Staphylococcal Endocarditis in Rabbits Treated with a Low Dose of Cloxacillin

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Rabbits with established staphylococcal endocarditis, injected twice at an interval of 2 h with either 0.5 mg of cloxacillin per kg or saline, were sacrificed 2.5 h after the second injection. Vegetations were excised, weighed, and cultured, and ultrathin sections were prepared and examined by light microscopy, transmission electron microscopy, and scanning electron microscopy. Several affected valves were examined histologically. Concentrations of cloxacillin in serum were determined 1 and 3 h after dosage. Staphylococci grown on membranes placed on agar containing 0.09 µg of cloxacillin per ml and in broth at the same cloxacillin concentration (one-third of the MIC) were examined by transmission electron microscopy. The mean numbers of CFU per gram of vegetations from control and treated rabbits were 2.28 × 10¹⁰ and 1.31 × 10¹⁰, respectively. Vegetations of treated rabbits contained staphylococci of normal size and form as well as organisms two to six times larger than normal with multiple cross walls. Larger bacterial cells were usually located in areas close to blood; cells of normal size were usually embedded in fibrin. The structures of these staphylococci and those grown on membranes in the presence of 0.09 µg of cloxacillin per ml were comparable but were different from those grown in broth containing this concentration of cloxacillin. Concentrations of cloxacillin in serum were 0.166 µg/ml at 1 h and 0.286 µg/ml at 3 h after dosage. The similarities in ultrastructure between staphylococci in vegetations of treated rabbits and staphylococci grown on membranes suggest that the vegetables contained approximately 0.09 µg of cloxacillin per g. Thus, antibiotic penetration from blood into vegetations and diffusion into fibrin were limited.

MATERIALS AND METHODS

Test organism. The Fitz strain of Staphylococcus aureus (CIBA-GEIGY AG, Basel, Switzerland), which has been shown in this laboratory to produce endocarditis in rabbits, was grown overnight in Mueller-Hinton broth (BBL Microbiology Systems, Cockeysville, Md.). A 1:500 dilution in saline (ca. 5 × 10⁶ CFU/ml) served as inoculum.

In vitro, membrane, and broth tests. The MIC of cloxacillin for the Fitz strain was 0.25 µg/ml as determined by a routine agar dilution technique (6). A 1:10 dilution of the overnight broth culture was prepared in Mueller-Hinton broth, and 0.1 ml was spread on a filter membrane (PHWP47005, 0.3-µm pore size; Millipore Corp., Bedford, Mass.) which was placed on agar containing 0.09 µg of cloxacillin per ml (one-third of the MIC) and incubated for 4 h at 37°C (8). A 1-ml portion of the 1:10 dilution was added to 9 ml of Mueller-Hinton broth containing 0.1 µg of cloxacillin per ml (final concentration, 0.09 µg/ml) and incubated for 4 h; the broth was then centrifuged at 900 × g for 30 min, and the supernatant was removed. Both the organisms on the membrane and those in the sediment were immersed in fixative and processed for electron microscopy (16).

Production of bacterial endocarditis. Endocarditis was produced in male chinchilla rabbits (2.5 to 2.9 kg) by using the modification by Sande and Irvin (15) of the catheter technique of Garrison and Freedman (4). One milliliter of the saline suspension of the staphylococci was injected intravenously 3 h after placement of the catheters. Animals were anesthetized and catheters were removed 48 h after inoculation. Rectal temperatures were measured twice daily.

Administration of antibiotics. In a preliminary experiment involving 14 rabbits, doses of 0.1, 0.3, 0.5, 0.7, 1, or 10 mg of cloxacillin (Beecham Laboratories, Bristol, Tenn.) per kg were administered subcutaneously 24 and 26 h after removal of the catheter. Animals were sacrificed 2 or 4.5 h after the first dose. Although there were large staphylococci on Gram stains of vegetations obtained at 2 h, the largest proportion of large cells was found in specimens taken 2.5 h after the second injection. These preliminary data led to an experiment on 45 rabbits with endocarditis, of which 30 received 0.5 mg of cloxacillin per kg in each of 2 doses, and 15 received saline only. This number of rabbits provided a reserve in case of manipulation accidents.

Measurement of cloxacillin in serum. Blood samples were collected from five treated and five control rabbits 60 to 70 min after the first injection, 60 to 70 min after the second injection, and before autopsy (2.5 h after the second injection). Cloxacillin concentrations in serum were measured by high-pressure liquid chromatography according to the method of Rudrik and Bawdon (14) with the following modifications. The wavelength of the detector was 229 nm, and the mobile phase for the assay was 25% acetonitrile, 15% 0.20 M...
FIG. 1. Ultrathin section of a vegetation from a rabbit with endocarditis treated with cloxacillin. (A) Large staphylococci (L) are situated close to areas showing erythrocytes. The normal-sized staphylococci (S) are located toward the center of the vegetation. Magnification, x300. (B) Detail with large cells. Magnification, x1,500.

FIG. 2. Staphylococci in vegetations from a rabbit treated with cloxacillin (A) and from a control rabbit (B). Magnification, x44,000. Bar = 1 μm. Approximate volumes of staphylococci are 4.51 μm³ (A) and 0.66 μm³ (B). (A is 6.75 times larger than control B).
ammonium acetate, and 60% water. The flow rate of the pump was 2.4 ml/min. The column was injected with 50 μl, and the detector was set at 0.005 absorbance units. Cloxacillin at 0.5 μg in serum gave a peak height of 7.6 cm. All data were calculated using least-square linear regression analysis. The correlation coefficient for the spiked standard curve was 0.99898. The slope was 14.88, and the intercept was 0.116.

Postmortem examinations. At 2.5 h after the last injection of cloxacillin, 22 treated and 10 control rabbits were sacrificed. The vegetations of 15 rabbits (12 treated and 3 controls) were excised, fixed, and processed for transmission electron microscopy (16) and scanning electron microscopy. Six ultrathin sections were prepared from the vegetations of each rabbit, stained with toluidine blue, and examined by light microscopy. The vegetations from four rabbits were selected for transmission electron microscopy, and those from two rabbits were selected for scanning electron microscopy. The entire valve from three treated rabbits with the vegetations was excised and fixed in 10% neutral Formalin. Paraffin sections were stained by hematoxylin-eosin and by Gram stain and examined histologically.

Number of bacteria. The vegetations of seven treated rabbits and seven controls were excised, weighed, homogenized in a tissue grinder, diluted in water, and planted on agar for the determination of the number of CFU.

RESULTS

In vitro. The staphylococci grown on membranes in the presence of 0.09 μg of cloxacillin per ml were 1.6 to 2.5 μm in diameter and contained multiple, thick cross walls; these cells were comparable to the large staphylococci grown on membranes in the presence of other beta-lactam antibiotics as described previously (9). The staphylococci grown in broth containing 0.09 μg of cloxacillin per ml were 1.2 to 1.6 μm in diameter. Most contained one thick cross wall, and a few contained two cross walls; these cells were comparable to staphylococci grown in broth containing oxacillin or penicillin (10, 13).

In vivo. On day 3 after inoculation, all rabbits had temperatures ranging from 39.7 to 40.4°C, with the majority between 40 and 40.2°C. The mean cloxacillin concentrations in serum were 0.166 μg/ml (standard deviation [SD], 0.059) 60 to 70 min after the first injection, 0.286 μg/ml (SD, 0.08) 60 to 70 min after the second injection, and 0.034 μg/ml (SD, 0.015) 2.5 h after the second injection.

All except two ultrathin sections of vegetations obtained from the treated rabbits (two injections of 0.5 mg/kg) contained staphylococci two to three times larger than normal (Fig. 1). These large staphylococci were found mostly in clusters ranging in size and number from a single area of ca. 10 μm² (20 to 25 organisms) to multiple areas covering one-fourth of the ultrathin section. The clusters of large staphylococci were usually located close to the lumenal margin of the vegetation. Most of the staphylococci embedded deeply in the fibrin were of normal size. As shown in the scanning electron micrograph (Fig. 2), large organisms were characterized by an irregular surface and a size four to six times that of organisms obtained from the saline-treated controls. As shown by transmission electron microscopy (Fig. 3), the large cells were 1.4 to 2.8 μm in diameter and contained multiple cross walls and a relatively normal peripheral cell wall. Histological preparations of vegetations from treated animals showed both large and normal-sized staphylococci. The large staphylococci were in microcolonies adjacent to the heart cavity, and staphylococci of normal size were predominantly embedded in fibrin (Fig. 4). Leukocytes were not observed on thin sections or on histological preparations.

The mean weights of vegetations from treated and control rabbits were similar: 0.140 (SD, 0.054)g and 0.146 (SD, 0.068)g, respectively. The mean numbers of CFU per gram of vegetation were also similar: 2.20 × 10¹⁰ (SD, 2.32) in control rabbits and 1.32 × 10¹⁰ (SD, 0.84) in treated rabbits (P > 0.1).

DISCUSSION

The number and sizes of clusters of large staphylococci produced by cloxacillin varied considerably from animal to animal. This is probably the result of differences in the rate of fibrin deposition in diverse vegetations (2). In vitro studies employing artificially produced fibrin clots exposed to penicillin (12) indicate that peak penicillin concentrations in the clots are significantly lower than concentrations in plasma. In most cases, the groups of large cells were located close to blood, and normal-sized organisms, not affected by the antibiotic, were located within the fibrin. This indicates that the antibiotic reaches the vegetation through the blood and diffuses into the vegetation. Since large staphylococci were produced on membranes by 0.09 μg of cloxacillin per ml, the presence of these bacterial forms in vegetations indicates an antibiotic concentration close to 0.09 μg/g. Since peak cloxacillin concentrations in serum 1 and 3 h after treatment were 0.166 μg/ml and 0.286 μg/ml, respectively, a cloxacillin concentration close to 0.1 μg/ml in a vegetation area adjac-
cent to blood is an indication of limited diffusion. That diffusion of cloxacillin deep into the vegetation is limited is also suggested by the presence of normal-sized staphylococci and the absence of large staphylococci within the fibrin clot. Measuring antibiotic concentrations in various areas of a vegetation has not been possible yet. With the aid of a computer and a mathematical model, however, the antibiotic levels near the center of vegetations with diameters of 0.5, 1.0, and 2.0 cm have been calculated to be 37, 22, and 18%, respectively, of the free concentration in serum (3). Polymorphonuclear cells were not observed in any ultrathin or histological section. This is consistent with previous observations of experimental endocarditis in rabbits (1).

The ultrastructure of the large staphylococci observed in vegetations is comparable to the ultrastructure of staphylococci grown on membranes in the presence of cloxacillin or other beta-lactam antibiotics but different from that of staphylococci grown in broth. It appears, therefore, that staphylococci exposed in vivo and on a membrane to a low concentration of a beta-lactam antibiotic assume a comparable ultrastructure.

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LITERATURE CITED


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