Eosinophils versus Neutrophils in Host Defense

KILLING OF NEWBORN LARVAE OF *TRICHINELLA SPIRALIS* BY HUMAN GRANULOCYTES IN VITRO

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ABSTRACT Eosinophil leukocytes have been reported to have a major role in host defense against invasive, migratory phases of helminth infestations, yet the relative larvicidal abilities of eosinophils and neutrophils have not been thoroughly examined. This study examined the killing of newborn (migratory phase) larvae of *Trichinella spiralis* during incubation by human granulocytes in vitro. The assay employed culture of larvae with cells, sera, and reagents in microtiter wells with direct counting of surviving larvae after incubation. Killed larvae appeared to be lysed. Verification of the microplate assay was obtained by demonstrating complete loss of infectivity of larvae incubated with leukocytes and immune serum. In the presence of optimal immune serum concentrations, purified neutrophils or eosinophils achieved ≧95% killing of larvae at cell:larva ratios of 2,000:1 or greater. Fresh normal serum prompted slight (19%) killing by leukocytes at a cell:larva ratio of 9,000:1. Cells plus heat-inactivated normal serum and all sera preparations in the absence of leukocytes killed <8% of the larvae. The activity of immune serum was opsonic. Cells adhered to larvae that had been preincubated in immune serum, and immunofluorescent studies indicated that such preopsonized larvae were coated with immunoglobulin (Ig)G. However, preopsonized larvae lost opsonic activity and surface IgG during incubation for 3 h in medium lacking immune serum.

The rate of killing was dependent on the cell:larva ratio; at high leukocyte concentrations (4,200:1), 99% were killed within 7 h; at lower cell:larva ratios, killing increased steadily during a 20-h incubation period. Killing was inhibited by 20 μg catalase, 5 μg/ml cytochalasin B, or 5 μM colchicine, but was unchanged by superoxide dismutase and was enhanced by azide or cyanide. Leukocytes from a patient with chronic granulomatous disease, lacking ability to mount a normal oxidative response, demonstrated a markedly suppressed larvicidal effect.

The data indicate that neutrophils are at least as effective as eosinophils in the killing of newborn larvae of *T. spiralis*. The killing appeared to be mediated by the oxidative metabolic burst with its generation of hydrogen peroxide.

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INTRODUCTION

Although the protective role of neutrophils has been accepted for over half a century, the function of eosinophil leukocytes remains controversial. Recent studies have emphasized two hypotheses of the role of the eosinophil: depending upon the method of study, they may have a role in host defense by an ability to kill invasive metazoan parasites; alternatively, they may appear as a homeostatic modulator of inflammation, serving to suppress the inflammatory (especially hypersensitivity) response and prevent its unnecessary spread. The dramatic stimulation of eosinophils by virtually any invasive or migratory metazoan parasitic infestation has long suggested that eosinophils may have a protective role, but direct evidence for this hypothesis was lacking until recently and data indicating that eosinophils are vital to host response or that eosinophils have an unusual ability (when compared with neutrophils or macrophages) to kill parasites are few and incomplete. Treatment of mice with antieosinophil serum causes ablation of circulating eosinophils and abrogates the immune response to *Schistosoma mansoni* or *Trichinella spiralis*, resulting in increased numbers of migratory, invasive larvae (1, 2). Treatment of mixed, normal leukocytes with antieosinophil serum

in vitro prevents antibody-dependent, leukocyte-mediated damage of schistosomula, as monitored by release of chromium 51 (3). However, although anti-eosinophil serum caused the death of only 10% of neutrophil preparations in vitro (3), the functional integrity of the remaining neutrophils has not been reported. Similarly, data confirming whether antieosinophil serum impairs neutrophil-dependent immune responses, such as to bacteria, is unavailable. Purified eosinophils can damage schistosomula (4), but the comparable cidal ability of purified neutrophils was not initially examined, and the same authors have recently reported that neutrophils may be more effective than eosinophils in the chromium release assay (5). Also, investigators employing other experimental models have reported killing of schistosomula by neutrophils (6, 7) or macrophages (8, 9). Recently, Kazura et al. (10) reported killing of newborn (migratory phase) larvae of *T. spiralis* by mouse peritoneal cells, but again the relative larvicidal ability of functionally intact neutrophils was not examined.

Invasive parasites are initially confronted by a great preponderance of neutrophils over eosinophils and the early phase (12 h) of the inflammatory reaction involves an influx of neutrophils with a small number of eosinophils (11). Most studies of killing of parasites in vitro have indicated that the effect may occur during the initial 12 h of incubation. As the granulomatous response evolves further, it becomes rich in eosinophils and macrophages. The distribution of eosinophils within granulomata varies, depending upon the parasite studied. In immune responses to schistosomula (12, 13) or schistosome eggs (14), eosinophils are found scattered throughout the lesions and are occasionally found in contact with parasites. This might suggest that the eosinophils are involved in parasite destruction. The strength of this interpretation is limited by the fact that: a larger proportion of eosinophils appear around the "penetration tracts" of schistosomula than around larvae (12, 15); conversely, only a small proportion of larvae appear in contact with eosinophils. The predominant cell in the early response to schistosomula is the neutrophil and the earliest histologic signs of parasite damage involve larval-neutrophil interactions (12, 13). Also, immunization with cercarial antigen results in a similar eosinophil-rich response but does not confer protection, i.e., the schistosomula are not killed (15). Moreover, in immune reactions to other parasites, the eosinophils appear most concentrated in the periphery of granulomata (11, 16–19). Most of these observations are not in accord with the hypothesis that the eosinophil is the primary killing cell in the host response. Although indirect, they would support the concept that the eosinophil is involved in the later hypersensitivity reaction to the parasite deposited in the tissue.

The present study examined the killing of newborn (migratory phase) larvae of *T. spiralis* by various preparations of human leukocytes in vitro. It examined the larvicidal abilities of eosinophils and neutrophils, opsonin requirements, and the kinetics of killing. Studies employing leukocytes obtained from a patient with chronic granulomatous disease and studies employing enzyme inhibitors provided some insight into the mechanism of the larvicidal event.

METHODS

Preparation of human leukocytes. Heparinized blood was obtained from normal volunteers and volunteers with eosinophilia resulting from allergic rhinitis, eosinophilic gastroenteritis, and the hypereosinophilic syndrome. Leukocytes were isolated using a modification of a method of Boyum (20). Blood was layered over an equal volume of 9.6% sodium metrizoate and 5% Ficoll (Isolymph, Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N. Y.). Sedimentation by gravity at room temperature was allowed to proceed for 30–40 min. Agglutination of the erythrocytes occurred at the interface, and the erythrocytes rapidly fell into the lower layer, leaving the leukocytes in their own plasma nearly free of erythrocyte contamination in the upper layer. The leukocyte-rich plasma was drawn off and the leukocytes washed in calcium and magnesium-free Hanks' balanced salt solution containing 0.1% gelatin (HBSS-g)[1] (Grand Island Biological Co., Grand Island, N. Y.). When indicated, granulocyte and mononuclear cell populations were separated by layering leukocyte-rich plasma over equal volume of Isolymph with centrifugation at 400 *g* for 40 min. The granulocyte-containing pellet was washed with Ca-Mg-free HBSS-g. Total leukocytes, differential (using Wright's stain) and absolute eosinophil counts (using Discombe's method [21]) were performed and the preparation diluted to the desired leukocyte concentration. In certain experiments eosinophils were purified by a modification of the method of Grover et al. (22). A linear gradient of 15–30% sodium metrizamide (Gallard-Schlesinger Chemical Mfg. Corp.) was prepared with a density gradient maker using HBSS-g supplemented with deoxyribonuclease (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.), 10 mg/liter as diluent. 10^8 leukocytes were layered over each 10-ml gradient and centrifuged at 900 *g* for 15 min. Fractions (0.5 ml) were removed by upward displacement with a tube-piercing device, and the number and purity of eosinophils in each fraction determined. In all preparations, >95% leukocytes were viable, as judged by trypan blue exclusion.

Sera. Rabbit trichinella-immune serum was obtained from rabbits 6–10 wk after oral infestation with 15,000 muscle stage trichinella larvae. In this report, unless specified otherwise, "immune serum" and "normal serum" refer to heat-inactivated preparations (55°C for 30 min).

Isolation of newborn larvae of T. spiralis. *T. spiralis*, a strain originally obtained from Adel A. F. Mahmoud of Case Western Reserve University, Cleveland, Ohio, was maintained by serial infestation of muscle stage larvae by oral inoculation of C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, Maine). Muscle stage larvae were isolated by digestion of infected muscle with acid-pepsin for 4 h

[1] *Abbreviations used in this paper*: CGD, chronic granulomatous disease; FCS, dialyzed fetal calf serum; HBSS-g, Hanks' balanced salt solution containing 0.1% gelatin; MEM, Eagle's minimal essential medium.

and quantitated as previously described (23). Newborn larvae were prepared by a modification of the method of Dennis et al. (24). Male Sprague-Dawley rats were inoculated orally with 10,000 muscle stage larvae. 6 d later the rats were starved overnight, sacrificed, and the entire small intestine removed, slit longitudinally, cut into 2-cm sections and placed in a modified Baermann apparatus containing 0.85% saline solution with 20 μg/ml streptomycin and 200 U/ml penicillin at 37°C. Adult worms were collected over a period of 4 h and washed four times by centrifugation and resuspension in saline at 37°C. The adult worms were then incubated in 25 ml of Eagle's Minimal Essential Medium (MEM) (Grand Island Biological Co.) containing 30% dialyzed fetal calf serum (FCS), 200 U/ml penicillin, 250 μg/ml gentamicin, and 100 U/ml mycostatin for 20 h at 37°C, in an atmosphere of 5% CO_2, 100% humidity, during which time the newborn larvae were shed. The medium containing adult and newborn larvae was then passed through a nylon mesh with 5-μm pore size (Small Parts Inc., Miami, Fla.). The isolated newborn larvae in the filtrate were sedimented for 2 min at 225 *g* at room temperature in 15 cm^3 conical centrifuge tubes, pooled, washed in MEM containing 10% FCS (MEM/FCS), and resuspended in 30 ml of MEM/FCS. The larvae were quantitated by direct counting of an aliquot of each preparation and diluted so that 100 μl would provide 30–40 larvae per larvicidal assay well.

Newborn larval killing assay. Killing of newborn larvae was studied by a modification of the method of Kazura et al. (10). To each well of flat-bottom microtiter plates (Linbro Chemical Co., Hamden, Conn.), the following were added: 100 μl suspension containing 30–40 larvae, 50 μl leukocyte preparation containing the indicated quantity of leukocytes, 10 μl serum (immune, fresh normal, or heat-inactivated normal) and sufficient MEM/FCS to provide a final volume of 200 μl. The number of viable larvae in each well was counted at the beginning of the experiment by direct observation with an Olympus CK inverted microscope (Olympus Corporation of America, New Hyde Park, N. Y.). Larval survival after varying periods of time (0–20 h) was determined by direct larval counts. In this assay the killed larvae were lysed; although coated to varying degrees by leukocytes, live larvae were readily visible.

In certain experiments specific reagents were added to the killing assay. These included catalase (65,000 U/mg; Boehringer Mannheim Biochemicals, Indianapolis, Ind.), superoxide dismutase (>3,000 U/mg; Truett Laboratories, Southwestern Drug Corp., Dallas, Tex.), sodium cyanide or sodium azide (Fisher Scientific Co., Pittsburgh, Pa.). Cytochalasin B (Aldrich Chemical Co., Milwaukee, Wis.) was dissolved at 2 mg/ml in dimethylsulfoxide. Control solutions containing equivalent concentrations of dimethylsulfoxide alone did not alter larval killing. Each of these reagents was diluted in MEM/FCS so that 10 μl would contain the desired concentration per assay well and was added to assay plates containing larvae immediately before addition of cells. Colchicine (Aldrich Chemical Co.) was handled similarly except that leukocytes were preincubated for 30 min at 37°C in the indicated concentration of colchicine in MEM/FCS before addition to the assay wells.

Opsonic activity of immune serum. Larvae were incubated in 10% immune or normal rabbit serum, washed three times in 100 vol MEM/FCS, resuspended to the desired concentration and added to wells containing leukocytes and MEM/FCS. Cell adherence and larval killing were examined after 3, 6, and 18 h incubation.

Coating of larvae with immunoglobulin was examined by immunofluorescence. Newborn larvae were incubated in 80% immune rabbit serum for 30 min at 37°C. They were washed three times, resuspended in MEM/FCS, and incubated at 37°C or 4°C. After 0, 3, and 5 h, the larvae were washed and incubated in 8% fluorescein-conjugated swine anti-rabbit IgG (Miles Laboratories, Elkhart, Ind.) for 15 min at room temperature, washed three times, resuspended in MEM/FCS, and examined for fluorescence under a Zeiss fluorescence microscope (Carl Zeiss, Inc., New York) equipped with a high pressure mercury vapor lamp, BG12 excitation filter and No. 44 and 53 barrier filters.

Larval infectivity. Larval viability after incubation under conditions similar to those of the microtiter assay was examined by the ability of the larvae to survive and attain muscle encystment after intravenous injection into normal mice. 50,000 larvae were incubated in 50 ml MEM/FCS alone or containing 2×10^8 normal mixed leukocytes and 5% immune or normal serum for 8 h at 37°C in an atmosphere of 5% CO_2 with gentle rotation. The larvae-cell mixtures were concentrated by centrifugation and resuspended so that 0.2 ml would contain 14% of the original mixture (or 7,000 larvae if there were 100% survival). 0.2 ml of the preparation was injected into the tail vein of normal C3H/HeJ mice. After 4 wk the mice were sacrificed, skinned, eviscerated, and the musculoskeletal tissues homogenized and digested for 4 h at 37°C in 10 ml of 1% acid-pepsin. The digested muscle was concentrated to 10 ml by centrifugation and thoroughly shaken; 0.2-ml aliquots were taken and the number of larvae present counted in sextuplicate.

Statistical analysis. In all experiments, each determination was expressed as the mean of three to six assay wells. Each experiment was repeated at least three times with similar results. Significance was examined employing the Student *t* test.

RESULTS

The microtiter plate direct assay of killing of newborn larvae of *T. spiralis* provided an excellent system for investigation of larvicidal events. Incubation of larvae in medium, with the medium containing immune or fresh serum or containing cells in the absence of immune serum resulted in <10% larval death, yet leukocytes with immune serum achieved 100% killing of larvae and the amount of killing was a function of the cell:larva ratio (Figs. 1 and 2). In the presence of optimal immune serum preparations, eosinophils and neutrophils were equally effective in the larvicidal assay (Fig. 1). Preparations of human peripheral blood mononuclear cells (mixed lymphocytes and monocytes) did not achieve killing of larvae in this system (9±2.8% [n = 12] killing after 24 h at 10,000 mononuclear cells per larva).

Killed larvae were apparently lysed, as no immobile larval forms were found, even at low concentrations at which the number of attached cells did not achieve coating of the larvae. That viable larvae were not hidden among cell clumps was verified by examination of larval infectivity. Larvae were incubated with leukocytes (cells:larva ratio of 3,000:1) and immune or normal serum, washed and injected into normal mice so that each animal received a preparation initially containing 7,000 larvae. 1 mo later, numbers of larvae achieving muscle encystment were determined. Of the

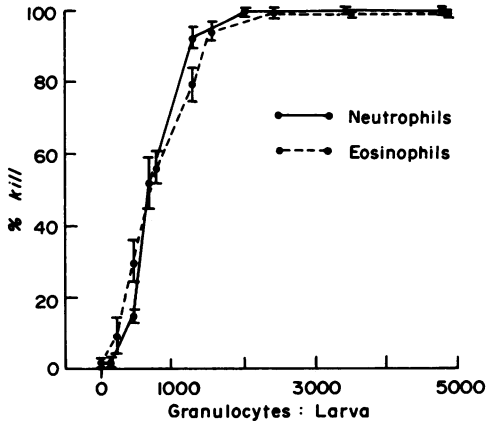

FIGURE 1 Killing of newborn larvae by three preparations of purified neutrophils (99%) or eosinophils (91–95%) after 18 h incubation in MEM/FCS with 5% heat-inactivated rabbit immune serum. This immune serum promoted optimal killing by both types of granulocytes. Mean±SE of nine determinations.

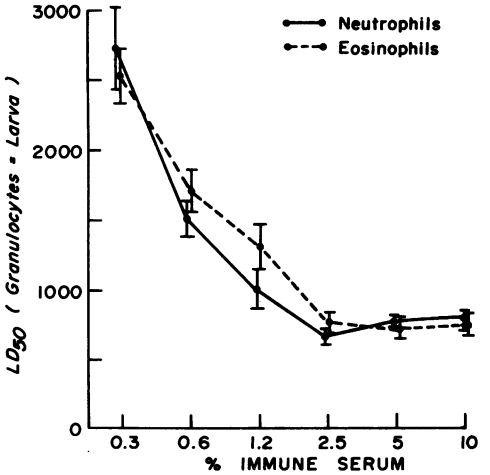

FIGURE 3 Effect of serial dilutions of rabbit immune serum on larval killing. The LD_{50} is the ratio of granulocytes per larva that killed 50% of the larvae after 18 h incubation. Mean±SE of triplicates.

larvae incubated with cells and normal serum, 2,080 ±276 (mean±SE, n = 6) were recovered; of those incubated with cells and immune serum, none was recovered.

Opsonic requirements. Maximal larval killing occurred in the presence of concentrations of heat-inactivated immune serum of 2.5% or greater (Fig. 3). With lower immune serum concentrations, killing by eosinophils or neutrophils decreased in parallel. Five immune sera were tested and all promoted comparable killing by neutrophils. Four of the sera provided opsonization for eosinophils comparable to that shown in Fig. 1. However, one immune serum preparation was associated with neutrophil-mediated killing as shown in Fig. 2 but diminished killing by six preparations of purified eosinophils (37±8.4% killing at a cell:larva ratio of 9,300:1)

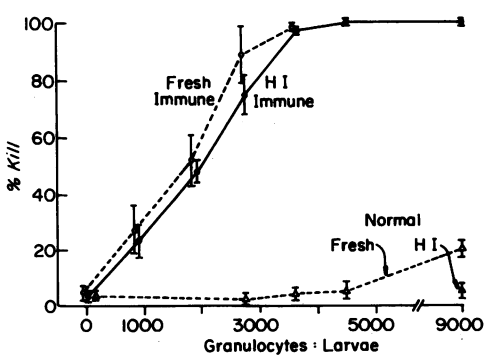

FIGURE 2 Killing of newborn larvae by normal, mixed granulocytes (98.9% neutrophils, 1.1% eosinophils) after 18 h incubation in MEM/FCS with addition of 5% fresh or heat-inactivated (HI) rabbit immune or normal serum and the indicated ratio of granulocytes per larvae. Mean±SE of five determinations.

The availability of complement in fresh sera did not significantly increase killing by neutrophils (Fig. 2) or eosinophils (data not shown) above that provided by heat-inactivated immune sera. Fresh normal sera had minimal opsonic activity; at a cell:larva ratio of 9,000:1, neutrophils achieved 19±3.1% (n = 5) and eosinophils achieved 14±2.8% (n = 5) killing, both of which were significantly greater ($P < 0.025$) than the kill observed in the presence of heat-inactivated normal sera (4±2.4% with neutrophils; 3±2.7% with eosinophils).

Purified eosinophils or neutrophils rapidly adhered to larvae in the presence of 5% immune serum. With each cell type, 85–96% of the larvae had five or more attached leukocytes after 3 h incubation (cells:larva of 1,000:1). The number of attached cells increased with further incubation but became impossible to quantitate because of lysis of an increasing proportion of the larvae. To determine whether these serum preparations enhanced larval killing by an opsonic effect, larvae were preincubated in heat-inactivated immune serum, washed, and placed in the assay chamber with normal leukocytes. After 3 h, a moderate number of leukocytes (>5) were found attached to the larvae; however, the quantity of adherent cells decreased with more prolonged incubation. After 18 h, very few cells were attached to larvae and only 15% of the larvae were killed at 5,000:1 cells per larva. The possibility that opsonins were being lost either by internalization or by shedding was examined by immunofluorescence. Larvae were preincubated with immune serum, washed, incubated for varying periods of time at 4° or 37°C, then examined for surface IgG by immunofluorescence.

Larvae studied immediately after preincubation with rabbit immune serum, or after 6 h incubation at 4°C, demonstrated bright, uniform, circumferential fluorescence. By 3 h of incubation at 37°C, all fluorescence had disappeared. Larvae preincubated in normal serum demonstrated no fluorescence at any time.

Time-course of killing. At a cell:larva ratio of 4,200:1, the increase in killing was essentially linear during the first 7 h of incubation, by which time 100% mortality had occurred (Fig. 4). At lower cell concentrations, killing was slower and continued to increase during a 20-h incubation.

Effect of inhibitors. Killing was inhibited by 1,300 U catalase, 5 μg/ml cytochalasin B, or 4 μM colchicine (Table I). Superoxide dismutase, 10 μg/ml (twice the concentration known to inhibit superoxide-mediated events in similar assay systems [25]), had no effect on larval killing. Sodium cyanide or azide significantly enhanced larval killing at 5 mM, concentrations expected to inhibit leukocyte (and presumably larval) peroxidases and catalase.

Killing ability of leukocytes from a patient with chronic granulomatous disease (CGD). Cells obtained from a patient with CGD demonstrated markedly impaired and delayed killing of newborn larvae (Fig. 5). A hydrogen peroxide-generating system was added to the CGD larvicidal assay to examine whether leukocyte enzymes such as peroxidase, known to be normal in CGD leukocytes (26), might act synergistically with hydrogen peroxide in larval killing. Larvae are killed in this assay by hydrogen peroxide generated by glucose-glucose oxidase with glucose oxidase concentrations of 0.12 mU/test or greater (27). Addition of 0.06 mU glucose oxidase to assays containing CGD leukocytes did not enhance larval killing (Table II).

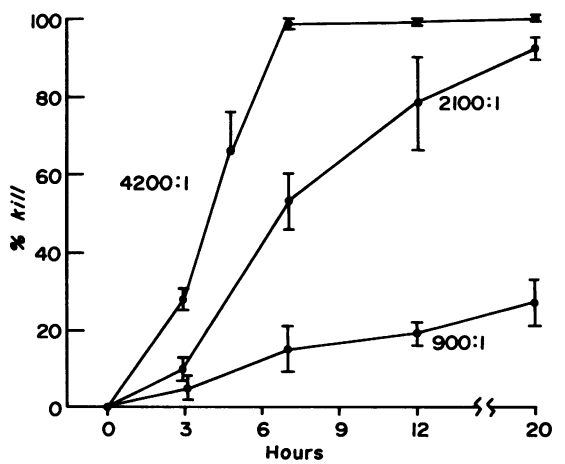

FIGURE 4 Rate of killing of larvae by granulocyte preparations during incubation in MEM/FCS with 5% heat-inactivated rabbit immune serum. Normal, mixed granulocytes were added at the ratios of 4,200, 2,100, and 900 granulocytes per larva. Higher cell concentrations (8,400:1) did not significantly increase the rate of killing above that observed at the ratio of 4,200:1. Mean±SE of triplicates.

After 20 h incubation, CGD cells achieved larval killing of 63 and 39% in two experiments (Fig. 5 and Table II). CGD cells kill bacteria that lack catalase and generate H_2O_2 (28); however, this did not appear to be the mechanism of delayed larval killing as the killing was not inhibited by catalase (Table II).

DISCUSSION

These studies demonstrate that human leukocytes are able to kill newborn (migratory phase) larvae of *T. spiralis* in a dose- and time-dependent manner during incubation in vitro. The relevance of responses to such migratory phase larvae and possible participation of eosinophils or neutrophils in the host response to trichinosis requires comment. Depending on the model examined, immunity to trichinosis may involve more rapid expulsion of adult worms from the intestine (29, 30), reduced fecundity of adult worms (30), and/or killing of migrating newborn larvae (31–35). Eosinophils or neutrophils could conceivable be involved in any of these immune mechanisms. However, studies of diverse metazoan parasites localized to the intestine have failed to demonstrate a reliable correlation with local or systemic eosinophil or neutrophil responses (36), and studies suggesting a direct role for the eosinophil or neutrophil in the immune response to trichinosis and schistosomiasis have only succeeded with examination of the tissue phases of these infections. The possibility that eosinophils or neutrophils may participate in reduced adult fecundity within intestinal tissues cannot be excluded. However, given the data regarding immunity to injected newborn larvae, it seems probable that this is a more likely site of participation of eosinophils or neutrophils in this immune response.

In the presence of optimal immune serum preparations, eosinophils and neutrophils demonstrated equivalent larvicidal abilities.

The opsonization of trichinella larvae by heat-inactivated immune sera is in accord with studies reporting IgG-mediated damage of schistosomula by eosinophils (37, 38). Schistosomula also fix complement and, in some assay systems, complement may also mediate killing of schistosomula (39). Newborn trichinella larvae apparently do not activate the alternate complement pathway (40), and the presence of complement provided minimal opsonic activity in the present studies.

The impaired larvicidal activity of CGD cells, the inhibition of killing by catalase, and the enhancement of killing by azide and cyanide suggest that products of oxidative metabolism, probably hydrogen peroxide, provide the major larvicidal activity. Although leukocytes from patients with CGD are unable to mount the

TABLE I
Killing of Newborn Larvae by Normal Granulocytes: Effect of Inhibitors

Inhibitor	cell:larva ratio of 3000:1	1000:1	No cells
	% kill after incubation		
None	81±9 (3)	27±4.1 (3)	3±1.7 (6)
Catalase, 1,300 μm	38±8 (3)	6±1.7 (3)	2±1.8 (6)
Heated catalase	87±12 (3)	34±7.2 (3)	5.2±3.4 (6)
Superoxide dismutase, 10 μg/ml	80±11 (3)	28±2.2 (3)	0.9±1.1 (6)
Cytochalasin B, 5 μg/ml	3±2.7 (3)	1±0.2 (3)	2.1±1.9 (6)
Colchicine, 4 μM	37±8 (3)	6±5.0 (3)	4.7±3.9 (6)
Cyanide, 5 mM	100±0 (3)	94±4.7 (3)	7±3.9 (6)
Azide, 5 mM	100±0 (3)	96±2.9 (3)	6.2±4.2 (6)

Incubation for 8 h with the indicated number of mixed, normal granulocytes per larvae in MEM/FCS with 5% rabbit immune serum and the indicated concentrations of added reagents. Mean±SE of determinations (number in parentheses).

normal oxidative metabolic response to membrane stimuli, other killing mechanisms are normal. Addition of a sublethal quantity of a hydrogen peroxide generator, such as glucose-glucose oxidase, improves the bactericidal activity of CGD cells (41) and provides important support for the bactericidal activity in intact cells of the myeloperoxidase-hydrogen peroxide-halide mechanism proposed by Klebanoff (42). Glucose-glucose oxidase did not improve killing of larvae by CGD cells. Moreover, azide and cyanide inhibit leukocyte peroxidase and bacterial killing by the peroxidase-hydrogen peroxide-halide mechanism (42, 43). Yet these agents enhanced larval killing by normal leukocytes. Azide and cyanide augment release of hydrogen peroxide by leukocytes (44, 45) that may result from increased production (46) or decreased destruction by enzymes such as catalase or peroxidase in the leukocytes, larvae, or medium (45). Such an increased release of hydrogen peroxide with direct larval toxicity would explain the enhanced killing of larvae in the presence of azide and cyanide.

Although the preceding data suggest that larval killing is directly due to hydrogen peroxide, nonoxidative larvicidal mechanisms may be present as well. The inhibitory effect of catalase was most evident after 8 h incubation and at cell:larva ratios of 5,000:1 or less. After 18 h incubation or at cell:larva ratios of 10,000:1, catalase had only a slight inhibitory effect. Studies of extracellular killing of fungi (47) or mammalian cells (48) by oxidative mechanisms have also reported a

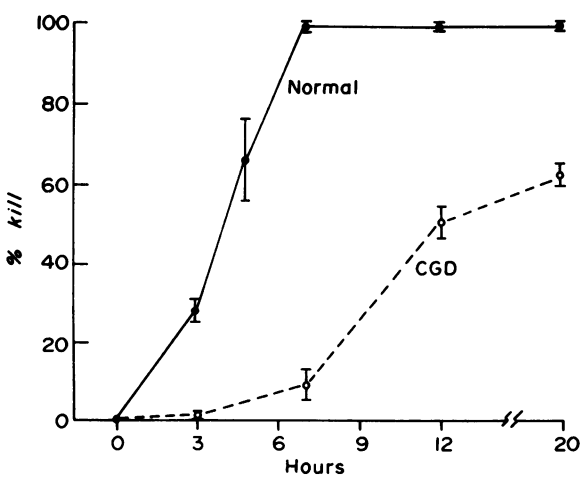

FIGURE 5 Killing of larvae by normal leukocytes and leukocytes obtained from a patient with CGD, each at a concentration of 4,200 granulocytes per larva. Incubation for the indicated times in MEM/FCS with 5% rabbit immune serum. Mean±SE of five determinations.

TABLE II
Effect of Catalase or Glucose Oxidase on Killing by CGD Leukocytes

	Hours of incubation 8	20
	% kill after incubation	
Cells alone	17±2.5 (3)	39±7.2 (3)
Cells + catalase, 1,300 U	24±6.4 (3)	46±3.5 (3)
Cells + glucose oxidase, 0.06 mU	11±5.1 (3)	38±10.7 (3)

Granulocytes from a patient with chronic granulomatous disease incubated in MEM/FCS (containing 5.6 mM glucose) with 5% rabbit immune serum alone or with addition of catalase or glucose oxidase of the indicated amount per 0.2 ml assay well. Mean±SE of triplicates.

limited ability of catalase to inhibit such events. This could suggest that the concentration of catalase at the cell-target interface might be inadequate to destroy all of the H_2O_2 generated; sufficient cells or prolonged exposure could then provide a lethal effect. Alternatively, it could suggest that mechanisms other than hydrogen peroxide participate to some degree in killing. The experiments with CGD leukocytes support the latter possibility. Although impaired, CGD cells killed approximately half of the larvae after prolonged incubation, which is comparable to the degree of killing of many catalase-positive bacteria (28) or mammalian tumor cells (47) by CGD leukocytes.

Killing of nucleated mammalian cells by granulocytes may be similar to the larval killing examined in the present study. Although mammalian cells are sensitive to the peroxidase-H_2O_2-halide system (49, 50) or to leukocyte cationic proteins (51), Clark and Klebanoff (48) reported that antibody-dependent granulocyte-mediated cytotoxicity to mouse lymphoma cells was dependent upon an intact oxidative metabolic burst but was independent of the peroxidase-H_2O_2-halide system. Extracellular killing by peroxidase-H_2O_2-halide mechanisms may be demonstrated in serum-free media (54, 55); however, the significance of this mechanism in vivo, where high protein concentrations would prevail, is uncertain. Also, Nathan et al. (54, 55) recently examined cytotoxicity to 10 mammalian cell lines by extracellular release of hydrogen peroxide by granulocytes or activated macrophages after stimulation by phorbol myristate acetate. Their data indicated the presence of direct cytotoxicity by hydrogen peroxide without necessary involvement of peroxidase-mediated mechanisms. The present studies suggest that the major event in larval killing may also involve direct larval toxicity by hydrogen peroxide. Studies employing hydrogen peroxide-generating systems and leukocyte lysates or granule preparations are consistent with this hypothesis (27).

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