Summary of Informal Discussions on the Role of Folic Acid Antagonists

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The two formal papers considered both the capacity for cellular uptake of amethopterin and the nature of enzymic inhibition in seeking to explain the selective effects which folate antagonists can exert on particular tissues or neoplasms. These considerations and questions concerning drug resistance were discussed at some length. Excerpts from the vigorous discussion about this class of compounds are grouped in relation to particular topics in the following summary.

Selective Transport or Differential Penetrability of Cells

Dr. Potter pointed out that in addition to the capacity of tissues to take up a drug, the period of exposure of tumors to circulating amethopterin and the rate of blood flow per unit weight of tissue are important considerations influencing the degree of enzyme inhibition occurring in different tissues in vivo. Although no direct estimates of blood flow were made in comparing the effects of amethopterin on the Murphy-Sturm lymphosarcoma and on the Walker carcinoma 256, Dr. Werkheiser commented that access of the drug to the sensitive enzyme seems to be more critical than actual enzyme amount, since the difference in enzyme content of these two tumors is relatively small and, in some ascites tumors, there is no correlation between the amount of folate reductase and the relative sensitivity to amethopterin.

Dr. Bertino cited evidence that amethopterin induced a state of folic acid deficiency as reflected by a fall in level of serum folates and posed the question, “Why does dietary deficiency of folic acid affect the growth of the Walker tumor, whereas amethopterin-induced folic deficiency does not?” Dr. Nichol commented that this paradoxical situation may be related to differences between the nutritional requirements in vivo of the two tumors used in this model system. The simple dietary deficiency seems to permit a more selective action against tissues which have poor capacity for uptake of folate compounds and avoids the limiting toxic effects of amethopterin. The effects of combining restricted intake of folic acid with milder amethopterin treatment or dosage with weaker folate antagonists to induce a controlled degree of deficiency certainly deserve further exploration. Dr. Burchenal recalled that in his early studies of an amethopterin-resistant AKR leukemia, treatment with 9-methyl or X-methyl folic acid definitely increased the period of survival. It is not known whether this treatment induced a deficiency of the vitamin or whether the analog was being metabolized by folate reductase to form an inhibitor of tetrahydrofolate. Dr. Rosen described an experiment in which animals fed folic-deficient diets bearing the Walker and Murphy-Sturm tumors bilaterally were given a single injection of folic acid. The total folic acid content of the tumors indicated that the Murphy-Sturm tumor takes up more folic acid than does the Walker tumor in the same animal. The concept based on earlier nutritional work that tumors have greater capacity than normal tissues for trapping amino acids does not seem applicable to folic acid and other vitamins which have been studied so far.

Dr. Fischer discussed the development of an in vitro system for studying the rate of amethopterin entry into L-5178 cells. By a centrifugation technic, the amount of amethopterin entering the cells could be measured after very short periods of exposure to the drug. In addition to the interpretation that a process of active transport is involved, Dr. Fischer stated that the transport of amethopterin apparently occurs by a system different from that for folic acid. Since 5-formyltetrahydrofolate could interfere competitively with the transport of amethopterin in this system, Dr. Fischer suggested that dihydrofolate might compete with amethopterin in vivo for a common transport system although limitations in extrapolating from in vitro to in vivo relationships were recognized. Dr. Fischer commented on preliminary studies to evaluate the capacity of leukemic cells to take up amethopterin with the objective of relating this parameter to the patient’s responsiveness to amethopterin treatment. Dr. Werk-
HEISER cited work of Dr. Hakala on the transport of amethopterin by Sarcoma 180 cells in culture. Interference with the transport of this drug by citrovorum factor occurred by a mechanism which did not appear to be competitive. DR. NICHOL added a comment concerning the transport system studied by Dr. Hakala in which a kinetic study has provided estimates of the four rate constants which determine the apparent drug uptake, i.e., the rates of cell entry and exit of amethopterin and its attachment to and dissociation from folate reductase. The interpretation of these data indicated that the capacity to pump the drug out of these cells is apparently greater than the capacity for drug uptake. Consequently, agents which can impede drug exit might potentiate the action of amethopterin.

DRUG-ENZYME RELATIONSHIPS

DR. WERKHEISER expressed his view that apparent discrepancies between his data and those presented by Dr. Bertino could be explained by differences in methodology and experimental conditions used in the study of the amethopterin-reductase relationships. Folic acid is used as substrate at about 5–7 times the $K_m$ concentration, whereas dihydrofolate is used at concentrations which are nearly 100 times the $K_m$ for dihydrofolate. Also, the enzyme concentration for assays with dihydrofolate are usually somewhat lower than with folate. Consequently, the kinetic relationships may be altered sufficiently so that an appreciable portion of the drug is actually free. Under these circumstances, an effect of pH could become more apparent.

DR. HUENNEKENS commented on the conditions for measurement of optimal reductase activity, indicating that dihydrofolate was preferable to folate as a substrate and that, under some conditions, the Bratton-Marshall assay measured only a fraction of the tetrahydrofolate as a product. Since other factors such as pH, salt concentration, and the presence of cations can influence the observed activity, some caution was advised in interpreting too precisely the degree and nature of the inhibition which is observed. Also, kinetic studies using dihydrofolate reductase from chicken liver which was purified some 1500-fold indicated that the inhibition by amethopterin was not stoichiometric, but, instead, some dissociation of the inhibitor apparently occurred. DR. WERKHEISER cited as an advantage of the procedure using folate as substrate that the period of incubation can be lengthened to more than 3 hours, if necessary, to obtain suitable activity, and under proper conditions of assay (avoiding high concentrations of mercaptoethanol) the Bratton-Marshall procedure has measured the quantitative conversion of folate to tetrahydrofolate.

RELATIONSHIP OF AMETHOPTERIN TOXICITY TO REDUCTASE ACTIVITY

DR. CONDIT reviewed several types of experiments which he interpreted as supporting the concept that the 4-amino folic acid antagonists have two or more metabolic sites of action. The competitive relationship between 5-formyltetrahydrofolate and amethopterin has been cited repeatedly as indicating some inhibition of the function of the folate cofactors. Also, doses of amethopterin several thousand times smaller than the LD$_{50}$ completely block the capacity of the liver of mice to form citrovorum factor for several days. Larger doses block this process for periods as long as 3 weeks. Similarly, the smallest dose of amethopterin (0.05 mg/kg) blocks this system in kidney for about 20 days and in intestine for from 8 to 15 days. Inhibition of the capacity to reduce folic acid based on the in vivo assay does not correlate with the dosage of amethopterin which induces toxicity or death. Also, based on the duration of the effect of amethopterin in man, as indicated by the urinary excretion of citrovorum factor following injection of folic acid, Dr. Condit had recommended an interval of 2 weeks between periods of treatment with amethopterin. However, in the treatment of patients with leukemia, he found that this schedule did not show any advantages over the more conventional treatment every day.

DR. GOLDIN referred to some of his experiments in which a dose of aminopterin (0.01 mg/kg) very much smaller than the LD$_{50}$ abolished the protective effect of prior administration of folic acid on aminopterin toxicity without at the same time changing the LD$_{50}$ of the drug. Dr. Goldin also commented on the ability of reduced forms of folic acid to prevent the toxicity of amethopterin. Dihydrofolate behaves like tetrahydrofolate or citrovorum factor with respect to prevention of the lethal action of amethopterin in mice when injected simultaneously with this drug, whereas folic acid has no such protective effect under these conditions—only upon prior injection. Also, the ability of folic acid to protect against amethopterin toxicity can be suppressed completely for weeks by intermittent injection of small sublethal doses of amethopterin, yet no signs of toxicity are associated with this impaired function. The difference in behavior of dihydrofolate and folate is not readily explained on the basis of inhibition of the reductase enzyme alone.

In reply, DR. BERTINO expressed his feeling that
the different observations on the effects of folate and dihydrofolate could be resolved on the basis of inhibition of one enzyme since, in his experiments, even a large dose of amethopterin did not completely inhibit dihydrofolate reductase activity when measured at pH 7.5 with dihydrofolate as substrate. On the other hand, with folic acid as substrate, the inhibition was apparently complete. Thus, the capacity of the liver to utilize folate for the formation of tetrahydrofolate is probably not equivalent to the use of dihydrofolate for this purpose, since a partially competitive relationship occurs in the latter case. Furthermore, in leukemic patients receiving methotrexate, an apparent induction of dihydrofolate reductase, rather than prolonged inhibition, is observed after several days. Dr. Werkheiser stated his impression that complete enzyme inhibition may occur in the animal even though the enzyme appears to be only partially inhibited in the dihydrofolate reductase assay in vitro since dilution of the enzyme reduces the effectiveness of amethopterin in the presence of high substrate concentrations. He also presented the possibility that the tissue concentration achieved by high doses of dihydrofolate may be large enough to displace amethopterin from the reductase even though folic acid itself could not do so. Dr. Werkheiser cited a discrepancy between the findings reported by Dr. Condit and his own observations on the regeneration of reductase activity in tissues following treatment of mice with amethopterin—even after a large dose of amethopterin (10 mg/kg), folate reductase activity in intestinal mucosa, although completely inhibited after 1 day, had returned to 50 per cent of its normal level by the 2d day. Dr. Hitchings pointed out that adequate control of the purity of dihydrofolate acid used in all of these investigations is a very important consideration, since some tetrahydrofolate can be formed during the reduction of folate to dihydrofolate.

Drug Resistance

Dr. Hutchison reviewed studies on the association between increased levels of dihydrofolate reductase in sublines of L-1210 resistant to amethopterin and the absence of a subtelocentric chromosome. The absence of this chromosome seemed to be associated with a stable genetic change persisting through many transfer generations. After prolonged passage, the chromosome in a subline triply resistant to amethopterin, 6-mercaptopurine, and 5-fluorouracil disappeared, and the activity of the reductase enzyme was elevated. A repeated selection of amethopterin-resistant sublines derived from an original line taken from the frozen tumor bank again indicated the same correlation between disappearance of this marker chromosome and elevated reductase activity. However, an exception was noted upon finding a third triply resistant line which retained the subtelocentric chromosome but which had reductase activity comparable to that of the parent L1210. The importance of maintaining each of the tumor lines in a frozen bank was emphasized. Dr. Hutchison also described experiments which showed that chromosomes isolated from L-1210 cells were taken up by fibroblast cells in culture and could be clearly seen in the cytoplasm. Such studies were viewed as preliminary to attempts to transform sensitive cells to resistant ones or to convert resistant cells to drug-sensitive lines. Dr. Nichol cited work published by Dr. Ishihara and Dr. Hakala in which marker chromosomes identified in amethopterin-resistant lines of S-180 were also present when these cells had regained sensitivity to the drug after being carried for many months in the absence of the drug. In this case, there was no correlation between the presence of the marker chromosomes and the level of resistance or the amount of folate reductase.

Dr. Jukes presented some speculative comments concerning the likelihood that changes in resistance to amethopterin may result from mutational alteration of the amino acids at the amethopterin binding site through changes in the coding sequences involving adenine in messenger RNA. On the basis of the concept that the very firm binding of the 4-aminofolate antagonists may require two points of attachment to the enzyme, perhaps due to carboxylic acid groups in the protein, the hypothesis was put forward that only one of these is necessary for the binding of folate or dihydrofolate to this site. Thus, by a change in the amino acid sequence, the binding of the di- amino antagonists would presumably be diminished, whereas the affinity of the normal substrate would be unchanged. With reference to the work of Dr. R. Litman concerning the increase in resistance to streptomycin associated with treatment of the transforming DNA with nitrous acid, Dr. Jukes reviewed the evidence that deamination of cytosine and adenine is the most prominent consequence of such chemical treatment and related the effect of nitrous acid to the modification of base sequences which can code for glutamic and aspartic acids. It was pointed out that the results of such a change in amino acid sequence could be to modify the structure of the reductase enzyme either at the binding site or by inducing a conformational change. Also, an effect on an operator gene, rather than a structural
gene, might occur so that the amount of the reductase enzyme would be increased.

If the increase in dihydrofolate reductase which occurs repeatedly in experimental systems in association with the development of resistance to amethopterin also occurs in man, then it may be possible to take advantage of this circumstance for the development of new agents which might be effective against amethopterin-resistant cells. Dr. Friedkin described the use of the reductase assay for the selection of compounds which are effective substrates for this enzyme with the hope that the metabolic product might inhibit some of the reactions involving tetrahydrofolate cofactors. Thymidylate synthetase was used as a second enzyme screen to test the effectiveness of such compounds as inhibitors of one of the several carbon transfer reactions. The iodo derivative of dihydrofolate was particularly effective as a substrate for the reductase enzyme, but unfortunately the corresponding tetrahydro derivative was ineffective in preventing thymidine synthesis. Dr. Kensler expressed the opinion that an inhibitor of a tetrahydrofolate-dependent system formed as a product of the reductase enzyme would probably have a relatively high $K_i$ and suggested that analogs of tetrahydrofolate bearing ring substituents would be of particular interest. Dr. Werkheiser commented that in addition to the problems concerning the development of resistance during drug treatment, the clinical evidence that the various folate analogs affect only a few out of the many forms of cancer directs our attention equally toward the basis for natural refractoriness to amethopterin. Some refractory or resistant neoplasms do not have markedly elevated levels of folate reductase, and there is evidence that altered permeability is associated with lack of response to the drug of some experimental tumors. Consequently, the two aspects of the problem of resistance concerning increased amounts of the reductase and altered permeability barriers appear equally important and deserving of much further study.
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