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A Reduced Microfilaraemia in Rats Sensitized to *Dirofilaria repens* Antigens

by

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INTRODUCTION

Previous studies (Ismail, Wijayaratnam and Amarasinghe, 1971) have shown that a moderate eosinophilia associated with a pronounced microfilaraemia occurs when live adult worms of *Dirofilaria repens* are transplanted intraperitoneally into rats. In the tropical pulmonary eosinophilia syndrome, although the majority of cases appear to be of filarial origin (Van Der Sar and Hartz, 1945 ; Buckley, 1958 ; Danaraj, Da Silva and Schacher, 1959), microfilariae are remarkably absent in the peripheral blood. The same is true in most cases of classical filariasis. The mechanisms responsible for the absence or suppression of a microfilaraemia in these syndromes are not known but a generally accepted view is that microfilariae, if produced, are trapped in the tissues and do not enter the blood. This paper deals with attempts to investigate this phenomenon by studying the eosinophilia, microfilaraemia and tissue response following transplantation of adult *D. repens* into rats previously sensitized to the same species.

MATERIALS AND METHODS

Two-month old female albino rats were used in all experiments. Live adult *D. repens* were obtained from the subcutaneous tissues of infected dogs. In the preparation of extracts the worms were lyophilized and a 3% extract made in ice-cold phosphate buffered saline, pH 7.2. The extract was mixed with an equal volume of incomplete Freund's adjuvant and used for inoculation.

Sensitization. Twentysix rats were divided equally at random into an experimental group and a control group. Each experimental animal was given 0.6 ml of worm extract in adjuvant into the foot pads and the dorsum of the neck. Each control animal was given 0.6 ml of adjuvant without the extract. A total of 5 inoculations was given to each animal at approximately fortnightly intervals. Although no tests were done to demonstrate sensitization, the signs observed in the experimental group—such as immediate erythema and oedema of the foot pads, ruffled appearance of the fur and rubbing of the face with the fore-limbs—after the 2nd or 3rd inoculation indicated that these animals were in fact sensitized to *D. repens*. These signs were not observed in any of the controls.

Transplantation of adult worms. Live female worms were transplanted intraperitoneally into all rats (both experimental and control) within a week after the final inoculation. The rats were grouped into pairs—an experimental (sensitized) and a control (non-sensitized) constituted a pair. Each member of a pair of rats received 4 female worms from the same infected dog so that the results would be comparable.

Measurement of eosinophilia, microfilaraemia and antibody levels. Absolute eosinophil counts, microfilarial counts, serological tests (complement-fixation and agar diffusion) and statistical analyses were carried out as previously described (Ismail *et. al.*, 1971) on 7 pairs of rats.

Peritoneal lavage. Rats were anaesthetized with ether and 10 ml of sterile 0.85% saline was injected intraperitoneally using a 10 ml glass syringe fitted with a 15-gauge needle. With the syringe in place the abdomen was massaged for 1 minute and the barrel of the syringe was detached from the needle. The outflow from the peritoneal cavity was then collected in a clean graduated centrifuge tube and the volume noted. In most instances the recovery was over 7.0 ml. To determine the number of microfilariae in the washings, the fluid in the centrifuge tube was well mixed and 0.05 ml was withdrawn, spread on a microscope slide, and the number of microfilariae counted. The total number of microfilariae recovered from the peritoneal cavity was then calculated. From an average of 3 determinations the total number of microfilariae in 10 ml (volume of saline injected into peritoneal cavity) was calculated. This was taken as an estimate of the number of microfilariae in the peritoneal cavity. Six pairs of rats were subjected to this procedure. The peritoneal cavity was lavaged weekly and the animals killed (in pairs) sometime between the 2nd and 8th week. The rats were then dissected and live worms, if any, were recovered.

Histological examination. At dissection portions of the liver, lung, spleen and lymph nodes were removed, fixed in 10% formol saline and embedded in paraffin wax. The tissues were sectioned at 10 μm thickness. Sections were stained in haematoxylin and eosin and examined for evidence of parasitic material and any host reaction. Tissues from 4 pairs of rats killed at 4, 5, 8 and 12 weeks respectively were processed. About 50 to 75 sections from each tissue were examined.

RESULTS

The eosinophil response and the level of microfilaraemia in 7 experimental and 7 control animals are summarized in Tables 1 and 2 respectively. There was a moderate increase in the number of circulating eosinophils in both groups but the response was not significantly different ($P>.05$) in the 2 groups. A single booster dose of worm extract given to the experimental rats at the end of the 4-month period failed to enhance this response. The sensitized rats, however, showed a marked decrease in the microfilaraemia (Table 3). In 5 of the 7 rats in this group the peak microfilarial response was between 6-28 microfilariae per 0.05 ml. In the other 2 rats there was a slight increase in the number of microfilariae towards the latter part of the experimental period and a peak response of 63 and 77 microfilariae per 0.05 ml blood respectively was encountered. In the control group a heavy microfilaraemia with a peak response ranging from 56 to 280 microfilariae per 0.05 ml was

TABLE 1.

Eosinophilia and microfilaraemia (mean values) in sensitized rats after introduction of *Dirofilaria repens* females intraperitoneally.

DAYS	EXPERIMENTAL GROUP (7 Rats) (sensitized to *Dirofilaria repens*)					
	Eosinophils/cu.mm			*Microfilariae/0.05 ml.*		
	Mean (SE)	95 % confidence intervals min.	max.	Mean (SE)	95 % confidence intervals min.	max.
Basal						
i	106 (15)	76	142			
ii	149 (9)	136	162			
Post-transp.						
5	180 (11)	154	206	< 1		
10	162 (24)	105	219	1 (0.3)	0	2
15	235 (32)	158	312	3 (0.7)	1	5
21	454 (103)	207	701	3 (1.1)	0	6
28	383 (117)	102	644	4 (1.8)	0	8
35	668 (175)	248	1088	8 (2.6)	2	14
42	439 (70)	271	607	7 (2.5)	1	13
49	616 (60)	452	760	12 (3.2)	4	20
60	504 (79)	315	693	24 (8.1)	5	43
84	571 (104)	322	820	15 (3.9)	6	24
96	444 (104)	195	693	14 (4.3)	4	24
124	423 (68)	260	586	9 (2.7)	2	16
*						
140	379 (183)	—	818	16 (7.9)	—	35
145	562 (47)	449	675	17 (3.4)	9	25
152	543 (153)	175	910	10 (3.7)	1	19
166	434 (87)	225	643	12 (4.2)	2	22

* Single booster dose of 0.6 ml *Dirofilaria* extract in adjuvant given to all rats during 125-135th day.

seen in 6 of the 7 rats. The microfilarial response in 1 of the controls was poor with a peak of 19 microfilariae per 0.05 ml. A plot of the mean microfilarial counts with their 95% confidence intervals indicated that the number of microfilariae in the peripheral blood was significantly lowered in the sensitized group from the 35th day up to the 124th day following transplantation (Fig. 1). After the 140th day the number of microfilariae in the blood of sensitized rats continued to be less than in the controls; the mean microfilarial count however, was significantly lower ($P<.05$) in the sensitized group only on 2 of the 4 days on which blood was examined.

A record of the number of microfilariae recovered from 6 pairs of rats by weekly peritoneal lavage and the number of adult worms recovered from them at dissection is indicated in Table 4. One pair of rats was examined for the first time and killed at 8 weeks; only visual estimates of the number of microfilariae recovered from the peritoneal cavity were made in this case. Less microfilariae were apparently produced in the sensitized rats but the results were rather inconsistent. Adult worms, as expected, survived longer in the non-sensitized rats. Live worms were recovered from 5 of the 6 non-sensitized rats examined. We have, in fact, maintained adult worms in the peritoneal cavity of normal rats for as long as 19 months. In the sensitized group no live adult worms were recovered from rats examined after the 4th week of transplantation.

TABLE 2.

Eosinophilia and microfilaraemia (mean values) in non-sensitized rats after introduction of *Dirofilaria repens* females intraperitoneally.

DAYS	CONTROL GROUP (7 Rats) (non-sensitized)					
	Eosinophils/cu.mm			*Microfilariae/0.05 ml.*		
	Mean (SE)	95 % confidence. intervals min.	max.	Mean (SE)	95 % confidence. intervals min.	max.
Basal						
i	72 (22)	19	125			
ii	90 (9)	69	111			
Post-transp.						
5	129 (14)	95	163	< 1		
10	251 (74)	74	428	3 (1.3)	0	6
15	348 (128)	41	655	4 (1.4)	1	7
21	439 (98)	204	674	17 (7.0)	1	33
28	406 (112)	137	675	34 (16.1)	0	68
35	529 (83)	330	728	46 (10.7)	21	71
42	645 (90)	429	861	64 (16.5)	25	103
49	491 (99)	253	729	72 (20.8)	23	122
60	431 (93)	208	654	87 (16.5)	47	127
84	506 (52)	381	631	121 (31.1)	47	195
96	449 (66)	291	607	69 (17.3)	28	110
124	441 (87)	232	650	38 (8.4)	18	58
*						
140	475 (53)	348	602	34 (8.7)	13	55
145	463 (97)	229	697	44 (6.3)	29	59
152	317 (73)	142	492	35 (13.9)	2	68
166	367 (75)	187	547	46 (3.9)	35	54

* Single booster dose of 0.6 ml adjuvant only given to all rats during 125-135th day.

TABLE 3.

Microfilaraemia in sensitized and non-sensitized rats during the 35th to 166th day after introduction of *Dirofilaria repens* females intraperitoneally

RATS Pair No.	EXPERIMENTAL (sensitized to *D.repens*) *Microfilariae/0.05 ml.*		CONTROL (non-sensitized) *Microfilariae/0.05 ml.*	
	Mean	Range	Mean	Range
1	1,8	0—6	58,7	15—123
2	13,2	4—27	5,8	2—13
3	26,1	4—63	114,7	33—280
4	5,6	0—12	108,8	49—271
5	27,0	2—61	46,6	6—133
6	4,8	0—8	42,8	4—108
7	11,1	0—28	33,7	13—56

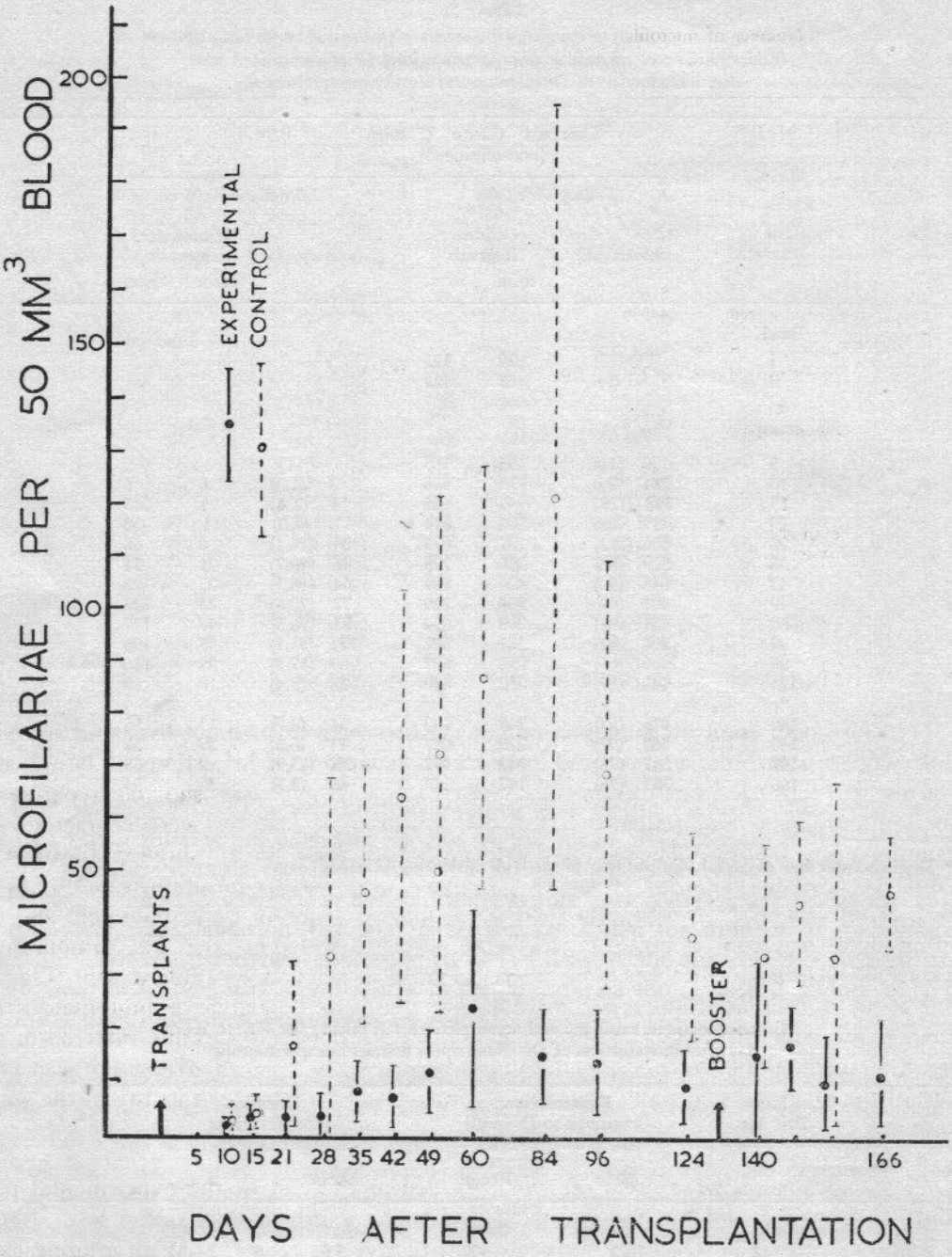

FIG. 1.

Microfilaraemia in sensitized and non-sensitized rats—mean counts and their 95% confidence intervals

TABLE 4.

Number of microfilariae recovered by weekly peritoneal lavage and number of adults recovered at time of killing.*

RATS	HOST STATUS	TIME IN WEEKS							
		1	2	3	4	5	6	7	8
Pair No. 8	Sensitized	129900	45450	15250	1660	650 (0)†			
	Non-sensitized	56970	32220	28630	11230	11590 (2)			
9	Sensitized	81660	23060	5230	1100 (1)				
	Non-sensitized	44570	7930	900	840 (0)				
10	Sensitized	154810	57590	92570	12790 (1)				
	Non-sensitized	85270	81050	163880	116330 (3)				
11	Sensitized	13940	0	0 (0)					
	Non-sensitized	73096	37600	800 (2)					
12	Sensitized	13330	1240	2880	0	0	0 (0)		
	Non-sensitized	289840	131460	144520	78640	112300	49010 (2)		
13	Sensitized								0 (0) ‡
	Non-sensitized	—	—	—	—	—	—		+++ (2)

* Number of adult worms transplanted — 4 females/rat

† Number of live adults recovered by dissection in parenthesis

‡ Only visual estimate of number of microfilariae recorded.

No histopathological changes were observed in sections of lung, liver and spleen but adult worms, microfilariae and host tissue reaction were seen in sections of lymph nodes in 2 of the 4 sensitized rats examined. The enlarged lymph nodes showed hyperplasia of the lymph follicles and reticular cells. The histological features were characterised by granulomatous lesions. The cellular infiltration in these areas showed lymphocytes, plasma cells, eosinophils, macrophages and many large foamy multinucleated giant cells (Figs. 2, 3). The giant cells contained 10-20 nuclei. Among these giant cells and often within them was seen as amorphous eosinophilic material (Fig. 4). The granulomatous lesions and cellular infiltration were observed both in relation to adult worms (Fig. 5) as well as to microfilariae (Fig. 6). In addition there were satellite granulomatous areas showing the same histological features without any evidence of parasitic material in them (Fig. 7). In the non-sensitized rats, although fragments of microfilariae were seen in sections of the lung and spleen, no abnormal cellular reactions were seen in any of the sections examined.

Serological tests were done on sera collected at the end of the experimental period from 12 sensitized and 12 non-sensitized rats. The complement-fixation test, using an alcoholic extract of *D. repens* as antigen, was negative for all sera. With a saline extract of the worm as antigen, precipitin bands were seen in agar diffusion plates with sera from all the sensitized rats and 10 of the 12 controls. No attempt was made to titrate the sera but with undiluted sera stronger reactions were obtained in the sensitized group.

DISCUSSION

These experiments have shown that when *D. repens* females are transplanted intraperitoneally into rats the degree of microfilaraemia is considerably lower in animals previously sensitized to *Dirofilaria* antigens than in non-senzitised controls. In 2 of 7 sensitized rats the number of microfilariae in the blood showed a slight increase during the latter part of the experimental period. The reason for this is not clear ; a decrease in circulating antibody may have been a contributory factor or, more probably, the 2 rats may not have been adequately sensitized. Although an increase in the eosinophil response in the sensitized animals was expected, there was no significant difference in the eosinophil response in the experimental and control groups.

There are 4 possibilities which could account for the reduced microfilaraemia. The adult worms may have undergone early death. Jordan (1955) suggested that the absence of microfilariae in the blood in filariasis is due to death or sterilization of the worms. This would account for the present findings except in the 2 instances where there was an increase in the number of microfilariae in the blood during the latter part of the experimental period. The second possibility is that the production of microfilariae has been suppressed and the third is that the microfilariae produced are rapidly cleared from the blood. There is some evidence to support the second and third possibilities. Wong (1964) found that when serum from a dog immunized against microfilariae was added to the culture medium used to maintain adult *Dirofilaria immitis* worms *in vitro*, the number of microfilariae produced was markedly reduced. When female worms whose fecundity had been interrupted by several days of incubation in immune serum were transferred to media containing no serum, they resumed production of microfilariae. She also showed that living microfilariae when repeatedly injected into dogs elicited an immune response which was demonstrated *in vivo* by the rapid removal of microfilariae from the peripheral blood. The fourth possibility is that the microfilariae are trapped in the tissues and do not enter the blood. In *Litomosoides carinii* infections in the cotton rat, Kershaw (1949) believed that a state of partial immunity develops against the worm which would account for the absence of microfilariae in the blood at a time when they were present in the pleural cavity. Ramakrishnan, Dalip Singh and Krishnaswami (1962) suggested that humoral antibodies destroy and prevent microfilariae from reaching the blood during the latent phase of *L. carinii* infections. In the present experiments circulating antibodies were detected in both the sensitized and non-sensitized groups. The sera, however, were not titrated but stronger precipitin bands were obtained in agar diffusion in the sensitized group. Whether circulating antibodies actually contributed to a lowering of microfilaraemia is difficult to say. Bagai and Subrahmanyam (1968) in their studies on the mechanism of acquired resistance in rats to *L. carinii* infections concluded that humoral antibodies play no part in reducing the number of microfilariae in the blood during the latent phase. Their results suggested, however, that a local tissue response, probably immunologic in nature, develops in the thorax which prevents the microfilariae from leaving this site and entering the circulation. In occult filarial syndromes the explanation for the absence of microfilariae in the blood may be one of the above 4 possibilities or a 5th alternative (which is outside the scope of this communication) where the infective filarial larvae concerned do not develop into sexually mature adults and microfilariae are therefore not produced.

It was in an attempt to determine the exact cause that peritoneal lavage, subsequent dissection of the animals and histological studies were carried out. Examination of the peritoneal washings indicated that although less microfilariae were apparently produced in the sensitized rats the results were rather inconsistent ; in 2 of the non-sensitized animals the number of microfilariae recovered decreased rapidly after the 2nd week. In one of these the 2 worms recovered on subsequent dissection were found outside the peritoneal cavity probably having made their exit from the peritoneal cavity through needle punctures made during peritoneal lavage. While this would account for the reduced number of microfilariae recovered from the 2 non-sensitized rats referred to above, it does not exclude the possibility that a similar migration of adult worms had not occurred in some of the sensitized animals. One cannot therefore say with certainty, on the basis of these findings, that production of microfilariae in the sensitised group was suppressed. Further experiments are undoubtedly necessary to establish this. Adult worms survived for a shorter period in the sensitized rats and this may to some extent explain the reduced microfilaraemia seen in this group.

The histopathological changes observed in these studies are very similar to those described in the lung and other organs in cases of tropical eosinophilia (Viswanathan, 1947 ; Danaraj, 1959 ; Danaraj, Pacheco, Shanmugaratnam and Beaver, 1966) and in other forms of occult filariasis (Pacheco and Schofield, 1968). Fragments of microfilariae have been demonstrated in these lesions in a few cases only. Recently Joshi, Udwadia and Gadgil (1969) examined lung biopsies in 26 cases of tropical eosinophilia and demonstrated a microfilaria in only one of them. The histological changes described are by no means peculiar to filarial infections; they have been seen, for instance, in experimental toxocariasis (Chaudhuri and Saha, 1959). But the presence of such changes, even in the absence of parasites, should strongly suggest a parasitic aetiology. The presence of satellite granulomatous foci suggests that the parasites need not be present in the lesions. What probably happens is that parasitic material such as the secretory or excretory products of the adult worms and microfilariae are carried by macrophages to other tissues and organs and induce the formation of focal granulomata at the new sites. Danaraj *et al.* (1966) found degenerating microfilariae in some cases of tropical pulmonary eosinophilia. On the basis of this finding these workers assumed that adult worms were present and produced microfilariae which on reaching the lung were destroyed in an intense tissue reaction. They also suggested that the host response did not have a marked effect on the adult worms which continued to produce embryos. The present results indicate very strongly that the host tissue reaction definitely contributes to the reduced microfilaraemia seen in these animals. The immune reaction is apparently directed against both the adult worms and the microfilariae. None of the histological sections examined from the non-sensitized group showed such cellular reactions.

Although it is generally accepted that tropical pulmonary eosinophilia has a filarial actiology, the species of filarial worms involved in natural infections has not been established. The clinical syndrome has been induced in volunteers by the inoculation of both animal and human filarial species (Buckley, 1958 ; Edeson, Wilson, Wharton and Laing, 1960 ; Buckley and Wharton, 1961). As parasitism involves a host as well as a parasite, Edeson

et al. (1960) suggested that the end result of any infection may be determined more by the host's response than by the strain of parasite. According to Lie and Sandosham (1968) the problem of human or animal origin of the parasites is no longer crucial in the aetiology of occult filariasis. They suggested that any person hypersensitive to a specific filarial antigen, no matter whether the parasite is of animal or human origin, can develop symptoms of occult filariasis. Our studies in which the characteristic histopathological changes have been observed to occur only in previously sensitized animals strengthens the view that it is the immune status of the host rather than the invasiveness of the parasite that determines the outcome of the interaction between the filarial parasite and the host. On the basis of this premise one may explain the probable pathogenesis of filarial infections in man as follows. In a non-sensitized host the parasite is well adapted to the host and microfilariae are produced which freely circulate in the blood. In sensitized persons the resulting host reaction may be directed towards interference with the maturation of infective larvae, suppression of producton of microfilariae or trapping of microfilariae in organs such as the lung, spleen, lymph nodes etc. all of which would contribute to a marked reduction or absence of microfilariae in the peripheral blood. Symptoms of classical filariasis or hypersensitive syndromes such as tropical pulmonary eosinophilia may be produced in such persons depending on the location of the parasite and the state of sensitization of the host.

SUMMARY

The mechanisms responsible for the absence or suppression of microfilaraemia in many cases of classical filariasis and in occult filarial syndromes are not definitely known. The present paper deals with attempts to investigate this phenomenon by studying the eosinophil, microfilarial and tissue responses following transplantation of adult *D. repens* intraperitoneally into rats previously sensitized to the same species of parasite. Although there was an eosinophilia in both sensitized and non-sensitized animals there was no significant difference in the response in the 2 groups. The degree of microfilaraemia in the the sensitized group was significantly lower than in the non-sensitized group. It could not be established by peritoneal lavage that there was a suppression of production of microfilariae in the sensitized group but adult worms survived for a shorter period in this group. Histopathological changes similar to those described in occult filarial syndromes were observed in lymph nodes of 2 of 4 sensitized rats examined. The histological features were characterised by granulomatous lesions in relation to both adult worms as well as microfilariae. No abnormal cellular reactions were observed in the non-sensitized rats. The results indicate strongly that the host-tissue reaction definitely contributes to the reduced microfilaraemia seen. These findings strengthen the view that it is the immune status of the host rather than the invasiveness of the parasite that determines the outcome of the interaction between the filarial parasite and the host.

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EXPLANATION OF PLATES

All plates represent photomicrographs of sections of lymph nodes taken from 2 rats 6 and 8 weeks respectively after transplantation of *D. repens* adults intraperitoneally.

PLATE I

FIG. 2. Cellular infiltration consisting of lymphocytes, plasma cells eosinophils, histiocytes and multinucleated giant cells. (H & E ×1000.)

Fig. 3. Close-up of foamy giant cells. (H & E, ×1000.)

Fig. 4. Large multinucleated giant cells showing amorphous eosinophilic material. (H & E ×1000.)

Fig. 5. Cellular reaction in relation to adult worms. (H & E, ×50.)

PLATE II

Fig. 6. Granulomatous lesions in relation to microfilariae. (H & E, ×1000.)

Fig. 7. A satellite granulomatous focus. No evidence of parasitic material. (H & E, ×170.)

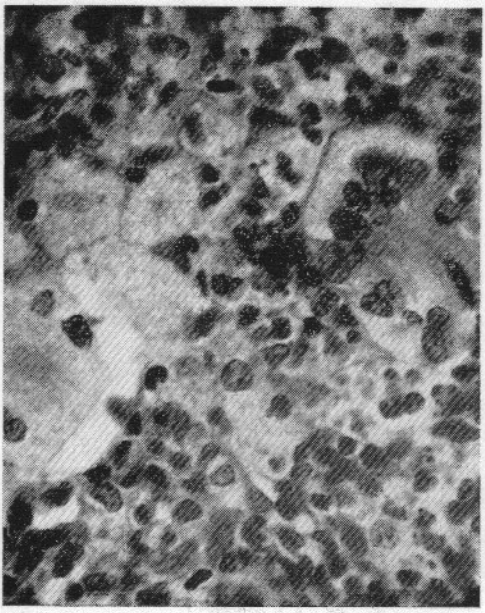

FIG. 2.

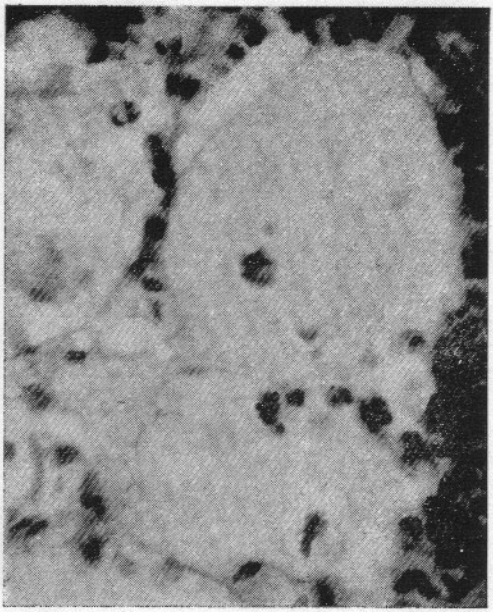

FIG. 3.

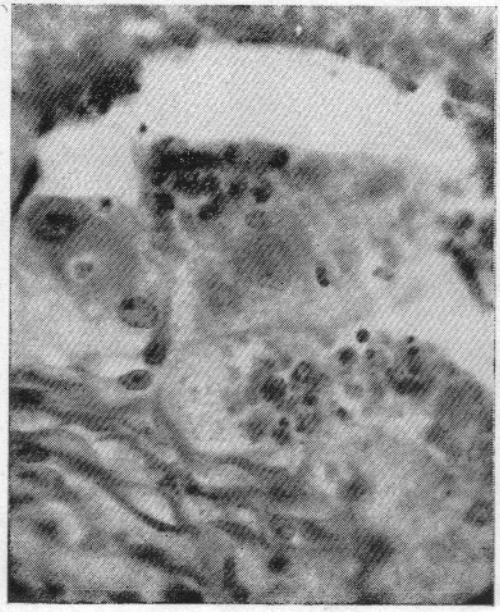

FIG. 4.

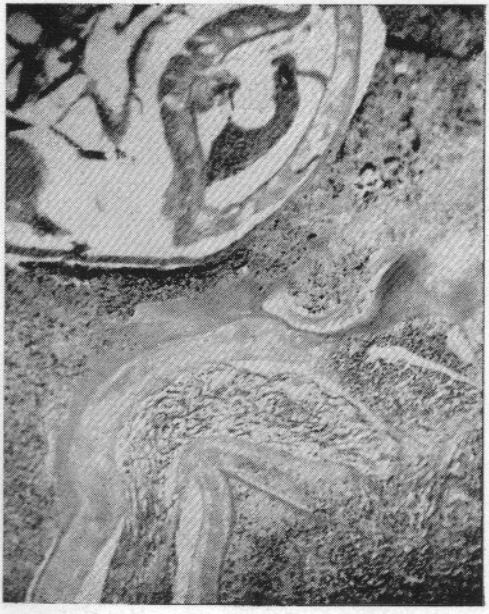

FIG. 5.

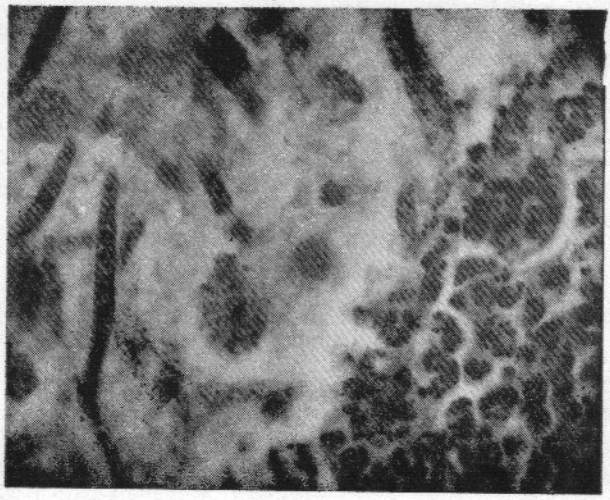

FIG. 6

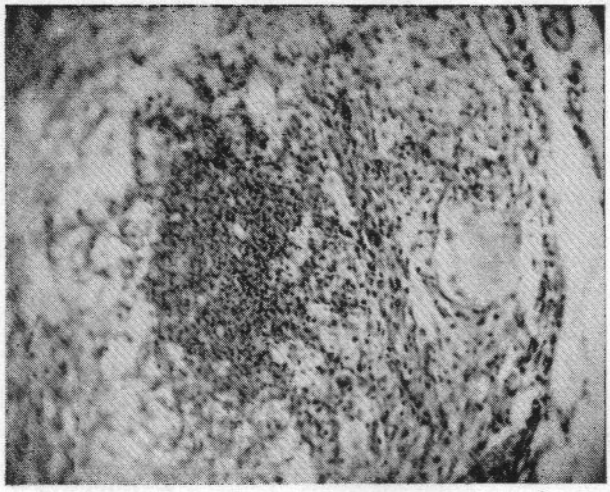

FIG. 7