Title: Soluble egg antigen of Schistosoma heamatobium induces HCV replication in PBMC`s from patients with chronic HCV infection.

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Author's response to reviews: see over
The authors should give a proper reply to the question why they have chosen to use S. haematobium derived SEA instead of S. mansoni. If SEA, like they claim, does not differ substantially between the two species, they should provide evidence for this statement.

Co-infection with HCV and Schistosoma mansoni has been reported by several authors to be associated with higher HCV titers, higher incidence of cirrhosis and hepatocellular carcinoma as well as higher mortality rate (references 2, 8, 9, 10). However similar studies on S. haematobium derived SEA has been scarcely reported. It is well established that S. haematobium infection is the endemic Schistosoma species in upper Egypt and anti HCV is still relatively high (Medhat A, Shehata M, Magder L S, Mikhail N, Abdel-baki L, Nafeh M, Abdel-Hamid M, G. Strickland T, and Fix AD. Am. J. Trop. Med. Hyg., 2002; 66(5), pp. 633–638: hepatitis C in a community in upper Egypt: risk factors for infection).

There are indirect evidences that SEA from both species are related:

- It was shown that immune responses of patients infected with S haematobium and S mansoni were found to be similar so that differentiation could not be established (Norden AP, Strand M. Exp Parasitol. 1984 Dec; 58(3):333-44: Schistosoma mansoni, S. haematobium, and S. japonicum: Identification of genus- and species-specific antigenic egg glycoproteins). Besides, most of the SEA ELISA screening tests used to diagnose Schistosomiasis failed to provide indication of the infecting species of Schistosoma in areas where both S. haematobium and S. mansoni are endemic due to cross reactivity (Turner P, Laloo K, Bligh J, Armstrong M, Whitty CIM, Doenhoff MJ and Chiodini PL. Journal of clinical pathology 2004; 57:1193-1196: serological speciation of human Schistosome infections by ELISA with a panel of three antigens).

*The authors should update their references with respect to the constituents of SEA. The cited paper dates from 1977. There are numerous more recent papers giving details on which constituents are found in SEA.

This 1977 paper was cited for the original method of SEA preparation. However, paper describing the SEA constituents are dated (1989-2002, references 12, 14, 26, 27, 28, 29).

*The question on a dose-dependent effect of SEA on PMBC proliferation and on viral replication remains unanswered.

Actually, a preliminary experiment was done on normal PBMC similar to what has been published by our team on uroepithelial cells (ElAwady et al., 2001) to explore the optimum concentration of SEA that induces cell proliferation. Cells were treated with 10, 50, 100 and 150 µg /ml culture of SEA. Results obtained were similar to previous studies on uroepithelial cells that 50µg/ml culture is optimal, since cell proliferation was nominal at lower concentrations whereas higher concentrations of SEA were toxic for the cells. These data were referred to as (results not shown) in the manuscript. Starting from this point we believed that analysis of the cell proliferative dose of SEA (50 µg/ ml) on viral replication was more logic to focus on.
Referee 2

* Clear rationale should be presented to explain why S. haematobium derived SEA was used instead of S. mansoni derived SEA. The authors mention that they have previously used haematobium derived SEA in urinary bladder cancer studies and state that the differences between both antigens are minimal. Here the setting is different therefore this point should be further discussed in the context of previous literature.

- Co-infection with HCV and *Schistosoma mansoni* has been reported by several authors to be associated with higher HCV titers, higher incidence of cirrhosis and hepatocellular carcinoma as well as higher mortality rate (references 2, 8, 9, 10). However similar studies on *S. haematobium* derived SEA has been scarcely reported. It is well established that *S. haematobium* infection is the endemic Schistosoma species in upper Egypt and anti HCV is still relatively high (Medhat A, Shehata M, Magder L S, Mikhail N, Abdel-baki L, Nafeh M, Abdel-Hamid M, G. Strickland T, and Fix AD. *Am. J. Trop. Med. Hyg.*, 2002; 66(5), pp. 633–638: hepatitis C in a community in upper Egypt: risk factors for infection).

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* Figure 2 needs to be improved and the S.D. should be shown in the figure.

Thanks for the referee. The figure was modified as requested.
Soluble egg antigen of *Schistosoma Haematobium* induces HCV replication in PBMC from patients with chronic HCV infection

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**Running head**: Soluble egg antigen induces HCV replication.

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Abstract:

**Background/Aim:** To examine, in vitro, the effect of soluble egg antigen (SEA) of *S. haematobium* on intracellular HCV RNA load in peripheral mononuclear cells (PBMC) as well as on cell proliferation in patients with chronic HCV infection. **Methods:** PBMC from 26 patients with chronic HCV infection were cultured for 72 hours in presence and absence of 50 µg SEA / ml medium. Intracellular HCV RNA quantification of plus and minus strands was assessed before and after stimulation. PBMC from five healthy subjects were cultured for 7 days, flow cytometric analysis of DNA content was used to assess the mitogenic effect of SEA on PBMC proliferation compared to phytoheamaglutinin (PHA). **Results:** Quantification of the intracellular viral load showed increased copy number /cell of both or either viral strands after induction with SEA in 18 of 26 patients (69.2%) thus indicating stimulation of viral replication. Flow cytometric analysis showed that mean ± S.D. of percent values of cell proliferation was induced from 3.2 ± 1.5% in un-stimulated cells to 16.7 ± 2.5 % and 16.84 ± 1.7 % in cells stimulated with PHA and SEA respectively. **Conclusion:** the present study supports earlier reports on SEA proliferative activity on PBMC and provides a strong evidence that the higher morbidity observed in patients co-infected with schistosomiasis and HCV is related, at least in part, to direct stimulation of viral replication by SEA.

**Key words:** hepatitis C virus, SEA, *schistosoma haematobium*, PBMC, viral replication, cell proliferation.
Introduction:

Hepatitis C Virus (HCV) is the major agent in non A non B hepatitis with serious complications ranging from chronic inflammatory disease to hepatic cirrhosis and end stage liver failure or hepatocellular carcinoma (HCC). It is estimated that 170 millions world wide are infected with HCV. Egypt has unusually high prevalence of hepatitis C resulting in high morbidity and mortality from liver disease. Approximately 20% of blood donors are seropositive for HCV antibodies [1, 2, 3, 4, 5]. Schistosomiasis is another hepatotropic infection with a major burden on Egyptian patient population particularly in rural societies [1, 6, 7]. Co-infections with Schistosoma mansoni were repeatedly shown to augment pathogenesis induced by HBV and HCV hepatitis [2, 8, 9]. Subjects co-infected with HCV accelerate advancement of liver disease to the chronicity of HCV infection, cirrhosis and hepatocellular carcinoma and high incidence of viral persistence [10]. However similar studies on Schistosoma haematobium derived SEA has been scarcely reported.

Several reports attempted to reveal the underlying mechanisms of the severity of coinfection. The majority of these have focused on the role of growth factor rich soluble egg antigen (SEA), in contrary to adult worm soluble antigens, on angiogenic processes and granuloma formation mediated by peripheral blood mononuclear cell (PBMC) [11] and endothelial cell proliferation [12] through distinct intracellular signaling pathway [13]. Such mitogenic activity of SEA was shown to be cell specific, dose dependent and involves up regulation of cell cycle engine promoting genes [14].
Although HCV titer was shown to be higher in patients co infected with HCV and Schistosomiasis than patients with HCV infection alone, direct data on the effect of SEA on intracellular HCV replication in PBMC derived from patients with chronic hepatitis C are yet unavailable. Thus the rational of this study is to examine, in vitro, the influence of SEA on intracellular HCV RNA load in PBMC from patients with chronic HCV infection. Careful study of cell proliferation response to SEA allows analysis of relative induction in intracellular viral load versus stimulation of cell proliferation.

**Subjects and Methods**

**Subjects**

Twenty six patients were enrolled in this study. All patients had detectable HCV antibodies by third generation ELISA (Dia Sorin, Torino, Italy). Serum concentrations of HCV RNA varied from $29 \times 10^3$ to $3 \times 10^6$. Twenty four patients had genotype 4 of HCV genome, while the remaining two had 1b. None of the patients received treatment for HCV. All the patients had undetectable levels of HBsAg, schistosoma and HIV antibodies. Five subjects served as controls for normal PBMC stimulation studies. None of the 5 control subjects had detectable sero markers for viral or parasitic infections.
**Methods**

Effect of SEA on cell proliferation

A lyophilized preparation of the soluble fraction of mature live *S.hematobium* eggs was supplied by Theodore Bilharz Research Institute, Imbaba, Egypt.

Ten ml blood were collected from each normal control subject and PBMC were separated from whole blood using Ficoll separating solution. Cells were washed with PBS three times. Mixtures were then centrifuged at 1600 rpm for 10 min to collect the washed cell pellet. After the last wash, cells were re-suspended in 1ml RPMI-1640, supplemented with 10% FCS, counted and adjusted with RPMI to be 0.75 million cells/ml media. Cells were then plated into a 6 well plate at 2 million cells per well. A preliminary experiment was done to explore the optimum concentration of SEA that induces cell proliferation. Cells were treated with 10, 50, 100 and 150 µg /ml culture of SEA. Results obtained proved that 50µg/ml culture is optimal (data not shown). To compare the stimulatory effect of SEA with phytoheamaglutinin (PHA), cells were stimulated with PHA at 5µg/ml culture medium, other two wells were stimulated with SEA at 50µg/ml medium, and the last two wells were left un-stimulated as control. Cells were cultured in a humidified atmosphere at 37°C, 5% CO₂ for 7 days. Media were changed every 48 hours. After 7 days, cells were washed, permeablized with 0.1% triton X-100 solution (v/v) for 6 min at 4°C, then stained with 50 µg/ml propidium iodide (PI) as a DNA-specific fluorochrome for 30 min at 4°C in a dark place. DNA index analysis was
performed on FACS Calibur flow cytometer equipped with logarithmic amplifiers.

Effect of SEA on HCV replication in PBMC:

Fifteen ml of blood were withdrawn from each patient, five of them were collected on EDTA and were used to extract total cellular RNA from PBMC (to assess the presence of intracellular HCV strands in PBMC), the remaining ten ml blood were collected on heparin to separate PBMC using Ficoll for in vitro experiments. Cells were washed 5 times with PBS, then re-suspended in RPMI 1640 supplemented with 10% FCS at a concentration of $0.75 \times 10^6$ / ml, plated on a 6 well plate, and cultured at $37^0C$, 5% CO$_2$ in absence or presence of SEA (50µg/ml culture). After 72hr, cells were counted (using haemocytometer), collected, washed seven times with PBS, then subjected to RNA extraction with guanidinium isothiocyanate according to Chomczynski and Sacchi\cite{15}.

Reverse transcription –polymerase chain reaction (RT-PCR)

Total cellular RNA was reverse transcribed to cDNA in 25 µl reaction mixture containing 20 U of AMV reverse transcriptase (promega, Madison, WI, USA), 200 µM of each dNTP, 25 pmoles of either antisense primer (1CH: 5’-GGT GCA CGG TCT ACG AGA CCT-3’) for plus strand or sense primer (2CH: 5’-AAC TAC TGT CTT CAC GCA GAA -3’) for minus strand. The first round PCR was done in a total volume of 50 µl containing 200 µM of each dNTP, 1 x reaction buffer (10mM Tris-HCl pH 8.8, 1.5 mM MgCl$_2$, 50mM KCl and 0.1% triton X-100), 2 U Taq polymerase (Finnzyme, Finland), 50 pmoles from each primer (2 CH and
P2: 5′-TGC TCA TGG TGC ACG GTC TA -3′). Amplification was performed by 35 cycles of thermal cycling, each consists of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min followed by a final extension step for additional 10 min. The reaction was then cooled to 4°C. Twenty percent of the reaction was taken for a second amplification round (35 cycles) with the internal pair of primers (D1: 5′-CGC AGA AAG CGT CTA GCC AT -3′ and D2: 5′-ACT CGG CTA GCA GTC TCG CG -3′). Cycling conditions on the thermal cycler were the same as the first round. Products of PCR were analyzed on 2% agarose gel electrophoresis and photographed.

Quantification of intracellular HCV RNA

Plus–strand RNA was transcribed in vitro (using sp7 primer and transcription in vitro kit, Promega, WI) using as template a cloned fragment containing the entire 5′ UTR in PGEM-T plasmid (unpublished data). 5′UTR RNA was quantified spectrophotometrically and standard amounts (2x10⁶ - 2x10⁷ copies) were reverse transcribed and amplified using the method described above. Amplified products from standard concentrations of 5′ UTR RNA and from infected PBMC were resolved on 2% agarose gel stained with ethidium bromide. Polaroid photographed gels were scanned and the intensity of the amplified bands were analyzed using Total Lab software (Phoretix, New Castle, UK). Number of copies of plus-strand RNA in each specimen was calculated on the standard curve. The later was made from serial dilutions of RNA prepared by in vitro transcription and the intensity units of scanned amplified bands using software units.
Results

Detection of HCV in circulating PBMC

Plus HCV RNA strand was detectable in sera and PBMC derived from all the 26 HCV patients. Both plus and minus RNA strands was detectable in PBMC of 21 patients, the remaining 5 patients had only plus strand detectable in their circulating PBMC (Table 1).

Effect of SEA on PBMC proliferation

Flow cytometric analysis showed that the mean ± S.D. of percent values of cell proliferation from 5 control un-stimulated cell cultures at day 7 was 3.2 ± 1.5 %. Stimulation of the 5 control cultures with PHA and SEA resulted in a significant (p< 0.01) increase in DNA index to 16.7 ± 2.5 % and 16.84 ± 1.7 % respectively (figure 1).

Effect of SEA on cultured PBMC count from HCV patients:

Stimulation of patients' PBMC in culture for 72 hours resulted in increased PBMC count in all cases ranging from 2 – 5 fold increase with a mean value of 4.26± 1.5 compared to non stimulated patients` cells that showed mean increase in cell count ranging from 1.3-2.3 fold only with a mean value of 1.75± 0.25 (Fig 2)

Effect of SEA on HCV replication in PBMC in vitro:
Quantification of the intracellular viral load (table 2) showed increased copy number /cell of both or either viral strands after stimulation with SEA in 18 of 26 patients (69.2%) thus indicating induction of viral replication (Fig 3, table 2). Five out of 21 (23.8%) patients lost preexisting viral strands upon culture. SEA stimulated cells of those patients showed restoration of either or both strands (Table 2).

**Discussion:**

In Egypt, viral hepatitis along with schistosomiasis is the major cause of chronic liver disease and liver cirrhosis \[16, 17, 18\]. The present study provides, on molecular bases, ample evidence that SEA treatment in vitro induces HCV replication and therefore may explain the higher disease severity in subjects co-infected with HCV and Schistosomiasis. The notion that intracellular HCV replication was induced in vitro by SEA in 69% of patients represents the first direct evidence on the role of this potent mitogen on HCV replication. It also suggests that individual host factors play distinct roles in determining viral response in vitro and perhaps the overall morbidity in patients co-infected with the two diseases. Several cellular proteins such as Elongation factor 3 (elfa 3), ribosomal proteins S5 and S9 and Eukaryotic translation initiation factor (eIf 3), the polypyrimidine tract binding protein (PTB), heterogenous nuclear ribonucleoprotein C (hnRNPC) and la antigen were shown to regulate translation of HCV polyprotein precursor and hence intracellular replication of viral genome \[19, 20, 21, 22\]. Since PBMC have a major contribution into the circulatory pool of HCV load the results presented herein may explain why HCV titers are higher in patients with co-infection than those infected with HCV alone \[23, 24\]. Furthermore we can not exclude similar role of SEA on hepatocytes
harboring HCV genome in co-infected patients. Culturing the PBMC resulted in loss of both plus and minus RNA strands in 23.8% of cases. Interestingly SEA was capable of restoring either one or both strands in those cultures. This strengthens our hypothesis that SEA acts as a direct inducer of viral replication and makes it a valuable stimulatory reagent if future plans are considered to use short term PBMC cultures for antiviral screening. Recently, combinations of mitogens and /or cytokines were found to be able of stimulating plus and minus RNA strands of HCV in PBMC and, in certain cases, affinity-purified T and B cells [25]. The exact mechanism whereby SEA stimulates HCV replication is not yet clear. A mechanism involving extracellular signaling of the growth factor components of SEA is perhaps implicated. In agreement with the current data, Kamal et al., [23] concluded that co-occurrence of the two diseases was characterized by higher HCV RNA titers, histological activity, incidence of cirrhosis and hepatocellular carcinoma (HCC) compared to HCV infection alone. In the present study, the 5 fold increase in DNA index after 7 days and the observed 2.5 fold increase in PBMC count after 72 hour exposure to SEA indicate that SEA mediated cell proliferation involves increased rate of cellular DNA replication. In this regard both SEA and the commercial cellular mitogen PHA exerted similar magnitudes of DNA replication in the studied controls within 7 days of culture. Reports on other cell systems from our laboratory [14] and others [26,27] demonstrated that SEA associated cell proliferation involved up-regulation of cell cycle controlling genes such as peripheral cell nuclear antigen (PCNA) and B-cell translocation gene 1 (BTG1). Previous studies [28] reported that most soluble egg antigen fractions elicited in vitro granuloma reactions by PBMC of almost all Schitosoma haematobium infected patients and in vivo via endothelial cell proliferation
Conversely, separated soluble adult-worm antigens failed to stimulate PBMC of infected patients to form granulomas suggesting that SEA contains unique factor(s) which are lacking in adult worm antigens. Furthermore, the role of SEA in PBMC proliferation has been well established as an initial step for angiogenesis and granuloma formation in Schistosomiasis patients by up-regulation of vascular endothelial cell growth factor. In HCC, the proliferative activity of vascular endothelial cells is suggested to play an important role in the positive regulation of tumor-associated vascular endothelial cell proliferation. However, in vitro stimulatory effect of SEA on PBMC from chronic HCV patients was scarcely investigated. The significance of PBMC in chronic hepatitis C patients is based on the fact that PBMC represent a large extrahepatic reservoir for HCV replication. We and others have shown that PBMC from chronic HCV patients do not only contain HCV genomes but also function to maintain genomic replication and to support viral core and envelope glycoprotein E1 expression in culture. The two mitogens tested in the present study appeared not to have the same function on intracellular HCV titers indicating that different mechanisms of mitogen-viral interactions are involved. Induction of viral replication does not always correlate with increased cell proliferation, as SEA induced cell proliferation was notable in all patients even those whom intracellular HCV replication was not induced. Taken together the dual stimulatory function on both PBMC and viral copies per cell, the magnitude of total viral pool response in SEA stimulated culture is obviously larger than the currently presented values, in table 2, as viral copies per cell. The stimulatory function of SEA on replication of DNA viruses was reported as early as 1989 when Ishii et
al., [37] reported that SEA from *Schistosoma japonicum* induced Epstein Barr virus RNA in lymphoblastoid cells.

In conclusion the present study suggests that SEA possesses potent in vitro proliferative activity on PBMC and provides the first evidence that SEA directly stimulates HCV replication in vitro. This may explain, at least in part, the higher morbidity observed in patients co-infected with Schistosomiasis and HCV.

**References**


