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Effect of Antibiotics on Protease Production by a Viridans Streptococcus

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A viridans streptococcus (*Streptococcus* MG *intermedius* 974) isolated from a confirmed case of subacute bacterial endocarditis was studied for the production of extracellular proteases during exponential growth and after penicillin (0.10 μg/ml) and/or streptomycin (20 μg/ml) treatment. Exponentially growing cultures produced a variety of extracellular proteases, as determined by the elution profiles of active proteins from Sephadex G-100 and Sepharose 4B columns. Examination of supernatant fluids from cultures of *S.* MG *intermedius* treated with penicillin or streptomycin for 12 h indicated a reduction of at least 50% in the number of different proteolytic species produced. However, some of the proteases produced by the cultures during penicillin or streptomycin treatment had significantly higher specific activities when compared with proteases produced by exponentially growing cells. The combination of penicillin and streptomycin further reduced both the number and the specific activities of the extracellular proteases on a cell dry weight basis.

Recent evidence indicates that the viridans streptococci appear to have a natural tolerance to penicillin and other cell wall inhibitors (3). Lysis of *Streptococcus sanguis* was not observed at minimum growth-inhibitory concentrations, and there was only a minor loss in the colony-forming ability of the cells (3). This tolerance of *S. sanguis* to antibiotics which act on the assembly of the cell wall was interpreted as being indicative of an autolysin-defective organism, because active murein hydrolases have been shown to correlate with the irreversible lytic effects of cell wall inhibitors (11, 12). However, the release of substantial amounts of extracellular glycerol lipids into the medium was also noted during antibiotic treatment (2). It appears in some bacteria, therefore, that inhibition of cell growth by "bactericidal" antibiotics directed against cell wall synthesis does not necessarily correlate with cell death or loss of the ability to elaborate extracellular products, some of which might delay or aggravate effective treatment of chronic diseases such as subacute bacterial endocarditis.

In another study Mattingly et al. (5) demonstrated that a cariogenic strain of *Streptococcus mutans* did not lyse when treated with penicillin, cycloserine, or vancomycin. Surprisingly, in addition to inhibiting peptidoglycan synthesis, these wall antibiotics markedly suppressed the synthesis of both RNA and protein. However, these antibiotic-treated cells retained metabolic activity because they were capable of synthesizing and catabolizing an intracellular polysaccharide during antibiotic treatment (5). Similarly, Straus et al. (9) demonstrated that another viridans streptococcus, *Streptococcus* MG *intermedius*, was capable of synthesizing and excreting considerable quantities of extracellular protein and carbohydrate- and glycerol-containing macromolecules under nongrowing conditions induced by deprivation of essential cell wall peptidoglycan (glutamate/glutamine) or nonwall (cystine) amino acids. Notably, a significant increase in the elaboration of extracellular proteases was observed when these cells were incubated under nutritionally limiting conditions.

Because extracellular protease production could conceivably contribute to tissue damage, even during prolonged antimicrobial chemotherapy (1) in diseases such as subacute bacterial endocarditis, and could partially account for the chromic nature of the disease, we investigated the potential of antibiotic (penicillin and/or streptomycin)-treated cultures of *S.* MG *intermedius* to elaborate extracellular proteases.

MATERIALS AND METHODS

Growth conditions. *S.* MG *intermedius* 974 was isolated from a confirmed case of subacute bacterial endocarditis and was supplied by R. Facklam, Center for Disease Control, Atlanta, Ga. Culture maintenance and growth in a chemically defined medium (FMC) have been described (9). In experiments in which the

incorporation of [^{3}H]leucine into protein was monitored, the specific activity of [^{3}H]leucine was increased by decreasing the normal concentration of leucine in the medium (100 to 30 μg/ml). Growth was monitored by measuring the absorbance in a Coleman Junior II spectrophotometer at 675 nm. The observed optical density was multiplied by 1,000 and converted to adjusted optical density (AOD) units so that readings would agree with Beer's law and be proportional to bacterial mass (10). Turbidities were quantitated by dry weight determinations of 10-ml samples of washed stationary and exponential-phase cell suspensions. Samples were dried at 84°C for 48 h and desiccated over $CaSO_4$ before weighing. It was ascertained that 1 AOD unit corresponded to 0.39 μg of cellular dry weight per ml.

Effect of antibiotic treatment on protein synthesis and cell viability. Exponential-phase cultures, grown in FMC and labeled with [^{3}H]leucine (407 mCi/mg, 0.50 μCi/ml) for at least eight generations, were chilled on ice and centrifuged at 10,000 × *g* for 20 min. The cells were then suspended in 1 liter of FMC containing 0.50 μCi/of [^{3}H]leucine per ml. The initial cell suspension was approximately 225 to 260 AOD units (10), which corresponded to a dry weight of 0.08 to 0.10 mg/ml. Penicillin G (0.10 μg/ml) and/or streptomycin (20 μg/ml) was added, and the cultures were incubated at 37°C for 12 h. The concentrations of penicillin and streptomycin employed were approximately twofold higher than the levels which prevented an increase in the turbidity of cultures of *S.* MG *intermedius* inoculated at an AOD of 1 AOD unit and incubated for 24 h at 37°C. Cell viability was determined in duplicate routine plate count procedures utilizing Todd-Hewitt agar (Difco Laboratories) plates which were incubated for 48 h at 37°C. For determination of [^{3}H]-labeled protein, 0.5-ml samples were precipitated with 5.0 ml of cold 10% trichloroacetic acid. The precipitates were collected on glass fiber filters (Whatman GMC, and the filters were washed three times with cold 10% trichloroaetic acid and two times with 95% ethanol. Filters were transferred to scintillation vials, and precipitates were solubilized with 0.5 ml of 90% NCS (Amersham/Searle) for 2 h at 50°C. After cooling to room temperature, 5 ml of a toluene-based scintillation cocktail (7) were added, and the samples were counted in a Searle Mark III scintillation counter equipped with computer conversion of counts per minute to disintegrations per minute.

Preparation and partial purification of extracellular proteases. After antibiotic treatment, the cells were collected by centrifugation, and supernatant fluids were rendered cell-free by filtration through a 0.65-μm filter. After filtration the supernatant fluids were dialyzed against distilled water at 4°C for 3 days with daily changes to remove all free compounds of low molecular weight. The materials in the supernatant fluids were then concentrated by lyophilization and dissolved in 6 ml of starting buffer. A 1-ml quantity was retained for analysis, and the other 5 ml was applied to an upward-flow Sephadex G-100 column (2.6 by 90 cm), which was eluted with 0.01 M tris (hydroxymethyl)aminomethane-hydrochloride, pH 8.0, containing 0.02% sodium azide. Fractions (4 ml) were collected after 100 ml had passed through the column. Elution of protein-containing material was followed with a Gilford Model 250 single-beam spectrophotometer at 280 nm and also radioisotopically by taking 0.5 ml from every third tube and processing as described above.

Material eluting in the void volume of the Sephadex G-100 column (peak I) was collected, concentrated by lyophilization, and dissolved in 6 ml of starting buffer. A 1-ml quantity was used for analysis, and the other 5 ml was applied to an upward-flow Sepharose 4B column (2.6 by 90 cm), which was eluted with 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 8.0, containing 0.02% sodium azide. Monitoring of this column was as described above.

Physical and chemical determinations. Protein concentrations and protease quantifications were performed as previously described (9). Proteolytic activities were also confirmed by using gelatin diffusion plates, as described by Schultz and Miller (8), and hide powder labeled with Remazole brilliant blue as described by Rinderknecht et al. (6).

RESULTS

Metabolic activity of antibiotic-treated viridans streptococci. The concentrations of the antibiotics employed were determined to be at least two-fold higher than the minimum growth-inhibitory concentration for this organism. Incubation of an exponentially growing culture of *S.* MG *intermedius* in a defined medium containing penicillin G (0.10 μg/ml) and/or streptomycin (20 μg/ml) resulted in a substantial reduction in the rate and extent of incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable material (Table 1). Under balanced growth conditions with adequate nutrients, protein, as well as other cellular macromolecules, doubled every 60 to 63 min in *S.* MG *intermedius* (unpublished data). Exponentially growing cultures inoculated at an AOD of approximately 225 AOD units (0.08 mg of cell dry weight per ml) and incubated for 12 h exhibited increases in culture turbidity, incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable material, and colony-forming units which ranged from 430 to 570% above the zero time level. Penicillin treatment, however, resulted in a 244% increase in protein above the zero time level after 12 h, and streptomycin reduced the level of protein synthesized during antibiotic treatment to an increase of about 100%. The combination of penicillin and streptomycin further reduced the level of trichloroacetic acid-precipitable protein to 63% above the zero time level after a 12-h incubation. Although cellular lysis was not apparent as determined by microscopic observation or by a decrease in cultural turbidity, both antibiotics, particularly the combination of penicillin and streptomycin, effectively reduced cellular viability during antibiotic treatment, with

only 0.01% of zero-time colony-forming units remaining after a 12-h incubation (Table 1).

Production of extracellular material by antibiotic-treated cells of *S.* MG *intermedius*. Although penicillin and/or streptomycin treatment resulted in decreased synthesis of total trichloroacetic acid-precipitable protein, numerous extracellular protein components were detected by polyacrylamide gel electrophoresis (data not shown). Further examination of the extracellular protein produced by *S.* MG *intermedius* was afforded by chromatographic fractionation of the material labeled with [^{3}H]leucine on gel filtration resins. Extracellular protein produced by exponential-phase cells grown in FMC was fractionated into four components on a Sephadex G-100 column as previously reported (9) (Fig. 1). To examine further the size and distribution of the [^{3}H]leucine-labeled material produced by *S.* MG *intermedius* that was above the fractionation range of the Sephadex G-100 column, peak I ($V_e = V_o = 148$ ml) was applied to a Sepharose 4B column and further resolved into at least four additional components with molecular weights greater than 150,000 (Fig. 2). Thus, *S.* MG *intermedius* produced at least seven extracellular [^{3}H]leucine-labeled components resolvable by a combination of Sephadex G-100 and Sepharose 4B column chromatography.

When an exponential-phase culture of *S.* MG *intermedius* was treated with 0.10 μg/of penicillin per ml (Fig. 1 and 2), significantly greater quantities of extracellular proteins were found in the supernatant fluid after 12 h than were observed in exponential-phase cultures grown in complete medium after a similar time period. The amount of extracellular protein in peak I from penicillin-treated cultures was approximately 10 times that recovered from a peak with a similar elution volume on Sephadex G-100 produced by untreated exponential-phase cells. Although cellular lysis was not apparent, as determined by microscopic observation or by a decrease in culture turbidity, leakage of intracellular material through an osmotically ruptured cytoplasmic membrane due to a weakened peptidoglycan layer cannot be ruled out.

Streptomycin (20 μg/ml) treatment for 12 h resulted in a significant decrease in the incorporation of [^{3}H]leucine into extracellular macromolecules previously found in peak I from Sephadex G-100 fractionation of exponentially grown cells (Fig. 1). Surprisingly, however, the amount of protein in the fourth peak from the Sephadex G-100 column produced by streptomycin-treated cells increased threefold over that observed in exponentially grown cells. Fractionation of the first peak from the Sephadex G-100 column produced by streptomycin-treated *S.* MG *intermedius* on a Sepharose 4B column also demonstrated the presence of at least four components with molecular weights greater than 150,000 (Fig 2).

The combination of penicillin and streptomycin treatment resulted in an increase of 63% in

TABLE 1. *Viability and synthesis of trichloroacetic acid-precipitable protein during antibiotic treatment*[a]

Incubation period (h)	Culture turbidity		[^{3}H]leucine incorporation		CFU/ml of culture[b]	% Survivors[b]
	AOD (AOD units)	% Change	Disintegrations/min	% Change		
Penicillin treatment						
0	225	0[c]	45,166	0[c]	2.1×10^8[c]	100.00[c]
6	330	47	136,032	201	2.3×10^6	1.10
12	350	56	155,511	244	1.1×10^6	0.53
Streptomycin treatment						
0	225	0	47,872	0	2.1×10^8	100.00
6	380	69	92,635	94	1.2×10^7	5.70
12	410	82	96,187	101	8.2×10^6	3.90
Penicillin plus streptomycin treatment						
0	257	0	57,910	0	2.4×10^8	100.00
6	477	86	90,708	57	2.5×10^6	1.04
12	477	86	94,196	63	2.2×10^4	0.01

[a] Cells were suspended in FMC containing penicillin (0.10 μg/ml) and/or streptomycin (20 μg/ml) at 37°C.

[b] Colony-forming units (CFU) were determined in duplicate, and the percent survivors was calculated from zero time values of CFU.

[c] The increase in untreated control cultures incubated for 12 h ranged from 430 to 570% for culture turbidity, [^{3}H]leucine incorporation, and colony-forming units.

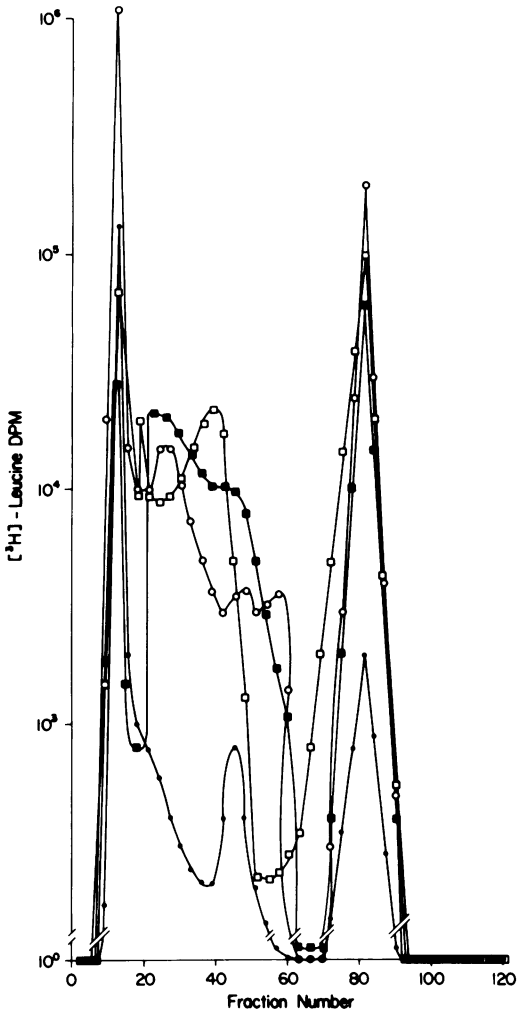

FIG. 1. *Fractionation of the material produced by exponential-phase (●), penicillin-treated (○), streptomycin-treated (■), and penicillin plus streptomycin-treated (□) cells of S. MG intermedius 974 on a Sephadex G-100 column (2.6 by 90 cm) as described in the text. Every third fraction was assayed for radioactivity in the form of [³H]leucine. The results are expressed on an equivalent cell dry weight basis (0.39 g). DPM, Disintegrations per minute.*

the total incorporation of [³H]leucine into protein (Table 1) after a 12-h treatment. However, at least seven resolvable [³H]leucine-labeled components were again obtained by the combination of Sephadex G-100 and Sepharose 4B column chromatography (Fig. 1 and 2). Thus, the addition of penicillin and/or streptomycin to exponential-phase cultures of *S.* MG *intermedius* resulted in greatly reduced rates and extents of protein snythesis, but continued elaboration of extracellular [³H]leucine-labeled material of varying molecular weights (from 5,000 to 3,000,000) was observed.

Protease production by antibiotic-treated cells of *S.* MG *intermedius*. A previous attempt to identify the nature of the protein material produced by exponential cells and by cells deprived of essential amino acids indicated that the proteolytic activity was associated with the majority of resolvable components (9). Because protease activity could potentially re-

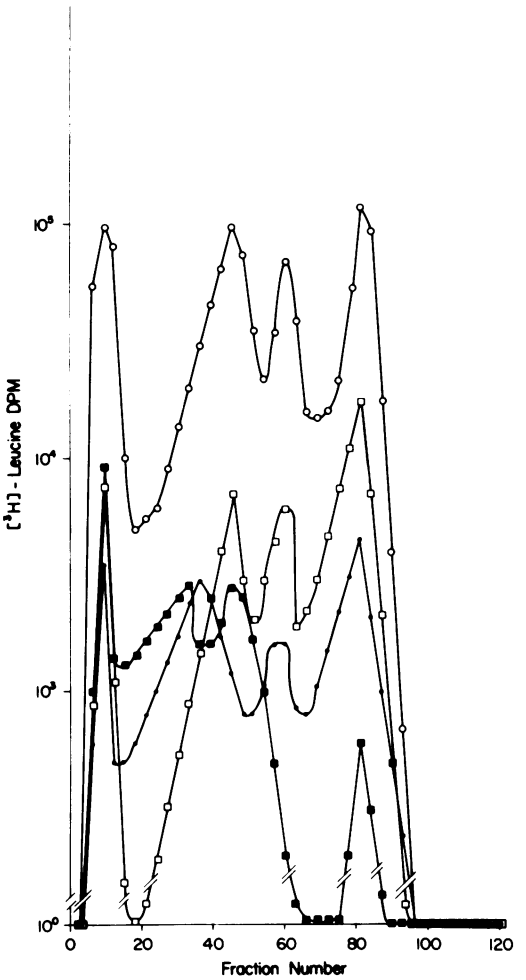

FIG. 2. *Fractionation of the Sephadex G-100 void volume material produced by exponential-phase (●), penicillin-treated (○), streptomycin-treated (■), and penicillin plus streptomycin-treated (□) cells of S. MG intermedius 974 on a Sepharose 4B column (2.6 by 90 cm) as described in the text. Every third fraction was assayed for radioactivity as described in the legend to Fig. 1. DPM, Disintegrations per minute.*

sult in the destruction of valvular material, we examined the extent to which the antibiotic-treated cells were able to continue producing cell-free proteases.

Table 2 shows that cells incubated in complete FMC were able to produce proteases of varying molecular weights. When exponential cells of *S.* MG *intermedius* were incubated in FMC containing penicillin or streptomycin, proteolytic enzymes were produced, but the number of different resolvable molecular species was reduced by at least 50%. Because the elution volumes of peaks possessing protease activity tended to vary among exponential-phase cells and penicillin and/or streptomycin-treated cells (Table 2), it is not possible to quantitatively compare the proteolytic activities in each peak from one treatment to another. However, it is readily apparent that several peaks of protease activity obtained after penicillin or streptomycin treatment were quite high. For instance, when the specific activities of the proteases were compared by Student's *t* test, peak I of the Sepharose 4B-fractionated material from streptomycin-treated cells (8,940 nmoles of peptide bonds cleaved per mg of protein per h per g of cell dry weight) had a significantly higher value than did any protease produced by balanced exponentially growing cells of *S.* MG *intermedius* ($P < 0.01$). Sepharose 4B peak II from penicillin-treated cells (6,170 nmol of peptide bonds cleaved per mg of protein per h per g cell dry weight) was also elevated when compared with proteases produced by untreated exponentially growing cells ($P = \sim 0.05$). These results may indicate that these antibiotics administered alone may result in a preferential synthesis of one or more particular proteolytic species. However, these results are in marked contrast to the protease activities measured after combined penicillin and streptomycin treatment. In the latter case, proteolytic activity was demonstrated in only two peaks, and the values were considerably decreased compared with cultures treated with only penicillin or streptomycin.

DISCUSSION

The data presented in this study demonstrates that viridans streptococci associated with subacute bacterial endocarditis maintain the ability to elaborate extracellular proteins when their multiplication has been arrested by antibiotic treatment. Several of these extracellular proteins produced under conditions of antibiotic treatment had proteolytic activity (Table 2). It is apparent, however, that under such conditions the number of different proteases was decreased by at least 50% when cells were treated with penicillin or streptomycin. Several of the proteases produced during either penicillin or streptomycin treatment had higher specific activities than proteases produced by the same organisms grown in complete antibiotic-free medium.

The increased excretion of extracellular macromolecules by antibiotic-treated streptococci (10-fold-greater protein levels in penicillin-

TABLE 2. *Proteolytic activities of compounds eluting as peaks on Sephadex G-100 and Sepharose 4B columns from material produced in complete FMC and FMC plus penicillin and/or streptomycin by S.* MG *intermedius* 974

Peak[a]	nmol of peptide bonds cleaved per mg of protein/h (sp act)[b]				Sp act per g cell dry weight			
	Control[c] (FMC)	FMC + penicillin	FMC + streptomycin	FMC + penicillin and streptomycin	Control[c] (FMC)	FMC + penicillin	FMC + streptomycin	FMC + penicillin and streptomycin
I	262	104	ND[d]	ND	672	774	ND	ND
II	491	297	280	ND	1,260	2,210	1,760	ND
III	183	ND	ND	ND	469	ND	ND	ND
IV	109	ND	ND	ND	281	ND	ND	ND
4B I	318	134	1,430	92	816	996	8,940	496
4B II	1,680	829	ND	247	4,310	6,170	ND	1,332
4B III	475	ND	ND	ND	1,220	ND	ND	ND
4B IV	215	ND	197	ND	553	ND	1,240	ND

[a] The first four peaks were from Sephadex G-100 fractionation, and the second four peaks were from Sepharose 4B fractionation. Elution volumes (in milliliters) at the respective peaks were: peak I, 148; peak IV, 424; peak 4B I, 136; peak II, 184 to 208; peak III, 244 to 316; peak 4B II, 244 to 280; peak 4B III, 280 to 340; and peak 4B IV, 426 to 436.

[b] Determined by the method of Lin et al. (4) using *N,N*-dimethyl gelatin as the substrate. All assays were performed in duplicate with inactivated enzyme blanks subtracted as described in the text.

[c] Grown to exponential phase.

[d] ND, Not detected (less than 90 nmol of peptide bonds cleaved per mg of protein per h or no linear increase seen with increasing time periods).

treated cells than in cells in the exponential phase of growth) is a phenomenon that has been observed in other gram-positive bacteria. Horne et al. (2) observed that inhibition of peptidoglycan synthesis by benzylpenicillin in several species of bacteria resulted in a large excretion of both nascent and preexisting lipids. Their studies with the group A streptococci, *Streptococcus pneumoniae, Staphylococcus epidermidis,* and *Bacillus subtilis* demonstrated that this lipid production occurred in the absence of detectable bacterial lysis. They also observed that this massive outpouring of lipid was induced only by those antibiotics that act by inhibiting peptidoglycan synthesis. Antibiotics that cause inhibition of protein or nucleic acid synthesis did not exhibit this effect. In another study on the release of extracellular material by antibiotic-treated streptococci, Horne and Tomasz (3) demonstrated that exposure to penicillin caused the release of lipoteichoic acid and peptidoglycan polymers from *S. sanguis.* They suggested that macromolecular components secreted after exposure to penicillin were most likely cell surface components, because they could detect little or no excretion of nucleic acids. However, in contrast to our data on the excretion of protein-containing material by *S.* MG *intermedius* after penicillin exposure, they were able to detect only a small secretion of extracellular proteins after antibiotic treatment in *S. sanguis* (3). However, the nature of the proteinaceous material released was not examined in this latter study (3).

The extracellular products with proteolytic activity that were separated by Sephadex G-100 fractionation of the supernatant fluid materials from exponential-phase cells grown in complete FMC are in good agreement with our previously published data (9). However, we now find that the Sephadex G-100 peak IV material from cells grown in complete FMC has proteolytic activity when previously we were unable to detect activity (9). It should be noted that this specific activity (109.6 nmol of peptide bonds cleaved per mg of protein per h) was the lowest specific activity detected in any of the proteases produced by the cells grown in FMC and was probably not produced in sufficiently detectable quantities in the previous study (9). This peak was also positive on both gelatin agar (8) and the hide powder-Remazole brilliant blue assay (6).

In conclusion, these studies have demonstrated that viridans streptococci are capable of elaborating potentially toxic and damaging compounds when their multiplication has been arrested by antibiotic treatment. Penicillin or streptomycin treatment was found to greatly reduce the number of proteases elaborated by these organisms, and there was preferential synthesis of some proteolytic compounds. The combination of penicillin and streptomycin resulted in the greatest reduction in proteolytic activity found in the supernatant fluids.

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