ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1995, p. 1281–1286 Vol. 39, No. 6
0066-4804/95/$04.00+0

Effects of Ciprofloxacin and Protamine Sulfate Combinations against Catheter-Associated *Pseudomonas aeruginosa* Biofilms

F. SOBOH,[1] A. E. KHOURY,[1,2,3] A. C. ZAMBONI,[1,3] D. DAVIDSON,[1,4] AND M. W. MITTELMAN[1,2,4]*

Centre for Infection and Biomaterials Research, The Toronto Hospital,[1] Department of Surgery[2] and Department of Microbiology,[4] University of Toronto, and Hospital for Sick Children,[3] Toronto, Ontario, Canada

Received 21 October 1994/Returned for modification 8 February 1995/Accepted 7 April 1995

Infection is a common complication associated with the use of transcutaneous and implanted medical devices. These infections are generally difficult to treat and frequently require removal of the biomaterial before the infection can be completely eradicated. The presence of a bacterial biofilm recalcitrant to treatment often mediates these infections. We studied the influence of a polycationic protein, protamine sulfate, on the efficacy of the fluoroquinolone ciprofloxacin against a clinical isolate of *Pseudomonas aeruginosa*. A *P. aeruginosa* biofilm was developed on 1-cm sections of red rubber catheter material and then treated with various combinations of protamine sulfate and ciprofloxacin. The present work demonstrated that ciprofloxacin in combination with protamine was more effective against biofilms than was ciprofloxacin alone. Protamine sulfate at 50 μg/ml combined with antibiotic at 0.5 μg/ml reduced the number of viable cells by an average of 98.97%, while protamine sulfate at 50 μg/ml alone resulted in an average 107.8% increase and antibiotic alone resulted in an average 58.6% reduction after 24 h. Furthermore, protamine sulfate, in combination with ciprofloxacin, inhibited *P. aeruginosa* in a dose-dependent fashion. It was further observed that treatment with the combination of protamine sulfate and ciprofloxacin had a more drastic effect on planktonic organisms as compared with the *P. aeruginosa* biofilms; the MBC was reduced to <0.05 μg/ml in the presence of 25 μg of protamine sulfate per ml. These findings were substantiated by ultrastructure studies of treated cells using scanning and transmission electron microscopy. The synergism between ciprofloxacin and protamine sulfate significantly enhanced the efficacy of ciprofloxacin against planktonic and biofilm *P. aeruginosa*.

The application of biomaterial substances used for tissue substitution and joint and heart replacement and as access for dialysis and drainage of body fluids has been hampered by infection problems (13). Microorganisms can attach to any biomaterial surface (7). These cells colonize the surface, reproduce, and produce extracellular polymeric substances which form a matrix surrounding the cells. This accretion of microorganisms and associated host and bacterial extracellular material is defined as a biofilm. *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Candida albicans*, and *Pseudomonas aeruginosa* are common infecting agents of prosthetic devices (7, 25). These organisms cause infections which are difficult to treat with otherwise clinically effective dosages of antibiotics (9, 18).

While planktonic bacteria are usually controlled by conventional doses of antibiotics, biofilm bacteria are notably resistant to antibiotic therapy. The insensitivity of biofilm bacteria to antibiotics is a function of cell wall composition, surface structure, and phenotypic variation in enzymatic activity (3, 22). It has also been suggested that the bacterial extracellular polymeric substance acts as an ion-exchange structure, binding charged antibiotic molecules, limiting their penetration into the biofilms (5); diffusion limitation has been shown to limit the penetration of an antibiotic to the biofilm-surface interface (23).

One approach to overcoming the intrinsic antimicrobial resistance of biofilm bacteria would be to enhance the penetration of agents through the biofilm matrix. We resolved to determine whether protamine sulfate, a surface-active, basic polypeptide presently used to reverse the anticoagulant effects of heparin, could aid antibiotics in penetrating a *P. aeruginosa* biofilm. Protamine is known to play a significant role in natural host defenses against infection and to exert antimicrobial activities (20). Parsons and coworkers have shown that protamine sulfate penetrates and disrupts the protective glycosaminoglycan layer lining the urinary bladder (19). The efficacy of vancomycin against *S. aureus* was enhanced by protamine sulfate in an infected rat model (24). *P. aeruginosa* is of particular interest as a pathogen of implanted medical devices, as it is often resistant to the commonly used antimicrobial agents, especially in nosocomial infections (11, 17).

The similarity of the glycosaminoglycan layer to biofilm extracellular accretions coupled with the antimicrobial activity of protamine sulfate led us to explore the usefulness of protamine sulfate as an adjuvant to antibiotics in the treatment of biofilm-related infections. The purpose of this study was to determine the effects of a surface agent on antibiotic efficacy against a clinically relevant bacterial biofilm. We evaluated the relative efficacies of ciprofloxacin, protamine sulfate, and protamine sulfate-ciprofloxacin combinations against *P. aeruginosa* biofilms associated with red rubber catheter surfaces.

MATERIALS AND METHODS

Bacterial strain and culture conditions. *P. aeruginosa* was isolated from a urine sample obtained from a patient with a symptomatic urinary tract infection. Identification was accomplished by routine microbiological procedures at Toronto Hospital (Toronto, Ontario, Canada). The MIC of ciprofloxacin was 0.5 μg/ml. Nutrient broth (Difco Laboratories, Detroit, Mich.) was used to culture the organism. The strain was stored in 20% glycerol in nutrient broth at −70°C.

Colonization of catheter sections. Polyurethane, red rubber catheters (0.6 cm in diameter and 40 cm in length) were used (Argyle, St. Louis, Mo.). Catheters were cut into 1-cm sections (colonization area = 2.2 cm^2), steam sterilized, and immersed in 100 ml of inoculated nutrient broth in beakers. A 100-μl volume of

* Corresponding author. Mailing address: Centre for Infection & Biomaterials Research, The Toronto Hospital, Bell G-631, 200 Elizabeth St., Toronto, Ontario M5G 2C4, Canada. Phone: (416) 340-4564. Fax: (416) 340-4607. Electronic mail address: mittelman@medac.med.utoronto.ca.

TABLE 1. MICs and MBCs of various treatment combinations of protamine sulfate and ciprofloxacin for planktonic bacteria

Protamine concn (μg/ml)	CFU/ml[a] at a ciprofloxacin concn (μg/ml) of:					
	5, 2.5, or 1	0.5	0.25	0.1	0.05	0
10,000	—	—	—	—	—	3.0×10^3
1,000	—	—	—	—	—	6.6×10^7
100	—	—	—	—	—	5.0×10^8
50	—	NG	NG	NG	NG	6.2×10^8
25	—	NG	NG	NG	NG	$>1.0 \times 10^9$
10	—	NG	NG	NG	1.0×10^2	$>1.0 \times 10^9$
5	—	NG	2.7×10^4	7.0×10^4	7.7×10^5	$>1.0 \times 10^9$
1	—	NG	$>1.0 \times 10^9$	$>1.0 \times 10^9$	$>1.0 \times 10^9$	$>1.0 \times 10^9$
0	NG	3.5×10^2	$>1.0 \times 10^9$	$>1.0 \times 10^9$	$>1.0 \times 10^9$	$>1.0 \times 10^9$

[a] NG, no growth; —, not done. The initial inoculum was 2.3×10^5 CFU/ml.

an overnight culture of *P. aeruginosa* washed two times in phosphate-buffered saline (PBS), pH 7.2, was used as an inoculum. The beakers were incubated at 37°C with shaking at 100 rpm for 48 h. At 12-h intervals, half of the bacterial suspension was discarded and replaced with sterile nutrient broth.

After the 48-h colonization period, catheter sections were rinsed with 10 ml of sterile PBS, pH 7.2, delivered via gravity through a 10-ml pipette. The catheter sections were then immersed in beakers containing 100 ml of nutrient broth or nutrient broth supplemented with protamine sulfate (Lyphomed, Markham, Ontario, Canada), 0.5 μg of ciprofloxacin (Miles Pharmaceuticals, West Haven, Conn.) per ml, or ciprofloxacin combined with protamine sulfate. The concentration of protamine sulfate ranged from 5 to 50 μg/ml. Catheter sections were removed, in triplicate, from each beaker at 0 (pretreatment), 4, 8, and 24 h and processed immediately to measure cell viability as described below.

Measurement of viability of sessile bacterial cells. The outer and inner surfaces of each catheter section were rinsed three times with 10-ml volumes of sterile PBS, pH 7.2, delivered via gravity through a 10-ml pipette to remove loosely adherent bacteria, which were not enumerated. Adherent bacteria were removed from the catheter sections by three cycles of 30-s sonication treatment in ice-cold PBS, followed by vortexing in PBS with 2-mm-diameter glass beads. The cells were diluted in PBS and then plated onto nutrient agar. Cultures were incubated for 48 h at 37°C. Preliminary studies which validated the recovery efficiency of the sonication-vortexing procedure were performed. The catheters were stained with acridine orange and observed under epifluorescent illumination to contain <10 cells per cm^2 (data not shown) following the cell removal procedures.

MIC-MBC assay of planktonic cells. A broth macrodilution assay was used to determine MICs. Briefly, 18-h nutrient broth cultures were washed three times in PBS and then suspended in PBS. A nutrient broth dilution series containing either protamine sulfate, ciprofloxacin, or the various concentrations of the combination was inoculated with 2.3×10^5 cells per ml. The suspensions were incubated at 37°C with shaking at 100 rpm. At 24 h, the tubes containing the MIC and 1 dilution higher were plated onto nutrient agar plates and incubated at 37°C for 24 h.

Electron microscopy. From each of the treatments, bulk-phase medium and catheter sections were taken at various time intervals for scanning electron microscopy (SEM). Planktonic bacteria were filtered through a 0.2-μm-pore-size polycarbonate membrane filter (Nuclepore, Pleasanton, Calif.) and then processed as described below. Specimens were fixed in 2.5% (vol/vol) glutaraldehyde in Dulbecco PBS (pH 7.2) for 1.5 h, rinsed with PBS, and then dehydrated through an ethanol series. Samples were critical point dried and gold-palladium coated. SEM examinations were made on a JSM-840 SEM (JEOL Ltd., Tokyo, Japan).

Data analysis. Assays were performed in triplicate for each of the four replicate experiments, and the results are presented as the mean ± standard deviation of the mean. The data were expressed in terms of percent surviving CFU per square centimeter. A one-way analysis of variance was used to assess the statistical significance of the various treatment effects.

RESULTS

The MICs of protamine sulfate and ciprofloxacin were >10,000 and 0.5 μg/ml, respectively. MICs and MBCs of various treatment combinations for planktonic bacteria are shown in Table 1. The addition of as little as 10 μg of protamine sulfate per ml reduced the MBC of ciprofloxacin by a factor of 4. In the presence of 25 μg/ml, the MBC was reduced at least 20-fold, from 1 to ≤0.05 μg/ml.

Both ciprofloxacin and protamine sulfate reduced the number of viable bacteria associated with the red rubber catheter surfaces. Ciprofloxacin at the MIC (0.5 μg/ml) resulted in decreases in viable counts on the catheter surfaces by averages of 77.5, 64.2, and 58.6% after 4, 8, and 24 h of treatment,

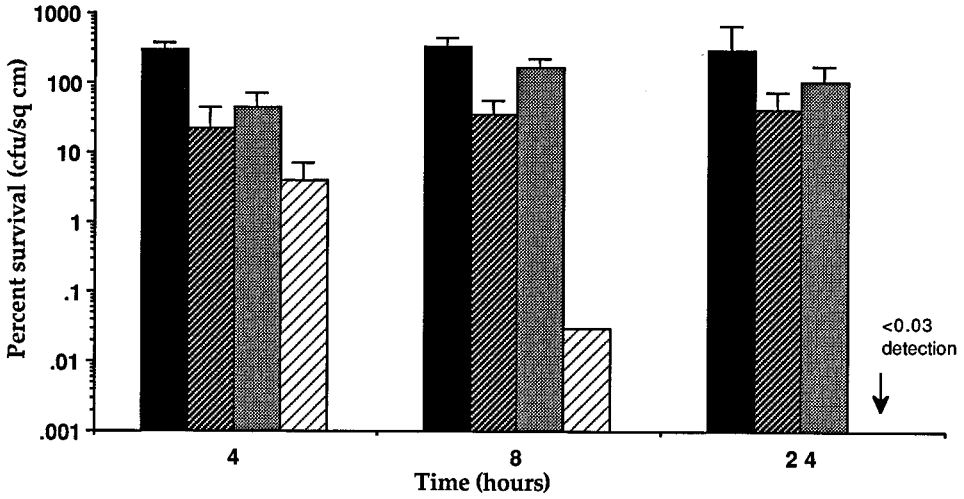

FIG. 1. From left in each group of bars, relative efficacies of nutrient broth, 0.5 μg of ciprofloxacin per ml, 50 μg of protamine sulfate per ml, and protamine sulfate-ciprofloxacin combinations (50 and 0.5 μg/ml, respectively) against 48-h *P. aeruginosa* biofilms as a function of contact time.

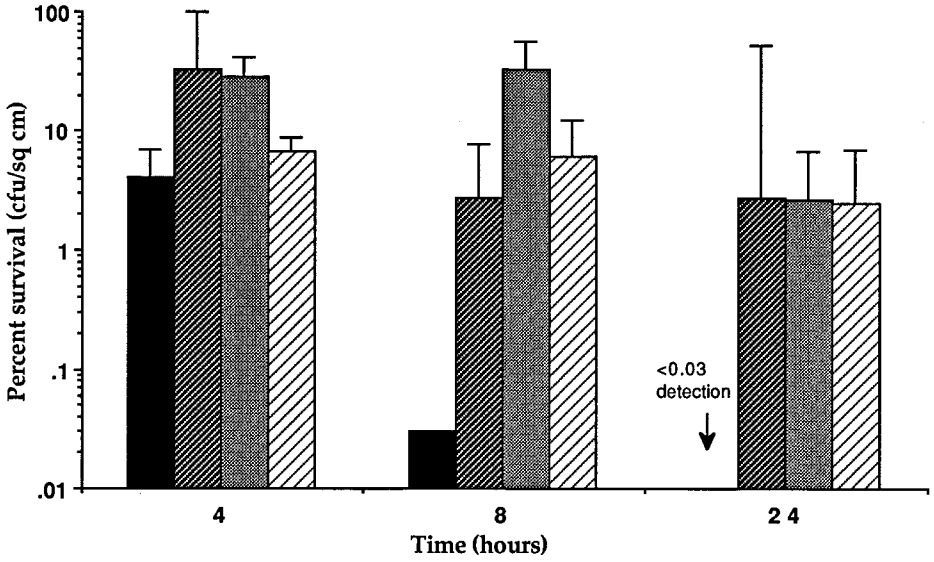

FIG. 2. Effects of protamine sulfate concentration (from left in each group of bars, 50, 25, 10, and 5 μg/ml) on the efficacy of 0.5 μg of ciprofloxacin per ml against 48-h *P. aeruginosa* biofilms as a function of contact time.

respectively (Fig. 1). A 50-μg/ml concentration of protamine sulfate alone reduced the viable cell count by an average of 55.8% after 4 h of treatment. However, after 8 and 24 h of incubation, the viable cell count increased to 171.8 and 107.8% of the time zero values, respectively. The combination of 0.5 μg of ciprofloxacin per ml and 50 μg of protamine sulfate per ml significantly ($P < 0.05$) reduced bacterial cell counts over the 24-h treatment period: viable cell numbers were reduced from 1.4×10^4 to $<5/cm^2$ (detection limit). This represented a >99.9% reduction in the viable count.

When lower concentrations of protamine sulfate were combined with ciprofloxacin at the MIC of 0.5 μg/ml, there was a pronounced decrease in efficacy (Fig. 2). A protamine sulfate concentration of 50 μg/ml provided the greatest effect ($P < 0.05$). While the additive effect of protamine sulfate and ciprofloxacin was calculated at 85.4% for the 50-μg/ml protamine concentration, a >98.7% reduction in viable cell numbers was observed when the combination treatment was applied. These numbers represent averages over the 24-h challenge period.

The results of SEM examinations of *P. aeruginosa* grown on catheter sections are shown in Fig. 3. Cells grown in nutrient broth (untreated control) possessed smooth cell walls, with an average length of 1.0 to 1.5 μm (Fig. 3a). Cells treated with ciprofloxacin were elongated to between 2.4 and >6 μm and demonstrated swollen, filamentous morphologies (Fig. 3b). Some of the cells exhibited lysed cell walls. In the presence of protamine sulfate alone, cells associated with the red rubber catheters were swollen, showing disruptions of the cell wall, with apparent protrusions of the cytoplasmic membrane (Fig. 3c). Some cavities were also observed in the cell wall. In the presence of both protamine and ciprofloxacin, the majority of the biofilm population exhibited extensive elongation and filamentation with multiple small and very large outer membrane vesicles and invaginations (Fig. 3d). The large invaginations predominated at the polar regions. Almost all cells in the upper exposed surfaces of the biofilm were much larger and demonstrated a more pronounced membrane disorganization, while bacteria in lower surfaces were less affected.

The results of SEM studies of planktonic *P. aeruginosa* are shown in Fig. 4. Changes in cell morphology and cell wall structure were similar to those observed in the biofilm SEM studies. However, planktonic cells appeared to be more drastically affected than did the biofilm cells. Ciprofloxacin treatment resulted in cells elongated to average lengths of 3.5 μm (Fig. 4b). Protamine sulfate treatment created significant disruptions in the cell wall (Fig. 4c), as did the combination of ciprofloxacin and protamine sulfate (Fig. 4d).

DISCUSSION

This work examined the potential effect of protamine synergism on the antimicrobial activity of ciprofloxacin against *P. aeruginosa* adhering to catheters. The results demonstrated that the combination of ciprofloxacin with protamine sulfate significantly enhanced the antimicrobial effect. Bacteria associated with surfaces demonstrate an increased resistance to antimicrobial therapy. This biofilm-mediated antimicrobial resistance has been described by several investigators for *P. aeruginosa* (12, 15, 18) and other organisms (4, 10). The results of this study confirmed the resistance of *P. aeruginosa* biofilm bacteria to ciprofloxacin relative to their planktonic counterparts. At the MIC of 0.5 μg/ml, biofilm cell numbers were not significantly reduced. The concentrations of ciprofloxacin required to eliminate biofilm organisms would likely be higher than could be safely administered in the clinical setting. The concentrations of antibiotics required to kill biofilm bacteria under in vitro or in vivo conditions can be in excess of 200 times the MIC (2, 8). For ciprofloxacin, the maximum achievable concentrations in serum in healthy male volunteers are in the range of 2 to 4 μg/ml (6). Our approach to overcoming biofilm-mediated resistance was to improve the efficacy of antimicrobial therapy with a surface-active agent, protamine sulfate.

It was clear that the effects of protamine were dose related. Ciprofloxacin or protamine sulfate, when used alone, had a more pronounced effect on planktonic bacterial cells than on sessile cells (Fig. 1; Table 1). These findings are similar to those recently described by Selan et al. (21), who showed that the proteolytic enzyme serratiopeptidase greatly enhanced the efficacy of ofloxacin against planktonic biofilm bacteria. Since

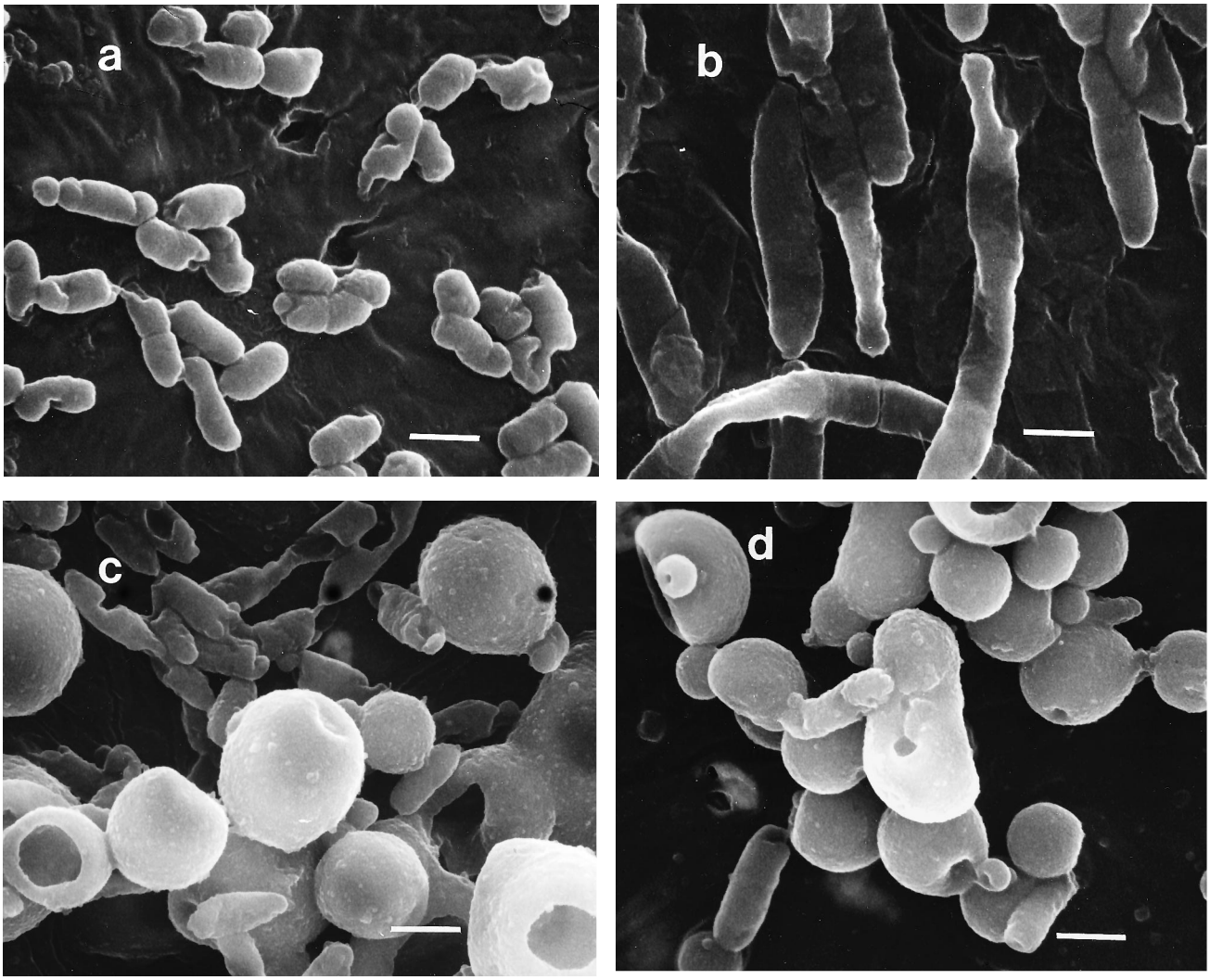

FIG. 3. Morphological responses of 48-h *P. aeruginosa* biofilms associated with catheter segments incubated at 37°C with shaking at 100 rpm exposed to antibiotic-free nutrient broth (a), 0.5 μg of ciprofloxacin per ml (b), 50 μg of protamine sulfate per ml (c), and 0.5 μg of ciprofloxacin per ml plus 50 μg of protamine sulfate per ml (d). The contact period was 24 h. Bars = 1 μm.

the additive antimicrobial effect of protamine sulfate and ciprofloxacin dosed separately was less than the combined effect (85.4 versus 98.7% reduction), our data suggest that the two compounds are acting in a synergistic fashion.

Protamine has been shown to possess growth-inhibiting properties against various microorganisms. Results of the present investigation also corroborate the antibacterial property of protamine sulfate when used alone. However, viable cell numbers were significantly reduced only during the first 4 h of incubation (Fig. 1), after which growth occurred. The observed synergistic effect may be due to an alteration in membrane permeability and a dilation of ionic channels by the protamine sulfate, which facilitates transport of the antibiotic to the cytoplasm (1, 16). Alternatively, the protamine sulfate may have denatured the complex extracellular polymeric structure of the *P. aeruginosa* biofilm, enhancing penetration of the ciprofloxacin through the biofilm. Elongation and membrane disorganization of *P. aeruginosa* produced by exposure to ciprofloxacin was seen in the present study. The morphological changes are the result of DNA gyrase inhibition (14). The formation of vesicles and vacuoles as well as other membrane disorganizations may represent the early stages of cell lysis.

The 50-μg/ml protamine sulfate concentration used in these studies is similar to that used in clinical practice during cardiac surgery (6). Binding of this compound to plasma proteins may reduce its effective concentration in the body, and there may be allergic responses in some patients. For this reason, application of protamine sulfate and other surface-active agents may be limited to topical and/or instillation applications. We are in the process of evaluating other surface-active agents for their ability to enhance the efficacy of systemically applied antibiotics.

In summary, we have demonstrated a significant, synergistic effect of protamine sulfate on the efficacy of ciprofloxacin against *P. aeruginosa* biofilms. If these in vitro findings can be confirmed in an in vivo model, the use of surface-active synergistic agents such as protamine sulfate may overcome the resistance of biofilm-related infections to current antibiotic therapies. This type of therapeutic approach could be particularly useful in the treatment of recalcitrant, biomaterial-related infections.

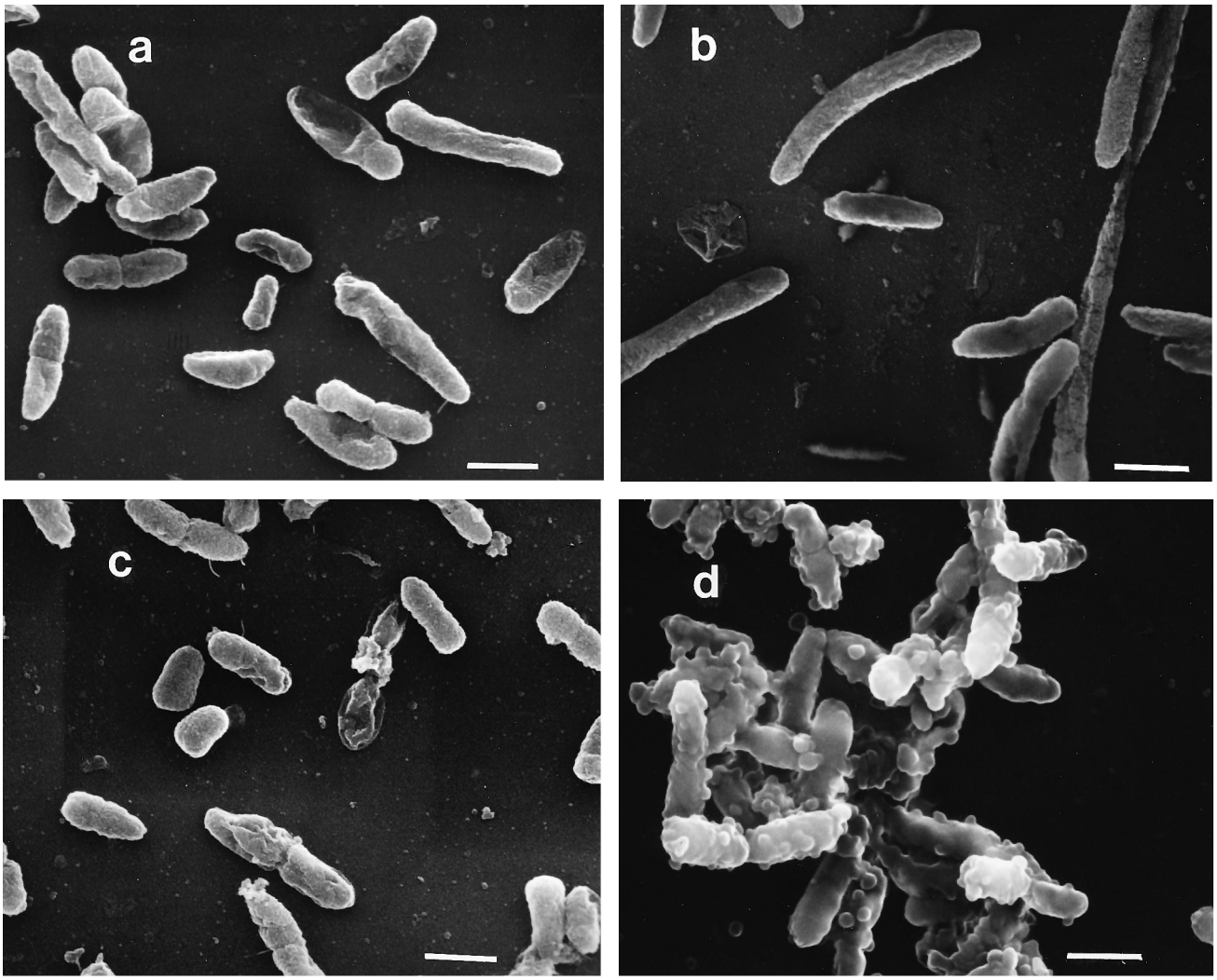

FIG. 4. Morphological responses of *P. aeruginosa* planktonic cells incubated at 37°C with shaking at 100 rpm exposed to antibiotic-free nutrient broth (a), 0.5 μg of ciprofloxacin per ml (b), 50 μg of protamine sulfate per ml (c), and 0.5 μg of ciprofloxacin per ml plus 50 μg of protamine sulfate per ml (d). The contact period was 24 h. Bars = 1 μm.

ACKNOWLEDGMENTS

This research was supported by grants from Miles-Canada, the Natural Sciences and Engineering Research Council of Canada, and The Hospital For Sick Children Research Institute.

We thank the reviewers for their helpful comments and suggestions.

REFERENCES

1. **Antohi, S., and A. Popescu.** 1979. Lethal effect of protamine and histone on competent *Bacillus subtilis* cells. Mol. Gen. Genet. **170:**345–349.
2. **Anwar, H., M. K. Dasgupta, and J. W. Costerton.** 1990. Testing the susceptibility of bacteria in biofilms to antibacterial agents. Antimicrob. Agents Chemother. **34:**2043–2046.
3. **Anwar, H., G. H. Shand, K. H. Ward, K. H. Ward, M. R. W. Brown, K. E. Alpor, and J. Gower.** 1985. Antibody response to acute *Pseudomonas aeruginosa* infection in a burn wound. FEMS Microbiol. Lett. **29:**225–230.
4. **Anwar, H., J. L. Strap, and J. W. Costerton.** 1992. Eradication of biofilm cells of *Staphylococcus aureus* with tobramycin and cephalexin. Can. J. Microbiol. **38:**618–625.
5. **Anwar, H., J. L. Strap, and J. W. Costerton.** 1992. Establishment of aging biofilms: possible mechanism of bacterial resistance to antimicrobial therapy. Antimicrob. Agents Chemother. **36:**1347–1351.
6. **Canadian Pharmaceutical Association.** 1995. Compendium of pharmaceuticals and specialties, p. 261–263, 1121–1122. Canadian Pharmaceutical Association, Ottawa, Canada.
7. **Christensen, G. D., L. M. Baddour, D. L. Hasty, G. H. Lowrance, and W. A. Simpson.** 1989. Microbial and foreign body factors in the pathogenesis of medical device infections, p. 59–70. *In* A. L. Bisno and F. A. Waldvogel (ed.), Infections associated with indwelling medical devices. American Society for Microbiology, Washington, D.C.
8. **Costerton, J. W., A. E. Khoury, K. H. Ward, and H. Anwar.** 1993. Practical measures to control device-related bacterial infections. Int. J. Artif. Organs **16:**765–770.
9. **Costerton, J. W., and E. S. Lashen.** 1984. Influence of biofilm on efficacy of biocides on corrosion-causing bacteria. Mater. Perform. **23**(2)**:**13–17.
10. **Farber, B. F., M. H. Kaplan, and A. G. Clogston.** 1988. *Staphylococcus epidermidis* extracted slime inhibits the antimicrobial action of aminoglycocide antibiotics. J. Infect. Dis. **161:**37–40.
11. **Furusawa, T., T. Uete, T. Kawada, and A. Okuma.** 1986. Resistance to cefsulodin and gentamicin in five areas of Japan between 1980 and 1983. J. Antimicrob. Chemother. **17:**755–762.
12. **Giwercman, B., E. T. Jensen, N. Høiby, A. Kharazmi, and J. W. Costerton.** 1991. Induction of β-lactamase production in *Pseudomonas aeruginosa* biofilm. Antimicrob. Agents Chemother. **35:**1008–1010.
13. **Gristina, A.** 1987. Biomaterial-centered infection: microbial adhesion vs. tissue integration. Science **237:**1588–1595.
14. **Hooper, D. C., and J. S. Wolfson.** 1991. Fluoroquinolone antimicrobial agents. N. Engl. J. Med. **324:**384–394.
15. **Hoyle, B. D., J. Alcantara, and J. W. Costerton.** 1992. *Pseudomonas aeruginosa* biofilm as a diffusion barrier to piperacillin. Antimicrob. Agents Chemother. **36:**2054–2056.
16. **Miller, B. F., A. Abrams, A. Dorfman, and M. Klein.** 1942. Antibacterial

properties of protamine and histone. Science **96:**428–430.
17. **Murray, B. E.** 1989. Problems and mechanisms of antimicrobial resistance. Infect. Dis. Clin. N. Am. **3:**434–439.
18. **Nickel, J. C., I. Ruseska, J. B. Wright, and J. W. Costerton.** 1985. Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. Antimicrob. Agents Chemother. **27:**619–624.
19. **Parsons, C. L., C. Stauffer, and J. D. Schmidt.** 1981. Impairment of antibacterial effect of bladder surface mucin by protamine sulfate. J. Infect. Dis. **144:**180–184.
20. **Richards, G. K.** 1976. Resistance to infection, p. 65–77. *In* S. O. Freedman and P. Gold (ed.), Clinical immunology. Harper & Row, New York.
21. **Selan, L., F. Berlutti, C. Passariello, M. R. Comodi-Ballanti, and M. C. Thaller.** 1993. Proteolytic enzymes: a new treatment strategy for prosthetic infections? Antimicrob. Agents Chemother. **37:**2618–2621.
22. **Shand, G. H., H. Anwar, J. Kadurugamuwa, M. R. W. Brown, S. H. Silverman, and J. Melling.** 1985. In vivo evidence that bacteria in urinary tract infections grow under iron-restricted conditions. Infect. Immun. **48:**35–39.
23. **Suci, P. A., M. W. Mittelman, F. P. Yu, and G. G. Geesey.** 1994. Investigation of ciprofloxacin penetration into *Pseudomonas aeruginosa* biofilms. Antimicrob. Agents Chemother. **38:**2125–2133.
24. **Teichman, J. M. H., V. E. Abraham, P. C. Stein, and C. L. Parsons.** 1994. Protamine sulfate and vancomycin are synergistic against *Staphylococcus epidermidis* prosthesis infection *in vivo*. J. Urol. **152:**213–216.
25. **Vas, S. I.** 1985. Peritonitis, p. 403–439. *In* K. D. Nolph (ed.), Peritoneal dialysis. Martinus Nijhoff, Boston.