Zeitschrift: Acta Tropica
Herausgeber: Schweizerisches Tropeninstitut (Basel)
Band: 42 (1985)
Heft: 2

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DOI: https://doi.org/10.5169/seals-313465

Acta Tropica *42*, 157–163 (1985)

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Activation of the alternative complement pathway in normal human serum by *Loa loa* and *Brugia malayi* infective stage larvae

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Summary

Infective stage larvae (L3) of *Loa loa* and *Brugia malayi* upon in vitro incubation with normal human serum activated the alternative complement pathway. C3 conversion products were detected on larval cuticles by eosinophil adherence and by immunofluorescence with C3c antiserum. No evidence for cuticle binding of IgG, IgA, IgM, Clq, or C4 was found by immunofluorescence. L3-induced C3 activation was inhibited by 10 mM EDTA but unaffected by 10 mM Mg^{++}-EGTA. Human sera deficient in C2, C4, or C6 incubated with L3 resulted in C3 activation. However, sera treated with zymosan or heated for 1 h, 56° C were unreactive with L3. Immunoelectrophoresis of fresh serum exposed to L3 for 1 h at 37° C showed C3 cleavage products. The results indicate that these nematode L3 activate the alternative complement cascade via cuticular surface components. Larval viability was unaffected by complement activation or by adherence of eosinophils.

Key words: *Loa loa; Brugia malayi;* eosinophil adherence; complement; alternative pathway.

Introduction

The helminthocytotoxic activity of eosinophils, neutrophils, and macrophages has been under active investigation in recent years (Capron et al., 1982; Joseph, 1982). The adherence to parasite surfaces by these cells appears to be mediated by specific antibodies through Fc cell receptors or by C3b receptors

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after classical complement activation (McLaren and Ramalho-Pinto, 1979; McKean et al., 1981; Capron et al., 1982). Additionally, numerous helminthic parasites (particularly larval stages) will induce the activation of the alternative complement pathway. The helminths known to active complement directly represent all classes including the cestodes *Taenia taeniaformis* (Hammerberg and Williams, 1978), *Echinococcus granulosus* (Rickard et al., 1977); the trematode *Schistosoma mansoni* (Machado et al., 1975; Sher, 1976); and the nematodes *Nippostrongylus brasiliensis*, *Trichinella spiralis* (Mackenzie et al., 1980), *Trichostrongylus vitrinus* (Stankiewicz et al., 1981), *Dirofilaria immitis* (Staniunas and Hammerberg, 1982), *Acanthocheilonema (Dipetalonema) viteae* (Haque et al., 1982) and *Brugia malayi* (Piessens and Dias Da Silva, 1982). Activation of the alternative complement pathway was followed by leukocyte adherence and subsequent parasite death in some instances. The combination of antibody-binding with or without classical complement activation and/or independent C3 activation is thought to promote leukocyte adherence in vitro thus constituting the primary protective mechanisms against invading helminthic larvae.

The present paper demonstrates that infective stage larvae (L3) of *Loa loa* and *B. malayi* readily activate the alternative complement pathway in normal serum and that this cascade of events promotes eosinophil adherence to cuticle surfaces.

Materials and Methods

Laboratory maintenance of the entire life cycle of *L. loa* outside endemic areas is described elsewhere (Orihel and Lowrie, 1975; Orihel and Moore, 1975). *L. loa* L3 were cryopreserved in liquid nitrogen as described by Lowrie (1983), then packed on dry ice and transported over night from Covington, Louisiana to Ann Arbor, Michigan. Upon their arrival they were stored in liquid nitrogen until needed. Thawed larvae were washed three times in medium by centrifugation. Larvae exhibiting normal motility and structural integrity were individually transferred using a fine dissecting pin, into tissue culture chambers or tubes containing 100 μl of medium. Subperiodic *B. malayi* L3 were harvested from experimentally infected *A. aegypti* using the technique of Ash and Schacher (1971). Normal human sera were obtained from 8 healthy adult volunteers (6 males and 2 females). Fluorescein-conjugated antisera to Clq, C4, and C3c were obtained from Accurate Chemical and Scientific Corp., Westbury, New York while fluorescein-conjugated antiserum to IgG, IgA, and IgM were obtained from Miles Laboratories, Inc., Elkhart, Indiana. Heat inactivation of complement was achieved by maintaining serum at 56° C for 1 h. Serum was also treated with zymosan, Sigma Chemical Co., Saint Louis, Missouri (15 mg/ml, 37° C, 30 min) to deplete the alternative complement cascade system.

Medium and chelating agents

HEPES-buffered RPMI 1640 medium containing 100 IU/ml penicillin and 100 μg/ml streptomycin was used for washing the larvae and in the adherence assay. Stock solutions (0.1 M, pH 7.2) of the chelating agents ethylenediaminetetraacetic acid (EDTA) and ethylene glycol-bis-N,N-tetraacetic acid (EGTA) were prepared in phosphate-buffered saline and used at a final concentration of 10 mM. The stock EGTA solution also contained 20 mM $MgSO_4$. Sera from C2, C4, and C6 deficient patients were provided by Dr. S. L. Kunkel, Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan.

Eosinophil-enriched leukocyte suspension

A metrizamide gradient was used to isolate an eosinophil enriched (>95%) leukocyte fraction from normal human peripheral blood (Vadas et al., 1979).

Adherence assay

The assay was performed in Lab-Tek (Miles Laboratories, Naperville, Illinois) flat bottomed slide chambers. To each well was added 100 μl of medium, 5 to 10 L3, 100 μl of serum, and 100 μl of eosinophil enriched cell suspension (adjusted to provide a cell-target ratio of 3×10^4 to 1), consecutively. The chambers were incubated at 37° C, 5% CO_2 for up to 48 h. Adherence of eosinophils and larval motility were evaluated at 6–, 24–, or 48 h with the aid of an inverted microscope. A larva was scored positive for cellular adherence if 10 or more eosinophils were distributed along its surface.

Solid-phase protein A adsorption

Immunoglobulin G was depleted from serum by treatment with staphylococcal protein A (IgGsorb, The Enzyme Center, Inc., Boston, Massachusetts). A 10:1 ratio (vol/vol) of protein A cell suspension to serum was incubated for 45 min at 4° C with frequent agitation. The mixture was centrifuged and the adsorbed serum was tested for adherence promoting activity.

Fluorescent antibody assay

Larvae were incubated in 12×75 mm polystyrene tubes containing each serum preparation, for 30 min at 37° C, washed 3 times by centrifugation in large volumes of medium then incubated for 1 h at 4° C in fluorescein-labeled antiserum. After an additional 3 washes in cold medium the larvae were examined with a Leitz epiilluminated UV microscope.

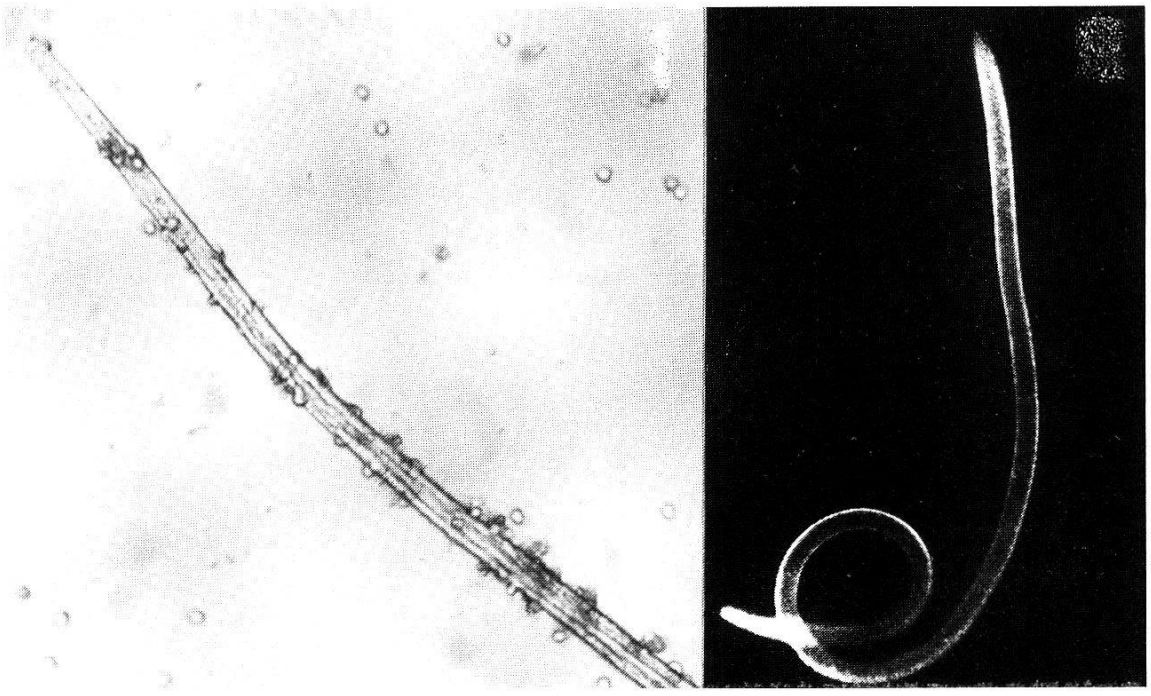

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Fig. 1. *Loa loa* L3 with adherent eosinophils after 6 h incubation in fresh normal serum containing 3×10^4 eosinophils per larva. $\times$ 105.

Fig. 2. *Loa loa* L3 opsonized with C3 after incubation in fresh normal human serum and fluorescein-conjugated goat anti-human C3c. $\times$ 70.

Table 1. The effect of serum treatments on adherence of eosinophils to *L. loa* and *B. malayi* L3

Human serum	Treatment			
	Fresh	Heated	Protein A	Zymosan
1	+	−	+	−
2	+	−	+	−
3	+	−	+	−
Pool	+	−	+	−

Table 2. Effect of serum treatment on binding of C3 activation products to *L. loa* and *B. malayi* L3 assayed by immunofluorescence

Human serum	Anti-C3c	Serum treatment		
	Fresh	Heated	EDTA	Mg^{++}EGTA
1	+	−	−	+
2	+	−	−	+
3	+	−	−	+
4	+	−	−	+
5	+	−	−	+
Pool	+	−	−	+
$-C_2$	+	−	ND*	ND
$-C_4$	+	−	ND	ND
$-C_6$	+	−	ND	ND

* ND = Not done

Conversion of C3

Immunoelectrophoresis in 1% agarose using goat anti-human C3 antiserum (Miles Laboratories) was employed to detect C3 conversion. Approximately 3,400 *L. loa* L3 were incubated in 200 μl of normal human serum at 37° C for 1 h. The larvae were pelleted by centrifugation and the supernatant serum was tested. As a control, an aliquot of normal serum without larvae was incubated for 1 h at 30° C and simultaneously evaluated with the larval treated serum by electrophoresis.

Results

The effect of IgG depletion (solid-phase protein A adsorption), complement depletion with zymosan, or heating of serum at 56° C for 1 h on the adherence of normal eosinophils to *L. loa* or *B. malayi* L3 was evaluated in three replicates of an in vitro adherence assay. Using the criterion of at least 10 adherent eosinophils, all larvae in normal sera and protein A-treated sera were positive for adherence (Fig. 1). Many larvae had more than 50 adherent cells while larvae in zymosan-treated or heated serum did not promote adherence (Table 1).

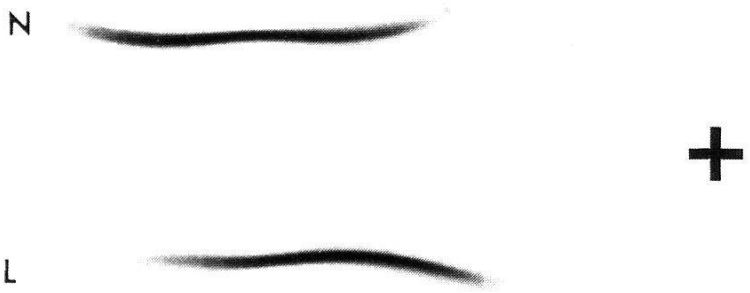

Fig. 3. Immunoelectrophoresis patterns of normal human serum (N) and serum treated with *L. loa* (L) for 1 h at 37° C. The trough contained goat antihuman antiserum.

These findings suggested that, in the absence of specific antibodies, adherence of eosinophils to L3 may have been mediated by activation of the alternative complement pathway. Direct evidence in support of this hypothesis was obtained in a series of immunofluorescence studies on L3 pretreated with fresh-, heated-, complement deficient-, or chelator-treated sera (Table 2). Larvae pretreated in fresh normal sera, or C2, C4, or C6 deficient sera fluoresced brightly with fluoresceinated anti-human C3 (Fig. 2). The addition of EDTA to fresh serum, inhibited the reaction while addition of Mg^{++}-EGTA had no effect. Human IgG, IgA, IgM, Clq, and C4 were not detected on the larvae by immunofluorescence after incubation of L3 with normal human sera or with the complement-deficient sera.

Immunoelectrophoresis of fresh human serum after incubation with larvae demonstrated that C3 had been degraded into faster migrating subunits (Fig. 3).

Discussion

These studies demonstrate that *L. loa* and *B. malayi* infective stage larvae activate the alternative complement pathway in normal serum in vitro. The data further show that activated C3 binds to larval surfaces promoting the adherence of eosinophils. Human eosinophils have been shown to possess surface receptors for both C3b and C3d (Gupta et al., 1976). It is unlikely that antibodies played a role in this phenomenon since IgG, IgA, and IgM were not demonstrable on the larvae by immunofluorescence and larvae incubated in serum which had been depleted of IgG promoted cellular adherence. Furthermore, adherence was abolished by serum treatments known to inactivate complement.

Complement-mediated leukocyte adherence to L3 of another filaria, *A. viteae*, has been demonstrated using normal rat and human serum but not

with normal hamster serum (Hague et al., 1982). These authors proposed that activation of the alternative complement pathway in serum from nonpermissive hosts (i.e. rats and humans) might be an important mechanism in natural resistance to filariae. Other workers have shown that *A. viteae* L3 do not activate the alternative complement pathway in normal sera from mice or jirds (Gass et al., 1979; Tanner and Weiss, 1981). However, it has since been shown that the intravascular, microfilaria stage of *B. malayi* and *D. immitis* activate complement in normal serum from their normal definitive hosts (Piessens and Dias Da Silva, 1982; Staniunas and Hammerberg, 1982). In light of our observation that *L. loa* and *B. malayi* L3 can also activate complement in sera from their normal host, we suggest that species differences in the functional potency of the alternative complement pathway (Gewurz et al., 1968) could explain some of these findings from the various hosts studied. Adherent eosinophils did not kill the L3 of either filarial species tested in the present work. The larvae exhibited typical motility and apparent structural integrity even after 48 h incubation. Absence of larval killing by eosinophils may have been a result of sub-optimal cell:target ratio and/or the apparent need for replenishment with fresh eosinophils (Haque et al., 1982). Thus the role of this phenomenon in resistance to infection or possibly in evasion of further recognition of the parasite by the host remains to be determined.

The mechanisms by which filariae activate complement have not been fully elucidated. However, Staniunas and Hammerberg (1982) have presented evidence that microfilariae release a complement activating substance into culture medium that is structurally related to polyanionic carbohydrates known to occur in the cuticle of filariae. These workers suggested that complement may be activated directly by virtue of the polyanionic nature of the molecules. An analogous mechanism for complement activation by infective stage larvae warrants investigation.

Acknowledgments

Supported in part by a grant from the Filariasis Component of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and by grant RR 00164–20, Division of Research Resources, NIH. Some materials used in this study were provided by the U.S.-Japan Cooperative Medical Science Program-NIAID.

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