ADHERENCE AND COLONIZATION BY BACTERIAL PATHOGENS IN EXPLANT CULTURES OF BOVINE MAMMARY TISSUE

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ABSTRACT

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Explant cultures of bovine mammary tissue taken from virgin heifers were used to examine adherence, colonization and cytopathogenesis of *Streptococcus uberis*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Staphylococcus aureus* and *Escherichia coli* in the putative target tissue. None of the five bacteria was able to adhere to healthy ductular epithelium but all showed a marked tropism for exposed connective tissue. *S. aureus* and *E. coli* induced a marked cytopathic effect in ductular epithelium after 6 hours in culture but the bacteria were not in close association with the affected tissue. No evidence could be found to support the hypothesis that adherence to epithelium might be the first stage in the pathogenesis of mastitis caused by these organisms.

Keywords: adherence, bacteria, cattle, cell culture, mammary gland, *Staphyloococcus*, *Streptococcus*

INTRODUCTION

It has been suggested by Bramley and Dodd (1984), by analogy with studies on bacteria in the gut and urogenital tract, that adhesion to epithelium may be an important process in the pathogenesis of mastitis. Frost and colleagues (1977) have shown that *Staphylococcus aureus* and *Streptococcus agalactiae* have a higher adherence index for ductular epithelium *in vitro* than have other less significant mammary gland pathogens and they proposed that specific adherence may be an important aspect of the pathogenesis of mastitis by these two bacteria. However, Anderson (1978), using the mouse mastitis model, could find no substantial evidence of adherence by *S. aureus*, *Staphylococcus epidermidis*, *Escherichia coli* or *S. agalactiae* to mammary gland epithelium.

Explant cultures of bovine respiratory tissue have been used successfully to study the attachment and pathogenesis of certain bovine mycoplasmas (Thomas and Howard, 1974; Thomas *et al.*, 1987). These cultures have the advantage compared to isolated cells *in vitro* that the putative target cell is in place in relation to other supporting tissues and viability can be readily assessed histologically. Explant cultures of mammary gland were therefore used to study adhesion, tropism and cytopathogenicity for mammary gland tissue of five bacterial pathogens including *S. aureus* and *S. agalactiae*.

MATERIALS AND METHODS

Explant cultures

Mammary tissue was obtained within 2 hours of slaughter from three virgin heifers aged 8–10 months. Thin slices of tissue, 2–3 mm thick, were taken aseptically from the region of the lactiferous sinus to include adjacent ductular and putative glandular tissue. Strips were removed to the laboratory in Eagle's based culture medium, without antibacterials (Stott *et al.*, 1976), cut into small pieces of 2–3 mm^3, set in six-well plastic Petri dishes (2 per well) and covered with 3 ml of medium. The cultures were then incubated at 37°C in a CO_2 incubator for 1–2 hours prior to inoculation with 10^8 colony-forming units (cfu) of washed bacteria in 0.1 ml and returned to the incubator.
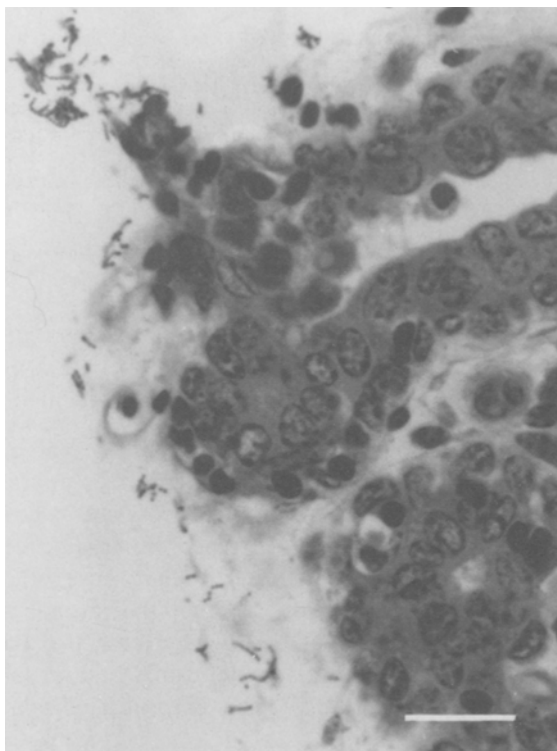

Figure 1. *Streptococcus agalactiae*, 6 hours after inoculation; typical long chains of streptococci adherent to peripheral connective tissue, none adherent to healthy ductular epithelium. Toluidine blue; bar = 20 μm
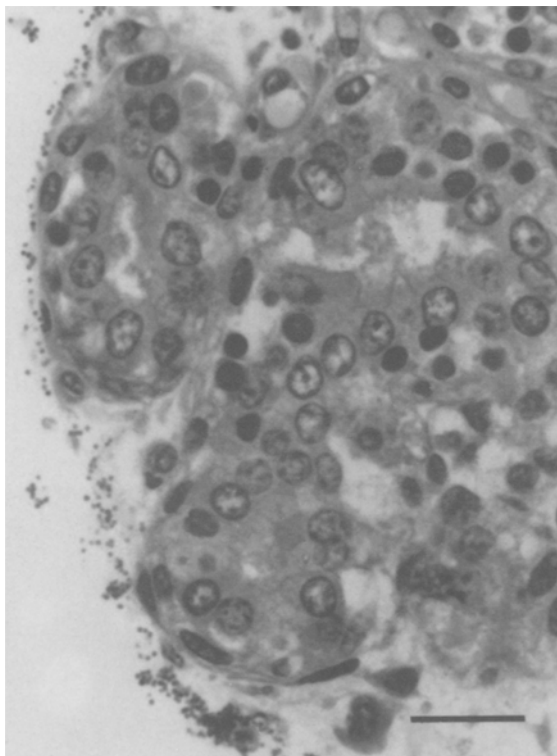
Figure 2. *Staphylococcus aureus*, cytopathic effect 6 hours after inoculation; bacteria adherent to periphery of culture, none in association with the putative ductular epithelium. Loss of ductular architecture, condensation of nuclear chromatin and pyknosis of epithelium. Compare with Figures 1 and 4. Toluidine blue; bar = 20 μm

Bacteria

S. uberis strain EF20, *S. dysgalactiae* strain CE127, *S. agalactiae* strain 411.07, *S. aureus* strain M60 and *E. coli* strain P4 were used to inoculate the cultures. These were originally obtained from clinical cases of bovine mastitis and were isolated at the National Institute for Research in Dairying, Shinfield, UK. Bacteria were stored at a density of approximately 10^8 cfu/ml at $-20°C$ in Todd–Hewitt broth containing 25% (v/v) glycerol and revived by growth in Todd–Hewitt broth at 37°C for 18 hours. Bacteria were harvested by centrifugation (3000 *g*, 15 min), washed twice in phosphate-buffered saline (PBS, pH 7.2) and resuspended in PBS to a density of 10^9 cfu/ml. All bacteria were tested in three separate experiments.

Sampling of explant cultures

Tissue samples were taken at 2, 6 and 18 hours after inoculation. Samples for bacteriology were weighed, washed in three changes of PBS to ensure that only

adherent bacteria were retained, then suspended in 3 ml ice-cold PBS and macerated for 2 minutes using an Ultra-Turrax (Janke and Kunkel, Germany) homogenizer. Bacterial counts were performed by dilution of the homogenate in PBS and inoculation of nutrient agar supplemented with 5% (v/v) washed bovine erythrocytes and 0.1% (w/v) aesculin. Colony counts were recorded after 18 hours' incubation at 37°C. Samples for histology were taken into formol sublimate (90% saturated mercuric chloride and 10% formaldehyde), dehydrated, mounted in paraffin wax, sectioned and stained by haematoxylin, eosin and Giemsa, or mounted in JB4 plastic, sectioned at 3 μm and stained with toluidine blue. Samples for scanning electron microscopy (SEM) were taken into 0.1 mol/L phosphate-buffered 3% glutaraldehyde (pH 7.4). After post-fixation in 0.1 mol/L phosphate-buffered 1% osmium tetroxide, the tissue was dehydrated through graded methanol to acetone and dried to critical point in CO_2. Samples were coated with platinum and examined in a Hitachi S520 scanning electron microscope at an accelerated voltage of 20 kV.
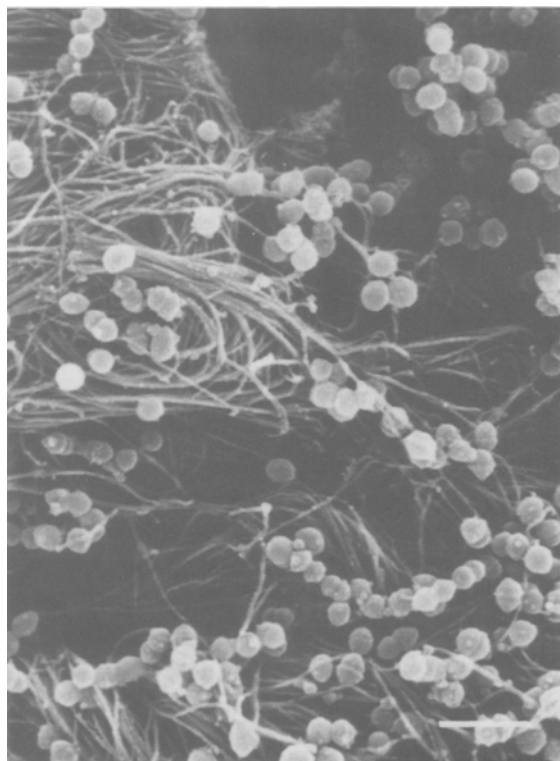

Figure 3. *Streptococcus dysgalactiae*, 18 hours after inoculation; large numbers of bacteria adherent to connective tissue. SEM; bar = 2 μm
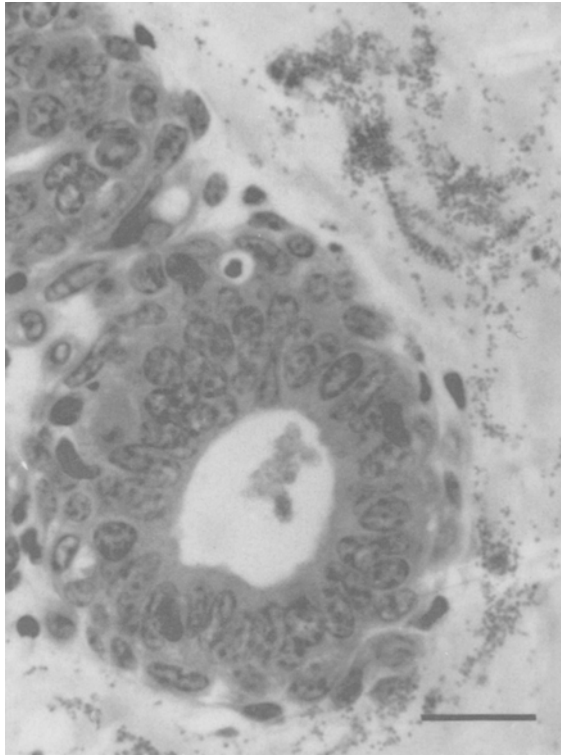
Figure 4. *Streptococcus dysgalactiae*, 6 hours after inoculation; large numbers of bacteria invading the connective tissue, none adherent to healthy duct epithelium. Toluidine blue; bar = 20 μm

RESULTS

Large numbers of bacteria could be seen by light microscopy in all cultures at 2, 6 and 18 hours after inoculation. Bacteria were re-isolated in pure culture from all three experiments. The numbers varied between 3.9 and 6.4 $\log_{10}$ cfu/mg and were consistently lower for *S. uberis* than for the other four bacteria. None of the bacteria increased in numbers during the course of the experiments.

All the bacteria appeared to have a preferential tropism for the exposed connective tissue at the periphery of the cultures (Figures 1, 2 and 3), the majority being located in this site. *S. uberis*, *E. coli*, *S. dysgalactiae* and *S. aureus* were locally invasive (Figure 4), penetrated the loose connective tissue and localized in the primitive lymphatic vessels. This was particularly evident for *S. uberis*, these organisms being observed lining vessels deep inside the cultures (Figure 5). SEM observations indicated that this adhesion occurred where the endothelium was coated with fibrin.
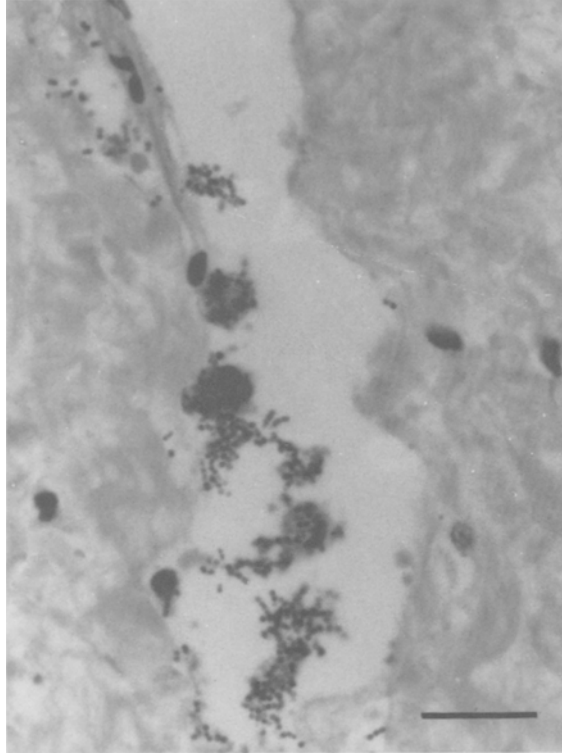
Figure 5. *Streptococcus uberis*, 6 hours after inoculation; bacteria in a putative lymphatic vessel and adherent to detached pyknotic cells. Toluidine blue; bar = 20 μm

All the bacteria also appeared to have a limited tropism for sloughed, effete epithelial cells from the large ducts (Figures 6 and 7) but none was observed adhering to the living epithelium of either large or small internal ducts. Only *E. coli* was observed to adhere to flattened, epithelial cells that were devoid of microvilli (Figure 8). These flattened cells were only recognized by SEM and may be a peculiar response to infection with *E. coli*. Staggered serial sections were used to follow the course of a large duct to the external surface of each culture. Bacteria were located in at least one section in the lumina of the large ducts of all cultures, thus confirming their accessibility to duct epithelium.

None of the streptococci induced a cytopathic effect in the tissues even after 18 hours, whereas *E. coli* and *S. aureus* induced a marked effect from 6 hours after inoculation. This effect was characterized by cytoplasmic vacuolation and nuclear condensation of the epithelium throughout the cultures, although bacteria were not closely associated with these changes (Figure 2). Control cultures and those inoculated with streptococci (Figures 1 and 4) contained a high proportion of viable ductular epithelium up to 6 hours after inoculation.

The failure to adhere to live epithelium, the limited tropism for dead epithelium and the marked tropism for connective tissue observed by one of us (L.H.T.) by light microscopy were independently observed and confirmed (by A.P.B.) by scanning electron microscopy.
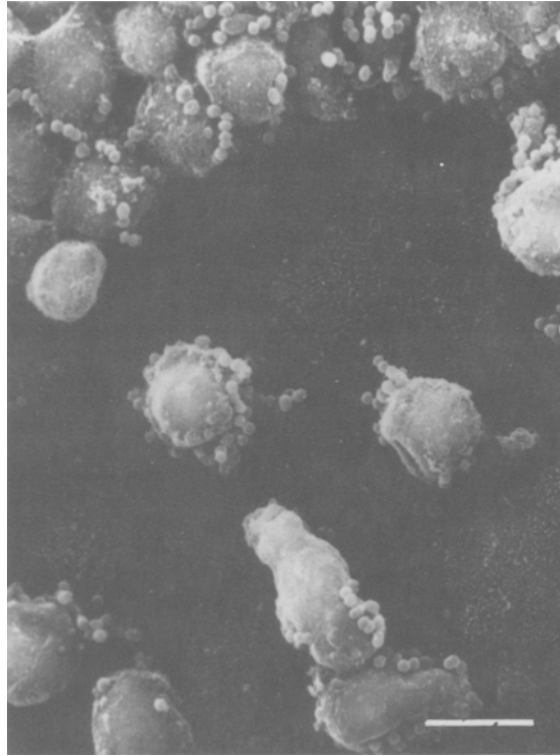
Figure 6. *Streptococcus uberis*, 18 hours after inoculation; bacteria only adherent to detaching epithelial cells, none adherent to the microvilli of healthy cells. SEM; bar = 6 μm

DISCUSSION

None of the five bacteria used in this study showed any tropism for live epithelium, whether it was lining large ducts or the smaller, more centrally located ducts from which the secretory tissue is derived. The failure of these recognized mammary pathogens to adhere to healthy mammary gland epithelium would appear to support the findings in mice by Anderson (1978) that adhesion is not an important factor in their pathogenicity. A limited tropism for sloughed, effete ductular epithelium was seen, particularly with the three species of streptococci, and this may explain the discrepancy between the results reported here and those previously published by Frost and colleagues (1977), who may have had a high proportion of effete cells in their *in vitro* test system. Up to 100% of cells extracted by the method used by those authors may stain with trypan blue (J.A. Leigh and others, unpublished observations). Alternatively the apparent invasion by *E. coli* of flattened epithelium could have resulted from phagocytosis by the epithelium (Figure 8).

Figure 7. *Streptococcus agalactiae*, 2 hours after inoculation; long chains of streptococci adherent largely to detaching epithelial cells. SEM; bar = 3 μm

The overwhelming majority of bacteria of all species were attached to the exposed connective tissue around the periphery of the cultures, which could be particularly relevant once the bacteria have broached the mammary epithelium. All the species tested were able to penetrate the stromal tissue of the cultures to a variable degree. *S. uberis* appeared able to utilize the putative venules or lymphatics for this purpose, perhaps through adherence to fibrin overlying the endothelium.

Failure to adhere to the epithelium of the centrally located small ducts may have been due to an inability of the bacteria to reach the tissue in question, although their observed invasiveness (Figure 4) makes this seem unlikely. Accessibility of bacteria to the epithelium of the larger ducts was ascertained by using staggered serial sections and observing free, non-adherent bacteria in the ductular lumina.

Failure to adhere to the epithelium may also have been related to the early stage of mammary development of the virgin heifers providing the material. The immature tissue used may not have provided the putative target tissue, that is the secretory glandular epithelium, necessary for bacterial adherence. However, as *S. uberis* is the most common cause of new infections in the dry cow (Bramley, 1984), this does not explain the inability of *S. uberis* to adhere.
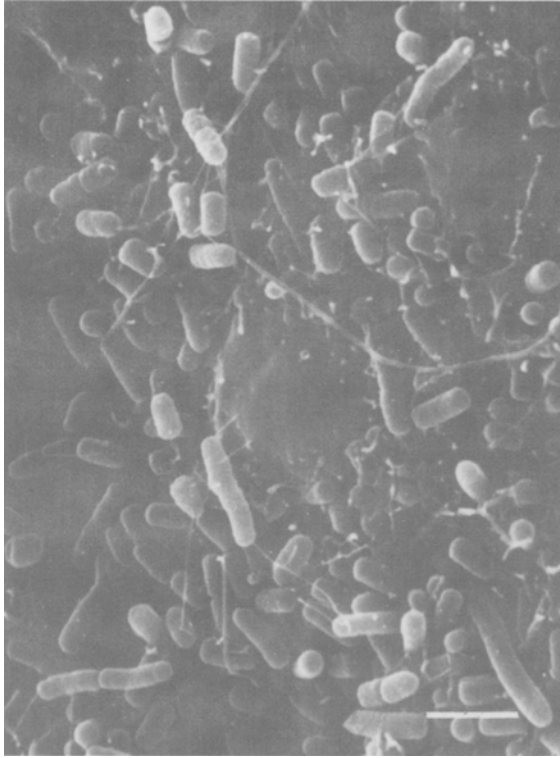
Figure 8. *Escherichia coli*, 6 hours after inoculation; large numbers of bacteria adherent to and apparently invading flattened epithelial cells. SEM; bar = 2 μm

Failure to demonstrate growth of bacteria during the experiments was probably due to the large number inoculated (10^8 cfu), which would have masked all but the most marked increase in numbers. In addition, the sampling method would have retained only strongly adherent bacteria, while non-viable detached cells would have been lost in the washing process.

Finally, failure to demonstrate adhesion could be the result of using organisms grown *in vitro*, which may lack essential adhesive properties.

For whatever reason, these preliminary experiments have failed to find evidence for adhesion by five bacterial pathogens to bovine mammary tissue and doubt is therefore thrown on whether this is an essential first step in the pathogenesis of bovine mastitis. If adhesion is not a factor, then the means whereby these mammary pathogens, particularly the non-toxin-producing bacteria such as *S. uberis*, induce disease remains to be explained.

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