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Effect of host portal and peripheral sera fractions on cell proliferation of *Schistosoma mansoni* schistosomules

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Abstract. Schistosomules of *Schistosoma mansoni* were incubated in medium containing hamster (highly susceptible host) portal or peripheral venous serum, or rat (poorly susceptible host) portal or peripheral venous serum or their fractions in the presence of bromodeoxyuridine (BrdU) in order to determine effects of host sera on cell proliferation. BrdU labeling indices (BLIs) were significantly increased in the presence of portal (33.05±0.70, $p<0.05$), but not in peripheral serum (19.1±0.85, $p>0.05$) of hamsters, compared to schistosomules cultured in presence of control medium (18.96±0.66). This stimulatory effect was substantially reproduced by fraction 4 (31.03±0.69, $p>0.05$) separated from hamster portal serum. In contrast, no significant differences were observed in the BLIs in rat portal or peripheral sera as well as their fractions when compared to control medium. Taken together, it was concluded that portal serum of hamster and its fraction which includes a low molecular weight protein (20 kDa) enhanced cell proliferation of *S. mansoni* schistosomules. This could explain the localization and preference of *S. mansoni* schistosomules and worms to portal-mesenteric venous system.

INTRODUCTION

It has been observed that different species of *Schistosoma* bear a remarkable predilection for particular anatomical sites (Cheever, 1965), resulting in complication in specific organ systems. In man, the preference of *Schistosoma haematobium* for the veins of the urinary system causes inflammation and fibrosis of the bladder and /or ureters (Warren, 1978a). Similarly, localization of *Schistosoma mansoni* and *Schistosoma japonicum* in the human portal-mesenteric venous system results in colonic and hepatic granulomatous disease with progressive hepatic fibrosis that becomes manifest as the morbid complications of portal hypertension (Warren, 1978b). It is unclear whether the site preference of *S. mansoni* could be dependent on a constituent of portal blood that is not present in the periphery. This might take the form of a substance that the parasite recognizes or requires to develop. In this regard, Wu *et al.* (1985) showed that portal serum and its fraction ranging between 2 and 50 kDa stimulate egg deposition of *S. mansoni* worms *in vitro*. The range of this low molecular weight substances was larger than would be expected for simple carbohydrates, amino acids or free fatty acids absorbed from gastrointestinal tract. Previous studies showed also that portal serum of human and hamster (susceptible hosts) and a 1-50 kDa fraction separated from human portal sera by ultrafiltration stimulate cell proliferation of *S. mansoni in vitro* (Shaker *et al.*, 1998; Draz, *et al.*, 2008). Also, mice subjected to partial portal vein ligation yielded fewer *S. mansoni* worms than intact controls, this data support the

concept that a leaky portal system is the main factor in resistance to reinfection in murine schistosomiasis (Medeiros & Andrade, 1986).

The purpose of this study was to examine the effects of whole portal and whole peripheral sera as well as their fractions of a highly susceptible host (hamster) and poorly susceptible host (rat) on cell proliferation of *S. mansoni* schistosomules *in vitro*. This study could help in identifying the host stimulating factor(s) which enhance the maturation of the parasite.

MATERIALS AND METHODS

Collection of portal and peripheral sera from hamsters and rats

Rats (*Rattus norvegicus*) were obtained from the animal house of the National Research Center, Cairo, Egypt. Golden hamsters (*Mesocricetus auratus*) were obtained from Schistosome Biological Supply Program in Theodore Bilharz Institute, Cairo, Egypt. All animals were housed under specific-pathogen-free conditions in an environmentally controlled room at 22 ± 3ºC and a 12:12-hr light/dark cycle, with food and water available *ad libitum*. All animals received humane care according to National Institute of Health (USA) guidelines. Portal and peripheral blood samples were collected, after overnight fasting, from 10 hamsters (body weight 120–180 g) as well as from 10 rats (body weight 110-170 g). Animals were anesthetized with thiopental diluted with deionized water to a concentration of 0.5 g/10 ml and administered (100 mg/kg) by intraperitoneal (i.p.) injection. Portal blood was obtained by withdrawal into a syringe via a 20-gauge needle by direct puncture facing the mesenteric venous bed. About 3–5 ml of portal blood were collected from each animal. Peripheral blood samples were obtained from the inferior vena cava from these animals. Whole blood samples were allowed to clot, and then they were spun down at 3,000 rpm for 15 min in a cooling centrifuge. Portal hamster serum samples were pooled and the same procedure was done with peripheral hamster sera and rat sera (either portal or peripheral). Sera were used either immediately or within 2 weeks of collection after storage at -70ºC.

Fractionation of portal and peripheral sera

Reagent and gel preparation for polyacrylamide gel electrophoresis (PAGE) were based on the manufacturer's instructions (Biorad, Richmond, CA, USA), according to Laemmli buffer system (Laemmli, 1970). Native polyacrylamide gel electrophoresis (PAGE) was performed in a vertical slab gel unit (Mini Protean II, Biorad). Pooled portal and peripheral sera were fractionated by native PAGE into four fractions according to electrophoretic mobility (fraction 1 has the least mobility while, fraction 4 has the highest mobility). Method in brief, 200 µl of serum was run in a stacking gel at 5% acrylamide concentration and separating gel with 12.5% acrylamide concentration. The electrophoresis of serum samples was run for about 2 hrs at 130 V. The electrode-running buffer was Tris base (3 g/liter), glycine (14.1 g/liter), pH 8.3 for both electrophoretic processes as well as for electroelution. Sodium dodecyl sulfate (SDS)-PAGE was done with the same acrylamide concentration but in presence of SDS. The method described by Merril (1990) was used for silver staining of the gels. After running the native gel, a slice (about 2 cm) was cut vertically along the gel and proteins were stained with coomassie. The stained gel slice was aligned with the remainder of the gel and then gel was divided into four portions corresponding to the stained proteins and then excised horizontally. Electroelution was done for gel slices using electroeluter Model 422 (Biorad, Richmond, CA, USA) according to manufacturer's instructions (Fig. 1). Native PAGE and electroelution was repeated nine times for each sample and the eluted protein fractions were pooled for each fraction separately and were concentrated to the original volume by dialysis through membranes (Spectrum Labs, Rancho Dominguez, California), with a 1-kDa exclusion limit against polyethylene glycol 20-kDa. SDS-PAGE is applied to an aliquot of the concentrated eluted serum protein

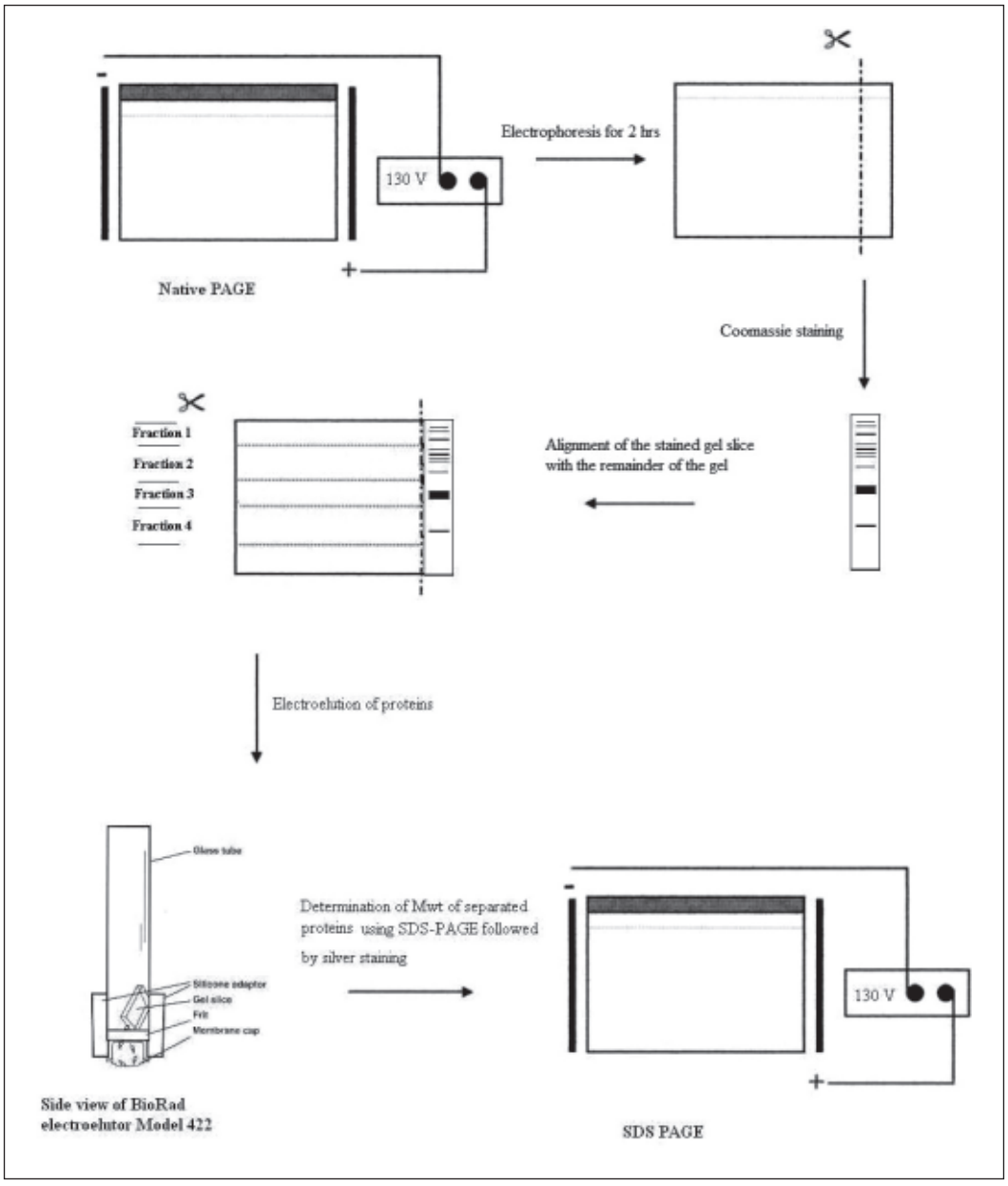

Figure 1. A schematic representation of the fractionation of serum samples. Adapted from Sa-Pereira *et al.*, 2000

fractions followed by silver staining for molecular weight determination of separated proteins of each eluted fraction.

Recovery of *S. mansoni* schistosomula from the hepatic portal system

Schistosoma mansoni cercariae were shed from *Biomphalaria alexandrina* snails, and hamsters were exposed, using a body immersion method (Liang *et al.*, 1987), to 500–1,000 cercariae for recovery of schistosomes from the hepatic portal system. Schistosomula were collected by sterile perfusion from infected golden hamsters 20 days after exposure (Duvall & DeWitt, 1967), the timing was selected to simulate the situation shortly after arrival of these organisms in the portal venous circulation.

Each hamster received the anesthetic-anticoagulant solution (100 mg/kg thiopental and 100 units/ml heparin) by i.p. injection. Animals were dissected to expose internal viscera. Liver perfusions were accomplished by passing an 18-gauge needle into the heart. The portal vein was transected by scissors close to its entrance to the liver. Schistosomula were collected in a nylon net with a mesh grade of 150 µm (Small Parts, Miramar, Florida) and washed twice with RPMI 1640 media and once with sterile culture media. They were kept in sterile dishes for *in vitro* cultivation.

Assay for *in vitro* cell proliferation

Twenty to thirty schistosomules per dish were incubated at 37°C in the following media for 2 days: (a) RPMI-1640 medium with penicillin (100 units/ml) and streptomycin (0.1 mg/ml) plus 10% fetal calf serum (FCS) {as negative control medium}; (b) the above medium + 100 µmol BrdU {as positive control medium}; (c) the medium of item (b) + 10% whole portal hamster serum; (d) the medium of item (b) + 10% of the fraction 1 of portal hamster serum. The experiment was also performed with the other fractions separated from portal hamster serum (fraction 2, 3, and 4); (e) the same steps from (c-d) were performed with peripheral hamster, portal rat, peripheral rat sera and their fractions. The worms were fixed in 70% ethanol in glycine buffer (pH 8.3). The fixed worms were embedded in paraffin blocks, cut longitudinally into sections of 5µ thick and stained with anti-BrdU by a BrdU labeling and detection kit (Roche Company, Mannheim, Germany). Identical sections were stained with hematoxylin and eosin, and the BrdU Labeling Index (BLI) determined according to the formula:

$$\text{BLI (\%)} = \frac{\text{Number of nuclei stained by anti-BrdU}}{\text{Total number of nuclei stained by hematoxylin}} \times 100$$

The stained nuclei were counted in a blinded fashion across the whole worm, and the minimum number of worms examined in the sample per time point was six.

Statistical analysis

An unpaired Student's t-test was used to determine significant differences between BLIs in schistosomula cultured in media containing various sera. In all cases, a P value <0.05 was selected as the minimal criterion for statistically significant difference.

RESULTS

***In vitro* cell proliferation assay for *S. mansoni* schistosomules cultured in media containing hamster portal and peripheral serum fractions**

Table (1) shows the BLIs for 20 day old schistosomules cultured for 2 days in media containing various hamster portal and peripheral serum fractions. The data revealed that the mean BLI of schistosomules in control medium (RPMI plus 10% fetal calf serum) was 18.96 %. However, in whole hamster portal serum, the BLI of 33.05% was substantially and significantly greater under identical conditions. Of interest was the fact that schistosomules cultured in hamster portal serum fraction 4 had an almost a high

Table 1. Bromodeoxyuridine labeling indices (BLIs) of 20 day old *S. mansoni* schistosomules cultured for 2 days in media containing portal and peripheral hamster sera and their fractions

Sera Source		RPMI	Whole sera	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Portal Hamster	Mean BLI % ± S.E.	18.96 ± 0.66	33.05 ± 0.70	18.9 ± 0.54	18.83 ± 0.70	19.21 ± 0.79	31.03 ± 0.69
	P value		P < 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05
Peripheral Hamster	Mean BLI % ± S.E.	18.96 ± 0.66	19.1 ± 0.85	18.86 ± 0.81	18.8 ± 0.87	18.99 ± 0.72	18.36 ± 0.58
	P value		P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05

BLI as whole hamster portal serum, 31.03%. While fractions 1, 2 and 3 of hamster portal sera had a BLI of 18.9%, 18.83% and 19.21% respectively which were not significantly different from control medium. In presence of whole hamster peripheral serum, the BLI of 19.1% was not significantly different from control medium. The mean BLI % for fractions 1, 2, 3 and 4 of peripheral hamster sera had a BLI of 18.86%, 18.8%, 18.99% and 18.36% respectively which were also not significantly different from control medium.

In vitro cell proliferation assay for *S. mansoni* schistosomules cultured in media containing rat portal and peripheral serum fractions

Table (2) shows the individual data of the BLI for 20 day old schistosomules cultured for 2 days in media containing various rat portal and peripheral serum fractions. The data revealed that the mean BLI of schistosomules in control medium (RPMI plus 10% fetal calf serum) was 18.96%. While, in presence of whole rat portal serum, the BLI of 22% was not significantly different from control medium. The mean BLI% for fractions 1, 2, 3 and 4 of rat portal sera had a BLI of 19.07%, 18.97%, 19.2% and 20.915% respectively which were not significantly different from control medium. In presence of whole rat peripheral serum, the BLI of 19.3% was not significantly different from control medium. The mean BLI% for fractions 1, 2, 3 and 4 of rat peripheral sera had a BLI of 18.88%, 18.84%, 18.92% and 18.45% respectively which were also not significantly different from control medium.

DISCUSSION

It is not clear whether the migration of schistosomes to the mesenteric circulation is mainly due to mechanical factors (trapping of young re-circulating schistosomules in the liver) or due to biochemical reasons (different composition of mesenteric versus systemic blood). In either case it is likely that schistosomes have adapted to the special biochemical environment of the mesenteric circulation. Experimental evidence for schistosome adaptation to mesenteric blood was provided by Wu *et al.* (1985) and Wu & Wu (1986) who showed that media containing portal serum from various mammalian sources have components that stimulate *S. mansoni* oviposition *in vitro*. This phenomenon was observed in the presence of portal serum from humans and hamsters (highly susceptible hosts), as well as rats and rabbits (poorly susceptible hosts), but not in peripheral serum from any of the studied hosts. Furthermore, a portal serum fraction with molecular weight ranged from 2- to 50-kDa was found to be the most effective one in stimulating oviposition (Wu *et al.*, 1985). Moreover, the immunity displayed by mice with a chronic infection was shown to be an artifact of a leaky hepatic portal system (Wilson, 2009), the collateral circulation probably diverted arriving immature worms back to the general circulation where they died as the result of being out of their natural habitat rather than from immune killing (Medeiros & Andrade, 1986). It was concluded that a stimulatory substance(s) is present in portal serum which stimulate

Table 2. Bromodeoxyuridine labeling indices (BLIs) of 20 day old *S. mansoni* schistosomules cultured for 2 days in media containing portal and peripheral rat sera and their fractions

Sera Source		RPMI	Whole sera	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Portal rat	Mean BLI % ± S. E.	18.96 ± 0.66	19.3 ± 0.22	19.07 ± 0.53	18.97 ± 0.55	19.2 ± 0.78	20.915 ± 0.23
	P value		P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Peripheral rat	Mean BLI % ± S. E.	18.96 ± 0.66	19.3 ± 0.70	18.88 ± 0.85	18.83 ± 0.47	18.92 ± 0.54	18.45 ± 0.63
	P value		P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05

oviposition of *S. mansoni* worms and that was absent in peripheral serum or in blood regardless of the susceptibility of animals to *S. mansoni* infestation. Also, Badr *et al.* (1999) reported that bile salts increase the number of *S. mansoni* eggs deposited *in vitro* monitored for two weeks, they attributed such finding to the presence of high concentration of bile salts in portal blood by 5-to 10- times more than systemic blood in fasting state in human.

Results of the current study demonstrated a significantly increased cell proliferation of *S. mansoni* schistosomules in the presence of hamster portal serum compared with peripheral serum. Consistently, it has been shown that both human and hamster portal sera stimulated cell proliferation of *S. mansoni in vitro* (Draz *et al.*, 2008). In an attempt to better defining the biochemical factor(s) that may be responsible for the increased proliferation in portal serum, we tested the effect of portal and peripheral sera fractions of both hamster as a highly susceptible host and rat as a poorly susceptible host on cell proliferation of *S. mansoni* schistosomules. The direct comparisons of the effects of various serum components on cell proliferation of schistosomules indicated that a substantial proportion of growth stimulatory activity in portal hamster serum can be attributed to fraction No. 4 which includes a 20 kDa protein together with some other high molecular weight proteins (Fig. 2). The observed proliferative effect of fraction No. 4 separated from portal hamster serum was not likely due to the presence of high molecular weight proteins but it is most likely due to the presence of low molecular weight protein (20 kDa) because fractions No. 1, 2, and 3 separated from hamster portal serum also contain the same high molecular weight proteins but there was no effect of these fractions on cell proliferation of *S. mansoni* schistosomules. These results

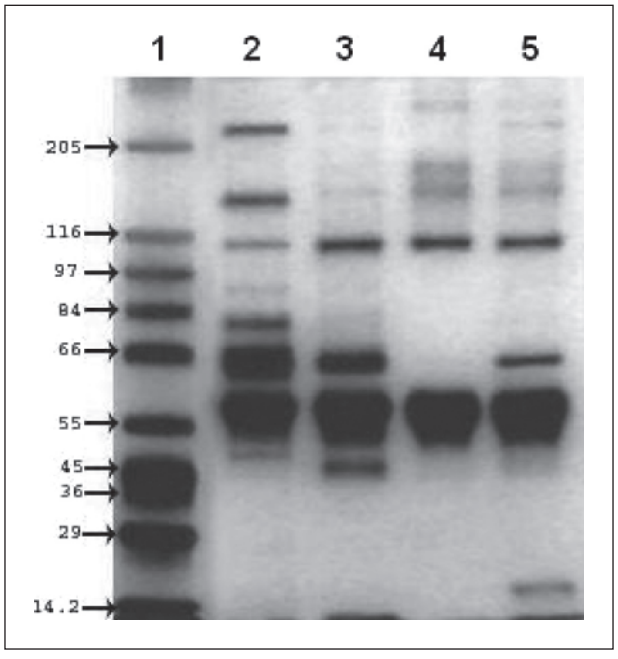

Figure 2. SDS-PAGE of hamster portal serum fractions separated by native electrophoresis (PAGE) and recovered by electroelution. Lane 1: M.Wt Marker (M.Wt 205, 116, 97, 84, 66, 55, 45, 36, 29 and 14.2 kDa), lane 2: Portal serum fraction No.1, lane 3: Portal serum fraction No. 2, lane 4: Portal serum fraction No. 3, lane 5: Portal serum fraction No. 4

are in agreement with our previous data done using sera of another susceptible host (human) which showed that human portal serum and its fraction ranged from 1- to 50- kDa enhanced cell proliferation of schistosomules compared with peripheral serum (Shaker *et al.*, 1998*)*. Consistently, it has been shown that gene expression profile of *S. japonicum* schitosomula maintained *in vitro* were significantly different from those obtained *in vivo* from lung tissue or adult worms. This could be due to the absence of host environmental factors for schitosomula cultured *in vitro* which influence the development of schistosomes (Chai *et al.*, 2006). *In vivo* sourced schistosomes is recommended for molecular, and biochemical studies, however schistosomula cultured *in vitro* in presence of host serum factors required for maturation could be also used.

Taken together, it was concluded that a stimulatory substance or substances for cell proliferation was present in hamster (highly susceptible host) portal serum. The stimulatory effect of portal serum could be attributed to fraction 4 which includes a protein of molecular weight (20 kDa). Further studies are needed to characterize the stimulatory factor(s) which enhance the maturation of the parasite, blocking the activity of this stimulatory factor(s) could help in curing the disease through prevention of *S. mansoni* maturation.

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