

COMPLEMENT-MEDIATED KILLING OF SCHISTOSOMULA
OF *SCHISTOSOMA MANSONI*
BY RAT EOSINOPHILS IN VITRO

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Recently there has been growing evidence for the role of the eosinophil in the effector mechanism of immunity to reinfection with schistosomes. Mice immune to *Schistosoma mansoni* are no longer able to resist reinfection after treatment with anti-mouse eosinophil serum (1). In vitro studies using human serum and eosinophils (2) or rat serum and cells (3), have demonstrated antibody-mediated damage to schistosomula by eosinophils. These cells adhere to IgG-coated schistosomula by Fc receptors (3), and peroxidase, from the matrix of the eosinophil granule, is secreted onto the surface of the worm (4).

Eosinophils have been shown to possess C3 receptors in addition to Fc (5, 6), and schistosomula are known to activate complement by the alternative pathway, binding C3 to their surface (7). It seemed appropriate, therefore, to investigate the adherence of rat eosinophils to schistosomula through the C3 receptor, and to monitor the effects of this interaction.

Materials and Methods

Parasite Cycle and Preparation of Schistosomula. A Puerto Rican strain of *S. mansoni* was maintained in laboratory bred *Biomphalaria glabrata* and outbred Parkes mice, as described elsewhere (8).

Schistosomula were prepared in vitro from cercariae by a mechanical method (9). Briefly, cercariae freshly shed from snails were concentrated by addition of penicillin-streptomycin, followed by chilling and spinning at 1,000 rpm for 15–30 s. 1 ml of deionized water was added to the pellet and the suspension was whirled in a Vortex mixer (Scientific Industries, Inc., Bohemia, N. Y.) for 1 min. This effected the rupture of tails from bodies, which were afterwards separated by sedimentation in Hanks' balanced salt solution. The cercarial bodies were then incubated at 37°C in RPMI-1640 (Flow Labs. Ltd., Ayrshire, Scotland) and 20 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethane sulfonic acid (Hepes)¹ for 3 h. The schistosomula were used on the day of preparation. Formalin-fixed schistosomula were prepared as previously described (10).

5-Day schistosomula were recovered from the lungs of CBA mice after exposure to 1,000

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¹ Abbreviations used in this paper: BSA, bovine serum albumin; EGTA, ethylene glycol tetraacetic acid; FCA, Freund's complete adjuvant; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid; IRS, immune rat serum; NRS, normal rat serum; S. D., Sprague-Dawley rats; VBS, Veronal-buffered saline.

cercariae. The method was performed as described previously (11) except that RPMI-1640, 20 mM Hepes pH 7.2 was used as the incubation medium.

Animals and Sera. Outbred female Sprague-Dawley (S.D.) and inbred female August rats were obtained from the National Institute for Medical Research, Mill Hill colony; they weighed 180–200 g each. S.D. rats exposed percutaneously to 500 cercariae of *S. mansoni* (8) 7–8 wk previously, were the source of immune rat serum (IRS). Preparation of IRS or of serum from normal S.D. rats (NRS) was as described previously (3). The sera were dispensed in small aliquots and stored in liquid N₂.

Preparation of Eosinophil-Rich Cell Populations. Normal August rats were killed with ether. Using sterile techniques, 20 ml of RPMI-1640 containing 20 mM Hepes buffer, pH 7.2, and heparin (25 IU/ml) was injected slowly into the abdominal cavity of each rat. The abdomen was massaged for 1 min and the fluid and cells were withdrawn, transferred to a 90-mm diameter plastic Petri dish, incubated at 37°C for 5 min, and then transferred to a new Petri dish. Incubation and transfer into fresh dishes was repeated three to four times until the eosinophils, counted with Discombes fluid (12), accounted for 50–60% of the total population of leucocytes. Before use in the assay system, the cells were washed twice in RPMI-1640 containing 20 mM Hepes and without heparin.

Culture Conditions and Cell Adherence Assay. These are basically as described previously (3), but inactivated fetal calf serum and heparin were omitted from the incubation medium. Briefly, 100- μ l aliquots of a suspension containing 100–150 schistosomula in RPMI-1640 were transferred into the wells of a flat-bottomed microtiter plate. 100 μ l of serum sample, at the appropriate dilution, followed by 100 μ l of an eosinophil-rich population of cells were added to each well. The final eosinophil:schistosomulum ratio was 3,000–5,000:1, except in experiments designed to determine the minimum number of cells necessary for killing. The scoring of cell adherence from + to ++++ was as previously described (3). The number of dead worms was estimated by direct visual observation and by the methylene blue dye exclusion test. Chromotrope 2R staining (13) was used to visualize and identify eosinophils.

Chemicals and Immunological Reagents. The requirements for Mg⁺⁺ and Ca⁺⁺ were investigated using the chelating agents EDTA and ethylene glycol tetraacetic acid (EGTA) at a final concentration of 10 mM (14). Zymosan A (Sigma Chemical Co., St. Louis, Mo.), at a final concentration of 15 mg/ml (5), was used to activate and deplete serum of complement through the alternative pathway.

Fluorescence Assay. About 500 schistosomula, in 50 μ l of RPMI-1640, 20 mM Hepes, and 1% bovine serum albumin (BSA) was incubated with 50 μ l of rat serum for 15 min at 37°C. After washing three times with RPMI-BSA, the schistosomula were incubated with 20 μ l of rabbit IgG anti-rat C3, or rabbit anti-rat IgG (Miles Laboratories Ltd., Stoke Poges, England) for 20 min at 4°C. After washing again in RPMI-BSA, 10 μ l of 1:3 fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories, Inc., Downingtown, Pa.) was added and the preparation was incubated for 20 min at 4°C. After washing with RPMI-BSA, the schistosomula were mounted on microscope slides and examined with a Leitz Orthoplan fluorescence microscope (E. Leitz, Inc., Rockleigh, N. J.).

Chemotaxis Assay. A modification of the Boyden chamber (15) was used to assess migration of cells in response to chemotactic factors generated during the interaction of schistosomula with NRS. The plunger of a 2-ml disposable syringe (Sabre; Gillette Surgical, Isleworth, England) was adapted by sawing off the sealed end to within 15 mm of the flanged open end. Millipore membranes with 0.65- μ m pore sizes were attached to this flanged end by wetting the flanges with chloroform to make them tacky. Each membrane chamber was placed in one of the wells of a flat-bottomed, 24-well disposable-tray (3008; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). 300 μ l of the sample to be tested for chemotactic activity was added to the well and 0.2 ml of cell suspension containing 1.5×10^7 cells was added to the chamber. Duplicate sample trays were incubated at 37°C in an atmosphere of 5% CO₂ for 3 h. The membranes were then washed carefully in RPMI-1640, fixed in methanol for 5 s, and washed in distilled water. Samples were stained in chromotrope 2R for 30 min, dehydrated in ethanol, cleared in xylene, and mounted in Drabkin's Solution (R. A. Lamb, London, England) according to the schedule of Boyden (15). Chemotaxis was evaluated by counting the number of cells which had migrated to the far side of the membrane in 10 randomly examined microscope fields.

Electron Microscopy. Schistosomula with attached cells were prepared for ultrastructural examination as described elsewhere (4). Briefly, the specimens were fixed sequentially in glutaraldehyde, osmium tetroxide, and uranyl acetate; they were then dehydrated in ethanol, and embedded in Araldite. Sections were stained with uranyl acetate and lead citrate and examined in a Philips-300 electron microscope (Philips Industries, Eindhoven, The Netherlands).

Preparation of Rabbit Anti-Rat C3. We used the method of Dr. Peter Lachmann (The Medical School, Cambridge, England) where activation of C3 is accomplished with inulin in the presence of Mg⁺⁺ and EGTA to prevent activation of C2 and C4 and also to avoid Ig binding.

Fresh S.D. rat serum containing 10 mM EGTA and 10 mM MgCl₂ was added to an equal volume of inulin (5 mg/ml) in Veronal-buffered saline (VBS). The mixture was incubated at 37°C for 30 min, washed first with cold VBS and then with 2 M NaCl followed by phosphate-buffered saline. Before injection into rabbits, the material was emulsified with an equal volume of Freund's complete adjuvant (FCA). The rabbits received two weekly injections, in different legs, of 1 ml of inulin-rat C3 FCA. After 6 wk the animals were bled weekly and the rabbit anti-rat C3 activity was tested by immunoelectrophoresis.

Rabbit IgG anti-rat C3 was purified by affinity chromatography using a Sepharose-4B-Protein A (*Staphylococcus aureus*) (Sepharose 4B; Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) column as previously described for the purification of mouse serum (16).²

Results

Eosinophil Adherence and Killing of Schistosomula. Schistosomula were incubated at 37°C in serial dilutions of fresh NRS and an eosinophil-rich cell suspension. After 1 h the schistosomula were found to be covered with cells. Specific staining with chromotrope 2R, showed that the adherent cells were mainly eosinophils. Adherence was also observed with heat-inactivated IRS, and it followed the same pattern as described elsewhere (3). Cell attachment did not occur at any time up to 18 h in control cultures containing eosinophils, heat-inactivated NRS, and schistosomula. Similar results were observed when formalin-fixed schistosomula were substituted for living schistosomula in the adherence assays. No adherence of eosinophils could be demonstrated either in fresh NRS or in heat-inactivated IRS, when 5-day schistosomula recovered from the lungs of mice were used as targets.

After an 18-h culture in the presence of eosinophils and either heat-inactivated IRS or fresh NRS, the majority of schistosomula were covered with cells and were immobile. The methylene blue dye exclusion test confirmed that these worms were dead.

In contrast, when schistosomula were incubated with heat-inactivated IRS or fresh NRS in the absence of eosinophils, <5% of organisms was found to be dead. Schistosomula incubated with eosinophils but without rat serum showed <25% mortality. The results obtained with these various test systems are presented in Fig. 1.

The ratio of eosinophils to target schistosomula necessary to cause death of the parasites was investigated. In all experiments, <25% of schistosomula was killed in heat-inactivated NRS, even with eosinophil to target ratios greater than 5,000:1. In the presence of fresh NRS or heat-inactivated IRS, killing was dependent on the ratio of eosinophils to target organisms. There was, however, a clear difference between the two systems; although the killer activity of

² Ramalho-Pinto, F. J., D. W. Dresser, and S. R. Smithers. 1977. Murine schistosomiasis mansoni: IgG dependent adherence of eosinophils to schistosomula *in vitro*. In preparation.

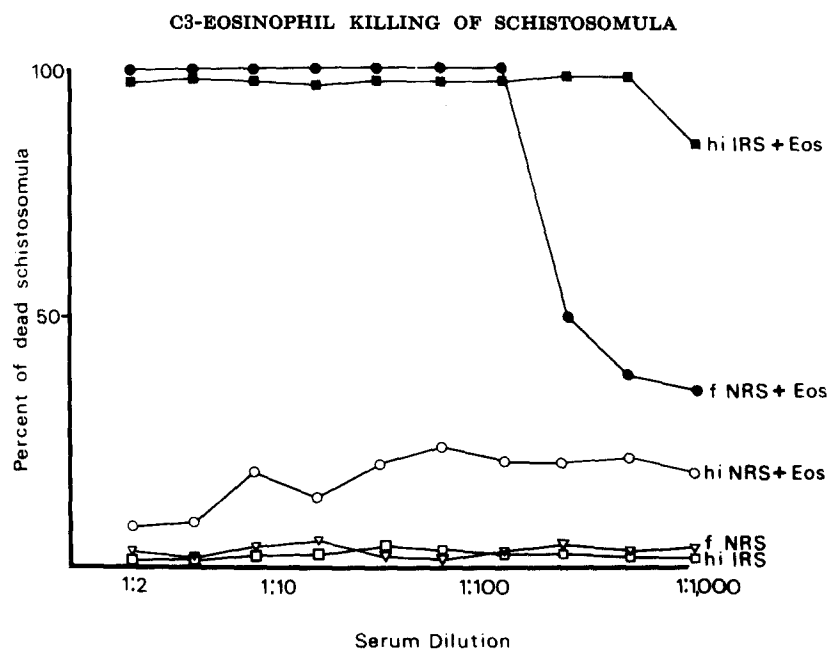


FIG. 1. Death of schistosomula after an 18-h culture in various concentrations of fresh NRS (f.NRS) or heat-inactivated IRS (h.i. IRS). Eosinophil:target ratio = 3,000-5,000:1.

eosinophils varied with each preparation, fewer eosinophils were needed to kill schistosomula in fresh NRS than were needed in heat-inactivated IRS. Fig. 2 illustrates one of our experiments; from the results it was calculated that an eosinophil:target ratio of 3,500:1 was necessary to kill 50% of schistosomula in heat-inactivated IRS, whereas in fresh NRS, only 900 eosinophils per schistosomulum were needed to kill the same percentage of parasites.

Mechanism of Adherence of Eosinophils to Schistosomula in Fresh NRS. Schistosomula incubated in fresh NRS for 15 min at 37°C and then washed extensively with RPMI-1640 were still able to bind eosinophils in vitro. In contrast, when 10 mM EDTA was added with the fresh NRS, no adherence could be observed. Using 10 mM EGTA with fresh NRS there was a negligible decrease in the number of adherent cells per schistosomulum. The adherence observed with heat-inactivated IRS was not blocked by the addition of EGTA or EDTA. When fresh NRS was incubated with zymosan for 60 min at 37°C before use in the adherence assay system, no cells bound to the schistosomula. These results indicate that eosinophil adherence to schistosomula depends upon the activation of complement by the alternative pathway.

By using the indirect fluorescent antibody technique, C3 molecules were detected on the surfaces of living and formalin-fixed schistosomula, but not on 5-day schistosomula after incubation with fresh NRS. C3 could not be detected on the surfaces of schistosomula which had been incubated in heat-inactivated IRS, in fresh NRS pretreated with zymosan, or in fresh NRS with 10 mM EDTA. Table I summarizes these results.

Ultrastructural Observations on C3-Mediated Adherence of Eosinophils to Schistosomula. Sections of schistosomula, recovered after 60 min in culture with eosinophils and fresh NRS, showed that the surfaces of the majority of

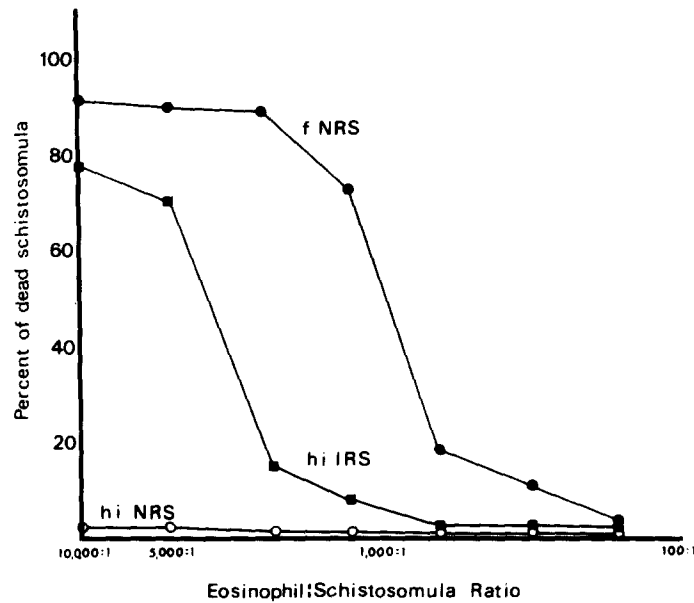


FIG. 2. Death of schistosomula after an 18-h culture in fresh NRS (f.NRS) or heat-inactivated (h.i. IRS) with various concentrations of eosinophils. Serum dilution = 1:100.

TABLE I

Adherence of Eosinophils to Schistosomula and the Presence of C3 or Anti-Schistosome Antibody at the Schistosomular Surface as Measured by the Fluorescent Antibody Test

Treatment	Reaction with FITC*		Eosinophil adherence
	Anti-C3	Anti-IgG	
Living schistosomula			
—	+++	—	+++
f.NRS (10 mM EDTA)	—	—	—
f.NRS (10 mM EGTA)	+++	—	+++
f.NRS (zymosan)	—	—	—
h.i. NRS (56°C, 60 min)	—	—	—
h.i. IRS (56°C, 60 min)	—	+++	+++
h.i. IRS (10 mM EDTA)	—	+++	+++
Formalin-fixed schistosomula			
f.NRS	+++	—	+++
h.i. IRS	—	+	+++
5-Day schistosomula			
f.NRS	—	—	—
h.i. IRS	—	—	—

* FITC, fluorescein-conjugated antiserum; f., fresh; h.i., heat inactivated.

parasites were covered with adherent eosinophils (Fig. 3). The basal plasma membrane of each cell was closely apposed to the tegumental outer membrane of the schistosomulum. Large cytoplasmic vacuoles were observed within many of the attached eosinophils, and the mechanism of vacuole formation by granule fusion was as described previously (4). In some cells, the vacuoles were connected to the basal plasma membrane of the eosinophil and they were releasing their granular contents onto the surface of the schistosomulum.

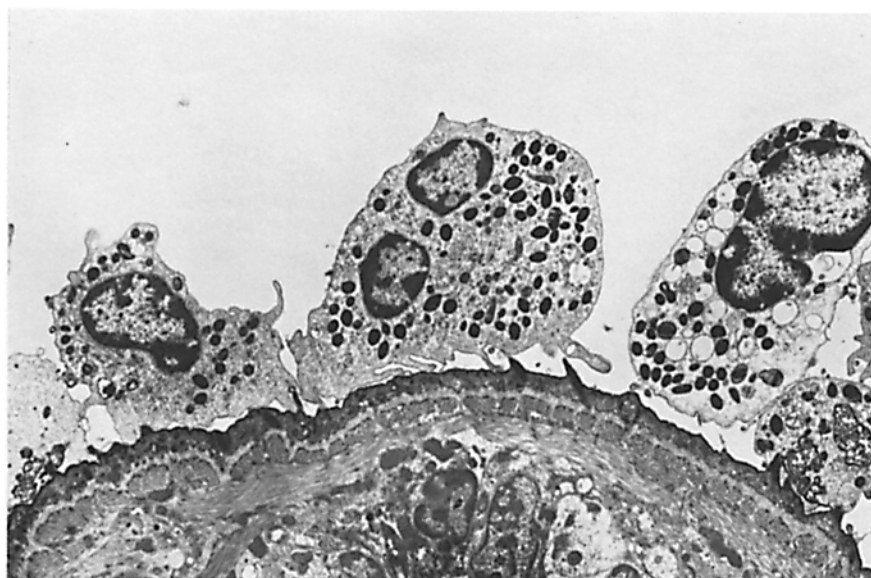


FIG. 3. Electron micrograph showing four eosinophils adhering to the surface of a schistosomulum after 4 h in culture with fresh NRS. $\times 4,400$.

Complement-Dependent Production of a Chemotactic Factor for Eosinophils. The results presented here indicate that cell adherence to schistosomula in fresh NRS is mediated by C3. Although both eosinophils and macrophages are known to possess C3 receptors (6, 7, 16) the evidence obtained from electron microscopy and chromotrope staining showed that the majority of adherent cells were eosinophils. This raised the possibility that a chemotactic factor for eosinophils was generated during the activation of complement. To investigate this possibility, a modification of the Boyden chamber was employed. The number of cells which had migrated through to the far side of the filter were counted at $\times 400$ in 10 randomly examined fields and the average number of cells per field was estimated. The results of two of the several experiments carried out are presented in Table II. They clearly show that only in conditions of complement activation (i.e. fresh NRS + schistosomula) did eosinophils migrate through the membrane.

Discussion

Previous *in vitro* studies have established that in the presence of anti-schistosome IgG antibodies, eosinophils adhere to schistosomula through their Fc receptors and kill the parasites (3). The present investigation has demonstrated an alternative method by which eosinophils adhere to and kill schistosomula; it depends upon the activation of complement by the alternative pathway at the schistosomular surface and the adherence of eosinophils through their C3 receptors. This complement-dependent mechanism can occur in the absence of anti-schistosome antibodies. In fresh NRS diluted to 1:125, and using an eosinophil-enriched peritoneal exudate from normal rats with a cell-to-target ratio of 3,000–5,000:1, eosinophils attached to schistosomula within 30

TABLE II
Eosinophil Chemotactic Activity of Fresh NRS and Schistosomula

Test medium in well (300 μ l)	Cell suspension in chamber (0.2 ml)	Migration <i>eosinophils/field</i>
Experiment 1		
RPMI-1640	1.5×10^7 cells	0.3
f.NRS*	"	1.6
RPMI-1640 + 400 schistosomula	"	1.4
f.NRS + 400 schis- tosomula	"	26.0
h.i. NRS + 400 schistosomula	"	3.0
Experiment 2		
f.NRS	0.75×10^6 cells	0.35
f.NRS + 400 schis- tosomula	"	10.0
h.i. IRS	"	0.25
h.i. IRS + 400 schis- tosomula	"	0.4

* f., fresh; h.i., heat inactivated.

min, and by 18 h, 100% of the parasites was dead. In heat-inactivated NRS or in the absence of cells, the majority of schistosomula remained alive.

It has also been shown that the eosinophil/target ratio required to kill the schistosomula is less when adherence is mediated by C3-C3 receptor interaction than when the cells adhere through IgG-Fc interaction. This enhanced killer activity might be explained by C3 promoting a closer association between target and killer cells than that mediated through Fc interaction. Alternatively, C3 may cause the eosinophil to release cytotoxic factors which are qualitatively or quantitatively different from those released after Fc binding. It is known that eosinophils secrete peroxidase and possibly other enzymes during Fc and C3 adherence to schistosomula (4; D. J. McLaren, unpublished observations). We believe however, the most likely explanation is that complement activation at the schistosomular surface generates eosinophil chemotactic factors, and that these promote a greater concentration of killer cells at the target surface.

We have not attempted to characterize the eosinophil chemotactic factors which induced the migration of rat eosinophils towards schistosomula incubated in NRS, but they are likely to differ from the mast cell-derived eosinophil chemotactic factor of anaphylaxis (17), and the lymphokine eosinophil stimulator promotor of Colley (18). It is more probable that the factors are themselves activated complement components; C3a, C5a, and C567 have all been shown to be chemotactic for eosinophils as well as for neutrophils (19).

Although these investigations were deliberately designed to distinguish between complement-mediated and antibody-mediated adherence of eosinophils to schistosomula, both will occur in the immune host. Three related mechanisms will facilitate eosinophil adherence: (a) binding to IgG-coated schistosomula

through Fc interaction; (b) adherence mediated by antibodies which can activate complement through the classical pathway (in these conditions, C3 bound to the Fc piece of the antibody would react with C3 receptors on the eosinophil: it is well known that antibodies which activate complement and are specific for the schistosomular surface are present in infected hosts [20]); and (c) a direct interaction between C3 receptors on the eosinophil and C3 on the parasite surface activated by the alternative pathway.

The preliminary observation that resistance to reinfection with *S. mansoni* depends, to some extent, upon an intact complement system (21) lends additional support to the suggestion that eosinophil adherence, mediated through complement activation, is a principle mechanism for destroying schistosomula in vivo. Finally, it is interesting that eosinophils could not adhere to 5-day schistosomula recovered from the lungs of mice, by either their C3 or Fc receptors. At this age, schistosomula no longer activate complement by the alternative pathway, nor do they bind anti-schistosome antibodies (22). The findings are in accordance with the observation that immunity to schistosomes is a form of concomitant immunity (23); although immune hosts may be able to destroy the young schistosomula of a challenge infection, older worms derived from a previous infection may persist unharmed (20).

Summary

Eosinophils from the peritoneal cavity of normal rats, in the presence of fresh normal rat serum (NRS), adhered to schistosomula of *Schistosoma mansoni* in vitro and killed the majority of parasites within 18 h. The reaction differed from the previously described antibody-mediated eosinophil adherence to schistosomula which occurs in heat-inactivated immune rat serum (IRS) and where adherence is mediated through Fc receptors. Adherence of eosinophils in fresh NRS was shown to be due to the activation of complement at the schistosomular surface by the alternative pathway, and it was effected through C3 receptors. The ability of eosinophils to kill schistosomula in C3-mediated adherence was greater than their ability to kill in Fc-mediated adherence. This enhancement of killer activity may be due to the generation by complement activation of eosinophil chemotactic factors which increase the concentration of cells at the target surface. It is suggested that eosinophil adherence mediated through complement activation could be the principal mechanism of destroying schistosomula in the host.

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