Author's response to reviews

Title: Effective Antiprotease-Antibiotic Treatment of Experimental Anthrax

Authors:

Serguei G Popov (spopov@gmu.edu)
Taissia G Popova (topova@gmu.edu)
Svetlana Hopkins (svetlana.hopkins@analex.com)
Raymond S Weinstein (alaskaray@aol.com)
Rebecca MacAfee (rmacafee@potomach.com)
Karl J Fryxell (kfryxell@gmu.edu)
Vikas Chandhoke (vchandho@gmu.edu)
Charles Bailey (cbailey2@gmu.edu)
Ken Alibek (kalibek@analex.com)

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Author's response to reviews: see over
Dear Editor

Please find below our response to the reviewers’ critiques. The authors would like to thank the reviewers for valuable comments, which helped improve the manuscript.

Reviewer 1 comments

**Minor Essential Revisions** (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

Just two minor points should be modified:

Page 3, line 1 (P3,L1): ...to M4 and M9 thermolysin and bacterial collagenase families, respectively.

P18L9: intraperitoneally

**Response:**

All suggested corrections have been made [pp.3. and 18].

Reviewer 2 comments:

**Comment 1**

General

The work presented by the authors in this manuscript can be roughly divided into two parts. In the first part, the authors prepared filtered supernatants from B. anthracis (Ba), B. cereus (Bc) and B. subtilis (Bs). They demonstrated that Ba and Bc supernatants contain proteases and have hemorrhagic activity when administered to mice by the sub-cutaneous route and are lethal when administered by the intratracheal route into the lungs of mice. This work is very preliminary. As presented here, it does not add significantly to our knowledge of B. anthracis pathogenesis nor does it contribute to the second part of the study. The authors need to identify those components of the supernatant responsible for the activities they describe, before presenting this work.
Response

In the overall evaluation of the first part of our study, we request the reviewer to take into account the following consideration. Back in 1954, the supernatants of B. anthracis cultures were used to discover anthrax toxins, but other virulence factors had been missed. In 1963, Bonventre and Eckert compared biologic activities of B. anthracis and B. cereus culture filtrates, however they could not explain why less virulent strain produced more toxic substances. In our study, we had to revisit the issue of supernatant toxicity, and we had to repeat the experiments with culture filtrates. In addition, the data presented in the first part of our study demonstrate that:

a) Secreted factors of B. anthracis (other than lethal and edema toxins) cause serious hemorrhage and may be useful targets in the development of effective anti-anthrax therapies.

b) This hemorrhagic activity is shared in common with the pathogenic B. cereus but is entirely absent from the closely related non-pathogenic species B. subtilis. It is thus not an artifact of cell culture, but rather is one of the pathogenic adaptations of B. anthracis.

c) The hemorrhagic factors of B. anthracis are metalloproteases and their hemorrhagic effects can be blocked in vivo with specific protease inhibitors.

The experiments in the first part also provided a necessary rational for the protease inhibition experiments in the second part of our study. Essentially, the ICUC approval of our animal experiments would be impossible without this evidence.

We completely agree with the Reviewer that the components of the supernatant responsible for the observed activities need to be identified and characterized. Our current research aims to address this goal. However, we realize that this is a task, which goes far beyond the scope of the presented work. One needs to take into account just a sheer number of the protease genes in the anthrax genome,
as well as possible microbial proteolytic pathogenic mechanisms. For example, several of the latter, which can be attributed to anthrax proteases, according to our data include: activation of kallikrein-kinin cascade, activation of host matrix metalloproteases, disruption of vascular permeability, shedding of extracellular signaling mediators, degradation of immunoglobulins, surfactant proteins, antibacterial peptides, plasma protease inhibitors, etc.

We have revised the text (p. 5, L 8,9) to clarify that we were looking for initial evidence of the possible virulence-enhancing role of B. anthracis proteases. Clearly, the reviewer 1 understood this connection. Our study for the first time demonstrates an important fact that the anti-protease therapy is effective in the animal model of anthrax infection, and identifies specific groups of proteases as targets for further development of therapeutic interventions. We have also revised the text of Discussion on pp. 19-21 to clarify these points, particularly the significance of this hemorrhagic effect in pathogenesis by B. anthracis.

Comment 2.

Major Compulsory Revisions
1) Page 20, lines 4-11: There is no evidence that LT plays an immunosuppressive role at early stages of B. anthracis infection. A large number of groups have demonstrated that LT suppresses secretion of cytokines from macrophages, monocytes, and dendritic cells in tissue culture and only when large amounts of purified LT are used. It is not clear that these observations are relevant to natural infection. A study by these authors and two studies by Pickering et. al. demonstrated that there is robust pro-inflammatory cytokine response in vitro and in vivo following infection with B. anthracis spores.

Response
Inhibition of the pro-inflammatory cytokines secretion from immunocompetent cells is one of the mechanisms of immunosuppression. Also, LT inhibitors increase the bactericidal capacity of macrophages against the engulfed spores, indicating that LT suppresses the macrophage innate immune response [refs 28
on p. 20]. Admittedly, the immunosuppressive capacity of LT is limited, and LT does not completely inhibit the pro-inflammatory host response. Nevertheless, the LT activity does help anthrax spores to survive in the hostile environment, which does contribute to the decrease in ID50. We have revised the text to clarify these points [changes on p.20, L. 11-14]

Comment 3
Page 23, lines 16-23 and Page 24, lines 1: Based on the data presented here, one can’t possibly assign a percent contribution of LT to anthrax lethality and one can’t conclude that LT doesn’t have a predominant role as a death-causing factor. Purified LT kills animals when administered alone, and passive administration of antisera against LT components protects against lethal challenge by B. anthracis.

Response
We found that in the conditions of our experiments a combination of antibiotic and phenanthroline protected 70% of animals after infection by the Sterne strain of B. anthracis (pXO1+). In addition, a combination of antibiotic and specific antiserum protected 90% of animals. None of these substances has a direct anti-LT activity. Admittedly, the passive administration of antisera against LT components is also effective, and so LT and hemorrhagic activity(s) must have a synergistic effect in promoting a fatal infection. We argue that the role of LT is likely to be permissive, but it is also possible that the hemorrhagic activity plays a permissive role.

Although the exact percentage contribution of LT to mortality is difficult to determine, we noticed that the efficacy of anti-LT therapy reported previously “corresponds well to the maximal expected contribution of LT to overall lethality based on our current data”. This is a correct statement but we agree with the reviewer that it may need further substantiation, and therefore we excluded it from the article.
Although LT kills animals, and anti-sera against its components confer some degree of protection, in our experiments secreted proteases kill mice better than LT, and passive administration of anti-sera against proteases also has protective effect against lethal challenge by B. anthracis.

We have revised the text to clarify the points discussed above [changes on page 24, first paragraph].

Comment 4

Figure 6. It appears that the a-M4AC serum confers protection when given alone and that the a-M4 AC, a-M9 and a-M4EP confer enhanced protection when given in combination with Ciprofloxacin, however, this analysis is confounded by the protection conferred by naïve serum. An analysis needs to be included to determine whether the differences observed between the protection conferred by each of these serums is statistically different than the protection conferred by the naïve serum in each experiment.

Response

We agree. We calculated statistical reliability of protection relative to naïve serum-antibiotic experiments and added corrections to the article (pp. 18, 19). Briefly, we confirmed that high dose sera effects are highly significant (98.4% to 99.8%, p<<0.05).

Comment 5

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

1) Page 20, line 7: Several groups have demonstrated the ability of purified LT to inhibit cytokine responses from macrophages, monocytes, and dendritic cells. They should be cited here.
Response
Reference to a comprehensive recent review dealing with cytokine response and LT, as well as the citation of the study by Pickering et al., have been added on p. 20.

Comment 6
Discretionary Revisions (which the author can choose to ignore)
1) Pages 10-11: The genomic analysis is poorly described. On page 11, line 4, the authors don’t define the “non-pathogenic” bacteria they used for comparison. I assume it was B. subtilis. Other than selecting proteases encoded almost exclusively by the B. cereus group, the authors don’t explain how their genomic analysis brought them to focus on the M4 family of proteases or the M9 family of collagenases.

Response
By non-pathogenic bacteria we meant B. subtilis, B. halodurans. Reference to these bacteria has been added on p.11. The M4 and M9 proteases are most probable tissue-damaging factors acquired by B.cereus group. The article says that “metalloproteases (MPs) from several bacterial species belonging to this family are capable of causing massive internal hemorrhages and other life-threatening pathologies [10, 13-16]” (p. 11).

Comment 7
2) Page 24, lines 1-3: Rather than speculate that a triple-component therapy (ciprofloxacin-phosphoramidon-caspase inhibitor) might be completely protective, the authors should do the experiment and present the results as part of this study. Particularly in light of the fact that they’ve already published on the caspase inhibitor.

Response
Unfortunately, performance of the suggested experiment on the scale necessary to rigorously demonstrate 100% protection against pXO1+ pXO2+ strains of *B. anthracis* is beyond our current financial resources. We agree that very specific speculations may not be appropriate under these circumstances. However, brief and general discussions of future strategies are appropriate, and so we have revised this comment to make the point that 100% protection against *B. anthracis* will likely require a combination of approaches [changes on page 24, first paragraph].