Inhibitory Effect of Disodium EDTA upon the Growth of Staphylococcus epidermidis In Vitro: Relation to Infection Prophylaxis of Hickman Catheters

JENNIFER L. ROOT,1 O. ROSS MCINTYRE,1,2* NICHOLAS J. JACOBS,3 AND CHARLES P. DAGHLIAN4

Department of Medicine1 and Department of Microbiology,3 Dartmouth-Hitchcock Medical Center, and Julius A. Rippel Electron Microscope Facility,4 Dartmouth College, and The Norris Cotton Cancer Center,2 Hanover, New Hampshire 03756

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Granulocytopenic patients with an intravascular catheter are at increased risk for infection with Staphylococcus epidermidis. During the intervals when the catheters are not being used for infusions, it is customary to maintain patency of the catheter lumen with a solution containing heparin. We show that heparin does not inhibit the growth of S. epidermidis isolated from the catheter of an infected patient. A 20-μg/ml solution of disodium EDTA, a chelating agent which effectively anticoagulates blood at this concentration, was shown to be bactericidal for an initial inoculum of 107 CFU of staphylococci per ml in 24 h. Vancomycin, an antibiotic which is often employed to treat Staphylococcus infections, was also bactericidal for initial inocula of 103 CFU/ml at doses of 6.7 μg/ml, a drug concentration in the therapeutic range. When 105 staphylococci per ml were cultured in the presence of catheter segments and disodium EDTA or vancomycin, subcultures of the catheters showed minimal or no growth, respectively. In contrast, when cultured with heparin alone, subcultures showed abundant growth. In view of its low cost, effectiveness as an anticoagulant, and bactericidal activity, EDTA should be studied as a replacement for heparin solutions for the maintenance of intravenous catheters in granulocytopenic patients.

Hickman and Broviac catheters offer long-term venous access for the treatment of patients who are seriously ill (2, 7). They are typically maintained by twice-daily injections of a heparin flushing solution. Reports of complications associated with the use of these catheters focus on thrombosis and infection (9, 10, 16). Among the latter, Staphylococcus epidermidis has emerged as the most significant opportunistic pathogen, especially in patients who are granulocytopenic (1, 19). Christensen et al. have shown that microbial colonization of Hickman lines is dependent upon the production of a slime coat by the bacteria (3). Recent studies suggest that this glyocalyx contributes to S. epidermidis virulence by protecting the organism from the bactericidal effects of empiric antibiotic therapy (4, 5).

Numerous approaches to infection prophylaxis have yielded inconclusive results. Pizzo and Schimpff report that total protected isolation of granulocytopenic patients reduces but does not eliminate completely the incidence of catheter-related sepsis (11). Similarly, the efficacy of systemic antimicrobial prophylaxis or selective suppression of alimentary flora is counterbalanced by the emergence of resistant bacteria, fungal superinfections, and drug-induced organ toxicity (11).

We address the issue of infection prophylaxis from the standpoint that bacterial adherence and colonization of Hickman catheters are prerequisite to S. epidermidis septicemia. Our model is based upon disodium EDTA, a chemical well known for its chelating properties in vivo and which has been used as an anticoagulant in vitro. EDTA has also been used as a topical antiseptic in gram-negative infections and more recently, in combination with other agents, for gram-positive infections (6). In this report, we investigate the antibacterial action of disodium EDTA against a catheter-associated S. epidermidis isolate, comparing its efficacy with those of heparin alone and a vancomycin-heparin preparation in vitro.

MATERIALS AND METHODS

Chemical agents. All agents were obtained commercially: vancomycin hydrochloride (Eli Lilly Industries, Inc., Indianapolis, Ind.); disodium EDTA (Fischer Scientific Co., New Lawn, N.J.); heparin sodium injection, B.P., bactericide free (Weddel Pharmaceuticals, Ltd., Wrexham, United Kingdom); heparin sodium injection, USP, preservative free (Squibb-Marsam, Inc., Cherry Hill, N.J.).

A concentration of 6.7 μg/ml was selected for all in vitro testing of vancomycin for two reasons. One, the desired trough level of vancomycin in serum for effective antibacterial action is 5 to 10 μg/ml (15). Two, this concentration is 1.5 times the MBC for the strain utilized, to ensure complete bactericidal activity.

A concentration of 20 mg/ml was selected for the in vitro testing of disodium EDTA because in preliminary experiments, this concentration was effective in preventing the growth of three noncatheter-related coagulase-negative staphylococcal blood isolates, one of which was methicillin resistant. In addition, this concentration was considered to be adequate for anticoagulating a Hickman catheter since standard phlebotomy Vacutainers (Becton Dickinson & Co., Lincoln Park, N.J.) contain 1.5 mg of EDTA per ml of whole blood.

Organism. S. epidermidis was isolated from the Hickman catheter of a septic bone marrow transplant recipient at the Dartmouth-Hitchcock Medical Center, Hanover, N.H. The organism was identified to species by the New Hampshire Public Health Laboratory in Concord. Antibiotic susceptibility was determined by standard clinical microdilution test panels (MicroScan).

* Corresponding author.
A technique described by Christensen et al. was used to determine slime production (3). Briefly, a colony of organisms from a blood agar plate was inoculated into 5 ml of Trypticase soy broth (TSB) and incubated at 37°C for 48 h. The vial contents were poured off, and the tubes were stained with 6 ml of 3% safranine. A positive slime test was established by demonstration of a safranine-stained film along the tube walls. A negative test was indicated by formation of a ring of film only at the liquid-air interface or no staining at all.

SEM. For scanning electron microscopy (SEM), the patient's catheter was bisected and fixed in 4% glutaraldehyde in sodium cacodylate buffer (742 mosM; pH 7.3) for 24 h. It was postfixed in 2% osmium tetroxide, dehydrated in ethanol, air dried, coated with gold palladium (150 Å; 15 nm), and examined in an AMR 1000 scanning electron microscope at 20 kV.

To study the effect of EDTA upon bacterial cell morphology, an inoculum of 10^3 CFU of S. epidermidis in TSB was pipetted into sterile polystyrene test tubes containing disodium EDTA at a final concentration of 20 mg/ml. The tubes were placed in a 37°C incubator. At 0, 10, and 24 h, the vial contents were transferred to sterile Eppendorf tubes and pelleted by centrifugation at 12,800 x g for 20 min (Brinkmann Eppendorf Centrifuge 5412). The supernatant was discarded, and the bacteria were suspended in 100 μl of 4% glutaraldehyde in 1,4-piperazinediethanesulfonic acid buffer (0.1 M; pH 7.3). The samples were rinsed in sodium cacodylate buffer (0.1 M; pH 7.4) and vacuum filtered on 13-mm, 0.45-μm-pore-size membrane filters (Millipore) with additional buffer. The moist samples were then postfixed on the filters with 1% osmium tetroxide in sodium cacodylate buffer. The samples underwent serial ethanol dehydration (75 to 95%), followed by immersion in trichorotrifloroethane (Freon 113) and rapid air drying. The bacteria were mounted on stubs, coated with gold palladium (15 nm), and examined in an AMR 10000 scanning electron microscope at 20 kV.

In vitro susceptibility and time-kill curve studies. A 24-h culture of S. epidermidis in TSB was serially diluted to approximately 10^3 CFU/ml. One milliliter of each bacteriabroth mixture was inoculated into sterile polystyrene test tubes previously filled with vancomycin hydrochloride and heparin sodium injection (B.P.) in 10 ml of each concentration of 6.7 μg/ml and 10 U/ml, respectively. Tubes were incubated at 37°C. At 0, 12, and 24 h, the culture tubes were analyzed for viable organisms after residual antibacterial agents were washed away by the following procedures. The tube contents were vortexed, and the solutions were transferred by sterile pipette to Corex centrifuge tubes. The bacteria were pelleted by centrifugation at 10,000 x g for 25 min (Sorvall SS-34 RC2-B Automatic Refrigerated Centrifuge). The supernatant was discarded, and 3 ml of TSB was added to each vial. The tubes were again centrifuged at 10,000 x g for 25 min. This washing procedure was carried out twice before the bacteria were suspended in sterile TSB at their original volumes. Then, three replicate samples of 0.1 ml of the test solutions were transferred by sterile pipette to three blood agar plates (Scott Laboratories, Inc., Fiskeville, R.I.) and spread with a wire loop. Colony counts and culture turbidity changes were determined by visual inspection. This experiment was repeated three times and the data were pooled.

The same protocol as described above was used to test disodium EDTA. One milliliter of TSB containing 10^3 CFU/ml was added to filter-purified disodium EDTA at a final concentration of 20 mg/ml. Incubation at 37°C for 24 h was interrupted at identical times as listed above for TSB washes and blood agar plating. This experiment was repeated three times and the data were pooled.

Quantitative recovery of bacteria using the above centrifugation and wash procedure was studied in control experiments not employing antibacterial agents.

Assays of affinity for catheters. (i) Experiment 1. An overnight culture of S. epidermidis in TSB was serially diluted to 10^3 CFU/ml. Into sterile polystyrene test tubes were placed 1 ml of microorganisms and the following chemical agents at the respective final concentrations given: disodium EDTA, 20 mg/ml; vancomycin, 6.7 μg/ml; heparin, 10 U/ml; vancomycin-heparin solution, 6.7 μg/ml-10 U/ml. One 1-cm segment from a sterile triple lumen right atrial catheter (Quinton Instrument Co., Seattle, Wash.) was immersed in each bacteria-antiseptic solution. The test vials were vortexed, and uniform intralumenal contact with the fluid was ensured by pipette suctioning of the catheter segment. After incubation (37°C; 24 h), the following procedure was used to determine the quantity of viable bacteria adherent to the catheter segment. The catheter piece was removed from the test solution and immersed in 3 ml of sterile TSB. The bacteria were removed from the catheter surfaces by vortexing, extraluminal scraping with a wire loop, and intraluminal scraping with an inoculating needle. The catheter segments were discarded, and the test solutions were centrifuged at 10,000 x g for 25 min to separate any viable bacteria from tramps of antibacterial agents which might interfere with subsequent growth. After removal of the supernatant, 3 ml of TSB was introduced and the vials underwent a second centrifugation. This wash procedure was repeated twice, and the bacteria were suspended in 0.5 ml of TSB. By sterile pipette, three replicates of 0.1 ml of each test culture were transferred to three blood agar plates and spread with a wire loop. The colonies were counted, and the data were expressed as CFU per milliliter of TSB in which the bacteria were suspended. This experiment was repeated three times.

(ii) Experiment 2. A second experiment was conducted to test the effect of various concentrations of EDTA on the adherence of viable staphylococci to catheter segments. Serial dilutions of an overnight culture of S. epidermidis were carried out to a final concentration of 10^3 CFU/ml in TSB. Samples (1 ml) of this broth were then transferred by sterile pipette to polystyrene test tubes containing various dilutions of disodium EDTA. The final concentrations of EDTA were 0.1, 5, 10, and 20 mg/ml. Into each vial was also placed one 1-cm segment of the triple lumen right atrial catheter. The tubes were vortexed, and the catheter tips were pipetted. After 24 h of incubation at 37°C, the same methods of TSB washings and agar plating as described above in Experiment 2 were performed. In addition, two replicates of 0.1 ml of the culture solutions in which the catheters had been incubated for 24 h were transferred by sterile pipette to two agar plates and spread with a wire loop. Colony counts of the media were compared with those found upon scraping of the catheter surfaces.

This experiment was repeated using an initial bacterial inoculum of 10^2 CFU/ml and disodium EDTA at final concentrations of 10, 20, 30, and 50 mg/ml.

Coagulation assay. The partial thromboplastin and prothrombin times of heparin-vancomycin mixed (10 U/ml-6.7 μg/ml) and heparin alone were determined by routine clinical laboratory methods. An Electra 700 Automatic Coagulation Timer was employed.
FIG. 1. S. epidermidis colonization of catheter removed from patient. (A) Lumen surface with abundant slime coat and debris; magnification, ×1,920; bar, 10 μm. (B) Enlargement of a small cluster of S. epidermidis seen in slime coat; magnification ×7,800; bar, 5 μm.

RESULTS

Characteristics of the organism. For this strain of S. epidermidis, the MICs of five antimicrobial agents were as follows: oxacillin, 1 μg/ml; vancomycin, ≤2 μg/ml; tetracycline, ≤1 μg/ml; amikacin, 4 μg/ml; nitrofurantoin, ≤16 μg/ml. The MBC of vancomycin was determined to be 4.0 μg/ml. The organism was resistant to erythromycin, ampicillin, penicillin, gentamicin, clindamycin, and chloramphenicol. The slime coat test was positive.

SEM. Bacterial colonization of both the luminal and external surfaces of our patient’s catheter was verified by SEM (Fig. 1).

The effect of EDTA on S. epidermidis morphology after incubation for 10 and 24 h was also studied. SEM revealed no distinct morphologic changes in cell surfaces. No cellular ballooning, distinct breaks in cell wall integrity, or scattered cellular debris were observed. The bacteria were indistinguishable from those in Fig. 1.

Time-kill studies. The killing curve of vancomycin-heparin against 10³ CFU/ml is shown in Fig. 2. Vancomycin reduced an initial bacterial concentration of 3.0 log₁₀ CFU/ml to 0.3 log₁₀ CFU/ml in 24 h, with a range of 0 to 1.0 log₁₀ CFU/ml. No vial content turbidity or slime coat production was evident.

Disodium EDTA exhibited a similar but lower rate of bactericidal activity for 10³ CFU of S. epidermidis per ml (Fig. 2). An initial inoculum of 3.0 log₁₀ CFU/ml was reduced to a mean of 1.4 log₁₀ CFU/ml after 24 h of incubation, with a wide range of 0 colonies to 1.9 log₁₀ CFU/ml surviving. No culture turbidity or slime coat production was detected in any of the cultures.

Separate studies confirmed that the centrifugation technique used for washing away residual antibiotic agents was sufficient to pellet the S. epidermidis and minimize bacterial loss in the supernatant. Using the protocol described in Materials and Methods, an initial inoculum of 2.90 log₁₀ CFU/ml was determined to be 2.92 log₁₀ CFU/ml after multiple washes and centrifugations in TSB alone. Thus, there is no possibility that the results shown in Fig. 2 were caused by a residual carryover of inhibitory agents to the agar plates used to determine viable cell counts.

Influence of catheter presence. The antimicrobial effect of disodium EDTA against 3.0 log₁₀ CFU/ml was not altered by the presence of catheter segments in vitro (Table 1). After 24

TABLE 1. Effects of EDTA, heparin, vancomycin, and vancomycin-heparin on adherence and growth of 10³ CFU of S. epidermidis along catheter surfaces after 24 h of incubation

<table>
<thead>
<tr>
<th>Agent (conc)</th>
<th>Mean (range) of viable bacteria (log₁₀ CFU/ml) after 24 h*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disodium EDTA (20 mg/ml)</td>
<td>1.0 (0–1.6)</td>
</tr>
<tr>
<td>Heparin (10 U/ml)</td>
<td>&gt;8.0</td>
</tr>
<tr>
<td>Vancomycin (6.7 μg/ml)</td>
<td>0</td>
</tr>
<tr>
<td>Vancomycin (6.7 μg/ml)-heparin (10 U/ml)</td>
<td>0</td>
</tr>
</tbody>
</table>

* Pooled data from three experiments.
TABLE 2. Dose-response relationship between 10^3 CFU of S. epidermidis per ml and various concentrations of disodium EDTA*

<table>
<thead>
<tr>
<th>EDTA concn (mg/ml)</th>
<th>Mean (range) of viable bacteria (log_{10} CFU/ml) after 24 h in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Catheter</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>1.4 (1.3-1.5)</td>
</tr>
<tr>
<td>5</td>
<td>1.4 (1.3-1.6)</td>
</tr>
<tr>
<td>0.1</td>
<td>&gt;8.0</td>
</tr>
</tbody>
</table>

* Bacterial counts isolated from the catheter surfaces are compared with those found in the culture medium after 24 h of incubation.

h of incubation, 1.0 log_{10} CFU/ml remained. It could not be definitively ascertained whether this concentration of surviving bacteria represented adherent colonies upon the catheter surface or free-floating bacteria which were picked up with small droplets of culture media when the catheter tip was initially transferred to 3 ml of sterile TSB. SEM of the lumenal and external surfaces of a second catheter segment incubated for 24 h in the presence of 10^3 CFU/ml and EDTA at a final concentration of 20 mg/ml failed to reveal any adherent bacteria.

Catheters cultured in vancomycin and the vancomycin-heparin solution showed no growth after 24 h of incubation. In sharp contrast, catheter segments which were incubated with heparin alone had >8 log_{10} CFU/ml at 24 h. These latter test solutions were also the only ones which became turbid with incubation. This experiment was repeated three times.

Dose-response study. The dose-response relationship between various concentrations of S. epidermidis and disodium EDTA incubated in the presence of catheter segments is shown in Tables 2 and 3. In this experiment, 20 mg of EDTA per ml maintained the sterility of the catheter segment against an initial inoculum of 10^3 CFU/ml but did not kill all the bacteria in the surrounding culture medium, which contained 0.7 log_{10} CFU/ml at 24 h. As the concentration of EDTA was decreased from 20 mg/ml, the number of surviving bacteria rose both in the culture medium and along the catheter surfaces. However, even at 5 mg/ml, EDTA was able to induce a 1.5-log reduction in the initial inoculum of 3.0 log_{10} CFU/ml. EDTA had no detectable antibacterial effect at 0.1 mg/ml.

EDTA was unable to maintain the catheter's sterility when faced with a 10^2-CFU/ml challenge. At very high concentrations, EDTA effected approximately a 3-log reduction in the number of viable bacteria. However, 1.5 log_{10} CFU/ml continued to thrive on the catheter surfaces after 24 h of incubation with 50 mg of EDTA per ml.

TABLE 3. Dose-response relationship between 10^6 CFU of S. epidermidis per ml and various concentrations of disodium EDTA*

<table>
<thead>
<tr>
<th>EDTA concn (mg/ml)</th>
<th>Mean (range) of viable bacteria (log_{10} CFU/ml) after 24 h in:</th>
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<tbody>
<tr>
<td></td>
<td>Catheter</td>
</tr>
<tr>
<td>50</td>
<td>1.5</td>
</tr>
<tr>
<td>30</td>
<td>1.1 (1.0-1.3)</td>
</tr>
<tr>
<td>20</td>
<td>1.8 (1.6-1.9)</td>
</tr>
<tr>
<td>10</td>
<td>2.3 (2.0-2.5)</td>
</tr>
</tbody>
</table>

* Bacterial counts isolated from the catheter surfaces are compared with those found in the culture medium after 24 h of incubation.

Coagulation assay. The anticoagulant action of heparin was not changed significantly by the presence of vancomycin. The partial thromboplastin/prothrombin times for heparin alone and heparin plus vancomycin were 13.8 s/120 s and 12.9 s/120 s, respectively.

**DISCUSSION**

These studies show that disodium EDTA, in a concentration sufficient to serve as an effective anticoagulant, prevents the growth of S. epidermidis and causes at least a 1.5-log reduction in viable organisms even in the presence of catheter segments. Heparin showed no similar bactericidal or bacteriostatic action.

The clinical significance of these findings rests upon elucidation of the mechanism of bacterial colonization of central venous catheters. If infectious complications are secondary to intraluminal colonization, as proposed in a recent study by Tenney et al. (18), EDTA as both an anticoagulant and antiseptic agent would have far-reaching impact. However, if extraluminal colonization along the intercutaneous catheter tract is the primary route, the role of EDTA would be of less importance and systemic antibiotic therapy would be necessary.

The mechanism of *Staphylococcus* killing by EDTA also awaits further exploration. The chelator forms extremely firm soluble complexes with divalent and trivalent metals (13), and it is most likely that in our experiments the concentration of one or more metal ions necessary for bacterial growth was reduced to below a critical level. Over the past 20 years, microbiologists have been studying the antibacterial action of disodium EDTA against gram-negative organisms. They have determined that in some gram-negative organisms, specifically *Pseudomonas aeruginosa* and *Salmonella typhimurium*, EDTA appears to attack the cell wall membrane by chelating cations, especially Mg2+, and thereby disrupting cellular integrity (8, 14). In other bacteria, most notably *Escherichia coli*, the mechanism of EDTA action is at a nuclear level and has been suggested to involve RNA transcription and synthesis (12). The SEM study described in this paper failed to reveal any morphologic evidence of enhanced cell wall fragility in the S. epidermidis under EDTA effect. This suggests that the mechanism of EDTA against gram-positive bacteria may be intracellular in origin, although definitive proof awaits further investigation.

When administered by rapid intravenous injection, disodium EDTA causes a prompt decline in the ionized calcium concentration in serum, and tetany results. Slow intravenous injection, however, is unassociated with significant hypocalcemia. For instance, Spencer (17) has studied serum and urinary calcium levels in four normocalcemic human subjects for 24 h following the intravenous administration of 4 g of disodium EDTA over a 4-h period. Slight and temporary reductions of serum calcium were noted, and urinary calcium excretion increased from 4- to 20-fold. The calcium excretion induced by the drug is offset by liberation of calcium from the large bone storage pool. Usual care for the catheter includes twice-daily flushes with 2.5 ml of heparin solution. If this volume were replaced with 20 mg of EDTA per ml, the daily dose of EDTA would amount to 100 mg. Thus, the amount of disodium EDTA which might reasonably be required to anticoagulate blood for 24 h in standard intravenous catheters is 1/40 of that given acutely in the 4-h infusions performed by Spencer. On these grounds it is possible that EDTA could prove to be a safe, inexpensive, and effective means of catheter maintenance.
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LITERATURE CITED

DISODIUM EDTA KILLING OF S. EPIDERMIDIS