

Schistosomula of *Schistosoma mansoni* Clear Concanavalin A from Their Surface by Sloughing

JOHN C. SAMUELSON, JOHN P. CAULFIELD, and JOHN R. DAVID

Department of Pathology and Medicine, Harvard Medical School, Boston, Massachusetts 02115; and
Division of Tropical Medicine, Brigham and Women's Hospital, Boston, Massachusetts 02115

ABSTRACT The lectin concanavalin A (Con A) was used as a model probe to study the behavior of molecules bound to the surface of recently transformed schistosomula of *Schistosoma mansoni*. Con A binding was saturable (150–180 pg/organism) and specifically competed by α -methyl mannoside. Both FITC-Con A and ^{125}I -Con A were lost from the surface of schistosomula with a halftime of 8–10 h in culture in defined medium. A comparable decrease in the binding of Con A to schistosomula cultured and then labeled with the lectin indicated that the labeling procedure itself was not inducing the observed change. Internalization of Con A was not seen by either fluorescence microscopy or electron microscope radioautography. In addition, 70–80% of the radioactivity lost from the parasite was recoverable by TCA precipitation from the culture medium as intact Con A (27,000 mol wt on SDS PAGE). Thus, the mechanism of clearance of bound Con A from the surface of cultured schistosomula is apparently by sloughing of Con A molecules intact into the culture media and not by endocytosis and degradation. Con A binding sites, visualized with hemocyanin by scanning electron microscopy, appeared homogeneously distributed over the surface of schistosomula when organisms were labeled at 4°C or after fixation with glutaraldehyde. However, Con A and hemocyanin formed aggregates on the surface of schistosomula when labeling was performed at 37°C, which suggests that lectin binding sites have lateral mobility within the plane of the membrane. These aggregates are likely independent of metabolism by the parasite because aggregation also occurs on the surface of organisms killed with azide.

The surface of schistosomula of *Schistosoma mansoni*, the larvae of a human pathogen, is a syncytium, called the tegument which is 2 μm deep and has an area of 20,000 μm^2 (16, 35). The tegument is connected to cell bodies deep within the organism and is covered by a surface membrane composed of two closely applied lipid bilayers, the inner and outer tegumental membranes (16). The outer membrane, which appears on the parasite surface shortly after transformation from cercariae, contains very few intramembrane particles (IMPs) (7, 17, 43) and forms 7–10- μm fusions with the plasma membrane of human neutrophils adherent to schistosomula via antibody and complement (7). In addition, the parasite acquires host molecules (40), including ABH blood group (14), Forssman (12), and histocompatibility antigens (37), on its surface. With development of the schistosomula in vivo or in vitro, the outer membrane binds decreasing amounts of antischistosomal antibodies (11, 13, 15, 19, 25, 36), which correlates with a decreased killing of the organisms by immunized hosts (45) or by immune reactions in vitro (11, 13, 25). All three of these phenomena, i.e., changes in the double membrane structure (26, 31), host

antigen acquisition, and loss of antigenicity, have been invoked to explain how the parasite avoids the host immune response.

Recently, we have shown that the amount of antischistosomal antibodies and complement bound to the surface of schistosomula decreases with a halftime of 5–6 h in culture (36). How these molecules are lost from the double membrane surface of the schistosomula is unclear. Theoretically, they could be cleared by endocytosis and degradation within the tegumental cell bodies or by sloughing of the molecules into the culture media. Here we used the lectin concanavalin A (Con A) as a model probe to study the loss of bound molecules from the surface of schistosomula. We chose the lectin because it binds well to schistosomula (27, 41, 43). In addition, since Con A is tetravalent, it can also be used to promote adhesion of blood cells to schistosomula (5, 9).

MATERIALS AND METHODS

Design of Con A Experiments

These experiments were designed to see whether Con A bound to the surface

of schistosomula behaved like antibody and complement, which bind to recently transformed schistosomula, then are cleared within hours in culture, and bind weakly to cultured schistosomula (36). In addition to measuring the rate at which Con A was being lost, we wished to determine the route by which bound Con A was cleared from the parasites and the electron microscopic distribution of the lectin on the organisms's surface. Con A bound to the schistosomula was demonstrated by three basic techniques: (a) fluorescein isothiocyanate-conjugated Con A (FITC-Con A) was examined by fluorescence microscopy and fluorimetry; (b) Con A radioiodinated with iodine-125 (^{125}I -Con A) was detected by use of a gamma counter and by electron microscope autoradiography (EMARG); and (c) unlabeled Con A was demonstrated with both scanning and transmission electron microscopy by binding *Busycon canaliculatum* hemocyanin to the lectin. With all techniques the same experimental approach was taken. Recently transformed schistosomula were labeled with Con A, cultured, and the amount of Con A remaining on the organisms was examined. In the case of ^{125}I -Con A, the amount and the molecular weight of the label in the culture media were also measured by SDS PAGE. In addition, to control for clearance of the lectin induced by the lectin itself, schistosomula were also cultured, then labeled, and examined.

Preparation and Culture of Schistosomula

A Puerto Rican strain of *S. mansoni* was maintained by passage through outbred mice and *Biomphalaria glabrata* snails. Schistosomula, the larval skin stage of the organisms, were prepared by two methods. Skin schistosomula were prepared by allowing cercariae to penetrate an excised rat skin into Earle's balanced salt solution containing 0.5% lactalbumin hydrolysate, pH 7 (Flow Laboratories, Inc., Rockville, MD), by methods previously described (7, 42). 3 h after skin penetration the preparation contained 0–10% tailed organisms and <5% dead organisms, as judged by the absence of gross movement and of flame cell motion. Mechanical schistosomula were prepared by a modification of the method of Ramalho-Pinto et al. (29). Cercariae were cooled to 4°C in an ice bath, passed through a metal screen to remove snail debris, concentrated by low speed centrifugation, and placed in cold minimal essential medium (MEM) (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY). The cercariae, 10–50 × 10³ organisms in 2 ml of MEM in 15-ml Falcon culture tubes, were treated for 1 min at full speed with a Genie Vortex in order to break the organisms into bodies and tails. The vortexed material was layered onto 60% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) in cold MEM and centrifuged at 600 g for 10 min (24). Tail, dead organisms, and snail debris remained on the top of the Percoll and were discarded. The pellet contained cercarial bodies, which were washed three times in MEM to remove Percoll and incubated for 3 h at 37°C in RPMI-1640 (Gibco Laboratories) to allow the bodies to complete their transformation into schistosomula. Mechanical schistosomula contained 0–2% tailed organisms and <2% dead organisms. Both mechanical and skin schistosomula were cultured at a concentration of 200–1,000 organisms/ml at 37°C under 5% CO₂ and 95% O₂ in RPMI-1640 with either 5% heat-inactivated fetal calf serum (RPMI-FCS) or 200 µg/ml bovine serum albumin (RPMI-BSA) (35). Both culture media contained 100 U/ml penicillin and 100 µg/ml streptomycin.

Con A Labeling

Standard conditions for labeling schistosomula with Con A involved incubating schistosomula, 3,000–5,000/ml of MEM, with Con A in a final concentration of 50 µg/ml for 30 min at 37°C, washing three times, and culturing as above. Saturation of Con A binding was determined by incubating schistosomula with 0–100 µg/ml Con A. The specificity of labeling was demonstrated by competing Con A binding with 100 mM α -methylmannoside.

Fluorescence Microscopy

Schistosomula were labeled with FITC-Con A, containing 2.5 fluorescein molecules/Con A molecule (Vector Laboratories, Inc., Burlingame, CA), and concentrated to 100 organisms/20 µl, and observed with a Leitz Orthoplan microscope equipped with a Ploem illumination system containing an H-2 cube and a 450-nm prefilter to reduce autofluorescence. For photography, schistosomula were immobilized with 5–10 mM eserine sulfate (Sigma Chemical Co., St. Louis, MO) (3) and photographed at 75–300 times primary magnification with Kodak Tri-X film.

To measure quantitatively FITC-Con A remaining bound to schistosomula after culture for 0–24 h, schistosomula were washed three times in MEM and fixed for 30 min at 4°C in 3% glutaraldehyde and 1% formaldehyde in 0.1 M cacodylate buffer, pH 7.4 (20), mixed 1:1 with the final wash buffer. Fixation did not alter the fluorescence signal. The fluorescence of 200-µm² area of individual schistosomula was then measured with a Leitz MPV-2 photometer attached to a Leitz Orthoplan microscope equipped as above (courtesy of Dr. Samuel Latt, Harvard Medical School). The percentage of Con A remaining bound to the

surface of cultured organisms was determined by the emission at a given time-point minus the emission of the mannoside-inhibited control divided by the emission of zero-time labeled schistosomula. At each time point the average emission of 15 organisms was determined and the individual variation was expressed by the standard deviation of the average.

Fluorimetry

The absolute amount of Con A bound to schistosomula labeled with FITC-Con A was determined by solubilizing 1,000 schistosomula at each time point in 300 µl of 2% SDS and measuring the fluorescence in a Perkin-Elmer fluorimeter model MPF-44B (Perkin-Elmer Corp., Instrument Div., Norwalk, CT) with an exciting wavelength of 490 nm and emitting wavelength of 520 nm. Values were compared with standard curves constructed from known concentrations of FITC-Con A in 2% SDS.

Preparation of ^{125}I -Con A

250 mg of Con A (Miles three times recrystallized) was cleared of 14,000- and 12,500-mol wt fragments by NH₄HCO₃ precipitation (10). The yield of 27,000-mol wt Con A was 75–100 mg after dialysis and lyophilization. 50 mg of this material was labeled with 5 mCi of Na¹²⁵I (New England Nuclear, Boston, MA) by lactoperoxidase-catalyzed oxidation while bound to Sephadex G-75 to protect the lectin's active site (28). The yield of ^{125}I -Con A was 25 mg with a specific activity of 5–6 × 10⁴ cpm/µg. ^{125}I -Con A was 99% precipitable by trichloroacetic acid (TCA) and 97% of the radioactivity migrated with the 27,000-mol wt peak on SDS PAGE. For EMARG ^{125}I -Con A was prepared by iodination with chloramine-T (18). This preparation of ^{125}I -Con A was 96% TCA precipitable and had a specific activity of 1 × 10⁶ cpm/µg.

SDS PAGE Analysis of ^{125}I -Con A-labeled Schistosomula and Culture Supernatants

Schistosomula, cultured in RPMI-BSA, were separated from the culture medium by centrifugation and the radioactivity in the pellets and in the supernatants was measured. The pellets were then washed twice in MEM and boiled for 2 min in 100 µl of sample loading buffer: 0.1 M Tris-HCl, pH 6.8, 5% B-mercaptoethanol, and 1% SDS (23). The supernatants were chilled to 4°C, precipitated in 10% TCA, centrifuged at 10,000 rpm for 10 min, washed twice in 2 ml of –20°C ethyl ether, resuspended in 100 µl of sample loading buffer and 6 M urea, sonicated, and boiled 2 min. Samples were loaded in 13-cm, 10%-polyacrylamide gels with 3% acrylamide stacks and run at a constant voltage of 50 mV while the samples were in the stacking gels and at 100 mV while the samples were in the running gels. Gels were cut into 4-mm slices with a Gilson gel slicer, and the radioactivity was determined. Standard proteins were run in parallel gels, fixed, and stained with Coomassie Blue.

Electron Microscopy

Con A bound to schistosomula was visualized with hemocyanin by both scanning and transmission electron microscopy (39). Immediately before fixation, Con A-labeled schistosomula were incubated with 50 µg/ml *B. canaliculatum* hemocyanin (isolated by the method of Karnovsky et al. [21]) for 10 min at 37°C in MEM and washed three times. Because Con A and hemocyanin might induce artifactual distributions of Con A binding sites on the surface of schistosomula, labeling with Con A and hemocyanin was also done at 4°C. In addition, Con A binding sites were stabilized before labeling by fixation in 0.1% glutaraldehyde in MEM for 10 min at room temperature, three washes, and reduction with 100 mM NaBH₄ for 10 min at room temperature. After labeling with hemocyanin, schistosomula were prepared for scanning and transmission electron microscopy as previously described (7). To visualize ^{125}I -Con A bound to schistosomula, EMARG was performed with Ilford L4 emulsion by the methods of Salpeter et al. (33).

RESULTS

Skin and mechanically prepared schistosomula bind equal amounts of Con A initially and lose bound Con A from their surface at the same rate and by the same route. The distribution of Con A bound to the surface of both preparations is also similar by fluorescence and electron microscopy, so that the results for both preparations will be presented together.

Con A binding to the surface of recently prepared schistosomula is saturable and specific. Lectin binding sites are nearly

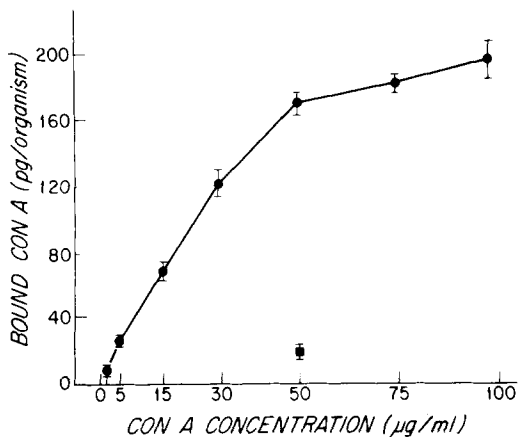


FIGURE 1 Dose response curve of ¹²⁵I-Con A binding to mechanical schistosomula. (●) Con A bound to schistosomula incubated for 30 min at 37°C with 1–100 µg/ml ¹²⁵I-Con A. Con A-binding is saturated after incubation with 50 µg/ml ¹²⁵I-Con A, so that this concentration of the lectin was used to label schistosomula in all other experiments. (■) Con A-binding to organisms incubated with 50 µg/ml Con A in the presence 100 mM α-methyl mannoside is 90% inhibited. Average of three experiments, each performed in duplicate. Bars indicate standard deviation.

saturated when schistosomula are incubated with 50 µg/ml ¹²⁵I-Con A (Fig. 1). The amount of Con A bound per organism at this dose is between 150 and 180 pg or ~10⁹ Con A molecules per organism. Con A binding reaches a maximum after 30 min at 37°C or 60 min at 4°C. Lectin binding is 90% decreased by coincubation with α-methyl mannoside which suggests that Con A binds specifically to sugars on the surface of schistosomula (Fig. 1). However, Con A bound to the surface of schistosomula is not released by subsequent incubation of labeled organisms with α-methyl mannoside. Standard conditions for labeling schistosomula with Con A in all experiments reported below is 30 min at 37°C with 50 µg/ml of the lectin.

Binding of FITC-Con A to Schistosomula

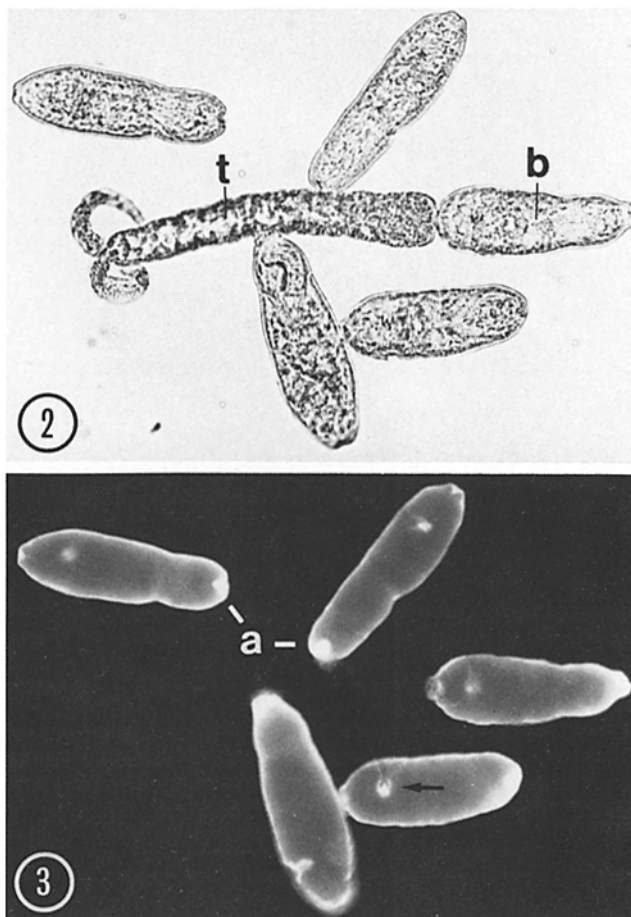
FITC-Con A binds to the bodies of schistosomula but not to cercarial tails which occasionally remain attached to organisms after skin penetration (Figs. 2 and 3). FITC-Con A binds all parasites in a preparation. Con A binds to the surface of living parasites and forms a rim around organisms when examined in cross section (Fig. 3). In tangential section or at low power, the fluorescence is more intense over the ventral sucker and at the anterior tip of the organisms (Fig. 3). Similarly, the spines of schistosomula labeled with Con A appear more fluorescent than the areas between the spines (see Fig. 1 in reference 9).

FITC-Con A remains on the surface of schistosomula that have been labeled and then cultured for 1–24 h with a distribution similar to that of recently transformed schistosomula shown in Fig. 3. No patching, capping, or endocytosis of the lectin is observed. The intensity of the fluorescence, however, decreases when schistosomula are cultured for a number of hours. The amount of Con A remaining bound to cultured schistosomula can be quantified by measuring the relative fluorescence of a 200-µm² area of individual schistosomula with a photometer attached to the microscope. Schistosomula lose 50% of bound Con A after 6 h of culture and 85% after 12 h of culture (Fig. 4). The form of the curve showing the loss of bound Con A from cultured schistosomula resembles that of an exponential decay (Fig. 4). Schistosomula cultured for 18 h

at 37°C and then labeled with FITC-Con A bind <20% of the lectin bound to recently transformed organisms (Fig. 4). FITC-Con A bound to the surface of schistosomula, measured with a fluorimeter after SDS solubilization of organisms, decreases from 150 pg/organism initially to 50 and 30 pg/organism after 12 and 24 h, respectively, (Fig. 5). The halftime of loss of FITC-Con A measured with a fluorimeter (8 h; Fig. 5) is slightly longer than the 6-h halftime measured with the fluorescence microscope and attached photometer (Fig. 4).

Binding of ¹²⁵I-Con A to Schistosomula

Recently transformed schistosomula bind 150 pg of ¹²⁵I-Con A/organism, the same amount as FITC-Con A (Fig. 5). 97% of this radioactivity runs as a single peak on SDS PAGE of 27,000 mol wt, characteristic of intact Con A monomer (Fig. 6). In culture, schistosomula lose bound radioactivity so that after 12 and 24 h only 55 and 35 pg ¹²⁵I-Con A/organism, respectively, remains bound (Fig. 5). SDS PAGE of solubilized cultured organisms shows that bound radioactivity still runs at 27,000 mol wt (Fig. 6). There is no increase in the radioactivity at the



FIGURES 2 and 3 Phase-contrast and fluorescence micrographs of the same group of skin schistosomula labeled with FITC-Con A. Note in Fig. 2 that one schistosomulum still has a cercarial tail (t) attached to its body (b). FITC-Con A binds to the bodies of all the schistosomula, but not to the tail (Fig. 3). The fluorescent lectin forms a rim around the schistosomular bodies when focused in cross section, indicating that the Con A binds only to the surface of the organisms. The fluorescence is generally homogeneous over the surface of the organisms but is more intense over the ventral sucker (arrow) and the anterior tip of the organisms (a), where the surface of the schistosomula is more convoluted. × 250.

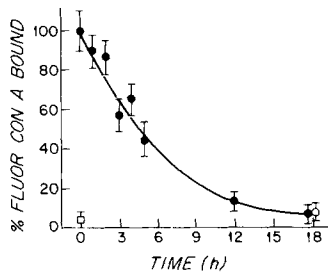


FIGURE 4 Representative experiment showing the loss of FITC-Con A from cultured skin schistosomula, measured with a fluorescence microscope and attached photometer. (●) Con A remaining on cultured organisms as a percentage of initial labeling. Con A is lost from the surface of schistosomula with a halftime of 6 h, so that <20% of the Con A

bound before culture remains on organisms cultured for 18 h. (□) Zero-time control with α -methyl mannoside shows that FITC-Con A binding is specifically inhibited by competition with the sugar. (○) Schistosomula cultured for 18 h and then labeled with FITC-Con A bind <20% of the lectin bound by freshly prepared organisms. Experiment was repeated three times. Each point represents the average of measurements on 15 organisms. Bars indicate standard deviations. The loss of Con A from the surface of cultured schistosomula is apparently exponential because the data are well fit (correlation coefficient = -0.96) by the equation: $\ln \text{Con A bound } (t) = \ln 100 - 0.13 \times t$, where $\ln \text{Con A bound } (t)$ equals the natural log of Con A bound expressed as a percentage of zero-time labeling at any given time and t equals hours in culture.

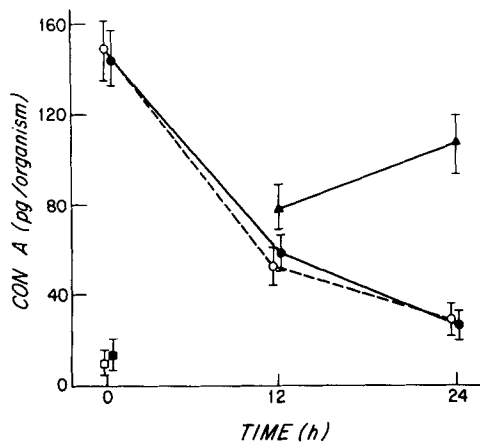


FIGURE 5 Loss of bound Con A from the surface of cultured skin schistosomula, measured with a fluorimeter and with a gamma counter. Schistosomula, labeled with FITC-Con A (○) or with ^{125}I -Con A (●), bind equivalent amounts of the lectin initially (150 pg/organism) and lose bound Con A at the same rate, so that only 55 and 30 pg Con A/organism binds to schistosomula cultured for 12 and 18 h, respectively. 70–80% of the radioactivity lost from cultured schistosomula is recoverable from the culture media, by precipitation with 10% TCA (▲). Note also that zero time binding of FITC-Con A (□) and of ^{125}I -Con A (■) is inhibited by coincubation with α -methyl mannoside. Average of three experiments, each performed in duplicate. Bars indicate standard deviation.

dye front or in the lower molecular weight ranges (slices 1–15 on Fig. 6 B and C) where one would expect to find proteolytic fragments of degraded Con A.

Radioactivity lost from schistosomula labeled with ^{125}I -Con A and cultured is quantitatively recoverable in the culture media (Fig. 5). 70–80% of this radioactivity in the culture media is precipitable with 10% TCA. On SDS PAGE the TCA precipitates of the culture media run as a single peak of 27,000 mol wt. As a fraction of Con A initially bound to schistosomula, ~30% and 50% of the Con A is recovered intact in the culture media after 15 and 24 h, respectively, while 10% and 20% of the radioactivity remains uncharacterized and 60% and 30% remains bound to the worms (Fig. 6).

To test the metabolic dependence of Con A loss from the surface of schistosomula in culture, organisms were labeled with Con A and incubated at 4°C for 15 h. Schistosomula lose between 10% and 35% of bound Con A at 4°C vs. an 72–85% loss at 37°C (range of three experiments with FITC-Con A and photometer). Similarly, schistosomula incubated for 15 h at 4°C before labeling with Con A bind between 74% and 90% of Con A bound initially (range of two experiments with the photometer and 1 experiment with ^{125}I -Con A). In contrast, schistosomula cultured for 15 h at 37°C and then labeled with Con A bind 20–38% of Con A bound initially (range of two experiments with photometer and four experiments with ^{125}I -Con A).

Scanning Electron Microscopy of Con A-Hemocyanin Bound to the Surface of Schistosomula

To observe the distribution of Con A on the surface of schistosomula with the electron microscope, hemocyanin was applied to Con A-labeled schistosomula immediately before fixation. Con A-hemocyanin binds homogeneously and densely over the entire surface of recently transformed schistosomula, including the spines and areas between the spines (Fig. 7). Hemocyanin does not bind to organisms incubated with Con A and α -methyl mannoside (Fig. 8). Schistosomula cultured for 12 h and then labeled with Con A followed by hemocyanin bind less hemocyanin than recently transformed schistosomula. The distribution of hemocyanin molecules on cultured schistosomula depends upon the temperature of labeling: cultured schistosomula labeled at 37°C have aggregates of hemocyanin on their surface as well as large areas clear of hemocyanin (Fig. 9). In contrast, cultured organisms labeled at 4°C have hemocyanin evenly distributed over spines and areas between the spines, with a density less than that of recently transformed organisms (Fig. 10). A homogeneous distribution

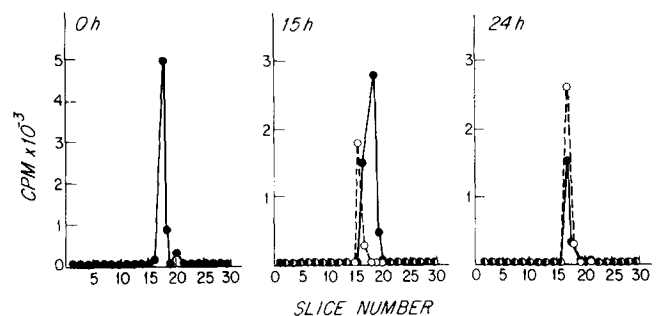
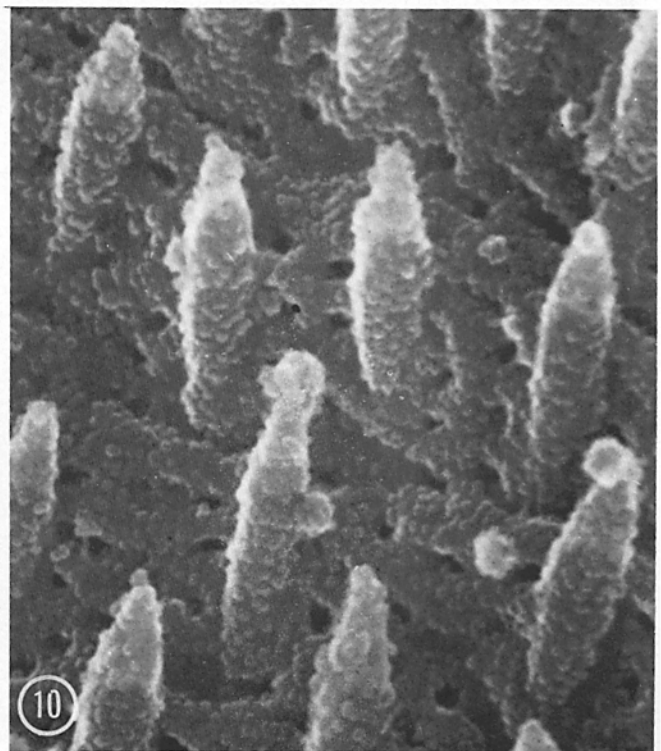
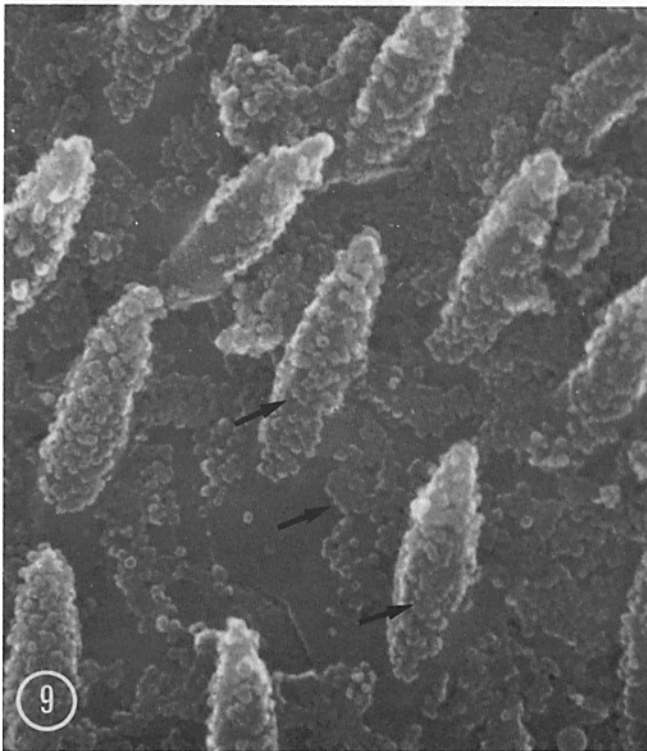
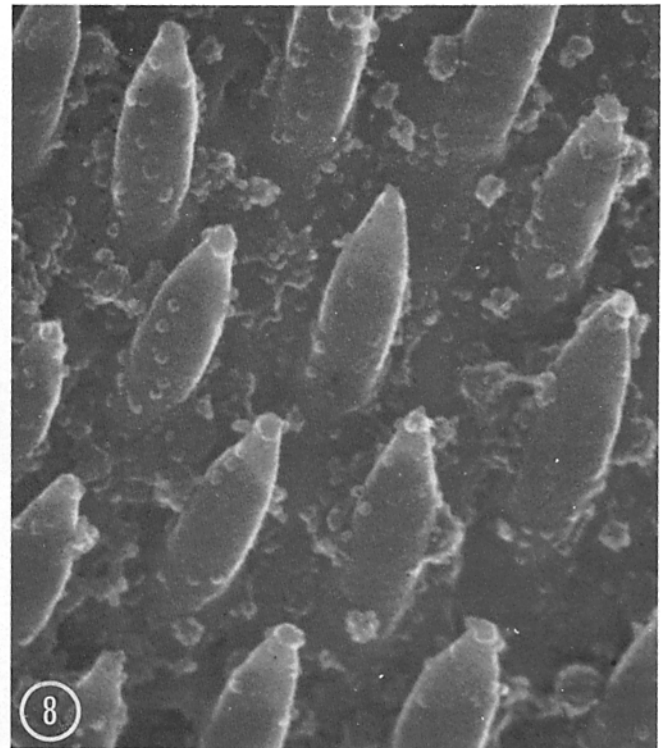
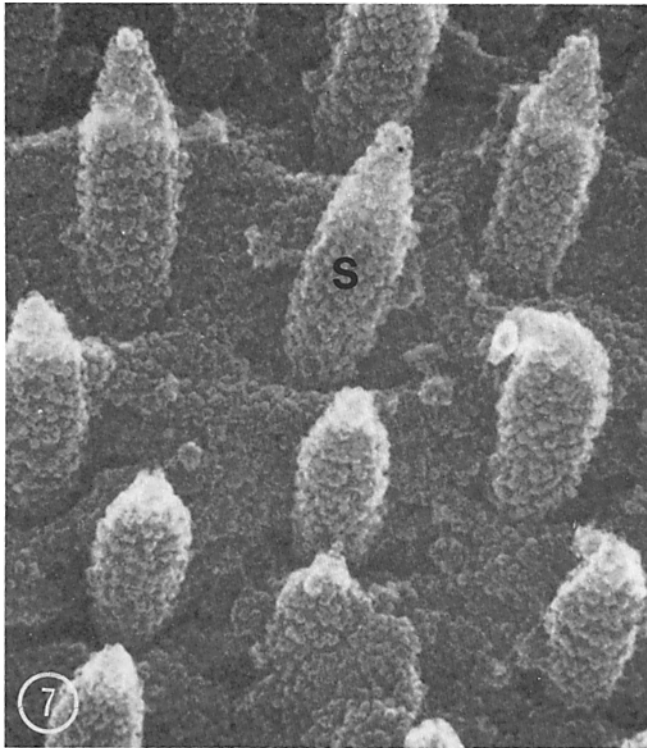


FIGURE 6 Representative experiment showing SDS PAGE of ^{125}I -Con A bound to schistosomula before and after culture, and of radioactivity recovered by TCA precipitation of culture media. (●) ^{125}I -Con A bound to 2,000 schistosomula before culture runs on SDS PAGE as a single 27,000-mol wt peak (left panel). (●) Radioactivity which remains bound to the same number of organisms cultured for 15 and 24 h (middle and right panels) also runs as single 27,000-mol wt peaks, characteristic of intact Con A. (○) TCA precipitates of the culture media of schistosomula which have lost bound Con A (middle and right panels) contain 70–80% of the radioactivity in the media, all of which runs at 27,000 mol wt. Con A is apparently shed intact from the surface of cultured schistosomula. SDS PAGE in tube gels containing 10% acrylamide, cut into 4-mm slices. Dye fronts at third slice. Slight shift to the right of radioactive peak of 15-h cultured schistosomula represents an error in the register of the gel slicer. Experiment repeated with similar results three times with tube gels and two times with slab gels and radioautography.



FIGURES 7-10 Scanning electron micrographs of skin schistosomula labeled with Con A and with hemocyanin immediately before fixation. High-power view of a fresh schistosomulum shows rectilinear hemocyanin molecules binding uniformly and densely over spines (S) and areas between the spines (Fig. 7). A control organism incubated with FITC-Con A in the presence of α -methyl mannoside shows little hemocyanin binding to the schistosomular surface (Fig.

8). A schistosomulum cultured for 12 h and then labeled at 37°C has aggregates of hemocyanin molecules (arrows) over the schistosomular surface between bare areas (Fig. 9). In contrast, a schistosomulum cultured for 12 h and then labeled at 4°C has an even and diffuse distribution of hemocyanin molecules on its surface (Fig. 10). $\times 40,000$.

of hemocyanin similar to that seen in Fig. 10 is seen on the surface of cultured schistosomula briefly fixed with glutaraldehyde, treated with NaBH_4 to quench reactive aldehydes, and then labeled with Con A and hemocyanin. Cultured schisto-

somula killed with 10^{-3} azide and then labeled have aggregates of Con A-hemocyanin on their surface when labeled at 37°C and diffuse, homogeneous hemocyanin when labeled at 4°C, as in Figs. 9 and 10, respectively. These results are summarized

TABLE I

Appearance of Con A-Hemocyanin on the Surface of Cultured *Schistosomula*

Labeling condition	Appearance of hemocyanin
Con A-hemocyanin, 37°C	Aggregated
Azide, then C-H, 37°C	Aggregated
C-H, 4°C	Diffuse
Fix, then C-H, 37°C	Diffuse
Azide, then C-H, 4°C	Diffuse

Schistosomula were cultured for 12 h and then labeled with Con A and hemocyanin (C-H) at 37°C or at 4°C after no pretreatment, pretreatment with 10^{-3} M azide to kill the organisms, or prefixation with 0.1% glutaraldehyde to immobilize Con A binding sites. The appearance of hemocyanin molecules on the surface of *schistosomula* by scanning electron microscopy is characterized as aggregated if it appears as in Fig. 9 or diffuse if it appears as in Fig. 10.

in Table I. Finally, *schistosomula* labeled with Con A, cultured for 12 h, and then labeled with hemocyanin at 37°C have aggregates of hemocyanin on their surface similar to those in Fig. 9.

Transmission Electron Microscopy of Con A-Hemocyanin and ^{125}I -Con A Bound to the Surface of *Schistosomula*

Transmission electron microscopy of recently transformed *schistosomula* labeled with Con A and hemocyanin shows a monomolecular layer of hemocyanin, which is closely applied to the pentalaminar tegumental surface membrane (Fig. 11). The appearance of the surface membrane and the underlying tegumental cytoplasm when Con A and hemocyanin are bound is indistinguishable from that of unlabeled organisms. Similarly, no changes in the morphology of the tegument are observed when cultured *schistosomula* have Con A and hemocyanin in either the aggregated or diffuse distribution such as seen in Figs. 9 and 10.

EMARG of ^{125}I -Con A labeled *schistosomula* was performed to determine whether bound Con A is endocytosed into the tegument and tegumental cell bodies underlying the muscle layer during culture. EMARG of ^{125}I -Con A bound to recently transformed *schistosomula* shows grains homogeneously rimming the tegumental membrane (see Fig. 5 in reference 9). *Schistosomula* labeled with ^{125}I -Con A and cultured for 18 h before fixation require exposures to emulsion three times as long as those of freshly labeled organisms to reach the same grain density (Fig. 12, and see Fig. 5 in reference 9). However, very few grains are apparent within the tegument or tegumental cell bodies of cultured parasites which suggests that there is no endocytosis of ^{125}I -Con A by the *schistosomula*.

DISCUSSION

The multivalent ligand Con A has been used as a model of IgG, and its binding to, distribution on, and clearance from the outer tegumental membrane of *schistosomula* have been examined. Con A binds specifically and saturably to the outer membrane from which it is sloughed intact into the culture medium. Con A binding sites are also apparently sloughed, because the parasites lose the capability to bind Con A as well. The loss of Con A which appears exponential suggests that Con A binding sites are continually remixing in a fluid membrane. Similarly, the aggregation of Con A and Con A binding sites by hemocyanin which occurs at 37°C indicates that the

lectin binding sites have lateral mobility in the plane of the membrane. This aggregation is probably independent of metabolism by the organisms because aggregation also occurs on the surface of *schistosomula* killed with azide.

Con A bound to the surface of cultured *schistosomula* is cleared with the same 5–6-h half-time as antibody and complement bound to *schistosomula*, when measured with a fluorescence microscope and attached photometer (36). Like antibody and complement, Con A also binds less well to organisms which have been cultured for 18 h and then labeled (13, 36). Together, these results suggest that the outer membrane of the *schistosomula* is itself being lost in culture, and that the character of molecules on the membrane surface is changing. Rates measured by this technique may underestimate the half-time of

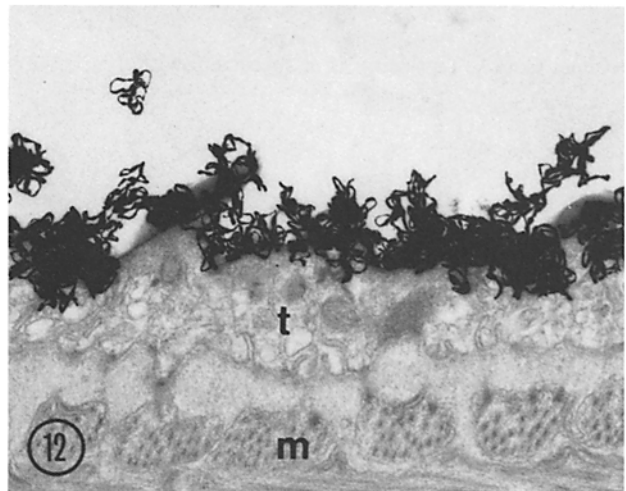
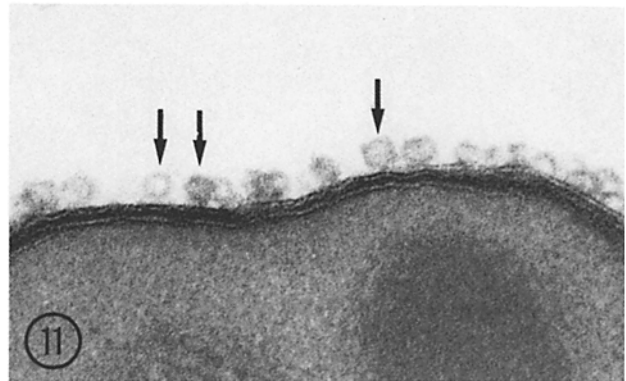


FIGURE 11 High-power transmission electron micrograph of *schistosomulum* labeled with Con A and hemocyanin. Hemocyanin molecules (arrows) form a monomolecular layer closely apposed to the pentalaminar (double lipid bilayer) tegumental surface membrane. No changes in the shape of the *schistosomulum* surface or in the underlying tegumentary cytoplasm are seen when Con A binds to fresh *schistosomula* or to cultured organisms. $\times 142,000$.

FIGURE 12 Electron microscope autoradiograph of a *schistosomulum* labeled with ^{125}I -Con A and cultured for 18 h. Silver grains cover the tegumental membrane of cultured *schistosomula*. Grains are not seen within the tegument (t) or within the tegumental cell bodies (not shown) which are located beneath the muscle layers (m). This suggests that there is no stable internalization of the lectin by the *schistosomula*. *Schistosomula* labeled with ^{125}I -Con A and cultured for 18 h before fixation, however, require three times as long exposure to emulsions to reach the same grain density as freshly labeled organisms (see Fig. 5 in reference 9), indicating that Con A has been lost from cultured *schistosomula*. $\times 18,000$.

molecules on the surface of schistosomula because autofluorescence interferes with the specific fluorescein signal when small amounts of lectin or antibodies are bound. The 8–10-h half-time of Con A loss measured with a gamma counter or a fluorimeter is likely a more accurate estimate of the rate of loss because the background signal from unlabeled organisms on these two instruments is less than that in the fluorescence microscope. Moreover, longer times of ~10 h are in agreement with the turnover half-times we measured of covalently labeled glycoproteins and glycolipids on the parasite surface (34). The longer half-time is also in agreement with the loss of antibody-dependent, eosinophil-mediated killing which occurs within days after transformation (13). On the other hand, other functional measurements of the turnover of molecules on the surface of schistosomula, such as decreased binding of antischistosomal antibodies with development *in vivo* or *in vitro* (11, 15, 19, 25, 37), resistance to immune-mediated killing with development *in vitro* or *in vivo* (11, 13, 25, 45), or decreased rosetting of erythrocytes by antierythrocyte antibodies bound to schistosomula by their F_c moiety (22), suggest half-times as long as 1–2 d (11, 13, 15, 19, 25, 37, 45) and as short as a few hours (22). These studies are difficult to compare to ours because the assay systems are so different.

Con A is apparently cleared from the surface of schistosomula by sloughing of molecules intact into the culture media, rather than by endocytosis and lysosomal degradation as is common in mammalian cells (44). The evidence for this conclusion comes from the failure to observe internalization of the lectin into the schistosomula by fluorescence microscopy and EMARG, and the recovery of the bulk of the radioactivity (70–80%) shed from the schistosomula into the culture media as intact Con A. This result is in agreement with the previous failure to observe internalization of cationized ferritin (47), thorotrast (6), or peroxidase (6) by schistosomula. The failure to recover 20–30% of the Con A originally bound to the parasite is presumably caused by absorption onto glass, inefficiency of precipitation, and so on. There is no evidence that this loss is due to proteolysis in that molecular weight fragments smaller than the Con A monomer are not seen by SDS PAGE. On the other hand, others have found proteolytic enzymes in the culture media and some degradation of nonspecific antibodies incubated together with the parasite (1).

The distribution of Con A binding sites on the surface of schistosomula is apparently homogeneous and diffuse because this is seen after the surface membranes have been stabilized either by cross-linking with aldehydes or at 4°C (44). The aggregates of Con A-hemocyanin seen on schistosomula labeled at 37°C are probably induced by the labeling procedure. Con A is tetravalent and hemocyanin contains a large number of mannose residues so that large complexes of Con A binding sites, Con A, and hemocyanin can form, provided the Con A binding sites are free to move on the surface (44). These aggregates are unlike cap formation on mammalian cells because they occur on organisms killed with azide (32) and because there is no gross rearrangement of cytoskeletal elements under the caps (2, 4, 21, 44). They are more analogous to “patching” or the aggregation of H-2 antigen (4) which occurs as a result of the multivalent ligands on the outside of the plasma membrane.

The aggregation also suggests that molecules may move laterally in the plane of the outer membrane (38). Such a notion of fluidity in the outer membrane is also supported by the exponential loss of Con A, which suggests that Con A

binding sites remaining on the surface of the schistosomula are mixing continuously with the rest of the membrane. In addition, neutrophil membranes fused to the parasite contain IMPs and are separated from the unfused outer membrane, which is virtually devoid of IMPs, by an occluding junctionlike structure (7). When this junctionlike structure is broken, there are no longer any differences in IMP concentration, which suggests that the fused and normal membranes have mixed (8). The fluidity of the outer membrane has been questioned by others (47) who observed patches, of cationized ferritin on the tips of the spines, similar to those shown here. They interpreted this distribution as evidence of bulk flow of the tegumental membrane from the pits, where membrane-containing vesicles fuse with the tegument (46), to the tips of the spines, where membrane fragments are often seen (47). Our data strongly suggest that the outer membrane is similar to other membranes (38), except that there is no evidence that it interacts with the cytoskeleton. This may be caused by the geometry of the tegumental membrane which is composed of two lipid bilayers. Any cytoskeletal interactions would necessarily have to span both bilayers rather than a single bilayer such as is seen in mammalian cells (2, 4, 21, 44).

The present paper and our companion paper (34) strongly suggest that schistosomula are shedding their outer membrane and that molecules bound to the membrane such as Con A, antibody (36), or complement (36) are shed along with it. This implies that any immune response depending on the specific antigens present on schistosomula immediately after transformation, on antibodies bound to those antigens, or on complement activated on the parasite surface by the alternative pathway (30), must be carried out in the first twelve to 24 h after the organism has penetrated the skin. Failure to carry out an immune attack in that time will probably result in the escape of the organism from susceptibility to these types of killing mechanisms, as has been demonstrated both *in vivo* (45) and *in vitro* (11, 13, 25, 30). Finally, any factors that lessen the amount of antibody binding to the parasite, such as sequestration in the epithelium as has been shown in the hamster cheek pouch (48), or low titers of specific antischistosomal antibody, will also abrogate granulocyte-mediated killing.

The authors wish to thank Mr. Jack Quinn for help maintaining the parasite life cycle and Ms. Ramona Gonski for excellent secretarial help.

This work was supported by National Institutes of Health (NIH) grant AI 16479 and NIH Medical Scientist Program grant GM 07753.

Received for publication 28 December 1981, and in revised form 30 March 1982.

REFERENCES

1. Auriault, C., M. A. Ouassii, G. Torpier, H. Eisen, and A. Capron. 1981. Proteolytic cleavage of IgG bound to the Fc receptor of *Schistosoma mansoni* schistosomula. *Parasite Immunol.* 3:33–44.
2. Ash, J. F., and S. J. Singer. 1976. Concanavalin-A-induced transmembrane linkage of concanavalin A surface receptors to intracellular myosin-containing filaments. *Proc. Natl. Acad. Sci. U. S. A.* 73:4575–4579.
3. Barker, L. R., E. Bueding, and A. R. Timms. 1966. The possible role of acetylcholine in *Schistosoma mansoni*. *Br. J. Pharmacol. Chemother.* 26:656–665.
4. Braun, J., K. Fugiwara, T. D. Pollard, and E. R. Unanue. 1978. Two distinct mechanisms for redistribution of lymphocyte surface macromolecules. I. Relationship to cytoplasmic myosin. *J. Cell Biol.* 79:409–418.
5. Butterworth, A. E., M. A. Vadas, D. L. Wassom, A. Dessein, M. Hogan, B. Sherry, G. J. Gleich, and J. R. David. 1979. Interactions between human eosinophils and schistosomula of *Schistosoma mansoni*. II. The mechanism of irreversible eosinophil adherence. *J. Exp. Med.* 150:1456–1471.
6. Carlisle, S., and L. S. Weisberg. 1978. *Schistosoma mansoni*: tracer studies in mice. *Exp. Parasitol.* 44:124–135.

7. Caulfield, J. C., G. Korman, A. E. Butterworth, M. Hogan, and J. R. David. 1980. The adherence of human neutrophils and eosinophils to schistosomula: evidence for membrane fusion between cells and parasites. *J. Cell Biol.* 86:46-63.
8. Caulfield, J. C., G. Korman, A. E. Butterworth, M. Hogan, and J. R. David. 1980. Partial and complete detachment of neutrophils and eosinophils from schistosomula: evidence for the establishment of continuity between fused and normal parasite membrane. *J. Cell Biol.* 86:64-76.
9. Caulfield, J. P., G. Korman, and J. C. Samuelson. 1982. Human neutrophils endocytose multivalent ligands from the surface of schistosomula of *S. mansoni* before membrane fusion. *J. Cell Biol.* 94:370-378.
10. Cunningham, B. A., J. L. Wang, M. N. Pflumm, and G. M. Edelman. 1972. Isolation and proteolytic cleavage of the intact subunit of Concanavalin A. *Biochemistry.* 11:3233-3239.
11. Dean, D. A. 1977. Decreased binding of cytotoxic antibody by developing *Schistosoma mansoni*. Evidence for a surface change independent of host adsorption and membrane turnover. *J. Parasitol.* 63:418-426.
12. Dean, D. A., and K. W. Sell. 1972. Surface antigens on *Schistosoma mansoni*. II. Adsorption of Forssman-like host antigen by schistosomula. *Clin. Exp. Immunol.* 12:525-540.
13. Desein, A., J. C. Samuelson, A. E. Butterworth, M. Hogan, B. A. Sherry, M. A. Vadas, and J. R. David. 1981. Immune evasion by *Schistosoma mansoni*: loss of susceptibility to antibody or complement-dependent eosinophil attack by schistosomula cultured in medium free of macromolecules. *Parasitology.* 82:357-374.
14. Goldring, O. L., J. A. Clegg, S. R. Smithers, and R. J. Terry. 1976. Acquisition of human blood group antigens by *Schistosoma mansoni*. *Clin. Exp. Immunol.* 26:181-187.
15. Goldring, O. L., A. Sher, S. R. Smithers, and D. J. McLaren. 1977. Host antigens and parasite antigens of murine *Schistosoma mansoni*. *Trans. R. Soc. Trop. Med. Hyg.* 71:144-148.
16. Hockley, D. J., and D. J. McLaren. 1973. *Schistosoma mansoni*: changes in the outer membrane of the tegument during development from cercaria to adult worm. *Int. J. Parasitol.* 3:13-25.
17. Hockley, D. J., D. J. McLaren, B. J. Ward, and M. V. Nermut. 1975. A freeze-fracture study of the tegument of *Schistosoma mansoni* (Platyhelminthes: Trematoda). *Tissue Cell.* 7:485-496.
18. Hunter, W. M., and F. C. Greenwood. 1962. Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature (Lond.)* 194:495-496.
19. Imohiesen, E. A. E., A. Sher, and F. von Lichtenberg. 1978. Early developmental changes of the schistosomula of *Schistosoma mansoni* *in vitro* and in mouse lung. *Parasitology.* 76:317-326.
20. Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell Biol.* 27(2, Pt. 2):137a (Abstr.).
21. Karnovsky, M. J., E. R. Unanue, and M. Leventhal. 1972. Ligand-induced movement of lymphocyte membrane molecules. II. Mapping of surface moieties. *J. Exp. Med.* 136:907-930.
22. Kemp, W. M., S. C. Merritt, M. S. Bogucki, J. G. Rosier, and J. R. Seed. 1977. Evidence for adsorption of heterospecific host immunoglobulin on the tegument of *Schistosoma mansoni*. *J. Immunol.* 119:1849-1854.
23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
24. Lazdins, J. K., M. J. Stein, J. R. David, and A. Sher. 1982. *Schistosoma mansoni*: rapid isolation and purification of schistosomula of different developmental stages by centrifugation on discontinuous density gradients of Percoll. *Exp. Parasitol.* 53:105-116.
25. McLaren, D. J., J. A. Clegg, and S. R. Smithers. 1975. Acquisition of host antigens by young *Schistosoma mansoni* in mice: correlation with failure to bind to antibody *in vitro*. *Parasitology.* 70:67-75.
26. Moser, G., D. Wassom, and A. Sher. 1980. Studies on the antibody-dependent killing of schistosomula of *Schistosoma mansoni* employing haptenic target antigens. *J. Exp. Med.* 152:41-53.
27. Murrel, K. D., D. W. Taylor, W. E. Vannier, and D. A. Dean. 1978. *Schistosoma mansoni*: analysis of surface membrane carbohydrates using lectins. *Exp. Parasitol.* 46:247-255.
28. Phillips, P. G., P. Furmanski, and M. Lubin. 1974. Cell surface interactions with Concanavalin A. *Exp. Cell Res.* 86:301-308.
29. Ramalho-Pinto, F. J., G. Gazzinelli, R. E. Howells, T. A. Mota-Santos, E. A. Figueiredo, and J. Pelligrino. 1974. *Schistosoma mansoni*: defined system for a step-wise transformation of cercariae to schistosomula *in vitro*. *Exp. Parasitol.* 36:360-372.
30. Ramalho-Pinto, F. J., D. J. McLaren, and S. R. Smithers. 1978. Complement mediated killing of schistosomula of *S. mansoni* by rat eosinophils *in vitro*. *J. Exp. Med.* 147:147-156.
31. Rumjanek, F. D., and D. J. McLaren. 1981. *Schistosoma mansoni*: modulation of schistosomular lipid composition by serum. *Mol. Biochem. Parasitol.* 3:239-252.
32. Rutishauser, V., I. Yahara, and G. M. Edelman. 1974. Morphology, motility, and surface behavior of lymphocytes bound to nylon fibers. *Proc. Natl. Acad. Sci. U. S. A.* 71:1149-1153.
33. Salpeter, M. M., and L. Bachman. 1972. Autoradiography. In Principles and Techniques of Electron Microscopy. Biological Applications. M. A. Hayat, editor. Van Nostrand Reinhold, New York. 2:221-278.
34. Samuelson, J. C., and J. P. Caulfield. 1982. Loss of covalently labeled glycoproteins and glycolipids from the surface of newly transformed schistosomula of *S. mansoni*. *J. Cell Biol.* 94:363-369.
35. Samuelson, J. C., J. P. Caulfield, and J. R. David. 1980. *Schistosoma mansoni*: post-transformational changes in schistosomula grown *in vitro* and in mice. *Exp. Parasitol.* 50:369-383.
36. Samuelson, J. C., A. Sher, and J. P. Caulfield. 1980. Newly transformed schistosomula spontaneously lose surface antigens and C3 acceptor sites during culture. *J. Immunol.* 124:2055-2057.
37. Sher, A., B. F. Hall, and M. A. Vadas. 1978. Acquisition of murine major histocompatibility complex gene products by schistosomula of *Schistosoma mansoni*. *J. Exp. Med.* 148:46-57.
38. Singer, S. J., and G. L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. *Science (Wash. D. C.)* 175:720-731.
39. Smith, S. B., and J.-P. Revel. 1972. Mapping of Concanavalin A binding sites on the surface of several cell types. *Dev. Biol.* 27:434-441.
40. Smithers, S. R., R. J. Terry, and D. J. Hockley. 1969. Host antigens in schistosomiasis. *Proc. R. Soc. Lond. B. Biol. Sci.* 171:483-494.
41. Stein, P. C., and R. D. Lumsden. 1973. *Schistosoma mansoni*: topographical features of cercariae, schistosomula and adults. *Exp. Parasitol.* 33:499-514.
42. Stirewalt, M. A., D. R. Mannick, and W. A. Fregeau. 1966. Definition and collection of schistosomules of *Schistosoma mansoni*. *Trans. R. Soc. Trop. Med. Hyg.* 60:352-360.
43. Torpier, G., and A. Capron. 1980. Intramembrane particle movements associated with binding of lectins on *Schistosoma mansoni* surface. *J. Ultrastruct. Res.* 72:325-335.
44. Unanue, E. R., W. D. Perkins, and M. J. Karnovsky. 1972. Ligand-induced movement of lymphocyte membrane molecules. I. Analysis by immunofluorescence and ultrastructural radioautography. *J. Exp. Med.* 136:885-906.
45. von Lichtenberg, F., A. Sher, and S. McIntyre. 1977. A lung model of schistosome immunity in mice. *Am. J. Pathol.* 87:105-20.
46. Wilson, R. A., and P. E. Barnes. 1974. The tegument of *Schistosoma mansoni*: observations on the formation, structure and composition of cytoplasmic inclusions in relation to tegumental function. *Parasitology.* 68:239-258.
47. Wilson, R. A., and P. E. Barnes. 1977. The formation and turnover of the membranocalyx on the tegument of *Schistosoma mansoni*. *Parasitology.* 74:61-74.
48. Wilson, R. A., and J. R. Lawson. 1980. An examination of the skin phase of schistosome migration using a hamster cheek pouch preparation. *Parasitology.* 80:257-266.