

Treatment of anthrax infection with combination of ciprofloxacin and antibodies to protective antigen of *Bacillus anthracis*

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Abstract

Currently there is no effective treatment for inhalational anthrax beyond administration of antibiotics shortly after exposure. There is need for new, safe and effective treatments to supplement traditional antibiotic therapy. Our study was based on the premise that simultaneous inhibition of lethal toxin action with antibodies and blocking of bacterial growth by antibiotics will be beneficial for the treatment of anthrax. In this study, we tested the effects of a combination treatment using purified rabbit or sheep anti-protective antigen (PA) antibodies and the antibiotic ciprofloxacin in a rodent anthrax model. In mice infected with a dose of *Bacillus anthracis* Sterne strain corresponding to 10 LD₅₀, antibiotic treatment with ciprofloxacin alone only cured 50% of infected animals. Administration of anti-PA IgG in combination with ciprofloxacin produced 90–100% survival. These data indicate that a combination of antibiotic/immunoglobulin therapy is more effective than antibiotic treatment alone in a rodent anthrax model.

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1. Introduction

Bacillus anthracis is considered one of the most probable biological threats that could be used as a weapon against military or civilian targets. Large-scale immunization against anthrax, similar to that of smallpox, is problematic because of the limited efficacy and reactogenicity of the anthrax vaccine. Currently, there is no effective treatment for inhalational anthrax, which is most likely to be seen in a biological attack, beyond the administration of antibiotics shortly after exposure. Time delay reduces the effectiveness of antibiotic treatment. In the recent anthrax outbreak, five out of 11 patients succumbed to inhalational anthrax despite antibiotic therapy (CDC MMWR). Therefore, there is a need for a safe and efficient treatment as a supplement to traditional antibiotic intervention.

Two main factors are important in anthrax infection:

bacterial proliferation and invasion of organ systems, and the cytotoxic effect of anthrax toxin, with eventual organ failure and death.

The two anthrax toxins are formed by three different proteins: protective antigen (PA) combines either with lethal factor (LF) to form lethal toxin (LeTx), or with edema factor (EF) to form edema toxin (EdTx). PA facilitates transport across the cell membrane of LF and EF, both of which are enzymes targeting substrates within the cytosol.

It has been demonstrated that treatment with hyperimmune serum containing polyclonal antibodies against PA was protective in mice [1] and guinea pigs [2] challenged with anthrax.

Our study was based on the premise that simultaneous inhibition of lethal toxin action and blocking of bacterial growth by antibiotics will be beneficial for the treatment of anthrax. It has been shown by Lincoln et al. [3] and Friedlander et al. [4] that anthrax toxin-based vaccination and hyperimmune serum treatment in combination with antibiotic treatment showed better efficacy than antibiotic therapy alone in primates. The effectiveness of antibody/

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antibiotic combinations has been demonstrated for various bacterial infections [5–8]. In this study, we tested the effects of a combination treatment using purified rabbit or sheep anti-PA antibodies and the antibiotic ciprofloxacin in a rodent anthrax model.

2. Materials and methods

2.1. Reagents

Recombinant *B. anthracis* LF and PA (rPA) were acquired from List Biological Laboratories (Campbell, CA, USA). Ciprofloxacin was obtained from ICN (Costa Mesa, CA, USA) and dissolved in sterile distilled water. Pyrogen-free phosphate-buffered saline (PBS) was purchased from Mediatech (Herndon, VA, USA).

2.2. Immunization of rabbits and sheep

Rabbits were immunized s.c. with rPA mixed with Freund's complete adjuvant (FCA) initially, and with Freund's incomplete adjuvant (FIA) at the time of boosting 3 and 6 weeks later. Rabbits were bled 1 week after the last immunization. Reciprocal endpoint titers in the rabbit serum were > 5.0 log. Polyclonal rabbit anti-PA IgG were isolated by protein A affinity chromatography, IgG purity was 95%. Immunization of rabbits and IgG purification were carried out by Spring Valley Laboratories (Woodbine, MD, USA). Endotoxin content in the rabbit IgG samples was less than 10 pg mg^{-1} as measured by a limulus amoebocyte lysate kit (BioWhittaker, Walkersville, MD, USA). Sheep were immunized s.c. with rPA mixed with FCA initially, and with FIA at the time of boosting 3 and 17 weeks later. Sheep were bled 5 weeks after the last immunization. Reciprocal endpoint titers in the sheep serum were > 5.0 log. Polyclonal sheep anti-PA IgG were isolated using ammonium sulfate precipitation followed by DEAE Sepharose column chromatography. Immunization of sheep was carried out by Spring Valley Laboratories, and IgG purification was performed at CBER, FDA (Bethesda, MD, USA), IgG purity was more than 90%. Endotoxin content in the sheep IgG samples was less than 10 pg mg^{-1} as measured by a limulus amoebocyte lysate kit.

2.3. Cells and cell culture

Murine RAW 264.7 monocyte-macrophage cell line ATCC TIB-71 was obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in phenol-free Dulbecco's modification of Eagle's medium/Ham's F-12 50/50 mix (DMEM) from Mediatech supplemented with 10% heat-inactivated fetal bovine serum, 100 U ml^{-1} , $100 \text{ } \mu\text{g ml}^{-1}$ penicillin–streptomycin, 0.1 mM non-essential amino acids, and 0.5 mM 2-mercaptoethanol at 37°C in 5% CO_2 .

The cells were harvested using Cellstripper[®] from Mediatech and were washed once with medium to remove the non-enzymatic dissociation solution. RAW 264.7 cells were plated in 96-well flat-bottomed tissue culture plates from Becton Dickinson (San Jose, CA, USA) at a concentration of 10^5 cells per well in the DMEM medium mentioned above and incubated overnight.

2.4. Cytotoxicity neutralization test

LeTx (LF = 16 ng ml^{-1} or 64 ng ml^{-1} ; PA = 500 ng ml^{-1}) was pre-incubated in DMEM with varying concentrations of the tested antibody for 1 h at 37°C in a 5% CO_2 atmosphere. The medium was removed and the solutions were added to the RAW 264.7 cells and further incubated for 4 h. Cell viability was estimated using an MTS kit from Promega (Madison, WI, USA). A μ Quant spectrophotometer from Bio-Tek Instruments (Winooski, VT, USA) was used to obtain readings.

2.5. Animals

Female DBA/2 mice, 10–12 weeks old, were used in this experiment. The mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and Charles River Laboratories (Wilmington, MA, USA).

2.6. Microorganisms

B. anthracis strain 34F2 (Sterne) was obtained from Colorado Serum (Denver, CO, USA). A starter culture was grown overnight at 37°C in NB medium from Invitrogen (Carlsbad, CA, USA) without antibiotic. Cells from this overnight culture were plated onto NB agar and incubated at 37°C . When a 99:1 ratio of spores to vegetative cells was detected by phase contrast microscopy, the spores were removed from the agar surface with water and pelleted by centrifugation at $5000 \times g$ at 4°C for 30 min. The spores were washed five times with sterile distilled water.

2.7. Intraperitoneal and subcutaneous administration

Groups of 10 DBA/2 mice were treated as follows: (i) PBS (pH 7.4); (ii) irrelevant purified IgG (10 mg kg^{-1}), obtained from a non-immunized sheep; (iii) ciprofloxacin (50 mg kg^{-1}); (iv) anti-PA IgG (10 mg kg^{-1}); (v) anti-PA IgG (10 mg kg^{-1})+ciprofloxacin (50 mg kg^{-1}).

In both studies, each mouse was inoculated with 2×10^7 spores ($200 \text{ } \mu\text{l}$) i.p. Vehicle control groups received PBS ($200 \text{ } \mu\text{l}$) i.p.

After 4 h post infection, all anti-PA IgG (and irrelevant IgG) treatment groups were given a dose of the antibody i.p. On days 1 and 2 post infection, two injections of antibodies were administered (one in the morning, one in late afternoon). Only one treatment a day of anti-PA IgG (and

irrelevant IgG) was given on days 3–9 post challenge. Ciprofloxacin was administered s.c. once daily in the morning, beginning on day 1 post infection and continuing until day 9. Vehicle control groups received PBS i.p. once a day for 9 days after inoculation with spores.

Mice were observed daily for 18 days post challenge, and the numbers of survivors were recorded. Survival curves were analyzed with GraphPad Software using the log rank test.

3. Results and discussion

The rabbit and sheep anti-PA IgG used in this study were characterized by their titers in enzyme-linked immunosorbent assays against rPA and exhibited high binding titers (data not presented). Murine RAW 264.7 monocyte-macrophage cell cultures were utilized for in vitro experiments to approximate macrophage-like traits essential for disease pathogenesis. The antibodies were checked for their ability to inhibit cytotoxicity of lethal toxin on the RAW 264.7 cells, and they demonstrated similar protective activity (Figs. 1 and 2). Non-specific rabbit and sheep IgG were not protective (data not presented). The in vitro experiments showed that the antibodies themselves were not toxic to the cells at all concentrations tested.

In vivo testing of the antibodies was carried out in mice using the i.p. route of challenge instead of the intranasal route, which is less accurate. Macrophages circulating within the peritoneum can ingest spores and carry them to regional lymph nodes similarly to alveolar macrophages resulting in systemic disease with similar pathogenesis. Intraperitoneal administration has been utilized previously for anthrax challenge in mouse models [1,9]. We compared treatment efficacy of ciprofloxacin administered to mice infected with the Sterne strain by different routes. While all routes of administration achieved some protection, the s.c. route had the highest efficacy followed by i.m., p.o. and i.p. administration (data not shown). Subcutaneous

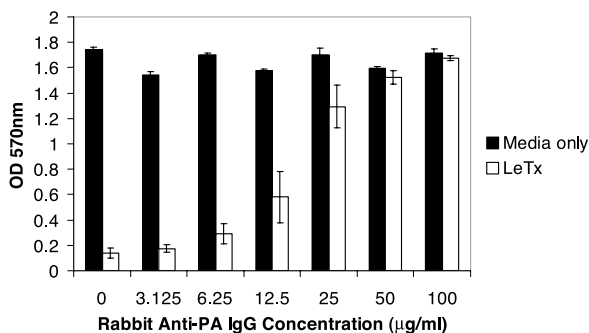


Fig. 1. Dose-dependent protection of RAW 264.7 cells from LeTx-induced cell death by rabbit anti-PA polyclonal antibodies. RAW 264.7 cells were incubated with increasing concentrations of rabbit anti-PA IgG with or without LeTx in triplicate. Cell viability was determined by the MTS colorimetric assay. Error bars represent 95% confidence intervals.

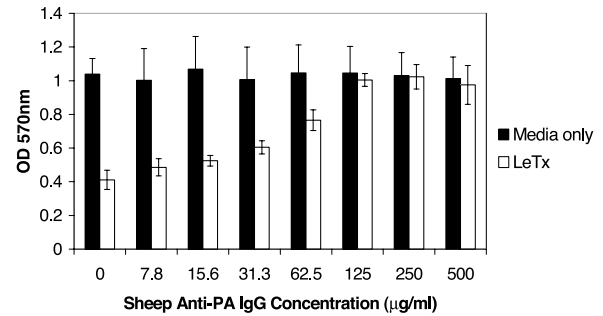


Fig. 2. Dose-dependent protection of RAW 264.7 cells from LeTx-induced cell death by sheep anti-PA polyclonal antibodies. RAW 264.7 cells were incubated with increasing concentrations of sheep anti-PA IgG with or without LeTx in triplicate. Cell viability was determined by the MTS colorimetric assay. Error bars represent 95% confidence intervals.

administration of ciprofloxacin was used in this study, and a dose of 50 mg kg⁻¹ was chosen since it is close to the highest dose acceptable for therapeutic use in humans, and it did not cause toxic effects in mice. Antibodies were administered via the i.p. route, which was utilized before [2,10] and is more convenient than the i.v. route with mice. Our IgG treatment schedule was chosen based on the fact that clearance rates for i.p. administered IgG in mice are rather rapid (~3 h) [11–13].

Data on in vivo efficacy of rabbit antibodies against PA in the absence and presence of antibiotic are presented in Fig. 3. In animals infected with a dose of anthrax corresponding to 10 LD₅₀, antibiotic treatment with ciprofloxacin alone resulted in 50% survival. Administration of rabbit anti-PA IgG alone resulted in 30% survival. In contrast, 100% of the mice survived if treated with antibody in

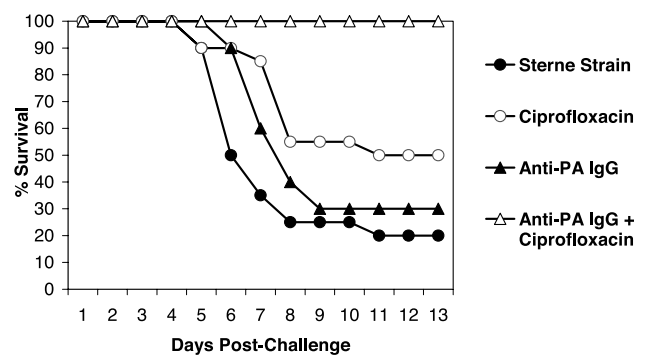


Fig. 3. Protection of DBA/2 mice against anthrax infection using ciprofloxacin in combination with rabbit antibodies against PA. Twelve-week-old female DBA/2 mice were inoculated with 2×10^7 spores of the *B. anthracis* Sterne strain by the i.p. route. Control mice received PBS i.p. 4 h after infection and then daily for 9 days. In the groups receiving antibody alone or with antibiotic, the mice were injected i.p. with anti-PA IgG 4 h after infection. The mice were given two injections of the antibody per day (morning and late afternoon) on days 1 and 2 post infection, and on days 3–9, mice were injected with antibodies once a day. Ciprofloxacin was also administered s.c. on days 1–9 post challenge in the groups receiving antibiotic alone or with antibody. The mice were monitored daily to determine survival. Survival curves were analyzed with GraphPad Software using the log rank test.

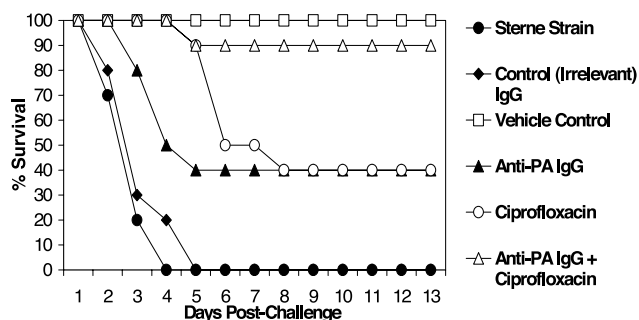


Fig. 4. Protection of DBA/2 mice against anthrax infection using ciprofloxacin in combination with sheep antibodies against PA. Twelve-week-old female DBA/2 mice were inoculated with 2×10^7 spores of the *B. anthracis* Sterne strain by the i.p. route. Control mice received PBS i.p. 4 h after infection and then daily for 9 days. In the groups receiving antibody alone or with antibiotic, the mice were injected i.p. with anti-PA IgG 4 h after infection and then daily for 9 days. On days 1 and 2 post infection, the mice were given two injections of the antibody per day (morning and late afternoon). On days 3–9, mice were injected with antibodies once a day. Ciprofloxacin was also administered s.c. on days 1–9 post challenge in the groups receiving antibiotic alone or with antibody. The mice were monitored daily to determine survival. Survival curves were analyzed with GraphPad Software using the log rank test.

combination with ciprofloxacin ($P=0.0012$ compared to the anti-PA IgG alone group; $P=0.0091$ compared to the ciprofloxacin alone group). Experiments with the use of sheep antibodies against PA produced similar results (Fig. 4). All mice left untreated or treated with irrelevant IgG died. Ciprofloxacin or antibody only treatment provided 40% survival, while a combination of the specific antibody with ciprofloxacin resulted in 90% survival ($P=0.0156$ compared to the anti-PA IgG alone group; $P=0.0324$ compared to the ciprofloxacin alone group).

These results indicate that a combination of antibiotic/immunoglobulin therapy was more effective than antibiotic treatment alone in a rodent anthrax model. Although it was shown that antibodies to PA have anti-spore as well as anti-toxin activities [14], we believe that systemic passive protection is likely a mechanism of IgG protection. According to our experience, most bacterial spores germinated within 2 h after infection, and we performed the first injection of IgG 4 h post infection. We think that the antibodies to PA serve mostly as anti-toxins in addition to antibiotics that inhibit bacterial replication but cannot protect against already released anthrax toxin. Our ultimate goal is to develop combined antibiotic/immunoglobulin therapy for reducing the mortality rate of infected persons.

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