Sensitivity of *Hartmannella* (*Acanthamoeba*) to 5-fluorocytosine, hydroxystilbamidine, and other substances

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SYNOPSIS The effect of 24 anti-amoebic and other chemotherapeutic compounds on six strains of hartmannellid amoebae was studied by tissue culture, agar diffusion, and liquid axenic culture.

Of the recognized anti-amoebic compounds only one inhibited growth of the amoebae, which were remarkable for their resistance.

Two compounds, hydroxystilbamidine isethionate and 5-fluorocytosine, showed some amoebicidal activity while some others were inhibitory.

Hartmannella and *Naegleria* have both been reported as causes of primary amoebic meningo-encephalitis but recent reports on the role of normally saprophytic amoebae in human disease have tended to emphasize *Naegleria* as the chief potential pathogen (eg, Carter, 1968; Apley, Clarke, Roome, Sandry, Saygi, Silk, and Warhurst, 1970). However, the ability of some hartmannellae to grow at 33-37°C, to produce degenerative changes in tissue culture, and to produce a lethal meningo-encephalitis in small laboratory mammals suggests that they should be regarded as facultatively pathogenic, as proposed by Griffin and others (Armed Forces Institute of Pathology, 1968).

The taxonomy of the hartmannellid group has not yet been resolved. Page (1967a and b, and personal communication) would give the amoebae used in this study the generic status *Acanthamoeba*: I do not feel competent to judge this point and have retained the nomenclature previously used. The potentially pathogenic nature of some hartmannellid amoebae suggested that an examination of their drug sensitivity would be worthwhile.

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Materials and Methods

AMOEBAE

Six strains of amoebae were used. They conformed to Warhurst and Armstrong's description (1968) of *Hartmannella castellanii*. Their sources are shown in Table I.

Strain Number and Origin	Source
29 Manchester	Postmortem bronchial swab
324 Cirencester	Throat swab
B147 London	Throat swab
565 Leeds	Throat swab
524 Coventry	Throat swab
M67 Middlesbrough	Tissue culture contaminant

Table I Sources of amoebae and geographical origins

COMPOUNDS UNDER TEST

Compounds were chosen because they had reputed antiprotozoal activity, because they were chemically related to known antiprotozoal agents, because they were constituents of tissue-culture

media that might be used for attempts at isolation of amoebae, or because their mode of action against other organisms suggested possible activity against amoebae.

Some compounds used for the treatment of entamoebiasis were not tested in this study *in vitro* because they depend for their activity on a chemical reaction within the intestinal tract, resulting in the local release of iodine, arsenic, etc.

Compounds were obtained as pure powders from the makers, or as pharmaceutical preparations for injection. Stock solutions were prepared, usually in distilled water, at a concentration of 1,000 µg/ml. Each was tested for anti-amoebic activity at an arbitrary concentration of 100 µg/ml. Compounds showing some effect at this concentration were tested by titration in at least two of the test systems used; those showing no effect or only slight inhibition at 100 µg/ml by all three methods were not tested further.

TISSUE CULTURE

HeLa cells were grown and maintained using conventional media and methods. Sensitivity tests were performed in media without any of the antibiotics normally used to control bacterial and mycotic growth.

AGAR DIFFUSION

Klebsiella-water-agar medium (Warhurst and Armstrong, 1968) was used; 1 ml of the tenfold concentration of the compound being tested was mixed with 9 ml of cooled, molten agar. Killed *Klebsiella* was used to avoid inconsistencies that might arise when the *Klebsiella* was itself inhibited by the compound under test.

AXENIC CULTURE

Several peptone media, with and without glucose, were tried (Neff, 1957; Adam, 1964). Best growth was obtained in a thin layer of medium containing 4% proteose-peptone (Difco) and 1% glucose, dissolved in tap water and sterilized by autoclaving (PPG medium).

In most cases a tenfold concentration of the compound was diluted with the medium and then inoculated with the amoebae. In some cases, however, the compound was added to a dense, actively growing culture.

Tests for combined drug action were performed using chess-board titrations to give varying concentration ratios in PPG medium in a divided Petri dish (Sneath and Stevens, 1967).

INCUBATION

All cultures were incubated at 37°C. Proteose-peptone glucose and agar-culture containers were enclosed in polythene bags to reduce evaporation.

EXAMINATION

Although growth could often be detected by naked-eye examination, especially in PPG medium, all cultures were examined microscopically after 24 hours and then at irregular intervals during the week after inoculation. Tests were controlled by inoculating each strain, in parallel with the test, into the same medium free from the compound under test. Results were recorded as heavy, moderate, or scanty growth; growth not detected, and, when marked differences occurred, approximate proportions of cysts to trophozoites. Equivocal results, especially those involving few survivors after exposure to inhibitory compounds, were checked by recovering the amoebae and subculturing to assess their viability.

INTERPRETATION

The relative amounts of growth recorded for tests and controls were interpreted as follows:

1 No effect

When test and control showed no difference in rate of multiplication, yield of trophozoites, or rate of encystment.

2 Inhibition without amoebicidal effect

When any of the following effects were noted in test cultures: (a) slower rate of multiplication, (b) lower yield of trophozoites, or (c) more rapid encystment.

3 Amoebicidal effect

When (a) cysts recovered from (2) failed to vegetate in medium free from the compound being tested, or (b) when the addition of a test compound to a dense, actively growing axenic culture of trophozoites caused loss of viability as indicated by subculture of trophozoites to fresh medium free from the compound being tested.

Results

Table II shows the results of preliminary screening of the test substances or combinations at concentration of 100 µg/ml of each compound. Only four substances, amphotericin B, paromomycin, hydroxystilbamidine isethionate, and 5-fluorocytosine, were sufficiently active in the screening tests to justify further studies. The purpose of the study was to investigate the sensitivity pattern rather than to determine minimum inhibitory concentrations (MICs). Although the strains of amoebae showed slight differences in their growth characteristics and MICs there was no difference in their sensitivity patterns, so drug effects on the six strains are not shown separately.

Antimetabolites acting on the folic acid cycle—sulphonamides, trimethoprim, pyrimethamine—

tend to act synergistically. Different sulphonamides may vary in their efficacy to potentiate other substances acting on the folic acid cycle or in their suitability for use in this way. In any such combination the degree of potentiation may be affected by the ratio of compounds used. Chess-board titrations of trimethoprim/sulphamethoxazole and pyrimethamine/sulphormethoxine,

each at concentrations of 100, 80, 60, 40, and 20 µg/ml, failed to show any potentiation of the inhibition indicated by the screen tests (Table II).

Sulphadiazine by itself was only feebly inhibitory against all the strains. However, Dr C. G. Cuthbertson (personal communication) found it to be effective in mouse protection tests with *Hartmannella* strains that he tested.

Amphotericin B was shown by Carter (1969), in laboratory studies, to be effective against *Naegleria* at a concentration of 0.075 µg/ml. In my tests (Table III) it was inhibitory to *Hartmannella* at 10 µg/ml, but during prolonged incubation, which causes inactivation of the drug, survivors multiplied vigorously. The effect was greater than could be explained by the effect of the bile salt incorporated with the drug to effect solution.

Paromomycin, an aminoglycoside of the neomycin group, is effective against *Entamoeba histolytica*. Although it had a marked inhibitory effect on *Hartmannella*, the surviving amoebae were viable and multiplied rapidly when sub-cultured to medium free from paromomycin.

Hydroxystilbamidine isethionate is a diamidine with properties similar to stilbamidine but with considerably less human toxicity. It is used in the treatment of various protozoal and mycotic infections and may be used for a prolonged period (Snapper, Schneid, McVay, and Lieben, 1952). It may be incorporated into cells and remain active for long periods (Snapper, Schneid, Greenspan, and Lieben, 1950). Titration in all three media showed marked inhibition by as little as 0.125 µg/ml for some strains (Table IV). A concentration of 100 µg/ml was amoebicidal but was toxic to the HeLa cells.

5-Fluorocytosine is a nucleotide analogue used in the treatment of infections due to cryptococci and candida, including cryptococcal meningitis (Watkins, Campbell, Gardner-Medwin, Ingham, and Murray, 1969). It was inhibitory at 12.5 µg/ml and amoebicidal at 100 µg/ml (Table IV).

Compound	Effect on <i>Amoebae</i> at 100 µg/ml ¹
Acetarsol	No effect
Diloxanide furoate	No effect
Oxytetracycline	No effect
Benzyl penicillin	No effect
Streptomycin sulphate	No effect
Neomycin	No effect
Kanamycin	No effect
Paromomycin	I (marked)
Chloroquin	Morphological changes only
Nystatin	No effect
Metronidazole	VI
Polymyxin B	I (feeble)
Sulphonamides ²	VI
Trimethoprim lactate	No effect
Pyrimethamine sulphate	No effect
Trimethoprim + sulphamethoxazole	I
Trimethoprim + polymyxin B	I
Pyrimethamine + sulphormethoxine	I
Trimethoprim + pyrimethamine	I
Amphotericin B	I (marked)
Hydroxystilbamidine	AC
5-Fluorocytosine	AC

Table II Preliminary screening tests of 24 compounds alone or in combination

¹In tests of two drugs together, each was used at a concentration of 100 µg/ml.
I = inhibition without amoebicidal effect
AC = amoebicidal
VI = variable inhibition reactions
— = not tested

²Separate tests on sulphanilamide, sulphadiazine, sulphasomizole, sulphafurazole, sulphathiazole, sulphamethoxazole, and sulphormethoxine

Compound	Effect on <i>Amoebae</i> at Concentration (µg/ml)		
	100	10	1
Amphotericin B	Marked inhibition	Inhibition	Feeble inhibition
Paromomycin	Marked inhibition	Feeble inhibition	No effect

Table III Further tests on the two inhibitory substances

Effect on <i>Amoebae</i> at Concentration (µg/ml)										
	100	50	25	12.5	6	3	1.5	0.7	0.35	0.125
Hydroxystilbamidine isethionate	AC	I	I	I	I	VI	VI	VI	VI	VI
5-Fluorocytosine	AC	I	I	VI	NE	NE	NE	—	—	—

Table IV Further tests on the two amoebicidal compounds

¹Meaning of abbreviations as indicated at foot of Table II.

Discussion

Sensitivity testing of amoebae *in vitro* is much more difficult than tests on bacteria. The longer period of growth, the varying ability of different strains of the same species to grow in the same medium, and the ability of amoebae to encyst, all contribute to the difficulty. The correlation of sensitivity patterns shown by the three test systems used suggest that the techniques, as outlined here, probably give a fair indication of the sensitivity of these organisms and should be of use in testing other compounds.

The results of the tests *in vitro* reported here cannot be extrapolated to indicate therapeutic action; for example, amphotericin had no amoebicidal activity in my tests, but Apley *et al* (1970) have reported its use, with encouraging

effect, for one proven and two probable cases of Naeglerial meningo-encephalitis.

Animal studies are in progress to determine whether the more active substances can protect mice against challenge infection of amoebae.

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