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Model of Experimental Chronic Osteomyelitis in Rats

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We describe here a Sprague-Dawley rat model for chronic osteomyelitis. *Staphylococcus aureus* and sodium morrhuate were implanted by either microdrilling or direct needle injection into the tibiae of rats. Of 107 rats, 87 (81%) developed osteomyelitis when a high-speed drill was used for implantation, and 27 (51%) of 53 rats developed osteomyelitis by direct needle inoculation (chi square = 9.81, $P < 0.01$). Demonstrated histopathological changes included the presence of resorption bays filled with osteoclasts. Quantitative microbiological monitoring of tibial count confirmed disease chronicity, yielding stable numbers of CFU ($10^{6.29 \pm 0.27}$) of *S. aureus* over 70 days. Infected animals became anemic and lost weight. The erythrocyte sedimentation rates and leukocyte counts were not elevated. Roentgenograms provided the best correlation with the number of organisms in infected tibiae ($r^2 = 0.80$). Rats with infected tibiae were treated with either oxacillin (120 mg/kg per day) or ceftriaxone (50 mg/kg per day). Treatment over 14 or 28 days reduced *S. aureus* counts in tibiae but did not reliably sterilize infected bones, suggesting that this model was resistant to prolonged antimicrobial therapy.

Chronic osteomyelitis remains a source of disability. The infection is often refractory to prolonged antimicrobial therapy.

Rabbit (1, 3, 7) and canine (4) models have been used to examine the pathophysiology and treatment of osteomyelitis. Rats have seldom been used even though a successful rat model was described by Zak, who implanted *Staphylococcus aureus* or *Pseudomonas aeruginosa* plus the sclerosing agent sodium morrhuate into rat tibiae (O. Zak, F. Zak, and R. Rich, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 21st, Chicago, Ill., abstr. no. 530, 1981). The model appeared amenable to therapeutic manipulation and revealed a purulent osteomyelitis with abscesses and sequestra.

We felt that this model is worthy of further investigation, since rats are better suited to therapeutic manipulations than rabbits (2). We describe here the development of an experimental chronic osteomyelitis model in rat tibiae, using *S. aureus* and sodium morrhuate at inoculum strengths comparable to those used in rabbits. Quantitative nondestructive and destructive techniques for osteomyelitis assessment were compared. The resistance of the model to antimicrobial manipulation was also assessed.

MATERIALS AND METHODS

Animals. Albino Sprague-Dawley rats (300 to 400 g) were used. The rats were individually caged, fed a standard pellet diet, and provided with water ad libitum.

Bacteria and preparation of inocula. Two isolates of *S. aureus* were used in these studies. The first isolate, phage type 52/52A/80, was kindly provided by C. W. Norden. This organism was used in experiments designed to study the assessment of osteomyelitis. The second isolate (OM-1), used in the antimicrobial studies, was obtained from a patient with osteomyelitis. The isolates were periodically passed through rabbits to maintain virulence (5). An analysis of the MBC of the OM-1 strain indicated resistance to penicillin (>50 μg/ml) but susceptibility to oxacillin and ceftriaxone (2.0 and 3.0 μg/ml, respectively).

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S. aureus strains were cultured with shaking for 18 h in 150 ml of tryptic soy broth (Difco Laboratories, Detroit, Mich.) at 37°C. A portion (100 μl) was transferred to 3 ml of broth and incubated for 3 h to obtain log-phase growth. The organisms were centrifuged, and the pellet was washed and reconstituted in isotonic saline to a concentration of 3×10^6/5 μl. The bacterial density of the inoculum (CFU per milliliter) was determined by a spectrophotometric standard curve and confirmed by plate count.

Sclerosing agent. Undiluted 5% sodium morrhuate (Eli Lilly & Co., Indianapolis, Ind.) was injected before the implantation of *S. aureus*. Isotonic sterile saline was used as a negative control.

To assess the growth inhibition of *S. aureus* by sodium morrhuate, the organism was streaked onto 5% sheep blood agar, and 6-mm sensitivity disks containing 5,000 μg of sodium morrhuate were applied. The plates were then examined for growth inhibition. Killing curves of *S. aureus* (2×10^7 CFU/ml) in the presence of 2.5% sodium morrhuate were also determined. Colony counts were performed immediately, at 15 min, and hourly for 2 h.

Implantation: needle and drill protocols. Rats were anesthetized with 500 μl of a 50% (vol/vol) mixture of ketamine hydrochloride (100 mg/ml; Bristol Laboratories, Syracuse, N.Y.) and xylazine hydrochloride (20 mg/ml; Miles Laboratories, Inc., Shawnee, Kans.) given intravenously. The right hind leg was shaved and scrubbed with Betadine (Purdue Frederick Co., Norwalk, Conn.). In the needle protocol, a 1-in (2.54-cm) 22-gauge needle was inserted percutaneously into the marrow in the metaphyseal region of the tibia. Sodium morrhuate (25 μl of a 5% stock preparation) was injected through the needle, followed by the standardized inoculum of organisms and saline (25 μl of each). In these and all syringe techniques, the use of mid-barrel markings eliminated dead-space volume dispensing problems.

In the drill protocol, the anterior tibial metaphysis was surgically exposed, and a high-speed Dremmel Moto-Tool with a 1-mm burr bit was used to create an aperture through the bone cortex that exposed the marrow. Approximately 30 s after the addition of 5 μl of 5% sodium morrhuate, *S.*

TABLE 1. Summary of parameters studied, including implantation protocols, *S. aureus* strains, inocula, and assessment types[a]

Implantation protocol	*S. aureus* strain used	Inocula (total no. of rats)	Assessment type (No. of rats)							
			Destructive			Nondestructive				
			Quantitative bone count	Gross pathology	Histopathology	Weight	Radiographs	HCT	WBC	ESR
Drill	52/52A/80	SA + SM (113)	107	107	6	30	20	34	34	34
		SA only (21)	18	18	ND	18	18	ND	ND	ND
		Saline only (12)	9	9	ND	12	9	ND	ND	ND
		SM only (3)	ND	ND	3	ND	3	ND	ND	ND
Drill	OM-1	SA + SM with oxacillin (12)	12	12	ND	ND	ND	ND	ND	ND
		SA + SM with ceftriaxone (24)	24	24	ND	ND	ND	ND	ND	ND
Needle	52/52A/80	SA + SM (53)	53	ND	ND	ND	ND	ND	ND	ND

[a] Abbreviations: SA, *S. aureus*; SM, sodium morrhuate; HCT, hematocrit; WBC, leukocyte count; ESR, erythrocyte sedimentation rate; and ND, not determined.

aureus (3×10^6 CFU/5 μl) was injected through a Hamilton microsyringe. Bone wax was used to prevent leakage.

Assessment of surgical technique was judged independently for each rat on a scale from 0 to 10. Low scores were given if needle positioning was in doubt or if leakage occurred after drilling. Rats with surgical scores below 7 were not studied further. Rats were sacrificed by CO_2 asphyxiation at day 35 or 70 after implantation.

Assessment. Evaluation techniques not requiring the sacrifice of the test rat were as follows: roentgenograms, weight loss, and hematologic tests (erythrocyte sedimentation rate, hematocrit, and leukocyte count). Osteomyelitis was confirmed at autopsy by gross tibial pathology, histopathology, and *S. aureus* count in tibial bone.

Roentgenograms were performed after 28 days. The presence of periosteal elevation, architectural distortion, widening of the bone shaft, and new bone formation were determined for each tibia. The mean composite score represents the total number of rats with each of these particular abnormalities divided by the total number of rats tested. Roentgenograms were interpreted in a coded, blind manner by physicians and radiologists unaware of the inocula. The percentage of tibial destruction was also estimated subjectively by roentgenography. Internal controls for precision by observer-blind repetitive reading disclosed the correlation of repetitive readings over time to be high ($r^2 = 0.94$).

Rat weights were monitored weekly. Erythrocyte sedimentation rate determinations were performed in Wintrobe sedimentation tubes on 1 cc of whole heparinized blood obtained by heart puncture or tail vein phlebotomy. Chamber leukocyte counts and hematocrits were performed by standardized laboratory methods (8).

The gross bone pathology was determined by grading bone destruction from 0 to 4. A score of 0 represented the absence of abscess, sequestrum, active bone formation, and erythema. A score of 1 indicated minimal erythema without abscess or evidence of new bone formation. A score 2 indicated erythema with a widening of the head and shaft of the bone with new bone formation. A score of 3 indicated abscess with new bone formation, sinus tract drainage, or grossly purulent exudate, and a score of 4 typically indicated severe bone resorption, abscess, and diaphyseal or total tibial involvement.

For histopathology studies, the tibiae were dissected free of soft tissue, fixed in phosphate-buffered 10% formaldehyde, dehydrated in graded acetone solutions, and embedded in methyl methacrylate without prior decalcification (9). Longitudinal sections (5 μm) were cut on a Jung model K sledge microtome and stained by a modification of the Masson technique (6). Several sections from each specimen were decalcified and stained with hematoxylin and eosin for examinmation under polarized light.

For quantitative bone bacterial counts, muscle and connective tissue were first removed from the tibiae. Tibiae were aseptically cross-sectioned at both ends with a high-speed circular saw. Proximal sectioning was performed between the metaphysis and the epiphyseal plate; distal sectioning was performed approximately 8 mm from the distal articulating surface. The resulting bone segments were weighed. Quantitative bacterial counts were determined for tibiae (including marrow) after snap freezing in liquid nitrogen and pulverization with chilled (−20°C) mortars and pestles. The resulting bone chips were vortexed in saline for 15 min, and serial dilutions were streaked in triplicate onto

TABLE 2. Roentgenographic assessment of chronic experimental osteomyelitis

Inoculum	No. of rats tested	Raised periosteum (%)	Destruction of architecture (%)	Widening of shaft (%)	New bone formation %	Concurrent presence of any two positive roentgenographic tests (%)	Composite score (±SD)	Bone destruction (%)
Saline	9	22	22	0	22	11	0.7 ± 0.7	6.3 ± 4.6
S. aureus-saline	18	61[a]	56[a]	50[a]	39[a]	61[a]	2.3 ± 1.5[b]	12.6 ± 10.2[b]
S. aureus-sodium morrhuate	20	60[a]	50[a]	45[a]	50[a]	65[a]	2.0 ± 1.5[b]	19.7 ± 13.5[b]

[a] $P < 0.05$ (Fisher's exact test).
[b] $P < 0.05$ (Student's t test).

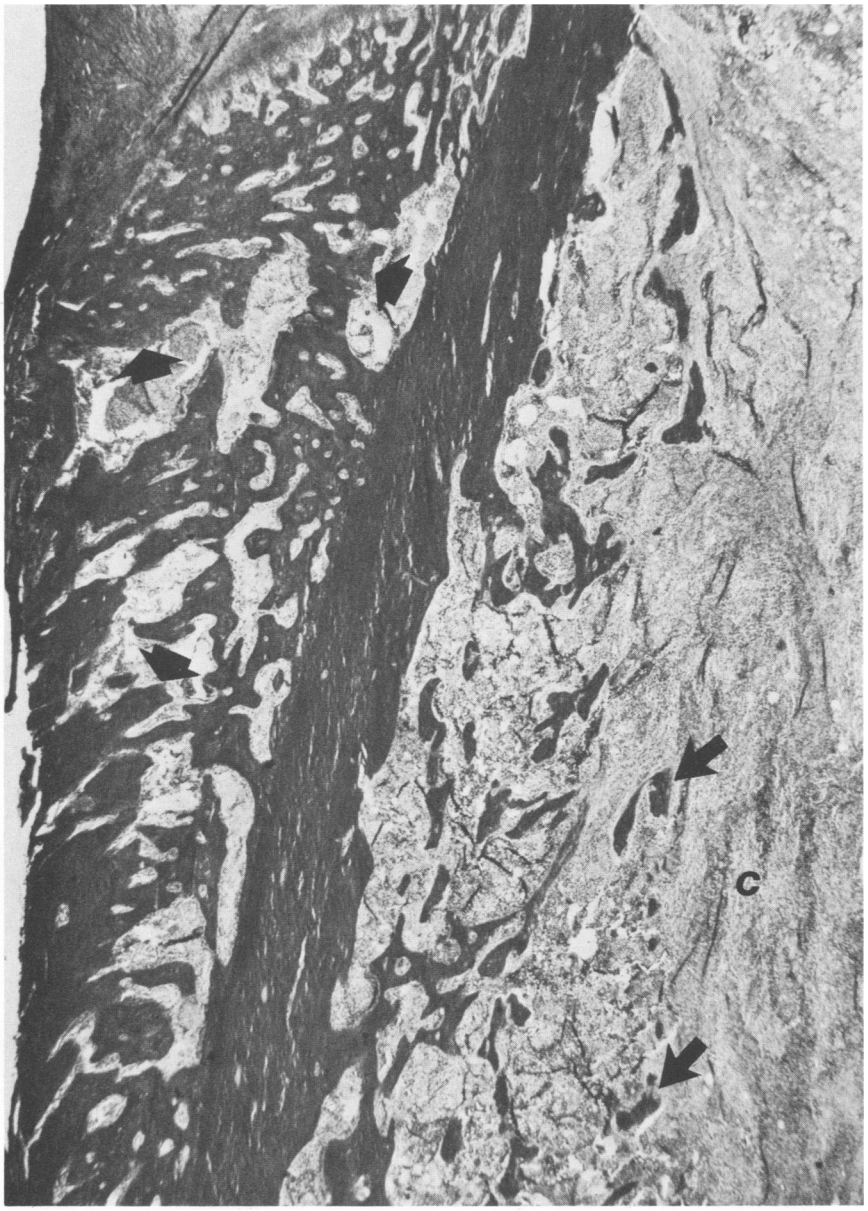

FIG. 1. Photomicrograph of an undecalcified longitudinal section of rat tibia taken 21 days postimplantation of *S. aureus* and sodium morrhuate. Marrow is on the right, and original cortical bone lies in the middle. Periosteal reactive bone formation (arrows on left), necrotic trabecular bone (arrows on right), and chronic inflammatory cells (C) are also seen (modified Masson stain; magnification, ×25).

tryptic soy agar plates. Bacterial counts are expressed as $\log_{10}$ per gram.

Antimicrobial studies. In other studies, rats were treated with oxacillin and ceftriaxone commencing 21 days after the surgical implantation of *S. aureus*. The rats included were those determined to have osteomyelitis by roentgenographs. The treatment regimens were oxacillin (120 mg/kg) every 12 h, ceftriaxone (50 mg/kg) every 24 h, or ceftriaxone (25 mg/kg) every 12 h. Earlier studies confirmed that these doses yielded mean peak concentrations in serum consistently six- to eightfold greater than the MICs for both antimicrobial agents. Antimicrobial injections were given subcutaneously for 14 or 28 days. The rats were sacrificed immediately after the termination of treatment or held for an additional 14 days to assess bacterial regrowth.

Multiple linear regression analysis and other statistical analyses were performed on a Cyber 170/750 mainframe computer (Control Data Corp., Minneapolis, Minn.) by using the Minitab and Statistical Package for the Social Sciences systems.

RESULTS

A total of 280 rats received surgical implants in nine separate experiments. Of these, 247 rats were scored as acceptable for analysis; 9 of these died prematurely.

Of the remaining 238 rats, 160 received *S. aureus* plus sodium morrhuate and were used to compare the infectivity rates of the needle and drill protocols: 53 animals were implanted with the inoculum by the needle protocol and 107 by the drill protocol. Six rats were used for histopathology studies (Table 1).

A group of 36 control rats implanted by the drill protocol was also studied. Of these, 21 received *S. aureus* only, 12 received saline only, and 3 received sodium morrhuate only.

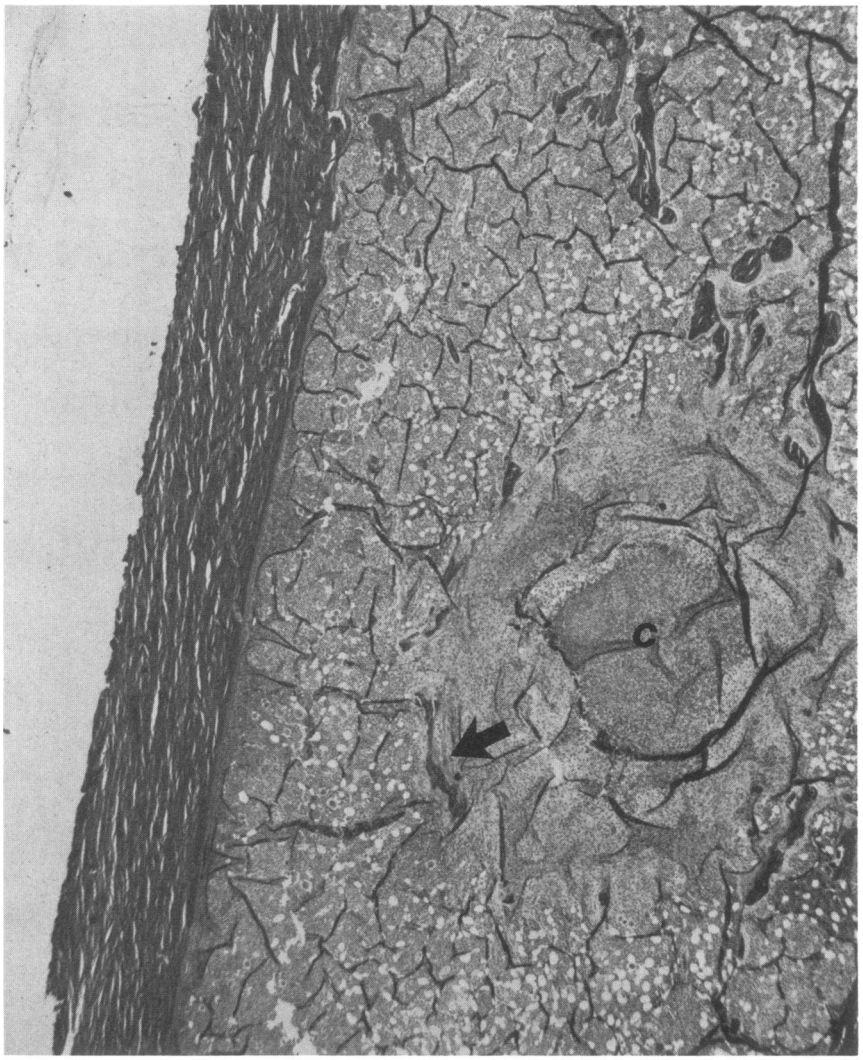

FIG. 2. Photomicrograph of histologic features of a tibia (oriented like that in Fig. 1) 21 days after instillation of sodium morrhuate alone. Only inflammatory cells (C) and some necrotic trabecular bone (arrow) are revealed. Reactive bone formation is absent (magnification, ×25).

An additional 36 rats implanted with *S. aureus* by the drill protocol were treated with antimicrobial agents.

Drill versus needle protocol. Low surgical confidence scores were seen with the needle protocol. Correct needle depth and positioning were difficult to control, and periodically the needles would slip out of or penetrate through bone. Leakage of the inoculum was a problem in the drill protocol. This problem was eliminated by using warmed bone wax before and after inoculation. Sterility requirements and suturing increased the time required for implantation considerably.

The drill protocol yielded higher infectivity rates. Rat tibiae implanted with *S. aureus*-sodium morrhuate yielded organisms from 27 (51%) of the 53 needle protocol rats and 87 (81%) of the 107 drill protocol rats (chi square = 9.81, $P < 0.01$). In further analyses only the results from the drill protocol were used.

Osteomyelitis disease parameters measured by nondestructive protocols. Roentgenographic results are presented in Table 2. Only 1 (11%) of the 9 rat tibiae implanted with saline only displayed two or more roentgenographic predictors, compared with 11 (61%) of the 18 *S. aureus*-saline-challenged tibiae ($P = 0.01$, Fisher's exact test) and 13 (65%) of the 20 *S. aureus*-sodium morrhuate-challenged tibiae ($P = 0.008$, Fisher's exact test). Composite roentgenographic scores increased when *S. aureus* was given with the sclerosing agent sodium morrhuate versus when the organism was given alone from 0.7 ± 0.7 to 2.31 ± 1.5 ($P < 0.01$, Student's *t* test).

The roentgenographic tibial involvement scores (percent tibia involved) were somewhat higher for *S. aureus*-sodium morrhuate-implanted rats than for those implanted with *S. aureus*-saline. Mean scores were 19.7 ± 13.5 (standard deviation) and 12.6 ± 10.2 ($P < 0.09$), respectively. The mean tibial involvement score for saline-injected rats was 6.3 ± 4.6%.

Rats implanted with saline only gained weight throughout the study. Rats implanted with *S. aureus*-saline lost weight initially but showed a net increase in weight by day 35. Rats implanted with *S. aureus*-sodium morrhuate lost weight over the 35 days of the study. Weight loss curves for rats receiving *S. aureus*-sodium morrhuate were different from those for the other two groups ($P < 0.03$, analysis of variance for repeated measures test).

Erythrocyte sedimentation rates, leukocyte counts, and hematocrits were determined for 34 rats receiving *S. aureus*-sodium morrhuate. These rats did not disclose elevated

erythrocyte sedimentation rates or leukocyte counts. The mean erythrocyte sedimentation rate rose from 0.8 ± 0.63 at day 0 to 2.2 ± 2.5 at day 35. Mean leukocyte counts changed from 7,700 ± 3,000 to 6,100 ± 2,000. Injected rats showed a marked decrease in mean hematocrit readings, which fell from 41 to 34% ($P < 0.05$).

Osteomyelitis disease parameters measured by destructive protocols. Undecalcified sections of rat tibiae inoculated with *S. aureus*-sodium morrhuate were examined 35 days postimplantation (Fig. 1). The metaphyseal area contained islands of necrotic trabecular bone which were devoid of osteocytes. The trabecular margins were serrated with resorption bays filled with osteoclasts. Hematopoietic marrow was replaced by numerous polymorphonuclear cells, fibroblasts, and macrophages. Clusters of cocci were also present. The endosteal surfaces were lined with wide osteoid seams rimmed by plump, cuboidal osteoblasts. With polarized light, the osteoid showed the haphazard birefringence of woven collagen architecture. Thick basophilic cement lines separated the endosteal osteoid from the underlying cortical bone. Inflammatory cells infiltrated the Volkmann's and haversian canals, dissecting between the periosteum and cortex. Exuberant periosteal new bone formation was seen at the sequestrated periosteal margins, findings typical of an involucrum. The epiphyseal cartilage was spared.

Tibiae from animals injected with sodium morrhuate alone (Fig. 2) showed some necrotic bone and inflammatory cells but had a paucity of reactive bone formation, an important indicator of chronic osteomyelitis (8a).

Examination of infected bone marrow by Giemsa stain disclosed that marrow from bones yielding counts exceeding $10^{6.0}$/ml had increased polymorph to lymphocyte ratios. Erythrocytic precursors were depressed. The macroscopic appearance of the bone marrow was a purulent, chalky white when *S. aureus* bone counts approached 10^6.

Gross pathology scores for rats inoculated with saline only disclosed a mean of 0.6 ± 0.5. Scores for *S. aureus*-saline-inoculated tibiae increased to 1.9 ± 1.4 ($P < 0.01$, Student's *t* test). Scores with *S. aureus*-sodium morrhuate were 3.4 ± 0.84 ($P < 0.001$). Rats receiving sodium morrhuate alone disclosed higher pathology scores than those receiving *S. aureus* but no sclerosing agent ($P < 0.01$).

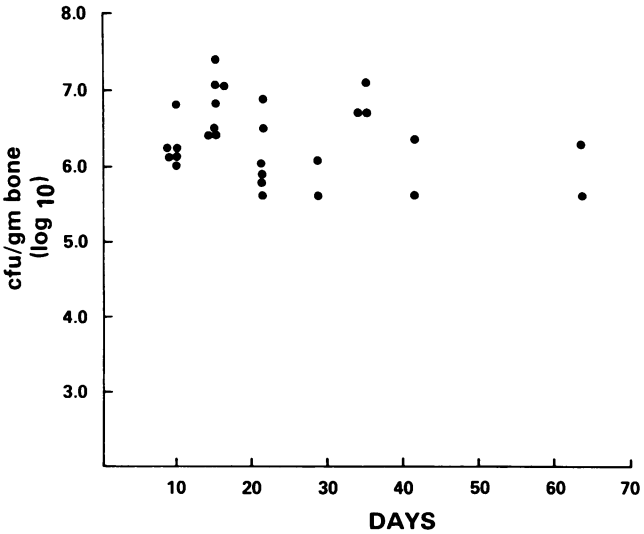

FIG. 3. Chronicity of infections as assessed by CFU ($\log_{10}$) per gram of rat tibia at various times postimplantation.

TABLE 3. Results of bone bacterial counts (geometric mean) after antimicrobial therapy as indicated

Treatment regimen[a]	Geometric mean CFU ($\log_{10}$)
Oxacillin (120 mg/kg every 12 h) for:	
14 days and immediate sacrifice	4.27[b]
14 days and sacrifice 14 days later	4.89[b]
28 days and immediate sacrifice	3.39[b]
28 days and sacrifice 14 days later	3.69[b]
Ceftriaxone (50 mg/kg every 24 h) for:	
14 days and immediate sacrifice	6.43
14 days and sacrifice 14 days later	4.07[b]
28 days and immediate sacrifice	3.50[b]
28 days and sacrifice 14 days later	4.25[b]
Ceftriaxone (25 mg/kg every 12 h) for:	
14 days and immediate sacrifice	5.95
14 days and sacrifice 14 days later	5.24
28 days and immediate sacrifice	4.57[b]
28 days and sacrifice 14 days later	5.27

[a] Three rats were used for each of the 12 regimens.
[b] Results were significantly lower than those for untreated control animals (Student's *t* test, $P < 0.05$).

Quantitative bone culture results were determined after animal sacrifice on day 35. The mean number of CFU per gram of tibia in 79 rats was $10^{6.7\pm0.59}$. Additional studies were performed over time, and the results are presented in Fig. 3. A group of 28 rats receiving *S. aureus*-sodium morrhuate disclosed a geometric mean tibial *S. aureus* count of $10^{6.28\pm0.27}$ ($n = 28$) over 70 days. Linear regression analysis disclosed an equation of $y = 10^{6.48} - [0.0068 \times (\log_{10}$ CFU/g)] with a slope which was not significantly different from 0, thus confirming bacterial chronicity (analysis of variance; $F = 1.13$, 26 *dof*; $r^2 = 4.4\%$).

Rat tibiae receiving *S. aureus*-saline yielded a geometric mean count of $10^{4.48\pm1.6}$. This was lower than the mean count for *S. aureus*-sodium morrhuate-implanted tibiae ($P < 0.01$, Student's *t* test).

Correlation of predictors. A stepwise multiple linear regression analysis correlating the independent variables of roentgenographs, weight loss, and erythrocyte sedimentation rates to colony counts per gram of bone yielded an equation of y (CFU/g) = 2.46 + 1.04 (roentgenogram score) + 0.003 (weight loss) − 0.193 (hematocrit value). Roentgenographs were the nondestructive predictor which best correlated with the tibial *S. aureus* counts ($r^2 = 0.80$). Erythrocyte sedimentation rate and weight loss were not significantly correlated ($r^2 = 0.074$ and 0.16, respectively).

The results of prolonged antimicrobial treatment are presented in Table 3. Each oxacillin-treated group yielded colony counts significantly lower than those of controls ($P < 0.05$, Student's *t* test). No significant differences were noted between the counts from animals sacrificed immediately or after 14 days. However, infection was not eradicated after 28 days of oxacillin treatment. Only 1 of the 12 treated rat tibiae was sterile. Ceftriaxone also caused a one to two log decline in the number of CFU of *S. aureus* in bone, and none of the tibiae was sterile. Ceftriaxone-treated rat bone count results were similar whether the rats were treated once or twice per day.

Studies were performed to determine whether sodium morrhuate solution influenced the growth of *S. aureus*. Agar diffusion assays did not indicate inhibition of bacterial

growth. However, the growth of *S. aureus* was totally inhibited in tryptic soy broth containing either 2.5 or 0.25% sodium morrhuate within 15 min.

DISCUSSION

In this study, we used the anterior superior tibia and clearly demonstrated the ability to establish a chronic infection yielding a large and stable number of *S. aureus* for a substantial period of time in the rat. The drill protocol was more predictable than the needle protocol. Therefore, we used the drill protocol for the remaining portion of this study. Histopathologic sections of the affected bones were analyzed by the relatively newly described technique of embedding sections in methyl methacrylate to preserve bone architecture. These sections provided clear evidence of chronic osteomyelitis, including new endosteal and trabecular bone formation, numerous osteoclasts, a dense infiltration of polymorphs and macrophages, and an increase in demineralized osteoid.

Several noninvasive parameters were used to determine their correlation with the presence of active disease. The clearest correlations were between bone dissolution and disease as assessed by roentgenograms or weight loss. Other parameters, including those examined both in the human disease and in the disease as modeled in other experimental animals, were less remarkable. For example, the erythrocyte sedimentation rate and leukocyte count did not change; however, infected animals usually developed anemia.

One important pathophysiologic quality of chronic osteomyelitis is its resistance to apparently appropriate single-antimicrobial-agent therapy, even when the agent is administered for a prolonged time. Two agents used to treat human disease were administered to rats in relatively high doses and for extended times after *S. aureus* incubation periods of 21 days. Although both agents achieved statistically significant decreases in bacterial counts, bone sterilization was uncommon, occurring in only 1 of 12 animals treated with oxacillin and in 0 of 24 animals treated with ceftriaxone.

A potential weakness of the rat model is the bone trauma induced by the drill. This trauma may be adjuvantive in the development of osteomyelitis through prostaglandin elaboration (3). To date, sclerosing agents, including sodium dodecyl sulfate and sodium morrhuate, have been used in animal models. An additional problem related to our studies is that sodium morrhuate has a toxic effect on *S. aureus* in vitro. However, the application of sodium morrhuate at least 30 s before *S. aureus*, thus allowing dilution of the sclerosing agent into bone cavities, apparently eliminated its toxicity to *S. aureus*. Some histopathologic changes were seen when sodium morrhuate alone was injected. Efforts are under way to eliminate the use of sodium morrhuate by exploring the adjuvantive effects of other methods or substances.

Despite these reservations, we feel that the rat model of chronic osteomyelitis offers several advantages. The animal has considerable tolerance for antimicrobial therapy, and both purchase and maintenance costs per animal are substantially less than when rabbits are used. Consequently, the number of experimental observations can be increased. Established chronic disease can be monitored roentgenographically. An interesting potential of the rat model is the opportunity it affords to evaluate osteomyelitis in inbred rat strains. This would allow a selective evaluation of the host-parasite relationship in immunologically defined settings.

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