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Antimicrobial Therapy for *Bacillus anthracis*-Induced Polymicrobial Infection in ^{60}Co γ-Irradiated Mice

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Challenge with both nonlethal ionizing radiation and toxigenic *Bacillus anthracis* spores increases the rate of mortality from a mixed bacterial infection. If biological weapons, such as *B. anthracis* spores, and nuclear weapons were used together, casualties could be more severe than they would be from the use of either weapon alone. We previously discovered that a polymicrobial infection developed in B6D2F$_1$/J mice after nonlethal (7-Gy) ^{60}Co γ irradiation and intratracheal challenge with *B. anthracis* Sterne spores 4 days after irradiation. In this present study, we investigated the survival of mice and the response of the polymicrobial infection during the course of antimicrobial therapy with penicillin G procaine, ofloxacin, trovafloxacin, or gatifloxacin. Survival was prolonged, but not ensured, when the mice were treated with either broad-spectrum ofloxacin or narrow-spectrum penicillin G for 7 days beginning 6 or 24 h after challenge. Survival was not prolonged when therapy was delayed more than 24 h after challenge. When these two antimicrobial agents were given for 21 days, the survival rate was increased from 0% for the controls to 38 to 63% after therapy. Therapy with trovafloxacin or gatifloxacin reduced the incidence of mixed infection and improved the rate of survival to 95% (trovafloxacin) or 79% (gatifloxacin), whereas the rate of survival for the controls was 5%. We conclude that the mixed infection induced by *B. anthracis* in irradiated mice complicates effective therapy with a single antimicrobial agent. To limit mortality following nonlethal irradiation and challenge with *B. anthracis* spores, antimicrobial therapy needs to be initiated within a few hours after challenge and continued for up to 21 days.

Accidental or war-related exposure to ionizing radiation, if combined with accidental or intended infection with *Bacillus anthracis* spores, could cause combined injuries that would be more severe than the injuries caused by either agent alone. Nonlethal doses of ionizing radiation increase susceptibility to exogenous bacterial infections (18). As a potential biological weapon, *B. anthracis* spores are a source of exogenous infection by inhalation (36). Inhalational anthrax is rare but usually fatal in humans because the disease progresses rapidly with few clinical signs or symptoms until just prior to death (1, 17), as demonstrated in several species of laboratory animals (16, 27).

It is likely that such combined injuries would be presented to civilian as well as military emergency medical services. Effective preventive and therapeutic measures need to be developed for such combined injuries. To do so, it is essential that the threats posed by the biological effects of ionizing radiation in combination with the effects of biological weapons, such as *B. anthracis* spores, or endemic infectious diseases be assessed in a practical laboratory animal model.

We needed a laboratory animal model that would not only demonstrate differences in responses to combined doses of both γ radiation and bacterial spores but would also demonstrate the efficacies of experimental therapies (7). We chose the hybrid B6D2F$_1$/J female mouse because it is both susceptible to *B. anthracis* Sterne and relatively resistant to γ radiation compared to other strains of mice. The susceptibilities of mice to *B. anthracis* Sterne when it is inoculated subcutaneously (s.c.) vary with the strain of mouse (39). Inbred mice are more susceptible to virulent strains of *B. anthracis* than to *B. anthracis* Sterne (40).

We previously determined that susceptibility to *B. anthracis* Sterne spores was increased, and we discovered that a polymicrobial infection developed when spores were inoculated either s.c. or intratracheally (i.t.) into sublethally γ-irradiated mice (6). The efficacies of therapeutic agents against *B. anthracis*-induced polymicrobial infection in irradiated animals remain unknown.

In the studies reported here, we evaluated antimicrobial therapy with penicillin G procaine (PEN G) and the quinolones ofloxacin (OFX), trovafloxacin (TVA), and gatifloxacin (GAT) against an i.t. *B. anthracis* Sterne challenge combined with nonlethal γ irradiation in a mouse model and evaluated the correlating microbiology of tissues and the antimicrobial susceptibilities of the isolated bacteria. We also evaluated the macrolides erythromycin (ERY), azithromycin (AZM), and clarithromycin (CLR) in this model. This evaluation would be valuable to physicians who would treat radiation casualties in combination with exposure to *B. anthracis*.

MATERIALS AND METHODS

Animals. Our research was conducted according to the principles enunciated in the *Guide for the Care and Use of Laboratory Animals* (26) in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All procedures involving animals were reviewed and approved by our institutional animal care and use committee. Disease-free, female

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B6D2F$_1$/J mice (*Mus musculus*) were obtained from Jackson Laboratories (Bar Harbor, Maine). The mice were held in quarantine for 2 weeks. Representative mice were examined by microbiological, serologic, and histopathologic methods to ensure the absence of specific bacteria, particularly *Pseudomonas aeruginosa*, and common murine diseases. Up to 10 mice were housed in sanitized polycarbonate boxes (46 by 24 by 15 cm) with a filter cover (MicroIsolator; Lab Products, Inc., Maywood, N.J.) on hardwood-chip bedding. During the experiments, four mice were housed per cage. The animal holding rooms were maintained at approximately 70°F and 50% (±10%) relative humidity with a 12-h light and 12-h dark full-spectrum lighting cycle. Conditioned fresh air was changed at least 10 times per hour. The mice were given feed (Wayne Lab Blox; Continental Grain Co., Chicago, Ill.) and acidified (pH 2.5) water freely and were used when they were 17 to 38 weeks of age. Cages, bedding, and water were changed twice weekly.

Bacteria. The suspension of *B. anthracis* Sterne spores was prepared by culture of the live veterinary anthrax spore vaccine (Colorado Serum Co., Denver, Colo.) inoculated into Schaeffer's sporulation medium (37), as described previously (6). Spore suspensions were heated at 65°C for 30 min prior to enumeration or inoculation. This attenuated strain possesses the pXO1 plasmid, which encodes the lethal factor, edema factor, and protective antigen, but not the pXO2 plasmid, which encodes the biosynthetic enzymes required for production of the poly-D-glutamic acid antiphagocytic capsule. James Rogers of the Biological Defense Research Program, Naval Medical Research Institute, Bethesda, Md., kindly confirmed the presence of pXO1 plasmid and the absence of pXO2 plasmid in our culture of *B. anthracis* Sterne.

The number of spores in the suspensions was determined by inoculating 10-fold dilutions, prepared in sterile water in sterile glass tubes, onto Trypticase soy agar (Becton Dickinson and Co., Cockeysville, Md.), which was incubated at 35°C for 18 to 24 h. The numbers of colonies per 100-μl inoculum were counted.

Antimicrobial agents. Antimicrobial therapy with PEN G (62.5 or 125 mg/kg of body weight administered intramuscularly [i.m.] or s.c.; Wyeth Laboratories, Inc., Philadelphia, Pa.) or OFX (40 mg/kg administered either per os [p.o.] or intraperitoneally [i.p.]; Floxin I.V.; Ortho-McNeil, Raritan, N.J.) was administered once daily for 7 or 21 days after challenge. Although ciprofloxacin (CIP) is at present a drug of choice for the treatment of anthrax, it requires administration every 12 h (q12h), whereas OFX offers the experimental advantage of once-a-day administration with the same antimicrobial coverage as CIP. TVA (20 mg/kg; Trovan I.V.; Pfizer Roerig, New York, N.Y.) and GAT (20 mg/kg; Tequin Injection; Bristol-Meyers Squibb Co., Princeton, N.J.) were given either s.c. or p.o. once daily for 21 days. The doses of quinolones were selected on the basis of the fact that only the *levo* isomer of OFX is active, so comparative doses of TVA and GAT would be 20 mg/kg. Doses in mice of 40 mg of OFX/kg and 20 mg of TVA/kg are seven times the recommended doses in humans, and the dose in mice of 20 mg of GAT/kg is 3.5 times the recommended dose in humans. Diluent was given to mice p.o. but not s.c. in order to avoid inoculating skin microorganisms, which could inadvertently increase mortality in control irradiated mice.

In a previous study, the lowest detected mean serum PEN G concentration in 6.5-Gy-irradiated B6D2F$_1$/J female mice was 0.6 μg/ml when concentrations were determined 1 and 24 h after administration on the fourth day of therapy (5). Mean serum OFX concentrations were also previously reported to be 2.6 ± 0.4 μg/ml at 1 h and 0.4 ± 0.2 μg/ml at 23 h, and those of CIP were 2.8 ± 0.5 μg/ml at 1 h and 0.2 ± 0.1 μg/ml at 11.5 h after administration on the fifth day of therapy in 8-Gy-irradiated mice (8).

Three macrolides were given either p.o. or s.c. for 14 days in doses scaled for mice, which were 10 times greater than the pediatric doses for humans (22, 24), and compared to water. AZM (a 100-mg/kg loading dose at the first administration and a 50-mg/kg maintenance dose thereafter; Zithromax for injection; Pfizer) was given either p.o. or s.c. CLR (150 mg/kg; BIAXIN Granules; Abbott Laboratories, North Chicago, Ill.) and ERY (500 mg/kg per day in divided doses of 250 mg/kg twice per day; E.S.S. Granules; Abbott Laboratories) were given only p.o.

Irradiation. Mice were placed in ventilated acrylic plastic boxes, and bilateral midline tissue was given a 7-Gy, nonlethal dose of ^{60}Co γ radiation at 0.4 Gy/min in the Armed Forces Radiobiology Research Institute ^{60}Co Whole-Body Irradiation Facility (11), as previously described in detail (19).

Microbiology and antimicrobial susceptibility. Mice that had either recently deceased within the previous 2 h or that had been euthanized by cervical dislocation were dissected aseptically to isolate bacteria from tissues. Spleens and both lungs were removed and crushed with a sterile cotton swab in a sterile dish. The apex of the hearts was cut. Specimens of spleen and lung tissues on swabs and heart blood from the cut surface of the heart were inoculated onto Columbia sheep blood agar (SBA; Becton Dickinson and Co., Cockeysville, Md.), colistin-naladixic acid sheep blood agar (CNA; Becton Dickinson and Co., Cockeysville, Md.), and MacConkey agar (MAC; Becton Dickinson and Co., Cockeysville, Md.). Inocula were streaked by the four-dilution streaking technique with a flamed wire loop to obtain the isolated colonies. The two sheep blood media, SBA and CNA, were incubated in 5% CO_2 at 35°C for 18 to 24 h. Cultures on MAC were incubated at 35°C for 18 to 24 h. If no growth was observed, the plates were reincubated for another 24 h. Colonies of all morphologies, except *B. anthracis*, isolated from each mouse were transferred to SBA for Gram staining and identification with an automated system (Vitek; bioMérieux, Inc.) at the clinical laboratory of the Uniformed Services University of the Health Sciences, Bethesda, Md.

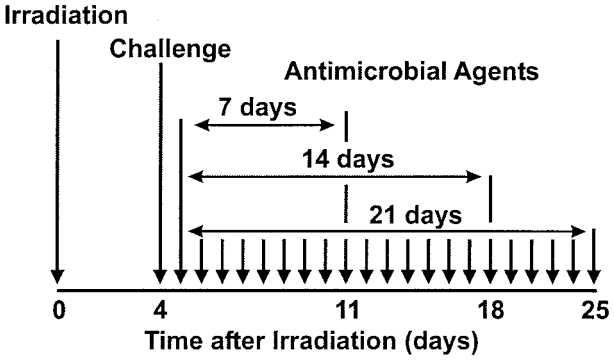

FIG. 1. General experimental design. Mice were irradiated and were then challenged by i.t. inoculation of *B. anthracis* Sterne spores 4 days later and given antimicrobial therapy daily for 7, 14, or 21 days beginning 6, 7.5, 24, 48, or 72 h after bacterial spore challenge.

A Vitek instrument was used to determine the susceptibilities of selected isolates to several antimicrobial agents to discern whether resistance appeared or increased with continuous therapy, as determined by measurement of the MIC. The results for susceptibility to PEN G, CIP, levofloxacin (LVX; the active isomer of OFX), and vancomycin (VAN) were reported for gram-positive bacteria. The results for susceptibility to ampicillin were reported for gram-negative bacteria because PEN G is not tested against gram-negative bacteria in the Vitek system. In addition, in that system CIP is used as the representative quinolone. β-Lactamase production was detected in many isolates by the acidometric method with Vitek cards. For the quinolones, we considered an MIC ≥4 μg/ml to indicate resistance.

Production of β-lactamase was determined in 20 colonies of *B. anthracis* Sterne grown on Mueller-Hinton agar medium for 24 h at 35°C. Growth from each colony was spread onto a cefinase disk (BBL231650; Becton Dickinson and Co., Sparks, Md.). The color reaction was recorded within 60 min.

B. anthracis is not included in the database of the Vitek (bioMérieux) software. Consequently, we assigned a pseudonym for isolates of *B. anthracis* Sterne in order to obtain and compare antimicrobial susceptibilities on a relative basis only within this study.

Survival measurement and data analysis. The 30-day survival rates for the experimental groups of mice were compared by the generalized Mantel-Cox procedure (Program 1L; BMD Statistical Software, Inc., Los Angeles, Calif.).

Experimental design. We evaluated the efficacies of four antimicrobial agents for the treatment of *B. anthracis* infection in irradiated mice (Fig. 1). Mice were given a nonlethal 7-Gy dose of ^{60}Co γ radiation. A challenge dose of *B. anthracis* Sterne spores was inoculated i.t. 4 days after irradiation. We inoculated a known, well-controlled dose of bacterial spores i.t. so that we could simulate an inhalational challenge accurately (6). Handling and care of spore-challenged mice were conducted according to the recommendations for biosafety level 2 of the Centers for Disease Control and Prevention. Antimicrobial therapy was initiated 6, 7.5, 24, 48, or 72 h after spore challenge and continued for 7 or 21 days. In order to observe survival only, mice were housed at 4 mice per cage in treatment groups of 16 mice per group and one control group of 12 mice. Heart blood of representative recently deceased mice was cultured for microorganisms to confirm the presence of *B. anthracis*. In parallel experiments to monitor the incidence of infection, mice were housed at seven or eight per cage in the same treatment groups used for survival experiments and reserved for microbiological assessment at scheduled intervals. For these mice, five mice per group were euthanized by cervical dislocation on days 1, 3, 5, 8, and 10 after spore challenge, that is, days 5, 7, 9, 12, and 14 after irradiation, respectively. Specimens of spleen and lung

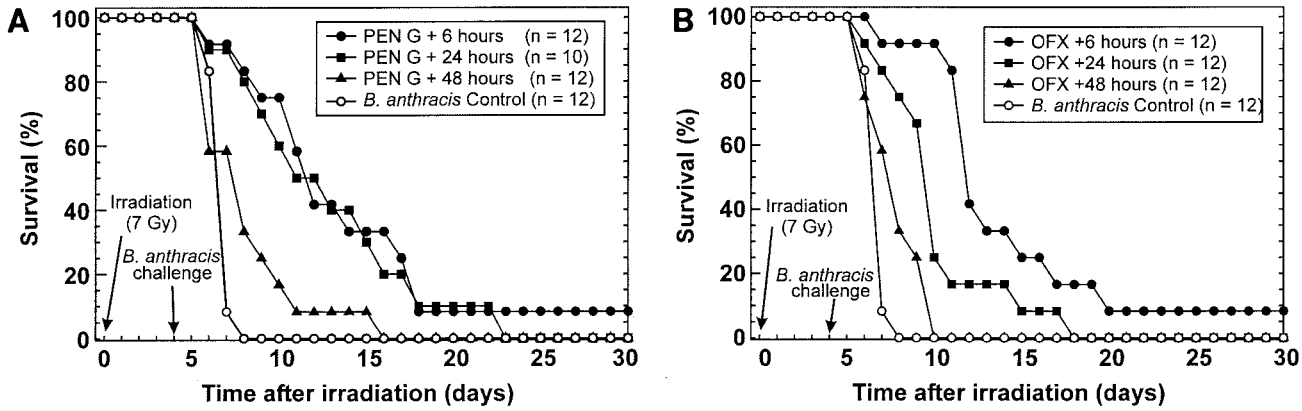

FIG. 2. Time of administration of PEN G (62.5 mg/kg i.m.) (A) or OFX (40 mg/kg i.p.) (B) for 7 days and rates of survival from *B. anthracis* Sterne challenge (7.8×10^8 CFU i.t.) in ^{60}Co γ-irradiated (7 Gy) mice. Therapy was started 6, 24, 48, or 72 h after challenge. Significant comparisons were as follows: $P < 0.01$ for the control versus PEN G started at 6 h, OFX started at 6 h, PEN G started at 24 h, and OFX started at 24 h; $P < 0.05$ for the control versus OFX started at 48 h; $P < 0.05$ for PEN G started at 48 h versus PEN G started at 24 h, OFX started at 24 h versus OFX started at 6 h, OFX started at 48 h versus OFX started at 24 h, and OFX started at 72 h versus OFX started at 48 h.

tissue and heart blood were inoculated onto bacteriological media. Bacteria were isolated and identified as described above.

RESULTS

Survival. We evaluated the survival of mice that were given antimicrobial therapy. The concentration of *B. anthracis* spores in challenge inocula varied slightly among experiments from 2.1×10^8 to 7.8×10^8 CFU/0.1 ml/mouse. Consequently, the rates of survival varied slightly among the groups of mice that were given the same therapeutic dose of an antimicrobial agent. Statistical evaluations between experimental groups in each experiment are presented for each figure.

(i) Experiment 1: determination of optimal time for initiation of 7-day therapy. We evaluated therapy with PEN G i.m. (62.5 mg/kg) or OFX i.p., which was started 6, 24, 48, or 72 h after challenge (7.8×10^8 CFU/0.1 ml i.t.), for 7 days. Mortality occurred later when therapy was started at 6 or 24 h ($P < 0.01$) than when therapy was started at 48 or 72 h (Fig. 2A and B). The rate of survival for the groups in which therapy was started at 72 h (data not shown) corresponded to that for the control ($P > 0.05$).

(ii) Experiment 2: comparison of two starting times for two antimicrobial agents given for 21 days. We (i) increased the duration of antimicrobial therapy to 21 days and compared two starting times, (ii) increased the dose of PEN G to 125 mg/kg, and (iii) administered OFX p.o. in this and subsequent experiments because we surmised that daily i.p. injections, even of an effective antimicrobial agent like OFX, might be deleterious in an irradiated mouse. When either PEN G s.c. or OFX p.o. was started 7.5 h after spore challenge (3.3×10^8 CFU/0.1 ml i.t.) and continued for 21 days, 63% (10 of 16) of the mice survived for more than 30 days, but when antimicrobial therapy with either agent was started 24 h after spore challenge, only 38% (6 of 16) of the mice survived (Fig. 3). All 12 control mice died less than 5 days after bacterial spore challenge. Deaths of mice occurred between days 2 and 14 after spore challenge when PEN G was started 7.5 h after spore challenge (median day of death after spore challenge [MD], day 12), between days 4 and 12 when OFX was started 7.5 h after spore challenge (MD, day 7), between days 1 and 14 when PEN G was started 24 h after spore challenge (MD, day 5), and between days 3 and 15 when OFX was started 24 h after spore challenge (MD, day 5).

(iii) Experiment 3: comparison of TVA to OFX and PEN G given for 21 days starting 24 h after challenge. When we treated irradiated and challenged (4.1×10^8 CFU/0.1 ml i.t.) mice once daily, with therapy beginning 24 h after challenge, with a combination of PEN G s.c. (125 mg/kg) and OFX p.o., 55% (11 of 20) of the mice survived, whereas 25% (5 of 20) of the mice given only PEN G, 21% (4 of 19) of mice given only OFX, and 3% of controls survived. However, 95 to 100% of mice treated with TVA survived (Fig. 4).

(iv) Experiment 4: comparison of GAT and TVA given for 21 days starting 24 h after challenge. Because newer quinolones offer broader coverage than other agents for mixed or polymicrobial infections, we evaluated GAT in comparison with TVA. These quinolones were administered once daily either s.c. or p.o. starting 24 h after spore challenge (4.5×10^8 CFU/0.1 ml i.t.) and continued for 21 days. Rates of survival (Fig. 5) were greater for mice given TVA (90%) by either route than for mice given GAT (79%) or no antimicrobial agent (5%).

(v) Experiment 5: comparison of AZM p.o. or s.c., CLR p.o., and ERY p.o. with TVA p.o. given for 14 days starting 24 h after challenge. We evaluated the macrolides AZM, CLR, and ERY in comparison with TVA because ERY, in particular, could be used instead of doxycycline prophylactically against anthrax in children because doxycycline discolors teeth. AZM and CLR accumulate intracellularly with a low concentration in blood serum, whereas ERY is distributed between blood serum and tissues. AZM, CLR, and TVA were given once daily and ERY was given twice daily starting 24 h after spore challenge (1.8×10^8 CFU/0.1 ml i.t.) and continued for 14 days These treatments were compared to sterile water as a control vehicle. Rates of survival (Fig. 6) were greater for mice given TVA (63%) than for mice given ERY p.o. (15%), AZM s.c.

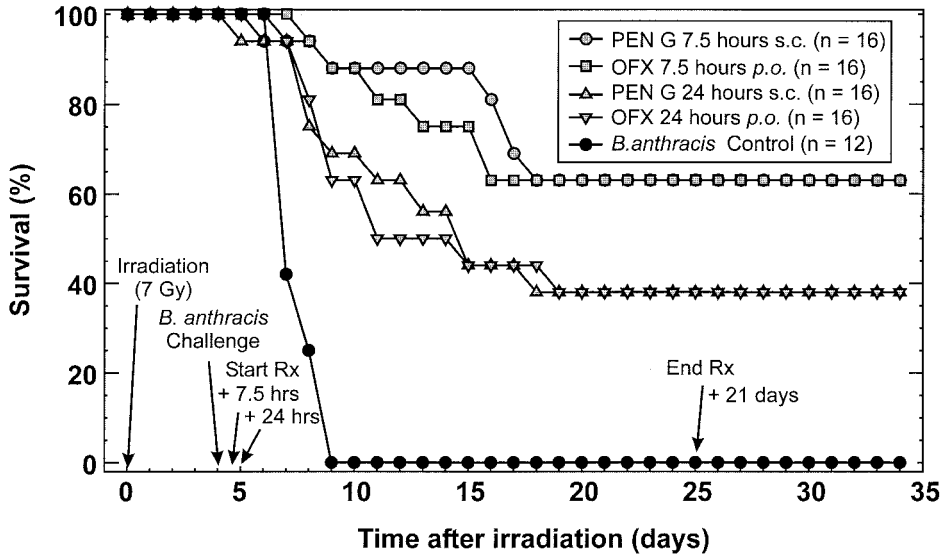

FIG. 3. Survival of mice treated with PEN G (125 mg/kg s.c.) or OFX (40 mg/kg p.o.) for 21 days after ^{60}Co γ irradiation (7 Gy) and challenge with *B. anthracis* Sterne spores (3.3×10^8 CFU i.t.). Therapy was started either 7.5 or 24 h after challenge. Significant comparisons were as follows: $P < 0.01$ for control versus PEN G started at 7.5 h, OFX started at 7.5 h, PEN G started at 24 h, and OFX started at 24 h.

(5%), AZM p.o. (0%), CLR p.o. (0%), water s.c. (5%), and water p.o. (0%).

Microbiology. Procedures for isolation of bacteria were performed with euthanized and recently deceased mice in experiments 2, 3, and 4. Following treatment with various antimicrobial agents, which have differential effects on gram-positive and gram-negative bacteria, as well as in control mice, the cumulative data show that a mixed, polymicrobial, infection occurred following challenge with *B. anthracis* Sterne spores in a large proportion of sublethally γ-irradiated mice (Tables 1 and 2). A majority of *B. anthracis* organisms isolated from mice were sensitive to PEN G and the two quinolones, CIP and LVX. A majority of the *Enterococcus faecalis* organisms isolated were resistant to CIP and LVX, but all gram-negative rods isolated were sensitive to CIP.

(i) Experiment 2: comparison of two starting times for two

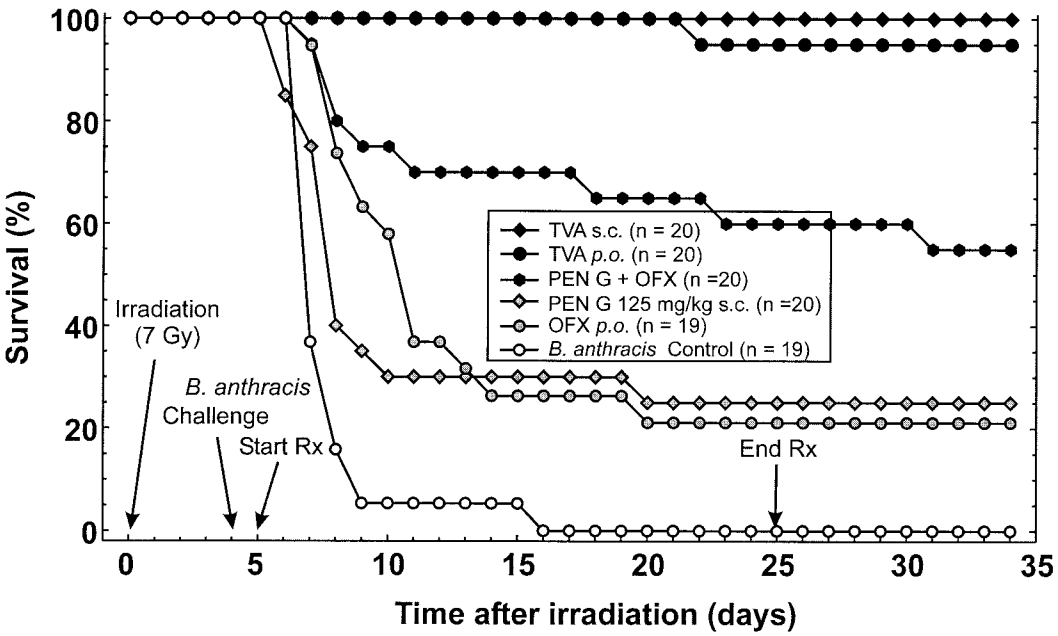

FIG. 4. Survival of B6D2F$_1$/J mice treated with antimicrobial agents (20 mg of TVA/kg p.o. or s.c., 40 mg of OFX/kg p.o., or 125 mg of PEN G/kg s.c.) for 21 days after ^{60}Co γ irradiation (7 Gy) and challenge with *B. anthracis* Sterne spores (4.1×10^8 CFU i.t.). Therapy was started 24 h after challenge. Significant comparisons were as follows: $P < 0.01$ for control versus TVA s.c., TVA p.o., PEN G plus OFX, and OFX p.o., for PEN G plus OFX versus TVA p.o., and for PEN G + OFX versus TVA s.c.; $P < 0.05$ for control versus PEN G s.c., PEN G s.c. versus PEN G plus OFX, and OFX p.o. versus PEN G plus OFX.

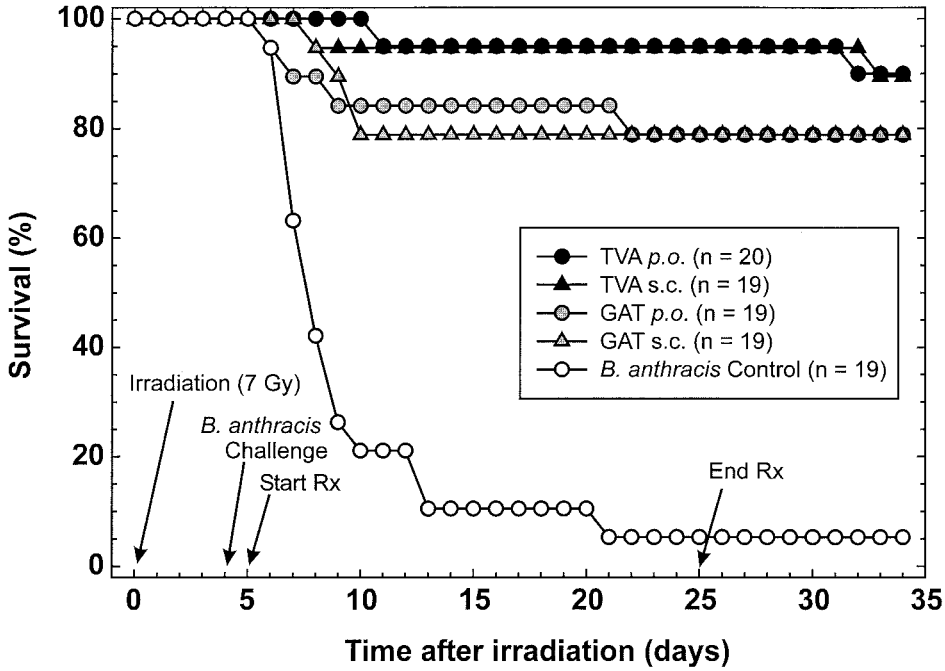

FIG. 5. Survival of B6D2F$_1$/J mice treated with GAT and TVA (20 mg/kg p.o. or s.c.) for 21 days after ^{60}Co γ irradiation (7 Gy) and challenge with *B. anthracis* Sterne spores (4.5×10^8 CFU i.t.). Therapy was started 24 h after challenge. Significant comparisons were as follows: $P < 0.01$ for control versus TVA s.c., TVA p.o., GAT s.c., and GAT p.o.

antimicrobial agents given for 21 days (Table 1). Of 60 euthanized mice from which spleen, lungs, and heart blood were cultured on a schedule, 23 were given PEN G (125 mg/kg s.c.), 21 were given OFX p.o., and 16 were used as controls following administration of a challenge dose of 3.3×10^8 CFU/0.1 ml i.t. Predominant genera or species that were isolated from euthanized mice, in addition to *B. anthracis*, were *E. faecalis* (17 of 25 mice), *Enterobacter cloacae* (10 of 25 mice), *Klebsiella pneumoniae* (5 of 25 mice), and *Lactobacillus* spp. (3 of 25 mice). Three species of *Staphylococcus* (5 of 25 mice) were isolated in small numbers, and *Enterococcus avium* (1 of 25 mice) and *Edwardsiella tarda* (1 of 25 mice) were isolated less frequently. The overall incidence of infection was 69% (11 of 16 mice) for euthanized control mice, whereas they were 70% (16 of 23

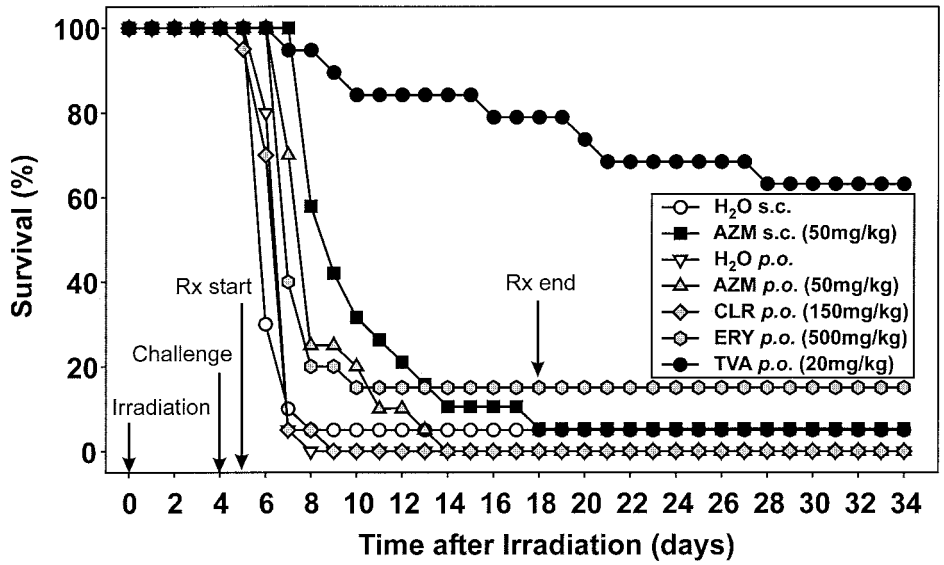

FIG. 6. Survival of B6D2F$_1$/J mice treated with macrolide antimicrobial agents (50 mg of AZM/kg p.o. or s.c., 150 mg of CLR/kg p.o., 500 mg of ERY/kg p.o., and 20 mg of TVA/kg p.o.) for 14 days after ^{60}Co γ irradiation (7 Gy) and challenge with *B. anthracis* Sterne spores (1.8×10^8 CFU i.t.). Therapy was started 24 h after challenge and continued for 14 days. Significant comparisons were as follows: ERY versus TVA ($P = 0.0001$).

TABLE 1. Cumulative incidence of bacteria isolated from tissues of $B6D2F_1/J$ mice after irradiation, i.t. challenge on day 4 with *B. anthracis* Sterne spores, and during treatment with an antimicrobial agent (experiment 2)[a]

Bacterium isolated	No. of mice with bacteria in tissues/no. of mice examined (%)			
	Control	PEN G[b] s.c.	OFX p.o.	Total
B. anthracis only	6/16 (38)	5/23 (22)	3/21 (14)	14/60 (23)
Other species only	0/16 (0)	1/23 (4)	1/21 (5)	2/60 (3)
Gram positive	0	0/1	1/1	
Gram negative	0	1/1	0/1	
Mixed (*B. anthracis* + other)	5/16 (31)	10/23 (43)	8/21 (38)	23/60 (38)
Other, gram positive	5/5	7/10	8/8	
Other, gram negative	4/5	9/10	0/8	
None	5/16 (31)	7/23 (30)	9/21 (43)	21/60 (35)

[a] The mice were irradiated with 7 Gy of ^{60}Co γ radiation and challenged with 3.3×10^8 CFU of *B. anthracis* Sterne spores. Lung, spleen, or heart blood was aseptically removed from mice euthanized on schedule on days 5, 7, 9, 12, and 14. Therapy was begun 7.5 h after challenge. The numerators show the proportion of animals from which general categories of bacteria were isolated relative to therapeutic agent or control. The numerators were differentiated further between gram-positive and gram-negative bacteria.
[b] Dose, 125 mg of PEN G/kg.

mice) for PEN G-treated mice and 57% (12 of 21 mice) for OFX-treated mice. We found that the total incidence of mixed infection among the experimental treatment groups of mice was 38%, the incidence of infection with *B. anthracis* only was 23%, the incidence of infection only with bacterial species other than *B. anthracis* was 3%, and the absence of infection was 35%. It is remarkable that no gram-negative bacteria were isolated between days 1 and 10 after spore challenge from euthanized mice that were treated with OFX, whereas they were isolated from 10 of 23 mice treated with PEN G and 4 of 16 control mice.

Bacteria were also isolated in heart blood from 19 of 22 recently deceased mice. The findings correlated with those for mice euthanized on schedule, with the exception that bacteria were isolated from seven of seven deceased control mice.

(ii) Experiment 4: comparison of GAT and TVA given for 21 days starting 24 h after challenge. Heart blood was cultured from 20 recently deceased mice, of which 1 was given GAT s.c., 4 were given GAT p.o., 2 were given TVA s.c., 3 were given TVA p.o., and 10 were used as controls following administration of a challenge dose of 4.5×10^8 CFU/0.1 ml i.t. (Table 2). Only gram-positive bacteria were isolated from heart blood from 5 of 10 mice that were given the quinolones. In addition to *B. anthracis*, *E. faecalis* (3 of 7 mice) and *Staphylococcus* species (4 of 7 mice), including *S. sciuri* and *S. aureus*, were isolated.

Antimicrobial susceptibility (experiments 2, 3, and 4). In scheduled cultures of samples obtained from euthanized mice from day 5 through day 14, 4 of 13 isolates of *B. anthracis* were resistant to PEN G (MICs, ≥16 μg/ml) and 9 of 13 isolates were sensitive to PEN G (MICs, ≤0.03 to 4 μg/ml), whereas all 13 isolates were sensitive to CIP (MICs, ≤0.5 μg/ml) and LVX (MICs, ≤1 μg/ml), the active isomer of OFX. The four isolates resistant to PEN G produced β-lactamase. Three of the four PEN G-resistant isolates were from PEN G-treated mice and one was from an OFX-treated mouse, whereas five of the nine PEN G-sensitive isolates were from PEN G-treated mice and four were from OFX-treated mice.

Of eight isolates of *E. faecalis* tested, one was resistant to PEN G (MIC, ≥16 μg/ml), five were resistant to CIP (MICs, ≥4 μg/ml), six were resistant to LVX (MICs, ≥8 μg/ml), and eight were resistant to VAN (MICs, ≥32 μg/ml). Of three strains of *E. faecalis* isolated from euthanized mice treated with OFX, one was resistant to CIP and two were resistant to LVX. There appeared to be no change in the sensitivity of this species during the course of therapy.

Of 47 isolates of *B. anthracis* in cultures from recently deceased mice from day 2 through day 29, 22 were resistant to PEN G (MICs, ≥16 μg/ml) and 25 were sensitive to PEN G (MICs, ≤0.12 μg/ml), whereas 46 isolates were sensitive to CIP (MICs, ≤0.5 to 2 μg/ml) and 35 of 35 isolates tested were sensitive to LVX (MICs, ≤1 to 4 μg/ml). Of the 22 PEN G-resistant isolates, 21 produced β-lactamase. Of the 47 isolates, 23 were from control mice; 12 of these isolates were among those that were resistant to PEN G, and 11 of those 12 produced β-lactamase. Six of the 47 isolates were from de-

TABLE 2. Incidence of bacteria isolated from heart blood of recently deceased $B6D2F_1/J$ mice after irradiation and i.t. challenge on day 4 with *B. anthracis* Sterne spores and during treatment with an antimicrobial agent for 21 days begun 24 h after challenge (experiment 4)[a]

Bacterium isolated	No. of mice with bacteria in blood/no. of mice examined (%)					
	Control	GAT s.c.	GAT p.o.	TVA s.c.	TVA p.o.	Total
B. anthracis only	8/10 (80)	0/1 (0)	0/4 (0)	1/2 (50)	0/3 (0)	9/20 (45)
Other species only	0/10 (0)	1/1 (100)	2/4 (50)	0/2 (0)	1/3 (33)	4/20 (20)
Gram positive		1/1	2/2		1/1	
Gram negative		0/1	0/2		0/1	
Mixed (*B. anthracis* + other)	2/10 (20)	0/1 (0)	0/4 (0)	0/2 (0)	1/3 (33)	3/20 (15)
Other, gram positive	2/2				1/1	
Other, gram negative	0/2				0/1	
None	0/10 (0)	0/1 (0)	2/4 (50)	1/2 (50)	1/3 (33)	4/20 (20)

[a] The mice were irradiated with 7 Gy of ^{60}Co γ radiation and challenged with 4.5×10^8 CFU of *B. anthracis* Sterne spores. The numerators show the proportion of animals from which general categories of bacteria were isolated relative to therapeutic agent or control. The numerators were differentiated further between gram-positive and gram-negative bacteria.

ceased PEN G-treated mice, and 5 of these 6 isolates remained sensitive to PEN G. Six of the 47 isolates were from OFX-treated mice and remained sensitive to CIP and LVX.

Of 23 isolates of *E. faecalis* from deceased mice, 13 were resistant to PEN G (MICs, ≥16 μg/ml) and 10 were sensitive to PEN G (MICs, 0.12 or 8 μg/ml), 15 isolates were resistant to CIP (MICs, ≥4 μg/ml) and 8 were sensitive to CIP (MICs, ≤0.5 and 2 μg/ml), and 8 of 24 isolates were resistant to VAN (MICs, ≥32 μg/ml). Of 14 strains of *E. faecalis* from mice that were treated with one of the three quinolones, 9 were resistant to CIP and 11 were resistant to LVX. This is a particularly important observation regarding the effective treatment of infection in an irradiated host because of the high incidence of translocated enterococci and the difficulty in treating enterococcal infections in immune-competent hosts. A total of four of four strains of *E. faecalis* isolated from control mice were resistant to both CIP and LVX. There appeared to be no changes in the susceptibilities of these species during the course of therapy.

B. anthracis Sterne possesses the ability to produce a β-lactamase. When the growth of 20 colonies on Mueller-Hinton agar medium was spread onto cefinase discs, a red color developed slowly in less than 60 min on 19 discs and a pale pink developed on 1 disk. These results indicate that the 20 colonies inherently produced a β-lactamase.

DISCUSSION

This report confirms and extends the findings from our previous study (6) to mice treated with antimicrobial agents. In the previous study we found not only that susceptibility to infection with *B. anthracis* Sterne spores is increased but also that intestinal microorganisms translocate and cause a mixed, polymicrobial, systemic infection together with *B. anthracis* infection (a unique finding in our experience with nonlethally γ-irradiated mice) that further complicates the difficult challenge of providing effective therapy for toxigenic *B. anthracis* infection. We would expect to find translocating intestinal bacteria only in lethally irradiated mice (19). Our initial investigation supports further study of the hypothesis that lethal toxin and/or edema toxin plays a role in the induction of mixed infection in nonlethally irradiated mice challenged with toxigenic *B. anthracis* Sterne but not in irradiated mice challenged with nontoxigenic *B. anthracis* Δ-Sterne-1 (6). Successful therapy must attack not only *B. anthracis* but also all microorganisms, which contribute to sepsis following irradiation.

We demonstrated empirically that GAT and TVA, representing recently developed quinolones, reduced the level of polymicrobial sepsis and increased the rate of survival remarkably in a neutropenic irradiated mouse model but that the macrolides ERY, AZM, and CLR did not. Calculation of pharmacokinetic (PK) and pharmacodynamic (PD) parameters from published data provides insight into the complex nature of treating the *B. anthracis*-induced polymicrobial sepsis.

The MICs of the quinolones for *B. anthracis* Sterne were reported to be 1.6 mg of alatrofloxacin/liter, 0.025 mg of GAT/liter, and 0.2 mg of OFX/liter (9, 12). Ng et al. (33) determined PK and PD parameters in mice for trovafloxacin given s.c. The maximum concentration in serum (C_{max}) of TVA was 3.3 mg/liter and the half-life ($t_{1/2}$) of TVA in serum was 8.6 h following administration of a dose of 10 mg/kg; the C_{max} of TVA was 7.1 mg/liter and the $t_{1/2}$ was 9.2 h following administration of a dose of 30 mg/kg. Andes and Craig (3) determined PK and PD parameters for gatifloxacin in mice. The C_{max} of GAT was 5.96 mg/liter and the $t_{1/2}$ of GAT in serum was 0.59 h following administration of a dose of 18.75 mg/kg s.c.; the C_{max} was 17.6 mg/liter and the $t_{1/2}$ was 1.1 h following administration of a dose of 75 mg/kg. Also, the mean concentrations of OFX in serum were reported to be 2.6 ± 0.4 μg/ml at 1 h and 0.4 ± 0.2 μg/ml at 23 h after administration p.o. on day 5 of therapy in 8-Gy-irradiated mice (8).

The MICs of the macrolides for *B. anthracis* Sterne were reported to be 12.5 mg of AZM/liter, 0.2 mg of CLR/liter, and 6.25 mg of ERY/liter (9). Administration of a single oral dose of AZM or CLR of 50 mg/kg produced maximum concentrations in the lungs of mice of 8.89 and 12.49 μg/g, respectively; $t_{1/2}$s of 9.99 h and 1.64 h, respectively; and areas under the concentration-time curves (AUCs) of 147.58 and 47.73 μg · h/ml, respectively (32). However, Tessier et al. (38) reported an AUC from 0 to 12 h ($AUC_{0\text{-}12}$) of 24 μg · h/ml, a C_{max} of 6.3 μg/ml, and a $t_{1/2}$ of 10.5 h after administration of a 150-mg/kg dose.

The application of PK and PD parameters to the results obtained with quinolones and macrolides in this model appears to offer a complex and equivocal explanation of efficacy. The inconsistent predictability is most likely due to the polymicrobial nature of the sepsis rather than infection only with *B. anthracis* Sterne. By using these available published parameters, on the basis of the guidelines of Andes and Craig (2) and Craig (13–15) for correlating efficacy between mice and humans, i.e., an AUC/MIC ≥100 or a C_{max}/MIC >8, the C_{max}/MIC ratios for GAT (238.40 after administration of a dose of 18.75 mg/kg) and OFX (13 after administration of a dose of 40 mg/kg) would predict efficacy, but the C_{max}/MIC for TVA (2.06 and 4.44 after administration of doses of 10 and 30 mg/kg, respectively) would not predict efficacy against *B. anthracis* alone, yet TVA and GAT were more efficacious than OFX against the *B. anthracis*-induced polymicrobial sepsis. Remarkably, GAT was effective despite a short $t_{1/2}$, which would predict that the concentration would decrease below the MIC in approximately 5 h, perhaps due to a prolonged postantibiotic effect. In the case of CLR, both C_{max}/MIC and AUC/MIC (62.45 and 238.65, respectively) after administration of a single oral dose of 50 mg/kg or C_{max}/MIC and $AUC_{0\text{-}12}$/MIC (31.5 and 120, respectively) after administration of a dose of 150 mg/kg would predict efficacy. In the case of AZM, however, neither C_{max}/MIC nor AUC/MIC (0.71 and 11.8, respectively, after administration of a dose of 50 mg/kg) would predict efficacy against *B. anthracis* alone, and neither of these two drugs was efficacious against the polymicrobial sepsis. These macrolides tend to accumulate in tissues intracellularly, whereas the bacteria, which cause sepsis, are extracellular.

We concluded further that antimicrobial therapy must be started promptly in less than 24 h after challenge with *B. anthracis* spores following nonlethal γ irradiation and that to limit mortality therapy must be continued for more than 7 days and up to 21 days because inactive spores germinate after termination of antimicrobial therapy in humans and experimental animals (28). For example, six monkeys died 6, 9, 12, 20, 28, and 73 days, respectively, after the 30-day antimicrobial

therapy was discontinued (23). Beginning 1 day after exposure to 4×10^5 spores of *B. anthracis* Volum 1B in an aerosol, Friedlander et al. (23) gave groups of 9 or 10 rhesus monkeys (*Macaca mulatta*) either procaine PEN G (180,000 U i.m. q12h), CIP (125 mg p.o. q12h), or doxycycline (30 mg p.o. q12h) for 30 days. The postchallenge therapy with the antimicrobial agents provided 70 to 90% survival of the monkeys. PEN G (4×10^6 IU i.v. q4h), doxycycline (100 mg i.v. q12h), or CIP (400 mg i.v. q12h) is at present the therapeutic drug of choice for the treatment of individual cases of inhalational anthrax, whereas CIP (500 mg p.o. q12h), amoxicillin (500 mg p.o. q8h), or doxycycline (100 mg p.o. q12 h) is recommended for the treatment of mass casualties (21, 25). The quinolones have advantages for the therapy of both endogenous and exogenous infections after irradiation (4, 10).

Following irradiation in mice, a period of 21 days allows initiation of the recovery of the depleted bone marrow progenitor cells and the innate immune responses that are depressed by irradiation. The recovering innate responses might then be adequate to control infection by extant spores that remain after antimicrobial therapy. We concluded that there is an evident advantage to early initiation of antimicrobial therapy for *B. anthracis* infections to enhance and prolong survival after mice are irradiated because the mice in which treatment was begun 7.5 and 24 after challenge are well separated (Fig. 3). Also, survival was prolonged but not increased when mice, which were irradiated and challenged i.t., were treated with either the broad-spectrum agent OFX or the narrow-spectrum agent procaine PEN G for 7 days beginning within 24 h after challenge; however, survival was not even prolonged when initiation of therapy was delayed for more than 24 h (Fig. 2). PEN G, OFX, AZM, CLR, and ERY did not prevent *B. anthracis* infection or mixed infection in irradiated mice, even though doses of the macrolides, in particular, were scaled optimally for mice. Serum AZM and CLR concentrations are low because these drugs accumulate in tissues, so they are not available where they are needed most to prevent or suppress sepsis. However, the newer quinolones, GAT and TVA, even though the doses of these quinolones were less than the optimal allometric scaling for mice, reduced the incidence of mixed infections caused by both gram-positive and gram-negative bacteria and significantly improved the survival rates to 79 and 95%, respectively.

The question arises, did *B. anthracis* develop antimicrobial resistance in animals treated with specific antimicrobial agents? Resistance to PEN G and ampicillin was common among isolates of *B. anthracis* from control mice as well as from those treated with PEN G or quinolones (Table 3). Many of these resistant strains produced a β-lactamase. On the other hand, resistance to CIP occurred in only one isolate from a control mouse.

Resistance to PEN G was evidently inherent in the original population of *B. anthracis* Sterne because not only was β-lactamase demonstrated in the stock culture but also (i) 48% of PEN G-resistant isolates from control mice produced β-lactamase, (ii) these isolates were cultured from deceased or euthanized mice throughout the course of therapy rather than only toward the end of therapy, and (iii) PEN G-sensitive strains predominated in cultures from PEN G-treated mice (Table 3). As noted by Lalitha and Thomas (29), resistance to PEN G in *B. anthracis* does not appear to be a significant clinical concern generally because most isolates of wild strains are sensitive to PEN G and resistant strains can be identified and treated with alternative antimicrobial agents. However, that approach requires time for isolation and identification of bacteria from clinical specimens, and based upon the need to initiate effective therapy promptly after inoculation in irradiated animals, there is not likely to be sufficient time to discover resistant strains from an irradiated human and change the therapy with assurance of clinical recovery. Also, the resistant derivatives of *B. anthracis* Sterne that we isolated from our euthanized and deceased mice had higher levels of resistance (MICs, ≥16 μg/ml) than the resistance seen clinically by Lalitha and Thomas (MICs, 0.125 μg/ml), and the MICs for most of the isolates from our mice (≤0.03 to 4 μg/ml) demonstrated that the isolates were sensitive to PEN G. Nevertheless, only 63% of the PEN G-treated mice survived. This observation suggests that PEN G does not provide adequate coverage for the polymicrobial infection and that, if a portion of the original *B. anthracis* population was resistant, it could be selected during the course of PEN G therapy and emerge to become predominant together with endogenous microorganisms.

TABLE 3. Antimicrobial resistance of *B. anthracis* organisms isolated from tissues of $B6D2F_1/J$ mice after irradiation, i.t. challenge with *B. anthracis* Sterne spores, and treatment with antimicrobial agents (experiments 2, 3, and 4)[a]

Antimicrobial treatment	No. of isolates of *B. anthracis* resistant to antimicrobial agents or producing β-lactamase/no. of isolates tested				
	AMP	PEN G	CIP	LVX	β-Lactamase
Control	12/21	12/23	1/23	0/21	11/23
PEN G	3/12	4/13	0/13	0/12	4/13
OFX	5/8	4/10	0/10	0/8	4/10
PEN G + OFX	0/1	0/1	0/1	0/1	0/1
GAT	0/4	5/11	0/11	0/4	5/11
TVA	1/2	1/2	0/2	0/2	1/1

[a] The mice were irradiated with 7 Gy of γ ^{60}Co radiation.

We previously demonstrated that *B. anthracis* could develop drug class-related resistance in vitro to several quinolones but only minimal resistance to doxycycline during sequential passage (9, 12), but we found no resistance to CIP or LVX in isolates of *B. anthracis* from mice during 21 days of therapy with OFX, GAT, or TVA. Although 98% of our *B. anthracis* isolates were sensitive to CIP throughout the course of our studies, only 63% of OFX-treated (p.o.) mice survived. We are concerned that 63 to 76% of *E. faecalis* strains isolated were resistant to CIP and LVX as well as PEN G. Such inherent resistance or such a propensity to develop resistance to quinolones among microorganisms in polymicrobial infections will pose a challenge to effective clinical management of *B. anthracis* infections with a single antimicrobial agent following nonlethal γ irradiation.

Mixed infections will likely require selective, multiple antimicrobial therapy for anthrax in an irradiated host. However, antimicrobial therapy alone still may not be sufficient to ensure survival. By augmenting specific antimicrobial therapy with nonspecific immunostimulation of natural host responses, the

clinical outcome would likely be improved even further (20, 30, 31, 34, 35, 41).

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