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Study on the Distribution of Microflariae in the Cotton Rat Infected with *Litomosoides carinii* after Treatment with DEC

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Abstract

The effect of DEC (diethylcarbamazine) on larval stage of filarial worm (*Litomosoides carinii*) was studied in detail by following up microfilaria density in peripheral blood of cotton rat (*Sigmodon hispidus*) after DEC treatment, and by comparing microfilaria density in blood with that in the lung, liver, spleen and kidney. In the first experiment, the fluctuation of microfilaria density in peripheral blood was observed by taking blood from tails, at 0 min, 30 min, 1 hr, 2 hrs, 4 hrs, 6 hrs, 8 hrs, 10 hrs, 12 hrs, 1 day, 3 days, and 7 days after DEC treatment. Microfilaria density showed rapid fall within 30 min after treatment, and then, increased slightly and fell down again between 4 hrs and 10 hrs. In the second experiment, the time of blood sampling was determined as 0 min, 30 min, 7 hrs, 10 hrs and 3 days after DEC treatment. Similar results to the first experiment in the time course of microfilaria density were observed again. While, it was observed that microfilaria density in peripheral blood showed 85.8% reduction on an average at 30 min after treatment, microfilariae accumulated in organs at that time, the relative microfilaria density in the liver, spleen, lung, and kidney being 77, 12, 9 and 8 times respectively in comparison with pretreatment level, followed by gradual decrease after that. However at 7 hrs after DEC treatment, the changes of microfilaria density in these four organs did not reveal clear coincidence with those in peripheral blood. From the results, it was proved that microfilariae accumulated mostly in the liver after DEC treatment, as has been suggested by many researchers and it was postulated that most of the disposal of microfilariae took place in this organ. But there is a remarkable difference between the number of microfilaria decreased in peripheral blood and that increased in the organs after DEC treatment.

Key words: **cotton rat,** *Litomosoides carinii,* DEC, filaricide, microfilaria distribution

Introduction

DEC (diethylcarbamazine) has been used as an antifilarial drug since the 1940s, and many examinations have been performed on the mode of action of the drug, the distribution of microfilaria after its administration and so on. The mechanisms of filaricidal effect of the drug is still largely unknown. But it has been shown by many researchers that microfilaria density in peripheral blood falls down abruptly forming bottom between 15 to 30 min after DEC administration (Zahner *et al.* 1978, Weiner *et al.* 1982, Tanaka *et al.* 1965, Mitsui *et al.* 1966).

DEC activity on microfilaria was observed in more detail, and it was found that microfilaria density increased temporarily at about 7 hrs after administration of DEC and then, decreased again. Moreover, microfilaria density in such organs as the liver, lung, spleen and kidney was examined following the time course. The contrast of microfilaria density in peripheral blood with that in organs made the understanding of DEC activity in more detail.

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Material & Method

[animals]

American cotton rats, *Sigmodon hispidus,* were used as host animals. They were infected with cotton rat filaria, *Litomosoides carinii,* by intrathoracic injection technique as described below.

A strain of cotton rat was imported into Japan and has been bred in the Institute of Medical Science, Tokyo University since 1954. After that they are now quite domesticated as laboratory animals. Their susceptibility to *L. carinii* infection is excellent, and seems to be higher than that of wild colonies.

Six and 25 cotton rats were used in the first and second experiment, respectively. The animals were reared on ordinary rat feeds and water.

[infection]

Cotton rats, at 6–10 weeks after birth were infected with *L. carinii* 7 day larvae by intrathoracic introduction. The infection method is as follows; first of all, tropical rat mites, *Ornithonyssus bacoti,* which have been kept in the special container devised by Oikawa *et al.* (1985), were fed on a cotton rat. The infected mites were kept in the container for 9 days at 28°C when infective larvae are established in those mites.

Then, two or three intact cotton rats were put into the container of infective mites, and exposed for 5 hrs. After that these cotton rats were transferred into individual cage and insecticide (pyrethroid) was applied on their skin so that the mites on the animals might be killed. They were kept for 7 days. On the seventh day after infection, they were anesthetized with chloroform and chest was opened, then chest cavity was washed with RPMI 1640. The washed out fluid contained several hundreds to thousands of 7 day larvae.

Under stereomicroscope, 7 day larvae were counted and a group of 20 larvae was put in each hole of 96 well microplate with capillary pipette. And cotton rats at 4 weeks after birth were infected with these larvae intrathoracically using the method of Tanaka *et al.* (1977). Cotton rats became microfilaremic after an incubation period of 6 weeks. The microfilaria density of peripheral blood in the rats was investigated 14 weeks after infection. Those cotton rats with microfilaria density at 200–500 per 1.0 μl were used for the experiment.

[preparation and staining of blood films]

Blood was taken from the tail vein using a filaria mélangeur, and a pair of blood films were prepared on a grass slide using 0.5 μl blood sample for each line.

Blood smears were dried overnight, dehemoglobinized by soaking in still water for a few minutes carefully, then dried and fixed in methanol. And, the stock solution of Giemsa was diluted with 20 to 25 times volume of phosphate buffer which was adjusted to pH 7.2. The slides were soaked in the diluted Giemsa's solution for about 30 min. After staining, the slides were rinsed gently in water, and dried in the air. The number of microfilaria was counted under light microscope (Sasa, 1976).

[DEC treatment]

The pure powder of DEC (diethylcarbamazine citrate) was provided from TANABE Pharmaceutical Company. Ten mg of DEC was dissolved in 1 ml of distilled water, and the solution was administered intraperitoneally at a single dose of 200 mg/kg body weight.

[preparation of tissue emulsion]

After each organ was cut using razor blade, each one block of them was weighed, put into petri dish which contained 5 ml of RPMI 1640. They were disintegrated as small as possible using tweezers and 22 G needle. The emulsion was filtered through a stainless net of 200 mesh to remove such comparatively larger parts as capsule, fibrous tissue and so on. The filtrate was put on a Improved Neubauer Hemacytometer (American Optical Corporation, Buffalo, N.Y. 14215 U.S.A.). And the number of microfilaria was counted under microscope.

[the first experiment]

Six infected cotton rats were used for the first experiments. DEC was administered intraperitoneally at dose of 200 mg/kg body weight. Blood samples were taken at 0 min (as control), 30 min, 1 hr, 2 hrs, 4 hrs, 6 hrs, 8 hrs, 10 hrs, 12 hrs, 1 day, 3 days, and 7 days after treatment as shown in Fig. 1.

[the second experiment]

Twenty five cotton rats were divided into 5 groups, each group containing 5 animals. They

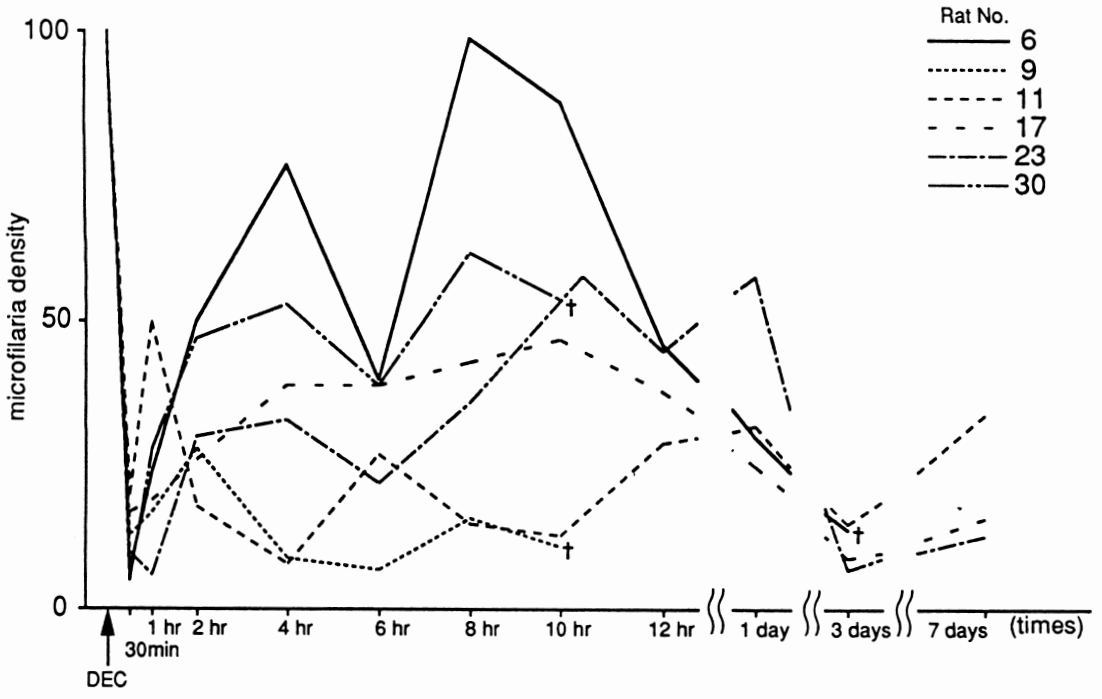

Fig. 1 Changes of microfilaria density after DEC treatment in peripheral blood

were administered with DEC intraperitoneally at a single dose of 200 mg/kg body weight 14 weeks after infection. Judging from the results obtained by the first experiment, blood samples were taken at 5 times; 0 min, 30 min, 7 hrs, 10 hrs, and 1 day after treatment. On the other hand, each time after blood taking, one group of the cotton rats was dissected under ether anesthesia and their lung, liver, spleen, and kidney were removed for preparation of tissue emulsion. The purpose of this experiment was to investigate the changes of microfilaria density in those organs and to compare the results with those in peripheral blood.

Results

1) Changes of microfilaria density in peripheral blood

In the first experiment, microfilaria density in peripheral blood showed a remarkable fall at 30 min after DEC treatment (Fig. 1). The values indicated 17.9–66.2 when original levels were expressed as 100. After that, microfilaria densities showed some fluctuation and it was noticed that most of them formed second peaks between 8 and 10 hrs.

In contrast, microfilaria density in peripheral blood became less fluctuant at 1 day, 3 days, 7 days after treatment. Especially after 3 days, density fell in the range at 8–15% of original level. After 7 days, it showed some scattering but there was no significant difference between the densities on day 3 and day 7.

In the second experiment, microfilaria density showed 85.5% reduction on an average at 30 min after treatment, and slight fluctuation was observed after that. The results were similar to those obtained in the first experiment.

2) The number of microfilaria in tissues (Fig. 2)

Before treatment, microfilaria density in the lung specimen was overwhelmingly high comparing with those in other organs. From 30 min though 7 hrs after treatment, it had a tendency to decrease in the lung, while in the liver, the density increased at 30 min and decreased after that.

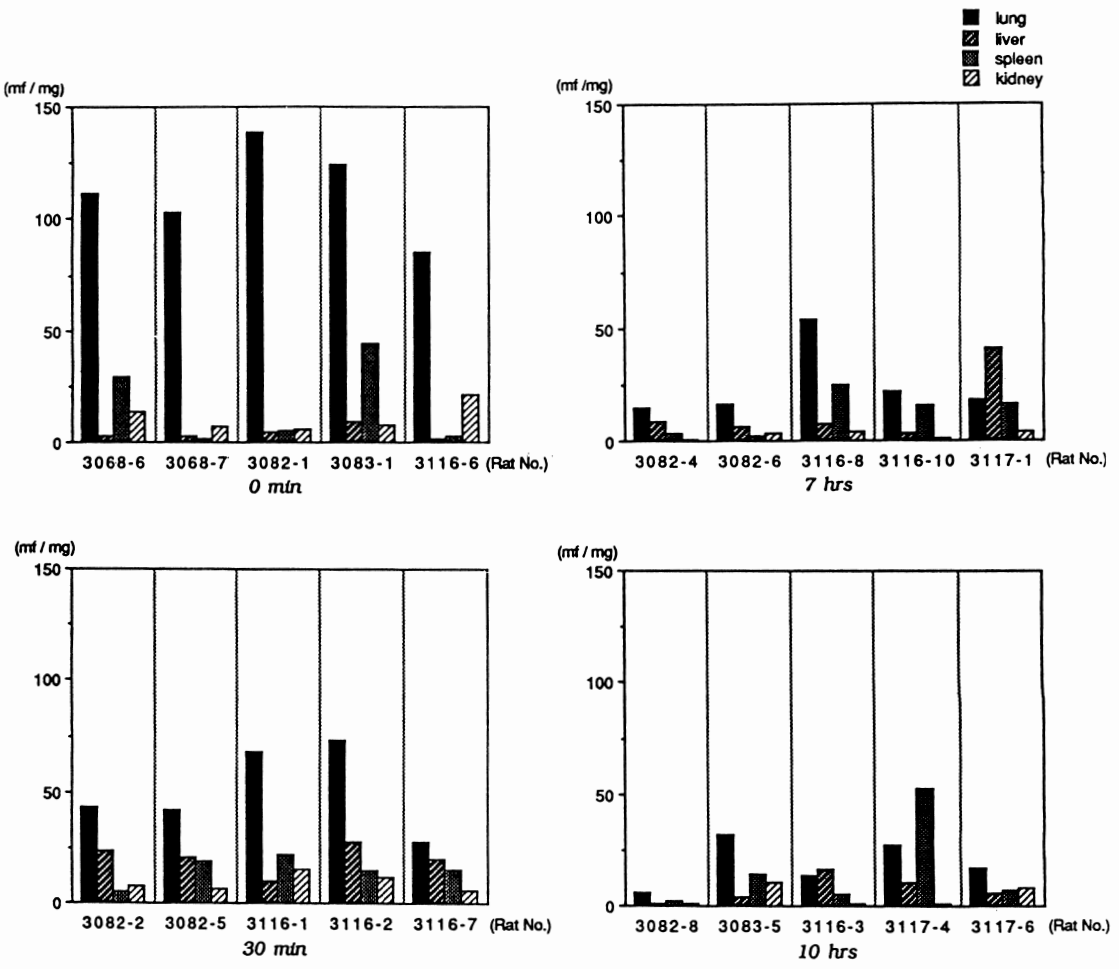

Fig. 2 Changes of microfilaria density after DEC treatment in organs

There was no difference of microfilaria density in the lung and liver specimens obtained at 7 and 10 hrs.

In the spleen, there was no clear change of microfilaria density all through the experiments. In the kidney, consistent reduction of microfilaria density was observed until 7 hrs after treatment. No microfilaria was detected in any tissue emulsion after 24 hrs by this method.

Table 1 summarized the average of the rate of motile microfilariae to the total number in those organs. Before treatment, the majority of microfilariae was observed to be moving, but the number of immotile microfilariae was increasing

Table 1. The rate of motile microfilariae to total number

time \ organ	lung	liver	spleen	kidney
0 min	96.0	100.0	100.0	96.0
30 min	63.3	76.0	33.3	100.0
7 hr	37.5	75.0	66.7	65.0
10 hr	100.0	75.0	75.0	100.0

at 30 min after treatment. At 7 hrs, the rate of immotile microfilariae to total number was similar to that observed at 30 min, but the movement of microfilariae was more sluggish. The rate of motile worms increased again at 10 hrs.

In the lung, the proportion of motile microfilariae decreased gradually by 7 hrs after treatment, and at 10 hrs all of them were observed to be moving again. In the liver, there was increase in the number of immotile microfilariae at 30 min and after that, it remained unchanged. Likewise the rate of immotile microfilariae was highest at 30 min in the spleen and thereafter it decreased gradually. In the kidney, some of the immotile microfilariae were observed at 7 hrs after treatment. But at the same time, it was noticed that the total number of microfilaria observed in the emulsion of kidney tissue was very small.

Discussion

In the present experiment, microfilaria density showed a rapid fall within 30 min after DEC treatment as has been reported by many researchers. While following microfilaria density after DEC treatment, it was noticed that the density in most experimental animals increased slightly within 4 to 10 hrs after DEC administration and decreased again.

There are many papers in which the changes of microfilaria density of *L. carinii* in peripheral blood after DEC treatment are described. However, there have been no papers which mentioned the fact that microfilaria density increased slightly within 4 to 10 hrs after DEC treatment. But the data presented in the following papers agreed with our findings. Tanaka *et al.* (1965), Mitsui *et al.* (1966), and Kobayashi *et al.* (1969), have described the changes of microfilaria density after single administration of DEC as was found in the present study.

In the case of Tanaka *et al.* (1965), the drug was given at 10, 30, 100, 300, 500 mg/kg body weight of infected cotton rats. In many cases, microfilaria density showed remarkable decrease between 10 to 30 min after treatment, then it increased slightly during 2 to 6 hrs and fell again after that. The data by Mitsui *et al.* (1966) which used 500 mg of DEC showed decrease of microfilaria density at 10 or 30 min after treatment, and it also showed slight increase between 4 and 6 hrs. The experiment of Kobayashi *et al.* (1969) which used 200 mg DEC, showed similar fluctuation of microfilaria density making a peak at 6 hrs. Thus, retrospective study on the data of many researchers revealed that most of the data which appeared in their reports coincide with that of the present study. The reason, that the transient increase of microfilaria density following steep decrease after DEC treatment has not been stressed by those researchers, might be due to the preconception that microfilaria density in peripheral blood simply decrease first and then increase after DEC treatment.

Further follow-up study of microfilaria density revealed that in the first experiment which used the animals at 9 months after infection, the increasing tendency from 10 hrs to 1 day was not clear, whereas, similar experiment using animals at 14 weeks after infection showed clear increase. The difference of the results between two experiments could be explained by the decrease of microfilaria production in the rats with old infection.

Weiner and Soulsby (1982) compared the microfilaria production after DEC treatment using *Mastomys natalensis* with new (at 10 days PI), and old infections (at 7 months PI), and microfilaremic animals without adult worms, prepared by intravenous injection of blood or culture-derived microfilariae. After single administration of DEC at 500 mg/kg per os, microfilaria density dropped abruptly in all treated groups within the first 15 min. Microfilaria density of animals with old infection remained low until day 12 after treatment when they began to rise, whereas that of animals with new infections began to rise immediately. Microfilaria density of animals without adult worms remained depressed for the duration of the experiment. And, they regard that the resurgence of microfilariae after treatment with DEC is the result of production of new microfilaria by female worms rather than release of trapped microfilaria by the host.

Based on their view, it could be mentioned that the delayed return of microfilaria in the old infection is caused by the lower production of microfilaria by the aged adult worms.

In the present paper, microfilaria density in internal organs of cotton rats was investigated for the purpose of clarifying the significance of fluctuation of microfilaria density in peripheral blood. Naturally, microfilaria density varies in each animal, and this made it impossible to compare the number of microfilariae in each organ of different animals directly. Therefore to make the comparison possible, the number of microfilaria of each organ was revised by dividing with total count of microfilariae in peripheral blood of the same animal, and the results and shown in Table 2.

From the table 2, the number of microfilariae seen in the lung specimen is much larger comparing with other organs before DEC treatment. It could be considered that since adult worms of *L. carinii* reside in the chest cavity in cotton rats and produce many microfilariae, most of them are inclined to enter into the lung tissues or vessels and to stay there rather than in other organs. However, even in the case of *Wuchereria bancrofti,* whose adult worms live in the lymphatic vessels or glands, there are many microfilariae in the lung (Hawking, 1960). In the light of this facts, the mere presence of adult worms in the thoracic cavity would not justify the presence of more microfilariae in the lung.

While, it would not be able to attribute the accumulation of microfilaria in the lung to the blood volume in the organ. Because in spite of the presence of more volume of blood in the spleen than in the lung, the number of microfilaria in the former was less than that in the latter. Thus, as Hawking (1960) described, the reason for dense microfilarial concentrations in the lung is less clear. He wrote that lungs are the most favorable site in the body for the microfilariae, perhaps because of the high oxygen tension etc.

The rate of the number of microfilaria in all these organs against the number of microfilaria of total blood increased remarkably at 30 min after treatment, the increase in the liver being the highest among them. The rate in the liver, spleen, lung, and kidney increased up to 77, 12, 9, and 8 times of pretreatment level, respectively. At this stage, the decrease of total number of microfilaria in blood was striking. These two facts, comparative increase in number of microfilaria in the organs, especially in the liver, and their decrease in peripheral blood, seem to be suggesting the role of the liver as principal organ to manage the shift of microfilariae. The results obtained in the present study are similar to the results by Hawking (1950, 1960), Mitsui (1966) and Tasaka (1965). But, a clear explanation about the cause of microfilaria accumulation in the liver has not yet been made.

However, discrepancy was noticed between the total number of microfilariae in the whole blood and total number of microfilaria in the four organs. That is to say, the number of microfilaria which disappeared from total blood

Table 2. Changes of number of microfilariae in each organ revised by total counts of microfilariae in peripheral blood

time	number of microfilariae in total blood* ($\times 10^6$)	$\frac{\text{number of microfilariae in each organ}}{\text{number of microfilariae in total blood}} \times 10^2$			
		lung	liver	spleen	kidney
0 min	2.35	3.31	1.15	0.31	0.74
30 min	0.14 (1.70)‡	29.20	88.38	3.70	5.66
7 hr	0.65 (2.74)‡	19.21	29.39	4.68	1.81
10 hr	0.14 (2.09)‡	8.59	20.11	5.08	4.26

* Total blood volume was calculated as 7.4% of body weight.
† All values are average from five animals.
‡ Values at pretreatment.

can be calculated as 1.56×10^6 at 30 min after DEC treatment. The total number of microfilaria detected in the four organs was 1.78×10^5 at the same time. That means, only one ninth of the microfilariae disappeared from the peripheral blood was detected in these organs. It is certain that removed and disposal of microfilariae in peripheral blood occur during the 30 min after treatment, but it is hard to consider that such a greater number of microfilaria could be removed only by these organs within this short period of 30 min. Therefore, the possibility still remains at present that respectable number of microfilaria is located outside of above mentioned four organs.

At 7 hrs, the number of microfilaria in total blood showed increase again. On the contrary, the number of microfilariae in the lung, liver, and kidney decreased up to approximately one-half to one-third of that at 30 min. For dynamics of DEC, Sakuma *et al.* (1967) have observed the time course of distribution of tritium labeled DEC in rats or cotton rats after intraperitoneal injection using whole body autoradiography. After the intraperitoneal injection, the radioactivity in most organs reached to the highest after about 20 min, and the activity decreased rather rapidly after 3 hrs.

Weiner *et al.* (1986) cultivated microfilariae and peritoneal exudate cells (PE cells) with various combinations of DEC, normal serum, serum obtained from DEC treated animals (DEC serum), and RPMI 1640. They observed trapping of microfilariae by PE cells with the coexistence of DEC serum. From their observations of *in vitro* experiments, they assumed that the total *in vivo* effect on microfilaria entails two parts, trapping and killing.

The adhesion of PE cells were regarded as the first step, and if the required components for killing were not present in the peritoneal exudate preparation, only trapping would normally take place, thus the cells began to come off and release microfilariae. Hawking had already suggested the existence of the two steps in 1950. Killing component has not been described in detail by Weiner *et al.* (1986). But Zahner *et al.* (1976, 1983) also reported that, DEC-mediated cell (polymorphonuclear cells) adherence is an important step in microfilaricidal effects of the drug leading to disintegration and finally elimination of the larvae.

In view of the results of these experiments, it could be considered that there is no effect of DEC at 7 hrs, as the drug is already metabolized or excreted though not completely. And, it could be possible that temporary increase of microfilaria density in peripheral blood might be caused by the releasing of microfilaria after the effect of DEC is lost.

A decreasing tendency was seen in the lung and liver at 10 hrs after DEC administration. However the number of microfilariae of the spleen showed less change. The reason for this finding in the spleen is not clear at present, but it may reflect the function of this organ to store ingredients of blood. In the kidney the number of microfilaria seems to be increased, but it is difficult to make any conclusion as the values in the kidney were too low to be compared.

The role of internal organs to manage microfilariae after treatment by DEC has been described in many papers. Hawking (1950) and Mitsui *et al.* (1966) have reported that initial effect of DEC treatment is to induce an accumulation of microfilaria in various organs, especially in the liver. Kobayashi *et al.* (1978) observed that microfilariae are found to be trapped by the fixed macrophages of the sinusoid in the liver. On the contrary, Zahner *et al.* (1978) reported that no major accumulation of microfilariae occurred in the inner organs in their study. However, it is clear from the present study that microfilariae are accumulated at least in the lung and liver after DEC treatment.

In the emulsion method to count microfilariae in the organs, the motile or immotile microfilariae were investigated. It was assumed beforehands that only immotile microfilariae would be observed after DEC administration. Actually, many immotile microfilariae were observed at 30 min after treatment, however, motile microfilariae were observed again at 10 hrs in the lung. In the spleen, motile microfilariae also decreased at 30 min after treatment, but they increased again after that.

From these experiments, more detailed changes of microfilaria density in peripheral blood after DEC administration was elucidated. And, it was confirmed again that microfilariae accumulated mostly in the liver after treatment, as has been postulated by other researchers. But there is a remarkable difference between the number of microfilaria vanished from peripheral blood and that accumulated in the organs after treatment.

Based on these results, the elucidation of the distribution of microfilaria in the whole body of experimental animals after treatment, and of the process in disposal by macrophage etc., could be suggested as the subject of study in future.

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