Lipid-Based Slow-Release Formulation of Amikacin Sulfate Reduces Foreign Body-Associated Infections in Mice

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Treatment and prophylaxis of uncomplicated infections with standard systemic antibiotics are usually successful. However, standard systemic antibiotic therapy alone is frequently unsatisfactory in certain circumstances, such as the presence of a foreign body (FB), necrotic tissue, overwhelming bacterial inoculum, or poor vascular supply to the involved tissues. We have developed a lipid-based sustained release formulation of amikacin sulfate (DepoFoam encapsulated amikacin sulfate [DEAS]) as a biodegradable, locally injectable antibiotic for such circumstances. The encapsulated drug is released over 7 to 10 days. We tested the efficacy of this formulation in an FB infection model in which Teflon tubes (length, 1 cm; outside diameter, 1.6 mm) were implanted into the subcutaneous tissue in mice and the local site was inoculated with 0.87×10^7 CFU of *Staphylococcus aureus* 3 days later. Inoculation was followed by either no treatment or a local injection of DEAS, free amikacin sulfate, non-drug-containing DepoFoam, or systemic free amikacin sulfate. All drug applications contained 1 mg of amikacin. One group was implanted with the FB and left unchallenged with bacteria and untreated as a sterile control group. All animals were sacrificed 10 days following FB implantation. FBs were retrieved from tissues by an aseptic technique and incubated in liquid culture media for 7 days. Local wound tissue was excised and processed to determine the number of CFU per gram of tissue. Treatment with local or systemic free amikacin had no effect on the number of infected FBs or on the log CFU in wound tissue compared with the untreated or non-drug-containing DepoFoam group. Compared with local free amikacin therapy, the number of infected FBs was reduced from 86 to 25% ($P = 0.02$) following treatment with DEAS, and log CFU per gram of tissue was significantly decreased from 4.8 ± 0.9 to 1.3 ± 0.6 ($P < 0.0005$). DEAS may have clinical utility as locally injected antibiotic in certain infections.

Standard systemic antibiotics are usually adequate in treatment of uncomplicated surgical infections. Good penetration into well-vascularized tissues, high bacterial sensitivity, and low host toxicity make these antibiotics safe and efficacious in uncomplicated infections. In certain situations, however, failures of systemic antibiotic treatment occur and are associated with considerable cost and morbidity (13, 26).

Common circumstances for antibiotic failures are infections in poorly vascularized infected tissues such as joint spaces, diabetic ulcers, irradiated tissues, or abscess cavities. Another circumstance is the presence of foreign bodies (FBs), which make adherent bacteria notoriously difficult to eradicate (6, 8, 9, 21). Yet another circumstance for antibiotic failure is the presence of necrotic tissue caused by injury, cauterization, or fibrin deposits (1).

In many of these situations direct local application of an antibiotic could be more effective than the standard systemic administration if therapeutic local antibiotic concentrations could be maintained for sufficient time to eradicate the organisms. However, standard antibiotics have short half-lives, and repeated injections or continuous infusions into local sites of infection are impractical. A slow-release antibiotic formulation applied directly into infection sites might maintain therapeutic local drug concentrations and avoid systemic exposure to potentially toxic agents. Selection of resistant organisms at remote sites could also be potentially avoided.

Slow-release gentamicin-impregnated polymer beads administered locally have been shown to be efficacious for some applications including infected orthopedic implants (18, 23), vascular prostheses (17), soft-tissue infections (14), and contaminated cavities (12). However, there are major disadvantages which limit their usefulness. Following completed release of the antibiotic, the residual polymer beads act as FBs which can promote infections unless they are removed. In addition, their removal usually requires an operative procedure under general or regional anesthesia. These disadvantages preclude gentamicin-impregnated polymer beads from prophylactic use.

We have developed a slow-release formulation of amikacin sulfate which consists of the drug encapsulated into a lipid-based drug delivery system, DepoFoam. This drug delivery system consists of microspheres of amphipathic lipids, which are biodegradable synthetic copies of cell membrane lipids, enclosing multiple nonconcentric aqueous chambers. The DepoFoam formulation has been tested as a slow-release drug delivery system for another molecule, cytarabine, in human clinical trials (3), and a randomized, multicenter pivotal clinical trial is ongoing for neoplastic meningitis (15) (DepoTech Corp., La Jolla, Calif.).

In this article, we report the efficacy of DepoFoam-encapsulated amikacin sulfate (DEAS) utilizing an FB infection mouse model (4). We hoped to demonstrate that maintenance of local bactericidal concentrations for a prolonged period would result in improved treatment compared with repeated systemic or local application of the free antibiotic.

MATERIALS AND METHODS

Preparations of DEAS. DEAS was prepared by the previously published method (16) with some modifications. Briefly, the aqueous phase containing 50 mg of amikacin sulfate (Bristol Meyer Squibb, Syracuse, N.Y.) per ml was mixed

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with an equal volume of lipid phase that contained previously published lipid ratios (16) of dipalmitoylphosphatidylglycerol, dioleoylphosphatidylcholine, cholesterol, and triolein in chloroform (all lipids were obtained from Avanti Lipids, Albaster, Ala.). The emulsion was then mixed into 5 volumes of 4% glucose and 40 mM lysine. This product was then exposed to a nitrogen flow of 50 liters/min for 20 min, during which the chloroform evaporates. Following this process, the particles are microspheres of bilayer lipid membranes enclosing multiple nonconcentric aqueous chambers with amikacin trapped within. These particles were then centrifuged at $200 \times g$ for 1 min, washed in 0.9% NaCl, and stored at 4°C in a 0.9% NaCl solution.

Analysis of DEAS formulations. For measurement of amikacin content, particles were lysed in 3 volumes of isopropyl alcohol. The amikacin released was assayed by particle concentration fluorescence immunoassay (PCFIA) using a fluorescence concentration analyzer (Idexx Corp., Portland, Maine (22). Fluorescein isothiocyanate-labelled amikacin and sheep polyclonal antibody against amikacin used in the assay were supplied by The Binding Site, San Diego, Calif.

The particle size distribution was determined with a particle size analyzer using laser light diffraction (Horiba, Irvine, Calif.). The unencapsulated-amikacin concentration was determined by separating the supernatant from the pellet following centrifugation and measurement of free amikacin in the supernatant by PCFIA. Preparations for animal trials were used within 1 week of manufacture. Drug contents in the pellet and supernatant were measured, and the concentration was adjusted to contain a total of 2 mg of amikacin per ml.

Bacteria. *Staphylococcus aureus* (ATCC 25923) was grown overnight in trypticase soy broth (TSB) (Becton Dickinson and Company, Cockeysville, Md.). Prior to injection, the concentration was adjusted to 1.7×10^7 CFU/ml by comparison with the McFarland equivalence turbidity standards (Remel, Lenexa, Kans.) and reconfirmed by serial dilution in TSB broth, plating on trypticase soy agar plates (Becton Dickinson and Company), and incubation for 48 h.

Animal experiments. Female outbred CF-1 mice obtained from Charles River Laboratories (Wilmington, Mass.) were housed four animals per cage and had free access to food and water. One to two days prior to the procedure the dorsal skin was shaved and depilated. On day 0 of the experiment, animals were anesthetized with Methofane (Abbott Laboratories, North Chicago, Ill.) and the back was disinfected with Betadine swabs and isopropyl alcohol. Through a 2-mm incision in the lower back the FB application system was introduced. It consisted of a needle that was covered by a 4-cm segment of Teflon high-pressure liquid chromatography tubing (inside diameter 0.76 mm; outside diameter, 1.57 mm) (Western Analytical, Temecula, Calif.). The distal 1 cm of the needle was covered by another segment of the same tubing of a 1-cm length. After the FB at the tip of the needle had been directed into a subcutaneous position in the neck, the Teflon sleeve of the applicator was held in place while the needle was removed, releasing the FB from the tip of the needle. Finally, the whole applicator system was removed from its subcutaneous location. The needle wound tunnel was then closed with a single suture close to the FB. Following this procedure, animals were returned to cages and wounds were allowed to heal for 3 days. On day 3, a 1/2-ml suspension of $1.7 \times 10^7 \pm 0.6 \times 10^7$ CFU of *S. aureus* per ml was injected at a position adjacent to the FB. Then, 0.5 ml of a DEAS suspension containing 1 mg of amikacin was injected in the same way. Control animals received either the same amount of amikacin in free form locally once or systemically daily for 7 days, with the first dose of systemic treatment given 10 min prior to bacterial challenge. The third control group received no bacteria and no drug in order to ensure the sterility of the procedure. The fourth control group received bacteria but no drug, and a fifth control group received non-drug-containing DepoFoam. Following the procedure the animals were housed again in cages of four, observed daily, and given free access to food and water.

Microbiological workup and evaluation of efficacy. On day 10 following injection of bacteria and antibiotics, animals were sacrificed, skin was thoroughly disinfected, and the FB was retrieved from the tissue by an aseptic technique. The FB was incubated for 7 days in TSB and the growth was assessed in a yes-no fashion for infection. The local tissue was excised aseptically, cut into small segments, and homogenized after 1:10 dilution with TSB in sterile bags in a stomacher 80 tissue homogenizer (Seward Medicals, London, United Kingdom). Serial dilutions were made from the tissue broth, and aliquots of 0.1 ml of the resulting broth were plated on TSB plates. Plates were counted for CFU after 48 h, and the results were expressed as log CFU per gram of tissue. When no bacterial growth was detected at the lowest dilution, a log value of 0 was assigned. Likewise, a log value of 7 was assigned when confluent bacterial growth occurred on the 10,000-fold dilution plate.

Residual total amikacin in the tissue after termination of the experiment on day 7 was measured in some of the animals that received DEAS by PCFIA. The effect of the residual tissue amikacin on the in vitro microbiological assay was tested by plating the undiluted tissue broth together with 10^3 CFU of *S. aureus*. Statistical significance of data was tested by Fisher's exact test (two-sided) for discrete data and by analysis of variance and the Kruskal-Wallis nonparametric, one-way (SAS Inc., Cary, N.C.) test for continuous parameters.

RESULTS

DEAS preparations. The DEAS formulation contained 6 ± 0.8 mg of amikacin sulfate per ml of suspension (four prepa-
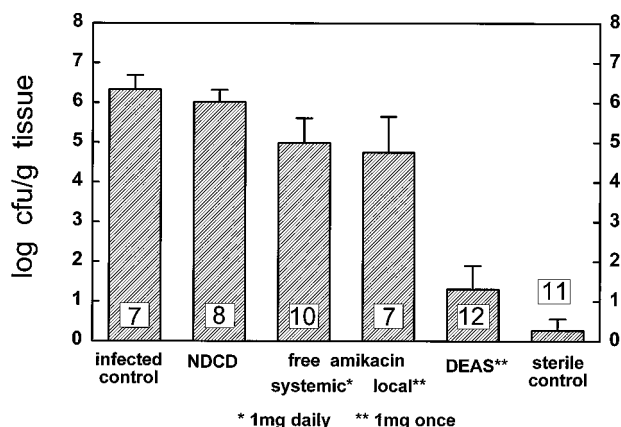

FIG. 1. Concentrations of *S. aureus* in tissues surrounding the FB after challenge with $0.87 \times 10^7 \pm 0.3 \times 10^7$ CFU of *S. aureus* and treatment with DEAS, non-drug-containing DepoFoam (NDCD), and free amikacin administered locally or systemically versus sterile controls. $P < 0.0005$ for DEAS versus the free drug administered locally or systemically (analysis of variance). Group sizes are indicated. Error bars, standard errors of the means.

rations). In fresh product, not more than 5% of total amikacin was found as free amikacin in the supernatant. The encapsulation efficiency, i.e., percent amikacin sulfate encapsulated into DepoFoam relative to the starting amikacin amount, was $48\% \pm 1.1\%$ (mean for four preparations). The median particle size was 20 ± 1 μm (length-weighted diameter by laser light diffraction).

Animal experiments and microbiology. There was no mortality in the animals following the infectious challenge.

The experimental results are shown in Fig. 1 and 2. Of seven animals in the group treated with local free amikacin, six (86%) developed FB infections. The mean ($\pm$ standard error of the mean) log CFU per gram of tissue cultured from the surrounding tissue was 4.8 ± 0.9. In contrast, only 3 of 12 animals (25%) treated with DEAS developed infection of the FB ($P = 0.02$) despite the massive initial inoculation with 0.87×10^7 CFU of *S. aureus*. The log CFU per gram of tissue was 1.3 ± 0.6 ($P < 0.0005$). The animals treated with the once-daily
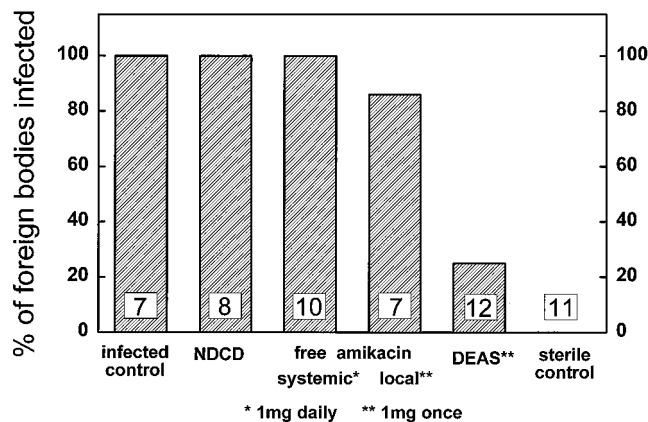

FIG. 2. Percentages of FBs infected after challenge with $0.87 \times 10^7 \pm 0.3 \times 10^7$ CFU of *S. aureus* and treatment with DEAS, non-drug-containing DepoFoam (NDCD), and free amikacin administered locally or systemically versus sterile controls. $P = 0.02$ for DEAS versus local treatment with the free drug (Fisher's exact test). Three of the animals in the infected group that extruded the FB through necrotic skin ulcers were scored as "infected". Group sizes are indicated.

administration of systemic free amikacin for 7 days developed FB infections at a rate of 100%; the log CFU per gram of tissue was 5 ± 0.6. In the group of infected controls all FBs were infected; the log CFU per gram of tissue was 6.3 ± 0.4. None of 12 FBs (0%) in the sterile control group was infected; the log CFU per gram of tissue was 0.3 ± 0.3.

The residual tissue amikacin concentration in animals treated with DEAS as determined by PCFIA was 86.5 ± 29.5 μg/g of tissue. The tissue broth of animals treated with DEAS did not show any inhibitory effect when plated after standard dilution (1:100) together with a fresh suspension of 10^3 CFU of *S. aureus* compared with tissue broth obtained from animals not treated with amikacin sulfate.

DISCUSSION

We have previously reported favorable results on the efficacy and pharmacokinetics of DepoFoam containing gentamicin sulfate (11, 12). Since then, we have continued our investigation of sustained-release antibiotic preparations. For the present work, the drug chosen was amikacin. Although gentamicin has a higher antimicrobial potency, amikacin has the advantage of a lower risk of resistance (7) and a broader spectrum of activity (10).

On the basis of our experience with the efficacy of gentamicin-containing DepoFoam, we modified a *Staphylococcus epidermidis* animal model of FB infections described in the literature (4) to an acute challenge with *S. aureus*. The presence of an FB is uniformly accepted as an adjuvant to infectious challenge. Obviously, the inoculum size of 0.87×10^7 CFU represents a massive challenge. A similar inoculum has been shown to cause abscess formation in rats even in the absence of promoting factors such as FB, necrotic tissue, or suture material (5). Even when cefazolin was administered as a prophylactic dose, 50% of the animals still developed abscesses. In another subcutaneous-infection model using guinea pigs, doses of 2×10^7 CFU of various strains of staphylococci were injected. Despite prophylaxis with 100 mg of various cephalosporins per kg of body weight, all bacterial concentrations produced infections (25). In another report, a dose of 10^2 CFU consistently led to infections of implanted Teflon FBs (2).

The extents of infection in animals treated with free amikacin either locally or systemically did not differ from each other or from the no-treatment, infected control group. These three groups had almost identical numbers of infected FBs and log CFU per gram. In contrast, animals treated with a single-dose of DEAS had fewer infected FBs and significantly lower log CFU per gram of tissue.

In three-quarters of the animals treated with a single dose of DEAS, the FB was sterilized and the log CFU per gram of tissue was decreased close to the levels of uninfected control animals. In the other quarter of the animals treated with DEAS, the failure to sterilize the contaminated FB was, in most cases, macroscopically undetectable. The most likely cause for DEAS failure in one-quarter of the animals is the massive inoculum size of 0.87×10^7 CFU of *S. aureus* which was utilized. Such an inoculum size associated with an FB (e.g., vascular catheter or graft or orthopedic implant) would be extremely unlikely in modern surgery. It is probable that, had we utilized a lower inoculum size, more relevant to surgical practice, we would have obtained much higher cure rates. Nevertheless, it is impossible to obtain a perfect animal model to mimic clinical practice, and human trials are necessary to prove or disprove clinical efficacy.

Since a fraction of the initial DEAS dose was found remaining at the local site at day 7, we asked the question whether the residual DEAS could interfere with the in vitro microbiological assay. To address this question, TSB containing 10^3 CFU of *S. aureus* was plated together with undiluted tissue broth from animals treated with DEAS. The experiment showed that there was no growth inhibition of the bacteria. This result demonstrated that the reduction of CFU observed is indeed attributable to in vivo killing of the bacteria and is not an in vitro artifact of bacterial growth inhibition in the culture plate. Obviously, the residual amount of amikacin is diluted to an extent that excludes interference with the assay. Likewise, there would be no interference with the sterility assay of FBs since they are transferred without any adherent tissue into a volume of 5 ml of TSB. A trace amount of amikacin on the surface of the FBs would be diluted well below the bacteriostatic concentration.

Other biodegradable drug delivery systems that do not require subsequent removal have been described. A collagen matrix impregnated with gentamicin has been shown to be efficacious when used for prevention of wound infection after excision of acutely infected pilonidal sinus and primary wound closure (24). Price et al. have shown that subcutaneous infections could be treated with a single dose of a locally applied liposome formulation of tobramycin in small unilamellar vesicles as well as by repeated local injections of tobramycin as a free drug (19). The efficacy of the tobramycin-containing unilamellar vesicles was reported to be equivalent to multiple local injections of free tobramycin.

Although toxicological studies of DEAS have not been completed, major problems are not expected. The side effects of the active ingredient, amikacin sulfate, have been well characterized, and the other components of the formulation occur naturally in the human body. Because the amikacin dose in DEAS for local administration was well below the usual daily dose of systemic free amikacin, serious toxicities such as nephrotoxicity and ototoxicity are highly unlikely. With further development and clinical testing in humans, DEAS may prove to be a useful addition to the surgeon's armamentarium in prophylaxis and treatment of localized infections.

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