Further Observations on the Folate-Binding Factor in Some Leukemic Cells

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ABSTRACT The lysates of peripheral cells as well as the serum from some patients with chronic myelogenous leukemia, contained a macromolecular factor which bound tritiated folic acid. Bound tracer folate filtered through Sephadex G-75 and G-100 columns with the early effluent and appeared with the inner volume through a Sephadex G-200 column. Bound tracer could not be extracted from solution by coated charcoal or the anion exchange resin Dowex 2-X8 and could not be reduced to tetrahydrofolate by folate reductase. The velocity of the binding reaction was very rapid and dissociation of bound tracer extremely slow. Binding decreased sharply below pH 5.0 and the binding factor as well as the folate-binder complex, resisted 56°C for 30 min. The binding factor in the leukemic lysate could be separated from endogenous folate reductase by filtration through a G-75 Sephadex column.

Competitive inhibition studies demonstrated little or no inhibition of binding of tritiated folic acid by formyltetrahydrofolate and methyltetrahydrofolate. Diopterin (pteroyldiglutamate), pteropterin (pteroyltriglutamate), methotrexate, and dihydrofolate inhibited binding of tracer folate but not as effectively as unlabeled folic acid.

The function of this folate binder is unknown. However, that it reacts with dihydrofolate suggests some relationship (physiologic or pathologic) to DNA synthesis since this folate cofactor is essential for the de novo synthesis of thymidylate from deoxyuridylate. In addition, these findings also suggest that the binding of methotrexate may, like folate, inhibit its reaction with folate reductase, and thus be a mechanism by which leukemic cells become resistant to this drug.

This work was presented in part at the annual meeting of the American Society for Clinical Investigation, 3 May 1970.

Dr. Rothenberg is a Career Scientist, New York City Health Research Council, Contract I-423.

Dr. daCosta is a Kresevich Foundation Research Fellow.

Received for publication 18 May 1970 and in revised form 13 August 1970.

INTRODUCTION

The cellular uptake of folic acid has been studied by several investigators (1–3) and there is suggestive but no direct evidence for an intracellular binding site other than folate reductase (Enzyme Commission 1.5.1.3), the enzyme which catalyzes the reduction of folate and dihydrofolate to tetrahydrofolate (4). In blood, as well, there is no definite evidence for a specific folate binder. When folic acid is added to serum, the per cent bound remains fairly constant over a wide concentration range (5), the binding is reversible (6), and the bound vitamin can easily be extracted with charcoal (7).

A previous communication from this laboratory (8) presented evidence that the lysate of cells from two patients with chronic myelogenous leukemia, bound tritiated folic acid. This binding factor has now been found in the cell lysates from two additional patients with this disease. Serum available for testing from three of these patients also contained a folate binder. The properties of this binding factor have been studied in detail and the findings form the basis of this report.

METHODS

Tritiated folic acid (FA-^{3}H or tracer FA) was purchased from Amersham/Searle Corp., Arlington Heights, Ill. The purity of this compound, established by coprecipitation with unlabeled folic acid (FA), varied from 70 to 90% and appropriate corrections were made for this nonfolate radioactivity (9). Unlabeled FA[1] was obtained commercially and the concentration of each stock solution was established spectrophotometrically using an $E^{1\%}_{1\text{ cm}}$ of 206 at 365 mμ. Dihydrofolic acid (FH_2) was prepared by the method of Blakely (10) and N^5-methyltetrahydrofolic acid (methyl FH_4) by the method of Keresztesy and Donaldson (11). The folate analogues, diopterin, pteropterin, and formyl-

[1] *Abbreviations used in this paper:* FA, Folic acid; AML, acute myeloblastic leukemia; ALL, acute lymphocytic leukemia; CML, chronic myelogenous leukemia; CLL, chronic lymphocytic leukemia; Km, Michaelis constant; MTX, methotrexate.

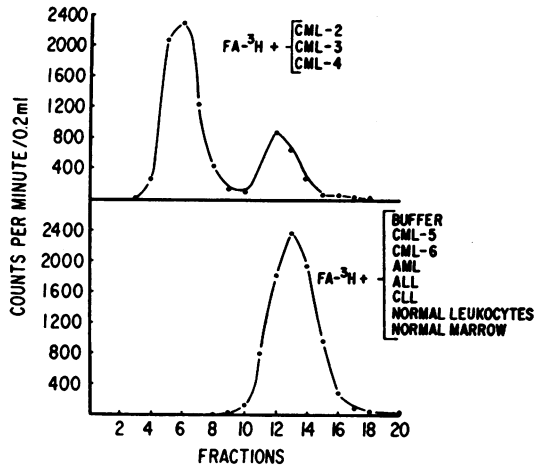

FIGURE 1 Radiochromatograms of FA-³H filtered through Sephadex G-75. Upper: most of the radioactivity of FA-³H incubated with either CML-2, CML-3, or CML-4 lysate appeared with the early effluent corresponding to the filtration of dextran blue. Lower: all the FA-³H filtered with buffer or after incubation with each of the indicated lysates appeared with the inner volume of the column.

tetrahydrofolate (formyl FH_4) were obtained by courtesy of Lederle Laboratories, Inc., Pearl River, N. Y.

Leukocyte lysates were prepared from the peripheral blood of patients with acute myeloblastic leukemia (AML), acute lymphocytic leukemia (ALL), chronic myelogenous leukemia (CML), chronic lymphocytic leukemia (CLL), and from normal peripheral blood and normal bone marrow as previously described (12).

Folate reductase was assayed by a radioenzymatic method using FA-³H as the substrate (9). The protein concentration of the lysates was determined by the method of Lowry, Rosebrough, Farr, and Randall (13).

The reaction between cell lysate or serum and FA was studied by incubating an aliquot (0.01–0.1 ml) of test sample with 0.05 ml of FA-³H (0.1–0.5 ng) in sufficient 0.05 M sodium citrate for a total volume of 0.5 ml and the mixture incubated for 30 min at room temperature. The following methods were then used to demonstrate binding of the tracer FA:

(*a*) The whole reaction mixture was filtered through G-75, G-100, or G-200 Sephadex columns using 0.05 M sodium citrate as the eluant and collecting 0.5 ml fractions. The sephadex gels were first hydrated with distilled water and columns (0.5 × 25 cm) of each prepared by gravity packing and equilibration with 0.05 M sodium citrate. (*b*) 0.5 ml of a 2.5% aqueous suspension of charcoal (Norit-A) previously coated with bovine albumin was added to the reaction mixture to adsorb the free FA-³H. The coated charcoal was freshly prepared by mixing equal volumes of a 1% solution of bovine albumin and a 5% suspension of charcoal (14), centrifuging the charcoal, washing it once with water, and resuspending it in sufficient distilled water to make a 2.5% suspension. (*c*) Approximately 100 mg of the anion exchange resin Dowex 2-X8 was added directly to the reaction mixture to bind the free FA-³H.

The radioactivity of aliquots of the column fractions and the charcoal and Dowex supernatants was assayed in 15 ml of a scintillation solution containing 5 g of 2,5-diphenyloxazole and 50 ml of BBS-3 solubilizer (Beckman Instruments, Inc., Fullerton, Calif.) per liter toluene use of a liquid scintillation counter (Nuclear-Chicago, Des Plaines, Ill.). Sufficient counts were accumulated for a counting error of 4% or less.

The effect of hydrogen ion concentration on the binding of FA-³H was studied using mixtures of 0.05 M citric acid and 0.05 M sodium citrate as the diluent for the reactions of pH 7.6 or below. 0.02 M Tris was used with either 0.1 N NaOH or 0.1 N HCl for pH values greater than 7.6. The effect of temperature was determined by heating binding lysate and lysate-bound FA-³H at 56°C. The specificity of the binding factor was studied by determining the competitive effect of unlabeled folic acid and other pteridine analogues on the binding of FA-³H. The rate of binding of FA-³H was studied by removing an aliquot of a reaction mixture at timed intervals and rapidly adding it to 0.5 ml of the coated charcoal suspension. Dissociation was studied by adding a swamping excess of unlabeled folic acid (0.15 mg) to FA-³H previously incubated with binding lysate, and at timed intervals determining the bound radioactivity by adding an aliquot of the reaction mixture to 0.5 ml of the charcoal suspension.

RESULTS

The filtration patterns of FA-³H through G-75 Sephadex with buffer alone and after incubation with the lysates of various leukemic cells, normal bone marrow, and normal leukocytes are shown in the radiochromatograms in Fig. 1. After incubation with CML-2, CML-3, or CML-4 cell lysates the major fraction of radioactivity filtered with the early effluent indicating that tracer folate was bound to a factor with a mol wt of at least 50,000. In contrast, after incubation with the lysates of other cells, or when filtered alone, all of the tracer appeared with the inner volume indicating that the folate was either free or bound to a factor with a mol wt of less than 50,000.

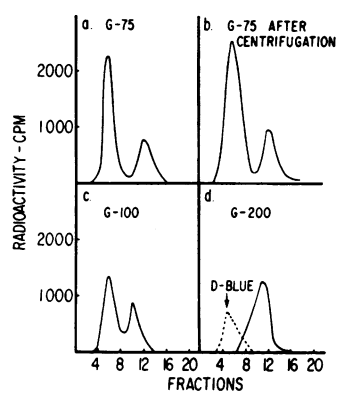

FIGURE 2 Radiochromatograms of FA-³H filtered through G-75 (*a*), G-100 (*c*), and G-200 (*d*) Sephadex columns after incubation with CML-2 lysate. The pattern in (*b*) was obtained when FA-³H was filtered through G-75 Sephadex after incubation with the supernate of CML-2 lysate after it had been subjected to centrifugation at 105,000 *g* for 60 min.

The radiochomatograms in Fig. 2 show that FA-^{3}H incubated with CML-2 lysate filtered with the early fractions through G-100 as well as G-75 but appeared with the inner volume through G-200 Sephadex. When CML-2 lysate was subjected to centrifugation at 105,000 *g* for 60 min and the supernate incubated with FA-^{3}H, the radioactivity again filtered with the excluded volume of G-75 Sephadex (Fig. 2*b*). Thus, this FA-binding factor appears in the soluble fraction of the cell cytoplasm and has a mol wt between 100,000 and 200,000.

The filtration of FA-^{3}H through G-75 Sephadex after incubation with serum from CML-2 and CML-3 patients is shown in Fig. 3. For these experiments the reaction mixture was treated with 200 mg of dry albumin coated charcoal to remove free FA-^{3}H before filtration. Most of the radioactivity not removed by charcoal appeared in the early fractions indicating that the serum from these patients also bound folic acid. The small fraction of radioactivity appearing with the inner volume was, for the most part, an impurity in the FA-^{3}H preparation which also could not be adsorbed from buffer solution with charcoal. Some of this nonadsorbable radioactivity may have also been bound to low molecular weight serum proteins, but it represented only a small per cent of the total radioactivity added. When other leukemic serums, obtained from patients without the binding factor in their cell lysates, and normal serums were incubated with FA-^{3}H and similarly treated with charcoal, the small fraction of unadsorbed radioactivity filtered with the inner volume of the column and no FA-^{3}H appeared with the early effluent.

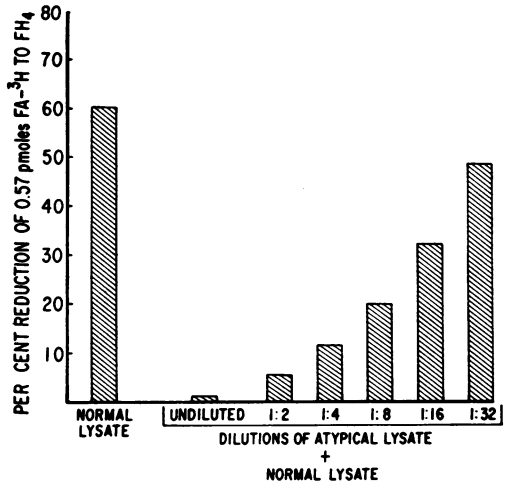

FIGURE 4 Inhibition by "atypical" CML-2–binding lysate of reduction of 0.57 pmoles of FA-^{3}H by folate reductase in a leukemic lysate considered "normal" because it did not contain any folate-binding factor. As CML-2 lysate was serially diluted with buffer before addition to the "normal" leukemic lysate. more of the FA-^{3}H was reduced to FH$_4$.

The binding of folic acid by this factor in cell lysates can inhibit its enzymatic reduction to FH$_4$ as demonstrated by the results of experiments shown in Fig. 4. When CML-2 lysate containing binding factor was added to a leukemic lysate which alone reduced 60% of 0.57 pmoles of FA-^{3}H, the reduction of the tracer FA to FH$_4$ decreased to 1.3%. This inhibitory effect decreased as

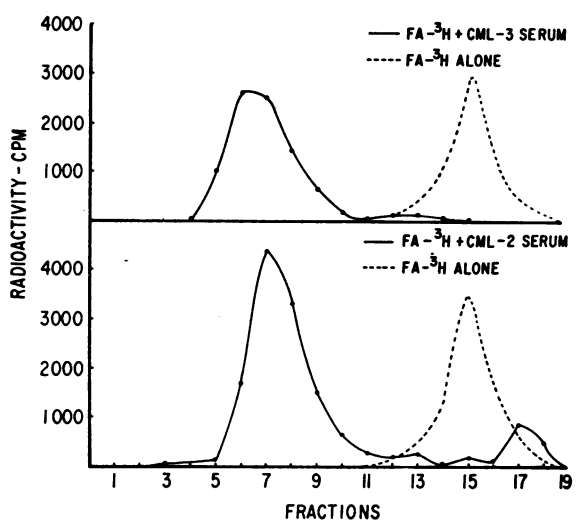

FIGURE 3 Radiochromatograms of FA-^{3}H filtered through G-75 Sephadex after incubation with serum from CML-2 and CML-3 patients. The reaction mixture was treated with dry protein–coated charcoal before filtration to remove most of the unbound radioactivity. The dotted lines show the elution of free FA-^{3}H.

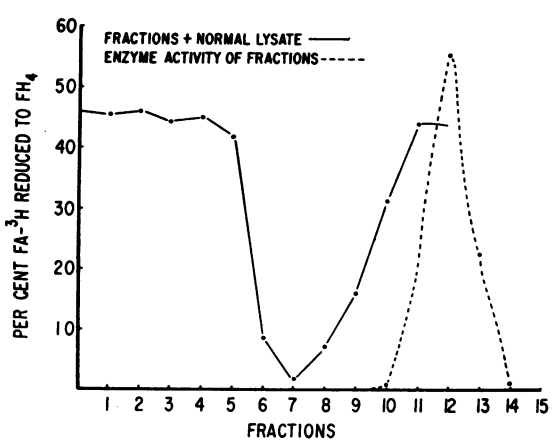

FIGURE 5 Separation of binding factor from folate reductase in CML-2 lysate. A sample of lysate was filtered through a G-75 Sephadex column and each fraction assayed for: (*a*) folate reductase activity using 0.57 pmoles of FA-^{3}H, and (*b*) inhibition of reduction of 0.57 pmoles of FA-^{3}H by a leukemic lysate which did not contain binding factor. Inhibition occurred with early effluent fractions (solid line) and endogenous folate reductase appeared with fractions which filtered later (dashed line).

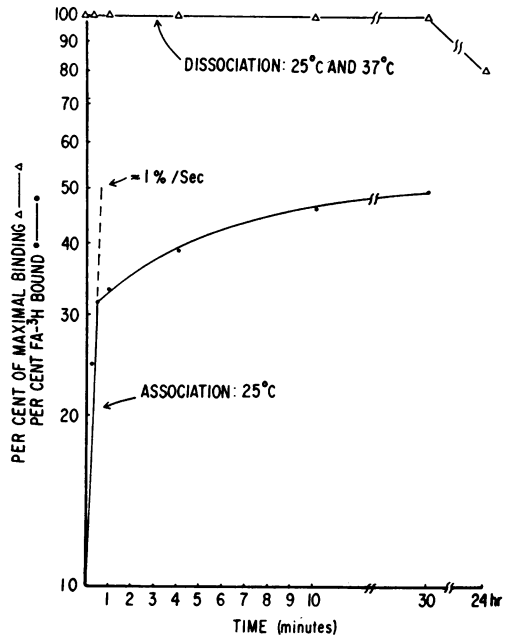

FIGURE 6 Rate of binding of FA-^{3}H by CML-2 lysate and dissociation of FA-^{3}H binder complex. The latter was studied by adding a swamping quantity of stable folic acid to the reaction mixture after it had incubated 30 min, and then determining the decrease in bound FA-^{3}H by the addition of the coated charcoal suspension.

the CML-2 lysate was serially diluted before mixing with the active leukemic lysate.

The FA binder in CML-2 lysate could be separated from endogenous folate reductase by filtration through a G-75 Sephadex column and assaying the effluent fractions for folate reductase and for "inhibition" of enzyme activity in a leukemic lysate which did not bind FA. As shown in Fig. 5, endogenous folate reductase activity appeared with the fractions of the inner volume. The early effluent fractions, however, inhibited the reductase activity of the test leukemic lysate. These inhibitory fractions corresponded to the early appearance of "bound" FA-^{3}H shown in Fig. 1. These findings indicate that this FA-binding factor and folate reductase are different molecules which can be separated on the basis of size.

The Michaelis constant (Km) for the reduction of folic acid to FH_4 by folate reductase in several leukemic cell lysates was determined by the addition of unlabeled folic acid to the tracer concentration of FA-^{3}H to establish different total substrate concentrations. The total folate reduced as a function of time was analyzed by the double reciprocal plot of Lineweaver and Burk (15) and the results are summarized in Table I. The apparent Km for folate reductase in those lysates containing the binding factor was higher than the 99% confidence limit for the range of Km values for enzyme activity in lysates which did not contain the binding factor. This high Km was clearly not due to any alteration of the enzyme but, was rather due to the binding of folate by this factor thus requiring additional free substrate to reach half maximal velocity of the enzyme.

The extraction of FA-^{3}H from solutions containing aliquots of lysates of different types of cells or different serums using coated charcoal or Dowex 2-X8 is summarized in Table II. 22–87% of the tracer could not be adsorbed from mixtures of FA-^{3}H with CML-2, CML-3 or CML-4 lysates, or with CML-1, CML-2 or CML-3 serums. In contrast, only 0–5.6% of radioactivity remained after similar treatment of mixtures containing lysates of other leukemic cells, normal leukocytes, normal bone marrow, or normal serum.

The effect of pH and temperature on the binding of FA-^{3}H by CML-2 lysate is summarized in Table III. Binding remained fairly constant between pH 6.0 and 12 but decreased sharply below pH 5.0. The binder as well as the binder-folate complex was resistant to the effects of 56°C temperature.

The competitive effect of various folate compounds on the binding of FA was studied by adding a quantity of CML-2 lysate, previously determined to bind approximately 60% of FA-^{3}H, to a mixture of tracer and two concentrations of unlabeled analogue. The unbound FA-^{3}H was then removed with the coated charcoal suspension. The results of these experiments are summarized in Table IV. Whereas unlabeled FA inhibited the binding of FA-^{3}H, the completely reduced analogues

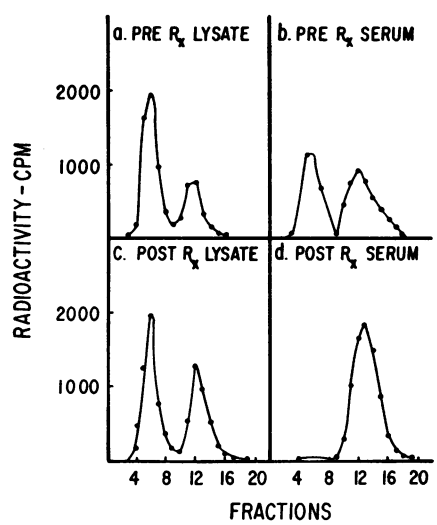

FIGURE 7 The effect of therapy on the binding factor in the cell lysate and serum from patient CML-3. Posttherapy the patient's peripheral granulocyte count decreased to 3500/mm^3 of blood. Although binding was still evident in posttreatment lysate by the radioactivity filtering with early effluent (*c*), it virtually disappeared from the posttreatment serum (*d*).

TABLE I

Km Values for Reduction of Folic Acid by Folate Reductase in Leukemic Lysates

Sample	Km
CML*	1.1×10^{-6} M
ALL	1.4×10^{-6} M
CML-5	1.0×10^{-6} M
AML	1.0×10^{-6} M
CML-6	1.9×10^{-6} M
MEAN ±SD‡	$1.3 \pm 0.4 \times 10^{-6}$ M
CML-2	20×10^{-6} M
CML-3	3.6×10^{-6} M
CML-4	19×10^{-6} M

* Blast crisis.

methyl FH_4 and formyl FH_4 were virtually without effect at the two concentrations tested. The unreduced folates, diopterin, pteropterin, and methotrexate, and the partially reduced analogue, FH_2 did compete for binding sites but not as effectively as unlabeled FA. A swamping amount of vitamin B12 did not inhibit binding of FA-^{3}H indicating that this folate binding site is different from the B12-binding site of CML cell fractions (16).

The rate of binding of FA-^{3}H by CML-2 lysate, and the dissociation of the formed complex is shown in Fig. 6. The forward velocity was extremely rapid with a rate of approximately 1.0%/sec. There was virtually no dissociation of the bound FA-^{3}H for the first 30 min of observation either at room temperature or 37°C, and even after 24 hr only 14% of the FA-^{3}H dissociated.

The effect of therapy on the FA binder in the cells and serum of patient CML-3 was studied after she was treated with busulfan. The radiochromatograms in Fig. 7 demonstrate that the binder was still evident in the cell lysate after treatment (peripheral leukocyte count of

TABLE II

FA-^{3}H Not Absorbed by Coated Charcoal or Dowex 2-X8 from Mixtures Containing Different Cell Lysates of Serum

Sample	FA-^{3}H not adsorbed*			
	Charcoal		Dowex 2-X8	
	Lysate	Serum‡	Lysate	Serum‡
	%	%	%	%
CML-1	—	86	—	—
CML-2	76	45	87	55
CML-3	72	22	78	28
CML-4	49	—	35	—
CML-5	0	0	0	0
CML-6	0	0	0	0
CML-7	4.8	0	4.5	0
ALL-1	5.6	0	1.1	—
ALL-2	0	0	—	—
AML-1	0	—	—	—
AML-2	0	0	0	0
CLL-1	0	—	—	—
CLL-2	0	0	0	0
Normal peripheral leukocytes (7)§	0	—	0	—
Normal bone marrow (2)	0	—	0	—
Normal serum (20)‖	—	0	—	0

* Calculated using radioactivity extracted from 0.05 M sodium citrate alone as 100% adsorption. Each value is the mean of duplicate determinations.
‡ 0.1 ml vol tested.
§ ( ), number of samples tested.
‖ A few values less than 0.5% were considered as zero.

TABLE III

Effect of pH and Temperature on Binding of FA-^{3}H by CML-2 Lysate

pH	Temperature	% of binding at pH 7.6 and 25°C*
	°C	
3	25	7.2
4	25	8.3
5	25	69.0
6	25	87.2
7.6	25	100
9	25	100
12	25	100
7.6	25	100
7.6	56, 25‡	94
7.6	25, 56§	100

* Results are mean of duplicate of experiments.
‡ Extract alone was treated at 56°C for 30 min and the binding of FA-^{3}H determined at 25°C using coated charcoal.
§ The mixture of lysate and FA-^{3}H was incubated at 25° C for 30 min and then at 56°C for 30 min and the bound FA-^{3}H was then determined using the coated charcoal.

TABLE IV

Inhibitory Effect of Folate Analogues and Vitamin B_{12} on Binding of FA-^{3}H by Binding Lysate

Test Compound	% of maximum binding*	
	1.8×10^{-9}M	3.6×10^{-9}M
Unlabeled FA	59	35
N^{10} methyl FA	67	48
Diopterin	60	50
Teropterin	62	50
Methotrexate	94	72
FH_2	65	58
Formyl FH_4	99	96
Methyl FH_4	100	100
	(2.26×10^{-4}M)	
Vitamin B_{12}	100	

* Calculated as a per cent of FA-^{3}H bound by lysate alone in absence of any unlabeled compound. Each value is the mean of duplicate determinations.

TABLE V
Hematologic Data on the Patients

Patient	Binding protein in lysate	Binding protein in serum	Hb	PCV*	Platelets/ mm³	WBC/ mm³	Blasts	Promyelocytes
			g/100 ml	%				
CML-1	+	+	8.6	27	490,000	199,500		
CML-2	+	+	13.6	39	505,000	123,000	14	9
CML-3	+	+	6.4	27.5	842,000	167,000	1	4
CML-4	+	Not done	12.4	38	447,000	47,900		
CML-5	—	—	9.5	26	952,000	382,470	3	37
CML-6	—	—	14.8		94,000	41,690		
CML-7	—	—	10.2			391,000	3	

* Packed cell volume.
‡ Leukocyte alkaline phosphatase (normal: 30–100).

3500/mm³) whereas the binder in serum decreased significantly and was almost not detectable (Figs. 7*b* and 7*d*). Since there were no immature cells in the peripheral blood after treatment, the binder must be present in the mature polymorphoneuclear cells of this disease.

The pertinent clinical data available for all the CML patients studied are summarized in Table V. It is apparent that there are no hematologic features which distinguish those patients who contain FA-binding factor in their cells from those who do not.

DISCUSSION

This report has characterized the properties of a folate-binding macromolecule recently found in CML cells (8). This factor has been found in the cells of four of seven CML patients and in the serum from three of these. In one patient treated with busulfan (CML-3) the binder virtually disappeared from the serum but was still identified in the cell lysate even when the total leukocyte count decreased to 3500 per mm³ of blood suggesting that the source of the serum binder is probably the leukemic cell, either as a result of leakage or cell death. Although the cells of one patient with ALL demonstrated binding of questionable significance (Table II), the lysates of cells from patients with other types of leukemia, normal bone marrows, normal peripheral leukocytes, or normal serums, did not bind FA-^{3}H.

This factor appears to have a mol wt between 100,000 and 200,000, is resistant to heating at 56°C for 30 min, and is minimally active below pH 5.0. It binds FA very rapidly and reacts with such unreduced folate analogues as diopterin, pteropterin, and methotrexate. It also binds FH_2 but not the completely reduced analogues, methyl FH_4 or formyl FH_4.

The finding of this folate binder in only some leukemic cells and serums might suggest that it is an abnormal factor peculiar to these particular diseases. However, the failure to demonstrate binding by all leukemic lysates (or even normal cells) does not prove that this factor is absent, for if it was saturated by endogenous substrate (i.e. dihydrofolate), it would not bind the exogenous FA-^{3}H used to detect it. In fact, the finding of high concentrations of this vitamin in leukemic cells (17) might be explained by such a binding factor which could result in intracellular folate accumulation even in the face of an energy-dependent efflux mechanism recently described by Lichtenstein, Oliverio, and Goldman (18).

The evidence for binding of FH_2 adds some logic to the properties of this factor which initially appeared to bind a form of folate less physiologic than the completely reduced folate cofactors which it did not bind. It is perhaps more reasonable to speculate that the function (physiologic or pathologic) of this factor is to bind FH_2, a key folate analogue in the synthesis of thymidylate from deoxyuridylate. As a pathologic factor, or even as a normal factor in excess, it could interfere with such synthesis and perhaps be responsible for a longer cell generation time (19) or a population of "dormant" cells unable to synthesize DNA at all (20).

Another and perhaps more practical consideration is the effect that this binding factor might have on the treatment of leukemia with methotrexate, since it also binds this drug, albeit with lesser affinity than it binds folic acid or FH_2. In a recent communication from this laboratory (21) we demonstrated that some leukemic cells after treatment with MTX contained free folate reductase while simultaneously containing an excess of drug which appeared to be bound, at least in part, to a macromolecule. If it was to this folate-binding factor

with Chronic Myelogenous Leukemia

Differential									
Myelocytes	Metamyelocytes	Polys	Eosinophiles	Basophiles	Lymphocytes	Monocytes	LAP‡	Ph chromosome	Serum B_{12}
									pg/ml
13	27	58			2		4		1744
2	33	35	2	2	1	2	12		1171
20	35	30	1		9		14	+	1267
11	27	40			8	9	8		1984
30		21	5	4			1	—	1144
21	6	47			16	9	0	+	1169
38	37	21			1		Low	+	

that the MTX was bound, then all leukemic cells which have the potential to synthesize this macromolecule could become resistant to this drug by binding it and sterically preventing it from inactivating folate reductase. It is interesting in this regard that Anton and Nichol in 1959 (22) found a strain of *Streptococcus faecalis* highly resistant to aminopterin which took up much greater amounts of folic acid and drug than did the sensitive strain, leading them to suggest that the antagonist was bound in some manner which rendered it inactive. If such was the case with MTX-resistant mammalian leukemic cells, an appropriate adjunct to therapy with this drug would be the concomitant administration of folic acid which would preferentially bind to this macromolecule, permitting the MTX to react with and inactivate folate reductase because of its much greater affinity for this enzyme than either FA or FH_2 (23).

The finding of significantly higher Km values for the reduction of folic acid or FH_2 by whole lysates containing this binding factor indicates that caution must be exercised when interpreting studies which demonstrate altered enzyme properties. Clearly any factor which effectively prevents a substrate from reacting with an enzyme will result in a higher concentration of the substrate necessary to achieve half maximal velocity of the enzyme. Although this phenomenon has not been identified in protozoan cells (24) recently reported to contain folate reductase with an altered Km, it has not been excluded.

The folate-binding pattern of this factor is similar to the antifolate antibody obtained by immunizing rabbits with folic acid coupled to methylated bovine albumin (25). Since an immunoassay with this antibody can reveal immunoreactive FA in serum and whole blood extracts (26), this macromolecule may have practical value as a binder for a competitive inhibition radioassay for folic acid in blood. This application is presently under investigation.

A more speculative consideration at this time is the potential value of such a binding factor in the treatment of neoplastic processes. Since the binding of FA prevents its enzymatic reduction, it is likely that reduction of FH_2 would be similarly prevented by this factor no matter how high the concentration of reducing enzyme. As MTX interrupts the cycle of thymidylate synthesis by inhibiting folate reductase and preventing recycling of FH_2 to FH_4, so this binder could similarly inhibit this process by binding this cofactor. Although the size of this binder would generally preclude its entry into most cells, there is evidence that some macromolecules can penetrate tumor cells (27). Nevertheless, this potential application will require more definitive knowledge as to the chemical nature, structure, and reacting site of this FA-binding factor.

ACKNOWLEDGMENTS

We are grateful to Dr. Robert Goldstein for his helpful suggestions during the course of this study and in the preparation of this manuscript. We would also like to thank Dr. Robert Levy for the samples of blood from patients CML-5, CML-6, and CML-7.

This work was supported by research grant CA 08976 from the National Cancer Institute, and from Training Grant AM 05423 from the U. S. Public Health Service.

Note added in proof: Since this manuscript has been in press we have identified a folate binding factor in the peripheral leukocytes of a patient with acute lymphocytic leukemia and from several pregnant women. The properties of this binding factor are now being studied to determine whether they are similar to the binder in CML cells described in this report.

REFERENCES

1. Johns, D. G., and I. H. Plenderleith. 1963. Folic acid-displacement in man. *Biochem. Pharmacol.* **12**: 1071.
2. Neal, G. E., and D. C. Williams. 1965. The fate of intravenously injected folate in rats. *Biochem. Pharmacol.* **14**: 903.
3. Jacquez, J. A. 1966. Permeability of Ehrlich ascites cells to folic acid, aminopterin, and amethopterin. *Cancer Res.* **26**: 1616.
4. Futterman, S. 1957. Enzymatic reduction of folic acid and dihydrofolic acid to tetrahydrofolic acid. *J. Biol. Chem.* **228**: 1031.
5. Johns, D. G., S. Sperti, and A. S. V. Burgen. 1961. The metabolism of tritiated folic acid in man. *J. Clin. Invest.* **40**: 1684.
6. Condit, P. T., and D. Grob. 1958. Studies on the folic acid vitamins. I. Observations on the metabolism of folic acid in man and on the effect of aminopterin. *Cancer* **11**: 525.
7. Metz, J., R. Zalusky, and V. Herbert. 1968. Folic acid binding by serum and milk. *Amer. J. Clin. Nutr.* **21**: 289.
8. Rothenberg, S. P. 1970. A macromolecular factor in some leukemic cells which binds folic acid. *Proc. Soc. Exp. Biol. Med.* **133**: 428.
9. Rothenberg, S. P. 1966. A rapid radioassay for folic acid reductase and amethopterin. *Anal. Biochem.* **16**: 176.
10. Blakley, R. L. 1960. Crystalline dihydropteroylglutamic acid. *Nature, (London).* **188**: 231.
11. Keresztesy, J. C., and K. O. Donaldson. 1961. Synthetic prefolic A. *Biochem. Biophys. Res. Commun.* **5**: 286.
12. Rothenberg, S. P. 1968. Radioenzymatic assays for folic acid, folic acid antagonists, and folate reductase. *In* Radioisotopes in Medicine: In Vitro Studies. R. L. Hayes, F. A. Coswitz, and B. E. P. Murphy, editors. *U. S. Atomic Energy Commission.* 413.
13. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**: 265.
14. Lau, K. S., C. Gottlieb, L. R. Wasserman, and V. Herbert. 1965. Measurement of serum vitamin B12 level using radioisotope dilution and coated charcoal. *Blood* **26**: 202.
15. Lineweaver, H., and D. Burk. 1934. The determination of enzyme dissociation constants. *J. Amer. Chem. Soc.* **56**: 658.
16. Meyer, L. M., E. P. Cronkite, I. F. Miller, C. W. Mulzac, and I. Jones. 1962. Co^{60} vitamin B12 binding capacity of human leukocytes. *Blood* **19**: 229.
17. Hoffbrand, A. V., and B. F. A. Newcombe. 1967. Leucocyte folate in vitamin B12 and folate deficiency and in leukemia. *Brit. J. Haematol.* **13**: 954.
18. Lichtenstein, N. S., V. T. Oliverio, and I. D. Goldman. 1969. Characteristics of folic acid transport in the L1210 leukemia cell. *Biochim. Biophys. Acta* **193**: 456.
19. Mauer, A. M., E. F. Saunders, and B. C. Lampkin. 1969. Possible significance of non-proliferating leukemic cells. *U. S. Nat. Cancer Inst. Monogr.* **30**: 63.
20. Clarkson, B. D. 1969. Review of recent studies of cellular proliferation in acute leukemia. *U. S. Nat. Cancer Inst. Monogr.* **30**: 81.
21. Rothenberg, S. P. 1969. Alteration of methotrexate by leukemic cells: loss of affinity for an anion exchange resin. *Cancer Res.* **29**: 2047.
22. Anton, A. H., and C. A. Nichol. 1959. Studies on the nature of resistance of Streptococcus faecalis to folic acid antagonists. *Biochem. Pharmacol.* **3**: 1.
23. Werkheiser, W. C. 1961. Specific binding of 4-amino folic acid analogues by folic acid reductase. *J. Biol. Chem.* **236**: 888.
24. Ferone, R., M. O'Shea, and M. Yoeli. 1970. Altered dihydrofolate reductase associated with drug-resistance transfer between rodent plasmodia. *Science (Washington).* **167**: 1263.
25. Rothenberg, S. P., F. Gizis, and B. Kamen. 1969. Antibodies against folic acid. I. In vitro biophysical effect. *J. Lab. Clin. Med.* **74**: 662.
26. daCosta, M., and S. P. Rothenberg. 1970. A radioimmunoassay for folic acid in serum and whole blood. *Clin. Res.* **18**: 453.
27. Ryser, H. J.-P. 1968. Uptake of protein by mammalian cells: an underdeveloped area. *Science (Washington).* **159**: 390.