SUPPRESSION OF MICROFILARICIDAL ACTIVITY OF DIETHYLCARBAMAZINE BY ANTI-LYMPHOCYTE SERUM IN COTTON RAT FILARIASIS

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INTRODUCTION

The mechanism of action of diethylcarbamazine (DEC) has been studied by Hawking (1950, 1962, 1963), Taylor (1960), Kobayashi *et al.*, (1969), Tanaka *et al.*, (1970), Matsuda *et al.*, (1974), and Takaoka *et al.*, (1974). It was reported that DEC does not kill adult worms of *Litomosoides* either *in vivo* or *in vitro* but has microfilaricidal activity *in vivo*. In the studies by Hawking (1950, 1962, 1973) and Hawking *et al.*, (1950), microfilaria (mf) of *Litomosoides* was found to be trapped by Kupffer cells in the liver and he presumed that DEC has opsonin-like activity (Hawking, 1950), but this has not yet been proven by *in vitro* experiments (Hawking, 1973). Kobayashi *et al.*, (1969) reported that microfilaricidal activity of DEC could be observed when the host animals produced humoral antibody in the experiment with mf from implanted adult *Litomosoides*. In their study, DEC was not effective against the inoculated mf even though humoral antibody was introduced by transfusing sera from infected animals. The sensitization of the host animal was a necessary condition to make the action of DEC effective. DEC showed activity against the inoculated mf of *Litomosoides* in the presence of spleen cells from the other infected cotton rats in the preliminary experiments reported by Tanaka *et al.*, (1970) and Takaoka *et al.*, (1974). In the report by Hashiguchi *et al.*, (1972), prednisolone was shown to have a slight antagonistic activity to DEC in cotton rat filariasis. Based on these previous studies, the role of cells in the host was presumed to be important in the mode of action of DEC. In the present study, anti-lymphocyte serum was given to cotton rats and the suppression of microcidal action of DEC was examined to determine the role of lymphocytes in the mechanism of action of DEC.

MATERIALS AND METHODS

Infection of cotton rats with *Litomosoides carinii*: Cotton rats, *Sigmodon hispidus*, 3 to 4 weeks old, were infected with *Litomosoides carinii* by bites of infective tropical rat mite, *Ornithonyssus bacoti*. The mites were previously fed with blood of infected cotton rats and kept in a small container for 2 weeks until engorged mf developed into infective larvae. The average density of larvae per mite was 5.6 and 30 mites were placed on the body surface of each cotton rat. Mf became detectable in the blood about 7 weeks after infection. Mf density increased rapidly and became stable 10 weeks after infection. In the study with ATS, microfilaraemia was produced by implanting adult *Litomosoides* into the peritoneal cavity of each clean cotton rat. In this case, mf appeared in the blood on the following day after implantation.

Preparation of anti-lymphocyte serum: Thymus and lymph nodes were collected from 3 to 4 week old cotton rats after sacrificing by total bleeding and kept separately in Tyrode's

solution. Using cells from thymus and lymph nodes, anti-thymocyte serum (ATS) and anti-lymphocyte serum (ALS) were produced in rabbits, respectively (Hirose *et al.*, 1970; Hirose and Kurosawa, 1971).

For ATS, adherent fragments and blood clots were removed from parenchyma and thymus was teased using forceps in Tyrode's solution. Thymus cells were then dispersed by rapid pumping in a Pasteur pipette and the cell suspension was filtered through a 80 mesh stainless steel wire net to remove coarse fragments. Cells were then washed in physiologic saline three times by centrifugation and finally the suspension of 10^7 cells/ml was prepared.

Rabbits weighing 3 to 4 kg were inoculated with 10 ml of cell suspension into ear arteries 3 times at 10 day intervals. Whole blood was collected 10 days after the last inoculation. The serum was isolated and inactivated at 56°C for 30 minutes. Hemagglutinin contained the prepared ATS was absorbed with red blood cells of cotton rats several times and sterilized through millipore filter.

ALS was also prepared from lymph nodes by the same procedures as described above. The prepared ALS and ATS were kept in small ampoules and stored in a freezer at −20°C.

Titration of activity of ALS and ATS: The activity of ALS and ATS was titrated by cytotoxicity test (Hirose and Kurosawa, 1971). Thymus cells and lymphocytes obtained from clean cotton rats were used for the test with the corresponding antisera. Cells were suspended at 5×10^6 cells/ml in veronal buffer containing 0.02% KCl and 0.1% glucose, pH 7.4 - 7.6, designated as K-GVB^{++}. For the test, the antiserum was diluted serially at 2 fold dilutions from 1 : 5 to 1 : 1280 with 1 drop (0.025 ml) of K-GVB^{++} solution in the wells of the microtiter plate. One drop of the above cell suspension and 1 drop of guinea pig complement (approximately 200 CH_{50}/ml) diluted at 1 : 3 were added to the diluted serum in each well. After incubating the prepared plates in an incubator at 37°C for 30 minutes and being shaken every 5 minutes, plates were centrifuged at 60 G for 5 minutes. The supernatant in each well was replaced by 2 drops (0.05 ml) of K-GVB^{++} by a micropipette. Immediately after mixing 1 drop of 0.3% trypan blue solution, cells from each well on a glass slide were observed microscopically within 3 minutes. The proportion of the damaged cells, which were stained blue, to the total cells observed was obtained and the serum dilution which gave 50% damage was regarded as the titer of test serum.

The titers of 2 lots of ALS were 1 : 60 and 1 : 120, respectively, and 1 out of 4 lots of ATS was 1 : 120 in titer and the other 3 were 1 : 240.

Treatment of animals with ALS and ATS: Cotton rats were injected intraperitonealy with 0.5 ml of ALS daily 3 times a week continuously before the test of DEC. Dose schedule was changed according to the purpose of experiments after treatment with DEC. With ATS at a titer of 1 : 240, the daily dose was reduced to 0.3 ml per animal.

Hemagglutination test with *Litomosoides* antigen: The levels of humoral antibody to *Litomosoides* in test animals were examined by hemagglutination test with antigen derived from adult *Litomosoides*. The blood was taken by retroorbital puncture with a Pasteur pipette with pointed tip. Obtained blood was mixed with 3 fold volume of physiologic saline and kept in a refrigerator overnight. The supernatant isolated by centrifugation was regarded as the serum diluted at 1: 8. Hemagglutination test was performed using formalinized sheep cells (Csizmas, 1960) by the microtiter technique (Kamiya and Tanaka, 1969).

Microfilaria density: Blood was sampled from cotton rat by a calibrated micropipette and 2 or more thick smears of 2.5 c.mm blood were made on a microscope glass slide. Smears of blood were haemolysed in water, stained with Giemsa solution and observed microscopically. Mf density was shown by the average of mf counts in a few smears.

RESULTS

Change of mf density by a single dose of DEC: In the study of suppression on the effect of DEC in *Litomosoides* infection in cotton rats, it was found that observation of the mf density for 4 to 7 days was necessary after the treatment with DEC since there was always an abrupt and transient reduction of the density even if the action of DEC was suppressed by the other treatment (Kobayashi *et al.*, 1969; Tanaka *et al.*, 1970).

The change of mf density after a single dose of 200 mg/kg of DEC was studied in cotton rats infected with *Litomosoides*. As shown in Fig. 1, mf density was reduced to about 20% of the initial level on the following day and to less than 10% after 4 days in most cases. The density then increased slightly in most cases but did not exceed 30%, 7 days after treatment. When the increase of mf density went higher than 30% of the initial level, the action of DEC was regarded as suppressed in the present study.

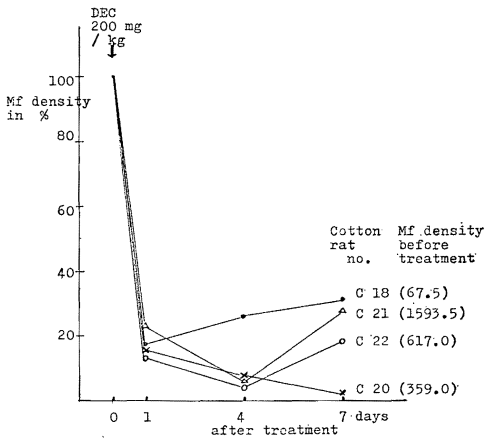

Fig. 1—Change of mf density of *Litomosoides carinii* in cotton rat by a single injection of DEC at a dose of 200 mg/kg.

Effect of ALS on DEC in the infected cotton rat: Several cotton rats were injected intraperitoneally with ALS. After the 2nd injection of ALS, cotton rats were infected with *Litomosoides* by the bite of the infective *Ornithonyssus*. The test of action of DEC was scheduled 10 weeks after infection but only two infected cotton rats (Nos. L4 & L5) survived the continuous injection of ALS (Table 1).

Table 1

Effect of a single does of 200 mg/kg of DEC on microfilaria of *Litomosoides* in the infected cotton rats which were continuously injected with ALS 3 times a week before infection until 2 weeks after treatment with DEC.

Weeks after infection		6	7	9	10*	Injection of DEC						11	12**
Cotton rat no.		Before treatment					After treatment						
		4w	3w	1w	0		1h	6h	1d	2d	3d	1w	2w
L4	mf	/	1.0	147.5	296.5		129.0	61.0	29.7	23.3	44.3	61.3	237.7
	HA	32>	/	32>	/		/	/	/	/	/	/	500
L5	mf	/	6.3	426.7	209.0		144.7	164.3	213.3	221.3	133.3	525.0	814.7
	HA	32>	/	32>	/		/	/	/	/	/	/	64

mf = mean count of microfilaria in 2.5 c.mm blood.
HA = reciprocal of hemagglutination titer with *Litomosoides* antigen.
* = immediately before injection of DEC.
** = ALS treatment discontinued.

Table 2

Effect of DEC on microfilaria of *Litomosoides* in an infected cotton rat which was continuously injected with ALS for 12 weeks and DEC was given 3 weeks after termination of ALS treatment.

Weeks after infection		12**	14	15*	Injection of DEC			16	18
Cotton		Before treatment				After treatment			
rat no.		3w	1w	0	5m	1h	1d	1w	3w
L5	mf	814.7	835.5	1025.5	986.0	960.5	917.5	997.0	794.5
	HA	64	/	1000	/	/	/	/	2000

* = immediately before injection of DEC.
** = ALS treatment discontinued.

In the test of DEC at a single dose of 200 mg/kg, the change of mf density was observed a few times on the day of DEC treatment and a few more times in a week. The change of mf density and HA titer are shown in Table 1.

In cotton rat No. L4, the initial mf density was 296.5. This decreased gradually after injection of DEC to 23.3 after 2 days and then gradually increased to 61.3 after 1 week. In cotton rat No. L5, the density was 209.0 before treatment and dropped to 144.7, 1 hour after DEC injection. It remained at 133.3 after 3 days and rose to 525.0 after 1 week. The action of DEC was restricted in these cotton rats especially in No. L5.

HA titers to *Litomosoides* were less than 1 : 32 before injection of DEC. These increased to 1 : 500 and 1 : 64 in Nos. L4 and L5, respectively, 2 weeks after DEC treatment when the treatment with ALS was discontinued.

Action of DEC after termination of ALS treatment: Cotton rat No. L5 which survived the previous test, was kept without giving any treatment for 3 weeks. During this period, mf density increased from 814.7 to 1025.5 and HA titer to *Litomosoides* went up to 1 : 1000 (Table 2). In the test of DEC, mf density fell slightly to 917.5 on the following day and went up to 997.0 after 1 week. In this case, DEC did not affect the mf though ALS was not given for 3 weeks and humoral antibody level remained high at 1 : 2000 by HA test.

Effect of ATS on the action of DEC on mf from implanted worms: The prepared ATS was more lethal to cotton rats than ALS and cotton rats did not survive continuous injection for several weeks. To shorten the period of injection, the effect of ATS was tested on mf from adult *Litomosoides* which was implanted to the peritoneal cavity. Mf density reached its peak several days after implantation. Action of DEC was examined 2 or 3 weeks after implantation since it was known that DEC showed its activity on mf from the implanted adults 2 weeks or later after implantation (Kobayashi *et al.*, 1969).

In 2 cotton rats (Nos. T2 & T3 in Table 3) treated with ATS for two weeks, mf density dropped immediately after injection of DEC but returned to the initial level or higher after 1 week. HA titer to *Litomosoides* remained at less than 1 : 32 for 2 weeks after injection of DEC and rose to 1 : 256 and 1 : 128, respectively, 4 weeks after injection of DEC.

Table 3

Effect of DEC on microfilaria from implanted *Litomosoides* in cotton rats which were treated with ATS during a period from the implantation of adult *Litomosoides* to the test of DEC.

Cotton rat no.		After implantation 1w*	2w*	Injection of DEC	After treatment with DEC 5m	1h	6h	1d	4d	1w	2w	3w	4w
T2	mf	14.5	11.5		2.0	6.5	15.5	15.0	12.5	13.0	13.5	9.5	/
	HA	/	32>		/	/	/	/	/	32>	32>	32	256
T3	mf	25.0	33.0		1.0	48.0	63.5	58.0	87.0	150.0	211.0	314.0	/
	HA	/	32>		/	/	/	/	/	32>	32>	32	128

* treatment with ATS.

In 2 cotton rats (T6 & T7) treated with ATS for 3 weeks before the test of DEC and until 1 week after the test, the change of mf density was nearly the same as in the previous two animals (T2 & T3) as shown in Table 4. It was shown that ATS also suppressed the action of DEC on the mf from implanted *Litomosoides*.

Action of DEC by treatment with ATS together with serum from infected animals: In two cotton rats (T6 & T7), DEC was again injected 10 days after the 1st test of DEC while ATS was being injected and 1 ml of serum from the infected cotton rats was also injected daily for 5 days starting from the previous day of the 2nd injection of DEC (Table 4). The mf density rose 4 days after injection of DEC. In this experiment, humoral antibody introduced from the infected animal did not influence the inhibitory action of ATS on DEC.

Table 4

Effect of DEC on microfilaria from implanted adult *Litomosoides* in cotton rats which were previously injected with ATS and continuously treated with ATS after the last test of DEC or together with serum from infected cotton rats until 3 days after the second test of DEC.

Days after implantation		22	23*	Injection of DEC			24	25	28	30
Time after treatment					1h	6h	1d	2d	5d	1w
Cotton rat no.	ATS	x						x	x	x
T6	mf /	18.0	57.5		37.5	19.5	26.5	33.0	/	/
T7	2.5 c.mm	14.0	13.0		12.5	6.5	6.0	6.5	/	8.0

Days after implantation		32	33*	Injection of DEC			34	35	36	37	
Time after treatment					1h	6h	1d	2d	3d	4d	
Cotton rat no.	ATS	x	x				x	x	x		
	immune serum	x	x				x	x	x		
T6	mf /	/	22.5		14.5	2.5	9.0	/	/	15.0(HA,32)	
T7	2.5 c.mm	/	7.0		4.5	5.0	3.5	/	/	25.0(HA,128)	

x = injected, HA = reciprocal of HA titer, * = immediately before injection of DEC.

From the result of the aforementioned experiments, it was apparent that ALS and ATS inhibit strongly microfilaricidal activity of DEC and it can be said therefore, that lymphocytes may play an important role in the mechanism of action of DEC.

DISCUSSION

Using a single injection of DEC at a large dose, mf density of *Litomosoides* in cotton rats was reduced to about 10% on the following day (Hawking, 1950; Hawking *et al.*, 1950; Tanaka, 1965). In the previous studies in this laboratory (Kobayashi *et al.*, 1969; Tanaka *et al.*, 1970; Hashiguchi *et al.*, 1972), a follow-up period for a few days after injection was not enough to study the suppressive effect on DEC. The dose regimen in the present study was a single injection of 200 mg/kg similar to the dosage used in the previous studies (Kobayashi *et al.*, 1969; Tanaka, 1965) because of simplicity in evaluating the suppressive effect.

With the administration of the sera from infected cotton rats (Kobayashi *et al.*, 1969) the density of inoculated mf dropped remarkably 1 hour after treatment with DEC but returned to the initial level sooner or later. The same change of mf density was observed in the infected cotton rats previously treated with prednisolone and injected with DEC. In these experiments, the final results of the suppressive effect had to be read 4 to 7 days after injection of DEC. In order to determine the suppressive effect of ALS on DEC, change of mf density was observed for 1 week and when mf density that was once reduced, went up higher than 30% of the initial density, the given pre-treatment was regarded as suppressive on the action of DEC.

To prove the immunosuppressive activity of ALS, the skin of clean cotton rat was transplanted to the test animals. The grafted skin survived without any rejection signs in the recipient animals while ALS was being given and also during the period of this experiment until 3 months after the termination of ALS treatment. It was thought, however, that in the present study, the skin grafts could not be a good indicator because the cotton rats used might have become unintentionally a nearly pure colony as a result of having been bred continuously for about 20 years in this laboratory and of having originated from a few litters.

Inoculated mf is a good material for experiments in studying the mode of action of DEC. Microfilaraemia could not be produced well by injecting mf into the tail vein of cotton rats but could be established by inoculating mf intraperitoneally. Using the second procedure of inoculating mf, Kobayashi *et al.*, (1969) tested the action of DEC 40 days after inoculation and DEC showed no action. After injecting serum from the infected cotton rats, the density of inoculated mf was reduced abruptly by DEC but was recovered gradually in a week. It was shown in that study that the presence of humoral antibody to filaria was not a satisfactory condition to make DEC effective and that observation for 1 week after injection of DEC was necessary to evaluate the suppressive action of pre-treatment.

In the preliminary studies by Tanaka *et al.*, (1970) and by Takaoka *et al.*, (1974), inoculation of 10^6 to 10^8 spleen cells from the infected cotton rat made DEC active on inoculated mf when it was given 2 days later. Sensitized spleen cells were found to be important components in the mechanism of action of DEC.

Microfilaraemia produced by adult *Litomosoides* implanted to the peritoneal cavity is also a useful material in the study of DEC. Immunological response in cotton rats after implantation of adult worms was studied by Fujita and Kobayashi (1969) and it was reported that the antibody became detec-

table by HA test 10 days after implantation. DEC was not active against the mf produced 5 days after implantation and became active only after 2 weeks or later corresponding to the immunologic response of the hosts. In view of the above results, DEC was examined 2 and 3 weeks after implantation of adult worms in the present study.

Suppressive effect on DEC was found in ATS as well as in ALS. In both experiments with ALS and ATS, DEC became inactive and the role of lymphocytes were found to be a necessary factor in the mode of action of DEC.

SUMMARY

Two cotton rats which were given anti-lymphocyte serum (ALS) 3 times a week were infected with *Litomosoides carinii*. In the test of diethylcarbamazine (DEC) at a single dose of 200 mg/kg, 10 weeks after infection, no microfilaricidal activity was observed. After this test, the injection of ALS was discontinued in a cotton rat and DEC was given again after 3 weeks, but still no effect was observed. Following the treatment with anti-thymocyte serum (ATS) in 4 cotton rats, adult *Litomosoides* were implanted into their peritoneal cavity and treatment with ATS was continued until the test of DEC or 1 week after the test. Action of DEC on microfilaria from the implanted worms was examined 2 or 3 weeks after implantation but DEC showed no activity either. After this experiment, although DEC was given again to the same animals which were continuously injected with ATS or together with sera from infected cotton rats, still no activity of DEC was observed. These results indicate that DEC was not effective when the host animals were treated with ALS or ATS irrespective of presence of humoral antibody against *Litomosoides* in the blood. It is considered that lymphocytes play an important role in the mode of action of DEC.

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