

In Vitro Adhesion of Eosinophils to Infective Larvae of *Wuchereria bancrofti*

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Summary. A reproducible *in vitro* test was developed to quantitatively study the adhesion of human eosinophils to *Wuchereria bancrofti* infective larvae. Eosinophils, regardless of the donor, selectively adhered to the larvae in the presence of immune serum. The reaction reached a maximum by 90 minutes at room temperature and remained unchanged up to 6 hours. The adherent eosinophils, however, did not induce any apparent morphologic change in the larvae.

The phenomenon appeared to require, primarily, IgG anti-larval antibodies. Heat-inactivation of the serum did not prevent the reaction from occurring, although addition of fresh normal serum enhanced the intensity of adhesion.

Maximal adhesion of eosinophils was obtained when the larvae were viable and in the presence of immune serum and fresh normal serum during incubation with the leucocytes.

Normal serum was found to induce this adhesion reaction. The responsible factor could be removed by absorption of normal serum with cotton. However, this procedure had no effect on the reactivity of sera from filariasis cases.

The reaction was almost totally inhibited by EDTA and citrate. The anti-inflammatory steroid, betamethasone, had a moderately inhibitory effect. An unexplained finding was an enhancing effect on the reaction when histamine was added to non-reactive normal serum.

INTRODUCTION

The *in vitro* adhesion of eosinophils and other leucocytes on the surface of various parasitic helminths has been described although few attempts have been made to standardize and measure the reaction (Pandit, Pandit and Iyer, 1929; Fros and Liqui Lung, 1953; Raghavan, Roy and Nair, 1958; Bang, Saha and Bandyopadhyay, 1962; Newsome, 1962; Soulsby, 1961, 1962; Wong, 1964). In general, these studies suggested that the phenomenon was immunologically mediated in the serum and leucocytes from infected or immunized hosts were required before cellular adhesion could occur. However, the organisms in most instances, were not isolated free of contaminants and quantitative data were not reported. It was also not known whether or not the eosinophils were specifically sensitized during infection of the host by the parasite.

Therefore, an *in vitro* test system was developed to study various parameters of the adhesion of human eosinophils to the infective larval stage of *Wuchereria bancrofti*, a common filarial parasite of man in Calcutta.

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MATERIALS AND METHODS

The adhesion tests

The reactions were conducted on glass microscope slides previously washed and treated with silicone (Siliclad, Clay-Adams Inc., New York). Serum (0.03 ml), infective larvae (1-10), phosphate-buffered saline (0.15 M, pH 7.2) or fresh normal serum (0.03 ml), and 0.03 ml of the cell suspension were added sequentially on the slide. The buffered saline also contained 1.5×10^{-4} M calcium ions and 5×10^{-4} M magnesium ions. A coverglass was fixed over the reaction mixture, the borders were sealed with heated paraffin and the preparation was incubated at room temperatures (23°-27°). The initial pH of the preparation was 7.5. Counts of the adherent eosinophils and other leucocytes were made with a Zeiss phase contrast microscope, at a magnification of approximately $\times 600$. All routine tests were read after 90 minutes incubation, except for the time course studies when counts were made on individual larvae every 10 minutes over the 1st hour, and then every 30 minutes over the following 5 hours. All data are expressed as total adherent leucocytes per larva. In all cases, eosinophils comprised at least 80-85 per cent of the adherent leucocytes.

Permanent preparations were made at various times of incubation by transferring the larvae on to slides lightly treated with albumin followed by methanol-fixing and staining with Hansel's stain (Lide Laboratories, St Louis) to enumerate the eosinophils.

Infective larvae of W. bancrofti

Infective (third stage) larvae of *W. bancrofti* were used throughout this study. The worms were allowed to develop in *Culex fatigans* mosquitoes, the natural vector in Calcutta, which were previously reared in the laboratory and fed on donors with known levels of microfilaraemia. The engorged mosquitoes were then placed in an air-conditioned environment and fed during the next 15-17 days, the time required for development of the ingested microfilariae to the third stage. The motile larvae were dissected free of the mosquitoes, washed thoroughly in physiological saline, and kept overnight suspended in phosphate saline in the cold. Normally, all larvae were used the following day for the adhesion test. On warming to room temperature, the larvae regained active movement within a few minutes. All larvae measured approximately 1.5-2.0 mm in length.

Suspension of eosinophils

Patients with a peripheral eosinophilia of at least 15 per cent served as donors. They had been diagnosed as suffering from filariasis, hookworm infection, ascariasis, trichuriasis, tropical pulmonary eosinophilia and other conditions unrelated to parasitic infection, including one case of eosinophilic leukaemia. Regardless of the source, all eosinophils were equally efficient in adhesion to the larvae under the conditions described. Leucocytes were obtained from 10 ml of venous blood by dextran sedimentation of the erythrocytes with ethylenediamine tetra-acetic acid (EDTA) as the anticoagulant (Lichtenstein and Osler, 1964). The cells were washed thrice with 50 volumes of 0.15 M phosphate-buffered saline. Total leucocyte counts, eosinophil counts (Speirs, 1952), and counts of cell viability with 0.05 per cent eosin Y (Hanks and Wallace, 1968) were determined. The leucocyte concentration was adjusted to provide at least 1×10^6 eosinophils/ml. Cell viability did not drop below 85 per cent. The cells were held in an ice bath and gently mixed with a silicone treated Pasteur pipette just prior to use.

Absorption of serum with cotton

Preliminary studies indicated the ability of serum from all sources to initiate the adhesion of eosinophils to the larvae. However, cotton was found to remove the adhesion-promoting factors in sera from subjects with no evidence of previous exposure to human filarial infections. The cotton was locally purchased (Jayer and Co., Calcutta, batches 240,241) and was stated to be thoroughly washed, absorbent and sterile.

Absorption was performed in a 100×16 mm test tube in an ice bath. Approximately 7–10 mg of cotton were immersed in 1 ml of serum. After 10 minutes, a wooden applicator stick (Tomac Brand, A. H. Thomas Co., Philadelphia) was used to remove the cotton by twisting and the excess serum was expressed by applying pressure with the cotton-wrapped stick against the inner wall of the tube. This procedure was repeated twice for 20 and 30 minutes, each time with clean aliquots of cotton. The absorbed serum, 0.5–0.6 ml, was then passed through a 0.3 or 0.45 μm Millipore filter to remove residual particulate matter and to clarify the serum, thus facilitating subsequent microscopic observation. The absorbed and filtered serum was stored at -70° .

Serum

Sera from 157 individuals were stored at -70° after collection and used in the test within 1 year's time. The sera were divided into the following groups, the figures indicate the total number studied: microfilaraemia (forty-seven); filarial lymphoedema or elephantiasis (thirty-eight); non-filarial helminthiasis including ascariasis, trichuriasis, hookworm infection (twenty); Indians without demonstrable helminthic infections (seventeen); and newly arrived Americans in India without evidence of parasitic infections (thirty-five). The patients with lymphoedema or elephantiasis (L-E) had no detectable microfilariae in the circulation. All patients with microfilaraemia (MF) were diagnosed as *W. bancrofti* infections. Some of the MF group had evidence of clinical filariasis, viz. lymphoedema, chyluria and lymphscrotum.

As a source of thermolabile serum factors including complement, serum was obtained from a donor (blood group AB, Rh positive) whose serum was previously found to be unreactive in the adhesion test. After treatment with cotton, aliquots were kept at -70° and used within 3 months of the collection date.

Prior incubation of larvae in serum

Larvae were incubated in sera from various groups for 1 hour at 37° , washed in phosphate saline, incubated in fresh normal serum for 30 minutes and finally washed. These larvae were then used in the test in the presence of only phosphate saline and washed leucocytes.

Alteration of larvae

In order to test the influence of live larvae on the adhesion of eosinophils, the larvae were treated in different ways.

A batch of larvae was held in the cold for 2 days after recovery and used in experiments compared to infective larvae 1 day after isolation.

Two groups of dead larvae were exposed to 2 per cent aqueous formalin or to 70 per cent ethanol for 10 minutes and then thoroughly washed in phosphate saline. A fourth batch was killed by three cycles of alternate freezing at -70° and thawing at room temperature. The treated larvae were compared with unaltered, 1-day-old isolated infective larvae in the adhesion test.

Treatment of serum with 2-mercaptoethanol (2-ME) or heat

Aliquots (0.5 ml) of various sera were placed in dialysis bags and treated with 0.1 M 2-ME and 0.02 M iodoacetamide as described by Sadun, Duxbury, Gore and Stechsulte (1967) or were heated at 56° for 4 hours. Each treated serum was compared in the test with an untreated aliquot of the corresponding serum.

Absorption of immunoglobulins

Because of the technical difficulty of obtaining sufficient numbers of infective larvae to conduct adequate specific absorption experiments and because of the possible role of the worm's metabolic products in the test, it was decided to attempt the individual removal of the three major immunoglobulins from serum with specific antisera. IgG, IgA and IgM were absorbed from test sera by adding 0.3 ml of each Ig-specific, anti-human goat antisera (Hyland Laboratories, Los Angeles) to 1-ml aliquots. Previous testing in Ouchterlony plates showed each of these antisera to produce a unique precipitin band against human serum. The mixtures were incubated for 1 hour at 37° and then for 72 hours at 2°. The precipitate was removed by centrifugation at 20,000 *g* for 45 minutes at 2°. The absorption was repeated eight to nine times. Traces of IgG and IgA were present at the end of the treatment but IgM was not detectable by gel diffusion. The absorbed sera were then compared in the test to 1-ml aliquots of the unabsorbed sera diluted with phosphate saline to the same final volume of the absorbed sera.

Chemical modification of eosinophil adhesion

Sodium azide, histamine dihydrochloride (Pfanstiehl Laboratories, Inc., Illinois), EDTA, tri-sodium citrate, heparin (Biological Evans, Ltd, Hyderabad, batch A7004), and di-sodium-phosphate betamethasone (Pfizer Ltd, Bombay, lot 720-96029) were made into appropriate stock solutions in phosphate saline and stored at 5–10°. Dilutions were made and added to equal volumes of cell suspensions. The mixtures were then added to the serum and larvae. In all instances the initial pH of the reaction mixtures with the chemicals ranged from 7.2 to 7.6. All stock solutions were used within 7 days of preparation.

Quantitation of immunoglobulins

IgG, IgA and IgM were quantitatively determined in antibody-agar plates (Fahey and McKelvey, 1965) obtained commercially (Hyland Laboratories, Los Angeles). The three immunoglobulins were measured in various sera before and after absorption with cotton.

Statistical analyses

The analysis of variance, Student's *t*-test, and chi-square test were applied as indicated in the results (Snedecor, 1956; Moroney, 1965).

RESULTS

NATURE OF THE REACTION

Untreated sera from all sources promoted the adhesion of eosinophils to infective larvae. However, absorption with cotton left normal (American) sera uniformly negative in the adhesion reaction, while sera from filariasis, similarly treated, were unaffected. Cotton-absorbed sera from non-filarial helminthiases and from parasite-negative Indian subjects

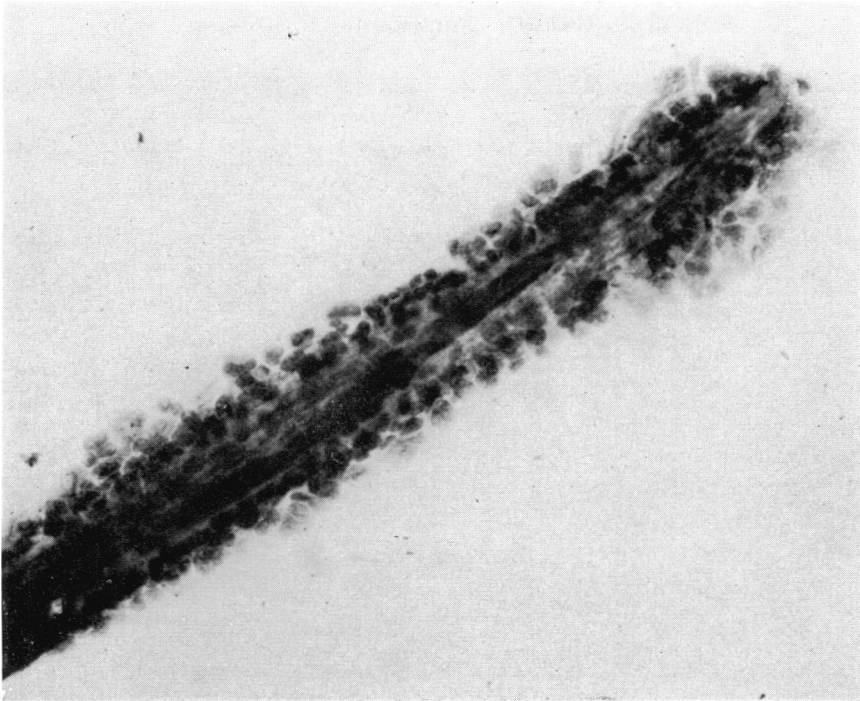


FIG. 1. Stained preparation of a larva of *Wuchereria bancrofti* with adherent eosinophils after 2 hours incubation with immune serum and leucocytes. The anterior third of the worm is shown. In this case, eosinophils comprised over 90 per cent of the attached cells. Hansel's stain, $\times 500$.

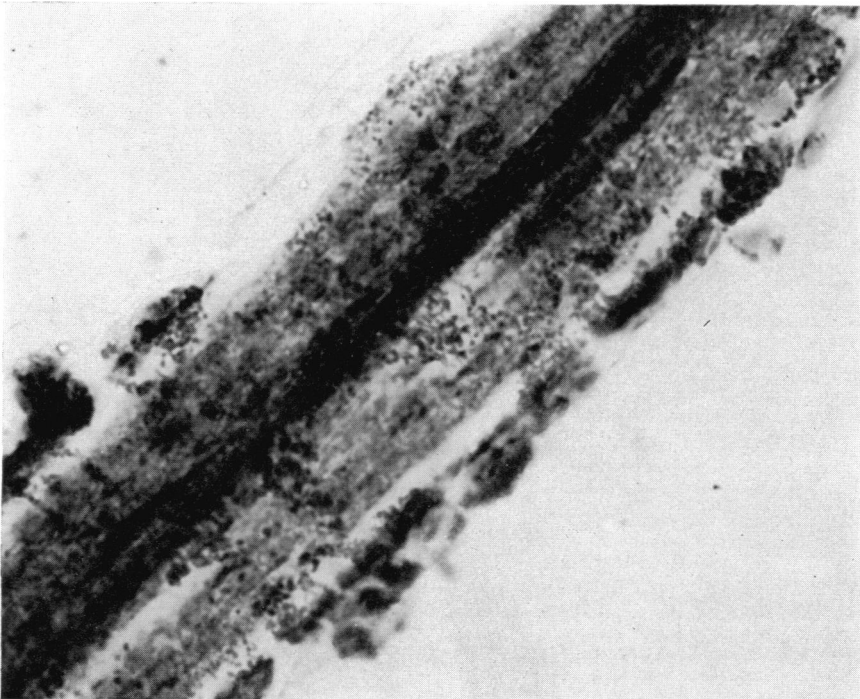


FIG. 2. Higher magnification of the mid-portion of the larva in Fig. 1. Adherent eosinophils are evident with their pseudopodia (containing granules) extended over the worm's surface. $\times 1250$.

TABLE 1
COMPARISON OF THE PER CENT EOSINOPHILS IN THE ORIGINAL LEUCOCYTE SUSPENSION WITH THE PER CENT EOSINOPHILS OF ADHERENT CELLS

Experiment No.	Diagnosis	Leucocyte suspension			Serum No.	Adherent leucocytes		
		Leucocytes/ml	Eosinophils/ml	Per cent eosinophils		Leucocytes/larva (mean)	Eosinophils/larva (mean)	Per cent eosinophils (mean)
63	Lymphoedema-elephantiasis	5.3×10^7	5.0×10^6	9.4	220*	353.3	346.2	98
					219	385.0	348.1	93
					123	456.0	437.8	96
					281	248.0	245.5	99
					PC33†	34.2	34.2	100
65	Microfilaraemia	3.1×10^7	3.4×10^6	10.9	237	256.0	250.9	98
					174	281.0	267.0	95
					039	277.0	268.7	97
					PC15	0.0	-	-
					Buffer	9.6	9.6	100
70	Normal Indian	2.3×10^7	2.9×10^6	12.6	257	228.0	205.2	90
					095	174.0	167.0	96
					130	283.0	277.3	98
					092	127.0	123.2	97
					PC43	2.0	2.0	100
					PC11	3.5	3.5	100

* All test sera were absorbed with cotton and heated for 30 minutes at 56°, fresh normal serum also was added to provide thermolabile factors.

† PC, Normal American sera.

showed a wide scatter of results in the test. In contrast, larvae suspended in buffer and washed leucocytes in all cases had few adherent cells, amounting to less than fifteen eosinophils per larva.

A stained preparation of the reaction in Fig. 1 indicates that eosinophils comprise almost all of the adherent leucocytes. Eosinophils can also be seen attached to other eosinophils and were not in direct contact with the cuticle of the larva. Such cells were not dislodged on movement of the worms. This inter-cellular leucocyte adhesion is similar to that which occurs subsequent to phagocytosis (Allison and Lancaster, 1964). A closer view of adherent eosinophils shows them, in many instances, spread out on to the larval surface with extended granule-containing pseudopodia (Fig. 2). Adherent leucocytes were found over the whole length of the helminth without selective aggregation at the excretory, genital, oral or anal openings. In control tests, the larval surface was consistently intact without cellular adhesion.

Observations of the phenomenon up to a maximum of 24 hours did not indicate any change in the general morphology of the larvae when compared to larvae incubated in non-reactive serum and leucocytes. In positive tests, a majority of the adherent eosinophils had detached from the larvae; some of the remaining eosinophils had only a few cytoplasmic granules.

Eosinophils, regardless of the sources, were equally reactive for adhesion to larvae in the presence of reactive sera (Table 1). The number of adherent eosinophils invariably exceeded 85 per cent of the total leucocytes attached to the larval cuticle even though eosinophils constituted no more than 15 per cent of the initial leucocyte suspension (Table 1). The other adherent leucocytes identified were neutrophils and mononuclear cells. An analysis of the difference in the proportion of eosinophils adherent from that in the leucocyte suspension was highly significant ($P < 0.001$, t -test). There were no differences between

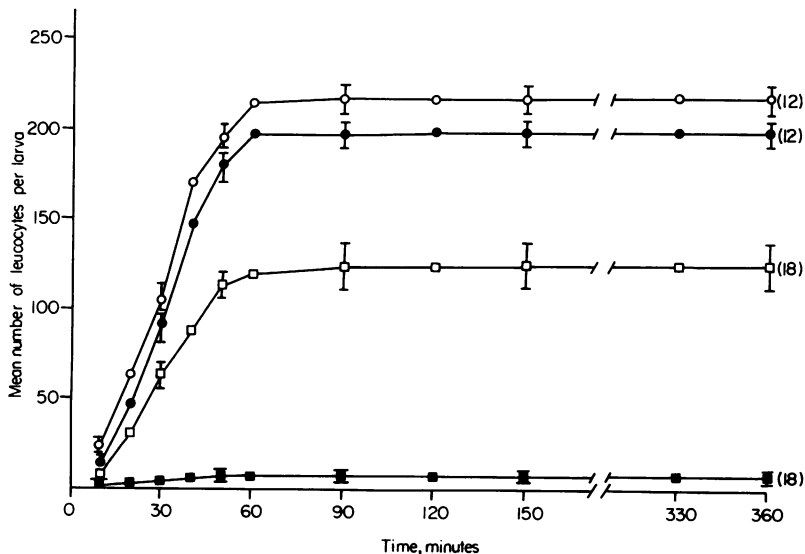


FIG. 3. Time course of the adhesion of eosinophils to filarial infective larvae with sera unabsorbed or absorbed with cotton. Nine sera from filariasis, absorbed (●) and unabsorbed (○) with cotton were compared to nine normal American sera, absorbed (■) and unabsorbed (□). These sera were not heated and fresh normal serum was not included in the reaction mixtures. The figures to the right of the curves are the number of larvae observed. The vertical bars represent ± 1 SE.

the sera from microfilaraemic patients and the sera from patients with lymphoedema or elephantiasis and these results are combined in Fig. 3. Further comparison of sera from non-filarial helminthiases and parasite-negative Indian subjects with sera from filariasis in similar time course studies showed the former groups of sera to have significantly less numbers of adherent eosinophils ($P < 0.01$, *t*-test).

The treatment with cotton had no effect on the quantitative level of the immunoglobulins, IgG, IgA or IgM in thirty-nine filarial sera, twelve non-filarial helminthic sera, nine non-parasitic Indian sera and in nineteen normal American sera. The mean levels of IgG in sera from all Indian subjects before (1791 mg/100 ml) or after (1479 mg/ml) absorption with cotton were higher than that found in American sera, 1438 and 1027 mg/100 ml, respectively. IgA and IgM showed no quantitative differences for the same groups of sera.

The quantitative levels of the antibodies mediating the adhesion of eosinophils were not investigated, although reactive serum diluted in normal serum to one in six was less reactive quantitatively. Further attempts to dilute such serum in saline produced erratic results in the test. A major difficulty in these attempts at titration may have been due to a requirement of eosinophils and other leucocytes for stabilizing factors in serum (Ketchel and Favour, 1955; Cline, Hanifin and Lehrer, 1968), though tests were not made for these.

EFFECT OF HEAT-INACTIVATED SERA ON THE ADHESION TEST

Addition of fresh non-reactive serum to reactive serum previously treated with cotton and heated (56° , 30 minutes), distinctly enhanced the magnitude of the adhesions when compared to the latter alone (Fig. 4). Similar findings also were found with normal Indian and non-filarial helminthic sera. However, addition of the fresh serum to normal American

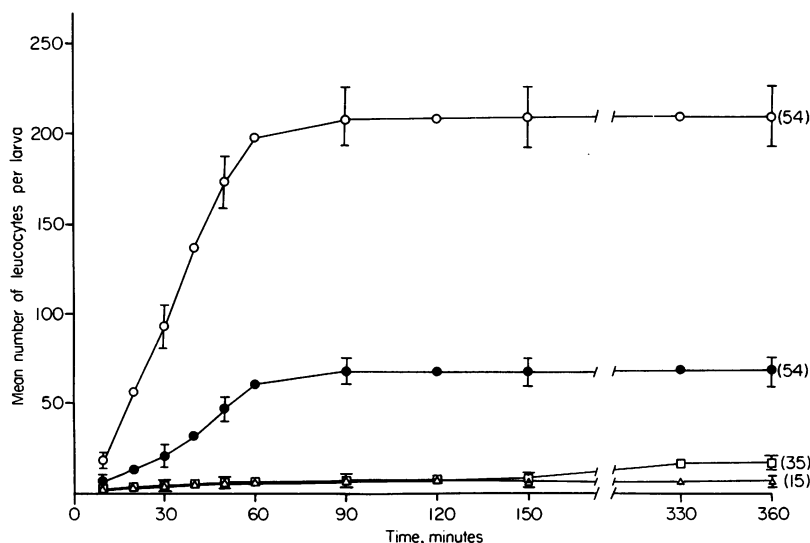


FIG. 4. Time course of the adhesion of eosinophils to filarial infective larvae showing the effect of fresh normal serum. All sera were heated for 30 minutes at 56° and tested with added fresh normal serum or phosphate-saline. Each serum was absorbed with cotton as described in the text. The symbols represent: thirty-one sera from filariasis, with added fresh normal serum (○) and with buffer (●); seventeen normal American sera with fresh normal serum (□); and eight trials with buffer and fresh normal serum (△). The figures to the right of the curves are the number of larvae observed. The vertical bars represent ± 1 SE.

serum or to buffer had little effect on cellular adhesion. The increase in adherent leucocytes observed when the fresh serum was added was significant ($P < 0.01$, *t*-test) for all groups of sera except for buffer and American sera.

SPECIFICITY AND REPRODUCIBILITY OF THE ADHESION OF EOSINOPHILS

Comparison of results obtained from filarial sera with that from non-filarial sera showed that the number of sera which allowed fewer than 100 adherent leucocytes per larva was significantly less in the latter sera than in the former ($\chi^2 = 36.2$, $P < 0.001$, for 1 degree of freedom). Thus, a positive serum was regarded as one which, after absorption with cotton and treatment with heat (56° , 30 minutes), would promote in the presence of thermolabile factors at least 100 leucocytes (>80 per cent eosinophils) to adhere to a larva after 90 minutes incubation at room temperature. Utilizing these criteria, Table 2 shows a comparison of the positive and negative sera found in the various groups and Fig. 5 shows these same groups of sera with the mean numbers of leucocytes per larva. There was no difference between the two groups of filariasis, but there were significant differences when these were compared to the results of the other groups ($P < 0.001$, *t*-test).

TABLE 2

COMPARISON OF VARIOUS GROUPS OF SERA IN THE EOSINOPHIL ADHESION REACTION (EACH SERUM WAS HEAT-INACTIVATED, ABSORBED WITH COTTON AND TESTED WITH ADDED FRESH NORMAL SERUM)

Serum group	Total No. of sera	No. of positive sera	No. of negative sera	Per cent positive
Buffer	18 (trials)	0	18 (trials)	0.0
Normal (American)	31	0	31	0.0
Normal (Indian)	17	5	12	29.4
Non-filarial helminthiases	14	9	5	64.3
Microfilaraemia	37	36	1	97.3
Lymphoedema-elephantiasis	26	25	1	96.2

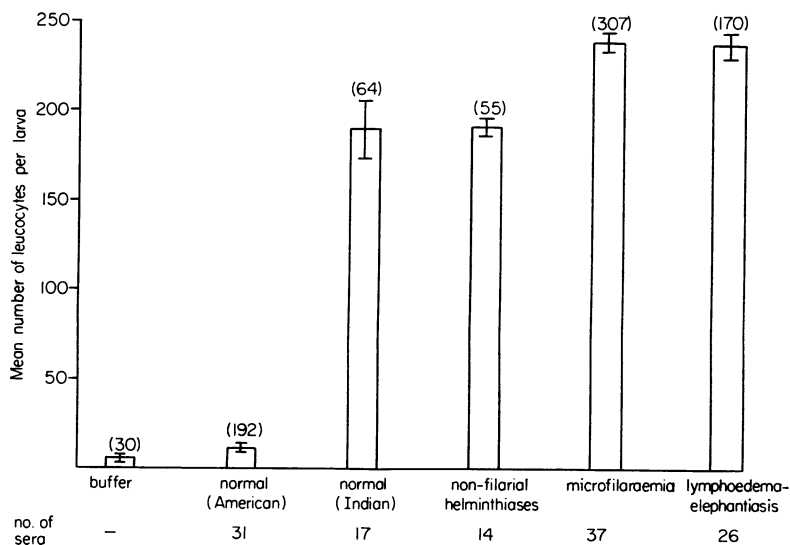


FIG. 5. Comparison of the mean numbers of adherent leucocytes per larva for all sera in each group indicated in Table 2. The figures above each bar are the total number of larvae observed. The vertical bars represent ± 1 SE.

Although there were significant variations in the numbers of adherent leucocytes in experiments conducted on different days with the same serum, variation in cell counts in any one experiment was usually no more than 40 per cent. However, a positive serum was always positive on repeated testing and conversely for a negative serum.

EFFECT OF MIXING EQUAL VOLUMES OF POSITIVE AND NEGATIVE SERA

Numerous non-specific factors in serum other than immunoglobulins and complement have been reported to possess antimicrobial activity (Skarnes and Watson, 1967; Shilo, 1959), although their anti-helminthic activity has not been determined. Thus, aliquots of two positive sera, a positive and a negative serum, and two negative sera were separately combined to determine whether the factors responsible for the adhesion of eosinophils could be augmented or inhibited by the various combinations. However, the counts of adherent eosinophils on the larvae, in the presence of any of eight pairs of positive sera, seven pairs of a positive and a negative serum, or of three pairs of negative sera, were neither enhanced nor markedly diminished when compared to each serum of the pair alone.

Eight sera from patients with non-infectious allergies* were tested to determine whether such conditions may have induced eosinophil adhesion-promoting factors. After absorption with cotton, these sera were uniformly negative with a mean number of 15.3 (± 1.3 SE) leucocytes per larva from eighty worms, ten per serum.

EFFECT OF INCUBATING LARVAE IN SERUM PRIOR TO TESTING

The metabolic products (excretory and/or secretory) of parasitic helminths have been shown to provoke antibody formation in experimental and natural infections (Thorson, 1963). Such antibodies have been considered by many to be of primary importance in the host response to many helminthic infections.

The presence of positive serum during incubation of the larvae and leucocytes was necessary for maximal cellular adhesion (Table 3). 'Pre-sensitized' larvae were markedly less reactive than larvae bathed in positive serum during the incubation for all groups excluding the normal American sera ($P < 0.001$, *t*-test).

TABLE 3

EFFECT OF PRIOR INCUBATION OF LARVAE WITH SERUM AND FRESH NORMAL SERUM ON THE ADHESION OF EOSINOPHILS

Serum group	Infective larvae (previously incubated in sera)			Control infective larvae		
	No. of sera	No. of larvae	Leucocytes/larva (mean \pm SE)	No. of sera	No. of larvae	Leucocytes/larva (mean \pm SE)
Normal American	7	23	0.3 \pm 0.17	5	17	1.8 \pm 0.89
Filariasis	20	89	65.9 \pm 4.29	15	48	256.7 \pm 11.22

The treated larvae were exposed to leucocytes in a buffer medium only. Controls were larvae incubated in aliquots of the same sera during exposure to leucocytes.

EFFECT OF LARVAL VIABILITY ON THE ADHESION OF EOSINOPHILS

The reactivity of leucocytes with larvae approximately 48 hours post-isolation was distinctly diminished in sera from filariasis compared to larvae 24 hours post-isolation (Table

* Sera were kindly provided by Dr L. M. Lichtenstein, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, U.S.A.

TABLE 4

COMPARISON OF INFECTIVE LARVAE, 24 HOURS POST-ISOLATION, WITH INFECTIVE LARVAE, 48 HOURS POST-ISOLATION, ON THE ADHESION OF EOSINOPHILS

Serum group	No. of sera	Larvae 48 hours post-isolation		Larvae 24 hours post-isolation	
		No. of larvae	Leucocytes/larva (mean \pm SE)	No. of larvae	Leucocytes/larva (mean \pm SE)
Normal American	2	10	4.2 \pm 0.14	5	2.0 \pm 0.84
Filariasis	7	35	93.5 \pm 6.38	22	161.3 \pm 13.30

4). Greater depressions of cellular adhesions were found with larvae killed by freezing and by formalin (Table 5). However, alcoholic treatment promoted cellular adhesion when the dead larvae were incubated with normal American serum and leucocytes ($P < 0.001$, t -test) (Table 5).

TABLE 5

EFFECT OF KILLING LARVAE BY FREEZING, FORMALIN OR ETHANOL ON SUSCEPTIBILITY TO THE ADHESION OF EOSINOPHILS

Serum group	No. of sera	Dead larvae		Live larvae	
		No. of larvae	Leucocytes/larva (mean \pm SE)	No. of larvae	Leucocytes/larva (mean \pm SE)
'Freeze-killed'					
Normal American	3	30	0.0 \pm 0.00	9	1.8 \pm 0.92
Filariasis	21	210	13.5 \pm 0.87	63	189.0 \pm 9.02
'Formalin-killed'					
Normal American	2	4	32.3 \pm 15.38	8	23.4 \pm 8.43
Filariasis	6	14	89.6 \pm 26.46	28	213.2 \pm 22.20
'Ethanol-killed'					
Normal American	2	10	190.5 \pm 8.98	10	9.0 \pm 1.34
Filariasis	5	50	193.3 \pm 5.18	30	164.9 \pm 11.06

EFFECT OF ABSORPTION OF IMMUNOGLOBULINS ON THE ADHESION OF EOSINOPHILS

Significant ($P < 0.001$, t -test) decreases of 50–92 per cent in counts of adherent leucocytes were found with all positive sera studied when IgG was absorbed (Table 6). Absorption of IgA and IgM diminished cellular adhesion for each of two sera suggesting the possible presence of anti-larval antibodies in these two classes.

EFFECT OF PROLONGED HEATING OR 2-ME TREATMENT OF SERA ON THE ADHESION OF EOSINOPHILS

Prolonged heating of twenty positive sera and eight negative sera did not alter the counts of adherent eosinophils compared to aliquots of serum heated for 30 minutes.

However, treatment with 2-ME reduced the reactivity in the test for two of seven sera: a microfilaraemic serum ($P < 0.001$, t -test); and a serum from a patient with ascariasis ($P < 0.01$). These results agree with the findings of the previous experiments in which anti-larval antibodies may reside in either IgA or IgM. The above results further indicate that classical reaginic antibody (thermolabile and mercaptoethanol sensitive) or IgE is not involved in the test.

TABLE 6
EFFECT OF ABSORPTION OF IMMUNOGLOBULINS ON THE ADHESION OF EOSINOPHILS

Serum No.	Serum group	No. of larvae	Immunoglobulin (Ig) absorbed			
			IgG	IgA	IgM	Control
			Leucocytes/larva (mean \pm SE)	Leucocytes/larva (mean \pm SE)	Leucocytes/larva (mean \pm SE)	Leucocytes/larva (mean \pm SE)
PC 7	Normal American	5	4.4 \pm 0.78	2.0 \pm 0.56	0.2 \pm 0.43	11.4 \pm 0.60
PC 4	Normal American	5	6.0 \pm 1.05	0.0 \pm 0.00	1.6 \pm 0.92	3.6 \pm 1.43
376	Microfilaraemia	5	120.0 \pm 8.48	266.0 \pm 3.84	249.0 \pm 5.36	246.0 \pm 6.69
316	Microfilaraemia	5	134.0 \pm 5.07	237.0 \pm 14.14	235.0 \pm 4.24	370.0 \pm 21.72
337	Microfilaraemia	5	131.0 \pm 4.97	242.0 \pm 5.01	226.0 \pm 7.40	271.0 \pm 3.84
387	Microfilaraemia	5	19.0 \pm 1.05	294.0 \pm 5.17	229.0 \pm 5.36	245.0 \pm 3.74
349	Lymphoedema-elephantiasis	5	125.0 \pm 6.63	278.0 \pm 7.01	268.0 \pm 8.55	244.0 \pm 3.84

EFFECT OF EDTA AND CITRATE ON THE ADHESION OF EOSINOPHILS

The use of high concentrations of citrate (Pandit *et al.*, 1929; Bang *et al.*, 1962), used as an anticoagulant, and EDTA at 1-4 mg/ml (Bang *et al.*, 1962) have been reported to inhibit the adhesion of eosinophils and other leucocytes to microfilariae of *W. bancrofti*.

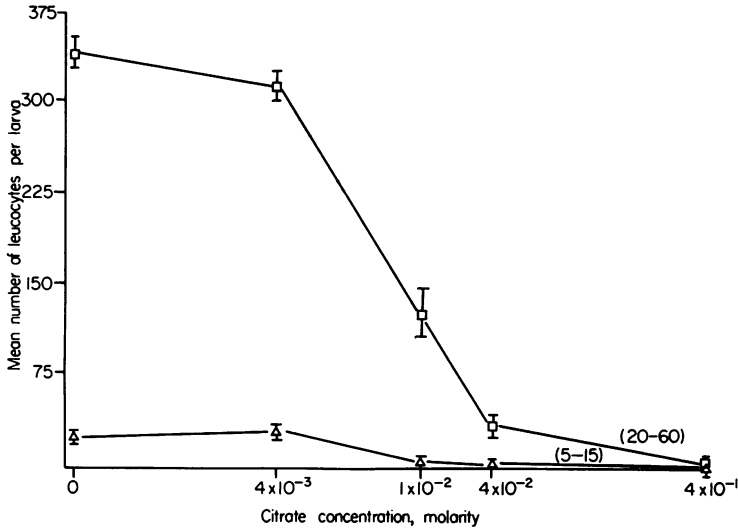


FIG. 6. Effect of sodium citrate on the adhesion of eosinophils. The symbols indicate: six sera from filariasis (□); and two normal American sera (△). The figures above the curves indicate the number of larvae observed. The vertical bars represent ± 1 SE.

A concentration of 6×10^{-3} M EDTA uniformly inhibited the attachment of eosinophils in all of the sera tested (Table 7). Citrate also inhibited the reaction (Fig. 6), the inhibition ranged from 58.9 per cent at 1×10^{-2} M to 100 per cent at 4×10^{-1} M. An analysis of variance showed that this concentration effect was significant ($P < 0.005$).

TABLE 7
EFFECT OF EDTA ON THE ADHESION OF EOSINOPHILS

Serum group	No. of sera	0.006 M EDTA		Buffer	
		No. of larvae	Leucocytes/larva (mean \pm SE)	No. of larvae	Leucocytes/larva (mean \pm SE)
Normal American	4	15	0.0 \pm 0.00	14	8.9 \pm 2.48
Normal Indian	2	7	0.0 \pm 0.00	6	49.8 \pm 9.60
Filariasis	13	87	0.2 \pm 0.07	50	196.8 \pm 19.79

EFFECT OF HISTAMINE ON THE ADHESION OF EOSINOPHILS

It has been reported that histamine stimulated specific chemotaxis of eosinophils in the skin of horses (Archer, 1963) although others have found no such effect in the human skin window (Feinberg, Feinberg and Lee, 1967; Felarca and Lowell, 1968). Therefore, histamine was used to determine what effect it might have on the adhesion of human eosinophils to the parasitic larvae. There was generally an increase in the number of adherent eosinophils especially evident with normal sera (Fig. 7).

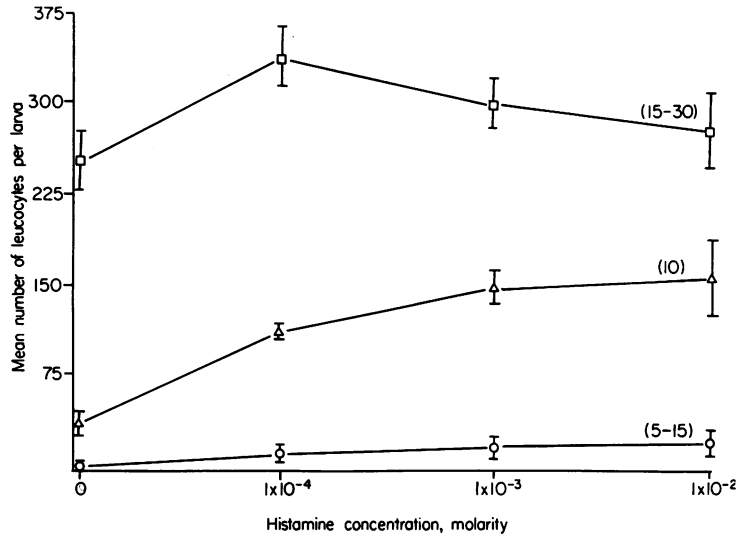


FIG. 7. Effect of histamine on the adhesion of eosinophils. The symbols represent: six sera from filariasis (□); two normal American sera (Δ); and buffer (○). The figure above the curves indicate the number of larvae observed. The vertical bars represent ± 1 SE.

EFFECT OF β -METHASONE ON THE ADHESION OF EOSINOPHILS

Since cortisol has been reported to inhibit emigration of human eosinophils into skin windows (Felarca and Lowell, 1969), β -methasone was used to see if the adhesion of eosinophils could also be inhibited. At the highest concentrations used, this anti-inflammatory steroid did partially suppress the reaction ($P < 0.01$, analysis of variance) (Fig. 8). There was no morphological change in the adherent eosinophils nor was there any alteration of eosinophil numbers or viability in the presence of the drug.

EFFECT OF SODIUM AZIDE AND HEPARIN ON THE ADHESION OF EOSINOPHILS

Sodium azide, $4 \times 10^{-3} M$, has been reported to inhibit the adhesion of macrophages to glass surfaces but had no effect on immune adherence (Nelson, 1965). No adverse effects were found on the adhesion of eosinophils with azide at 4×10^{-2} and $4 \times 10^{-3} M$ with six positive and two negative sera.

Heparin has similarly been reported to have no effect on immune adherence (Nelson, 1965) although the adhesion of eosinophils to microfilariae was partially inhibited by this agent in high concentrations (Bang *et al.*, 1962). In the present experiments, heparin in concentrations from 0.1 to 100 international units per millilitre had no inhibitory effect on the test with seven positive sera and three normal sera.

DISCUSSION

The results reported in this communication suggested that human eosinophils selectively attached to filarial larvae in comparison to the other leucocytes in the reaction mixture. Previous reports of others have not included data on the numbers of eosinophils either in the cellular suspensions or in the leucocytes adherent to microfilariae of *W. bancrofti* (Fros and Liqiu Lung, 1953; Bang *et al.*, 1962) and to larvae of *Ascaris suum* (Soulsby, 1961,

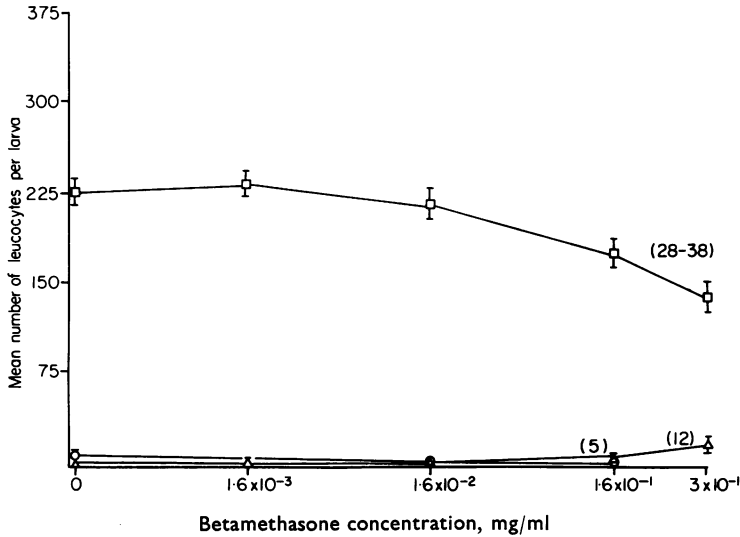


FIG. 8. Effect of β -methasone on the adhesion of eosinophils. The symbols indicate: two normal American sera (Δ); six sera from filariasis (\square); and buffer (\circ). The figures above the curves indicate the number of larvae observed. The vertical bars represent ± 1 SE.

1962, 1963). However, Reddy, Parvathi and Sivaramappa (1969) briefly reported finding viable and dead larvae of *Drancunculus medinensis* recovered from patients with guinea-worm arthritis, with adherent leucocytes, approximately 30 per cent being eosinophils. The eosinophils in the aspirated synovial fluid comprised less than 10 per cent of the total leucocytes in three of the four cases presented.

Eosinophils did not appear to be specifically sensitized *in vitro* by a helminthic infection. The eosinophils, which came from patients with various helminthic infections were non-reactive for adhesion to the larvae except when a reactive serum was present. There were also no apparent differences in the quantitative responses of eosinophils from cases of filariasis in comparison to those from patients with other helminthic infections or from patients without demonstrable helminths. These findings are at variance with the reports of Soulsby (1961, 1962) in which it was stated that eosinophils from an immunized rabbit was a prerequisite for adhering to sensitized *Ascaris* larvae.

Under different experimental conditions and with different parasitic helminths, other types of leucocytes consisting of either macrophages, or neutrophils, or pyronophilic mononuclear cells have been found to adhere *in vivo* or *in vitro* to adult blood flukes of the *Schistosoma* spp. (Newsome, 1962), to *Ascaris* larvae (Crandall, Crandall and Arean, 1967; Soulsby, 1967; Jeska, 1969), and to *Haemonchus contortus* larvae (Jeska, 1969).

It was not known whether the 60–90 minutes required for maximal adhesion was necessary for the physiological activation and migration of the eosinophils or whether an accumulation of immune complexes derived from the metabolic activity of the larvae occurred during this time, although the present experiments suggest the latter possibility. There are no published reports on the time course of the adhesion of eosinophils to a helminth though others have noted that *in vitro*, the reaction occurred within a few minutes to 90 minutes (Bang *et al.*, 1962; Soulsby, 1963). Studies on the *in vitro* chemotaxis of human eosinophils have indicated that maximum migration towards chemotactic factors generated by immune complexes occurred at 90 minutes (Ward, 1969).

The necessity for patient's serum and the inhibition found with IgG absorption suggest that the adhesion of eosinophils to the filarial larvae was mediated by anti-larval antibodies. The decreased reactivity of the two IgA and IgM absorbed sera indicated that other Ig classes may also mediate the adhesion of eosinophils.

The reactivity in this test of sera from individuals without clinical or laboratory evidence of filariasis indicated the possible presence of cross-reacting antibodies induced by other nematode infections or that such sera were from subjects with inapparent infections of *W. bancrofti* or filarial species other than those normally found in man. The endemicity of *W. bancrofti* in the Calcutta region (Rozeboom, Bhattacharya and Gilotra, 1968) and the high degree of specificity of homologous larval antigens for bancroftian filariasis, in this same region, in fluorescent antibody tests (Chowdhury and Schiller, 1962) and in skin tests (Higashi and Chowdhury, 1968) suggest the latter possibilities. However, the specificity of the adhesion of eosinophils to the larvae by specific absorption and isolation of the anti-larval antibodies in reactive serum was not determined.

The reactive serum appeared to contain anti-larval antibodies directed against the worm's metabolic products as well as against the cuticle indirectly suggested by three findings. First, it was found that when infective larvae were kept in a cold environment for 48 hours after isolation from mosquitoes, the leucocyte counts on these worms in the adhesion test were 53–67 per cent of the controls, larvae used within 24 hours after isolation. Second, infective larvae incubated in reactive serum washed prior to addition of the leucocytes had three- to five-fold fewer adherent eosinophils than the controls, larvae incubated with leucocytes in the presence of reactive serum. Third, when the metabolic activity of the infective larvae was abolished by freezing or formalin-treatment, the dead larvae had less than 50 per cent as many cells attached as on normal larvae.

An unexpected finding was that when larvae, killed by ethanol, were used in the test, increased cell counts on the larvae resulted, especially in the presence of normal serum. The reason for this effect was not determined, although it may have been a result of the solvent nature of the alcohol acting on the surface of the larvae allowing the exposed cuticle to be reactive with eosinophils. Non-specific enhancement of ethanol-fixed tissue sections of mammalian organs due to increased diffuse absorption of fluorescent antibodies has been described (David, Ruth and Law, 1966). Moreover, formalin-fixed sections did not prevent specific staining by the fluorescent antibodies.

The adhesion-promoting factors in normal American sera were most probably non-specific as absorption with cotton removed these substances without affecting either the quantitative levels of IgG, IgA and IgM or the reactivity of sera from filariasis. It is not known whether these factors are similar to the many antimicrobial substances described in serum and tissues (Skarnes and Watson, 1957; Shilo, 1959). However, Boyden, North and Faulkner (1965) stated that absorption of normal serum with cellulose removed specific, natural antibodies which in combination with bacteria induced chemotaxis of neutrophils.

It thus appears that cotton absorbed non-specific factors for the adhesion of eosinophils without affecting the antibodies for the larvae, the specific factors for adhesion of eosinophils. Turk (1959) has suggested similarly that serum contained non-specific factors, absorbed by polysaccharides, and specific anti-bacterial antibodies, refractory to polysaccharide absorption, each of which was capable of inducing the immune adherence of erythrocytes to bacteria.

The apparent quantitative enhancement of the adhesion of eosinophils by as much as 50

per cent when fresh normal serum was added to a heat-inactivated reactive serum suggests a role for heat labile factors which may include complement. Previous work (Soulsby, 1962, 1963) on the adhesion of eosinophils to *Ascaris* larvae has suggested that complement (fresh normal serum) had no effect but no quantitative data on cell counts were presented. However, adhesion of leucocytes to schistosomes had been noted to be distinctly reduced if the antisera were heated for 30 minutes at 56°; addition of a 1 : 4 dilution of fresh guinea-pig serum restored the reactivity (Newsome, 1962). It appears from the above that the adhesion of leucocytes in several systems is enhanced by fresh serum but as in the present study identification of the thermolabile factors is lacking.

In studies of the adhesion of leucocytes to non-helminthic substrates (Henson, 1969), complement was found to enhance the adhesion of guinea-pig neutrophils and eosinophils to antibody-coated erythrocytes.

The almost complete inhibition of the cellular adhesion by EDTA and citrate confirms previous reports with microfilariae of *W. bancrofti* as the substrate for the eosinophils (Pandit *et al.*, 1929; Bang *et al.*, 1962). Furthermore, this inhibition is consistent with the possible cation-dependent leucocytic phagocytosis and intercellular adhesion by human and rabbit neutrophils (Allison and Lancaster, 1964). No information exists on the mechanism of the adhesion of eosinophils, but Bryant (1969) has found that selective removal of calcium ions did not prevent the adhesion of neutrophils to glass and to one another. However, the adhesion of eosinophils to the larvae differs from phagocytosis and adhesion to glass surfaces since in the present study, sodium azide had no effect on the former but is known to inhibit the latter (Nelson, 1965). In addition, heparin did not have any effect on the adhesion of eosinophils, although in another report (Bang *et al.*, 1962) it was stated to partially inhibit the reaction with microfilariae.

Betamethasone did have a significant inhibitory effect on the magnitude of the adhesion of eosinophils. The steroid may have acted by preventing the eosinophil from migrating and attaching to the parasite since these cells were not lysed by the steroid but have been found to be inhibited from migrating into skin windows by such drugs (Felarca and Lowell, 1969).

An unexpected finding was an enhancing effect on the adhesion of eosinophils by histamine, especially evident when normal serum was used. Although histamine had no effect in the absence of serum, it was not possible under the conditions of the experiment to determine if the amine had reacted with the larvae, the eosinophils, or the serum.

Despite the marked selectivity of eosinophils in adhering to the filarial larvae, the nature of the eosinophil's function(s) remains unknown. No apparent changes in the worm's general morphology were observed up to 24 hours incubation although others have reported that microfilariae (Pandit *et al.*, 1929) and adult schistosomes (Newsome, 1962) were killed as a result of leucocytic adhesions.

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