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Cercarial Glycocalyx of *Schistosoma mansoni* Activates Human Complement

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Human complement activation by cercariae and schistosomula of the human parasite *Schistosoma mansoni* was studied in vitro. Cercariae are composed of tails which are shed after infection of the host and bodies which transform into the larvae or schistosomula after infection. After incubation in fresh normal human serum (NHS), cercarial tails bound more anti-C3 antibodies than did cercarial bodies (CB), and the tails were rapidly lysed, while the attached CB remained intact. Complement activation by cercariae was dependent on the alternative pathway but was independent of antibody, as shown by C3 deposition by hypogammaglobulinemic human sera. By transmission microscopy, the fibrillar glycocalyx on both CB and tails was stained by NHS but not by heat-inactivated serum (HI-NHS). The glycocalyx was labeled with periodate and tritiated borohydride, and parasites were incubated in NHS and HI-NHS. After solubilization, the labeled glycocalyx on organisms incubated in NHS but not HI-NHS bound anti-C3 antibodies. Of the CB incubated with eserine sulfate to prevent transformation, 78% ± 10% were dead after culture for 24 h in NHS. In contrast, 21% ± 12% of the CB were dead after culture in HI-NHS. Schistosomula incubated in NHS bound 37% of the amount of anti-C3 antibodies bound by cercariae but were not killed by NHS. In conclusion, the cercarial glycocalyx activated human complement, and schistosomula were less susceptible to killing than cercariae because they had less glycocalyx and activated less complement.

Cercariae, the stage of *Schistosoma mansoni* which infects humans, are covered by a syncytium called the tegument. The tegument is bounded by a single-unit membrane and a 1- to 2-μm-thick, carbohydrate-rich glycocalyx, which is antigenic (9, 10, 23). Recently we have shown that this glycocalyx is a meshwork of 15- to 30-nm fibrils, which is stained for transmission microscopy by antischistosomal antibodies, ruthenium red, or tannic acid (17). When cercariae are radiolabeled by periodate oxidation and tritiated borohydride reduction (Per-^{3}H), the single species that is labeled has a molecular weight of $>5 \times 10^6$ and is excluded from Sepharose 2B after reduction (17). The labeled material has a pI of 5 and is also antigenic (17). When cercariae penetrate the host skin, they transform to schistosomula, a process which can be duplicated in vitro (14). During transformation, two-thirds of the radiolabeled glycocalyx is shed into the medium (17), tails are lost, and a new double-unit membrane is formed over the tegument (7). Cercariae are lysed by complement in fresh normal human serum (NHS) activated by the alternative pathway (1, 12, 22). Whether the fibrillar, high-molecular-weight glycocalyx activates complement is not known. In contrast, schistosomula after transformation are no longer killed by human complement alone (4, 5, 19). Why schistosomula are more resistant to complement than cercariae are is unclear.

The goals of the present experiments were to determine whether the glycocalyx activated complement and to correlate bound complement with parasite damage. First, cercariae were incubated with NHS as a source of complement, and the amount and distribution of complement on the parasite surface were studied by fluorescence and electron microscopy. Second, the glycocalyx was radiolabeled, parasites were incubated with NHS, and solubilized material was immunoadsorbed with anti-human C3 antibodies attached to Affigel beads. Third, the lysis of cercarial bodies (CB) and schistosomula cultured with NHS was compared and correlated with measurements of C3 bound to the parasites.

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MATERIALS AND METHODS

Abbreviations. HI-NHS, heat-inactivated NHS; ES, eserine sulfate; RhαC3, rhodamine-conjugated goat immunoglobulin G to human C3; F1.TSC, fluorescein thiosemicarbazide.

Parasite preparation. A Puerto Rican strain of *S. mansoni* was maintained in *Biomphalaria glabrata* snails and CBA/j mice (Jackson Laboratories, Bar Harbor, Maine). Cercariae, CB, and 3-h mechanical schistosomula were prepared and purified on Percoll gradients (Pharmacia, Inc., Piscataway, N.J.) by methods previously described (14, 17). To inhibit swimming, secretion, and transformation, cercariae and CB were incubated with 1 mM ES (17).

Incubations of parasites with fresh NHS. Fresh NHS was used as a source of complement and was obtained from volunteers uninfected with *S. mansoni*. NHS was heat inactivated for 60 min at 56°C (HI-NHS). Hypogammaglobulinemic human sera, obtained from three agammaglobulinemic patients before treatment with gamma globulin and containing 90 to 300 mg of immunoglobulin per 100 ml, were generously donated by Fred Rosen, Children's Hospital Medical Center, Boston, Mass. Serum (200 μl) was added to 1,000 to 5,000 organisms per ml of medium and incubated for 30 min at 37°C. Media used for incubations included artificial pond water (17); 10 mM EDTA; 10 mM Mg^{2+} and 10 mM ethylene glycol tetraacetate (EGTA); 10 mM Ca^{2+} and 10 mM Mg^{2+}; and RPMI 1640. All the media were buffered with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.2; M. A. Bioproducts, Walkersville, Md.).

Fluorescence microscopy. After incubation with NHS, par-

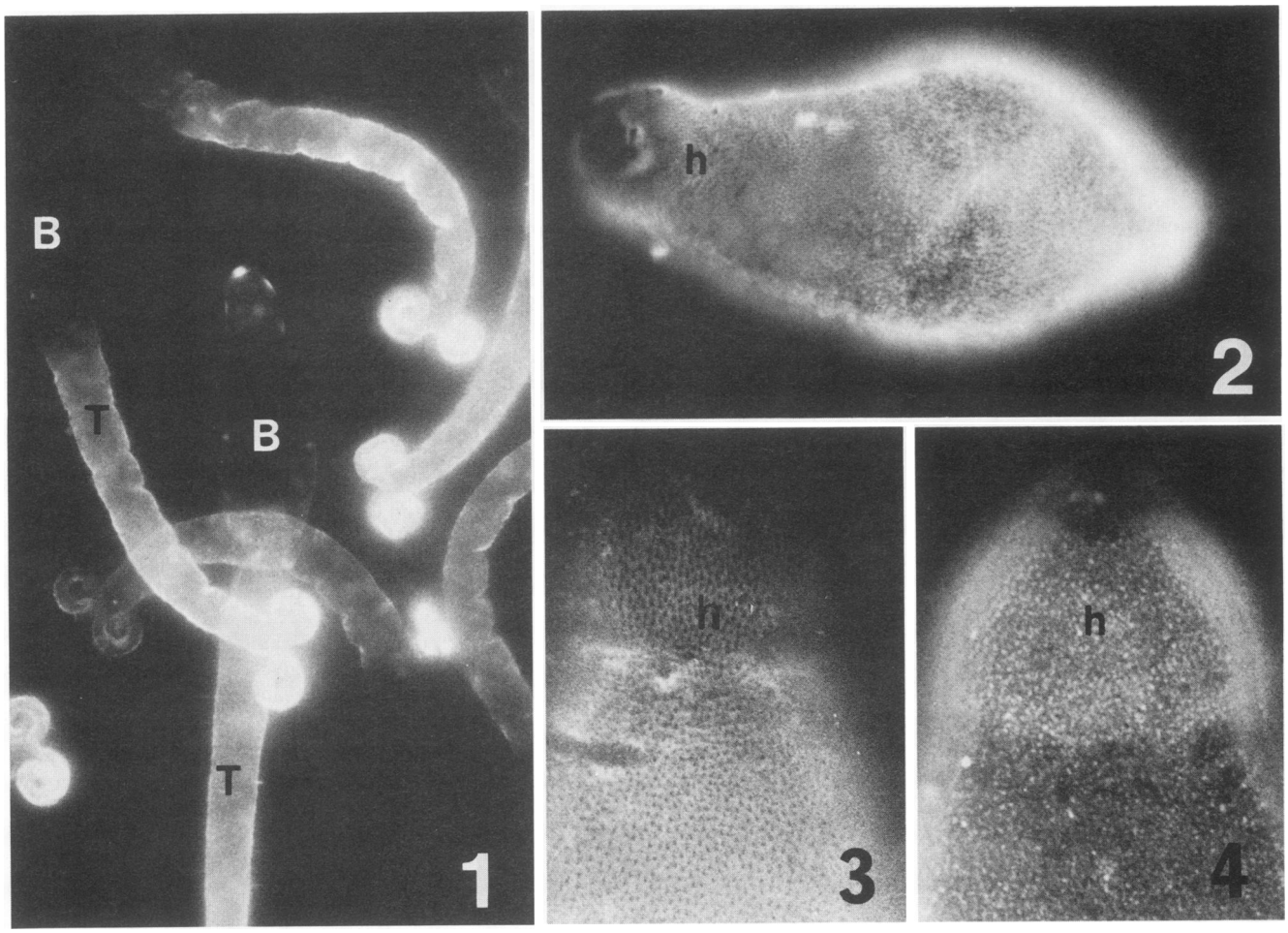


FIG. 1 to 4. Fluorescence microscopy of parasites incubated with NHS and then stained with RhαC3. Tails (T) on cercariae activated more complement and were brighter than bodies (B; Fig. 1). A CB (Fig. 2) was brighter over the posterior body (right) than the head (h), and the spines were dark against a bright background (Fig. 3). A schistosomulum (Fig. 4) was less bright than a CB and had bright spines against a dark background. Parasites incubated with HI-NHS and then RhαC3 were dark (not shown). Figure 1, ×240; Fig. 2, ×375; Fig. 3 and 4, ×900.

asites were washed four times, incubated with RhαC3 (Cappel Laboratories, Cochranville, Pa.) for 30 min at room temperature, and washed. Parasites were observed with a Leitz Orthoplan fluorescence microscope, and the relative fluorescence of bound RhαC3 was measured with an attached photometer (17). RhαC3 was not measured with the photometer on the tails because they were too rapidly lysed to obtain repeatable measurements. Alternatively, the glycocalyx on cercariae was labeled with periodate and F1.TSC by methods previously described (17), and then parasites were incubated with NHS.

Electron microscopy. Parasites incubated with NHS or HI-NHS were washed four times and fixed in 1% formaldehyde and 3% glutaraldehyde (M. J. Karnovsky, J. Cell Biol. **27**:137a, 1965), postfixed in osmium, and prepared for scanning and transmission microscopy as previously described (17).

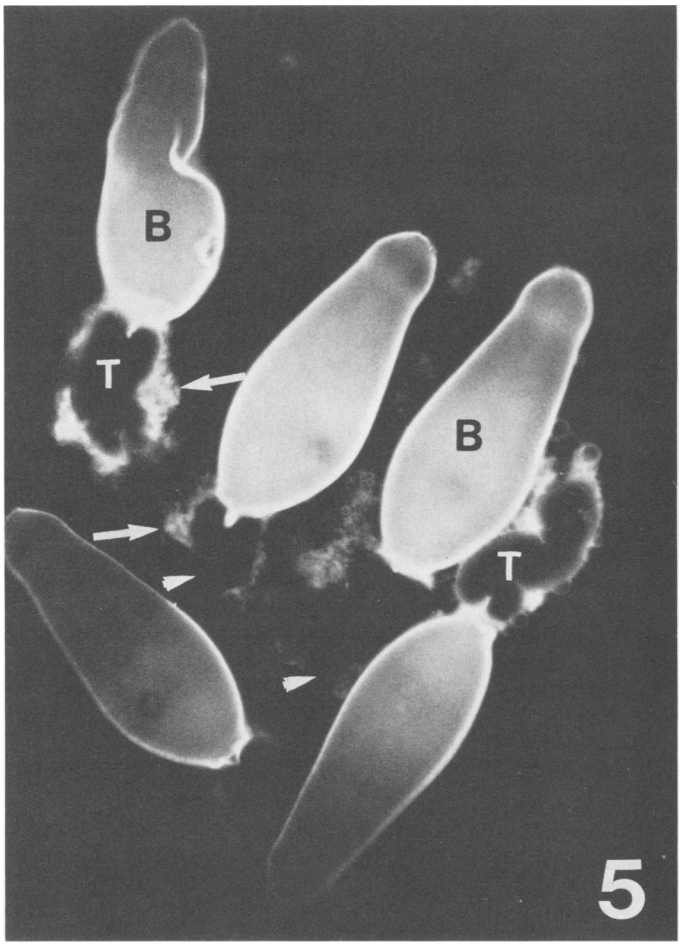
Immunoadsorption of radiolabeled glycocalyx with anti-human C3 antibodies. The glycocalyx on cercariae was radiolabeled by Per-^{3}H as previously described (17). Parasites were then incubated with NHS or HI-NHS for 30 min at 37°C, washed six times, and solubilized (10,000 organisms per 200 μl) in 0.5% Nonidet P-40 for 60 min at room temperature. Solubilized material was incubated for 60 min at room temperature with 100 μl of Affigel beads (Bio-Rad Laboratories, Richmond, Calif.) conjugated to goat anti-human C3 immunoglobulin G, washed four times, boiled in sodium dodecyl sulfate, and chromatographed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (17).

Killing assays. Parasites (200 to 500 organisms per ml) were cultured in medium containing 200 μl of either NHS or HI-NHS per ml of RPMI (18). ES was added to CB and to some schistosomula. Parasites were concentrated by centrifugation after 15 h at 37°C, and the percentage killed was determined. Parasites were considered dead when there was extensive surface blebbing, granularity of the internal organs, or absence of flame cell motion as seen by interference-contrast microscopy (17), or all three conditions.

Repetition of experiments. Each experiment was repeated a minimum of three times.

RESULTS

Fluorescence microscopy. When cercariae were incubated in NHS, tails activated more complement than did bodies and appeared brighter when stained by RhαC3 (Fig. 1). The posterior of CB was brighter than the anterior (Fig. 2), while the spines were black against a bright background (Fig. 3). Schistosomula preincubated with NHS bound less RhαC3 than cercariae, and the spines were bright rather than dark (Fig. 4). The relative fluorescence of bound RhαC3 mea-



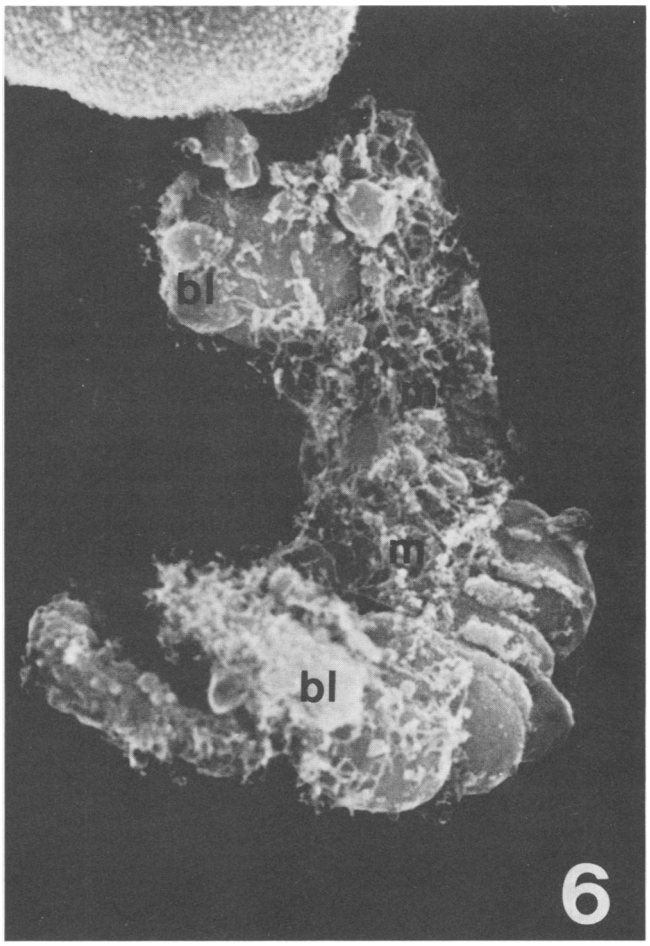


FIG. 5 and 6. Fluorescence and scanning microscopy of cercarial tails lysed by NHS. Tails (T) on cercariae labeled with Per-F1.TSC and then incubated for 30 min with NHS had fluorescent glycocalyx which was clumped in some areas (arrows; Fig. 5) and absent in other areas (arrowheads). At the same time, the glycocalyx covering bodies (B) of cercariae remained intact. A cercarial tail lysed by NHS (Fig. 6) had large blebs (bl) and microvilli (m) over the tegument. In HI-NHS, tails remained intact by fluorescence and scanning microscopy (not shown). Figure 5, ×230; Fig. 6, ×1,600.

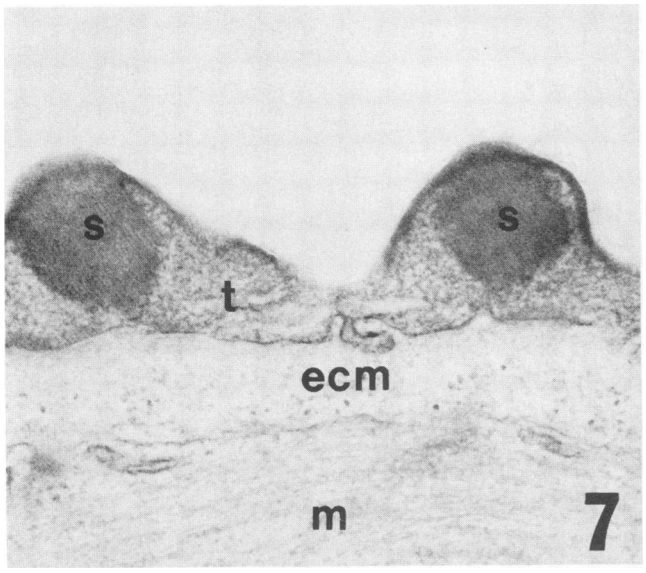
sured with a photometer on CB was similar when parasites were incubated with NHS diluted in media containing Ca^{2+}-Mg^{2+}, EGTA and Mg^{2+}, or RPMI 1640. In contrast, CB incubated with NHS diluted in EDTA or with HI-NHS bound <5% of the measured RhαC3 bound by CB in RPMI (for both, $P < 0.01$; Student's t test). Similarly, when organisms were incubated with three different hypogammaglobulinemic sera, tails were brighter than CB, which were brighter than schistosomula. These results showed that complement was activated by the alternative pathway and that antibody probably was not involved. Three hours after mechanical transformation, schistosomula incubated with NHS bound 37% ± 17% of the amount of RhαC3 bound by CB ($P < 0.01$).

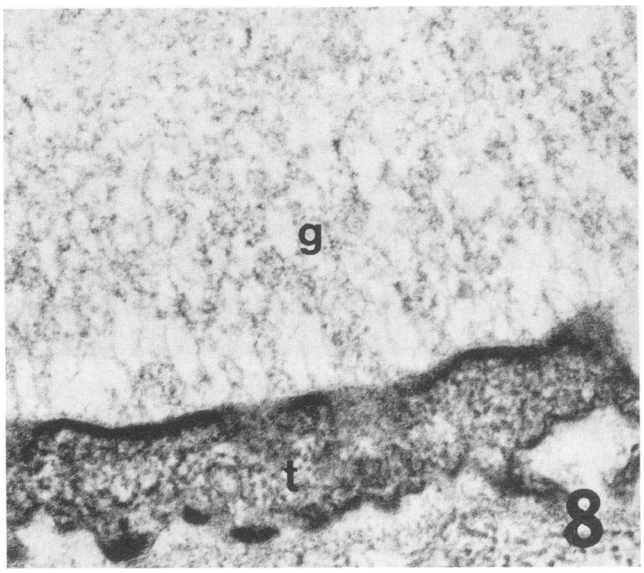
The tails of cercariae were damaged by incubation in NHS or hypogammaglobulinemic sera for 30 to 60 min, and the glycocalyx, which had been covalently labeled with fluorescein, was disrupted (Fig. 5). The glycocalyx on the tail was clumped in some regions and absent in others. However, the glycocalyx and tegument of the body remained intact. By scanning microscopy, many microvilli and large blebs were seen over the tail (Fig. 6).

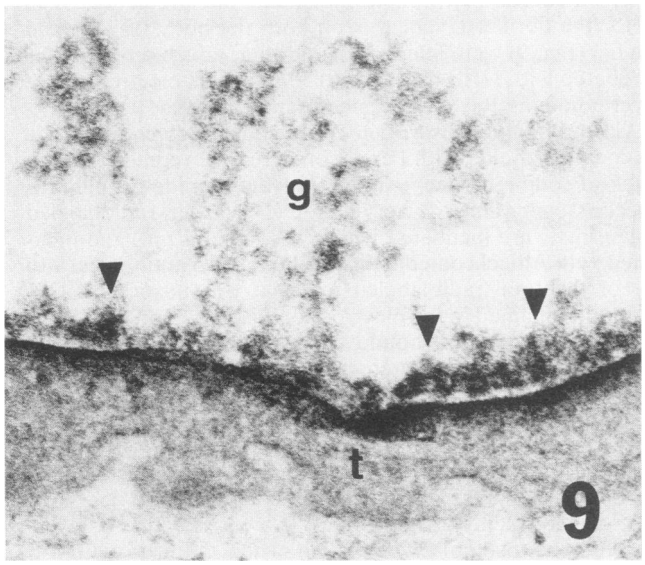
Transmission microscopy of complement bound to cercariae. The glycocalyx was not seen on cercariae incubated with HI-NHS and then prepared for transmission microscopy (Fig. 7). In contrast, when parasites were preincubated in NHS, the fibrillar glycocalyx on both the body (Fig. 8) and the tail (Fig. 9) were stained. In addition, tails but not bodies incubated with NHS had a 30- to 50-nm-thick electron-dense layer adjoining the surface membrane (Fig. 9).

Anti-C3 immunoadsorption of radiolabeled glycocalyx. The glycocalyx labeled with Per-^{3}H on cercariae remained in the stack of sodium dodecyl sulfate-polyacrylamide gel electrophoresis containing 5% acrylamide (17). When radiolabeled organisms were incubated with NHS, solubilized, and incubated with Affigel coated with anti-human C3 antibodies, 1 to 2% of the total radiolabeled material was adsorbed. This material remained in the electrophoresis stack and was 5.4 ± 1.0 times the amount bound to anti-C3-coated beads from radiolabeled organisms preincubated with HI-NHS (average of three experiments).

Killing of parasites by complement. After 15 h,78% ± 26% of CB cultured in the presence of ES and NHS were dead, while only 21% ± 12% of CB cultured in ES and HI-NHS were dead ($P < 0.01$; Fig. 10). In contrast, 12% ± 8% of NHS-treated schistosomula and 16% ± 9% of HI-NHS-treated schistosomula were killed when cultured with ES (Fig. 10). The schistosomular killing rates were not statisti-







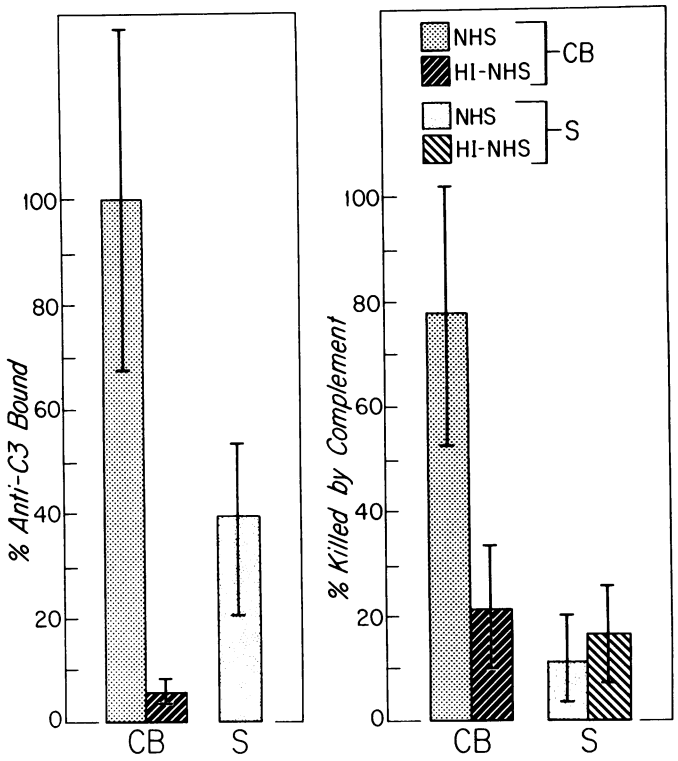


FIG. 10. Complement binding to and killing of CB and schistosomula. CB treated with ES to prevent transformation and preincubated with NHS bound 25 times the amount of RhαC3 as CB preincubated with HI-NHS (left). Schistosomula (S) preincubated with NHS bound 37% ± 19% (mean ± standard deviation) of the RhαC3 bound by CB (left). More than 78% of CB were killed by complement in NHS after 15 h, while only 21% were killed by HI-NHS (right). In contrast, 11% of ES-treated schistosomula were killed by NHS, and 16% were killed by HI-NHS (right). Bars mark the standard deviations of four to eight experiments.

cally different from each other or from those of CB incubated with HI-NHS (Student's *t* test for four experiments). Similarly, 4 and 3% of schistosomula incubated without ES were killed by NHS and HI-NHS, respectively, suggesting that ES was mildly toxic.

DISCUSSION

In these experiments the cercarial glycocalyx was shown to activate human complement. In addition, the failure of complement to kill schistosomula was correlated with a 63% decrease in the binding of C3 by schistosomula compared with cercariae.

Complement activation by the cercarial glycocalyx. The glycocalyx on the surface of cercariae and schistosomula appears to activate human complement by the alternative

FIG. 7 to 9. Transmission microscopy of glycocalyx staining by NHS. The tegument (t) of a cercaria (Fig. 7) contained paracrystalline spines (s) and overlaid extracellular matrix (ecm) and muscle (m). The glycocalyx covering a cercaria preincubated with HI-NHS before fixation was not visualized (Fig. 7). In contrast, the fibrillar glycocalyx (g) was seen on the body (Fig. 8) and the tail (Fig. 9) of cercariae preincubated with NHS as a source of complement before fixation. On the tails in NHS, there was also densely stained material close to the tegumental membrane (arrowheads; Fig. 9). Figures 7 and 8, ×65,000; Fig. 9, ×130,000.

pathway. First, C3 was deposited on the surface of parasites incubated in NHS or hypogammaglobulinemic sera in media containing Mg^{2+}-Ca^{2+} or Mg^{2+}-EGTA but not in media containing EDTA. Second, RhαC3 bound to the posterior body more than to the head, with the spines in relief, which is similar to the distribution of the glycocalyx labeled with Per-F1.TSC on CB (17). Third, by transmission microscopy the glycocalyx was stained by NHS but not by HI-NHS. This result is similar to the staining of the glycocalyx by immune but not by nonimmune antibodies (17). Fourth, Per-^{3}H-labeled glycocalyx was precipitated by anti-C3 antibodies when parasites were preincubated with NHS but not HI-NHS. Finally, schistosomula, which lose two-thirds of the glycocalyx during transformation (17), bound 37% of the C3 bound by CB.

The glycocalyx forms a 1- to 2-μm-thick fibrillar coat on cercariae (7, 10, 17) and is the predominant material labeled on cercariae by either Per-^{3}H or by Iodogen-catalyzed iodination (17). The glycocalyx appears then to be the major moiety on the cercarial surface and is likely the major moiety responsible for complement activation. Why tails, which have 55% of the fluorescence of CB when the glycocalyx is labeled with F1.TSC (17), bound more RhαC3 than CB is unclear. One possibility is that moieties other than the glycocalyx activate complement on tails. Because Per-^{3}H labels only the high-molecular-weight glycocalyx on cercariae (17), the immunoadsorption experiment cannot demonstrate other molecules which may activate complement. A second possibility is that antibodies bind to tails but not CB and contribute to alternative pathway complement activation, as has been demonstrated in multiple bacterial systems (15). However, this is unlikely because tails bound more anti-C3 than CB did and were more rapidly lysed by sera from hypogammaglobulinemic patients.

The glycocalyx has been shown to be carbohydrate rich (9, 17, 23), high molecular weight, acidic (17), and antigenic (10, 17, 23). It may be similar to carbohydrates on bacteria which activate complement by the alternative pathway (6, 8, 25).

Killing of cercariae and schistosomula by complement. Tails bound more RhαC3 than did CB, which bound more RhαC3 than schistosomula. Similarly, tails were more susceptible to complement-mediated lysis than were CB, which were more susceptible than schistosomula. Parasite killing, therefore, was roughly proportional to the amount of C3 bound. These results extend previous studies of cercarial killing by complement (1, 12, 22) by showing that tailless CB inhibited from transforming by ES (17) are also very susceptible to complement. These results parallel previous in vitro studies showing that (i) consumption of complement components is correlated with parasite killing (12) and (ii) the development of resistance by schistosomula to eosinophil-mediated attack is correlated with decreased binding of complement by the organisms (4). Moreover, it is unlikely that the new double membrane formed on the surface of schistosomula protects the organisms from lysis, because schistosomula are killed by complement activated via bound antibody (2, 11, 13, 24).

The effect of alternative pathway complement on the survival of parasites in vivo is disputed. In one study, 65% of parasites penetrating the skin of nonimmune mice are not recovered after 24 h and are presumed dead (21). In contrast, other workers (3), using radioautography to localize radiolabeled organisms penetrating nonimmune mice, show that >80% of the organisms migrate successfully from skin to lungs. Furthermore, depletion of C3 by cobra venom factor causes a significant decrease in the resistance of nonimmune mice to percutaneous skin infection (20). However, decreased killing of parasites is not seen in mice genetically deficient in C5 (16). Despite their disagreement, all these animal studies show that many cercariae transform to schistosomula and escape killing by host complement. The present experiments suggest that the shedding of the cercarial glycocalyx during transformation may be an important mechanism by which the parasites evade the host complement system.

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