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Research Article

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Host Granulomatous Response in Schistosomiasis Mansoni

Antibody and Cell-mediated Damage of Parasite Eggs In Vitro

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Abstract. In chronic schistosomiasis mansoni the major pathologic lesions are granulomas surrounding eggs deposited in host tissues. Parasite ova release antigenic material that sensitize the host, resulting in the development of delayed-type hypersensitivity granulomas. The objectives of the present study were to assess the ability of components of the host granulomatous response to induce biochemical and biologic alterations in eggs in vitro, and to correlate these with the capacity of ova to induce granulomas in vivo. An assay of egg tricarboxylic acid cycle activity was developed by use of 2-[^{14}C]acetate as substrate and measurement of accumulation of released $^{14}CO_2$. Addition of human granulocytes (96% neutrophils, 4% eosinophils) to eggs (cell/egg ratio 1,000:1) and heat-inactivated normal human serum reduced predicted egg $^{14}CO_2$ generation by 15.6±3.0%. This effect was greater in the presence of sera of subjects with schistosomiasis (25.6±2.8% reduction) or when complement was present (24.4±4.0%). Autologous eosinophils and neutrophils were equally effective in decreasing egg 2-[^{14}C]acetate metabolism (25.6 and 21.4% reductions, respectively). Since the biological role of schistosome eggs relates to their ability to hatch and produce miracidia, we evaluated the effect of granulocytes and sera on this function. The hatching rate of eggs incubated with normal serum was 52.8±3.3 miracidia/100 eggs; this value decreased to 37.0±2.6 when granulocytes were added ($P < 0.01$). Granulocytes plus antibody- or complement-containing sera led to hatching rates of 23 and 20 miracidia/100 eggs. When ova were pre-incubated with granulocytes and various sera and injected into mice, the areas of egg-induced pulmonary granulomas measured 8 d later were reduced 32 to 45% as compared with lesions elicited by parasite eggs not exposed to granulocytes. Exposure of antigen-coated Sepharose beads to granulocytes and immune serum before injection into mice also led to a reduction in granuloma formation as compared with beads pre-incubated with serum alone. These data indicate that granulocytes in conjunction with antibodies and complement inflict biologically relevant toxic effects on eggs that are manifest in vivo by a decreased ability to elicit granulomas.

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Introduction

In chronic schistosomiasis mansoni, the major pathological lesions consist of granulomas which develop around eggs deposited in the liver, intestines, and other tissues of infected hosts. The immunological basis of granuloma formation and its importance in morbid complications, such as hepatomegaly and portal hypertension have been extensively studied (1). In contrast, the role of the various cellular constituents of the host granulomatous response in determining the magnitude of tissue destruction and elimination of parasite eggs is not well understood. Eosinophils comprise >50% of granuloma cells in the early phases of their development. The remaining cellular elements include lymphocytes, macrophages, giant cells, and fibroblasts (2). Inspection of serial histopathological sections of hepatic granulomas showed that eosinophils adhere tightly to the egg surface (3). Degranulation of eosinophils around schistosome ova and invasion of the egg shell also have been observed (3). In vitro, murine eosinophils, but not neutrophils or other leukocytes, have been demonstrated to attach to schistosome eggs and produce morphological alterations in their structure (4–7). Eosinophil-mediated egg damage depended upon the presence of cytophilic antibodies (5) and was enhanced by the addition of lymphokines (6). Recent in vivo studies of murine schistosomiasis mansoni confirmed that the eosinophil

is an important effector cell in egg destruction (8). When mice infected with *Schistosoma mansoni* were depleted of eosinophils by repeated administration of anti-eosinophil serum, they were observed to have smaller granulomas with fewer eosinophils than did control animals. This was accompanied by the retention of an increased number of eggs in the livers of eosinophil-depleted mice and, consequently, more extensive tissue damage. Furthermore, in these studies we found that the absence of eosinophils in host granulomas resulted in a marked decrease in the rate of egg destruction. Further investigations of the possible deleterious effects of eosinophils and other leukocytes on parasite ova have been hampered by the multicellular nature of the target eggs, their hard shell, the lack of biologically relevant, quantifiable measures of parasite damage.

We describe here the effects of human eosinophils, neutrophils, and mononuclear cells on biochemical, parasitological, and immunological functions of *S. mansoni* eggs in vitro. Since schistosome eggs metabolize 2-[^{14}C]acetate at a rate higher than that reported for human granulocytes, we used this difference in apparent TCA cycle activity to study the metabolic effects of human effector cells on *S. mansoni* eggs in vitro. Both neutrophils and eosinophils significantly inhibited conversion of 2-[^{14}C]acetate to $^{14}CO_2$ by eggs. This metabolic alteration in schistosome ova was associated with impairment of biologically relevant egg functions, including hatching of miracidia and elicitation of pulmonary granulomas after injection into mice. Granulocyte-mediated egg damage occurred in the presence of heat-inactivated normal human serum and was enhanced by the addition of sera containing anti-egg antibodies or complement.

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 a Puerto Rican strain of *S. mansoni* (9). Freshly dissected organs were perfused with cold (4°C) 1.7% NaCl 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 (10). The partially purified eggs were then layered over 40 ml of a Percoll solution (Sigma Chemical Co.) prepared by mixing one part stock Percoll with one part 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 (11). Preparations containing ≥85% mature eggs (i.e., eggs with a fully developed miracidium) were washed three times in complete RPMI-1640 medium (Gibco Laboratories, Gibco Div., 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 Biological Inc., Lenexa, KS) and suspended to the desired concentration.

Preparation of leukocytes. Blood was drawn from normal donors (age 22 to 40 yr) with no prior exposure to *S. mansoni* into 50-ml syringes containing 10 U heparin/ml (Upjohn Co., Kalamazoo, MI). Granulocytes and mononuclear cells were obtained by density gradient centrifugation over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) followed by dextran sedimentation (Sigma Chemical Co.) and hypotonic lysis of red cells (12). Purified preparations of eosinophils and neutrophils were obtained by discontinuous metrizamide (Nyegaard Co., Oslo) gradient centrifugation (13). Cells were washed three times in Hanks' balanced salt solution (KC Biological Inc.) and suspended to the desired concentration in complete RPMI 1640 medium. Cell counts were performed with a Coulter counter (Coulter Electronics, Inc., Hialeah, FL). Cell types were determined by microscopic examination of smears stained with Wright's-Giemsa. Viability in each of the preparations was ≥95% as judged by exclusion of trypan blue.

Assessment of S. mansoni egg $^{14}CO_2$ generation from 2-[^{14}C]acetate. The apparent TCA cycle activity of *S. mansoni* eggs was measured by $^{14}CO_2$ production after incubation with 2-[^{14}C]acetate (14, 15). Triplicate samples of eggs (1.0×10^3 to 5.0×10^3) were incubated in 10 ml Erlenmeyer flasks containing 4 ml of complete medium to which 2 μCi of 2-[^{14}C]acetate (specific activity, 30 mCi/mmol; New England Nuclear, Boston, MA) was added. Aliquots of 2% heat-inactivated normal human serum (hiNHS)[1] (56°C for 1 h) were also added to each sample. The flasks were fitted with an airtight rubber stopper and center well (Kontes Glass Co., Vineland, NJ) containing a piece of electrofocusing paper 60 mm^2 in area (LKB Produkter, Uppsala, Sweden). After 12, 24, 48, and 96 h of incubation at 37°C, 0.1 ml of NCS tissue solubilizer (Amersham Corp., Arlington Heights, IL) and 1 ml of 10% TCA (Sigma Chemical Co.) were injected into the center well and main flask, respectively. After the flasks were stored for 6 h at 37°C, the electrofocusing papers were removed and placed into 2 ml of scintillant fluid (aqueous counting scintillant, Amersham Corp.). Samples were left overnight before the evolved $^{14}CO_2$ was counted in a liquid scintillation counter (Nuclear Chicago Corp., Des Plaines, IL). To evaluate the contribution of various metabolic pathways to production of $^{14}CO_2$ from 2-[^{14}C]acetate by schistosome eggs, we studied the effect of puromycin (protein synthesis inhibitor), sodium fluoride (inhibitor of glycolysis), and fluorocitric acid (irreversible inhibitor of TCA cycle) (all obtained from Sigma Chemical Co.). The metabolic inhibitors to be tested were dissolved in the appropriate solvent immediately before use and then added to the incubation flasks.

Effect of antibodies, complement, and human granulocytes on S. mansoni egg $^{14}CO_2$ generation from 2-[^{14}C]acetate. The ability of anti-schistosome antibodies and complement to alter 2-[^{14}C]acetate metabolism by eggs was evaluated by comparing the amount of $^{14}CO_2$ generated in the presence of 2% hiNHS with that generated by ova incubated with heat-inactivated immune human serum (hiIHS), fresh normal human serum (fNHS), or fresh guinea pig serum (fGPS) (Cordis Laboratories Inc., Miami, FL).

To study the effect of granulocytes on apparent TCA cycle activity of schistosoma ova, the amount of $^{14}CO_2$ generated by eggs or cells alone was compared with that of mixtures of cells and eggs (15). Cells (5.0×10^3 to 5.0×10^6) were added to 5.0×10^3 eggs in 4 ml complete medium containing 2 μCi 2-[^{14}C]acetate and 2% human serum. Sera used in these studies included hiNHS, hiIHS (obtained from 5 Kenyans and 1 Brazilian with schistosomiasis mansoni), fNHS, and fresh immune human serum (fIHS). fNHS was employed as a complement source in these experiments since it, unlike fGPS, did not reduce egg

1. *Abbreviations used in this paper:* fGPS, fresh guinea pig serum; fIHS, fresh immune human serum; fNHS, fresh normal human serum; hiIHS, heat-inactivated immune human serum; hiNHS, heat-inactivated normal human serum; SEA, soluble antigens of *S. mansoni* eggs.

acetate metabolism. The use of fNHS thus allowed assessment of the effect of granulocytes on eggs independent of damage directly mediated by serum factors (see Results). At the same time that cells and eggs were mixed, duplicate flasks containing an equal number of cells or eggs alone (with the appropriate serum) were set up. After 24 h of incubation at 37°C, TCA cycle activity was stopped by the addition of TCA and the preparations treated as described above. Results were expressed as the percentage reduction of predicted TCA cycle activity as calculated with the formula: percentage reduction in egg $^{14}CO_2$ generation = {[cpm (cells) + cpm (eggs) − cpm (cells + eggs)]/[cpm (cells) + cpm (eggs)]} × 100.

Additional control experiments were done to ascertain that granulocyte inhibition of schistosome egg acetate metabolism was due to a direct effect of the cells on the parasites rather than to scavenging of TCA cycle intermediates released by ova. Duplicate flasks containing cells (5×10^6) and eggs (5×10^3) with 2% hiIHS were set up as described above. The same number of granulocytes were also placed inside dialysis tubing (molecular weight cut-off, <10,000) (Fisher Scientific Co., Fairlawn, NJ) and incubated with or without eggs in medium containing 2% hiIHS. The percentage reduction in egg $^{14}CO_2$ generation was calculated as above, and results for flasks containing granulocytes permitted to contact eggs were compared with those for cells physically separated from ova by the dialysis membrane.

In vivo granuloma formation. Eggs incubated with human granulocytes or mononuclear cells for 24 h were washed three times in complete medium, adjusted to 2,000 ova per 0.5 ml 0.9% NaCl, and injected intravenously into CF1 mice. At 8 d, the animals were killed, and their lungs were inflated with buffered 10% formalin and processed for determining granuloma area with a πMc particle measurement computer (Millipore Corp., Bedford, MA) (16). Results are expressed as the mean area of granulomas per group of experimental animals.

Soluble antigens of *S. mansoni* eggs (SEA) (1 mg/ml phosphate-buffered saline, pH 7.0) were also prepared (17) and conjugated to Sepharose 4 B beads by the cyanogen bromide method as directed by the manufacturer (Pharmacia Fine Chemicals). The SEA-conjugated beads (10% vol/vol in RPMI) were then incubated for 24 h with 2% hiIHS in the absence or presence of 5×10^6 granulocytes. Sepharose bead suspensions were subsequently washed three times in RPMI to remove nonadherent serum factors and cells. The particles were then injected intravenously into CF1 mice and lung granuloma diameters around embolized beads determined as described above. Beads with a similar diameter in histologic sections were selected for analysis.

Hatching of miracidia from S. mansoni eggs. Eggs were incubated alone or with cells (granulocytes or mononuclear cells) at a cell/target ratio of 1,000:1 and 2% vol/vol of various sera in 4 ml complete medium as described above. At the end of the 24- to 48-h incubation period, the eggs were suspended in 5 ml spring water and exposed to light for 1 h, and the released miracidia were fixed by addition of 0.1 ml of 1% aqueous iodine solution (Fisher Scientific Co.). 200 miracidia and eggs were then counted by microscopic inspection to determine the percentage hatching. Results are expressed as the hatching rate (percent) which was calculated by the following formula: [(number of miracidia)/(number of miracidia + number of unhatched mature eggs)] × 100%.

Statistics. The *t* test was used to assess the significance of difference between observed means.

Results

Generation of $^{14}CO_2$ from 2-[^{14}C]acetate by S. mansoni eggs. The amount of $^{14}CO_2$ generated by eggs from 2-[^{14}C]acetate depended upon the number of ova and duration of incubation. Incubation of 1×10^3 or 2×10^3 eggs with 2 μCi of 2-[^{14}C]acetate and 2% hiNHS resulted in 153 and 128% increases in the amount of $^{14}CO_2$ accumulated between 12 and 24 h. When 5×10^3 eggs were incubated for 12, 24, 48, and 96 h, the mean counts per minute $^{14}CO_2$ accumulated were 31,078; 56,377; 86,893; and 100,164, respectively. The increase in $^{14}CO_2$ generated between 24 and 96 h was significant only with the higher ova numbers (Fig. 1). When 5×10^3 eggs were subjected to three cycles of freezing and thawing and incubated with 2-[^{14}C]acetate, the amount of $^{14}CO_2$ generated was from 4 to 18% of that released from an equal number of live eggs at each time interval studied (Fig. 1).

The effects of various metabolic inhibitors on apparent egg TCA cycle activity are shown in Table I. Generation of $^{14}CO_2$ from 2-[^{14}C]acetate was reduced 28 to 72% by 10 μM of 1 mM fluorocitric acid. Sodium fluoride, an inhibitor of glycolysis, reduced egg $^{14}CO_2$ generation by 14, 30, and 37% at concentrations of 10 μM, 100 μM, and 1 mM, respectively. In contrast, puromycin (1–10 μg/ml), an inhibitor of protein synthesis, had no detectable effect on $^{14}CO_2$ generation from 2-[^{14}C]acetate.

Effects of sera and granulocytes on egg $^{14}CO_2$ generation. Conversion of 2-[^{14}C]acetate to $^{14}CO_2$ by eggs varied when different serum donors were used. In the presence of 2% hiNHS obtained from six individuals, $^{14}CO_2$ generation by a single batch of eggs varied from 68,000 to 94,000 cpm (Table II). When hiIHS was used (obtained from 1 Brazilian and 5 Kenyans with *S. mansoni* infection), the mean counts per minute for $^{14}CO_2$ generation by eggs was 18% less than the mean value with hiNHS ($P < 0.01$) (Table II). Eggs incubated with pooled hiIHS generated 70,158 cpm vs. 73,290 cpm with pooled hiNHS. These values were not significantly changed by inclusion of hiGPS. Using fNHS as a complement source in cell-egg mixtures resulted in a 10 to 18% decrease in $^{14}CO_2$ generation as compared with results when autologous hiNHS

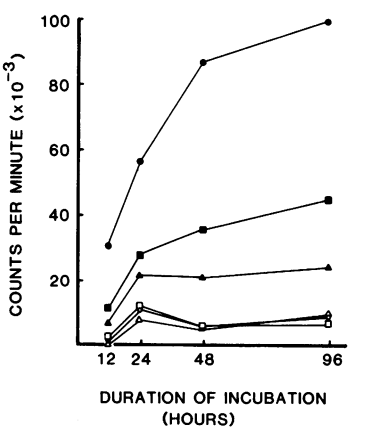

Figure 1. Relation of duration of incubation to amount of $^{14}CO_2$ generated by *S. mansoni* ova from 2-[^{14}C]acetate. Varying numbers of live or dead eggs (subjected to freezing and thawing) were incubated in RPMI with 2% hiNHS for 12–96 h, and the amount of $^{14}CO_2$ generated from 2 μCi 2-[^{14}C]acetate was determined as described in Methods. Live eggs: 1×10^3 (— ▲ —); 2×10^3 (— ■ —); 5×10^3 (— ● —). Dead eggs: 1×10^3 (— △ —); 2×10^3 (— □ —); 5×10^3 (— ○ —).

*Table I. Production of $^{14}CO_2$ from 2-[^{14}C]Acetate by Schistosoma mansoni Eggs: Effects of Fluorocitric Acid, Sodium Fluoride, and Puromycin**

Drug	Dose	cpm (*mean±SE*)‡	% Reduction cpm as compared with control
None (control)	—	33,097±2,434‡	—
Fluorocitric acid	1 mM	9,225±986	72
	100 μM	19,533±4,270	41
	10 μM	23,910±3,580	28
Sodium fluoride	1 mM	20,869±1,824	37
	100 μM	23,090±4,808	30
	10 μM	28,426±6,782	14
Puromycin	10 μg/ml	29,548±3,796	11
	5 μg/ml	27,705±3,016	16
	1 μg/ml	35,942±1,218	0

* For each variable, flasks containing 5.0×10^3 eggs in RPMI supplemented with 2% hiNHS were incubated for 24 h at 37°C with 2 μCi of 2-[^{14}C]acetate. Generation of $^{14}CO_2$ was determined as described in Methods.
‡ Results represent the mean of four separate experiments for each of these values.

in three of eight serum donors (data not shown); there were no differences when heat-inactivated and fresh sera of the other five normal donors were compared. Addition of fGPS as a complement source to hiNHS reduced the mean amount of $^{14}CO_2$ accumulated by 39% ($P < 0.01$) (Table II). 5×10^3 eggs incubated in 2% hiIHS plus 2% fGPS (antibody plus complement) generated 34% less $^{14}CO_2$ than did ova incubated in 2% hiIHS plus 2% hiGPS (41,370 vs. 63,123 cpm, $P < 0.01$) (Table II).

Addition of granulocytes to *S. mansoni* eggs reduced conversion of 2-[^{14}C]acetate to $^{14}CO_2$ independently of a direct serum effect. Since there was variability in the amount of $^{14}CO_2$ produced by granulocytes of different donors (12,000 to 50,000 cpm per 5×10^6 cells) and by different batches of eggs (40,000–90,000 cpm per 5×10^3 eggs), results of multiple experiments were normalized and expressed as a percentage reduction in $^{14}CO_2$ accumulated for eggs plus granulocytes as compared with the sum of the two components incubated separately. The possible contribution of serum factors to the reduction in $^{14}CO_2$ generation was minimized in these studies by using pooled immune or normal human serum, which resulted in similar amounts of egg acetate metabolism. Raw data from two experiments are shown in counts per minute in Table III; normalized data are described in Fig. 2. In the presence of hiNHS, granulocytes of donor 1 reduced apparent egg $^{14}CO_2$ generation by 1%; cells of donor 2 decreased egg 2-[^{14}C]acetate conversion to $^{14}CO_2$ by 12%. When hiIHS was used, the percentage reductions with cells of donor 1 and 2 increased to 16 and 22, respectively (Table III). An increase in the level of the granulocyte effect was observed in the presence of hiIHS vs. in the presence of hiNHS with cells of 13 of 15 donors (Fig. 2) (mean of 15.3% with hiNHS vs. 25.6% hiIHS, $P < 0.01$). The use of fNHS or fIHS as a serum source also increased the ability of granulocytes to reduce egg $^{14}CO_2$ generation. With fNHS or fIHS, granulocytes of donor 1 decreased egg $^{14}CO_2$ generation by 27% (41,616 vs. 57,334 cpm) and 35% (36,528 vs. 56,194), respectively. A similar effect was observed with cells of donor 2 (Table III). In experiments with cells of seven donors, the percentage reduction in predicted $^{14}CO_2$ accumulation with fNHS was 24.4±4.0 ($P < 0.05$ vs. value with granulocytes plus hiNHS). There was a 25.7±4.6% decrease in egg acetate metabolism with granulocytes and fIHS ($P < 0.02$).

The effect of various cell/target ratios on the reduction of egg $^{14}CO_2$ generation from 2-[^{14}C]acetate was subsequently examined (Fig. 3). Aliquots of 5×10^3 eggs and 2% hiIHS were incubated for 24 h with increasing numbers of granulocytes

*Table II. Effect of Various Sera on Schistosoma mansoni Egg Conversion of 2-[^{14}C]Acetate to $^{14}CO_2$**

	$^{14}CO_2$ accumulated in 24 h			
	Serum source			
Serum donor number	hiNHS	hiIHS + hiGPS	hiNHS + fGPS	hiIHS + fGPS
1	83,631	73,248	54,462	29,642
2	73,584	58,335	49,982	41,664
3	70,224	59,778	42,556	46,112
4	68,426	61,995	40,926	44,472
5	94,234	67,623	38,928	46,580
6	70,336	57,761	55,837	39,748
Mean±SE	76,739±4,147	63,123±2,495‡	47,115±2,968‡	41,370±2,578‡§

* *S. mansoni* eggs (5×10^3) were suspended in RPMI-1640 containing 2% vol/vol of serum with 2 μCi of 2-[^{14}C]acetate, and accumulated $^{14}CO_2$ was measured at 24 h (15). ‡ $P < 0.01$ vs. eggs suspended in hiNHS. § $P < 0.01$ vs. eggs in hiIHS.

*Table III. Granulocyte-mediated Reduction in Schistosoma mansoni Egg Conversion of 2-[^{14}C]Acetate to $^{14}CO_2$**

Experiment number	Serum source	Cells alone	Eggs alone	Cells + eggs	% Reduction as compared with sum predicted
		cpm	*cpm*	*cpm*	
1	hiNHS	12,178	41,782	53,505	1
	hiIHS	13,668	39,638	45,028	16
	fNHS	13,060	44,274	41,616	27
	fIHS	12,002	44,192	36,528	35
2	hiNHS	22,946	42,546	57,382	12
	hiIHS	21,376	58,626	62,032	22
	fNHS	26,176	54,502	42,983	47
	fIHS	19,488	38,172	42,890	26

* *S. mansoni* eggs (5×10^7) were suspended in RPMI containing 2% vol/vol of serum with 2-[^{14}C]acetate, and accumulated $^{14}CO_2$ was measured at 24 h (15).

obtained from six different donors. At a cell/parasite ratio of 1:1 there was a mean reduction in $^{14}CO_2$ generation of 10.0±6.0% ($P > 0.05$ vs. eggs and sera without cells). Increasing the cell/parasite ratio to 100:1 resulted in a significant reduction of $^{14}CO_2$ accumulation to a mean of 42.0±5.5% ($P < 0.01$). A further increase of the cell/egg ratio to 1,000:1 was not, however, associated with more reduction in $^{14}CO_2$ generation (Fig. 3). The effect of duration of cell/egg contact was evaluated next. Granulocytes from four donors were incubated with eggs (1,000:1 cell/egg ratio) for various intervals before the amount of $^{14}CO_2$ accumulated was determined. After 3 h of incubation, granulocytes significantly reduced egg $^{14}CO_2$ generation, by 39.4±4.9%. Increasing the time of incubation up to 48 h did not result in a greater reduction in $^{14}CO_2$ release (data not shown).

The reduction in egg $^{14}CO_2$ generation was lower when granulocyte contact with ova was prevented by placing the cells in dialysis tubing. The accumulation of $^{14}CO_2$ by 5×10^3 eggs suspended in 2% hiIHS was 67,010 cpm (mean of duplicate determinations); granulocytes (5×10^6) incubated separately generated 48,051 cpm (24-h incubation period). When cells and ova were incubated together, there was 72,744 cpm of $^{14}CO_2$ generated (37% reduction as compared with the sum of each incubated separately). Incubation of ova with the same number of granulocytes enclosed in dialysis tubing resulted in the accumulation of 98,855 cpm of $^{14}CO_2$ (14% reduction).

Comparison of the ability of eosinophils and neutrophils to reduce egg $^{14}CO_2$ generation from 2-[^{14}C]acetate. When purified suspensions of eosinophils (85–100% purity) or neutrophils (98–100% purity) were mixed with eggs at a ratio of 100:1 and 2% fIHS, no difference between the two cell types in the mean percentage reduction of predicted $^{14}CO_2$ accumulation was observed (Table IV). There was also no difference when results of experiments with neutrophils and eosinophils from the same donors were compared (Table IV). When the cell/egg ratio was decreased to 50:1, neutrophils failed to reduce predicted $^{14}CO_2$ accumulation (68,198 cpm vs. 70,150 cpm for the sum of cells and eggs incubated separately); eosinophils and eggs at a similar cell/target ratio also did not decrease

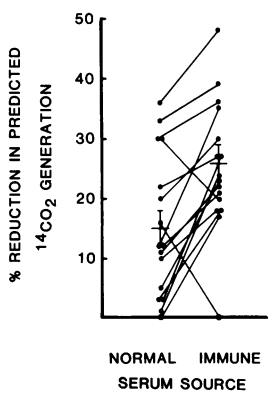

Figure 2. Granulocyte-mediated reduction in predicted $^{14}CO_2$ generation from 2-[^{14}C]acetate by *S. mansoni* eggs. Granulocytes and eggs (1,000:1 ratio) were incubated in RPMI with 2% hiNHS or 2% hiIHS for 18–24 h with 2-[^{14}C]acetate, and $^{14}CO_2$ generation was determined as described in Methods. Lines connecting dots are results of experiments with cells from the same donor performed at the same time.

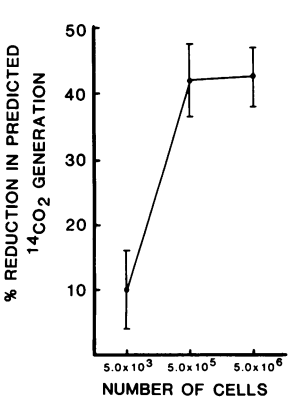

Figure 3. Effect of cell/egg ratio on amount of $^{14}CO_2$ generated from 2-[^{14}C]acetate. *S. mansoni* eggs (5×10^3) were incubated with increasing numbers of cells and 2% hiIHS for 18 h, and the amount of $^{14}CO_2$ generated from 2-[^{14}C]acetate was determined. Results are given as percentage reduction in predicted $^{14}CO_2$ generation, as described in Methods.

Table IV. Effect of Purified Neutrophils and Eosinophils on Schistosoma mansoni Egg $^{14}CO_2$ Generation from 2-[^{14}C]Acetate

	% Reduction of $^{14}CO_2$ generation from 2-[^{14}C]acetate*			
Donor	Neutrophils	% Cell purity‡	Eosinophils	% Cell purity‡
1	31	100	23	100
2	18	100	25	91§
3	15	100	27	85§
4	23	98	28	93§
5	20	100	25	95§
Mean±SE	21.4±2.7		25.6±0.9	

* Cells and eggs were incubated in triplicate at a ratio of 100:1 in the presence of 2% fIHS. Values were calculated as indicated in Methods (15).
‡ Cytocentrifuge preparations of cells were stained with Wright's-Giemsa, and differential counts were performed on a total of 500 cells.
§ The remainder of cells in these preparations were neutrophils.

*Table V. Granuloma Areas in the Lungs of CF1 Mice Injected with Schistosoma mansoni Eggs: Effects of Pre-Incubation with Granulocytes and Various Sera**

Serum source	Eggs	Eggs + granulocytes (Granuloma area [μm^2] mean±SE)	*P* value
hiNHS + hiGPS	52,738±3,290 (6)‡	29,064±4,726 (6)	<0.01
hiIHS + hiGPS	48,158±4,040 (5)	32,926±2,872 (6)	<0.02
hiNHS + fGPS	46,768±3,202 (6)	28,118±1,718 (5)	<0.001
hiIHS + fGPS	39,181±6,350 (5)	28,814±2,531 (6)	NS

* Eggs or egg-granulocyte mixtures were incubated for 24 h and injected intravenously into CF1 mice. 8 d later, the lungs were removed, and the sites of pulmonary granuloma were measured with a IIMc particle counter (21).
‡ Number of mice per group.

$^{14}CO_2$ generation (62,200 cpm for the cell-egg mixture vs. 63,290 cpm for each incubated separately). In addition, cell/target ratios of 10:1 and 1:1 did not reduce egg conversion of 2-[^{14}C]acetate to $^{14}CO_2$. Experiments assessing the effect of mononuclear cells on egg $^{14}CO_2$ generation from 2-[^{14}C]acetate were not performed, as the high level of TCA cycle activity in this cell population (>50,000 cpm per 5×10^6 cells) precluded meaningful comparison to eggs incubated alone.

In vivo granuloma formation around S. mansoni eggs and antigen-coated beads. Eggs (>85% mature) were incubated for 24 h with granulocytes and various sera, and injected intravenously into CF1 mice; 8 d later the lungs were removed for granuloma measurements. Eggs pre-incubated with hiNHS, in the absence of cells, elicited granulomas with a mean area of 52,783±3,290 μm^2 (standard error). The substitution of hiNHS by hiIHS or addition of fGPS to hiNHS or hiIHS had no significant effect on the size of pulmonary granulomas (Table V). In contrast, the addition of granulocytes (cell/target ratio 1,000:1) to eggs incubated in hiNHS and hiGPS resulted in a significant reduction (45%) of granuloma area ($P < 0.01$). Similar granulocyte-induced decreases in granuloma area were shown after incubations with serum containing antibodies (32%, $P < 0.02$) or complement (40%, $P < 0.001$) (Table V). No additive effect of granulocytes on reduction of granuloma size was seen in experiments where both antibodies and complement were included in incubations. A comparison of the effect of granulocytes and mononuclear cells on granuloma formation was subsequently performed. Eggs pre-incubated in medium containing 2% hiIHS elicited granulomas with a mean area of 30,595±1,486 μm^2. Addition of granulocytes (98% neutrophils, 2% eosinophils) or mononuclear cells (70% lymphocytes, 24% monocytes, and 5% neutrophils) decreased with the respective granuloma areas to 15,497±748 μm^2 (50% reduction, $P < 0.005$) and 19,699±1,163 μm^2 (46% reduction, $P < 0.001$).

When Sepharose beads conjugated with SEA were pre-incubated with hiIHS, washed, and subsequently injected into mice, the area of pulmonary granulomas surrounding these particles was 19,000±1,500 μm^2 (mean±SE of measurements in six animals). Pre-incubation of beads with hiIHS plus granulocytes resulted in the elicitation of smaller granulomas (mean area of 15,000±900 μm^2, $P < 0.01$).

Effect of granulocytes and mononuclear cells on hatching of S. mansoni eggs. The hatching rate after incubation of schistosome eggs in 2% hiNHS for 24 h was 52.8±3.3 miracidia/100 eggs. This value did not significantly change when the incubation period was extended to 48 h (55.0±7.6 miracidia/100 eggs). Exposure of eggs to granulocytes and 2% hiNHS (cell/egg ratio 1,000:1) reduced the hatching rate by 30% ($P < 0.01$) (24 h of incubation). Egg hatching was further decreased by granulocytes when hiIHS, fNHS, or fIHS was used instead of hiNHS (Table VI) (results of 15 experiments). Eggs incubated with each of these sera in the absence of cells released 42 to 50 miracidia/100 eggs (single experiment).

The granulocyte effect on egg hatching depended upon the cell/egg ratio. At ratios of 30:1, 60:1, and 125:1, the percentage

*Table VI. Effect of Granulocytes on Hatching of S. mansoni Eggs**

Incubation conditions	Hatching rate‡
	(miracidia released/100 eggs)
hiNHS alone	52.8±3.3
Granulocytes + hiNHS	37.0±2.6§
Granulocytes + hiIHS	23.4±3.5§
Granulocytes + fNHS	20.4±4.8§
Granulocytes + fIHS	20.0±5.4§

* Eggs (5×10^3) were incubated with or without granulocytes (5×10^6) with 2% vol/vol sera for 24 h, washed, and exposed to spring water to determine hatching, as described in Methods.
‡ Results of each experiment represent the mean±SE of 15 experiments.
§ Values are significantly less than for hiNHS alone, $P < 0.01$.

reductions in the presence of 2% hiNHS were 12, 24, and 31%. When the ratios were increased to 250:1, 500:1, and 1,000:1, these values were, respectively, 31, 28, and 41% (results of a single experiment with duplicate determinations). Incubation of eggs with mononuclear cells (cell/egg ratio 1,000:1) and 2% hiNHS decreased the hatching rate to 42.8±2.8 miracidia/100 eggs (mean±SE of five experiments; $P < 0.01$ vs. value for eggs incubated with hiNHS alone).

Discussion

The role of host leukocytes in the destruction of the invasive stages of multicellular parasites has been extensively evaluated over the past decade. Despite the large size of the microbial invaders and their failure to be ingested by effector cells, granulocytes and mononuclear cells alone or in association with antibodies and/or complement have been shown to kill migratory larvae of several helminths (18). Less attention has, however, been directed at the residing stages of these multicellular infectious agents. In schistosomiasis, adult worms parasitize the portal or vesical venous system; eggs are deposited intravascularly and attempt to move to the lumen of the gut or urinary tract. Some of these ova get trapped in host tissues such as the liver and intestines and elicit delayed-hypersensitivity granulomas made up of several cell types (2). Although there is evidence that eggs are destroyed within these granulomas (3), a satisfactory examination of the contributing factors leading to egg damage has been lacking.

The tissue-residing egg of *S. mansoni* measures ~160 × 60 μm and contains a metabolically active multicellular miracidium. Antigenic materials released in host tissues throughout the life span of the egg (~2–4 wk) are crucial in the development of granulomas, which characterize the pathology of schistosomiasis (1, 19, 20). Assessment of host defense mechanisms against such a formidable target necessitated the development of quantifiable biochemical measurements that could be confirmed by biologically relevant, parasite-related functions. In the present series of studies, we employed an assay of the apparent TCA cycle activity of schistosome ova. This was then correlated with the two major biological functions of parasite eggs, namely induction of granuloma formation and hatching.

The ability of *S. mansoni* eggs to metabolize 2-[^{14}C]acetate with subsequent production of $^{14}CO_2$ was shown to depend upon egg viability and duration of incubation, and was also a direct function of the number of parasites used. Fluorocitric acid (1 mM), an irreversible inhibitor of TCA cycle activity, reduced the generation of $^{14}CO_2$ by 72%. Significant but less inhibition was obtained when the glycolytic inhibitor sodium fluoride was added to the medium. Although eggs have previously been reported to have little glycolytic activity (14), it is known that pyruvate (a product of glycolysis) is oxidized in the formation of acetyl coenzyme A, which then reacts enzymatically with oxaloacetate to form citrate in the TCA cycle (21). Sodium fluoride could therefore have inhibited glycolysis and the formation of pyruvate, which would in turn diminish activity of the egg TCA cycle.

To assess the possible importance of humoral factors in damaging *S. mansoni* eggs, the conversions of 2-[^{14}C]acetate to $^{14}CO_2$ in the presence of various sera were compared. Antibody-containing sera of individuals with schistosomiasis mansoni (hiIHS) decreased egg acetate metabolism by 18%. When fNHS was used as a complement source, there was not a significant reduction of TCA cycle activity; fGPS, on the other hand, led to a 39% decrease ($P < 0.01$). The increased ability of fGPS vs. fNHS to inhibit conversion of 2-[^{14}C]acetate to $^{14}CO_2$ by eggs may be related to the presence of a greater amount of complement in fGPS. In addition, there may be different amounts of unlabeled acetate and/or other metabolic precursors of the TCA cycle in each sera.

Granulocytes reduced apparent TCA cycle activity independently of a direct toxic effect of serum factors. At a 1,000:1 cell/target ratio, granulocytes inhibited egg acetate metabolism by 15% in the presence of pooled hiNHS. The degree of inhibition increased when pooled hiIHS or fNHS was included (these sera did not reduce acetate metabolism when leukocytes were omitted). We further demonstrated that purified populations of eosinophils and neutrophils were equally efficient in decreasing parasite TCA cycle activity. Although neutrophil-mediated toxicity was not observed by Vadas et al. (13), several investigators have found that neutrophils as well as eosinophils damaged multicellular organisms (22–27). James and Colley showed that murine leukocytes adhered to *S. mansoni* eggs in vitro; morphologic destruction of parasites was only seen after incubation with eosinophil-rich preparations (4–7). The discrepancy between our results and those of James and Colley may be related to several factors, such as the method of detecting parasite mortality (metabolic assay vs. morphology), source of leukocytes (human vs. murine), and the purity and origin of cellular preparations (peripheral blood vs. peritoneal exudate).

Multiple in vitro studies have shown that granulocytes and other cell types can deliver products that may eventually kill *S. mansoni* (22–24, 28–30). These effector mechanisms have been related to the release of oxidants (24), basic and cationic proteins (31, 32), enzymes (33), or other undefined substances (30). Most important to our understanding of schistosomiasis, however, is the biological relevance of these in vitro assays of parasite damage. In the present study, we found that granulocyte-mediated alterations of egg TCA cycle activity were associated with an impaired ability of ova to release miracidia, the parasite stage that infects the obligatory snail vector. The in vitro model of granulocyte-egg interaction described here may thus be useful for further delineation of the subcellular basis of host effector cell-mediated damage, as it culminates in a deleterious effect on a biologically important and quantifiable egg function.

The host granulomatous response mounted around *S. mansoni* eggs is a delayed hypersensitivity reaction to soluble

antigens, which are continuously secreted by ova; depletion of antigens from eggs before exposure to the host results in the formation of smaller granulomas than when control ova are used (20, 34). In the present study, it was found that pre-incubation of eggs with human granulocytes or mononuclear cells with or without antibody- or complement-containing sera resulted in reduction of granuloma formation upon subsequent injection of the ova into mice. Diminished elicitation of these pathologic lesions appeared to be related to a deleterious effect of human granulocytes on the immunogenicity of eggs, as similar treatment of antigen-coated Sepharose beads also led to smaller granuloma size as compared with granuloma size when preparations pre-incubated without leukocytes before injection into mice were used. Corroborative evidence that alterations in egg antigen secretion are responsible for decreased granuloma formation has been provided in cell-free studies of eggs exposed to artificial oxidant generating systems. Ova pre-incubated with acetaldehyde-xanthine oxidase or hydrogen peroxide elicited significantly smaller granulomas after injection into mice than did eggs not pre-incubated in these cell-free systems (Kazura, J. W., P. de Brito, J. Rabbege, and M. Aikawa, manuscript submitted for publication).

We have demonstrated previously in vivo that the functional role of the host granulomatous reaction is the destruction of parasite eggs (8). Eosinophils and mononuclear phagocytes, the predominant cells of such lesions, were shown in the current in vitro study to inflict biologically relevant alterations in egg function. The mechanism of interference with egg metabolic and immunologic activities is currently unknown, although studies with other multicellular targets suggest that both oxidative and nonoxidative processes may be involved. How these mediators are delivered and effect damage of the viable miracidium within the hard egg shell are currently under investigation.

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