Comparison of clarithromycin and ciprofloxacin therapy for *Bacillus anthracis* Sterne infection in mice with or without $^{60}$Co gamma-photon irradiation

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Biological agents and ionizing radiation lead to more severe clinical outcomes than either insult alone. This study investigated the survival of non-irradiated and $^{60}$Co-gamma-irradiated mice given therapy for inhalation anthrax with ciprofloxacin (CIP) or a clinically relevant mixture of clarithromycin (CLR) and its major human microbiologically important metabolite 14-hydroxy clarithromycin (14-OH CLR). All B6D2F1/J 10-week-old female mice were inoculated intratracheally with $3 \times 10^8$ c.f.u. of *Bacillus anthracis* Sterne spores 4 days after the non-lethal 7 Gy dose of $^{60}$Co gamma radiation. Twenty-one days of treatment with CLR/14-OH CLR, 150 mg kg$^{-1}$ twice daily, or CIP, 16.5 mg kg$^{-1}$ twice daily, began 24 h after inoculation. Pharmacokinetics indicate that the area under the curve (AUC) for 14-OH CLR on the concentration-versus-time graph was slightly higher in gamma-irradiated than non-irradiated animals. Neither drug was able to increase survival in gamma-irradiated animals. CIP and CLR/14-OH CLR therapies in non-irradiated animals increased survival from 49 % (17/35 mice) in buffer-treated animals to 94 % (33/35) and 100 %, respectively ($P < 0.001$). *B. anthracis* Sterne only was isolated from 25–50 % of treated mice with or without irradiation. Mixed infections with *B. anthracis* Sterne were present in 50–71 % of gamma-irradiated mice but only in 5–10 % of mice without irradiation.

INTRODUCTION

Anthrax is a zoonotic disease caused by the spore-forming organism *Bacillus anthracis*. Human infections normally result from contact with contaminated animals or animal products; human-to-human transmission has never been reported. In 2001, however, in an apparent act of bioterrorism, anthrax spores were spread through the mail system in the USA causing an outbreak of infections (Dewan et al., 2002). *B. anthracis* is considered to be a likely biological warfare and terrorist agent because of the high mortality associated with inhaled anthrax spores and the excellent stability of anthrax spores (Christopher et al., 1997; Franz et al., 1997; Inglesby et al., 1999; Pile et al., 1998; World Health Organization, 1970). In fact, *B. anthracis* spores can persist in tissues of infected animals and germinate after levels of antibiotic fall in the blood (Friedlander et al., 1993).

In addition to biological threats, the risk of accidental or terrorism-related exposure to sublethal gamma or mixed-field (gamma and neutrons) radiation is a potential threat. Ionizing radiation damages the haematopoietic and gastrointestinal systems. Prompt, sublethal irradiation increases susceptibility to bacterial infections, including *B. anthracis*, by decreasing the number of circulating mature white blood cells and the number of epithelial cells in the intestine (Alper, 1979). Furthermore, disruption of stable gut flora with radiation results in overgrowth of pathogens, translocation and invasion of bacteria, and life-threatening infections. In a previous study, we demonstrated that sublethally gamma-irradiated mice have an increased susceptibility to *B. anthracis* Sterne and endogenous bacteria infections, with corresponding increases in mortality (Brook et al., 2001a).

Ciprofloxacin (CIP) is one of three antimicrobial agents (CIP, doxycycline and penicillin) approved by the USA Food and Drug Administration (FDA) for the treatment of a *B. anthracis* infection (Bartlett, 2002). The FDA approved it specifically for this indication in August 2000 based on efficacy data in rhesus monkeys (Friedlander et al., 1993).
However, CIP- and doxycycline-resistant \emph{B. anthracis} have been produced relatively easily in the laboratory (Brook et al., 2001b). Penicillin is not recommended alone because of the presence of inducible beta-lactamas in isolates from the 2001 attack on the USA Postal Service (Centers for Disease Control and Prevention, 2001). It is therefore necessary to explore the effectiveness of other antimicrobials that are active \emph{in vitro} against \emph{B. anthracis}. Exploring the efficacy of a macrolide in the treatment of \emph{B. anthracis} infection is of importance in children because of issues with the use of CIP or doxycycline in this age group (Benavides & Nahata, 2002).

In this study, we evaluated the efficacy of a mixture of clarithromycin (CLR) and its metabolite 14-hydroxy clarithromycin, 14-OH (i.v.) in a clinically relevant ratio, against an intratracheal challenge with \emph{B. anthracis} Sterne in mice with or without irradiation. Irradiation-induced damage to host defence and other systems is a very good model for simulating low host defence. The results from this study can therefore help guide clinicians to alternative treatment options for anthrax, especially in adolescents, elderly people and patients exposed to a combination of \emph{B. anthracis} and ionizing radiation.

**METHODS**

**Animals.** Female B6D2F1/J mice, supplied by Jackson Laboratories, were used in this study. Cross-breeding between the inbred DBA/2J and C57BL/6 strains produces the B6D2F1/J strain, whose response to ionizing radiation is relatively homogeneous yet has been well characterized at the Armed Forces Radiobiology Research Institute (AFRRI). Animals were quarantined and confirmed to be free of common murine pathogens. For the duration of the experiment the animals were housed in a segregated room in accordance with specifications outlined in the 'Guide for the Care and Use of Laboratory Animals' (National Research Council, 1996). The animal facility and programme are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International, and all experimental procedures were performed in compliance with 9CFR, Animal Welfare Act and the AFRRI policies regarding animal care and use. Animals were housed with a 12:12 h light/dark cycle in polycarbonate cages with a filter cover on hardwood chip bedding. Autoclaved commercial rodent feed and acidified water (pH 2.8) were provided ad libitum.

**Bacteria.** \emph{B. anthracis} Sterne is an uncapsulated strain of \emph{B. anthracis}; although attenuated in its ability to cause mortality, it maintains the ability to produce three proteins (protective antigen, lethal factor and oedema factor) that act in binary combinations to produce two important toxins (lethal toxin and oedema toxin) and thus maintains its virulence. The susceptibility of the Sterne strain to CIP or CLR is similar to that of encapsulated strains (Friedlander et al., 1993; Centers for Disease Control and Prevention, 2001).

\emph{B. anthracis} Sterne spores were harvested from batch fermentations in Schaeffer's sporulation medium (Schaeffer et al., 1965) seeded with live spore veterinary vaccine. Frozen spore stocks were kept in 10% (v/v) glycerol/water at −70 °C. The concentration was adjusted by dilution with sterile water to achieve 3×10⁶ c.f.u. ml⁻¹. This final concentration approximates the number of \emph{B. anthracis} Sterne spores required to cause 50% mortality (LD₅₀) in untreated B6D2F1/J mice without irradiation; an LD₅₀ of 1.8×10⁶ c.f.u. was observed in untreated B6D2F1/J mice with irradiation (T. B. Elliott, personal communication). The number of inoculated organisms was verified by tube dilution in sterile water and culture on tryptic soy agar. A 0.1 ml volume of suspension was inoculated i.t. into each mouse 4 days after irradiation. The method used for i.t. inoculation was originally described by Saffiotti et al. (1968) and previously described in detail (Brook et al., 2001a).

**Antimicrobials.** CLR and its metabolite were provided in powder form by Abbott Laboratories. They were combined 3:1, suspended in a solution of a 1:10 ratio of 95% ethanol in sterile 0.1 M phosphate buffer (pH 6-5), and then sonicated for 30 min. The mixture was freshly prepared and administered at a dose level of 150 mg kg⁻¹, or 3-75 mg per mouse, in 0-2 ml of diluent, twice daily for 21 days. A previous study showed that this regimen provides human-simulated exposures in the murine pneumonia model for the parent compound CLR (Tessier et al., 2002). For administration \textit{per os}, special feeding cannulas were used with small ball tips, and then cleaned and sterilized in self-sealing pouches.

CIP was provided as CIPRO I.V. or 40 ml sterile 1% solution of 400 mg CIP. The experimental dose for mice was 16.5 mg kg⁻¹, or 0.41 mg per mouse, in a 0-1 ml subcutaneous injection, twice daily. Onyeji et al. (1999) determined that administration of a single subcutaneous dose of 14-25 mg CIP kg⁻¹ in mice with renal impairment induced with 10 mg uranyl nitrate kg⁻¹ was required to achieve a value similar to humans for 24 h area under the curve (AUC) on the concentration-versus-time graph (12–19 h mg l⁻¹). We used a slightly higher dose of CIP since uranyl nitrate was not used.

**Plasma concentrations of CLR and 14-OH CLR.** One hundred 12-week-old animals were divided evenly into 0 Gy non- and 7 Gy gamma-irradiated groups. Forty-eight animals in each group received 150 mg CLR/14-OH CLR kg⁻¹ \textit{per os}, twice daily, for 4 days. At various times after the eighth dose, mice were anaesthetized by methoxyflurane inhalation for approximately 2.5–3 min in a carbon-filtered fume hood. Needles and syringes were rinsed with heparin before withdrawing up to 1 ml of blood by cardiac puncture at one time with a 23-gauge needle. There were six mice per time point at 0.25, 0.5, 1, 2, 4, 6, 8 and 11 h after the last administration of antimicrobial agent. Two mice were not given CLR/14-OH CLR and provided negative control data for the assay.

Blood was placed in sterile 500 μl Capiject tubes with inert gel silica particles and the tubes were microcentrifuged for plasma separation. Plasma samples were stored frozen at −70 °C until assayed using a validated HPLC methodology at the Center for Anti-Infective Research and Development, Hartford Hospital (Tessier et al., 2002).

**Experimental design.** Two hundred and eighty-nine 10-week-old mice were assigned to either a non- or a gamma-irradiated group. All mice were placed in ventilated acrylic plastic restrainers. Animals in the irradiation group were irradiated to 7 Gy at 0.4 Gy min⁻¹ mid-line tissue from bilaterally positioned 60Co sources as described previously (Elliot et al., 1995). Irradiation to 7 Gy without anthrax does not cause any mortality in the B6DF1/J mouse although changes in the intestinal flora are seen (Elliott et al., 1995). All animals were then housed as described above.

Mice were assigned to receive buffer, CLR/14-OH CLR or CIP and further assigned to sets for the purpose of determining survival or culturing bacteria from selected tissues (Table 1). Four days after the irradiation date all mice were challenged i.t. with \emph{B. anthracis} Sterne spores. Antimicrobial therapy was given twice daily for 21 days beginning 24 h after i.t. spore challenge.

Those mice in the survival sets were observed for signs of disease and survival for 30 days after spore challenge. Mice in the microbiological culture set were used to assess the extent of the \emph{B. anthracis} Sterne infection by culture of tissues on days 3, 5, 7, 10 and 12 after spore
Table 1. Allocation of female B6D2F1/J mice to radiation and treatment groups

All mice were challenged i.t. with B. anthracis Sterne. Abbreviations: p.o., *per os*; s.c., subcutaneously.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Irradiation and treatment</th>
<th>No. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Survival set</td>
</tr>
<tr>
<td>1</td>
<td>Irradiation + buffer (p.o.)</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>Irradiation + CLR/14-OH CLR (p.o.)</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>Irradiation + CIP (s.c.)</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>No irradiation + buffer (p.o.)</td>
<td>35</td>
</tr>
<tr>
<td>5</td>
<td>No irradiation + CLR/14-OH CLR (p.o.)</td>
<td>35</td>
</tr>
<tr>
<td>6</td>
<td>No irradiation + CIP (s.c.)</td>
<td>35</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>165</td>
</tr>
</tbody>
</table>

challenge. At the scheduled intervals, specimens of lung, liver and heart blood were removed aseptically from five mice in each microbiological culture set. Tissues were crushed with a sterile swab and spread onto Columbia sheep blood agar (SBA), Columbia colistin-nalidixic acid sheep blood agar (CNA) and xylose-lysine-desoxycholate agar (XLD). Inocula were streaked for isolation of colonies with a sterile bacteriological loop. Isolated micro-organisms were identified by the Vitek identification system (bioMerieux Vitek).

*B. anthracis* Sterne was identified by colonial morphology and MICs were determined using a macrodilution method in brain heart infusion (Brook et al., 2001b).

Statistical analysis. Survival analysis used the Fisher’s Exact Test (StatXact 5 software; CYTEL Software). The AUC between 0 and 11 h for CLR (AUCCLR) and for the metabolite (AUC14-OH CLR) in gamma-irradiated animals was compared to similar areas for non-irradiated animals over the same time interval after treatment using a data-based simulation method for statistical inference known as the ‘bootstrap’ (Efron & Tibshirani, 1993). This represents a repeated statistical sampling of the measured CLR and 14-OH CLR serum concentrations for six gamma-irradiated animals at each of the eight time points after treatment was performed. The resampled values were averaged, and an AUC calculated using the trapezoidal rule. This process was repeated for non-irradiated time points for both CLR and 14-OH CLR treatments. Differences in the AUC between animals with and without irradiation for both CLR and 14-OH CLR treatments were calculated. This process was repeated 1500 times. The bootstrap was carried out with a FORTRAN 77 program with random numbers generated using the Unix library.

RESULTS AND DISCUSSION

Survival

Treatment with either CLR/14-OH CLR or CIP increased the survival of non-irradiated mice challenged with *B. anthracis* Sterne spores. Thirty-day survival of non-irradiated mice increased from 49% (17/35) in buffer-treated animals to 94% (33/35) in CLR/14-OH CLR-treated animals (*P* < 0.001) and 100% (35/35) in those treated with CIP (*P* < 0.001, Fig. 1).

There was one survivor (1/20) in the buffer-treated, gamma-irradiated group but no survivors (0/20) in both the CLR/14-OH CLR- and the CIP-treated groups (*P* = 1.0). This study confirms our previous findings demonstrating an adverse effect in mice by combining exposure to ionizing radiation and i.t. challenge with *B. anthracis* Sterne spores (Brook et al., 2001a). Therapy of gamma-irradiated mice infected with *B. anthracis* Sterne would require antimicrobials with a broader spectrum of activity that are effective against *B. anthracis* Sterne as well as endogenous Gram-positive and Gram-negative translocated flora (Brook & Elliott, 1991; Elliott et al., 2002). The ability of both CLR/14-OH CLR and CIP to prevent mortality in non-irradiated mice despite the presence of translocated organisms that were not susceptible to these antimicrobials is explained by their efficacy against *B. anthracis* Sterne and the ability of the unimpaired immune system to control the translocated endogenous flora.

![Fig. 1](http://jmm.sgmjournals.org)
Plasma CLR and 14-OH CLR concentration

CLR is metabolized to 14-OH CLR in the liver of humans. It is this metabolite that is responsible for the antimicrobial activity of the drug. Mice cannot convert CLR to 14-OH CLR and, as a result, we simulated the situation in humans by administering both CLR and 14-OH CLR in a clinically relevant 3:1 ratio.

The AUCCLR was 11.884 h μg ml⁻¹ in mice with irradiation and 12.076 h μg ml⁻¹ in mice without irradiation (Table 2). A difference of 0.192 with a bootstrapped standard error of 1.241 gave a ratio of 0.155, which does not suggest a significant difference from zero. The percentage of 1500 iterated values less than zero was 42.1, which also suggests no difference in AUCCLR between non-irradiated and gamma-irradiated animals.

The AUC14-OH CLR was 7.433 h μg ml⁻¹ in mice with irradiation and 6.385 h μg ml⁻¹ in mice without irradiation (Table 2). A difference of 1.358 with a bootstrapped standard error of 0.681 was detected for the metabolite. Their ratio, equal to 1.241, promoting the dissimilarity in the AUC14-OH CLR and the percentage of bootstrapped differences less than zero was 2/3, promoting the dissimilarity in the AUC14-OH CLR between non-irradiated and gamma-irradiated animals.

There is slightly more rapid absorption of CLR and 14-OH CLR in gamma-irradiated mice, as indicated by a maximum plasma concentration at 15 min (Table 2). The increased initial absorption may be due to the degenerative changes in the gut mucosa following irradiation that makes the intestinal wall more permeable to the antimicrobials. However, the faster absorption and the increased AUC of the metabolite in gamma-irradiated animals did not reduce the incidence of infection due to translocated micro-organisms or increase survival.

Antimicrobial resistance

The MIC of CLR/14-OH CLR was 0-250 μg ml⁻¹ and the MIC of CIP was 0-08 μg ml⁻¹ against B. anthracis Sterne. There was no change in the MIC of either antimicrobial when tested against B. anthracis Sterne recovered from mice 12 and 30 days after initiating the 21 day treatment. Our MICs are similar to other reports and to other encapsulated and unencapsulated B. anthracis strains (Athanana et al., 2004; Brook et al., 2001b; Frenan et al., 2003; Centers for Disease Control and Prevention, 2001). Animal mortality may then be attributed to the lack of CLR/14-OH CLR and CIP activity against a portion of the polymicrobial pathogens that emerge after exposure to ionizing irradiation and a B. anthracis Sterne challenge.

Microbiology

Animals without irradiation. Despite the clinical success of therapy in mice without irradiation, B. anthracis Sterne was still present in the organs of treated animals. B. anthracis Sterne alone was recovered from 44 % (8/18), 50 % (10/20) and 25 % (5/20) of mice in the buffer, CLR/14-OH CLR and CIP treatment groups, respectively (Table 3). CIP appeared to provide a slight advantage over CLR/14-OH CLR in clearing the B. anthracis Sterne infection among mice without irradiation based on isolation of B. anthracis Sterne; however, this difference did not produce an increase in animal survival.

We found Enterococcus gallinarum mixed with B. anthracis Sterne in 6 % (1/18) of buffer-treated animals and in 5 % (1/20) of CIP-treated animals (Table 3). Mixed infections with Staphylococcus hominis (1/20) or Staphylococcus sciuri (1/20) were found in 10 % (2/20) of CLR/14-OH CLR-treated animals. Species of Enterococcus (Enterococcus faecalis and Enterococcus gallinarum) and Enterobacter cloacae were found alone in buffer-treated animals (Table 3). We found three animals with either Enterococcus faecalis or Enterococcus gallinarum and one animal with S. hominis in the CIP treatment group. In one CLR/14-OH CLR-treated animal only Staphylococcus constellatus was found.

Translocation with mixed infections has not been observed in animals without irradiation and without anthrax challenge. The observed B. anthracis Sterne infection with enteric organisms in 7 % (4/58) of mice infected with B. anthracis Sterne spores without irradiation suggests the capability of B. anthracis toxins alone to induce translocation in the non-irradiated host. This conclusion is consistent with our previous study, in which translocation occurred in up to 8 % (2/25) of non-irradiated mice challenged with toxigenic B. anthracis Sterne but not in gamma-irradiated mice challenged with non-toxigenic B. anthracis Δ-Sterne-1 (Brook et al., 2001a).

Animals with irradiation. In the present study we found B. anthracis Sterne alone in 29 % (2/7), 38 % (3/8) and 36 % (4/11) of gamma-irradiated mice in the buffer, CLR/14-OH...
Table 3. Number of B6D2F1/J mice in each treatment group with bacteria isolated from liver, lung or heart blood in the first 12 days after challenge

<table>
<thead>
<tr>
<th>Bacteria isolated</th>
<th>With irradiation</th>
<th>Without irradiation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer (n = 7)</td>
<td>CLR/14-OH CLR (n = 8)</td>
</tr>
<tr>
<td>B. anthracis Sterne only</td>
<td>2 (29%)</td>
<td>3 (38%)</td>
</tr>
<tr>
<td>Mixed (B. anthracis Sterne + other)</td>
<td>5 (71%)</td>
<td>4 (50%)</td>
</tr>
<tr>
<td>Other, Gram-positive</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Other, Gram-negative</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Other species only</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Gram-positive</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gram-negative</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>0 (0%)</td>
<td>1 (13%)</td>
</tr>
</tbody>
</table>

†Enterococcus faecalis and Enterobacter cloacae isolated from the same mouse.

CLR and CIP treatment groups, respectively. Many more mixed infections were found in irradiated animals than in animals without irradiation. Enterobacter cloacae, Enterococcus faecalis and S. sciuri were isolated with B. anthracis in 71% (5/7) of control animals. Only Gram-negative organisms (i.e. Enterobacter cloacae and Klebsiella pneumoniae) were found with B. anthracis Sterne in 50% (4/8) of animals treated with CLR/14-OH CLR, and only Gram-positive organisms (Enterococcus faecalis, Enterococcus gallinarum and S. sciuri) were found with B. anthracis Sterne in 64% (7/11) of the CIP treatment group (Table 3).

These patterns of antimicrobial activity are consistent with other reports that have found CLR to be active in vitro against Gram-positive micro-organisms but a poor inhibitor of Gram-negative micro-organisms (Anzueto & Norris, 2004) and CIP to be active in vitro against Gram-negative micro-organisms but less effective against Gram-positive micro-organisms (Dalhoff & Schmitz, 2003). We currently do not have an explanation for the emergence of three Gram-positive infections in animals treated with CLR/14-OH CLR without irradiation. Our observations demonstrate, however, the importance of treating not only the primary infection but subsequent enteric infections that are likely to emerge in an immunocompromised host.

Translocation of intestinal micro-organisms and subsequent polymicrobial infections in gamma-irradiated animals occur also in mice given lethal doses of ionizing irradiation alone without inoculation of B. anthracis Sterne (Brook & Elliott, 1991; Elliott et al., 1995). Furthermore, bacterial translocation of enteric organisms in irradiated mice occurred more often if challenged with B. anthracis Sterne than not (36% vs 3%; Brook et al., 2001a). This provides further evidence that B. anthracis Sterne is capable of reducing the threshold for translocation.

The poor survival and rapid mortality among gamma-irradiated mice challenged with B. anthracis Sterne limited the microbiological assessment of antimicrobials. Most of the mortality occurred within 2–3 days, illustrating the lethality of combining both ionizing radiation and B. anthracis. Initiating antimicrobial treatments within 24 h of challenge and continuing for at least 21 days optimizes survival of the host because persistent inactive spores germinate when antimicrobial therapy is finished (Elliott et al., 2002; Friedlander et al., 1993; Knudson, 1986). A period of 21 days following non-lethal gamma irradiation in mice also allows time to begin recovering from the depression of bone marrow progenitor cells and from the depressed innate immune system (Elliott et al., 1990; Brook & Ledney, 1992).

The persistence of B. anthracis Sterne in mice with and without irradiation underlines the practical difficulty in treating animals challenged with spores. This finding supports the rationale for prophylaxis and long-term therapy for inhalation anthrax that is recommended for humans and validates this animal model. Similar survival and microbiological results were observed when we repeated this experiment. The trend toward survival in both experiments with the administration of CLR/14-OH CLR in non-irradiated hosts supports the utility of CLR in the treatment of anthrax.

In conclusion, we found CLR/14-OH CLR therapy to be an effective agent against B. anthracis Sterne in animals without irradiation. Furthermore, oral administration of CLR/14-OH CLR was equal to subcutaneous CIP in the treatment of B. anthracis Sterne infections in hosts with or without irradiation; however, both antimicrobials were ineffective at increasing survival in gamma-irradiated animals. Further studies are needed to assess the in vivo effectiveness of other antimicrobials for treating infection in individuals with low host defence or after radiation injury.

ACKNOWLEDGEMENTS

The authors acknowledge and appreciate the technical support of David...
REFERENCES


