A Macromolecular Factor in Some Leukemic Cells Which Binds Folic Acid[1] (34489)

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(Introduced by S. A. Berson)
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An extremely sensitive radioenzymatic assay for folate reductase (EC 1.5.1.3.) has recently been developed in this laboratory using high specific activity ^{3}H-folic acid as the substrate (1). Unlike spectrophotometric methods (2, 3), which have been in general use to study this enzyme and which require sufficiently high substrate (folate or dihydrofolate) and reduced nicotinamide-adenine dinucleotide phosphate (NADPH) to give reliable optical density changes, this radioassay can measure directly the reduction of as little as 0.1 of a $\mu\mu$mole of ^{3}H-folate to tetrahydrofolic acid (FH_4). The method is advantageously adapted to measure maximal enzyme activity because a decrease in the percentage of ^{3}H-folate converted to FH_4 occurs by competitive inhibition when stable folic acid, but, paradoxically, when the total concentration and enzyme-reacting sites are saturated (4).

In the course of studying the activity of this enzyme in different human leukemia cells, using this radioassay procedure, an unexpected and unusual observation was made. The cell lysates from two patients with chronic myelogenous leukemia appeared to have little or no enzyme activity when the reaction mixture contained 0.57 $\mu\mu$moles ^{3}H-folic acid, but, paradoxically, when the total folate concentration was increased by the addition of stable folic acid, reduction of the ^{3}H-folic acid then became apparent. This report will demonstrate that this phenomenon was due to the binding of the tracer substrate by a macromolecular factor in these leukemic cell lysates.

Materials and Methods. The preparation of cell lysates and details of the enzymic reaction have been previously reported (5, 6). Briefly, the leukemic cells were separated from heparinized whole blood by either spontaneous sedimentation or slow centrifugation at 4°, washed thrice with cold 0.15 *M* NaCl, resuspended in 3 vol of 0.05 *M* sodium citrate and frozen at −65°. Enzyme activity was measured in the supernate after thawing and centrifugation of the cell debris. High specific activity ^{3}H-folic acid (Amersham/Searle) was used as the isotopically labeled substrate. The total substrate concentration in the enzymic reaction mixtures was increased by the addition of stable folic acid (Sigma). The reaction mixtures contained 0.57 $\mu\mu$moles ^{3}H-folic acid, 0.06 μmoles NADPH, stable folic acid as indicated, and 0.18 μmoles 2-mercaptoethanol in 0.4 ml of 0.02 *M* citrate buffer, pH 4.8. The reaction was started by the addition of 0.1 ml of the leukemic cell lysate, and after 30 min at 37° the reaction was stopped by the addition of 0.2 ml of a 0.27 M solution of stable folic and 0.2 ml of a 5% solution of $ZnSO_4 \cdot 7H_2O$. After centrifugation of the folate precipitate, the radioactivity of the ^{3}H-FH_4 in an aliquot of the supernatant solution was assayed by liquid-scintillation counting, using a scintillation solution described below, and the percentage of ^{3}H-folic acid reduced was calculated (1).

Gel-filtration studies were carried out using Sephadex G-75 in a 22 × 0.8-cm column equilibrated with 0.05 *M* sodium citrate. A 0.5-ml volume containing ^{3}H-folic acid (1.14

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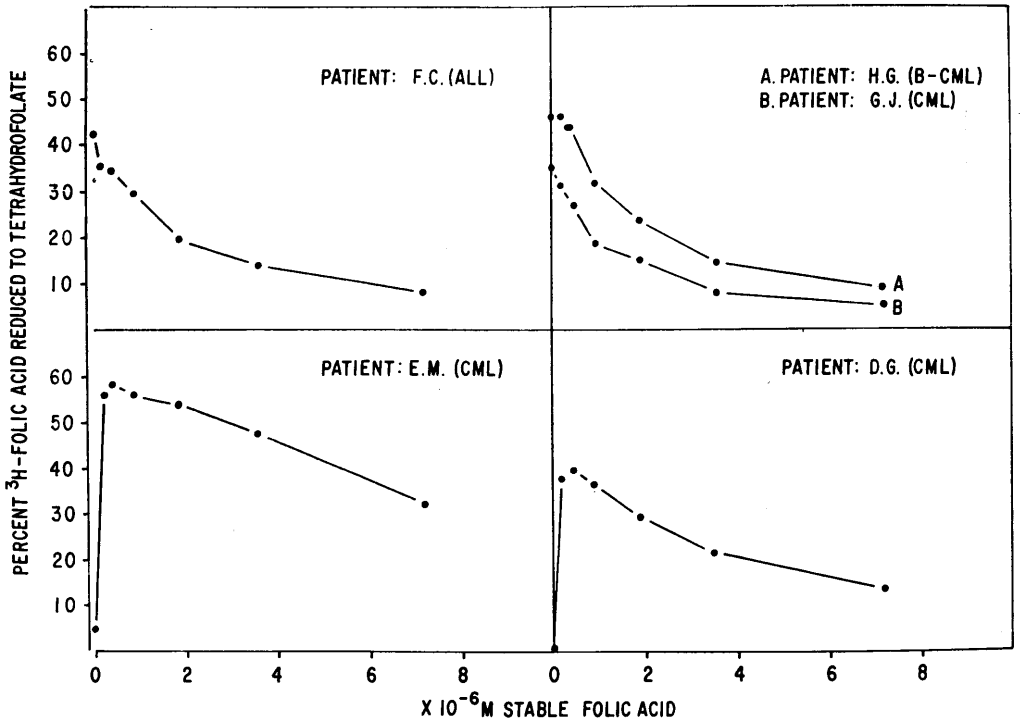

FIG. 1. Percentage of ^{3}H-folic acid reduced to FH_4 with increasing concentrations of stable folic acid. With the cell lysates from patients E. M. and D. G., significant reduction of the tracer substrate was not evident until stable folic acid was added. CML = chronic myelogenous leukemia. ALL = acute lymphoblastic leukemia. B-CML = blast crisis of chronic myelogenous leukemia.

$\mu\mu$moles) and 0.05 ml of leukemic cell lysate in 0.05 *M* sodium citrate was filtered through the column, and 10 drop fractions were collected. The radioactivity in 0.2 ml of each fraction was determined by liquid-scintillation counting using a toluene scintillation solution containing 5 g/liter of 2, 5-diphenyloxazole (PPO) and 100 ml/liter of BBS-3 solubilizer (Beckman Instruments).

Results. The percentage of ^{3}H-folic acid reduced to FH_4 at increasing concentrations of stable folate by cell lysates prepared from five patients with leukemia is shown in Fig. 1. With lysates from patients G. J., H. G., and F. C., as expected, the percentage of ^{3}H-folic acid converted to FH_4 was at or near the highest value at the tracer folic acid concentration (zero value on abscissa for stable folic acid) and then decreased as the concentration of stable folic acid increased, indicating that the substrate was saturating the enzyme-reacting sites. However, with lysates from patients E. M. and D. G. (both of whom had chronic myelogenous leukemia) only 5% and zero, respectively, of the ^{3}H-folic acid was reduced at the tracer concentration ($1.14 \times 10^{-9} M$), whereas 56 and 38%, respectively, of the ^{3}H-folic acid was converted to FH_4 when the substrate concentration was raised by the addition of stable folic acid ($0.22 \times 10^{-6} M$). A further increase in the stable folic acid concentration then decreased the percentage of ^{3}H-folate reduced to FH_4 by competing for enzyme-reacting sites. This observation was confirmed by several replicate determinations on samples of cell taken at different times from each patient.

This peculiar activity of the cell lysates from these two patients with chronic myelogenous leukemia was also evident when they were mixed with lysate from the cells of patient G. J. which contained "normal" enzyme activity. This patient had chronic myelo-

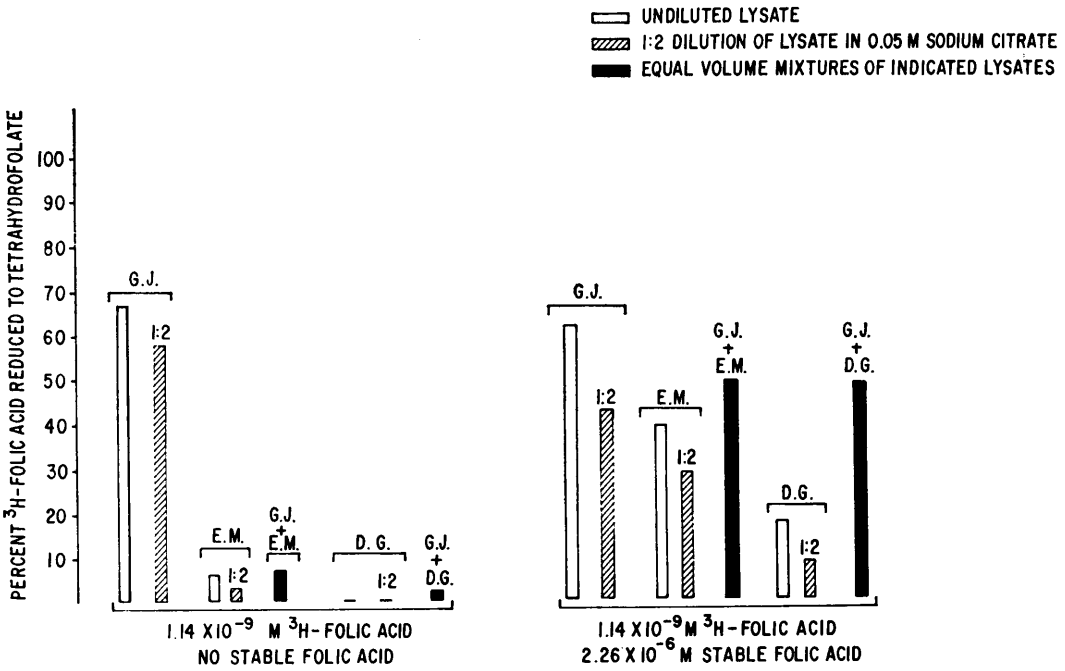

FIG. 2. Percentage of ^{3}H-folic acid reduced to FH_4 by mixtures of leukemic cell lysates from patients E. M. and D. G. with the cell lysate from patient G. J. This sample from patient G. J. was taken at a later date than the sample assayed for the data in Fig. 1, and it had higher enzyme activity. Note that at low substrate concentration (no stable folic acid) the lysates from patients E. M. and D. G. were inhibitory. When stable folic acid was added, however, this inhibition was no longer apparent.

genous leukemia but was entering a subacute blast crisis at the time of study. Figure 2 shows the marked decrease in enzyme activity of the lysate from G. J. at the tracer substrate concentration when mixed with the lysates from either E. M. or D. G. However, as noted with the individual lysates, when the substrate concentration was raised to 2.26×10^{-6} *M* by the addition of stable folic acid the inhibition observed at the tracer concentration was no longer apparent.

When lysates from the leukemic cells from patients E. M. and D. G. were incubated with ^{3}H-folic acid and subjected to gel filtration on Sephadex G-75, as shown in Fig. 3, the major peak of radioactivity appeared in the excluded volume in contrast to the filtration of free ^{3}H-folic acid or ^{3}H-folic acid incubated with the "normal" leukemic cell lysate from patient G. J., where all the radioactivity appeared later with the inner filtration volume. As shown in Fig. 4, however, when lysate from patient E. M. was added to a mixture of the tracer folic acid and 20 $\mu\mu$moles of stable folic acid and similarly filtered through the gel, the major peak of radioactivity then appeared later with the inner volume.

Discussion. The results of these experiments indicate that the cells of some patients with chronic myelogenous leukemia contain a macromolecular factor (mol wt 50,000 or greater) which inhibits the enzymic reduction of folic acid by binding this substrate. The reduction of ^{3}H-folic acid to FH_4 became evident at higher folic acid concentrations only because the stable folic acid added to the reaction system to raise the total substrate concentration competitively inhibited the tracer folate from binding to this factor permitting it to react with the enzyme.

This peculiar behavior of these cell lysates with respect to the enzymic reduction of folic acid may explain the elevated folate levels

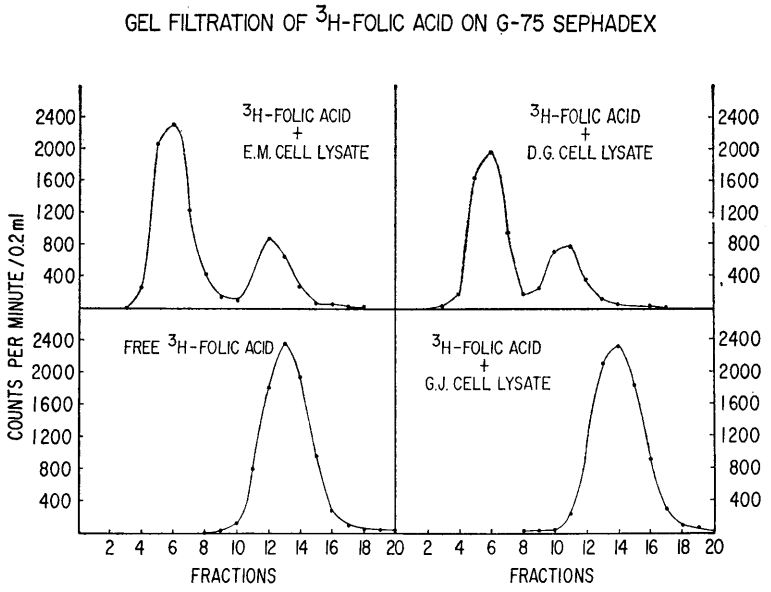

FIG. 3. Gel filtration on Sephadex G-75 of free ^{3}H-folic acid and ^{3}H-folic acid incubated with the leukemic cell lysates from patients E. M., D. G., and G. J. Early filtration peaks of radioactivity occurred only when the ^{3}H-folic acid was preincubated with the lysates from patients E. M. and D. G. indicating that the folate was bound to a factor greater than 50,000 mol wt.

found in this type of leukemia (7), since a high concentration of substrate would have to accumulate in the cells to saturate this binding factor before any folate would be available for enzymic reduction. On the other hand, if some cells fail to achieve this critical concentration of folic acid (or dihydrofolate, since this may also be bound), a deficiency of tetrahydrofolate coenzymes may result and be responsible for the giant metamyelocytes and multisegmented polymorphonuclear cells occasionally seen in this disease.

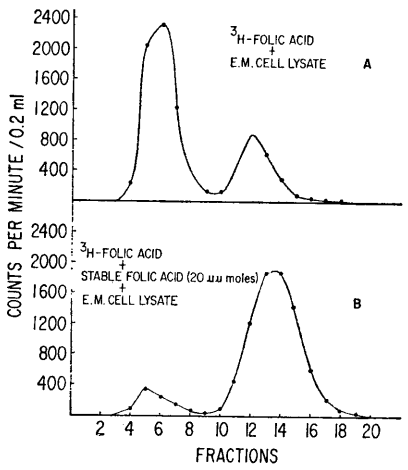

FIG. 4. Gel filtration on Sephadex G-75 of lysate from patient E. M. incubated with ^{3}H-folic acid alone (A) and with a mixture of ^{3}H-folic acid and stable folic acid (B).

Summary. The reduction of 0.57 μμmoles of ^{3}H-folic acid to tetrahydrofolic acid by folate reductase in the cell lysates from two patients with chronic myelogenous leukemia was not apparent until the total substrate concentration of the reaction mixture was raised by the addition of stable folic acid. In addition, these leukemic cell lysates inhibited the reduction of tracer ^{3}H-folic acid by a leukemic cell lysate containing "normal" folate reductase activity. Gel-filtration experiments demonstrated that this phenomenon was due to binding of the tracer folate to a large molecule because ^{3}H-folic acid preincubated with these peculiar leukemic cell lysates and filtered through Sephadex G-75 appeared in the early or excluded volume indicating that the substrate was bound to a factor with a molecular weight of at least 50,000. This binding of ^{3}H-folic acid could be competitively inhibited by stable folate.

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