ABSTRACT

The aim of this work was to establish a simple but reliable method of collecting epithelial cells from tissue lining the gland cistern of the bovine mammary gland and to investigate the ability of cultured epithelial cells to express major histocompatibility complex class II antigens. Bovine mammary gland epithelial cells were isolated and grown as monolayers on five substrates in vitro. The cells were identified as epithelial in origin by immunofluorescent staining with anticytokeratin monoclonal antibodies.

Major histocompatibility complex class II antigens were induced on epithelial monolayers by incubation with supernatants from bovine peripheral blood mononuclear cells stimulated with concanavalin A or incubation with recombinant bovine interferon-γ. These results suggest that, in bovine mammary gland epithelium, recombinant bovine interferon-γ, acting alone, can induce class II expression. This observation permits future investigation of the putative role of bovine mammary gland epithelial cells as accessory cells in the initiation of local immune responses and their involvement in peptide transport.

(Key words: major histocompatibility complex class II antigens, epithelium, bovine)

Abbreviation key: Con A = concanavalin A, FITC = fluorescein isothiocyanate, HBSS = Hanks balanced salt solution, MHC = major histocompatibility complex, PBM = peripheral blood mononuclear cells, P-S = penicillin-streptomycin.

INTRODUCTION

The ductular epithelium of the mammary gland is the first tissue to become exposed to pathogens following invasion of the teat duct in the early stages of mastitis. The interaction of the invading microorganism with the epithelium is, therefore, extremely important in the pathogenesis of infection.

Major histocompatibility complex (MHC) class II antigens are heterodimeric, integral membrane glycoproteins that have been described on a wide variety of cells involved in the induction and regulation of immune responses (26). In addition to being expressed on classical antigen-presenting cells (8), class II antigens have been described on epithelial tissue in various organs and species (4, 7). In the mammary gland, class II antigens have been described on the epithelium of guinea pigs (28), humans (20), and cattle (10). Other species, such as sheep (17) and mice (11), constitutively display class II antigens only on cells within the connective tissue of the gland, not on the epithelium.

Class II expression can be induced or modified in various tissues by lymphokines (23) and by immunological stimuli (2). Interferon-γ, alone and in concert with other cytokines, plays a central role in regulation of class II expression (24).

As part of our investigation (10) into the local immune events in the pathogenesis of mastitis in cows, we have shown that class II
antigens are induced on epithelium and on cells within the mammary gland connective tissue by in vivo intramammary infusion of formalin-killed Streptococcus uberis. The greatest epithelial class II expression was around the gland cistern, an area that was also heavily infiltrated by inflammatory cells following infusion with S. uberis. A possible explanation is that lymphokines, including interferon-γ, were released by primed T-cells in the mammary gland and that these lymphokines were responsible for induction of class II. For this investigation, we developed a reliable culture system for bovine mammary epithelium to enable analysis of induction of epithelial cell MHC class II antigens by T-cell cytokines in vitro.

MATERIALS AND METHODS

Cows

Mammary gland tissue was collected from 8 multiparous cows and 4 primiparous cows of various dairy breeds, immediately following commercial slaughter. Three of the cows were lactating, but the remaining 5 multiparous cows and all primiparous cows were nonlactating. The age of the cows was not ascertained. Blood was taken from normal Friesian cows in the University of Bristol dairy herd.

Tissues

Udders from lactating and dry cows were used for tissue collection. Epithelial cells were collected from two mammary gland quarters per cow (16 individual samples from multiparous cows; 8 individual samples from primiparous cows). The intact mammary gland, including skin, was removed from cows immediately following slaughter. Only udders considered to be of normal size, color, and appearance and producing grossly normal mammary secretions were selected. The gland was cleaned thoroughly, and, to expose the epithelial lining of each quarter, the teat orifice was incised, and the cut was extended through the teat into the gland cistern. The epithelial surface was rinsed several times with Hanks balanced salt solution (HBSS; Flow Laboratories, Irvine, Scotland) containing 100 U/100 µg/ml of penicillin and streptomycin mixture (P-S; Flow Laboratories).

Blood was withdrawn from the jugular vein of cows into heparinized vacutainers (Becton Dickinson Labware, Plymouth, England).

Media

Complete medium, optimized for epithelial cell isolation and maintenance, consisted of RPMI 1640 (Flow Laboratories) containing 10% fetal calf serum (Imperial Laboratories, Andover, Hampshire, England), 2 mM L-glutamine (Flow Laboratories), 1 mM sodium pyruvate (Imperial Laboratories), 2.5 µg/ml of fungizone (Gibco, Paisley, Scotland), 100 U/100 µg/ml of P-S, .04 mg/ml of gentamicin (Flow Laboratories), 1x nonessential AA (Flow Laboratories), .5% lactalbumin hydrolysate (10% solution, Gibco), .008 mM L-lysine (Sigma, Poole, Dorset, England), .002 mM L-methionine (Sigma), 1 µg/ml of hydrocortisone (Sigma), and 2.5 µg/ml of insulin (Sigma).

The medium used for concanavalin A (Con A) stimulation of peripheral blood mononuclear cells (PBM) consisted of RPMI 1640 (Flow Laboratories) supplemented with 10% fetal calf serum (Imperial Laboratories), 2 mM L-glutamine (Flow Laboratories), 20 mM HEPES (Flow Laboratories), 100 U/100 µg/ml of P-S (Flow Laboratories), and .04 mg/ml of gentamicin (Flow Laboratories).

Isolation of Epithelial Cells

Epithelial cells were isolated according to the method of Wanasinghe (27) within 2 h of tissue collection. The epithelial cells were removed by gentle stroking and twisting motions with a sterile pipette brush. The entire epithelial area exposed, from the teat duct to the mammary ducts, was brushed, and care was taken not to brush the same areas repeatedly to avoid collection of cells underlying the epithelial layer. The cells were dislodged by twirling the brush in HBSS containing 100 U/100 µg/ml of P-S (HBSS plus P-S), and the process was repeated several times until the HBSS plus P-S appeared cloudy. The cells were then washed three times in HBSS plus P-S and resuspended in HBSS plus P-S, and the live cell count was determined by Trypan blue exclusion before resuspension in complete medium.

To investigate whether the yield and viability of epithelial cells varied between
primiparous and multiparous cows, the yield and viability of the groups were compared by the Student's t test. Significance was at \( P < 0.05 \).

**Culture of Epithelial Cells**

Isolated bovine mammary gland epithelial cells were grown on various substrates: 1) polystyrene flat-bottomed 24-well tissue culture plates (Falcon; Becton Dickinson Labware), 2) glass coverslips (13 mm in diameter; Merck Ltd., Poole, Dorset, England) placed on the base of 24-well tissue culture plates (Falcon; Becton Dickinson Labware), 3) inorganic membrane inserts (Anopore; Nunc Tissue Culture Inserts, Gibco) placed on the base of 24-well tissue culture plates (Falcon; Becton Dickinson Labware), 4) polyester membranes (Melinex Film; Agar Scientific, Stansted, Essex, England) placed on the base of 24-well tissue culture plates (Falcon; Becton Dickinson Labware), and 5) collagen gels formed on the base of 24-well tissue culture plates (Falcon; Becton Dickinson Labware). Cell cultures were established from 2 cows (4 quarters) on each of the first four substrates and from 6 cows (12 quarters) on the fifth substrate.

Collagen gels were prepared by a modification of the method of Yang et al. (30). Rat tail collagen (type VII; Sigma) was sterilized by immersion in 100% ethanol for 1 h. The collagen was centrifuged, and the ethanol was decanted. The collagen was dissolved in freshly prepared 1% acetic acid (filtered through a 0.2-μm filter; Sartorius Ltd.; Epsom, Surrey, England) to give a final concentration of 2 mg/ml. Two parts of 10× HBSS were mixed with 1 part of 0.34 M NaOH and filtered through a 0.2-μm filter (Sartorius Ltd.); the mixture was added to the dissolved collagen at a 1:5 (vol/vol) ratio of HBSS-NaOH to collagen. The resulting mixture was added in 100-μl volumes to the wells of 24-well plates (Falcon; Becton Dickinson Labware), which were then polymerized by incubation at 37°C for 90 min.

With all substrates used, cells were added to the wells in a volume of 0.5 ml. The live cell concentration varied from \( 2 \times 10^5 \) to \( 2 \times 10^6 \) cells/ml. All cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂. After 24 h of incubation, 0.5 ml of fresh complete medium was added to each well, resulting in a final culture volume of 1 ml.

Every 3 d, one-half of the volume of medium was removed from each culture well and replaced with the same volume of fresh complete medium. The cells were subcultured when the cells had become confluent. The cell sheet was detached using a sterile rubber policeman, pipetted vigorously to break up cell clumps slightly, resuspended in four times the original volume of complete medium, and plated onto the same substrates, as described.

**Antibodies**

**Identification of Epithelial Cells.** To ascertain that cells in culture were of epithelial origin, monolayers were stained by indirect immunofluorescence using a monoclonal antibody reacting with cytokeratins. Cytokeratins are prekeratinlike polypeptides that act as general epithelial cell markers (12). The monolayers were similarly stained with a monoclonal antibody against vimentin, a polypeptide produced by cells of fibroblastic origin (16).

Three anticytokeratin monoclonal antibodies from clones K8.12, K4.62, and K8.13 [cytoskeletal antisera sampler pack (cytokeratin-vimentin); Sigma] were added to separate wells of confluent cells in 100-μl volumes at a final dilution of 1:20 (vol/vol) in PBS. Antivimentin monoclonal antibody from clone VIM 13.2 [cytoskeletal antisera sampler pack (cytokeratin-vimentin); Sigma] was added to wells in the same volume, but at a final dilution of 1:200 (vol/vol). As controls, the anticytokeratin and antivimentin monoclonal antibodies were replaced with PBS, 1% normal mouse serum, or mouse anti-bovine IgG monoclonal antibody in some wells.

**Identification of Class II Antigens.** The antibovine MHC class II monoclonal antibody, ILA-21 [IgG₂a isotype, ascites, directed against an invariant region; W. I. Morrison, 1991, personal communication; (19)], a kind gift from International Laboratory for Research on Animal Diseases (Nairobi, Kenya) was added to the monolayers in 100-μl volumes at a final dilution of 1:1000 (vol/vol) in PBS. As controls, the anticytokeratin and antivimentin monoclonal antibodies were replaced with PBS, 1% normal mouse serum, or mouse anti-bovine IgG monoclonal antibody in some wells.
Immunofluorescent Staining of Cell Monolayers

The culture medium was removed from confluent cell monolayers, and the cells were fixed in 1 ml of cold 95% ethanol per well for 10 min. Ethanol was removed, and cells were washed in three changes of PBS. Monoclonal antibodies were incubated on cells at 20°C for 90 min. Monolayers were washed in three changes of PBS and then incubated with 100-μl volumes of sheep anti-mouse IgG-fluorescein isothiocyanate [FITC, (whole molecule; Sigma)] at a final dilution of 1:120 (vol/vol) in PBS for 60 min. As a control, the FITC conjugate was replaced with PBS in some wells. The monolayers were washed again in three changes of PBS and were examined for fluorescence using a Leitz-Diavert microscope [E. Lietz (Instruments) Ltd., Luton, Bedfordshire, England]. Photographs of fluorescent cells were taken with a 400 American Standard Association film using exposure times of 2, 3, 4, and 5 min.

Induction of Class II Antigens In Vitro

Cytokines. The PBM were stimulated in culture with the T-cell mitogen Con A (Type IV; Sigma). Heparinized blood was diluted 1:1 (vol/vol) in HBSS (Flow Laboratories) containing 4 mM HEPES (Flow Laboratories), underlaid with 20 ml of separation medium (J-Prep; 1.077 g/ml; J. Bio, Les Ulis, France), and centrifuged at 800 x g for 40 min at 20°C. The cells at the interface were aspirated and washed three times. Cells were resuspended at a concentration of 1 x 10^6 cells/ml in medium. Cell suspension (20 ml) was added to each of two tissue culture flasks (75 cm²; Becton Dickinson Labware). The Con A was added to one flask at a final concentration of 5 μg/ml. The second flask contained only cells in complete medium, and the third flask contained complete medium with Con A at 5 μg/ml, but no cells. All flasks were incubated at 37°C in a humidified atmosphere with 5% CO₂. At 24, 48, and 72 h after establishment of the cultures, the cell suspension was removed from the flasks and centrifuged to pellet the cells. The supernatants were collected, divided into aliquots, and stored at −20°C until required.

Half of the volume of medium (500 μl) was removed from each culture well containing confluent epithelial cells; 500 μl of medium remained in each well. Supernatants from 24-, 48-, or 72-h cultures were thawed and added to the monolayers at a final dilution of 1:5 (vol/vol) into the medium remaining on the cell monolayers. The supernatants were incubated on the monolayers at 37°C in a humidified atmosphere with 5% CO₂ for 24 and 48 h.

Recombinant Bovine Interferon-γ. Bovine interferon-γ (10⁴ U/ml), expressed in Escherichia coli, was kindly donated by R. Collins, Institute for Animal Health (Compton, Berkshire, England). A sample of the supernatant from the untransfected E. coli host was used as a control for the interferon-γ.

The medium (900 μl) was removed from each culture well containing confluent cells. Interferon-γ and control supernatants were added at final concentrations of 20 U of interferon-γ/ml or 2.5 U of interferon-γ/ml into 100-μl volumes of medium remaining on the cell monolayers. The monolayers and supernatants were incubated at 37°C in a humidified atmosphere with 5% CO₂ for 24 h.

RESULTS

Isolation and Culture of Epithelial Cells

Isolation of epithelial cells from multiparous cows with large well-developed udders or from primiparous cows with small glands was equally successful, although the cells were easier to collect from mature cows because of the larger surface area of epithelial tissue available. Cell yields were similar in primiparous and multiparous cows; mean yields per mammary gland quarter were 39.6 x 10⁶ and 34.8 x 10⁶ epithelial cells, respectively (Table 1). Cells from primiparous cows were, on average, 79% viable, and cells from multiparous cows were 80.5% viable (Table 1). No significant differences occurred in cell yield or viability between primiparous and multiparous cows (Table 1). Cultures were successfully established from lactating and dry udders, although the cells from lactating glands divided more rapidly and reached confluence slightly earlier.

Epithelial cells were successfully grown on several substrates. Cell doubling times were shorter, and cells reached confluence earlier, when cultures were established on collagen.
TABLE 1. Epithelial cell yield and cell viability from primiparous and multiparous cows.

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<th>Primiparous</th>
<th>Multiparous</th>
<th>t Test</th>
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<tbody>
<tr>
<td>Cell yield1</td>
<td>59.6 ± 10.7</td>
<td>34.8 ± 5.6</td>
<td>.39</td>
<td>.7NS</td>
</tr>
<tr>
<td>Cell viability2</td>
<td>79.0 ± 2.6</td>
<td>80.5 ± 1.6</td>
<td>.49</td>
<td>.6NS</td>
</tr>
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1Epithelial cell yield per mammary gland quarter × 10^6.
2Epithelial cell viability: percentage of live cells calculated as those cells able to exclude trypan blue.

Use of an inverted microscope revealed that the isolated cells adhered to the collagen within minutes of addition to the wells. The cells usually reached confluence on collagen 10 to 14 d after primary isolation, but cells grown on the three different types of insert appeared to grow more slowly, requiring 2 to 3 wk to reach confluence (data not shown). Cells grown directly on the base of plastic tissue culture wells also required 2 to 3 wk to reach confluence, on average 5 d later than cells grown on collagen-coated wells. However, when the cell monolayers were subcultured, the cells grew rapidly on the base of the plastic tissue culture wells, reaching confluence in 10 to 14 d, perhaps reflecting adaptation of the cells to the plastic substrate (data not shown).

In all cases, small islets of cell growth could be identified 2 to 4 d following primary isolation. Cell growth was most rapid when the seed cell concentration was 2 x 10^5/ml and slower with cell concentrations of 2 x 10^5/ml. Cell growth usually started at the perimeter of large clumps of epithelial cells adhering to the surface. Growth then extended concentrically around the area. For this reason, no attempt was made to break cell clumps into single-cell suspensions in either primary isolation or subculturing stages. Primary cultured cells were usually polygonal or cuboidal, which is typical of epithelial cells in culture (21), and monolayers displayed a mosaic or cobblestone appearance (Figure 1). Cell morphology varied as cultured mammary gland epithelial cells formed islets interspersed with ridges of cells with a more elongated appearance (3, 18). Monolayers were usually subcultured within 5 d of confluence. If cells were cultured for longer, deterioration, such as vacuolation, was detected visually.

Cells were generally subcultured onto the plastic base of tissue culture wells or onto collagen-coated tissue culture wells. Cells divided rapidly and usually reached confluence 7 to 10 d following subculture. The optimal split ratio for subculture was 1:4. Subculture could be carried out approximately four times, after which the cell monolayer showed visible signs of deterioration and, eventually, cell death.

Identification of Epithelial Cells

Primary culture and subcultured cells were identified as epithelial by immunofluorescence with anticytokeratin monoclonal antibodies. All three anticytokeratin antibodies stained the cell monolayers, although the fluorescence was strongest with clone K8.13 (Figure 2). Monolayers in which the anticytokeratin antibody was replaced with PBS, mouse serum, or anti-bovine IgG monoclonal antibody or those in which the FITC-conjugate was replaced with PBS showed no staining.

Cells stained with the antivimentin antibody showed no fluorescence, indicating that the cells were not of fibroblastic origin.

Figure 1. Primary culture epithelial monolayer grown on collagen, d 11 of culture: unstained, phase contrast. The cultured cells show a cobblestone or mosaic arrangement. ×400.
Induction of Class II Antigens In Vitro

Experiments performed to investigate the in vitro induction of class II antigens were carried out on primary cell monolayers grown on collagen gels, on first subculture cells grown on collagen, or on the base of tissue culture wells. Results were similar on monolayers grown on all surfaces, but, in order to control for possible variation in class II expression on cells of differing passage numbers, all treatments, including controls, were carried out on the same cell isolate.

Cytokines. Incubation of the cell monolayers with Con A-induced cytokine supernatant induced class II expression on the epithelial cells. Most of the epithelial cells in the monolayer expressed class II. Class II antigens were successfully induced by supernatants produced after 24, 48, and 72 h of culture of PBM with Con A. The staining was most intense with the 24-h cultures, although the 48- and 72-h cultures were also strongly stained. Immunofluorescent staining was evident after 24 and 48 h of incubation of the supernatants on the cell monolayers, and the fluorescence appeared to be maximal after 48 h. Figure 3 shows a cell monolayer stained for class II following 48 h of incubation with the 24-h Con A supernatant. Control supernatants from flasks containing 1) cells only in complete medium and 2) complete medium and Con A at 5 μg/ml, but no cells were incubated on the monolayers; in these cases, no class II staining was detected by immunofluorescence.

Recombinant Bovine Interferon-γ. Incubation of bovine interferon-γ on cell monolayers for 24 h at concentrations of 20 and 2.5 U of interferon-γ/ml resulted in the induction of class II molecules on the epithelial cells, as demonstrated by immunofluorescence. Stronger class II staining was identified when the higher concentration of interferon-γ was incubated on the cells (Figure 4). When the lower concentration of interferon-γ was added to the monolayers, class II expression was detected, but immunofluorescence was faint (Figure 5). When untransfected E. coli supernatant was incubated on the monolayers, no class II was detected by immunofluorescence.

DISCUSSION

Many methods for isolation of mammary gland epithelial cells have been described, including growth of cells from mammary gland secretions (15) or isolation of cells by enzymatic methods (18, 30). The simple isolation method chosen in this study involved collection of cells directly from the epithelial surface of the mammary gland. Surface epithelial cells were collected without disruption of the underlying tissue, which resulted in less contamination by mesenchymal cells than methods involving the use of enzymes.

Franke et al. (12) showed that epithelial cells in culture could produce prekeratin-like
polypeptides called cytokeratins and demonstrated their presence in a variety of epithelial cells, including those from the bovine mammary glands. We identified cell monolayers as epithelial in origin by immunofluorescent staining with anticytokeratin antibody.

Expression of MHC class II molecules on macrophages is a prerequisite for presentation of antigen to primed T cells (25). Interferon-γ, a T-cell lymphokine, can induce class II molecules on many cell types in vitro, including smooth muscle (24), endothelium, and dermal fibroblasts (22). These class II-expressing cells can be functionally recognized by T cells, and the induction of class II antigens on cells otherwise expressing low or undetectable amounts of the antigen increased the number of functional or effective antigen-presenting cells (29).

Previous studies (6) have demonstrated the induction of class II on cultured gut epithelial cells by Con A-stimulated spleen cells and intraepithelial lymphocytes. Leukocytes infiltrating mammary parenchymal tissues following intramammary infusion of interferon-γ showed an increase in numbers of cells expressing class II (1). In the present study, Con A-stimulated PBM produced cytokine-containing supernatants 24, 48, and 72 h after addition of Con A, which induced expression of class II antigens on the mammary gland epithelial monolayer after 24 and 48 h of culture. Class II expression was maximal when the 24-h supernatant was incubated on the epithelial cells. This finding suggests that the cytokine responsible for class II induction is interferon-γ, which stimulates cellular responses in cattle within 6 to 24 h of incubation (9, 14). The 48- and 72-h supernatants were also able to induce class II expression, and interleukin-2 or other cytokines that act by induction of interferon-γ and, therefore, have a lag time may also be present in the supernatants (13).

Recombinant interferon-γ alone induced class II expression on the epithelial monolayer after 24 h of culture. In contrast, the cytokine-induced supernatants stimulated maximal class II expression after 48 h of culture on the epithelial monolayers. This difference may reflect sub- or supraoptimal amounts of interferon-γ in the supernatants or the slower kinetics of class II stimulation of other cytokines induced by Con A. The addition of monoclonals specific for cytokine to the epithelial monolayers treated with cytokine supernatant in future studies will allow investigation of the relative importance of different cytokines in class II induction.

In vivo, interferon-γ is produced by T cells, which accumulate in the mammary gland in response to intramammary infusion of bacteria (10, 17). Although bovine mammary gland epithelium has low constitutive class II expression, class II molecules can be induced by in vivo infusion of killed S. uberis bacteria (10).
Therefore, in addition to the secretory function of mammary epithelium, class II-expressing cells may play a role in processing and presentation of antigens to T cells in the local mammary gland environment, as in gut epithelium (5), where class II induction is a product of the effect of T-cell and non-T-cell cytokines (4).

CONCLUSIONS

This simple method of bovine epithelial cell isolation and culture allows investigation of the interaction between mastitis pathogens and the host tissue. Induction of MHC class II antigens on epithelial cells in vitro will facilitate future study of the role of these cells in local immune responses in the bovine mammary gland.

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REFERENCES

24 Stemme, S., G. Faber, and G. K. Hansson. 1990. MHC class II expression in human vascular smooth
muscle cells is induced by interferon-gamma and modulated by tumour necrosis factor and lymphotoxin. Immunology 69:243.