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Direct and Indirect Effects of Soluble Extracts of *Schistosoma mansoni* Eggs on Fibroblast Proliferation In Vitro

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The possibility that soluble products of *Schistosoma mansoni* eggs might participate in the pathogenesis of hepatic fibrosis in schistosomiasis was investigated. Both crude saline extracts of eggs (soluble egg antigen [SEA]) and a partially purified SEA fraction contained activity which stimulated guinea pig and human dermal fibroblasts to proliferate in vitro, as measured by uptake of [^{3}H]thymidine. Maximum activity was present in fractions which eluted from Sephacryl S-200 with an apparent molecular weight of ≤12,500 and in fractions which had an estimated pI 8, as determined by preparative isoelectric focusing of partially purified SEA. Activity in crude SEA was not removed by chromatography on concanavalin A-Sepharose 4B. When concanavalin A-binding glycoproteins lacking intrinsic fibroblast-stimulating activity were incubated with spleen cells from infected or uninfected mice, fibroblasts-stimulating activity was detected in the culture supernatants. Thus, SEA contains two functionally distinct molecular species. One of these directly stimulates fibroblasts, whereas the other induces the release of a fibroblast-stimulating activity from lymphocytes or macrophages or both. Since these fibroblast-stimulating factors might be elaborated in the livers of infected individuals, these observations suggest a potential role of soluble schistome products in the pathogenesis of hepatic fibrosis in schistosomiasis.

Although hepatic fibrosis underlies important morbidity and mortality in schistosomiasis mansoni infections, very little is known about hepatic fibrogenesis in this disease. Dunn and coworkers (2) have demonstrated that isolated hepatic egg granulomas from *Schistosoma mansoni*-infected mice could incorporate radiolabeled proline into collagen in vitro, presumably representing the synthetic activity of granuloma fibroblasts. Evidence accumulated in our laboratory (9, 10) indicates that these isolated egg granulomas could spontaneously elaborate soluble factors which stimulate dermal fibroblasts in vitro to proliferate and synthesize collagen. These observations suggest a potential molecular link between granuloma formation and hepatic fibrosis. We postulated that granuloma-derived fibroblast-stimulating activity might be a soluble product secreted by the chronic inflammatory cells comprising the granulomas. Indeed, such activity has also been identified in culture supernatants of in vitro-stimulated lymphocytes (7) and macrophages (3, 5, 8). The additional possibility that the granuloma-derived activity originated within the schistosome egg was investigated in the studies we report here.

MATERIALS AND METHODS

Animals. Outbred albino female CF_1 and inbred female C57BL/6 mice weighing 18 to 20 g each were obtained from Charles River Breeding Laboratories, North Wilmington, Mass.

Preparation and fractionation of SEA. Crude soluble egg antigen (SEA) was prepared by the method of Boros and Warren (1) from eggs isolated from the livers and intestines of CF_1 mice infected 7 to 8 weeks earlier by a subcutaneous injection of 200 cercariae of a Puerto Rican strain of *S. mansoni*. The crude preparation was dialyzed overnight against 1,000 volumes of 10 mM sodium phosphate-buffered 0.15 M saline (pH 7.4), clarified by centrifugation for 90 min at 105,000 × *g*, and stored frozen at −30°C. Protein concentrations were estimated by the method of Lowry et al. (4).

Crude SEA was fractionated by three different methods. A 2-mg amount of crude SEA in 2 ml of 10 mM sodium phosphate-buffered 0.15 M saline was subjected to gel filtration chromatography on a calibrated column (1.5 by 90 cm) of Sephacryl S-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.)

equilibrated at 4°C with 10 mM sodium phosphate-buffered 0.15 M saline. Fractions of 2.5 ml were collected at a flow rate of 15 ml/h. The protein content of the column effluent was monitored at 280 nm, and selected fractions were pooled and concentrated five-fold by vacuum dialysis (M_r cufoff, ≤5,000) at 4°C against RPMI 1640 medium. Concentrated material was filter sterilized (0.22 μm; Millipore Corp., New Bedford, Mass.), stored at 4°C, and tested (7 to 14 days later) at 1:10 dilution.

Preparative flatbed isoelectric focusing (pH 3 to 9) was carried out by mixing 4.2 mg of a non-concanavalin A (ConA)-Sepharose binding fraction of SEA (ConA fraction I; see below) in a solution containing 4.5 ml of Ampholine (LKB, pH 3.5 to 10), 0.5 ml of Ampholine (pH 5 to 8), 4 g of Ultrodex gel (LKB), and enough distilled water to bring the final volume to 100 ml. This slurry was poured onto a glass tray (22 by 10 cm) and partially dried with a low-speed fan. The flatbed was run on a cooling plate (approximately 4°C) at a constant power of 8 W (~500 V) for approximately 19 h. Fractions were cut at 0.5-cm intervals and mixed with 1 ml of distilled water, and the pH was determined. Material was recovered from the gel by the addition of 2 ml of water and centrifugation (1,000 × *g*, 10 min) and dialysis for 48 h against 20 volumes of RPMI 1640 medium (4°C; three changes of medium). Filter-sterilized fractions were stored at −10°C until tested.

Crude SEA was fractionated by affinity chromatography on a ConA-Sepharose 4B column essentially as described by Pelley et al. (6). Thus, crude SEA (25 mg of protein) was dialyzed against 1,000 volumes of a buffer (pH 7.4) containing 10 mM sodium phosphate, 0.5 M sodium chloride, 10^{-4} M $CaCl_2$, 10^{-4} M $MnCl_2$, and 0.02% sodium azide and applied to a column (1.5 by 28 cm) of ConA-Sepharose equilibrated at 4°C with the same buffer. Unadsorbed material (ConA fraction I) was eluted with three bed volumes of the equilibration buffer. Bound egg glycoproteins (ConA fraction II) were subsequently eluted with two bed volumes of the same buffer containing 0.1 M α-methyl-D-mannoside (grade III; Sigma Chemical Co., St. Louis, Mo). The column effluent was monitored for proteins at 280 nm, and selected fractions were pooled and concentrated at 4°C with an Amicon type 12 stirred ultrafiltration cell fitted with a YM-5 membrane (an ultrafiltration membrane with a ~5,000 M_r cutoff; Amicon Corp., Lexington, Mass.). Concentrated ConA fractions were stored at −30°C and exhaustively dialyzed before use against RPMI 1640 culture medium to remove the sodium azide.

Spleen cell cultures. Spleen cell suspensions were prepared from individual C57BL/6 mice which were either uninfected or had been infected intraperitoneally with 50 cercariae of a Puerto Rican strain of *S. mansoni* 8 weeks previously. Spleens were minced in cold Hanks balanced salt solution, and cells were dissociated by passage through a stainless steel mesh and a no. 25 gauge needle. Erythrocytes were hypotonically lysed, and twice-washed splenocytes were suspended in RPMI 1640 medium supplemented with 4 mM L-glutamine and 100 U of penicillin and 100 μg of streptomycin per ml. Spleen cell suspensions were further supplemented with 0.25% heat-inactivated fetal bovine serum and incubated at a density of 10^7 viable cells per ml in 3-ml samples in upright 25-cm^2 polystyrene tissue culture flasks (no. 25100; Corning Glass Works, Corning, N.Y.). Ten micrograms of the ConA fraction II per milliliter (final concentration) or no antigen were added to parallel cultures at the onset of incubation. After incubation for 48 h at 37°C in a humidified atmosphere of 5% CO_2–95% air, culture supernatants were obtained after centrifugation of 4°C (200 × *g*, 10 min) and then concentrated fivefold by vacuum dialysis at 4°C against RPMI 1640 medium, filter sterilized, and tested in fibroblast cultures.

In related studies, the in vitro proliferative response of splenic lymphocytes to the antigen preparations was tested. Spleen cell suspensions (prepared as described above) were suspended at a density of 10^6 viable cells per ml in supplemented RPMI 1640 medium also containing 10% heat-inactivated fetal bovine serum, and 200 μl of the cell suspension was added to each flat-bottomed well of 96-well culture plates (Linbro, Hamden, Conn.). Antigen in medium or medium alone was added at a volume of 20 μl per well. Cultures were incubated for 6 days at 37°C in a 5% CO_2–95% air atmosphere. Each well was pulsed with 0.5 μCi of [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) for the final 4 h of incubation. Cells were harvested onto glass fiber filters with a multiple sample automated cell harvester (MASH II; Microbiological Associates, Bethesda, Md.). Filters were placed in a toluene-based cocktail (Ready-Solv; Beckman Instruments, Inc., Fullerton, Calif.) and counted for 2 min each in a liquid scintillation counter (model LS 7500; Beckman). The mean counts per minute was determined from triplicate samples.

Fibroblast proliferation assay. The ability of parasite extracts and spleen cell culture supernatants to stimulate fibroblast proliferation was assessed with methods previously described (9). Briefly, primary newborn human foreskin or guinea pig dermal fibroblasts (passages 4 through 10) were plated at a subconfluent density of 2.5 × 10^5 cells per 16-mm-diameter well of 24-well culture plates (Cluster 24; Costar, Cambridge, Mass.) in supplemented culture medium containing 10% heat-inactivated fetal bovine serum. After overnight incubation at 37°C in a 5% CO_2–95% air atmosphere, cells were extensively washed with Hanks balanced salt solution, and cultures were continued for 24 h in serum-free medium (1 ml per well) before the addition of 100 μl of medium, SEA preparation, or spleen cell culture supernatants. Except where otherwise indicated, fibroblast cultures were further incubated for 24 h, and each well was then pulsed with 0.5 μCi of [^{3}H]thymidine for the final 4 h of incubation. Cells were trypsinized (0.25%) and harvested onto glass fiber filters as described above. Uptake of the radiolabel by triplicate fibroblast cultures was assessed by liquid scintillation counting. Our previous studies have confirmed that this assay is a measure of cell proliferation (9). Furthermore, we have detected no important differences in the response of human and guinea pig fibroblasts to a variety of stimuli (D. Wyler, unpublished data). During the course of the studies described here, we found it desirable to switch from rodent to human fibroblasts because of the greater ease of establishing and maintaining the latter cultures.

RESULTS

Direct stimulation of fibroblasts by SEA. Crude SEA stimulated [^{3}H]thymidine uptake in a dose-

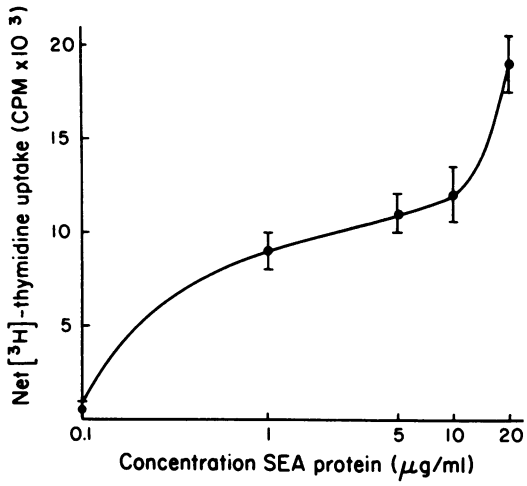

FIG. 1. In vitro proliferative response of guinea pig fibroblasts to crude SEA. The net uptake of [^{3}H]thymidine was determined by subtracting the mean counts per minute in triplicate cultures with medium only from counts per minute in cultures stimulated with SEA. The mean ± standard error of the mean of net counts per minute is shown for triplicate determinations.

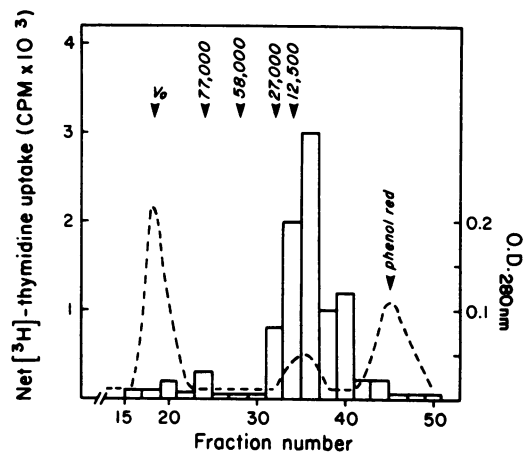

FIG. 3. Gel filtration chromatography (Sephacryl S-200) of 2 mg of crude SEA. Protein content was estimated by UV absorbance at 280 nm (---). The proliferative response of guinea pig fibroblasts (counts per minute) to pooled fractions is shown by bars and represents mean net counts per minute (see Fig. 1) calculated from triplicate determinations; standard error of the mean was ≤15% of the mean. Elution positions of molecular-weight standards are shown. Phenol red was run with SEA, and its elution is revealed by the third peak. O. D., Optical density.

dependent manner when added directly to fibroblast cultures (Fig. 1). This effect could not be attributed to contamination of the SEA preparation with endotoxin, since the direct addition of lipopolysaccharide (*Salmonella enteritidis*, Difco Laboratories, Detroit, Mich.) at concentrations between 1.0 ng and 10 μg/ml failed to stimulate [^{3}H]thymidine uptake by guinea pig fibroblasts (data not shown). The kinetics of the response were assessed by incubating fibroblasts with crude SEA fraction for 24, 48, or 72 h and determining the uptake of [^{3}H]thymidine during a terminal 4-h pulse. Maximum response was observed after 48 h (Fig. 2).

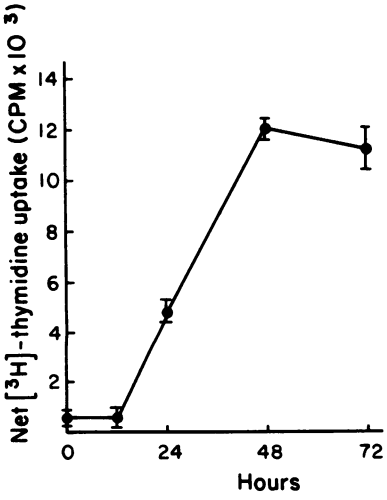

FIG. 2. Kinetics of proliferative response of guinea pig fibroblasts to 1 μg of crude SEA per ml. Net [^{3}H]thymidine uptake is defined in the legend to Fig. 1. The mean ± standard error of the mean of net counts per minute is shown for triplicate determinations.

Crude SEA was resolved into two major protein peaks by gel filtration chromatography (Fig. 3). Fibroblast-stimulating activity was present in fractions having apparent molecular weights (M_r) in the range of ≤12,500 to 27,000, with maximal activity observed in fractions having an M_r of ≤12,500. Because activity was not lost upon vacuum dialysis (M_r cutoff, ≤5,000), the apparent M_r of the factor was estimated to be 5,000 to 12,500.

To determine whether the fibroblast-stimulating activity in SEA might be due to the ConA-binding glycoprotein antigens in *S. mansoni* eggs (6), crude SEA was fractionated by ConA affinity chromatography. When both the unbound fraction (ConA fraction I) and the bound egg glycoproteins (ConA fraction II) were tested in the fibroblast assay, only ConA fraction I contained fibroblast-stimulating activity (Table 1). At the concentrations tested, ConA fraction II failed to significantly stimulate net [^{3}H]thymidine uptake by fibroblasts.

When ConA fraction I was further fractionated by preparative isoelectric focusing, fibro-

TABLE 1. Response of human fibroblasts to crude or ConA-Sepharose affinity chromatography-purified *S. mansoni* SEA

Protein concn (μg/ml)	Net [^{3}H]thymidine uptake by fibroblasts stimulated with SEA in following fraction[a]:		
	Crude	I[b]	II[c]
0.1	150	0	0
1	7,101	5,280	338
10	11,134	12,391	445

[a] Net uptake was calculated as the difference between the mean uptake of triplicate cultures to which SEA was added and to which medium alone was added (7,278 ± 467). Standard error of the mean was ≤15% of the mean in all cases.

[b] Fraction I is the material which did not bind to ConA (fall-through).

[c] Fraction II is the ConA-bound material eluted with α-methyl-mannoside buffer (see text).

blast-stimulating activity was present as a single peak in fractions with a pI in the range of 7.8 to 8.0 (Fig. 4).

Indirect stimulation of fibroblasts by SEA. Since ConA fraction II had no direct stimulating effect on fibroblasts at protein concentrations as high as 10 μg/ml, this glycoprotein fraction was tested to determine whether it might trigger the in vitro release of soluble fibroblast-stimulating factors from murine spleen cells. Although only spleen cells from sensitized (infected) mice responded to SEA in an in vitro blastogenesis assay (Table 2), spleen cells from both infected and control (uninfected) animals elaborated fibroblast-stimulating activity into culture supernatants when incubated for 48 h in the presence of ConA fraction II (Table 3). The magnitude of the fibroblast response to culture supernatants from control and sensitized spleen cells was not significantly different ($P > 0.8$ comparison of mean ± standard error of the mean net counts per minute for each group; see Table 3). The addition of 10 μg of ConA fraction II per ml to unstimulated spleen cell cultures for the final 15 min of incubation failed to produce supernatants which contained fibroblast-stimulating activity (data not shown).

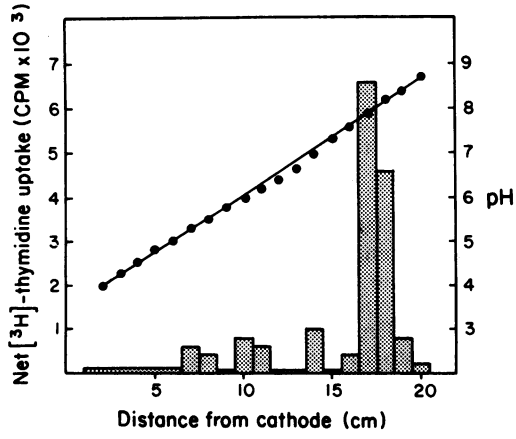

FIG. 4. Preparative isoelectric focusing of ConA fraction I of SEA. ●, pHs of fractions. The proliferative response of human fibroblasts (counts per minute) is shown by bars and represents mean net counts per minute (see Fig. 1) calculated from triplicate determinations; standard error of the mean was ≤15% of the mean.

TABLE 2. Representative in vitro blastogenic response of mouse spleen cells to ConA fraction II

Spleen cell source	Response (cpm) to stimulation with[a]:	
	Medium only	SEA (10 μg/ml)
Uninfected		
1	88 ± 4	76 ± 1
2	65 ± 10	66 ± 11
3	55 ± 7	104 ± 23
Infected		
1	277 ± 42	7,822 ± 528
2	241 ± 35	7,862 ± 27
3	358 ± 110	19,034 ± 1,951

[a] Mean ± standard error of the mean; triplicate determinations.

DISCUSSION

The results of these studies clearly indicate that SEA contains a biologically active molecule(s) capable of directly stimulating fibroblasts in vitro (Fig. 1). This activity resides in a molecule(s) with an M_r of ≤12,500 (Fig. 3) and a pI ~8 (Fig. 4) which does not bind to ConA-Sepharose. These characteristics distinguish the SEA-derived fibroblast-stimulating activity from that present in culture supernatants of isolated hepatic granulomas, which has an estimated M_r in the range of 30,000 and a pI ~5.5 to 6.5 (D. J. Wyler and L. J. Rosenwasser, J. Immunol., in press). The observation that the ConA-binding glycoproteins (ConA fraction II) lack direct fibroblast-stimulating activity in vitro (Table 1) indicates that the activity resides in molecules distinct from those antigens previously characterized (6) which have properties important in immune responses in schistosomiasis mansoni.

Specifically, it is thought that the egg glycoproteins are responsible for delayed hypersensitivity responses (such as lymphokine production) which underlie granuloma formation in *S. mansoni* infections. Thus, the observation that sensitized spleen cells appear to elaborate a fibroblast-stimulating factor when cultured in the presence of ConA fraction II (Table 3) is

TABLE 3. [^{3}H]thymidine uptake of human fibroblasts stimulated with culture supernatants from mouse spleen cells stimulated with ConA fraction II

Spleen cell source[a]	Fibroblast response (mean ± SEM) to supernatants[b]		
	Additives to spleen cell cultures		Δ[c]
	Medium only	Fraction II (10 μg/ml)	
Uninfected			
1	934 ± 167	3,906 ± 606	2,972
2	609 ± 74	4,359 ± 638	3,750
3	3,988 ± 216	10,864 ± 637	6,876
4	4,731 ± 311	10,094 ± 1,381	5,363
Infected			
1	571 ± 15	3,281 ± 169	2,710
2	2,077 ± 153	8,155 ± 121	6,078
3	511 ± 48	7,076 ± 207	6,565
4	239 ± 23	3,048 ± 359	2,809
5	733 ± 223	3,160 ± 76	2,427
6	420 ± 45	6,202 ± 148	5,482
7	780 ± 24	10,427 ± 207	9,647

[a] Supernatants were tested at 1:10 dilution. Results are from triplicate determinations.
[b] Spleen cells from individual mice were tested.
[c] Net counts per minute (SEA-stimulated minus medium control).

consistent with the idea that these glycoprotein antigens may induce release of fibroblast-stimulating lymphokines such as those previously described (7). It is noteworthy that cultivation of control spleen cells in the presence of ConA fraction II also resulted in the release of fibroblast-stimulating activity into culture supernatants, particularly since this fraction did not stimulate blastogenesis of control cells (Table 2). Alternatively, ConA fraction II might have stimulated release of activity from macrophages, cells which are known to be capable of secreting fibroblast-stimulating factors when appropriately stimulated in vitro. However, when we incubated normal resident peritoneal macrophages in the presence of ConA fraction II for 48 h, we were unable to detect appreciable fibroblast-stimulating activity in the resulting culture supernatants (193 cpm [net]), although supernatants from such macrophages stimulated with latex beads did contain stimulating activity (greater than threefold increase in counts per minute compared with medium control counts per minute). These observations not only fail to demonstrate a direct macrophage-stimulating effect of ConA fraction II but also serve to exclude the possibility that macrophage-derived proteinase activity altered egg material in a manner which rendered it biologically active.

In related studies, we observed that saline extracts of *S. mansoni* adult worms and cercariae failed to directly stimulate fibroblast proliferation when tested at concentrations between 0.01 and 10 μg of protein per ml. These observations suggest that the fibroblast-stimulating activity in schistosomes is stage specific and specificially detectable in the stage which is primarily associated with a fibrotic response in the host.

These in vitro findings may help to explain the pathogenesis of hepatic fibrosis in schistosomiasis mansoni and suggest that some soluble products of the schistosome egg, if secreted in vivo, might directly stimulate local fibroblast proliferation in the granuloma. If this material also diffused out into the portal perivascular tissue of humans, it might participate in development of the periportal clay-pipestem pattern of fibrosis characteristic of disease in humans. In addition, there appear to be components of SEA (ConA fraction II) which do not directly stimulate fibroblasts but may trigger elaboration of fibroblast-stimulating factors by cells present in the granuloma. Perhaps this involves elaboration of macrophage-activating factor by lymphocytes, with subsequent stimulation of macrophages to release the fibroblast-stimulating factor. In this regard, it is of interest that we have determined that macrophages isolated from granulomas constitutively secrete fibroblast-stimulating activity into culture supernatants (greater than sevenfold increase in counts per minute compared with medium control counts per minute), whereas no such activity was detected in similar supernatants of normal resident peritoneal macrophages. Presumably, the granuloma macrophages were activated for this secretory role in vivo. Further investigations of SEA-induced production or release of fibroblast-stimulating factors may help elucidate the mechanism by which the immunological network of the granu-

loma regulates hepatic fibrosis in schistosomiasis.

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