Effect of Specific Antibody on Adherence of *Staphylococcus aureus* to Bovine Mammary Epithelial Cells

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*Staphylococcus aureus* is a major pathogen in the bovine mammary gland. The ability of *S. aureus* to adhere to epithelial cells in the ductules and alveoli of the bovine mammary gland is believed to add greatly to its virulence and may be necessary for colonization. Two in vitro methods were developed for the purposes of quantifying adherence and of determining the effect which specific antibody may have in inhibiting the adherence of this organism. Both methods utilize bovine mammary epithelial primary cells as targets for labeled bacteria. In one assay, the bacteria are labeled with [methyl-3H]thymidine and incubated on the primary epithelial monolayers. The second assay involves labeling the bacteria with biotin. An enzyme-linked immunosorbent assay is then performed with streptavidin conjugated to horseradish peroxidase. Both methods have proven to be reliable and allow for the testing of many criteria in one assay. Cows were immunized with a whole-cell vaccine, and immune serum and milk were collected. The bacteria were then incubated in the presence of serum or milk as a test for antiadherent capability. By using the methods described, distinct antiadherent activity in both serum and milk was demonstrated.

The topic of bacterial adherence has attracted a considerable amount of attention in the past decade and has prompted investigations by many laboratories (4, 20, 32). As a result, this phenomenon is widely accepted as a primary step in the successful colonization of a host. *Staphylococcus aureus* is a major pathogen in the bovine mammary gland and is responsible for significant monetary losses annually to dairy farmers (13). Adherence by this organism in the bovine mammary gland has been studied, and its occurrence has been well established (8, 22, 36).

For a pathogenic organism to colonize and subsequently survive, it must be able to adhere to the host tissues (20). The failure to do so may result in the organism being swept away in the fluids bathing the various tissues, and this is particularly true for *S. aureus* in the mammary gland. Many steps must occur for the successful colonization of the gland. First, *S. aureus* must gain access through the teat canal, and then it must survive the flushing action of the fluids within the gland and of the milking process. This is accomplished by adhering to the epithelial cells lining the ductules and alveoli of the gland (8). Once the pathogen becomes established, it can proliferate and metastasize to other regions of the gland. When this occurs, *S. aureus* causes chronic and, at times, acute infection which is difficult to cure and may lead to the culling of the animal. The ability of *S. aureus* to adhere to the epithelial cells in the ductules and alveoli of the gland is believed to add greatly to its virulence (36).

The efforts of our laboratory are directed toward the understanding of this disease and its prevention through an immunological approach. Studies are conducted to increase our knowledge concerning the immune defense of the bovine mammary gland against bacterial pathogens. One aspect under investigation has been the inhibition of *S. aureus* adherence by specific antibody. Because of the cost and limited number of animals available for in vivo studies and the need to quantify adherence at a cellular level, an in vitro method was required.

The first purpose of this study was to develop a simple and reliable method to study bacterial adherence in the bovine mammary gland. Two such methods which use epithelial cells, isolated from the bovine mammary gland, grown in culture are presented. In one assay, *S. aureus* has been radiolabeled; in the other, the bacteria have been biotinylated as a means of quantifying adherence. The second purpose of this study was to examine the effect of bovine antibody on the adherence of *S. aureus* to bovine mammary epithelial cells. The methods described proved to be reliable in their ability to enable the measurement of bacterial adherence. By using these techniques, it has been found that antibody from serum and milk was able to inhibit the adherence of *S. aureus*.

**MATERIALS AND METHODS**

**Bacteria.** *S. aureus* 2-8 and A37 were isolated from clinical cases of bovine mastitis and were found to be adherent. Upon isolation, they were subcultured twice on modified Staph 110 medium (Difco Laboratories, Detroit, Mich.), lyophilized, and frozen on slants at −70°C. *Staphylococcus* sp. strain M was provided by Lisa Dunkle (St. Louis University School of Medicine) and is a nonadherent strain.

**Collection of epithelial cells.** Udders from freshly slaughtered lactating cows which appeared clinically normal both in tissue appearance and in milk production were selected. They were washed with sterile saline, and a sample of the ductular tissue located high in the gland was aseptically removed. Samples from different cows were used for each isolation and digestion for establishment of a primary cell line.

**Establishment of primary cell cultures.** Bovine mammary epithelial primary cells were established on the basis of the methods of Stamper (28). The dissected ductular tissue was rinsed several times with sterile phosphate-buffered saline

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(PBS) (pH 7.4) to wash away any milk and lacerated with opposing scalpels in a petri dish cover. Enzyme digestion mixture containing Dulbecco’s modified Eagle’s medium (DMEM) (4.5 g/liter of glucose) (Life Technologies, Inc., Gaithersburg, Md.), 5% fetal bovine serum (FBS) (Hyclone, Logan, Utah), 5 μg of insulin per ml (Sigma Chemical Co., St. Louis, Mo.), 200 U of collagenase per ml (Sigma), 100 U of hyaluronidase per ml (Sigma), and 5× antibiotics (Life Technologies, Inc.) was added to the diced tissue (10 ml/g of tissue) and incubated at 37°C for 6 h on a tube rotator. The suspension was then centrifuged (600 × g) for 10 min, resuspended in fresh enzyme digestion medium, and incubated overnight at 37°C. After 18 h of enzyme digestion, the single-cell suspension was washed twice with fresh basal growth medium consisting of a 1:1 mixture of DMEM (4.5 g/liter of glucose) and Ham’s F12 (Life Technologies, Inc.), supplemented with 5% FBS (Hyclone), 1× antibiotics (Life Technologies, Inc.), 5 μg of insulin per ml (Sigma), 5 μg of hydrocortisone per ml (Sigma), and 5 ng of epidermal growth factor per ml (Sigma). The suspension was then filtered through a 0.45-μm filter (Teflon Inc., Elmsford, N.Y.) and plated in basement at a density of 4 × 10^6 cells per ml in T25 tissue culture flasks (Corning Glass Works, Corning, N.Y.). After the cells grew to confluence, they were passaged by a brief rinse with saline A (8.0 g of NaCl, 0.2 g of KCl, 1.15 g of NaHPO₄, 0.2 g of KH₂PO₄, per liter), treated with trypsin-EDTA solution (Life Technologies, Inc.) for 20 min to detach them from the plastic and were plated. This procedure was repeated once more after the cells were again confluent. At this point, the mammary epithelial cells were trypsinized, washed with DMEM, and resuspended in DMEM-20% FBS-10% dimesyl sulfoxide (Sigma) at a concentration of 4 × 10^6 cells per ml. They were then frozen and stored in liquid nitrogen for subsequent use. These primary cells were tested for the presence of mycoplasmas with a commercial test (Gen-Probe, San Diego, Calif.).

Characterization of epithelial monolayers. The cells used in this study were characterized on the basis of specific staining against cytokeratins and vimentin. This was accomplished by a two-stage double-staining technique. A polyclonal rabbit antisem (DAKO, Santa Barbara, Calif.) against a broad range of cytokeratins of various molecular weights was used with a peroxidase antiperoxidase kit (PAP) (DAKO) with 3,3-diaminobenzidine (DAB) (DAKO) as the chromogen. This was followed with a monoclonal antibody against porcine vimentin (DAKO) in conjunction with an alkaline phosphatase-anti-alkaline phosphatase kit (APAAP) (DAKO) with fast red as the chromogen. In this way, the epithelial cells were stained brown and the fibroblasts were stained red. Controls included calf skin fibroblasts from primary cells, generously provided by Ronald Minor (New York State College of Veterinary Medicine, Cornell University).

Radiolabelling of bacteria. For the assays involving radio-labeled bacteria, 0.02 ml of a 4-h starter culture of each bacterial sample was used to inoculate 0.5 ml of modified Staph 110 medium containing 100 μCi of [methyl-3H]thymidine per ml (New England Nuclear, Boston, Mass.). The samples were incubated at 37°C overnight and then washed three times with fresh medium by centrifugation at 1,100 × g for 10 min to remove excess [3H]thymidine. Each sample was sonicated (Braun-sonic; B. Braun Instruments, San Francisco, Calif.) at 100 mW for 15 s to disperse the bacterial clumps. The number of colony-forming units (CFU) were determined for each suspension. The bacterial samples were subsequently washed and resuspended in Earle’s basic salt solution (Life Technologies, Inc.).

Adherence assay with radiolabeled bacteria. The procedure used for the adherence assay with radiolabeled bacteria is a modification of the method by Dunkle et al. (5). The bovine mammary mammary cells were thawed into a T25 tissue culture flask (Corning) and grown to confluence. They were then passaged into 12-well tissue culture plates (Corning) and grown with sterile Earle’s basic salt solution (Life Technologies, Inc.). One milliliter of a 0.5% bovine serum albumin (BSA) (ICN Biochemicals, Inc., Costa Mesa, Calif.) solution was added to each well and incubated for 30 min at room temperature. After the BSA was aspirated, 0.3 ml of each bacterial suspension was then placed in duplicate wells on the tissue monolayers and allowed to incubate for 4 h at 37°C. At that time, the supernatant, comprising the nonadherent fraction of bacteria, was aspirated, washed twice, and collected on a filter paper. The monolayer with the adherent fraction was then trypsinized, aspirated, and filtered. The well was then washed twice, and the supernatant was collected on the same filter. After being dried the filters were placed in vials, scintillation fluid was added, and the radioactivity of the samples was counted (Beckman LS 3801; Beckman Instruments, Irvine, Calif.). Bacterial adherence was expressed as the percent radioactivity in the adherent fraction of the total radioactivity in the sample. With each assay, both adherent and nonadherent strains were included as positive and negative controls, respectively.

Biotinylation of S. aureus. An overnight culture of each bacterial strain was prepared in modified Staph 110 medium. The culture was then diluted 1:4 in fresh medium and incubated for 4 h at 37°C, washed twice in 0.1 M carbonate buffer (pH 8.2) to an optical density of 0.7 (3.7 × 10^6 CFU per ml) at 620 nm. A total of 12.5 μl of a fresh solution of amino-hexanoyl-biotin-n-hydroxysuccinimide ester (10 mg/ml in dimethyl sulfoxide) (Zymed, San Francisco, Calif.) per ml of the washed and adjusted bacterial suspension was added, and the mixture was incubated for 2 h at room temperature. The bacteria were then washed three times and resuspended in PBS to a transmittance of 5% (7.0 × 10^6 CFU per ml) at 620 nm. Prior to the adherence assay, equal volumes of each sample were centrifuged and developed with the Strepavidin-peroxidase conjugate and 2,2’-azino-di(3-ethylbenzthiazoline sulfonic acid) (ABTS) as the substrate. The difference in optical density was then used to adjust the values of the bacterial samples from the adherence assay to control for any variation in the biotinylation of the samples.

ELISA for adherence. The procedure used for the enzyme-linked immunosorbent assay (ELISA) was adapted from the methods of Ofek et al. (21). The bovine mammary epithelial cells were thawed into T25 tissue culture flasks (Corning) and grown to confluence. They were then passaged to 96-well tissue culture plates (Nunc, Thousand Oaks, Calif.), grown to confluence, fixed with 2.5% glutaraldehyde (Fisher Scientific, Rochester, N.Y.) for 20 min, and washed with PBS (pH 7.2). Prior to the adherence assay, 100 μl of a 0.5% BSA solution was placed in each well to prevent nonspecific binding of bacteria, and the plates were incubated for 30 min at 37°C. After the BSA was aspirated from the wells, 100 μl of the biotinylated bacterial sample prepared as described above was placed in each well and incubated for 2 h at 37°C. In some cases, the bacterial sample was treated prior to...
being plated with various antibody preparations. After the incubation period, the monolayer was gently washed twice with PBS and then fixed again with 2.5% glutaraldehyde. A 1:2,500 dilution of a streptavidin-peroxidase conjugate (Zymed) was added for 5 min, and the monolayer was washed and developed with ABTS as the chromogen for 7 min. The supernatants were then transferred to ELISA microtiter plates, and the optical density at 410 nm was read with a microtiter plate reader (Bio-Tek Instruments, Winoosky, Vt.). Controls consisted of bacteria added to wells without epithelial monolayers and of the streptavidin and substrate added to wells with epithelial cells but without biotinylated bacteria.

Antibody preparation and treatment of bacteria. Normal serum and milk were collected from a cow which demonstrated a low titer as determined by ELISA against the *S. aureus* 2-8. This strain cross-reacts with a large percentage of the strains isolated from mastitic cows (19, 23). The cow was subsequently immunized with a whole-cell vaccine of strain 2-8 via the submammary lymph node, and the immune serum and milk were collected when the antibody titer was high. Immediately after collection, the milk sample was centrifuged at 1,100 × g for 20 min, and the excess fat was aspirated. The sample was further clarified by centrifugation at 28,000 × g for 90 min and stored at −70°C for later use. In preparation for the adherence assay, the biotinylated bacterial samples prepared as above were treated with an equal volume of a 1:50 dilution of immune serum or FBS (final concentration 1:100), either immune or normal milk (final concentration 1:2), or PBS. These preparations were incubated for 30 min at 37°C before and then during the adherence assay.

Preparation of bacterial extracts. *S. aureus* capsular ex- tracts were prepared as follows. An overnight culture of A37 grown on Staph 110 agar plates was removed from the agar with sterile glass scrapers and suspended in a 50 mM Tris buffer (pH 7.4) (Life Technologies, Inc.) containing the protease inhibitors EDTA, phenylmethylsulfonyl fluoride (Sigma), leupeptin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), pepstatin A (Boehringer Mannheim Biochemicals), and aprotinin (Sigma) at final concentrations (per liter) of 1.5 g, 0.0871 g, 0.5 mg, 0.7 mg, and 0.02 mg, respectively. Sterile glass beads were added, and the mixture was shaken for 1 h on a horizontal shaker. This preparation was strained through sterile cheesecloth and centrifuged for 30 min at 10,000 × g. The supernatant was then filtered and stored at −70°C.

ELISA. Antibody titers were tested by ELISA. The bacterial extracts (100 µl) diluted in 0.06 M carbonate buffer (pH 9.6) were pipetted into wells of a 96-well Immulon microtiter plate (Dynatech Laboratories, Inc., Chantilly, Va.) and incubated for 4 h at 37°C and then overnight at 4°C. The plates were washed three times with PBS-0.05% Tween 20 (pH 7.2), and bovine serum or milk diluted in PBS-0.05% Tween 20 was added and incubated for 1 h at 37°C. The plates were washed again, goat anti-bovine antibody, with both heavy and light chain specificity, conjugated to horse-radish peroxidase (Jackson Immuno-Research Laboratories, West Grove, Pa.) was added, and the plates were incubated for 40 min. Finally, the plates were washed and developed with ABTS.

Statistical analysis. The results have been analyzed by using Student’s *t* test.

### RESULTS

**Primary cell cultures.** Bovine mammary epithelial primary cells were prepared for use as targets in the adherence assays because methods employing cell suspension proved unsatisfactory. The bovine mammary epithelial primary cells grew well in the conditions established. A variety of prepared media and different supplemental additives for the purpose of culturing human, bovine, and rat mammary epithelial cells had been reported by several investigators (3, 18, 23, 39, 40) at the time the present studies began. Several of these factors were investigated (data not shown) to determine the optimum conditions for the growth of bovine mammary epithelial cells for use in this work: FBS, horse serum, calf serum, insulin, hydrocortisone, prolactin, epidermal growth factor, and cholera toxin. The spacial arrangement of the epithelial cells in culture has also been previously shown to be important (15), and the use of collagen gels has been demonstrated previously to aid in the three-dimensional growth of the cells (38, 40).

The factors required for inclusion in the medium, as described in Materials and Methods, were based on two main criteria. First, the supplement had to have an important effect in the growth of the mammary epithelial cells, and second, the additive had to effectively enhance the adherence of the bacteria. Prolactin has been shown to be important in the synthesis of α-lactalbumin but was eliminated in these studies because it was not required for the sustained growth of the cells (10). There was also some doubt as to the effectiveness of prolactin with organoid cultures, because the environment in which the cells are grown may affect the expression of prolactin receptors or the mode of action of prolactin may require more than just having mammary cells present (15). Cholera toxin has been reported previously to enhance epithelial growth and inhibit fibroblast growth (38) and also to raise cyclic AMP levels (15). However, in culture, its activity required that the epithelial cells be grown on rat tail collagen or fibroblast feeder layers. The collagen matrix did not prove to substantially meet either criterion initially established, and thus the use of cholera toxin as well was eliminated. A comparison of the sources of serum did not produce any significant differences; however, FBS was chosen because it proved to be the most effective at sustaining the epithelial cell’s growth. The remaining supplements of insulin, hydrocortisone, and epidermal growth factor also proved to most effectively meet the criteria for inclusion in the growth medium.

Figure 1 shows the confluent monolayers after three days of growth plated at a density of 2 × 10^5 cells per ml. It was possible to passage the primary cells 12 or 13 times before they began to die. At no time during their growth were fibroblasts evident. The characterization of these primary cells, based on specific staining against cytokeratins and vimentin, confirmed this observation. The staining was accomplished in such a way that the epithelial cells were stained brown and the fibroblasts were stained red (color photographs are not shown). On the bases of the specific staining and of the cell morphology in culture, these bovine mammary primary cells appear to be epithelial in nature. These cells were also tested for the presence of mycoplasmas and were found to be negative, thus eliminating any possible effect that contamination by this organism may have on the adherence of the various bacterial samples.

**Adherence assay with radiolabeled bacteria.** The assay with radiolabeled bacterial cells was the first of two assays developed which utilized the bovine mammary epithelial
cells as targets for the bacteria. In this assay, the bacteria were labeled with \(^{3}H\)thymidine as a means of quantifying adherence. To improve the sensitivity of the assay, it was important to maximize the amount of label taken up by the bacteria. Various concentrations of \(^{3}H\)thymidine (20 to 100 \(\mu Ci\)) in the growth medium were used, with the highest concentration giving the best results (data not shown). The sensitivity of the assay was also improved by increasing the surface area of the monolayer, decreasing the volume of bacterial cell suspension added, and lengthening the incubation time of the assay. The increased surface area allowed for a greater number of bacterial cells to adhere. The smaller volume of bacterial suspension allowed for more of the total amount of the bacteria to come in contact with the monolayer, as did the increased incubation time. However, because live epithelial cells were used in this experiment, incubation periods over 4 h had a deleterious effect on the cells; they began to round up and detach from the monolayer.

Three other factors critical for consistent results included the dispersal of bacterial clumps, the careful washing of the nonadherent bacteria from the epithelial monolayers, and the use of a blocking agent to prevent nonspecific adherence. It was necessary to sonicate the bacterial suspensions briefly to disperse any clumps which may have formed during growth. This was important to ensure that the results reflect the adherence of single cells and not clumps associated with a minimal cell interaction. The number of colony forming units reached a maximum after 15 s, as it seemed to take this long to begin to disrupt the clumps. After about 20 to 25 s, the number of colony-forming units began to drop because of lysis of the bacterial cells. Cell monolayers and adherent bacteria were fixed, stained, and examined by light microscopy after the completion of the adherence assay. Single \(S.\) \(aureus\) cells attached to the epithelial monolayer were observed, confirming their dispersal by the sonication. It was also apparent that the bacteria were not reaggregating during the adherence assay. Washing was a critical step in this assay as well as in the ELISA. Only gentle washes were required to remove the nonadherent bacteria. Any direct flushing or inconsistency in the technique resulted in a variation of the results. The most consistent results were achieved when the PBS was allowed to flow across the monolayer. It was important to use the epithelial primary cells at confluence to minimize exposed plastic surfaces. A few small spaces which formed during growth did exist in the epithelial monolayer. BSA at 3, 1, and 0.33% and gelatin at 1 and 0.2% were investigated as blocking agents to prevent the nonspecific binding of the \(S.\) \(aureus\). BSA was the most effective at this inhibition, and only very low concentrations (0.33%) were required.

For each experiment, positive and negative controls were tested as a means of comparison of the unknown samples. Figure 2A shows the results of these two strains assayed on seven separate days. The results reflect the reproducibility with this assay when performed from day to day. The difference in the adherence values between the two strains is very significant \((P < 0.0001)\). Similar results were observed when this assay was performed five times on the same day.
Each bacterial sample was treated independently of the others. Figure 2B illustrates the results of this experiment and further demonstrates this assay to be a reliable method for quantifying bacterial adherence. The difference between the adherence values of the adherent and nonadherent strains is again very significant ($P < 0.0001$). All bovine $S. aureus$ isolates tested gave results similar to those of the positive control.

The adherence values of the two experiments illustrated in Fig. 2 were produced by using the same two strains of $Staphylococcus$ spp. and experimental conditions. However, the values in panel A are 40% higher for both strains than those in panel B. The one variable between these two experiments was the origin of the epithelial primary cells used. Separate primary cells isolated from two different cows were used in each experiment. The variation resulting from individual cows demonstrates the problem of using cells freshly isolated for each experiment. Day-to-day comparisons become very difficult when separate sources of mammary epithelial cells are used and emphasize the distinct advantage of using one consistent isolation of epithelial primary cells. From this observation, all subsequent assays were performed with cells prepared from one large digestion so that the monolayers would be identical from experiment to experiment.

**ELISA for adherence.** Though the assay with radiolabeled bacteria proved reliable, it was still found to be limiting in the number of samples or variables which could be tested at any one time. The use of biotinylation to label the bacteria proved to be a great improvement in that 96-well plates could be used. A time-dependent study was again performed in an effort to optimize the sensitivity of the ELISA, in which the biotinylated bacteria was used, and to establish it as a reliable method for measuring bacterial adherence. Figure 3 shows the results of this study at 1-, 2-, and 3-h incubation times. While 4 h of incubation gave the best results in the assay of radiolabeled bacteria, 2 h of incubation was optimum with the ELISA.

A comparison of the use of viable epithelial monolayers and cells which are fixed prior to the adherence assay was also made. There was very little difference between the two methods, with the fixed cells giving slightly better results (data not shown). This allowed 96-well tissue culture plates to be prepared in advance, and this convenience was a distinct advantage. In addition, a comparison of the results (optical density) for samples in the same tissue culture plates and for the supernatants transferred to ELISA plates was made. Results became more consistent and reliable when they were read in the ELISA plates because the interference by and inconsistencies of the epithelial monolayers were eliminated.

The adherent strain, A37, behaved in a dose-dependent fashion with this assay. The most concentrated suspension (neat) was about $7 \times 10^8$ CFU per ml. A concentration of $3.5 \times 10^8$ CFU per ml was used for all studies using this method, which correlates to the second dilution. Figure 4 shows the results of the titration of strain A37.

This assay was validated by repeating the experiment three times in the same day with strain 2-8 versus strain M. Three separate overnight suspensions of each strain were plated in triplicate, and the means of each sample were determined. Based on a 95% confidence interval, the difference between the means of these three values is statistically significant ($P < 0.0016$). The relative adherence values for these strains with this assay were nearly identical to those with the assay with radiolabeled bacteria.

**Collection of bovine antisemum and immune milk.** Once the assay with radiolabeled bacteria had been developed and bacterial adherence has been demonstrated, specific antibody was introduced into the system to see whether the observed adherence could be inhibited. Cows were used as a source for this antibody, and several were screened to find the lowest preimmunization titer possible. Serum and milk samples were taken prior to the cow's immunization with the whole-cell vaccine. The selected cow was boosted twice after the initial immunization, samples were taken after each booster, and the antibody titer of each sample was determined. Antibody titers increased from 40 to 1,280 (expressed as the inverse of the dilution) in serum and from 10 to 20 in milk. As can be observed, a much higher titer is produced in serum than in milk. This may be due to the route of immunization and low production of local immunoglobulin in the mammary gland. In general, immunoglobulin levels in serum are 40-fold higher than those in milk. The presence of...
specific antibody in normal serum may be the result of a previous and likely exposure to \textit{S. aureus}. In vivo, this titer may not be significant; however, in vitro, this created some confusion in the interpretation of results of the inhibition of \textit{S. aureus} adherence by antibody. As a consequence, FBS was used as a negative control in the adherence studies.

\textbf{Inhibition by antibodies isolated from serum.} The normal and immune samples were introduced into the adherence assays to examine the effect that specific antibody would have on bacterial adherence. It has been shown that both normal serum and milk contain antibodies against \textit{S. aureus} because of its existence as a common pathogen in the environment. However, tests conducted with immune versus normal serum do show significant experimental differences, demonstrating that the immunization and subsequent increase in antibody titers improved the antiadherent capability of the serum. This is demonstrated in Fig. 5 by the ELISA for adherence with \textit{S. aureus} A37.

In this experiment, FBS was used as a control against any effects which the serum components may have on adherence of the bacteria. The slight increase in adherence demonstrated by FBS at the lower dilutions may be a result of some serum factors which enhance or help mediate bacterial adherence. This effect diminishes as the serum concentration decreases. Serial dilutions of the PBS and immune serum had a similar effect on their antiadherent capacity. It was expected that at some point the results with the immune serum would equal those with FBS, and this proved to be the case. PBS represents the level of adherence expected without any inhibition due to specific antibody or other serum factors. Distinct antiadherent activity in the immune serum is observed to a dilution of 1:800.

\textbf{Inhibition by antibodies isolated from milk.} The results of the inhibition of adherence by antibodies in milk are shown in Fig. 6. As noted above, normal milk does inhibit the adherence of the different strains of \textit{S. aureus}. This may be a result of both the presence of specific antibodies due to a previous exposure to \textit{S. aureus} and a nonspecific masking effect by factors in the milk. These factors may coat the bacterial cell, preventing an adhesin from coming in contact with the epithelial cells, or may change the surface charge of the bacterium, affecting its ability to interact with the host cells. Regardless, all strains in the presence of milk, normal or immune, showed a decrease in adherence. In addition, an increase in this inhibition by the immune milk above that by the normal milk was observed and can only be accounted for by the increase in specific antibody. The only strain which did not show this effect was the nonadherent control strain M with which the antibody did not cross-react. Also, there was a difference in the adherent abilities of the various strains of \textit{S. aureus} tested. Strain 2-8 was significantly more adherent than strain A37.

\textbf{DISCUSSION}

Bacterial adherence in several different systems has been established and serves as a model of the importance of this phenomenon in the process of bacterial pathogenesis (6, 14, 26, 29, 31, 37). Bacterial adherence has been shown to occur through a direct interaction between the bacterium and host cell, as well as through the involvement of other host factors, such as fibronectin, which helps to mediate the process (16, 30, 34, 35). In this paper, we have addressed the occurrence of this phenomenon by \textit{S. aureus} in the bovine mammary gland and have investigated the role which antibody plays in the inhibition of this process. We and others have demonstrated the adherence of \textit{S. aureus} to bovine mammary epithelial cells (8, 22, 36). The pathogenicity of \textit{S. aureus} in the bovine mammary gland is enhanced by its ability to adhere to the epithelial cells lining the ductules and alveoli.

In order to evaluate this process, an in vitro assay which allows for the measurement of the adherence is required. Several factors need to be considered when designing and selecting a procedure for this purpose: the assay needs to be as simple to use as possible, the results of the assay must be reliable in their reproducibility, it should allow for the
application of many samples at once, and it should be relevant to the in vivo situation. The methods which have been developed by many investigators to address this phenomenon satisfy these criteria to various degrees.

There are several differences in the procedures which have been developed. One major difference in these methods is the type of target cells used. Some studies employ cell suspensions, many requiring dispersion of fresh cells for each assay (7, 8, 12, 24, 25, 33, 36). This requirement results in the use of a different source each time, can be a time-consuming task, and introduces individual variation of target cells. With the bovine mammary gland, a source of donors is also limiting. The advantage, however, is that they are the closest to the actual in vivo situation; the cells are fully differentiated and developed. In other studies, researchers utilized cell lines in culture (1, 5, 9, 17). This method has the convenience of having identical target cells on hand for each experiment but may have the disadvantage of being the least representative of the in vivo situation. By definition, cell lines are derived from transformed cells and may be abnormal or arrested at various stages of differentiation, which might affect the expression of receptors on the tissue culture cells as a result of a different stage of differentiation and development.

Another area which varies between the adherence assays are the methods employed in the quantification of the attached bacteria. Many have used direct light microscopy to examine and count the adherent bacteria (2, 7, 17, 36). Though this method provides for a close examination of the attachment of the bacterial cells, it can be quite cumbersome, limiting the number of samples which can be tested at one time. It also allows for some subjectivity to be introduced in the counting. This method does have the advantage, however, of allowing visual examination of each bacterium-host cell interaction and determination of whether the attachment has occurred as single cells or clumps.

A second method used, and one applied here, is the radiolabeling of the bacteria (5, 22, 27). This procedure provides an easy and accurate method of labeling and measuring adherent bacteria and of comparing the adherence capability of various strains of bacteria. However, proper precautions must be taken to ensure that the radiolabel is not incorporated into the bacterial cell membrane. If clumps are present, the levels of adherence could be biased in a way favoring a strain in which cells tend to clump. One disadvantage of this method is that large amounts of the labeled bacteria are required for accurate results. This requirement limits the number of samples which can be tested at any one time.

A third method also employed in this study is the use of biotinylation to directly label the bacterial cell (21). This is the simplest method to perform and allows for the testing of the largest number of samples at any one time because of the use of 96-well microtiter plates. This technique thereby provides the capability of examining a variety of factors which affect the adherence of a particular sample, all on one plate. Preliminary studies have shown that S. aureus cell surface extracts will block adherence. The adherence of different bacterial strains can also be compared by using this method when the extent of biotinylation of different samples is measured. The methods presented have proven to be valuable tools in the study of bacterial adherence by S. aureus to bovine mammary epithelial cells and, combined with a genetic approach, may be helpful in determining the factor on the S. aureus cell surface mediating this adherence. We address the possibility of preventing bovine mastitis caused by S. aureus through the stimulation of antibodies capable of blocking its adherence. The best defense against staphylococcal mastitis may be effective neutralization of new infections.

Intermediate to the use of cell suspensions and lines are primary cell cultures, which were used in the methods presented here. They provide the advantages of the cell lines in that once isolated and established, they are convenient to use and remain closer to the actual cells in vivo; they have not been selected for or undergone transformation. However, it is important to note that any cell growing in culture is placed in an unnatural environment. Methods devised to mimic the in vivo environment have had only limited success.

The effect which this has on experimental results depends on the application of the cells. The bovine mammary primary cells worked well in the way in which they were applied in the present study. Certainly, the effort to minimize the number of passages is important. However, the actual levels of adherence expressed in this paper may not truly reflect the levels in the mammary gland. Another report has described much higher levels of adherence than observed in the present studies (5). The higher levels of adherence may directly reflect the higher expression of receptors on the tissue culture cells as a result of a different stage of differentiation and development.

Another area which varies between the adherence assays are the methods employed in the quantification of the attached bacteria. Many have used direct light microscopy to examine and count the adherent bacteria (2, 7, 17, 36). Though this method provides for a close examination of the attachment of the bacterial cells, it can be quite cumbersome, limiting the number of samples which can be tested at one time. It also allows for some subjectivity to be introduced in the counting. This method does have the advantage, however, of allowing visual examination of each bacterium-host cell interaction and determination of whether the attachment has occurred as single cells or clumps.
S. aureus to bovine mammary epithelial cells in vitro has been demonstrated. It should be noted that although every effort was made to simulate the actual environment under which bovine mastitis occurs, the results of these experiments must be interpreted carefully until in vivo tests can be performed. Further studies performed with purified antigens for the production of antibody may help to improve the results. A molecular approach to the analysis of the S. aureus adhesins may be required to identify the molecule responsible for its adherence.

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REFERENCES