Characteristics of Folic Acid-binding Protein in Folate-deficient Serum

By Samuel Waxman and Carol Schreiber

Folate-deficient serum contains a specific folic acid-binding protein (FABP) that has a rapid association and slow dissociation rate for the binding of $^3$H-pteroylglutamic acid ($^3$H-PGA). FABP is also present in normal serum but in lesser amounts. The molecular weight of FABP is less than 100,000 and may represent more than one protein. FABP elutes as a beta globulin and is recovered in the transferrin band region in polyacrylamide gel electrophoresis. FABP binds oxidized folyl-, mono-, and poly-glutamates in preference to reduced folates. FABP retards the delivery and uptake of $^3$H-PGA into HeLa cell monolayer cultures. The characteristics of FABP suggest it to be a membrane-derived intracellular folate storage protein and perhaps an important regulator of folate uptake into the cell and a storage site for folyl polyglutamates. The characteristics of serum FABP suggest it to be similar to beta lactoglobulin (the folate-binding protein isolated from cow’s milk).

PREVIOUS WORK has shown that the folate in serum is, for the most part, removed by dialysis and is rapidly cleared in vivo from the serum following infusion. In vitro studies have shown serum to be a poor binder of folic acid. However, more recent studies have revealed that a small fraction of endogenous serum folate is nondialyzable and is bound to a large molecular weight protein fraction. The existence of a large molecular weight protein that avidly binds folic acid in both human and cow’s milk, the bone marrow of some patients with chronic myeloid leukemia, and in some pregnant women further suggests that a specific binding protein exists for folic acid.

We have reported that serum from patients with folate deficiency contains folic acid-binding protein (FABP), which binds $^3$H-pteroylglutamic acid ($^3$H-PGA). This FABP is measurable in early folate deficiency and is not measurable immediately following folic acid replacement. The characteristics of FABP in serum, milk, and bone marrow and its possible physiologic significance in folate metabolism form the basis of this report.

MATERIALS AND METHODS

$^3$H-PGA, 40 Ci/mM, was purchased from Amersham Searle Corp., Arlington Heights, Ill. The purity of this compound varied from 90% to 95%, as determined by descending paper chromatography.

*From the Cancer Chemotherapy Laboratory, Division of Oncology, Department of Medicine, Mount Sinai School of Medicine of the City University of New York, New York, N.Y. 10029.


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Samuel Waxman, M.D.: Assistant Clinical Professor of Medicine, and Head, Cancer Chemotherapy Laboratory, Division of Oncology, Department of Medicine, Mount Sinai School of Medicine of the City University of New York, New York, N.Y. 10029. Carol Schreiber: Research Assistant, Cancer Chemotherapy Laboratory, Division of Oncology, Department of Medicine, Mount Sinai School of Medicine of the City University of New York, New York, N.Y. 10029.

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raphy in 0.1 M phosphate buffer pH 7.4. Stable d,L N-5-methyltetrahydrofolic acid (methyl-THFA) was prepared by Nixon and Bertino.\textsuperscript{13,14} C-Labeled teropterin, and stable pteric acid, dihydrofolic acid, pteridine-6-carboxylic acid, and teropterin were kindly provided by Dr. Charles Baugh and Dr. Carlos Krumdieck. Formyltetrahydrofolic acid was obtained from Lederle Laboratories, Inc., Pearl River, N.Y. The HeLa cell line was provided by the laboratory of Dr. I.A. Jaffe.

Serum was obtained from normals and from patients with folic acid deficiency and was stored at −10°C. Cow’s milk (Carnation Instant Powdered Milk), and mother’s milk were assayed for binding of folic acid and analogues. Folate levels were measured by both \textit{Lactobacillus casei} microbiologic assay,\textsuperscript{12} as well as by radioisotopic assay.\textsuperscript{13}

FABP was measured by incubating 0.4 ml test material (serum or 10\textsuperscript{o}, dialyzed powdered milk suspension) with \textsuperscript{3}H-PGA (0.5 ng) and 0.1 M phosphate buffer, pH 7.4, in a final volume of 1.3 ml for 30 min at room temperature. Hemoglobin-coated charcoal was added to the mixture to adsorb unbound \textsuperscript{3}H-PGA.\textsuperscript{14} A similar reaction mixture containing stable PGA (5 ng) was simultaneously run to determine the amount of radioactive impurity (non-\textsuperscript{3}H-PGA) in each assay remaining after charcoal adsorption. This was subtracted from the test reaction to determine FABP. Freeze-thawing did not diminish FABP capacity over many months. In some experiments, the \textsuperscript{1}H-PGA bound to serum after charcoal adsorption was submitted to dialysis against 0.9\textsuperscript{o} saline (1000 x volume) for 48 hr at 4°C to measure avidity of the serum-bound \textsuperscript{1}H-PGA.

Serum incubated with \textsuperscript{3}H-PGA (charcoal-treated and untreated) was filtered through Sephadex G-75 columns (0.5 x 25 cm) and Sephadex G-200 columns (2.5 x 100 cm) and was then eluted with 0.05 M phosphate, 0.5 M NaCl pH 7.4 buffer. In some studies, the column was eluted with glycine-HCl buffer, pH 3.0, in an attempt to separate \textsuperscript{3}H-PGA from FABP. Proteins were measured by absorption optical density at 280 \textmu m.

DEAE-cellulose chromatography of serum incubated with \textsuperscript{3}H-PGA (1 ng/ml) was done in 5-ml columns eluted with 0.06 M phosphate buffer, pH 6.3, to separate beta globulins from alpha globulins (eluted with 1 M NaCl).

Serum labeled with \textsuperscript{3}H-PGA (charcoal-treated and untreated) was studied by disc gel polyacrylamide electrophoresis using the method of Ornstein and Davis.\textsuperscript{15,16} In some experiments, the time was lengthened and the monomer concentrations were increased to sharpen band preparation. Stable PGA (50 mg) was added to the bathing buffer to provoke mass action dissociation of the \textsuperscript{1}H-PGA bound to serum. The gels were sliced, and radioactivity was eluted by swelling the gel with Hyamine (hydroxide of Hyamine 10-X, Packard Instrument Co., Downers Grove, Ill.) for 24 hr at 37°C.

The effect of pH on the binding of \textsuperscript{3}H-PGA to serum was studied in the range of pH 1.6-11.7 using 0.05 M citrate buffers (with HCl or NaOH as needed). In normal serum, experiments were designed to uncover FABP that was possibly not measurable because of saturation with endogenous folate. To dissociate endogenous folate from the serum FABP, pH was lowered by dialysis to 3.9 against 0.05 M citric acid; serum was then dialyzed against 0.05 M Na citrate to return pH to 6.9 for binding of added \textsuperscript{3}H-PGA to measure FABP. Serum was also treated with 6 M urea and was dialyzed and measured for FABP. The effect of temperature of \textsuperscript{1}H-PGA binding to serum was studied at 4°C, 37°C, after 56°C heat inactivation and after boiling for 30 min in a water bath.

The specificity of binding \textsuperscript{3}H-PGA to serum and cow’s milk was studied by the inhibitory effect on \textsuperscript{1}H-PGA binding by ten times equimolar amounts of folic acid analogues added to the binding substance sequentially, or simultaneously with \textsuperscript{3}H-PGA. Conjugase activity was assayed in this system using \textsuperscript{14}C-teropterin by the method of Krumdieck and Baugh.\textsuperscript{17}

The physiologic role of serum and milk FABP was assessed in HeLa cell monolayers maintained in MEM Eagles (Minimal Essential Media; Associated Biomedic Systems, Inc., Buffalo, N.Y.) with 10\textsuperscript{o}, fetal calf serum and 4 mM glutamine. Uptake of \textsuperscript{3}H-PGA into HeLa cell monolayers was measured at 37°C in Hank’s balanced salt solution (HBSS). This was compared to the uptake of \textsuperscript{3}H-PGA preincubated with 5\textsuperscript{o}, normal albumin, normal serum, folate-deficient serum, cow’s milk, and human milk, all of which were previously dialyzed for 48 hr in 0.9\textsuperscript{o}, saline at 4°C to remove endogenous folate. After timed uptake, the plates were washed three times with HBSS, and the cells were scraped and dissolved in Hyamine. \textsuperscript{3}H-PGA efflux from the HeLa cell monolayer was studied by a timed washout in fresh HBSS following a 1-hr pulse uptake of \textsuperscript{3}H-PGA. The effect of Dilantin (100 \mu g/ml) on uptake and efflux of \textsuperscript{3}H-PGA was also studied.
Radioactivity was measured in 20 ml of scintillation mixture (toluene containing 0.7%, 2,5-diphenyloxazole (POO) and 10% bio-Solv BBS-3, Beckman Instruments, Fullerton, Calif., or toluene with 0.6% POO, 0.03% dimethyl-POPOP and 25% ethanol for the Hyamine samples.) and was counted in a Beckman LS-250 liquid scintillation counter to a counting error of 1% or less.

RESULTS

Sephadex Gel Filtration Patterns

The Sephadex G-75 filtration patterns of normal serum (containing almost no FABP) and folate-deficient serum (containing FABP sufficient to bind almost 75% of the added 3H-PGA), after incubation with 3H-PGA, are shown in Fig. 1. 3H-PGA in the normal serum mixture or in buffer appeared in the inner volume, indicating that it was either unbound or bound to a factor with a molecular weight less than 50,000. In contrast, the 3H-PGA in the folate-deficient serum filtered with the early effluent indicating that it was bound to a factor with a molecular weight of at least 50,000; except for 25% that filtered with the inner volume and presumably represented unbound 3H-PGA. Charcoal adsorption of the reaction mixture prior to filtration removed the 3H-PGA activity recovered in the inner volume.

3H-PGA bound to several folate-deficient serums was recovered near the

![Graph showing Sephadex G-75 filtration patterns of normal and folate-deficient serum incubated with 3H-PGA.]

Fig. 1. Sephadex G-75 filtration patterns of normal and folate-deficient serum incubated with 3H-PGA.
albumin region when filtered through Sephadex G-200 (Fig. 2). $^3$H-PGA added to normal serum was retained on the column and was recovered in a separate later fraction. Two peaks of $^3$H-PGA activity were found in Sephadex G-200 filtration of $^3$H-PGA bound to the serum of two patients with alcoholic folate deficiency (an early large molecular peak in the region of the macroglobulins and a late peak in the albumin region). Thus, FABP appears to have a molecular weight of nearly 50,000 and may represent more than one protein, some of which have a molecular weight greater than 100,000.
DEAE-Cellulose Chromatography

DEAE-cellulose chromatography of $^3$H-PGA bound to serum (after charcoal adsorption) is shown in Fig. 3. FABP was found in the beta globulin fractions (fractions 1-7) and was over eight times greater in quantity in folate-deficient serum than in normal serum. When $^3$H-PGA was added to buffer or normal serum and was not charcoal treated, the $^3$H-PGA was recovered in the saline effluents (fractions 8-11). This $^3$H-PGA activity was adsorbed by coated charcoal.

Polyacrylamide Disc Gel Electrophoresis Studies

$^3$H-PGA incubated with folate-deficient serum and electrophoresed in polyacrylamide disc gel showed a peak of noncharcoal adsorbable radioactivity in the transferrin band region (Fig. 4). This peak of radioactivity was not found in normal serum incubated with $^3$H-PGA. A charcoal adsorbable peak of radioactivity in the prealbumin region accounted for all of the radioactivity recovered from $^3$HPGA added with buffer, normal serum, and the $^3$H-PGA in excess of FABP in the folate-deficient serum. When swamping amounts of stable PGA were added to the bathing buffer during electrophoresis, the radioactivity in the transferrin band region was removed (Fig. 5). However, the radioactivity in the prealbumin region was not affected by similar conditions.
This suggests that the \(^3\)H-PGA activity found in the transferrin band region was bound to FABP and that this complex could be dissociated by large amounts of PGA under the conditions of this electrophoretic environment.

**Effects of pH and Temperature on \(^3\)H-PGA Binding to Serum**

Serum binding of added \(^3\)H-PGA was very rapid. The binding of \(^3\)H-PGA to serum was the same from 4°C to 37°C (Table 1). Heating at 56°C for 30 min did not destroy FABP. However, boiling the serum for 30 min destroyed FABP. The subsequent addition of tenfold stable PGA did not displace \(^3\)H-PGA once bound to serum. Continuous dialysis at 4°C did not remove \(^3\)HPGA bound to serum.

\(^3\)H-PGA binding to serum was maximal at pH 7.5, was nearly unchanged from pH 6.1–8.0, and decreased rapidly below pH 5 (Fig. 6). Normal serum was treated several ways in an attempt to uncover FABP that was possibly not measurable with exogenous \(^3\)H-PGA because of saturation with exogenous folate. The pH of the incubation mixture was lowered to less than 5; the mixture was dialyzed, and \(^3\)H-PGA added. The incubation mixture was then re-
turned to pH 7.4, but FABP did not increase in normal serum. The addition of 6 M urea to serum followed by dialysis did not uncover additional FABP in normal serum, but it did dissociate significant amounts of $^3$H-PGA from the FABP of folate-deficient serum.

**Effect of Folate Analogues on $^3$H-PGA Binding to Serum**

The competitive effect of various folate analogues on the binding of $^3$H-PGA to folate-deficient serum and cow’s milk is shown in Table 2. The FABP in

<table>
<thead>
<tr>
<th>Temperature</th>
<th>% $^3$HPGA bound/0.4 ml Serum</th>
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<tbody>
<tr>
<td>$4^\circ$C</td>
<td>48.5</td>
</tr>
<tr>
<td>$25^\circ$C</td>
<td>45.3</td>
</tr>
<tr>
<td>$37^\circ$C</td>
<td>49.0</td>
</tr>
<tr>
<td>$56^\circ$C</td>
<td>46.2</td>
</tr>
<tr>
<td>Boiling</td>
<td>5.2</td>
</tr>
<tr>
<td>Following dialysis for 48 h†</td>
<td>42.3</td>
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<tr>
<td>Following addition of PGA (5 ng)</td>
<td>40.5</td>
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</tbody>
</table>

† $^3$H-PGA (0.5 ng) added

**Figure 5.** Polyacrylamide disc gel electrophoresis separation in bathing buffer containing stable PGA, 50 mg/ml.

- Buffer
- Normal serum
- Normal serum plus charcoal
- Folate deficient serum
- Folate deficient serum plus charcoal

**Table 1.** Effect of Temperature, Dialysis, and Displacement on $^3$H-PGA Binding to Folate-Deficient Serum

- Continuous dialysis against 0.9% saline: 1000 X volume, at $4^\circ$C.
serum and milk was similarly affected by these analogues. Oxidized folates were more effective inhibitors of $^3$H-PGA binding than reduced folates (PGA was greater than dihydrofolic acid that was greater than tetrahydrofolic acid). Teropterin (a folyl polyglutamate) was as effective as PGA in inhibiting $^3$H-PGA binding in this system where there was no measurable conjugase activity. Methyl-THFA, although a poor inhibitor, was more effective an inhibitor than formyl-THFA. Pteric acid inhibited $^3$H-PGA binding, whereas pteridine-6-carboxylic acid barely inhibited $^3$H-PGA binding. Methotrexate was less an inhibitor than was PGA or dihydrofolic acid.

**Effect of FABP on $^3$H-PGA Delivery to HeLa Cells**

The effects of FABP on folate delivery to HeLa cell monolayer culture are shown in Fig. 7. The $^3$H-PGA bound to FABP in folate-deficient serum, human milk, and cow's milk was not available for uptake in HeLa monolayer culture. $^3$H-PGA added in HBSS, and to a lesser extent in the presence of normal serum, was delivered to HeLa cells and was increased with time of incubation over a 3-hr period. The presence of Dilantin, 100 µg/ml 2 hr prior to and following the addition of $^3$H-PGA to the HeLa cell culture, did not affect $^3$H-PGA uptake. Efflux of $^3$H-PGA from the HeLa cell did not appear to be significant over a 60-min washout in HBSS and was not altered by the presence of FABP or Dilantin in the medium.

<table>
<thead>
<tr>
<th>Table 2. Per Cent Inhibition of $^3$H-PGA Binding to Milk and Serum by Folate Analogues</th>
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<tbody>
<tr>
<td>Milk</td>
</tr>
<tr>
<td>PGA</td>
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<tr>
<td>Teropterin</td>
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<tr>
<td>Dihydrofolic acid</td>
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<tr>
<td>Methyl-THFA</td>
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<tr>
<td>Formyl-THFA</td>
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<tr>
<td>Methotrexate</td>
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<tr>
<td>Pteroic acid</td>
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<tr>
<td>Pteridine-6-carboxylic acid</td>
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*Tenfold equimolar amounts of analogue to $^3$H-PGA added.
† $^3$H-PGA (0.5 ng) added with folate analogue to milk or serum.
‡ $^3$H-PGA (0.5 ng) added after folate analogue mixed with milk or serum.
DISCUSSION

The results of this study permit the conclusion that folate-deficient serum contains a specific folic acid-binding protein (FABP). FABP has a rapid, pH-dependent and temperature-independent association and a slow dissociation rate for the binding of PGA. FABP is destroyed by boiling for 30 min, and FABP binding of PGA is inhibited by 6 M urea. FABP appears to be present in normal serum in less amounts than in folate-deficient serum and does not increase following various attempts to remove endogenous folate bound to FABP.

The molecular weight of FABP is less than 100,000 and may represent more than one protein. When submitted to DEAE-cellulose chromatography, FABP is eluted in the beta globulin fraction and is over eight times greater in quantity in folate-deficient serum than in normal serum. Polyacrylamide disc gel electrophoresis showed FABP to be in the transferrin band region and that the PGA-FABP complex could be dissociated by large amounts of PGA during electrophoresis.

The FABP of serum and cow's milk was similarly affected by folate analogues. FABP binds oxidized folate monoglutamates and polyglutamates in preference to reduced folates. Methyl-THFA is bound poorly but more so than other reduced folates. An intact pteridine-p-aminobenzoic acid bridge structure is required for binding to FABP. Methotrexate is a significant inhibitor of 3H-PGA binding to FABP, although it is a less effective inhibitor than PGA or dihydrofolinic acid.

The physiologic role of FABP was assessed in HeLa cell monolayer culture and revealed that 3H-PGA bound to FABP of serum or of human and cow's milk was not available for uptake or delivery to the cell. Moreover, the presence of FABP in the culture medium did not significantly affect 3H-PGA efflux from the cell.

The characteristics of FABP we have described are similar to those of the folate-binding factor described by Rothenberg and da Costa in some chronic myelogenous leukemic cells and serum, and in the leukocyte lysates of some pregnant women with low serum folates. FABP may represent the unsaturated
folate-binding capacity of the recently described high molecular weight globulin factor that contains 5% of the endogenous folic acid activity of normal serum and considerably less in folate-deficient serum. This correlates well with the high FABP in folate-deficient serum and barely measurable FABP in normal serum, since FABP is measured by the binding of exogenous $^3$H-PGA. FABP is probably also the same serum folate binder of nondialyzable endogenous folic acid described by Retief and Huskisson. FABP can be separated from dihydrofolate reductase by its larger size on gel filtration and pH optimum for binding.

Serum and mother’s milk have similar FABP characteristics, except the FABP content of mother’s milk is greater. Cow’s milk FABP, has similar PGA binding characteristics to the FABP of human serum and milk. Recent work has shown the FABP of cow’s milk to be beta lactoglobulin, a protein of molecular weight 35,000. We find a similar molecular weight for most of the FABP of folate-deficient serum. The additional high molecular weight FABP (greater than 100,000) in some folate-deficient serums may represent aggregation of the 35,000 molecular weight protein. We are presently attempting to determine by immunologic means whether serum FABP is the same or is distinct from the milk beta lactoglobulin.

The characteristics of FABP suggest it to be an intracellular folate storage protein and an important regulator of folate uptake into the cell and perhaps a storage site for folyl polyglutamates. In the normal cell, FABP may be saturated with folate and can bind only a small fraction of free folate as it traverses the cell. In folate deficiency, net folate uptake may increase because of the availability of unsaturated FABP, as well as increased dihydrofolate reductase in the cell to bind free folate.

The presence of unsaturated FABP in the cell could be responsible for intracellular folate accumulation, despite the proposed energy-dependent, active efflux mechanism described by Lichtenstein et al. It is also possible that FABP binds intracellular folyl polyglutamates and perhaps prevents enzymatic degradation by conjugase, which is present in many human tissues. Whether FABP plays an important role as a regulator of DNA synthesis because of its ability to bind dihydrofolic acid remains to be determined.

FABP is probably mainly saturated with endogenous folate in normal serum and, thus, is not readily measurable with exogenous $^3$H-PGA. In folate deficiency, serum FABP may be partially unsaturated and, thus, is measurable with exogenous $^3$H-PGA. The time of cellular release of FABP is unknown. The larger amounts of FABP in milk than in serum suggests that it may be a membrane-derived protein. A folate-binding factor similar to FABP has been described in some lysates of chronic myelogenous leukemia (CML) cells. It is of interest that CML cells are known to contain and release B$_{12}$-binding proteins, some of which retard vitamin B$_{12}$ delivery to the cell. Thus, it is possible that the granulocyte may be a source of serum FABP.

Several questions remain to be answered, such as the mechanism of release of folate bound to FABP. It appears that dissociation is slow and will occur in the presence of large amounts of PGA. FABP should be looked for in intestinal mucosa and secretions to determine if FABP is important in folate absorption.
CHARACTERISTICS OF FOLIC ACID-BINDING PROTEIN

It is possible that FABP may be important in the understanding of folate malabsorption associated with sprue and in patients taking Dilantin or oral contraceptives. The intracellular location of FABP, once ascertained, will help interpret its physiologic role. The isolation of serum FABP, as recently done in cow’s milk by affinity chromatography, will be an important further step in the study of FABP.

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