Role of Granulocyte Oxygen Products in Damage of Schistosoma mansoni Eggs In Vitro

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Abstract

The objectives of this study were to describe the ultrastructure of granulocyte-Schistosoma mansoni egg interaction and to determine the role of reduced oxygen products as effectors of cell-mediated damage to the parasite target. Granulocytes attached to the parasites and closely applied their plasma membranes to the microspicules of the egg shell 30 min after mixing in the presence of immune serum. By 4 h, the egg shell was fractured and granulocyte pseudopodia extended toward the underlying miracidium. Granulocyte attachment to eggs resulted in release of \( \text{O}_2 \) (0.30–0.52 nmol/min per 2 \times 10^6 cells) and accumulation of \( \text{H}_2\text{O}_2 \) (0.14–0.15 nmol/min) in the presence of antibody or complement. Granulocytes reduced egg tricarboxylic-acid cycle activity and hatching by 28.3±0.9 and 35.2±2.8%, respectively (cell-egg ratio of 1,000:1). Exogenous superoxide dismutase (10 \( \mu \text{g/ml} \) inhibited granulocyte toxicity for egg metabolic activity (3.0±2.1% reduction in acetate metabolism vs. 28.3±0.9% decrease in controls without superoxide dismutase, \( P < 0.0005 \) and hatching (12.5±1.8% reduction, \( P < 0.0005 \), whereas catalase and heparin had no effect. Inhibitors of myeloperoxidase (1 mM azide, cyanide, and methimazole) augmented granulocyte-mediated toxicity of egg tricarboxylic-acid cycle activity (44–58% reduction in activity vs. 31 and 35% reduction in controls), suggesting that \( \text{H}_2\text{O}_2 \) released from cells was degraded before reaching the target miracidium. Oxidants generated by acetaldehyde (2 mM)-xanthine oxidase (10 mM/\( \mu \text{l} \) also decreased egg metabolic activity and hatching by 62.0±9.0 and 38.7±7.3%, respectively. Egg damage by the cell-free system was partially prevented by superoxide dismutase (26.5±4.2% reduction in egg tricarboxylic-acid cycle activity) and completely blocked by catalase (0% reduction in activity). These data suggest that granulocyte-mediated toxicity for \( S. \text{mansoni} \) eggs is dependent on release of \( \text{O}_2 \) or related molecules. These oxygen products, unlike \( \text{H}_2\text{O}_2 \), may readily reach the target miracidium where they may be converted to \( \text{H}_2\text{O} \) or other microbialid effectors.

Introduction

Attachment of granulocytes (PMN)\(^1\) to ligand-coated non-ingestible surfaces may result in cellular spreading, activation of the respiratory burst, and secretion of lysosomal contents. This process has been referred to as “frustrated phagocytosis” and may have physiologic relevance in eosinophil- and neutrophil-mediated host defense against infections by multicellular organisms (1–6). Eosinophils are a major component of the granulomatous response around \( S. \text{mansoni} \) eggs and are involved in eliminating this parasite stage from host tissues (7, 8). Destruction of parasite ova is clearly important to the host as the morbid complications of schistosomiasis mansoni (hepatosplenomegaly and portal hypertension) are largely related to the granulomatous and fibrotic responses to eggs deposited in the liver (9).

\( S. \text{mansoni} \) eggs represent a formidable target for PMN and mononuclear cells. Unlike the lipid-rich outer membranes of unicellular microbes (10) or the syncytium-bound schistosomula (11), the surface of schistosome ova consists of a hard “tanned” proteinaceous shell with multiple pores and microspicules (12–14). Within the shell but not in direct physical contact with mammalian tissues is the live miracidium, which releases enzymes and antigens (15, 16). To damage the enclosed miracidium, host effector mechanisms must either physically breach the hard egg shell and/or deliver toxic products outside the shell in areas removed from their possible target. In this situation, effective PMN microbicidal activity may require the generation of high concentrations of toxic mediators or the release of small amounts of these substances over a long period of time. We have recently developed an in vitro assay to assess the biologically relevant effects of human PMN on \( S. \text{mansoni} \) ova (17). Mixed granulocyte preparations or purified neutrophils and eosinophils were found to significantly impair energy metabolism (apparent tricarboxylic-acid cycle activity) of ova in a dose-dependent manner (dimunition of egg metabolic activity was observed at cell-egg ratios of 100:1 and 1,000:1 but not at a 1:1 ratio); hatching of eggs (release of miracidia) was also reduced. These alterations were associated with a diminished capacity of eggs to induce delayed-type hypersensitivity granulomas in vivo.

The purposes of the present study are to delineate the ultrastructural features of the interaction of schistosome eggs and human granulocytes and define the biochemical basis of the deleterious effect of PMN on eggs. Our results indicate that neutrophils and eosinophils initially attach to the outer surface of the egg shell and interdigitate their plasma membranes with the parasite’s microspicules. By 4 h of incubation, this interaction results in fracture of the entire thickness of the shell and the development of PMN pseudopodia that project toward the enclosed miracidium. PMN-egg contact is associated

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1. Abbreviations used in this paper: A, absorbance; fNHS, fresh immune human serum; GPO, glutathione peroxidase; hNHS, heat-inactivated immune human serum; hiNHS, heat-inactivated normal human serum; PMA, phorbol myristate acetate; PMN, granulocytes; SOD, superoxide dismutase; TCA, tricarboxylic acid.
with extracellular release of $O_2^\cdot$ and $H_2O_2$. Damage to ova by PMN is decreased by exogenous superoxide dismutase (SOD) and unaffected by catalase or heparin. Hydrogen peroxide ($H_2O_2$) and oxygen products derived from acetaldehyde-xythane oxide also reduce egg tricarboxylic acid cycle activity and hatching; SOD and catalase inhibit the deleterious effects of acetaldehyde-xythane oxide on eggs. Inhibitors of myelo-peroxidase (azide, cyanide, and methimazole) augment PMN-mediated egg damage, suggesting that $H_2O_2$ released from adherent leukocytes is degraded by a peroxidase-catalyzed reaction before reaching the target miracidium. Granulocyte host defense against schistosome ova may thus be dependent on PMN release of superoxide ($O_2^\cdot$). This molecule, unlike $H_2O_2$, may readily proceed past the egg shell and reach the viable miracidium, where it can be converted to $H_2O_2$ or other microbicidal oxygen species.

**Methods**

**Isolation of S. mansoni eggs.** Parasite eggs were obtained from the livers and intestines of CF1 mice (Carworth Farms, New City, NY) infected with 200 cercariae of S. mansoni 6 wk earlier (18). Freshly dissected organs were perfused with cold (4°C) 1.7% NaCl containing penicillin (200 U/ml) and streptomycin (200 μg/ml) (KC Biologicals, Lenexa, KS) and macerated in a blender (Waring Products Div., Dynamics Corp. of America, New Hartford, CT). The resulting suspension was digested for 2 h at 37°C in 0.5% trypsin (Sigma Chemical Co., St. Louis, MO) and sieved through a sterile Brown capsule (19). The partially purified eggs were layered over 40 ml of a Percoll solution (Sigma Chemical Co.) prepared by mixing one part of stock Percoll with one part of 1.7% NaCl. After sedimentation by gravity for 10 min, the pellet containing S. mansoni eggs free of cellular debris was washed and eggs were enumerated. The percentage of mature eggs was determined according to the method described by Pellegrino et al. (20). Preparations containing >85% mature eggs (i.e., eggs with a fully developed miracidium) were washed three times in RPMI 1640 (Gibco Biologicals, Grand Island, NY) containing 200 U/ml of penicillin, 200 μg/ml of streptomycin, 25 mM Heps buffer (Sigma Chemical Co.), and 2 mM L-glutamine (KC Biologicals) (complete medium) and suspended to the desired concentration in complete medium or Hank's balanced salt solution without phenol red (HBSS) (Gibco Laboratories).

In some experiments, miracidia were obtained by exposure of freshly isolated eggs to spring water for 30 min. Miracidia, intact eggs, and empty shells were visualized with a dissecting microscope; groups of each were separated and aspirated with a Pasteur pipette. Miracidia, "empty" egg shells, and intact eggs were then suspended to 5 × 10^6 per 4 ml of complete medium and assayed of [2-14C]acetate metabolism were performed as described below.

**Preparation of leukocytes.** Blood was obtained from normal donors with no exposure to S. mansoni and anticoagulated with 10 U heparin/ml (Upjohn Co., Kalamazoo, MI). Mixed granulocyte populations were prepared by density gradient centrifugation over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) followed by dextran sedimentation (Sigma Chemical Co.) and hypotonic lysis of erythrocytes (21). The purified granulocyte preparations were washed three times in HBSS without Ca++ and Mg++ (Gibco Laboratories) and suspended to the desired concentration in complete medium or in Ca++-Mg++ replete HBSS (Gibco Laboratories). Cell counts were performed with a counter (Coulter Electronics Inc., Hialeah, FL). Viability in each of the preparations was >95% as judged by exclusion of trypan blue.

**Electron microscopy.** Eggs (2 × 10^6) with or without PMN (2 × 10^6) and 2% heat-inactivated immune or normal serum were incubated together at 37°C for 30 min, 2, 4, and 18 h. The preparations were then washed three times in 0.9% NaCl, fixed in a solution of 0.1 M caccodylate buffer containing 2.5% glutaraldehyde and 4% sucrose, and postfixed in 1% osmium tetroxide. Samples were then dehydrated and embedded in Epon 812 (22). The resulting blocks were cut with a Porter-Blum MT-2 ultramicrotome with a diamond knife (DuPont Instruments–Sorvall Biomedical Div., Newtown, CT). Sections 1 μm thick were stained with 1% toluidine blue and studied by light microscopy to select proper areas for electron microscopy. Thin sections mounted on 200-mesh copper grids and stained with 1% uranyl acetate and lead citrate were examined with a 100 electron microscope (Elmiskop; Siemens Corp., Iselin, NJ).

$O_2^\cdot$ and $H_2O_2$ release from ova-stimulated granulocytes. Triplicate preparations of 2 × 10^6 PMN were mixed with 2 × 10^6 S. mansoni eggs in round-bottomed borosilicate tubes (12835-3, American Scientific Products Div., American Hospital Supply Corp., McGaw Park, IL) or 5-ml polystyrene tubes (No. 14-956-10, Fisher Scientific Co., Pittsburgh, PA) containing 1.2 mg of cytochrome c (Sigma Chemical Co.) with or without 10 μg of SOD (Sigma Chemical Co.) in 1 ml of HBSS. Human serum in a final concentration of 2% was added from the following sources: (a) heat-inactivated normal serum (hiNHS); (b) heat-inactivated pooled immune serum (hiIHS) (obtained from Brazilians or Kenyans with documented S. mansoni infection); (c) fresh normal serum (fNHS); or (d) fresh immune serum (fIHS). The cell-parasite-serum mixtures were incubated for 0–30 min at 37°C in a Dubnoff shaker, transferred to cuvettes, and SOD-inhibitable reduction of ferricytochrome c was measured at 37°C for 10 min at 1-min intervals in a spectrophotometer (DU-8; Beckman Instruments, Inc., Fullerton, CA) (23). A constant rate of SOD-inhibitable reduction of cytochrome c was observed after 2 min of incubation in the cuvette and continued for an additional 8 min. The rate of extracellular $O_2^\cdot$ release was calculated from this value and expressed as nmol O$_2^\cdot$/min per 2 × 10^6 cells, unless otherwise indicated. To determine the efficiency of various concentrations of cytochrome c in measuring $O_2^\cdot$ release, 0.6, 1.2, 2.4, and 3.6 mg of cytochrome/ml were used in preliminary experiments (24). More cytochrome c was also added after the initial 10-min determination of $O_2^\cdot$ release to ascertain whether a sufficient amount of cytochrome c was included in the original mixture to scavenge all the $O_2^\cdot$ generated.

Phorbol myristate acetate (PMA) (0.1 μg/ml) (Consolidated Midland Corp., Brewster, NY) diluted in dimethyl sulfoxide (Sigma Chemical Co.) was added after the 10-min measurement of $O_2^\cdot$ production to determine the capacity of cells to respond to further stimulation of $O_2^\cdot$ production. This value was compared with $O_2^\cdot$ production by a similar number of cells not mixed with eggs.

To assess whether the detected $O_2^\cdot$ was released from PMN adherent to eggs and/or from cells remaining unattached, PMN with eggs attached were separated from nonadherent PMN. PMN (10^6)-egg (5 × 10^6) mixtures with 2% fNHS plus 2% hiIHS were prepared, incubated at 37°C for 10 min, and eggs were separated from PMN by allowing the mixtures to settle for an additional 10 min at 4°C. The upper 0.9 ml (primarily containing PMN without eggs) and lower 0.1 ml (primarily containing eggs, with and without adherent PMN) were separated, washed three times in HBSS, and finally placed in 1 ml of HBSS containing 1.2 mg of cytochrome c/ml with or without 10 μg of SOD/ml. Cytochrome c reduction at 10, 30, and 60 min was determined and the results were expressed as nanomoles of O$_2^\cdot$ generated/2 × 10^6 cells for each time interval.

To determine the ability of soluble factors derived from egg-serum mixtures to induce release of $O_2^\cdot$ by PMN, 5 × 10^6 ovum or sera (from the various sources indicated above) were incubated together for 18 h. Supernatants were harvested and 10, 50, or 100 μl was added to PMN before measurement of ferricytochrome c reduction.

Extracellular release of $H_2O_2$ by PMN was measured by the scopoletin method (25) using an Amino-Bowman spectrophotometer (American Instrument Co., Travenol Laboratories, Silver Spring, MD). Cells (2 × 10^6) and eggs (2 × 10^6) were incubated in 1 ml of HBSS with 2% serum (same sources used for the $O_2^\cdot$ assay) in round-bottomed borosilicate tubes for 10 min in a shaking water bath held at a temperature of 37°C. The cell-egg mixtures were then washed in excess
cold (4°C) HBSS and the rate of H2O2 accumulation was determined as previously described (4).

Assays of PMN-induced egg damage. Damage to S. mansoni eggs was assessed by (a) the level of tricarboxylic acid (TCA) cycle activity, (b) release of miracidia (hatching), and (c) capacity to induce granolomas in vivo. An index of TCA cycle activity was obtained by determining the amount of 14CO2 generated from [2-14C]acetate (17, 26, 27). In studies of PMN-induced alterations in egg metabolism, triplicate samples of eggs (5 x 106), PMN (5 x 106), and PMN-egg mixtures (all containing 2% hiHIS) were incubated in 10 ml Erlenmeyer flasks containing 4 ml of complete medium to which 2 μCi of [2-14C]acetate (sp act 30 mCi/mmol, New England Nuclear, Boston, MA) was added. The flasks were fitted with an airtight rubber stopper and center well (Kontes Co., Vineland, NJ) containing a piece of electrotococating paper (LKB Produktor, Uppsala, Sweden) 60 mm2. After 24 h of incubation at 37°C, 0.1 ml of NCS tissue solubilizer (Amer sham Corp., Arlington Heights, IL) and 1 ml of 10% trichloroacetic acid (Sigma Chemical Co.) were respectively injected into the center well and main flask. The flasks were stored for 6 h at 37°C, the papers were then removed and placed in 2 ml of scintillant fluid (ACS, aqueous counting scintillator; Amer sham Corp.). Samples were left overnight before counting the evolved 14CO2 in a liquid scintillation counter (Nuclear Chicago, Chicago, IL). Results were expressed as the percentage of reduction of TCA cycle activity for eggs mixed with PMN compared with eggs incubated alone and calculated with the formula: % reduction in predicted egg 14CO2 generation = [cpm (cells) + cpm (eggs)] - cpm (cells + egg)]/cpm (cells) + cpm (eggs)) x 100. Three preparations of catalase (5,000 U/ml) (Sigma Chemical Co.; Worthington Biochemical Corp., Freehold, NJ; Calbiochem-Behring Corp., LaJolla, CA), SOD (10 μg/ml) (Sigma Chemical Co.), or heparin (preservative-free, 500 U/ml) (Sigma Chemical Co.) were used in triplicate to evaluate their effects on PMN-induced alterations of egg [2-14C]acetate metabolism. The enzymatic activity of each catalase preparation was determined by its ability to degrade H2O2 (28). Sodium azide, sodium cyanide, or methimazole (1 mM) (Sigma Chemical Co.) were also added to PMN-egg mixtures to determine whether inhibition of myeloperoxidase affected PMN-induced changes in egg acetate metabolism (29).

Hatching was determined by exposing eggs to light for 1 h; four drops of 1% aqueous iodine were then added to stop hatching. Eggs were sedimented at 100 g x 5 min, resuspended in 0.15 M NaCl, and transferred to a Sedgewick-Rafter chamber (Curtin-Matheson, Cleveland, OH) for light microscopic inspection. 200 miracidia and eggs were counted to determine the hatching rate (percentage of eggs that released a miracidium).

For measurements of in vivo granuloma formation, aliquots of eggs were incubated with H2O2 or acetaldehyde-xanthine oxidase for 24 h (see below), washed three times in complete medium, suspended to 2,000 in 0.5 ml of 0.15 M NaCl, and injected intravenously into CFI mice. At 8 d, animals were sacrificed, and their livers were removed and processed for determining granuloma area with a ×40 light microscope measurement computer (Millipore Corp., Bedford, MA) (30).

Effects of oxidant-generating systems and scavengers on egg viability. The effects of H2O2 on egg TCA cycle activity and hatching were determined by addition of reagent H2O2 ( Fisher Scientific Co., Fairlawn, NJ). The concentration of H2O2 was measured with a spectrophotometer with ε250 = 81 M-1 cm-1 (31). To assess the toxicity of H2O2, O2, OH, and possibly O2, acetaldehyde (2.0 mM) (Eastman Kodak Co., Rochester, NY) and xanthine oxidase (10 μU/ml) (type II, Sigma Chemical Co.) were incubated with 5 x 106 eggs for 18 h at 37°C in studies of TCA cycle activity (32). The hatching rate of eggs was determined after exposure to acetaldehyde-xanthine oxidase for 2, 6, 18, 24, and 48 h. The rate of O2 generation for this substrate and enzyme was measured by the SOD-inhibitable reduction of ferricytochrome c. The effect of degradative enzymes or inhibitors of oxidants generated by acetaldehyde-xanthine oxidase was also evaluated. H2O2 and O2 were destroyed by the addition of catalase (5,000 U/ml) and SOD (10 μg/ml), respectively. The parasite cytotoxic effects of OH and O2 were investigated by addition of diazobicyclooctanate (Aldrich Chemical Co., Milwaukee, WI), mannitol, sodium benzoate, or histidine (all from Sigma Chemical Co.) (33, 34).
Figure 1. (A) Schistosoma mansoni egg incubated in 2% heat-inactivated immune serum for 30 min. The egg shell has multiple spicules on its outer surface and covers the underlying vitelline membrane. The multicellular miracidium is situated below the vitelline membrane (x 2,600). (B) Granulocyte-egg interaction in the presence of 2% heat-inactivated immune serum, 30 min of incubation. The granulocyte (PMN) is closely applied to the shell of the egg, which has multiple spicules extending from its surface (x 2,600). E, egg; S, egg shell; Vm, vitelline membrane.

cmpared with hiNHS) (Table I). Significant quantities of O$_2^-$ release by PMN were also observed when cells and eggs were preincubated at 37°C for 5, 10, and 30 min before measurement of cytochrome c reduction (Table I). At all durations of preincubation studied (5, 10, and 30 min), PMN O$_2^-$ release induced by eggs was greater with fNHS, hiIHS, or fIHS than with hiNHS (Table I).

The calculated rate of O$_2^-$ release was not different when higher concentrations of cytochrome c were used. PMN mixed with eggs and fIHS were calculated to release 0.53 and 0.49 nmol O$_2^-$/min per 2 × 10$^6$ cells at cytochrome c concentrations of 2.4 and 3.6 mg/ml, respectively (results are the mean of triplicate samples for two experiments). When the cytochrome c concentration was increased from 1.2 to 2.4 mg/ml after the 10-min measurement of O$_2^-$ release, a further increase in the rate of SOD-inhibitable reduction of cytochrome c was also not detected. Addition of 0.1 μg of PMA/ml to cells and eggs incubated with fIHS for 10 min increased O$_2^-$ release from 0.52±0.10 to 4.63±0.58 nmol/min (mean±SE of four experiments). A similar number of PMN not exposed to eggs released 5.23±0.49 nmol O$_2^-$/min per 2 × 10$^6$ cells after addition of 0.1 μg of PMA/ml.

When PMN adherent to eggs were separated from nonadherent PMN and O$_2^-$ release was measured, the PMN adherent...
to eggs released a total of 0.84, 2.84, and 3.44 nmol O₂ per 2 × 10⁶ cells after 10, 30, and 60 min, respectively. (These values are given as the total amount of detectable O₂ accumulated over the entire time interval rather than as nanomoles released/minute because the rates were not constant and lower than that observed when PMN and egg were incubated together as described for Table I). At corresponding time intervals, non-adherent PMN released a total of <0.06, 0.07, and 0.08 nmol O₂. There were 9.6 × 10⁶ cells in the nonadherent population; the remaining cells (0.4 × 10⁶) were assumed to be adherent to the parasite egg. When PMA was added to adherent PMN, O₂ release increased to 6.30 nmol/10 min.

 Supernatants (10, 50, or 100 μl) of eggs incubated with 2% hiNHS, fNHS, or hiIHS for 18 h did not induce PMN O₂ production (<0.06 nmol/min per 2 × 10⁶). Furthermore, reduction of ferricytochrome c by eggs incubated without granulocytes did not occur. Inclusion of SOD (10 μg/ml) in any of the cell-egg-sera mixtures resulted in >90% inhibition of ferricytochrome c reduction.

 Egg-induced release of H₂O₂ by PMN was also observed. The rate of H₂O₂ release for PMN (2 × 10⁶) preincubated for 10 min with eggs (10⁶) and 2% hiNHS or 2% hiIHS was 0.03±0.01 nmol/min per 2 × 10⁶ cells (mean±SE of four experiments). When fNHS or fIHS were utilized, H₂O₂ accumulation increased, respectively, to 0.14±0.03 and 0.15±0.01 nmol/min (P < 0.05 and <0.005). H₂O₂ release was not detectable in the presence of catalase (1,250 U/ml).

 PMN-mediated reduction in predicted egg TCA cycle activity and hatching: effects of heparin, exogenous scavenger enzymes, and peroxidase inhibitors. In preliminary studies, ¹⁴CO₂ generated in cell-ova mixtures was found to be primarily related to egg and not PMN metabolism of labeled acetate. PMN (5 × 10⁶) incubated alone or with dead (freeze-thawed) eggs and 2% fIHS generated 3,000-10,000 cpm ¹⁴CO₂ (range of five experiments) compared with 15,000-55,000 cpm for mixtures containing live eggs (range of 12 experiments). Generation of ¹⁴CO₂ from [2-¹⁴C]acetate by eggs was associated with metabolism of acetate by the miracidium and not the egg shell. The values for ¹⁴CO₂ accumulation by “intact” eggs, freshly released miracidia, and “hatched” eggs (a mixture that contained 20%
Figure 3. A granulocyte (PMN) and eosinophil are attached to an egg whose shell has been disrupted in multiple areas. PMN has a cellular projection that extends inward toward the miracidium (× 2,600). (Inset) Fragment of egg shell that has been ingested by a PMN (× 8,300). Eos, eosinophil; S, egg shell.

intact eggs as well) were, respectively, 10,268, 14,442, and 1,512 cpm per 5 × 10^3 parasites (mean of two experiments with triplicate determinations in each).

Incubation of eggs (5 × 10^3) with PMN (5 × 10^6) and 2% HiHs led to 28.3±0.9% reduction in predicted 14CO2 generation from [2-14C]acetate and 35.2±2.8% decrease in hatching. Heparin (500 U/ml), a substance that prevents binding of eosinophil major basic protein to helminths (38), did not decrease these PMN-induced alterations in egg function (Table II). In contrast, inclusion of 10 μg of SOD/ml in the PMN-egg mixtures prevented the effect of PMN on egg TCA cycle activity and markedly diminished granulocyte-mediated reduction in hatching (35.2% decrease in hatching induced by PMN without SOD vs. 12.5% in the presence of SOD, P < 0.0005) (Table II). Catalase (5,000 U/ml) did not alter PMN-induced changes in egg acetate metabolism and hatching; catalase plus SOD had an effect similar to SOD alone (Table II).

To assess the possibility that myeloperoxidase or eosinophil peroxidase released from PMN catabolized H2O2 and thereby prevented this molecule from damaging the underlying miracidium, the effects of peroxidase inhibitors on PMN-induced suppression of egg acetate metabolism were measured. In two experiments, PMN induced 35 and 31% reduction in the amount of 14CO2 generated from [2-14C]acetate compared with the quantity predicted from PMN and eggs incubated separately (Table III). In experiment 1, inclusion of 1 mM azide, cyanide, or methimazole in the cell-parasite mixtures led to 44–48% suppression of egg acetate metabolism compared with 35% in
controls without peroxidase inhibitors. Similar enhancement of PMN-mediated toxicity was observed in experiment 2 (Table III).

Changes in egg activities induced by H$_2$O$_2$ and oxidants generated by acetaldehyde-xanthine oxidase. Exposure of eggs to 10 μM, 100 μM, and 1 mM H$_2$O$_2$ reduced egg generation of $^{14}$CO$_2$ from [2-14C]acetate by 24.8-81.2% compared with control ova (Table IV); hatching was not inhibited by 10 or 100 μM H$_2$O$_2$ but was decreased by 70.5±6.1% in the presence of 1 mM H$_2$O$_2$ ($P<0.0025$ vs. control, Table IV). Acetaldehyde (2 mM) or xanthine oxidase (10 μU/ml) had no effect on acetate metabolism or hatching when these substances were individually added to egg suspensions. In contrast, the combination of acetaldehyde and xanthine oxidase reduced apparent TCA cycle activity by 62.0±9.0% at 18 h incubation ($P<0.0025$, Table IV). Hatching was also decreased 38.7% by exposure to acetaldehyde-xanthine oxidase for 18 h (Table III). After 2 and 6 h of exposure to acetaldehyde-xanthine, hatching was decreased by 20 and 37%, respectively, compared with controls. There were no further decreases in hatching when eggs were incubated for 24 or 48 h (40 and 33% inhibition, respectively) (results are the mean of duplicate experiments at each time point). Acetaldehyde and xanthine oxidase generated O$_2$ at a maximal rate of 1.18 nmol/min within the first 3 min of mixing. After 10 min, the rate decreased to 0.18 nmol/min. Production of O$_2$ was not detectable 2 h after acetaldehyde and xanthine oxidase were mixed. When 10 μg of SOD/ml or 5,000 U of catalase/ml were added to acetaldehyde-xanthine oxidase, the deleterious effects of this oxidant-generating system on egg activities were decreased. The percentage reductions in TCA cycle activity and hatching induced by acetaldehyde-xanthine oxidase in the presence of SOD were 26 and <1%; catalase alone or when added with SOD completely abrogated the effects of acetaldehyde-xanthine oxidase (0% reduction in TCA cycle activity and hatching) (Table IV). SOD and catalase did not prevent egg toxicity of acetaldehyde-xanthine oxidase if the scavenger enzymes were boiled for 30 min before addition to the egg suspensions (data not shown). Mannitol, benzoate, or histidine did not reverse the effects of acetaldehyde-xanthine oxidase on egg TCA cycle activity and hatching (Table IV) and did not affect these egg functions in the absence of the oxidant generating system (data not shown). Diazobicyclooctane (1–10 mM) had a direct toxic effect on egg TCA cycle activity (74.0% reduction in $^{14}$CO$_2$ generation from 2-

### Table I. PMN O$_2$ Release Induced by S. mansoni Eggs: Relation of Duration of Incubation to Level of O$_2$ Production*

<table>
<thead>
<tr>
<th>Time of preincubation before measurement of O$_2$ release min</th>
<th>Rate of PMN O$_2$ release according to source of serum added†</th>
<th>hiHHS</th>
<th>TNHS</th>
<th>hiHHS</th>
<th>TNHS</th>
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<td></td>
<td>nmol/min per 2 × 10$^6$ cells</td>
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* PMN (2 × 10$^6$) and S. mansoni eggs (2 × 10$^6$) were mixed in the presence of 2% vol/vol sera and preincubated at 37°C for the times indicated before O$_2$ release was measured for a period of 10 min (23). † Results represent the mean±SE of experiments with PMN of four to six donors.

### Table II. Effects of Heparin and Antioxidant Scavenger Enzymes on PMN-induced Alterations in S. mansoni Egg Acetate Metabolism and Hatching

<table>
<thead>
<tr>
<th>Inhibitors added to PMN-egg mixtures</th>
<th>Percent reduction in egg acetate metabolism induced by PMN*</th>
<th>$P$ value</th>
<th>Percent reduction in hatching induced by PMN†</th>
<th>$P$ value</th>
</tr>
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<tbody>
<tr>
<td>No addition (control)</td>
<td>28.3±0.9</td>
<td>&lt;0.0025 vs. PMN and eggs incubated separately</td>
<td>35.2±2.8</td>
<td>&lt;0.0025 vs. eggs incubated without PMN</td>
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<tr>
<td>Heparin (500 U/ml)</td>
<td>43.3±7.3</td>
<td>&gt;0.10 vs. control</td>
<td>30.0±5.3</td>
<td>&gt;0.10 vs. control</td>
</tr>
<tr>
<td>SOD (10 μg/ml)</td>
<td>3.0±2.1</td>
<td>&lt;0.0005 vs. control</td>
<td>12.5±1.8</td>
<td>&lt;0.0005 vs. control</td>
</tr>
<tr>
<td>Catalase (5,000 U/ml)</td>
<td>31.7±2.0</td>
<td>&gt;0.10 vs. control</td>
<td>29.3±4.5</td>
<td>&gt;0.10 vs. control</td>
</tr>
<tr>
<td>Catalase (5,000 U/ml) + SOD (10 μg/ml)</td>
<td>8.3±4.3</td>
<td>&lt;0.0025 vs. control</td>
<td>7.0±2.3</td>
<td>&lt;0.0005 vs. control</td>
</tr>
</tbody>
</table>

* PMN (5 × 10$^6$) and eggs (5 × 10$^6$) were incubated together with 2% hiHHS and the percentage of reduction in egg $^{14}$CO$_2$ generation induced by PMN was calculated by comparison with the sum predicted from PMN and eggs incubated separately with 2% hiHHS (17, 27). Results represent the mean±SE of five experiments. † PMN (5 × 10$^6$) and eggs (5 × 10$^6$) were incubated together with 2% hiHHS and the hatching rate (miracidia released per 100 eggs) was determined after 24 h. This value was compared with the hatching rate of control eggs incubated in 2% hiHHS without PMN. The percent reduction induced by PMN was calculated from these values (17, 27). Results represent mean±SE of three experiments.
Table III. Effect of Peroxidase Inhibitors on PMN-induced Suppression in Acetate Metabolism of S. mansoni Eggs*

<table>
<thead>
<tr>
<th>Peroxidase inhibitor added</th>
<th>Contents of culture vessels</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm</td>
<td>cpm</td>
<td>cpm</td>
</tr>
<tr>
<td>None (control)</td>
<td>PMN alone</td>
<td>10,738</td>
<td>17,614</td>
</tr>
<tr>
<td></td>
<td>Eggs alone</td>
<td>11,607</td>
<td>16,500</td>
</tr>
<tr>
<td></td>
<td>PMN + eggs</td>
<td>14,493</td>
<td>23,600</td>
</tr>
<tr>
<td></td>
<td>(% reduction in cpm</td>
<td>(35%)</td>
<td>(31%)</td>
</tr>
<tr>
<td></td>
<td>induced by PMN§)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azide (1 mM)</td>
<td>PMN alone</td>
<td>6,514</td>
<td>15,832</td>
</tr>
<tr>
<td></td>
<td>Eggs alone</td>
<td>13,463</td>
<td>22,005</td>
</tr>
<tr>
<td></td>
<td>PMN + eggs</td>
<td>11,288</td>
<td>15,820</td>
</tr>
<tr>
<td></td>
<td>(% reduction in cpm</td>
<td>(44%)</td>
<td>(58%)</td>
</tr>
<tr>
<td></td>
<td>induced by PMN§)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyanide (1 mM)</td>
<td>PMN alone</td>
<td>8,024</td>
<td>13,305</td>
</tr>
<tr>
<td></td>
<td>Eggs alone</td>
<td>8,889</td>
<td>13,613</td>
</tr>
<tr>
<td></td>
<td>PMN + eggs</td>
<td>8,778</td>
<td>13,710</td>
</tr>
<tr>
<td></td>
<td>(% reduction in cpm</td>
<td>(48%)</td>
<td>(49%)</td>
</tr>
<tr>
<td></td>
<td>induced by PMN§)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methimazole (1 mM)</td>
<td>PMN alone</td>
<td>9,945</td>
<td>19,801</td>
</tr>
<tr>
<td></td>
<td>Eggs alone</td>
<td>13,972</td>
<td>18,005</td>
</tr>
<tr>
<td></td>
<td>PMN + eggs</td>
<td>13,062</td>
<td>17,400</td>
</tr>
<tr>
<td></td>
<td>(% reduction in cpm</td>
<td>(45%)</td>
<td>(54%)</td>
</tr>
<tr>
<td></td>
<td>induced by PMN§)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* PMN (2 x 10⁶) and eggs (2 x 10⁶) were incubated together or separately with 2% hiHIS and the amount of ¹⁴CO₂ generated from [2-¹⁴C]acetate after an 18-h period measured, as described in Methods (17, 26, 27).

‡ Mean of duplicate determinations for all values in this column.
§ This value was calculated by the formula: cpm (PMN alone) + cpm (eggs alone) - cpm (PMN + eggs)/(cpm (PMN alone) + cpm (eggs alone)) x 100.

14C]acetate (and hatching (60% reduction) and was thus not utilized in further studies.

Effect of cell-free oxidants on egg-induced granuloma formation. The area of egg-induced granulomas in mice injected with S. mansoni ova was 27,671±2,280 μm² (mean±standard error of the mean granulomas from six mice each injected with 2,000 eggs). Preincubation of eggs for 24 h with 1 mM H₂O₂ resulted in a mean granuloma area of 7,236±610 μm² (P < 0.001 compared with controls). Exposure of eggs to 100 μM or 10 μM H₂O₂ had no significant effect on granuloma area. The mean granuloma area induced by eggs preincubated with 2 mM acetaldehyde and 10 mM xanthine oxidase/ml was 24,600±2,780 μm² (results of injection of 2,000 eggs each into six mice; P > 0.10 compared with controls). Inclusion of 10 μg of SOD/ml with this oxidant-generating system and eggs resulted in a granuloma area of 27,500±6,170 μm² (mean±SE of mean granulomas from six mice). This value was not significantly different (P > 0.05) from that obtained in mice injected with control egg preparations or ova exposed to acetaldehyde-xanthine oxidase.

Catalase, SOD, and GPO activities of eggs. Catalase was undetectable in egg extracts (<0.025 U/mg of protein, results of experiments with five different egg preparations). Intact eggs also did not degrade H₂O₂. The A₃₂₀ of 0.5 and 1.0 mM solutions of H₂O₂ were 0.0412 and 0.0830, respectively, before and after incubation for 30 min with 10⁴, 10⁵, or 2 x 10⁴ freshly isolated live S. mansoni eggs. Control solutions of 0.5 and 1.0 mM H₂O₂ not exposed to parasite ova had A₃₂₀ of 0.0403 and 0.0805, respectively. SOD at a mean level of 0.74±0.01 U/mg of protein was present in soluble ova extracts (four separate experiments). Superoxide production as measured by ferricytochrome c reduction was not detectable when intact eggs (1-5 x 10⁴) or parasite extracts (1-100 μg of protein) were added to 1 or 10 mM acetaldehyde (in the absence of exogenous xanthine oxidase). GPO activity was detected at a level of 8.6 U/mg of parasite protein (mean value of two experiments).

Discussion

The microbicidal function of PMN primarily occurs in confined subcellular spaces where toxic mediators are highly concentrated around the invading microorganism. In contrast, killing of nonphagocytosable helminths such as S. mansoni schistosomula is initiated at extracellular sites of intimate contact between the PMN plasma membrane and parasite surface (39, 40). PMN-mediated host defense against schistosome ova represents, however, an unusual and little-studied model of the effector function of leukocytes. The metabolically active and antigen-producing miracidium is enclosed within a hard nonliving shell and does not migrate through or directly interact with the surrounding host environment. Despite these qualities, schistosome eggs are actively eliminated by the host in a process that is at least in part effected by eosinophils (7, 8). In the present in vitro study, electron microscopic analysis of human PMN interaction with eggs showed that both neutrophils and eosinophils attach to the parasite shell, interdigitate closely with the protruding micropiscules, and eventually cause its fracture. Cellular projections are subsequently extended inward toward the miracidium within the shell. Damage to ova thus may occur in a two-step sequence: the first involves intimate contact between the PMN and egg shell leading to its physical interruption and the second is associated with approximation of PMN pseudopodia to the miracidium. Ultrastructural analysis of egg granulomas in the livers of mice with S. mansoni infection indicate that a similar process may occur in vivo (12, 41). In these studies, partially disintegrated eggs and shell remnants were observed in the phagosomes of adjacent cells.

PMN interaction with eggs and serum containing antiparasite antibodies or complement resulted in metabolic activation of these cells with release of O₂ and H₂O₂. Oxidants were primarily produced by cells attached to ova, as adherent PMN generated ~20 times more O₂ than PMN not associated with eggs. In addition, supernatants of eggs preincubated with fnHS or hiHIS failed to stimulate PMN oxidant production. These data suggest that activation of the respiratory burst by S. mansoni ova is dependent on direct contact between PMN and ligand-coated parasites. Fluid-phase immune complexes or activated complement components that may be generated in mixtures of eggs and sera or present in the sera of infected subjects do not appear to be sufficient to stimulate PMN oxidant production (42, 43).
Further evaluation of PMN respiratory burst activity induced by eggs showed that O₂⁻ release persisted for at least 30 min after cells and parasites were mixed. Addition of the soluble activator PMA led to the generation of greater amounts of O₂⁻. This pattern of oxidant production, characterized by the release of submaximal amounts of reduced oxygen products over a relatively long period of time, has also been noted for PMN-schistosomula interaction and may be peculiar to granulocyte interaction with multicellular helminths (3, 4). In this situation, multiple PMN attack to the surface of the nonphagocytosable helminth and attack discrete and small areas of the organism's surface, unlike bacteria and protozoa, which are sequestered rapidly into phagocytic vacuoles. In the case of the S. mansoni ovum, the metabolically active miracidium lies beneath the egg shell and is physically separated from the attached PMN. Quantities of effector molecules sufficient to damage the miracidium might only be achieved if the PMN generates and releases these molecules at a moderate rate over a long period of time. If large amounts of oxygen radicals or other microbicidal substances were rapidly produced, burst activity might be terminated before the cell damages its helminthic target (44). Furthermore, the viability of neighboring adherent PMN or other host tissues might be affected by exposure to high oxidant fluxes (45).

To assess the importance of oxygen-derived molecules as effectors of leukocyte-mediated host defense against ovum, the ability of O₂⁻ and H₂O₂ degradative enzymes to inhibit egg damage were examined. SOD, an enzyme that degrades O₂⁻, inhibited the deleterious effects of PMN on egg TCA metabolism and hatching. In contrast, catalase, an enzyme that degrades H₂O₂, did not affect these PMN-induced alterations in egg function. This difference in the abilities of SOD and catalase to inhibit leukocyte-mediated parasite damage may be related to the unusual anatomic relationship that pertains to PMN-egg interaction. When PMN attach to the ligand-coated egg shell, the cells presumably release myeloperoxidase/eosinophil peroxidase as well as generate O₂⁻ and H₂O₂. Whereas low molecular weight-reduced oxygen products may pass readily through the porous egg shell, large molecules such as myeloperoxidase or eosinophil peroxidase are unlikely to reach the miracidium at a rapid rate. These peroxidases may catalyze PMN-derived H₂O₂ at the egg shell or in other areas sufficiently far from the target miracidium to prevent peroxide toxicity. Superoxide, on the other hand, may escape degradation and proceed past the egg shell to reach the metabolically active miracidium, where it or more toxic oxygen radicals may damage this portion of the ovum. The observation that the rate of detectable extracellular accumulation of H₂O₂ is relatively lower than that of O₂⁻ (0.15 vs. 0.52 nmol/min per 2 × 10⁶ PMN in the presence of fIHS) is consistent with peroxidase-catalyzed degradation of H₂O₂. It was also noted that azide, cyanide, and methimazole increased the level of PMN-mediated damage to egg TCA cycle activity by 26 to 87%, suggesting that cellular-derived H₂O₂ in the absence of these peroxidase inhibitors is catalyzed before reaching the target miracidium.

The capacity of various oxygen products to damage schistosome ova was confirmed in studies employing cell-free oxidant-generating systems. Egg TCA cycle activity was significantly reduced by ≥10 μM H₂O₂. Acetaldehyde-xanthine oxidase, which generated H₂O₂, O₂⁻, and OH⁻, also decreased the level of activity of this metabolic pathway in eggs (32). Addition of SOD, which degrades O₂⁻, partially inhibited the toxicity of this oxidant-generating system whereas catalase, which degrades H₂O₂, completely prevented egg damage. These results are consistent with a major role for H₂O₂ and/or derivatives of this molecule as mediators of egg damage. In the case of PMN, where large amounts of cellular-generated H₂O₂ may be degraded before reaching the miracidium, O₂⁻ released from PMN may be converted to oxygen radicals with greater microbicidal activity via intraparastic dismutation.
and/or iron-dependent Haber–Weiss reactions (46–49). The failure of OH· and O2 scavengers such as mannitol, benzoate, and histidine to protect eggs against damage in cell-free oxidant-generating systems does not exclude a role for these reduced oxygen products, as the accessibility of the scavengers to sites within the ovum is not known.

Egg TCA cycle activity, hatching, and ability to induce granulomas in vivo were used to assess parasite damage in the present study. Conversion of [2-14C]acetate to 14CO2 was noted to be more susceptible to inhibition by artificially generated oxygen products than the latter two activities of the egg. These differences in the susceptibility of various egg functions to the deleterious effects of reduced oxygen products may be related to several factors. First, slight reduction in the level of egg acetate metabolism may be more readily detected and quantifiable than changes in hatching or granuloma formation. Second, TCA cycle activity represents a well-defined biochemical pathway that may be altered by damage to one or a few key enzymes. Hatching, on the other hand, is the culmination of multiple biologic processes. Severe damage to several of these processes may be needed to be manifest as a decrease in the release of miracidia. Finally, granuloma formation is the result of a complex series of host cellular reactions to antigens released from eggs. Reduction in size of these pathologic lesions may be dependent on nonoxidative mechanisms of egg injury that are not present in cell-free oxidant-generating systems.

The susceptibility of eggs to damage mediated by oxygen-derived molecules was assessed by measuring the levels of several antioxidant scavenger enzymes. Ova contained only a moderate amount of SOD (0.74 U/mg of protein). The possible importance of intraparasitic peroxidases in determining oxidant sensitivity was examined by determining the levels of endogenous catalase and GPO. Whereas catalase was not detected in egg extracts and live eggs failed to degrade H2O2, S. mansoni ova contained a high level of GPO activity (8.6 U/mg). An important functional role for GPO compared with catalase as a biologic defense mechanism has been noted in other studies of oxidant-mediated membrane damage. Lipid peroxidation of membranes mediated by low and physiologic levels of H2O2 was inhibited by GPO but not by catalase (50), a finding consistent with the observation that the Km of GPO for H2O2 is significantly lower than the value for catalase (51, 52). Further elucidation of the role of these mechanisms in egg damage induced by oxygen-derived molecules will require characterization of S. mansoni egg GPO, assessment of parasite glutathione metabolism, and measurements of intraparasitic H2O2 and OH·.

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References


