia coli*, and human bone marrow cells. The enzyme from the transplanted mouse tumors, livers, and human bone marrow cells was partially purified by ammonium sulfate fractionation of the extracts obtained after centrifugation of the homogenized tissue. The protein that precipitated between 50 and 80% saturation was dissolved in 0.05 M potassium phosphate buffer, pH 7.5, and then stored frozen until use. The enzyme from *E. coli* was prepared in a similar manner except that the cells were broken by grinding with alumina.

Precipitin reaction. Qualitative precipitin reactions were carried out in agar gel using the Ouchterlony method (18) adapted to a microtechnique with commercially available plates (Hyland Laboratories, Los Angeles).

Complement fixation. All complement fixation tests were performed by the technique of Weir (19), and dilutions were recorded to a 2+ end point with a standardized optimal antigen dilution.

Determination of enzyme activity. Enzyme activity was measured by a spectrophotometric method utilizing the decrease in absorbance that occurs at 340 mμ when NADPH and H_2-folate are converted to NADP and H_4-folate, respectively (11). Absorbance readings were made automatically with a Gilford 2000 multiple sample absorbance recorder attached to a Beckman DU spectrophotometer. One unit of enzyme activity is defined as that amount reducing 1 μmole of substrate per hour under the conditions of the standard assay (pH 7.5, 25°).

RESULTS

Precipitin reaction. At intervals after the booster injection, serum was taken from both rabbits and tested by the Ouchterlony plate method against various enzyme preparations. The serum taken from rabbit I, 10 days after the booster injection, gave a distinct precipitin reaction with both purified and concentrated impure dihydrofolate reductase (Fig. 1). A similar but much weaker reaction was observed with serum from rabbit II. Two distinct precipitin bands were detected with the relatively impure ammonium sulfate enzyme or when Sephadex enzyme was used, whereas a single band was observed in the presence of the purified hydroxylapatite enzyme. Serum from rabbit I was used in all subsequent experiments.

Inactivation of the enzyme. Incubation

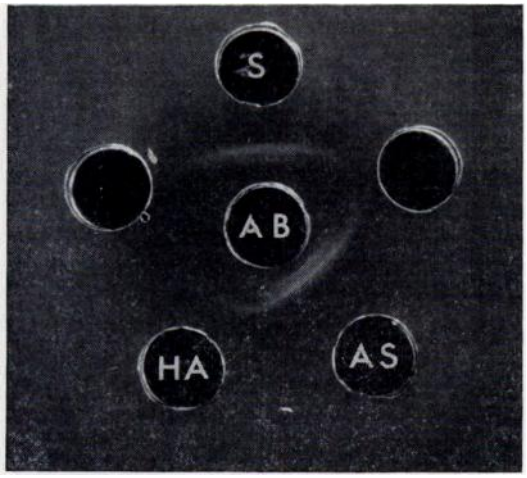

FIG. 1. *Precipitin reaction of rabbit antiserum to L1210 dihydrofolate reductase*

Serum (AB) from rabbit I (10 μl) was placed in the center well. Enzyme (10 μl) from various steps during purification (11) was put in the surrounding wells: AS, ammonium sulfate enzyme; S, Sephadex enzyme; HA, hydroxylapatite enzyme.

of dihydrofolate reductase with the antibody preparation caused a time- and temperature-dependent inhibition of enzyme activity. The data shown in Fig. 2 indicate the dependence of enzyme inhibition on the amount of antibody. The percentage inactivation increased as the amount of antibody increased. Of interest is the "titrating" type of inhibition produced, indicating that the binding between antibody and enzyme was extremely tight. In support of this conclusion, attempts to separate enzyme and antibody from the enzyme-antibody complex have thus far been unsuccessful. High salt concentrations followed by Sephadex gel filtration, as well as conditions of low pH, have been used.

Table 1 shows the effect of incubation temperature on the inactivation of dihydrofolate reductase by antibody. The degree of inactivation increased as temperature increased. Although the enzyme was 60% denatured by heat, at 50° it was still possible to observe further inactivation in the presence of antibody.

Complement fixation. Positive complement fixation at a 1:16 dilution was found using antigen (L1210 R enzyme) diluted 1:16 and 1:32.

Species specificity. Precipitin reactions were observed between the antibody and dihydrofolate reductase obtained from several mouse tumor enzymes, including L1210 S (parent line), 5178Y, Sarcoma 180, and the Ehrlich ascites carcinoma. None could be detected when mouse, rat, or rabbit liver was used as the enzyme source, or with human bone marrow enzyme. Enzyme activity from those sources giving precipitin reactions was also inactivated by antibody (Table 2). Enzyme activity from all the mouse tumors was completely inactivated, whereas the enzymes from *E. coli* and liver (mouse, rat, guinea pig, and rabbit) were not affected by equal amounts of antibody. Partial inhibition of human bone marrow dihydrofolate reductase was obtained under these conditions.

Protection against inactivation. Previous studies using the L1210 R enzyme showed

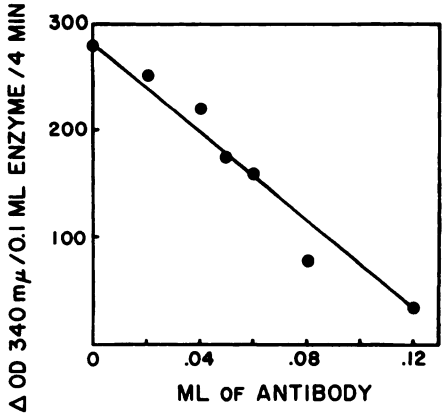

FIG. 2. *Inhibition of L1210 R dihydrofolate reductase activity as a function of antiserum concentration*

Partially purified L1210 R dihydrofolate reductase (Sephadex enzyme), 0.1 ml, giving a control rate of 0.280 absorbance unit/4 min measured at 340 mμ (11), was incubated with 50 μmoles of potassium phosphate buffer, 100 μmoles of KCl, varying amounts of antiserum or control rabbit serum, and water in a total volume of 0.9 ml. After a 20-min interval, 0.1 ml of a solution containing 0.08 μmole of NADPH, 0.03 μmole of dihydrofolate, and 10 μmoles of 2-mercaptoethanol was added to assay residual enzyme activity. The activities obtained were corrected for the decrease in enzyme activity observed (less than 10%) in the absence of antiserum.

TABLE 1
Effect of temperature on inactivation of enzyme by antibody

After incubation of 0.2 unit of enzyme with 0.2 ml of antiserum in the presence of 100 μmoles of Tris-HCl, pH 7.5, and 150 μmoles of KCl in a volume of 0.9 ml for 30 min at the indicated temperature, NADPH and dihydrofolate were added and enzyme activity was determined as described in EXPERIMENTAL PROCEDURE. Control tubes were incubated with an equivalent amount of 5% albumin in 0.9% NaCl instead of antibody. Activity did not appreciably decrease (less than 5%) as a result of the incubation, except at 50°, where activity of the control (less antibody) decreased by 60%.

Temperature of incubation	Inactivation
	%
0°	4
28°	54
37°	83
50°	78

that both substrates (folate and dihydrofolate) and the coenzyme NADPH bind strongly to the free enzyme (20). Therefore, substrates and cofactors were evaluated for their ability to protect the enzyme against inactivation by antibody (Table 3).

Both substrates, folate and dihydrofolate, as well as the cofactor NADPH, give appreciable protection at 10^{-5} M. At 10^{-6} M, NADPH protected somewhat better than dihydrofolate, which in turn seemed to protect better than folate. At 10^{-5} M, of the pyridine nucleotides tested, only NADPH gave significant protection, although at 10^{-4} M NAD, NADH, and NADP all protected the enzyme from antibody inactivation.

By using appropriate assay conditions, it was possible to test the ability of the folate antagonist, methotrexate, to protect the enzyme from inactivation by the antiserum, despite the fact that methotrexate itself inactivates the enzyme. A concentration of 1.1×10^{-8} M was used, and incubation of the enzyme and methotrexate with antibody at pH 7.5 was performed at 37° for 30 min; the subsequent assay was then carried out at a high pH (9.0), at which pH methotrexate dissociates from the enzyme (21). Protection by methotrexate was not established. Since higher concentrations of methotrexate could not be tested in this way because of inhibition at pH 9.0, methotrexate protection was studied in another manner. Ouchterlony plates were made with agar containing 10^{-6} and 10^{-7} M methotrexate. At both concentrations of methotrexate, the antibody did not form a precipitin line with a concentrated ammonium sulfate enzyme, a dialyzed ammonium sulfate enzyme, or hydroxylapatite enzyme (about 60% pure), while controls not containing methotrexate in the agar did. This test, although less sensitive than the kinetic tests, showed that methotrexate prevented the precipitin reaction and probably pro-

TABLE 2
Specificity of antibody inactivation

Enzymes (50–80% ammonium sulfate fractions) and 0.2 ml of antiserum (rabbit I) were incubated for 20 min at 37° in the presence of 100 μmoles of Tris-HCl, pH 7.5, and 150 μmoles of KCl in a volume of 0.9 ml. After incubation, residual enzyme activity was assayed by adding 0.1 ml of a solution containing 0.08 μmole of NADPH, 0.04 μmole of dihydrofolate, and 10 μmoles of 2-mercaptoethanol to the incubation mixture and determining the decrease in absorbance at 340 mμ for an additional 5 min. The percentage inhibition produced was obtained by comparing the rates obtained when control mixtures were incubated with an equivalent amount of serum from a nonimmunized rabbit; no significant decrease in enzyme activity was measurable during the incubation in the presence of normal rabbit serum.

Enzyme source	Inhibition
Mouse tumors	%
L1210	>95
L1210 R	>95
L5178Y	>95
Ehrlich ascites carcinoma	>95
Sarcoma 180	>95
Animal livers	
Chicken	<5
Mouse	<5
Guinea pig	<5
Rabbit	<5
Miscellaneous	
E. coli	<5
Human bone marrow	36

TABLE 3
Protection of dihydrofolate reductase by substrates and cofactors

Enzyme (L1210 R, 0.31 unit of Sephadex enzyme) was incubated for 15 min at 37° together with 0.2 ml of rabbit antiserum in the presence of the indicated concentrations of the protecting agents. Tris-HCl buffer, pH 7.5, 100 μmoles, and KCl, 150 μmoles, were also present. The volume of solution was 0.9 ml. Residual enzyme activity was determined as described in Table 1; the control contained 0.2 ml of 5% albumin in 0.9% NaCl instead of antibody.

Protecting agent	Concentration of protecting agent			
	3.8×10^{-7} M	1×10^{-6} M	1×10^{-5} M	1×10^{-4} M
	% of control			
Substrates				
Folate		18	74	
Dihydrofolate		42	95	
Cofactors				
NAD			18	100
NADH			20	100
NADP			29	100
NADPH	28	70	85	100

tected the enzyme from inactivation at a lower concentration than was needed for protection with NADPH or dihydrofolate.

DISCUSSION

Dihydrofolate reductase from a methotrexate-resistant subline of the L1210 lymphoma is an enzyme of low molecular weight (approximately 20,000). It contains tyrosine and tryptophan, although the number of these residues per molecule has not been exactly determined (20). The availability of a specific stoichiometric inhibitor, methotrexate, allows an estimate of purity of the protein to be made. Since only 1–2 mg of highly purified (70–90%) enzyme can be prepared from 200 mice, the number of rabbits used and the amount of enzyme employed for immunization were necessarily limited. Nevertheless, despite the relatively small amount of antigen employed for immunization, rabbit I developed an antibody that (a) formed a precipitin reaction with the enzyme, (b) inactivated the enzyme activity in a time- and temperature-dependent manner, and (c) fixed complement. Except to show that the antibody could be precipitated by 50% saturated ammonium sulfate, and that it is eluted from DEAE-cellulose with the characteristics of 7 S γ_2-globulin, no further characterization of the antibody has been carried out.

Of interest was the species specificity and organ specificity of the antiserum. Thus, a battery of partially purified mouse tumor enzymes were equally inhibited by equal amounts of antiserum, while the enzymes obtained from livers of many species, including the mouse, chicken, guinea pig, and rabbit, were not inhibited. Of significance may be the lack of inhibition produced using the mouse liver enzyme: this appears to be the first experimental evidence indicating that two different forms of dihydrofolate reductase may exist in one species. The partial inactivation of the enzyme from human bone marrow[4] produced by quantities of antiserum sufficient to inactivate the mouse tumor enzymes may be attributable to antibody heterogeneity, enzyme heterogeneity, or, most probably, to a difference in amino acid content or conformation of the human blood cell enzyme in comparison to the mouse tumor enzyme. This finding is of some interest, in that the enzymes from these sources have thus far been indistinguishable by several physical and kinetic properties.[5] As expected, the enzyme from *E. coli* was not inhibited by the antibody, since this enzyme has many physical and kinetic properties that differ from both the liver and tumor enzymes (3).

The protection afforded by pyridine nucleotides, especially NADPH, and the substrates folate and dihydrofolate, correlates reasonably well with the ability of these substances to bind to the enzyme. Recent studies have shown, by fluorometric measurements, that either NADPH, folate, or dihydrofolate can bind to free enzyme (20). These substances may protect the

[4] Purified dihydrofolate reductase obtained from "induced" leukocytes and erythrocytes from patients treated with methotrexate is similarly partly inhibited by antibody.

[5] Unpublished observations.

enzyme from antibody inactivation and from denaturation by heat (22), urea (22, 23), guanidine HCl (22), or proteolysis (24, 25) by protecting the active site or by causing a conformational change that stabilizes this protein. Studies of the use of the antibody to measure "turnover" of dihydrofolate reductase, in particular to investigate the phenomenon of enzyme "induction" (26), are in progress. Antibody to dihydrofolate reductase may also be of help in the study of intracellular enzyme localization using fluorescent antibody techniques.

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