Chronic Staphylococcal Osteomyelitis: a New Experimental Rat Model

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A rat model of chronic staphylococcal osteomyelitis was developed. Fibrin glue (5 μl) and Staphylococcus aureus (2 × 10⁶ CFU/5 μl) were inoculated into the proximal metaphysis of the tibia. The rats were killed at intervals of between 1 and 6 months, and the tibias were removed. Induced lesions were evaluated by radiographic, macroscopic, and histological examinations and bacterial counts. Roentgenograms revealed osteomyelitis in more than 90% of the tibias. Gross bone pathology revealed skeletal deformation, new bone formation, abscesses, and draining skin fistulas in more than 80% of cases. Histological examination revealed osteomyelitis in more than 90% of cases, and bacterial counts were positive in 86% of cases. Only fibrin glue (5 μl) was inoculated into controls. Controls showed no osteomyelitic lesions, and counts were negative in seven of eight control tibias. The main feature of this model is the use of fibrin glue instead of the sclerosing agents and foreign bodies used in other models. The model reproduces lesions similar to those of human posttraumatic osteomyelitis and can be reliably used in pathophysiological and therapeutic studies.

Osteomyelitis is an infective process in bone and bone marrow. Despite progress in the field of diagnosis and therapy of infectious diseases, some aspects of the pathogenesis, early diagnosis, and therapy of osteomyelitis are still unclear because of the multiple variables involved. It is therefore necessary to follow a methodological path which is both clinical and experimental. Therefore, animal models of osteomyelitis are able to reproduce the clinical and gross pathological phases of the disease must be developed.

Animal models of acute and chronic osteomyelitis have been developed with rats (24, 25, 31), rabbits (1, 17-19, 22, 26, 27, 30), dogs (11, 13), guinea pigs (23), and chickens (12). In these models, osteomyelitic lesions are induced by use of sclerosing agents and foreign bodies with bacterial strains. Sclerosing agents probably cause alterations of the marrow circulation (10), and intramedullary foreign bodies could constitute an inert substratum to which Staphylococcus aureus adheres tenaciously and produces the extracellular hexopolysaccharide matrix called the glycocalyx (18, 19). The aim of our study was to develop a rat model of chronic staphylococcal osteomyelitis with pathophysiological, clinical, and gross pathological characteristics similar to those of the human disease by using fibrin glue instead of sclerosing agents or foreign bodies.

MATERIALS AND METHODS

Animals. Seventy-six male outbred Wistar rats (250 to 350 g) were used. Seven of these died of anesthetic complications. The rats were individually caged, fed a standard pellet diet, and supplied with water ad libitum. They were divided into two groups, A and B, consisting of 61 and 8 animals, respectively. Group A was treated with fibrin glue and S. aureus, and group B (control) was treated with fibrin glue alone.

The two groups were each divided into four subgroups as a function of the time interval between surgery and death (Table 1). The animals were killed by CO₂ asphyxiation.

Bacterial strain and preparation of inocula. The S. aureus strain used in our model was isolated from a clinical specimen of a patient with osteomyelitis. This strain was identified by biochemical tests and MIC assays against several drugs by use of the Sceptor system (Becton Dickinson). No strains other than this one were tested in our model.

An S. aureus colony was inoculated on nutrient agar (Difco) slants and incubated at 37°C for 24 h. Subsequently, a subculture was made in nutrient broth (Difco) for 18 h at 37°C. This culture was then diluted to obtain 2 × 10⁶ CFU/5 μl.

Fibrin glue. Reconstituted lyophilized human fibrin (Tisucol 0.5 kit; Immuno, Vienna, Austria) was used. Five microliters of fibrin glue was inoculated into each tibia.

Experimental protocol. The rats were anesthetized intraperitoneally with 2 mg of diazepam (Valium; Roche, Milan, Italy) and intramuscularly with 2.5 mg of ketamine (Ketal; Parke-Davis, Milan, Italy) per 100 g of body weight. Both hind legs were shaved and disinfected with polyvinylpyrrolidone-iodine (Betadine; Chinoin, Milan, Italy). The anterior tibial musculature of each leg was surgically exposed, and a hole was drilled through the cortex by use of a high-speed drill with a 0.6-mm-diameter bit.

Group A was inoculated with fibrin glue (5 μl) and S. aureus (2 × 10⁶ CFU/5 μl), and group B was inoculated with fibrin glue alone (5 μl). The cortical bone was then closed with bone wax to avoid leakage of the inoculum, and the muscle fascias and skin were sutured.

Assessment. After the animals had been killed, the lesions were assessed in a blinded manner on the basis of roentgenograms, gross bone pathology, histological examination, and microbiological analysis. Two reviewers helped the authors with the radiographic assessment, gross bone pathology, and histological examination. Gross bone pathology was determined when the tibias were removed. Radiographic assessment and histological examination were performed at the end of the experiment.

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Both tibias were removed. One, chosen at random, was used for microbiological analysis, and the other was used for radiographic and histological investigations.

Gross bone pathology was determined in both tibias according to the grading of Rissing et al. (25): 0, absence of abscesses, sequestra, active bone formation, and erythema; 1, minimal erythema, without abscesses or new bone formation; 2, erythema, with widening of the head and shaft and new bone formation; 3, abscesses, with new bone formation, sinus tract drainage, or grossly purulent exudate; 4, severe bone resorption, abscesses, and diaphyseal or total tibial involvement.

For microbiological analysis, muscle and connective tissue were removed from the tibia, which was then crushed with bone rongeurs and pulverized with a mortar and pestle under sterile conditions. The powder was suspended in 5 ml of saline, and serial dilutions of up to 10^6 were prepared. One milliliter of each dilution was added to 9 ml of nutrient agar, placed in an incubator at 37°C, and cultured in triplicate in petri dishes. All colonies were counted after 24 and 48 h of incubation, and the mean number of microorganisms per milliliter of solution was calculated.

Standard-view roentgenograms of the tibia were taken. For each tibia, periosteal reaction, bone deformation, widening of the diaphysis, osteolysis, and osteosclerosis were determined. The frequency of each of these alterations was recorded.

For histological analysis, the samples were fixed in neutral buffered formalin solution, decalcified in 4 N formic acid–sodium citrate (10%), embedded in paraffin, and cut into 6-μm longitudinal sections. The sections were stained with hematoxylin and eosin and Masson’s stain. The following alterations, characteristic of osteomyelitis, were assessed: bone marrow inflammation, cortical alterations, subperiosteal, medullary, and intracortical abscesses, and sequestra.

RESULTS

Radiographic examination. Radiographic findings for the 61 tibias from group A are shown in Table 2. At least two radiographic alterations were observed in 90.2% and at least three were observed in 70.5% of the tibias from group A. Periosteal reactions were observed in 43 of the 61 tibias from group A (70.5%), bone deformation was observed in 31 tibias (50.8%), widening of the shaft was observed in 52 tibias (85.2%), osteolysis was observed in 60 tibias (98.4%), and osteosclerosis was observed in 49 tibias (80.3%).

One month after surgery, the radiographic images showed osteolytic areas at the site of operation, initial periosteal reactions and, less frequently, abscesses in the soft tissues communicating with the bone marrow cavity (Fig. 1A). Moreover, a marked alteration of the bone, with widening of the shaft, was observed.

Two months after surgery, the osteolytic areas were more extensive and displayed a thick sclerotic border (Fig. 1B). Three and 6 months after the operation, new bone formation was accentuated (Fig. 1C and D). At 6 months, there was sometimes radiographic evidence of sequestra (Fig. 1D).

For six of the eight tibias from group B, roentgenograms showed small osteolytic areas surrounded by a thin sclerotic border at the site of operation. In no case was there a periosteal reaction, bone deformation, or widening of the shaft.

Gross bone pathology. Gross bone pathology was studied for the 122 tibias (61 rats) from group A and the 16 tibias (8 rats) from group B. The results for group A are shown in Table 3.

In group A, 80.4% of tibias had a score >1 and showed signs of osteomyelitis, whereas only 19.6% had a score of 0 or 1, without signs of osteomyelitis. The mean score assigned to group A tibias was 2.2.

A score of 1 was assigned to 4 group B tibias (25%), and a score of 0 was assigned to the remaining 12 (75%). The mean score for controls was 0.25.

For group A, macroscopic examination during the first 3 months after surgery revealed large abscesses either confined to the subperiosteal site or, in cases in which there was a periosteal rupture, invading the soft tissues (Fig. 2A). These abscesses communicated with the bone and sometimes drained externally by way of bright red fistulas. The skin was atrophic and adhered to the underlying layers.

Six months after surgery, gross bone pathology was characterized by marked metadiaphyseal alterations, with erythema, new bone formation, and small intracortical abscesses. In some cases, the bone collapsed and caused accentuated axial deviation of the skeletal segment.

Histological examination. Histological examination was performed on the 61 tibias from group A and the 8 tibias from group B. Osteomyelitis was observed in 55 of the 61 tibias from group A (90.2%). In particular, osteomyelitis was observed in 8 of the 9 tibias from subgroup A1 (88.9%), 9 of the 10 tibias from subgroup A2 (90.0%), 10 of the 12 tibias from subgroup A3 (83.3%), and 28 of the 30 tibias from...
subgroup A4 (93.3%). Samples from group B did not show histological signs of osteomyelitis.

Extraperiosteal, subperiosteal, intracortical, and intramedullary abscesses were observed in group A. Three areas could be clearly distinguished in the abscesses: central, medial, and peripheral. The central area contained polymorphonuclear cells, fibrinoid necrosis, and an occasional foam cell (Fig. 2B and C and 3A). The medial area showed mononuclear cells, identified as lymphocytes, monocytes, and macrophages, and polymorphonuclear cells, including eosinophils. The peripheral area was composed of fibrous tissue and enclosed the abscesses. No granulomas were observed.

Sequestra, i.e., irregular fragments of infected necrotic bone surrounded by polymorphonuclear cells, were observed within the abscesses (Fig. 3B). A progressive increase in the frequency of sequestra with increase in the time interval between the induction of the lesion and histological examination was observed. In particular, no sequestra were observed in subgroup A1, but sequestra were observed in 2 of the 10 tibias from subgroup A2, 4 of the 12 tibias from subgroup A3, and 12 of the 30 tibias from subgroup A4. Osteoclasts were sometimes seen in areas of resorption on the surface of the sequestra.

Hematopoietic marrow was replaced by polymorphonuclear cells, fibroblasts, and macrophages. The experimental lesions were confined to the tibial metaphysis and only rarely extended distally, without ever completely involving the diaphysis. The epiphysis was always intact because of the protective effect of the growth cartilage.

In the early stages of infection, the metadiaphyseal cortex was partially destroyed, creating a passage between the bone marrow and the abscesses. Six months after the operation, new bone formation was observed and the cortex was repaired in a deformed fashion.

Microbiological analysis. Bacterial counts were performed for the 61 tibias from group A and the 8 tibias from group B.

The results for group A are shown in Table 4.

One tibia from group B had a positive culture with a bacterial count of 5 x 10^5 CFU/ml. The strain isolated was identified by use of the Sceptor system as S. epidermidis. In an MIC assay with the same drugs as those tested for the S. aureus strain used in our model, the isolated strain showed a different sensitivity pattern. Thus, the positive culture for a tibia from group B was probably due to contamination during surgery.

**DISCUSSION**

This study was aimed at developing a rat model of chronic staphylococcal osteomyelitis with pathophysiological, clinical, radiographic, and histological characteristics similar to those of human disease. We chose the rat because purchase and housing costs are low and this animal tolerates surgical trauma and long-term high-dose antibiotic therapy. Moreover, the rat tibia is sufficiently small to allow pulverization for bacterial counts, and inbred strains can be used for
experimentally studying the immunological aspects of the pathogenesis of osteomyelitis. The main disadvantage of the rat model is the size of the animal, which considerably limits the possibility of experimenting with complex surgical techniques.

A few rat (24, 25, 31), rabbit (1, 17-19, 22, 26, 27, 30), and canine (11, 13) models of osteomyelitis used sclerosing agents or foreign bodies. Sclerosing agents (e.g., sodium morrhuate or barium sulfate) probably cause alterations of the marrow circulation, according to some authors (10). Intramedullary foreign bodies (e.g., silicone catheters, intramedullary nails, and polyvinyl alcohol sponges), alone or in combination with sclerosing agents, could constitute an inert substratum to which S. aureus adheres tenaciously and produces the extracellular hexopolysaccharide matrix called the glycocalyx (18, 19).

In this model, we used fibrin glue instead of sclerosing agents or foreign bodies. Fibrin glue is a biocompatible material which does not cause aseptic bone necrosis and does not constitute an inert substratum, because of its resorption in a few days. S. aureus could bind to this molecule through a receptor-like structure, so fibrin glue could constitute an optimal medium for bacterial growth in loco, thus trapping bacteria and preventing sepsis. The adhesive and hemostatic properties of fibrin glue justify its wide use in surgery (29). Furthermore, some authors have attributed to fibrin glue the property of stimulating and accelerating repair osteogenesis via a mechanism of the osteoconductive type (2, 3, 4, 5, 6, 16).

Radiographic, macroscopic, microscopic, and microbiological analyses were carried out on the tibias removed after the animals had been killed. Since it has been demonstrated (25) that there is no relationship between clinical and blood chemistry parameters and the progression of experimental osteomyelitic lesions, no clinical or laboratory assessments were done for the live animals.

An analysis of the results permits us to suggest that this model of chronic osteomyelitis could correspond to the clinical and biological aspects of human disease.

The kind and the development of radiographic alterations of the tibias from group A were similar to those observed in human disease. Alterations of the bone structure were more frequent in the early stages of disease, and osteolytic areas of increasing dimensions were observed, depending on the progression of the lesions. The osteolytic lesions were surrounded by sclerotic borders, which were thicker in subgroup A4. Sequestra were detected less frequently radiographically than by histological examination because of the limited resolution power of roentgenograms. In fact, in human osteomyelitis, radiographic images very often do not reveal sequestra, which are clearly identified by computerized tomography.

Gross bone pathology examination in the early stages of disease revealed extensive destructive lesions involving the soft tissues, with draining skin fistulas. In later stages, new bone formation replaced the destructive process, analogous to that observed in human osteomyelitis.

Histological examination revealed osteomyelitis in more than 90% of cases, demonstrating the reliability of the model. Whereas in the first months following induction of the lesions large abscesses associated with osseous necrosis were prevalent, 6 months after surgery, osteogenetic processes predominated. New bone formation often took place along abnormal load-bearing lines and resulted in deformation of the bone. Osteomyelitis became chronic because the sequestra could be neither resorbed, in that they were

FIG. 2. (A) Tibia from subgroup A2. Note the voluminous abscess in the soft tissues which communicated with the bone by way of a fistula. (B) Longitudinal section of a tibia from subgroup A2 showing the central area of the abscess. Note polymorphonuclear cells and fibrinoid necrosis. Magnification, X336. (C) Longitudinal section of a tibia from subgroup A2 showing the detail of the central area of the abscess, containing polymorphonuclear cells. Magnification, X840.
isolated from the vital connective tissue, nor expelled. Sequestra were detected in only a few cases, most likely because many of them had been missed in the preparation of histological sections.

The lesions were confined to the tibial metaphysis and only rarely extended distally, without ever completely involving the diaphysis.

Osteoclasts were always observed in fragments of infected necrotic bone, contradicting the thesis of Collins, according to whom osteoclasts are a cell population rarely found in the course of osteomyelitis (9). It has been hypothesized on the basis of in vitro studies that in infective bone disease (and probably also in physiological conditions) there could be mechanisms of bone resorption mediated by polymorphonuclear cells and monocytes (14, 15, 20, 21). However, it is known that bone marrow monocytes can differentiate into osteoclasts. At present there is no clear evidence that cells other than osteoclasts can resorb either normal or pathological bone.

Microbiological analysis, showing a high percentage of positive cultures (86%), also confirms the reliability of the model.

The chronic osteomyelitis described in this study is localized and can be classified as type 3A, according to the classification of Cierny et al. (7, 8). Given the methods of induction of the disease, our model can be considered posttraumatic chronic osteomyelitis, according to the classification of Waldvogel and coworkers (28). Because of the high level of positivity of induced osteomyelitic lesions and the probable analogy with human disease, we believe that this model could be reliably used in pathogenetic and therapeutic studies.

### REFERENCES


### TABLE 4. Microbiological analysis of group A tibias

<table>
<thead>
<tr>
<th>Group or subgroup (total no. of tibias)</th>
<th>CFU (10⁶/ml)</th>
<th>No. of tibias with the following culture result for S. aureus (% of total)</th>
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<tbody>
<tr>
<td></td>
<td>(mean ± SD)</td>
<td>Positive</td>
</tr>
<tr>
<td>A (61)</td>
<td>Not determined</td>
<td>52 (85.2)</td>
</tr>
<tr>
<td>A1 (9)</td>
<td>2.95 ± 3.16</td>
<td>8 (88.9)</td>
</tr>
<tr>
<td>A2 (10)</td>
<td>1.78 ± 2.90</td>
<td>10 (100)</td>
</tr>
<tr>
<td>A3 (12)</td>
<td>2.63 ± 3.15</td>
<td>10 (83.3)</td>
</tr>
<tr>
<td>A4 (30)</td>
<td>0.77 ± 1.34</td>
<td>24 (80.0)</td>
</tr>
</tbody>
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*FIG. 3. (A) Longitudinal section of a tibia from subgroup A3 showing the detail of an abscess. Note the exuberant fibrinoid necrosis containing phlogistic cells. Magnification, X1,000. (B) Longitudinal section of a tibia from subgroup A4. Note the voluminous sequestrum surrounded by polymorphonuclear cells. The infected necrotic bone has large lacunas filled by purulent exudate. Magnification, X200.*
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