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Dextranase Enhances Antibiotic Efficacy in Experimental Viridans Streptococcal Endocarditis

AZZAM SALEH MGHIR, ANNE CLAUDE CREMIEUX,* RONAN JAMBOU, MARTINE MUFFAT-JOLY, JEAN JACQUES POCIDALO, AND CLAUDE CARBON

Institut National de la Santé et de la Recherche Médicale Unité 13, Hôpital Claude-Bernard Bichat, Paris, France

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In endocarditis, exopolysaccharide production by viridans streptococci has been associated with delayed antimicrobial efficacy in cardiac vegetations. We compared the efficacies of temafloxacin alone and in combination with dextranase, an enzyme capable of hydrolyzing 20 to 90% of the bacterial glycocalyx, in a rabbit model of endocarditis. In in vivo experiments, rabbits were infected intravenously with 10^8 *Streptococcus sanguis* organisms and were treated 6 days later with temafloxacin (50 mg/kg of body weight intramuscularly twice a day) alone or combined with dextranase (1,000 U per rabbit per day intravenously). After 4 days of treatment (day 11), the animals were sacrificed and vegetations were quantitatively cultured. For ex vivo experiments, rabbits were infected as stated above and, on day 11, vegetations were excised aseptically and incubated in vitro in rabbit serum alone (control) or with temafloxacin or temafloxacin plus dextranase at concentrations similar to peak levels in plasma. In vitro, dextranase alone had no antimicrobial effect. In vivo and ex vivo, temafloxacin combined with dextranase was more effective than temafloxacin alone ($P < 0.05$). Our results suggest that dextranase is able to increase the effects of temafloxacin by reducing the amount of bacterial glycocalyx in infected vegetations, as confirmed in vitro by electron microscopy showing a markedly reduced amount of glycocalyx and a more clearly visible fibrin matrix.

Previous studies have suggested that the difficulty in curing endocarditis could be due to heterogeneous diffusion of some antibiotics into fibrin vegetations (5) or to the reduced metabolic activity of organisms inside vegetations, especially the older ones (11, 12, 14). However, other factors may account for the difficulty in curing endocarditis, among them the secretion of exopolysaccharide by some organisms inside vegetations.

Viridans streptococci still represent a major cause of native valve endocarditis. These streptococci are known to produce an exopolysaccharide (glycocalyx) composed predominantly of dextran in the human mouth and have been shown to produce dextran in experimental cardiac vegetations (18). This production is strain dependent and can be quantified by histochemical techniques (18) or tryptophan quantitative assay (9). Glycocalyx-producing strains form larger vegetations (18). Also, the presence of glycocalyx surrounding viridans streptococci within cardiac vegetations adversely affects penicillin efficacy (8, 20). Experimental vegetations infected by exopolysaccharide-producing strains of viridans streptococci are more difficult to sterilize than those infected by exopolysaccharide-deficient strains (20). Moreover, combining penicillin with dextranase, an enzyme capable of hydrolyzing 20 to 90% of the bacterial glycocalyx, has been shown to facilitate sterilization of experimental vegetations infected with a glycocalyx-producing strain of *Streptococcus sanguis* (8).

Glycocalyx could alter the efficacy of penicillin by increasing the size of the vegetations and thus alter penicillin diffusion into the core of the vegetations, as suggested by a previous study which showed that this antibiotic diffuses with a slight gradient between the periphery and the core of the vegetations (5). Alternatively, the low growth rate of biofilm-enclosed bacteria in vegetations could explain the relative resistance of these bacteria to the bactericidal activity of β-lactams (14, 15). Finally, exopolysaccharide may interact with penicillin and render it biologically inactive or may interfere with its action on the bacterial cell wall, an interaction previously noted between *Staphylococcus epidermidis* slime and glycopeptide (13). Should this be the case, exopolysaccharide interference in antimicrobial action should be visible in in vitro tests.

To better understand the influence of exopolysaccharide production on antibiotic efficacy, it appeared interesting to evaluate whether this interaction could be observed with antibiotics other than β-lactams, in vitro and in infected vegetations. For this purpose we compared, in a rabbit model of *S. sanguis* vegetations similar to that used for penicillin (8), the efficacies of temafloxacin alone and combined with dextranase in in vivo and ex vivo experiments. Temafloxacin, a fluoroquinolone, was selected because its efficacy in another experimental model of viridans streptococci was similar to that of penicillin, and its diffusion into vegetations was demonstrated to be homogeneous (5).

MATERIALS AND METHODS

Test strain. A strain of *S. sanguis* I (ATCC 10556) (16) was used in this study. This strain was shown to be a high glycocalyx producer in vitro. Strain cultures were kept frozen in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) with 15% glycerol.

In vitro antibiotic susceptibility tests. MICs and MBCs were determined in triplicate by using the tube macrodilution method and Mueller-Hinton broth. The inocula were diluted from log-phase cultures to obtain a final concentration of 10^5 CFU/ml. The MIC was defined as the lowest concentration of antibiotic that prevented turbidity in the test tube after 24 h of incubation, and the MBC was defined as the lowest concentration of antibiotic that reduced the original organism count by 99.9%.

Time-kill curves were plotted for log-phase inocula of 10^7

* Corresponding author. Mailing address: Hôpital Bichat, 46, rue Henri-Huchard, 75877 Paris Cedex 18, France. Phone: 33 (1) 40 25 70 00. Fax: 33 (1) 40 25 88 45.

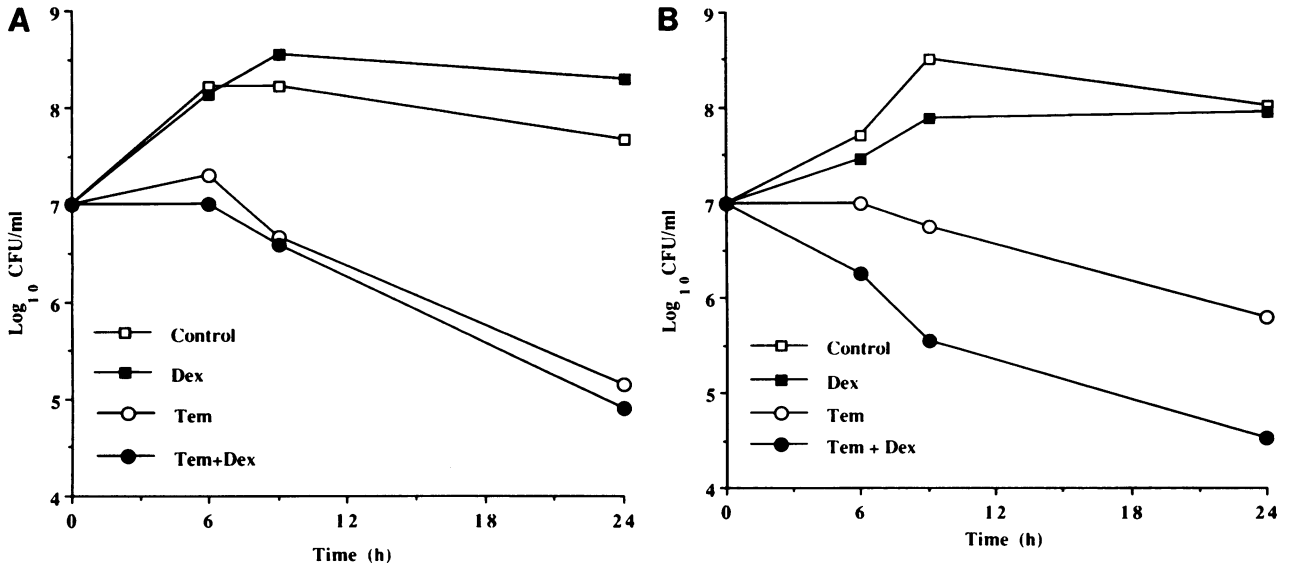

FIG. 1. Time-kill curves showing the effect of temafloxacin alone or combined with dextranase on *S. sanguis* grown in Mueller-Hinton broth (A) or rabbit serum (B). Tem, temafloxacin (7.4 μg/ml); Dex, dextranase (25 U/ml).

CFU/ml in Mueller-Hinton broth or in rabbit serum containing temafloxacin (Abbott Laboratories, Rungis, France) alone, dextranase alone, or temafloxacin plus dextranase. The concentrations of temafloxacin and dextranase were similar to the peak levels in serum obtained in the series of treated animals in previous experiments done with the same doses (6, 8).

Dextranase was obtained from Sigma (La Verpilliere, France) as a lyophilized powder with activity ranging from 250 to 500 U/mg.

Experimental endocarditis. Aortic valve endocarditis was induced in New Zealand White female rabbits weighing between 2 and 3 kg by a modified version of the method of Perlman and Freedman (19). Briefly, a polyethylene catheter was inserted through the right carotid artery into the left ventricular cavity and was left in place throughout the experiment. Twenty-four hours after placement of the catheter (day 1), rabbits were inoculated via the marginal ear vein with 10^8 CFU of *S. sanguis* in 1 ml of saline. This resulted in endocarditis in 100% of the inoculees when the catheter was correctly placed, as demonstrated by positive blood cultures on day 7 and by the presence of infected vegetations on the aortic valves at the time of sacrifice.

Ex vivo studies. Fifteen untreated rabbits were killed on day 11 by pentobarbital injection. Infected vegetations were excised and incubated in vitro for 24 h at 37°C in rabbit serum alone (control) or serum containing temafloxacin (7.4 μg/ml) alone or in combination with dextranase (25 U/ml). As for time-kill curves, these concentrations were similar to the peak levels obtained in the sera of treated animals (6, 8).

Therapeutic studies in vivo. Six days after infection (day 7), animals were treated with temafloxacin (50 mg/kg of body weight twice a day, given intramuscularly) alone or combined with dextranase (1,000 U per rabbit once daily intravenously). The dose of temafloxacin was the same as that used in a previous experimental study in which the concentrations obtained in plasma were comparable to those achieved in humans (6). The dose of dextranase was similar to that previously used by Dall et al. (8) in combination with penicillin. Each treatment regimen was administered for 4 days. Animals were killed on day 11, 12 h after the last dose of temafloxacin and 24 h after the last dose of dextranase. Untreated control rabbits were killed at the time corresponding to the end of therapy (day 11).

All vegetations from each rabbit were excised, separately weighed, and homogenized in 0.5 ml of saline and quantitatively cultured on blood agar plates for 24 h at 37°C. Drug carryover was avoided by serial dilutions and spreading of the subculture (50 μl) on agar plates. The vegetations were considered to be sterile when the culture showed no growth after 48 h of incubation at 37°C, and the number of CFU was recorded as the lowest detectable bacterial count from 2.0 to 2.3 $\log_{10}$ CFU/g of vegetation, depending on the weight of the vegetations.

Electron microscopy. *S. sanguis* organisms were grown in rabbit serum-supplemented culture medium (Mueller-Hinton broth) at 37°C with or without dextranase (25 U/ml) for 24 and 48 h. A control sample of bacteria was grown without rabbit serum under the same conditions. Bacteria were pelleted by centrifugation and washed twice in 0.2 M cacodylate buffer (pH 7.3). Samples were then fixed in 2.5% glutaraldehyde overnight and postfixed in ruthenium-osmium by the method of Dall et al. (8).

After excision, *S. sanguis*-infected vegetations from control rabbits were grown under the same conditions with and without dextranase and processed as described above.

Statistical analysis. Results are expressed as means ± standard deviations. Bacterial counts in vegetations and vegetation weights from the various experimental groups were compared by an analysis of variance. The Bonferoni method was applied for group-to-group comparisons. The number of rabbits with sterile vegetations was compared by using χ^2 analysis with Yates' continuity correction. A P value of <0.05 was considered significant.

RESULTS

In vitro studies. The MIC and MBC of temafloxacin for the study strain were 2 and 4 μg/ml, respectively. Time-kill curves in Mueller-Hinton broth (Fig. 1A) and in serum (Fig. 1B) showed that temafloxacin alone was slowly bactericidal. Dex-

TABLE 1. Results of treating of *S. sanguis* endocarditis with temafloxacin alone or combined with dextranase

Treatment[a]	Vegetation wt (mg)	$\log_{10}$ CFU/g of vegetation (mean ± SD)	No. of rabbits with sterile vegetations/ total no.
None (control)	77 ± 31	9.70 ± 0.50	0/5
Temafloxacin	55 ± 30	6.96 ± 1.23[b]	0/6
Temafloxacin + dextranase	29 ± 13[c]	3.80 ± 1.67[d]	4/11

[a] Rabbits were treated intramuscularly for 4 days with temafloxacin (50 mg/kg twice a day) alone or combined with dextranase (1,000 U per rabbit given intravenously once daily).
[b] $P < 0.05$ versus control.
[c] $P = 0.01$ versus control and $P = 0.12$ versus temafloxacin alone.
[d] $P < 0.001$ versus control and temafloxacin alone.

tranase alone had no antimicrobial effect. In Mueller-Hinton broth, temafloxacin kill curves were unchanged by the addition of dextranase. In contrast, in serum, the addition of dextranase increased the killing rate of temafloxacin (1.2 and 2.5 $\log_{10}$ decreases at 24 h with temafloxacin and the combination, respectively).

In vivo and ex vivo experiments. All five control rabbits had infected vegetations with a high mean bacterial count per gram of vegetation (Table 1). Temafloxacin alone significantly reduced bacterial counts in vegetations compared with those of control animals ($P < 0.05$); however, no animal had sterile vegetations. When dextranase was combined with temafloxacin, bacterial counts in vegetations were significantly reduced compared with those for temafloxacin alone ($P < 0.001$), and 4 of 11 animals had sterile vegetations. Also, the combination of dextranase and temafloxacin significantly reduced the mean vegetation weight compared with that of the control ($P < 0.01$).

Similar results were obtained in ex vivo experiments (Table 2). Bacterial counts were significantly reduced in vegetations incubated in serum containing temafloxacin and dextranase compared with vegetations incubated in temafloxacin alone ($P < 0.05$), and four of five vegetations were sterile with this combination.

Electron microscopy. Electron microscopy of ruthenium red-stained *S. sanguis* cells grown in rabbit serum showed bacterial cells surrounded by glycocalyx (Fig. 2A). When dextranase was added, the amount of glycocalyx was markedly reduced (Fig. 2B). Glycocalyx was not visible when bacteria were grown without rabbit serum (data not shown).

TABLE 2. Antibacterial effect of temafloxacin alone or combined with dextranase on *S. sanguis*-infected vegetations incubated in vitro in serum

Treatment[a]	$\log_{10}$ CFU/g of vegetation (mean ± SD)	No. of sterile vegetations/total no.
None (control)	8.75 ± 0.80	0/5
Temafloxacin	6.08 ± 2.72[b]	1/5
Temafloxacin + dextranase	2.46 ± 0.67[c]	4/5[d]

[a] Vegetations were incubated in vitro for 24 h at 37°C in rabbit serum alone (control) or containing temafloxacin (7.4 μg/ml) alone or combined with dextranase (25 U/ml).
[b] $P = 0.08$ versus control.
[c] $P < 0.01$ versus control and $P < 0.05$ versus temafloxacin alone.
[d] $P = 0.053$ versus control.

In infected vegetations incubated in serum (Fig. 3A), bacteria were surrounded by exopolysaccharide and embedded in a fibrin matrix made of exopolysaccharides and fibrin. When vegetations were incubated in rabbit serum with dextranase (Fig. 3B), the amount of glycocalyx was markedly reduced and the fibrin matrix could be seen more distinctly.

DISCUSSION

Ours results suggest that dextranase is able to increase the efficacy of temafloxacin in vitro, ex vivo, and in vivo by reducing the amount of bacterial glycocalyx in vegetations, as shown by electron microscopy. This effect was not observed in vitro when the bacteria were grown without rabbit serum, and, under this growth condition, glycocalyx secretion could not be detected electron microscopically. The beneficial effect of dextranase could not be due to an antibiotic effect of this enzyme, since we showed that it had no antimicrobial effect in vitro on killing. Moreover, Dall et al. (8) previously showed that a 5-day treatment with dextranase alone at the same dosage had no effect on vegetation titers in left-sided, *S. sanguis* experimental endocarditis. Nor could the beneficial effect of dextranase be attributed to the enhancement of spontaneous, polymorphonuclear cell-mediated intravegetation clearance of bacteria. Previous experimental studies have shown that, in left-sided endocarditis, in contrast to right-sided endocarditis (1), polymorphonuclear cell-mediated intravegetation clearance of bacteria is not observed. The absence of an in vivo effect of dextranase alone (8) tends to exclude a direct effect of dextranase on spontaneous clearance of bacteria from vegetations. Thus, the effect of dextranase should be explained, as for penicillin (8), by the partial digestion of the glycocalyx by dextranase which would increase the susceptibility of glycocalyx-producing streptococci to temafloxacin.

In a similar experiment, Bayer et al. (2) showed that alginase enhanced the efficacy of amikacin in left-sided experimental endocarditis caused by mucoid *Pseudomonas aeruginosa.*

Our results and those of others suggest that the presence of a glycocalyx may compromise antimicrobial efficacy. Several hypotheses could explain this deleterious effect. First, exopolysaccharide may interact with the antibiotic or interfere with its target on the bacteria. This interaction should be nonspecific, which would explain the results obtained with penicillin and temafloxacin. Second, exopolysaccharide could alter antibiotic efficacy by increasing the size of vegetations, thus affecting antibiotic diffusion toward the core of the vegetation. We showed that vegetations in rabbits treated with temafloxacin plus dextranase were significantly smaller than those in control rabbits. Compared with vegetations in rabbits treated with temafloxacin alone, the difference was not significant. We previously found that temafloxacin diffusion was homogeneous throughout the vegetation (6). Thus vegetation size should not constitute an impediment for the efficacy of this antibiotic, unlike penicillin. Third, as suggested by others (15), the bacteria embedded in biofilm have lower metabolic activity. Reducing the amount of exopolysaccharide could lead to an acceleration of their metabolic rate and better activity of antibiotics, for example, cell wall and DNA synthesis inhibitors (15). The influence of the metabolic state of the bacteria on penicillin efficacy has been suggested for nutritionally variant streptococci in endocarditis (7). Procaine penicillin G was significantly less effective when therapy was started on day 11 (10 days postinfection) rather than on day 7. This could be explained by the altered metabolic rate of the intravegetational bacteria surrounded by a thick layer of exopolysaccharide as seen electron microscopically on day 11 but not on day 7 (14).

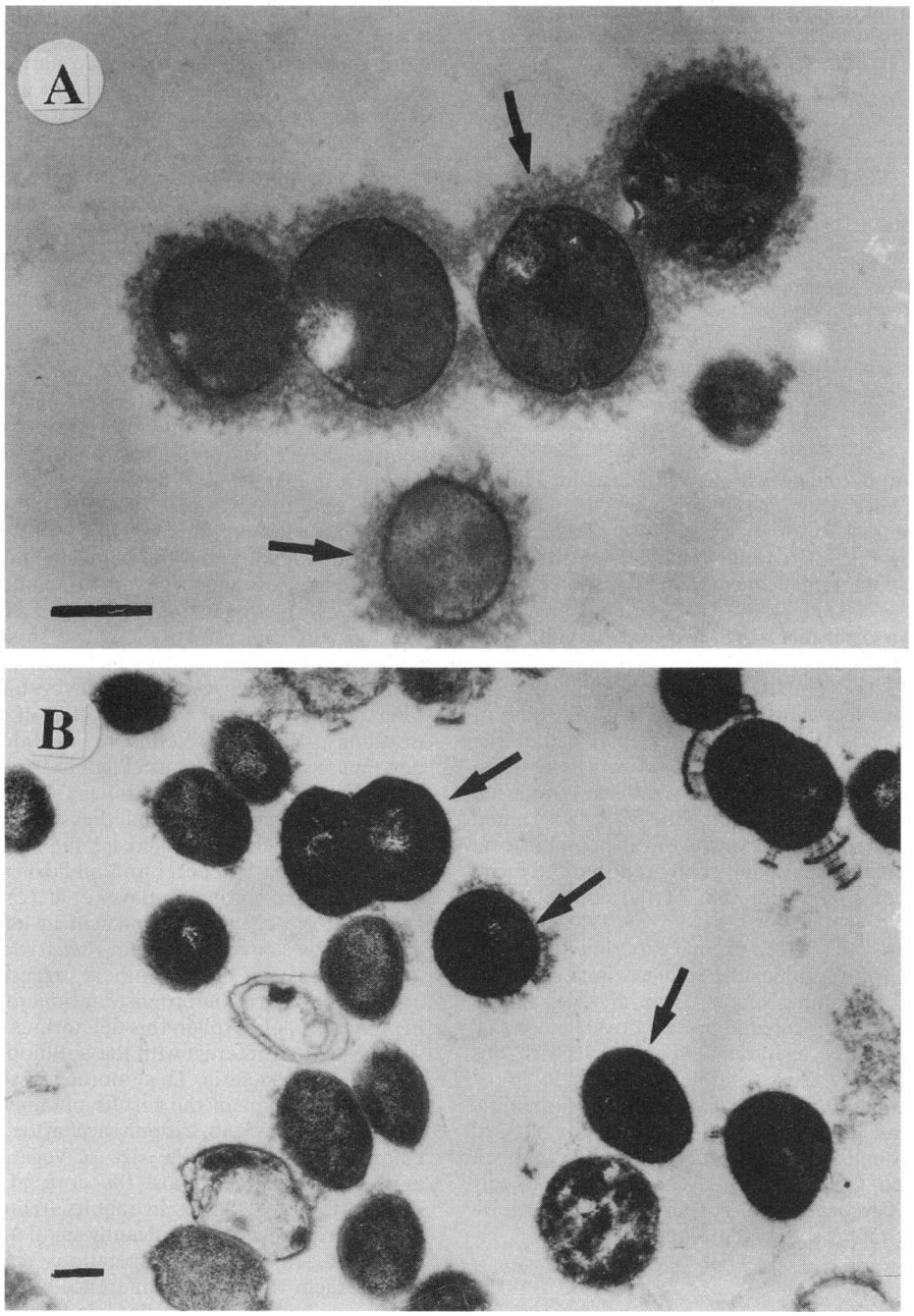

FIG. 2. Transmission electron micrograph of ruthenium red-stained *S. sanguis* grown in rabbit serum. (A) Control, showing abundant cell-adherent glycocalyx (arrows). (B) With dextranase. The amount of glycocalyx is markedly reduced (arrows). Bars = 125 mm.

As in the present study, another explanation for this difference of efficacy of penicillin between day 7 and day 11 could be the larger size of vegetations on day 11 than on day 7.

Other attempts to enzymatically reduce the size of vegetations in order to improve the response to antimicrobial agents have been made. Two investigations (3, 10) evaluated the influence of fibrinolytic treatment on the efficacy of penicillin in left-sided, *S. sanguis* experimental endocarditis. In both studies, in spite of a significant reduction in the size of vegetations, the efficacy of penicillin was not enhanced by fibrinolysis of experimental *S. sanguis* vegetations. These results suggested that reduction of the size of the vegetations by itself, in the present study as in others (7), is not sufficient to explain the enhancement of penicillin efficacy. Therefore, it seems that modification of the metabolism of bacteria might play the crucial role.

In conclusion, the efficacy of temafloxacin, as for penicillin, can be significantly potentiated by the removal of exopolysaccharide in vegetations. This effect could be mediated by modification of the metabolic state of bacteria within the vegetations. Dextranase has previously been used in humans, as a local dental application (17). Further experiments should

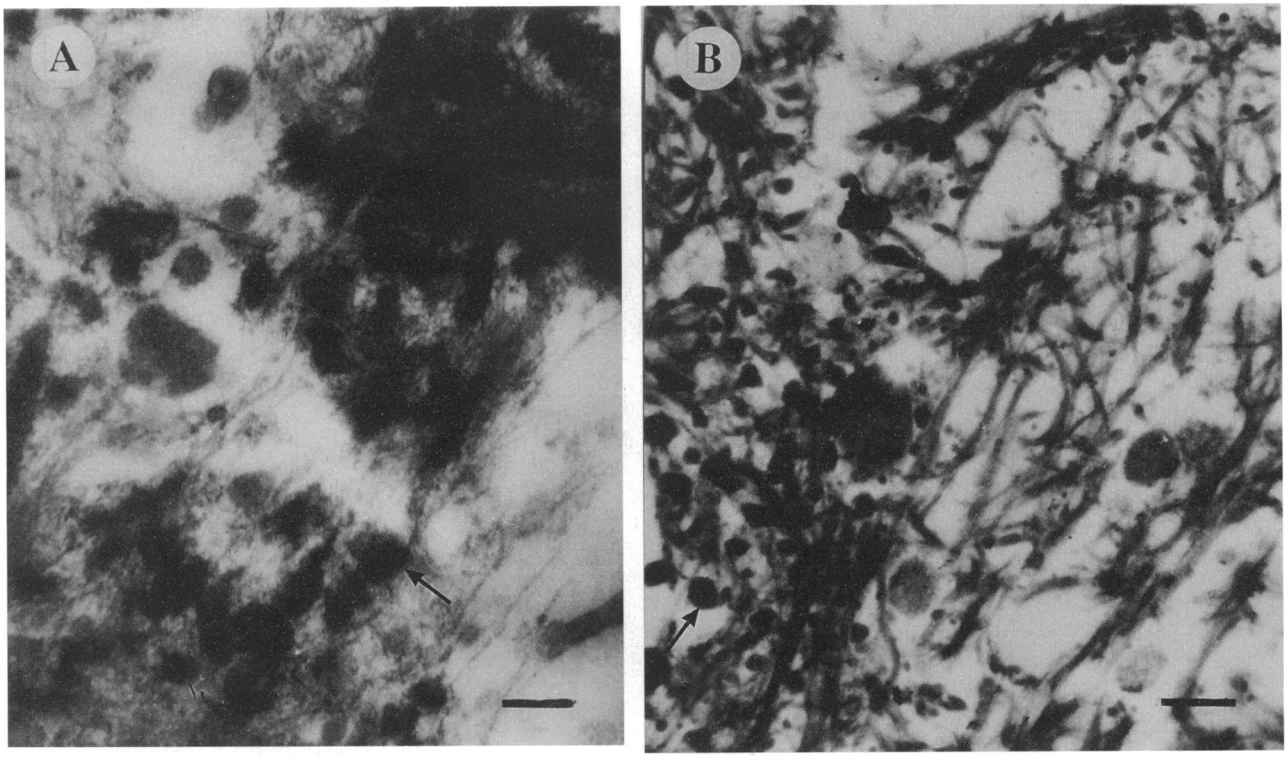

FIG. 3. Transmission electron micrograph of *S. sanguis*-infected vegetations incubated in rabbit serum. (A) Control. There is abundant cell-adherent glycocalyx (arrow), and the fibrin matrix is poorly visualized. (B) With dextranase. The amount of glycocalyx is markedly reduced (arrow), and the fibrin matrix appears much more clearly.

explore adjunctive therapy which could digest the bacterial glycocalyx and render the antibiotic more effective in eradicating glycocalyx-enveloped bacteria. Since bacterial growth conditions in vegetations share some similarities with those encountered in prosthetic device infections (4), such a therapeutic approach could also be beneficial in curing those infections.

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