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Phagocytosis of *Staphylococcus aureus* by Cultured Bovine Aortic Endothelial Cells: Model for Postadherence Events in Endovascular Infections

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We examined the interaction of *Staphylococcus aureus* with cultured bovine aortic endothelial cells as a model for the initial events in the pathogenesis of endovascular infections. Confluent monolayers of cultured endothelial cells were incubated with *S. aureus*. Cell-associated bacteria were measured by washing away nonadherent organisms, disrupting the monolayers, and performing quantitative cultures. Phagocytosis was differentiated from adherence by treating the cells with lysostaphin; approximately 60% of cell-associated bacteria was found to be intracellular. Phagocytosis could be blocked by using cytochalasin B, which interferes with microfilament function. Addition of fibronectin resulted in a 63% increase in adherence of *S. aureus* to the endothelial cells but did not increase ingestion. Transmission electron microscopy demonstrated a sequence of events similar to that which occurs during ingestion by professional phagocytes, including: (i) adherence of bacteria to the endothelial cell; (ii) formation and elongation of surface extensions of the endothelial cell to surround the adherent bacteria; and (iii) complete enclosure within apparent phagosomes. Phagocytosis of bacteria by endothelial cells, followed by intracellular persistence, may be an important postadherence event in the pathogenesis and pathophysiology of endovascular infections.

The importance of bacterial adherence followed by invasion has been demonstrated for infections of intestinal epithelial cells and other mucosal surfaces (9, 12). For the initiation of infective endocarditis, bacteria must first adhere to the cardiac valve before colonization and invasion can occur (20, 28). Specific adherence of *Staphylococcus aureus* to endothelial cells has been demonstrated (4, 14, 26). Once attached, bacterial species invade host tissues to exert their pathogenic activity. However, the means by which *S. aureus* invades cardiac structures to initiate infective endocarditis remains obscure. Recently, some insight into the mechanism of invasion has been achieved by the observation that endothelial cells may ingest *S. aureus* (14). We further investigated the phagocytic capacity of endothelial cells and found that cultured bovine aortic endothelial cells actively phagocytose *S. aureus* but do not appear to be capable of killing the bacteria.

MATERIALS AND METHODS

Bacteria. *S. aureus* ATCC 25923 and ENDO were used throughout all experiments. These strains have been described previously (15, 17) and gave similar results. Bacteria were grown overnight to logarithmic phase for all quantitative studies and to stationary phase for all electron micrographic experiments. The organisms were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) at 37°C, harvested by centrifugation at 2,800 × *g* for 5 min, washed with sterile saline, and resuspended to desired concentrations in Hanks balanced salt solution (HBSS), pH 7.4, with 0.5% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.). Bacterial concentrations were estimated spectrophotometrically and confirmed by plate and Petroff-Hausser chamber counts. Solutions were sonicated briefly to assure that bacterial clumps were not present.

Isolation and culture of bovine aortic endothelial cells (6). Freshly obtained bovine aortic segments were flushed clean, filled with a solution of 0.1% collagenase (Sigma) in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline, and suspended for 10 to 15 min in a 37°C bath of sterile HEPES-buffered saline. Cells were collected by centrifugation at 220 × *g* for 5 min and resuspended in Dulbecco modified Eagle essential medium (M. A. Bioproducts, Walkersville, Md.) containing 20% heat-inactivated fetal calf serum (Hazelton-Dutchland, Denver, Pa.) and gentamicin (50 μg/ml; Sigma) for distribution to tissue culture dishes. Endothelial cell growth factor (Sigma; 25 μg/ml) was added to maintain the endothelial cell morphology of the cultured cells. Cell cultures were incubated in 5% CO_2 at 37°C. The endothelial cell nature of the cells was verified by the typical cobblestone morphology and immunofluorescence staining for factor VIII-related antigen (6, 7). All studies were performed with cells that had been passaged between two and eight times.

Preparation of fibronectin. Fibronectin was prepared from fibronectin- and fibrinogen-rich plasma protein fractions as described previously (17).

Phagocytosis assay. Phagocytic assays were performed in 24-well tissue culture plates (Becton Dickinson Labware, Oxnard, Calif.) containing a 15-mm-diameter round glass cover slip (Carolina Biological Supply Co., Burlington, N.C.) on which endothelial cells had been grown to confluence. Each confluent cover slip contained 2×10^5 to 3×10^5 endothelial cells as verified by trypsin release and visual counts in a hemacytometer. Before each assay, the cells were gently washed three times with 1 ml of HBSS-bovine serum albumin. Phagocytosis was initiated by adding *S. aureus* to yield a final volume of 1 ml. The effect of fibronectin on adherence and phagocytosis was evaluated by

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TABLE 1. Phagocytosis of *S. aureus* by cultured bovine aortic endothelial cells[a]

Condition of *S. aureus*	10^4 *S. aureus* CFU ± SEM	% Endothelial cell-associated *S. aureus* cells[b]
Adherent + ingested (untreated control)	1.44 ± 1.34	100
Ingested (lysostaphin treated)	0.94 ± 1.02	65
Adherent (cytochalasin B treated)	0.67 ± 1.15	47
Remaining cell associated (cytochalasin B + lysostaphin treated)	0.19 ± 0.19	13

[a] All experiments were performed on confluent monolayers of bovine aortic endothelial cells incubated for 210 min with 3.0×10^5 *S. aureus* cells at 37°C as described in Materials and Methods. Data are means from three experiments performed in triplicate.

[b] Results are expressed as percentages of the untreated controls after nonadherent bacteria were removed with three washes of HBSS.

adding 0.25 ml of Tris-buffered saline (pH 7.4) that contained 1.2 mg of fibronectin per ml. To investigate the effect of interfering with endothelial cell microfilament function, some wells were treated with 10 μg of cytochalasin B (Sigma) per ml. Incubation was carried out at 37°C for 3.5 h, the time period being based on preliminary observations that demonstrated that endothelial cell-associated *S. aureus* reached an equilibrium by 3.5 h. The wells were then washed, and alternate wells were treated with a lysostaphin (Sigma; 10 μg/ml) solution for 20 min at room temperature. Control wells containing bacteria but no endothelial cells were also treated with lysostaphin to verify the efficiency of the lysostaphin treatment. The wells were again washed, and cover slips were removed from the wells, placed into sterile vials containing 1 ml of sterile water, and gently sonicated to disrupt any intact endothelial cells. Serial dilutions were made in sterile water, and samples were cultured in tryptic soy agar (Difco) pour plates for bacterial quantitation. Each condition was studied in triplicate, and the mean of these three observations represents one experiment.

Electron microscopy methods. Bovine aortic endothelial cells were cultured to confluence on 13-mm-diameter Thermanox plastic cover slips (Lux Scientific Corp., Newberg Park, Calif.) in wells of a 24-well tissue culture plate. Cover slips were washed, and *S. aureus* (10^8 CFU/ml) was added to all wells with additional HBSS-bovine serum albumin to bring the volume in each well to 1 ml. Incubation continued for 3.5 h at 37°C, after which nonadherent bacteria were removed by washing each well with HBSS. Endothelial cells in half of the wells were immediately fixed with 2% glutaraldehyde (Ted Pella, Inc., Tustin, Calif.) in phosphate-buffered saline for 1 h. The remaining cells were treated for 20 min with lysostaphin (10 μg/ml), after which they were washed with HBSS, given fresh medium, and allowed to incubate for various times before glutaraldehyde fixation. Postfixation was carried out in 1.0% osmium tetroxide (Pella) for 1 h. Specimens were stained in aqueous uranyl acetate (Pella), followed by dehydration in increasing concentrations of ethanol. Then specimens were stained in lead acetate, rinsed in absolute ethanol, and infiltrated with increasing concentrations of Spurr resin (Pella). Simultaneously, blank blocks of Spurr resin were polymerized in a flexible Teflon (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) flat embedding mold (Pella) at 70°C for 18 h. The cover slips that had been infiltrated with resin were cut in half, and the half-cover slips were placed cell-side-down on the blank resin blocks and allowed to polymerize at 70°C for 18 h. The cover slip-resin block was then cooled on dry ice, and the cover slip was peeled off, leaving the endothelial cells polymerized into the resin block. These blocks were placed in Beem embedding capsules (Pella), which were filled with Spurr resin and polymerized at 70°C for 18 h. Thin sections were cut with an LKB 8800 ultramicrotome (LKB Instruments, Inc., Rockville, Md.). Thin sections on grids were stained with uranyl acetate followed by Reynolds lead citrate (Pella). Specimens were examined in a Philips 300 or a JEOL 100 CX transmission electron microscope.

RESULTS

Quantitative endothelial cell phagocytosis assay. Results of a representative endothelial cell phagocytosis assay are shown in Table 1. At the end of 3.5 h, cultured bovine aortic endothelial cells had actively phagocytosed *S. aureus*. In the absence of added cytochalasin B or lysostaphin, $1.44 \pm 1.34 \times 10^5$ *S. aureus* cells were endothelial cell associated. After treatment with lysostaphin to remove adherent bacteria, 65% of the endothelial cell-associated bacteria remained viable, presumably in an intracellular or subcellular location. Lysostaphin treatment of cover slips exposed to bacteria in the absence of endothelial cells resulted in >99% killing (data not shown).

To determine whether the entry of *S. aureus* into endothelial cells was an active process dependent on microfilament function, treatment of the endothelial cells with the phagocytic inhibitor cytochalasin B was performed. This treatment resulted in a decrease of cell-associated *S. aureus* to 47% of the untreated controls. Of cell-associated bacteria, 13% survived combined treatment with both cytochalasin B and lysostaphin.

Effect of fibronectin on adherence and phagocytosis of *S. aureus*. Adherence and phagocytosis were studied in connection with a physiological concentration of fibronectin (Table 2). In the absence of added fibronectin, 59% of *S. aureus* cells were still found to be cell associated after lysostaphin treatment and represent sequestered bacteria. The presence of 300 μg of fibronectin per ml stimulated a 63% increase in the number of adherent and ingested *S. aureus* cells. However, after incubation with lysostaphin, the number of remaining cell-associated *S. aureus* cells was very similar whether or not fibronectin had been added ($4.9 \pm 2.0 \times 10^3$ versus $4.5 \pm 0.9 \times 10^3$, respectively).

Ultrastructural analysis of endothelial cells. Phagocytosis

TABLE 2. Effects of added soluble fibronectin on adherence and phagocytosis of *S. aureus* by cultured bovine aortic endothelial cells[a]

Condition	10^3 *S. aureus* CFU ± SEM		% Ingested *S. aureus*
	Adherent + ingested (no treatment)	Ingested (lysostaphin treated)	
No added fibronectin	7.7 ± 2.1	4.5 ± 0.9	59
Added fibronectin	12.4 ± 3.1[b]	4.9 ± 2.0	39

[a] Fibronectin (300 μg/ml) was added to confluent monolayers of cultured bovine aortic endothelial cells before the addition of 3.0×10^5 bacteria, as described in Materials and Methods. Data are from a single representative experiment.

[b] Significantly different compared with no added fibronectin ($P < 0.01$) by the two-sided *t* test.

of *S. aureus* by cultured bovine aortic endothelial cells appeared to proceed in a sequential fashion. Adherence of *S. aureus* to the endothelial cell surface could be observed. Some endothelial cells developed cup-shaped processes underlying the adherent *S. aureus* (Fig. 1A). Endothelial cell appendages elongated and began to wrap around the *S. aureus* cells (Fig. 1B). In some cells, bacteria were completely enclosed in what appear to be phagosomes, some of which contained tetrads, but large clumps of *S. aureus* cells were not seen. Bacteria were not observed in subcellular locations. The observation that apparently intracellular staphylococci were still observed despite lysostaphin treatment helped to exclude the possibility that bacteria were simply lying in deep crypts in the surfaces of the endothelial cells. In addition, when the lysostaphin-treated monolayers were reincubated for 18 h, intact intracellular *S. aureus* organisms were demonstrated (Fig. 1C).

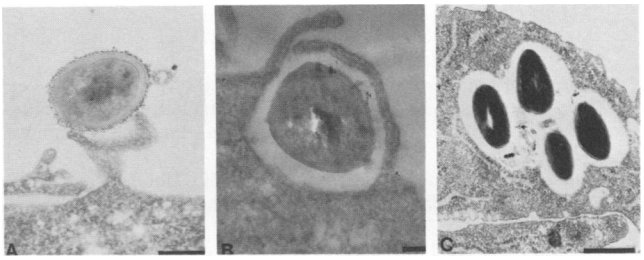

FIG. 1. Transmission electron micrographs demonstrating the sequence of events during the phagocytosis of *S. aureus* by bovine aortic endothelial cells. Bars: 500 nm (A and C); 100 nm (B). (A) Adherence of *S. aureus* to the endothelial cell and formation of a cup-shaped process on the endothelial cell membrane underlying the adherent bacteria. (B) Elongation of endothelial cell appendages to enclose the *S. aureus*. (C) Multiple bacteria enclosed in a phagosome of a lysostaphin-treated cell culture after 18 h of incubation.

DISCUSSION

Our results confirm and extend the observations of Ogawa et al. (14) that endothelial cells can ingest *S. aureus*. This phagocytosis appears to proceed in a sequential fashion as is observed with professional phagocytes (5). Bacterial adherence to the endothelial cell is followed by the appearance of endothelial appendages which elongate and eventually enclose the bacteria into apparent phagosomes. We found that phagocytosis of *S. aureus* by bovine aortic endothelial cells is an active process that can be blocked by the phagocytic inhibitor cytochalasin B. A similar sequence of ultrastructural events has been recently demonstrated for endothelial phagocytosis of *Rickettsia prowazekii* (27) and *Candida* species (19).

Endocytosis is a normal function of endothelial cells to fulfill nutritional requirements (2). The ability of endothelial cells to ingest particulate materials was appreciated by the demonstration that they could phagocytose latex beads (21). Kramer (10) has recently suggested that the phagokinetic response of endothelial cells which are challenged with fibronectin-coated materials or extracellular matrix fragments is an attempt to maintain a nude, nonthrombogenic lumenal surface.

Although definitive data implicating fibronectin in the pathogenesis of endovascular infections are not yet available, considerable circumstantial evidence suggests that fibronectin is important. Expression of specific fibronectin receptors (3, 11, 17) correlates with the relative ability of clinical isolates of *S. aureus* to produce invasive infections (15). Endothelial cells synthesize substantial amounts of fibronectin (8) although their lumenal surfaces are free of fibronectin (1). Vercellotti et al. (26) have demonstrated an enhanced adherence of fibronectin-treated *S. aureus* cells to monolayers of endothelial cells, a result similar to that found in the present study. Fibronectin has also been shown to mediate the binding of *S. aureus* cells to clots (22) and collagen-fibronectin matrices (16, 24). Thus, fibronectin may be involved in the pathogenesis of intravascular infections via specific interactions between the *S. aureus*-fibronectin receptor and host fibronectin found in blood clots (13), vegetations (22), intercellular matrix exposed after endothelial trauma (13), and on intact endothelium. The larger quantities of fibronectin found in thrombi and in the intercellular matrix, as compared with the endothelial surface, correlates well with the relative frequencies of *S. aureus* endocarditis developing on damaged endothelium, as compared with intact endothelium.

Fibronectin did not enhance phagocytosis of *S. aureus* by bovine aortic endothelial cells. This may be due to a relatively limited phagocytic capacity of endothelium such that adherence is not the rate-limiting step. Additionally, fibronectin may not be a complete opsonin for endothelial cells which may require other factors to more effectively phagocytose. Others have demonstrated that although fibronectin promotes the adherence of *S. aureus* to professional phagocytes, it does not facilitate phagocytosis (18, 23, 25).

Despite a considerable amount of literature addressing the issue of adherence in the pathogenesis of bacterial infections, limited data are available concerning postadherence events. This report is one of the first to attempt to investigate this problem. We hypothesize that endothelial cells actively phagocytose *S. aureus* as an extension of their normal endocytic activity or as an attempt to maintain a nonthrombogenic surface. Persistence or intracellular multiplication of *S. aureus* (or both) may occur with eventual lysis and death of the endothelial cell, resulting in exposure of thrombogenic extracellular matrices. Subsequent events may include local thrombosis and growth of vegetations. This hypothetical sequence would help explain: (i) the frequent occurrence of staphylococcal infection of vascular surfaces; (ii) the presence of thrombi at sites of microvascular *S. aureus* infections; (iii) the relative inefficiency of the immune response of the host in endovascular infections due to an inability of phagocytes to reach sequestered, intracellular bacteria; and (iv) the slow response to β-lactam antibiotics which may have difficulty reaching intracellular *S. aureus*.

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