DELAYED HYPERSENSITIVITY-TYPE GRANULOMA FORMATION AND DERMAL REACTION INDUCED AND ELICITED BY A SOLUBLE FACTOR ISOLATED FROM SCHISTOSOMA MANSONI EGGS*‡

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Granulomatous inflammation plays a major role in the pathogenesis of a wide variety of diseases caused by infectious agents, including mycobacteria (tuberculosis, leprosy); fungi (coccidioidomycosis, histoplasmosis); worms (schistosomiasis, filariasis); and possibly viruses and protozoa (1, 2). Although it has long been suspected that the granuloma may be a manifestation of delayed hypersensitivity, significant data supporting this concept have been gathered only in the past 15 yr (2). As recently as 1967 a review of granulomatous hypersensitivity concluded that the evidence was still insufficient to include the granuloma in the spectrum of delayed responses (2).

Since then, the *Schistosoma mansoni* egg granuloma, a quantifiable model of granulomatous inflammation (3), has been established as a form of delayed hypersensitivity. Mice previously exposed to schistosome eggs develop larger granulomas more rapidly than control unsensitized animals (4). The anamnestic reaction is specific in relation to other worm genera (4), to the two other schistosome species infective to man (5), and even to the different life cycle stages of *S. mansoni* itself (6). Sensitization is transferrable from infected to uninfected inbred mice by lymph node and spleen cells, but not by serum (4). Finally, the granulomatous response is strongly inhibited by a series of immunosuppressive measures known to be particularly effective against delayed hypersensitivity (7-9) and is unaffected by measures directed primarily against antibody-mediated reactions (9, 10).

The schistosome egg granuloma is a local inflammatory response composed mainly of macrophages and their derivatives, lymphocytes and eosinophils (11). Immuno-fluorescent studies have shown that the parasite eggs emit specifically stainable antigens through submicroscopic pores in their shells (11, 12). These antigens are taken up by phagocytic cells clustered about the eggs (12).

The present investigation was initiated to determine the source of the antigens which elicit granuloma formation and to attempt to isolate and identify them. Using an indirect method based on the theory that antigens which elicit granuloma formation might induce sensitization, it was determined sequentially that intact dead eggs, egg homogenates, and the clear supernatant fluid after high speed centrifugation of the homogenates, were sensitizing. It was also shown that intact living eggs secrete active soluble antigens both in vitro and in vivo and that, on hatching, eggs release similar sensitizing material.

The availability of these highly active soluble materials provided an opportunity to isolate and characterize the specific antigen or antigens responsible for the induction of sensitization. In addition, the direct elicitation of granuloma formation by the soluble antigens was accomplished by adsorbing them to bentonite and injecting the particles into the pulmonary microvasculature of sensitized mice. Finally, the delayed hypersensitivity etiology of the schistosome egg granuloma and the unique nature of the soluble egg antigens were confirmed by the induction and elicitation of delayed footpad swelling by microgram quantities of the material unmixed with adjuvant, and by the failure to detect circulating antibodies with a sensitive hemagglutination test using the soluble material as antigen. In summary, soluble antigens were isolated from *Schistosoma mansoni* eggs which, in minute amounts and without the addition of adjuvants, both induced and elicited granuloma formation and delayed dermal reactions in the absence of detectable circulating antibody.

Materials and Methods

Quantitative Measurement of Granuloma Formation.—Schistosome eggs were isolated by a modification of the method of Coker and von Lichtenberg (13, 14) from the livers of female Swiss albino mice (CF 1, Carworth Farms, New City, N.Y.) exposed 8 wk previously to 150 cercariae of a Puerto Rican strain of *Schistosoma mansoni*. 1000 eggs suspended in 0.5 ml of 0.9% saline solution were injected via a tail vein into the microvasculature of the lungs of similar mice (3, 4). 8 days after egg injection, groups of mice were anesthetized, 1 ml of 10% buffered formalin was injected intratracheally into each animal, and the lungs were removed and placed in a container of the same solution. Three sections from each lung, 5 μ in thickness and at least 250 μ apart, were stained with hematoxylin and eosin and examined for the presence of eggs. The size of each egg, including the reaction around it, was determined by measuring two diameters at right angles to each other with a Cooke AEI Image-Splitting Eyepiece (Cooke, Troughton, and Simms, Inc., Walden, Mass.). The mean diameter of large numbers of such lesions was then calculated for each experimental group.

Sensitization.—Granuloma formation reaches its peak in unsensitized mice at 16 days but mice sensitized by an earlier intraperitoneal injection of eggs form larger granulomas more rapidly, peak size occurring at 8 days. The greatest difference between unsensitized and sensitized animals is therefore found at 8 days (4). Each of the sensitizing substances described below was injected intraperitoneally in a concentration equivalent to 1000 eggs (unless stated otherwise) 1 wk before the intravenous injection of eggs, and the lungs were removed 8 days after the second injection. The results were compared to those in control unsensitized animals and animals sensitized by intact living eggs, proven viable by the hatching of active motile miracidia.

Dead Eggs, Homogenates, Soluble Egg Antigens (Fig. 1-part 1).—Sensitization was attempted by the intraperitoneal injection of (a) eggs killed by heating and freeze-thawing, (b) homogenates prepared by sonication and grinding, and (c) the clear supernatant fluid resulting from high speed centrifugation of the homogenate prepared by grinding. Eggs were killed by heating in a Castle Thermatic Sixty Autoclave (Wilmot Castle Co., Rochester, N.Y.) for 15 min at 15 lb pressure (121°C) (15), or by five cycles of freezing in an alcohol dry bath and thawing under running tap water. Viable eggs were suspended in an 0.9% sodium chloride solution, placed in an ice bath and then disrupted by sonication, using a 400 w Blackstone ultrasonic probe (Blackstone Ultrasonics, Inc., Sheffield, Pa.) set at maximum vibrations. After 15 min only 0.3% of the eggs remained intact. For sensitization, this material was used both directly and mixed with equal portions of Freund's complete adjuvant (Difco 486331), (Difco, Inc., Detroit, Mich.). In addition, eggs in a concentration of 50,000 per ml of phosphate buffered saline, pH 7.4 (16), were ground in a TenBroeck glass tissue homogenizer maintained in

Living *Schistosoma mansoni* eggs
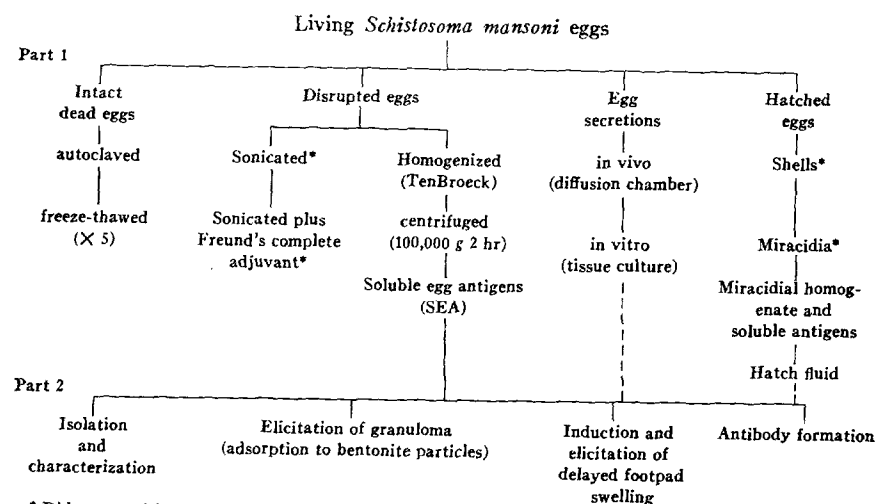

* Did not sensitize.

Fig. 1. Experimental Plan: Part 1. Flow diagram showing materials derived from *Schistosoma mansoni* eggs which were injected intraperitoneally into mice in order to sensitize them to granuloma formation around intact living eggs injected intravenously into the pulmonary microvasculature 1 wk later. Part 2. Further investigations of soluble egg antigens.

an ice bath until no intact eggs were seen. This material was centrifuged in a Beckman Model L (Beckman Instruments, Inc., Fullerton, Calif.) preparative ultracentrifuge at 4°C for 2 hr at 100,000 g. Both the homogenate and the clear supernatant fluid produced by centrifugation were used for sensitization. For storage the supernatant fluid was decanted, quick-frozen in a dry ice-ethanol bath and maintained at −30°C. The stored material was later tested in concentrations ranging from 100–25,000 egg equivalents.

Egg Secretions In Vitro and In Vivo (Fig. 1-part 1).—The sensitizing effect of secretions from living eggs was then examined. For in vitro studies, eggs obtained aseptically from the livers of infected animals were suspended to a concentration of 25,000 per ml in tissue culture medium 199 (Flow Laboratories, Inc., Rockville, Md.) to which had been added 100 units of penicillin and 100 μg each of streptomycin and kanamycin per ml of medium (final pH 7.4). 2 ml portions of this suspension were dispensed into screw-capped tubes which were filled with 5% CO_2 and 95% air, closed tightly, and put on a roller drum for 72 hr at 37°C. At the end of the incubation (readjustment of pH was not necessary) the contents of the tubes were pooled, the eggs were

removed by centrifugation at 1500 rpm for 10 min, and the clear supernatant fluid was injected intraperitoneally into mice in 0.5 ml volumes.

For in vivo studies 1000 eggs in 0.1 ml of sterile 0.9% saline solution were inserted into diffusion chambers constructed from plexiglass rings and membranes of 0.45μ pore size (Millipore Corporation, Bedford, Mass.). The chambers were moistened with saline and placed into the peritoneal cavity of anesthetized mice. 15 days later when the lungs were removed from the mice, the chambers were examined; no leakage was observed.

Egg Components-Shells, Miracidia, Hatch Fluid (Fig. 1-part 1).—Immediately following their removal from mice infected for 8 wk, livers were homogenized in a Waring Blendor and the eggs were separated by a combination of sedimentation, centrifugation and filtration. They were washed in 1.7% saline solution and suspended in spring water in a conical measuring flask. The room was darkened and a beam of light was directed at the spout; the phototropic miracidia were collected with a capillary pipette. The techniques used for homogenization of miracidia in a TenBroeck tissue grinder and the isolation of the miracidial soluble substance by centrifugation were identical with those described above for whole eggs.

The shells and remaining unhatched eggs were then centrifuged. At this point the ratio of shells to intact eggs was 1:10. Separation of shells from eggs was carried out by a modification of the sucrose density gradient method described by von Lichtenberg and Raslavicius (15). The final yield was 98.7% shells. The few intact eggs were all immature.

In order to obtain the fluid which is released by hatching, eggs were suspended in spring water (Solon Springs Bottling Co., Solon, Ohio) at a concentration of 20,000 per ml. They were maintained at room temperature (23°C) and were placed under a strong light. As a control, a similar concentration of eggs was maintained in cool 0.9% saline solution in the dark. After 40 min both solutions were passed through Millipore filters of 3μ pore size. The clear filtrate was immediately cooled in an ice bath, adjusted to 0.9% salinity by the addition of sodium chloride, and injected intraperitoneally into mice in 0.8 ml volumes.

Isolation and Characterization of Soluble Egg Antigens (Fig. 1-part 2).—After each of the following procedures the materials were tested for sensitizing activity as described above. The soluble egg antigens prepared from homogenized eggs were incubated in a water bath for 2 hr at 23°, 37°, and 56°C. In addition, SEA[1] was exposed to three different enzymes obtained from bovine pancreas: trypsin (2 $\times$ crystallized-Sigma Chemical Co., St. Louis, Mo.), ribonuclease (prepared by Sephadex chromatography-Worthington Biochemical Corporation, Freehold, N. J.) and deoxyribonuclease (1 $\times$ crystallized-Worthington). The enzymes were dissolved in phosphate buffered saline pH 7.2 (0.15 M Na_2HPO_4-286 ml, 0.15 M KH_2PO_4-90 ml, and 0.15 M NaCl-376 ml). The enzymes and SEA were mixed to a final concentration of $10 \mu g$ of enzyme and $50 \mu g$ of SEA per ml and were incubated for 30 min at 23°C.

Disc electrophoresis in 7% polyacrylamide gel at pH 9.5 was performed with a Canalco (Canal Industrial Corp., Rockville, Md.) model 12 disc electrophoresis apparatus, and preparative zone electrophoresis was carried out in a water-cooled electrophoresis cell using a matrix of powdered polyvinyl chloride (Pevikon, Mercer Chemical Corp., New York) with 0.025 M tris (hydroxymethyl) aminomethane buffer, pH 8.6 as described by Daniel and Ferguson (17).

Elicitation of Granuloma Formation with Soluble Egg Antigens Adsorbed to Bentonite Particles (Fig. 1-Part 2).—Indian Head bentonite (325 mesh) obtained from Johns-Manville (Lampoc, Calif.) was washed several times in 0.9% saline solution and sieved through a Browne capsule (from the Case Western Reserve University Medical School) containing 100 and 320 mesh filters (14). The mean diameter of the particles was 70 ± 4 μ. A suspension of 20,000 particles was incubated for 30 min at 15–16°C with 1 ml of the soluble egg antigens (18). The protein content of the solution, 600 μg per ml as determined by the Lowry method (19), was reduced

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[1] *The following abbreviations are used in this paper:* SEA, soluble egg antigens; BSA, bovine serum albumin; PAS, periodic acid–schiff.

by 25% after incubation with the particles. The coated particles were washed twice in phosphate buffered saline (pH 7.2) at 1,500 rpm in a refrigerated centrifuge and suspended in 0.9% saline solution containing 6000 particles per ml. 0.5 ml solutions were injected into the tail veins of unsensitized mice and mice sensitized by intraperitoneal injections of intact living schistosome eggs or soluble factor. Lungs were removed 8 days after injection.

As controls, uncoated bentonite particles and particles coated with protein by the suspension of 20,000 particles in 10 mg per ml of crystallized bovine serum albumin (Pentex Biochemical., Kankakee, Illinois) were injected intravenously into unsensitized and egg-sensitized mice. Lungs were removed at 8 days. The reaction around uncoated bentonite particles was further studied by removal of lungs at 1, 2 and 4 days after injection of the particles.

Delayed Footpad Swelling (Fig. 1-Part 2).—Mice were sensitized with either 1000 eggs injected intraperitoneally or the equivalent in soluble egg antigens. For elicitation of the footpad reaction, soluble egg antigens equal to 10 μg protein in 0.03 ml phosphate buffered saline were injected into the right hind footpad using an 0.25 ml tuberculin syringe and a 27 gauge needle. As a control, the same volume of phosphate buffered saline was injected into the left hind footpad (20, 21).

Reactions were measured 6, 24, and 48 hr later with a micrometer (Lufkin Rule Co., Saginaw, Michigan) to 100th of a mm. Two readings were taken from each footpad. The mean difference in thickness between the right and left footpads was taken as the net swelling. Significance of the differences was evaluated by the *t* test. At the peak of the footpad reaction (24 hr), the hind feet of representative control and treated mice were amputated and fixed in 10% buffered formalin. Decalcification was performed by immersion in 30% formic acid solution containing 10% Win-3000 granular resin. Sections were prepared and stained with hematoxylin and eosin.

Antibody Determinations (Fig. 1-Part 2).—A passive hemagglutination test (22) was utilized in which sheep red blood cells were fixed with 1% glutaraldehyde, tanned with a 1:2000 solution of tannic acid and sensitized at pH 7.2 with soluble egg antigens: 1 ml of 2.5% erythrocytes was mixed with 1 ml of a 1:5 dilution of SEA equivalent to 10,000 eggs and containing 60–90 μg of protein. Heteroagglutinins were absorbed from the antisera with washed sheep red blood cells before the test. Antisera obtained from mice with *Schistosoma mansoni* infections of 8 wk duration gave titers of 1:2560 to 1:5120. Sera were tested 7 and 15 days after sensitization with soluble factor or intact eggs. In addition, mice sensitized with soluble factor were injected intravenously 7 days later with eggs; serum collected 8 days thereafter was also tested.

RESULTS

Granuloma-Sensitizing Properties of S. mansoni Eggs and Materials Prepared from them.—Granuloma formation around *Schistosoma mansoni* eggs injected into the microvasculature of the lungs of mice previously sensitized by an intraperitoneal injection of intact living eggs is greatly accelerated and augmented in comparison with the granulomatous reaction in unsensitized animals. The maximal difference between the lesions in the sensitized and unsensitized mice occurs at 8 days when the mean granuloma volume in the sensitized animals is more than 3 times that in the unsensitized animals. As can be seen in Fig. 2, there is a wide separation between the mean granuloma volumes of the animals considered to be unsensitized and those deemed to be sensitized, there is no overlap of two standard deviations of the means of all unsensitized versus sensitized lesions, and the difference between the overall means is statistically

highly significant. Granulomas representative of the means of all unsensitized and sensitized reactions are shown in Fig. 3. In accordance with past observations, mice previously exposed to intact living schistosome eggs responded at 8 days with lesions typical in size to those seen in sensitized animals (Table I).
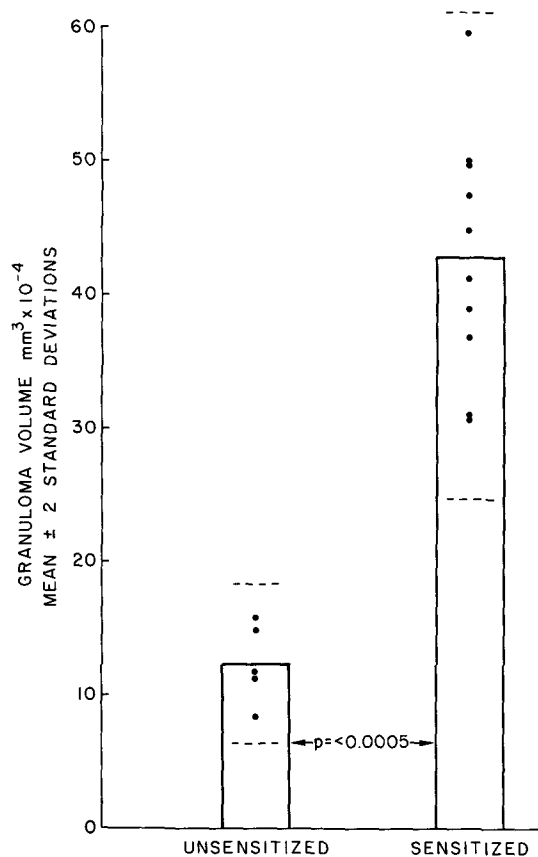

FIG. 2. Comparison of mean granuloma volumes around *Schistosoma mansoni* eggs in the lungs of groups of unsensitized mice and mice sensitized by intraperitoneal injections of eggs or materials prepared from them.

Sensitization by Dead Eggs, Egg Homogenates and Soluble Egg Antigens (Table I).—Intact eggs killed by autoclaving or freeze-thawing fully retained their ability to sensitize mice to granuloma formation around eggs subsequently injected into the microvasculature of the mouse lungs.

Disruption of the eggs by sonication, however, rendered them incapable of sensitizing mice, even when the material was combined with complete Freund's adjuvant. In contrast, complete homogenization of the eggs by grinding in a

TenBroeck tissue grinder maintained at 0°C did provide material which sensitized (without Freund's adjuvant) in doses equivalent to the usual number of intact eggs used for sensitization. Centrifugation of the egg homogenate at 100,000 g for 2 hr at 4°C provided a clear nonparticulate supernatant fluid which
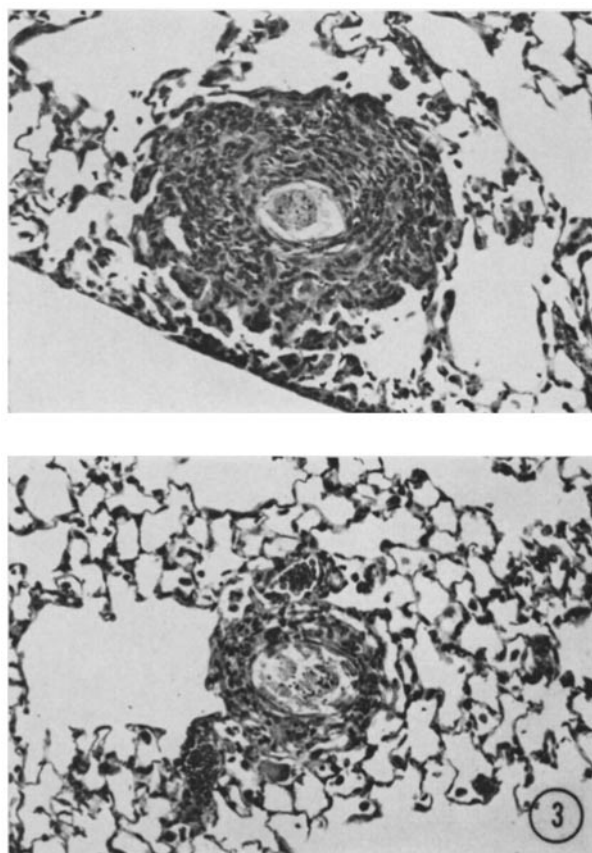

Fig. 3. Granulomas representative of the collective means of granuloma determinations in sensitized (top) as compared to unsensitized (bottom) mice as shown in Fig. 2. After collective means were determined, slides were searched to find representative lesions which were marked and photographed. Hematoxylin and eosin stain × 185.

in a concentration equivalent to 1,000 eggs was fully effective in sensitizing mice to granuloma formation. This material was active over a wide range of concentrations (Fig. 4). Further studies with these soluble antigens are described later in this paper.

Sensitization by Egg Emissions (Table II).—Following the demonstration that

a soluble material isolated from ground schistosome eggs sensitizes mice to granuloma formation, experiments were designed to determine whether the material was secreted or excreted in an active form by intact living eggs. In vitro emission of soluble egg antigens was substantiated by the activity of tissue

TABLE I

Granuloma Formation Around S. mansoni Eggs Injected Intravenously into Lungs of Mice, After Previous Sensitization with Live, Killed, and Homogenized Eggs, and Soluble Antigens Isolated from Homogenized Eggs

Sensitizing material	No. of mice	Lesions measured	Mean granuloma diam*	Increase in mean granuloma diam relative to egg diam‡	Granuloma vol§
		(No.)	(μ ± SE)	(%)	(mm³ × 10⁻⁴ ± SE)
Unsensitized control	8	101	130.36 ± 4.50	117	11.60 ± 1.19
Intact live eggs	6	136	224.82 ± 11.40	275	59.50 ± 9.05
Dead eggs					
Autoclaved	5	100	211.75 ± 8.00	253	49.71 ± 5.90
Freeze-thawed	6	73	211.92 ± 8.40	253	49.83 ± 5.90
Sonicated eggs	6	76	100.98 ± 7.08	68	5.39 ± 1.13
Sonicated eggs + Freund's complete adjuvant	3	51	112.02 ± 8.22	87	7.36 ± 1.62
Homogenized eggs‖	5	98	191.40 ± 8.56	219	36.71 ± 4.92
Supernatant fluid after centrifugation of homogenized eggs	16	319	198.19 ± 6.75	230	41.03 ± 4.16

* Granuloma size measured 8 days after intravenous injection of eggs.
‡ Egg diam alone—60 μ.
§ Calculated from the mean granuloma diam.
‖ TenBroeck tissue grinder, at 0°C.

culture medium which had contained eggs for 3 days. Similar activity was demonstrated in vivo by eggs maintained intraperitoneally in diffusion chambers.

Sensitization by Egg Components (Table III).—In order to localize the source of the granuloma sensitizing material the eggs were separated into three components simply by letting them hatch: shells, miracidia (the embryonic organisms) and so-called "hatch fluid." As under the best of circumstances only a portion of the eggs hatch, the egg shells were separated from unhatched eggs by a series of

sucrose density gradient centrifugations. Egg shells (morphologically intact except for the small fissure through which the miracidium escaped) were incapable of sensitizing mice; surprisingly, so were the live miracidia. For reasons to be presented in the discussion, miracidia were then homogenized in a TenBroeck grinder in a manner identical to that of the whole eggs; both the homogenate
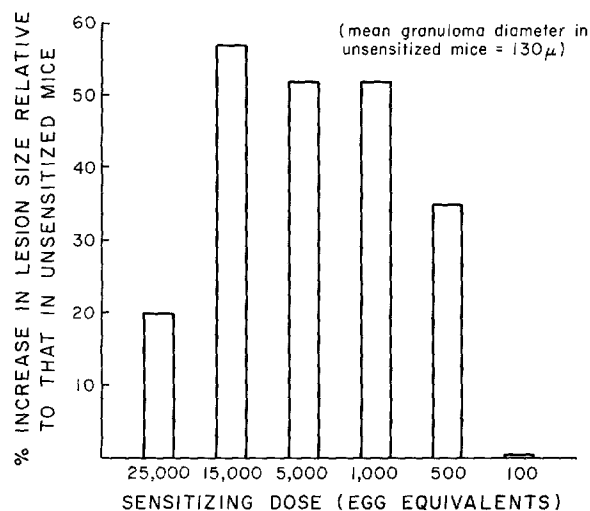

FIG. 4. Degree of sensitization induced by intraperitoneal injection of different dosages of soluble egg antigens.

TABLE II

Granuloma Formation Around S. mansoni Eggs Injected Intravenously into Lungs of Mice, After Previous Sensitization with Soluble Antigens Emitted by Intact Eggs

Soluble sensitizing material	No. of mice	Lesions measured	Mean granuloma diam*	Increase in mean granuloma diam relative to egg diam‡	Granuloma vol§
		(No.)	($\mu \pm S.E$)	(%)	($mm^3 \times 10^{-4} \pm SE$)
In vivo (diffusion chamber)	5	83	208.46 ± 7.81	247	47.43 ± 5.32
In vitro (tissue culture)	11	151	180.20 ± 7.31	200	30.64 ± 3.72
Control (tissue culture medium)	2	35	117.26 ± 4.73	95	8.44 ± 1.02

* Granuloma size measured 8 days after intravenous injection of eggs.
‡ Egg diam alone—60 μ.
§ Calculated from the mean granuloma diam.

and the soluble supernatant fluid remaining after high speed centrifugation sensitized mice, although 2–4 times the usual egg equivalent amount was needed (Table III).

The fluid that escaped at the time of hatching, when filtered free of unhatched eggs, shells and miracidia, was as capable of sensitizing mice as the soluble material isolated from homogenized whole eggs. The fluid from eggs maintained in hypertonic saline to prevent hatching was inactive.

TABLE III

Granuloma Formation Around S. mansoni Eggs Injected Intravenously into Lungs of Mice After Previous Sensitization with Egg Components

Sensitizing material	No. of mice	Lesions measured	Mean granuloma diam*	Increase in mean granuloma diam relative to egg diam‡	Granuloma volume§
		(No.)	($\mu \pm SE$)	(%)	($mm^3 \times 10^{-4} \pm SE$)
Egg shells	11	148	140.81 ± 7.70	135	14.97 ± 2.54
Intact miracidia	12	220	143.92 ± 7.03	140	15.67 ± 2.29
Homogenized miracidia	6	111	180.62 ± 5.22	201	30.85 ± 2.67
Supernatant fluid after centrifugation of homogenized miracidia‖	3	75	204.48 ± 7.90	241	44.77 ± 5.18
Hatch fluid	17	266	195.17 ± 7.20	225	38.92 ± 4.30
(saline control)	11	242	128.84 ± 6.26	115	11.20 ± 1.63

* Granuloma size measured 8 days after intravenous injection of eggs.

‡ Egg diam alone–60 μ.

§ Calculated from the mean granuloma diam.

‖ Amount used for sensitization is equivalent to 4000 intact miracidia.

Further Investigations of Soluble Antigens Derived from Schistosoma mansoni Eggs

The clear supernatant fluid remaining after high speed centrifugation of a homogenate of intact living schistosome eggs has been designated soluble egg antigens (SEA). Preliminary experiments revealed that incubation with trypsin and RNAase destroyed SEA activity but DNAase had no effect. SEA was stable for 2 hr at 23° and 37°C but was destroyed at 56°C. Its activity remained unimpaired after many months of storage at −30°C. When the frozen stored material was thawed, a flocculent precipitate was observed. Removal of the precipitate by centrifugation decreased the protein concentration, but the activity of the

supernatant fluid remained unimpaired. The protein concentration of this material prepared at a concentration of 50,000 eggs per ml was 300–450 μg per ml. Mice were fully sensitized by SEA at an egg equivalent concentration of 1000, which equals 6–9 μg of protein. Complete sensitization was observed over an egg equivalent range of 1000–15,000; activity dropped off at higher concentrations (25,000) and lower concentrations (500), with no sensitization whatsoever at 100 egg equivalents (Fig. 4).

Disc electrophoresis of SEA revealed eight bands on staining with Coomassie blue. Preparative zone electrophoresis of SEA then provided active material in a 1 cm strip 4 cm from the origin and 12 cm from the cathode. Disc electropho-

TABLE IV

Granuloma Formation Around Bentonite Particles with or without Adsorbed Antigens Injected Intravenously into the Lungs of Unsensitized Mice and those Sensitized with Soluble Egg Antigens or Intact Eggs

Particles injected	Mean granuloma diameter*		
	Unsensitized mice	Mice sensitized with soluble egg antigen	Mice sensitized with intact eggs
	μ ± SE	μ ± SE	μ ± SE
Bentonite alone	54.78 ± 1.72 (66)‡	54.86 ± 2.99 (34)§	63.97 ± 1.77 (97)‡
Bentonite coated with bovine serum albumin	52.32 ± 1.68 (75)‡	55.62 ± 2.31 (35)§	65.47 ± 2.81 (60)‡
Bentonite coated with soluble egg antigen	77.61 ± 2.14 (84)‡	93.71 ± 3.71 (64)‡	110.40 ± 3.24 (110)‡

* Granuloma size measured 8 days after intravenous injection of bentonite particles.

‡ Number of lesions measured in tissue samples from 6 mice.

§ Number of lesions measured in tissue samples from 3 mice.

resis of both this material and hatch fluid revealed, in each case, three bands which were similar in mobility.

Elicitation of Granuloma Formation by SEA Bound to Bentonite Particles.— Bentonite particles in the size range of schistosome eggs, injected intravenously into the microvasculature of the lungs of mice, stimulated only minimal reactions which were foreign body in type, with onset at less than 24 hr, peak reaction at 24 hr and gradual decline in reaction to 8 days, at which point the cells were almost exclusively macrophages (Table IV).

The reaction around bentonite with adsorbed bovine serum albumin (BSA) or SEA was similar at 8 days to that around bentonite particles alone. In contrast, bentonite SEA injected into mice sensitized 1–2 wk previously by the intraperitoneal injection of SEA or intact living eggs resulted in large lesions (Table IV). They were even larger in a few egg-sensitized animals from which the lungs were removed at 4 days (142.56 ± 5.59 μ). The cellular composition of
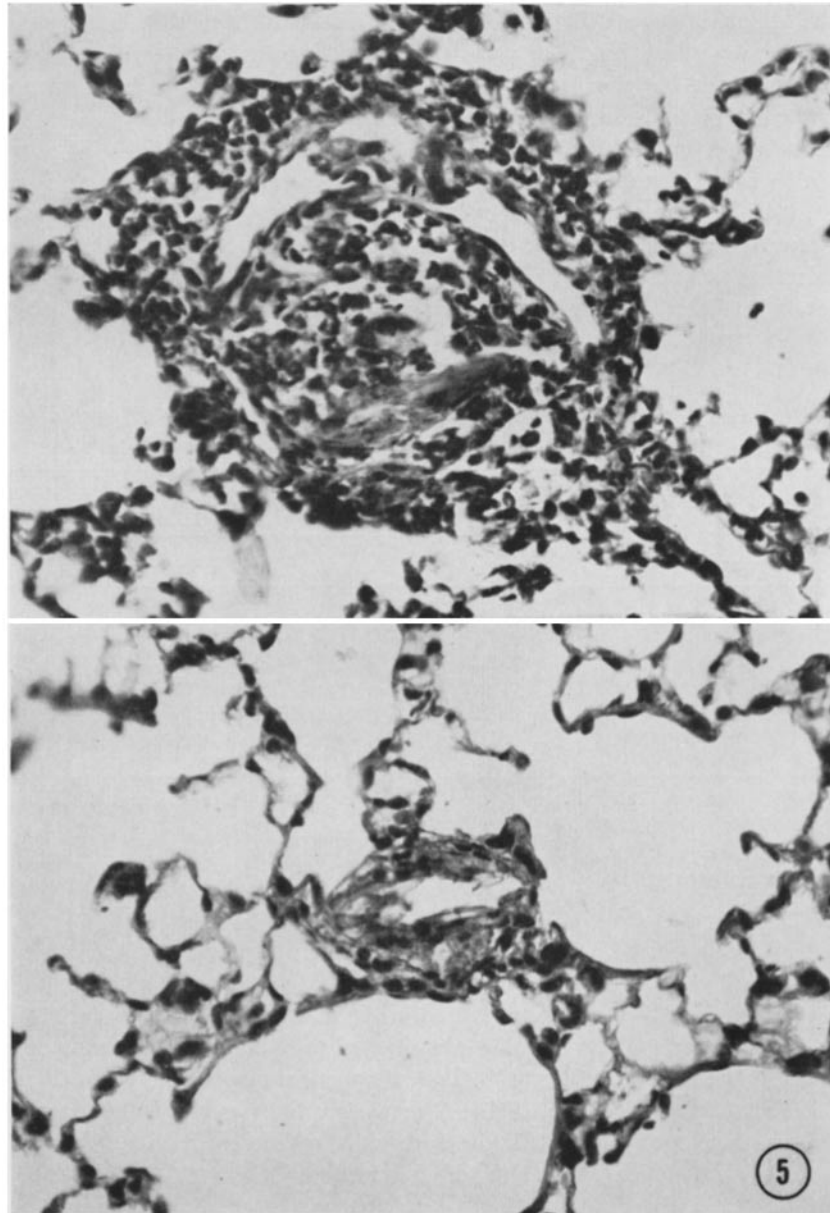
Fig. 5. Representative granulomas formed around bentonite particles, with (top) and without (bottom) adsorbed soluble egg antigens, injected into the pulmonary microvasculature of mice sensitized by a previous intraperitoneal injection of intact *Schistosoma mansoni* eggs. Hematoxylin and eosin stain × 375.

the lesions in the sensitized mice differed markedly from that in the control animals described above, and was characterized by large numbers of eosinophils, macrophages, lymphocytes, and giant cells (Fig. 5). Egg-sensitized mice displayed no cross-reactivity to bentonite-BSA.

Induction and Elicitation by SEA of Delayed Footpad Swelling.—Mice were sensitized with either intact living schistosome eggs or an equivalent amount of SEA. They were challenged 1 or 2 wk later by the footpad injection of SEA equivalent to somewhat over 1000 eggs (10 μg protein). Some increased footpad thickness was observed by 6 hr, the peak reaction occurred at 24 hr and it declined by 48 hr (Table V). Intact eggs were more highly sensitizing than SEA,

TABLE V

Elicitation of Footpad Swelling with Soluble Egg Antigens (SEA) in Mice Sensitized with Intact Eggs or SEA

Sensitizing material	No. of eggs or egg equiv- alents	No. of ani- mals	Mean increase in footpad thickness* at intervals after injection of SEA		
			6 hr	24 hr	48 hr
			(mm ± SE)	*(mm ± SE)*	*(mm ± SE)*
Viable eggs	1000	10	0.33 ± 0.04‡	0.66 ± 0.09‡	0.15 ± 0.03§
Viable eggs	5000	10	0.34 ± 0.04‡	0.57 ± 0.08‡	0.19 ± 0.05§
SEA	1000	10	0.24 ± 0.04‡	0.42 ± 0.05‡	0.15 ± 0.04§
SEA	5000	10	0.16 ± 0.03‡	0.27 ± 0.04‡	0.06 ± 0.01§

* Right paw thickness (SEA) minus left paw thickness (phosphate buffered saline). Before injection paws averaged 2.62 ± 0.03 mm in thickness.
‡ *t*-test, $P = < 0.0005$.
§ *t*-test, $P = < 0.05$.

but in both cases SEA had the capacity to elicit significant delayed footpad swelling (*P* value <0.0005 at most time intervals). The larger sensitizing dose did not produce greater reactivity and in the case of SEA actually resulted in less pronounced footpad swelling. Histological examination of the footpad revealed infiltration of the dermis by large numbers of mononuclear cells (Fig. 6).

Induction of Antibodies by SEA.—A highly sensitive passive hemagglutination test was developed utilizing SEA as antigen. Mice infected with *S. mansoni* for 8 wk (egg production begins at 5 wk) had antibody titers of 1:2580 to 1:5160. No detectable circulating antibody was observed in mice injected intraperitoneally with 1000 eggs or egg equivalents of SEA at 7 and 15 days after injection. Challenge, with intact eggs, of mice sensitized 7 days previously by SEA or intact eggs still did not result in detectable titers by 15 days.
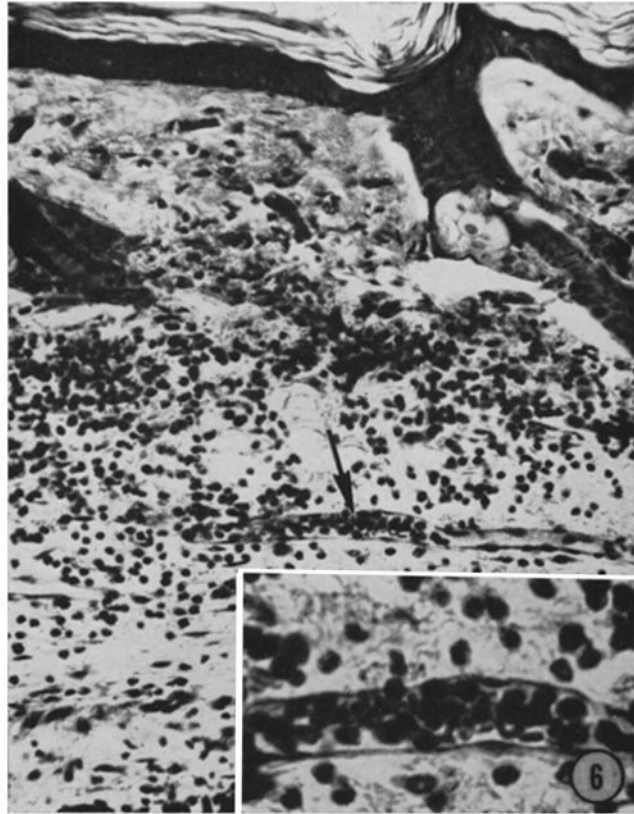
Fig. 6. Section from the footpad of a mouse sensitized by an intraperitoneal injection of 1000 living *Schistosoma mansoni* eggs and tested 1 wk later by an intradermal injection of soluble egg antigens in a concentration equivalent to about 1,000 eggs. The tissue was removed and fixed at the height of footpad swelling (24 hr). Hematoxylin and eosin stain × 250. The arrow indicates an area shown at higher magnification (× 460) showing that the infiltrate consists primarily of round cells.

DISCUSSION

A granuloma is a circumscribed inflammatory reaction usually formed around a nidus consisting of either a foreign body or an infectious agent (1, 2, 23). Investigation of the foreign body reaction is relatively easy as insoluble particulate substances can be injected into the tissues or microvasculature where they remain fixed as discrete objects (23). In contrast, the organisms usually responsible for infectious granulomas, for example, mycobacteria or fungi, tend to multiply and disseminate following injection into experimental animals. Schisto-

somiasis, however, is a granulomatous disease in which the organism which elicits the inflammatory reaction (the schistosome egg) neither multiplies nor disseminates. In the past decade von Lichtenberg has established the schistosome egg granuloma as a model for the study of the infectious granuloma (3). Using his methodology we have been able to demonstrate that the schistosome egg granuloma is an immunologic reaction of the delayed hypersensitivity type (4, 7–10). Initial attempts to isolate the egg antigens responsible for this reaction were based on von Lichtenberg's experiments which revealed that eggs emit antigenic material and that granulomas form around dead eggs as well as live eggs, but not around egg shells and miracidia separated by hatching. He concluded, on the basis of these data, that while viability was not necessary, the eggs had to be intact to elicit granuloma formation (3).

We decided to test this hypothesis by determining whether completely disrupted eggs had granulomatous activity. As the particles were too small to be retained in the arterioles of the lungs, an assay method was developed based on the ability of intraperitoneally injected materials to induce anamnestic responses around intact living schistosome eggs subsequently injected into the lungs (4). Using this method it was shown that intact dead eggs sensitize and that this activity was not lost after homogenization in a glass tissue grinder. Since neither viability nor morphological integrity was necessary for sensitization, it became possible to investigate the source of the sensitizing material within the egg and to isolate and characterize the material biochemically. These studies were greatly facilitated by the demonstration that after high speed centrifugation of the homogenized eggs the clear nonparticulate supernatant fluid was antigenic, providing material which we have termed the soluble egg antigens (SEA). Subsequently, the presence of SEA was demonstrated in vivo by the use of diffusion chambers, and soluble antigen was obtained in vitro from eggs maintained in tissue culture medium for 3 days, and from the fluid released at the time of egg hatching.

When eggs were separated into three components, shells, miracidia, and hatch fluid, by allowing them to hatch in spring water, the initial results were puzzling as neither the shells nor the miracidia induced sensitization. While von Lichtenberg had shown that these components do not elicit granuloma formation, he also observed that the miracidia were attacked by neutrophils and rapidly destroyed (15). This suggested that the miracidial antigens might not be available to cells which initiate immunologic reactivity. In order to increase the availability of the antigens, miracidia were homogenized; both the homogenate and the soluble antigens derived from it proved to be sensitizing. As twice the usual dosage of these materials was needed it appeared that significant amounts of antigen were lost in the hatching process. The hatch fluid was then tested and found to be highly sensitizing.

The emission of soluble antigens by the living intact eggs and their release at

the time of hatching suggested a biological role for SEA. Schistosome eggs which are laid in the mesenteric venules pass through the intestinal tissues into the lumen. They hatch on entering fresh water releasing free-swimming miracidia which penetrate into snails; each of these processes is believed to involve enzymatic secretions (24). Andrade and Barka, using histochemical techniques, found high levels of aminopeptidase in the miracidial cephalic glands (25) and Kloetzel demonstrated a protease diffusing from the eggs (26). Thus it is possible that the granuloma-sensitizing factor is an enzyme secreted as a normal biological function of the schistosome egg.

The chemical nature of the soluble egg antigens has not yet been determined. Previous studies have shown antigenic diastase-resistant PAS-positive material and sulfhydril-disulfide–rich protein in the egg and its secretions (25, 27). Chemical analysis of SEA revealed significant levels of protein, carbohydrate and nucleic acids, but enzymatic inactivation studies were inconclusive. Further such analyses have been deferred until the material can be isolated in pure form. Partial purification without loss of activity has already been achieved by preparative zone electrophoresis. These materials are now undergoing molecular sieving on Sephadex columns.

The availability of highly active soluble antigens afforded an opportunity to examine granuloma elicitation directly by coating SEA onto bentonite, a colloidal hydrated aluminum silicate. Bentonite has the property of adsorbing antigens, and has been used in a variety of immunologic systems for the detection of antibodies or as an adjuvant (18, 28). Bentonite, either alone or with adsorbed antigens (SEA or bovine serum albumin), resulted in small foreign body type granulomas similar to those which occur around plastic beads (23). Intravenous injection of the SEA-coated bentonite into mice previously sensitized by intact eggs or SEA resulted in large granulomas consisting of macrophages, eosinophils and giant cells, which closely resembled the reactions around intact schistosome eggs. This response appeared to be specific as it did not occur in schistosome egg-sensitized mice injected with bentonite coated with bovine serum albumin. The ability of SEA to elicit granuloma formation as well as induce sensitization confirmed our original assumption that the same antigens were involved in both processes. The artificial hypersensitivity type of granuloma produced by adsorption of soluble antigens to bentonite particles might provide a valuable method for the investigation of granuloma-inducing microorganisms, obviating the problems of multiplication and dissemination.

The isolation of the soluble egg antigens led to studies of their role in the induction and elicitation of the classical delayed skin reaction. Delayed footpad swelling (20, 21) was elicited by the soluble antigens in mice sensitized by intact eggs or by minute amounts of the soluble material itself. The peak reaction occurred at 24 hr and was made up almost entirely of mononuclear cells. Skin test studies now in progress with guinea pigs, the standard laboratory animal

for the investigation of hypersensitivity, have shown similar results. The demonstration that SEA causes delayed skin responses confirms the delayed hypersensitivity etiology of the schistosome egg granuloma by associating it with the most accepted from of this reaction. In addition, the development of the skin test provides a rapid assay system for the various fractions of SEA provided by electrophoresis and molecular sieving.

While sensitization to delayed skin reactions, as measured by footpad swelling, has been achieved in the mouse by infection with living bacteria (20, 21), the induction of delayed hypersensitivity by purified antigens, even when incorporated into complete adjuvant, remains controversial (29-31). This contrasts markedly with our demonstration that microgram quantities of SEA in aqueous solution induce delayed cutaneous responses in mice.

Finally, circulating antibody was not detected by a highly sensitive passive hemagglutination test using the soluble egg material as antigen during the 15 day period in which our investigations were performed. Other workers, using a fluorescent antibody test, found only a slight rise in antibody titer 2-3 wk after the intraperitoneal injection of large numbers of intact schistosome eggs (32). These findings strongly suggest that antibody-mediated reactions play little or no role in the schistosome egg granuloma, and are consistent with previous observations that immunosuppressive measures which inhibit antibody formation have no effect on the schistosome egg granuloma (9, 10).

Induction of delayed hypersensitivity without concomitant antibody formation was demonstrated by Uhr, Salvin, and Pappenheimer (33) and by Salvin (34) by the injection into guinea pigs of minute amounts of antigen-antibody complexes or antigen mixed with incomplete adjuvant. Similar results were observed in our systems, but without the addition of antibody or adjuvant to the antigen. In this regard our findings resemble the Jones-Mote reaction, a transient cell-mediated response observed in guinea pigs sensitized by the intraperitoneal injection of a relatively large dose of antigen (250 μg) (35) and in humans sensitized by repeated intradermal injections of minute amounts of protein (36). Attempts to demonstrate a Jones-Mote type of hypersensitivity in mice, however, have thus far been unsuccessful (37). Although sensitization induced with the soluble egg antigens appears to be relatively transient, the results are virtually identical with those following sensitization with intact eggs which lasts for at least 4 months (4).

In summary, a unique substance has been isolated from *Schistosoma mansoni* eggs which has the property of both inducing and eliciting delayed hypersensitivity in minute amounts without adjuvant and without inducing detectable circulating antibody. Evidence was gathered as to the source of this material in the schistosome egg, and its biological role was suggested. Further evidence that the schistosome egg granuloma is a form of delayed hypersensitivity was provided by its correlation with delayed skin reactions, and by the absence of

circulating antibody. The relevance of this work to other forms of infectious granulomas awaits study, but the methodology developed in the course of the present experiments should facilitate the investigation of this important problem.

SUMMARY

A soluble material has been isolated from schistosome eggs which in minute quantities without the addition of adjuvant induces sensitization to a delayed hypersensitivity type of granuloma forming around intact schistosome eggs. This material is secreted by intact eggs and is found in high concentration in the fluid released during the hatching process. When adsorbed to bentonite particles this substance elicits hypersensitivity type granuloma formation in specifically sensitized animals. The granuloma sensitizing factor also both induces and elicits delayed footpad swelling in mice. Quantities which sensitize with respect to these delayed type reactions do not induce antibody formation detectable by a sensitive hemagglutination technique within the duration of the above experiments.

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