Polycationic Peptides as Prophylactic Agents against Methicillin-Susceptible or Methicillin-Resistant Staphylococcus epidermidis Vascular Graft Infection

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Several polycationic peptides isolated from animals, plants, and bacterial species possess a broad spectrum of antimicrobial activity. A rat model was used to investigate the efficacies of two peptides, ranalexin and buforin II, in the prevention of vascular prosthetic graft infections. The effect of peptide-soaked collagen-sealed Dacron was compared to that of rifampin-soaked collagen-sealed Dacron in the rat model of graft infection caused by methicillin-susceptible rifampin-susceptible Staphylococcus epidermidis and methicillin-resistant rifampin-susceptible S. epidermidis. Graft infections were established in the back subcutaneous tissue of 240 adult male Wistar rats by implantation of 1-cm² Dacron prostheses, followed by topical inoculation with 2 × 10⁷ CFU of S. epidermidis. The study included a control group (no graft contamination), two contaminated groups that did not receive any antibiotic prophylaxis, two contaminated groups to which perioperative intraperitoneal cefazolin prophylaxis (30 mg/kg of body weight) was administered, six contaminated groups that received a peptide- or rifampin-soaked graft, and six contaminated groups that received a peptide- or rifampin-soaked graft and perioperative intraperitoneal cefazolin prophylaxis (30 mg/kg). The grafts were sterilized 7 days after implantation, and the infection was evaluated by using sonication and quantitative agar culture. Overall, the efficacies of the polycationic peptides against the methicillin-susceptible and methicillin-resistant strains were not significantly different from that of rifampin. Nevertheless, the combinations of ranalexin- and buforin II-coated grafts with cefazolin treatment demonstrated efficacies significantly higher than that of the combination of rifampin-coated grafts and cefazolin treatment against the methicillin-resistant strain.

Vascular prosthetic graft infection is one of the most feared complications that the vascular surgeon treats, frequently resulting in prolonged hospitalization, organ failure, amputation, and death. The patient can develop late-appearing signs of infection as commonly as early postoperative infection (1, 3). Coagulase-negative staphylococci are among the most common pathogens that cause biomaterial infections. In particular, Staphylococcus epidermidis, a commensal organism of the skin, is the most frequent cause of late-appearing vascular graft infection in humans (1–3, 21). Effective strategies for the prevention of prosthetic infection vary from device to device. The centerpiece of therapy is prophylactic systemic antibiotics (3, 7). In addition, in the case of vascular grafts, antimicrobials, such as rifampin, bound at high concentrations to prosthetic grafts have been proposed as adjunctive prophylaxis (6, 9, 15, 19, 20, 22, 24, 26). In recent years several polycationic peptides, compounds that comprise a diverse class of molecules, have been isolated from a wide range of bacteria, plants, insects, fish, amphibians, birds, mammals, and humans (4, 5, 11). In mammals, including humans, they are the predominant protein species in the neutrophil, and they are also found on the surfaces of the tongue, trachea, lungs, and upper intestine and are thought to be a major antibacterial defense on mucosal surfaces (11, 12). Recent reports have demonstrated that the site for the antibacterial action of the peptides is the cytoplasmic membrane, where they cause the formation of ion-channel pores that span the membrane without requiring a specific target receptor. Therefore, these compounds must initially be able to cross or disintegrate the outer membranes of gram-negative bacteria and the peptidoglycan (10, 11, 12, 25). The essential property of the polycationic peptides is their net positive charge at neutral pH (usually +4, +5, or +6) by virtue of their possession of the amino acids arginine and lysine (11). In addition, these compounds are amphipathic molecules: they have both a hydrophobic face, comprising nonpolar amino acid side chains, and a hydrophilic face of polar and positively charged residues (11, 12). The selective antibiotic activity of the cationic peptides is determined by their mode of interaction with the bacterial surface: typically, their positively charged residues interact with the negatively charged lipids of the bacterial membranes. On the other hand, the low anionic lipid content of the eukaryotic cells leads to the selectivity of the activity of the peptides for bacteria (10, 11, 12). The surfaces of several synthetic materials used by microbiologists and surgeons, such as polystyrene, polyethylene terephthalate (Dacron), and polytetrafluoroethylene, bind cationic molecules, and this property has been evaluated and used during in vitro and in vivo studies (13, 17; R. E. W. Hancock, Laboratory methods, 1998 [http:...
Materials and Methods

Organisms. A commercially available methicillin-susceptible (MS) quality control strain of S. epidermidis, strain ATCC 12228, and one clinical isolate of methicillin-resistant (MR) S. epidermidis (Se56-99) were used.

Drugs. Bupafilon II, ranalexin, rifampin, ceftazolin, and oxacillin were obtained from Sigma-Aldrich S.r.l. (Milan, Italy). Bupafilon II and ranalexin were dissolved in distilled H₂O at 20 times the required maximal concentration. For in vitro studies, serial dilutions of the peptides were prepared in 0.1% acetic acid containing 0.2% bovine serum albumin in polypropylene tubes (Hancock, Laboratory methods). Rifampin was dissolved in 50% methanol-50% acetone at a concentration of 1 mg/ml. Cefazolin and oxacillin were dissolved in sterile distilled water at a concentration of 1 mg/ml. Solutions were made fresh on the day of assay or were stored at −80°C in the dark for short periods. The concentration range tested for each antibiotic was 0.25 to 256 μg/ml.

Susceptibility Testing. The antimicrobial susceptibilities of the strains were determined by using the broth microdilution method by the procedures outlined by the National Committee for Clinical Laboratory Standards (14). The MIC was taken as the lowest antibiotic concentration at which observable growth was inhibited. However, the MICs of bupafilon II and ranalexin were determined by the procedures recently proposed for the testing of antimicrobial peptides (Hancock, Laboratory methods). Particularly, since cationic peptides bind to polystyrene, 96-well polystyrene plates (polystyrene plates) were used for all assays. The plates and the plates were incubated for 18 h at 37°C in air. The plates were shaken throughout the study. The MIC was considered the lowest peptide concentration that reduced growth by more than 50% of that in the control well. The viable count in each well was determined by preparing 10⁻² dilutions and plating 10 μl of each dilution onto Mueller-Hinton agar plates to obtain overnight cultures. Experiments were performed in triplicate.

Rat Model. Adult male Wistar rats (weight range, 300 to 350 g) were studied. The animals were divided into the control group (no graft contamination) and two groups composed of eight groups (groups MS1 to MS8 and MR1 to MR8) for each of the staphylococcal strains. Each of the series included one contaminated group (groups MS1 and MR1) that received intraperitoneally isotonic sodium chloride solution (0.9% w/v) and a group (groups MS2 and MR2) to which a viable intraperitoneal ceftazolin prophylaxis (30 mg/kg of body weight), was administered, three contaminated groups (groups MS3 to MS5 and MR3 to MR5) that received a bupafilon II-, a ranalexin-, or a rifampin-soaked graft, respectively, and three contaminated groups (groups MS6 to MS8 and MR6 to MR8) that received a bupafilon II-, a ranalexin-, or a rifampin-soaked graft, respectively, and perioperative intraperitoneal ceftazolin prophylaxis (30 mg/kg). Each group consisted of 15 animals. The rats were anesthetized with ether, the hair of the inbacks was shaved, and the skin was cleansed with 10% povidone–iodine solution. One subcutaneous pocket was made on each side of the median line with 1.5-cm incision. Aseptically, 1-cm² sterile collagen-sealed Dacron grafts (Albograft; Sorin Biomedica Cardio, S.p.A., Saluggia VC, Italy) were implanted into the pockets. Prior to implantation, the Dacron graft segments were impregnated with 10 μg of bupafilon II per ml (groups MS2, MS5, MR2, and MR5), 10 μg of ranalexin per ml (groups MS3, MS6, MR3, and MR6), and 5 μg of rifampin per ml (groups MS4, MS7, MR4, and MR7). Antibiotic soaking was done immediately before implantation by placing the grafts for 20 min in a sterile solution of the agents mentioned above. Groups MS1, MS8, MR1, and MR8 received nonantibiotic-impregnated Dacron graft segments. In addition, the effect of preoperative intraperitoneal ceftazolin administered 30 min before implantation at the standard dose of 30 mg/kg was evaluated in groups MS5 to MS8 and MR5 to MR8. The pockets were closed by means of skin clips, and sterile saline solution (1 ml) containing S. epidermidis ATCC 12228 or the MR strain S. epidermidis Se56-99 at a concentration of 2 × 10⁶ CFU/ml was inoculated onto the graft surface by using a tuberculin syringe to create a subcutaneous fluid-filled pocket (2). The animals were returned to individual cages and were thoroughly examined daily. All grafts were explanted 7 days following implantation.

Assessment of Infection. The explanted grafts were placed in sterile tubes, washed with sterile saline solution, placed in tubes containing 10 ml of phosphate-buffered saline solution, and sonicated for 5 min to remove the adherent bacteria from the grafts. Quantitation of viable bacteria was done by preparing serial dilutions (0.1 ml) of the bacterial suspensions in 10 mM sodium HEPES buffer (pH 7.2) (Sigma-Aldrich) to minimize the carryover effect and by culturing each dilution on blood agar plates. All plates were incubated at 37°C for 48 h and were evaluated to determine the presence of colonies. All organisms were quantitated by counting the numbers of CFU per plate. The limit of detection for this method was approximately 5 × 10⁴ CFU/cm² of graft tissue.

Statistical Analysis. MICs are presented as the geometric means of three separate experiments. Quantitative culture results for all groups are presented as the mean ± standard deviation, and the statistical comparisons between groups were done by analysis of variance of the log-transformed data by the Tukey-Kramer honestly significant difference test. Significance was accepted when the P value was ≤0.05.

Results

According to the broth microdilution method, the bupafilon II and ranalexin MICs for S. epidermidis ATCC 12228 and S. epidermidis Se56-99 were 2 and 4 mg/liter and 2 and 8 mg/liter, respectively. The two strains were similarly susceptible to rifampin (MICs, 0.25 mg/liter for both organisms), while they demonstrated different patterns of susceptibility to the beta-lactam antibiotics. Actually, S. epidermidis ATCC 12228 was susceptible to oxacillin and ceftazolin (MICs, 0.5 and 2 mg/liter, respectively), while S. epidermidis Se56-99 was resistant (MICs, 8 and 32 mg/liter, respectively).

None of the animals included in the control group (no graft contamination) had anatomic or microbiologic evidence of infection. On the contrary, all 30 rats included in groups MS1 and MR1 demonstrated evidence of graft infection, with quantitative culture results showing 3.1 × 10⁵ ± 6.0 × 10⁵ and 1.8 × 10⁶ ± 3.3 × 10⁶ CFU/cm² of graft, respectively, although there were no local signs of peri graft inflammation. Groups MS2 and MR2 (with bupafilon-coated Dacron grafts) and groups MS5 and MR5 (with bupafilon-coated Dacron grafts plus intraperitoneal ceftazolin treatment) showed no evidence of staphylococcal infection, with negative quantitative culture results. For the 30 rats with ranalexin-coated Dacron grafts (groups MS3 and MR3), the quantitative graft cultures demonstrated bacterial growth (1.2 × 10³ ± 5.0 × 10⁴ and 2.3 × 10⁶ ± 6.5 × 10⁶ CFU/cm² of graft, respectively). On the contrary, none of the 30 rats with ranalexin-coated grafts plus intraperitoneal ceftazolin treatment (groups MS6 and MR6) had evidence of infection. Cultures of the grafts from groups MS4 and MR4 (with rifampin-coated grafts) showed results similar to those for the animals treated with ranalexin-coated grafts (1.9 × 10³ ± 4.5 × 10⁴ and 2.4 × 10³ ± 7.0 × 10⁴ CFU/cm² of graft, respectively). Nevertheless, the results showed that the use of rifampin-coated grafts and ceftazolin treatment (groups MS7 and MR7) was less effective than the use of ranalexin-coated grafts and ceftazolin treatment. Actually, infection occurred in groups MS7 and MR7, although with low bacterial numbers (4.0 × 10² ± 1.0 × 10² and 8.0 × 10² ± 2.0 × 10⁴ CFU/cm² of graft, respectively). The results for groups MS8 and MR8 (with intraperitoneal ceftazolin treatment and a Dacron graft without antibiotic impregnation) confirmed the efficacy of preoperative ceftazolin against the MS staphylococcal strains (6.5 × 10³ ± 3.5 × 10⁵ CFU/cm² of graft) and, on the contrary, its poor efficacy against the MR strains (1.5 × 10⁷ ± 4.7 × 10⁶ CFU/cm² of graft). There were significant differences in the results for the quantitative bacterial graft cultures when the data obtained for all treated groups were compared with those obtained for the untreated groups. On the contrary, no statistically significant difference was observed between groups MR1 and MR6. Data on the quantitative culture results and from statistical comparisons of the groups are summarized in Table 1.
Actually, in the present study, rifampin exerted equal in vitro activity against the two strains. In addition, rifampin and ranalexin, polycationic peptides, were previously bound to an albumin-sealed Dacron graft for the prevention of graft infections is dependent on the pharmacokinetics of antibiotic penetration into tissue and maintenance of adequate levels in tissue after administration of a single dose, with maintenance of those levels for 2 to 3 h. In order to evaluate the presence of a positive interaction with the drugs bonded to the Dacron graft, rifampin was also tested against MR S. epidermidis, although we presumed that it would be ineffective when used alone. Indeed, recent studies demonstrated that the polycationic peptides present properties of synergy with lipophilic and amphiphilic agents such as rifampin, macrolides, fusidic acid, and novobiocin. Actually, those reports indicated that they allow maximal entry of several hydrophobic substrates into the cell (11, 12, 23). Moreover, recent investigations demonstrated a positive interaction between peptides and beta-lactams: it might be due to increased access of the peptides to the cytoplasmic membrane following breakdown of the peptidoglycan by beta-lactams. On the other hand, the peptides, by triggering the activities of bacterial murein hydrolases, might cause degradation of the peptidoglycan and enhance the activities of the beta-lactams (10, 11, 12).

Taken together, the results of this study demonstrated that the use of preoperative intraperitoneal cefazolin or an antibiotic-soaked Dacron graft can result in significant bacterial growth inhibition even if high concentrations of organisms are topically inoculated into the Dacron prostheses. Statistical analysis demonstrated that any prophylactic antibiotic treatment was useful; nevertheless, only rifampin II was able to inhibit the bacterial growth completely, even though rifampin II bonded to the Dacron graft was used alone. On the other hand, ranalexin was also shown to be highly effective. Actually, ranalexin was demonstrated to be as effective as rifampin and, when combined with intraperitoneal cefazolin, produced complete suppression of both MS and MR staphylococcal strains.

Similar to the other agents tested, neither rifampin II nor ranalexin showed any noteworthy toxicity. Actually, none of the animals included in any group died or had clinical evidence of drug-related adverse effects, such as local signs of perigraft inflammation, anorexia, vomiting, diarrhea, or behavioral alterations.

Bifurin II and ranalexin are polycationic peptides derived from amphibian tissues: the first was derived from Bufo bufo gargarizans, the second was isolated from Rana catasbeiana, and the third was isolated from the skin of the American bullfrog (Rana catesbeiana) (8, 16). The strong in vivo antibacterial efficacies of the two peptides chosen for this study well suit the remarkable resistance of frogs and toads to infection after external injury, despite the contaminated environments in which these animals live (5, 11). The widespread use of several antimicrobial agents both in therapeutic regimens and in prophylactic regimens resulted in a dramatic increase in the prevalence of multidrug-resistant organisms, such as MR staphylococci. In fact, the short doubling times and genetic plasticity of bacteria permit these organisms to rapidly prove whether specific mutations enhance their ability to grow in inhospitable environments. Mutations that confer resistance help bacteria survive attacks from antibiotics used clinically. Nevertheless, as a consequence of the mode of action of the peptides, the emergence of peptide-resistant mutants should be an unlikely event, since alteration of the membrane structure to prevent insertion and channel formation is far more difficult than remodeling of target en-

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* Each group consisted of 15 animals; MS1 to MS8, groups of animals infected with MS S. epidermidis ATCC 12228; MR1 to MR8, groups of animals infected with MR S. epidermidis Se56-99.

** The Dacron graft segments were impregnated with 10 mg of buforin II per ml (groups MS2, MS3, MR2, and MR3), 10 mg of ranalexin per ml (groups MS3, MS6, MR3, and MR6), and 5 mg of rifampin per ml (groups MS4, MS7, MR4, and MR7).

† Cefazolin, 30 mg/kg.

‡ Statistically significant compared with group MS1.

§ Statistically significant compared with group MR1.

Statistically significant compared with groups MS3, MS4, MS7, and MR8.

Statistically significant compared with groups MR3, MR4, MR7, and MR8.

Statistically significant compared with group MS8.
zymes. Today, despite the speculated modes of action of the peptides, proof of their clinical benefits are lacking. However, the antistaphylococcal in vitro activity and the prophylactic in vivo efficacy demonstrated in the present study make these molecules potentially useful for preoperative antimicrobial chemoprophylaxis. Future research based on animal and human models is needed to elucidate their in vivo efficacies and toxicities and their utility in clinical practice.

REFERENCES