Brief Definitive Report

ROLE OF ARGINASE IN KILLING OF SCHISTOSOMULA
OF *SCHISTOSOMA MANSONI**

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Infectious agents such as *Toxoplasma gondii*, Bacillus Calmette-Guérin (BCG), and *Corynebacterium parvum* induce nonspecific resistance in mice against the helminth *Schistosoma mansoni* (1, 2). Recently, we reported that BCG infection protects C57BL/ 6J but not BALB/cJ mice from schistosomiasis; this acquired resistance was inherited in a dominant fashion and did not segregate with the mouse H-2 locus (3). To examine the effector mechanisms responsible for this protection, we have utilized an in vitro assay in which adherent peritoneal exudate cells (PEC) from BCG- or *C. parvum*-treated C57BL/6J mice killed a significant fraction of schistosomula of *S. mansoni* (4). An antihelminthic activity also was detected in the supernates of macrophages cultured with the parasite. Although the nature of the active supernate factor is unknown, studies of tumor and microbial killing by activated macrophages (Mϕ) suggest that arginase (5, 6) and/or products of oxidative metabolism (7–10) may be the mediators of cytotoxicity. In our studies, we used the in vitro assay of schistosomula killing to investigate the cellular and biochemical basis for genetically restricted, *C. parvum*-induced resistance to *S. mansoni*. The evidence presented here favors a major role for arginase as the mediator of increased schistosomula killing by activated Mϕ.

Materials and Methods

Mice and C. parvum Treatment. C57BL/6J and BALB/cJ female mice weighing 18–20 g each were purchased from The Jackson Laboratory, Bar Harbor, Maine. Killed *C. parvum* was obtained from Burroughs Wellcome Co., Research Triangle Park, N. C. (lot No. CA-732). The preparation was mixed thoroughly and a dose of 0.2 ml (1.4 mg) was inoculated intraperitoneally into each mouse. Control mice were untreated.

Cell Preparations. PEC were obtained from *C. parvum*-treated mice 7 d after inoculation or from control animals by peritoneal lavage with Hanks' balanced salt solution (HBSS; Flow Laboratories, Inc., Rockville, Md.) that contained 10 U/ml heparin. The cells were washed twice and suspended in RPMI-1640 or Fischer's exact medium (KC Biological, Inc., Lenexa, Kans.) supplemented with 50 U/ml penicillin, 5 μg/ml gentamicin, 2 mM L-glutamine, and 10% heat-inactivated fetal calf serum (FCS; KC Biological, Inc.). Cell counts were adjusted to the desired cell density and aliquots of 0.5 ml were placed in 16-mm Linbro tissue culture plate wells (Linbro Chemical Co., Hamden, Conn.) and incubated for 3 h at 37°C (4). Each well then was washed three times with fresh medium at 37°C to remove nonadherent cells. Greater

TABLE I

Schistosomula Killing, Arginase Release, and H_2O_2 Production by Control and C. parvum-activated Macrophages

Mouse strain	Treatment	Percent schistosomula killing		Arginase	H_2O_2
		Macrophage monolayers	Culture supernates		
				U/ml	*nmol/5 min per 2 × 10^6 cells*
C57BL/6J	Controls	5 ± 4*	2 ± 2‡	0.9 ± 0.1§	0.06 ± 0.01‖
C57BL/6J	C. parvum	30 ± 5¶	11 ± 1**	3.8 ± 0.8¶	0.23 ± 0.01¶
BALB/cJ	Controls	7 ± 1	3 ± 2	1.1 ± 0.2	0.11 ± 0.01
BALB/cJ	C. parvum	6 ± 2	2 ± 1	0.7 ± 0.2	0.39 ± 0.01¶

* Mean killing of schistosomula by macrophages ± SEM for four separate experiments.

‡ Mean killing of schistosomula by supernates from macrophages cultured with shistosomula for 24 h ± SEM for three experiments.

§ Mean arginase released into the supernates of macrophages cultured with shistosomula for 24 h ± SEM for four experiments.

‖ Mean H_2O_2 release from macrophages cultured with schistosomula for 10 min, detached, and washed, with measurement of H_2O_2 production over the next 10 min ± SEM for two experiments.

¶ Significantly greater than controls, $P < 0.02$.

** Significantly greater than controls, $P < 0.05$.

than 95% of the adherent cells phagocytosed latex particles, <5% were T and B lymphocytes, and <1% were neutrophils. To count adherent cells, wells were washed with cold calcium- and magnesium-free HBSS (Flow Laboratories, Inc.) and the dislodged cells enumerated in a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.). In preliminary experiments, we ascertained that a larger fraction of PEC from *C. parvum*-treated mice adhered to the plastic wells than did resident PEC. To produce monolayers of comparable density, resident PEC were cultured at 8 × 10^6/ml and PEC from *C. parvum*-treated animals were cultured at 6 × 10^6/ml.

Assay of Schistosomula Killing. Cercariae of a Puerto Rican strain of *S. mansoni* were allowed to penetrate mouse skin, and the resulting schistosomula were counted and adjusted to 4,000 organisms/ml in medium with 10% FCS (4). Aliquots of 0.05 ml, which contained 200 schistosomula, were added to each well and the plates incubated at 37°C. After 24 h, viability of 100 schistosomula was determined by methylene blue dye exclusion, which has previously been shown to correlate with the infectivity of schistosomula for mice (11). Killing of schistosomula by adherent monolayers was expressed as the mean percent dead organisms in triplicate wells after subtraction of background mortality. The Student's *t* test was used to evaluate the significance of observed differences.

In other experiments, supernates from 24-h cultures of *C. parvum*-activated Mφ and schistosomula were aspirated and centrifuged at 600 *g* for 15 min to remove cells and organisms. Control supernates were obtained from similar cultures of resident nonactivated Mφ. Aliquots of 0.5 ml of these supernates were added to 200 fresh schistosomula and incubated for 24 h at 37°C; killing of the organisms was determined as above.

Biochemical Assays and Reagents. Arginase levels in supernates of cultures of Mφ with and without schistosomula were determined by spectrophotometric analysis of urea production from arginine (12). To measure H_2O_2 production, adherent cells were dislodged with calcium- and magnesium-free HBSS. H_2O_2 release by the cell suspensions (with and without schistosomula) was assayed by the scopoletin method (13) in a spectrofluorometer (American Instrument Co., Travenol Laboratories, Inc., Silver Springs, Md.). In some experiments, phorbol myristate acetate (0.1 μg/ml) (Consolidated Midland Corp., Brewster, N. Y.) was added to these suspensions. Bovine arginase (55 U/mg), L-arginine, superoxide dismutase, and catalase were purchased from Sigma Chemical Co., St. Louis, Mo.

Results

Mφ from control C57BL/6J mice killed 5 ± 4% of the schistosomula at 24 h; those from BALB/cJ mice killed 7 ± 1% (Table I). Mφ from *C. parvum*-treated C57BL/6J
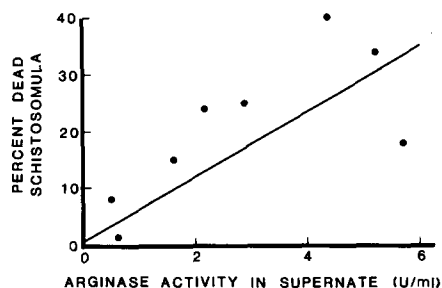
FIG. 1.   Relationship between schistosomula killing by *C. parvum*-treated Mφ of C57BL/6J mice and arginase content of culture supernates. Each data point represents the mean killing and arginase release for a single experiment. Correlation coefficient = 0.72 ($P < 0.025$).

mice showed increased killing of 30 ± 5% of schistosomula ($P < 0.005$). However, Mφ from similarly treated BALB/c mice did not kill more schistosomula than Mφ from control animals of the same strain. Supernates of activated C57BL/6J Mφ cultured with schistosomula killed 11 ± 0.7% of fresh organisms, supernates from control C57BL/6J Mφ cultures killed 2 ± 2% ($P < 0.05$). When supernates of BALB/cJ Mφ were tested in the same manner, no differences were apparent between killing by those from *C. parvum*-treated and control mice (2 ± 1% and 3 ± 2%) (Table I).

Arginase levels in the supernates of cultures of Mφ from untreated C57BL/6J mice and BALB/cJ mice incubated with schistosomula were 0.9 ± 0.1 and 1.1 ± 0.2 U/ml, respectively (Table I). Mφ from *C. parvum*-treated BALB/cJ mice produced 0.7 ± 0.2 U/ml of arginase. In contrast, the mean arginase concentration in the supernates of Mφ from *C. parvum*-treated C57BL/6J mice was 3.8 ± 0.8 U/ml; this level is significantly higher than all other groups ($P < 0.005$). Furthermore, a linear relationship was observed between schistosomula killing by *C. parvum*-activated C57BL/6J macrophages in individual experiments and the arginase levels in the supernates; the correlation coefficient was 0.72 ($P < 0.025$) (Fig. 1).

To define whether arginase release merely reflected macrophage activation, or if the enzyme was an important effector molecule in schistosomula killing, we studied the effect of arginine depletion and supplementation. Activated C57BL/6J Mφ were cultured with schistosomula either in RPMI-1640 medium (L-arginine content = 200 μg/ml) or in Fischer's exact medium (L-arginine content = 15 μg/ml); killing was 18 ± 0.4 ($P < 0.02$) and 34 ± 0.6 ($P < 0.01$), respectively. Killing of schistosomula by Mφ from control C57BL/6J mice was not affected by similar changes in the incubation medium (8 ± 2.4% in RPMI-1640 medium compared to 6 ± 1.9% in Fischer's medium). This difference was not explained by differences in background mortality (7 ± 2% dead in supplemented Fischer's medium alone and 8 ± 1% in RPMI-1640 medium. The addition of exogenous L-arginine to activated Mφ cultured with the parasites in Fischer's medium inhibited schistosomula killing in a dose-dependent fashion. L-Arginine at 200 and 300 μg/ml reduced schistosomula killing from a mean of 34 ± 0.6% to respective means of 24 ± 1% and 8 ± 1% ($P < 0.01$ and 0.001). The addition of 1,000 μg/ml of L-arginine to active supernates reduced their ability to kill fresh schistosomula from 12 ± 2% to 0 ± 2% ($P < 0.001$). Furthermore, in two separate experiments, the addition of 5.5 U/ml of bovine arginase (a concentration similar to that observed in supernates of activated C57BL/6J Mφ) to schistosomula incubated in Fischer's medium increased killing by 129% ($P < 0.01$) and by 47% ($P < 0.05$), respectively.

Baseline production of H_2O_2 by peritoneal Mϕ from control and *C. parvum*-treated mice ranged from 0.07 to 0.12 nmol/5 min per 2 × 10^6 cells. Mϕ from C57BL/6J and BALB/cJ mice produced similar levels of H_2O_2. When schistosomula were incubated for 10 min with Mϕ monolayers, and the cell-parasite mixture suspended and washed, activated Mϕ from C57BL/6J and BALB/cJ mice showed increased H_2O_2 release of 0.23 ± 0.01 and 0.39 ± 0.01 nmol/5 min per 2 × 10^6 cells, respectively ($P < 0.001$). Phorbol myristate acetate also increased H_2O_2 generation by activated C57BL/6J Mϕ to 1.6 ± 0.04 nmol/5 min per 2 × 10^6 cells and by activated BALB/cJ Mϕ to 3.1 ± 0.3 nmol/5 min per 2 × 10^6 cells. Although H_2O_2 production by Mϕ incubated with schistosomula and phorbol myristate acetate was greater in the case of activated BALB/cJ than C57BL/6J Mϕ ($P < 0.01$), schistosomula killing was increased only in the latter. Furthermore, killing of the parasite by activated C57BL/6J Mϕ was not inhibited by superoxide dismutase (5,000 U/ml) or catalase (1,000–10,000 U/ml) when added to the cell suspension simultaneously with the schistosomula or 3 h before incubation with the organisms.

Discussion

Activated macrophages possess cytostatic and cytolytic effects against a variety of tumors and infectious agents including schistosomula of *S. mansoni*. The latter organism measures ~400- × 50-μm and is not phagocytosed as it is killed by the activated macrophages (4). In view of the special nature of Mϕ-multicellular organism interaction, it was of interest to explore the biochemical basis for parasite death. Recently, arginase and peroxidative products have been implicated as likely mediators of activated Mϕ cytotoxicity against single-cell targets such as tumors and protozoa (5–10). In our studies, we evaluated the role of these mediators in schistosomula killing by activated Mϕ. Mϕ from *C. parvum*-treated C57BL/6J but not from *C. parvum*-treated BALB/cJ mice showed increased killing of schistosomula. The production of the putative mediators, arginase and H_2O_2, by Mϕ from these two strains was then compared. Although Mϕ from *C. parvum*-treated C57BL/6J mice produced fourfold more arginase than controls, no comparable change in arginase production was observed in Mϕ from *C. parvum*-treated BALB/cJ mice. Furthermore, in individual experiments, the increased levels of arginase in the supernates of activated C57BL/6J macrophages correlated with the degree of Mϕ-mediated killing of schistosomula. The use of medium rich in L-arginine, and the addition of this amino acid to the cultures, significantly reduced parasite killing by *C. parvum*-activated C57BL/6J Mϕ and their supernates. In contrast, killing was enhanced in arginine-poor medium. Exogenously added bovine arginase at concentrations similar to those found in supernates of activated C57BL/6J Mϕ resulted in an equivalent degree of parasite mortality. These experiments show that in vitro killing of schistosomula by *C. parvum*-activated Mϕ parallels, and presumably provides the basis for, the genetically restricted induction of nonspecific resistance to schistosomiasis in vivo (3). Moreover, arginase appears to be an important mediator of in vitro schistosomula killing by activated Mϕ.

We also examined the release of H_2O_2 by the activated macrophages of the two mouse strains and its relationship to parasite mortality. The level of H_2O_2 production stimulated by schistosomula or phorbol myristate acetate dissociated from parasite killing. Moreover, catalase and superoxide dismutase did not affect schistosomula killing by activated Mϕ. These data militate against a central role for hydrogen

peroxide and superoxide anion in the killing of schistosomula of *S. mansoni*. Recent studies by Murray and Cohn (14) and Murray et al. (15) showed differences in the susceptibility of phagocytosable pathogens to oxidative metabolites; *T. gondii* is more resistant to H_2O_2 than *Trypanosoma cruzi*. Survival of *S. mansoni* schistosomula has been shown not to be affected by H_2O_2, although the addition of purified eosinophil peroxidase and halide ions led to significant parasite mortality (16). The primacy of arginase as mediator of *S. mansoni* schistosomula killing presumably reflects a unique macrophage-parasite relationship as well as target susceptibility.

Summary

Nonspecific resistance to the multicellular organism *Schistosoma mansoni* can be induced in mice by several infectious agents. We utilized the observed genetic restriction of such acquired resistance to study the mediators of killing of the larval stage of *S. mansoni* in vitro. Adherent peritoneal cell monolayers from *Corynebacterium parvum*-treated C57BL/6J but not from *C. parvum*-treated BALB/cJ mice killed an increased proportion of schistosomula in 24 h. Activated macrophages (Mϕ) from both strains exhibited enhanced H_2O_2 production after incubation with the parasites or phorbol myristate acetate. Thus H_2O_2 production was not associated with schistosomula killing. Moreover, schistosomula killing was unaffected by catalase or superoxide dismutase. In contrast, activated C57BL/6J (but not BALB/cJ) Mϕ released fourfold more arginase into supernates than control Mϕ. Schistosomula killing by these Mϕ correlated with arginase content of the supernates, was exaggerated in arginine-poor medium, and could be blocked by the addition of arginine. Exogenous bovine arginase added to Fischer's medium without macrophages produced comparable parasite mortality. Our data suggest that arginase is a critical mediator of in vitro killing of this multicellular organism by activated macrophages.

References

1. Mahmoud, A. A. F., K. S. Warren, and G. T. Strickland. 1976. Acquired resistance to infection with *Schistosoma mansoni* induced by *Toxoplasma gondii*. Nature (Lond.). **263**:56.
2. Civil, R. H., K. S. Warren, and A. A. F. Mahmoud. 1978. Conditions for Bacille Calmette-Guérin induced resistance to infection with *Schistosoma mansoni* in mice. *J. Infect. Dis.* **137**:550.
3. Civil, R. H., and A. A. F. Mahmoud. 1978. Genetic differences in BCG-induced resistance to *Schistosoma mansoni* are not controlled by genes within the major histocompatibility complex of the mouse. *J. Infect. Dis.* **120**:1070.
4. Mahmoud, A. A. F., P. A. S. Peters, R. H. Civil, and J. S. Remington. 1979. *In vitro* killing of schistosomula of *Schistosoma mansoni* by BCG and *C. parvum* activated macrophages. *J. Immunol.* **122**:1655.
5. Kung, J. T., S. B. Brooks, J. P. Jakway, L. L. Leonard, and D. W. Talmage. 1977. Suppression of in vitro cytotoxic response by macrophages due to induced arginase. *J. Exp. Med.* **146**:665.
6. Currie, G. A. 1978. Activated macrophages kill tumor cells by releasing arginase. *Nature (Lond.).* **273**:758.

7. Johnston, R. B., Jr., C. A. Godzik, and Z. A. Cohn. 1978. Increased superoxide anion production by immunologically activated and chemically elicited macrophages. *J. Exp. Med.* **148:**115.

8. Nathan, C. F., L. H. Brokner, S. C. Silverstein, and Z. A. Cohn. 1979. Extracellular cytolysis by activated macrophages and granulocytes. I. Pharmacologic triggering of effector cells and the release of hydrogen peroxide. *J. Exp. Med.* **149:**84.

9. Nathan, C. F., S. C. Silverstein, L. H. Brukner, and Z. A. Cohn. 1979. Extracellular cytolysis by activated macrophages and granulocytes. II. Hydrogen peroxide as a mediator of cytotoxicity. *J. Exp. Med.* **149:**100.

10. Nathan, C. F., N. Nogueira, C. Juangbawich, J. Ellis, and Z. A. Cohn. 1979. Activation of macrophages in vivo and in vitro. Correlation between hydrogen peroxide release and killing of Trypanosoma cruzi. *J. Exp. Med.* **149:**1056.

11. Kassis, A. I., M. Aikawa, and A. A. F. Mahmoud. 1978. Mouse antibody-dependent eosinophil and macrophage adherence and damage to schistosomula of Schistosoma mansoni. *J. Immunol.* **122:**398.

12. Geyer, J. W., and D. Dabich. 1971. Rapid method for determination of arginase activity in tissue homogenates. *Anal. Biochem.* **39:**412.

13. Root, R. K., J. Metcalf, N. Oshino, and B. Chance. 1975. H_2O_2 release from human granulocytes during phagocytosis. I. Documentation, quantitation, and some regulating factors. *J. Clin. Invest.* **55:**945.

14. Murray, H. W., and Z. A. Cohn. 1979. Macrophage oxygen-dependent antimicrobial activity. I. Susceptibility of Toxoplasma gondii to oxygen intermediates. *J. Exp. Med.* **150:**938.

15. Murray, H. W., C. W. Juangbawich, C. F. Nathan, and Z. A. Cohn. 1979. Macrophage oxygen-dependent antimicrobial activity. *J. Exp. Med.* **150:**950.

16. Jong, E. C., A. A. F. Mahmoud, and S. J. Klebanoff. 1979. Toxic effect of eosinophil peroxidase on schistosomula of Schistosoma mansoni. *Clin. Res.* **27:**479 (Abstr.).