Author's response to reviews

Title: Effective Antiprotease-Antibiotic Treatment of Experimental Anthrax

Authors:

Serguei G Popov (spopov@gmu.edu)
Taissia G Popova (tpopova@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.

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, even if it may look like they had come from 1950th. In addition, the data presented in the first part of our study serve a very important purpose to demonstrate that:

a) secreted factors of B. anthracis (other that lethal and edema toxins) could be lethal to experimental animals and could be considered as targets in the development of effective anti-anthrax therapies.

b) hemorrhagic factors of B. anthracis belong to metalloproteases, and that the inhibitors chosen for animal experiments abolish hemorrhagic effect.

c) each of the anti-serum against M4 and M9 metalloproteases used in our experiments targets a discrete set of enzymes.

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.

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 important targets for further development of therapeutic interventions. We believe that our findings are firmly established and need to be reported without a delay to stimulate research in this direction.

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. Please take into account that we do not argue with the fact that the immunosuppressive capacity of LT is limited, as well as we do not say that LT completely inhibits the pro-inflammatory host response. Nevertheless, the LT activity helps a certain
fraction of anthrax spores survive in the hostile environment. Ultimately, this contributes to the decrease in LD50, which is a measure of virulence.

Comment 2
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. In addition, a combination of antibiotic and specific antiserum protected 90% of animals. None of these substances has a direct anti-LT activity. Doesn’t it “argue against” LT as a predominant death-causing factor, at least in these specific conditions?

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.

The final argument of the reviewer regarding LT as a major death-causing factor is surprising, taking into account the results we present. 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. Our data demonstrate that B. anthracis
produces a number of toxic substances, and therefore we made our conclusion not on the mere fact of toxicity but on the extent of protection provided by a particular therapy.

Comment 3
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
This is an important comment. We calculated statistical reliability of protection relative to naïve serum-antibiotic experiments and added corrections to the article (pp. 18, 19). Briefly, high dose sera effects are highly significant (98.4% to 99.8%), while low dose protection differs from naïve one with 89% reliability.

Comment 4
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
The citations have been added.

Comment 5
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, and B. clausii. Reference to these bacteria has been added. 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 6**

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**

We apologize for not being able to continue experiments with caspase inhibitors taking into account that only direct animal and husbandry costs of the current study have exceeded $200,000, and a projected cost of caspase inhibitors alone is above $20,000.