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STUDIES ON ISOMETAMIDIUM: THE EFFECT OF ISOMETAMIDIUM, HOMIDIUM AND PYRITHIDIUM ON THE INFECTIVITY OF TRYPANOSOMES FOR MICE

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During the course of an investigation into the antitrypanosomal activity of isometamidium, some phenanthridinium compounds were administered to mice infected with *Trypanosoma rhodesiense* or *T. congolense;* trypanosomes from these mice were subinoculated into fresh mice at various times after treatment, and the effect of the compounds on infectivity was noted. *In vivo/in vivo* experiments of this kind have been carried out in the past by Lock (1950) with dimidium bromide and *T. congolense* and by Ormerod (1951) with antrycide and *T. equiperdum.* These authors also carried out *in vitro/in vivo* experiments similar to those described below, as did Hawking (1939) with suramin and *T. rhodesiense.* But so far no one seems to have compared the activities of compounds against different species of trypanosome by either of these methods.

METHODS

The Lugala II strain of *T. rhodesiense* was used. This was isolated in Uganda in 1955 and received by us from EATRO in 1958. Since then it has been passaged by blood inoculation through mice, which it kills in 3 to 4 days. The strain of *T. congolense* was an old laboratory one which produces an acute or subacute infection in mice, killing them in 7 to 20 days (Brown, Hill & Holland, 1961).

Mice were infected intraperitoneally with approximately 750,000 trypanosomes each in 0.2 ml. of citrate-saline, as in a normal therapeutic experiment. They were treated with homidium, isometamidium or pyrithidium (Prothidium) 1 or 2 days later, when there were 1 to 10 trypanosomes per high power field in the peripheral blood-stream. One mouse was left untreated, as a control, in each experiment. At 2, 24 or 48 hr after treatment blood was taken from the tail of the treated mice with a syringe and a No. 18 hypodermic needle, diluted with citrate-saline and injected intraperitoneally into previously untreated and uninfected mice. Three such mice were subinoculated from each donor. Bloods from different donors were not mixed. Three control mice were similarly infected from the untreated control donor referred to above.

Beginning 4 to 5 days after subinoculation, the peripheral blood of the donor and subinoculated mice was examined three times a week for up to 28 days after subinoculation. Failure to detect trypanosomes during this period in a donor was taken as an indication that it had been cured, or in a subinoculated mouse as an indication that the trypanosomes in the inoculum had been non-infective due to their exposure to the drug before subinoculation.

Some preliminary experiments with *T. congolense* and isometamidium failed to reveal any difference between the effects on infectivity of the intravenous and subcutaneous administration of the drug, so the subcutaneous route was used in the later work with both parasites and all three compounds.

A few *in vitro/in vivo* experiments were carried out with isometamidium and *T. congolense*. In these the trypanosomes were exposed *in vitro*, in citrate-saline or horse serum, to known concentrations of the drug at room temperature for 6 hr. They were then washed and centrifuged three times in citrate-saline and injected intraperitoneally into groups of five to ten mice, allowing 65,000 trypanosomes per mouse as in a prophylactic experiment (Brown *et al.*, 1961). Control mice were infected in each experiment with trypanosomes which had been allowed to stand for 6 hr in drug-free medium.

TABLE 1

THE *IN VIVO/IN VIVO* ACTIVITY OF HOMIDIUM

Doses and CD50s were subcutaneous. * Combined results of several experiments. † In a normal therapeutic test

Trypanosome	Period of exposure (hr)	Dose of homidium (mg/kg)	Donor mice (proportion cured*)	Subinoculated mice (proportion protected*)
T. rhodesiense	24	75·0	1/1	3/3
†CD50=2·5 mg/kg		7·5	4/4	7/12
		0·75	3/6	12/17
		0·075	0/4	0/12
	2	75·0	3/3	0/9
		7·5	2/2	0/6
		0·75	1/2	0/6
T. congolense	24	75·0	4/4	0/12
†CD50=0·2 mg/kg		7·5	2/2	0/6
		0·75	1/1	0/3
		0·075	0/1	0/3

RESULTS

The results obtained in the *in vivo/in vivo* experiments after 24 or 2 hr exposure to homidium, isometamidium or pyrithidium are given in Tables 1, 2 and 3.

Homidium had much less effect on *T. rhodesiense* after 2 hr than after 24 hr, and it had little effect on *T. congolense* even after 24 hr; the prepatent period was prolonged but the infectivity was not destroyed. Because of this, the CD50 was checked after these experiments had been completed in case any drug resistance had developed, but none was found.

Lock (1950) was unable to infect mice with *T. congolense* taken from the tail blood of a mouse given an intravenous injection of 10 μg of dimidium bromide 24 hr previously. In view of our results with homidium we repeated this experiment, but could not confirm Lock's results. A slight prolongation of the prepatent period was obtained when trypanosomes were subinoculated 24 hr after the intravenous or subcutaneous injection of 10 μg/20 g (0.5 mg/kg), and a greater prolongation was obtained after a subcutaneous dose of 50 mg/kg, but the trypanosomes were still infective.

Tables 2 and 3 show that pyrithidium and isometamidium had less effect on the infectivity of *T. congolense* after 2 hr than after 24 hr, as might be expected, but the difference was less marked in the case of *T. rhodesiense*.

Although *T. congolense* had normally disappeared from the blood-stream of the treated donor mice within 2 days, it was possible on one occasion to subinoculate three mice from a donor given 0.075 mg/g of homidium 48 hr previously. The subinoculated mice failed to develop an infection. Although the parasitaemia in the donor was very low, much

TABLE 2

THE *IN VIVO/IN VIVO* ACTIVITY OF PYRITHIDIUM

Doses and CD50s were subcutaneous. * Combined results of several experiments. † In a normal therapeutic test

Trypanosome	Period of exposure (hr)	Dose of pyrithidium (mg/kg)	Donor mice (proportion cured*)	Subinoculated mice (proportion protected*)
T. rhodesiense	24	7·5	3/3	8/9
† CD50=2·5 mg/kg		0·75	0/4	5/11
		0·075	0/2	0/6
	2	7·5	1/3	8/9
		0·75	0/3	1/9
T. congolense	24	7·5	2/2	5/5
† CD50=0·2 mg/kg		0·75	4/4	11/12
		0·075	1/3	2/9
		0·0075	0/2	0/6
	2	7·5	3/3	8/9
		0·75	3/3	2/9

less than one trypanosome per high power field, it is unlikely that this was responsible for the failure, since equally low parasitaemias in untreated mice give rise to infections when tail blood is subinoculated. Attempts to repeat this experiment with the same and lower doses were unsuccessful because the trypanosomes disappeared from the bloodstream of the donor mice in less than 48 hr.

With *T. rhodesiense*, the trypanosomes persisted for longer in the treated mice and there was no difficulty about subinoculating 48 hr after treatment. On the one occasion when this was done the results with homidium, pyrithidium and isometamidium were no different from those obtained in the same experiment after 24 hr. The extra period of exposure to the drugs had no additional effect on the trypanosomes.

It can be seen from the results with *T. rhodesiense* and pyrithidium (0.75 mg/kg) in Table 2 and isometamidium (0.075 mg/kg, 2 hr exposure) in Table 3 that, although none of the donor mice was cured, trypanosomes subinoculated from these donors sometimes

TABLE 3

THE *IN VIVO/IN VIVO* ACTIVITY OF ISOMETAMIDIUM

Doses and CD50s were subcutaneous. * Combined results of several experiments. † In a normal therapeutic test

Trypanosome	Period of exposure (hr)	Dose of isometamidium (mg/kg)	Donor mice (proportion cured*)	Subinoculated mice (proportion protected*)
T. rhodesiense	24	7·5	1/1	3/3
† CD50=0·2 mg/kg		0·75	2/2	5/5[1]
		0·075	2/4	11/12
		0·0075	0/3	0/9
	2	7·5	3/3	9/9
		0·75	3/3	9/9
		0·075	0/3	6/9
T. congolense	24	7·5	1/1	3/3
† CD50=0·01 mg/kg		0·75	5/5	10/12
		0·075	3/3	8/9
		0·0075	2/4	0/12
	2	75·0	3/3	9/9
		7·5	3/3	8/9
		0·75	3/3	1/9

failed to establish an infection in untreated mice. The opposite condition, in which the donor mice were cured but the subinoculated ones became infected, may be seen with various drugs and doses in Tables 1, 2 and 3.

The results of the *in vitro/in vivo* experiments with isometamidium and *T. congolense* are shown in Table 4. The compound was less effective when the trypanosomes were suspended in horse serum than when they were suspended in citrate-saline. In similar experiments with homidium and *T. congolense* ten out of ten mice were protected after exposure to 10 μg/ml. in citrate-saline, but none of nine mice after exposure in horse serum. This finding supports the results obtained with isometamidium.

TABLE 4

ACTIVITY OF ISOMETAMIDIUM AGAINST *T. CONGOLENSE* (6 HR EXPOSURE)

* The combined results of several experiments. † Blood concentration probably about 0·2 μg/ml. (Hill & McFadzean, 1963). ‡ Included for comparison

Method	Concentration or dose	Donor mice (proportion cured*)	Subinoculated mice (proportion protected*)
In vitro/in vivo in citrate-saline at room temp.	1·0 μg/ml.	—	5/5
	0·1 μg/ml.	—	17/17
	0·01 μg/ml.	—	4/18
	0·001 μg/ml.	—	0/18
In vitro/in vivo in horse serum at room temp.	10·0 μg/ml.	—	9/9
	1·0 μg/ml.	—	28/67
	0·1 μg/ml.	—	0/30
	0·01 μg/ml.	—	0/20
In vivo/in vivo in mice‡	75·0 mg/kg†	3/3	9/9
	7·5 mg/kg	3/3	6/9
	0·75 mg/kg	4/4	4/12
	0·075 mg/kg	3/3	1/9

DISCUSSION

The results indicate that homidium may be taken up more slowly by trypanosomes, or by the host, than are pyrithidium and isometamidium. In the *T. rhodesiense* infections the difference between the 2 hr exposure and the 24 hr exposure was much greater with homidium than it was with the other two compounds ; and in the *T. congolense* infections a maximum effect was not obtained with less than 48 hr exposure to homidium, although 24 hr was sufficient for the other compounds.

In all the experiments with homidium and isometamidium *T. rhodesiense* probably took up the drugs more rapidly than *T. congolense*, as shown by the equal or greater effect of the compounds against *T. rhodesiense* by the *in vivo/in vivo* technique, although they were more active against *T. congolense* in a normal therapeutic test. With isometamidium the effect was particularly clear after 2 hr exposure. Had the rate of uptake been the same for each species, the *in vivo/in vivo* activities would presumably have corresponded with the activities in a conventional therapeutic test. Pyrithidium was slightly more active against *T. congolense in vivo/in vivo* than against *T. rhodesiense*, particularly after 24 hr, but it was over ten times as active in a therapeutic test, which indicates that like the other compounds it may have been taken up more rapidly by *T. rhodesiense*. Also, the difference between exposure for 2 hr and exposure for 24 hr was greater with *T. congolense* than with *T. rhodesiense* for both pyrithidium and isometamidium, which suggests

that relatively more compound was taken up in the first 2 hr by *T. rhodesiense* than by *T. congolense*. In spite of this more rapid uptake, *T. rhodesiense* persisted in the peripheral blood-stream for longer after the host was treated than did *T. congolense*.

In terms of the hypothesis put forward by Newton (1957) to explain uptake of homidium by *Strigomonas oncopelti*, a greater rate of uptake of the three compounds by *T. rhodesiense* compared with *T. congolense* could be due to a differential affinity of the compounds for the primary binding sites of the two species, to their unequal rate of removal to the secondary binding sites, or to a combination of both these factors. The fact that *T. rhodesiense* multiplies more rapidly than *T. congolense* in the mouse suggests that the compounds might be removed more quickly to the secondary binding sites in the former species if they interfere with deoxyribonucleic acid metabolism, as homidium does in *Strigomonas oncopelti* (Newton, 1957, 1964).

The development of parasitaemia in mice subinoculated from a donor which had received a curative dose of a compound presents no difficulties, since the trypanosomes were presumably subinoculated before they had been irreversibly affected ; but it is more difficult to explain the reverse phenomenon, where trypanosomes failed to infect mice although the donor was not cured. In some of the experiments where this happened the trypanosomes disappeared from the peripheral blood of the donor mice but a relapse occurred after 14 or more days, but in other experiments the donor died without the peripheral blood ever having been cleared. It is possible, particularly in the first set of circumstances, that at the time of inoculation all the trypanosomes in the peripheral blood-stream had been irreversibly affected by the drug, but some more resistant ones were present in the blood of the internal organs.

The experimental results quoted in Table 4 show that a higher concentration of isometamidium is necessary *in vitro* than *in vivo* to produce a given effect on the infectivity of the trypanosomes. It is true that the blood concentration in mice is only very approximate, since Hill & McFadzean (1963) calculated from a bioassay that the blood concentration of isometamidium in mice 1 to 3 hr after a subcutaneous injection of 160 mg/kg was about 0.4 μg/ml. But the blood concentration after a dose of 0.75 mg/kg, which had some effect on the infectivity, is bound to be much lower than this, whereas a similar effect *in vitro* in horse serum was produced only by a concentration of about 1.0 μg/ml.

This difference between the concentrations needed *in vitro* and *in vivo* to produce the same effect could be due to the reduced growth rate of *T. congolense in vitro*. If isometamidium interferes with deoxyribonucleic acid metabolism, as homidium does in *Strigomonas oncopelti* (Newton, 1957), the reduced growth rate could mean that sufficient drug to inhibit infectivity was removed from the primary binding sites to the secondary sites only when the concentration at the former was extremely high.

The marked difference between the *in vitro* activity of isometamidium in citrate-saline and in horse serum may be due to a different partition coefficient between the primary binding sites and citrate-saline compared with the primary binding sites and horse serum. Since amidine groups are known to have a strong affinity for protein molecules, it is distinctly possible that a smaller proportion of the total isometamidium present would be atached to trypanosomes suspended in horse serum than to those suspended in citrate-saline.

SUMMARY

1. *In vivo/in vivo* experiments were carried out in which mice were infected with *Trypanosoma rhodesiense* or *T. congolense,* treated with homidium, pyrithidium or isometamidium 1 to 2 days later, and the trypanosomes in the peripheral blood-stream were subinoculated into fresh mice 2, 24 or 48 hr after treatment. The effect of the compounds on the infectivity of these trypanosomes was noted.

2. Although the compounds were ten to twenty times more active against *T. congolense* than against *T. rhodesiense* in a normal therapeutic test, this was not so in the *in vivo/in vivo* experiments. In particular, homidium was about ten times more active against *T. congolense* in a therapeutic test, but in the *in vivo/in vivo* experiments it was over 100-times more active against *T. rhodesiense.*

3. It was considered that these differences might be due to the differential affinity of the compounds for the primary binding sites of the two species of trypanosome, to their unequal rate of removal to the secondary binding sites, or to a combination of these two factors.

4. Some *in vitro/in vivo* experiments with isometamidium and *T. congolense* were also carried out, in which trypanosomes were exposed to the drug *in vitro* and then inoculated into mice after being washed in citrate-saline. The same concentration of drug was more active in citrate-saline than in horse serum, and in both media the compound was less active than it was *in vivo.* The possible reasons for this are discussed.

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