EXPERIMENTAL OCCULT DIROFILARIASIS IN DOGS WITH REFERENCE TO IMMUNOLOGICAL RESPONSES AND ITS RELATIONSHIP TO TROPICAL EOSINOPHILIA IN MAN

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INTRODUCTION

Although infection by a filarial worm is usually manifested by the appearance of its embryonic form, microfilaria, in the peripheral blood or skin, fllariasis without microfilaremia has been reported in humans and other animals. This is a form of occult filariasis which has recently been experimentally produced using the system of the heartworm, *Dirofilaria immitis*, in dogs as a laboratory model (Wong *et al.*, 1973). Essentially, this is accomplished by inoculation of infective larvae of *D. immitis* in dogs which had previously been sensitized (or immunized) by multiple injections of microfilariae (mf) of the same species. Dogs so infected were found to harbour many mature adults in the heart and pulmonary arteries. The presence of these worms was detected by angiocardiography (Fig. 1) or at necropsy. Microfilariae which had apparently been shed by gravid worms were found to be trapped in the lung, inciting the formation of granulomatous lesions (Fig. 2). Initial cellular infiltrations consisted mainly of polymorphonuclear leukocytes with many eosinophils (Fig. 3). These were later replaced by monocytic cells. Other laboratory findings on these dogs included a chronic eosinophilia (often with leukocytosis) in the peripheral blood, and miliary interstitial densities detectable by chest radiograms. Since immune responses of the host apparently play a major role in this form of infection, the development of serum antibodies to the various life cycle stages of the filarial

This work was supported in part by grants R22-AI-09741 and RR-00169 from the National Institutes of Health, Bethesda, Maryland, U.S.A.

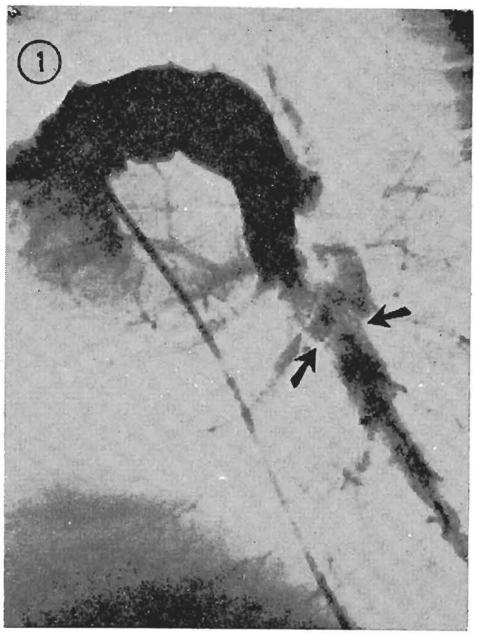

Fig. 1—Positive print of an angiocardiogram of a group A dog showing the presence of adult worms in the pulmonary artery (arrows).

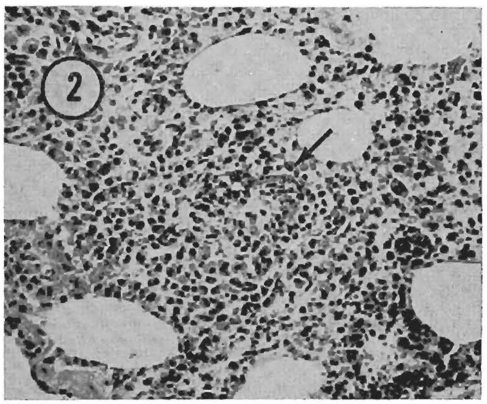

Fig. 2—Section of lung showing small lesion surrounding a trapped microfilaria (X95).

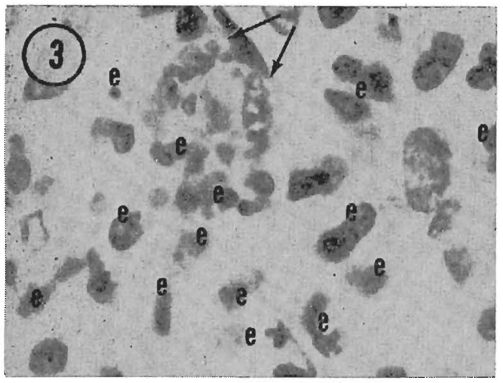

Fig. 3—Section of lung from a group A dog showing a recently trapped microfilaria (arrows) and some eosinophils (e) (X790).

worm—microfilaria (mf), infective-stage larva (L_3), and adult—was studied. The purpose of this paper is to report the data obtained from this study and to analogize occult dirofilariasis in dogs with a type of tropical eosinophilia in man which is called "Eosinophilic Lung" (Danaraj *et. al.*, 1959) in Malaysia.

MATERIALS AND METHODS

Details of the experiment used to develop the occult dirofilariasis in dogs were described elsewhere (Wong *et al.*, 1973). The experimental design and resulting microfilaremias are summarized as shown below.

Serum antibody studies were performed throughout this experiment using the indirect fluorescent antibody technique (IFA). The tube test method using mf and L_3 antigens (details are published in a previous report by Wong and Guest, 1969), as well as adult antigens, was utilized in this study. Formalin-phosphate-buffered saline fixed adult worms were cryostat sectioned at 14 microns in thickness. All antigens were tested in the same tubes. A positive reaction was based on interpretation of the cuticle fluorescence. Nonspecific fluorescence observed on the membrane lining and glycogen portion of adult muscle cells was recorded.

Two of the five dogs with occult infection were sacrificed 9 and 10 months after L_3 inoculation. Three were treated with microfilaricide (dithiazanine or diethylcarbamazine) and/or adulticide (thiacetarsamide sodium) 16 to 18 months post-inoculation. One of the three dogs died of congestive heart failure. The other two were later re-infected with approximately 200 L_3 each.

RESULTS

All dogs in the test groups became sensitized to *D. immitis* mf. No control dogs became sensitized. This paper is concerned mainly with the five dogs in group A which developed occult infection; therefore, only antibody titres of these dogs are presented here.

Antibodies to mf: Dogs injected with living mf concentrates of *D. immitis* produced detectable antibodies to them upon the disappearance of the artificially produced microfilaremia (Table 1). Titres increased after the second and third injections of living mf and continued to be detectable throughout the entire period of observation. Peak antibody levels (up to 1: 4,096 in Dogs 1 and 2) appeared around 6-8 months p.i., coinciding with the first production of microfilariae.

Antibodies to L_3: Antibody titres to L_3, as detected by the IFA technique, remained at low to moderate levels. In most instances, low titres were observed in sera having significantly high titres to mf. This occurred in

Dog Grp.	No. of Dogs	mf injected	L_3 inoc.	Microfilaremia	mf Detected (Mos. Post-Inoc.)
A	5	*D. immitis*	*D. immitis*	0/5	Never
B	5	*D. immitis*	*B. pahangi*	5/5	2.1 - 2.5
C	5	None	*D. immitis*	5/5	6.2 - 6.8

Table 1

Reciprocal titres of IFA to Mf and L_3 in sera of 5 dogs with occult dirofilariasis.

Dog No.	Anti-gens	Sensitization with Mf.				L_3 Inoc	Months Post L_3 Inoculation																			
		*−4	−3	−2	−1	0	+1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
		Mf ↓	Mf ↓		Mf ↓	L_3 ↓																		Rx ↓		
1	L_3	0	0	4	4	8	8	8	32	32	16	16	0	32	32	8	8	8	8	ND	32	8	8	8	8	8
	Mf.	0	32	64	512	512	128	512	512	512	512	2048	512	4096	4096	2048	2048	2048	2048	ND	2048	2048	4096	1024	1024	1024
			Mf ↓		Mf ↓	Mf L_3 ↓																Rx ↓				
2	L_3			0	0	8	8	0	8	0	0	0	8	32	32	8	8	8	8	8	8	32	32	8	8	8
	Mf.		8	8	8	512	128	128	512	2048	128	512	512	4096	4096	512	2048	2048	128	2048	512	1024	32	256	64	64
		Mf ↓		Mf ↓		Mf L_3 ↓																		Rx ↓		
3	L_3	0	0	0	8	0	0	0	0	0	0	0	0	32	8	8	8	0	8	8	8	8	32	32	64	8
	Mf.	0	8	8	128	32	32	32	32	ND	32	8	32	512	128	128	32	32	32	ND	32	128	256	2	64	8
			Mf ↓		Mf ↓	Mf L_3 ↓																				
4	L_3			4	8	8	0	0	0	128	0	0	0	0	0	0										
	Mf.		0	4	128	128	32	32	64	128	32	64	128	256	1024	1024		Sacrificed								
		Mf ↓		Mf ↓		Mf L_3 ↓																				
5	L_3	0	0	0	0	0	8	0	0	0	2	2	2	0	0											
	Mf.	2	8	32	2	2	16	8	256	64	32	8	128	256	64			Sacrificed								

↓ = injection or inoculation
* = months pre-L_3 inoculation
Rx = treatment with filaricide

four of the five dogs even before inoculation with infective larvae (Table 1).

To provide a better visual pattern of antibody production over a period of two years, the data obtained from sera of Dog 1 is presented in graph form (Fig. 4).

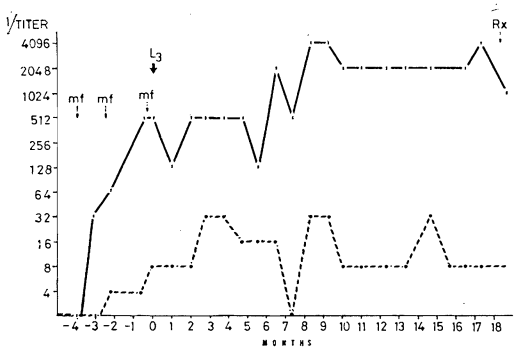

Fig. 4—Dog 1 IFA titres to mf (solid line) and L_3 (broken line) over a 22-month period. Small arrows = mf injections; big arrow = L_3 inoculation.

Antibodies to the adult worm: The membrane lining and glycogen portion of muscle cells in adult section were seen to fluoresce in low titres in most sera tested, including control sera obtained before either the injection of mf or the inoculation of L_3. These antibody titres increased with an increase in mf antibody titres. Strongly positive reactions also showed fluorescence at the following sites : along the cuticle, on the hypodermis and gut wall linings, and in the reproductive organ and ova. Sections of female worms appeared to have a stronger reactivity than those of male worms. Strongly positive reactions were seen most often in sera obtained after the administration of adulticide.

DISCUSSION

The clinical and laboratory findings of the experimentally produced occult dirofilariasis in these dogs are reminiscent of those of the human cases of "Eosinophilic Lung." The filarial etiology of "Eosinophilic Lung" has been proven by the presence of antibodies to filarial antigens (Danaraj *et al.*, 1959; Wong and Guest, 1969), its response to treatment with diethylcarbamazine, a microfilariacide (Danaraj, 1959), and more specifically, by detection of microfilarial lesions in lung biopsies from patients with "Eosinophilic Lung" (Danaraj *et al.*, 1966). It has been suggested that the filariids responsible for this type of infection are aberrant animal filarial species.

Antibodies to microfilarial antigens are not demonstrable in sera of dogs with circulating microfilariae. This phenomenon was reported in a previous study (Wong, 1964), and was again observed among the control dogs (group C) in this experiment. It appears that such sera are in a state of antigen excess, so that even though antibodies may be present, they are masked by the massive amount of circulating antigens. This has been borne out by the observations that sera of blood containing large numbers of mf could be used as antigens in tests for anti-mf antibodies in immune sera in either Schultz-Dale or passive cutaneous anaphylaxis reactions (Guest and Wong, 1965; Guest *et al.*, 1967).

Apparently, sensitization or immunity to the microfilarial stage of a filarial species does not prevent a subsequent infection by infective larvae or their maturing to the adult stages. It does, however, inhibit the circulation of microfilariae produced. The existence of this immunity to the microfilarial stage is certainly one of the main causes of occult filariasis. That this "stage" immunity is long-lasting is suggested by the observations on Dog 1 when it was experimentally re-infected several months after treatment with adulticide. Subsequent infection again produced no circulating microfilaria, and, as shown in monthly chest radiograms, the lung lesions intensified six months after the second inoculation of L_3 (experiment in progress).

The significance of antibodies to L_3, especially of the levels detected in this experiment, has not been elucidated. Experiments using large numbers of abnormal, irradiated *D. immitis* larvae in natural hosts (dogs) and normal larvae in unnatural hosts (monkeys) indicated that antibodies to L_3 appeared when most of the L_3 were dying or dead (Wong *et al.*, 1974; Wong, 1974).

The nonspecific reactions at the glycogen portion and membrane lining of muscle cells in the adult sections were probably due to cross-reactivity of shared antigens among the different stages of the filariid, or possibly even with other tissue nematodes. The interpretation of the IFA positive reaction against adult filarial antigen is thus made difficult. The fluorescence on the cuticle, however, appeared to be directly related to antibodies elicited by dying or dead adult worm and thus may be useful in the diagnosis of human dirofilariasis.

Low titre antibodies to mf were distinctly differentiated from those to other stages, but high titres cross-reacted with other stages. The presence of shared antigens among the various filarial stages may contribute to the tipping of a very delicate microfilarial antigen-antibody balance *in vivo* and could account for the sudden disappearance of microfilaremia observed in infected dogs.

Indications are that mf antibodies are species-specific. This was demonstrated *in vivo* by the successful establishment of microfilaremia in the five group B dogs in this experiment which were challenged with a heterologous species, *Brugia pahangi* (Materials and Methods). Additionally, in sera collected in 1964 from 13 proven "Eosinophilic Lung" patients in Malaysia using the IFA technique against four different species of mf, viz. *Wuchereria bancrofti*, *Brugia malayi*, *B. pahangi* and *D. immitis*, it was found that sera of nine patients were positive only to *W. bancrofti* mf, three only to *B. malayi* mf, and one only to *B. pahangi*. The latter species, *B. pahangi*, although regarded as an animal filaria, has been observed to produce patent infection with microfilaremia in man (Edeson *et al.*, 1960), and in at least one volunteer, occult infection leading to tropical pulmonary eosinophilia ("Eosinophilic Lung") (Buckley, 1958). None of the total of 69 serum samples collected from these patients before and after treatment reacted with *D. immitis* mf. In comparison, sera from four asthmatic patients with eosinophilia were also tested and were found to be negative for antibodies to any of the mf used. This finding again supports the theory that the etiological agent directly responsible for the disease entity "Eosinophilic Lung" is the filarial worm progeny, the microfilaria. It thus stands to reason that it is only those filarial species which can produce patent infection, and not animal filariae which normally produce only abortive, or at most, nonpatent infections in man, which are involved.

The question naturally arises: How did the sensitization or immunization to microfilariae occur in nature? According to Beaver's review (1970), congenital microfilaremias have been reported from as far back as 90 years ago and as recently as 1966 by Mantovani and Jackson. It is quite conceivable that puppies which have congenital microfilaremia develop hypersensitivity rather than tolerance to the microfilaria of that filarial species. This may explain the occurrence of 5% to 10% of the heartworm infected dogs without microfilaremia reported in the U.S.A. and Japan (Jackson, 1973; Kume, 1970). It is quite conceivable that similar occurrences in human babies may lead to similar sequelae. It would be interesting to obtain an *accurate* figure of the frequency of congenital microfilaremia in human populations in an endemic area, since it is the usual practice to exempt the

pregnant women and infants in a mass survey on filariasis and thus the occurrence of congenital microfilaremia may have been overlooked. However, it would be naive to suggest that all patients with occult filariasis are born of microfilaremic mothers. Exposure to mf through blood transfusions, although unlikely, is another possibility. Moreover, cross-reactivities of shared antigens among tissues from various stages of the same or different worm species may also play a role in upsetting the microfilarial antigen-antibody balance.

In conclusion, although the understanding of the exact pathogenesis of occult filariasis is as yet somewhat incomplete, the experimental findings presented here strongly suggest that hypersensitivity or immunity to the microfilarial stage of a filarial infection is at least one of the main etiological factors in an occult infection.

SUMMARY

Dirofilariasis without microfilaremia was experimentally produced in dogs sensitized with living mf injections and challenged with homologous infective larvae. Serum antibodies against mf, L_3, and adult stages of *D. immitis* were studied using the IFA tube test technique over a period of two years. Stage and species-specific antibodies to mf appeared with the disappearance of microfilaremia, and titres heightened at the end of prepatency. These findings were discussed and analogized to the etiology of "Eosinophilic Lung", a form of occult filariasis in man.

ACKNOWLEDGEMENTS

The experimental design for this study was essentially conceived when the author was studying with Dr. P. C. Beaver and the late Dr. G. Pacheco at Tulane University, New Orleans, Louisiana. The IFA tests would not have been successful without the excellent preparation and generous supply of the anti-dog conjugate serum from Dr. A. Sulzer, National Center for Disease Control, Atlanta, Georgia. The sera and clinical data from E.L. patients were kindly supplied by Dr. H.O. Wong of the Faculty of Medicine, University of Malaya. K. Brown, S. Karr, P. Lee and K. C. Lim provided technical assistance.

REFERENCES

BEAVER, P.C., (1970). Filariasis without microfilaremia. *Amer. J. Trop. Med. Hyg.*, *19*(2) : 181.

BUCKLEY, J.J.C., (1958). Occult filarial infection of animal origin as a cause of tropical pulmonary eosinophilia. *E. Afr. Med. J.*, *35* : 493.

DANARAJ, T.J., (1959). The treatment of eosinophilic lung (tropical eosinophilia) with hetrazan (a preliminary report). *Proceedings of the Alumni Ass., Malaya*, *9*(3) : 172.

DANARAJ, T.J., DA SILVA, L.S. and SCHACHER, J.F., (1959). The serological diagnosis of eosinophilic lung (tropical eosinophilia) and its etiological implications. *Amer. J. Trop. Med. Hyg.*, *8*(6) : 640.

DANARAJ. T.J., PACHECO, G., SHANMUGARATNAM, K. and BEAVER, P.C., (1966). The etiology and pathology of eosinophilic lung (tropical eosinophilia). *Amer. J. Trop. Med. Hyg.*, *15*(2) : 183.

EDESON, J.F.B., WILSON, T., WHARTON, R.H. and LAING, A.B.G., (1960). Experimental transmission of *Brugia malayi* and *B. pahangi* to man. *Trans. Roy. Soc. Trop. Med. Hyg.*, *54* : 229.

GUEST, M.F. and WONG, M.M., (1965). Schultz-Dale reaction with sera of eosinophilic lung patients--A preliminary report. *Med. J. Malaya*, *20*(2) : 146.

GUEST, M.F., WONG, M.M. and CHIN, L.G., (1967). Passive cutaneous anaphylaxis using a microfilarial antigen-antibody system. *Med. J. Malaya, 21*(4) : 379.

JACKSON, W.F., (1963). Diagnosis and treatment of heartworm disease in dog. *Allied Vet., 35* : 131.

KUME, S., (1970). Epizootiology of canine heartworm disease in the Tokyo area: Diagnosis and treatment. *In*: Bradley, R.E., ed., Canine Heartworm Disease: A Discussion of the Current Knowledge. University of Florida Press, Gainesville, p. 38.

MANTOVANI, A. and JACKSON, R.F., (1966). Transplacental transmission of microfilaria of *Dirofilaria immitis* in the dog. *J. Parasit., 52* : 116.

WONG, M.M., (1964). Studies on microfilaremia in dogs. II. Levels of microfilaremia in relation to immunologic response of the host. *Amer. J. Trop. Med. Hyg., 13*(1) : 66.

WONG, M.M., (1974). Experimental dirofilariases in the macaques. I. Susceptibility and host responses to *Dirofilaria immitis*, the dog heartworm. *Trans. Roy. Soc. Trop. Med. Hyg.*, (In press).

WONG, M.M. and GUEST, M.F., (1969). Filarial antibodies and eosinophilia in human subjects in an endemic area. *Trans. Roy. Soc. Trop. Med. Hyg., 63*(6) : 179.

WONG, M.M., SUTER, P.E., RHODE, E.A. and GUEST, M.F., (1973). Dirofilariasis without circulating microfilaria: A problem in diagnosis. *J. Amer. Vet. Med. Ass., 163*(2) : 133.

WONG, M.M., GUEST, M.F. and LAVOIPIERRE, M.J., (1974). *Dirofilaria immitis*: Fate and immunogenicity of irradiated infective stage larvae in beagles. *Exp. Parasit., 35* : 465.