Antiviral Effects of Aphidicolin, a New Antibiotic Produced by Cephalosporium aphidicola

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Aphidicolin is an antibiotic of novel structure produced by the mold Cephalosporium aphidicola. It is a potent inhibitor of cellular deoxyribonucleic acid synthesis, and it also strongly inhibits the growth of herpes simplex virus both in tissue culture and in the rabbit eye. Aphidicolin is active against iododeoxyuridine-resistant herpes virus, and does not itself readily induce the formation of drug-resistant strains of herpesvirus.

In 1972, Brundret et al. (1) described the structure of an antibiotic produced by Cephalosporium aphidicola Petch. The antibiotic was named aphidicolin and has the structure shown in Fig. 1. During the biological evaluation of aphidicolin, it was shown to have a marked inhibitory effect on the growth of deoxyribonucleic acid (DNA) viruses. This paper describes these effects in vitro and in vivo.

MATERIALS AND METHODS

Aphidicolin. C. aphidicola Petch was grown on a medium containing Czapek-Dox salts plus 5% cere- lose and 0.1% yeast extract. Aphidicolin was extracted and purified by methods of Hesp et al. (manuscript in preparation). The solubility of aphidicolin in water is extremely low and in the order of a few micrograms per milliliter.

Tissue cultures. Human embryonic lung cells and primary calf kidney cells were grown in stoppered tubes (3 by 0.5 in, approximately 7.5 by 1.25 cm) medium containing 8% inactivated calf serum. When confluent, monolayers were changed to Eagle medium containing 2% calf serum or 0.2% bovine plasma albumin.

Viruses. The following strains of viruses were used: Herpes simplex types 1 and 2 (clinical isolates ob- tained from the Public Health Laboratory, Manches- ter, England), influenza A₂ (DSP), influenza A₁ (CAM), influenza A₃ (Hong Kong), rhinovirus type 2, coronavirus type 229E, parainfluenza type 1, and vaccinia. Virus infectivity was titrated in tissue culture tubes of appropriate cells.

The methods for measuring the effects of aphidicolin and other drugs on the DNA and ribonucleic acid RNA synthesis of tissue culture cells by uptake of [H]-thymidine and [H]-uridine have already been described (2).

Herpetic keratitis in rabbit eyes. Separately caged Dutch female rabbits (1-2 kg) were used throughout. One eye in each of six rabbits formed an experimental group.

The cornea was anesthetized with one drop of 4% Xylocaine and the epithelium was lightly scratched in a cross-hatch pattern by using a 27-gauge needle, care being taken not to disturb the stromal layer. A 0.1-ml virus suspension was then instilled into the lower cul-de-sac and the lids were gently rubbed together for several seconds. The corneal epithelium was examined daily after staining with fluorescein and washing with phosphate-buffered saline. Corneal lesions were graded 0 to 4, grade 0 representing no abnormality and grade 4 representing complete corneal involvement. The allocation of scores was done by one person throughout the whole of this study and was organized so that he had no knowledge of the therapy each animal was receiving.

In order to make a statistical comparison between the response of groups of animals on different treatments, the following procedure was used. The lesion scores for each group of animals were summed from the first day of treatment up to day 9 postinfection. Scores beyond 9 days were not included because after this time the lesions in the control groups usually began to heal (at least with herpes simplex type 1), and the difference between the control and the treated group became less.

The mean sum of the lesion scores was calculated for each group, and the difference between the control group mean and each drug-treatment mean was tested for statistical significance by means of Student's t test. The significance of each comparison at the 1% level of confidence is given in the figure legends.

RESULTS

Spectrum of antiviral activity. Aphidicolin was not visibly toxic to confluent human embryonic lung cells at concentrations up to 200 μg/ml for periods of up to 7 days. In human lung cultures infected with approximately 100 mean tissue culture doses (TCD₅₀) of various viruses, aphidicolin caused a 50% inhibition of growth of herpes types 1 and 2 at 0.2 μg/ml and a 50%
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Inhibition of vaccinia growth at 4 µg/ml. It was not active against adenovirus type 5. In the same test system, it showed no activity against rhinovirus type 2 or coronavirus type 229E. In confluent primary calf kidney cultures, aphidicolin was not active against influenza A, A1, or A2, or parainfluenza type 1.

Mode of action. The specific inhibition of DNA viruses by aphidicolin suggested that the antibiotic is an inhibitor of DNA synthesis. We therefore compared the effects of aphidicolin on the nucleic acid synthesis of cultured human lung cells with two well-known inhibitors of DNA synthesis: 5-iodo-2-deoxyuridine (IUdR) and cytosine arabinoside (CA). The results (Fig. 2) show that aphidicolin, like IUdR and CA, is a powerful inhibitor of cellular DNA synthesis. None of the compounds had any effect on cellular RNA synthesis at 12.5 µg/ml, the highest concentration tested. Parallel cultures were infected with approximately 100 TCD50 of herpes simplex type 1, treated with the same range of concentrations of IUdR, CA, or aphidicolin, and harvested 24 h later to measure virus yields.

It is clear that aphidicolin depresses virus yield and cellular DNA synthesis in parallel, and it is therefore probable that it exerts its antiviral effects by inhibiting herpes virus DNA synthesis. IUdR, on the other hand, is antiviral at concentrations much lower than those which inhibit cellular DNA synthesis, suggesting a considerable degree of specificity of IUdR for viral, rather than cellular, DNA synthesis. The figures for CA show that cellular DNA synthesis is affected rather more than is virus DNA synthesis.

The activity of aphidicolin against ocular herpes. Aphidicolin was active against experimental herpes type 1 infections of the rabbit eye. When applied as aqueous drops hourly for 7 h each day for 5 days, aphidicolin at 1 mg/ml...
was somewhat less effective against an ocular challenge of 400 eye mean infective dose (ID₅₀) than was IUdR at 1 mg/ml (Fig. 3). By increasing the concentration of aphidicolin to 10 mg/ml, or by reducing the challenge virus to 40 eye ID₅₀, the activity of aphidicolin could be made comparable to that of IUdR at 1 mg/ml.

In human cases, the corneal herpetic lesion is often well advanced before therapy is begun; to simulate these conditions, groups of rabbits were infected with 400 eye ID₅₀ of type 1 herpes and left untreated for 48 or 72 h before beginning 5 days of eye drops of either 1 mg of IUdR per ml or 10 mg of aphidicolin per ml. When treatment was begun 48 h after infection, both drugs reduced the maximal lesion score to approximately half that achieved in the controls (Fig. 4a). Treatment begun 72 h after infection was less effective, and neither drug produced a statistically significant reduction (at the 1% level of confidence) in the development of lesions.

In an experiment where rabbits were infected in the eye with herpes simplex type 2 and left for 72 h before treatment was begun, the disease was well controlled by 5 days of treatment with either drug (Fig. 5).

**Activity of aphidicolin against IUdR-resistant herpes simplex type 1.** The growth of our experimental strain of herpes simplex type 1 virus in vitro was reduced by 50% in the presence of 0.2 to 0.4 μg of IUdR per ml. However, when virus which had been grown in the presence of 0.4 μg of the drug per ml was harvested and tested again for susceptibility to IUdR, it then required 12 μg/ml to produce 50% inhibition. The virus was thus 30 times less sensitive to inhibition after one passage in the presence of the drug, but further passages in the drug did not increase the resistance. The development of IUdR-resistant strains of herpes virus is well known (5, 6) and may be one reason why IUdR therapy of human ocular herpesvirus is occasionally found to be ineffective (7). Unlike IUdR, aphidicolin does not readily give rise to drug-resistant strains of virus. After six passages in the presence of 0.8 μg of aphidicolin per ml, there was no decrease in the drug-susceptibility of the virus. Also, IUdR-resistant herpes virus is fully susceptible to aphidicolin.

As may be expected from these results, when an IUdR-resistant strain of herpesvirus type 1 was inoculated into the rabbit eye, the severity of the keratitis could be reduced with aphidicolin, but not with IUdR (Fig. 5).

The effects of combined IUdR and
Aphidicolin and herpetic encephalitis. The relatively low animal toxicity of aphidicolin makes it possible to test the compound systemically in guinea pigs inoculated intracerebrally with herpes simplex virus. Pairs of guinea pigs were dosed subcutaneously or intraperitoneally with a suspension of aphidicolin at 200 mg/kg from the day of virus inoculation (100 guinea pig mean lethal dose intracerebrally) for 12 days. The drug dose was reduced to 100 mg/kg on day 6 because the animals looked unwell and were not gaining weight at the same rate as were the infected, but undosed, controls. However, there was no evidence of any protective effect of the compound in either the time of onset of symptoms or the mortality rate.

Derivatives of aphidicolin. Because of the low solubility of aphidicolin in water, the drug used at 1 and 10 mg/ml in the rabbit eye experiments was in the form of a fine suspension. Two water-soluble derivatives were made, the C17, C18 diphosphate and the C17, monohemisuccinate sodium salt, but only the latter showed any activity in vitro, and this was only about one-quarter of the activity of aphidicolin itself (Table 1). The monohemisuccinate ester sodium salt is highly soluble in water, but when it was tested in the rabbit eye, a concentration of 30 mg/ml was needed to achieve a therapeutic effect comparable to a suspension of 1 mg/ml of aphidicolin. It seems, therefore, that conferring water-soluble properties on the molecule does not increase its efficacy.

A large number of derivatives of aphidicolin have been synthesized (Hesp et al., manuscript in preparation) and tested in vitro, but those which showed activity against herpes-virus were much less active than was aphidicolin itself. Only the C17 monoacetate was comparable in activity, and this was possibly because it may be hydrolyzed to aphidicolin in the test system.

| Drug                                    | Amt (µg/ml) causing 50% Cyto-
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<th>toxnic conc</th>
<th>50% Virus inhibition</th>
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<tr>
<td>Aphidicolin</td>
<td>&gt;200</td>
<td>0.25</td>
</tr>
<tr>
<td>Aphidicolin C17, C18 bis primary phosphate (tetraammonium salt)</td>
<td>&gt;45</td>
<td>Not active</td>
</tr>
<tr>
<td>Aphidicolin C17, monohemisuccinate (sodium salt)</td>
<td>&gt;45</td>
<td>1</td>
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aphidicolin on the growth of herpesvirus in vitro seem to be simply additive, and the effects of the two drugs together on the progress of herpetic keratitis in the rabbit cornea is only slightly greater than those either drug alone.
DISCUSSION

Although IUdR is probably the most widely used drug in the treatment of human herpetic infections, it is far from an ideal medicine. It is an inhibitor not only of virus growth but also of cellular DNA synthesis (albeit at a higher concentration), and it is also incorporated into mammalian DNA (4). Herpes simplex rapidly becomes resistant to it in vitro and may also do so in vivo (7), and it is not without toxicity when administered systemically to humans (3). There is therefore a need for better anti-herpes drugs, and as part of our research program for developing such agents, we have evaluated aphidicolin, a novel antibiotic produced by C. aphidicola. The antibiotic is highly active against herpes virus growth in vitro, and this activity could not be improved by considerable chemical manipulation of the parent molecule. Indeed, most chemical modifications gave a large reduction in the antiviral activity.

In therapeutic experiments in the rabbit eye, the curative effects of 1 mg of aphidicolin and IUdR per ml were equivalent against 40 eye ID₅₀ of herpesvirus. Although virtually equimolar amounts of aphidicolin and IUdR were used in these experiments (the molecular weights being 354 and 338, respectively), because aphidicolin is so poorly soluble in water, the effective concentration in true solution would be very low. Thus, on a molar basis, aphidicolin is probably considerably more potent than IUdR, and its effectiveness is limited only by its extremely low solubility in water. Unfortunately, chemical modification of aphidicolin to increase its water solubility led to a considerable loss of intrinsic anti-herpes activity.

The basis of the anti-herpes activity of aphidicolin is almost certainly an inhibition of virus DNA synthesis, although this has not been demonstrated directly. The concentration of antibiotic required to reduce herpesvirus growth by 50% in vitro is very close to that required to inhibit cellular DNA synthesis by 50%. Thus, the action of the antibiotic is not specific for virus DNA synthesis.

The high intrinsic potency of aphidicolin, its ability to inhibit the growth of IUdR-resistant herpesvirus, and the apparent lack of the development of drug resistance are all valuable properties of a potential anti-herpes drug. However, because its action is not specific for virus-directed DNA synthesis, aphidicolin may not offer any advantages for clinical use over other DNA antagonists.

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LITERATURE CITED


