Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 5983–5987, August 1986
Cell Biology

Receptor-mediated folate accumulation is regulated by the cellular folate content

(5-methyltetrahydro[^{3}H]folate binding/folate-binding factor)

BARTON A. KAMEN AND ANTONIETA CAPDEVILA

Department of Pediatrics and Pharmacology, The University of Texas Health Science Center at Dallas, Dallas, TX 75235-9063

Communicated by Alfred G. Gilman, April 22, 1986

ABSTRACT **Cultured monkey kidney cells are shown to possess a cell-surface receptor that binds 5-methyltetrahydro[3′,5′,7,9-$^3H_4$]folic acid (5-methyltetrahydro[^{3}H]folic acid) with high affinity (K_d = 3 nM at 4°C). The cell-surface binding capacity for [^{3}H]folic acid or [^{3}H]methotrexate is equivalent to that for 5-methyltetrahydro[^{3}H]folate, but the K_d values are 0.4 and 20 nM, respectively. These nonlabeled folates also inhibit cellular binding and accumulation of 5-methyltetrahydro[^{3}H]folate, whereas specific moieties of folic acid (i.e., *p*-aminobenzoylglutamic acid, pteroic acid, and glutamic acid) had no effect. Surface-bound folate(s) can be released by incubation of the cells at pH 3.5. At 37°C the bound material is slowly transferred into cells, where it becomes resistant to acid release. Within 4 hr the cells internalize about 3–4 times as much folate as is bound to the surface in the steady state. The amount of receptor activity increases markedly when cells are depleted of folate through growth in folate-depleted medium. Binding of folate was inhibited by an antibody raised against a soluble plasma folate-binding protein, suggesting that the cell-surface receptor and the circulating folate-binding protein are immunologically related. These data indicate that cells possess a high-affinity, high-specificity folate receptor whose expression is regulated by the folate content of the cell. They also suggest that a small molecule such as folate can enter cells by receptor-mediated endocytosis.**

Folates are a class of pteridine compounds that are essential for normal growth and maturation. Reduced folic acid coenzymes are involved in one-carbon transfer reactions such as those necessary for the biosynthesis of methionine, serine, deoxythymidylic acid, and purines. Although these folate-mediated reactions have been studied extensively and many of the key enzymes that catalyze these reactions have been purified and characterized (1), little is known about the overall mechanism of cellular folate homeostasis. The normal plasma folate concentration in humans is 10–20 nM. However, tissue concentrations are 3 orders of magnitude higher (2–30 nmol/g of wet weight). Even when the plasma concentration of folate is experimentally increased 20- to 40-fold above normal, the steady-state tissue concentration remains constant (2). Therefore, most likely folate enters cells by a high-affinity uptake process that can be regulated.

To better understand folate metabolism, many investigators have utilized tissue culture systems. The generally accepted model is that folate uptake is mediated by a membrane carrier that either facilitates the diffusion of folate across the membrane or actively transports the molecule into the cell (3). Another possibility is that folate is internalized by high-affinity receptors on the cell surface by a process that is similar to the receptor-mediated endocytosis of macromolecules. In the latter model, as in the case of vitamin B-12, folate might first bind to a plasma carrier protein that in turn binds to surface receptors before internalization into the cell. In fact, a soluble plasma protein and a membrane binder that have a high affinity for folates have been characterized, but a transport function has not yet been defined (4–6). Studies by Kolhouse and associates suggest that a soluble, high-affinity folate binder in milk is immunologically related to the high-affinity binding sites for folic acid on human placenta (7).

To address the question of how folate is taken up by cells, we have studied folate binding and internalization in MA104 cells, a monkey kidney cell line adapted for growth in low-folate medium. Using radiolabeled 5-methyltetrahydrofolate as a ligand, we present evidence that these cells contain a folate-regulated high-affinity surface receptor for folates, that tightly bound folates can be removed from this surface receptor with pH 4.0 buffer, and that this receptor mediates the internalization of folate without a requirement for any plasma folate-binding factor. We also show that the activity of the receptor is regulated by the folate content of the cell.

MATERIALS AND METHODS

Buffers and Tissue Culture Medium. Dulbecco's phosphate-buffered saline (PBS) was obtained from Hazelton Laboratories (Denver, PA). Medium 199 (M199) with Earle's salts and folic acid was purchased from GIBCO and supplemented with glutamine (GIBCO). RPMI 1640 medium without folic acid was formulated in our laboratory. Organic components were purchased from Sigma; the inorganic salts were obtained from Fisher Scientific. The RPMI medium was supplemented with adenine sulfate (10 mg/liter), thymine (0.3 mg/liter), xanthine (0.3 mg/liter), and hypoxanthine (0.3 mg/liter) (Sigma), and it was buffered with Hepes (25 mM, Sigma) and sodium bicarbonate (2 g/liter). All tissue culture medium contained fetal bovine serum (Hazelton Laboratories). To prepare folate-deficient medium, the serum was treated with activated charcoal (Norit A, Fisher Scientific) as detailed elsewhere (8) and filtered through a 0.25-μm pore filter (Millipore). Trypsin solution (0.5 g/liter) containing EDTA (0.2 g/liter) was purchased from GIBCO.

Determination of Radioactivity. Scintillation fluid was prepared by mixing 2 liters of toluene (Fisher Scientific) containing 9 g of Preblend 2a70 (Research Products International, Mt. Prospect, IL) with 1.2 liters of Triton X-100 (purchased from the same supplier). Radioactivity was quantitated in a Packard Tri-Carb 460 at a counting efficiency of 42%.

Tissue Culture. MA104 cells were maintained in M199 containing 5% serum (vol/vol) and passaged or harvested for study by treatment for 5 min with 1 ml of the trypsin solution at 37°C. Dislodged cells were collected by gentle aspiration and, when necessary, concentrated by low-speed centrifu-

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Abbreviation: FBP, folate-binding protein.

gation (5 min at 200 × g). Routinely, cells were seeded at 2–2.5 × 10^5 cells per T 25 flask (Costar, Cambridge, MA) in 5 ml of medium. For growth in low-folate medium, the complete M199 was replaced 12–16 hr after plating with the folate-different RPMI medium.

Analysis of Cell Folate Binding and Accumulation. Cell binding of radioactive folate was determined by incubating cells with ligand as indicated after the cells were washed with 5 ml of cold PBS. Incubations were done on a rocking platform (Bellco Glass) at the indicated temperature and conditions in a final volume of 2–3 ml. Prior to harvesting cells for analysis of radioactivity, we aspirated the incubation medium, and the cells were washed with two 5-ml aliquots of cold PBS. Nonspecific binding was determined by measuring radioactivity bound in the presence of a 500-fold excess nonlabeled 5-methyltetrahydrofolate.

Since folate rapidly dissociates from specific, high-affinity binding factors when in acid pH (9) and an acidic saline wash has also been used to separate epidermal growth factor from cell-surface receptors (10), we used an acidic saline wash to remove surface-bound folate. After rinsing with PBS, cells were incubated 2 min at 4°C with 1.5 ml of 0.15 M sodium chloride adjusted to pH 3.5 with acetic acid and then rinsed with 1.5 ml of cold PBS. The cells then were released from the plate with trypsin/EDTA, and the radioactivity in the wash and cells was determined by liquid scintillation counting.

Folate Analogs and Radiolabeled Compounds. 5-Methyltetrahydro[3′,5′,7,9-$^3H_4$]folic acid (5-methyltetrahydro[^{3}H]folic acid) was synthesized from high-specific activity [3′,5′,7,9-$^3H_4$]folic acid ([^{3}H]folic acid; 18–20 Ci/mmol from Moravek Biochemicals, City of Industry, California; 1 Ci = 37 GBq) by enzymatic reduction to tetrahydrofolate using *Lactobacillus casei* (Biopure Fine Chemicals, Boston, MA, 0.07 units of enzyme per mg of dry weight) followed by methylation according to established methods (11). The folates were separated from the other reaction mixture components by using a 0.5 × 2.5 cm column of Dowex 50X4, 200–400 mesh, ammonium form, according to Duch *et al.* (12); however, we omitted ascorbate from the buffer. The lyophilized eluate from the Dowex chromatography containing all folates was dissolved in 200 μl of 0.1 M Tris, pH 8.5/0.05 M mercaptoethanol, and the folates were separated by HPLC using a μBondapak C_{18} column (0.39 × 15 cm, Waters). Aliquots containing the 5-methyltetrahydro[^{3}H]folic acid were pooled, lyophilized, and dissolved in 0.05 M mercaptoethanol to yield a final folate concentration of 4–5 μM and then stored at −70°C under nitrogen. This methodology resulted in a 40–50% recovery of the starting [^{3}H]folic acid as 5-methyltetrahydro[^{3}H]folate, having a specific activity of 8000–16,000 cpm/pmol, depending on the specific activity of the [^{3}H]folic acid.

[^{3}H]Folic acid and [^{3}H]methotrexate (Moravek Biochemicals, 10–20 Ci/mmol) were used as obtained from the manufacturer. Purity was >98% on days of experiments as assessed by HPLC or ion-exchange chromatography (13).

Methotrexate and 5-formyltetrahydrofolate were obtained from Lederle Laboratories (Pearl River, NJ). Other folate analogs were purchased from Sigma and used without further purification.

Preparation of Antibody and Antibody Assay. Rabbit anti-porcine plasma folate-binding protein (FBP) was prepared by immunizing a rabbit subcutaneously with 5 μg of FBP suspended in complete Freund's adjuvant. The rabbit was given three weekly injections and then a similar booster injection approximately 3 weeks later. Approximately 2 weeks later, serum was harvested for analysis of antibody. Thereafter, the animal was bled once or twice weekly until the antibody titer began to wane.

Binding of FBP by antiserum was assessed by determining the amount of FBP precipitated by protein–staphlococcus A beads (Calbiochem) after incubation of the binding protein with serum from the immunized rabbits. The methodology was similar to that detailed by others (14). The antisera used in the experiments presented precipitated FBP with or without bound folate. One microliter of the antiserum precipitated approximately 0.1 pmol of purified FBP. Prior to use in tissue culture experiments, serum was treated with charcoal to remove folate. The antisera had ≤ 0.1 nM folate and did not contain measurable folate-binding activity.

FBP. FBP was prepared from approximately 40 liters of porcine plasma obtained from a local processing plant. It was purified by techniques of acidification, affinity chromatography on methotrexate-Sepharose and Sephadex G-100 (Pharmacia) chromatography as detailed (15). Based upon a molecular size of 38,500 Da, the [^{3}H]folic acid-binding capacity, and the protein concentration, the specific activity of the FBP preparation was 0.8 pmol of folate bound per pmol of protein.

Folate Assay. Folate was measured by radioligand-binding assay by using sequential saturation analysis and extraction techniques detailed elsewhere (16). Commercial β-lactoglobulin (Sigma) was used as the source of folate binder. The assay has an absolute sensitivity of 0.1 pmol and detects folate polyglutamates without need for conjugase treatment. Folate complete and depleted medium were ≈50 nM and ≤0.4 nM in folate, respectively.

RESULTS

MA105 cells grown in folate-deficient medium have a normal growth rate for 3 days and typically will undergo several rounds of division. At the end of this growth period, they contain 0.5 pmol of folate per 10^6 cells, whereas a companion set of cells grown in the presence of folate contain 3–5 pmol of folate per 10^6 cells. Cells grown in the folate-depleted medium exhibited a saturable binding of 5-methyltetrahydro[^{3}H]folate at 4°C (Fig. 1). Maximum binding was observed at 20 nM folate, and half-maximal binding was found to be at 2–3 nM. In the presence of excess unlabeled 5-methyltetrahydrofolate, binding of radiolabeled folate was inhibited >90% (Fig. 1).

To better understand the relationship between the cellular concentration of folate and the ability of cells to bind 5-methyltetrahydro[^{3}H]folate to surface membrane-binding sites, cellular folate concentration and surface binding sites were measured in MA104 cells at various times after being incubated in folate-deficient medium. At the time of initial plating in folate-depleted medium, the concentration of folate

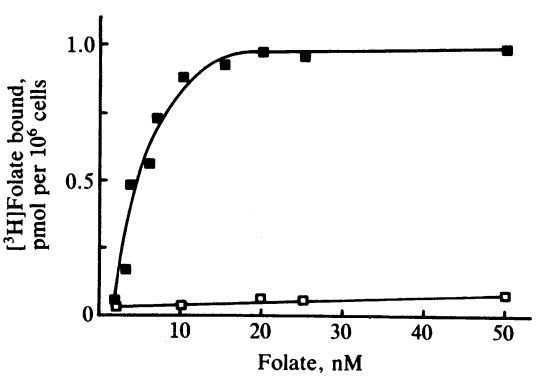

FIG. 1. Cell binding of 5-methyltetrahydro[^{3}H]folate at 4°C as a function of extracellular folate. Folate-deficient MA104 cells were incubated with 5-methyltetrahydro[^{3}H]folate for 1 hr at 4°C in the presence (□) or absence (■) of a 100-fold excess of 5-methyltetrahydrofolate to distinguish specific and nonspecific binding.

was 3 pmol per 10^6 cells and the cells exhibited very little binding of 5-methyltetrahydro[^{3}H]folate at 4°C (Fig. 2). However, after approximately 2 days of incubation in low-folate medium, the folate concentration declined to <1 pmol per 10^6 cells, and there was a dramatic increase in the amount of 5-methyltetrahydro[^{3}H]folate bound.

In addition to binding the physiologic (plasma) form of folate, 5-methyltetrahydrofolate, folate-depleted MA104 cells also bound [^{3}H]folic acid and [^{3}H]methotrexate (Fig. 3). Although each of these ligands bound to the cells in a concentration-dependent manner and each reached saturation for binding at 1 pmol per 10^6 cells, the half-maximal binding was quite different. Whereas half-maximal binding for 5-methyltetrahydrofolate was 2–3 nM, half-maximal binding for folic acid and methotrexate were approximately 0.4 and 20 nM, respectively. These results suggest that each of these folate analogs binds to the same surface receptor but with different affinities. This interpretation was confirmed by testing the ability of folic acid analogs to compete for 5-methyltetrahydro[^{3}H]folate binding to the MA104 cells at 4°C. Folic acid was the most effective, followed by 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, and then methotrexate. Pteroic acid, glutamic acid, and *p*-aminobenzoylglutamic acid, molecular components of folate, did not block binding even when present in a 100-fold excess (data not shown).

If this receptor is involved in the uptake of folates, then it should mediate its internalization. To answer this question, we compared the ability of folate-depleted cells to take up 5-methyltetrahydro[^{3}H]folate at 4°C and at 37°C. Uptake due to surface binding was distinguished from that due to internalization by assessing the amount of radiolabeled ligand that could be released from the cells at 4°C with an acid wash. When folate-depleted cells were allowed to incubate in the presence of 5-methyltetrahydro[^{3}H]folate at 4°C for 4 hr (Fig. 4), the level of uptake was maximal by 30 min. Virtually all of the bound 5-methyltetrahydro[^{3}H]folate could be released by acid wash. When cells were incubated with 5-methyltetrahydro[^{3}H]folate at 37°C, the amount bound to the surface (acid releasable) also was maximal within 30 min and was similar to the amount measured at 4°C. However, at 37°C acid-resistant material continued to accumulate over a 4-hr period. After 4 hr of incubation at 37°C, the cells internalized an amount of 5-methyltetrahydrofolate that was equivalent to 3–4 times the amount bound to surface receptors at 4°C. Interestingly, this amount is equal to the concentration of folate in cells grown in complete medium. Moreover, cells incubated for 24 hr at 37°C in the presence of 5-methyltetrahydro[^{3}H]folate internalized only 1 pmol of folate more

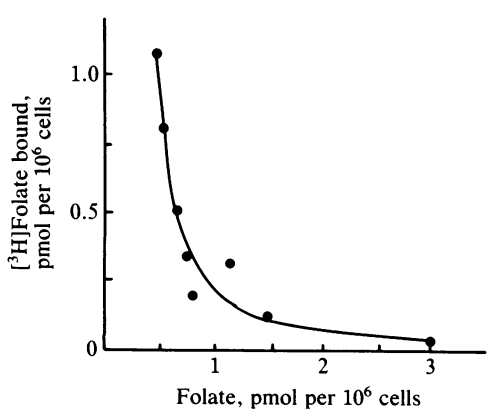

FIG. 2. Cell content and binding of 5-methyltetrahydro[^{3}H]folate as a function of intracellular folate. Cell folate content and binding of 5-methyltetrahydro[^{3}H]folate (20 nM) at 4°C by MA109 cells grown for 12–96 hr in folate-deficient medium to deplete intracellular folate was measured. Binding was measured as in Fig. 1.

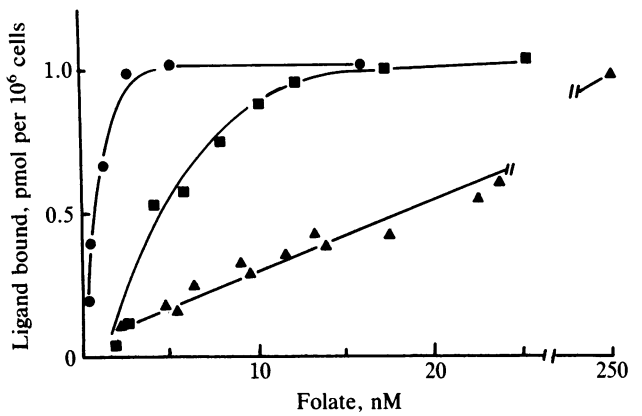

FIG. 3. Cell binding of radiolabeled folic acid, 5-methyltetrahydro[^{3}H]folate, and methotrexate as a function of extracellular ligand concentration. After MA104 cells were grown for 72 hr in folate-deficient medium, the binding of ligand at indicated concentrations was determined by using a 1-hr incubation at 4°C ●, [^{3}H]Folic acid; ■, 5-methyltetrahydro[^{3}H]folate; ▲, [^{3}H]methotrexate.

than that which was accumulated after 4 hr of incubation (Fig. 4). The amount of acid-labile radiolabel also declined, suggesting a decrease in the number of surface receptors.

Additional evidence that the receptor was responsible for folate accumulation at 37°C was obtained by measuring acid-resistant radioactivity in cells either incubated simultaneously with 5-methyltetrahydro[^{3}H]folate (40 nM) and folic acid (10 nM) for 2 hr at 37°C or first incubating with folic acid (5 nM) for 1 hr at 4°C, removing free folic acid and then incubating with 5-methyltetrahydro[^{3}H]folic acid (40 nM) for 2 hr at 37°C. In each case, the acid-resistant radioactivity was reduced (95% and 65%, respectively) compared to cells incubated with only 5-methyltetrahydro[^{3}H]folic acid. Thus, when the receptor was saturated with the higher-affinity ligand (folic acid), the net accumulation of the 5-methyltetrahydro[^{3}H]folate was decreased.

Previous investigators have demonstrated that high-affinity binding sites for [^{3}H]folate on the surface of KB cells can be inhibited with antibodies to purified cell-membrane FBPs (17). Therefore, we performed experiments to see whether the high-affinity binding sites on the surfaces of MA104 cells were related to a plasma folate-binding factor. When 5-methyltetrahydro[^{3}H]folate was preincubated with purified porcine plasma FBP and then tested for the ability of the

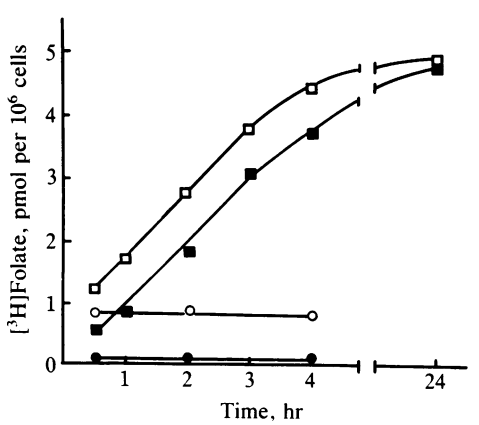

FIG. 4. Cell binding and accumulation of 5-methyltetrahydro[^{3}H]folate at 4°C and 37°C by folate-deficient cells. MA104 cells, grown for 3 days in folate-depleted medium were incubated with 20 nM 5-methyltetrahydro[^{3}H]folate for the times indicated, and then total radioactivity (○, □) and acid-resistant radiolabel (●, ■) were determined at 4°C (○, ●) and 37°C (□, ■).

radioligand to bind to the surface of MA104 cells, virtually no binding was observed (Table 1).

Antibodies against purified porcine plasma FBP inhibited the binding of 5-methyltetrahydro[^{3}H]folate to the surface of MA104 cells in a concentration-dependent manner (Fig. 5). These results suggest that the soluble FBP in no way mediates the uptake of folate by these cells but that the soluble and membrane folate-binding factors share epitopes.

DISCUSSION

The characteristics of 5-methyltetrahydro[^{3}H]folate binding to the surface of MA104 cells suggests that these cells contain a high-affinity receptor for this ligand. This receptor also is capable of binding similar amounts of folic acid and methotrexate, ligands that compete for 5-methyltetrahydrofolate binding to the surface of these cells. On the other hand, specific moieties of the folic acid molecule did not compete for 5-methyltetrahydro[^{3}H]folate binding sites, supporting the specific nature of the receptor.

This high-affinity binding for 5-methyltetrahydrofolate was clearly regulated by cellular folate concentration. Cells grown in the presence of folate bound <10% of the amount of folate, compared to cells grown in the absence of folate for 3 days. Moreover, there was a direct correlation between the ability of cells to bind 5-methyltetrahydrofolate and the cellular folate content (Fig. 2). It is this phenomenon that may at least partially explain the apparent K_m values of >1 μM for folate and methotrexate commonly measured in most cells studied *in vitro* (3). Folate-repleted cells do not contain significant "unsaturated" folate receptors; thus, the measured K_m values may reflect the concentration of extracellular compound necessary to either exchange radiolabeled folate with the endogenous bound material or to be transported via a general anion-transport system as detailed by Henderson and Zevely (18).

The membrane receptor described here was able to mediate the internalization of 5-methyltetrahydrofolate. Over the course of 4 hr of incubation in the presence of 20 nM 5-methyltetrahydro[^{3}H]folate, the cells accumulated 3–4 times more folate than could bind to the surface receptors. Little additional folate accumulated after this time, even though cells still contained approximately the same number of high-affinity receptors as the starting cells (Fig. 4). Therefore, folate receptor activity can be regulated acutely. Further, when the cells are incubated in a continuous presence of folate, there is a down-regulation of receptor number (Fig. 4).

The high-affinity receptor on the surfaces of MA104 cells behaves as if it is capable of internalizing folate by receptor-mediated endocytosis. The kinetics of internalization are similar to those found for other receptors involved in receptor-mediated endocytosis; receptors appear to recycle because cells internalize 3–4 times more folate than can bind to the cell surface, and the binding of [^{3}H]folate to the receptor is sensitive to pH, a common property of receptors involved in receptor-mediated endocytosis (19). Therefore, the itinerary for folate uptake may be (*i*) binding to surface receptor, (*ii*) internalization by endocytosis, and (*iii*) release from the receptor in the acidic endosome.

Table 1. Effect of FPB on binding of 5-methyltetrahydro[^{3}H]folate

Ligand	Total cpm incubated*	Cell binding,† cpm per 10^6 cells
5-Methyltetrahydro[^{3}H]folate	120,000	8000
5-Methyltetrahydro[^{3}H]folate binder complex	118,000	250

*Approximately 5 nM folate in a 3-ml volume. Values are means ± 5%.

†Cells grown for 3 days in RPMI folate-deficient medium were incubated for 1 hr at 4°C with 5-methyltetrahydro[^{3}H]folate or an equivalent amount of [^{3}H]folate–FPB complex. The [^{3}H]folate–FBP complex was formed by incubating the FBP in the presence of excess 5-methyltetrahydro[^{3}H]folate for 15 min at room temperature and then removing unbound folate with charcoal (8).

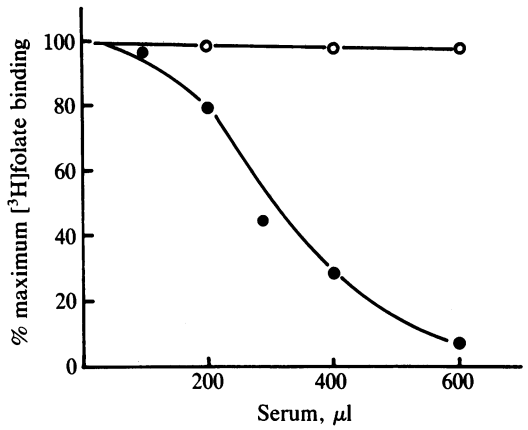

FIG. 5. Effect of rabbit anti-porcine plasma FBP on 5-methyltetrahydro[^{3}H]folate binding to MA109 cells. Cells were incubated for 1 hr at 4°C with immune (●) or preimmune (○) sera, and then 5-methyltetrahydro[^{3}H]folate (10 nM) was added for an additional hr and binding of radiolabeled ligand was determined. Maximum binding (100%) was the value obtained in the absence of any type of rabbit serum.

The 5-methyltetrahydrofolate binding site on the surface of MA104 cells clearly is related to soluble folate-binding factors that occur in plasma. However, this binding factor seems not to be involved in mediating the internalization of folate. Rather, it may represent membrane receptors for folate that have been shed from cells. Other membrane receptors involved in receptor-mediated endocytosis can be shed into the medium in response to specific manipulation (20).

The results of these studies provide a framework for analyzing the role that this receptor plays in the regulation of folate uptake by normal cells. Understanding the biology of this receptor should lead to a better understanding of normal and abnormal folate metabolism and homeostasis.

The helpful criticism and advice of R. G. W. Anderson cannot be overstated. The patience of D. Miller in preparing the manuscript is appreciated. The work was supported by The American Cancer Society (Grant CH228). B.A.K. is a Scholar of the Leukemia Society of America.

1. Kisliuk, R. L. (1984) in *Folate Antagonists as Therapeutic Agents*, eds. Sirotnak, F. M., Burchall, J. J., Ensminger, W. B. & Montgomery, J. A. (Academic, Orlando, FL), pp. 1–68.
2. Winick, N. J., Krakower, G. R. & Kamen, B. A. (1985) in *Proceedings of Second Workshop on Folyl and Antifolyl Polyglutamates*, ed. Goldman, I. D. (Praeger, New York), pp. 297–307.
3. Sirotnak, F. M. (1985) *Cancer Res.* **45**, 3992–4000.
4. Kamen, B. A. & Caston, J. D. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4261–4264.
5. McHugh, M. & Cheng, Y. C. (1979) *J. Biol. Chem.* **254**, 11312–11318.
6. Selhub, J. & Franklin, W. A. (1984) *J. Biol. Chem.* **259**, 6601–6606.
7. Antony, A. C., Utley, C., Van Horne, K. C. & Kolhouse, J. F. (1981) *J. Biol. Chem.* **256**, 9684–9692.
8. Kamen, B. A. & Caston, J. D. (1974) *J. Lab. Clin. Med.* **83**, 164–171.
9. Kamen, B. A. & Caston, J. D. (1975) *J. Biol. Chem.* **250**, 2203–2205.
10. Haigler, H. T., Maxfield, F. R., Willingham, M. C. & Pastan, I. (1980) *J. Biol. Chem.* **255**, 1239–1241.

11. Horne, D. W., Briggs, W. T. & Wagner, C. (1977) *Anal. Biochem.* **83,** 615–621.
12. Duch, D. S., Bowers, S. W. & Nichol, C. A. (1983) *Anal. Biochem.* **130,** 385–392.
13. Moran, R. J., Werkheiser, W. & Zakrzewski, S. F. (1976) *J. Biol. Chem.* **251,** 3569–3575.
14. Kessler, S. W. (1981) *Methods Enzymol.* **73,** 442–458.
15. Kamen, B. A. & Caston, J. D. (1980) *Methods Enzymol.* **66,** 678–686.
16. Zettner, A. & Duly, P. E. (1974) *Clin. Chem.* **20,** 5–14.
17. Antony, A. C., Kane, M. A., Portillo, R. M., Elwood, P. C. & Kolhouse, J. F. (1985) *J. Biol. Chem.* **260,** 14911–14917.
18. Henderson, G. B. & Zevely, E. M. (1982) *Biochem. Int.* **4,** 493–502.
19. Brown, M. S., Andersen, R. G. W. & Goldstein, J. L. (1983) *Cell* **32,** 663–667.
20. Cone, R. E. (1977) in *The Lymphocyte Structure and Function: Part II*, ed. Marchalonis, J. J. (Dekker, New York), pp. 567–592.