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The Eosinophil Response in Rats to Extracts, Larvae and Adult Worms of *Dirofilaria repens**

by

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Although filarial worms have been incriminated as the most likely causative agents of tropical pulmonary eosinophilia, the species responsible has not been established. Many investigators have suggested that filarial worms of animal origin are responsible (Danaraj, Da Silva and Schacher, 1959; Webb, Job and Gault, 1960 ; Donohugh, 1963) but the induction of symptoms of tropical pulmonary eosinophilia in volunteers with filarial infective larvae of both animal and human origin (Buckley, 1958; Buckley and Wharton, 1961) and the detection of antibodies against microfilariae of *Wuchereria bancrofti* in some cases of tropical pulmonary eosinophilia (Jayewardene and Wijayaratnam, 1968) suggest that both animal and human filarial species may be involved.

Dirofilaria repens is a common filarial parasite of dogs in Ceylon; of 547 stray dogs in the city of Colombo and its suburbs examined by us during the course of this study, 116 (21.2%) harboured this worm. In 1961 Dissanaike estimated the infection rate in the Dehiwela-Mt.Lavinia region to be about 60% while an incidence of 30% was reported by Crawford in 1934 (quoted by Dissanaike, 1971). Human infections with *Dirofilaria* species have been reviewed by Faust (1957), O'Grady, Fawcett and Buckley (1962) and Beaver and Orihel (1965). More recently 2 cases of human infection with *Dirofilaria tenuis* were reported by Jung and Espenan (1967) and Pacheco and Schofield (1968) in which the adult female worms recovered contained microfilariae. Dissanaike (1971) reports 7 cases of *D.repens* infection from man in Ceylon and suggests that in endemic areas of Bancroftian filariasis a number of these cases may be missed because the clinical manifestations may be recorded as having been produced by *W.bancrofti*.

It was of interest to examine the reaction to *D.repens* in an abnormal host. In this study adult worms, infective larvae and extracts of this parasite were investigated for their ability to induce an eosinophilia in rats.

MATERIALS AND METHODS

Two-month old female albino rats were used in all experiments. Adult worms were dissected out from the subcutaneous tissues of infected dogs and either kept in sterile 0.85% saline until required for transplantation or stored in the deep freeze (-17°C) to be used later

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for preparation of extract. In the transplantation experiments rats were anaesthetized with ether and live adult worms were introduced either subcutaneously or intraperitoneally within 2 hours of collection. Abdominal incisions were also made in the controls but no worms were transplanted.

In the preparation of extracts of *D. repens* the worms were lyophilized, weighed, ground with fine sand (BDH) and extracted with ice-cold phosphate buffered saline, pH 7.2 (PBS). Ten ml of PBS was added to every 0.3 gm. of lyophilized worms. The mixture was centrifuged in the cold (8000 rpm at −4 °C) for 20 minutes. The supernatant was mixed with an equal volume of incomplete Freund's adjuvant and used for animal inoculation after the addition of a mixture of penicillin and streptomycin to a final concentration of 500 units of each per ml of extract. The supernatant was also used, when necessary, as antigen for serological tests.

Infective larvae were obtained by feeding laboratory bred *Aedes aegypti* mosquitoes on infected dogs or on experimental rats showing a heavy microfilaraemia. The mosquitoes were dissected about 10-14 days after their blood meal. The infective larvae were suspended in saline, counted and divided into 4 equal lots to be inoculated into 4 rats subcutaneously using a 1 ml syringe fitted with a 19-gauge needle.

For eosinophil and microfilarial counts, blood was obtained from the tail as follows. Rats were immobilised in a restrainer, the tip of the tail cut with a pair of sharp scissors and the first drop of blood wiped away. Subsequent drops were drawn into appropriate pipettes. To avoid any diurnal variation in eosinophils or microfilariae, blood samples were drawn by one of us (MMI) in the 10-11 a.m. period. Eosinophil counts were made in duplicate using standard haematological techniques with phloxine as the diluent (Pilot, 1950). Blood for microfilarial counts was drawn into 0.05 ml pipettes, discharged on to a microscope slide, spread out and allowed to dry. The film was then dehaemoglobinised in tap water and the number of microfilariae in the entire film counted under the low power objective.

Complement-fixation tests as described by Danaraj and his co-workers (1959) were carried out on all experimental and control sera using a 1 % alcoholic extract of *D. repens* instead of *D. immitis* as antigen. Studies using sera from cases of tropical pulmonary eosinophilia indicate that antigens prepared from these 2 species of *Dirofilaria* give identical antibody titres (Ismail, unpublished). All sera were also tested for antibody against saline extracts of *D. repens* by diffusion in agar plates using the Ouchterlony method (Kabat and Mayer, 1964).

EXPERIMENTAL PROTOCOL

Experiment 1. Twelve rats, 6 experimental and 6 control, were used. Two pre-inoculation eosinophil counts were made from each rat during the fortnight preceding inoculation. Each experimental animal then received 1 ml of worm extract (approximately 3.0 mg.

protein) in adjuvant while each of the controls received 1 ml adjuvant without the extract. A total of 5 inoculations was given to each animal at fortnightly intervals. Eosinophil counts were done at various times during the follow-up period.

The follow-up period in this and subsequent experiments (except experiment 2) was 3 months at the end of which the rats were bled by cardiac puncture and the sera stored at -17 °C. for subsequent serological examination.

Experiment 2. Of 8 rats used, 4 received subcutaneous inoculations of *Dirofilaria* infective larvae suspended in 0.5 ml saline. Each experimental rat was given a total of 4 inoculations—containing 13, 16, 33 and 42 larvae respectively—at 1-4 week intervals. Each of the 4 controls received 0.5 ml saline only at each inoculation. Pre– and post inoculation eosinophil counts were done as in experiment 1 but the period of follow-up was 14 months after the first inoculation.

Experiment 3. Live adult *D. repens* were transplanted subcutaneously into 6 rats and intraperitoneally into another 6 rats, each animal receiving 4-5 females and 1-2 males. Six rats served as controls and did not receive transplants. Two pre-transplantation eosinophil counts were done but both eosinophil and microfilarial counts were done during the post-transplantation period. At the end of 3 months, 4 rats from each of the 2 experimental groups were dissected and a search made for adult worms.

Experiment 4. Adult worms (4-5 females and 1 male) killed by overnight freezing at -17° C. were introduced intraperitoneally into each of 4 rats while 4 rats served as controls. Eosinophil and microfilarial counts were done as in experiment 3.

Statistical Analysis. The mean eosinophil counts and the standard errors of the means were computed in both the experimental and control series of animals. To determine whether the eosinophil response in the experimental groups was significantly different from that of the control groups, 95% confidence intervals for the means of each group were established. Correlation coefficients were computed to determine whether the number of eosinophils circulating in the blood was significantly correlated to the number of microfilariae circulating at the same time.

RESULTS

None of the rats which received inoculations of *Dirofilaria* extract showed a consistent increase in the eosinophil level in the blood. The eosinophil responses in this group and in the controls which received adjuvant only were not significantly different at the 5% level during the entire follow-up period (Table 1).

The eosinophil response in the group of rats which received 4 inoculations of infective larvae and that of their controls is recorded in Table 2. Although the eosinophil levels in the experimental group were somewhat higher than in their controls, the differences were not significant ($P > .05$) except for the values recorded on one post-inoculation day.

TABLE 1

The eosinophil response in rats inoculated with an extract of *Dirofilaria repens*

DAYS	Inoc. Schedule	EXPERIMENTAL GROUP (inoc. with *Dirofilaria* extract in adjuvant) No. of rats	*Eosinophils/cu.mm* Mean	(SE)	95% confidence intervals min.	max.	CONTROL GROUP (inoc. with adjuvant only) No. of rats	*Eosinophils/cu.mm* Mean	(SE)	95% confidence intervals min.	max.
Basal											
i		6	162	(57)	19	305	6	22	(3)	14	30
ii		6	66	(27)	3	129	6	34	(6)	20	48
	1st inoc.	6									
Post-inoc.											
3		6	86	(21)	34	138	6	36	(6)	22	50
7		6	139	(41)	37	241	6	66	(15)	29	103
11		6	82	(18)	37	127	6	58	(13)	26	90
14	2nd inoc.	6									
18		6	49	(5)	37	61	6	55	(22)	3	107
25		6	70	(22)	15	125	6	54	(21)	2	106
28	3rd inoc.	6					6				
29		6	90	(23)	33	147	6	65	(20)	15	115
36		6	159	(41)	59	259	6	84	(2)	79	89
42	4th inoc.	6					6				
43		6	193	(39)	96	290	6	140	(33)	58	222
52		5	177	(25)	109	245	6	81	(21)	27	134
60	5th inoc.	5					6				
62		5	161	(14)	123	199	6	129	(26)	64	194
72		5	178	(33)	89	267	6	101	(27)	34	168
96		5	222	(28)	146	298	6	119	(35)	32	206

TABLE 2

The eosinophil response in rats given repeated inoculations of infective larvae of *Dirofilaria repens*

DAYS	Inoc. Schedule larvae/rat	EXPERIMENTAL GROUP (received infective larvae in saline) No. of rats	*Eosinophils/cu.mm* Mean	(SE)	95% confidence. intervals min.	max.	Blood for microfilaria	CONTROL GROUP (received saline only) No. of rats	*Eosinophils/cu.mm* Mean	(SE)	95% confidence. intervals min.	max.
Basal												
i		4	94	(28)	8	180		4	119	(35)	11	227
ii		4	71	(6)	53	89		4	118	(36)	6	230
	13	4						4				
Post-inoc.												
7		4	136	(34)	31	241		4	125	(39)	3	246
9	16	4						4				
14		4	212	(42)	82	342		4	115	(18)	57	173
28		4	170	(42)	40	300		4	119	(33)	17	221
35	33	4						4				
42		4	140	(18)	85	195		4	131	(22)	63	199
54		4	389	(117)	19	759		4	141	(37)	30	252
64	42	4						4				
71		4	277	(50)	122	432		4	119	(48)	—	268
86		4	252	(47)	107	397	Negative	4	145	(20)	83	207
110		4	293	(45)	154	432		4	177	(16)	127	227
148		4	288	(37)	174	402	Negative	4	102	(22)	34	170*
186		4	225	(52)	173	277		4	163	(39)	42	284
240		3	255	(47)	110	400	Negative	4	188	(32)	89	287
306		3	251	(45)	112	490	Negative	3	95	(31)	—	191
351		2	199		—		Negative	3	115	(46)	—	273
421		2	198		—		Negative	3	131	(18)	68	194

*denotes difference between experimental and control groups significant at the 5% level.

None of the rats showed microfilariae in the peripheral blood at any stage. Two of the infected rats died during the course of this experiment; no worms were recovered from these rats and the two dissected at the end of the follow-up period.

The results of the experiment in which live worms were transplanted into rats are summarized in Table 3 and 4. A moderate increase in eosinophils in the blood was seen in all 6 rats which received intraperitoneal transplants. The eosinophilia came on during the

TABLE 3

The eosinophils response in rats into which live *Dirofilaria repens* adults were transplanted

DAYS	INTRAPERITONEAL GROUP (6 rats †)				CONTROL GROUP (6 rats †)				SUBCUTANEOUS GROUP (6 rats †)			
	Eosinophils/cu.mm				*Eosinophils/cu.mm*				*Eosinophils/cu.mm*			
	Mean	(SE)	95% confidence. intervals min.	max.	Mean	(SE)	95% confidence. intervals min.	max.	Mean	(SE)	95% confidence. intervals min.	max.
Basal												
i	156	(23)	99	213	91	(17)	48	134	139	(31)	62	216
ii	140	(32)	60	220	128	(21)	76	180	160	(14)	122	198
Post-transp.												
2	132	(29)	59	205	89	(8)	69	109	104	(35)	17	191
4	143	(48)	23	263	96	(31)	18	174	111	(36)	21	201
7	248	(72)	68	428	113	(13)	80	146	205	(53)	73	337
12	297	(77)	105	489	105	(29)	32	178	360	(126)	76	644
16	466	(68)	296	636*	76	(15)	38	114	374	(57)	232	516*
22	373	(117)	84	662	137	(80)	57	217	270	(64)	110	430
28	345	(66)	180	510*	111	(17)	69	153	329	(47)	211	446*
34	475	(128)	165	795*	105	(22)	50	160	198	(30)	123	273
40	389	(113)	107	671	132	(12)	102	162	193	(30)	118	268
48	514	(90)	289	739*	102	(23)	44	160	341	(116)	51	631
56	611	(132)	281	941*	93	(16)	53	133	232	(42)	127	337
64	589	(90)	364	814*	133	(17)	90	176	287	(41)	164	390
72	648	(117)	356	940*	132	(12)	102	162	380	(46)	265	495*
80	555	(79)	356	751*	133	(19)	85	181	307	(81)	104	510
90	527	(59)	380	674*	119	(23)	61	177	231	(46)	116	346

† Number of animals in each group

* Significantly higher ($P < .05$) than mean eosinophil count of controls.

2nd and 3rd week after transplantation and was most prominent after the 4th week. Microfilariae appeared in the blood as early as the 2nd day. The number of microfilariae was highest during the latter part of the experimental period and a peak response ranging from 147 to 251 microfilariae per 0.05 ml blood was seen in 5 of the 6 animals. Although the number of eosinophils and microfilariae in the blood were significantly correlated after the 12th day of transplantation ($r = .33$, $r^2 = .11$, $P < .05$), the coefficient of determination r^2 indicates that only 11% of the total variation could be attributed to a linear relationship between these two responses. The correlation must, therefore, be viewed with caution. A typical response (Rat HB) is shown in Fig 1. Although there was some variation in

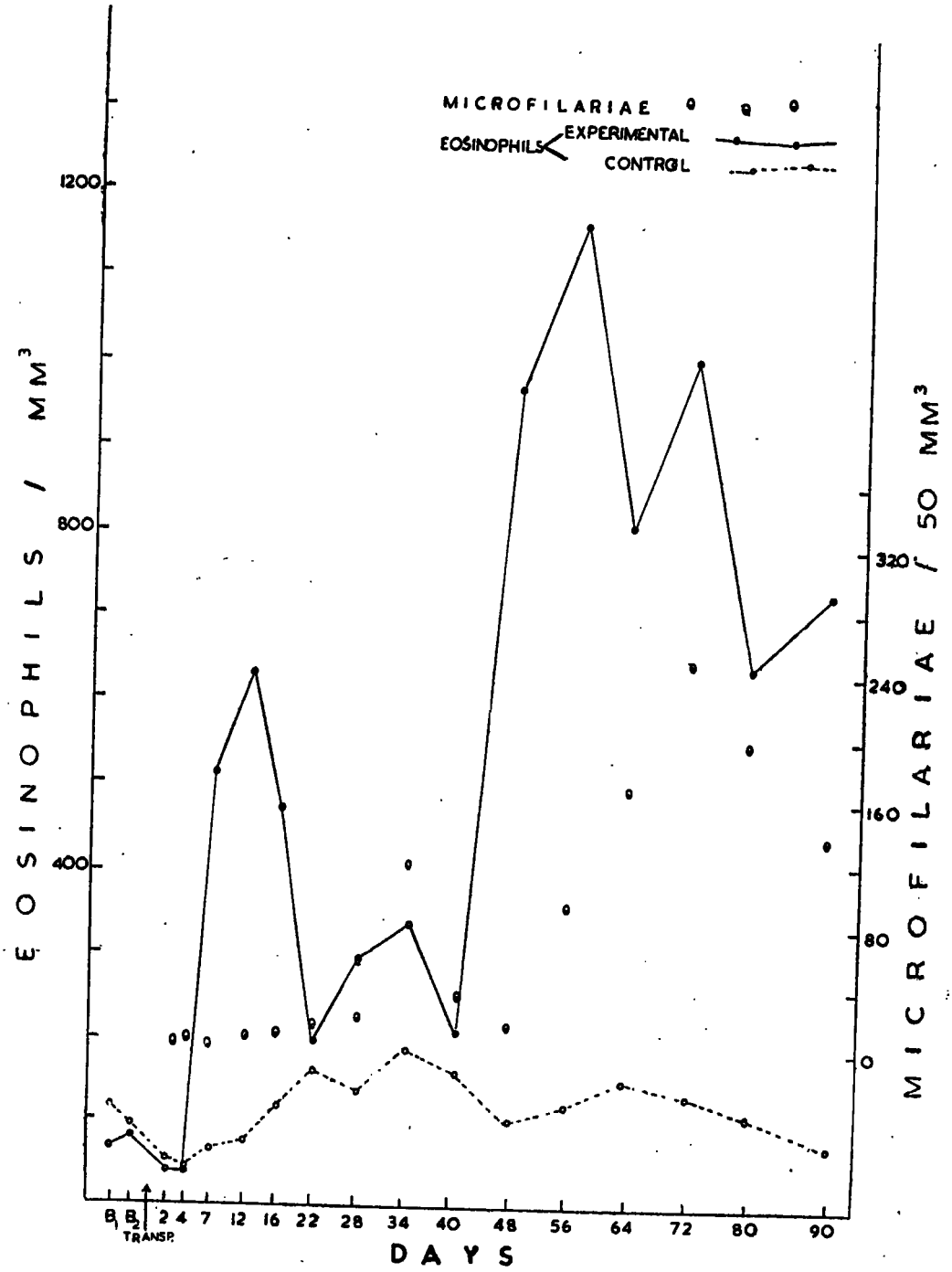

FIG. 1

The eosinophil and microfilarial response in a rat (Rat HB) after transplantation of *D. repens* adults intraperitoneally

TABLE 4

The microfilaraemia in rats into which live *Dirofilaria repens* adults were transplanted

DAYS AFTER TRANSPLANTATION	INTRAPERITONEAL GROUP (6 rats ‡) *mf*/0.05 *ml* Mean	Range	SUBCUTANEOUS GROUP (6 rats ‡) *mf*/0.05 *ml* Mean	Range
2	0	—	0	—
4	2.3	0—8	1.5	0—3
7	2.5	0—9	1.1	0—3
12	1.8	1—6	3.1	0—11
16	7.3	0—17	2.1	0—4
22	8.7	1—18	6.9	0—13
28	20.3	2—57	13.7	2—31
34	44.7	6—120	24.0	4—90
40	43.7	2—127	27.0	1—106
48	34.5	11—192	35.0	2—132
56	86.5	21—127	20.3	2—84
64	98.3	7—216	40.0	0—183
72	143.5	5—251	44.5	3—171
80	116.5	28—198	14.0	1—35
90	108.0	19—156	25.0	1—109

‡ Number of animals in each group.

the individual eosinophil counts, the mean counts were significantly raised in the experimental group when compared with the controls on all but 2 days after the 12th day of transplantation (Fig. 2).

In the group which received subcutaneous transplants, only 1 of 6 rats showed a consistent elevation of the eosinophil level. After the 12th day, the mean eosinophil counts were significantly raised above those of the controls only on 3 of the 11 days on which counts were made (Fig. 3). The microfilaraemia in this group was rather low—only 2 rats showed over 30 microfilariae per 0.05 ml blood at any stage. The eosinophils and the number of microfilariae in the blood were not significantly correlated ($r = .13, P > .05$). At least 1 live worm was recovered from each of the 4 rats of the 'intraperitoneal group' dissected at the end of 3 months (Table 5). In the 'subcutaneous group' only a single live worm was recovered. It is interesting to note that this worm was found in a subcutaneous nodule very similar to those seen in infected dogs. Two rats in this group had no trace of any worms.

TABLE 5

Record of adult worms recovered from rats 3 months after transplantation

GROUP	Rat No.	FEMALE WORMS Number Introduced	Number Recovered live	dead	MICROFILARIAE per 0.05 ml (after 12th day) Mean	Range
Intraperitoneal transplants	GR	5	1	2	49	2—150
	GY	5	1	0	10	0—28
	HR	4	2	0	87	13—159
	HB	5	2	0	101	6—251
Subcutaneous	FY	4	0	1	2	0—5
	HG	5	0	0	5	0—12
	GB	5	0	0	12	2—28
	GG	5	1	1	33	4—106

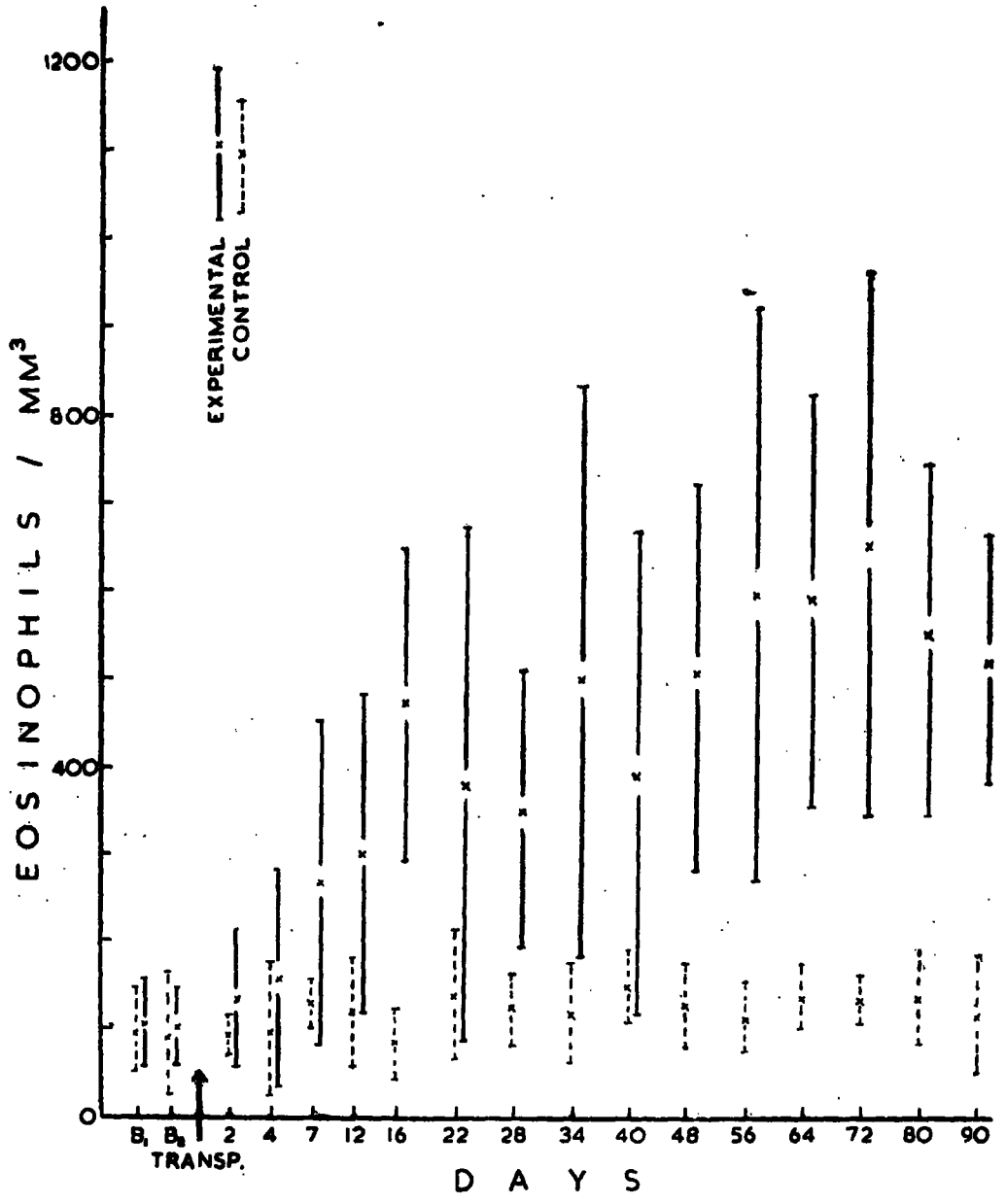

FIG. 2

Intraperitoneal transplants—mean eosinophil counts and their 95% confidence interrvals

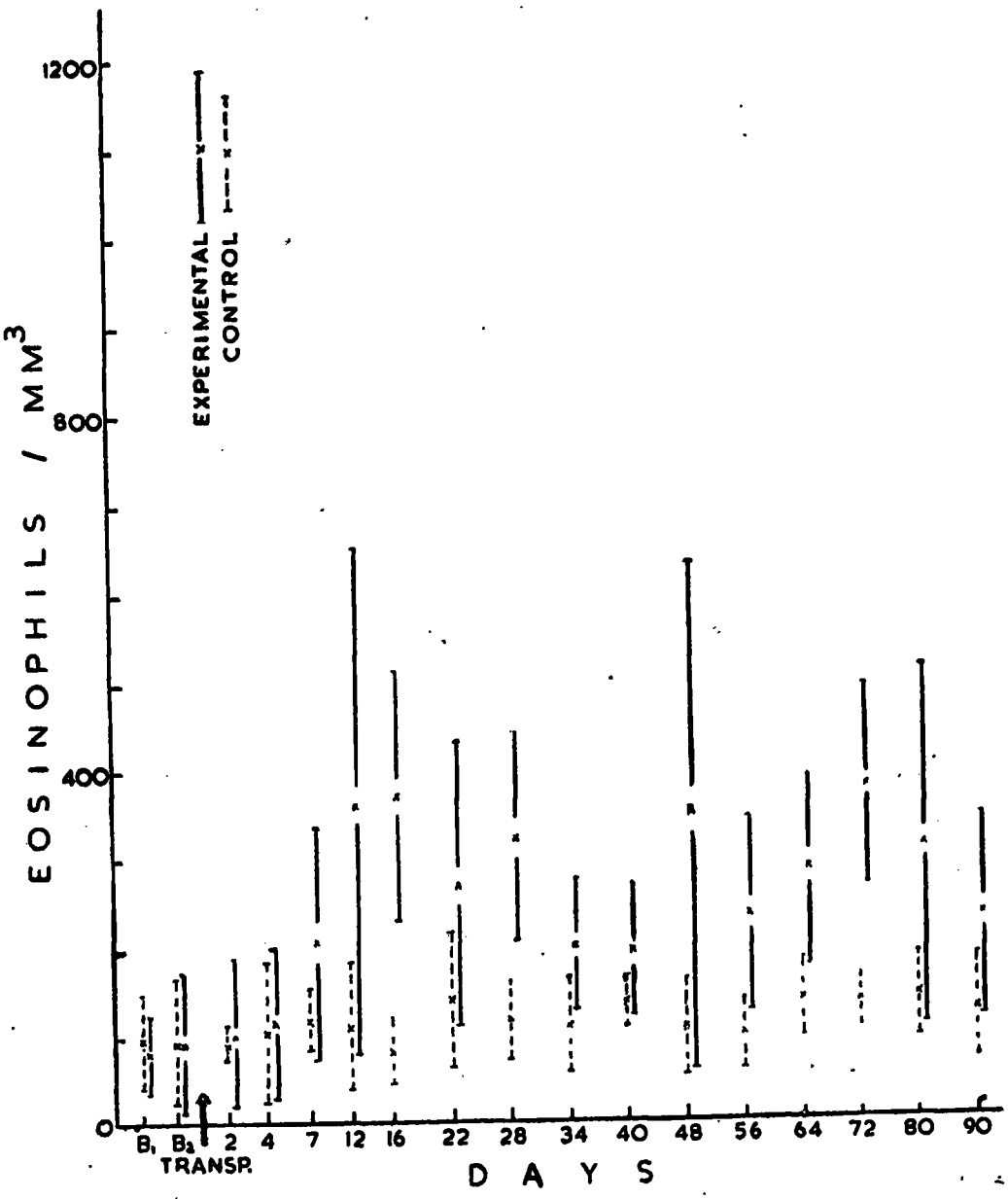

FIG. 3

Subcutaneous transplants—mean eosinophil counts and their 95% confidence intervals

The eosinophil response in the rats into which worms killed by freezing were introduced intraperitoneally was not significantly different ($P > .05$) from the response in the controls (Table 6).

TABLE 6

The eosinophil response in rats after introduction intraperitoneally of *Dirofilaria repens* adults killed by freezing.

	Experimental Group					Control Group					
Days	No. of rats	*Eosinophils/cu mm*		95% confidence intervals		No. of rats	*Eosinophils/cu mm*		95% confidence intervals		Difference in Eosin. Response* Exptl : Control
		Mean	(SE)	min.	max.		Mean	(SE)	min.	max.	
Basal	4	168	(39)	47	289	4	174	(32)	75	273	—
Post-transp.											
5	4	186	(42)	56	316	4	203	(22)	135	271	—
11	4	287	(101)	—	600	4	168	(45)	29	307	—
21	4	222	(43)	89	355	4	186	(54)	19	353	—
35	4	178	(37)	63	293	4	172	(28)	85	259	—
52	4	317	(29)	229	409	4	202	(43)	69	335	—
87	4	213	(64)	15	411	4	166	(27)	83	249	—

* — denotes difference not significant at the 5% level.

All sera were negative in the complement-fixation tests. Precipitin bands were, however, seen in agar diffusion plates with all sera taken from the experimental groups except in those inoculated with infective larvae and in 3 of the 4 rats into which killed worms were introduced. No attempt was made to titrate the sera. No bands were seen with any of the control sera.

DISCUSSION

Although it has been suggested that eosinophilia is exclusively due to the death of adult worms and microfilariae (Galliard, 1954), the results of the present experiments indicate that death of the worms *per se* does not apparently initiate an eosinophil response. A moderate but significant eosinophilia was seen in rats into which live worms were introduced intraperitoneally. The eosinophilia came on during the second and third week after transplantation suggesting that the worms have to be alive for a sufficient length of time to effectively initiate an eosinophil response. Live worms, in fact, were recovered from this group at the end of 3 months. In the group which received subcutaneous transplants, there was no significant eosinophilia. Most of the rats showed a low microfilaraemia and had no trace of the adult worms at the end of 3 months suggesting early death of the worms. Furthermore there was no increase in eosinophils in any rat into which killed worms were introduced intraperitoneally. A prolonged release of antigenic material, for example the secretions of the adult worms and their offspring, are presumably necessary for this response.

Repeated inoculations of *Dirofilaria* extract failed to increase the level of eosinophils significantly although there was demonstrable antibody production. It is generally accepted that eosinophilia is closely associated with the immune response but the characteristics of the antigenic stimulus and the nature of its release are apparently important factors.

The inoculation of infective larvae of animal filarial species experimentally into man has been shown to produce an eosinophilia (Buckley, 1958; Edeson, Wilson, Wharton and Laing, 1960). An increase in eosinophils in rats exposed to infective larvae of *D. repens* was, therefore, expected but, although a few of the rats showed an increase in eosinophils on some days, this was never consistent. In the early part of this experiment the numbers of larvae inoculated were small because mosquitoes fed on infected dogs generally gave poor yields of infective larvae. This problem was overcome to some extent and better yields of infective larvae were obtained during the latter phase of this experiment by feeding mosquitoes on rats which had previously received intraperitoneal transplants. The absence of a significant increase of eosinophils inspite of repeated inoculations of infective larvae suggests that these larve underwent little or no development in the rat and probably died early. The absence of microfilariae in the blood and the failure to recover any adults after a 14 month follow-up supports this view. In the experiments on human volunteers (Buckley, 1958 ; Edeson *et al.*, 1960) all of them developed an eosinophilia 12 to 14 weeks after inoculation of infective larvae. In these cases the larvae presumably underwent some further development and in one volunteer microfilariae were observed in the peripheral blood.

The technique of transplanting adult filarial worms into small laboratory animals has been used to carry out studies on morphology, life cycles, periodicity and transmission (Wharton, 1946 ; Bertram, Unsworth and Gordon, 1946 ; Kershaw, 1949a ; Webber, 1954 ; Williams, 1955 ; Nelson, 1962 ; Ansari, 1964 ; Furmaga, 1964 ; Dissanaike and Ponnudurai, 1968 ; Gooneratne, 1969 ; Niles and Kulasiri, 1970) and to investigate host-parasite relationships (Kershaw, 1949b ; Ramakrishnan, Dalip Singh and Krishnaswami, 1962 ; Bagai and Subrahmanyam, 1968). In the present studies *D. repens* adults were transplanted into rats in an attempt to induce a hypereosinophilia. This is probably the first record of this species being successfully transplanted into small laboratory animals.

The eosinophilia produced by intraperitoneal transplants was associated with a pronounced microfilaraemia. Although a significant correlation coefficient was obtained for these two parameters, this was probably due to factors other than a linear relationship between these two responses. It is of interest to note that microfilariae were detected in the peripheral blood of recipient rats on the second day after transplantation. This is in agreement with the experience of Williams (1955) who observed microfilariae in the peripheral blood of rats 56 hours after *Setaria cervi* adults were transplanted intraperitoneally ; Kershaw (1949a), Ramakrishnan *et al.* (1962) and Bagai and Subrahmanyam (1968) who detected microfilariae in recipient cotton rats within a day or two after transplantation of *Litomosoides carinii* adults and Niles and Kulasiri (1970) who transplanted *Cardiofilaria nilesi* adults intraperitoneally into chickens and observed microfilariae in the peripheral blood on the following day. In the majority of cases of classical filariasis and in the tropical pulmonary

eosinophilia syndrome microfilariae are absent in the peripheral blood. If microfilariae are, in fact, produced in these cases they are believed to be immunologically trapped in the tissues and prevented from entering the peripheral circulation. It will be interesting, no doubt, to extend this study and investigate the eosinophil response and microfilaraemia in rats previously sensitized to *Dirofilaria* antigens and then exposed to the parasite.

SUMMARY

Filarial worms of both animal and human origin appear to be involved in the aetiology of tropical pulmonary eosinophilia. The majority of human infections with animal filariae are caused by species of *Dirofilaria*. In this study the ability of infective larvae, antigenic extracts and adult worms of *D. repens* to induce an eosinophilia in rats has been investigated. A moderate, but significant, eosinophilia associated with a pronounced microfilaraemia was produced in the group in which adult worms were transplanted intraperitoneally. Worms transplanted subcutaneously resulted in a low microfilaraemia and an inconsistent eosinophilia. Repeated inoculations of *Dirofilaria* extract or infective larvae did not induce a significant increase in eosinophils. Death of the worms *per se* does not apparently initiate an eosinophil response; a prolonged release of antigenic material in the secretions of the adult worms and microfilariae are presumably necessary for the eosinophil response.

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