# STUDIES OF THE ANTIBODY-DEPENDENT KILLING OF SCHISTOSOMULA OF *SCHISTOSOMA MANSONI* EMPLOYING HAPTENIC TARGET ANTIGENS

## I. Evidence That the Loss in Susceptibility to Immune Damage Undergone by Developing Schistosomula Involves a Change Unrelated to the Masking of Parasite Antigens by Host Molecules*

By GINA MOSER,‡ DONALD L. WASSOM, and ALAN SHER§

*From the Departments of Medicine and Pathology, Harvard Medical School and the Robert B. Brigham Division of the Affiliated Hospitals Center, Inc., Boston, Massachusetts 02115; and the Department of Medicine and Immunology, Mayo Foundation, Rochester, Minnesota 55901*

Mice infected with *Schistosoma mansoni* acquire a partial resistance to subsequent challenge infection (1). A number of studies have suggested that this immunity is directed in large part against the early schistosomulum during the period immediately after its entry into the host (1–3). For example, it has been shown that within 24 h after intravenous injection into mice, newly transformed schistosomula lose their susceptibility to rejection by passively transferred antibody (1).

An intriguing mechanism for explaining the resistance of older parasites to immune killing was proposed by Smithers et al. (4). These investigators demonstrated the presence of substances of host origin on the integument of adult schistosomes and speculated that host molecules might promote the survival of the parasites by masking target antigens on the worm surface. In support of their hypothesis, a correlation was demonstrated between the acquisition of host molecules by schistosomula and the inability of the larvae to bind antibodies directed against parasite antigens (5, 6). Nevertheless, no direct evidence has been obtained supporting a protective role for host molecules, and other changes in the integument could account for the ability of older parasites to survive in immune hosts.

In this paper, a new approach for analyzing the mechanisms responsible for the loss in susceptibility to immune damage undergone by developing schistosomula is introduced. Parasites were surface labeled with haptenic target groups and tested at various stages of their development for their ability to be killed by hapten-specific effector mechanisms. Immune damage to the hapten-conjugated worms was assayed by measuring the survival of these parasites after either intravenous injection into

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hapten-immunized mice or in vitro culture with anti-hapten antibody in combination with either complement (C)[1] or human eosinophils. The above are adaptations of assays of antibody-dependent damage to schistosomula, which have been extensively utilized by workers in the field (1, 7). The experiments were designed for testing the specific hypothesis that the increased resistance of older schistosomula to immune attack is caused by an intrinsic change in the larvae which occurs independently of the masking of parasite antigens by the host molecules. Thus, if the absorption of host molecules is the principal mechanism by which lung-stage schistosomula escape antibody-dependent damage, then it should be possible to bypass the host molecule disguise of these parasites by superimposing nonparasite haptenic groups on their surface, thereby rendering them susceptible to immune damage by antihapten-directed antibodies. Alternatively, if hapten-conjugated lung worms behave like unlabeled lung worms and are found to be less susceptible than early stage schistosomula to immune killing, it would strongly suggest that a change in the parasite surface unrelated to the masking of parasite antigens by host molecules is responsible for the resistance of the larvae to immune attack.

Trinitrophenyl (TNP) groups were chosen as the haptenic moieties for the surface labeling of schistosomula in these experiments. This hapten has been used as a schistosomulum surface label for the investigation of helper T cell responses to the larvae (8–10). The labeling procedure utilized in that work killed the majority of the parasites (10), and therefore was not suitable for the present study. Described below is a modified TNP labeling procedure, which results in only a minimal loss in viability of the parasites. Using this technique, we have obtained evidence which suggests that an intrinsic change in the tegument of schistosomula plays a role in the resistance of older larvae to immune damage.

## Materials and Methods

*Laboratory Hosts.* Female DBA mice (6- to 9-wk-old) were obtained from The Jackson Laboratory, Bar Harbor, Maine. Outbred female $CF_1$ mice (18–20 g) and male CD rats (175–250 g) were supplied by Charles River Breeding Laboratories (Wilmington, Mass.).

*Parasite. Schistosoma mansoni* (Puerto Rican strain) is routinely maintained in our laboratory by passage through *Biomphalaria glabrata* snails and outbred $CF_1$ mice (11). Skin-stage (3-h-old) schistosomula were prepared in vitro from cercariae using an adaptation of the skin penetration method (12, 13). Lung-stage (5-d-old) schistosomula were prepared from mice infected subcutaneously with 2,000–3,000 cercariae, or injected intravenously with an equivalent number of skin-stage schistosomula (14). 4–6 d after infection, the mice were sacrificed, and viable schistosomula were recovered and purified from chopped lung tissue according to techniques which have been already described (2, 14, 15). Both the subcutaneous and the intravenous routes of infection resulted in a 15–30% recovery of lung-stage parasites and were found to yield organisms with similar properties in the assays used in this paper.

*Surface Labeling of Schistosomula.* Parasites were coupled with TNP using a modified version of a procedure previously described (9, 10). 2,4,6-trinitrobenzenesulfonic acid (TNBS) (ICN Nutritional Biochemicals, Cleveland, Ohio) was dissolved in Hanks' balanced salt solution (HBSS) and the solution adjusted to pH 7.0–7.2 with NaOH. Schistosomula were washed three times in HBSS by centrifugation (10 s, 400 *g*) and resuspended to a concentration of 1,500/ml. 1 vol of the parasite suspension was incubated at 37°C with an equal volume of the TNBS solution in a tube covered with foil. The mixture was allowed to react either for 15 min using

[1] *Abbreviations used in this paper:* BGG, bovine gamma globulin; C, complement; FCS, fetal calf serum; FITC, conjugated with fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; HI, heat-inactivated; MEM/H, Eagle's minimum essential medium buffered with Hepes; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TNP, trinitrophenyl.

2 mM TNBS, or for 45 s using higher concentrations of TNBS (1.56–25 mM). (The shorter incubation time was usually found to result in a lower loss in parasite viability and was therefore used throughout this study unless otherwise indicated.) The reaction was stopped by adding an excess of Hepes-buffered Eagle's minimum essential medium (MEM) (Grand Island Biological Co., Grand Island, N. Y.), containing 100 U/ml penicillin, 100 μg/ml streptomycin, 1% L-glutamine, and 20% fetal calf serum (FCS) (MEM/H–20% FCS) to the tube. The hapten-coupled parasites were then washed three times with MEM/H–10% FCS and two additional times with sterile medium for in vitro culture, or resuspended in MEM–2% FCS for injection into mice.

*Induction of Anti-TNP Antibodies and Preparation of Rat Anti-Schistosome Sera.* Anti-TNP antibodies were raised in New Zealand White rabbits by intramuscular injection with 3 mg of $TNP_{59}$bovine gamma globulin (BGG) (16) emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) followed by three boosts at 2-wk intervals with antigen at the same dose in incomplete Freund's adjuvant. $CF_1$ mice were immunized subcutaneously with TNP-BGG or unsubstituted BGG in Freund's adjuvant using a similar protocol employing 0.2 mg of antigen/injection (17). Sera from mice and rabbits actively immunized against TNP-BGG were found to contain average reciprocal hemagglutination titers (measured against TNP horse or sheep erythrocytes) of $2^8$ and $2^9$–$2^{13}$, respectively (18). Rat anti-schistosome sera was obtained from CD rats 8 wk after percutaneous infection with 1,500 cercariae each (19). All antibody pools were heat-inactivated (HI) for 30 min at 56°C before use.

*In Vivo Assay of Immunity.* Immunity to challenge infection was measured by determining the recovery of adult worms from the hepatic portal system 6 wk later, according to procedures previously described (19). The level of immunity to challenge infection was calculated according to the formula:

$$\text{percent immunity} = \frac{\bar{C} - \bar{E}}{\bar{C}} \times 100,$$

where $\bar{E}$ equals the mean recovery from experimental (TNP-BGG-immunized) and $\bar{C}$, the mean recovery from control (BGG-immunized) mice (seven to nine per group). The Student's *t* test was used to analyze the statistical significance of the differences in worm recoveries between the two groups.

*In Vitro Assay of Damage to Schistosomula by Antibody Plus C.* Antibody-dependent C-mediated damage to schistosomula was assayed in U-bottom microtiter wells using a modified version of a procedure described elsewhere (20). The duplicate assay wells contained 0.05 ml of schistosomula (100/well), 0.05 ml of diluted immune serum or HI normal rabbit (Gibco Diagnostics, GibcoInvenex Div., Chagrin Falls, Ohio) or rat serum, plus 0.1 ml of diluted guinea pig C or HI C, or medium (MEM/H–10% FCS) alone. After incubation for 18 or 63 h at 37°C, the organisms were transferred to slides previously coated with 0.1% toluidine blue and the percentage of dead larvae determined by microscopic examination (20, 21).

*In Vitro Assay of Damage to Schistosomula by Antibody Plus Eosinophils.* Antibody-dependent eosinophil-mediated damage to schistosomula was assayed using techniques similar to that above except that purified human eosinophils (0.1 ml) were substituted for guinea pig C and the cultures incubated for 18 or 36 h (20). The eosinophils were purified from the blood of one human volunteer by discontinuous metrizamide gradient centrifugation and ranged in purity from 83 to 96%. The percentage of dead larvae as well as the percentage of larvae with >5 adherent eosinophils were determined at the end of the culture period (20). In some experiments, the cell-parasite reactions were stopped by the addition of Karnovsky's fixative (22), and the percentage of adherent eosinophils which were degranulated scored by microscopic examination (× 1,000) with Nomarski optics on 10 worms/sample.

*Quantitative Immunofluorescence.* The amount of anti-TNP antibody bound to skin- and lung-stage schistosomula was compared by means of quantitative immunofluorescence. This method was chosen instead of radioimmunoassay because it measures individual worms and consequently avoids potential inaccuracies as a result of the presence of contaminating host tissue in lung worm preparations to which anti-TNP antibodies might also bind. That the measurements accurately reflect the relative amount of fluoresceinated ligand bound to the worm surface has

been confirmed in a separate series of experiments involving the reaction of lectin with the larval membrane (J. Samuelson. Personal communication.).

In the procedure, schistosomula were incubated with either rabbit anti-TNP sera (1:5), normal rabbit sera (1:5), or medium alone, washed and then reacted with fluorescein-conjugated (FITC) goat antirabbit IgG (1:10) (N. L. Cappel Laboratories Inc., Cochranville, Pa.) according to the indirect immunofluorescence protocol already described (11). The amount of fluorescence emitted/200 $\mu m^2$ area of a worm was measured in relative units by a Leitz MPV-2 photometer attached to a Leitz Orthoplan microscope equipped with a Ploem illuminator for fluorescein (E. Leitz, Rockleigh, N. J.). Approximately 20 readings (1 reading/worm) were taken per sample and averaged. Specific fluorescence was determined by subtracting the mean background values (TNP schistosomula plus normal rabbit serum plus FITC goat antirabbit IgG) from the mean experimental values (TNP schistosomula plus rabbit anti-TNP antibody plus FITC goat antirabbit IgG).

## Results

*Viability of TNP-labeled Schistosomula.* The viability of TNP-conjugated schistosomula was analyzed by either morphological examination 18–36 h after culture or portal recovery of these parasites 6 wk after intravenous injection. In general, the survival of TNP schistosomula both in vivo and in vitro was the same or slightly lower than that of uncoupled parasites. Thus, the portal recovery of TNP skin-stage schistosomula from unimmunized mice was ~35% vs. a recovery value of ~36% for uncoupled larvae. Similarly, after 36 h of culture, TNP-conjugated and unconjugated larvae exhibited mortality levels ranging from 5 to 20% and from 3 to 20%, respectively. Although these mortality values varied somewhat from experiment to experiment, lung-stage schistosomula, both coupled and uncoupled, consistently exhibited a lower loss in viability than skin-stage schistosomula.

To test for the possibility that the TNP labeling procedure induced sublethal damage to the parasites which renders them more sensitive to immune killing, the susceptibility of labeled and unlabeled schistosomula to damage by rat anti-schistosome antibody and C was compared. As indicated in Table I, TNP schistosomula,

TABLE I

*Susceptibility of TNP Schistosomula to Killing by Rat Anti-Schistosome Antibody Plus C*

| Incubation time | Concentration of TNBS used for labeling | Percent (mean) dead schistosomula | | |
|---|---|---|---|---|
| | | C + ab* | C − ab‡ | ([C + ab] − [C − ab]) |
| *h* | *mM* | | | *% specific kill* |
| 18 | 0 | 43.0 | 10.1 | 32.9 |
| | 6.25 | 38.4 | 9.0 | 29.4 |
| | 12.5 | 36.2 | 12.4 | 23.8 |
| | 25.0 | 45.5 | 14.3 | 31.2 |
| 63 | 0 | 76.9 | 19.5 | 57.4 |
| | 6.25 | 64.6 | 10.8 | 53.8 |
| | 12.5 | 63.4 | 19.4 | 44.0 |
| | 25.0 | 73.8 | 23.2 | 50.6 |

* Reaction mixtures contained 12.5% rat anti-schistosome sera and 25% guinea pig sera as a source of C.

‡ Reaction mixtures contained 12.5% normal rat sera and 25% guinea pig sera.

prepared with TNBS concentrations ranging from 6.25 to 25 mM, exhibited a sensitivity to anti-schistosome antibody-dependent C-mediated killing which did not differ significantly from that of unlabeled worms, even in cultures maintained for as long as 63 h. Therefore, as judged by this criterion, reaction with TNBS does not predispose the larval integument to immune damage.

*Relative Measurement of Anti-TNP Antibody Bound to TNP Schistosomula.* Before comparing the susceptibility of labeled skin- and lung-stage worms to damage by anti-TNP effector mechanisms, it was necessary to show that the amount of anti-hapten antibody bound to these TNP-conjugated larval stages was comparable. The technique of quantitative immunofluorescence was used for these measurements. As shown in Table II, lung-stage schistosomula labeled for 45 s with 25 mM TNBS or for 15 min with 1 mM TNBS, were found to emit either the same or a statistically greater amount of immunofluorescence than skin-stage worms. These results suggest that lung-stage schistosomula bind as much if not more anti-TNP antibody on their surface as do skin-stage schistosomula. In other experiments (not shown), TNP 3-h-old and TNP 5-old schistosomula were found after 36 h of culture to retain 96.4 and 81.2% of their TNP groups, respectively, as measured by microfluorimetry. Similarly, no significant loss of bound anti-TNP antibody was observed from either larval stage during the same culture period.

*Susceptibility of TNP Schistosomula to Rejection in TNP-immunized Mice.* Mice actively immunized against either TNP-BGG or BGG were challenged intravenously with either TNP-labeled skin- or lung-stage schistosomula. Recovery of the challenge infection was measured 6 wk later at the adult stage. As shown in Fig. 1 A, TNP 3-h-old worms were readily rejected by TNP-BGG immunized mice, as evidenced by a 96% reduction ($P < 0.001$) in the recovery of the parasites from TNP-BGG vs. BGG-immunized mice. In contrast, a much smaller percentage (46%) ($P < 0.02$) of the TNP 5-d-old schistosomula were rejected by the same TNP-immunized animals (Fig. 1 B), indicating that older worms are partially susceptible to hapten-specific killing, but clearly far less so than worms newly transformed from cercariae.

*Susceptibility of TNP Schistosomula to Anti-TNP Antibody-dependent C-mediated Killing In Vitro.* It has been previously shown that newly transformed schistosomula are readily

TABLE II

*Quantitation of TNP Groups by Microfluorimetry*

| Conditions for TNP coupling | Experiment | Specific fluorescence (in relative units)* | | |
|---|---|---|---|---|
| | | 3-h schistosomula | 5-d schistosomula | *P* values |
| A‡ | 1 | 49.8 | 72.3 | <0.05 |
| | 2 | 53.7 | 101.9 | <0.001 |
| B§ | 1 | 68.9 | 63.1 | NS‖ |
| | 2 | 80.5 | 79.5 | NS |

* See Materials and Methods. Note: unlabeled parasites plus anti-TNP antibody plus FITC goat antirabbit IgG (control for nonspecific binding of antibody) gave fluorescence readings similar to those obtained for the background group (8–27).

‡ Parasites surface labeled for 15 min with 1 mM TNBS.

§ Parasites surface labeled for 45 s with 25 mM TNBS.

‖ NS, not statistically significant.

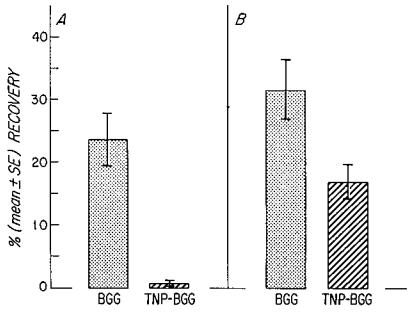


FIG. 1. Rejection of TNP schistosomula by $CF_1$ mice actively immunized against TNP. 3-h-old schistosomula (150/mouse) (A) and 5-d-old schistosomula (110/mouse) (B) were surface labeled with 1 mM TNBS for 15 min and used to challenge mice actively immunized against either TNP-BGG or BGG. Percent recovery refers to the percent of challenge schistosomula recovered as adult worms.

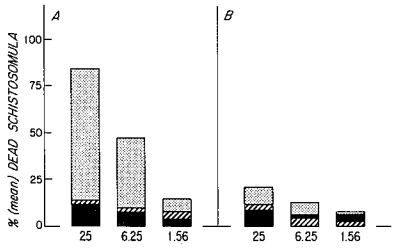


FIG. 2. Antibody-dependent C-mediated killing of TNP schistosomula. 3-h-old (A) and 5-d-old (B) schistosomula, surface labeled with either 25, 6.25, or 1.56 mM TNBS (45 s), were cultured with either rabbit anti-TNP antibody (12.5%) plus C (25%) (▩), normal rabbit serum plus C (▨), or medium alone (■). Parasite killing was assayed after 18 h of culture.

killed in vitro by antischistosome antibody plus C (7, 13). The susceptibility of TNP skin- and lung-stage schistosomula to TNP-specific killing by this C-mediated mechanism was compared in the following experiment. Schistosomula, labeled with different concentrations of TNBS (25, 6.25, and 1.56 mM), were exposed to anti-TNP antibody plus C or control media and examined after 18 h of culture. TNP skin-stage schistosomula were found to be extremely susceptible to C-mediated killing (Fig. 2). Furthermore, the level of killing was highly dependent on the concentration of TNBS used for labeling the larvae. In contrast, TNP lung-stage schistosomula were essentially refractory to C-mediated damage, even when labeled with the highest concentration (i.e., 25 mM) of TNBS tested.

To make certain that the insusceptibility of lung worms to complement-mediated killing was not caused by insufficient quantities of effector elements, cultures were set up in which the concentration of antibody and/or C was doubled. As shown in Fig. 3, 3-h-old schistosomula, labeled with either 12.5 or 25 mM TNBS and incubated with different dilutions of C (½ or ¼) and/or anti-TNP antibody (¼ or ⅛), were in all cases readily killed and most effectively at the higher concentrations of C. In contrast, lung-stage schistosomula remained almost totally resistant to TNP-specific killing, even when the concentration of C was increased to 50% and the concentration of antibody to 25%.

*Susceptibility of TNP Schistosomula to Anti-TNP Antibody-dependent Eosinophil-mediated*

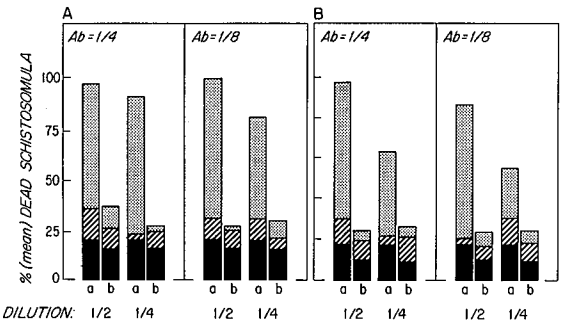


FIG. 3. Effect of varying the concentration of antibody and C on the killing of TNP schistosomula. 25 mM (A) or 12.5 mM (B) TNBS was used to surface label 3-h-old (a bars) and 5-d-old (b bars) schistosomula. Parasites were cultured for 18 h with either rabbit anti-TNP antibody plus C (▩), normal rabbit serum plus C (▨), at the dilutions indicated, or medium alone (■). Mortality was assessed as in Fig. 2.

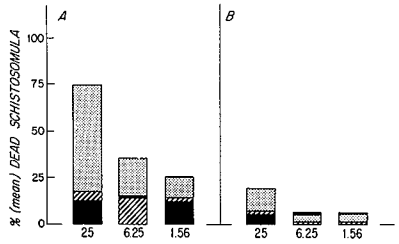


FIG. 4. Antibody-dependent eosinophil-mediated killing of TNP schistosomula. 3-h-old (A) and 5-d-old (B) schistosomula, surface labeled with either 25, 6.25, or 1.56 mM TNBS were cultured with either rabbit anti-TNP antibody (12.5%) plus human eosinophils (effector cell:parasite target = 2,000:1) (▩), normal rabbit serum (12.5%) plus eosinophils (▨), or medium alone (■). Parasite mortality was determined after 36 h of incubation.

*Killing In Vitro.* Another well-studied example of an antibody-dependent effector mechanism which kills schistosomula in vitro, is that mediated by human eosinophils (7, 20). In an adaptation of this assay to haptenated schistosomula, skin-stage parasites were found to be readily killed in vitro by anti-TNP antibodies and eosinophils, the level of killing being dependent on the concentration of TNBS (25, 6.25, and 1.65 mM) used for labeling (Fig. 4 A). In contrast, lung-stage schistosomula were shown to be highly resistant to killing, even when labeled with 25 mM TNBS (Fig. 4 B).

In this experiment, the adherence of effector cells to the larvae was also quantitated. As indicated in Fig. 5, after 36 h of culture, the adherence of eosinophils to lung-stage schistosomula was found to be greater than the adherence to skin-stage schistosomula at each of the three concentrations of TNBS used for labeling. This observation indicates that the resistance of the lung worms to eosinophil mediated damage is not a result of a failure in the binding of effector cells to the parasites.

To confirm that the failure of lung-stage schistosomula to be killed by antibody and effector cells was not caused by a deficiency of effector elements in the cultures, the concentration of eosinophils and/or anti-TNP antibody was doubled in the assay. As shown in Fig. 6, TNP lung-stage schistosomula remained resistant to killing, even when the effector cell/target ratio was increased to 4,000/1 and anti-TNP antibody increased to 25% in the cultures. It has been argued that temperature-dependent cell

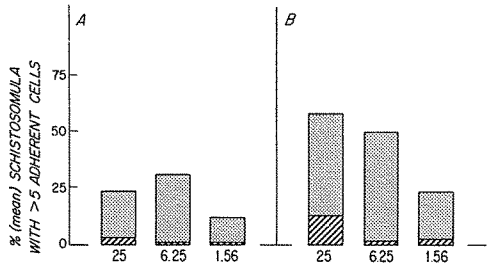


FIG. 5. Antibody-dependent adherence of eosinophils to TNP schistosomula. The adherence of effector cells to TNP 3-h-old (A) and 5-d-old (B) schistosomula as determined for the experiment depicted in Fig. 4. ▩, TNP schistosomula incubated with anti-TNP antibody plus eosinophils. ▨, TNP schistosomula incubated with normal rabbit serum plus eosinophils.

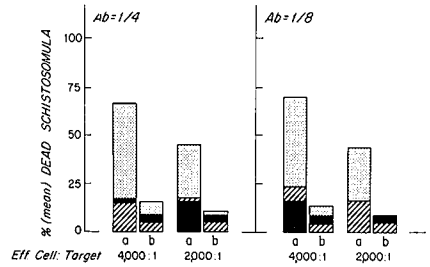


FIG. 6. Effect of varying the concentration of anti-TNP antibody (Ab) and eosinophils on the killing of TNP schistosomula. 3-h-old (a bars) and 5-d-old (b bars) schistosomula, surface labeled with 25 mM TNBS, were incubated with either rabbit anti-TNP antibody (diluted ¼=25% or ⅛=12.5%) plus eosinophils (effector [Eff.] cell:target=4,000:1 or 2,000:1) (▩), normal rabbit serum plus cells (▨), or medium alone (■). Parasite mortality was assayed after 36 h incubation.

degranulation is a crucial step in the antibody-dependent killing of schistosomula by eosinophils (21, 23). To confirm that the insusceptibility of TNP lung-stage schistosomula to killing by eosinophils was not caused by the failure of the cells to degranulate on the parasite surface, the percentage of degranulated eosinophils adhering to skin- vs. lung-stage TNP-labeled worm targets was compared. In two different experiments (Table III), the percentage of degranulation of cells on lung-stage worms was found not to differ significantly from the values determined for skin-stage larvae. In addition, both eosinophil degranulation and parasite killing were shown to be temperature dependent. However, in the case of lung worms, eosinophil degranulation was not accompanied by parasite killing.

## Discussion

Previous studies have suggested that the newly transformed schistosomulum is a major target in the rejection of challenge infections by mice with acquired resistance to *S. mansoni* (1–3). That the susceptibility of schistosome larvae to immune attack is age dependent has also been suggested by in vitro studies in which schistosomula after overnight culture were shown to become resistant to killing mediated by either antibody and C (24) or antibody plus eosinophils.[2]

[2] Dessein, A., J. C. Samuelson, A. E. Butterworth, M. E. Hogan, B. A. Sherry, M. A. Vadas, and J. R. David. 1979. Immunologic studies on *Schistosoma mansoni* larvae. I. Loss of susceptibility to antibody or complement-dependent eosinophil attack by schistosomula cultured in medium free of macromolecules. Manuscript submitted for publication.

TABLE III

*Degranulation of Eosinophils on the Surface of TNP Schistosomula*

| Experiment | TNP larvae | Percent degranulated eosinophils adhering to larvae* | | Percent (mean) larvae with >5 adherent eosinophils* | | Percent (mean) specific kill‡ ([cells + ab] − [cells − ab]) | |
|---|---|---|---|---|---|---|---|
| | | 37°C | 4°C | 37°C | 4°C | 37°C | 4°C |
| | | *mean ± SD* | | | | | |
| 1 | Skin | 52.7 ± 12.8 | 3.9 ± 7.8 | 88.8 | 100 | 80.5 | ND§ |
| | | 53.9 ± 21.7 | 6.1 ± 3.6 | | | | |
| | Lung | 61.6 ± 16.7 | 5.5 ± 4.9 | 100 | 100 | 15.2 | ND |
| | | 65.1 ± 15.3 | 6.1 ± 3.5 | | | | |
| 2 | Skin | 70.0 ± 14.9 | 3.6 ± 4.1 | 100 | 95 | 87.8 | 0 |
| | | 65.8 ± 14.4 | 4.7 ± 4.5 | | | | |
| | Lung | 74.6 ± 7.6 | 3.0 ± 3.4 | 100 | 100 | 11.3 | 1.8 |
| | | 72.2 ± 14.1 | 3.2 ± 3.5 | | | | |

* Results from duplicate samples were scored after 18 h of incubation.
‡ Samples were assayed after 36 h of incubation.
§ ND, not done.

In the present study, we have compared the susceptibility of developing schistosomula to immune killing in a model system employing TNP target antigens and anti-TNP antibodies. The major conclusion of this report is that TNP-coupled lung-stage (5-d-old) schistosomula are far less susceptible than TNP-labeled skin-stage (3-h-old) schistosomula to killing by anti-TNP effector mechanisms both in vivo (Fig. 1) and in vitro (Figs. 2–4 and 6). This difference was observed even though TNP lung-stage larvae appeared to bind the same amount or more anti-TNP antibody than TNP skin-stage larvae before culture (Table II) and were found to retain most of their TNP as well as bound anti-TNP antibody after 36 h of culture.

In our in vivo experiments, TNP skin worms were shown to be extremely susceptible, whereas TNP lung-stage worms were found to be partially (although far less) susceptible to immune killing in TNP-immunized mice (Fig. 1). Rejection of schistosomula in these animals is likely to involve an antibody-dependent mechanism, because sera from the mice were found to contain high titers of anti-TNP antibodies and because in separate studies (G. Moser and A. Sher. Unpublished observations.) we have been able to transfer significant immunity against TNP schistosomula with as little as 0.015 ml of mouse anti-TNP serum per recipient.

The susceptibility of TNP larvae to antibody-dependent damage was tested directly in our in vitro experiments. In contrast with TNP skin-stage schistosomula, TNP lung-stage schistosomula were found to be highly resistant to rabbit anti-TNP antibody-dependent damage mediated by either C or eosinophils. That the insusceptibility of lung worms to TNP-specific killing mechanisms in vitro was not a result of a deficiency in the number of TNP target antigens on the surface of the parasite or to an insufficient concentration of effector elements in the cultures was demonstrated by showing that doubling the TNBS used in labeling or the concentrations of antibody, C, or eosinophils did not significantly alter the killing of the parasites (Figs. 3 and 6). It could be argued, however, that the failure of lung worms to be killed by antibody plus C is caused by the inactivation of C components by the parasite during culture. Although this possibility cannot be ruled out, it seems unlikely in light of recent

results, where it was shown that during a 45-min incubation, lung-stage schistosomula deplete neither C3 nor Factor B from human serum.[3] Similarly, it is improbable that the insusceptibility of lung-stage parasites to killing by anti-TNP antibody plus eosinophils was a result of the blocking of the reaction by the parasite, because the adherence of eosinophils to the lung worms was in fact greater than that to skin-stage schistosomula (Fig. 5) and the degranulation of these effector cells was quantitatively comparable on both parasite targets (Table III).

On the basis of the above evidence, we propose that the insusceptibility of lung worms to immune damage must be caused at least in part by an intrinsic change in the larval surface which is independent of the masking of parasite antigens by host molecules. Our results, however, do not exclude a possible function for host molecules in blocking the interaction of toxic effector elements (e.g., C activation products, eosinophil major basic protein, etc.) with the parasite. Morphological studies have already demonstrated the presence of structural changes in the integument of schistosomula during the early phase of their development (25, 26). It is unlikely that the formation of a double unit membrane is involved in the loss of susceptibility to immune damage because this second lipid bilayer is already present when the worms are still susceptible to immune attack (25). Recent freeze-fracture studies have revealed a redistribution of intramembranous particles along with a change in the type of inclusion body in the integument of maturing worms, which occurs during the development of resistance to cytotoxic attack (27). However, a direct causal relationship between these changes in the ultrastructure of the schistosome membrane and the resistance of the parasite to damage has not been established.

In addition to the integumental change discussed in this study, other processes have been described which probably contribute to the transition in susceptibility to immune damage undergone by developing schistosomula. Dean has argued that schistosomula cultured in vitro become resistant to killing by antibody and C because of a loss in their antigenicity (i.e., reactivity with host antibody) which occurs independently of host molecule acquisition (24). That schistosomula do indeed spontaneously and permanently lose their surface antigens recognized by antischistosome antibody during culture in chemically defined media has recently been confirmed by quantitative immunofluorescence (28). Because either of these changes (i.e., an intrinsic structural change in the integument or a loss in surface antigens) in itself could account for the resistance of lung worms to immune damage, the relevance of host molecule acquisition to the survival of the parasites is uncertain. Nevertheless, it is possible that acquired host molecules (e.g., human A, B, H, and Lewis blood group antigens) (29) and mouse major histocompatibility complex gene products (11) may play a protective role at a later (i.e., postlung) stage in the parasite's development, or may have some other immunoregulatory function. In this regard, it should be recalled that in the experiments of Smithers et al. (4), adult schistosomes recovered from mice were readily rejected when transferred to recipient monkeys immunized against mouse tissue, a finding which indicates that adult worms are indeed susceptible to immune damage and that host molecule uptake might be responsible for their long-term survival during natural infection. Thus, it should be emphasized that the findings presented here apply only to the antibody-dependent killing of schistosomula during

[3] Dias da Silva, W., and M. D. Kazatchkine. Activation of the alternative pathway of human complement by schistosomula of *Schistosoma mansoni*. *Exp. Parasitol.* In press.

the early stages of their development in the host. Recent evidence suggests that there may be additional killing mechanisms operating against later (i.e., postlung) developmental stages of the parasite in mice immunized by chronic infections (1, 3). The factors governing the susceptibility of the parasite to this putative late mechanism of killing have not yet been eludicated.

Finally, it should be noted that previous investigations of the killing of schistosomula have employed heterogenous antibodies directed against multiple antigens on the parasite surface. As a result, they have been of limited value for quantitatively analyzing the specific antigen-antibody interactions involved in parasite damage. This difficulty can now be overcome by the use of haptenated schistosomula as described here. The approach may have general application for analyzing the immune killing of a variety of different parasite targets in addition to schistosomula.

## Summary

A method was developed for coupling a hapten, trinitrophenyl (TNP), to the surface of schistosomula of *Schistosoma mansoni* which results in a minimal loss in their viability as judged by morphological examination in vitro and survival after injection in vivo. Skin-stage (3-h-old) and lung-stage (5-d-old) schistosomula surface labeled in this manner were then compared for their susceptibility to killing by anti-TNP antibody-dependent effector mechanisms both in vivo and in vitro. TNP skin-stage larvae were readily rejected in mice actively immunized against TNP bovine gamma globulin and were highly susceptible to anti-TNP-dependent killing mediated either by complement or purified human eosinophils in vitro. In contrast, TNP-lung-stage schistosomula, which were shown by microfluorimetry to bind anti-TNP antibody to approximately the same extent as skin-stage schistosomula, were found to be resistant to killing by the same in vivo and in vitro mechanisms. These findings suggest that the insusceptibility of postskin-stage schistosomula to antibody-dependent killing must result at least in part from an intrinsic structural change in the integument of the parasite and cannot be caused solely by the masking of parasite antigens by acquired host molecules, a mechanism of immune evasion previously proposed for schistosomes.

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