The Efficacy of Post-Exposure Therapy Against Glanders in Mice

David M. Waag

Bacteriology Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD

Running Head: Post-Exposure Therapy for Glanders

#Address correspondence to: david.m.waag.ctr@mail.mil
Burkholderia mallei, the causative agent of glanders, is a CDC Tier 1 Select Agent for which there is no preventive vaccine and antibiotic therapy is difficult. In this study, we show that a combination of vaccination using killed cellular vaccine and therapy using moxifloxacin, azithromycin, or sulfamethoxazole/trimethoprim can protect BALB/c mice from lethal infection even when given 5 days after infectious challenge. Vaccination only, or antibiotic therapy only, was not efficacious. Although antibiotics evaluated experimentally can protect when given before or 1 day after challenge, this time course is not realistic in the cases of natural infection or biological attack, when the patient seeks treatment after symptoms develop or after a biological attack has been confirmed and the agent identified. Antibiotics can be efficacious after a prolonged interval between exposure and treatment, but only if the animals were previously vaccinated.

Key words: glanders, B. mallei, cellular vaccine, azithromycin, moxifloxacin, sulfamethoxazole, trimethoprim

Introduction.

Glanders, caused by the etiological agent B. mallei, is a very severe disease in humans, characterized by extreme pain, prostration, fever, and abscesses that can be found primarily in the spleen and liver, but also in any organ and tissue throughout the body (1, 2). Although glanders was originally described over two thousand years ago, effective countermeasures have not been sufficiently developed to provide protection for
those at risk. The lack of suitable medical countermeasures, high infectivity by aerosol exposure, severe clinical symptoms, and a high rate of lethal infections in untreated cases resulted in classifying this microorganism as a CDC Tier 1 Select Agent (3).

Although poorly delineated, glanders disease symptoms can be acute (a more fulminant and rapidly fatal clinical course, including fever, malaise, myalgia, fatigue, inflammation, and swelling of the face and limbs and the development of painful nodules involving the face, arms, and legs (2, 4)) or chronic (multiple subcutaneous abscesses, enlarged lymph nodes, nodules which may ulcerate in respiratory and alimentary mucosa, clinical history of remission and exacerbation, necrotic foci in bones, and nodules in the viscera (5). In the absence of effective treatment, glanders is almost always fatal.

Localized infection is unusual. Since cases of human glanders are rare, the compiled knowledge of this disease occurred mostly before the antibiotic era. Most human cases resulted from contact with infected equids, although the most recent cases were laboratory acquired (6, 7).

Organisms usually enter the body through abrasions, through mucous membranes, or by inhalation. The incubation period is variable, ranging from less than a day to several weeks (1, 7). The infection will eventually become systemic.

In vitro susceptibility testing has shown that B. mallei is sensitive to a variety of antibiotics, including sulfonamides, aminoglycosides, ciprofloxacin, novobiocin, several tetracyclines, imipenem, and ceftazidime (8-10). Limited information exists regarding effective antibiotic treatments. The most studied cases of human glanders followed laboratory exposures. Sulfonamides were used successfully in the first six U.S. laboratory-acquired infections (7). The most recent case had disseminated disease and
developed abscesses of the spleen and liver. This patient was gravely ill, requiring ventilator assistance before improving on a prolonged course of several antibiotics, including imipenem and doxycycline (6). A 6-month course of doxycycline and azithromycin followed and the patient recovered completely.

There is no evidence for immunity against glanders by virtue of previous infection (11). No glanders vaccine candidates described to date have provided consistent sterilizing immunity in animal studies, but some candidates were shown to extend the time to death after infectious challenge (12-14). Studies in my laboratory have also shown that killed cellular glanders vaccines can protect a majority of mice for at least 3 weeks after aerosol challenge, but mice typically remain infected (data not shown).

Amemiya et al. found that nonviable B. mallei failed to protect mice from a parenteral live challenge (15). They examined heat-killed B. mallei, irradiation-inactivated B. mallei, and an irradiation-inactivated B. mallei capsule mutant in the BALB/c model of glanders, and found a mixed T-cell helper (Th)1- and Th2-like immune response to all of the nonviable cell preparations. It was suggested that nonviable B. mallei cell preparations did not protect mice in the study because of the induction of a mixed cytokine response and increased IgG1 versus IgG2a subclass response.

In this paper, we have combined 2 imperfect countermeasures against glanders (antibiotic therapy and vaccination) and show that this strategy can provide protection from acute lethal disease and also has the potential of eliminating residual infection after challenge.
Materials and Methods.

Experimental Animals.

Specific pathogen-free female BALB/c mice (10 mice per group) (Charles River - NCI, Frederick, MD), weighing between 20 - 25 gram and approximately 6-8 weeks old, were housed in transparent plastic cages with microisolator tops in a biosafety level 3 facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The mice were given commercial rodent feed and tap water ad libitum. In conducting research with animals, the investigators adhered to the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011. The USAMRIID IACUC approved all animal experiments described in this paper.

Microorganisms.

_Burkholderia mallei_ (ATCC 23344; China 7 strain) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Prior to animal challenge, this strain was serially passaged three times in hamsters and designated GB15.1–3. In some experiments, GB15.1-3 isolated from a human case of glanders (designated strain FMH23344) was used as challenge agent (6). _B. mallei_ was propagated in glycerol (4%) tryptone broth (GTB; Difco) and on glycerol (4%) tryptone agar (GTA).

Experimental Design.

Mice (10 per group) were vaccinated intraperitoneally (IP) with 100µg of heat-killed (80°C for 3h) _B. pseudomallei_ strain 1026b. Four weeks later, mice were boosted with
the same dose and route of candidate vaccine. Six weeks after the boost, mice were
challenged with *B. mallei* by small particle aerosol (see below).

After the index death, mice were treated with moxifloxacin (moxi), azithromycin
(Az), or sulfamethoxazole / trimethoprim (ST). Moxifloxacin therapy (16 mg/kg; Q12h;
IP) was initiated on day 3 or day 5 after challenge and administered for 3, 5 or 10 days.
Azithromycin therapy was initiated on day 3 or day 5 after challenge (15mg/kg; Q12h;
IP) and administered for 5 or 10 days. Sulfamethoxazole (200mg/kg) / Trimethoprim
(40mg/kg; Q12h, IP) therapy was initiated on day 5 after challenge and administered for
5 or 10 days. Antibiotic dosing was based on appropriate pharmacokinetic (PK) values.
Survival of individual mice within groups was monitored for at least 21 days after
challenge.

**Aerosol exposure.**

Mice were exposed in a whole body chamber to an aerosol generated with a
Collison nebulizer (approximately 1 µm diameter particle size) (16). Prior to use in
challenge experiments, 25ml of GTB was inoculated with 20 ul of *B. mallei* stock culture
and incubated overnight in a shaking incubator (200 rpm) at 37°C. The concentration of
microorganisms in the overnight bacterial culture was estimated using absorbance at 660
nm and a standard curve. The culture concentration was confirmed using plate counts.
The bacterial culture was then diluted to the appropriate concentration predetermined to
deliver the approximate desired challenge dose. After the aerosol exposure, actual doses
were then calculated after determining the concentration of bacteria in all-glass impingers
(determined using plate counts) and the respiratory volume and respiratory rate in mice of
similar age and weight. Challenge doses ranged from 1-5 LD$_{50}$. One LD$_{50}$ is approximately 1000 cfu of *B. mallei* when delivered by small particle aerosol to BALB/c mice.

**Presence of bacteria in spleens.**

In order to estimate whether vaccinated, challenged, and treated survivors harbored residual bacterial infection, mice surviving the 21 day observation period were euthanized with CO$_2$, and the presence of bacteria in the spleens was determined. Spleens were excised and dissociated in 5 ml of HBSS and 0.1 ml of cell suspension was plated in triplicate on GTA; or spleens were cut longitudinally with a sterile scissors and “stamped” on the surface of GTA 3 times. Plates were incubated at 37°C for 72h and spleens were scored “infected” or “not infected” based on bacterial growth. Typically, spleens with no obvious signs of abscess were dissociated and plated on GTA, while spleens with abscess were “stamped” on GTA to confirm the presence of viable bacteria. The sensitivity of the plating method for bacterial detection was approximately 50 microorganisms per spleen and greater.

**Statistical analysis.**

In order to determine whether differences in survival between untreated (HBSS inoculation) and treated (vaccine and/or antibiotic therapy) groups were significant after challenge, P values were calculated using the logrank test (Prism 6; GraphPad software).

**Results.**

**Mice treated with azithromycin.**
Azithromycin was successfully used to treat a case of human glanders and was the first antibiotic we tested (17). In this trial, vaccinated mice were challenged by aerosol with approximately 5 LD$_{50}$ of \textit{B. mallei} China 7. Antibiotic therapy was initiated the fourth day after challenge (the day of the index death) and continued for 3 or 5 days. Ninety percent of the mice given azithromycin for 3 days survived, whereas 70 percent of mice treated for 5 days survived (Figure 1a). Forty percent of the mice receiving only vaccine survived through the 21 day observation period, but all the unvaccinated and untreated mice died. Survival for the groups given vaccine and azithromycin were significantly higher (P<0.01) than the group given HBSS. Vaccine given without therapy also aided survival (P<0.05).

Since BALB/c mice challenged with \textit{B. mallei} tend to remain persistently infected, we were interested in knowing whether mice surviving the 21 day observation period were infected. In a similar study to that described above, mice were vaccinated, challenged with 3 LD$_{50}$ of \textit{B. mallei} and azithromycin therapy initiated 2 or 3 days after aerosol challenge for a duration of 3 or 5 days. Mice were euthanized 64 days after challenge. We found that all spleens from surviving control mice (N=3) and from all vaccinated mice (N=2) harbored \textit{B. mallei} (Figure 1b). However, 29, 44, 20, and 57 percent, respectively, of vaccinated mice given azithromycin 2 days after challenge for a duration of 3 (N=7) or 5 days (N=9); and vaccinated mice given azithromycin 3 days after challenge for a duration of 3 (N=5) or 5 days (N=7) had no detectable \textit{B. mallei} in the spleens. Because of the sensitivity of our assay, we were unable to detect bacteria in spleens if less than 50 microorganisms were present. Therefore, we were unable to determine whether spleens were actually sterile (no microorganisms present) and did not
In order to assess whether increased survival in the vaccinated and treated mice was due solely to azithromycin, we conducted a similar study and included groups of unvaccinated mice that received azithromycin. Mice received vaccine only, azithromycin only, or vaccine and azithromycin. Mice were challenged with 3 LD$_{50}$ of *B. mallei* beginning 5 days after aerosol challenge for a duration of 5 or 10 days. The index death occurred 3 days after challenge. We did not observe greater than 10 percent survival in control mice, mice given only vaccine, or mice given only azithromycin for 5 or 10 days after challenge (Figure 1c). In contrast, at least 80 percent of the vaccinated and treated mice survived (P<0.0001). Eighteen weeks after challenge, 67 percent of surviving vaccinated mice treated with azithromycin for 5 days (N=6) and 71 percent of surviving vaccinated mice treated for 10 days (N=7) had undetectable *B. mallei* in spleens (data not shown).

**Mice treated with moxifloxacin.**

When vaccinated mice were treated with moxifloxacin beginning 3 days after challenge with 1.5 LD$_{50}$ of *B. mallei* for a duration of 3 or 5 days, all vaccinated and treated mice survived (P<0.0001), whereas 90 percent of the control mice and 40 percent of the mice given vaccine only (P<0.05) died (Figure 2a). To investigate whether moxifloxacin alone can facilitate survival after delayed onset treatment, a similar experiment was conducted but some groups of mice were only given moxifloxacin. Vaccinated mice were challenged (3 LD$_{50}$ of *B. mallei*) and treatment was initiated 5 days after challenge (index death on day 3). Ten percent or less of the control mice, mice
given vaccine only, or unvaccinated mice given moxifloxacin survived until the end of
the observation period (Figure 2b). At least 80 percent of the vaccinated mice that were
treated with moxifloxacin survived ($P<0.0001$), whereas no protection ($P>0.05$) was
provided to unvaccinated mice given moxifloxacin.

In another test of the ability of moxifloxacin to extend survival in vaccinated
mice, we used the *B. mallei* challenge strain isolated from a human glanders patient
(FMH23344; 1 LD$_{50}$) and extended the observation period through day 39 because of the
relatively low challenge dose. All control mice and 60 to 70 percent of mice given
moxifloxacin only died during the observation period ($P>0.01$; Figure 2c), while 100
percent of the vaccinated mice given either moxifloxacin regimen survived ($P<0.0001$).
Eighty percent of the mice given vaccine only survived through the observation period
($P<0.0001$). When spleens from survivors were examined for sterility on day 44 to 47
after challenge, at least 50 percent of the mice treated with moxifloxacin for 10 days
[vaccinated ($N=10$) and unvaccinated ($N=2$)] had no detectable microorganisms in the
spleens (Figure 2d). Thirty-three percent of the vaccinated mice treated with
moxifloxacin for 5 days had undetectable levels of bacteria in spleens ($N=9$). As
mentioned earlier, we were unable to distinguish whether spleens were truly bacteria-
free, or whether a small undetectable number of residual microorganisms remained.
Furthermore, addition tissues and organs were not sampled to determine whether they
were infected.

**Mice treated with sulfamethoxazole and trimethoprim.**

Sulfamethoxazole / trimethoprim was similarly tested for efficacy in vaccinated
mice after clinical signs had developed. Mice were given the same challenge strain and
dose described in the previous experiment. Only 20 and 30 percent of the unvaccinated
mice given sulfamethoxazole / trimethoprim 5 days after challenge for 5 or 10 days,
respectively, survived (P>0.05; Figure 3a). However, at least 90 percent of the
vaccinated mice survived if they received either regimen of sulfamethoxazole /
trimethoprim (P<0.0001). When spleens from surviving mice were examined for residual
microorganisms, only vaccinated mice treated for 10 days yielded sterile spleens in over
half of the survivors (5 of 9 mice) (Figure 3b).

Discussion.

Antibiotics have been tested for efficacy against glanders in rodent models by
administering them shortly after infectious challenge (10, 18). While such testing may
identify antibiotics effective against human disease, this methodology that treats infection
before symptoms arise is quite different than occurs in actual human infection. Most
commonly, patients seek treatment after the onset of disease symptoms, which could be
days or weeks after infection. Furthermore, in the case of attack with a biological agent,
the interval between infection and appropriate treatment delivery could be prolonged due
to delayed identification of the biological agent, or logistical problems and general
confusion inherent in a mass casualty situation. Therefore, antibiotics need to be
efficacious when administered after the onset of clinical symptoms and beyond.

While candidate vaccines tested to date against glanders can be somewhat
protective in extending the time to death after infection, none have been shown to deliver
sterile immunity (12). We previously determined that immunization with heat-killed B.
*pseudomallei* whole cells could protect approximately 50 to 80 percent of BALB/c female mice from an aerosol challenge using $3 - 5 \text{LD}_{50}$ of *B. mallei* (ATCC 23344) for a period of 21 days (data not shown). However, surviving mice would typically remain infected, as determined by *B. mallei* recovered from the spleens, and surviving mice would die or require euthanasia within 60 days of challenge. Experiments described here were to determine if vaccination and antibiotic therapy were useful in enhancing survival after challenge compared to each treatment alone; and whether this “combination therapy” could be useful in reducing residual infection. Because treatment of human diseases generally begins several days after infection, treatment was not initiated until mice exhibited clinical signs and the index death had occurred. Typically that index death occurred in the negative control group as vaccination tended to provide protection from death within the first week after challenge.

The majority of human glanders cases occurred before the antibiotic era and the mortality rate was above 90% (19). There have been several cases of human glanders since the 1940s, primarily in laboratory workers, that were successfully treated with antibiotics (6, 7, 20). Six cases were successfully treated with sulfadiazine in 1944-45 (7). In a recent case of laboratory-acquired glanders, the patient received imipenem and doxycycline intravenously for one month followed by oral azithromycin and doxycycline for six months (6). This treatment regimen was successful and there was no relapse of disease.

Azithromycin, moxifloxacin, and sulfamethoxazole / trimethoprim were chosen for evaluation in our murine animal model because published reports suggested they could be useful in treating glanders. *In vitro* antibiotic testing identified several
antibiotics with potential for therapeutic benefit when used in humans (8). In that study, B. mallei strains demonstrated susceptibility to aminoglycosides, macrolides (including azithromycin), quinolones, doxycycline, piperacillin, ceftazidime, and imipenem. While there are no reports demonstrating the therapeutic efficacy of moxifloxacin against glanders in humans, efficacy has been demonstrated in hamsters (21) and in mice challenged by aerosol and treated shortly after infection (S. Demons, unpublished data). Sulfamethoxazole / trimethoprim has been reported to be efficacious against aerosol infection when tested in mice (18). Current recommendations for treating glanders are ceftazidime or meropenem for initial intensive therapy, and sulfamethoxazole / trimethoprim or amoxicillin / clavulanic acid for eradication therapy (22).

While azithromycin, moxifloxacin, and sulfamethoxazole / trimethoprim are reported to be efficacious against B. mallei in vitro and when given shortly after challenge before clinical symptoms begin, our testing demonstrated that they only delivered marginal efficacy when administered to clinically ill mice. However, these therapies were efficacious when given to previously vaccinated mice. While the mechanism of this enhanced protection was not investigated, vaccination might stimulate immune responses that restrict bacterial growth, allowing antibiotics to more effectively control infection. Specifically, vaccination could result in the production of gamma interferon, which acts synergistically with antibiotic therapy to inhibit bacterial growth (23). Although we did note residual infection in some mice given combination therapy, optimization of therapeutic and vaccination strategies might result in greater patient survival and lack of residual infection.
Acknowledgements.

Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Research was conducted under an IACUC approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.

This research was funded by the Defense Threat Reduction Agency under USAMRIID project number 923663.
References


**Figure Legend.**

Figure 1A-C. Azithromycin therapy.

Figure 1A. Azithromycin efficacy in previously immunized mice. Mice were given HBSS (diamonds) or immunized with glanders vaccine (HKBp) (triangles) followed by *B. mallei* aerosol challenge (ATCC 23344; 5 LD<sub>50</sub>). Azithromycin therapy (Az) was initiated 4 days after challenge for durations of 3 (small squares) or 5 days (large squares). Line breaks (//) indicate change in scale of x-axis.

Figure 1B. Lack of residual infection after azithromycin therapy. Mice were given HBSS or immunized with glanders vaccine (HKBp) followed by *B. mallei* aerosol challenge (ATCC 23344; 3 LD<sub>50</sub>). Azithromycin therapy (Az) of immunized mice was initiated 2 (+2d) or 3 days (+3d) after challenge for durations of 3 or 5 days.

Figure 1C. Azithromycin efficacy in immunized and unimmunized mice. Mice were given HBSS (diamonds) or immunized with glanders vaccine (HKBp) (triangles) followed by *B. mallei* aerosol challenge (ATCC 23344; 3 LD<sub>50</sub>). Azithromycin therapy (Az) was initiated 5 days after challenge for durations of 5 days (unimmunized mice – small circles; immunized mice – small squares) or 10 days (unimmunized mice – large circles; immunized mice – large squares). Note: Line breaks (//) indicate change in scale of x-axis.
Figure 2A-D. Moxifloxacin therapy.

Figure 2A. Moxifloxacin efficacy in previously immunized mice. Mice were given HBSS (diamonds) or immunized with glanders vaccine (HKBp) (triangles) followed by *B. mallei* aerosol challenge (ATCC 23344; 1.5 LD$_{50}$). Immunized mice were given moxifloxacin therapy (Moxi) beginning 3 days after challenge for durations of 3 (small squares) or 5 days (large squares). Note: Line breaks (/) indicate change in scale of x-axis.

Figure 2B. Moxifloxacin efficacy in immunized and unimmunized mice. Mice were given HBSS (diamonds) or immunized with glanders vaccine (HKBp) (triangles) followed by *B. mallei* aerosol challenge (ATCC 23344; 3 LD$_{50}$). Moxifloxacin therapy (Moxi) was initiated 5 days after challenge for durations of 5 (unimmunized mice – small circles; immunized mice – small squares) or 10 days (unimmunized mice – large circles; immunized mice – large squares). Line breaks (/) indicate change in scale of x-axis.

Figure 2C. Moxifloxacin efficacy in immunized and unimmunized mice. Mice were given HBSS (diamonds) or immunized with glanders vaccine (HKBp) (triangles) followed by *B. mallei* aerosol challenge (*B. mallei* FMH23344; 1 LD$_{50}$). Moxifloxacin therapy (Moxi) was initiated 5 days after challenge for durations of 5 (unimmunized mice – small circles; immunized mice – small squares) or 10 days (unimmunized mice – large circles; immunized mice – large squares). Note: Line breaks (/) indicate change in scale of x-axis.
Figure 2D. Lack of residual infection after moxifloxacin therapy. Mice were given HBSS or immunized with glanders vaccine (HKBp) followed by *B. mallei* aerosol challenge (*B. mallei* FMH23344; 1 LD<sub>50</sub>). Moxifloxacin therapy (Moxi) was initiated 5 days (+5d) after challenge for durations of 5 or 10 days.

Figure 3A-B. Sulfamethoxazole / trimethoprim therapy.

Figure 3A. Sulfamethoxazole / trimethoprim efficacy in immunized and unimmunized mice. Mice were given HBSS (diamonds) or immunized with glanders vaccine (HKBp) (triangles) followed by *B. mallei* aerosol challenge (*B. mallei* FMH23344; 1 LD<sub>50</sub>). Sulfamethoxazole / trimethoprim (ST) therapy was initiated 5 days after challenge for durations of 5 (unimmunized mice – small circles; immunized mice – small squares) or 10 days (unimmunized mice – large circles; immunized mice – large squares). Line breaks (//) indicate change in scale of x-axis.

Figure 3b. Lack of residual infection after sulfamethoxazole / trimethoprim therapy.

Mice were given HBSS or immunized with glanders vaccine (HKBp) followed by *B. mallei* aerosol challenge (*B. mallei* FMH23344; 1 LD<sub>50</sub>). Sulfamethoxazole / trimethoprim (ST) therapy was initiated 5 days (+5d) after challenge for durations of 5 or 10 days.