Studies on the Uptake of Synthetic Conjugated Folates by Human Marrow Cells

By A. V. Hoffbrand, Edith Tripp, Catherine M. Houlihan, and J. M. Scott

Pteroyltriglutamate shows little ability (between 1/10 and 5/10) compared to pteroylmonoglutamate (folic acid), to enter human marrow cells and to act as a coenzyme in intracellular DNA synthesis. This was shown by comparing the effectiveness of these two forms of the vitamin at stimulating folate-dependent pyrimidine incorporation into DNA in vitro in the bone marrow cells and lymphocytes of patients with megaloblastic anemia. It, therefore, appears that human hemopoietic cells (like *Streptococcus faecalis* rather than *Lactobacillus casei*) are unable to take up efficiently polyglutamy forms of folate. The suggestion that polyglutamyl analogues of the antifolates might be more effective chemotherapeutic agents than corresponding monoglutamates, while biochemically possible, would appear to be precluded because of failure of transport of these compounds into human hemopoietic cells.

It has been appreciated for some time that folate occurs in natural materials largely with three or more glutamic acid residues attached to the pteroyl portion of the molecule. During absorption of food folates, these folate polyglutamates are enzymatically hydrolyzed to the equivalent monoglutamate derivatives. It is still uncertain where this hydrolysis occurs, but the finding that the enzyme involved, γ-glutamyl carboxypeptidase (folate conjugase, pteroylpoly glutamate hydrolase), is found within the lysosomes of the small intestinal cells raises the possibility that conjugated folates may enter the intestinal cell as such before hydrolysis to the monoglutamate form.

Some bacterial cells (e.g., *L. casei*) are capable of transporting triglutamate forms of folate, while others (e.g., *S. faecalis*) cannot transport compounds with more than two glutamate moieties. No equivalent information is available, however, about transport of conjugated folates into animal or human cells. The demonstration that polyglutamyl analogues of at least some antifolates are more effective in vitro than the corresponding monoglutamate forms and the suggestion that antifolate polyglutamates might be used in chemotherapy make the question of the cellular transport of conjugated folates more urgent.

Previous work has shown that dU (deoxyuridine) does not inhibit the uptake
of $^3$HTdR (tritiated thymidine) by megaloblasts in vitro to the same extent as it inhibits the uptake of $^3$HTdR by normoblasts.\cite{7-9} The explanation for this difference is thought to be as follows (Fig. 1). Exogenous dU is taken up by normal cells and is phosphorylated to deoxyuridine monophosphate (dUMP); dUMP is then methylated to thymidine monophosphate (TMP) under the action of the enzyme thymidylate synthetase, and TMP is then phosphorylated to its triphosphate derivative (TTP). TTP allosterically inhibits thymidine kinase, the enzyme responsible for uptake and phosphorylation of exogenous $^3$HTdR. Addition of dU extracellularly is, therefore, thought to reduce the uptake of $^3$HTdR by normoblasts through raising their intracellular level of TTP. On the other hand, folate deficiency per se (or disturbed folate metabolism caused by vitamin $B_{12}$ deficiency) is thought to inhibit thymidylate synthetase, since a folate coenzyme is required for this reaction. The overall conversion of dU to TTP, and so the degree of inhibition of $^3$HTdR uptake by exogenous dU, is, therefore, less for megaloblasts than for normoblasts.

Metz et al.\cite{8} showed that folic acid added in vitro would completely correct the dU blocking in megaloblasts, whether the megaloblastic hemopoiesis was due to folate or to vitamin $B_{12}$ deficiency. In the present studies, the ability of PteGlu$_3$ (pteroyltriglutamate) and PteGlu (pteroylglutamic acid, folic acid) to correct dU blocking in megaloblasts was compared.

**MATERIALS AND METHODS**

Studies were carried out on bone marrow cells and peripheral lymphocytes of six patients with megaloblastic anemia. Five patients had untreated pernicious anemia. Their hemoglobin levels ranged from 5.3 to 8.7 g/100 ml; serum vitamin $B_{12}$ levels were 30–65 pg/ml and serum folate levels were 2.9–18.9 ng/ml. The sixth patient (subject 6, Table 1) had nutritional folate deficiency (hemoglobin 6.3 g/100 ml, serum folate 0.9 ng/ml, serum vitamin $B_{12}$ 125 pg/ml).

Short-term bone marrow cultures and cultures of lymphocytes transformed with phyto-
hemagglutinin (PHA) were set up as previously described.

In the present studies, the cells were washed three times with cold (4°C) phosphate-buffered saline (pH 7.4) to remove all traces of plasma, and the volume was reconstituted with tissue culture medium 199 (Wellcome Laboratories) before addition of folate compounds.

The ability of the folate compound to enter the cell and take part in intracellular biochemical reactions was assessed by the dU (deoxyuridine) suppression assay. These assays were carried out as previously described.

Microbiologic Assays
The L. casei and S. fecalis assays were carried out as described previously.

Chemical Synthesis of Pteroyltriglutamate (PteGlu)

The solid-phase synthesis method, described originally by Krumdieck and Baugh, was used with some technical modifications. The pteroic acid used was purified by the method of Houlihan et al., and during the synthesis the acetylation step introduced into the original method was employed. This step has the effect of blocking any unreacted incomplete chains during the synthesis. This blocking precludes them from reacting in the final step, namely the coupling of pteroic acid. The result is that lower folate homologues cannot be synthesized, and the resultant product is uniform and contains a constant number of glutamyl residues.

Authenticity of the Prepared PteGlu

For these studies, it was essential that the PteGlu was pure, that it contained the number of glutamyl radicals stated, and that these radicals were linked correctly, namely, in the γ configuration. As pointed out above, the introduction of the acetylation step makes the synthesis of a lower homologue impossible. In addition, in the DEAE chromatography used in the purification of the product, the di- and monoglutamyl derivatives are completely separated. Reanalysis of the purified product by the same system or by thin-layer chromatography failed to detect any contaminants that, had they been present at a level of 1%, would easily be detected. Furthermore, the ability of the purified PteGlu to support the growth of S. fecalis was less than 1% of its activity for L. casei. That the final product had the correct natural configuration was demonstrated by the fact that pteroylpolyglutamate hydrolase, which is known to have an absolute requirement for the γ configuration, completely hydrolyzed the PteGlu to the monoglutamate, folic acid (PteGlu).

RESULTS

Bone Marrow

At approximately equimolar concentrations of $2.7 \times 10^{-4}$ M, both PteGlu and PteGlu completely corrected the dU blocking of $^3$HTdR uptake in all bone marrow cultures (Table 1). However, only slight correction could be obtained in one of three cultures with PteGlu at a concentration of $1.35 \times 10^{-4}$ M and none with lower concentrations, whereas PteGlu still showed some correction in all six cultures tested at $2.7 \times 10^{-5}$ M, in both cultures tested at $5.4 \times 10^{-6}$ M, and in three of five cultures tested at $2.7 \times 10^{-6}$ M.

Lymphocytes

Fewer studies were performed, but the results were similar to those obtained with bone marrow. At a concentration of $2.7 \times 10^{-4}$ M, PteGlu and PteGlu both corrected dU blocking of $^3$HTdR uptake in one culture. In this culture PteGlu still corrected at a concentration of $2.7 \times 10^{-5}$ M, whereas PteGlu
Table 1. Effect of Folic Acid (PteGlu) and Pteroylglutamate (PteGlu₃) on the Blocking by Deoxyuridine (dU) of Uptake of $³$H-Thymidine ($³$HTdR) Into DNA by Bone Marrow Cells and Lymphocytes From Patients With Megaloblastic Anemia

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<th>1.35 × 10⁻⁴</th>
<th>2.7 × 10⁻⁴</th>
<th>5.4 × 10⁻⁴</th>
<th>2.7 × 10⁻⁴</th>
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*Amount of PteGlu and PteGlu₃ added to lymphocyte cultures (final volume 3.4 ml) was 8.1 times that for the bone marrow cultures (final volume 0.42 ml).
failed to correct at a concentration below $2.7 \times 10^{-4} \text{ M}$ (Table 1). In a second culture, similar results were obtained with PteGlu, but PteGlu3 failed to correct even at the highest concentration, $2.7 \times 10^{-4} \text{ M}$.

*Salmonella* fecalis assays of the culture supernatants at the end of the incubation period showed no increase over the initial assay value, suggesting that no hydrolysis of PteGlu3 to PteGlu or PteGlu had occurred during the incubation periods, or if this hydrolysis had occurred, it was extremely slight.

**DISCUSSION**

These results indicate that the ability of pteroyltriglutamate (PteGlu3) to enter human hemopoietic cells and to participate in DNA synthesis is very significantly less than that for the monoglutamate, folic acid. In these studies, PteGlu corrected the dU blocking of $^3$H-thymidine uptake by megaloblasts only about 1%-5% as well as PteGlu. Since triglutamate forms of the vitamin have shown to be, if anything, more active than the monoglutamate at the enzymatic level in the few instances when this has been studied, it seems likely that the failure of PteGlu3 in the present system is due to a permeability problem. Nevertheless, PteGlu3 did have a marginal effect, and this is probably not due to contamination of the starting material with the monoglutamate. To explain the results on this basis, a contamination of the triglutamate with between 1% and 5% of monoglutamate would have to exist. This would have been well within the detection limits of the chromatography methods used to authenticate the purity of the triglutamate, and no such impurity was found. Moreover, with the improved method of synthesizing the polyglutamate used, lower homologues are inactivated during the synthesis, and the resulting product has an unambiguous number of glutamyl radicals, in this instance, three.

Thus, it is possible either that a small amount of triglutamate transport takes place at the highest concentrations used ($1.35 - 2.7 \times 10^{-4} \text{ M}$), or that a small amount of enzymatic breakdown of the added triglutamate by pteroyl-polyglutamate hydrolase released by cell lysis during the incubation takes place, and the monoglutamate so produced enters the cells. We have no entirely conclusive evidence which of these possibilities is correct, but assays with *Salmonella* fecalis showed that no difference in the supernatant folate activity occurred before and after the incubation. Thus, if any hydrolysis of pteroyltriglutamate had occurred, it could only have been slight. In any event, it is clear that the addition of two further glutamyl radicals to folic acid greatly, if not totally, renders it incapable of participating in intracellular biochemical reactions when added to the fluid surrounding intact human hemopoietic cells. It seems likely that this will also pertain to antifolates of similar structure to folic acid, and thus, the usefulness of pteroylglutamyl analogues of such antifolates, while sound biochemically, may be ineffective in practice.

**REFERENCES**


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