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 O_2^- (0.30–0.52 nmol/min per 2×10^6 cells) and accumulation of H_2O_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 μg/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 H_2O_2 released from cells was degraded before reaching the target miracidium. Oxidants generated by acetaldehyde (2 mM)–xanthine oxidase (10 mU/ml) 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. mansoni* eggs is dependent on release of O_2^- or related molecules. These oxygen products, unlike H_2O_2, may readily reach the target miracidium where they may be converted to H_2O_2 or other microbicidal effector molecules.

Introduction

Attachment of granulocytes (PMN)[1] to ligand-coated noningestible 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 *Schistosoma 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. 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. 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; fIHS, fresh immune human serum; fNHS, fresh normal human serum; GPO, glutathione peroxidase; hiIHS, heat-inactivated immune human serum; hiNHS, heat-inactivated normal human serum; PMA, phorbol myristate acetate; PMN, granulocytes; SOD, superoxide dismutase; TCA, tricarboxylic acid.

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with extracellular release of O_2^- 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-xanthine oxidase also reduce egg tricarboxylic acid cycle activity and hatching; SOD and catalase inhibit the deleterious effects of acetaldehyde-xanthine oxidase on eggs. Inhibitors of myeloperoxidase (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^-). 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* 8 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 Laboratories, Grand Island, NY) containing 200 U/ml of penicillin, 200 μg/ml of streptomycin, 25 mM Hepes buffer (Sigma Chemical Co.), and 2 mM L-glutamine (KC Biologicals) (complete medium) and suspended to the desired concentration in complete medium or Hanks' 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 5×10^3 per 4 ml of complete medium and assays of [2-^{14}C]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 (2×10^3) 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 cacodylate 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^- and H_2O_2 release from ova-stimulated granulocytes. Triplicate preparations of 2×10^6 PMN were mixed with 2×10^3 *S. mansoni* eggs in round-bottomed borosilicate tubes (T1285-3, American Scientific Products Div., American Hospital Supply Corp., McGaw Park, IL) or 5-ml polypropylene 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^- release was calculated from this value and expressed as nmol O_2^-/min per 2×10^6 cells, unless otherwise indicated. To determine the efficiency of various concentrations of cytochrome *c* in measuring O_2^- 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^- release to ascertain whether a sufficient amount of cytochrome was included in the original mixture to scavenge all the O_2^- 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^- production to determine the capacity of cells to respond to further stimulation of O_2^- production. This value was compared with O_2^- production by a similar number of cells not mixed with eggs.

To assess whether the detected O_2^- was released from PMN adherent to eggs and/or from cells remaining unattached, PMN with eggs attached were separated from nonadherent PMN. PMN (10^7)-egg (5×10^3) 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^- 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^- by PMN, 5×10^3 ova and 2% 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 Aminco-Bowman spectrofluorometer (American Instrument Co., Travenol Laboratories, Silver Spring, MD). Cells (2×10^6) and eggs (2×10^3) were incubated in 1 ml of HBSS with 2% serum (same sources used for the O_2^- 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 H_2O_2 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 granulomas in vivo. An index of TCA cycle activity was obtained by determining the amount of $^{14}CO_2$ generated from [2-^{14}C]acetate (17, 26, 27). In studies of PMN-induced alterations in egg metabolism, triplicate samples of eggs (5×10^3), PMN (5×10^6), and PMN-egg mixtures (all containing 2% hiIHS) were incubated in 10-ml Erlenmeyer flasks containing 4 ml of complete medium to which 2 μCi of [2-^{14}C]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 electrofocusing paper (LKB Produkter, Uppsala, Sweden) 60 mm^2. After 24 h of incubation at 37°C, 0.1 ml of NCS tissue solubilizer (Amersham 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 scintillant; Amersham Corp.). Samples were left overnight before counting the evolved $^{14}CO_2$ 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 $^{14}CO_2$ generation = [cpm (cells) + cpm (eggs) − cpm (cells + eggs)]/[cpm (cells) + cpm (eggs)] × 100. Three preparations of catalase (5,000 U/ml) (Sigma Chemical Corp.; 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 some experiments to evaluate their effects on PMN-induced alterations of egg [2-^{14}C]acetate metabolism. The enzymatic activity of each catalase preparation was determined by its ability to degrade H_2O_2 (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* × 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 H_2O_2 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 CF1 mice. At 8 d, animals were sacrificed, and their lungs were removed and processed for determining granuloma area with a πMc particle measurement computer (Millipore Corp., Bedford, MA) (30).

Effects of oxidant-generating systems and scavengers on egg viability. The effects of H_2O_2 on egg TCA cycle activity and hatching were determined by addition of reagent H_2O_2 (Fisher Scientific Co., Fairlawn, NJ). The concentration of H_2O_2 was measured with a spectrophotometer with $\epsilon_{230} = 81\ M^{-1}\ cm^{-1}$ (31). To assess the toxicity of H_2O_2, O_2^-, $OH^\cdot$, and possibly $^1O_2$, acetaldehyde (2.0 mM) (Eastman Kodak Co., Rochester, NY) and xanthine oxidase (10 mU/ml) (type II, Sigma Chemical Co.) were incubated with 5×10^3 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 O_2^- 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. H_2O_2 and O_2^- were destroyed by the addition of catalase (5,000 U/ml) and SOD (10 μg/ml), respectively. The parasite cytotoxic effects of $OH^\cdot$ and $^1O_2$ were investigated by addition of diazobicyclooctane (Aldrich Chemical Co., Milwaukee, WI), mannitol, sodium benzoate, or histidine (all from Sigma Chemical Co.) (33, 34).

Determination of SOD, catalase, and glutathione peroxidase activities in S. mansoni eggs. Ova were isolated as described above, suspended in 0.1 M phosphate-buffered saline (pH 7) containing 0.1% Triton X-100 (Sigma Chemical Co.), and homogenized in an ice bath with a motor-driven Ten-Broeck apparatus for 10 min. The homogenized suspension was cleared of particulate debris by centrifugation at 20,000 *g* for 30 min and the protein concentration of the supernatant was measured (35). SOD activity was determined by inhibition of ferricytochrome *c* reduction by acetaldehyde-xanthine oxidase with bovine SOD (Sigma Chemical Co.) as standard (36). Catalase was measured by the decrease in the absorbance of reagent H_2O_2 (Fisher Scientific Co.) at 230 nm in a spectrophotometer (DU-8; Beckman Instruments, Inc.) (28). Bovine catalase (Sigma Chemical Co.) was utilized as a standard. Activity of glutathione peroxidase (GPO) was measured by a modification of method of Hopkins and Tudhope (37); 2.2 mM *t*-butylperoxide (MCB Reagents, E. Merck, Darmstadt, Federal Republic of Germany) was substituted for H_2O_2. GPO activity was measured as a decrease in absorbance, A_{340}, for NADPH with ϵ_{340} of 6.22 $M^{-1}\ cm^{-1}$. One unit of GPO activity was defined as the amount that causes the oxidation of 1 μmol of reduced glutathione per minute at 25°C.

H_2O_2 degradation by live intact eggs was also assessed. Ova (10^3, 10^4, or 2×10^4) were suspended in clear HBSS containing 0.5 or 1 mM H_2O_2 and incubated at 37°C for 30 min. The parasite eggs were then removed by centrifugation (300 *g* for 10 min) and the absorbance of the H_2O_2 solution at 230 nm was measured (28). The values obtained before and after incubation with ova were compared. In addition, control H_2O_2 solutions not containing eggs were also set up and their absorbances at 230 nm were determined at the beginning and end of the 30-min incubation period.

Statistics. Student's *t* test was used for assessing the significance of difference between observed means.

Results

Ultrastructure of PMN-egg interaction. A low-power (× 2,600) electron micrograph of an egg incubated for 30 min with hiIHS or hiNHS and no PMN is shown in Fig. 1 *A*. The egg shell consists of dense material with multiple spicules on its outer surface and pores through its thickness. The vitelline membrane and miracidium are located on the internal aspect of the shell. When PMN were added, multiple sites of cell attachment to the eggs were observed with hiIHS; rarely did cells adhere to eggs when hiNHS was used. Cell-parasite contact occurred within 30 min. The surfaces of PMN were closely applied to the egg shell and surrounded the multiple spicules (Fig. 1 *B*). By 4 h of incubation, complete fracture of the egg shell was observed in some sections (Fig. 2). Eosinophils and neutrophils developed projections that extended through the shell toward the miracidium (Fig. 3). Portions of the egg shell fragments appear to have been ingested by the cells (Fig. 3, inset).

Egg-induced release of O_2^- and H_2O_2 from granulocytes. PMN (2×10^6) mixed with eggs (2×10^3) and 2% hiNHS released 0.08 nmol O_2^-/min per 2×10^6 cells. There were no significant differences when PMN and eggs were mixed in glass or polypropylene tubes. When fNHS, hiIHS, or fIHS were substituted for hiNHS, the rate of SOD-inhibitable ferricytochrome *c* (1.2 mg/ml) reduction (measured immediately after addition of eggs to cells) increased respectively by 275 ($P < 0.005$), 188 ($P < 0.0025$), and 550 percent ($P < 0.0025$

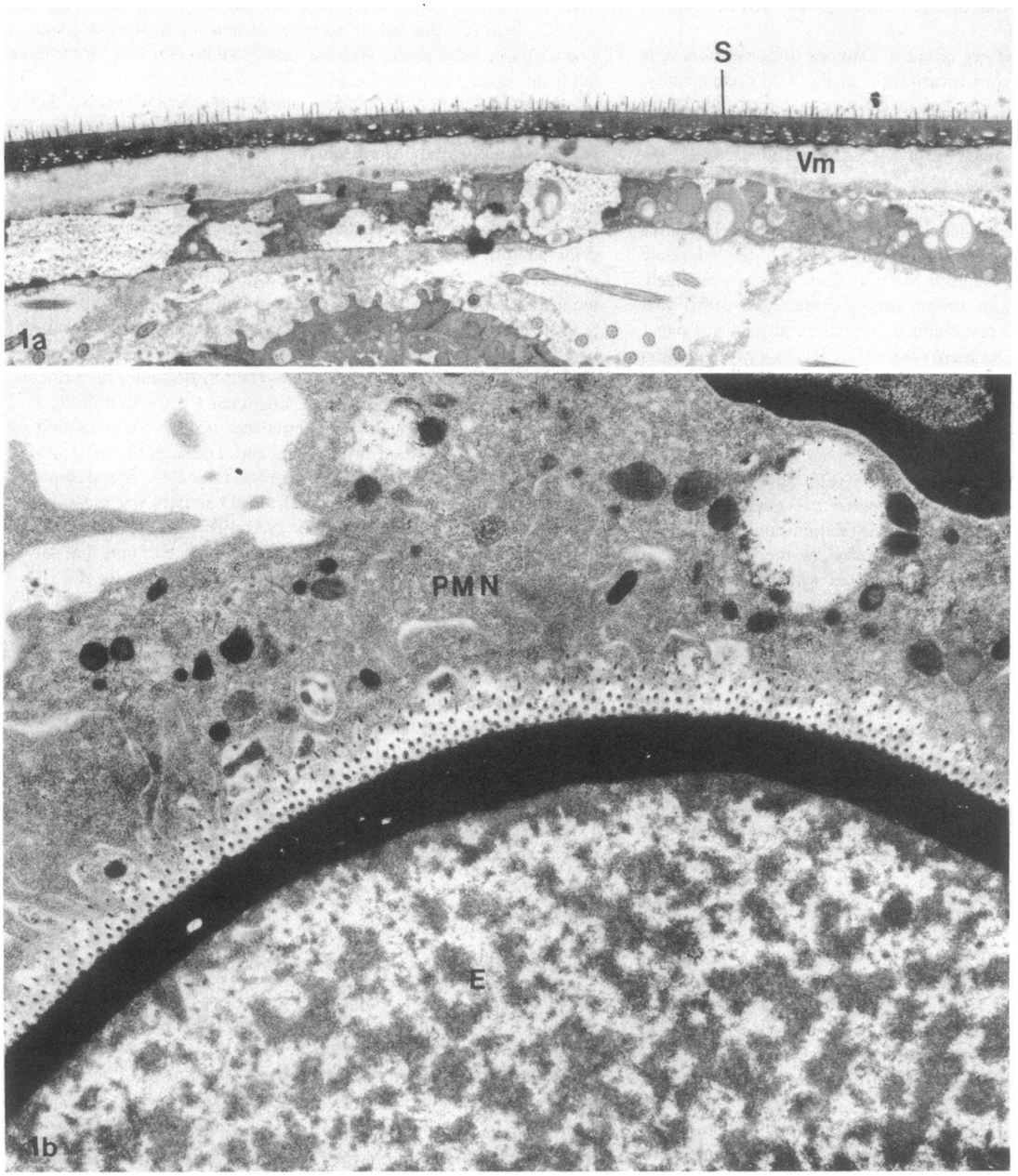

Figure 1. (*A*) *Schistosoma mansoni* egg incubated in 2% heat-inactivated immune serum for 30 min. The egg shell has multiple spicules on its outer surface and covers the underlying vitelline membrane. The multicellular miracidium is situated below the vitelline membrane (× 2,600). (*B*) Granulocyte-egg 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 (× 2,600). E, egg; S, egg shell; Vm, vitelline membrane.

compared 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

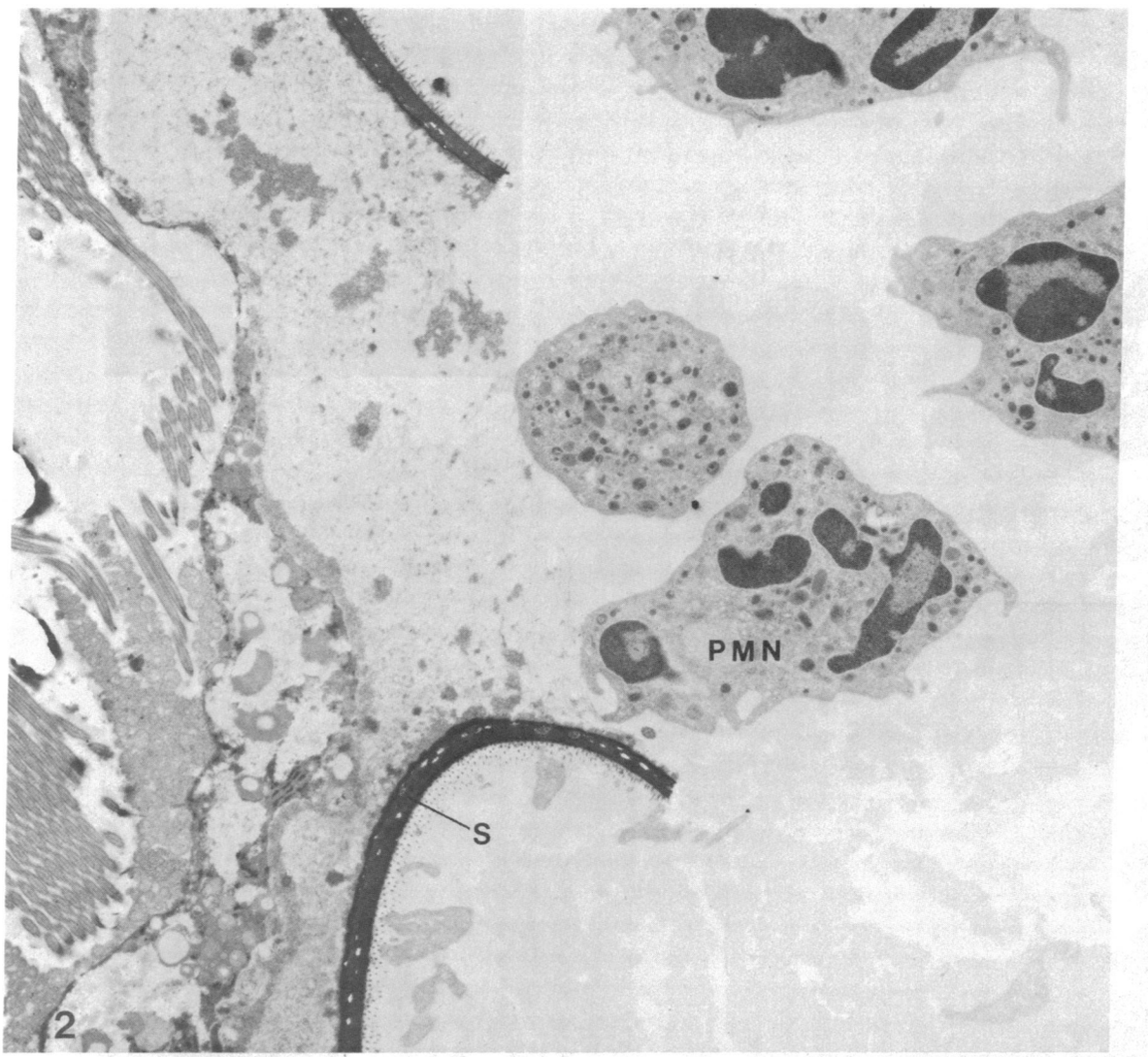

Figure 2. Granulocyte-egg interaction in the presence of 2% heat-inactivated immune serum, 4 h of incubation. The egg shell is fractured and granulocytes (*PMN*) are attached to the underlying structures (× 2,600).

to eggs released a total of 0.84, 2.84, and 3.44 nmol O_2^- per 2 × 10^6 cells after 10, 30, and 60 min, respectively. (These values are given as the total amount of detectable O_2^- 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, nonadherent PMN released a total of <0.06, 0.07, and 0.08 nmol O_2^-. There were 9.6 × 10^6 cells in the nonadherent population; the remaining cells (0.4 × 10^6) were assumed to be adherent to the parasite ova. When PMA was added to adherent PMN, O_2^- 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 O_2^- production (<0.06 nmol/min per 2 × 10^6). 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 H_2O_2 by PMN was also observed. The rate of H_2O_2 release for PMN (2 × 10^6) preincubated for 10 min with eggs (10^3) and 2% hiNHS or 2% hiIHS was 0.03±0.01 nmol/min per 2 × 10^6 cells (mean±SE of four experiments). When fNHS or fIHS were utilized, H_2O_2 accumulation increased, respectively, to 0.14±0.03 and 0.15±0.01 nmol/min ($P < 0.05$ and <0.005). H_2O_2 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, $^{14}CO_2$ generated in cell-ova mixtures was found to be primarily related to egg and not PMN metabolism of labeled acetate. PMN (5 × 10^6) incubated alone or with dead (freeze-thawed) eggs and 2% fIHS generated 3,000–10,000 cpm $^{14}CO_2$ (range of five experiments) compared with 15,000–55,000 cpm for mixtures containing live eggs (range of 12 experiments). Generation of $^{14}CO_2$ from [2-^{14}C]acetate by eggs was associated with metabolism of acetate by the miracidium and not the egg shell. The values for $^{14}CO_2$ accumulation by "intact" eggs, freshly released miracidia, and "hatched" eggs (a mixture that contained 20%

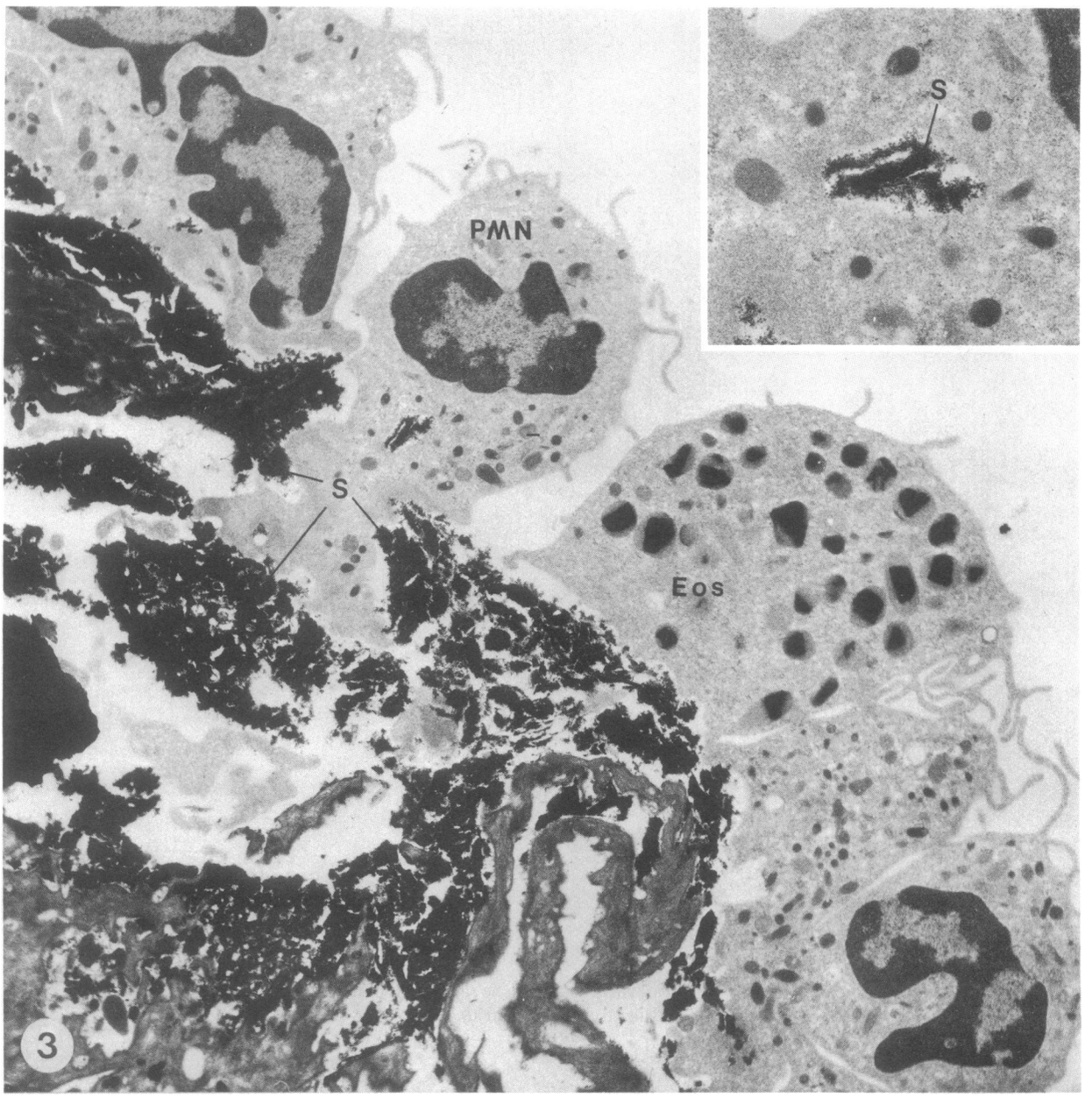

Figure 3. A granulocyte (*PMN*) and eosinophil are attached to an egg whose shell has been disrupted in multiple areas. PMN has a cellular projection that extends inward toward the miracidium (× 2,600). (*Inset*) Fragment of egg shell that has been ingested by a PMN (× 8,300). Eos, eosinophil; S, egg 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% hiIHS led to 28.3±0.9% reduction in predicted $^{14}CO_2$ generation from [2-^{14}C]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 H_2O_2 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 $^{14}CO_2$ generated from [2-^{14}C]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

*Table I. PMN O_2^- Release Induced by S. mansoni Eggs: Relation of Duration of Incubation to Level of O_2^- Production**

Time of preincubation before measurement of O_2^- release	Rate of PMN O_2^- release according to source of serum added‡			
	hiNHS	fNHS	hiIHS	fIHS
min	*nmol/min per 2 × 10⁶ cells*	*nmol/min per 2 × 10⁶ cells*	*nmol/min per 2 × 10⁶ cells*	*nmol/min per 2 × 10⁶ cells*
0	0.08±0.01	0.30±0.01	0.23±0.02	0.52±0.10
5	0.06±0.02	0.32±0.04	0.22±0.03	0.43±0.06
10	0.08±0.02	0.34±0.04	0.18±0.03	0.27±0.02
30	0.07±0.01	0.21±0.04	0.20±0.02	0.21±0.02

* PMN (2×10^6) and *S. mansoni* eggs (2×10^3) 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.

controls without peroxidase inhibitors. Similar enhancement of PMN-mediated toxicity was observed in experiment 2 (Table III).

Changes in egg activities induced by H_2O_2 and oxidants generated by acetaldehyde-xanthine oxidase. Exposure of eggs to 10 μM, 100 μM, and 1 mM H_2O_2 reduced egg generation of $^{14}CO_2$ from [2-^{14}C]acetate by 24.8–81.2% compared with control ova (Table IV); hatching was not inhibited by 10 or 100 μM H_2O_2 but was decreased by 70.5±6.1% in the presence of 1 mM H_2O_2 ($P < 0.0025$ vs. control, Table IV). Acetaldehyde (2 mM) or xanthine oxidase (10 mU/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 II. Effects of Heparin and Antioxidant Scavenger Enzymes on PMN-induced Alterations in S. mansoni Egg Acetate Metabolism and Hatching

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

* PMN (5×10^6) and eggs (5×10^3) were incubated together with 2% hiIHS 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% hiIHS (17, 27). Results represent the mean±SE of five experiments. ‡ PMN (5×10^6) and eggs (5×10^3) were incubated together with 2% hiIHS 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% hiIHS 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**

Peroxidase inhibitor added	Contents of culture vessels	Amount of $^{14}CO_2$ generated from [2-^{14}C]acetate	
		Experiment 1	Experiment 2
		cpm	*cpm*
None (control)	PMN alone	10,738‡	17,614‡
	Eggs alone	11,607	16,500
	PMN + eggs	14,493	23,600
	(% reduction in cpm induced by PMN§)	(35%)	(31%)
Azide (1 mM)	PMN alone	6,514	15,832
	Eggs alone	13,463	22,005
	PMN + eggs	11,288	15,820
	(% reduction in cpm induced by PMN§)	(44%)	(58%)
Cyanide (1 mM)	PMN alone	8,024	13,305
	Eggs alone	8,889	13,613
	PMN + eggs	8,778	13,710
	(% reduction in cpm induced by PMN§)	(48%)	(49%)
Methimazole (1 mM)	PMN alone	9,945	19,801
	Eggs alone	13,972	18,005
	PMN + eggs	13,062	17,400
	(% reduction in cpm induced by PMN§)	(45%)	(54%)

* PMN (2×10^6) and eggs (2×10^3) were incubated together or separately with 2% hiIHS and the amount of $^{14}CO_2$ generated from [2-^{14}C]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)] × 100.

^{14}C]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 μm^2 (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 H_2O_2 resulted in a mean granuloma area of 7,236±610 μm^2 ($P < 0.001$ compared with controls). Exposure of eggs to 100 μM or 10 μM H_2O_2 had no significant effect on granuloma area. The mean granuloma area induced by eggs preincubated with 2 mM acetaldehyde and 10 mU xanthine oxidase/ml was 24,600±2,780 μm^2 (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 μm^2 (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 H_2O_2. The A_{230} of 0.5 and 1.0 mM solutions of H_2O_2 were 0.0412 and 0.0830, respectively, before and after incubation for 30 min with 10^3, 10^4, or 2×10^4 freshly isolated live *S. mansoni* eggs. Control solutions of 0.5 and 1.0 mM H_2O_2 not exposed to parasite ova had A_{230} 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 ($1-5 \times 10^3$) 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 microspicules, 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 anti-parasite antibodies or complement resulted in metabolic activation of these cells with release of O_2^- and H_2O_2. Oxidants were primarily produced by cells attached to ova, as adherent PMN generated ~20 times more O_2^- than PMN not associated with eggs. In addition, supernatants of eggs preincubated with fNHS or hiIHS 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).

Table IV. Effects of Oxidants Generated in Cell-free Systems on Apparent TCA Cycle Activity and Hatching of S. mansoni Eggs

Oxidants/scavengers added to 5×10^3 eggs	Percent reduction of $^{14}CO_2$ generation from [2-^{14}C]acetate*	*P* value	Percent reduction in hatching*	*P* value
None (control)	0	—	0	—
H_2O_2 (10 μM)‡	24.8±4.5	<0.005 vs. control	8.5±8.5‡	>0.10 vs. control
H_2O_2 (100 μM)	28.0±13.0	<0.09 vs. control	12.0±8.5‡	>0.10 vs. control
H_2O_2 (1 mM)	81.2±4.3	<0.0005 vs. control	70.5±6.1‡	<0.0025 vs. control
AC (2 mM)‡	2.1±1.8	—	13.7±1.7‡	—
XO (10 mU/ml)	3.5±1.7	—	0‡	—
AC-XO	62.0±9.0	<0.0025 vs. AC or XO alone	38.7±7.3‡	<0.05 vs. AC or XO alone
AC-XO + SOD (10 μg/ml)	26.5±4.2	<0.005 vs. AC-XO	0.7±0.7‡	<0.0025 vs. AC-XO
AC-XO + catalase (5,000 U/ml)	0	—	0	—
AC-XO + mannitol (10 mM)§	60.0	—	40.2§	—
AC-XO + benzoate (10 mM)	58.7	—	39.8§	—
AC-XO + histidine (10 mM)	64.5	—	34.8§	—

Abbreviations used in this table: AC, acetaldehyde; SOD, superoxide dismutase; XO, xanthine oxidase. * 5×10^3 eggs were incubated in 2% vol/vol hiNHS for 18 h and conversion of [2-^{14}C]acetate to $^{14}CO_2$ and hatching measured as described in Methods (17, 26, 27). ‡ Results represent mean±SE of three separate experiments for studies with H_2O_2 and with acetaldehyde-xanthine oxidase. § Results represent mean of two experiments with mannitol, benzoate, or histidine.

Further evaluation of PMN respiratory burst activity induced by eggs showed that O_2^- 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 O_2^-. 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 attach 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 ova, the ability of O_2^- and H_2O_2 degradative enzymes to inhibit egg damage were examined. SOD, an enzyme that degrades O_2^-, inhibited the deleterious effects of PMN on egg TCA metabolism and hatching. In contrast, catalase, an enzyme that degrades H_2O_2, 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 O_2^- and H_2O_2. 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 catabolize PMN-derived H_2O_2 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 H_2O_2 is relatively lower than that O_2^- (0.15 vs. 0.52 nmol/min per 2×10^6 PMN in the presence of fIHS) is consistent with peroxidase-catalyzed degradation of H_2O_2. 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 H_2O_2 in the absence of these peroxidase inhibitors is catabolized 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 H_2O_2. Acetaldehyde-xanthine oxidase, which generates H_2O_2, O_2^-, and $OH^\cdot$, also decreased the level of activity of this metabolic pathway in eggs (32). Addition of SOD, which degrades O_2^-, partially inhibited the toxicity of this oxidant-generating system whereas catalase, which degrades H_2O_2, completely prevented egg damage. These results are consistent with a major role for H_2O_2 and/or derivatives of this molecule as mediators of egg damage. In the case of PMN, where large amounts of cellular-generated H_2O_2 may be degraded before reaching the miracidium, O_2^- released from PMN may be converted to oxygen radicals with greater microbicidal activity via intraparasitic dismutation

and/or iron-dependent Haber–Weiss reactions (46–49). The failure of $OH^{\cdot}$ and $^1O_2$ 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-^{14}C]acetate to $^{14}CO_2$ 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 eggs 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 H_2O_2, *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 H_2O_2 was inhibited by GPO but not by catalase (50), a finding consistent with the observation that the K_m of GPO for H_2O_2 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 H_2O_2 and $OH^{\cdot}$.

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