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# EXPERIMENTAL HEPATIC AMOEBIASIS AND ITS APPLICATION TO CHEMOTHERAPEUTIC STUDIES

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Induction of hepatic amoebiasis with two strains of *Entamoeba histolytica* has been attempted by direct inoculation of trophozoites into the livers of rats, hamsters, and guinea-pigs, and by intravenous injection into guinea-pigs. A high incidence of amoebic abscesses was obtained in hamsters. Rats and guinea-pigs were relatively less susceptible. The two strains of *Entamoeba histolytica* differed in virulence. Serial liver passage increased their virulence for the hamster liver, and also increased invasiveness to the rat caecum. The chemotherapeutic effects of chloroquine, emetine, and some anilides were determined. The procedure finally adopted was based on mean survival times of treated and untreated animals. Chloroquine and emetine were effective under conditions which greatly favoured the drugs, but the anilides showed little effect.

Early attempts in the laboratory to induce hepatic amoebiasis were successful, using dogs and cats (Cleveland and Sanders, 1930a; Kondo, 1939), but rats and guinea-pigs are relatively insusceptible (Rao, 1951; Yoshimura, 1952; Maegraith and Harinasuta, 1953, 1954a, 1954b), though hamsters gave more encouraging results (Reinertson and Thompson, 1951; Neal and Vincent, 1955, 1956). Successful treatment of hepatic amoebiasis in hamsters was described by Thompson and Reinertson (1951) and by Neal and Vincent (1955).

In this laboratory it was hoped to use hepatic amoebiasis for further chemotherapeutic studies on a series of compounds found active *in vitro*, and in experimental intestinal amoebiasis in rats (Bristow, Oxley, Williams, and Woolfe, 1956). As we normally used rats and guinea-pigs for studies of intestinal amoebiasis, initial attempts were made to establish hepatic abscesses in these species. Later, hamsters were employed with much greater success.

## METHODS

Rats, hamsters, and guinea-pigs of both sexes were used. The rats were approximately four weeks old and weighed about 60 g. The hamsters were five to six weeks old and weighed 45 to 55 g. The guinea-pigs were adult.

Two strains of *Entamoeba histolytica* of human origin were used. These were strain M (obtained originally from Dr. R. A. Neal of the Wellcome Foundation), and strain R (obtained from the Liverpool School of Tropical Medicine). Strain M had been in culture for some years; strain R was of more recent origin. All strains and substrains were maintained in culture with their respective bacterial flora at 37°. The cultures were subcultured every other day, alternately on a diphasic medium (egg-slope with a serum-saline (1:6) overlay at *p*H 7.3) and on a monophasic medium (liver-Marmite-serum) at *p*H 7.4. Rice starch was added to each tube.

To infect the animals, the technique of Reinertson and Thompson (1951) was followed. Initially 20,000 to 50,000 *E. histolytica* trophozoites with the concomitant bacteria from a 48 hr. culture were inoculated in a volume of 0.05 ml. Preliminary tests revealed a difference in infectivity of the two strains to rats, and thereafter strain R, the more infective of the two, was usually used. The inoculum was standardized at 8,000 trophozoites in 0.02 ml. and was thickened with rice starch to control "run back."

In some experiments prophylactic inoculations of bacteria were given to raise the resistance of the hamsters, using the method of Reinertson and Thompson (1951), before the amoebae and bacteria were injected. The inoculum, however, usually contained very few bacteria. It was obtained by culturing the amoebae plus bacteria with 0.02 mg./ml. of oxytetracycline in the monophasic medium for 24 hr. under $N_2$ in a McIntosh and Fildes jar. After counting the amoebae and standardizing the inoculum, sterile starch was added to thicken the medium. This method reduced very considerably the number of bacteria in the culture. Further bacterial control was carried out by subcutaneous injection of antibiotics (penicillin, 2 mg./kg., and streptomycin, 50 mg./kg.) into the inoculated animals. However,

this never produced bacteriological sterility of the amoebic abscesses.

For the experiments on the chemotherapy of experimental hepatic amoebiasis, hamsters infected with strain R of *E. histolytica* were used throughout. After a few preliminary experiments using 8,000 to 25,000 amoebae in the inoculum, the dose was standardized at 8,000 amoebae.

The hamsters were divided into groups for treatment and then selected at random (Thompson and Reinertson, 1951). In early experiments they were killed after four to six days and their livers examined for the presence of active amoebae in wet smears in sections. The effects of therapy were determined by comparison of treated with control animals.

Emetine hydrochloride was given intraperitoneally, and chloroquine sulphate and fumagillin were given orally. Emetine was administered once a day, and the other drugs twice daily, over a period of four days. An initial dose was given 6 hr. before inoculation, a second at the time of inoculation, and the remainder in the morning and afternoon on the following three days. This regimen proved inadequate and was finally altered to three doses before, and three to five after, inoculation. All hamsters were examined for the presence of active trophozoites in the liver abscesses as soon as practicable after death. Often, active amoebae were recovered from abscesses many hours after death of the animals. The lesions could not be cut out and weighed as described by Thompson and Reinertson (1951). We thus could not readily detect compounds which might partially suppress the abscesses, and mean survival time was therefore adopted as an alternative method of evaluation.

New drugs tested were nearly all anilides, analogues of diloxanide (Entamide, dichloroacet-*p*-hydroxy-*N*-methylanilide). All compounds were given in aqueous solutions or suspensions, by oral intubation under ether anaesthesia.

## RESULTS

### *Inoculation with Strain M*

The incidence of lesions and recovery of *E. histolytica* in rats, hamsters, and guinea-pigs is given in Table I. Forty-eight out of eighty-four inoculated rats showed lesions when killed and examined four to fifteen days after inoculation. The lesions were from 0.1 cm. to 1.0 cm. in diameter and all had a definite wall. The small lesions were thick walled with creamy pus at the centre. Those intermediate in size also had thick walls but had a thick greenish paste in the centre. Eight of the lesions were large with a thin wall, and were full of a brown pus. Bacteria were present in all abscesses, but active amoebae were recovered only from the large thin walled abscesses, and only in those examined between four and six days after inoculation. Inoculation of the bacterial flora alone gave abscesses which were small, hard and thick walled and contained whitish pus.

TABLE I

INTRAHEPATIC INOCULATION WITH *E. HISTOLYTICA*

The inocula marked with an asterisk were given into the mesenteric or portal veins.

| Strain | Species | No. of Amoebae Inoculated | Source of Trophozoites | Proportion with Lesions | Proportion in which *E. histolytica* were Found |
|---|---|---|---|---|---|
| M | Rats | 20,000–50,000 | Culture | 48/84 | 8/84 |
| | Hamsters | 20,000–50,000 | ,, | 22/33 | 14/33 |
| | | Approx. 6,000 | Liver lesion | 50/59 | 45/59 |
| | Guinea-pigs | 20,000–50,000 | Culture | 10/12 | 0/12 |
| MLVe | Hamsters | 20,000–30,000 | ,, | 11/12<br>8/12 | 8/12<br>3/12 |
| R | ,, | 10,000–40,000 | ,, | 20,22 | 18/22 |
| | Guinea-pigs | 20,000–60,000 | ,, | 16/16 | 0/16 |
| | | 20,000–60,000* | ,, | 8/20 | 0/20 |
| | | Approx. 5,000 | Liver lesions | 8/14 | 0/14 |
| RL1 | Hamsters | 20,000 | Culture | 12/12 | 12/12 |
| RL2 | ,, | 20,000 | ,, | 12/12 | 12/12 |
| RL5 | ,, | 20,000 | ,, | 12/12 | 12/12 |

In hamsters, the amoebic abscesses were quite different. The hamsters were killed and examined three to twenty-one days after inoculation. A few of the abscesses were of the walled-off type found in rats, but the majority were not encapsulated. Most of them were about 1 cm. in diameter, but often the whole of the inoculated lobe was affected with spread into other lobes. Active amoebae were recovered from individual abscesses from three to fifteen days after inoculation. These abscesses appeared as pale areas in the liver, many containing a reddish-brown paste or liquid full of bacteria and dead liver cells. While there was no wall at the periphery of the abscess, there was a sharp division between dead liver cells and healthy liver tissue. Amoebae were seldom found in the interior of the abscess, but they were usually found, in large numbers, at the junction between the abscess and healthy tissue. Twenty-two out of thirty-three inoculated hamsters had lesions, and active amoebae were recovered from fourteen of these.

These results showed that hamsters were more susceptible than rats, but the incidence of amoebic abscesses was too low to be used as a reliable test

for drug action. It has been shown that the virulence of strains of *E. histolytica* for rat and hamster liver and the rat caecum can be increased by passage through the rat intestine (Yoshimura, 1952), through the dog intestine (Thompson, McCarthy and Reinertson, 1954) and through the liver (Cleveland and Sanders, 1930b; Neal and Vincent, 1956). Instead of culturing the amoebae between liver passages, we attempted to raise the incidence of amoebic abscesses by repeated serial passage direct from liver to liver of hamsters before recovering the amoebae into culture for inoculation. Infected livers with active trophozoites were ground up in normal saline and 0.02 ml. of the suspension inoculated into the livers of fresh hamsters. The number of amoebae in the inoculum was considerably less than that used for the earlier experiments. Cleveland and Sanders (1930b) were apparently unable to accomplish direct liver passage and attributed their lack of success to too few trophozoites in the inoculum.

Table I shows that serial passage through the liver enhanced the infectivity of the amoebae, the incidence of amoebic abscesses being raised from 42% (14/33) to an overall incidence of 76% (45/59). After fourteen liver-to-liver passages the incidence rose to 95%. It proved difficult, however, to maintain in cultivation the amoebae recovered after the fourteenth passage, and they died after a few subcultures. A few hamsters were inoculated from cultures recovered from five to seven liver passages. The incidence of amoebic abscesses in these inoculations was 90%.

A small number of guinea-pigs were also inoculated with strain M, but no amoebic abscesses were obtained. Ten out of twelve guinea-pigs inoculated showed lesions, but no amoebae were recovered from these. The lesions were usually very small and hard and contained a little whitish pus. They were normally walled off from the rest of the liver, and appeared to be typical bacterial abscesses.

Some work was also done with strain MLVe (a liver-passaged substrain of strain M provided by Dr. R. A. Neal) because strain M had become very attenuated and was nearly non-invasive in the rat intestine. Strain MLVe infected the hamster liver better than did the original strain M, but the incidence of amoebic abscesses was still low (Table I), and fell fairly rapidly in the course of eight weeks.

*Inoculation with Strain R*

In the early stages of the experiments with strain M, when the incidence of amoebic abscesses was low, we obtained strain R which had been used by Maegraith and Harinasuta (1954a and b) in their work on guinea-pigs. This strain immediately proved highly virulent for the hamster liver. The type of lesion was almost invariably rapidly spreading without a wall. The abscesses were pale with a definite periphery, the interior consisting of a thin paste or liquid containing dead liver cells and bacteria—apparently the "anchovy paste" in human hepatic amoebiasis. Amoebae were found in large numbers round the periphery of the abscess, where there was an abrupt transition to healthy liver tissue. The infection killed the hamsters within six to ten days.

In guinea-pigs, though lesions were obtained both after intrahepatic inoculation and after injection into the mesenteric or portal vein, no amoebic abscesses were found. The abscesses were small and hard and appeared to be only bacterial, since no amoebae were recovered from any of these lesions, in contradistinction to the results of Maegraith and Harinasuta (1953, 1954a).

Direct liver-to-liver passage in hamsters was also carried out with this strain, and after one passage (RL1) the incidence of lesions was 100%. Strangely enough, the amoebae of the original strain R were not very invasive in the rat intestine. After five liver passages the amoebae were recovered into cultivation *in vitro* and were then very invasive in the rat. This substrain, RL5, has been in cultivation *in vitro* without liver passage for over a year. While it is still invasive for the rat intestine, its virulence is slowly declining.

*Chemotherapy of Hepatic Amoebiasis*

Emetine and chloroquine were used as control drugs. Table II shows the results of initial experiments with emetine, chloroquine, and fumagillin. The treatment failed to suppress the abscesses. Inoculation with smaller numbers of *E. histolytica* gave unreliable infections.

TABLE II

TREATMENT OF HEPATIC AMOEBIASIS (STRAIN R) WITH EMETINE, CHLOROQUINE, AND FUMAGILLIN

One dose given before, and the remainder after, inoculation. All doses given twice daily, except emetine (once a day). All hamsters were killed and examined five to six days after inoculation. A positive result means that amoebae were found in the lesion.

| Drug | Dose (mg./kg.) | No. of Doses | No. of Amoebae Inoculated | Treated: Proportion Positive | Untreated: Proportion Positive |
|---|---|---|---|---|---|
| Emetine | 6 | 3 | 10,000 | 6/11 | 5/5 |
| | 4 | 3 | 8,000 | 7/10 | 10/10 |
| Chloroquine sulphate | 100 | 6 | 8,000 | 9/11 | 10/10 |
| | | | 6,000 | 6/8 | 6/6 |
| | | | 3,000 | 2/8 | 4/6 |
| Fumagillin | 5 | 6 | 8,000 | 10/10 | 10/10 |
| | 2·5 | 6 | 8,000 | 10/10 | 10/10 |
| | 1·25 | 6 | 8,000 | 10/10 | 10/10 |

Table III shows the results of treatment with diloxanide and some of its esters. Several other analogues of diloxanide were also tested in the same way without any effect.

TABLE III

TREATMENT WITH DILOXANIDE AND SOME OF ITS ESTERS

Three doses before and three to five doses after inoculation, two doses given daily. All hamsters killed and examined six days after inoculation. Chloroquine controls were given 100 mg./kg. for the same number of doses as the drug on test. A positive result means that amoebae were found in the lesion. The drug marked with an asterisk is 7-chloro-4-(4-diethylamino-1-methylbutylamino)quinolinium di(4-dichloroacet-*N*-methyl amido)phenyl phosphate.

| Drug | Dose (mg./kg.) | No. of Doses | No. of Amoebae Inoculated | Treated: Proportion Positive | Chloroquine Controls: Proportion Positive | Untreated: Proportion Positive |
|---|---|---|---|---|---|---|
| Diloxanide | 100<br>100 | 6<br>8 | 5,000<br>4,000 | 4/6<br>3/5 | 2/6<br>0/4 | 4/6<br>6/6 |
| Diloxanide furoate | 100 | 6 | 5,000 | 4/6 | 2/6 | 4/6 |
| Diloxanide benzoate | 100 | 6 | 5,000 | 4/6 | 2/6 | 4/6 |
| Diloxanide stearate | 100 | 6 | 5,000 | 3/6 | 1/6 | 6/6 |
| RD5269* | 100<br>100 | 6<br>8 | 5,000<br>4,000 | 3/5<br>2/5 | 2/5<br>1/5 | 5/5<br>3/4 |

Table IV gives the results of tests with some compounds using mean survival times as the criterion of effectiveness. Emetine was found to be effective, while chloroquine was not consistently so. Fumagillin was inactive and somewhat toxic at the doses used. Several analogues of diloxanide were tried, but none had any significant effect. One piperazine salt of a diloxanide ester was also tested. In addition a number of compounds, including some of those given in Tables III and IV, were tested using liver-passaged strains RL2, RL3, and RL5. These substrains were rather more virulent than the original strain R, and all animals were therefore examined five days after inoculation. The rapid spread of the infection made these substrains unsuitable for testing drugs.

TABLE IV

MEAN SURVIVAL TIMES AFTER DRUG TREATMENT

All doses given twice daily, three before, the remainder after inoculation, except emetine which was given once a day with two doses before and two after inoculation. All hamsters inoculated with about 8,000 amoebae. A positive result means that amoebae were found in the lesions. RD5269, see Table III.

| Drug | Dose (mg./kg.) | No. of Doses | Proportion Positive | Mean Survival Time (Days) | |
|---|---|---|---|---|---|
| | | | | Treated | Untreated |
| Emetine .. | 6 | 5 | 0/10 | 7·5 | 10 |
| | 4 | 5 | 0/6 | >33·0 | 10 |
| | 2 | 5 | 0/6 | >33·0 | 10·25 |
| Chloroquine | 400 | 5 | Toxic | 0·75 | 8·0 |
| | 200 | 6 | ,, | 1·25 | 8·0 |
| | 100 | 6 | 9/52 | >22·0 | 7·5 |
| | 50 | 8 | 10/10 | 8·0 | 6·5 |
| Fumagillin | 25 | 6 | 6/8 | 4·5 | 10·25 |
| | 10 | 6 | 6/8 | 6·2 | 10·25 |
| Diloxanide | 200 | 8 | 6/7 | 7·1 | 6·4 |
| | 100 | 8 | 3/4 | 7·75 | 6·5 |
| | 25 | 8 | 3/4 | 7·4 | 6·5 |
| RD5269 .. | 100 | 6 | 4/5 | 12·6 | 9·0 |
| Piperazine diloxanide sulphate | 100 | 6 | 4/6 | 14·4 | 9·5 |
| | 100 | 8 | 2/5 | >16 | 7·4 |
| | 25 | 8 | 6/7 | 8·3 | 7·4 |

## DISCUSSION

Both rats and guinea-pigs are relatively insusceptible to hepatic amoebiasis. In our experiments with these species all the lesions in the liver after intrahepatic inoculation were encapsulated. Fibrosis round the margin of the liver abscess is reported in most of the experimental work published. This fibrosis occurs only rarely in amoebic abscesses in man (Craig, 1934). It may be that this wall of granulation tissue which limits the lesion is primarily a reaction against the bacteria, and only incidentally against the amoebae.

With hamsters, however, we found that a capsule or wall was seldom formed at the periphery of the abscess when amoebae were present and actively multiplying. When amoebae were found they were near the periphery of the abscess next to healthy liver tissue. When, rarely, the lesions were walled off, the appearance was that of a purely bacterial abscess, and if amoebae were present they were probably survivors of the original inoculum. This probably happens also in rats and guinea-pigs.

We found that the lesions set up in hamsters with strain R almost invariably had liquefied centres, and adhered to the peritoneum and diaphragm. This was found by Neal and Vincent (1956) after infection with one of their liver-passaged strains; they recovered from the lesions large numbers of amoebae which grew readily *in vitro*. On the other hand, Reinertson and Thompson (1951) found that the lesions caused in hamsters by their strain of *E. histolytica* were rarely purulent macro- or micro-scopically, although some bacteria were present, and the lesions were rarely liquefied. These authors consider that this indicated, *inter alia*, that the lesions were caused by the amoebae, and not by the associated bacteria. The lesions we obtained approximated more closely to those described in man.

There was a marked difference in virulence both in the same strain of *E. histolytica* towards

different species, and in the virulence of different strains towards the same host. The virulence of *E. histolytica* for both the rat liver and the rat caecum can be increased in a number of ways (Yoshimura, 1952; Thompson *et al.*, 1954; Cleveland and Sanders, 1930b; Neal and Vincent, 1956), the simplest probably being liver-to-liver passage. The infectivity of strain M towards the hamster liver was increased in this way, as was its invasiveness to the rat caecum. This recovery of invasiveness to the rat caecum after liver passage (Neal and Vincent, 1956) suggests that any strain of *E. histolytica* cultivated *in vitro* for use in a screening test in intestinal amoebiasis should be periodically passaged through the liver, so that it retains its ability to produce ulceration in the gut. Different strains, however, might require to be passaged at different intervals of time in order to maintain their invasiveness, as shown by the strain MLVe of Dr. R. A. Neal and our strain RL5. In the same way, consistently high incidence of infection towards the liver can be maintained by periodic liver passage of the strain cultivated *in vitro*.

The use of hamsters for the detection of agents effective in treating hepatic amoebiasis is certainly practicable, as shown by our results with emetine and chloroquine. The disadvantage, as pointed out by Thompson and Reinertson (1951), is that the test is a very severe one owing to the rapidity of spread of the lesions and early death of the animals. Reducing the number of amoebae inoculated delays the death of the hamsters, but this advantage is offset by less uniformity and dependability of the infections. In order to use mean survival times to demonstrate the pronounced effectiveness of emetine and chloroquine it was necessary to use large doses and to time them carefully.

In spite of the difficulties of the test, it is felt that it is a useful procedure for the investigation of selected drugs.

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