

# Role of Cell-generated Hydrogen Peroxide in Granulocyte-mediated Killing of Schistosomula of *Schistosoma mansoni* In Vitro

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**ABSTRACT** Human as well as murine granulocytes have been shown to kill the larval stages of helminth parasites; the mechanism of this cell-mediated cytotoxicity is, however, poorly understood. The present study was designed to assess the role of peroxidative processes in killing of schistosomula of *Schistosoma mansoni* by human granulocytes in vitro. The rate of  $H_2O_2$  production by human neutrophils, eosinophils, and basophils was measured upon incubation with schistosomula alone or in the presence of specific antibody or complement. Opsonized parasites (antibody and/or complement) increased the rate of  $H_2O_2$  production by neutrophils, eosinophils, and basophils by respective percentages of 500, 500, and 371. The rate of  $H_2O_2$  release was directly related to the number of granulocytes and to the proportion of cells attached to the surface of the schistosomula. Increased hydrogen peroxide release occurred by 10 min of incubation and was demonstrable up to 16 h after addition of leukocytes to schistosomula. The primary source of this oxygen product was found to be the granulocytes adherent to the schistosomula and not those that remained unattached. Hydrogen peroxide production by neutrophils and eosinophils was quantitatively similar (schistosomula coated with antibody plus complement stimulated  $5 \times 10^6$  neutrophils and eosinophils to release  $H_2O_2$  at respective rates of 0.35 and 0.40 nmol/min). Granulocyte-mediated parasite killing correlated with rate of  $H_2O_2$  generation; both processes were inhibited by catalase. To define further the role of oxidative metabolites, neutrophils and eosinophils of two subjects with chronic gran-

ulomatous disease were used; marked reduction of granulocyte-mediated parasite mortality was observed.

Peroxidase was required for  $H_2O_2$ -mediated killing. Addition of the peroxidase inhibitors azide (1 mM), cyanide (1 mM), or aminotriazole (1 mM) to neutrophil-schistosomula mixtures significantly reduced parasite cytotoxicity ( $P < 0.01$ ); similar reduction was observed when eosinophils were used ( $P < 0.01$ ). Fixation of halide (iodide) to trichloroacetic acid-precipitable protein (2.4–6.0 nmol/h per  $10^7$  neutrophils) was demonstrated in the presence of granulocytes, opsonins, and parasites; this process was completely inhibited by 1 mM azide.

These data indicate that contact between the surfaces of human granulocytes and schistosomula results in release of cellular hydrogen peroxide and iodination. The generation of  $H_2O_2$  and its interaction with peroxidase appear to be crucial in effecting in vitro granulocyte-mediated parasite cytotoxicity.

## INTRODUCTION

Our knowledge of host defenses against infectious microorganisms such as bacteria and fungi has progressed to an understanding of the basic biochemical events attendant to microbial killing (1, 2). For example, the bactericidal activity of neutrophils is initiated by surface contact with the microorganism, followed by ingestion, phagolysosomal fusion, and production of the toxic reduced states of oxygen (2). In contrast, despite the world-wide prevalence and clinical morbidity of helminth infections (3), relatively little is known about the processes by which mammalian cells combat these organisms. Helminths represent markedly different challenges from bacteria and other unicellular organisms. They are much larger, many of them consisting of more than 1,000

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cells, and pass through several developmental stages within the host (4). Each phase of the parasite life cycle has distinct morphologic, biochemical, and antigenic characteristics (5). Furthermore, many helminths have developed elaborate mechanisms to evade host resistance, such as masking their surface or acquisition of host antigens (6). Recently several *in vitro* systems describing the interaction of the components of the host immune system and multicellular parasites have been developed to elucidate the mechanisms of resistance against these organisms (7-10). Initially human eosinophils were demonstrated to damage the multicellular schistosomula of *Schistosoma mansoni* in the presence of specific anti-parasite antibodies (7). It is now established that close cell-parasite contact and degranulation precede eosinophil-mediated deaths of helminths such as *S. mansoni* (8) and *Trichinella spiralis* (9). Furthermore, human neutrophils (10) and mononuclear cells (11) have been shown to damage the larval stage of *S. mansoni*. Granulocyte-mediated killing has been associated with the deposition of peroxidase-reactive material on the surface of the parasite (12). Cytochalasin B, an agent that impairs microfilament function (13), inhibits eosinophil-mediated helminth cytotoxicity (14). Further investigations of granulocyte-mediated killing of schistosomula indicated that major basic protein, a moiety localized specifically to the crystalloid granules of the eosinophil, is capable of damaging these organisms in cell-free systems (15).

The peroxidative pathway is a major defense system used by granulocytes against several microorganisms and tumor cells (1, 2, 16). Although the role of this microbicidal system in host defense against non-phagocytosable helminths is unclear, artificial generation of  $H_2O_2$  in a cell free system has been shown to kill the multicellular newborn larvae of *T. spiralis* (17). The extensive areas of cell-parasite contact characteristic of leukocyte-schistosomula interaction (8) might be expected to result in significant cell-membrane perturbation. Both the eosinophil and neutrophil undergo a respiratory burst when their membranes are appropriately stimulated (1). This results in the production of  $H_2O_2$  and other activated states of oxygen that may then be used along with lysosomal peroxidase to kill ingested microorganisms (18). In the current studies, we investigated whether cell-parasite interaction results in the production of reduced states of oxygen such as  $H_2O_2$  and the role of this molecule in leukocyte-mediated parasite killing. Our data show that granulocyte-schistosomula interaction leads to extracellular release of  $H_2O_2$ ; this was associated with killing of the organisms. The three subtypes of granulocytes (neutrophils, eosinophils, and basophils) were demonstrated to undergo a respiratory burst on contact with opsonized schistosomula

and to kill a significant proportion of the organisms. In contrast, neutrophils and eosinophils from patients with chronic granulomatous disease (incapable of generating hydrogen peroxide) (19) had a marked impairment in parasite killing *in vitro*. Granulocyte-mediated parasite cytotoxicity was significantly reduced by the heme enzyme inhibitors azide, cyanide, and aminotriazole indicating the involvement of cell peroxidase, and was associated with fixation of halide to the proteins in the reaction mixture.

## METHODS

**Cell preparations.** Blood was obtained from normal volunteers with no recent history of viral, bacterial, or parasitic infections and from five patients with eosinophilia due to extrinsic asthma. Two subjects with chronic granulomatous disease (CGD)<sup>1</sup> were also studied.

Neutrophils were isolated by Hypaque-Ficoll density gradient centrifugation and hypotonic lysis of erythrocytes (20). Eosinophils of normal subjects and CGD patients were purified by centrifugation on discontinuous metrizamide gradients (Nyegaard, Oslo, Norway) (21), whereas those of individuals with eosinophilia were separated after sedimentation of erythrocytes in 5% dextran solution (Sigma Chemical Co., St. Louis, Mo.) (22). Basophils were obtained from a 23-yr-old man in the accelerated phase of chronic myelogenous leukemia. These cells were separated from other blood elements by dextran sedimentation. All cell preparations were washed three times in Hanks' balanced salt solution (KC Biological Inc., Lenexa, Kan.) and cell counts performed with a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.).

**Assay of  $H_2O_2$  release from parasite-stimulated leukocytes.** Extracellular release of  $H_2O_2$  by leukocytes was measured by the scopoletin method (23) using an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Travenol Laboratories Inc., Silver Spring, Md.). The assay was standardized by addition of known quantities of reagent grade  $H_2O_2$ , measured spectrophotometrically at an absorption of 230 nm using an extinction coefficient of  $81 M^{-1} cm^{-1}$  (24). Scopoletin, horseradish peroxidase, and catalase were obtained from Sigma Chemical Co., and  $H_2O_2$  for standard curves from Fisher Scientific Co. (Pittsburgh, Pa.). Triplicate preparations of  $5.0 \times 10^6$  leukocytes were mixed with 1 ml of RPMI 1640 (KC Biological, Inc.) containing 2,500 schistosomula of a Puerto Rican strain of *S. mansoni* (prepared by penetration of mouse skin) (25) in round bottom borosilicate tubes (T1285-3, Scientific Products Div., American Hospital Supply Corp., McGaw Park, Ill.). A final concentration of 20% human serum was added from the following sources: (a) heat-inactivated normal serum; (b) equal volumes of heat-inactivated (2 h at 56°C) pooled immune serum (obtained from Kenyans with documented *S. mansoni* infection) (26) and heat-inactivated normal serum; (c) equal volumes of fresh normal serum and heat-inactivated normal serum; (d) equal volumes of heat-inactivated immune serum and fresh normal serum. The cell-parasite-serum mixtures were incubated at 37°C for various time intervals in a Dubnoff shaker. The tubes were then removed and plunged into an ice bath; the cell-parasite mixture was washed three times (5 min at 100 g) in Krebs-Ringers phosphate buffer containing

<sup>1</sup> Abbreviations used in this paper: CGD, chronic granulomatous disease; PMA, phorbol myristate acetate.

5.5 mM glucose and resuspended in 2 ml of the same buffer. The contents of the tubes were then transferred to cuvettes, mixed with scopoletin (1–2  $\mu\text{M}$  final concentration) and 10  $\mu\text{g}$  horseradish peroxidase added after warming the mixtures to 37°C. Changes in fluorescence were recorded over 10 min on an X-Y recorder (American Instrument Co.). The rate of  $\text{H}_2\text{O}_2$  accumulation was derived from linear reduction in fluorescent signal during this time. Phorbol myristate acetate (PMA) (0.1  $\mu\text{g}/\text{ml}$ ) (Consolidated Midland Corp., Brewster, N. Y.) diluted in dimethyl sulfoxide (Sigma Chemical Co.) was added to determine the capacity of the cells to respond to further stimulation of  $\text{H}_2\text{O}_2$  production (27). Catalase (2,500–5,000 U/ml) was used in some experiments to inhibit accumulation of  $\text{H}_2\text{O}_2$  and to assess its effects on parasite mortality.

The role of cell attachment in parasite-stimulated  $\text{H}_2\text{O}_2$  production was subsequently investigated. Cell-parasite mixtures ( $5 \times 10^6$  neutrophils with 2,500 schistosomula and antibody plus complement) were incubated for 10 min and washed as described above. The tubes were then held vertically for 10 min, which allowed the schistosomula with adherent cells to settle by gravity to the bottom while the unattached cells remained in suspension. Hydrogen peroxide production rates by the leukocytes in the bottom 0.1 ml and upper 0.9 ml of the 1.0 ml mixtures were then measured. The effect of PMA on  $\text{H}_2\text{O}_2$  production by the leukocytes attached to the schistosomula was also determined.

**Assay of granulocyte-mediated schistosomula killing.**  $2.5 \times 10^5$  granulocytes in 0.1 ml RPMI were added to tissue culture wells (76-933-05, Linbro Chemical Co., Hamden, Conn.) containing 50 schistosomula in 0.1 ml RPMI with 20% human serum (11). The serum combinations were similar to those used in the assay of  $\text{H}_2\text{O}_2$  production. Percent parasite death was assessed by exclusion of methylene blue dye after a 24-h incubation period (28). Schistosomula viability was confirmed by injecting the organisms after different incubations into groups of female CF1 mice (Carworth Farms, New City, N. Y.) and recovery of adult worms from the portal circulation by perfusion 3 wk later (29).

**Halide fixation and role of peroxidase in parasite killing.** Fixation of halide during granulocyte-parasite interaction was monitored by the conversion of radiolabeled iodide to a TCA-precipitable form (30). Schistosomula ( $5 \times 10^3$ ), neutrophils ( $10^7$ ), and sera (10% heat-inactivated immune plus 10% fresh normal) were added to 0.5 ml of a reaction mixture containing 4 mM sodium phosphate buffer, pH 7.4; 1.28  $\times$  1 cM NaCl; 1.2  $\times$  1 cM KCl; 1 mM  $\text{CaCl}_2$ ; 2 mM  $\text{MgCl}_2$ ; 80  $\mu\text{M}$  NaI (40 nmol; 0.05  $\mu\text{Ci}$   $^{125}\text{I}$ ) (New England Nuclear, Boston, Mass.); and 2 mM glucose. After incubation at 37°C for 30 min in a shaking water bath, the reaction was stopped by the addition of 0.1 ml 1 cM sodium thiosulfate and 1.0 ml cold 10% TCA. The precipitate was collected by centrifugation at 2,000 g for 5 min, washed four times in 10% TCA, and radioactivity counted in a Searle model 1185 gamma counter (Searle Radiographics Inc., Des Plaines, Ill.). A blank tube containing schistosomula without serum or cells was prepared for each experiment along with a standard containing the total amount of  $^{125}\text{I}$  added. Results are expressed as nanomoles of iodide fixed to schistosomula in a TCA-precipitable form per  $10^7$  cells per hour, and were calculated with the formula:

$$\frac{\text{counts per minute experimental} - \text{counts per minute blank}}{\text{counts per minute standard}} \times 40 \times 2.$$

The total amount of iodide in the mixtures was 40 nmol

and the results were standardized to 60 min by multiplying by 2.

The role of peroxidase in granulocyte-mediated parasite cytotoxicity was subsequently evaluated. Azide (1 mM), cyanide (1 mM), or aminotriazole (1 cM) (30) (Sigma Chemical Co.) were added to neutrophil or eosinophil-schistosomula mixtures containing immune plus fresh serum. Parasite death was determined after 24 h of incubation and compared to killing in the absence of these enzyme inhibitors.

**Statistical analysis.** Student's *t* test was used to analyze the statistical significance of the observed differences.

## RESULTS

**Stimulation of granulocyte  $\text{H}_2\text{O}_2$  release by schistosomula.** Incubation of human neutrophils, eosinophils, or basophils with opsonized schistosomula (antibody or complement) led to significant release of  $\text{H}_2\text{O}_2$  (Fig. 1). Base-line production of  $\text{H}_2\text{O}_2$  by neutrophils incubated with unopsonized schistosomula (95% of the cells in these preparations were neutrophils as judged by examination of Wright's stain smears) was  $0.07 \pm 0.02$  nmol/min per  $5 \times 10^6$  cells (Fig. 1). Addition of antibody, complement, or antibody plus complement to the cell-parasite mixtures resulted in respective increases in the rate of  $\text{H}_2\text{O}_2$  production of 428, 328, and 500% (Fig. 1). Each increase is significant at the 1% level when compared to base line. Furthermore, schistosomula incubated in Krebs-Ringer buffer without cells did not release measurable quantities of  $\text{H}_2\text{O}_2$ .

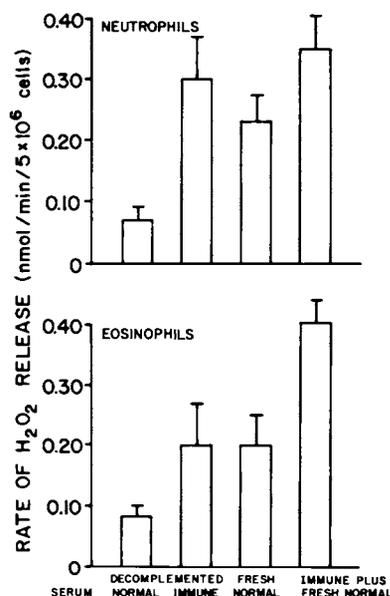


FIGURE 1 Schistosomula-induced release of  $\text{H}_2\text{O}_2$  from granulocytes. 5 million neutrophils or eosinophils were mixed with 2,500 schistosomula and various serum combinations. After incubation at 37°C for 10 min, the rate of  $\text{H}_2\text{O}_2$  release was measured. Results represent the mean of 8–10 experiments.

We subsequently examined the effect of cell:parasite ratio on the rate of  $H_2O_2$  release (Fig. 2). At a ratio of 125:1 ( $0.3 \times 10^6$  neutrophils:2,500 schistosomula),  $0.06 \pm 0.02$  nmol  $H_2O_2$ /min was released. Increasing the number of neutrophils to  $0.6 \times 10^6$  (cell:parasite ratio of 250:1) resulted in an increase of  $H_2O_2$  production of  $0.55 \pm 0.07$  nmol/min ( $P < 0.01$ ). Further increases in the number of neutrophils to 1.25, 2.5, and  $5.0 \times 10^6$  was associated with respective increases of the rate of  $H_2O_2$  production to  $0.80 \pm 0.07$ ,  $1.37 \pm 0.12$ , and  $1.13 \pm 0.12$  nmol/min ( $P < 0.01$ ). In these experiments we also evaluated the percentage of parasites with five or more adherent neutrophils (Fig. 2). When  $< 10\%$  of the schistosomula had five or more adherent cells, stimulation of  $H_2O_2$  release was not observed (cell:parasite ratio 125:1). Addition of increasing numbers of cells was associated with a parallel increase in the percentage of schistosomula with five or more adherent cells as well as in the rate of  $H_2O_2$  release.

Hydrogen peroxide release by human eosinophils was similarly stimulated on incubation with the opsonized parasites. Base-line production of  $H_2O_2$  by eosinophil-schistosomula mixtures (cells were obtained from subjects with eosinophilia and consisted of 70–80% eosinophils, 10–20% neutrophils, 10–20% mononuclear cells) was  $0.08 \pm 0.02$  nmol/min per  $5 \times 10^6$  cells (Fig. 1). Addition of antibody, complement, or antibody plus complement led to respective rates of  $H_2O_2$  production of  $0.20 \pm 0.07$ ,  $0.20 \pm 0.05$ , and

$0.40 \pm 0.05$  nmol/min per  $5 \times 10^6$  cells (Fig. 1). These rates represent a corresponding percent increase of 250, 250, and 500; each value is significantly higher than base line ( $P < 0.01$ ). As  $H_2O_2$  production by these eosinophil preparations may in part be due to the 10–20% contaminating neutrophils, the capacity of more highly purified cell preparations to release  $H_2O_2$  was examined. Eosinophils and neutrophils of four normal subjects were separated and purified by discontinuous metrizamide gradient centrifugation. These preparations consisted of  $> 90\%$  of the respective cell types. Cells ( $2.5 \times 10^6$ ), schistosomula (2,500), and immune plus fresh sera were then mixed and  $H_2O_2$  release determined. The respective rates of  $H_2O_2$  release by eosinophils and neutrophils of three of the donors were similar, as were the means of the entire group ( $0.16 \pm 0.03$  nmol/min per  $2.5 \times 10^6$  neutrophils and  $0.19 \pm 0.03$  nmol/min per  $2.5 \times 10^6$  eosinophils) (Table I). Decreasing the proportion of eosinophils to 80% and increasing that of neutrophils to 20% did not alter the rate of  $H_2O_2$  release. These data indicate that eosinophils and neutrophils of the same individual release  $H_2O_2$  at similar rates when stimulated by opsonized schistosomula.

Base-line release of  $H_2O_2$  by the basophil preparations (consisting of 85% basophils and 15% myelocytes, promyelocytes, and blasts) was  $0.07 \pm 0.03$  nmol/min per  $5 \times 10^6$  cells. Addition of schistosomula with immune plus fresh sera to the cell preparation resulted in an increase of  $H_2O_2$  production to  $0.26 \pm 0.04$

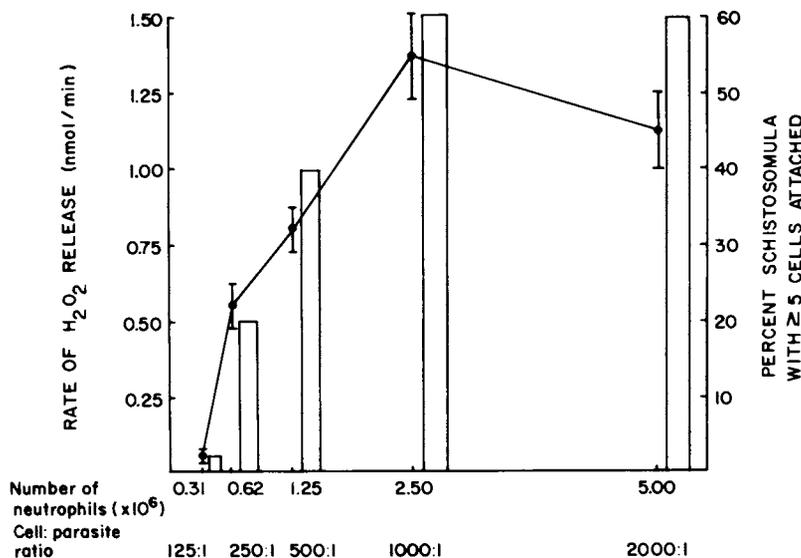


FIGURE 2 Relationship of schistosomula-stimulated  $H_2O_2$  production to number of neutrophils. Varying numbers of neutrophils were mixed with 2,500 schistosomula in media containing 10% heat-inactivated immune serum (antibody) and 10% fresh normal serum (complement). After incubation at  $37^\circ C$  for 10 min, the rate of  $H_2O_2$  release was determined (results of three experiments represented by points on lines). Percent schistosomula with  $\geq 5$  cells attached was assessed by light microscopy (results represented by bars).

TABLE I  
Parasite-stimulated H<sub>2</sub>O<sub>2</sub> Production by Neutrophils and Eosinophils of Healthy Subjects

Cell donor	Rate of H <sub>2</sub> O <sub>2</sub> release by neutrophils		Rate of H <sub>2</sub> O <sub>2</sub> release by eosinophils	
	nmol/min/2.5 × 10 <sup>6</sup> cells	% cell type	nmol/min/2.5 × 10 <sup>6</sup> cells	% cell type
J.K.	0.12	97	0.27	94
A.M.	0.10	95	0.14	96
R.O.	0.23	96	0.20	97
N.M.	0.20	95	0.17	96
Mean ± SE	0.16 ± 0.03		0.19 ± 0.09	

Each value represents the mean of duplicate determinations. Neutrophils and eosinophils were separated and purified on a discontinuous metrizamide gradient (21) and added to 2,500 schistosomula with 10% heat-inactivated immune and 10% fresh normal serum.

nmol/min per 5 × 10<sup>6</sup> cells ( $P < 0.005$  compared to base line).

Accumulation of extracellular H<sub>2</sub>O<sub>2</sub> was blocked by catalase. When the enzyme (2,500 U/ml) was added to neutrophil- or eosinophil-schistosomula mixtures (containing 5 × 10<sup>6</sup> cells and 2.5 × 10<sup>3</sup> parasites) with antibody and complement, extracellular H<sub>2</sub>O<sub>2</sub> accumulation was not detectable. Boiled catalase, on the other hand, did not alter the rate of H<sub>2</sub>O<sub>2</sub> release.

*Effect of duration of incubation on granulocyte H<sub>2</sub>O<sub>2</sub> generation.* To evaluate the effect of duration of cell-parasite contact on stimulation of H<sub>2</sub>O<sub>2</sub> release, neutrophils (5 × 10<sup>6</sup>) from the same donor were incubated with schistosomula and immune plus fresh sera or heat-inactivated normal serum (controls) for various time intervals before determination of H<sub>2</sub>O<sub>2</sub> accumulation. No increase of H<sub>2</sub>O<sub>2</sub> release was observed after 30 s of incubation. At 10 min, the rate was significantly increased by 500% (0.35 ± 0.05 vs. 0.07 ± 0.02 nmol/min in controls,  $P < 0.01$ ). An increase in H<sub>2</sub>O<sub>2</sub> accumulation was also noted after 4, 8, and 16 h of incubation (Table II). Neutrophils incubated with opsonized schistosomula for 24 h did not release H<sub>2</sub>O<sub>2</sub> at a rate greater than that of the control preparations (Table II).

*Source of H<sub>2</sub>O<sub>2</sub> release and effect of PMA* Replicate neutrophil-opsonized parasite mixtures (5 × 10<sup>6</sup> cells with immune plus fresh sera) were prepared and incubated for 10 min; the rate of H<sub>2</sub>O<sub>2</sub> release by these preparations was 1.00 nmol/min. In parallel tubes, the parasites and attached cells were allowed to settle by gravity; the cells in the bottom 0.1 ml of these mixtures (consisting of 1 × 10<sup>6</sup> neutrophils and schistosomula) released H<sub>2</sub>O<sub>2</sub> at 0.8 nmol/min, while those in the upper 0.9 ml (4 × 10<sup>6</sup> cells and no

TABLE II  
Relationship of Duration of Cell-Parasite Interaction to Neutrophil H<sub>2</sub>O<sub>2</sub> Production

Duration of incubation	Rate of neutrophil H <sub>2</sub> O <sub>2</sub> release*		Significance †
	Cells and unopsonized schistosomula*	Cells and opsonized schistosomula*	
	nmol/min/5 × 10 <sup>6</sup> cells		
30 s	0.07 ± 0.03	0.06 ± 0.02	$P > 0.1$
10 min	0.07 ± 0.02	0.35 ± 0.05	$P < 0.01$
4 h	0.06 ± 0.03	0.20 ± 0.03	$P < 0.01$
8 h	0.04 ± 0.01	0.15 ± 0.02	$P < 0.01$
16 h	0.09 ± 0.02	0.17 ± 0.03	$P < 0.01$
24 h	0.09 ± 0.02	0.14 ± 0.02	$P > 0.03$

\* Results represent mean ± SE for triplicate determinations. Unopsonized schistosomula were incubated in heat-inactivated normal serum and opsonized parasites were incubated in immune plus fresh sera.

† Significance of difference between cells incubated with unopsonized vs. opsonized schistosomula.

schistosomula) generated extracellular H<sub>2</sub>O<sub>2</sub> at a rate of 0.2 nmol/min ( $P < 0.01$  for lower vs. upper preparation). When the rate of H<sub>2</sub>O<sub>2</sub> was adjusted to a per cell basis, adherent neutrophils released H<sub>2</sub>O<sub>2</sub> at a rate 16-fold higher than those that remained in suspension. Addition of PMA (0.1 μg/ml) to neutrophils attached to the opsonized parasites led to a further increase of H<sub>2</sub>O<sub>2</sub> release to 2.1 nmol/min per 10<sup>6</sup> cells.

*Parasite killing by leukocytes.* The level of parasite killing correlated well with the rate of H<sub>2</sub>O<sub>2</sub> production; both were inhibited by catalase. 9% schistosomula were killed in the presence of decompartmented normal serum and neutrophils or eosinophils. Parasite killing by neutrophils was enhanced by the addition of immune and fresh serum to 38–40% (Table III). This enhancement of parasite cytotoxicity was associated with a 428 and 328% increase of H<sub>2</sub>O<sub>2</sub> release (Fig. 1). Addition of both opsonins led to a further increase in parasite killing to 50% and enhancement of H<sub>2</sub>O<sub>2</sub> release by 500%. Similar trends were observed with eosinophils and basophil preparations (Table III). Catalase inhibited leukocyte-mediated schistosomula killing and the release of H<sub>2</sub>O<sub>2</sub> when it was added at the beginning of the incubation period. In six separate experiments, when the enzyme (5,000 U/ml final concentration) was included in neutrophil-parasite mixtures containing immune plus fresh sera, schistosomula death at 24 h was significantly reduced to 29 ± 3% compared to 45 ± 5% with boiled catalase ( $P < 0.01$ ). Eosinophil-mediated killing was also significantly decreased by the enzyme (15 ± 3% with fresh catalase vs. 38 ± 3% with boiled catalase,  $P < 0.001$ ). In contrast, addition of the enzyme four hours after neutrophils and schistosomula were mixed failed to

**TABLE III**  
*Dependence of Granulocyte-mediated Killing of Schistosomula on Antibody and Complement\**

Cell type	Source of serum added			
	Heat-inactivated normal (control)	Heat-inactivated immune	Fresh normal	Immune plus fresh
	% schistosomula dead			
Neutrophil	9±2	38±3	40±4	50±4
Eosinophil	9±2	35±3	33±4	43±4
Basophil	12±2	ND	ND	43±3

\* Cells were incubated with schistosomula at a 5,000:1 cell:parasite ratio with 20% final serum concentrations for 24 h. Results represent mean±SE for eight experiments. The values for each cell type with opsonins are significantly greater ( $P < 0.001$ ) than their respective controls. The combination of immune plus fresh serum was associated with a significantly higher parasite cytotoxic effect than with either serum component alone for neutrophils ( $P < 0.025$ ) but not for eosinophils ( $P = 0.07$ ). ND, not done.

reduce parasite killing (37±4% parasite cytotoxicity vs. 39±5% with boiled catalase).

Assessing parasite mortality by uptake of methylene blue in vitro was confirmed by the results of in vivo injection of the organisms into mice. A mean of 18.8±1.6 adult worms per mouse were recovered from six animals injected with 200 schistosomula after 24 h incubation with neutrophils, antibody, and complement. In contrast, a mean of 34.8±4.3 worms per mouse was recovered from animals injected with control organisms ( $P < 0.005$ ).

*Cytotoxic effect of CGD leukocytes.* Granulocytes obtained from two individuals with CGD demonstrated a decreased capacity to kill schistosomula. Neutrophil-mediated killing of parasites opsonized with antibody, complement, or a combination of the two was not significantly greater than that observed when CGD neutrophils were mixed with heat-inactivated normal serum (control) (Table IV). Neutrophils obtained simultaneously from a normal subject killed significantly more parasites than did CGD cells (52–57% killing with normal vs. 0–13% with CGD cells,  $P < 0.001$ ). CGD, eosinophils also killed significantly less schistosomula than normal cells (19 and 25% parasite cytotoxicity with CGD eosinophils vs. 53% with normal cells,  $P < 0.002$ ). The eosinophils of CGD donors, however, killed a higher proportion of the opsonized organisms (19 and 25%) than in the control wells (9%) ( $P < 0.01$ ) (Table IV).

*Halide fixation and role of peroxidase in parasite killing.* The interaction of neutrophils and opsonized schistosomula resulted in the conversion of radio-labeled iodide to a TCA-precipitable form (Table V).

**TABLE IV**  
*Capacity of Neutrophils and Eosinophils of CGD Subjects to Kill Schistosomula*

Cell type	Subject	Serum			
		Heat-inactivated normal (control)	Immune plus fresh	Heat-inactivated immune	Fresh normal
		% schistosomula dead at 24 h			
Neutrophil	CGD 1	9	0	13	14
	CGD 2	11	13	14	11
	Control	11	57	52	54
Eosinophil	CGD 1	9	19	ND	ND
	CGD 2	9	25	ND	ND
	Control	9	53	ND	ND

Cell:lava ratio = 5,000:1. Each value represents the mean of triplicates. Eosinophils and neutrophils were purified on a discontinuous metrizamide gradient (21) and added to schistosomula with 20% final serum concentrations. ND, not done.

Neutrophils of three normal donors induced significantly more iodide fixation (133 to 633%) to proteins in the reaction mixture in the presence of opsonized than unopsonized parasites; this reaction was inhibited by 1 mM azide (1.0 vs. 4.2 nmol iodide fixed/h per  $10^7$  cells without azide). Addition of halide 1 mM to 1  $\mu$ M KI to neutrophil-parasite mixtures did not affect the level of parasite killing.

When peroxidase inhibitors were added to neutrophil or eosinophil preparations and schistosomula, a 30–50% decrease in parasite killing was observed (Table VI). In contrast, eosinophil-mediated killing was not altered by cyanide but was inhibited

**TABLE V**  
*Iodination of Protein in Neutrophil-schistosomula Mixtures*

Schistosomula	Iodide fixation		
	Cell donor		
	1	2	3
	nmol iodide fixed/ $10^7$ cells/h		
Opsonized	2.4±0.4	1.4±0.2	6.0±1.3
Unopsonized	0.6±0.1	0.6±0.2	2.2±0.1
P value*	<0.0005	<0.03	<0.005

$10 \times 10^6$  neutrophils were incubated with  $5 \times 10^8$  opsonized (immune plus fresh serum) or unopsonized (heat-inactivated normal serum) schistosomula in the presence of  $^{125}$ I for 30 min. The radioactivity in the TCA precipitate was measured and converted to nanomoles iodide as described in the text. Results represent the mean±SE of three experiments.

\* Significance of difference for opsonized vs. unopsonized parasites.

69 and 56% with azide and aminotriazole, respectively (Table VI).

The effect of these peroxidase inhibitors on the rate of  $H_2O_2$  release from neutrophils stimulated with opsonized (immune plus fresh sera) schistosomula was subsequently examined. Azide (1 mM) and cyanide (1 mM) increased the rate of  $H_2O_2$  production from 0.38 nmol/min per  $5 \times 10^6$  cells (mean of two experiments) to 2.82 and 3.24 nmol/min per  $5 \times 10^6$  cells, respectively.  $H_2O_2$  was not measurable by the scopoletin method in the presence of aminotriazole (addition of exogenous  $H_2O_2$  failed to decrease fluorescence).

## DISCUSSION

Multicellular parasites present a unique challenge to mammalian host defense mechanisms because of their size and complex structure. For example, the schistosomula of *S. mansoni* measure  $50 \times 400 \mu\text{m}$  and consist of more than 1,000 cells. Furthermore, the parasite is limited by a heptalaminar membrane which undergoes biochemical and immunologic alterations as it migrates within the host or during in vitro cultivation (31, 32). This organism with its complex surface represents, therefore, a formidable biochemical and ultrastructural barrier to host defenses. Helminth infections also elicit a number of immunologic and nonimmunologic reactions which distinguish it from responses to bacteria and other microorganisms. Eosinophilia both in the blood and sites of

tissue migration rather than neutrophilia is the hallmark of invasive worm infections (33). Activation of the complement system via the alternative pathway (34) and significant elevations of anti-parasite IgG (35) and IgE (36) have also been observed. The role of these various components in defense against the invading helminths has only recently been studied. In vitro leukocyte-mediated killing of schistosomula of *S. mansoni* in which eosinophils and neutrophils act as effector cells has been demonstrated (7, 10). Since the bactericidal function of these cells is dependent on their capacity to generate activated states of oxygen (1, 2), we explored whether the anti-helminthic effect of human granulocytes might also be related to this system.

In this investigation, we measured the ability of various granulocytes to kill schistosomula of *S. mansoni*. All three granulocyte subtypes (neutrophils, eosinophils, and basophils) killed a significant proportion of the parasite larvae in the presence of antibody or complement. Several other investigators have found that neutrophils as well as eosinophils were capable of effecting damage to these multicellular organisms. Schistosomula of *S. mansoni*, newborn larvae of *T. spiralis*, and microfilariae of *Onchocerca volvulus* have all been shown to be killed by neutrophils as well as eosinophils in the presence of antibody or complement (9, 10, 37-42). Parasite mortality was assessed in our studies by in vitro dye uptake and infectivity to susceptible hosts in vivo. In contrast, one study has shown that eosinophils and neutrophils equally attach to *S. mansoni* schistosomula and release  $^{51}\text{Cr}$ , but only eosinophils lead to parasite destruction (21). This discrepancy may be related to several factors such as the method of detecting parasite mortality, the manner in which the cytotoxicity assay was performed, and the source and concentration of immune sera. In our studies, granulocyte-parasite interaction was performed in flat bottom wells rather than round bottom tubes, and cytotoxicity was assayed by methylene blue dye exclusion, which has been shown in this and previous studies (28) to correlate with parasite infectivity to susceptible hosts. These variabilities in geometry of the reaction and opsonic activity of sera might result in differences in the degree of cell-parasite contact and delivery of cytotoxic mediators to the helminth surface.

Simultaneous with these investigations, the capacity of schistosomula to stimulate  $H_2O_2$  production by human granulocytes and the role of this cell-generated product of oxidative metabolism in parasite cytotoxicity was assessed. Each of the leukocyte types released large quantities of extracellular  $H_2O_2$ ; the production of this molecule was triggered upon interaction with opsonized parasites. Accumulation of extracellular  $H_2O_2$  was dependent on the relative numbers of granulocytes and schistosomula. A minimum cell:parasite ratio

TABLE VI  
Effect of Peroxidase Inhibitors on Granulocyte-mediated Schistosomula Killing

Cell preparation	Inhibitor	Percent parasites killed at 24 h
Neutrophils	None	42.3±4.0
	Azide (1 mM)	24.9±3.2*
	Cyanide (1 mM)	29.4±2.1
	Aminotriazole (1 mM)	18.4±1.0
Eosinophils	None	56.2±5.0
	Azide (1 mM)	17.3±2.6
	Cyanide (1 mM)	44.8±6.2
	Aminotriazole (1 mM)	24.8±5.1

Neutrophils and eosinophils of three normal individuals were prepared by discontinuous metrizamide gradient centrifugation of blood and incubated with schistosomula (cell:larva ratio 5,000:1) in the presence of immune plus fresh serum. Results represent the mean±SE of triplicates. Peroxidase inhibitors had no effect on parasites that were incubated without cells. Cell viability was >70% (trypan blue exclusion) at 24 h for each preparation.

\* Values for each cell type incubated with inhibitors were significantly less ( $P < 0.01$ ) than control mixtures (inhibitor omitted) except for eosinophils and cyanide ( $P > 0.1$ ).

of 250:1 resulted in enhanced  $H_2O_2$  accumulation, whereas a cell:parasite ratio of 125:1 was not associated with increased  $H_2O_2$  release (Fig. 2). To assess the importance of cell-parasite contact in stimulation of  $H_2O_2$  production, neutrophils adherent to opsonized parasites were separated from unattached cells and the rates of  $H_2O_2$  production compared. Although there were fewer leukocytes which adhered to the schistosomula ( $1 \times 10^6$  vs.  $4 \times 10^6$ ), they released  $H_2O_2$  at a rate 16 times that of the latter when compared on a cell for cell basis. These data suggest that initiation and perhaps maintenance of cell-parasite contact is required for stimulation of  $H_2O_2$  release. This physical interaction, however, did not result in maximal stimulation of cellular peroxidative metabolism. Addition of the soluble activating agent, PMA, led to further release of  $H_2O_2$ .

The rate of  $H_2O_2$  release was comparable when eosinophils or neutrophils were incubated with the opsonized organisms. Although the nature of the stimulus to release  $H_2O_2$  in our experiments is different, these results contrast with that of those who have found that eosinophils challenged with soluble or particulate activators released  $H_2O_2$  at a greater rate than neutrophils (43, 44). This discrepancy may be due to several factors. We, like others, obtained eosinophils from patients with a variety of clinical conditions associated with eosinophilia. Eosinophil membrane receptors for IgG and C3 have been shown to undergo alterations in various disease states (45). This in turn might lead to variability in the capacity of eosinophils of different donors to be stimulated to produce  $H_2O_2$  by antibody or complement-coated schistosomula. Variations in the relative purity of the eosinophil preparations or the presence of other contaminating cells also may contribute to differences in  $H_2O_2$  production reported by various investigators. In the present study, these problems were addressed by comparing  $H_2O_2$  production from highly purified (>90%) populations of eosinophils and neutrophils obtained from normal subjects. The relative rates of parasite-stimulated  $H_2O_2$  release by eosinophils and neutrophils of the same individual were similar. Another index of granulocyte oxidative metabolism, hexose monophosphate shunt activity, has also recently been shown to be identical for autologous eosinophils and neutrophils (46).

Several lines of evidence indicated that  $H_2O_2$  was directly involved in parasite killing by leukocytes. Catalase inhibited eosinophil- and neutrophil-mediated parasite cytotoxicity as well as accumulation of  $H_2O_2$  in the extracellular milieu. For both cell types, this inhibition was incomplete, possibly because the enzyme failed to penetrate the area sequestered between the leukocyte and helminth surface. The initial 4 h of granulocyte-schistosomula interaction, when  $H_2O_2$  production is greatest, appears to be crucial in

effecting the cytotoxic reaction, as addition of catalase after this time failed to reduce parasite killing. When eosinophils and neutrophils of subjects with CGD were tested for their capacities to kill schistosomula, it was found that both granulocyte types had a decreased killing capacity. Whereas CGD neutrophils totally lacked the ability to effect parasite death, eosinophils killed a significant but reduced number of organisms. These data suggest that peroxidative mechanisms of parasite cytotoxicity are exclusively involved in neutrophil-mediated damage, whereas additional non-peroxidative processes may be employed by eosinophils. Granulocytes of individuals with CGD have also been found to be impaired in their capacity to destroy the multicellular newborn larvae of *T. spiralis* (41). Furthermore, the eosinophil major basic protein may be an additional mediator (15).

Hydrogen peroxide-mediated killing of the multicellular schistosomula was dependent on peroxidase, since inhibition of this enzyme with azide, cyanide, or aminotriazole (30) reduced granulocyte-mediated parasite death. The dependence of  $H_2O_2$ -mediated parasite killing on peroxidase is further supported by the simultaneous increase of  $H_2O_2$  in the presence of peroxidase inhibitors. Neutrophil-mediated parasite cytotoxicity was reduced by all three agents, whereas eosinophil-mediated killing was inhibited by azide and aminotriazole and unaffected by 1 mM cyanide. Insensitivity of eosinophil peroxidase to cyanide has previously been reported (47). Our results are similar to those of Jong et al. (48) who reported that small quantities of  $H_2O_2$  plus eosinophil or neutrophil peroxidase and halide ion kill schistosomula in a cell-free system. In the present investigations, we have demonstrated that granulocyte-larva interaction was associated with fixation of halide (iodide) to protein; this was not demonstrable in the presence of the heme enzyme inhibitor azide. The iodination reaction was moderately enhanced (two- to fourfold) by opsonized schistosomula. This contrasts with the greater degree of stimulation (10- to 60-fold) reported with smaller targets such as opsonized zymosan (30). These observations are in agreement with the finding that  $H_2O_2$  release from granulocytes was also not maximally stimulated by opsonized parasites.

In conclusion, this study demonstrates that large nonphagocytosable helminths such as *S. mansoni* larvae stimulate the generation of hydrogen peroxide by human granulocytes. This biochemical process, in concert with the enzyme peroxidase, may explain the parasite cytotoxic effect of these cells. Thus, the  $H_2O_2$ -peroxidase-halide system, which is an integral component of the human defense mechanism against unicellular organisms (1, 2, 49, 50) may play a crucial role in limiting invasion by multicellular parasites as well.

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