Identification of a High-Molecular-Weight Nonfunctional Protein in L1210 Leukemia Cells with Common Antigenic Determinants to Dihydrofolate Reductase

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ABSTRACT

The concentration of immunoreactive protein in the cytosol of L1210 cells measured using a specific radioimmunoassay for dihydrofolate reductase was substantially greater than the concentration of active enzyme which was measured by the binding of [3H]methotrexate. When the cytosol was subjected to gel filtration, two immunoreactive proteins were separated, a high-molecular-weight (Mr 318,000) protein which did not have catalytic activity and which did not bind [3H]methotrexate and a smaller protein (Mr ~20,000) which did reduce [3H]folic acid to tetrahydrofolate and did bind [3H]methotrexate. The nonfunctional high-molecular-weight protein neutralized the inhibitory effect of the antisera on active dihydrofolate reductase. There was no spontaneous disaggregation of the big species into smaller subunits nor did 8 M urea alone, dithioe-rythritol, or RNase alter its apparent molecular weight. Trypsin, however, digested both the nonfunctional and active immunoreactive forms of the enzyme. Isoelectric focusing of the cytosol separated two nonfunctional immunoreactive isoproteins, each having the same isoelectric points as the two active isoenzymes of dihydrofolate reductase (pis of 8.0 and 8.5).

Studies in rapidly replicating and stationary-phase L1210 cells showed that the concentration of the nonfunctional immunoreactive protein increased rapidly, reaching a peak on Day 2 of log growth at which time active enzyme was at a nadir, and then decreased rapidly, reaching a nadir on Day 4, at which time active enzyme was at a peak.

The identical isoelectric points for the inactive and active immunoreactive proteins and the reciprocal concentration of each form in logistically growing cells suggest that the immunoreactive large species may be a precursor of the active enzyme.

INTRODUCTION

The cytoplasm of human chronic myelogenous leukemia cells, in the absence of functional DHFR activity, contains a protein which cross-reacts with DHFR from L1210 murine leukemia when tested against a monospecific antiserum raised against the purified L1210 enzyme (18). This protein also reacts with antiserum to purified calf liver DHFR, and by RIA using this antiserum the concentration of this nonfunctional immunoreactive protein in the cytoplasm of different types of human leukemia cells was 10- to 400-fold greater than the concentration of active DHFR (26). Gel filtration studies also demonstrated that this nonfunctional immunoreactive protein has an apparent molecular weight of 41,000 (26), which is virtually twice the molecular weight of the active enzyme (4). A similar RIA has now been used to quantify and characterize the immunoreactive forms of DHFR in L1210 cells. These cells also contain a high-molecular-weight nonfunctional protein which has antigenic homology with active DHFR. It has been characterized by gel filtration and denaturant experiments, and its concentration, relative to active DHFR, was measured in L1210 cells in log and stationary growth phases in vivo.

MATERIALS AND METHODS

[3H]MTX (specific activity, >5 Ci/mmol), [3H]folic acid (specific activity, 24 Ci/mmol), and sodium [125I]iodide were purchased from Amersham/Searle Corp., Arlington Heights, Ill. NADPH, dihydrofolinic acid, phenylmethylsulfon fluoride, sodium bisulfite, diithionytritol, bovine serum albumin, trypsin coupled to agarose, bovine pancreatic RNase, yeast RNA, and 1-cyclohexyl-3-carboximide metho-p-toluenesulfonate were purchased from the Sigma Chemical Co., St. Louis, Mo. The activity of RNase was established by the hydrolysis of yeast RNA (6). MTX was obtained through the courtesy of Lederle Laboratories, Inc., Pearl River, N. Y., and was purified by the method of Gallelli and Yokoyama (13). Sephadex G-75, Sephadex G-200, and Sepharose 4B were obtained from Pharmacia Fine Chemicals, Piscataway, N. J. Trasylol was obtained from the Mobay Chemical Corp., New York, N. Y. Rabbit anti-chicken y-globulin was purchased from Miles Laboratories Inc., Elkhart, Ind. All other chemicals were of reagent grade.

DHFR from L1210 leukemia cells was purified to homogeneity by affinity chromatography, and antiserum to this protein was raised in chickens as described previously (18). DHFR from calf liver was purified to homogeneity by affinity chromatography as described previously (26), using a modification of the method of Kaufman and Kemerer (20). The concentration of active enzyme was measured by titration at pH 4.8 with [3H]MTX (25). The functional activity of DHFR was measured by radioenzymatic assay using [3H]folic acid as the substrate (24) and by the binding of [3H]MTX (25).

The purified calf liver DHFR was radiolabeled with [3H] by the method of Hunter and Greenwood (17) and then purified through Sephadex G-75 (26). Only the labeled protein which eluted from this column in the fractions corresponding to the elution position of the [3H]MTX-DHFR complex was used as the tracer antigen in the RIA.

RIA. The RIA used for these analytical studies was a sequential noncompetitive procedure (15). The DHFR standards and the sample

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3 Dr. Iqbal's collaboration in this work was accepted by New York University as part of his doctoral requirement under the mentorship of Dr. Albert Gordon.

The abbreviations used are: DHFR, dihydrofolate reductase; RIA, radioimmunoassay; MTX, methotrexate.

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RIA. The RIA used for these analytical studies was a sequential noncompetitive procedure (15). The DHFR standards and the sample
being assayed were incubated separately with diluted antiserum in 0.5 ml of 0.02 M Tris-HCl buffer, pH 7.4, containing 0.05% gelatin, 250 KIU of Trasylol, and 0.1 mg of bovine serum albumin. After 48 hr at 4°C, 0.1 ml of the supernatant containing [3H]-DHFR (approximately 15,000 cpm) was added to each reaction, and the incubation was continued for an additional 16 hr at 4°C. Preliminary tests were carried out to determine the dilution of antiserum which bound approximately 40 to 50% of the [125I]-DHFR in the absence of competing antigen. Antibody-bound DHFR was precipitated by the addition of 10 μl of anti-chicken IgG followed in 30 min by 0.1 ml of 10% polyethylene glycol. The precipitate which formed after 15 min incubation at room temperature was pelleted by centrifugation at 4°C and washed with the cold 0.02 M Tris-HCl buffer. The radioactivity in the precipitate was then measured (Beckman Auto-Gamma counter), and sufficient cpm were accumulated for a counting error of ±3%.

A dose-response standard curve was constructed by plotting on the ordinate the ratio of antibody-bound [125I]-labeled DHFR in the reactions containing the unlabeled competing antigen (or sample being assayed) to antibody-bound [125I]-DHFR in the reaction containing no unlabeled competing antigen (B/Bo) versus, on the abscissa, the corresponding amount of unlabeled DHFR in the standard reactions. The B/Bo ratio was similarly determined for the unknown samples, and the concentration of immunoreactive protein was then obtained by referring this value to the standard curve.

Preparation of Cytoplast and Cytosol from L1210 Leukemia Cells. The ascitic fluid from C57BL × DBA/2 F~ (hereafter called BD2F1) mice, inoculated 6 to 7 days previously, was collected, and the leukemia cells were washed 3 times with cold 0.15 M sodium chloride. The contaminating RBC were lysed by diluting 1 volume of a suspension of cells with 1.5 M sodium chloride with 3 volumes of cold distilled water followed in 30 sec by 1 volume of 0.6 M sodium chloride. The cells were washed once with 0.15 M sodium chloride to remove the free hemoglobin, and the leukemic cells were then packed by centrifugation at 1400 × g for 15 min.

The cytoplasm of these cells was prepared using 2 different methods. For one preparation, the packed cells (0.5 to 1.0 ml) were diluted with 3 volumes of 0.06 M sodium citrate, pH 7.4, and the suspension was frozen at −70°C. To obtain the cytoplasm, the cells were frozen and thawed an additional 3 times, and the particulate was pelleted by centrifugation at 30,000 × g for 30 min. In the text, this supernatant will be referred to as diluted cytoplasm. The second cytoplasm preparation was obtained by freezing and thawing the packed cells (1 ml) without dilution. The cytoplasm was similarly obtained by centrifugation at 30,000 × g for 30 min, and this supernatant will be referred to as undiluted cytoplasm. For some studies, the cell cytosol was prepared by subjecting the lysed cells to centrifugation at 100,000 × g for 60 min.

Gel Filtration. Sephadex G-75 was equilibrated with 0.05 M Tris-HCl buffer, pH 7.4, and packed into a column (1.5 × 75 cm) using a constant flow rate of 12 ml/hr. Sephadex G-200 and Sepharose 4B were equilibrated with 0.05 M Tris-HCl buffer, pH 7.4, containing Trasylol (1000 KIU/liter), phenylmethylsulfonyl fluoride (3.5 mg/liter), and 0.02% sodium azide and packed into a column (2.5 × 100 cm) using a constant flow rate of 12 ml/hr.

Dextran blue and standard marker proteins (indicated in the legends of the charts) were used to calibrate the columns. Each fraction was assayed for immunoreactive protein by RIA and for active enzyme either by catalytic reduction of [3H]Hoc acid (24) or by the binding of [3H]MTX (25).

Studies of the Immunoreactive Proteins. A number of studies were carried out to determine the structural properties of the immunoreactive proteins. The products of each experiment were subjected to gel filtration, and each fraction was assayed by RIA and by the binding of [3H]MTX.

For one experiment, the fractions comprising the void volume of the Sephadex G-75 column and which contained a high-molecular-weight immunoreactive protein were pooled and concentrated to 1 ml by negative pressure. Crystalline urea was then added to a concentration of 8 M, and the mixture was filtered through Sephadex G-200 as described in the legend of Chart 4A except that the eluting buffer also contained 8 M urea. Each fraction was dialyzed for 72 hr against 0.01 M Tris-HCl buffer, pH 7.4, before RIA.

For a second experiment, dithioerythritol was added to 1 ml of cytosol to a concentration of 10 mM (5). The mixture was then filtered through Sephadex G-75 as described in the legend of Chart 3, except that the eluting buffer contained 0.65 mM dithioerythritol.

For a third study, 2 ml of L1210 cytosol were concentrated to 1.2 ml by evaporation in a dialysis tubing at 4°C. Crystalline urea and dithioerythritol were added to the sample to final concentrations of 8 M and 40 mM, respectively. This mixture was incubated at room temperature for 1 hr and then placed in a boiling water bath for 3 min. The sample was then dialyzed overnight at 4°C against 0.05 M Tris-HCl buffer, pH 7.4, containing 5 mM dithioerythritol and then chromatographed through Sephadex G-200 as described in the legend of Chart 4A.

For a fourth experiment, RNase (1 mg) was incubated with 1 ml of cytoplast for 100 min at room temperature. The mixture and a control reaction similarly incubated without RNase were filtered through Sephadex G-200, and each fraction was assayed for immunoreactive protein and functional enzyme activity.

Finally, in this series of experiments, 1 ml of L1210 cytosol was incubated for 120 min at 37°C with 7.2 units of trypsin coupled to polyacrylamide. The enzyme was pelleted by centrifugation, and the supernatant cytosol was chromatographed through Sephadex G-75 as described in the legend of Chart 3.

Isoelectric Focusing. The cytosol of L1210 cells was analyzed by isoelectric focusing in a standard LKB 110-ml column packed with 1% (w/v) amphoteries (LKB, Inc.) at a pH range of 3.5 to 10 in a zero to 50% sucrose gradient. The sample (1 ml) was dialyzed against distilled water for 6 hr to remove electrolytes prior to chromatography. The dialed sample and 25% of the total amphoteries used were added to the solution which was adjusted to contain 50% sucrose ("densed solution"). The remaining amphoteries (75%) were mixed with the "light solution." The electrode solution for the anode (23 ml) was 50% sucrose in 0.19 M HCl. The electrode solution for the cathode (20 ml) was 0.074 M ethylenediamine prepared in distilled water. A fraction of this solution, enough to completely cover the respective electrode, was placed on top of the column. The column was run under constant voltage at 4°C. The potential was 300 V in the initial 12-hr period, 600 V in the subsequent 24-hr period, and 800 V in the final 12-hr period. The column was eluted at a constant flow rate of 100 ml/hr, and 0.9-ml fractions were collected. The pH measurements were carried out at room temperature. The volume of each fraction was neutralized by the addition of 0.5 ml 1 M potassium phosphate buffer, pH 7.4. All fractions were then dialyzed for 20 hr against 0.02 M Tris-HCl buffer, pH 7.4, and assayed for the binding of [3H]MTX and immunoreactive protein.

In Vivo Growth of L1210 Cells in BD2F1 Mice. L1210 cells were harvested on the 4th day following i.p. inoculation into BD2F1 mice. The cells were washed with cold 0.15 M sodium chloride, and the viability (99%) was established by trypan blue exclusion. A group of 40 BD2F1 mice was inoculated i.p. with 5 × 10⁶ cells. On Days 2 through the 7, the cells from several mice were harvested from the peritoneal cavity as described by Hollocoat (16) (the number of mice sacrificed each day is shown in Chart 8A). The cells were pooled, counted, and washed; the packed volume was measured, the viability was determined to be greater than 95% by trypan blue exclusion; and the cytosis was prepared as described above. In addition to measuring the immunoreactive and functional DHFR, the total protein was measured by the method of Lowry et al. (21).

RESULTS

The interaction of the chicken antiserum with DHFR could be demonstrated indirectly by the inhibition of the binding of...
[\textsuperscript{3}H]MTX and directly by the immunoprecipitation of \textsuperscript{125}I-labeled enzyme. As little as 10 \mu l of the antiserum, preincubated with the purified enzyme, blocked the binding of 500 pg of [\textsuperscript{3}H]MTX, which represents the inactivation of 22 ng of enzyme protein (Chart 1). The results of this experiment indicate that at least one class of antibodies is directed at the antigenic site(s) at or near the active center of the enzyme.

This chicken antiserum to the L1210 DHFR does not react with \textsuperscript{125}I-labeled purified folate-binding protein from chronic myelogenous leukemia cells (Table 1) (12). Whereas the homologous rabbit antiserum to this protein precipitated 70% of the radioactivity, the chicken antiserum to DHFR precipitated virtually no radioactivity.

The direct immunoprecipitation of \textsuperscript{125}I-DHFR was exploited to develop the quantitative RIA. Chart 2 shows the dose-response curve obtained using either the purified DHFR or enzyme partially purified by gel filtration of cytoplasm through Sephadex G-75, as the competing unlabeled antigen. The concentration of unlabeled enzyme in these preparations was computed as the molar equivalent of the quantity of [\textsuperscript{3}H]MTX bound at pH 4.8 (27).

Although the competitive effect of the purified and partially purified DHFR appear to be equal as standards for the RIA, the total immunoreactive protein concentration in the cytoplasm measured by reference to this standard curve was substantially greater than the concentration of active enzyme which was measured by the binding of [\textsuperscript{3}H]MTX (Table 2). This observation suggested that gel filtration of the cytoplasm through Sephadex G-75, which was the method used to prepare partially purified DHFR, probably separated some immunoreactive but nonfunctional protein and the active enzyme. Indeed, this proved to be the explanation as shown by the chromatogram in Chart 3. Two immunoreactive proteins could be identified: one appearing in the void volume and which had no catalytic activity for the reduction of \textsuperscript{[3]}Hfolic acid; and a smaller immunoreactive peak which eluted later and which corresponds to the elution position of the active enzyme. It should be appreciated that the concentration of the nonfunctional high-molecular-weight form of the immunoreactive protein shown in the chromatogram is a relative value which indicates the antigenic sites in these column fractions which correspond to the immunoreactivity of the standard active enzyme used in the RIA. It is not a quantitative expression for the mass concentration of this protein.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Functional DHFR (ng/mg protein)</th>
<th>Nonfunctional immunoreactive protein (ng/mg protein)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>248</td>
<td>172</td>
</tr>
<tr>
<td>2</td>
<td>113</td>
<td>324</td>
</tr>
<tr>
<td>3</td>
<td>116</td>
<td>199</td>
</tr>
<tr>
<td>4c</td>
<td>95</td>
<td>110</td>
</tr>
<tr>
<td>a</td>
<td>These are cytoplasms prepared from different peritoneal collections of the BD2F1 mice.</td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>Measured by the binding of \textsuperscript{[3]}HMTX as described in the text.</td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>This sample was stored at -40 \degree C for about 36 months before being assayed.</td>
<td></td>
</tr>
</tbody>
</table>

Chart 4A shows the separation of the nonfunctional immunoreactive protein and functionally active DHFR by gel filtration of cytosol through Sephadex G-200. A major peak of nonfunctional immunoreactive protein appears close to the void volume with some heterogeneity evident by the trailing of some smaller immunoreactive components. The second major immunoreactive peak coelutes exactly with functional DHFR, which was titrated by the binding of [\textsuperscript{3}H]MTX and which is equivalent to the concentration of the immunoreactive enzyme measured by RIA.

An apparent mass of 318,000 daltons for the high-molecular-weight immunoreactive protein was computed from its elution through Sepharose 4B (Chart 4B) using the void volume,
Two ml of sample were applied to the column (1.5 x 75 cm) and eluted at a flow rate of 12 ml/hr. One-ml fractions were collected, and an aliquot was assayed for immunoreactive protein by RIA and for active enzyme by the reduction of [3H] folic acid. The column was calibrated using dextran blue (D.B.), cytochrome c, and [3H]2O. See text for the explanation for expressing the concentration of nonfunctional immunoreactive protein in ng of DHFR.

Chart 3. Gel filtration of diluted L1210 cytosol through Sephadex G-75. Two ml of sample were applied to the column (1.5 x 75 cm) and eluted at a flow rate of 12 ml/hr. One-ml fractions were collected, and an aliquot was assayed for immunoreactive protein by RIA and for active enzyme by the reduction of [3H] folic acid. The column was calibrated using dextran blue (D.B.), cytochrome c, and [3H]2O. See text for the explanation for expressing the concentration of nonfunctional immunoreactive protein in ng of DHFR.

Chart 4. A. gel filtration of L1210 cytosol through Sephadex G-200. Two ml of cytosol were applied to the column (2.5 x 100 cm) and eluted at a flow rate of 12 ml/hr, and 1.4-ml fractions were collected. Each fraction was assayed for immunoreactive protein by RIA and for the binding of [3H]MTX. Dextran blue (D.B.) and [3H]2O were used to determine the void volume and total column volumes, respectively. B. gel filtration of L1210 cytosol through Sepharose 4B. Two ml of cytosol were applied to the column (2.5 x 100 cm) and eluted as described in A. In addition to dextran blue (not shown), active DHFR, [3H]2O, and [125I]labeled thyroglobulin were used to calibrate the column.

Thyroglobulin (M, 669,000), and active DHFR (M, 20,000) as column markers (2, 22).

The high-molecular-weight nonfunctional immunoreactive protein which was separated from the active enzyme by filtration through Sephadex G-200 also neutralized the blocking effect of the antiserum on the binding of [3H]MTX by purified enzyme (Table 3). This experiment indicates that this nonfunctional large species of immunoreactive protein also has some antigenic homology with the functional enzyme at or near its active site.

Multimolecular variant forms of an enzyme (or other functional protein) may occur as a consequence of a number of biochemical phenomena (19). These include protein aggregation due to hydrophobic, hydrogen, ionic, or disulfide bonding; polymerization of active subunits; attachment to some carrier polypeptide (7) or other macromolecule; and coupling to some prosthetic carbohydrate or lipid adduct. Accordingly, a number of preliminary experiments were carried out to identify which, if any of the above, characterize this high-molecular-weight immunoreactive protein.

To determine whether this high-molecular-weight protein might be the consequence of an equilibrium between spontaneously aggregating and disaggregating units of active enzyme as

\[ n[E] \rightleftharpoons [E] \]

two experiments were carried out. First, the fractions from the Sephadex G-75 column containing the large species of immunoreactive protein were pooled, concentrated under negative pressure, and rechromatographed through Sephadex G-75. The chromatogram in Chart 5A shows that there was no disaggregation of the large species into a smaller immunoreactive component corresponding to the elution position of functional enzyme.

For the second experiment, cytosol from a fresh sampling of L1210 cells was prepared and passed immediately through a small-affinity column of MTX coupled to Sepharose 4B. The column was washed free of protein, the active enzyme was eluted with a solution of dihydrofolate (18), and a fraction of the eluate was applied immediately to the Sephadex G-75 column. The chromatogram in Chart 5B shows that virtually all the nonfunctional large species of immunoreactive protein has been removed, and only the immunoreactive peak which corresponds to the fractions which bind [3H]MTX remains. There was no spontaneous aggregation of the active enzyme into the large species of immunoreactive protein.

The denaturation experiments using 8 M urea with and without dithioerythritol and with and without boiling did not disassociate the high-molecular-weight nonfunctional protein (Chart 6). Treatment of the cytoplasm with RNase also did not alter the high-molecular-weight protein (data not shown), indicating

Table 3

<table>
<thead>
<tr>
<th>Experiments</th>
<th>[3H]MTX bound (pg)</th>
<th>Inhibition (%)</th>
<th>Neutralization (%)</th>
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<tbody>
<tr>
<td>1. DHFR + [3H]MTX</td>
<td>519</td>
<td>43</td>
<td>100</td>
</tr>
<tr>
<td>2. Antiserum (4 µl) + DHFR + [3H]MTX</td>
<td>297</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>3. Antiserum + immunoreactive protein (18 ng) + DHFR + [3H]MTX</td>
<td>485</td>
<td>7</td>
<td>84</td>
</tr>
<tr>
<td>4. Antiserum + immunoreactive protein (72 ng) + DHFR + [3H]MTX</td>
<td>519</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

* The reaction was carried out in the Tris-HCl buffer used for gel filtration through Sephadex G-200 and contained, in 0.5 ml, 600 pg [3H]MTX, NADPH (0.024 µmol), and sufficient purified L1210 DHFR to bind approximately 500 pg of the inhibitor. To determine the immunoinhibition, the antiserum was incubated with the enzyme for 15 hr at 4 °C before the addition of the [3H]MTX. To determine the neutralization of the antiserum, an aliquot of the fraction from the Sephadex G-200 column containing the nonfunctional immunoreactive protein was incubated with the antiserum for 15 hr before the addition of the enzyme. For Experiment 1, the enzyme was incubated alone for 15 hr before the addition of the [3H]MTX. These values were determined by RIA.
that it is not nascent polypeptide chains attached to polysomes (1). Exposure to trypsin effectively destroyed 80% of the large species and 100% of the low-molecular-weight active enzyme.

Isoelectric focusing of the L1210 cytosol is shown in Chart 7. There are 2 pH's, pH 8.0 and pH 8.5.

Chart 7. Isoelectric focusing of the cytosol of L1210 cells. The conditions of the procedure are described in the text. The pH ampholyte range was 3.5 to 10.0. •, nonfunctional component of the immunoreactive protein, computed by subtracting the concentration of active DHFR which was measured by the binding of [3H]MTX (○). There are 2 pH's, pH 8.0 and pH 8.5.

7. Two immunoreactive isoproteins were identified with isoelectric points corresponding to those of the active enzyme (pl 8.0 and 8.5). It is of interest that the major immunoreactive isoprotein peak focusing at pH 8.5 contains a lower proportion of functional enzyme than does the minor immunoreactive isoprotein focusing at pH 8.0, which contains a higher proportion of functional enzyme.

Chart 8 shows the in vivo growth of L1210 cells in BD2F1 mice.
mice and the analysis of the cytosol of these cells for nonfunctional immunoreactive protein, functional enzyme, and soluble protein on the days corresponding to log and stationary growth. The nonfunctional immunoreactive protein increased rapidly, peaking at Day 2 and then rapidly decreased reaching a nadir on Day 4, whereas the active enzyme initially decreased during early log growth reaching a nadir on Day 2, before increasing to a maximum on Days 3 and 4. Hillcoat (16) observed similar changes in the concentration of active dihydrofolate during in vivo log growth. As expected (16), the total soluble protein also increased during log growth of the cells. During stationary growth, between Days 5 and 7, the concentration of nonfunctional immunoreactive protein and the active enzyme appear approximately equal.

The cytosols of the cells on Day 0 and Day 4 of this study have similar values for the concentration of nonfunctional immunoreactive protein (45 and 48 ng/10^7 cells, respectively) and functional enzyme (87 and 72 ng/10^7 cells, respectively). This is expected because the inoculating cells were taken from Day 4 of logarithmically growing cells. However, the protein concentration of the cytosol was substantially lower on Day 0 (0.23 ng/10^7 cells) than on Day 4 (0.39 ng/10^7 cells). Although the precise reason for this finding has not been established, it could reflect the fact that the Day 4 inoculum (experimental Day 0 cells) was prepared from stationary-phase cells whereas the experimental Day 4 cells are derived from log-phase cells. In addition, the inoculum cells were obtained from 2 mice, whereas the Day 4 experimental cells were obtained from 7 mice so that the difference in protein concentration could be simply a statistical variation.

**DISCUSSION**

RIA, in addition to sensitivity, provides a number of advantages when measuring and characterizing proteins with a known function: (a) by measuring the mass concentration of the protein, it can complement the traditional method of measuring a functional protein, such as an enzyme, which is based on functional activity alone (9); (b) RIA can provide the analytical tool to identify modified forms of the protein which retain antigenicity but which have lost function. Indeed, our original purpose for developing the RIA was to identify the catabolic products of DHFR, an enzyme which appears to be susceptible, at least in some human cells, to rapid intracellular degradation (3). However, neither the nonfunctional immunoreactive DHFR identified in human leukemia cells (26) nor this species of nonfunctional immunoreactive enzyme in L1210 cells is likely to be a product of intracellular catabolism, since such a process would produce immunoreactive fragments which are smaller than the active enzyme.

When RIA is used to study and quantify an apparent immunoreactive factor which is reacting with the antiseraum, it is necessary to ensure that inhibition of the antibody binding of the tracer-labeled antigen by the unknown sample is due to a molecule with antigenic site(s) which are the same as the homologous antigen and not to some other nonspecific factors in the biological material (29). There can be little doubt about the validity of the RIA used in this study to measure active DHFR because the concentration of functional enzyme isolated by gel filtration was the same whether measured by RIA or the binding of [3H]MTX. There is also substantial evidence to conclude that the high-molecular-weight nonfunctional immunoreactive protein separated from the active enzyme by gel filtration has antigenic determinants in common with the purified L1210 DHFR which was used to raise the antiseraum: (a) the immunoreactive material was not lost when these fractions were dialyzed overnight as would frequently occur with low-molecular-weight “nonspecific inhibitors” in an RIA; (b) the gel filtration containing the high-molecular-weight material also neutralized the inhibitory effect of the antiseraum on the activity of DHFR indicating that this big species of immunoreactive protein also has antigenic homology with the functional enzyme at or near its active site; (c) it is highly unlikely that “nonspecific inhibitors” in the RIA would focus at the same pH as did the 2 active isoenzymes when analyzed by isoelectric focusing.

High-molecular-weight forms of DHFR have been identified in some species of *Trypanosoma* and *Crithidia* (14) and *Plasmodium berghei* (11). Unlike the high-molecular-weight species identified in the L1210 cells, these forms of enzyme are catalytically active. In fact, these organisms do not have the low-molecular-weight DHFR characteristic of either mammalian cells or bacteria, and it is therefore not a form of intracellular molecular heterogeneity.

High-molecular-weight variant forms of intracellular enzymes are not unique. For example, a high-molecular-weight form of adenine deaminase has been characterized as the active enzyme coupled to a carrier protein (7). A high-molecular-weight variant of terminal deoxynucleotide transferase has also been characterized (8). Both of these variant enzymes, however, are catalytically active, thus differing from the high-molecular-weight protein in the L1210 cells.

Further studies are clearly necessary to define the nature of this high-molecular-weight immunoreactive protein, particularly a comparison of its constituent amino acids with those of the active form of DHFR. The results of the isoelectric focusing, however, minimize the possibility that this large species of immunoreactive protein is a form of active enzyme coupled to some carrier polypeptide or other macromolecule because such complexes usually have isoelectric points which differ from the isolated subunits (28). The most plausible explanation for the similar pl’s of these 2 immunoreactive species of DHFR is that the large species is a polymeric form of the enzyme, because only then would its net charge be equal to the net charge of a single active subunit. An estimated molecular weight of 318,000 for the high-molecular-weight species in this instance would mean a polymer composed of 16 subunits of active enzyme, each having a molecular weight of 20,000 (23). The denaturant and thiol-resistant properties of this form of immunoreactive protein indicate that its intermolecular bonding is covalent and therefore that it is not likely to be a posttranslational modification of the active enzyme. It is of interest in this regard that Dolnick and Bertino (10) recently identified several species of mRNA for DHFR, one having a size greater than 23.7 kilobases. Their studies did not exclude the possibility that this large species of mRNA could translate for a high-molecular-weight nonfunctional variant of DHFR.

The early rise and subsequent decrease in the concentration of the nonfunctional immunoreactive DHFR during *in vivo* log growth of the L1210 cells merits some discussion. This finding provides additional evidence that this immunoreactive protein is a real phenomenon because it is extremely unlikely that the concentration of some nonspecific inhibiting factor would vary.
in the cytosol with such precise inverse synchrony with the concentration of functional DHFR during log growth of the cells. This inverse relationship of the peak and nadir concentrations of these immunoreactive proteins, taken with the similar pls of each pair of immunoreactive isoproteins, supports the notion that the nonfunctional form of immunoreactive enzyme protein may be a precursor form of the active enzyme.

The de novo pathway for the synthesis of TMP is required for the replication of most cells, and active DHFR is essential to maintain the tetrahydrofolate pool required for this process. Any interference with the mechanism for transformation of an inactive precursor to the active enzyme could, therefore, be the molecular basis for disorders characterized by impaired cell replication, similar to the megaloblastic anemias in which this pathway is impaired as a consequence of folate or cobalamin deficiency.

DHFR has also been the target enzyme for antifolate drugs in order to block de novo TMP synthesis. An inactive precursor of DHFR which does not react with MTX could provide a mechanism by which cells can bypass this metabolic block to DNA synthesis. For example, during time periods between the administration of MTX, when the intracellular concentration of the drug is low, this precursor pool could be a readily available source of active enzyme, particularly during phases of rapid cell proliferation when its concentration in the cells appears to be high. The covalent structure of this high-molecular-weight species would require some proteolytic mechanism to dissociate the active subunits, and this would not be unlike the process by which the high-molecular-weightzymogen form of some hormones (e.g., proinsulin) is converted to the active hormone molecule.

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Identification of a High-Molecular-Weight Nonfunctional Protein in L1210 Leukemia Cells with Common Antigenic Determinants to Dihydrofolate Reductase

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