Species-related Innate Resistance to *Schistosoma mansoni*

ROLE OF MONONUCLEAR PHAGOCYTES IN SCHISTOSOMULA KILLING IN VITRO

CHARLES A. PECK, MURRAY D. CARPENTER, and ADEL A. F. MAHMOUD, *Division of Geographic Medicine, Department of Medicine, Case Western Reserve University and University Hospitals, Cleveland, Ohio 44106*

ABSTRACT Resistance to infection with the multicellular parasite *Schistosoma mansoni* has been previously demonstrated to vary among several host species. The current investigation was designed to examine the basis for this species-related resistance in vitro. Adherent peritoneal macrophages or peripheral blood mononuclear cells from several species of host animals were incubated with *S. mansoni* schistosomula for 18–24 h; parasite viability was then assayed by methylene blue exclusion. Peritoneal exudate macrophages from susceptible species, such as mice (C57Bl/6) and hamsters killed, respectively, 6.6±2 and 8.0±2% of incubated schistosomula. In contrast, cells from resistant species: rats, guinea pigs, and rabbits, killed 21±2.3, 15±4.6, and 17±5.5%, respectively. Furthermore, blood monocytes from rabbits resulted in a mean of 25.9±2.8% dead organisms. Schistosomula killing by mononuclear phagocytes obtained from resistant species (rats or rabbits) was dependent on the cell/parasite ratio. Significant schistosomula mortality resulted from culture supernatants of rat macrophages or rabbit monocytes. Killing by cells from both species was significantly reduced upon addition of L-arginine, while catalase reduced killing only by rat macrophages. We conclude that mononuclear phagocytes may play a key role in species-related innate resistance to schistosomiasis; their in vitro schistosomulicidal activity parallels the known in vivo susceptibility of the donor species. Killing is mediated by lysosomal enzymes (arginase) and by products of oxidative metabolism; the predominant mechanism depends on the specific animal species.

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INTRODUCTION

Susceptibility to infection with the multicellular helminth of the genus Schistosoma has been shown to depend on the species of the parasite as well as that of the host (1–3). For example, man is susceptible to infection with *Schistosoma mansoni*, *Schistosoma haematobium*, and *Schistosoma japonicum*, whereas cercariae of avian schistosomes can only penetrate skin and soon die resulting in a syndrome known as cercarial dermatitis or swimmers' itch (4, 5). Similar observations have been reported using laboratory animals; the susceptibility to infection with the human parasite *S. mansoni* has been shown to be species related (1, 2). Mice and hamsters are very susceptible with ~30–40% of infective cercariae maturing into adult worms but rabbits and rats are resistant with no more than a 5% maturation rate. Attempts to infect fox or muskrats showed that they are completely resistant (1).

Understanding the basis of variability of host resistance to *S. mansoni* may help elucidate some of the biologically relevant defense mechanisms. Although a multitude of in vitro systems of schistosomula killing (the larval stage of the parasite) have been described, little is known of their contribution to resistance in vivo. In the mouse, in vivo experiments have delineated a role for the eosinophil (6), combination of antibody and complement (7), and for activated macrophages (8–10) in resistance to *S. mansoni*. In man, the inability to correlate antibody-dependent eosinophil-mediated killing of schistosomula with intensity

of infection (11) as an index of susceptibility has frustrated attempts to define which system, if any, contributes to human protective responses to schistosomiasis. Recently, we have demonstrated that adherent peripheral blood monocytes from normal human donors kill a significant proportion of schistosomula in vitro (12). Furthermore, the schistosomulicidal activity of these cells was found to be inversely proportional to the intensity of infection in the donors (13). The cytotoxic activity of host monocytes may therefore reflect a biologically relevant mechanism involved in resistance to schistosomiasis and in determination of intensity of infection.

In this study, we examined the role of mononuclear phagocytes in host resistance to *S. mansoni* in several laboratory animals. These animals were chosen on purpose, to represent different known in vivo degrees of susceptibility to infection. Schistosomula killing by peripheral blood monocytes or peritoneal exudate macrophages from a certain host species in vitro was found to parallel the known in vivo degree of that host's resistance to infection. In addition, schistosomula killing by the mononuclear phagocytes of the resistant species (rabbits and rats) appears to be mediated by a combination of products of oxidative metabolism and secretion of the lysosomal enzyme arginase.

METHODS

Animals. Female C57Bl/6J, BALB/cJ, and CBA mice weighing 18 to 20 g each were purchased from Jackson Laboratory, Bar Harbor, ME. New Zealand female rabbits weighing 4 kg were obtained from Gutman Laboratories, Thompson, OH. White, female outbred CF1 mice, Syrian hamsters, Fisher rats, and guinea pigs (weighing 200–250 g each) were purchased from Charles River Laboratories, North Wilmington, MA.

Peritoneal macrophage preparations. Resident peritoneal macrophages were obtained by lavage with Hanks' balanced salt solution (HBSS) to which 10 U/ml heparin was added. Cells were washed twice and then suspended in supplemented RPMI 1640 medium (KC Biologicals, Inc., Lenexa, KS) containing 50 U/ml penicillin, 5 μg/ml gentamicin, 2 μM L-glutamine, and 10% heat-inactivated fetal calf serum. To obtain "elicited" macrophages, an aliquot of 10% aqueous solution of proteose peptone was injected intraperitoneally into each animal; 2 ml per mouse, 3 ml per hamster, 10 ml per rat or guinea pig, and 25 ml per rabbit. Cells were harvested 4 d later, washed twice at 1,000 rpm for 15 min, and suspended in supplemented RPMI 1640 or Fisher's exact medium (KC Biologicals, Inc.). Cell numbers were adjusted to the desired density by counting in a Coulter counter (Coulter Electronics, Inc., Hialeah, FL) and viability was determined by trypan blue exclusion. Aliquots of 0.5 ml of cell suspension were incubated for 3 h at 37°C in 16-mm Linbro tissue culture plates (Linbro Chemical Co., Hamden, CT). Each well was then washed with fresh medium at 37°C to remove nonadherent cells (8). In some wells the adherent cells were washed with 0.1% xylocaine-HBSS and enumerated in a Coulter counter. Greater than 95% of these adherent cells were found to phagocytose latex particles.

Peripheral blood mononuclear cells. Normal human volunteers from Cleveland and rabbit peripheral blood mononuclear cells (PBMC)[1] were obtained by Ficoll-Hypaque sedimentation of heparinized blood (14). Adherent cell monolayers were prepared as described above; they contained >90% esterase-positive cells or monocytes (15). A nonadherent lymphocyte-enriched preparation was obtained from washing of the dense monolayers and contained <1% monocytes. These were used as controls for parasite incubations in some experiments.

Parasite cytotoxicity. Adherent mononuclear phagocyte monolayers were cultured in supplemented medium for 24 h before the addition of parasites. Schistosomula of a Puerto Rican strain of *S. mansoni* were freshly prepared by skin penetration and adjusted to 4,000 organisms/ml. Aliquots of 50 μl were added to each well containing cells or medium. Plates were then incubated at 37°C in a humidified 5% CO_2 atmosphere for 18–24 h. Evaluation of schistosomula viability was performed by exclusion of methylene blue; percent mortality was calculated as the proportion of dead/total schistosomula counted. This method has previously been shown to correlate with schistosomula ability to mature into adults upon injection into susceptible hosts (12, 16). Duplicate incubations were performed for each variable; viability was counted after coding the wells. All percent dead organisms are expressed after subtracting mortality in control cultures with resident macrophages or medium alone that did not exceed 5% in any single experiment. The Student's *t* test was used to evaluate the significance of observed differences.

In some experiments, the contents of each well (cells and parasites) were aspirated, washed twice, and smears were made. After fixation, duplicate slides were stained with Giemsa or for nonspecific esterase (15) to identify the type of cells adherent to schistosomula.

Mediators of schistosomula killing. To examine the role of soluble mediators in parasite killing, the contents of wells from 24-h cultures of schistosomula with either rat or rabbit mononuclear phagocytes were aspirated and centrifuged at 600 *g* for 15 min. Control supernatants were obtained from similar cultures without organisms or from culture with cell preparations of species known not to result in significant parasite killings, e.g., mouse or man. Aliquots of 0.5 ml of these supernatants were added to 200 freshly prepared schistosomula and incubated for 24 h at 37°C; killing of the organisms was determined as above.

Blocking of parasite killing by mononuclear phagocytes or their culture supernatants was also attempted using either scavengers of oxygen products (17) or blockers of enzymes previously shown to kill *S. mansoni* or tumor cells (18, 19). The effects of several concentrations of catalase, superoxide dismutase, sodium azide, and L-arginine (Sigma Chemical Co., St. Louis, MO) were studied.

RESULTS

Peritoneal macrophage-mediated schistosomula killing. Adherent peritoneal exudate cells (PEC) obtained from proteose peptone-treated rats, guinea pigs, or rabbits, but not C57Bl/6J mice or hamsters, killed significant proportions of schistosomula at 24 h (Fig. 1). At a cell concentration of 6×10^6 well (equivalent

[1] *Abbreviations used in this paper*: PBMC, peripheral blood mononuclear cells; PEC, peritoneal exudate cells.

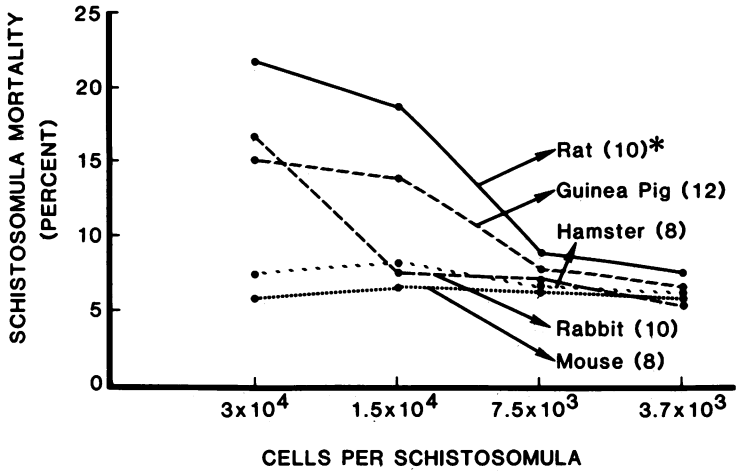

FIGURE 1 Effect of various cell/parasite ratios on schistosomula killing by proteose peptone-induced peritoneal macrophages of five animal species. Adherent cell monolayers were cultured for 24 h with 200 schistosomula in supplemented RPMI 1640 medium with 10% fetal calf serum. Data are expressed as mean percent killing; ( ) refer to number of animals in each group.

to a cell/target ratio of 3.0×10^4:1), the mean parasite death in four separate experiments by adherent macrophages of rats, guinea pigs, and rabbits was 21±2.3, 15±4.6, and 17±5.5%, respectively. All values are significantly different from mean survival of schistosomula in supplemented medium (5±1.1%) at the 1% level. In contrast, similar numbers of C57Bl/6J mice or hamster PEC failed to induce significant schistosomula killing; the mean parasite death was 6.6±2 and 8.0±2%, respectively. In experiments using PEC from two other inbred strains of mice, Balb/cJ and CBA, and one outbred strain (CF_1) no significant parasite mortality was detected; the mean dead schistosomula was 3.8±1.2, 7.4±3.0, and 5.5±2.0%, respectively. Furthermore, killing by PEC of resistant species was associated with adherent cells as an equivalent number of nonadherent cells from rats or rabbits had no significant effect on schistosomula viability.

The relationship of cell/target ratio to subsequent killing of the parasites was further examined (Fig. 1). Decreasing cell/target ratio of rat adherent macrophages from 3.0 ± 10^4:1 to 1.5×10^4:1 did not change parasite killing significantly (21±2.3 vs. 19±1.4%). At a ratio of 7.5×10^3 cells to one organism, the mean parasite killing was 8.7±3%, significantly higher than the mortality in supplemented medium ($P < 0.05$). Schistosomula killing by rat peritoneal macrophages at a cell/target ratio of 3.7×10^3:1 was not significantly different from parasite mortality in supplemented medium alone. Similar observations were obtained using adherent peritoneal macrophages from guinea pig and rabbits. The only detectable difference when using rabbit macrophages was that decreasing cell/target ratio to 1.5×10^4:1 led to loss of significant parasite killing. In all experiments using peritoneal macrophages, the number of cells adhering to the bottom of each well did not differ significantly among species (Table I). Cell/target ratios were, however, corrected for the percentage of adherent cells.

Microscopic examination of cell-parasite preparations indicated that >97% of cells that attached to schistosomula were macrophages. Fig. 2 shows a Giemsa-stained preparation of schistosomula incubated for 24 h with rat adherent PEC. Macrophages formed more than one cell layer around schistosomula: the granularity of the organisms and disruption of some parts of its membrane are morphologic indications of parasite destruction.

PBMC-mediated schistosomula killing. Rabbit PBMC killed significantly more schistosomula than human PBMC at a cell/target ratio of 3.0×10^4:1 (25.9±2.8 vs. 11.2±2%, $P < 0.01$). At cell/target ratios of 1.5×10^4:1 and 7.5×10^3:1 killing by rabbit monocytes was reduced to 6.6±1.6 and 7.1±2.1%, respectively. This did not differ from killing in supplemented medium alone (5.5±1.2%). It is interesting to note that on per cell basis rabbit PBMC killed significantly more organisms than PEC (25.9±2.8 and 15.6±1.5%, respectively, $P < 0.05$).

The proportion of adherent PBMC of man or rabbits remained constant throughout the range of cell/target ratio used. At total cell counts of 6×10^6/well, the adherent cell/target ratio for rabbit and human was 1.0×10^4:1 and 1.1×10^4:1 (Table I).

Mediators of schistosomula killing. Supernatants removed from cultures of schistosomula and the two most cytotoxic cell populations, i.e., rat PEC and rabbit PBMC were added to fresh organisms. These super-

TABLE I

Comparison between the Total Number of PEC or PBMC Added to Linbro Wells, Those Which Adhered and the Resulting Cell Parasite Ratios. Cells were Obtained from the Peritoneal Cavities of Protease Peptone-treated Mice, Guinea Pigs, Rabbits, or Rats. PBMC Were Obtained from Rabbits or Normal Human Volunteers. There Was No Significant Difference in Cell/Parasite Ratio in Any of the Species Tested

	PEC				PBMC	
	Mouse	Guinea pig	Rabbit	Rat	Rabbit	Human
Total cell numbers added to Linbro wells	6×10^6	6×10^6	6×10^6	6×10^6	6×10^6	6×10^6
Number of adherent cells (%)	2.9×10^6 (48)	3.0×10^6 (50)	2.7×10^6 (45)	2.8×10^6 (48)	2.0×10^6 (33)	2.2×10^6 (37)
Number of schistosomula	200	200	200	200	200	200
Adherent cell/target ratio	1.5×10^4	1.5×10^4	1.4×10^4	1.4×10^4	1.0×10^4	1.1×10^4

natants were obtained from 24-h cultures of ~2.8 $\times 10^6$ adherent cells with 100 schistosomula (Table II). Rat macrophage supernatant when added to fresh organisms killed 19.2±0.8% schistosomula, while rabbit PBMC supernatant killed 21.4±4.2% of the organisms. Both values are significantly different from killing by mouse PEC supernatant (6.1±1%) at the 1% level. Furthermore, viability of organisms was not affected by supernatants of schistosomula cultured alone in RPMI 1640 for 24 h indicating that parasite death per se does not lead to release of toxic products.

We subsequently examined the possible toxic mediators responsible for parasite mortality. Rat peritoneal macrophages were prepared in Fisher's medium (L-arginine content 15 μg/ml). To these cultures schistosomula were added followed by different concentrations of L-arginine. Although 100 or 200 μg/ml exogenous L-arginine had no effect on parasite mortality, killing was significantly reduced by 400 or 800 μg/ml, to respective means of 2.4±7 and 2.3±8% ($P < 0.01$) (Fig. 3). Blocking of parasite killing by culture supernatants was also achieved by addition of L-arginine; an amount of 800 μg/ml reduced killing from a mean of 19.2±1.3 to 3.3±1.8% ($P < 0.001$). Similarly, killing of schistosomula by rabbit PBMC cultured in Fisher's medium (L-arginine content 15 μg/ml) and RPMI 1640 medium (L-arginine content 200 μg/ml) was 28.9±1.7 and 23.6±2.3%, respectively. The background mortality was similar in both media (5.4±0.2%). Addition of exogeneous L-arginine to rabbit PBMC and schistosomula cultured in Fisher's medium inhibited parasite killing; L-arginine at 400 and 800 μg/ml reduced killing from a mean of 28.9±1.7% to respective means of 8.5±1 ($P < 0.05$) and 5.8±0.7% ($P < 0.01$) (Fig. 3).

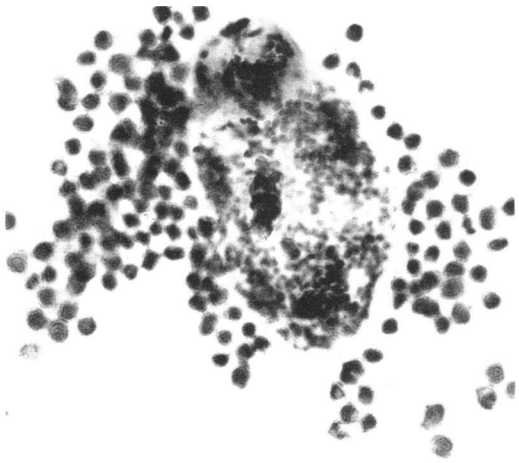

FIGURE 2 Photomicrograph of *S. mansoni* schistosomula following 24 h incubation with rat adherent PEC. Smears were fixed and stained with Giemsa. Morphologically, >97% of cells adherent to the parasite were identified as macrophages. Damage of schistosomula is indicated by prominent granularity and disruption of parasite surface in some areas.

Because of the known negative surface charge of schistosomula, we examined whether pH changes due to L-arginine had a direct deleterious effect on the parasites. We incubated schistosomula in media enriched with two other basic amino acids, L-glutamine and L-isoleucine; the pH of each of these amino acid solutions was adjusted to 7.30. Killing of schistosomula by rat PEC or rabbit PBMC was not significantly different when concentration of 100 or 800 μg/ml of either of the two amino acids was used.

Subsequently, the role of oxidative metabolism in parasite killing was investigated. Rat macrophage-mediated killing was significantly inhibited by catalase (5,000 U/ml) from a mean of 22.4±1.5 to 3.5±1.3% ($P < 0.001$), while superoxide dismutase had no significant effect. Heated catalase in similar concentrations did not inhibit rat PEC-mediated killing of schistosomula or decrease viability of the parasites. Sodium

TABLE II

Comparison of Schistosomula Killing by Adherent Cell Monolayers or Culture Supernatants. 200 Schistosomula Were Incubated for 24 h with Adherent Monolayers. Supernatants Were Collected, Concentrated by PM-10 Filter to One-fifth Volume and Tested for Cytotoxicity on Fresh Organisms

Incubations	Killing by cell monolayers	Killing by culture supernatants (mean±SE)	Killing by supernatant effluent
	%		%
Proteose peptone-induced rat PEC + schistosomula	18.7±2.8*	19.2±0.8‡	6.0±1.15§
Rabbit PBMC + schistosomula	34.6±5.8*	21.4±4.2‡	4.2±1.2§
Proteose peptone-induced mouse PEC + schistosomula	6.1±1%	4.8±0.7%‡	5.2±0.5
RPMI 1640 medium + schistosomula	4.23±1.24*	5.73±0.98‡	ND

* Difference between parasite killing by rat PEC or rabbit PBMC and in controls incubated with medium alone is significant at 0.1% level.

‡ Difference between killing by supernatants from rat PEC culture or rabbit PBMC and those from mouse PEC or controls is significant at 0.1% level.

§ Difference between killing by supernatant from rat PEC or rabbit PBMC cultures and their effluent from PM-10 filter is significant at 1% level.

azide (10^{-3} M) suppressed killing by rat macrophages from a mean of 16.4±2.5 to 5.6±1.8% ($P < 0.01$). Since rabbit PBMC are known to lack cytochemically detected myeloperoxidase in their granules (20), it was interesting to study the effects of peroxidative pathway inhibitors on schistosomula killing. At a cell/target ratio of 1.5×10^4:1, rabbit adherent PBMC killed a mean of 25±5.5% schistosomula. Addition of catalase (5,000 U/ml) or superoxide dismutase (5,000 U/ml) had no effect on parasite mortality.

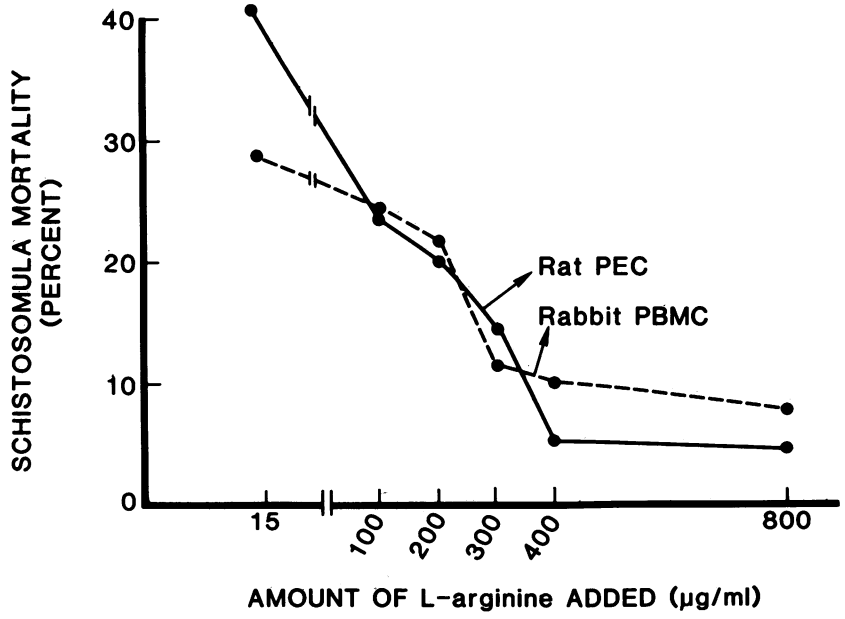

FIGURE 3 Blocking of schistosomula killing by rat PEC or rabbit PBMC (cell/target ratio 3×10^4:1) by various amounts of L-arginine. Adherent cell monolayers were cultured for 24 h with 200 schistosomula and 10% fetal calf serum; L-arginine was added at the initiation of incubations. Data are expressed as mean percent killing of parasites based on three separate experiments.

DISCUSSION

Susceptibility to *S. mansoni* infection varies significantly among different mammalian host species (1–3). Mice and hamsters are very susceptible, whereas rats and rabbits are extremely resistant; no patent infection can be detected in muskrats or fox. The mechanism and mediators of this innate species-related resistance to schistosomiasis is not known. Of the multiple in vitro models for schistosomula killing acquired in experimental animals, antibody alone or in association with complement, eosinophils, or macrophages have been shown to play an important role in acquired resistance in vivo (6–10). A common feature of all these models is that they involve mechanisms dependent on prior exposure to the schistosome worms. They, therefore, describe acquired rather than innate host defenses.

Although innate resistance to schistosomiasis is a poorly defined phenomenon, it seems to have a significant biological role. For example, avian schistosomes can only penetrate human skin and then die (5). Similarly, most human schistosome cercariae succumb in the subcutaneous tissues of rabbits, guinea pigs, and muskrats (1, 2). The major difficulty in studying innate resistance is the absence, until recently, of an in vitro model. When we described mononuclear phagocyte-mediated killing of schistosomula in the absence of specific antibody or complement (12), it was suggested that this system may be helpful in studies of resistance to primary exposure to the parasite. Further support of a significant role of monocytes was obtained from our field studies; human monocyte-mediated killing of schistosomula is inversely proportional to intensity of infection (13). Monocytes from heavily infected individuals were particularly deficient in this cytotoxic effect.

To examine the hypothesis that mononuclear phagocytes play an important role in innate host defense, we took advantage of the unique species-related resistance to schistosomiasis. Our results show that the known in vivo variability in resistance can be reproduced in vitro by studying the cytotoxic effects of the mononuclear phagocytes. Proteose peptone-induced adherent peritoneal macrophages from rats, as well as guinea pigs and rabbits, killed significant numbers of schistosomula. In contrast, mouse or hamster PEC did not impair parasite viability, reflecting the known susceptibility of these two species to infection. Similar variability in schistosomula killing by PBMC was observed; rabbit cells killed two and a half times as many parasites as human monocytes. Over 95% of the adherent cells used in our investigations were macrophages or monocytes as evidenced by latex phagocytosis, morphologic criteria, and nonspecific esterase staining. These cells were demonstrated to adhere to the parasite surface and to cause morphologically evident damage to its structures. The difference in schistosomula killing observed by cells of different species was not due to variations in cell/target ratios; all data were corrected for the number of cells adhering to wells. Rat peritoneal exudate macrophages have been previously shown to cause considerable release of ^{51}Cr from labeled schistosomula in the absence of specific antibody (21). Furthermore, nonadherent mononuclear cells (mainly lymphocytes) from the two most resistant strains examined, rats and rabbits, had no effect on parasite viability.

The mononuclear phagocytes have been demonstrated to possess microbicidal (17, 22) as well as cytotoxic capabilities against tumor cells (23, 24); its activity against multicellular parasites is, however, poorly defined (25, 26). Klebanoff (20) has recently reviewed the basis for monocyte-mediated microbicidal effect; some of these cells possess a myeloperoxidase different from that obtained from neutrophils. The monocytes are capable of generating active products of oxidative metabolism such as hydrogen peroxide or hydroxyl radical that may be lethal to the schistosomes (27). While rat mononuclear phagocyte-mediated parasite killing was inhibited by catalase, rabbit monocyte cytotoxicity was not blocked by this enzyme, which may be related to the lack of cytochemically demonstrable peroxidase in their granules (20). The mononuclear phagocytes possess, in addition, nonoxygen-dependent systems, thought to contribute to its cytotoxicity to tumor cells (19, 28). Recently, we have shown that arginase plays an important role in activated murine macrophage-mediated killing of schistosomula (18). In this study, addition of L-arginine, but not other basic amino acids, blocked parasite killing by rat or rabbit mononuclear phagocytes or their culture supernatants. The exact nature of this arginase-related cytotoxicity is not clear; it may affect the parasite directly by interfering in its metabolism (29) or lead to accumulation of toxic products such as urea or ammonia. Finally, the observation that parasite killing can be achieved by culture supernatants suggest that it is mediated by long-acting substances and may argue against a sole role for products of oxidative metabolism known to be short lived (30).

In conclusion, our data suggest that the mononuclear phagocytes are involved in innate species-related resistance to schistosomiasis. The wide spectrum of susceptibility of different animal species to *S. mansoni* infection enabled us to examine and compare the relative effectiveness of these cells and the mediators of cytotoxicity. The extent of schistosomulicidal effect of mononuclear phagocytes in vitro is shown to parallel the known variability in resistance to schistosomiasis in vivo. Furthermore, our results suggest that the mononuclear phagocytes of different animal species

use several cytotoxic pathways against schistosomula of *S. mansoni*.

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