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Antileishmanial Effect of Allopurinol

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Allopurinol (4-hydroxypyrazolo[3,4-d]pyrimidine) has been shown to inhibit the growth of *Leishmania braziliensis* in vitro at concentrations which are attainable in human tissues and body fluids. This compound is believed to act by interdicting the de novo synthesis of pyrimidines, probably through the formation of allopurinol ribotide. Its lack of toxicity makes it a potential candidate for animal experimentation and it may serve as a prototype for other agents with similar mechanisms of action.

Leishmaniasis constitutes a public health problem of international importance. Although knowledge of the epidemiology and parasitology of the disease has progressed satisfactorily, therapeutic efforts have met with only qualified success. The principle reasons for this are a lack of knowledge of the metabolism of the causative organisms and the toxicity of the therapeutic agents employed. Pentavalent antimonials, diamidines, and the polyene macrolide, amphotericin B, have been used, but toxic reactions frequently result in early cessation of therapy.

An additional difficulty lies in the different morphological forms that exist during the life cycles of the *Leishmania* spp. The form found in the insect vector has a flagellum and is highly motile. When inoculated into man it is engulfed by macrophages, takes on a more rounded appearance, and losses this flagellum. It is referred to as an amastigote. Studies on the pathogenicity of these organisms perforce require examination of both forms: the vector forms, analogous to those grown in liquid culture, and the amastigotes, which must be grown in an animal or in tissue culture.

Our investigations into the metabolism of the *Leishmania* spp. and related protozoans (11, 12; J. J. Marr, Comp. Biochem. Physiol., in press) have led to the exploration of their pteridine biochemistry. *Crithidia fasciculata*, a protozoan frequently used as a model for studying the metabolism of the pathogenic protozoa, uses folate as an irreversible biopterin precursor (6). Trager has demonstrated that *Leishmania tarentolae* has a pteridine pattern similar to that of *C. fasciculata* (17). Although none of the pathogenic species of *Leishmania* has been shown to possess a pteridine requirement, the close phylogenetic relationship of the pathogens to the above organism led us to consider this possibility. It was hypothesized that the study of these pathways might provide a rational approach to chemotherapy since xanthine oxidase is active in the interconversion of pteridines of the biopterin series (13, 18). Although the pterdine requirements of man are not known with certainty, it is likely that preformed pteridines are necessary (S. Kaufman, personal communication). This would imply that an agent which interdicts pteridine interconversion might be effective against *Leishmania* spp. and have little effect on man. Allopurinol (4-hydroxypyrazolo[3,4-d]pyrimidine), an inhibitor of xanthine oxidase, has received wide use in man in the treatment of hyperuricemia. This agent was chosen for experimental study since it produces few toxic reactions, and its pharmacology has been well described (14). We report here the successful demonstration in vitro of an antileishmanial effect of allopurinol at concentrations which can be obtained in human serum.

MATERIALS AND METHODS

The experimental organism, *Leishmania braziliensis*, was obtained from Robert Yeager, Department of Parasitology, Tulane University, New Orleans, La. It was cultured in Tobie diphasic blood agar medium (16) in 50-ml Erlenmeyer flasks with a 5-ml rabbit blood agar base and 4 ml of Locke solution. The initial concentration of organisms in each flask was adjusted to 4×10^4 to 5×10^4 organisms per ml of Locke solution. Allopurinol (4-hydroxypyrazolo[3,4-d]pyrimidine) (Sigma Chemical Co., St. Louis, Mo.) was added to the flasks in varying concentrations, and the flasks were placed on a gyratory incubator shaker (100 rpm) at 27 C for 5 to 6 days. All cell counts were made on a hemocytometer, and only motile forms

were counted. The concentration of allopurinol in the liquid phase was determined spectrophotometrically at 254 nm on a Healh double-beam spectrophotometer, model 707 C, by the method of Elion et al. (5).

Since allopurinol is water-soluble and will partition itself between the solid and liquid phases, we repeated the experiments in a liquid medium to achieve a more precise control of allopurinol concentration. The liquid medium was that of Collier and Lourie (2) and consisted of defibrinated rabbit blood, sterile distilled water, 6.3% NaCl solution, and fresh, unheated rabbit serum. The medium was dispensed into 50-ml Erlenmeyer flasks, 10 ml per flask. The initial concentration of organisms was 4×10^4 to 5×10^4 organisms per ml. The flasks were placed on the shaker (120 rpm) at 37 C for 5 to 6 days, and counted as above. The final concentration of allopurinol was confirmed spectrophotometrically.

Xanthine oxidase was assayed by the method of Lowry (10) in which 2-amino-4-hydroxypteridine is oxidized by xanthine oxidase to isoxanthopterin. The formation of the product was measured by fluorescence using a Turner fluorometer connected to a Heath linear recorder. The substrate was purchased from the Sigma Chemical Co. A crude preparation of *L. braziliensis* was made by growing the cells as described above, centrifuging at $1,500 \times g$ for 10 min, resuspending the pellet in phosphate buffer, 0.2 M, pH 7.2, and centrifuging again. This pellet was sonically treated in ice using a Bronson Sonifier with a microprobe attachment for 30 s at a power output setting of 3. This procedure gave complete breakage. The suspension was centrifuged as above, and the supernatant was used as a source of xanthine oxidase. *C. fasciculata* was grown and harvested as previously described (11), and a crude extract was prepared as above. The assay system was verified using milk xanthine oxidase (Sigma Chemical Co.).

RESULTS

Ten experiments have been done using allopurinol, and all have been both quantitatively and qualitatively similar. Two typical experiments employing diphasic blood-agar medium are shown in Fig. 1 and 2. Allopurinol inhibited the growth of *L. braziliensis* at concentrations as low as 5 μg/ml. Cultures grown in the absence of allopurinol showed up to 100-fold multiplication, and microscope examination demonstrated healthy, vigorously motile organisms with few rounded forms. As the concentration of allopurinol was increased, the number of actively motile organisms decreased, and rounded, nonmotile, degenerating forms were observed until, at 20 μg/ml or greater, virtually all the organisms were nonmotile and apparently degenerating. These resembled the amastigote forms of the organisms.

Since allopurinol equilibrates between the solid and liquid phases of the diphasic medium, the experiments were repeated without adding agar to the medium. This allowed a more precise control of the concentration of the drug and still permitted good growth of the organisms. The results are identical to those described above (Fig. 3).

To determine if the drug was killing the organisms or inhibiting their growth, samples were taken from the flasks, placed in fresh medium with no allopurinol, and examined for growth. Organisms taken from flasks containing less than 25 μg of allopurinol per ml grew readily; those from the flasks containing 25 μg or more per ml grew very slowly, but after a prolonged time they eventually grew as well as the control. Allopurinol appears to be leishmanistatic.

Having demonstrated the effectiveness of allopurinol in the inhibition of the growth of *L. braziliensis*, we examined cell-free preparations of the organism for xanthine oxidase activity. This was done to investigate the site of action of the drug. Although the milk xanthine oxidase had considerable activity in this assay system, no activity could be demonstrated in crude extracts of *L. braziliensis*. This was repeated several times, using various preparations of the organism, without success. Extracts of *C. fasciculata*, a nonpathogenic protozoan in which the effect of allopurinol was first described, were also assayed for xanthine oxidase activity and none was found. These data would imply that, although a good inhibitor of xanthine oxidase, the effectiveness of allopurinol in preventing the growth of *L. braziliensis* may lie in its capacity to form allopurinol ribonucleotides (see Discussion).

DISCUSSION

Allopurinol has been shown to inhibit the growth of *C. fasciculata* and was postulated to interrupt the pathway from folic acid to biopterin or inhibit pyrimidine biosynthesis in this organism (6). The data presented here, although demonstrating the leishmanistatic effect of allopurinol on *L. braziliensis*, do not indicate a site of action for the compound. However, our experiments have shown that neither *L. braziliensis* nor *C. fasciculata* has a xanthine oxidase. This would imply that the drug probably does not act by inhibiting the interconversion of pteridines in either of these two organisms. If pteridine biosynthesis is not involved, then the most likely site(s) of inhibition is in pyrimidine biosynthesis.

None of the *Leishmania* species grown in a defined media require exogenous pyrimidines, implying that the enzymes required for biosyn-

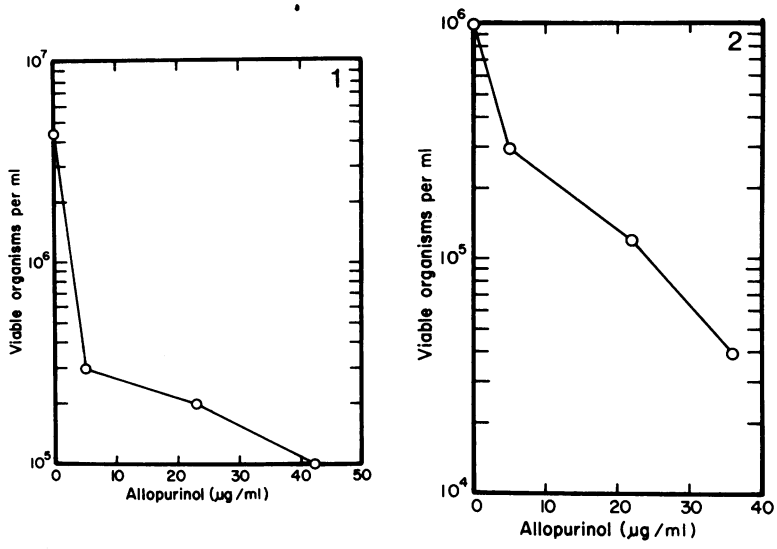

FIG. 1 and 2. *Effect of allopurinol on the growth of Leishmania braziliensis in diphasic blood-agar medium. The points are the average of four counts.*

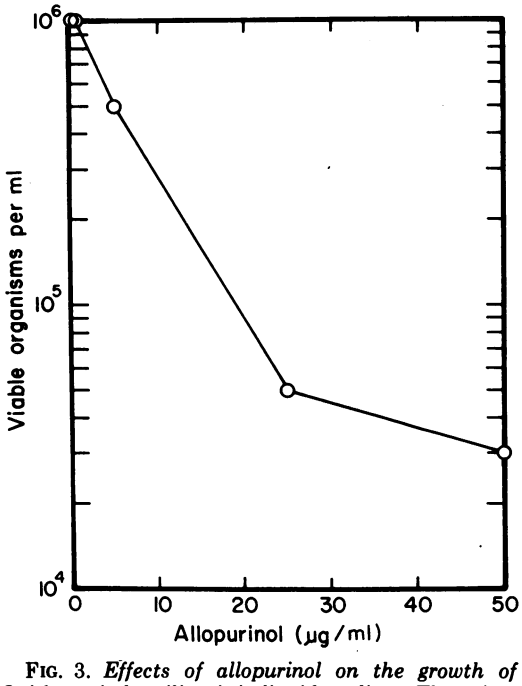

FIG. 3. *Effects of allopurinol on the growth of Leishmania braziliensis in liquid medium. The points are the average of four counts.*

thesis are present. The incorporation of [2-^{14}C]orotic acid into pyrimidine coenzymes and nucleic acid pyrimidines has been shown in trypanosomes (7). Both allopurinol and oxipurinol are known to inhibit orotate phosphoribosyltransferase and orotidylic decarboxylase in man (1). These enzymes carry out the final two reactions in the synthesis of uridine monophosphate. They also inhibit the de novo synthesis of purines and pyrimidines in human diploid cells (9). This may be due to the depletion of phosphoribosylpyrophosphate (PRPP), an essential substrate for the synthesis of both purines and pyrimidines or to the formation of allopurinol ribonucleotide, which inhibits PRPP aminotransferase and orotidylic decarboxylase (9).

It is not possible to state the mechanism of the leishmanistatic action of allopurinol at this time, but experiments are in progress to determine if allopurinol ribonucleotide is formed by this organism and if the enzymes of pyrimidine biosynthesis are inhibited by it. Since xanthine oxidase is absent in this organism, oxipurinol or oxipurinol-7-ribonucleotide is probably not involved in the antileishmanial effect of allopurinol.

It is recognized that the morphology of *Leishmania* grown in liquid culture is not identical to that of those found within mammalian cells in tissue culture or in the human disease. These latter forms are more rounded and no longer possess a flagellum. Metabolic studies done on both phases of these organisms have indicated that they are biochemically similar, although the oxygen consumption of the leptomonad (culture) form is greater. This difference is probably a quantitative rather than a qualitative phenomenon (3, 8, 15). Detailed studies on the biosynthesis of pyrimidines in

both morphological forms are lacking, but the assumption that it too will be similar is not unreasonable.

These experiments demonstrate for the first time that allopurinol has an in vitro antileishmanial effect at concentrations attainable in human tissues and body fluids. It is known to be nontoxic for man, and this makes it a potential candidate for clinical trial. As an inhibitor of pteridine and pyrimidine biosynthesis, it may also serve as a prototype for other agents with similar mechanisms of action.

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ADDENDUM IN PROOF

A recent paper by Dewey and Kidder (J. Protozool. **20**:678, 1973) has shown that allopurinol ribotide inhibits the enzyme orotidine-5'-phosphate decarboxylase obtained from the protozoan *Crithidia fasciculata*. The free base, allopurinol, did not inhibit this enzyme. These findings in *Crithidia* are in agreement with our own findings using *Leishmania braziliensis*, and support the concept that allopurinol ribotide is the active form of this compound in vivo. Neither *C. fasciculata* nor *L. braziliensis* has been shown to have xanthine oxidase.

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