Adherence of Staphylococcus aureus to Cultured Bovine Mammary Epithelial Cells

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ABSTRACT

Bovine mammary secretory cells, isolated at necropsy, were cultured in vitro and used as a model to study the mode of adherence of Staphylococcus aureus to mammary epithelium. Cultured cells were characterized by their morphology and physiology as secretory epithelial cells. Cells showed characteristic growth patterns when grown on polystyrene, fibronectin, laminin, collagen, and reconstituted basement membrane from the Engelbreth-Holm-Swarm murine sarcoma. Cells cultured on collagen formed confluent monolayers and were the most suitable for bacterial adherence studies. Cultured cells stained intensely for cytokeratin and for specific milk proteins, i.e., α-casein, β-casein, α-lactalbumin, β-lactoglobulin, and lactoferrin. The effect of frozen storage for 10 mo on cell viability or presence of milk proteins was minimal. Staphylococcus aureus showed large affinity for extracellular matrix components, i.e., fibronectin, laminin, and collagen. Adherence to confluent cell monolayers was minimal. In preconfluent cell monolayers, most S. aureus adhered more readily to the exposed matrix than to the epithelial cells. Overnight exposure to staphylococcal α-toxin greatly increased adherence of S. aureus to confluent monolayers. However, whether bacteria adhered to α-toxin damaged cells or to exposed matrix is not clear. Unencapsulated S. aureus adhered in larger numbers than did encapsulated S. aureus. (Key words: Staphylococcus aureus, adherence, mammary, epithelium)

INTRODUCTION

Cultured bovine mammary secretory cells are appropriate models 1) to study lactogenesis and galactopoiesis (13, 17, 29), 2) to study cell to cell and cell to extracellular matrix interactions (4, 11, 35), and 3) to characterize plasma membrane receptors for cytokines, hormones, and growth factors (31, 32) and their role on cell proliferation and function (12, 26, 30, 37). Cultured bovine mammary secretory cells also provide a model to study various pathological processes, i.e., interactions of bacteria and epithelium. Adherence of bacteria to mammary epithelium may be an initial phase in the pathogenesis of mastitis (9, 40); however, some controversy surrounds this hypothesis (1, 38). Several Staphylococcus aureus receptors for basement membrane components have been identified (8, 20, 34). Although a hemagglutinating protein that promotes binding to epithelial cells has been identified on S. aureus (19), limited evidence suggests that S. aureus may adhere to damaged cells (10, 38). In the...
present study, bovine mammary secretory cells were isolated at necropsy, characterized in vitro, and cultured on various extracellular matrices to determine 1) the most suitable extracellular matrix for bacterial adherence studies, 2) the mode of adherence of S. aureus to mammary epithelium, 3) the role of toxin damage in bacterial adherence, and 4) the role of bacterial capsule in adherence to mammary epithelial tissue.

**MATERIALS AND METHODS**

**Collection of Mammary Tissue and Isolation of Epithelial Cells**

Three clinically healthy Holstein-Friesian lactating cows with udders free of infection were used. Quarters were checked for bacterial infection 48 and 24 h prior to slaughter. Immediately after slaughter at the USDA abattoir (Beltsville, MD), the udder was removed, washed with Betadine® (Perdue Frederick Co., Norwalk, CT), and rinsed with 70% ethanol. Tissue samples were taken within 30 min after slaughter. An incision was made dorsal to the gland cistern. Secretory tissue was taken in 50-g sections, avoiding collection of large blood vessels and ducts. The tissues were immersed in a solution containing 500 ml of Hanks balanced salt solution (HBSS) (Sigma Chemical Co., St. Louis, MO), 1 ml of 1 M HEPES buffer (Sigma Chemical Co.), and 10 ml of antibiotic-antimycotic solution (10,000 U of penicillin/ml, 10 mg of streptomycin/ml, and 25 µg of amphotericin B/ml; Sigma Chemical Co.) (HBSSo). The tissues were kept at room temperature (22°C) and quickly returned to the laboratory for further processing. After the tissue samples were washed twice in HBSSo, they were placed in a sterile 100-mm Petri dish, moistened with HBSSo, and sliced into 5-g sections. The sections were then washed with HBSSo and minced with scissors. The minced tissue was transferred to a flask containing 50 ml of HBSSo and rocked for 5 min at room temperature. Large clumps of tissue were allowed to settle, and the supernatant was decanted. This procedure was repeated until the supernatant was clear. The HBSSo was replaced by 5 ml of HBSSo supplemented with collagenase type 3 from Clostridium histolyticum (360 U/ml; 184 U/img of dry weight; Worthington Biochemicals, Freehold, NJ) per gram of tissue and rotated at 37°C. Every 30 min, the mixture was filtered through a 200-µm nylon mesh (Spectrum, Los Angeles, CA) to collect dispersed cells. This procedure was repeated at 30-min intervals for 5 h, after which the residual tissue was discarded. The filtrates, composed mainly of individual cells and small cell clumps, were centrifuged at 100 × g for 5 min and washed twice in HBSSo to eliminate enzyme residue. The cells were counted and checked for viability using trypan blue exclusion and then were either cultured or cryopreserved.

**Culture Medium**

Culture medium contained 40% RPMI 1640 (JRH Biosciences, Lenexa, KS), 40% Dulbecco’s modified Eagle’s medium (DMEM; JRH Biosciences), 10% fetal bovine serum (FBS; JRH Biosciences), 2% antibiotic-antimycotic solution, 1 mM sodium pyruvate (Sigma Chemical Co.), 2 mM L-glutamine (JRH Biosciences), and 40 mM HEPES buffer was routinely used for cell culture in our laboratory. Bovine insulin (5 µg/ml; Sigma Chemical Co.), hydrocortisone (1 µg/ml; Sigma Chemical Co.), bovine prolactin (1 µg/ml; courtesy of D. J. Bolt, USDA, Animal Hormone Program, Beltsville, MD), murine epidermal growth factor (5 ng/ml; Sigma Chemical Co.), and bovine transferrin (10 µg/ml; Sigma Chemical Co.) were added to the culture medium (13, 27, 33, 35, 39). The medium was stored at 4°C.

**Culture of Epithelial Cells**

Mammary secretory epithelial cells isolated from each cow were plated and subcultured on collagen, laminin, fibronectin, reconstituted basement membrane from the Engelbreth-Holm-Swarm murine sarcoma (EHS), and polystyrene.

**Culture on Collagen Matrices.** Two classes of collagen type I were used, rat-tail tendon collagen [obtained as described (28)] and calf-skin collagen (Sigma Chemical Co.). To prepare thin and thick rat-tail collagen gels, 50 and 150 µl of titrated rat-tail collagen/cm² of growth surface were added, and the collagen was allowed to gel at room temperature. Thin calf-skin collagen gels were prepared by addi-
tion of 100 µl of 1% calf-skin collagen in 1N acetic acid/cm² of growth surface and overnight incubation at 4°C. Excess collagen was decanted, and the plates were allowed to dry overnight at 4°C. Collagen gels were used immediately or stored at 4°C covered with HBSS, until needed. Approximately 2 × 10⁵ cells in 1 ml of culture medium and 5 × 10⁵ cells in 5 ml of culture medium were overlaid on collagen gels in 24-well plates and 60-mm culture dishes and incubated at 37°C in 5% CO₂. Culture medium was replaced every 48 h. Cultures were examined microscopically and photographed daily. Cells were processed for high magnification light microscopy as described previously (24). To obtain cultures on floating collagen gels, the gels were detached by lifting the edges of the gels with a sterile pasteur pipette. During successive changes of media, care was taken to avoid damaging the shrinking gels. To embed cells in collagen, a pellet containing 5 × 10⁵ cells was gently mixed with rat-tail collagen (.3 ml at 4°C), placed in a well of a 24-well plate, and allowed to gel at room temperature for 15 min. Culture medium (5 ml) was added and incubated as described.

Culture on Polystyrene, Laminin and Fibronectin. Approximately 2 × 10⁵ cells in 1 ml of culture medium and 5 × 10⁵ cells in 5 ml of culture medium were plated on polystyrene 24-well plates and 60-mm tissue culture dishes. Approximately 5 × 10⁵ cells in 5 ml of culture medium were plated on 60-mm culture dishes coated with murine laminin and human fibronectin (Collaborative Biomedical Products, Bedford, MA). Cultures were incubated as described.

Culture on EHS. The EHS (Matrigel™; Collaborative Biomedical Products) was stored at -20°C and then thawed overnight at 4°C and kept on ice to avoid gelation. The EHS (1.6 mg/cm² of growth surface) was added to wells of 24-well plates and incubated at 37°C for 30 min (to allow EHS to solidify). Approximately 5 × 10⁵ cells in 1 ml of culture medium were plated on each EHS gel and cultured as described.

Lumina of multicellular structures formed on EHS were demonstrated as described for murine mammary epithelial cells (3). Briefly, culture medium was removed, and cultures were washed twice with HBSSₐ. A solution containing HBSS plus 2.5 mM ethylene glycol-bis(β-aminoethylether) (EGTA) (Sigma Chemical Co.) and 10 mM HEPES buffer was added (1 ml per well) and incubated at 37°C for 20 min. This solution was replaced with HBSS (1 ml) containing 1.2 mM EGTA and 1% trypan blue and incubated at room temperature for 15 min. The culture was washed several times with HBSSₐ and examined with phase contrast microscopy.

Recovery of Cells from Cultures. After two washes with HBSSₐ, trypsin-EDTA was added (5 ml per 60-mm culture dish and .5 ml per well of 24-well plates) and incubated at 37°C until most of the cells appeared in suspension. The cell suspension was pipetted into tubes containing an equal volume of HBSSₐ containing 50% FBS to stop trypsinization. The cells were washed twice with HBSSₐ and resuspended in culture medium to the desired concentration.

Cryopreservation and Thawing

Cells were collected by centrifugation at 100 × g for 5 min and resuspended in 70% culture medium, 20% FBS, and 10% dimethyl sulfoxide at 4°C (21, 23). Aliquots (1 ml; 5 × 10⁵ to 10⁶ cells/ml) were dispensed in 2-ml vials. Vials were frozen at −20°C for 15 min and then transferred to −70°C or to liquid nitrogen for long-term storage. Cells were thawed in a water bath at 37°C and washed twice with HBSSₐ to remove the freezing solution.

Immunohistochemistry

Cultured cells (1 ml of 10⁵ cells/ml of growth medium) were plated on 4-well polystyrene chamber slides (Nunc, Inc., Naperville, IL), uncoated and coated with collagen and EHS, and were incubated at 37°C in 5% CO₂. The cultures were rinsed twice with HBSSₐ and fixed with Bouin’s solution (Sigma Chemical Co.) for 15 min at room temperature with rocking. After rinsing with .01 M PBS, 1 ml of a 1/20 (vol/vol) gammaglobulin-free horse serum (Gibco, Grand Island, NY) in .01 M PBS with .05% Tween 20 (Sigma Chemical Co.) was added to each chamber and incubated for 30 min at room temperature with rocking. After washing with PBS, rabbit antibodies to
BACTERIAL ADHERENCE TO MAMMARY CELLS

human cytokeratin (cytokeratin peptide specificity not determined; Biogenex, San Ramon, CA), human vimentin (Chemicon, Temecula, CA), and bovine milk proteins, i.e., α-casein, β-casein, α-lactalbumin, β-lactoglobulin, and lactoferrin (courtesy of J. E. Butler, University of Iowa, Medical School, Iowa City, IA) were added and incubated for 1 h at room temperature with rocking. Specificity of antibodies to the respective milk proteins was verified by ELISA. The antibodies were removed and the cultures were washed three times with PBS. Fluorescein-labeled goat anti-rabbit IgG (Kirkegaard & Perry, Gaithersburg, MD) was added and incubated in the dark for 45 min at room temperature with rocking. Cultures were washed three times with PBS. Chamber walls were separated from the slides, and the slides were mounted with aqueous mounting medium (Biogenex). Care was taken to avoid drying of the cultures during staining. Freshly isolated, subcultured, and cryopreserved cells were immunostained for cytokeratin and for each milk protein in triplicate. Human lip fibroblasts (ATCC, Rockville, MD) served as a negative control for cytokeratin and milk protein staining. Omission of the primary antibody also served as a negative control. Immunostained preparations were examined and photographed using a fluorescence microscope and an Orthomat-W camera (Leitz, Midland, ON, Canada).

Culture and Fluorescein Labeling of S. aureus

Staphylococcus aureus Smith strain variants, compact (Cp), unencapsulated; diffuse (Df), rigid capsule; and diffuse large clearing (Dfle), exceptionally large flaccid capsule (14), were grown in trypticase soy broth at 37°C and killed with 3% formalin for 18 h at room temperature. Bacteria were washed three times with PBS (pH 7.4) and adjusted to $1 \times 10^9$ organisms/ml of PBS. A fivefold volume of .5 M carbonate-bicarbonate buffer (pH 9.5) and a twofold volume of .03% fluorescein isothiocyanate in carbonate-bicarbonate buffer were added, and the mixture was rocked at room temperature for 2 h. The slurry was washed three times in veronal-buffered saline, and the pellet was suspended to the original volume in PBS.

Assays for Adherence of S. aureus

Determination of S. aureus Adherence to Extracellular Matrix Components.

Wells of 4-well polystyrene chamber slides were coated (15 μg/cm²) with fibronectin, laminin, collagen, and EHS, incubated for 1 h at 37°C to allow for matrix attachment, and stored at 4°C with PBS until needed. Aliquots (1 ml; $10^8$ bacteria/ml of PBS) of fluorescein-labeled S. aureus Smith variants were added to uncoated and coated wells. Each adherence assay was performed in triplicate; one well (no bacteria) was the control. After incubation at 37°C for 90 min with rocking, the unattached bacteria were removed by being washed twice with PBS. Chamber walls were separated from the slide, and the slides were mounted with aqueous mounting medium. The slides were examined and photographed as described.

Determination of S. aureus Adherence to Intact and Damaged Cell Monolayers.

Secretory cells were grown on uncoated slides and 4-well polystyrene chamber slides coated with fibronectin, laminin, collagen, and EHS as described. After preconfluent and confluent monolayers were washed three times with HBSS, fluorescein-labeled S. aureus Smith variants were added and incubated, and the preparations were processed as described. To determine adherence of S. aureus to damaged epithelium, confluent cell monolayers were incubated overnight with 300 μg of staphylococcal α-toxin (courtesy of N. L. Norcross, Cornell University, Ithaca, NY) prior to determination of bacterial adherence. This concentration of α-toxin caused tissue damage in explants of mammary secretory tissue (6). Determination of S. aureus adherence to preconfluent and confluent cell monolayers on various matrices and to monolayers damaged by α-toxin was made in duplicate.

RESULTS

Large numbers of viable secretory cells were obtained from enzymatic digestion of bovine mammary tissue collected at necropsy compared with other sampling techniques, i.e., brushing of the epithelium to dislodge epithelial cells and centrifugation of bulk milk. Varying collagenase digestion time determined cell type and viability; in the three cows, cells
collected during h 1 of digestion were nonviable single cells; after 1 to 3 h of digestion, collected cells contained predominantly single epithelial cells and numerous epithelial cell clumps that readily attached and proliferated in culture; after 3 h of digestion, increasing numbers of cells that resembled fibroblasts were present in the cell suspension.

**Cell Culture on Collagen Matrices.** Freshly isolated cells from the three cows and subcultured cells plated on collagen gels proliferated in culture to approximately $3 \times 10^6$ cells per dish at confluency. Freshly isolated cells began to attach and to divide on thin collagen gels within 2 to 3 h after plating. Cell clumps proliferated into islands with a dense core of cells from which cell projections radiated (Figure 1a). By 7 d, cells surrounding the dense core increased, and the core atrophied to a dense amorphous mass. The islands continued to expand and eventually fused into a confluent monolayer. Two cell types were observed: 1) small round or polygonal cells, with minimal cytoplasm and numerous mitotic figures, in the interior of the island and 2) large cells, with frequent vacuoles, multiple nuclei, and no mitotic figures, on the periphery of the island (Figure 1b). At 10 to 14 d, primary cultures on thin collagen gels reached 80% confluency and then were dissociated and subcultured. Cells recovered from primary cultures grew on thick collagen gels showed growth patterns similar to those on thin collagen gels. Freshly isolated and subcultured cells both formed compact monolayers of cuboidal cells. Cell monolayers varied in thickness; some cells projected deep into the collagen (Figure 2a). After the cells reached confluence, thick collagen gels detached and then folded and shrunk to approximately one-third their original size. Cells recovered by trypsinization from thick collagen gels showed numerous microvilli, lipidlike vacuoles, and secretory vesicles, similar to freshly isolated cells (Figure 2b). Thus, the characteristics of the secretory epithelial cells were maintained through the culture process.

Cells embedded in collagen formed three-dimensional elongated structures resembling mammary ducts (Figure 2c) and stellated structures of varying size (Figure 2d).

Cells collected at 5 h of digestion of secretory tissue contained approximately 50% fibroblast-like cells as determined by cell shape and staining with vimentin. When the fibroblast-like cells were cocultured with epithelial cells on collagen, they formed loose layers among the epithelial islands (Figure 3). After several hours, fibroblastic cells organized in thick bundles that fused, forming three-dimensional netlike structures with interspersed epithelial islands.

**Cell Culture on Polystyrene, Fibronectin, and Laminin.** Freshly isolated cells attached poorly to polystyrene and failed to form confluent monolayers. Although subcultured cells proliferated and formed confluent monolayers on polystyrene, they grew slower and had a flatter appearance than those cultured on collagen. Cells cultured on fibronectin and laminin grew slower than cells cultured on collagen but formed tight cobblestonelike monolayers at 6 d.

**Cell Culture on EHS.** Freshly isolated cell clumps and individual cells cultured on EHS attached to the gel immediately after plating and coalesced to form larger three-dimensional structures in which cell clumps were indistinguishable (Figure 4a). By d 7, the multicellular structures resembled ducts draining secretory alveoli (Figure 4b). Cross sections of the multicellular structures in the early stages of development, already after plating, showed compact multilayered structures (Figure 4c). In the intermediate stages of development, two or three external layers of cuboidal and flat cells surrounded a mass of internal cells at various
stages of degeneration (Figure 4d). After 1 wk in culture, cross sections showed hollow structures lined with a monolayer of cuboidal cells, closely resembling mammary gland alveoli (Figure 4d). Monolayers often formed in the space between aggregates. When cells were

Figure 1. Phase contrast photomicrographs of freshly isolated and subcultured bovine mammary secretory epithelial cells on thin collagen gels; a) after 3 d, epithelial islands formed with small polygonal cells radiating from a central cell clump, ×100; b) after 3 d, islands were composed of small polygonal cells in the center and large multinucleated and often vacuolated cells on the periphery, ×100; c) 12 h after first subculture, islands of dividing cells (arrowheads) were present, but with no central clumps, ×210; d) 24 h after subculture, islands continued to grow and fuse, the large peripheral cells making first contact, ×100; e) 48 h after subculture, at confluence, monolayers were composed of small and large cells, ×100; and f) 5 d after subculture, monolayers were composed of small polygonal cells in a tightly packed pavement with domed areas (arrowheads) of various sizes, ×100.
plated at high concentrations (10⁶ cells per well), they formed cordlike structures that fused into a network resembling a honeycomb (Figure 4e). Treatment with EGTA and staining with trypan blue revealed lumina in all of the multicell aggregates by 2 wk (Figure 4f).

**Cryopreservation.** After cryopreservation of subcultured cells for 10 mo, cell viability was 90%, and no difference occurred in the presence of milk proteins. Prior to culture, approximately 70 and 90% of freshly isolated and subcultured cells, respectively, were viable at thawing. Also, the patterns of milk protein staining were similar before and after freezing.

**Immunohistochemistry.** Cultured secretory cells contained interconnecting bundles of cytokeratin filaments in the cytoplasm that extended into neighboring cells via desmosomes. The cytokeratin network was more dense around the nucleus, cytoplasmic vesicles, and in the periphery of the cell (Figure 5, a and a'). Human lip fibroblasts and bovine mammary fibroblastlike cells were negative for cytokeratin and positive for vimentin. None of the cells that stained for cytokeratin stained for vimentin. Cultured epithelial cells stained positive for α-casein, β-casein, α-lactalbumin, β-lactoglobulin, and lactoferrin. Cells on collagen stained more intensely for milk proteins in domed areas. No differences occurred in protein patterns between small and large cells. Milk proteins were consistently present during culture. However, the immunostaining technique used did not allow for quantitative meas-

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Figure 2. Phase contrast photomicrographs of bovine mammary secretory epithelial cells cultured on or embedded in thick collagen gels; a) 3 d after the first subculture, monolayer with irregular thickness, ×100; b) cells recovered from trypsinization of the thick collagen gel showed numerous microvilli, lipidlike vacuoles, and secretory vesicles, ×500; c) and d) cells embedded in collagen for 1 wk formed three-dimensional multicellular tubular (c, ×210) and stellate (d, ×100) structures that resembled mammary ducts.

Journal of Dairy Science Vol. 77, No. 4, 1994
assays for adherence of S. aureus. Fluorescein-labeled S. aureus attached to 4-well chamber slides coated with extracellular matrix components (i.e., fibronectin, laminin, collagen, and EHS) in larger numbers than to confluent monolayers of secretory epithelial cells. Bacterial adherence was similar for collagen, fibronectin, and laminin. Adherence of S. aureus to polystyrene was also higher than that to cultured cells. Adherence patterns were similar for the three cows. When S. aureus were incubated with preconfluent cell monolayers cultured on collagen and EHS, bacteria adhered primarily to the exposed matrix, and only a few scattered bacteria adhered to the epithelial cells (Figure 6, a and a'). Bacteria adhered more readily to confluent cell monolayers damaged by α-toxin than to healthy confluent monolayers (Figure 6, b and b'). Bacterial adherence was greatest in the areas damaged by α-toxin. However, whether the bacteria attached to the damaged cells or to the exposed extracellular matrix was difficult to determine. The unencapsulated S. aureus variant (Cp) adhered to the extracellular matrix components, to healthy confluent cell monolayers, and to monolayers damaged by α-toxin in larger numbers than the encapsulated variants (Df and Df1), of which Df was the least adherent (Figure 7).

**DISCUSSION**

Bovine mammary secretory cells collected at necropsy grew well in culture and withstood cryopreservation. The number of passages were kept to a minimum (<5) to avoid genetic drift and loss of in vivo cell characteristics. Extensive genetic drift could alter cell surface characteristics and expression of receptors. After the first passage, cells attached and proliferated faster, became more morphologically homogeneous, and were less affected by cryopreservation. However, growth and milk protein staining patterns of subcultured cells were similar to those of freshly isolated cells, indicating that, although subcultured cells became more adapted to the in vitro environment, they did not lose their morphological and functional characteristics. Cultured cells were not cloned in order to preserve the heterogeneity of the in vivo mammary epithelial cell population.

Two morphologic cell types were observed, but the presence of cytokeratin filaments in all cultured cells characterized them as epithelial cells. Because the large cells present on the periphery of the islands disappeared after reaching confluency, they were thought to be epithelial cells in early growth phase. Presence of similar morphologic cell types was reported.
Figure 4. Photomicrographs of freshly isolated bovine mammary secretory epithelial cells cultured on the reconstituted basement membrane from the Engelbreth-Holm-Swarm murine sarcoma (EHS); a) immediately after plating, cell clumps and individual cells attached to EHS coalesced, forming multicell aggregates of varying sizes, ×100; b) after 1 wk, cell aggregates formed three-dimensional structures that resembled secretory alveoli, ×25; c) cross-sections of multicell aggregates in early stage of development showed compact structures of several layers of cells, ×500; d) cross section of multicell aggregates in intermediate stage of development formed two or three external cell layers of cuboidal and flat cells surrounding a mass of cells at various stages of degeneration (large cell aggregate); final stages of development showed hollow structures lined with a single layer of cuboidal cells that resembled secretory alveoli (small cell aggregate), ×500; e) cells plated at high concentration after 12 h formed three-dimensional honeycomblike networks, ×100; f) after 2 wk, cell aggregates showed large dark internal areas indicative of lumen after treatment with ethylene glycol-bis(β-aminoethylether) and trypan blue, ×25.
Figure 5. Phase contrast (a to c) and immunofluorescence (a' to c') photomicrographs of bovine mammary secretory epithelial cells cultured on thin collagen gels; a and a') cytokeratin filaments were more concentrated around the nucleus and cell periphery, x400; b and b') α-casein concentration varied among cells, x400; c and c') small and large cells contained α-casein granules that were more concentrated around the nuclei, vacuoles, and cell margins, x400. Phase contrast (d) and immunofluorescence (d') photomicrographs of bovine mammary secretory epithelial cells cultured on the reconstituted basement membrane from the Engelbreth-Holm-Swarm murine sarcoma; multiscell aggregates appeared intensely stained for α-casein, x400.
in bovine (5, 21), rat (22), and canine (36) mammary epithelial cell cultures. Cells were observed at various degrees of differentiation. Cells were identified as fully differentiated "lactating" mammary secretory cells because of the presence of major milk proteins (13, 35) with a predominance of α-casein, the dominant milk protein in vivo (18). Lipid synthesis, determined by the presence of triacylglycerols in cell culture homogenates using gas chromatography (L. D. Wood, 1992, unpublished data), further characterized the cultured cells as differentiated mammary secretory cells.

Cells cultured on increasingly complex substrata formed increasingly complex structures. For example, cells cultured on polystyrene proliferated slowly and formed two-dimensional monolayers of flat cells. Cells cultured on substrata containing a single component of the in vivo extracellular matrix attached and proliferated more rapidly and produced monolayers of small and large cuboidal cells. Cells cultured on floating collagen gels became cuboidal and tended to form three-dimensional structures, which resulted in folding of the gels. Cells embedded in collagen formed three-dimensional ductlike structures that were lined with one or two layers of cells closely resembling mammary ducts (23, 36). The EHS, a close facsimile to the secretory cell basement membrane, is uniquely suited for culturing mammary secretory cells. Others (3, 15) found that cells on EHS became columnar and formed a continuous basement membrane.

Figure 6. Phase contrast and fluorescence photomicrographs of cultured bovine mammary secretory epithelial cells incubated with fluorescein-labeled Staphylococcus aureus Smith strain variant Compact for 90 min at 37°C with rocking; a) phase contrast of cells on collagen showing exposed collagen; a') fluorescence photomicrograph of the same area showing S. aureus adhered to exposed collagen, ×400; b and b') fluorescence photomicrographs of S. aureus adhered to a healthy confluent cell monolayer (b) and S. aureus adhered to a cell monolayer damaged by overnight exposure to α-toxin (b'), ×290.

Journal of Dairy Science Vol. 77, No. 4, 1994
In the present study, bovine secretory cells cultured on EHS formed multicellular hollow structures lined by a layer of cuboidal cells closely resembling mammary alveoli. Results were similar for rodent mammary cells (7). Coculture of fibroblastlike cells and secretory cells also resulted in three-dimensional structures.

Two-dimensional cell monolayers on thin collagen gels were the best suited for bacterial adherence studies because they exposed a uniform surface for measurement of adhered bacteria and allowed for proper washing of unadhered bacteria. Cell monolayers on fibronectin, laminin, and polystyrene contained gaps between cells where the subcellular matrix, for which bacteria have great affinity, was exposed. These gaps are not likely to occur in the healthy mammary gland; thus, the use of these models would be misleading. Cell monolayers on thick collagen gels became detached and folded after confluency, making the measurement of adhered bacteria difficult. Several cell culture conditions promoted three-dimensional configurations (i.e., floating collagen gels, embedding in collagen, EHS, and coculture with fibroblastlike cells) that closely resembled bovine mammary secretory tissue. However, these configurations were not suited for bacterial adherence studies because they exposed the subcellular matrix between the three-dimensional structures. Also, bacteria were trapped in the three-dimensional structures, preventing the removal of unadhered bacteria.

Confluent cell monolayers can be used to study bacterial adherence to healthy and damaged epithelium. On healthy cell monolayers, bacteria are exposed to the apical cell surface, as in the healthy mammary gland. The basolateral cell surfaces are exposed only after α-toxin damage, as in the injured mammary gland. Also, the use of cell monolayers at various degrees of confluency can be utilized to study bacterial adherence to epithelial cells versus basement membrane.

Various in vitro models have been used to study bacterial adherence. Mammary explants have been used (38), but their complex structures make observation and measurement of the interactions between bacteria and host difficult. Also, because mammary explants expose tissues that are not exposed to bacteria under normal conditions, use of explants to study bacterial adherence could be misleading. Cells in suspension have also been used to study bacterial adherence to mammary epithelium (25). The use of cells in suspension
may be misleading, because the basal surface of the cells with associated extracellular matrix material is exposed to the bacteria. This exposure is not likely to occur in healthy mammary epithelium in vivo and would yield an overestimate of bacterial adhesion. In the current study, flow cytometry was used to compare \textit{S. aureus} adherence to cultured cells in suspension with adherence to cell monolayers. \textit{Staphylococcus aureus} adhered to the suspended cells in larger numbers than to cell monolayers. This difference was attributed to exposed basal cell surface, attached basement membrane, or damaged cells (10).

Fluorescein was used to label bacteria because it binds to cytoplasmic proteins and does not impair cell migration and localization (16). In the present study, \textit{S. aureus} adhered more readily to extracellular matrix components than to the apical surface of healthy secretory cells in culture. Preferential adherence of bacteria to exposed connective tissue has also been reported (38) in explant cultures of bovine mammary tissue. The small number of bacteria that adhered to intact confluent monolayers are thought to adhere to aged cells. The increase in \textit{S. aureus} adherence to monolayers damaged by \textalpha-toxin suggests that damage to the epithelial cell surface or exposed basement membrane is prerequisite to adherence of \textit{S. aureus} in the mammary gland.

Unencapsulated \textit{S. aureus} adhered more readily than the encapsulated variants, suggesting that the bacterial cell wall has a high affinity for extracellular matrix proteins and for damaged cells and that this affinity is blocked by the exopolysaccharide capsule. Thus, the dynamics of capsule formation may be critical to bacterial adherence. Most \textit{S. aureus} present in the environment are devoid of capsule. But, upon entering the mammary gland, the organisms undergo rapid growth, after which they form a capsule (25). During rapid growth, toxins are formed, which could damage the epithelium and expose the subcellular matrix. Thus, unencapsulated \textit{S. aureus} would be exposed during this period to damaged epithelial cells and subcellular matrix.

Although the model used in the current study gave an insight into bacterial adherence to mammary epithelium, caution must be exercised when these results are extrapolated to the in vivo environment. Concentration of bacteria and \textalpha-toxin required for in vitro studies are probably larger than those in most mammary gland infections. Also, cultured cells may express different receptors than do cells in the mammary gland (2).

\section*{CONCLUSIONS}

The culture conditions used in the present study induced and maintained characteristic cell growth and presence of major milk proteins, thus providing close facsimiles to in vivo bovine mammary secretory epithelium. Among all culture substrata tested, cells cultured on collagen formed confluent monolayers that were most suited for bacterial adherence studies. This study showed that resistance of healthy epithelium to bacterial adherence is compromised by \textalpha-toxin damage. However, whether bacteria adhered to cells damaged by \textalpha-toxin or to exposed matrix is not clear. Also, this study showed that relative degree of capsule formation affects attachment of \textit{S. aureus} to mammary epithelium.

\section*{REFERENCES}

BACTERIAL ADHERENCE TO MAMMARY CELLS


Journal of Dairy Science Vol. 77, No. 4, 1994