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Immunological and Biochemical Characterization of the Antigens from Hatch Fluid of *Schistosoma japonicum* Eggs

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Introduction

Soluble egg antigen (SEA) of *Schistosoma mansoni* is a complex mixture of antigens which evoke immune responses of hosts such as granulomatous hypersensitivity (Boros *et al.*, 1977; Pelley *et al.*, 1976b), other delayed-type responses (Boros *et al.*, 1977; Carter and Colley., 1979) and antibody production (Pelley *et al.*, 1976a). *S. mansoni* SEA is derived from eggs which contain miracidia. Bogitsh and Carter (1975) suggested that SEA might contain materials secreted by miracidia. Hatch fluid (HF) of *S. mansoni* eggs was shown both to induce and elicit granulomatous hypersensitivity (Boros and Warren, 1970). Hamburger *et al.* (1976) repored that *S. mansoni* HF contained considerable amount of major serological antigen (MSA_1), as well as MSA_2 and MSA_3.

SEA from eggs of *Schistosoma japonicum* was also suggested to be active immunologically (Warren *et al.*, 1975) and serologically (Long *et al.*, 1981a), and some antigens of *S. japonicum* eggs have been isolated (Tracy and Mahmoud, 1982; Carter and Colley, 1981; Long *et al.*, 1981b). However, *S. japonicum* HF has not yet been studied. we report here the serological reactivity and some characteristics of HF from *S. japonicum* eggs by means of double immunodiffusions and polyacrylamide gel electrophoresis.

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Materials and Methods

Preparation of crude antigens: Female ddY mice were infected percutaneously with *Schistosoma japonicum* (Japanese strain). Eight to nine weeks later, eggs were harvested from intestines of infected mice as follows. The intestines suspended in a fivefold volume of 0.85% NaCl were homogenized in a Waring blender. The homogenate was passed through a wire sieve of 50 mesh with excess 0.85% NaCl. The suspension was centrifuged at $500 \times g$ for 2 min, then the sediment was resuspended in 0.85% NaCl. These procedures were repeated five times. Almost all the eggs were detected in the sediment after centrifugation. The eggs were washed through a 100-mesh wire sieve two additional times. The sediment obtained was used as isolated eggs. The extent of purification was monitored microscopically. The isolated eggs were homogenized in phosphate-buffered saline (PBS), pH 7.2. The homogenate was stirred at 4°C overnight and centrifuged at $100,000 \times g$ at 4°C for 1 h. The resulting supernatant was used as crude soluble egg antigens (SEA). Hatch fluid (HF) was obtained by allowing 6×10^5 to 1.1×10^6 isolated eggs to hatch in 100 ml tap water for 24 h. The fluid was centrifuged at $1,700 \times g$ for 10 min. The sediment containing the eggshells and miracidia

was discarded. The supernatant was used as the source of HF and sterilized by passage through a 0.45 μm Millipore filter and concentrated by vacuum dialysis against PBS at 4°C. HF which had been prepared from 5×10^6 eggs contained 1.5 to 9.4 mg of protein (6 experiments).

Antisera: Infection sera were collected from mice infected 7 or 16 weeks earlier by percutaneous infection of *S. japonicum* cercariae. Mice were bled by heart puncture, and the pooled sera were centrifuged at 1,700 $\times g$ for 20 min and stored at −20°C.

Polyacrylamide gel electrophoresis: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 7.5% disc gels by the method of Weber and Osborn (1969). The samples were dissolved in SDS (0.1%) buffer with 2-mercaptoethanol (2-ME). Analytical disc gel electrophoresis was performed by the method of Davis (1964). After electrophoresis, the gels were stained for protein with Coomassie blue. Duplicate gels were stained for carbohydrate with the periodic acid-Schiff (PAS) reagent by the method of Zacharius *et al.* (1969).

Double immunodiffusion: Immunodiffusion analysis was performed by the method of Ouchterlony and Nilsson (1978) with 1.2% agar (Difco, Detroit, MI, USA) prepared in PBS. The precipitin lines were observed after an overnight diffusion at 25°C.

Physicochemical treatments of HF: HF (6.75 mg/ml) was treated by the addition of equal volumes of various reagents. a) Periodate oxidation of HF was carried out with 0.016–10 mM sodium metaperiodate (final concentration) in PBS, and the reaction mixture was kept in dark for 18 h. The reaction was stopped by dialysis against PBS. b) HF was boiled in a water bath for 5 or 30 min, followed by chilling on ice. c) HF was treated with 5% 2-ME (final concentration) for 18 h at 25°C followed by dialysis against PBS. d) HF was treated with 0.1 M glycine-HCl buffer (final concentration), pH 2.5, for 30 min at 25°C followed by neutralization.

Protein determination: The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Results

Immunodiffusion analysis of HF

HF was tested for serological reactivity with sera from mice infected with *S. japonicum* for 7 weeks (Fig.1). Double immunodiffusion tests indicated that HF prepared in the present experiments produced one sharp precipitin line on reaction with the sera. Crude SEA also produced one precipitin line. All precipitin lines produced were fused with each other. However, repeated freezing and thawing weakened the reactivity of HF.

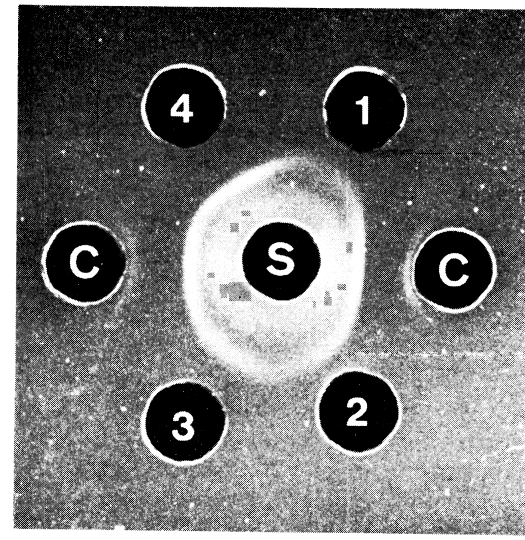

Fig. 1 Double immunodiffusion analysis of hatch fluid from *S. japonicum* eggs. The center well (s) contains sera from mice infected for 7 weeks with *S. japonicum*. Well Nos. 1, 2, 3 and 4 contain HF (6.75 mg/ml). HF in well 1 is the sample which have been frozen and thawed repeatedly. Well c contains crude SEA. Each well contains 20 μl.

Protein composition of HF

HF was subjected to SDS-PAGE and stained with Coomassie blue. Spectrophotometric scanning at 560 nm indicated that HF contained a minimum of 12–14 polypeptides, whereas SEA contained 18–20 polypeptides. The scan of typical gel is shown in Fig. 2. One of the major bands in HF, having rel-

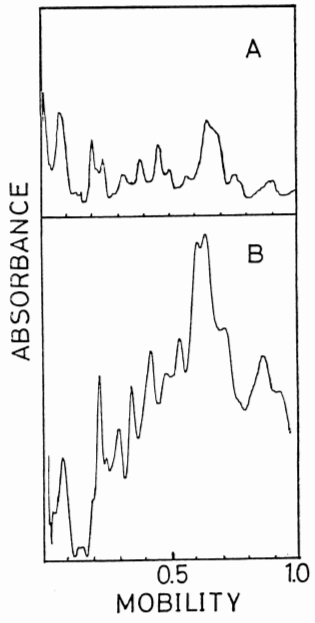

Fig. 2 SDS-polyacrylamide gel electrophoretic analysis of HF and SEA. A. 13μg of HF. B. 30 μg of SEA. Gels were stained with Coomassie blue and scanned at 560 nm.

ative mobility (Rf) of 0.07, correlated well with a band in SEA (Rf=0.07). This polypeptide represented 13% of the total HF area and 3.5% of the total SEA area under the peaks in the gels reacted with Coomassie. When the duplicate gels were stained for carbohydrate with PAS, SEA indicated at least 5 distinct bands with one band being very broad (Fig. 3B). In contrast, HF displayed one major band, having Rf of 0.07, and three minor bands (Fig. 3A). With both samples, an intensely PAS-positive material at the origin of each gel was detected.

To determine the electrophoretical profile of intact HF, untreated HF (without reduction) was subjected to analytical disc gel electrophoresis by the method of Davis (1964). At least eight bands were detected with Coomassie blue (Fig. 4). These bands had Rf of 0.05, 0.12, 0.13, 0.16, 0.23, 0.36, 0.71 and 0.76. The Coomassie blue staining pattern of SEA indicated 12-15 distinct protein bands. Two of major bands with Rf of 0.05 and 0.12 in SEA correlated well with the bands found

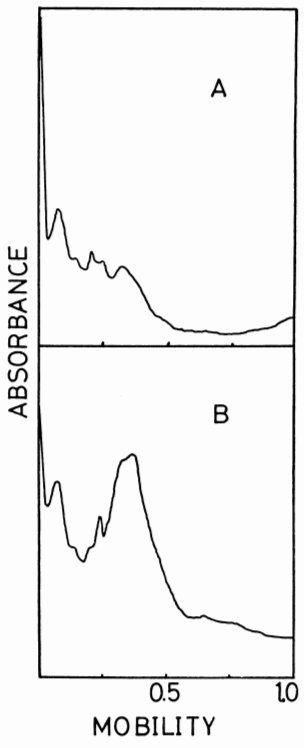

Fig. 3 Electrophoretic comparison of the PAS-reacting materials in HF and SEA. Materials were subjected to electrophoresis on SDS-polyacrylamide disc gels and stained with PAS. A. 13 μg of HF. B. 30 μg of SEA. Absorbance scans are 565 nm.

in HF, but the other major bands with Rf of 0.28 and 0.30 were not detected in HF.

The effect of various treatments on the serological reactivity of HF

Double immunodiffusion analysis of HF developed with 16-week infected mice sera indicated one precipitin line. HF (6.75 mg/ml) was subjected to various treatments. Treatments of HF by periodate (more than 2 mM) lost its substantial reactivity (Fig. 5). However, one minor precipitin line appeared between periodate-treated (more than 0.4 mM) HF and 16-week infected mice sera. This minor band was also observed after disapperance of main line. Fig. 6 shows the result of treatment of HF by 2-ME. Mild reduction of HF by 5% 2-ME at 25°C for 18 h lost its serological reactivity. Treatment of

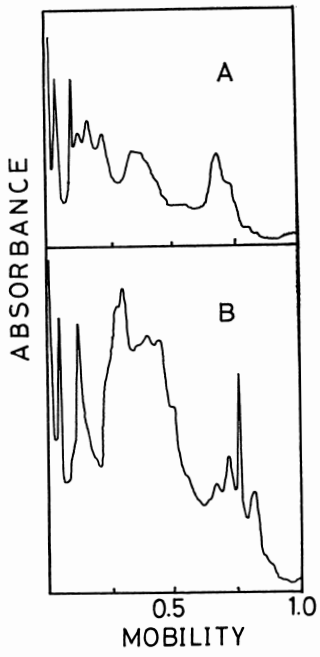

Fig. 4 Analytical disc gel electrophoresis of HF and SEA run on acrylamide gels. A. 13 μg of HF. B. 30 μg of SEA. Gels were stained with Coommassie blue and scanned at 560 nm.

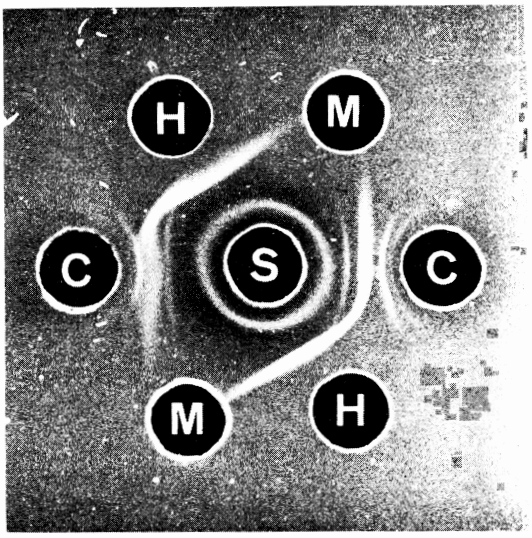

Fig. 6 Effect of 2-mercaptoethanol (2-ME) on serological reactivity of HF. The center well (S) contains serum from mice infected for 16 weeks with *S. japonicum*. Well M contains HF treated with 2-ME. Well H contains untreated HF. Well C contains SEA. Each well contains 20 μl.

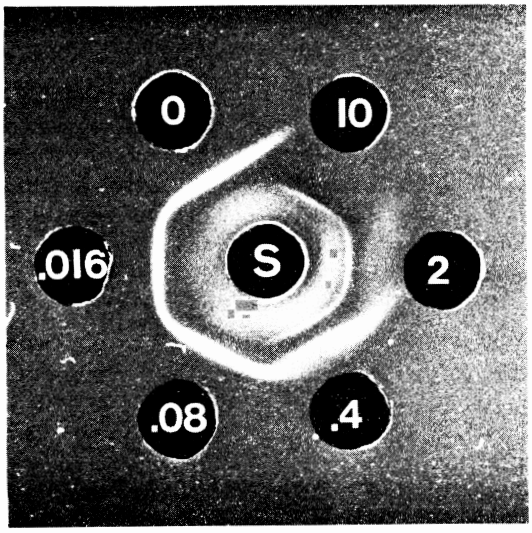

Fig. 5 Effect of periodate oxidation on serological reactivity of HF. The center well (S) contains sera from mice infected for 16 weeks with *S. japonicum*. The outer wells contain HF treated with 0.016, 0.08, 0.4, 2 and 10 mM sodium metaperiodate. Untreated HF (0) served as a control. Each well contains 20 μl.

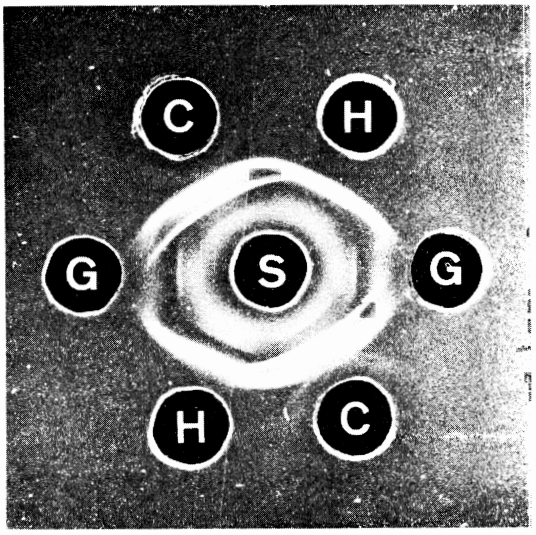

Fig. 7 Effect of glycine-HCl on serological reactivity of HF. The center well (S) contains the sera from mice infected for 16 weeks with *S. japonicum*. Well G contains HF treated with glycine-HCl buffer, pH 2.5. Well H contains untreated HF. Well C contains SEA. Each well contains 20 μl.

HF by heat at 100°C for 5 or 30 min also destroyed antigenicity of HF. When HF was treated with 0.1M glycine-HCl buffer, main precipitin line disappeared but a faint line was observed (Fig. 7).

Discussion

In the present study, we found that *S. japonicum* HF contained precipitating antigens which reacted with 7-week *S. japonicum* infected mice sera. A single major antigen was detected by double immunodiffusion against the sera. However, HF could be resolved into 12-14 Coomassie blue-reactive polypeptides when subjected to SDS-PAGE. Two of these polypeptides gave a strong reaction for carbohydrate with the PAS reagent. These results indicate that HF, as well as SEA, is a complex mixture of proteins and glycoproteins. Carter and Colley (1981) have isolated a highly purified antigen from *S. japonicum* SEA by an immunoaffinity column coupled with 7-week infected mice sera, which gave only one band on SDS-PAGE. One of the major polypeptides in HF, having Rf of 0.07, may be identical to the purified antigen by Carter and Colley (1981), because electrophoretical profile and strong PAS-reactivity of both antigens are closely similar to each other. This polypeptide (Rf=0.07) accounted for 13% of HF and 3.5% of SEA. Therefore, HF is suggested to contain one of major egg glycoprotein antigens, which induce antibody production at the early stage of infection, at a higher concentration.

Owhashi and Ishii (1981, 1982) reported that *S. japonicum* egg extracts contained allergens and eosinophil chemotactic factors. Whether these substances are contained in HF has not yet been studied. However, an intensely PAS-positive material detected at the origin of gel after electrophoresis of HF might possibly be responsible for an eosinophil chemotactic factor which had a high molecular weight of more than 900,000 and was stained with Coomassie blue and PAS.

Periodate oxidation, heating at 100°C and treatment with 2-ME were found to be effective in destroying the serological reactivity of antigens of HF. The extensive loss of serological reactivity after these treatments suggests that the antigenicity of HF is associated with both carbohydrate and peptide moieties of antigens.

Hamburger *et al.* (1982) reported that periodate oxidation of a purified glycoprotein antigen from *S. mansoni* eggs (MEG) resulted in a loss of molecular mass and of serological reactivity. They have demonstrated that the molecular weight of MEG dropped after mild periodate treatment from 70,000 to 30,000. In the present study, after periodate treatment, HF lost its substantial serological activity against 16-week infected mice sera. However, double immunodiffusion of periodate-treated HF gave an additional faint precipitin line which had not been observed on reaction with untreated HF. Whether the additional faint precipitin line is due to dropping of large molecular antigen to small molecule is not yet known. However, it should be noted that antigenic substance which is activated by treatment of periodate is present in HF.

Many antigens have been isolated from helminths by immunoaffinity chromatography (Carter and Colley, 1979 ; Harrison *et al.*, 1979 ; Hillyer and Satiago de Weil, 1977 ; Suzuki *et al.*, 1975 ; Taylor and Butterworth, 1982). Glycine-HCl has been used routinely for the elution of antigen by antibody-affinity chromatography with CNBr-activated Sepharose 4B. Hillyer and Santiago de Weil (1977) have isolated *Fasciola hepatica* antigens cross-reacted with *S. mansoni* by immunoaffinity columns and glycine-HCl, because only the antigens were stable to the acid buffer whereas most of immunological reactivity was destroyed by the buffer (Hillyer and Rodriguez Ramos, 1980). However, our result showed that antigenicity of *S. japonicum* HF was unstable to the acid buffer. Thus, when performing affinity chromatography with HF antigens, acid buffer would not be the useful method for eluting the antigens.

The importance of the antigens which react with 7-week *S. japonicum* infected mice sera

was demonstrated by Warren *et al.* (1978). They reported that double immunodiffusion of SEA developed with serum from chronically infected humans yielded a single precipitin line. The precipitin line showed complete identity with the primary precipitin line developed with mice sera at 7 weeks post infection. Since HF was demonstrated to contain these antigens and to be prepared easily in this study, immunodiagnosis such as enzyme-linked immunosorbent assay using HF would be a potential tool. Kamiya (1981) has developed an intraoval precipitin (IOP) test in schistosomiasis japonica by using formalin fixed tissue section. The antigens detected between a miracidium and an eggshell may by partially identical to that of HF.

On the other hand, schistosome eggs trapped in the tissues of hosts have been considered to excrete antigenic materials which evoke strong humoral and cellular responses (Smithers and Doenhoff, 1982). Therefore, the antigens of HF may play a role in granuloma formation and other immunological response of the host. Further studies of the HF antigens would help to explain some of the clinical manifestation of the schistosome infection.

Summary

Hatch fluid (HF) of *Schistosoma japonicum* eggs was characterized with double immunodiffusion tests and polyacrylamide gel disc electrophoresis (PAGE). HF exhibited a single precipitation band upon immunodiffusion against sera from mice infected with *S. japonicum* for 7 weeks. With SDS-PAGE, at least 12–14 protein bands (Coomassie blue staining) and at least 4 glycoprotein bands (PAS reactive) were present in HF. Analytical PAGE of HF displayed at least 8 protein bands. The effect of various treatments on its serological reactivity was determined by double immunodiffusions. The serological reactivity of HF was unstable to heat at 100°C for 30 min, to 2-mercaptoethanol and to glycine-HCl buffer (pH. 2.5). Periodate oxidation also resulted in a substantial loss of serological reactivity. These results show that HF is a complex mixture of proteins and glycoproteins, and suggest the importance of both carbohydrate and peptide of major antigens of HF to its serological reactivity.

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日本住血吸虫虫卵の孵化液由来抗原の免疫生化学的性状

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日本住血吸虫虫卵の孵化由来抗原（HF）の性状を，二重免疫拡散法，ポリアクリルアミドゲル電気泳動法（PAGE）を用いて検討した．

HFは，日本住血吸虫感染マウス血清（7週）とのオクタロニー法で，1本の沈降線を形成し，この沈降線は，可溶性虫卵粗抗原（SEA）が感染血清との間に形成した沈降線と融合した．SDS-PAGEでは，HFには，12～14本のタンパクバンド及び4本のPAS陽性バンドが検出された．非還元下でのPAGEでは，HFは8本のタンパクバンドに分離した．

HFの抗原性は易熱性で，過ヨウ素酸処理，メルカプトエタノール，酸処理（pH 2.5）により失活した．

以上の結果から，HFはタンパク質及び糖タンパク質を含む異質性の成分から成り，感染初期マウス血清と反応する抗原を含むことが明らかとなった．さらに，その抗原活性部位には，タンパク部位及び糖部位が関与することが示唆された．