Mechanism of destruction of "Brugia malayi" microfilariae in vitro : the role of antibody and leucocytes

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Mechanism of destruction of *Brugia malayi* microfilariae in vitro: the role of antibody and leucocytes[1]

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Summary

Human and monkey leucocytes in serum from previously patent *Brugia malayi* infected monkeys adhered to and caused cytotoxicity to microfilariae of *B. malayi* in vitro. The phenomenon also occurred in human serum from confirmed cases of bancroftian and malayan elephantiasis. Adherence of leucocytes was not seen in serum from uninfected monkeys, monkeys with patent *B. malayi* infection and serum from rats infected with *Breinlia booliati* but now amicrofilaraemic. Immune serum failed to promote adherence of leucocytes to L_3 of *B. malayi.* The activity was lost when the serum was either incubated with Protein A-bearing *Staphylococcus aureus* or heat inactivated at 56° C for 30 min. Light and electron microscopic observations regarding the type of adhering cells and sequence of events following adherence are described.

Key words: *Brugia malayi;* microfilaria in vitro destruction.

Introduction

Amicrofilaraemia or the sudden disappearance of microfilariae in a patent infection has been observed in many filarial infections (Ramakrishnan et al., 1962; Weiss, 1970; Denham et al., 1972). The exact course of events which lead to such an occurrence is not fully understood.

Antibody dependent cell-mediated effector mechanisms in the immunity to parasitic helminths is being increasingly recognised (Dean et al., 1974; Sher et al., 1975; Butterworth et al., 1975; Perez and Smithers, 1977; Mackenzie et

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al., 1978; Vadas et al., 1980). Evidence that similar effector mechanisms are operational for the termination of microfilaraemia in filarial infections have been presented recently (Subrahmanyam et al., 1978; Tanner and Weiss, 1978). However, there has been neither consistency with regard to the class of antibody involved, nor to the type of the effector cells. It appears that these factors vary with respect to the animal models studied. The termination of microfilaraemia in albino rats with *L. carinii* has been shown to be an IgE dependent mechanism involving a mixed population of effector cells (Mehta et al., 1980). In *D. viteae* infected hamsters this phenomenon has been associated with IgM; the predominant effector cells being identified as neutrophils (Tanner and Weiss, 1978; Rudin et al., 1980). In human filarial infections, however, IgG fractions of sera from elephantiasis cases have been shown to promote killing of microfilaria by enriched eosinophils (Subrahmanyam et al., 1978).

In our laboratory we had embarked on longitudinal studies on the development of immune responses in rhesus monkeys following infection with *B. malayi*. In the hope that responses in monkeys would better mimic the human situation, as compared to smaller animals, we investigated the ability of peripheral leucocytes and antibodies to kill microfilariae of *B. malayi* using both light and electron microscopy.

Material and Methods

Culture medium

RPMI 1640 buffered with HEPES (N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (Flow Lab., USA) containing 100 units of penicillin per ml and 100 μg of streptomycin per ml was used.

Filarial infection

Brugia malayi (subperiodic type) infective larvae (L_3) were obtained from laboratory-bred *Aedes togoi* mosquitoes dissected 12 days after feeding on a wild-caught Malayan Long Tailed Monkey *(Macacca iris)* with a natural infection. Uninfected Indian rhesus monkeys *(Macacca mulatta)* were each inoculated with 100 infective larvae. Albino rats were infected with *Breinlia booliati* from infected *Aedes togoi* (Ho et al., 1976).

Preparation of microfilariae

Brugia malayi microfilariae were obtained from the peritoneal washings of experimentally infected jirds (McCall et al., 1973). The suspension, rich in microfilariae, was drawn into a syringe and passed through a millipore filter fitted with an 8 μm membrane. Microfilariae were then washed by passing sterile phosphate-buffered saline (pH 7.2) through the filter. The filter containing the microfilariae was dipped into culture medium at room temperature. The active microfilariae passed into the medium, while the host cells remained on the filter. The medium was centrifuged, the microfilariae resuspended in medium and counted in a haemocytometer. Microfilariae of *Breinlia booliati* were obtained by incubating adult worms, recovered from infected rats, in culture medium at 37° C.

Sera

Serum was obtained from normal, infected, mircofilaraemic and post-microfilaraemic monkeys (within 1 week after the animals became amicrofilaraemic). The serum was stored in small

aliquots at –20° C. Human sera from patients infected with *Wuchereria bancrofti* were obtained from Sri Lanka (Courtesy of Dr. S. Dissanayake).

Absorption of IgG by Protein A

Staphylococcus aureus containing Protein A (National Collection of Type Cultures No. 8532) was grown overnight in nutrient broth with added B-glycerophosphate. Thrice washed bacteria were treated with 0.5% formaldehyde for 3 h and heated at 80° C for 4 min. The bacteria were washed, resuspended in phosphate buffered saline (PBS), pH 7.2, and stored at 4° C until use. For the absorption procedure, the bacterial suspension was centrifuged and serum added in proportion of 1 volume of *Staphylococcus aureus* to 4 volumes of serum, and held at 4° C overnight.

Preparation of effector cells

Freshly drawn heparinised blood from normal and infected animals was mixed with plasmagel to sediment the erythrocytes. The leucocytes from the plasma layer were washed and resuspended in culture medium. Adherent cell populations were removed by incubating in plastic tissue culture flasks (Costar Inc., Cambridge, Mass., USA) at 37° C for 2 h. During this incubation period, the culture medium was supplemented with 10% heat inactivated fetal calf serum. The cells were washed twice in medium, counted in a haemocytometer and the cell concentration adjusted to 10^6/ml. In subsequent experiments requiring either enriched granulocytes or mononuclear cells, the cell suspension was layered on to Ficol-Isopaque and centrifuged (Boyum, 1968). Where only mononuclear cells were used for adherence assay, the step requiring incubation in plastic was omitted. The cells at the interface consisted of mononuclear cells (95–98% purity) while the pellet was mainly granulocytes (90% purity).

Adherence assay

The in vitro adherence assay was carried out in 38 × 7 mm sterile plastic tubes (Costar Inc., Cambridge, USA). Each tube contained a mixture of 0.1 ml of microfilariae, 0.1 ml of the appropriate serum sample (10% final dilution) and 0.1 ml of effector cells. The final volume was made up to 0.4 ml with medium. The microfilariae to cell ratio was maintained at 1:3000. Incubation was at 37° C in a CO_2 incubator, with occasional agitation. The assay was set up in triplicate. The degree of adherence was determined at different time intervals by pipetting a sample onto a slide and viewing it under a microscope. The test was considered positive when more than 5 cells were seen attached to a microfilaria. In some experiments L_3 of *B. malayi* were used instead of microfilariae. 100 L_3 were added to each assay tube. The L_3 to cell ratio was 1:4000. For transmission electron microscopic studies, ten times the concentration of microfilariae and effector cells were included in the cultures and the total volume was 1 ml, the final serum concentration remaining at 10%.

Transmission electron microscopy

Cultures were centrifuged (500 *g* for 10 min) into a pellet and the supernate was removed. Cold 5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2) was layered onto the pellet. Then, 2 to 3 min later the pellet was gently broken up by repeated pipetting to obtain better penetration of the fixative. After fixation overnight at 4° C, the specimens were washed in two changes of 0.1 M PBS (pH 7.2) and were post fixed in 2% osmium tetroxide in 0.1 M phophate buffered saline for 2 h at 4° C. The samples were dehydrated in single changes (15 min) of 25%, 50%, 75%, 90% ethanol and 3 changes of absolute alcohol followed by one 15 min change of propylene oxide. Embedding in low viscosity epoxy resin was done by the procedure of Spurr (Spurr, 1969). Ultra thin sections were cut on a Sorval Porter-Blum MT-26 ultramicrotome with a glass knife and mounted on clean copper grids covered with formvar film coated with carbon. The sections were double stained with alcoholic uranyl acetate (10 min) followed by Reynolds lead citrate (10 min).

Table 1. Adhesion of peripheral blood leucocytes to microfilariae of *B. malayi*

Type and source of cells	Source of serum for incubation	Microfilariae (%)* with adherent cell (+S.D.)	
		6 h	16 h
Monkey PBL	Uninfected monkey	2 ± 2.6	2 ± 2.6
	Inoculated, amicrofilaraemic monkey	4 ± 3	6 ± 2.4
	Microfilaraemic monkey	6 ± 1.8	6 ± 2
	Post-microfilaraemic monkey	48 ± 2.6	95 ± 2.4
Monkey mononuclear cells	Post-microfilaraemic monkey	2 ± 3	4 ± 3
Monkey granulocytes	Post-microfilaraemic monkey	60 ± 2.6	91 ± 2.2
Human granulocytes	Post-microfilaraemic monkey	56 ± 1.8	95 ± 3
Rat granulocytes	Post-microfilaraemic monkey	45 ± 2.4	88 ± 2.6

* Percentages expressed as number per 100 microfilariae counted.

Results

Sera from monkeys which had lost circulating microfilariae (post-microfilaraemic) caused intense adherence of peripheral blood leucocytes (PBL) to the microfilariae of *B. malayi*. In contrast, adherence of PBL was absent with sera from uninfected monkeys, microfilaraemic monkeys infected with *B. malayi*, and monkeys which did not become infected despite repeated inoculations (Table 1). Post-microfilaraemic serum (subsequently termed immune serum) alone, in the absence of PBL, affected neither the viability nor the motility of microfilariae incubated for a period of 24 h. The addition of PBL, however, resulted in adhesion of cells to the microfilariae within the first 45 min of incubation (Fig. 1). The number of cells adhering increased with time and by 16 h the microfilariae became covered with clusters of PBL (Fig. 2). Increased adherence was usually associated with decreased motility and vacuolation of the microfilariae. At 16 h, most of the microfilariae became immobilised and granular. However, approximately 5%, although completely covered with cells, remained alive even after 24 h of incubation.

Purified mononuclear cells (Table 1) failed to adhere. Enriched granulocytes, however, adhered in large numbers. In Giemsa stained preparations, neutrophils (approximately 75%) were seen to be the predominant adhering cells followed by eosinophils.

Heating immune serum at 56° C for 30 min significantly inhibited the adhesion phenomenon (Table 2). The activity could be restored, however, by the addition of fresh normal serum. Immune serum incubated overnight with *Staphylococcus aureus* containing Protein A completely abrogated adhesion. This suggests that the adhesion promoting factor is IgG.

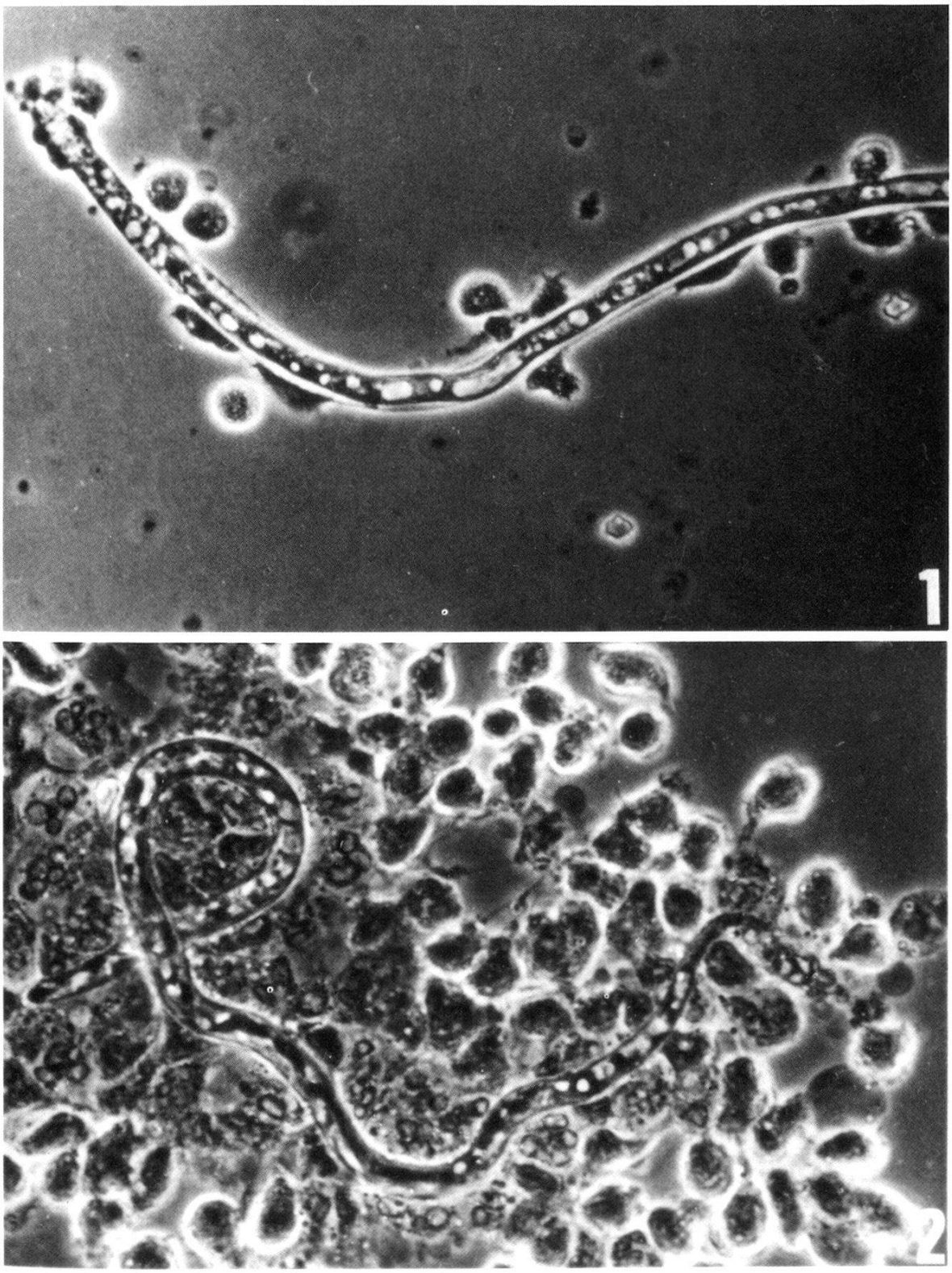

Fig. 1 und 2. Phase contrast micrographs: (1) After 4 h incubation with immune serum the adhering cells flatten against the sheath of the microfilaria. Magnification × 1,000. (2) After 12 h incubation the microfilaria become trapped within clusters of granulocytes. Magnification × 1,000.

Table 2. The role of complement and nature of immunoglobulin in adherence

Treatment of immune serum	*B. malayi* microfilaria (%) with adherent cells (±S.D.) at 16 h incubation
Unheated	96±1.8
Heat-inactivated (56° C for 30 min)	18±3
Heat-inactivated and fresh normal serum	76±3
Absorbed with *Staphylococcus aureus*	9±2.6

Percentages expressed per 100 microfilariae counted.
Monkey granulocytes were the effector cells.

The specificity of the reaction is shown in Table 3. Immune serum, sera from patients with either bancroftian or brugian elephantiasis could promote adhesion to microfilariae of *B. malayi* but not to microfilariae of *B. booliati.* On the other hand, immune serum from rats infected with *B. booliati* promoted adhesion of PBL to microfilariae of *B. booliati.*

Immune serum from monkeys did not promote adhesion of PBL to L_3 of *B. malayi* (Table 3). L_3 remained active without any observable changes in morphology even after 16 h incubation with immune serum and PBL.

Phase contrast and transmission electron microscopy

The sequence of events following attachment was studied by both phase contrast and electron microscopy. The cell became attached to the surface of the sheath of the microfilaria, the plasma membrane of the cell fitting closely against the contour in the form of a template. The granulocyte then flattened against the sheath and spread intimately over the surface (Figs. 1 and 3). With time, morphological changes were observed in the microfilariae. Under the phase contrast microscope that part of the worm with adherent cells showed vacuolation of the internal structures. Corresponding degenerative changes were observed under the electron microscope. The internal structures appeared to undergo lysis with accumulation of material interposed between the sheath and cuticle. Damage to both sheath and cuticle was evident (Fig. 4). The greater the degeneration of the microfilaria, the greater was the amount of material

Fig. 3 and 4. Electron micrographs of cell adhesion after six hours of incubation with immune serum: (3) Cross section showing two neutrophils attached to the sheath, completely surrounding the microfilaria. The adherent neutrophils have degranulated. The internal morphology of the microfilaria appears normal. Magnification ×13,200. (4) Advanced stage of degeneration of the microfilaria. The internal tissues of the microfilaria are almost completely lysed, with corresponding accumulation of electron dense material between the sheath (s) and cuticle (c). Rupture of the sheath and cuticle evident (★). Magnification ×26,000.

Fig. 5. Electron micrograph showing a longitudinal section of a microfilaria (mf) with an adherent degenerate eosinophil (e). Following cell rupture, the granules (g) are released on to the sheath (s) of the microfilaria. After 6 h of incubation with immune serum. Magnification ×9,000.

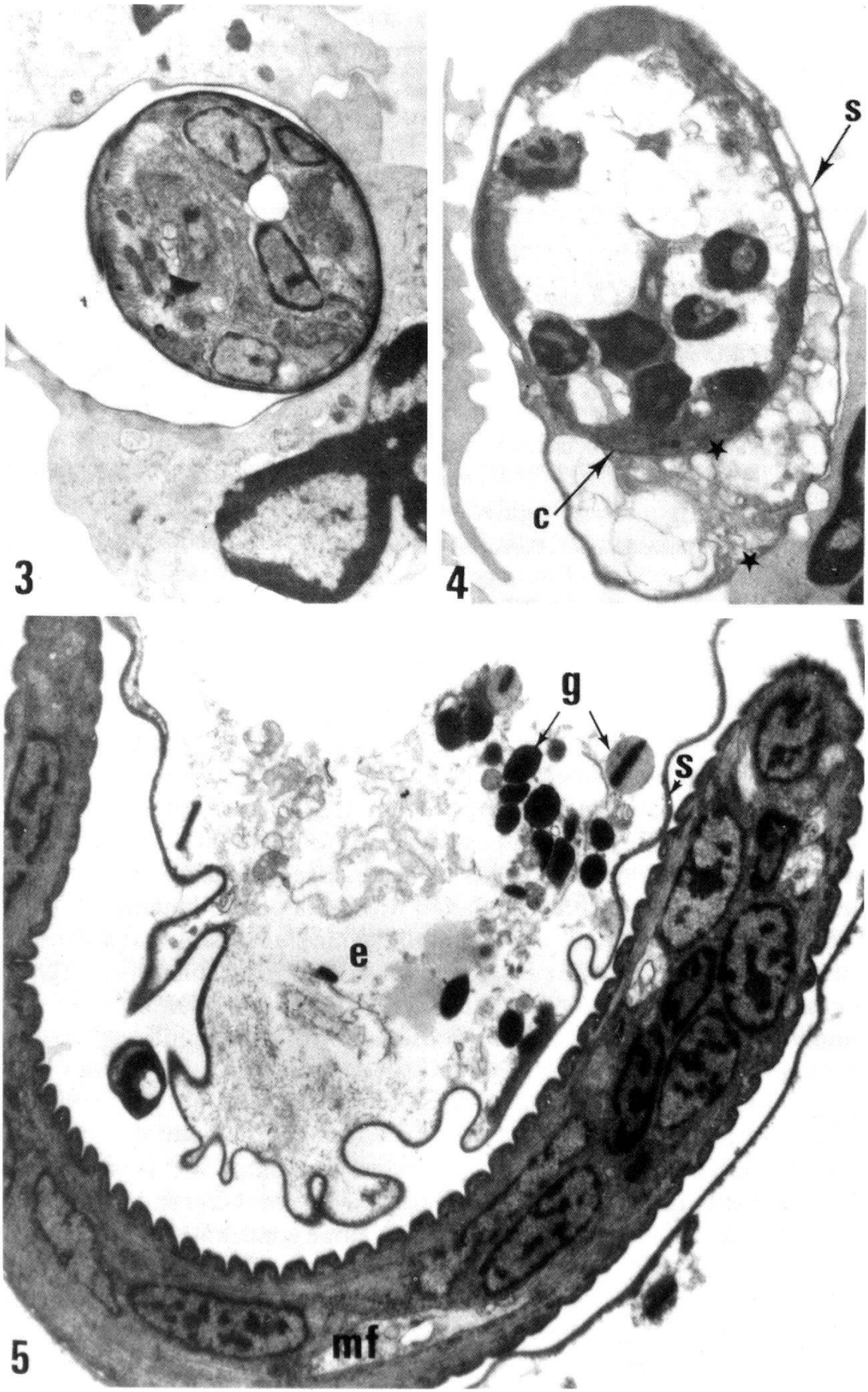
3
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Table 3. Specificity of the adherence reaction

Source of serum	Filarial larvae (%)[a] with adherent cells at 16 h post incubation (+S.D.)		
	B. malayi microfilaria	*B. booliati* microfilaria	*B. malayi* L_3
Uninfected monkey	4±3.1	4±2.6	2±1.3
Immune monkey	96±1.8	2±3	1±4.2
Rabbit immunized[b] against mf of *B. malayi*	100±0	4±1.8	5±1.7
Human elephantiasis (W. bancrofti)[c]	94±2.6	2±2.6	N.D.
Human elephantiasis *(B. malayi)*[c]	94±2.6	1±1.4	N.D.
Immune rat	1±1.8	86±3.1	N.D.
Normal rat	4±3	4±3	N.D.

[a] Percentage expressed per 100 microfilariae/L_3 counted, each value representing a mean of 6 serum samples from each group.
[b] Only two samples were tested.
[c] All human sera were positive for microfilarial antibodies (titre 1:128) as detected by the immunofluorescent test.

Monkey granulocytes were the effector cells.
N.D. = Not done.

accumulated. Neither penetration of cellular processes into the microfilariae nor cells with phagocytized structures of the worms were seen.

Under the electron microscope, the predominant effector cell type was seen to be the neutrophil, followed by the eosinophil. Adherent cells, whether neutrophils or eosinophils, were seen to be in various stages of degranulation. The sequence of events leading to degranulation was similar to both neutrophil and eosinophil. One striking feature of eosinophil degranulation was that a sizeable number of cells were seen to be destroyed on or after degranulation (Fig. 5). This was especially marked on 4 h of incubation with immune serum. However, such autolysis on degranulation was not observed with neutrophils.

Discussion

In filarial infections, microfilarial remission is accompanied by the detection of antibodies to the surface of microfilariae (Wong and Guest, 1969; Weiss, 1978). However, evidence that these antibodies alone contribute to the elimination of microfilariae, in a patent infection, is lacking. The in vitro studies in *B. malayi* infected monkeys reported here suggest that antibody dependent cell-mediated mechanisms could be responsible for the amicrofilaraemic state. In the presence of serum from monkeys which had lost circulating microfilariae, PBL adhered to and killed microfilariae of *B. malayi*. Sera from uninfected

monkeys and inoculated but uninfected monkeys did not promote such adhesion. This confirms similar phenomenon reported in *D. viteae* infections in hamsters (Tanner und Weiss, 1978; Rudin et al., 1980), *L. carinii* infections in rats (Mehta et al., 1980) and in human bancroftian elephantiasis (Subrahmanyam et al., 1978).

The role of immunoglobulin in promoting in vitro adhesion in filarial infections has now been established (Mehta et al., 1978; Tanner and Weiss, 1978; Haque et al., 1981). What is interesting is that the type of antibody involved appears to vary with the different animal model studied. While IgM is implicated in hamsters (Tanner and Weiss, 1978) and IgE in rats (Mehta et al., 1980; Haque et al., 1981), IgG apparently is responsible for opsonic properties in humans (Subrahmanyam et al., 1980). Protein A from *Staphylococcus aureus* is known to bind non-specifically to the F_c portion of IgG (Forsgren and Sjoquist, 1966). In our experiments, pre-incubation of immune serum abrogated the ability of PBL to adhere to microfilariae in vitro indicating that the adhesion promoting factor was IgG. The monkey being closer to man in the evolutionary ladder, probably mimics the human situation. Work done with human and baboon systems in schistosomiasis also point to IgG as the cytophilic antibody (Anwar et al., 1979).

Heating immune serum at 56° C for 30 min significantly reduced the number of microfilariae with attached cells. Activity could be restored, however, by the addition of fresh normal serum. Since adhesion reaction is IgG mediated, the non-specific heat labile factors required for adhesion could be complement components. Requirement of complement for an IgG dependent neutrophil mediated cytotoxicity has been reported in experimental schistosomiasis (Dean et al., 1974, 1975). Neutrophil was the predominant effector cell in our experiments, as seen both by light and electron microscopy. Receptors for complement and IgG have been demonstrated on human neutrophils and eosinophils (Rabellino and Metcalf, 1975; Anwar and Kay, 1977). The density of neutrophil receptors for IgG and complement, as shown by either rosette technique or immunofluorescence, was approximately two and a half times greater than in eosinophils (Kay, 1976). This could explain the requirement of complement for IgG dependent adherence involving neutrophils. On the other hand, eosinophil-mediated reactions are complement independent (Kay, 1976).

Electron micrographs confirmed that the predominant adhering cell type was the neutrophil, although eosinophils were also involved. The percentage of eosinophils adherent to microfilariae reflected the original ratio of eosinophils to neutrophils in the test system. In *B. malayi* infected monkeys, amicrofilaraemia is not usually accompanied by eosinophilia (Wong et al., 1977). In recent years in vitro work with purified eosinophils point to a pre-eminent role for these cells in parasite killing. In the light that neutrophils also contribute to in vitro killing of parasites, the importance of neutrophils in parasite immunity has probably been underestimated.

The sequence of events following adhesion, of either neutrophils or eosinophils, can be summarized as follows: after the cells flatten and spread intimately on to the sheath of the microfilaria, they degranulate, the secretion granules fusing to form larger electron dense bodies. Degenerative changes of the cuticle and internal tissue accompany such attachment and degranulation. A comparable sequence of events following antibody dependent adherence involving both neutrophils and eosinophils have been described for other parasitic helminths (Morseth and Soulsby, 1969; Dean et al., 1975; McLaren et al., 1977; Glauert et al., 1978; Rudin et al., 1980). The secretion granules of both neutrophils and eosinophils are rich in a variety of enzymes (Becker and Henson, 1973). Neutrophils release both alkaline phosphatase and myeloperoxidase while eosinophil secretions are rich in peroxidase, major basic protein and cationic proteins (Kay, 1976). The lytic changes seen in the structures of the microfilaria after degranulation of the leucocytes suggest that the damage to microfilariae could be brought about mainly by a combination of these enzyme-rich contents of the granules. Like Rudin et al. (1980), we did not observe the invasion of internal tissues by pseudopodia and processes of leucocytes after cuticular damage similar to those reported for the larvae of ascarids and schistosomes.

The inability of immune serum to cause adhesion of leucocytes to L_3 is puzzling. This is probably suggestive of stage specific immunity in filariasis. Stage specific antibody dependent cell-mediated destruction has been reported in *Trichinella spiralis* infections (Grove and Kazura, 1978). Recently, it has been reported that immune serum did not promote adhesion of hamster cells to L_3 of *D. viteae*, both in vivo as well as in vitro situations (Tanner and Weiss, 1981).

The role antibody dependent cell-mediated mechanisms play in the intact animal in filarial immunity is not clear. Microfilariae are very actively motile larvae. In all probability such mechanisms may serve to trap circulating microfilariae making it easier for their destruction and elimination by the lymphoid organs.

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