Drug Activity of Bithionol against the Different Life Cycle Stages of *Paragonimus ohirai* and *P. miyazakii* using Diffusion Chambers

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INTRODUCTION

Surgical implantation of diffusion chambers seems to provide a better understanding on the interaction of the implanted parasite and the host animals. Up to date, diffusion chambers have been used to culture in vivo several kinds of parasites, and to immunize animals against helminths or protozoa by surgical implantation of the chambers containing these organisms (Huff et al., 1960; Crandall and Arean, 1965; Petithory and Rousset, 1965; Despommier and Wostmann, 1968; Greenblatt and Shelton, 1968; Rickard and Bell, 1971; Zussmann et al., 1971; Logan and Hanson, 1974).

Diffusion chamber allows exchange of various materials between the implanted parasites and the host. Moreover, if desired, chambers may be easily retrieved for examination of the implanted organisms and fresh chambers may be implanted at intervals (Logan and Hanson, 1974). On the basis of these advantages, the present study was designed to test drug activity against the various developmental stages of the lung flukes, *Paragonimus* in rats.

Bithionol was chosen as the test drug in this investigation. This drug has been used for the first time for chemotherapy of animal or human paragonimiasis by Yokogawa et al. (1961 a, b), and it has been well known that Bithionol is the most effective drug for paragonimiasis among the drugs used hitherto. However, the effect of Bithionol on extra-pulmonary paragonimiasis, such as subcutaneous, cerebral or intraperitoneal paragonimiasis is not so clearly ascertained.

The present work was undertaken to determine whether diffusion chamber is suitable for obtaining information on the relation between the survival rates of the worms and the oral administration of Bithionol in vivo. In addition, attempts were made to secure whether Bithionol could kill the various developmental stages of the flukes in diffusion chambers implanted intraperitoneally or subcutaneously in rats.

MATERIALS AND METHODS

The animals :

Adult albino rats of the Wistar King strain weighing around 200 g were used. They were fed with a commercially prepared diet and water was provided ad libitum.

The parasites :

The organisms studied were *P. ohirai* and *P. miyazakii*. Metacercariae of *P. ohirai* and *P. miyazakii* were collected from the naturally infected sesarmid crabs, *Sesarma* (*H.*) *dehaani* and potamonid crabs, *Potamon* (*G.*) *dehaani*, respectively. They were washed several times in sterile saline, containing $\times 10^6$ units penicilin and 1 g streptomycin sulfate per liter. Fourteen-day-old *P. ohirai* and 43-day-old *P. miyazakii* (nonoviferous; young adult) and adult (oviferous) *P. ohirai* were collected from the experimentally infected rats and then washed in the saline mentioned above. In all series of the experiments, the stages and the numbers of the worms kept in one chamber were 20 in metacercariae, 5 in young adult worms and 4 in adult worms; 2 chambers were surgically implanted in each rat.

Diffusion chambers :

Diffusion chambers were made of diffusion chamber ring (Plexiglass U-100, diameter: 10 mm, thickness: 2 mm) and millipore filter (pore size: 14 μ). They were closed after the introduction of the worms with sterile saline and then the chambers were implanted intraperitoneally or subcutaneously in the rat. Two or three animals were used for each contol (non-treated with Bithionol) and treated group.

Drug used :

The drug utilized in the experiment, Bithionol, is put out as a tablet of 200 mg by the Tanabe Seiyaku Co., Ltd in Japan as Bitin. The tablets were powdered and administered *per os* to the experimental animals with normal saline using an injection syringe with a slender vinyl tube. The animals were given 400 mg/kg of Bithionol 3 to 12 times respectively every other day, before and after the implantation of the diffusion chambers.

Examination of the worms :

The diffusion chambers implanted were removed several times during 3 to 30 days after the implantation, according to the schedules as shown in Tables I and II. The contents and worms in the chambers were washed with sterile saline in petri dishes and examined under a binocular microscope. All the worms removed from the chambers were maintained alive in the saline at 37°C for 15 to 30 minutes to secure their vitalities and then the survived worms were reintroduced into newly prepared chambers and implanted again into the same animals.

RESULTS

At the examination of the chambers removed from the rats, gelatinous matrix (or fibrous mass) surrounding the worms was found in the all control chambers. While such material was scarcely recognized in the treated group with 400 mg/kg of Bithionol, most of the chambers in the treated group contained the muddy substances. Pertinent data are summarized in Tables I and II.

The survival rates of the worms described herein indicate the rate of the number of living worms found in the chamber to the total numbers of the worms used.

TABLE I. Influence of Bithionol on each stage of *P. ohirai* in the chambers implanted intraperitoneally or subcutaneously in rats

Stages of worms	Group	No. of worms encham-bered	Dose of Bithionol	No. (%) of survival worms					
				day 3 (×3*)	5(×4)	7(×5)	10(×7)	15(×9)	20(×12)	30
Metacercariae (excysted)	Treated									
	IP**	80	400 mg/kg		48(60.0)	32(40.0)	11(13.8)	2(2.5)	0	
	IP	80	400 mg/kg		53(66.3)	38(47.5)	15(18.8)	3(3.8)	0	
	SC***	80	400 mg/kg		8(10.0)	3(3.8)	0			
	Non-treated									
	IP	80	—		57(71.3)	41(51.3)	33(41.3)	22(27.5)	19(23.8)	
	SC	80	—		46(57.5)	41(51.3)	28(35.0)	22(27.5)	19(23.8)	
Young adult (14-day-old)	Treated									
	IP	50	400 mg/kg		0					
	SC	20	400 mg/kg	2(10.0)	0					
	Non-treated									
	IP	20	—		17(85.0)	15(75.0)	14(70.0)			
	SC	20	—	12(60.0)	9(45.0)	8(40.0)	7(35.0)			
Adult (oviferous)	Treated									
	IP	24	400 mg/kg	0						
	SC	24	400 mg/kg	0						
	Non-treated									
	IP	24	—	20(83.3)			12(50.0)	7(29.2)	7(29.2)	1(4.2)
	SC	24	—	19(79.2)			19(79.2)	17(70.8)	16(66.7)	13(54.2)

* Times of the administration of Bithionol.
** The chamber implanted intraperitoneally.
*** The chamber implanted subcutaneously.

TABLE II. Influence of Bithionol on each stage of *P. miyazakii* in the chambers implanted intraperitoneally or subcutaneously in rats

Stages of worms	Group	No. of worms encham-bered	Dose of Bithionol	No. (%) of survival worms					
				day 3 (×3*)	5(×4)	7(×5)	10(×7)	15(×9)	20(×12)
Metacercariae (excysted)	Treated								
	IP**	80	400 mg/kg		3(3.8)	2(2.5)	1(1.3)	1(1.3)	1(1.3)
	SC***	80	400 mg/kg		12(15.0)	2(2.5)	1(1.3)	0	
	Non-treated								
	IP	80	—		44(55.0)	42(52.5)	37(46.3)	35(43.8)	31(38.8)
	SC	80	—		56(70.0)	56(70.0)	48(60.0)	41(51.3)	39(48.8)
Young adult (43-day-old)	Treated								
	SC	10	400 mg/kg	0					
	Non-treated								
	SC	10	—	10(100.0)				8(80.0)	8(80.0)	7(70.0)

* Times of the administration of Bithionol.
** The chamber implanted intraperitoneally.
*** The chamber implanted subcutaneously.

Effect of Bithionol on *P. ohirai*:

Effect on the metacercarial stages:

The metacercariae of *P. ohirai* could excyst in the chambers implanted intraperitoneally or subcutaneously in rats. Their survival terms in the chambers were slightly prolonged compared with those of the young adult (14-day-old) and adult worms in the chambers implanted. In the non-treated control group, the survival rates of the worms were 71.3 % in the chambers implanted intraperitoneally and 57.5 % in the subcutaneously implanted chambers

in day 5; then these survival rates of the worms gradually decreased to 27.5 % in day 15 and then 23.8 % in day 20, respectively, showing the same figures.

Effect on the young adult and adult stages :

In the treated group with 400 mg/kg of Bithionol, no worms of the young adult (14-day-old) and adult stages of *P. ohirai* survived for 5 days in the former stages and 3 days in the latter ones after the implantation. However, both of the young and adult worms in the non-treated control group showed the significantly high survival rates. Especially, the adult worms in the non-treated control group survived for 30 days after the implantation; the survival rate of the worms was higher in the subcutaneously implanted chambers than in the intraperitoneally implanted chambers.

Effect of Bithionol on *P. miyazakii* :

Effect on the metacercarial stages :

P. miyazakii metacercariae also excysted in the diffusion chambers implanted intraperitoneally or subcutaneously in rats. In the case of the rats treated with 400 mg/kg of Bithionol, the survival rate of the worms was 3.8 % in the chambers implanted intraperitoneally and 15.0 % in the subcutaneously implanted chambers in day 5. In this treated group, only one of the worms implanted subcutaneously in rats survived for 10 days after the implantation, while in the worms implanted intraperitoneally the one worm survived in day 20. On the other hand, in the non-treated control group the survival rates of the worms were significantly higher than those of the treated group, showing the survival rates, 55.0 % to 38.8 % in the intraperitoneally implanted chambers and 70.0 % to 48.8 % in the chambers implanted subcutaneously throughout the experiment.

Effect on the young adult stages :

The young adult worms (non-oviferous) used were 43-day-old after the infection *per os*. In the non-treated control group, the majority of the worms survived more than 20 days after the implantation, while no living worms were found in day 3 in the treated group with 400 mg/kg of Bithionol. Thus, Bithionol could also kill 43-day-old *P. miyazakii* in the chambers implanted subcutaneously in rats.

DISCUSSION

In these trials, the survival rates of the worms in the chambers in non-treated control group were significantly higher than those in treated group with 400 mg/kg of Bithionol in any case of the experiments throughout the study. In the treated group, 43-day-old non-oviferous *P. miyazakii* and adult *P. ohirai* kept in the diffusion chambers in rats were killed with Bithionol in 3 days after the implantation. Bithionol was also effective against the young adult (14-day-old) stages of *P. ohirai*; all of the worms in the diffusion chambers implanted intraperitoneally or subcutaneously in rats were killed in 5 days after the implantation. On the contrary, in the non-treated control group a considerable number of young adult *P. ohirai* survived, showing the rate of 85.0 % to 70.0 % in the chambers implanted intraperitoneally and 45.0% to 35.0 % in the subcutaneously implanted chambers, during 5 to 10 days after

the implantation. While, Bithionol was less effective against the metacercarial stages of both *P. ohirai* and *P. miyazakii* than the other stages of the flukes examined. In this case, furthermore, *P. miyazakii* metacercariae had a tendency to be more sensitive against Bithionol when compared with *P. ohirai* metacercariae.

Thus, both *P. ohirai* and *P. miyazakii* kept in diffusion chambers in rats were killed with Bithionol given in doses of 400 mg/kg every other day. Moreover, the effects of Bithionol against the various developmental stages of *P. ohirai* and/or *P. miyazakii* in the chambers were found to be quite different. Limited data also indicate that Bithionol has similar activity against *P. ohirai* infection in rats, in the case of oral administration with the drug (Araki, 1968). Such finding was demonstrated by Yokogawa et al. (1956) working with *P. westermani* in vitro using Chloroquine, *viz.*, they tried in vitro screening test with various drugs against excysted larvae of *P. westermani*, and reported that the LD_{50} of Chloroquine in the adult worms was one tenth of the larvae.

In the non-treated control group, on the other hand, each stage of *P. ohirai* and *P. miyazakii* in the chambers survived showing relatively high survival rates through the experiment. These findings are especially interesting in light of the cultivation in vivo on the genus, *Paragonimus*; in the adult stages of *P. ohirai* in the chambers implanted subcutaneously, 13 (54.2%) out of the 24 worms survived for 30 days after the implantation. Unfortunately, in this non-treated control group each stage of *P. ohirai* and/or *P. miyazakii* could not show any developmental signs in the chambers implanted intraperitoneally or subcutaneously in rats. In the case of adult *P. ohirai*, moreover, the eggs in the uterus disappeared on day 10 after the implantation. Despommier and Wostmann (1968) working with *Trichinella spiralis* obtained partial development of this parasite in diffusion chambers implanted into rats intraperitoneally. They state: "The fibrous mass found in the chambers may serve as food for developing larvae or it may create favorable conditions for larval morphogenesis." Similar materials were also recognized in the diffusion chambers in the non-treated control group of the present experiment. There is no evidence, however, that the materials have any effect against *P. ohirai* or *P. miyazakii* in the diffusion chambers. According to Rickard and Bell (1971), activated embryos of *Taenia taeniaeformis* and *T. ovis* kept in diffusion chambers and implanted intraperitoneally in rats and lambs, respectively, for 3 weeks, developed at rates comparable with those reported in natural infections. While, Zussman et al. (1971) carried out the experiment to study the long-term in vivo survival of implanted adult *Schistosoma mansoni* in the mouse peritoneal cavity using diffusion chambers, but they failed due to the early death of the worms. In their investigation, no implanted worms survived more than 7 days; the majority of the worms had been in poor condition after 3 days and died in 5 days.

As mentioned above, the survival and/or growth and development of helminths in diffusion chambers surgically implanted into host animals, appears to be different according to the species of the parasites. However, if comfortable factors for the organisms could be found out by improving the experimental conditions, diffusion chambers would provide more advantages in studing on the relation between the implanted parasites and the host animals, and also between the parasites and the drugs in vivo.

SUMMARY

Metacercarial, young adult (non-oviferous) and adult (oviferous) stages of *Paragonimus ohirai* and/or *P. miyazakii* were kept in diffusion chambers, which were implanted intraperitoneally or subcutaneously in rats. The rats used were devided into two groups; one group did not receive the treatment with Bithionol as the control, while the other received the oral administration of Bithionol before and after the implantation of the chambers. In the non-treated control group, the worms of each stage of *P. ohirai* and *P. miyazakii* in the diffusion chambers implanted surgically in rats, survived showing relatively high survival rates throughout the experiment. On the other hand, in the treated group both *P. ohirai* and *P. miyazakii* kept in the diffusion chambers in rats were killed with Bithionol given in doses of 400 mg/kg every other day. In this case, the effects of Bithionol against the various developmental stages of the flukes in the chambers were found to be quite different. The drug was less effective against the metacercarial stages of the parasites than the other stages examined. Moreover, Bithionol showed similar killing effect against both flukes in the chambers implanted intraperitoneally and/or subcutaneously. Thus, the present study confirmed the effect of Bithionol against *P. ohirai* and *P. miyazakii* in the diffusion chambers implanted intraperitoneally or subcutaneously in rats. Besides, it was suggested that the diffusion chamber would be suitable for obtaining information on the interaction of the implanted flukes and the drug.

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