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Antimicrobial Therapies for Pulmonary *Klebsiella pneumoniae* Infection in B6D2F$_1$/J Mice Immunocompromised by Use of Sublethal Irradiation

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***Klebsiella pneumoniae* is a common cause of nosocomially acquired pneumonia in immunocompromised patients. Previously, we established a pneumonia model using *Klebsiella pneumoniae* in B6D2F$_1$/J mice sublethally irradiated with 7-Gy ^{60}Co γ-radiation and inoculated intratracheally. In the study reported here, we investigated survival of mice following 10 days of antimicrobial therapy with ceftriaxone, gentamicin, gatifloxacin, and a ceftriaxone-gentamicin combination given once daily. Survival was significantly prolonged in response to all therapies. However, survival of mice was 95% when treated with the ceftriaxone-gentamicin combination followed by ceftriaxone alone (75%), and gatifloxacin (80%), whereas survival for controls was 0%. In addition, resistance to any of the treatments did not develop during the study. We conclude that an immunocompromised status does not alter the Infectious Disease Society of America's primary recommendation for treating community-acquired *K. pneumoniae* pneumonia using a third-generation cephalosporin, with or without an aminoglycoside.**

In mammals, sublethal doses of γ-photon radiation reduce natural innate defenses to infection by creating a neutropenic state (8, 17). Because of depressed host defenses and impaired hemopoiesis, susceptibility of animals to endogenous and exogenous organisms is enhanced following whole-body irradiation (17, 18).

Klebsiella pneumoniae is one of the most common gram-negative bacteria that infects granulocytopenic patients (4, 36). Most *K. pneumoniae* infections are nosocomial (34). *Klebsiella pneumoniae* accounts for a substantial percentage of hospital-acquired pneumonia, septicemia, soft tissue infections, and urinary tract infections (34). *Klebsiella* species cause eight percent of all hospital-acquired infections (15). In the United States, they comprise three to seven percent of all nosocomial bacterial infections, placing them among the eight most important pathogens in hospitals and second only to *Escherichia coli* as the most common cause of gram-negative sepsis (23).

Klebsiella pneumoniae infection has been demonstrated in a sublethally irradiated mouse by use of the subcutaneous (s.c.) and oral (p.o.) routes of inoculation as well as by direct inoculation into wounds (7, 17, 18, 27). We have developed a novel model for creating a pulmonary infection by the intratracheal (i.t.) inoculation of *K. pneumoniae* in mice immunocompromised by use of sublethal irradiation (26).

The recommended antimicrobial therapy for management of *Klebsiella*-induced pneumonia is third-generation cephalosporins, with or without aminoglycosides (4). Carbapenems also are used as preferred treatments. In the study reported here, the fluoroquinolone, gatifloxacin, was selected as alternative antimicrobial therapy because it can be administered orally, whereas carbapenems must be administered via infusion. Fluoroquinolones are efficacious for treating *K. pneumoniae* sepsis in sublethally irradiated mice (6). The intratracheal route of inoculation was selected to induce a pulmonary infection resembling pneumonia by use of a known, controlled dose of bacteria.

We evaluated several antimicrobial agents individually and one combination to determine the most efficacious therapy and whether resistance developed during the course of treatment. We found that 95% of mice given a combination of ceftriaxone sodium and gentamicin sulfate s.c. once daily for 10 days survived for the entire 34-day study period. Single antimicrobial therapies with ceftriaxone, gentamicin, and gatifloxacin also prolonged survival significantly.

Materials and Methods

Animals. Specific-pathogen-free, nine- to 12-week-old female C57BL/6 x DBA/2 F$_1$ hybrids (B6D2F$_1$/J) mice were obtained from Jackson Laboratories (Bar Harbor, Maine). The mouse colony was extensively monitored by Jackson Laboratories and found to be free of *Klebsiella* species, cilia-associated respiratory bacillus, *Pasturella pneumotropica*, *P. multocida*, *Staphylococcus aureus*, *Bordetella bronchiseptica*, *Corynebacterium kutscheri*, *Streptobacillus monoliformis*, *Streptococcus* species, *Mycoplasma*

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arthriditis, *M. pulmonis*, *Citrobacter rodentium*, *Pseudomonas* species, *Salmonella* species, *Clostridium piliforme*, *Helicobacter* species, ectromelia virus, mouse encephalomyelitis virus (GD VII), K virus, Korean hemorrhagic fever (Hantaan) virus, lactate dehydrogenase-elevating virus, lymphocytic choriomeningitis virus, mouse minute virus, mouse adenovirus of mice, mouse cytomegalovirus, mouse hepatitis virus, mouse parvovirus, mouse thymic virus, pneumonia virus of mice, polyoma virus, reovirus 3, rotavirus, Sendai virus, *Encephalitozoon cuniculi*, *Pneumocystis carinii*, *Psorergates simplex*, *Syphacia* species, *Hymenolepsis* species, *Spironucleus muris*, *Giardia muris*, amoebae, trichomonads, fleas, lice, and fur mites.

All animals were kept in quarantine for 10 days before the study and were transferred to a room with a 12-h light-dark cycle. Animals were housed in 11.5 × 7.5 × 5-in. polycarbonate boxes with filter covers (Micro-Isolator™, Lab Products Inc., Maywood, N.J.), and autoclaved maple hardwood chip bedding (Harlan Sani-Chips, Harlan Teklad, Madison, Wis.) was changed twice weekly. All animal handling and husbandry procedures were performed under a laminar flow hood. A commercially formulated rodent diet (Rodent Diet [W] 8604, Harlan Teklad) and acidified water (pH 2.5 to 3.0) were provided ad libitum. All husbandry and experimental procedures were performed in compliance with the *Guide for the Care and Use of Laboratory Animals* (24) and the Armed Forces Radiobiology Research Institute (AFRRI) policies regarding animal care and use. The experimental protocol was approved by the AFRRI Institutional Care and Use Committee.

Irradiation. A Gray (Gy) is the International System of Units base unit for absorbed energy and equals one joule per kilogram. Groups of four mice per box were placed in perforated acrylic plastic restrainers and given a 7-Gy sublethal dose of ^{60}Co γ-photon radiation at a dosage of 0.4 Gy/min mid-line tissue (MLT) from bilaterally positioned ^{60}Co sources in the AFRRI ^{60}Co Irradiation Facility (11, 32). Results of previous studies with this mouse strain consistently indicated that mortality occurred only at doses > 7.75 Gy (28).

Dose determinations using acrylic mouse phantoms were made by use of a 0.5-cm^3 tissue-equivalent ionization chamber, with its calibration traceable to the National Institute of Standards and Technology. The dose uniformity of the radiation field was ± 3%.

Bacteria. Inocula containing *K. pneumoniae* AFRRI 7 with serotype-5 capsule, a clinical isolate, were prepared as described (31). The concentration was determined by inoculating tenfold serial dilutions in cold, sterile, saline solution onto trypticase soy agar (TSA) medium (Becton Dickinson and Company, Cockeysville, Md.). The stock suspension was stored at 4 to 8°C overnight. The following day, a suspension of the desired bacterial concentration was prepared for inoculation into mice. The actual number of bacteria inoculated into mice was verified by culture of serial tenfold dilutions of the challenge inoculum on TSA.

Bacterial inoculation procedure. Four days after irradiation, when circulating white blood cells are at their nadir and susceptibility to infection is greatest (17), 3.7 × 10^5 colony-forming units (cfu) of *K. pneumoniae*/0.1 ml (experiment 1), and 3.5 × 10^5 cfu/0.1 ml (experiment 2), which approximates the lethal dose that will kill 95% of the animals in 30 days ($LD_{95/30}$), as determined by a probit analysis from a previous study (26), were inoculated intratracheally into each mouse as described (5). The i.t. route of instillation was originally described by Saffiotti and co-workers (35). Briefly, mice were anesthetized by i.p. injection of a mixture of 30.3 mg of ketamine/kg, 0.15 mg of acepromazine/kg, and 3.3 mg of xylazine/kg, delivered in a volume of 0.1 ml/20 g of body mass, using a 25-gauge needle. As soon as the animal was anesthetized, it was positioned on a slanted aluminum holding board, with its back on the board and its mouth held open by hanging the lower incisor teeth on a wire arc, while retaining the upper incisors by use of a rubber band. The *K. pneumoniae* suspension was inoculated into mice through a blunt, 1.5-in., 22-gauge pipetting needle, gently curved at an angle of 135°. The blunt tip of the needle was inserted under the epiglottis to uncover the larynx, then was lightly pushed into the tracheal lumen. The needle was inserted to the mid-trachea, a volume of 0.1 ml of *K. pneumoniae* suspension was gently injected, and the needle was withdrawn. To ensure that suspension was not expelled, the pharynx was inspected for a short time while the animal was kept on the board.

Antimicrobial agents. Standard injectable solutions of gentamicin (Abbott Laboratories, North Chicago, Ill.), gatifloxacin (Tequin Injection; Bristol-Meyers Squibb Co., Princeton, N.J.), and the reconstituted powder formulation of ceftriaxone (Rocephin; Roche Laboratories, Inc., Nutley, N.J.) with known potency were used for this study. Freshly prepared and diluted gatifloxacin was administered in a volume of 0.2 ml by oral gavage, using a ball-tipped, 20-gauge feeding tube fitted to a 1.0-ml syringe, whereas diluted gentamicin and ceftriaxone were administered in a volume of 0.1 ml by s.c. injection, using a 25-gauge needle attached to a 1.0-ml syringe. All antimicrobial agents were given once every 24 h at 10:00 a.m., using the following doses: gatifloxacin, 20 mg/kg; gentamicin, 7.5 mg/kg; and ceftriaxone, 75 mg/kg. One group of control animals was given 0.2 ml of sterile distilled water (H_2O) p.o., and another group was given 0.1 ml s.c. All therapies were started five days after irradiation (one day after bacterial challenge), and were administered for 10 consecutive days.

We chose a dose of 75 mg of ceftriaxone/kg/d, because currently it is the recommended human pediatric dose (37) and because this dose was also used effectively in the study of immunmodulators and antimicrobials for treatment of acquired infections in irradiated mice (18, 28). Although once-daily dosing is not optimal, this regimen allowed us to detect the contributions of two drugs in combination to improve survival. The dosage of 7.5 mg of gentamicin/kg/d was chosen on the basis of previous studies of *K. pneumoniae* in irradiated mice and is 1.25 to 1.5 times greater than the 5- to 6-mg/kg once-daily recommended dosage in humans (3, 28, 30). This dosage is comparable to 8 mg of gentamicin/kg used to study prolonged postantibiotic effects (PAE) in neutropenic mice (19, 22). We intentionally used a sub-optimal dose of gentamicin so as to detect differences between the combination therapy and gentamicin or ceftriaxone alone. The dosage of gatifloxacin was selected on the basis of comparison with 40 mg of ofloxacin/kg, of which only the *levo* isomer of ofloxacin is active, so a comparative dosage of gatifloxacin would be 20 mg/kg. In mice, 40 mg of ofloxacin/kg and 20 mg of gatifloxacin/kg is 3.5 times the recommended dosage in humans (16).

Microbiological culture and antimicrobial susceptibility testing. Bodies of mice that had either recently died (within two hours) or had been euthanized by use of cervical dislocation were dissected in aseptic manner to isolate bacteria from tissues. Lungs were removed and crushed by use of a sterile cotton swab in a sterile dish. The apex of the heart was cut. Lung specimens

and heart blood samples were inoculated on 5% sheep blood agar (SBA), colistin-nalidixic acid in 5% SBA (CNA), and xylose-lysine-desoxycholate (XLD) media (BD Diagnostics, Sparks, Md.). The XLD plates were incubated at 35°C, and CNA and SBA media were incubated in 5% CO_2 and at 35°C. Selected microorganisms were identified and tested for antimicrobial susceptibility by use of a Vitek automated system (bioMérieux, Inc., Hazelwood, Mo.) at the Uniformed Services University of the Health Sciences, Bethesda, Md.

The minimum inhibitory concentration (MIC) was used to discern whether resistance appeared in *K. pneumoniae* isolated from mice during continuous therapy. The MICs of ceftriaxone, gatifloxacin, and gentamicin against stock *K. pneumoniae* AFRRI 7 were determined by use of the Etest agar gradient diffusion method, described by the manufacturer (AB Biodisk North America Inc., Piscataway, N.J.), and a macrodilution procedure. The MIC was defined as the lowest concentration inhibiting visible growth after 24 h by use of macrodilution. Because direct evaluation in Vitek cards was not readily available for ceftriaxone and gatifloxacin, comparisons were made to related antimicrobials to determine whether resistance developed during the treatment period. The quinolones, ciprofloxacin and ofloxacin, represented gatifloxacin.

Antimicrobial susceptibilities of the following cephalosporins were evaluated as surrogates for ceftriaxone, a third-generation cephalosporin, which was not readily available in Vitek cards: first generation, cephazolin and cephalothin; second generation, cefoteten and cefoxitin; third generation, cefotaxime and ceftizoxime; and fourth generation, cefepime and ceftazidime. Of these cephalosporins, cefotaxime correlates closest with ceftriaxone in spectrum of activity (37).

Serum concentration of antimicrobials. Because serum concentration for oral administration could not be ascertained in current scientific literature and to determine serum gatifloxacin concentration in mice after its administration, blood was collected from six non-irradiated and six irradiated mice at timed intervals. Because of its short half-life (2), gatifloxacin concentration in the serum was measured at 15 and 30 min, and two, three, and four hours on the fifth day of therapy. Serum concentration of gatifloxacin was determined, using *E. coli* ATCC 25922 as the test microorganism, grown on Mueller-Hinton agar. Ceftriaxone and gentamicin concentrations were measured at one hour in six irradiated and six non-irradiated mice to determine whether there was a difference in serum concentrations. Serum ceftriaxone concentration was determined using *Bacillus subtilis* ATCC 6633 as the test microorganism. The sensitivity of the biological assays was ≤ 0.1 μg/ml. Mouse plasma concentration for gentamicin was determined by use of the CEDIA Gentamicin II assay (Roche Diagnostics Corp., Indianapolis, Ind.), a homogeneous enzyme immunoassay using recombinant DNA technology. The sensitivity of the assay was ≤ 0.3 μg/ml.

Experimental design. For experiment 1, one-hundred forty 15- to 18-week-old mice were distributed randomly to seven groups of 20 mice. Randomization schemes were generated, using computerized statistical software (SPSS for Windows, Version 10.0, SPSS, Inc., Chicago, Ill.). All groups were given 7-Gy of ^{60}Co γ-photon radiation. Four groups were given antimicrobial therapies of ceftriaxone, gentamicin, the ceftriaxone-gentamicin combination, or gatifloxacin. One group did not receive treatment, and two groups, which were given the vehicle (sterile water for injection) by either the s.c. or p.o. route, served as controls. Mice were irradiated on day 0 and challenged on day 4 with 3.7×10^5 cfu of *K. pneumoniae*/0.1 ml, i.t. (370 $LD_{50/30}$). Beginning on day 5, treatments were initiated, then continued for 10 days. Gastric gavage needles were used to administer oral treatments. For s.c. injections, a special holder was used to expose the shaved-of-hair hindquarters of the mouse. The caudal aspect of the mouse was sprayed with 70% alcohol and wiped with clean gauze prior to injection of antimicrobial agent. Survival (numbers and percentage) was recorded daily for 30 days after bacterial challenge (34 days after irradiation). Heart blood and lungs from moribund or recently deceased mice were cultured for the presence of microorganisms. Morbundity was subjectively determined by the appearance of recumbency, dyspnea, kyphosis, ruffled coat, and/or reluctance to move when stimulated. A 30-day period is the standard period of evaluation of mice that are given doses of ionizing radiation, which, in turn, induces the hemopoietic syndrome. This experiment was repeated to document reproducibility.

For experiment 2, two-hundred forty-five 15- to 18-week-old mice were distributed randomly to seven groups of 25 to 45 mice (groups with higher expected mortality had more animals to ensure sufficient results for microbiological sampling) and were treated as described in experiment 1. Mice were challenged with 3.5×10^5 cfu of *K. pneumoniae*/0.1 ml, i.t. To evaluate the microbiological status of mice during the infectious process, on days 5, 6, 8, 11, and 15, five animals were removed from each group and euthanized before administration of treatment. The lungs and heart blood were submitted for culture. Isolated bacteria were identified and evaluated for antimicrobial susceptibility to determine whether antimicrobial resistance developed during therapy. An initial antimicrobial susceptibility test of the original *K. pneumoniae* AFRRI 7 stock was performed to track changes in susceptibility. Lungs and heart blood of recently deceased (within two to three hours) mice also were submitted for culture.

Statistical analysis. The mean and SEM were determined and the Mann-Whitney test (SPSS 10.0 for Windows) was done for each drug to compare serum antimicrobial concentrations at various radiation doses. Thirty-day survival for experimental groups of mice was compared, using the generalized Mantel-Cox procedure (Program 1L; BMD Statistical Software, Inc., Los Angeles, Calif.).

Results

Survival. When irradiated and challenged with 3.7×10^5 cfu of *K. pneumoniae*/0.1 ml, i.t. (370 $LD_{50/30}$, the lethal dose at which 50% of the animals die in 30 days), and treated once daily with antimicrobial agents for 10 days in experiment 1 (Fig. 1), 95% of mice that were given ceftriaxone plus gentamicin, 80% given gatifloxacin, 75% given ceftriaxone, and 10% given gentamicin survived, compared with no (0%) survival among the control groups. Survival of animals of the treated groups was significantly ($P < 0.05$) greater than that of untreated mice or mice treated only with sterile water. Mortality occurred between one and two days after bacterial challenge in untreated mice, between five and 13 days in ceftriaxone-treated mice, at 14 days in ceftriaxone plus gentamicin-treated mice, between three and nine days in gentamicin-treated mice, and between four and six days in gatifloxacin- treated mice. Table 1 lists the statistical differences between paired antimicrobial therapies. When this experiment was repeated, good reproducibility was documented.

Antimicrobial susceptibility. The MICs of antimicrobial

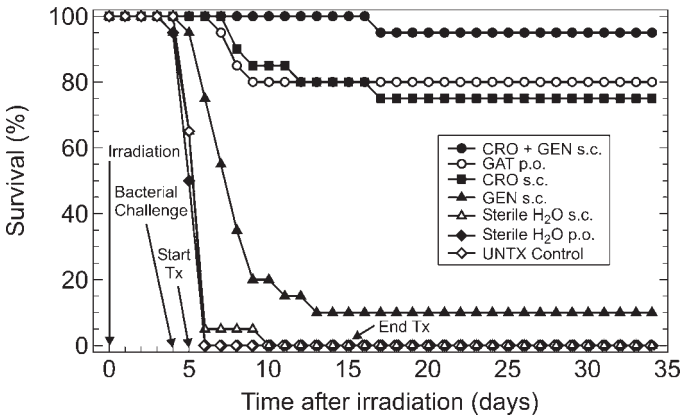

Figure 1. Survival of mice following treatment with antimicrobial agents after 7-Gy ^{60}Co γ-irradiation and intratracheal challenge with 3.7×10^5 colony-forming units of *Klebsiella pneumoniae* (370 $LD_{50/30}$).

agents were determined by use of Etest strips and macrodilution for the original stock culture of *K. pneumoniae* AFRRI 7. By using Etest strips, the MICs were: gentamicin (≤ 0.75 to 1.0 μg/ml), ceftriaxone (≤ 0.032 μg/ml), and gatifloxacin (≤ 0.032 μg/ml); by using macrodilution, they were: gentamicin (≤ 2.0 μg/ml [two-fold greater than in the Etest]), ceftriaxone (≤ 0.0625 to 0.125 μg/ml [two- to four-fold greater than in the Etest]), and gatifloxacin (≤ 0.8 μg/ml [25-fold greater than in the Etest]). There was no change in susceptibility of *K. pneumoniae*, isolated from treated animals during the course of therapy, to any evaluated antimicrobial agent determined by use of the Vitek system: cephalosporins cefotaxime (≤ 4 μg/ml), which most closely correlates with ceftriaxone (40), cephazolin (≤ 8 μg/ml), cefepime (≤ 4 μg/ml), cefoteten (≤ 16 μg/ml), ceftazidime (≤ 8 μg/ml), cephalothin (≤ 2 μg/ml), ceftizoxime (≤ 8 μg/ml), and cefoxitin (≤ 2 μg/ml); quinolones ciprofloxacin (≤ 0.5 μg/ml) and ofloxacin (≤ 1 μg/ml), which represented gatifloxacin; and the aminoglycoside, gentamicin (≤ 0.5 μg/ml).

Serum concentrations. Mean serum concentrations of all three drugs were higher at one hour in 7 Gy-irradiated mice, compared with 0 Gy-irradiated mice, although these biological differences were not statistically significant ($P > 0.05$, Table 2). Due to its short half-life, mean serum concentration of gatifloxacin was determined in samples from six mice at multiple times. The maximal concentration of gatifloxacin in the serum (C_{max}) was 1.37 ± 0.13 μg/ml at 0.5 h.

Microbiological results. Irradiated and challenged (3.5×10^5 cfu/0.1 ml, i.t [i.e., 350 $LD_{50/30}$]) mice were evaluated microbiologically at scheduled intervals. Clinical signs of disease (i.e., any combination of recumbency, dyspnea, ruffled coat, kyphosis, or reluctance to move when stimulated) were observed only in the untreated and H_2O-treated groups on day 8. Clinical signs of disease were not observed in the antimicrobial treatment groups during the course of therapy.

Klebsiella pneumoniae was isolated from the lungs of 10 to 20% of antimicrobial-treated mice, compared with 73% in controls, which indicated that antimicrobial therapy was effective at reducing *K. pneumoniae* in lungs, and from heart blood of 0 to 10% of those mice, compared with 45% in controls (Table 3). After day 6, gram-positive bacteria, including enterococci, staphylococci, and streptococci, were isolated in sparse numbers (10 or fewer cfu) from 11 of 75 (15%) treated animals, but only one of 51 (2%) controls. Bacteria were not isolated from 65% of antimicrobial-treated animals, whereas, in comparison, bacteria were not isolated from 43% of controls.

Table 1. Levels of statistical significance evaluated by use of Mantel-Cox between paired therapies against 3.7×10^5 colony-forming units of *Klebsiella pneumoniae*/0.1ml given intratracheally to 7-Gy ^{60}Co γ-photon-irradiated B6D2F_1/J mice

P value	Paired therapies[a]
$P < 0.001$	CRO + GEN vs. UNTX
	CRO + GEN vs. H_2O s.c.
	CRO + GEN vs. H_2O p.o.
	CRO + GEN vs. GEN
	GAT vs. UNTX
	GAT vs. H_2O s.c.
	GAT vs. H_2O p.o.
	GAT vs. GEN
	CRO vs. UNTX
	CRO vs. H_2O s.c.
	CRO vs. H_2O p.o.
	CRO vs. GEN
	GEN vs. UNTX
	GEN vs. H_2O s.c.
	GEN vs. H_2O p.o.
$P > 0.05$	CRO + GEN vs. GAT
	CRO + GEN vs. CRO
	GAT vs. CRO
	H_2O s.c. vs. H_2O p.o.
	H_2O s.c. vs. UNTX
	H_2O p.o. vs. UNTX

[a]CRO = ceftriaxone, GEN = gentamicin, GAT = gatifloxacin, UNTX = untreated.

In untreated and H_2O, s.c.-treated mice, *K. pneumoniae* was the only isolate found in each of five mice sampled on days 5, 6, and 8 after challenge (Table 4). On days 11 and 15, bacteria were not found in H_2O, s.c.-treated mice. *Klebsiella pneumoniae* was isolated from all treatment groups on day 6, but only low numbers of microorganisms (10 or fewer cfu per agar plate) were isolated from tissues in the following treatment groups: ceftriaxone plus gentamicin, ceftriaxone, and gatifloxacin (Table 4). On day 8, *K. pneumoniae* was isolated from ceftriaxone plus gentamicin, ceftriaxone, and control groups, but not from the gatifloxacin and gentamicin groups. On day 11, *K. pneumoniae* was isolated only from the group given H_2O, s.c. On day 15, *K. pneumoniae* was isolated from only one mouse of the ceftriaxone and gentamicin groups, but 10 or fewer colonies of *K. pneumoniae* were isolated from the ceftriaxone-treated mouse. No untreated mice survived after day 8. Only one mouse survived on day 15 in the group given H_2O, p.o., but all remaining groups had sufficient animals for sampling on day 15.

Klebsiella pneumoniae was not isolated on or after day 8 from mice given gatifloxacin, although sparse numbers (10 or fewer colonies) of other microorganisms (i.e., *Streptococcus* sp. and *Enterococcus* sp.) were isolated from one mouse each on day 8 but were not detected on days 11 and 15. *Enterococcus faecalis* was isolated on days 11 and 15 from mice in the ceftriaxone plus gentamicin, ceftriaxone, and gentamicin groups, but only a few colonies of this species were isolated from the ceftriaxone plus gentamicin group on days 11 and 15. On day 11, ten or fewer colonies of *E. faecalis* were isolated from only one mouse of the ceftriaxone group, but this species was not detected in mice of the gentamicin group on day 11 or 15, or of the ceftriaxone group on day 15.

On day 8, bacterial growth was not detected in 60% or more of the animals in the ceftriaxone, gentamicin, and gatifloxacin groups. On day 11, bacterial growth was not detected in 60% or more of mice in all treatment groups. With the exception of the

Table 2. Serum concentrations of antimicrobial agents in mice

Antimicrobial agent	Dose of radiation (Gy)	Time after administration (h)	Serum concentration (μg/ml ± SEM)	*P* value[a]	Reference	C_{max}/MIC[b,c]
CRO	0	0.5	83.0		(Kazragis et al.)	698.0[d]
	0	0.5	91.5		(Sauve et al.)	
	0	1	72.55 ± 4.13			
	7	1	80.92 ± 7.01	0.485		
GEN	0	0.25	9.8		(Fantin et al.)	4.9
	0	1	2.75 ± 2.70			
	7	1	3.00 ± 2.80	0.589		
GAT	0	0.25	0.96 ± 0.08			
		0.5	1.37 ± 0.13			42.8
		1	0.28 ± 0.03			
		2	0.23 ± 0.03			
		3	< 0.2			
		4	< 0.2			
	7	0.25	1.28 ± 0.14			
		0.5	1.60 ± 0.28	0.699		50.0
		1	0.58 ± 0.02			
		2	0.27 ± 0.03			
		3	< 0.2			
		4	< 0.2			

[a]Mann-Whitney test comparing non-irradiated with irradiated serum values.
[b]C_{max}/MIC = correlation for antimicrobial efficacy (i.e., C_{max}/MIC > 8).
[c]MIC determined by use of macrodilution procedure.
[d]C_{max} of 87.25 μg/ml after dose of 75 mg of CRO/kg extrapolated between 83 μg/ml (Kazragis et al.) and 91.5 μg/ml (Sauve et al.).
See Table 1 for key.

Table 3. Cumulative incidence of bacteria isolated from tissues of female B6D2F_1/J mice at scheduled intervals prior to daily administration of antimicrobial agents in experiment 2[a]

Bacterium isolated	Cumulative No. of mice given treatments[b] (%)				
	Controls	CRO+GEN[c] s.c.	CRO s.c.	GAT p.o.	GEN s.c.
Klebsiella pneumoniae only					
Lung	37/51 (73)	3/20 (15)	4/20 (20)	2/20 (10)	2/20 (10)
Heart blood	23/51 (45)	0	1/20 (5)	0	2/20 (10)
Other species only					
Gram-positive					
Lung	1/51(02)	4/20 (20)	1/20 (5)	2/20 (10)	0
Heart blood	1/51(02)	2/20 (10)	1/20 (5)	0	2/20 (10)
Gram-negative					
Lung	0	0	0	0	0
Heart blood	0	0	0	0	0
None					
Lung	13/51 (25)	13/20 (65)	15/20 (75)	16/20 (80)	17/20 (85)
Heart blood	22/51 (43)	17/20 (85)	18/20 (90)	19/20 (95)	17/20 (85)

[a]Mice were given a 7-Gy dose of ^{60}Co γ-photon radiation, then challenged i.t. on day 4 with 3.5×10^5 cfu of *K. pneumoniae*.
Antimicrobial therapy began approximately 24 h after bacterial challenge. Lung and heart blood were aseptically removed from mice that were euthanized on scheduled days 5, 6, 8, 11, and 15.
[b]Numerators indicate the number of animals from which general categories of bacteria were isolated relative to therapeutic agent or control. Numerators were differentiated further between isolated gram-positive and gram-negative bacteria. Denominators indicate total number of mice examined per treatment.
See Table 1 for key.

ceftriaxone plus gentamicin and the H_2O, p.o. groups, bacterial growth was not detected in 80% of all sampled mice in all groups.

Discussion

We documented that ceftriaxone, alone or in combination with gentamicin, and gatifloxacin reduced numbers of *K. pneumoniae* and increased survival in a neutropenic irradiated mouse model. Although all four antimicrobial therapies promoted significant ($P \leq 0.001$) increases in survival over that of untreated controls, ceftriaxone alone or the quinolone gatifloxacin improved survival approaching that of ceftriaxone plus gentamicin. An immunocompromised status does not appear to change the general therapeutic recommendations for therapy of *K. pneumoniae* infection.

Low numbers of gram-positive organisms were isolated in culture from tissues of two to 20% of the animals in each group (Table 3). Of those, the ceftriaxone plus gentamicin- and the gatifloxacin-treated groups had the highest percentage of isolated gram-positive organisms. These drugs are most effective against gram-negative bacteria. These groups also had some of the highest survival rates, which indicates that organisms other than *K. pneumoniae* did not add to mortality. Other gram-negative organisms were not isolated in culture. Clinical signs of disease (i.e., recumbency, dyspnea, kyphosis, ruffled coat, and/or reluctance to move when stimulated) were not present in any of the mice from which other organisms were isolated. When gram-positive organisms were isolated, there often were only 10 or fewer colonies, suggesting insignificant bacteremia or transient colonization. Therefore, we believe it is unlikely that other bacteria could have substantially contributed to the mortality detected. Further, *Pneumocystis carinii*, which, as an opportunist, might induce a debilitating infection in an immunocompromised host mouse, was not detected in stained, histologic sections in the

Table 4. Bacteria isolated from female B6D2F$_1$/J mice at scheduled time intervals prior to daily administration of antimicrobial agents in experiment 2 after 7-Gy ^{60}Co γ-photon radiation and intratracheal challenge with 3.5×10^6 cfu of *K. pneumoniae* ($LD_{95/30}$)

Sample day	Bacteria isolated	No. of mice with bacteria in lung and/or heart blood/No. of mice examined (%)						
		UNTX	H_2O s.c.	H_2O p.o.	CRO + GEN s.c.	CRO s.c.	GEN s.c.	GAT p.o.
5	*K. pneumoniae*	5/5 (100%)[a]						
6	*K. pneumoniae*	5/5 (100%)	5/5 (100%)	5/5 (100%)	3/5 (60%)[c]	1/5 (20%)[c]	2/5 (40%)	2/5 (40%)[b]
	Enterobacter cloacae	1/5 (20%)[b]						
	None				2/5 (40%)	4/5 (80%)	3/5 (60%)	3/5 (60%)
8	*K. pneumoniae*	5/5 (100%)[c]	5/5 (100%)[c]	5/5 (100%)[c]	5/5 (100%)	2/5 (40%)		
	Enterococcus faecalis							1/5 (20%)[b]
	Streptococcus equinus							1/5 (20%)[b]
	None					3/5 (60%)	5/5 (100%)	3/5 (60%)
11	*K. pneumoniae*	NS		2/5 (40%)				
	E. faecalis				1/5 (20%)[b]	1/5 (20%)[b]		
	Staphylococcus epidermidis					1/5 (20%)[b]	1/5 (20%)[b]	
	None		5/5 (100%)	3/5 (60%)	4/5 (80%)	3/5 (60%)	4/5 (80%)	5/5 (100%)
15	*K. pneumoniae*	NS[e]				1/5 (20%)[b]	1/5 (20%)	
	E. faecalis				3/5 (60%)[b]			
	Staphylococcus sciuri			1/1 (100%)[b]				
	Streptococcus equinus			1/1 (100%)				
	S. oralis						1/5 (20%)	
	S. uberis				1/5 (20%)[b]			
	None		5/5 (100%)		1/5 (20%)	4/5 (80%)	4/5 (80%)	5/5 (100%)

[a]Numerator indicates no. of mice from which a bacterial species was isolated; denominator indicates total No. of mice sampled prior to initial treatment on each day in each treatment group.
[b]Ten or fewer colonies isolated per agar plate.
[c]Clinical signs of disease present (recumbency, dyspnea, kyphotic, ruffled coat, reluctant to move when stimulated).
NS = no mice available for sampling because no mice survived.
See Table 1 for key.

model used in this study (26).

A negative aspect of the ceftriaxone plus gentamicin combination is that it involved two subcutaneous injections. The parenteral route may repeatedly expose the post-irradiation, immunodepressed animal to a potential source of exogenous infection (local or systemic) and to the possibility of local bleeding due to thrombocytopenia (8), although a localized antimicrobial agent would reduce the likelihood. Oral administration in mice is preferred, which is an advantage of gatifloxacin over ceftriaxone alone or of the ceftriaxone and gentamicin combination because it is a non-invasive route and offers practicality for mass casualties.

The oral route and frequency of dosing were chosen because fluoroquinolones are well absorbed (13), wide dosing intervals are possible, and they, as well as aminoglycosides, induce PAE (12). The β-lactam drugs are not associated with substantial PAE on growth suppression of gram-negative organisms when the drug is removed (9); however, some studies (10, 22) have indicated that some cephalosporins induce a PAE. Ceftriaxone was associated with PAE of 50 to 60 min at MIC against *K. pneumoniae* in vitro (10). A PAE ranging between 105 and 120 min for the ceftriaxone-gentamicin combination against *K. pneumoniae* (10), may have contributed to success in decreasing mortality in the mice of our study. A PAE of 3.8 h and a half-life of 19.5 min were documented for gentamicin alone in the neutropenic mouse against *K. pneumoniae* (22). Fantin and co-workers (19) reported PAE of 6.2 ± 1.4 h for gentamicin against *K. pneumoniae* when administered into the thigh of neutropenic mice. In that study, it was concluded that prolonged PAEs are consistently observed in vivo for aminoglycosides against Enterobacteriaceae, and that this duration is enhanced in the presence of neutrophils and by pharmacokinetic properties simulating those observed in humans. This result probably reflects the in vivo relevance of postantibiotic leukocyte enhancement (19). Due to radiation-induced neutropenia, the PAE should be less apparent in irradiated animals treated for bacterial infections.

The optimal duration of therapy is yet to be determined. Our experiments were designed to detect differences in survival between drugs alone and in combination. Although minimal mortality occurred after termination of therapy on day 15 (Fig. 1), longer duration of therapy might have prevented mortality noticed after that day. Therapy prevented bacterial sepsis and reduced bacterial load in the lungs (Table 3). Antimicrobial treatment controlled gram-negative bacteria in lung and heart blood, but did not entirely control gram-positive infection after day 6 (Tables 3 and 4). Use of antimicrobial agents eliminated infection by any microorganism in 65% of mice (Table 3).

Calculation of pharmacokinetic (PK) and pharmacodynamic (PD) parameters provides insight into the treatment of *K. pneumoniae* pneumonia. Andes and Craig determined that C_{max} of gatifloxacin was 5.96 mg/L at 0.25 h after 18.75 mg/kg given s.c. (2). In comparison, we found C_{max} of 1.37 μg/ml at 0.5 h after a dose of 20 mg/kg given p.o in non-irradiated mice. The difference is explained by the differing routes of administration. Compared with oral dosing, subcutaneous administration allows faster and higher C_{max}. Extrapolation from reported studies allows a determination of C_{max} for ceftriaxone and gentamicin. For ceftriaxone, Sauve and co-workers reported C_{max} of 91.5 μg/ml following an injected dose of 100 mg/kg at 0.5 h after administration (38) and Kazragis and co-workers documented C_{max} of 83 μg/ml following an injected dose of 50 mg/kg, s.c., at 0.5 h after administration (25). Therefore, we would anticipate C_{max} of 87.25 μg/ml in a non-irradiated mouse given a dose of 75 mg/kg, s.c. For gentamicin, C_{max} of 9.8 mg/L in non-irradiated mice was reported at 0.25 h after a dose of 8 mg/kg, s.c. (19), which is a dose similar to that (7.5 mg/kg) used in our study.

Craig and co-workers (1, 2, 14) established guidelines for correlating drug efficacy between mice and humans (i.e., C_{max}/MIC > 8). The C_{max}/MIC for ceftriaxone was calculated using the ex-

trapolated C_{max} and the highest MIC (macrodilution procedure). The C_{max}/MIC for gentamicin was calculated using C_{max} reported by Fantin and co-workers (19) divided by the highest MIC (macrodilution procedure). The expected C_{max}/MIC for gentamicin would be slightly lower than the calculated value due to the slightly lower dose used in this study. Serum concentrations of ceftriaxone and gatifloxacin greatly exceeded the MICs in irradiated mice. The high C_{max}/MIC for gatifloxacin and ceftriaxone would predict efficacy, but the low C_{max}/MIC for gentamicin would not predict efficacy against *K. pneumoniae* alone (Table 2). This may explain why gentamicin failed to decrease mortality effectively even though survival was significantly ($P < 0.001$) greater than that of controls. Perhaps, due to prolonged PAE, gatifloxacin was effective despite a short half-life, which would predict the concentration to decrease below the MIC in approximately five hours. The doses selected may serve as a guide for establishing human dosage regimens because it is likely that the magnitude of the PK and PD parameters required for efficacy would be similar in various animal species (14).

The high mortality in gentamicin-treated mice of our study may be attributable to serum concentration less than the recommended therapeutic level of 5 to 10 μg/ml (3) and the comparatively high MIC of 1.0 to 2.0 μg/ml, as well as variation in mouse strain and route of inoculation. These factors contributed to the difficulty in treating *K. pneumoniae* infections with gentamicin alone in our study despite a PAE (19, 22).

Aminoglycosides penetrate poorly in respiratory tract secretions and epithelial lining fluid (20), which also may help explain why gentamicin was not effecive against intratracheal challenge with *K. pneumoniae,* but was effective against subcutaneous challenge. On the other hand, Leggett and co-workers (29) contradict this concept by suggesting a more prolonged clearance of aminoglycosides from the respiratory tract than from the thigh of neutropenic mice and greater respiratory tract penetration, which may explain enhanced activity of aminoglycosides in the lungs. Rapid elimination rate and low serum concentrations indicate the most likely cause for low survival in gentamicin-treated mice in our study, which was comparable to the result reported by Madonna and co-workers (30).

Studies by Schentag and co-workers (39, 40) suggested that use of loading doses to avoid underdosing, with appropriate dosing intervals for aminoglycosides and fluoroquinolones, will result in greater antimicrobial activity and fewer treatment failures. To obtain high bactericidal effects from aminoglycosides and fluoroquinolones, high peak concentrations are more important than sustained values, whereas the β-lactams have a time-dependent effect, and sustaining high concentrations at infection sites is more important (13, 14, 21, 41). Although it was not our intention to use an optimal dose so as to detect differences between the combination therapy and gentamicin or ceftriaxone alone, future studies may demonstrate increased survivability if higher doses, loading doses, and/or more frequent dosing intervals are used for gentamicin in particular. However, antimicrobials, such as gentamicin, that induce long PAEs against microorganisms may be administered with longer dosing intervals without loss of efficacy and even with lower frequency of adverse reactions (22, 29). The long PAE of aminoglycosides, especially when used in combination with a β-lactam antibiotic, supports alternative dosing regimens (i.e., larger doses with longer dosing intervals [10]). Although long dosing intervals would be an advantage to treat infections after irradiation in humans, multiple daily dosing of aminoglycosides is more effective in small rodents infected with Enterobacteriaceae than is single daily dosing (33, 41).

The findings of this study suggest that ceftriaxone plus gentamicin, followed by ceftriaxone alone or gatifloxacin as an alternative, is the most effective antimicrobial therapy for treatment of acquired *K. pneumoniae* pneumonia in an immunocompromised host. Antimicrobial resistance did not develop during the treatment period. Applications for our findings range from treating immunocompromised patients with acquired immune deficiency syndrome that have a nosocomially acquired infection to treating radiation victims with acquired pneumonia. Further studies to evaluate optimal regimens with longer treatment periods and larger doses of gatifloxacin and gentamicin are warranted to determine whether survival can be further increased.

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