INFECTION AND IMMUNITY, Sept. 1987, p. 2155–2163
0019-9567/87/092155-09$02.00/0

Vol. 55, No. 9

Ingestion of *Staphylococcus aureus* by Bovine Endothelial Cells Results in Time- and Inoculum-Dependent Damage to Endothelial Cell Monolayers

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Received 9 January 1987/Accepted 11 June 1987

Cultured endothelial cells phagocytize *Staphylococcus aureus*, but the resultant effects are unknown. Monolayers of cultured bovine endothelial cells with or without [^{3}H]adenine label were exposed to 100, 10, or 1 *S. aureus* organism per endothelial cell for 3.5 h. Lysostaphin was then applied to all cultures to destroy extracellular but not phagocytized *S. aureus*. In cultures treated for only 20 min with lysostaphin, *S. aureus* multiplied exponentially after a 9- to 12-h lag period. In cultures treated continously with lysostaphin, numbers of *S. aureus* remained constant or decreased. These results indicate that *S. aureus* became extracellular and multiplied but did not multiply intracellularly. In parallel experiments, the release of ^{3}H-adenine from prelabled endothelial cell monolayers was assayed to indicate cytotoxicity. Results indicated that the loss of ^{3}H-adenine from endothelial cell monolayers depended on the following: (i) the size of the *S. aureus* inoculum, (ii) the strain of *S. aureus*, and (iii) the length of time after exposure to *S. aureus*. *S. aureus* endocarditis and persistent septicemia could arise, at least in part, from ingestion of *S. aureus* by host endothelium. The intracellular location would afford *S. aureus* protection from host defenses and antibiotics. Eventual damage to endothelial cells could expose collagen, thus resulting in platelet adherence and vegetation formation. Intracellular *S. aureus* would be continuously released into the circulation, possibly accounting for the persistent bacteremia that is found in *S. aureus* endovascular infections.

Staphylococcus aureus is an important pathogen which is responsible for significant morbidity and mortality, causing infections of soft tissues, joints, bones, and the cardiovascular system. *S. aureus* is the second most common causative agent, after streptococci, of infectious endocarditis. *S. aureus* endocarditis is difficult to eradicate, requiring 4 to 6 weeks of antibiotic therapy. The persistence of *S. aureus* at endovascular sites is not well understood. Possible explanations include *S. aureus* tolerance of the antibiotics used and possible sequestration of bacteria in devitalized tissue (5).

A widely accepted model for the pathogenic mechanism of infectious endocarditis was first proposed over 50 years ago (6). This model states that the development of endocarditis begins with the formation of nonbacterial thrombotic vegetations caused by congenital or rheumatic valvular heart disease (6). Then, during an episode of bacteremia, bacteria attach to the vegetations and multiply within their interstices (6). Consistent with this model, a recent study has indicated that initial bacterial colonization occurs on vegetations (22). In contrast, studies have shown that *S. aureus* adheres to cultured endothelial cells (18, 27), and various reports indicate that 31 to 70% of *S. aureus* endocarditis occurs in patients having no known preexisting heart disease (11, 25, 28). Although some of these patients may have had undetected valvular abnormalities, in some cases, an alternative model may be in order. Indeed, the recent discovery that cultured endothelial cells phagocytize *S. aureus* (7, 18) suggests that *S. aureus* could be phagocytized by the endothelium in vivo, with the ingested *S. aureus* possibly playing a role in the initiation of *S. aureus* infectious endocarditis and the persistence of *S. aureus* septicemia.

The consequences of ingestion of *S. aureus* by endothelial cells are largely unknown. Related literature is limited to one article published in 1943 by MacNeal et al., who reported that after intravenous challenge with streptococci, endothelial cells of the endocardium and aorta of rabbits contained intracellular streptococci (15). Reportedly, in some areas of the endothelium, intracellular streptococci appeared to have multiplied and resulted in the vegetations of endocarditis, but in most cases intracellular streptococci appeared to have been destroyed (15). The present study was undertaken to determine the consequences of ingestion of *S. aureus* by cultured bovine aortic endothelial cells. Experiments were carried out to determine whether *S. aureus* multiplies within endothelial cells and whether endothelial cell monolayers harboring intracellular *S. aureus* suffer damage. The results indicate that intracellular *S. aureus* does not multiply but that endothelial cells harboring intracellular *S. aureus* are damaged in a time- and inoculum-dependent manner.

(A preliminary report of these findings was presented at the 26th Interscience Conference on Antimicrobial Agents and Chemotherapy, New Orleans, La. [J. M. Vann and R. A. Proctor, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 267, 1986].)

MATERIALS AND METHODS

Cell culture medium. Dulbecco modified essential medium with 4,500 mg of D-glucose per liter and 584 mg of L-glutamine (GIBCO Laboratories, Grand Island, N.Y.) per liter and containing 20% defined supplemented bovine calf serum (Hyclone Laboratories, Logan, Utah), hereafter referred to as DMEM, was the medium used for culturing of

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bovine aortic endothelial cells. Gentamicin (Sigma Chemical Co., St. Louis, Mo.) (50 μg/ml) and antibiotic-antimycotic solution containing penicillin (100 μg/ml), streptomycin (100 μg/ml), and amphotericin B (250 ng/ml) (Sigma) were included in the medium for cell culturing but not in the medium which came into contact with *S. aureus* during the experiments.

Isolation and culturing of bovine aortic endothelial cells. Segments of freshly obtained adult bovine aorta were provided by a local slaughterhouse and transported to the laboratory in sterile *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline containing 50 μg of gentamicin per ml (HBS). After side arteries were sealed, aorta segments were filled with 0.1% collagenase in HBS and suspended in a 37°C bath of HBS for 20 min (9). Collagenase solutions containing cells were withdrawn, diluted in DMEM, and centrifuged at 220 × *g* for 5 min. Cell pellets were resuspended in DMEM for distribution to 100-mm tissue culture plates (Corning Glass Works, Corning, N.Y.). Cell cultures were incubated at 37°C in a 5% CO_2 atmosphere. The criteria used for identifying endothelial cells were typical cobblestone morphology and positive immunofluorescence staining for von Willebrand factor-factor VIII-related antigen (10), a glycoprotein which is the major component of the antihemophilic factor complex. Cultures containing contaminating fibroblasts or smooth muscle cells were cloned successfully (9) or discarded. Cell cultures were passaged with 0.2 mM EDTA (Sigma) in phosphate-buffered saline to loosen cells from tissue culture dishes. EDTA solutions containing endothelial cells were diluted in DMEM and centrifuged at 220 × *g* for 5 min. Cell pellets were resuspended in DMEM for distribution to 100-mm tissue culture plates or, for experiments, to 24-well tissue culture plates (Corning) containing 15-mm round glass cover slips (Carolina Biological Supply Co., Burlington, N.C.).

***S. aureus* strains and culturing.** Two clinical isolates of *S. aureus* were used in these studies: *S. aureus* ENDO was a blood isolate from a fatal case of endocarditis; *S. aureus* 6850 was isolated from a patient with a skin abscess which had progressed to staphylococcemia, hematogenous osteomyelitis, septic arthritis, and multiple systemic abscesses. *S. aureus* was grown to the logarithmic phase in tryptic soy broth (Difco Laboratories, Detroit, Mich.) at 37°C, harvested by centrifugation, washed three times in sterile Hanks balanced salt solution (HBSS; pH 7.4; 10× stock solution obtained from GIBCO), and resuspended in HBSS. To disperse clusters of *S. aureus*, we sonicated suspensions with a model W-370 sonicator (Heat Systems Ultrasonics, Inc.) equipped with a water-jacketed cup horn. *S. aureus* suspensions in sealed plastic test tubes were immersed in the water flowing over the cup horn for 30 s with the sonicator operating at a power output setting of 10. This treatment does not reduce *S. aureus* viability. *S. aureus* suspensions were then diluted appropriately, counted in a hemacytometer, and diluted with DMEM to provide the inoculum desired for each experiment.

Assessment of possible intracellular-proliferation of *S. aureus* ingested by endothelial cells. Cultures of bovine endothelial cells grown to confluence on 15-mm round glass cover slips in 24-well tissue culture plates were allowed to ingest various inocula of *S. aureus* for 3.5 h at 37°C in 5% CO_2. Lysostaphin (Sigma), which destroys extracellular but not intracellular *S. aureus*, was then applied (4). Incubation of endothelial cell cultures containing intracellular *S. aureus* was then continued. The number of CFU of *S. aureus* was subsequently assayed at various times.

To begin each experiment, we determined the number of endothelial cells on cover slips. This was done by using a light microscope equipped with a calibrated eyepiece grid which defined a known area of the subject at 40×. Endothelial cells within the grid were counted, and this procedure was performed on five areas of each of two cover slips. The number of endothelial cells on cover slips was calculated by using a conversion factor between the area of the grid and the area of the cover slip. These estimates typically were between 2.3×10^5 and 3.7×10^5. On the basis of the estimated number of endothelial cells. *S. aureus* suspensions were diluted to obtain approximate inocula of 100, 10, or 1 *S. aureus* organism per endothelial cell. Endothelial cell cultures were washed three times with HBSS, followed by the addition of 0.9 ml of DMEM and 100 μl of the appropriate suspension of *S. aureus*. Endothelial cells were then allowed to ingest *S. aureus* for 3.5 h, a time previously found to allow maximal association of *S. aureus* with endothelial cells (7). Cultures were washed once with sterile HBSS, followed by the application of 1.0 ml of filter-sterilized lysostaphin (10 μg/ml in DMEM) to destroy extracellular *S. aureus*.

Three different protocols for lysostaphin treatment were used. In the first, lysostaphin remained present continuously until the cultures were harvested 0, 3, 6, 9, 12, 24, and 48 h later. In the second, lysostaphin was added for 20 min and was then removed. Cultures were then washed with three changes of HBSS, and incubation was continued in DMEM. The third lysostaphin protocol was the same as the second, except that an additional 20 min of lysostaphin treatment was applied at the time of harvest. Cultures were harvested to determine the number of CFU of *S. aureus* by removing cover slips containing endothelial cell monolayers and plunging them into vials of sterile distilled water. In the second protocol, in which lysostaphin was applied initially for 20 min only, the culture supernatant was added to the endothelial cell lysate. Lysates were sonicated to disperse clumps of *S. aureus*. Appropriate serial 10-fold dilutions were plated on tryptic soy agar (Difco) spread plates. Spread plates were incubated at 37°C for 24 h, and colonies of *S. aureus* were counted. One serial 10-fold dilution series was performed on each well, with duplicate aliquots of each dilution being applied to spread plates. Experimental conditions in wells were duplicated at each time point. Thus, in one experiment, each datum point is the average of four numerical values. Each experiment was performed three to seven times.

Effectiveness of lysostaphin. For determination of what proportion, if any, of extracellular *S. aureus* survived lysostaphin treatment, paraformaldehyde-fixed endothelial cell monolayers were incubated with *S. aureus* for 3.5 h and then treated with lysostaphin for 20 min or mock treated, and the number of CFU of *S. aureus* was determined. The number of CFU of *S. aureus* in lysostaphin-treated conditions was 0.04 ± 0.02% ($n = 11$) of the number of CFU of *S. aureus* in mock-treated conditions. Thus, treatment with lysostaphin for 20 min was 99.96% effective. Under the same conditions, the application of lysostaphin for 24 or 48 h killed all *S. aureus*.

Assessment of possible lysostaphin artifacts. The possibility was assessed that lysostaphin becomes associated with monolayers of endothelial cells on cover slips, especially during prolonged incubation, causing an artifactual decrease in the CFU of *S. aureus*. Lysostaphin was radioiodinated with ^{125}I by the chloramine-T method. The concentration of iodinated protein was determined by the method of Lowry et al. (14). Polyacrylamide gel electrophoresis of ^{125}I-lysostaphin, followed by autoradiography for 4 h, revealed one

highly radioactive band which migrated at 25,000 daltons, a value which is consistent with that reported for lysostaphin. Clean cover slips or endothelial cells grown to confluence on cover slips in 24-well plates were incubated at 37°C in 5% CO_2 for 20 min or 24 h with 1.0 ml of ^{125}I-lysostaphin (10 μg/ml). When incubation times were concluded, wells were washed three times with HBSS, solubilized, and counted in an Auto-Gamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Experiments with ^{125}I-lysostaphin showed that very small percentages of added lysostaphin became associated with endothelial cells and glass cover slips (see Results). Further experiments were conducted to determine whether lysostaphin associated with cells and cover slips was present in an active form in quantities sufficient to confound results. Clean cover slips or confluent endothelial cells on glass cover slips were incubated in 1.0 ml of DMEM with or without 10 μg of lysostaphin per ml for 20 min or 24 or 48 h. At the conclusion of incubation times, wells were washed three times with HBSS, and cover slips were plunged into sterile distilled water. *S. aureus* (5×10^6) was added to these solutions containing cover slips, and the solutions were then sonicated. After incubation for 1 h at room temperature, these solutions were diluted and applied to spread plates to determine the CFU of *S. aureus*.

Assessment of possible loss of integrity of endothelial cells harboring ingested *S. aureus*. Cultures of bovine aortic endothelial cells grown to confluence on 15-mm round glass cover slips in 24-well plates were labeled with 8-^{3}H-adenine (specific activity, 20 to 25 Ci/mmol) or 2,8-^{3}H-adenine (specific activity, 25 to 50 Ci/mmol) (Amersham Corp., Arlington Heights, Ill., and ICN Radiochemicals, Irvine, Calif., respectively) (1). Labeled cell cultures were allowed to ingest a range of inocula of *S. aureus* for 3.5 h, followed by the application of lysostaphin treatments. Incubation of endothelial cell cultures harboring intracellular *S. aureus* was continued, and the ^{3}H content of endothelial cell monolayers was assayed at subsequent time intervals.

Labeling of endothelial cells with ^{3}H-adenine was accomplished by replacing the cell culture medium with 1.0 ml of DMEM containing 1.0 μCi of ^{3}H-adenine per ml for approximately 14 h (1). Wells were washed three times with HBSS and then incubated in 1.0 ml of DMEM for 1 to 2 h. Cell culture supernatants, which typically contained 60,000 dpm of spontaneously released radioactivity, were then withdrawn and replaced with 0.9 ml of DMEM. At this point, monolayers of endothelial cells typically retained 25% of the radioactivity originally applied to the culture. Appropriate suspensions of *S. aureus* (100 μl) were then added to the wells. Control cultures were not exposed to *S. aureus*. After 3.5 h of incubation, lysostaphin was applied to the cultures. Some cultures were then incubated with lysostaphin for 20 min and washed free of lysostaphin with three changes of HBSS, followed by continued incubation in 1.0 ml of DMEM. At various time points, these cultures were washed free of any radioactivity released from the cells with three changes of HBSS. The remaining cultures were incubated with lysostaphin until they were washed free of released radioactivity and lysostaphin. Cover slips were then placed in scintillation vials, and cells were solubilized by the addition of 1.0 N NaOH for 10 min at room temperature. The solution was neutralized with 1.0 N HCl, followed by the addition of Aquasol (Du Pont New England Nuclear Corp. Research Products, Boston, Mass.), and scintillation counting was carried out in a Tri-Carb liquid scintillation counter (Packard). Experimental conditions in wells, including control wells, were duplicated at each time point. Each experiment was performed four times. Data are expressed as follows: percent control ^{3}H content = (disintegrations per minute in infected monolayers/disintegrations per minute in control monolayers) × 100.

Statistical tests. Sample means were tested for significant differences with a two-tailed Student *t* test for sample means, with pooled variance, which does not assume that the samples have equal variance (23).

RESULTS

Assessment of possible proliferation of *S. aureus* CFU in endothelial cell cultures. In initial experiments, the CFU of *S. aureus* in endothelial cell cultures (cell monolayers plus culture supernatants combined) began increasing exponentially 9 to 12 h after the elimination of extracellular *S. aureus* with lysostaphin (Fig. 1). This result indicates that *S. aureus* did not multiply intracellularly for at least the first 9 to 12 h and contrasts with the delay of only 3 h before exponential growth began when *S. aureus* was incubated in DMEM (results not shown). We investigated whether there was an explanation besides the intracellular multiplication of *S. aureus* for the exponential increase in *S. aureus* CFU which occurred in the cultures 9 to 12 h after exposure to lysostaphin.

Multiplication of extracellular *S. aureus* surviving exposure to lysostaphin for 20 min. It was possible that some of the small numbers of extracellular *S. aureus* which survived exposure to lysostaphin for 20 min escaped subsequent ingestion and multiplied extracellularly to an extent that would account for the entire increase in CFU observed. To test this possibility, we fixed endothelial cells in 0.5% paraformaldehyde in phosphate-buffered saline for 1 h and then washed them. Fixed and unfixed endothelial cells were incubated with *S. aureus* for 3.5 h. The cultures were then exposed to lysostaphin for 20 min and washed, and incubation was continued for 48 h. After 48 h, the number of CFU of *S. aureus* in cultures containing fixed endothelial cells was less than 1% (0.92 ± 0.30%; $n = 8$) of the number of CFU of *S. aureus* in cultures containing viable endothelial cells. Thus, extracellular multiplication of *S. aureus* surviving exposure to lysostaphin did not account for the increases in CFU shown in Fig. 1.

Lack of intracellular proliferation of *S. aureus* in endothelial cell cultures. It was possible that the observed increase in *S. aureus* CFU in endothelial cell cultures resulted from the release of ingested *S. aureus* from within endothelial cells followed by extracellular multiplication. To investigate this possibility, we altered the experimental protocol such that (i) in addition to the initial 20-min exposure to lysostaphin, cultures were again exposed to lysostaphin for 20 min just before harvesting, or (ii) the lysostaphin applied initially was not removed until the time point. These procedures eliminated extracellular *S. aureus* from determinations of CFU in cultures. In 48 h, no significant net increases in the CFU of intracellular *S. aureus* occurred after additional lysostaphin treatments at various time points (data not shown) or after incubation in the continuous presence of lysostaphin (Fig. 2). This result indicates that *S. aureus* did not multiply intracellularly in endothelial cells. When an additional lysostaphin treatment was applied, statistically significant reductions in *S. aureus* CFU occurred between 0 and 12 h when the original inoculum was 100 *S. aureus* 6850 organisms per endothelial cell ($P = 0.032$; $n = 3$) and between 3 and 24 h when the original inoculum was 10 *S. aureus* 6850

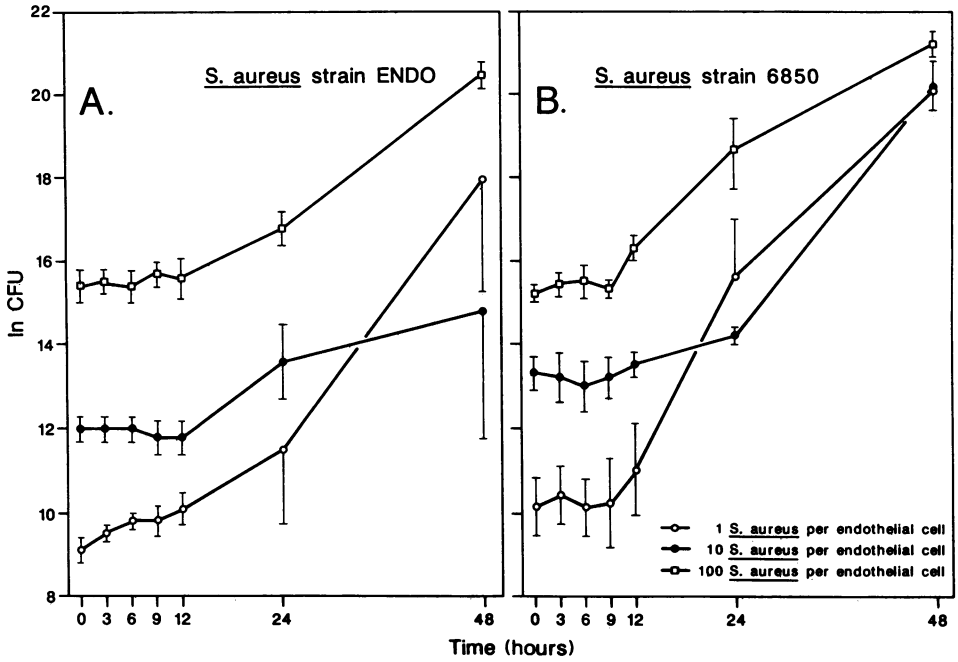

FIG. 1. Change in the number of *S. aureus* CFU in endothelial cell cultures with time. Cultures were incubated with 100, 10, or 1 *S. aureus* organism per endothelial cell for 3.5 h and then exposed to lysostaphin for 20 min, followed by the removal of lysostaphin. Subsequently, the combined CFU of both intracellular and extracellular *S. aureus* in the cultures remained nearly constant for 9 to 12 h and then began to increase exponentially. Bars indicate the standard error of the mean. Note that the data are expressed as the natural logarithm, not $\log_{10}$, of CFU of *S. aureus*.

organisms ($P = 0.038$; $n = 3$) or 100 *S. aureus* ENDO organisms ($P < 0.005$; $n = 3$) per endothelial cell (data not shown). When incubation of endothelial cell cultures harboring intracellular *S. aureus* was continued in the presence of lysostaphin, significant reductions in *S. aureus* CFU occurred when the original inoculum per endothelial cell was as follows: 100 *S. aureus* 6850 organisms, $P = 0.0005$ and $n = 5$; 1 *S. aureus* 6850 organism, $P < 0.0001$ and $n = 7$; and 100 *S aureus* ENDO organisms, $P = 0.0047$ and $n = 3$ (Fig. 2). With an original inoculum of 100 *S. aureus* organisms per endothelial cell, a 550-fold reduction in intracellular *S. aureus* 6850 CFU occurred, while the reduction in intracellular *S. aureus* ENDO CFU was only 30-fold (Fig. 2). Furthermore, inoculum size correlated positively with the

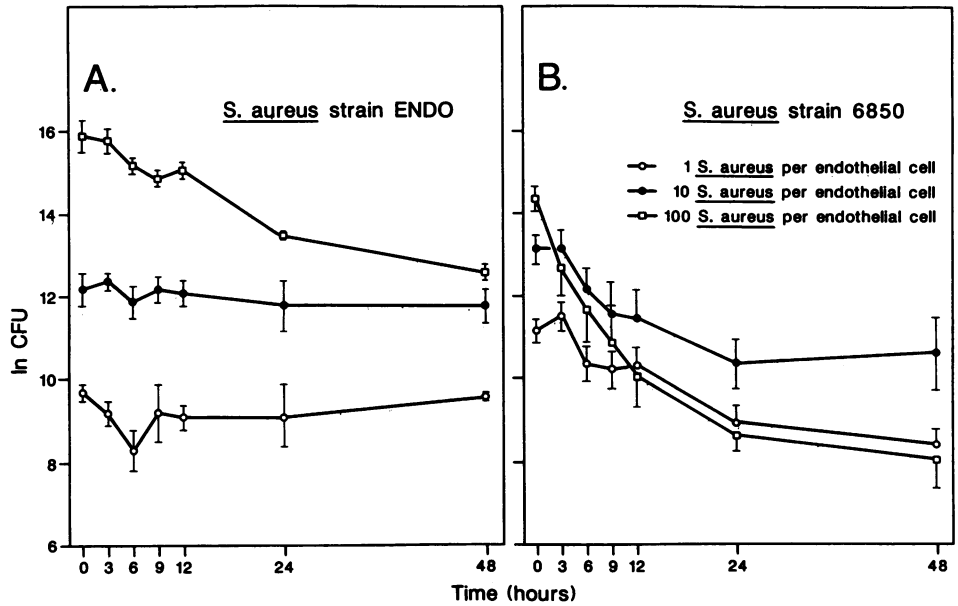

FIG. 2. Change in the number of intracellular *S. aureus* CFU in endothelial cell cultures with time. Cultures were incubated with 100, 10, or 1 *S. aureus* organisms per endothelial cell for 3.5 h, and then lysostaphin was added to the cultures and remained present continuously until each time point. Bars indicate the standard error of the mean.

TABLE 1. Association of ^{125}I-lysostaphin with glass cover slips and endothelial cell monolayers grown to confluence on glass cover slips

Sample[a] (no. examined)	^{125}I-lysostaphin associated with samples (% cpm added ± SEM) at:	
	20 min	24 h
15-mm Round glass cover slip (3)	0.054 ± 0.015	0.14 ± 0.022
Confluent endothelial cell monolayer on cover slip (3)	0.048 ± 0.010	0.22 ± 0.028
Endothelial cell monolayer exposed to viable *S. aureus*[b] (3)	0.82 ± 0.13	0.50 ± 0.09
Endothelial cell monolayer exposed to heat-killed *S. aureus*[b] (2)	1.3 ± 0.60	0.50 ± 0.05

[a] Samples were incubated with 10 μg of ^{125}I-lysostaphin.

[b] Confluent endothelial cells on glass cover slips were inoculated with 100 viable or heat-killed *S. aureus* organisms per endothelial cell. At 3.5 h later, 10 μg of ^{125}I-lysostaphin in 1 ml of DMEM was added.

rate and magnitude of reduction in the CFU of intracellular *S. aureus* (Fig. 2). The reductions in *S. aureus* CFU observed with lysostaphin present continuously in the incubation medium (Fig. 2) were greater than those observed with 20-min applications of lysostaphin at 0 h and at various time points (data not shown). Significant reductions in *S. aureus* CFU did not occur with an original inoculum of 1 or 10 *S. aureus* ENDO organisms per endothelial cell (Fig. 2), which indicates that incubation in the continuous presence of lysostaphin did not result in a reduction in intracellular *S. aureus* CFU.

Assessment of possible lysostaphin artifacts. Further studies were conducted to examine the possible association of lysostaphin with endothelial cells and the resultant effects. Very small percentages of added ^{125}I-lysostaphin associated with endothelial cells (Table 1). Similar amounts of radioactivity became associated with clean glass cover slips and cover slips containing endothelial cell monolayers after incubation with ^{125}I-lysostaphin for 20 min (Table 1). After incubation with ^{125}I-lysostaphin for 24 h, radioactivities associated with cover slips and cover slips containing endothelial cell monolayers were 2.6 and 4.5 times greater, respectively, than after incubation with ^{125}I-lysostaphin for 20 min (Table 1). In contrast, when ^{125}I-lysostaphin was added to endothelial cells phagocytizing viable or heat-killed *S. aureus*, the amount of associated radioactivity was lower after 24 h than after 20 min of incubation with ^{125}I-lysostaphin (Table 1). These data indicate that the amount of lysostaphin associated with glass and perhaps endothelial cells not exposed to *S. aureus* increased with time of exposure. The addition of lysostaphin to endothelial cells phagocytizing viable or heat-killed *S. aureus* resulted in internalization of a small amount of lysostaphin (Table 1), indicating that ^{125}I-lysostaphin was taken up concomitantly with *S. aureus*. Subsequently, the amount of intracellular lysostaphin decreased with time despite the continued presence of lysostaphin in the cell culture medium (Table 1). Although the proportions of lysostaphin which became associated with endothelial cells and cover slips were small, it was important to determine whether lysostaphin which had become associated with cover slips or endothelial cells could kill *S. aureus*. The results of three experiments (Table 2) indicate that lysostaphin associated with cover slips or endothelial cells was inactive or not present in sufficient quantities to cause a reduction in *S. aureus* CFU (Table 2).

Assessment of possible loss of integrity of endothelial cells harboring ingested *S. aureus*. In the studies described above which showed that intracellular *S. aureus* did not proliferate when lysostaphin was precent continuously after zero time, a reduction in the numbers of intracellular *S. aureus* was observed. We hypothesized that intracellular *S. aureus* damaged endothelial cells, resulting in the release of intracellular *S. aureus*, which was subsequently destroyed by lysostaphin. This hypothesis was tested by assaying the retention of ^{3}H-adenine by endothelial cells at various times after lysostaphin treatment of cultures exposed to *S. aureus*. After incubation for 48 h following a 20-min treatment with lysostaphin, the ^{3}H-adenine label retained in endothelial cell cultures harboring viable intracellular *S. aureus* 6850 decreased to 5.0×10^3 dpm, in contrast to mock-treated control cultures, which experienced a low rate of spontaneous release of label and retained 3.5×10^5 dpm (Fig. 3). Microscopic examination of cultures which retained only 5×10^3 dpm revealed few intact endothelial cells remaining. The plots of disintegrations per minute versus time in Fig. 3 indicate that larger inocula of *S. aureus* 6850 caused a more rapid release of ^{3}H-adenine. Endothelial cell cultures which ingested heat-killed *S. aureus* 6850 retained label at a level approximately 100% that of mock-treated control cultures (Fig. 4C). When lysostaphin treatment was applied for 20 min at zero time, the percent control ^{3}H content of endothelial cell cultures decreased significantly in 48 h in cultures exposed to original inocula of 1 ($P = 0.008$), 10 ($P = 0.0033$), or 100 ($P = 0.0031$) *S. aureus* 6850 organisms per endothelial cell (Fig. 4). When lysostaphin was present continuously, the percent control ^{3}H content of endothelial cell cultures exposed to original inocula of 100 *S. aureus* 6850 organisms per endothelial cell decreased significantly ($P = 0.019$) (Fig. 4). The loss of ^{3}H-adenine label from cultures treated only initially with lysostaphin was significantly greater than the loss of ^{3}H-adenine label from cultures treated continuously with lysostaphin when the original inocula were 1 ($P = 0.0015$) or 10 ($P < 0.0001$) *S. aureus* 6850 organisms per endothelial cell but not 100 *S. aureus* 6850 organisms per endothelial cell ($P = 0.087$) (Fig. 4). In contrast, the loss of ^{3}H-adenine label from cultures inoculated with *S. aureus*

TABLE 2. Staphylocidal activity associated with cover slips or endothelial cells as a result of incubation in lysostaphin

Sample[a] (no. examined)	% of *S. aureus* remaining viable as compared with controls (mean ± SEM)[b] at:		
	20 min	24 h	48 h
Cover slip (3)	98.4 ± 7.3	103.9 ± 15.6	95.9 ± 11.3
Endothelial cells on cover slip (3)	101.9 ± 3.2	93.9 ± 17.8	97.5 ± 13.9
Endothelial cells exposed to dead *S. aureus*[c] (3)	79.7 ± 11.0	133.7 ± 20.0	99.9 ± 4.1

[a] Cover slips, cover slips containing confluent monolayers of endothelial cells, or endothelial cells which had been exposed to *S. aureus* were incubated with lysostaphin or under control conditions (without lysostaphin) for the indicated times and then plunged into distilled water. *S. aureus* (5×10^6) was added, and the CFU of *S. aureus* were determined 1 h later. In all experiments, each day a freshly grown suspension of *S. aureus* was added to preparations for the determination of *S. aureus* CFU.

[b] Percent viable *S. aureus* = (CFU with lysostaphin/CFU without lysostaphin) × 100.

[c] Heat-killed *S. aureus* was incubated with endothelial cell cultures for 3.5 h before the addition of lysostaphin. The monolayer was treated, and the CFU of newly added viable *S. aureus* were determined as described in footnote *a*.

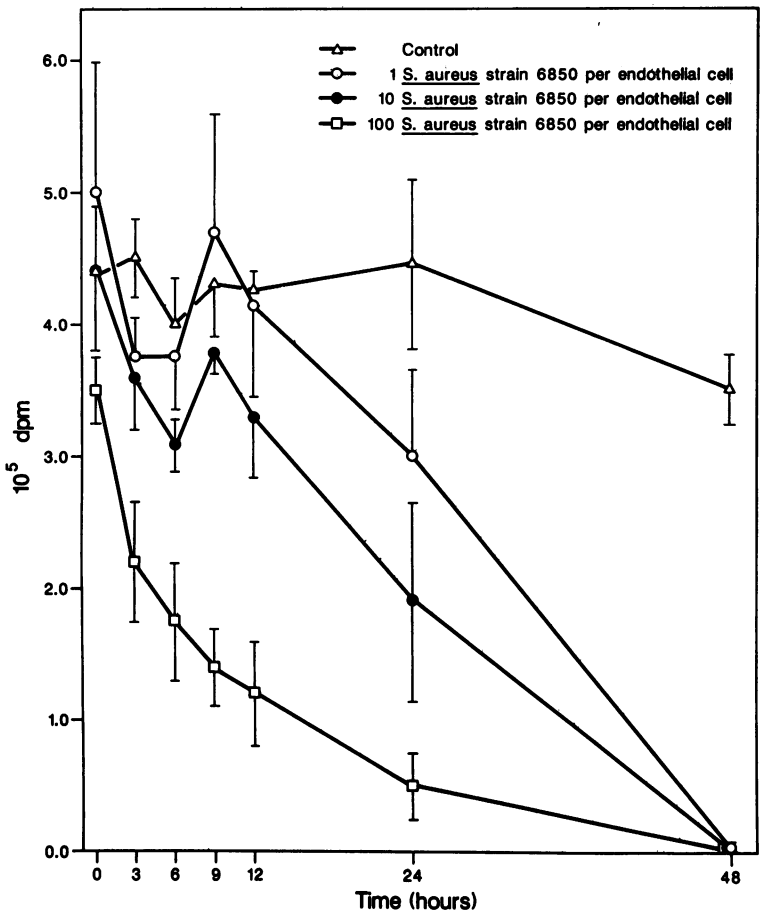

FIG. 3. Change in ^{3}H-adenine content in labeled endothelial cell cultures with time after ingestion of *S. aureus* 6850. Endothelial cell cultures were exposed to 0, 1, 10, or 100 *S. aureus* 6850 organisms per endothelial cell for 3.5 h, cultures were treated with lysostaphin for 20 min, and incubation was continued. Data are expressed simply in terms of disintegrations per minute retained by endothelial cell monolayers. Bars indicate the standard error of the mean.

ENDO and treated only initially with lysostaphin was nearly identical to the loss of ^{3}H-adenine label from cultures treated continuously with lysostaphin (Fig. 5). Furthermore, the reduction in percent control ^{3}H content between 0 and 48 h with an original inoculum of 100 *S. aureus* ENDO organisms per endothelial cell was not statistically significant (P = 0.081 for continuous lysostaphin treatment; P = 0.15 for zero-time lysostaphin treatment) (Fig. 5). That cultures incubated in the continuous presence of lysostaphin lost ^{3}H-adenine supports the concept that intracellular *S. aureus* causes damage to endothelial cells and supports the hypothesis that the reduction in the CFU of intracellular *S. aureus* (Fig. 2) was due to the release of *S. aureus* from damaged endothelial cells. The loss of ^{3}H-adenine label from cultures inoculated with *S. aureus* 6850 and treated continuously with lysostaphin (Fig. 4) was not significantly different from that from cultures inoculated with *S. aureus* ENDO (Fig. 5).

DISCUSSION

The continuous application of lysostaphin to endothelial cell cultures which had ingested *S. aureus* differentiated possible intracellular multiplication from the release of intracellular *S. aureus*, extracellular multiplication, and reingestion of *S. aureus*. The results of these experiments indicate that intracellular *S. aureus* CFU do not increase over time and that intracellular multiplication of *S. aureus* within endothelial cells does not occur. Indeed, with certain inocula, *S. aureus* CFU were found to decrease with the continuous application of lysostaphin. It was important to examine the possible effects of lysostaphin on the intracellular survival of *S. aureus* within endothelial cells because controversy exists over whether lysostaphin affects the intracellular survival of *S. aureus* within leukocytes. Some reports have indicated that lysostaphin does not enter human phagocytic cells (8, 21, 24). In other reports, however, lysostaphin has been reported to adhere to and enter human polymorphonuclear leukocytes (4, 19, 26). In the present study, small amounts of lysostaphin were found to become associated with bovine endothelial cells on cover slips. However, the small amounts of lysostaphin actually associated with cover slips and endothelial cells did not demonstrate the ability to reduce the CFU of *S. aureus*.

Despite the lack of intracellular multiplication of *S. aureus*, intracellular *S. aureus* damaged endothelial cell monolayers. That endothelial cell monolayers were damaged was evident when we related the endothelial cell monolayer ^{3}H-adenine content data (Fig. 4 and 5) to the results indicating that *S. aureus* was released from within endothelial cells and was present extracellularly unless constantly eliminated by lysostaphin (Fig. 1 and 2). The extent of damage to endothelial cell monolayers was affected by the *S. aureus*

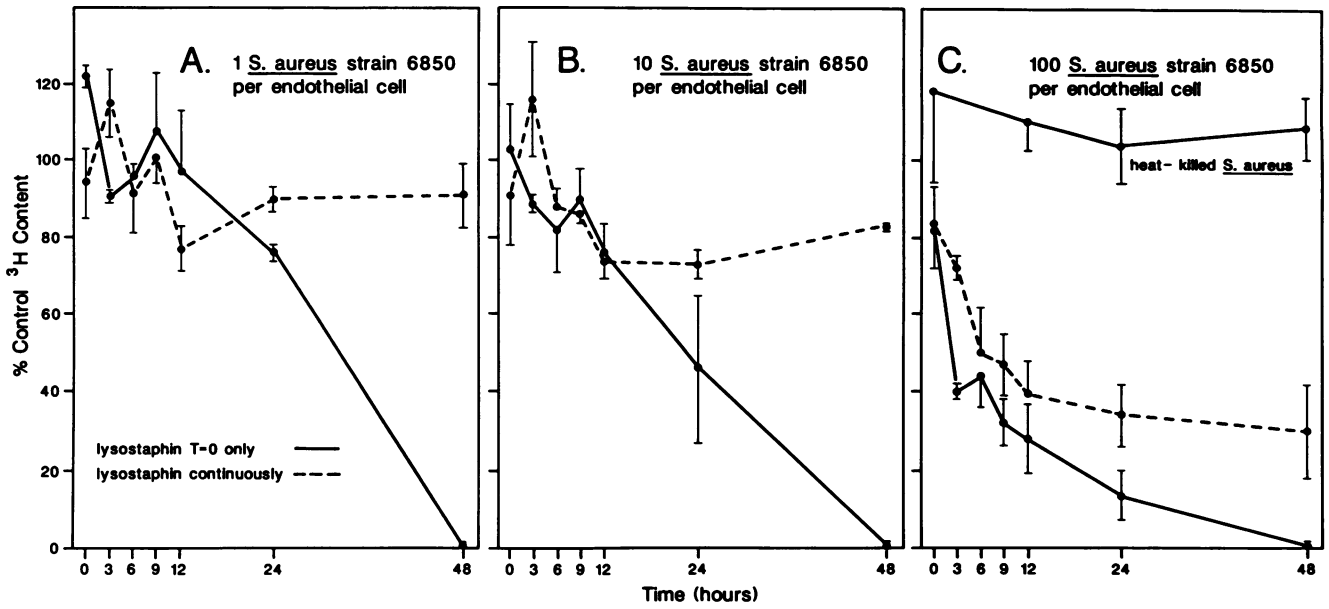

FIG. 4. Change in ^{3}H-adenine content in labeled endothelial cell cultures with time after ingestion of *S. aureus* 6850. Endothelial cell cultures were exposed to 1, 10, or 100 viable *S. aureus* 6850 or 100 heat-killed *S. aureus* 6850 organisms per endothelial cell for 3.5 h and then exposed to lysostaphin for 20 min only or continuously until each time point. Data are expressed as follows: percent control ^{3}H content = (disintegrations per minute in infected monolayers/disintegrations per minute in control monolayers) × 100. Bars indicate the standard error of the mean.

strain, the inoculum size, and the continuous elimination of extracellular *S. aureus*. Inocula of 100 *S. aureus* organisms per endothelial cell caused extensive damage to endothelial cell monolayers (Fig. 4C and 5C). Inocula of 1 or 10 *S. aureus* organisms per endothelial cell resulted in smaller initial burdens of intracellular *S. aureus*, but endothelial cells were not extensively damaged (Fig. 4A and B and 5A and D). However, under conditions in which extracellular *S. aureus* was not continuously eliminated, released *S. aureus* was reingested during incubations, increasing the intracellular load of *S. aureus*. With inocula of 1 or 10 *S. aureus* 6850 organisms per endothelial cell, there was a difference in the extent of monolayer damage at 24 and 48 h between conditions in which extracellular *S. aureus* was or was not

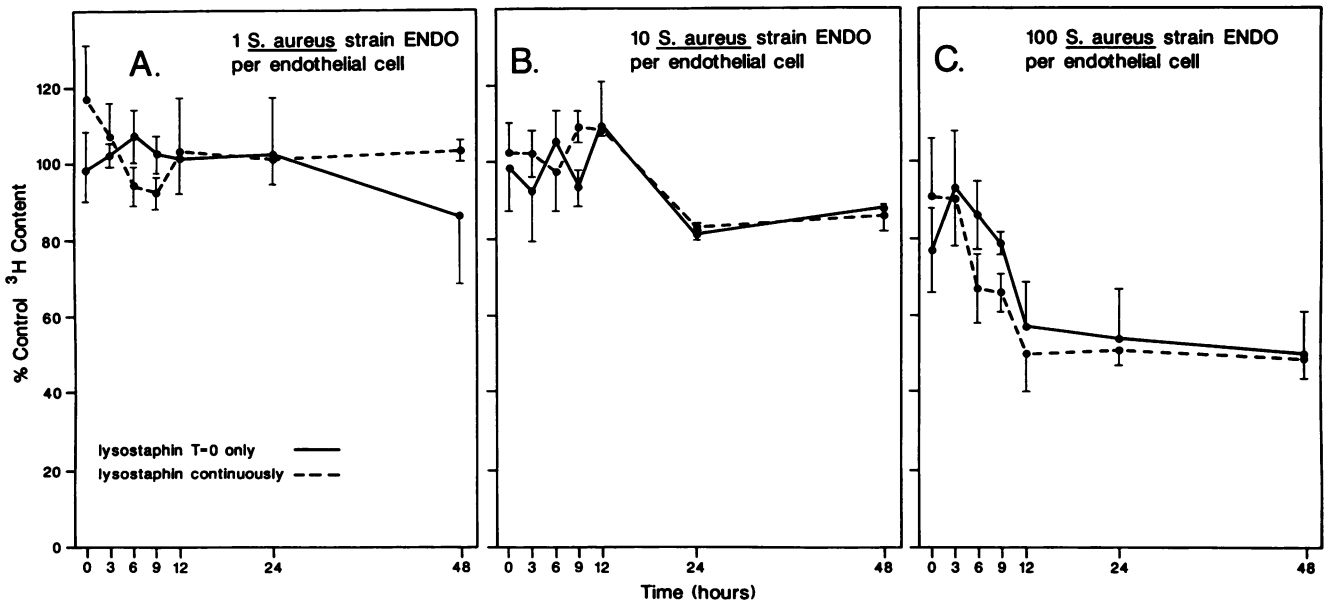

FIG. 5. Change in ^{3}H-adenine content in labeled endothelial cell cultures with time after ingestion of *S. aureus* ENDO. Endothelial cell cultures were exposed to 1, 10, or 100 *S. aureus* ENDO organisms per endothelial cell for 3.5 h and then exposed to lysostaphin for 20 min only or continuously until each time point. Data are expressed as described in the legend to Fig. 4. Bars indicate the standard error of the mean.

continuously eliminated (Fig. 4). This difference did not occur with *S. aureus* ENDO (Fig. 5). In addition, the CFU of intracellular *S. aureus* ENDO decreased much less than did the CFU of intracellular *S. aureus* 6850 (Fig. 2). These results indicate that *S. aureus* 6850 has a greater cytotoxic effect on bovine endothelial cells than does *S. aureus* ENDO. Hence, the ability of different strains of *S. aureus* to damage endothelial cell monolayers may vary. It is interesting to note that an inoculum of 100 *S. aureus* ENDO organisms per endothelial cell caused a rapid loss of ^{3}H-adenine during the first 12 h, followed by a more moderate rate of ^{3}H-adenine loss (Fig. 5). Possible explanations for this apparent change in the rate of ^{3}H-adenine release from endothelial cell monolayers include the following: (i) rapid damage to a possible subpopulation of endothelial cells which ingested above-average numbers of *S. aureus*, followed by a more gradual loss of ^{3}H-adenine from endothelial cells which phagocytized fewer *S. aureus*, (ii) rapid damage to a possible subpopulation of endothelial cells which was more susceptible to the cytotoxic activity of *S. aureus*, followed by gradual damage to more resistant endothelial cells, and (iii) a reduction in the metabolic or cytotoxic activity or both of intracellular *S. aureus* owing to changes occurring in *S. aureus* after 12 h in the apparently bacteriostatic intracellular environment. As these results were obtained with bovine endothelial cells, endothelial cells of humans and other species may be found to be more or less sensitive to the cytotoxic effects of intracellular *S. aureus* than are bovine endothelial cells.

In a population of non-drug addicts, 33% of patients with *S. aureus* bacteremia developed infectious endocarditis (17). Mortality rates for *S. aureus* native valve endocarditis in non-intravenous drug addicts are estimated to be between 25 and 40% (12). *S. aureus* is often difficult to eradicate from endovascular sites, possibly because of *S. aureus* tolerance of concentrations of antibiotics expected to be bactericidal (20). Additionally, it is thought that when sequestered within devitalized tissues (5), vegetations and occult foci of infection (13), and leukocytes (2), *S. aureus* may be protected from cell-wall-active antibiotics because it is not replicating yet is able to seed the bloodstream. Expanding this list, our results suggest that endothelial cell phagocytosis could result in the sequestration of *S. aureus*, protecting it from antibiotics and host defenses. Also, since *S. aureus* did not divide when ingested, it would be poorly responsive to cell-wall-active antibiotics. Seeding of the bloodstream would occur upon lysis of endothelial cells harboring *S. aureus*. Lysis of the endothelium harboring *S. aureus* would have the additional consequence of exposing procoagulant subendothelia collagen, resulting in platelet adherence and formation of a vegetation. It is also possible that endothelial cell phagocytic events or the influence of *S. aureus* metabolic products on endothelial cells results in the expression of procoagulant activity on the endothelial cell surface.

Endothelial cell phagocytosis of *S. aureus* could explain the occurrence of *S. aureus* endocarditis in patients having no detectable preexisting heart disease. This hypothesis is supported by the work of MacNeal et al. (15) and Ogawa et al. (18). Ogawa et al. demonstrated that cultured human valvular endothelial cells phagocytize *S. aureus* (18). MacNeal et al., who observed endothelial cell phagocytosis of streptococci in the endocardium and aorta of rabbits, reported that in some areas streptococci within endothelial cells appeared to have multiplied and resulted in the vegetations of endocarditis (15). Phagocytosis of streptococci by cultured endothelial cells has been previously reported (S. Chaudhary, T. Alred, S. L. Gatchel, T. Maciag, and R. P. Tewari, Abst. Annu. Meet. Am. Soc. Microbiol. 1986, D39, p. 72); however, the resultant effects on the cultured cells are unknown.

In the present study, the failure of *S. aureus* to multiply within endothelial cells was unexpected. This result indicates that endothelial cells possess some means of inhibiting the replication of intracellular *S. aureus*. It is possible that endothelial cells possess some bactericidal capacity and could perhaps inactivate organisms less virulent than *S. aureus*. MacNeal et al. reported that most streptococci within endothelial cells of rabbits appeared to have been destroyed (15), but more definite evidence is needed. Despite the apparent bacteriostatic intracellular environment, endothelial cells harboring *S. aureus* were damaged. *S. aureus* is also known to persist within leukocytes (2). Hence, endothelial cells and leukocytes harboring *S. aureus* may contribute to the persistence of *S. aureus* in endovascular infections. The ability of rifampin, a lipid-soluble antibiotic, to penetrate leukocytes and kill intracellular *S. aureus* has already been studied (16). Similar results might be expected if rifampin were applied to endothelial cells harboring *S. aureus*. Thus, the findings of the present report have potential applications to the therapy of *S. aureus* endovascular infections.

Damage to heart valve endothelium caused by *S. aureus* in vitro has been studied by Cooper et al. with scanning electron microscopy (3). However, the possibility that endothelial cell damage could have been caused by intracellular *S. aureus* was not addressed at that time (3). The present report represents the first detailed study of the consequences of the ingestion of *S. aureus* by cultured endothelial cells and provides the groundwork for investigation of the pathogenic effects of intracellular *S. aureus* within endothelial cells. Although further work is necessary, the phagocytosis and persistence of *S. aureus* in endothelial cells suggest a mechanism for the infection of normal heart valves, for the persistent bacteremia seen in endovascular infections, and for the relative ineffectiveness of antibiotics and host defenses in clearing the infections.

ACKNOWLEDGMENTS

We thank Jan Feyzi for performing statistical tests and Sherideen Stoll for help in preparing the manuscript.

This work was supported by funds from Public Health Service grant AI 16881 from the National Institutes of Health and by grants from the Department of Medicine research fund.

LITERATURE CITED

1. **Andreoli, S. P., R. L. Baehner, and L. M. Bergstein.** 1985. *In vitro* detection of endothelial cell damage using 2-deoxy-D-^{3}H-glucose: comparison with chromium 51, ^{3}H-leucine, ^{3}H-adenine and lactate dehydrogenase. J. Lab. Clin. Med. **106**:253–261.
2. **Baughn, R. E., and P. F. Bonventre.** 1975. Phagocytosis and intracellular killing of *Staphylococcus aureus* by normal mouse peritoneal macrophages. Infect. Immun. **12**:346–352.
3. **Cooper, M. D., C. Jeffery, D. L. Gall, and A. S. Anderson.** 1985. Scanning electron microscopy studies of staphylococcal adherence to heart valve endothelial cells in organ culture: an *in vitro* model of acute endocarditis. Scanning Electron Microsc. **1985 III**:1231–1237.
4. **Easmon, C. S. F., H. Lanyon, and P. J. Cole.** 1978. Use of lysostaphin to remove cell-adherent staphylococci during *in vitro* assays of phagocyte function. Br. J. Exp. Pathol. **59**:381–385.
5. **Faville, R. J., D. E. Zaske, E. L. Kaplan, K. Crossley, L. D. Sabath, and P. G. Quie.** 1978. *Staphylococcus aureus* endo-

carditis. J. Am. Med. Assoc. **240**:1963–1965.
6. **Grant, R. T., J. E. Wood, and T. D. Jones.** 1927–1929. Heart valve irregularities in relation to subacute bacterial endocarditis. Heart **14**:247–261.
7. **Hamill, R. J., J. M. Vann, and R. A. Proctor.** 1986. Phagocytosis of *Staphylococcus aureus* by cultured bovine aortic endothelial cells: model for postadherence events in endovascular infections. Infect. Immun. **54**:833–836.
8. **Hand, W. L., and N. L. King-Thompson.** 1986. Contrasts between phagocyte antibiotic uptake and subsequent intracellular bactericidal activity. Antimicrob. Agents Chemother. **29**: 135–140.
9. **Jaffe, E. A.** 1984. Culture and identification of large vessel endothelial cells, p. 1–13. *In* E. A. Jaffe (ed.), Biology of endothelial cells. Kluwer-Nijhoff Publishing, Norwell, Mass.
10. **Jaffe, E. A., L. W. Hoyer, and R. L. Nachman.** 1973. Synthesis of antihemophilic factor antigen by cultured human endothelial cells. J. Clin. Invest. **52**:2757–2764.
11. **Kaplan, E. L., H. Rich, W. Gersony, and J. Manning.** 1974. A collaborative study of infective endocarditis in the 1970's. Circulation **59**:327–335.
12. **Karchmer, A. W.** 1985. Staphylococcal endocarditis. Laboratory and clinical basis for antibiotic therapy. Am. J. Med. **78**(Suppl. 6B):116–127.
13. **Levison, M. E.** 1976. Pathogenesis of infective endocarditis, p. 29–42. *In* D. Kay (ed.), Infective endocarditis. University Park Press, Baltimore.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
15. **MacNeal, W. J., M. J. Spence, and A. E. Slavkin.** 1943. Early lesions of experimental endocarditis lenta. Am. J. Pathol. **19**: 735–749.
16. **Mandell, G. L., and T. K. Vest.** 1972. Killing of intraleukocytic *Staphylococcus aureus* by rifampin: *in vitro* and *in vivo* studies. J. Infect. Dis. **125**:486–490.
17. **Mirimanoff, R. O., and M. P. Glauser.** 1982. Endocarditis during *Staphylococcus aureus* septicemia in a population of non-drug addicts. Arch. Intern. Med. **142**:1311–1313.
18. **Ogawa, S., V. Hatcher, E. Yurberg, M. Levitt, and F. Lowy.** 1985. Bacterial adherence to human endothelial cells in vitro. Infect. Immun. **50**:218–224.
19. **Pruzanski, W., S. Saito, and D. W. Nitzan.** 1983. The influence of lysostaphin on phagocytosis, intracellular bactericidal activity, and chemotaxis of human polymorphonuclear cells. J. Lab. Clin. Med. **102**:295–305.
20. **Rajashekaraiah, K. R., T. Rice, V. S. Rao, D. Marsh, B. Ramakrishna, and C. A. Kallick.** 1980. Clinical significance of tolerant strains of *Staphylococcus aureus* in patients with endocarditis. Ann. Intern. Med. **93**:799–801.
21. **Schaffner, W., M. A. Melly, J. N. Hash, and H. G. Koenig.** 1966. Lysostaphin: an enzymatic approach to staphylococcal disease. *In vitro* studies. Yale J. Biol. Med. **39**:215–230.
22. **Scheld, W. M., J. A. Valone, and M. A. Sande.** 1978. Bacterial adherence in the pathogenesis of endocarditis. J. Clin. Invest. **61**:1394–1404.
23. **Snedecor, G. W., and W. G. Cochran.** 1980. Statistical methods, p. 91, 96–99. Iowa State University Press, Ames.
24. **Tan, J. S., C. Watanakunakorn, and J. P. Phair.** 1971. A modified assay of neutrophil function: use of lysostaphin to differentiate defective phagocytosis from impaired intracellular killing. J. Lab. Clin. Med. **78**:316–322.
25. **Thompson, R. L.** 1982. Staphylococcal infective endocarditis. Mayo Clin. Proc. **57**:106–114.
26. **van den Broek, P. J., F. A. M. Dehue, P. C. J. Leijh, M. T. van den Barselaar, and R. van Furth.** 1982. The use of lysostaphin in *in vitro* assays of phagocyte function: adherence to and penetration into granulocytes. Scand. J. Immunol. **15**:467–473.
27. **Vercelloti, G. M., D. Lussenhop, P. K. Peterson, L. T. Furcht, J. B. McCarthy, H. S. Jacob, and C. F. Moldow.** 1984. Bacterial adherence to fibronectin and endothelial cells: a possible mechanism for bacterial tissue tropism. J. Lab. Clin. Med. **103**:34–43.
28. **Watanakunakorn, C.** 1973. Some salient features of *Staphylococcus aureus* endocarditis. Am. J. Med. **54**:473–481.