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In Vivo Assessment of the Antimicrobial Activity of a Calcium-Deficient Apatite Vancomycin Drug Delivery System in a Methicillin-Resistant *Staphylococcus aureus* Rabbit Osteomyelitis Experimental Model[▽]

G. Amador,[1,2] H. Gautier,[3] V. Le Mabecque,[2] A. F. Miegeville,[2] G. Potel,[1,2] J.-M. Bouler,[3] P. Weiss,[3] J. Caillon,[1,2]* and C. Jacqueline[2]

CHU de Nantes,[1] Université de Nantes,[2] Faculté de Médecine, UPRES EA3826, UFR de Médecine, 1 Rue Gaston Veil, Nantes 44035, France, and INSERM UMRS 791, Laboratory of Osteoarticular and Dental Tissue Engineering 1, Place Alexis Ricordeau, Nantes 44042, France[3]

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The antimicrobial activities of calcium-deficient apatite loaded with different concentrations (25, 100, and 500 μg/mg) of vancomycin as a filling biomaterial were evaluated in a methicillin-resistant *Staphylococcus aureus* (MRSA) rabbit acute osteomyelitis model. Bacterial counts in bone, bone marrow, and joint fluid samples treated with forms of the apatite were compared to those in tissue samples receiving a constant intravenous vancomycin infusion after 4 days. This study demonstrates that using a calcium-deficient apatite loaded with vancomycin dramatically decreases the bacterial counts in bone and marrow.

Calcium-deficient apatites (CDA) are mineral groups lacking calcium but similar in structure and function to those biological components found in bone and other calcified tissues. These synthetic matrices have osteoconductive properties and generate processes of resorption-substitution, which serve to quicken the healing process (2). CDA, as a filling biomaterial, can be loaded with an antimicrobial drug, vancomycin, to form a drug delivery system used in osteomyelitis treatment (8). Osteomyelitis is characterized by severe bone loss, secondary to an acute or chronic inflammatory response caused by a bacterial invasion (1, 5). Bone infections sometimes require surgical debridement, followed by extensive antibiotic treatment (7, 12). *Staphylococcus aureus* is the most common form of bacteria found in osteomyelitis patients and, as such, has become increasingly resistant to the often prescribed antibiotic treatment, consisting of antistaphylococcal penicillins (6, 9, 10). The options for treatment of bone infections due to methicillin-resistant *S. aureus* (MRSA) are limited by pharmacokinetic factors (such as penetration into bone tissues). At least 6 weeks of parenteral therapy is usually needed to reach efficient concentrations *in situ* (3). Among the glycopeptides, vancomycin is considered only as a reference molecule but is restricted to intravenous (i.v.) administration (4, 11). Bolus injection is not possible, resulting in the use of prolonged infusion over at least an hour or, more recently, continuous infusion. If continuous infusion is used, a serum steady-state concentration of approximately 20 to 25 times the MIC should be targeted. Studies show that these values are often required in severe MRSA infections (11). The aim of this work was to assess the *in vivo* activity of CDA loaded with vancomycin versus that of constant infusion of vancomycin (VIV) in an acute osteomyelitis model.

* Corresponding author. Mailing address: EA 3826, Faculte de Médecine, 1 Rue Gaston Veil, 44035 Nantes, France. Phone and fax: (33) 240-412-854. E-mail: jocelyne.caillon@univ-nantes.fr.

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Female New Zealand White rabbits (weight, 2.0 to 2.5 kg) were anesthetized with intramuscular ketamine and xylazine, and experimental osteomyelitis was established in the distal right femur. A Jamshidi bone marrow biopsy needle was inserted between the two femoral condoyles through epiphysis, physis, and metaphysis to reach the medullary canal. The *Staphylococcus aureus* strain used in this study was a MRSA strain isolated from a blood culture with a MIC of 1 μg/ml for vancomycin. One milliliter of a 10^9-CFU/ml bacterial suspension was injected into the knee cavity. Animals were randomly assigned to nine different treatment groups: the vancomycin group (VIV) (constant i.v. vancomycin infusion to reach a 20× MIC serum steady-state concentration), CDA (CDA alone), CDA+V (unloaded CDA in combination with constant vancomycin infusion), CDA25 (CDA loaded with 25 μg/mg of vancomycin), CDA25+V (CDA loaded with 25 μg/mg in combination with constant vancomycin infusion), CDA100 (CDA loaded with 100 μg/mg of vancomycin), CDA100+V (CDA loaded with 100 μg/mg of vancomycin in combination with constant vancomycin infusion), CDA500 (CDA loaded with 500 μg/mg of vancomycin), and CDA500+V (CDA loaded with 500 μg/mg of vancomycin in combination with constant vancomycin infusion). Loaded CDA were made to release 80% of the total vancomycin introduced by wet granulation over the first 4 days. The release was monitored and verified through high-performance liquid chromatography (HPLC) assays. On day 3, joint fluid (JF), femoral bone marrow (BM), and epiphyseal bone (BO) samples were obtained, placed immediately on ice, weighed, homogenized in 0.5 ml of saline buffer, and plated on Trypticase soy agar and Chapman plates by using a spiral system (Interscience, St. Nom La Breteche, France). Afterward, a surgical debridement of the infected tissues and a rinse with sterile saline solution were performed. Aliquots of 100 mg of CDA were introduced in the femoral osseous gap for the CDA, CDA+V, CDA25+V, CDA25, CDA100, CDA100+V, CDA500, and CDA500+V groups. Constant infusion of vancomycin started on day 3 and continued for 4

TABLE 1. Differences between day 7 and day 3 bacterial counts in joint fluid (JF), bone marrow (BM), and bone (BO) and relationship to mortality rates

Treatment	No. of animals	Mean ± SD $\Delta\log_{10}$ no. of CFU/g of tissue[a]			Mortality rate (%)
		JF	BM	BO	
VIV	6	−0.03 ± 0.93	−0.73 ± 1.84	−0.61 ± 0.76	16.6
CDA	9	−0.49 ± 0.32	0.56 ± 0.31	0.22 ± 0.25	44.4
CDA+V	6	0.30 ± 0.43	0.75 ± 0.35	−0.35 ± 0.9	28.6
CDA25	7	−0.56 ± 0.95	−1.89 ± 1.29	−1.97 ± 1.05	0
CDA25+V	5	0.68 ± 1.32	−3.14 ± 0.86*	−2.40 ± 1.14†	0
CDA100	9	−0.05 ± 1.03	−3.41 ± 1.43	−2.35 ± 1.45†	0
CDA100+V	7	−0.90 ± 0.39	−4.03 ± 1.33*	−4.63 ± 1.28*§	0
CDA500	7	−0.29 ± 1.16	−4.08 ± 1.27†	−3.57 ± 1.32*	12.5
CDA500+V	11	−1.69 ± 0.62‡	−4.21 ± 1.16*	−2.87 ± 1.13†	20

[a] Results shown are differences between day 7 and day 3 counts. *, $P < 0.01$ for comparison with VIV, CDA, and CDA+V; †, $P < 0.05$ for comparison with VIV, CDA, and CDA+V; ‡, $P < 0.05$ for comparison with VIV; §, $P < 0.05$ for comparison with CDA25+V, CDA100, CDA500+V, and CDA500.

days for the VIV, CDA+V, CDA25+V, CDA100+V, and CDA500+V groups. On day 7, animals were euthanized by a lethal intravenous bolus of thiopental, and joint fluid, bone marrow, and bone samples were collected. Dilutions (10^{-2} and 10^{-4}) of the samples were made to eliminate potential carryover effects. Surviving bacteria in JF, BM, and BO samples were counted after a 48-h aerobic incubation at 37°C. The counts were normalized in the $\log_{10}$/g form and compared to day 3 data. Results were expressed as the difference between $\log_{10}$/g day 3 and $\log_{10}$/g day 7 bacterial counts. A Student-Newman-Keuls test after a one-way analysis of variance (ANOVA) was performed (GraphPad Prism software), and differences were deemed to be statistically significant if P was ≤ 0.05.

In vivo bacterial counts are summarized in Table 1. Constant vancomycin infusion (VIV) did not demonstrate significant antibacterial activity in any of the three compartments (JF, BM, and BO) tested over the 4-day treatment. Unloaded CDA showed no intrinsic activity or inhibition of the development of osteomyelitis, even in combination with a constant infusion of vancomycin. No difference was found with the reference group. CDA loaded with 25 μg/mg of vancomycin (CDA25) without combination with systemic vancomycin infusion did not show sufficient activity. However, CDA loaded with 25 μg/mg of vancomycin in combination with constant infusion was active in bone and bone marrow samples, with −2.40 $\log_{10}$ and −3.14 $\log_{10}$, respectively ($P < 0.05$). CDA loaded with an increased concentration of vancomycin (100 μg/mg) without systemic infusion showed significant antimicrobial activity in bone and marrow but not in joint fluid samples. The addition of a constant infusion of vancomycin to the CDA100 group showed the best results in bone (−4.63 $\log_{10}$) and bone marrow (−4.03 $\log_{10}$) samples. CDA loaded with 50% vancomycin showed results similar to those observed for CDA100 in bone marrow samples but not in bone samples, even with the addition of constant infusion. The bacterial counts in the joint fluid showed a linear response with loading, with the exception of the CDA500+V group. The impact of the constant infusion on the bacterial counts is summarized in Fig. 1.

The acute osteomyelitis model provides a rapid assessment of antibiotic activity in comparison with that obtained with the chronic model (12). This study showed that constant intravascular vancomycin infusion was not able to decrease bacterial counts after a 4-day treatment. Unloaded CDA alone or in concert with vancomycin infusion demonstrated no antimicrobiological activity. Low concentrations of antibiotic (25 μg/mg) loaded into the CDA were deemed efficient at treating the bone and marrow when used in combination with systemic infusion. Fifty micrograms per milligram of vancomycin incorporated via wet granulation was the maximum level usable in clinical practice, with the CDA matrix showing limits for drug incorporation. This was determined as the highest level of loading that did not compromise the osteoconductive properties of this artificial mineral compound. Nevertheless, the greatest reductions in bacterial counts *in situ* in bone and bone marrow samples were obtained with lower concentrations (CDA100) combined with constant infusion of vancomycin. Increased concentration of incorporated vancomycin (500 μg/mg) did not show significant enhancement. The mortality rate, as an indirect indicator of the efficiency of the treatment, confirms these data. Bone infections are very difficult to treat, and localized treatments in addition to systemic approaches are becoming more relevant as a means to limit the bacterial invasion and improve the prognosis of osteomyelitis. CDA loaded with 100 μg/mg of vancomycin may be the most effective antibiotic concentration for avoiding the adverse effects and toxicity of vancomycin.

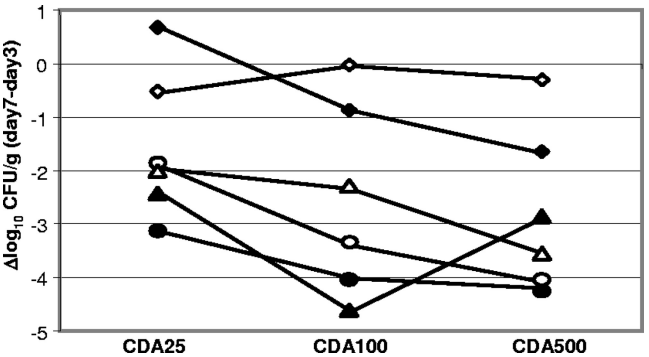

FIG. 1. Effect of constant vancomycin infusion (black forms) on bacterial counts in joint fluid (diamonds), bone marrow (circles), and bone (triangles) samples for CDA25, CDA100, and CDA500.

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