Author's response to reviews

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:

Referee 1
1) Please explain why S. haematobium derived SEA was used instead of S. mansoni derived SEA.

We have been focusing during the past 5 years on the role of S.hSEA. in bladder cancer (El Awady et al., 2001: Schistosoma hematobium soluble egg antigens induce proliferation of urothelial and endothelial cells. World J Urol. 2001 Aug; 19(4): 263-6). Our research was focusing on the proliferative activity of S.hSEA, which was repeatedly shown to be involved as a potent mitogen. In fact differences between SEA derived from both species was minimal.

2) Detail how this SEA was obtained and specify its major constituents. Discuss which of these constituents might be responsible for the observed results.

Thanks for the referee for this note, this information will be added in the materials and methods section. A lyophilized preparation of the soluble fraction of mature live S. hematobium eggs was supplied by Theodore Bilharz Research Institute, Imbaba, Egypt. It was prepared as described in Brown et al., 1977: "Partial purification of antigens from eggs of S.mansoni that Elicit delay of hypersensitivity" J Immunol 119: 1275-1278.

SEA constituents are mainly growth factors (Brown et al., 1977). A detailed analysis of the protein /glycoprotein. fraction of SEA constituent and the effect of each fraction is currently underway and will be reported later as a separate study. Our aim in the current study was to provide an in vitro evidence that SEA crude preparation may be associated with enhanced HCV replication in PBMC (a major cellular target for viral replication).

3) Give results of control experiments on PBMC proliferation in vitro and on viral replication using another unrelated stimulant than SEA or Phytohaemagglutinin.

Our idea was to compare the stimulatory power of a commercial mitogen as PHA with the crude SEA (the focus of our study which has clearly shown that both mitogens induced cell proliferation but only crude SEA was able to induce intracellular genomic replication.

4) Provide evidence of a dose-dependent effect of SEA on PBMC proliferation and on viral replication.

In fact the dose dependent effect of SEA on another cell type (uroepithelial cells G82 transitional cell carcinoma has been previously reported by our group (El Awady et al., 2001).

Referee 2
Study design:

* It is not clear how the patients were selected. Were they consecutively enrolled?
* Baseline demographic and clinical characteristics of subjects were not shown. The authors did not mention important information such as the mean disease duration and stage of liver disease at enrollment and if some already have cirrhosis. These criteria are particularly important since there are many factors that influence the immune responses. It is known that the magnitude and breadth of T cell responses differ
markedly with the stage of liver disease.

The patients were selected on the following basis which was included in the manuscript: "All patients had detectable HCV antibodies by third generation ELISA (Dia Sorin, Torino, Italy). None of the patients received treatment for HCV. All the patients had undetectable levels of HBsAg, schistosoma and HIV antibodies".

The described data were not meant to address epidemiological information. Therefore, patient selection criteria was to recruit patients with active viral replication in PBMCs. It was mentioned already in the manuscript that patients had chronic active hepatitis with no co- infection with any other factor, none of them had cirrhosis at time of enrollment.

Thanks for the referee, detailed demographic data of patients were added to table (1).

The present work is designed to test the influence of SEA on the genomic replication of HCV and cell proliferation in cultured PBMCs and does address analysis of the magnitude and breadth of T-cell responses.

What we mean by host factors is cellular transcription factors such as Elongation factor 3 (elfa 3), ribosomal proteins S5 and S9 and Eukaryotic translation initiation factor (Elf3) that are known to regulate translation of HCV polyprotein precursor and hence expression of HCV enzymes required for replication. This is different from T cell surface markers which is out of the focus of current study.

* It is stated that the patients were coinfected with HCV and schistosomiasis. It is not clear whether it is S. hematobium or S. mansoni. It is not mentioned if it is only urinary or intestinal schistosomiasis or it is hepatic schistosomiasis and whether they were treated or untreated. There is no mention on whether schistosomiasis was active or not and if the schistosomiasis preceded HCV infection. These data are very important because the immune responses are affected more with active and hepatic S. mansoni infection. Absence of these data represent a major weakness of the study.

The present study does not include patients co-infected with HCV and Schistosomiases. Therefore, questions related to whether Schistosoma infection is urinary, intestinal or hepatic, treated or untreated, active or not, prior to HCV infection or not and history of both diseases in our patient population is not applicable.

* Sample size: The number of patients recruited is extremely small (only 26 patients). This is not adequate given that this study is cross-sectional and limited number of immunological and virological experiments at one time point and the T cell responses are hard to detect in chronic hepatitis C. Also enrolling a larger cohort of chronic hepatitis C patients is not hard in a community with high prevalence of HCV. A larger sample would definitely increase the impact and credibility of the findings.

These are actually 26 different cultures, each one contains 8 time points including intracellular viral load, and the load of the replicating intermediate, besides the cell proliferation (before and after SEA and PHA). It is expensive and time consuming study.

T-cell response analysis is not included in this study. The host factors we are referring to are those intracellular transcription factors (mentioned above) which interact with the viral genome to direct the viral replication.

Methods:

1. It is not shown how the blood was collected and whether the blood was on heparin or EDETA

The blood was collected on heparin for culture purposes and on EDTA for direct PCR (modified statement was added to the paper).

2. The amount of blood drawn (10ml) may not be sufficient for production of enough PBMCs for the flowcytometry and proliferative assays particularly that the authors mention that 2 million cells were plated a 2 million cells per well. What was the average numbers of cells produced. This information is crucial. Typically, at least 20 ml are required to perform the assays mentioned in the study. These details are obviously important since the quality of functional assays may differ depending on the number of cells and the conditions in which the cells are processed.
. 10 ml of blood (heparinized) should theoretically produce from 50 x10^6 - 70 x 10^6. Since we need to use equal number per each culture, we counted the no. of cells so that 2x10^6 cells were plated per cell so that we have enough cells to plate 25 wells, we used triplet wells per each point (whether SEA treated or untreated) that comes to 16x10^6 cells total. For Flow cytometry of DNA index, only 500x10^3 cells are sufficient for propidium iodide (PI) staining.

3. Where the assays for all patients performed at the same time with the same laboratory and experimental settings?
   . Yes, the experiments were done in the same lab and experiment setting, however, it is almost impossible to culture 26 primary cultures in one day.

4. What is the rationale for using only Schitosoma heamatobium SEA?
   . We have been previously focusing on the role of SEA from S. heamatobium in uroepithelial cell proliferation and bladder carcinoma (El Awady et al., 2001: Schistosoma hematobium soluble egg antigens induce proliferation of urothelial and endothelial cells. World J Urol. 2001 Aug; 19(4): 263-6). In fact biological differences between SEA derived from both species was minimal since both elicited similar effects on the same cells as mitogens and angiogenic factors.

5. Cells were incubated for 7 days. This duration is long since proliferation occurs within 48 hours.
   . The duration has to be longer than a single doubling time to observe a meaningful difference between treated and untreated cells.

6. The authors tested the effect of SEA on cell proliferation only in 5 healthy control subjects. This is very confusing because it is not clear how the authors measured PBMCs proliferation in chronic HCV patients? How could the authors demonstrate the influence of SEA on the cell proliferation if they did not perform proliferation assay, ELISpot or FACs to the chronic HCV patients?
   . Thanks for the referee. We have re-stated this part in the manuscript so that it can be clearer to the reader.

7. T cell responses are hard to detect in chronic hepatitis C. The technologies to detect them have led to important improvements especially by introducing ELISPOT assays using overlapping peptides or known CD4 and CD8 epitopes on pre selected subpopulations of PBMCs. The assays used in this study are not state of the art and represent a weakness in the stud. ELISPOT assays for CD4 and CD8 responses are much more sensitive and produce more reproducible results than the tests used in this study.
   . Study of the T-cell response is not the focus of current study. The main target of SEA effect in this study is the genomic replication of HCV within PBMCs as one of the large cellular reservoirs for viral life cycles. ELISPOT assays for CDs response is not within the frame of this study. Simple and direct report on a growth factor rich component of schistosome as an inducer for HCV replication is required at this stage. The exact soluble antigen(s) involved in such stimulation and its interaction with specific cellular proteins involved in efficient replication of HCV genome will follow shortly in another publication.

8. FACS analysis: the specificities of FACs analysis are not mentioned.
   . FACS was done to study cell proliferation via intercalation of Propidium Iodide with the DNA, this is a well established and approved method for DNA index analysis (see references).

9. In general the immunological component of the study is much weaker than the virological component.
   . This study does not include immunological studies.
10. Primers: The genotypes represented in this report are G4 in 24 patients and G1b in only 2 patients. Although the primers used were from a conserved region, yet some differences exist between G4 and G1 given that commercial kits are based on G1. In order not to miss positive results it would it have been better to design and use specific primers for genotype 4. Do the authors have experience with such primers? If so, did the carry experiments comparing the sensitivity and specificity of G4 primers with the commercially available primers?

Results:

1. Several important data such as the clinical background of patients is not mentioned. This is not made explicit, and some more information is needed.

   The clinical data requested by the referee were included in the manuscript.

2. The authors repeat here that the predominant genotype among the studied cases is G4 and this has been already mentioned in the methods section.

   This statement will be removed.

3. The authors detected negative strand in 21 patients while only plus strand was detected in 5 patients. How do the patients explain that? Could be this attributed to potential technical aspects of the experiment? Were the testing for negative strands repeated for these 5 patients?

   Thanks for the referee. This part will be rephrased since (+) strand was detected in all 26 cases while (-) strand was detected in 21 of them.

4. The authors mention that stimulation of the 5 control cultures with PHA and SEA resulted in a significant increase in DNA index to 16.7 +/- 2.5 % and 16.84 +/- 1.7 % respectively. How did they prove that the difference is significant? What is the P value? In general the statistics need to be reviewed by a biostatistician - it seems to me that a lot of post-hoc analysis were applied to the data sets.

   Both PHA and SEA stimulated DNA index 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. However the magnitude of response is not significantly different between the two mitogens. P values will be included in the manuscript.

5. Again the authors mention that stimulation of patients' PBMC in culture for 72 hours resulted in increased PBMC count in all cases ranging from 1.3 - 4 fold increase with a mean value of 4.26 +/- 1.5. What does this mean value indicate? This is an ambiguous statement. Is it mean fold increase? If so how come the range is 1.3-4 folds and the mean is 4.26???

   Editorial error was corrected in the revised version of the paper.

6. Proliferation assays, ELispots or FACs were not shown to be performed in chronic patients. How did the authors get these results? There is plenty of missing information that need to be clarified.

   Proliferation in CHC patients were performed by cell counting after trypan blue staining. Thanks for the referee this part has been rephrased in the paper to be more clear.

7. The paradoxical finding that after 24 hours of PBMC cultures, 5 cultures (23.8%) lost both plus and minus RNA strands which were restored after addition of SEA is interesting, however, how could that be explained?
In those 5 cultures the intracellular viral load was so low that it has been lost after culturing PBMC. Due to the potent stimulatory effect on HCV intracellular replication, the synthesis of both RNA strands was dramatically increased so it could be detected after stimulation.

8. The authors mention that the goal of the study was to test the effect of soluble egg antigen (SEA) on intracellular HCV RNA load in peripheral mononuclear cells (PBMC’s) as well as on cell proliferation in patients with chronic HCV infection. While the answers for these goals were not properly presented in the results section.

The treatment of cultured PBMC with SEA induced intracellular viral load in 70% of cultures and increased cell no in 100% of cultures (please look up tables 1,2 and figure 2)

Discussion:

1. The discussion includes previous published data and repetition of the results, speculations. There is only limited analysis of the data or findings in the manuscript. The authors do not state how this improves upon previously published cross sectional reports, which have already suggested these findings. While the issue of explaining the role of schistosomiasis on HCV replication is of substantial interest, the authors have not provided credible information that would enhance our understanding of how this happens.

2. The authors state that the dual stimulatory function on both PBMCs and viral copies per cell, the magnitude of total viral pool response in SEA stimulated culture is obviously larger than the presented values. Given that the tests used for measuring PBMCs stimulation are not efficient and given the small samples size, it is not possible to make such statement and thus the interpretation of the results is hardly acceptable.

Although the direct cell counting is a well established method, however such statetment has been rephrased in the discussion.

3. Although the finding that SEA was capable of restoring either one or both strands in cultured cells from those patients, is interesting the authors do not provide convincing explanation or even speculation.

As explained before the intracellular viral load was so low that it has been lost after culturing PBMC. Due to the potent stimulatory effect on HCV intracellular replication, the synthesis of both RNA strands was dramatically increased so it could be detected after stimulation.

4. The conclusion is not supported by the data.

Thanks for the referee, the conclusion is now supported by the data.