Journal of Dairy Research (2002) **69** 345–355. © *Proprietors of Journal of Dairy Research 2002*
DOI: 10.1017/S0022029902005551 *Printed in the United Kingdom*

In vitro differentiation of a cloned bovine mammary epithelial cell

By MICHAEL T. ROSE[1]*†, HISASHI ASO[1], SHINICHI YONEKURA[2], TOKUSHI KOMATSU[1], AKIHIKO HAGINO[2], KYOUHEI OZUTSUMI[1] and YOSHIAKI OBARA[2]

[1] *Department of Animal Physiology, National Institute of Animal Industry, P.O. Box 5, Tsukuba-Norindanchi, Ibaraki 305-0901, Japan*
[2] *Laboratory of Animal Physiology, Tohoku University, Aoba-ku, Sendai 981-8555, Japan*

(*Received 14 February 2001 and accepted for publication 21 January 2002*)

Summary. The aim of the study was to establish *in vitro* a bovine mammary epithelial cell (MEC) clone, able to respond to mitogenic growth factors and to lactogenic hormones. Mammary tissue from a 200-d pregnant Holstein cow was used as a source of MEC, from which a clone was established through a process of limiting dilution. When plated on plastic, the cells assumed a monolayer, cobblestone, epithelial-like morphology, with close contact between cells. Inclusion of IGF-1 and EGF in the media significantly increased the number of cells 5 d after plating. All cells stained strongly for cytokeratin and moderately for vimentin at young and old passage stages, indicating the epithelial nature of this cell clone. When the cells were plated at a high density on a thin layer of a commercial extracellular matrix preparation (Matrigel), lobular, alveoli-like structures developed within approximately 5 d, with a clearly visible lumen. When cells were plated onto Matrigel in differentiation media (containing lactogenic hormones), detectable quantities of α-casein were present in the media and particularly on the lumen side of the structures. Omission of one of the lactogenic hormones (insulin, prolactin or hydrocortisone) reduced α-casein release to the limit of detection of the assay used. Lactoferrin was also produced when the cells were plated on Matrigel, again principally on the lumen side of the lobules, though this was independent of the lactogenic hormones. By passage 40, the cells had senesced, and it was not possible to induce α-casein or lactoferrin production. This study notes the establishment of a functional bovine mammary epithelial cell clone, which is responsive to mitogenic and lactogenic hormones and an extracellular matrix.

Keywords: Casein, lactoferrin, lactogenesis, matrigel.

Accurate replication *in vitro* of bovine mammary gland function would be of great benefit to studies on the control of milk synthesis and secretion. Progress towards this end has been slow, however, partly because of the biological variability in the cell preparations researchers have been able to produce. There are a number of possible *in vitro* mammary incubation methods. Attempts simply to culture MEC on plastic usually result in a loss of mammary specific functions within a couple of days

* Present address: Institute of Rural Studies, Llanbadarn Campus, University of Wales, Aberystwyth, SY23 3AL, UK.
† For correspondence; e-mail: mir@aber.ac.uk

(Larson, 1976). Incubation of slices of mammary tissue (Baumrucker & Stremberger, 1989) and mammary alveoli organoids (complete organoids taken from a lactating animal; Baumrucker *et al.* 1988) have shown that tissue-specific functions can be supported only for a limited period *in vitro* (Keys *et al.* 1992). Primary cell cultures have also been used with some success for short-term experiments; with an appropriate hormonal milieu and extracellular matrix, primary MEC can be stimulated to re-group and re-differentiate into structures that resemble alveolar organoids and secrete milk proteins (Talhouk *et al.* 1993). A number of immortal bovine MEC lines also exist (Schmid *et al.* 1983; Gibson *et al.* 1991; Huynh *et al.* 1991; Huynh and Pollak, 1995; Zavizion *et al.* 1996). However, in general, the level of milk protein synthesis of these transformed bovine cell lines is either very low or non-existent, and if it does occur, it is independent of some or all of the *in vivo* factors (hormones or extracellular matrix) known to regulate lactogenesis (Huynh *et al.* 1991). It seems, therefore, that these cell lines are not entirely appropriate for investigations of mammary gland function.

Here we report the establishment of a functional bovine MEC clone. Cell function is unaffected by cryopreservation and it is responsive to mitogenic and lactogenic hormones, and an appropriate extracellular matrix. The cell clone is not immortal, however, and after ~ 40 passages of growth on plastic, the cells visibly senesce and are unable to replicate further.

Materials and methods

All chemicals were obtained from Sigma (St. Louis, MO, USA), unless otherwise stated.

Establishment of cell clone

Several pieces of mammary tissue, ~ 1 cm^3 in size, were aseptically removed from the mammary gland of a 200-d pregnant Holstein cow immediately after slaughter, and placed in serum-free Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing penicillin and streptomycin (PS; 50 IU/ml and 50 μg/ml, respectively), which was then placed on ice. Within 20 min the mammary tissue pieces were then washed with serum-free cold DMEM and minced using surgical scissors. Approximately 2 g minced tissue was placed in 25 ml DMEM containing type II collagenase (600 units/ml), 10% fetal calf serum (FCS; Cytosystems, Castle Hill, Australia), and PS, and incubated at 37 °C for 30 min. The digesta were filtered through a steel mesh (pore size 105 μm) and the filtrate was placed in a 50-ml tube and centrifuged at 400 ***g*** at 4 °C for 7·5 min. The cells were washed a further three times with serum-free DMEM and were then resuspended in DMEM containing 10% FCS. Cells were counted and plated in DMEM containing 10% FCS on collagen-coated plastic (Nunc, Roskilde, Denmark) at 2×10^5 cells/cm^2. All cultures were maintained at 37 °C and 5% CO_2 in a humidified incubator. Plating efficiency was low but the adherent cells were highly proliferative. After ~ 6 d the cells were passaged and re-plated on collagen-coated plastic.

On the first passage, some of the primary culture cells were diluted to ~ 5 cells/ml in DMEM containing 10% FCS and PS, and 200 μl was aliquoted into each well of five collagen-coated 96-well plates (Sumilon, Tokyo, Japan). Each well was microscopically checked for cell growth 2 d after plating. Wells for which it was certain there was a single colony of rapidly growing cells with epithelial-like morphology were marked and passaged when they reached ~ 50% confluence. In

this way, three MEC clones were established, one of which was selected for further study.

Cell culture and growth on plastic

The growth medium (GM) used for the clone consisted of DMEM supplemented with 17·5% FCS, sodium acetate (5 mM), apo-transferrin (10 μg/ml) and PS (50 IU/ml and 50 μg/ml, respectively). Cells were passaged every 5 d. On passage, cells were washed with phosphate-buffered saline (PBS), and then treated with a sucrose/EDTA buffer (pH 7·25; 0·45 M sucrose, 3·6 g EDTA/l, 1 g bovine serum albumin (BSA)/l in PBS) for 3 min. Cells were then treated with trypsin and EDTA (0·5 g/l and 0·3 g/l, respectively), and centrifuged at 400 ***g***. Cells were counted and plated into plastic flasks (Nunc) at $1{\cdot}2 \times 10^4$ cells/cm^2.

Cell proliferation on plastic using normal GM (17% FCS) was determined with a haemocytometer on each of 8 d after plating. In a similar way, growth rate using GM with only 5% FCS, as well as growth with the 17·5% and 5% FCS media supplemented with either insulin-like growth factor-1 (IGF-1; 100 ng/ml), epidermal growth factor (EGF; 10 ng/ml) or both IGF-1 and EGF was determined. Lastly, the effect of individually omitting the sodium acetate, transferrin, FCS and PS from the normal GM on cell proliferation on day 5 of culture was also examined. Growth rates were determined in triplicate using 3·5-cm plastic dishes (Nunc).

Karyotype

The karyotype of the cells was determined every fifth passage from passage 15 to passage 40, using a method slightly modified from that of Sun *et al.* (1973).

Immunofluorescence of cytoskeletal proteins

To characterize cytoskeletal elements, the clone was cultured on an FCS-coated Labtek 8-chamber slide (Nunc), using GM for between 5 and 8 d, with a change of medium every other day. During this time, the cells formed confluent monolayers. The cells were then fixed at −40 °C in acetone and methanol (1 : 1, vol/vol) for 2 min, and blocked with BSA (30 g/l) for 2 h at 37 °C. Cells were treated with either anti-pan-cytokeratin (Progen, Biotecknik, Düren-Gürzenich, Germany), anti-type VII cytokeratin (Roche Diagnostics, Mannhein, Germany), anti-bovine vimentin (Progen) or anti-bovine-desmin (Progen) for 2 h at 37 °C. Antibodies were used according to manufacturers' recommendations and diluted in PBS, 5 g BSA/l. Slides were then incubated with an appropriate FITC-labelled second antibody for 1 h at 37 °C.

Enzyme linked immunosorbant assay

Six specific monoclonal antibodies (MoAb) against bovine α-casein and eight MoAb specific for bovine lactoferrin were produced using a modified method from Nomura *et al.* (1997). A sandwich ELISA system was then developed to allow detection of α-casein and lactoferrin down to approximately 1 ng/ml. Briefly, anti-α-casein or anti-lactoferrin MoAb was fixed to the walls of polystyrene micro-titre plates by dissolving in a 0·05 M-carbonate buffer, pH 9·6 (30 μg protein/ml) and incubating at 4 °C overnight. The wells were blocked with 100 μl BSA (30 g/l) for 2 h, and then 100 μl standard (1–1000 ng/ml), sample or blank was placed in the appropriate well and the plate incubated for 24 h at 4 °C. The plate was then blocked for 3 h with BSA (30 g/l). Biotin-conjugated anti-α-casein or anti-lactoferrin MoAb (as appropriate) was added to the wells and the plates were incubated overnight at 4 °C. Wells were then incubated for 30 min with a horseradish peroxidase-conjugated

avidin/biotin solution (ABC-PO kit, Vector Lab Inc., Burlingame, CA, USA). Finally, 100 μl *o*-phenylenediamine (1·5 mg/ml) dissolved in citrate-phosphate buffer (pH 5·0) was added and incubated at 37 °C for 15 min. The reaction was stopped with 9 M-H_2SO_4, and the colour intensity determined as the absorbance difference between 450 and 405 nm. No cross-reaction was detected by either assay for lactoferrin or α-casein (as appropriate), transferrin, α-lactalbumin or β-lactoglobulin. There was a low level of cross-reaction (approximately 10 %) between the α-casein assay and β- and κ-casein, which was probably due to α-casein contamination of the β- and κ-casein (which were $\sim$ 80–90 % pure; Sigma).

Inducement of milk protein production

All experiments were done in triplicate. Cells at passages 20, 25, 30, 35 and 40 were initially suspended in differentiation media (DMEM supplemented with 5 % FCS, 2 μg/ml insulin, 5 μg/ml hydrocortisone, 2 μg/ml prolactin from Chemicon International, Temecula, CA, 10 ng/ml β-oestradiol, and 10 ng/ml progesterone), and then plated onto a thin film of Matrigel (Becton Dickinson, Bedford, MA, USA; allowed to solidify at room temperature for 30 min in 3·5-cm petri dishes) at $2{\cdot}5 \times 10^5$ cells/cm^3. β-Oestradiol and progesterone supplements were omitted from day 2 after plating, which simulated withdrawal of oestradiol and progesterone at parturition. Differentiation media (DM) was then replaced every other day and the culture was maintained for up to 16 d. Cells plated onto a Matrigel film, but with GM rather than DM, was used as the control. Spent media was stored at −25 °C until further analysis. After 8 d of culture, the cells had formed alveoli-like lobule structures. The content of the lumen of these structures was isolated by incubating the cells with 2 ml calcium-free and serum-free media containing EDTA. This sequestered the calcium and caused the tight junctions between the cells to break open releasing the contents of the lobules. This was then stored at −25 °C until analysed for α-casein and lactoferrin; results were expressed as nanograms of protein per 10000 cells plated. The effect of omitting insulin, hydrocortisone and prolactin from the DM on α-casein and lactoferrin production was also investigated.

Statistical analysis

Values are presented as the mean $\pm$ SEM. Data were analysed using Student's *t* test or analysis of variance, as noted in the text.

RESULTS

Growth on plastic

When plated on plastic, the cells assumed a monolayer, cobblestone, epithelial-like morphology, with close contact between the cells (Fig. 1*a*). Even though the cells were derived from a single parent cell, at confluence there appeared to be two cell types present. Islands of small cobblestone-like cells were surrounded by larger, elongated cell types, which developed at points of contact between expanding colonies of cells. When cells were cultured on plastic for extended periods of time and at high density, dome-like structures appeared. Cell numbers at passage 18 ($\pm$ SE $\times 10^5$ cells) in 3·5-cm plastic dishes on day 5 of culture following the individual omission of sodium acetate, transferrin, FCS and PS from the normal GM were: $5{\cdot}71 \pm 0{\cdot}32$, $5{\cdot}96 \pm 0{\cdot}20$, $1{\cdot}56 \pm 0{\cdot}17$ and $6{\cdot}40 \pm 0{\cdot}40$, respectively, relative to a control (GM) value of $6{\cdot}33 \pm 0{\cdot}35 \times 10^5$ cells on day 5 of culture. Omission of FCS significantly reduced final cell number ($P < 0{\cdot}01$; Student's *t* test), but none of the other omissions had any significant effect.

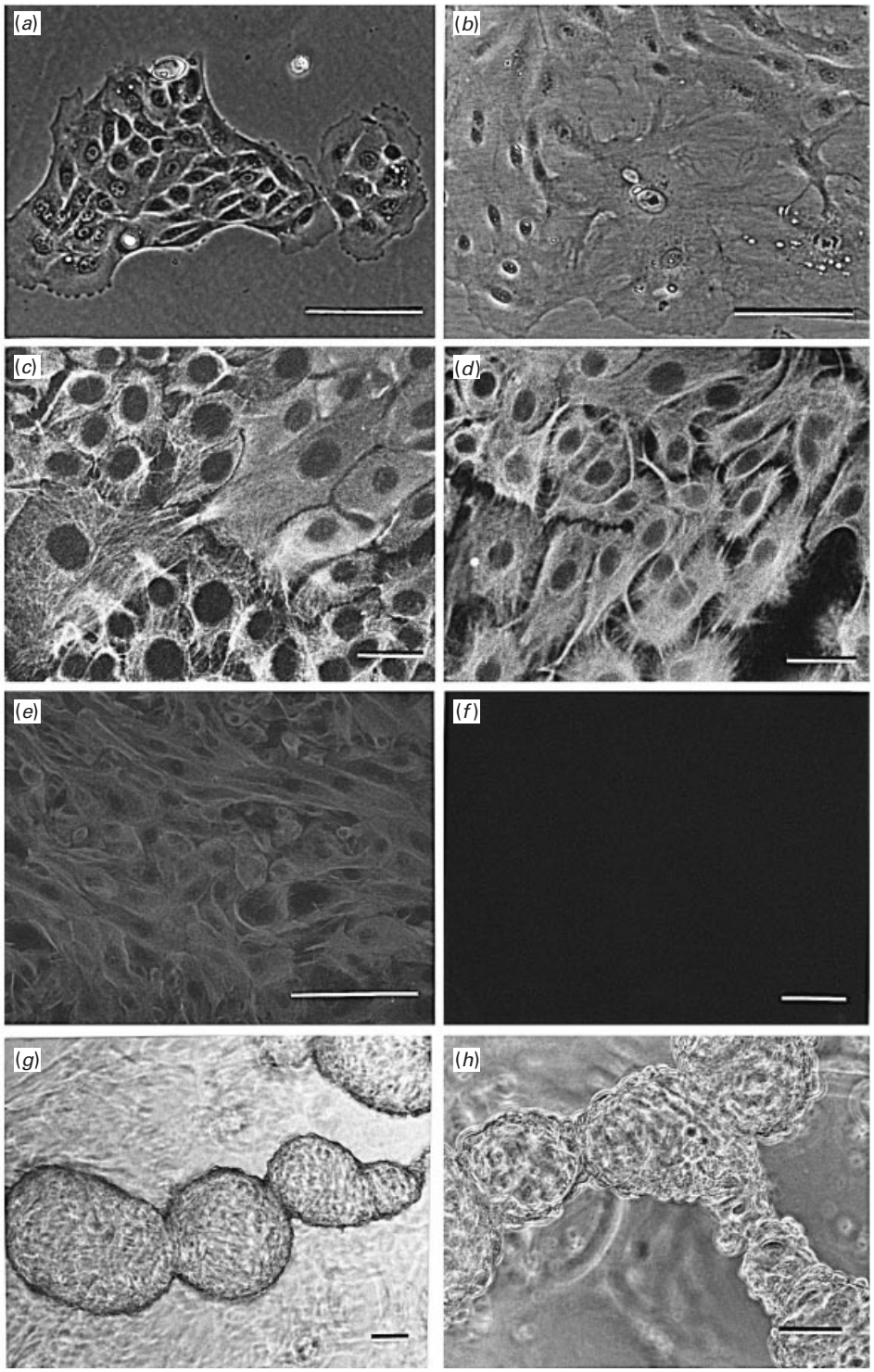

Fig. 1. Cloned bovine mammary epithelial cells (MEC) growing rapidly at passage 15 (*a*) and senescing at passage 37 (*b*). MEC fixed and stained for anti-pan-cytokeratin (*c*), for anti-VII-cytokeratin (*d*), for anti-vimentin (*e*) and failure to stain for anti-desmin (*f*). MEC on a thin film of Matrigel growing in differentiation media on day 8 of incubation (*g* and *h*). In all cases, bars represent 50 μm.

https://doi.org/10.1017/S0022029902005551 Published online by Cambridge University Press

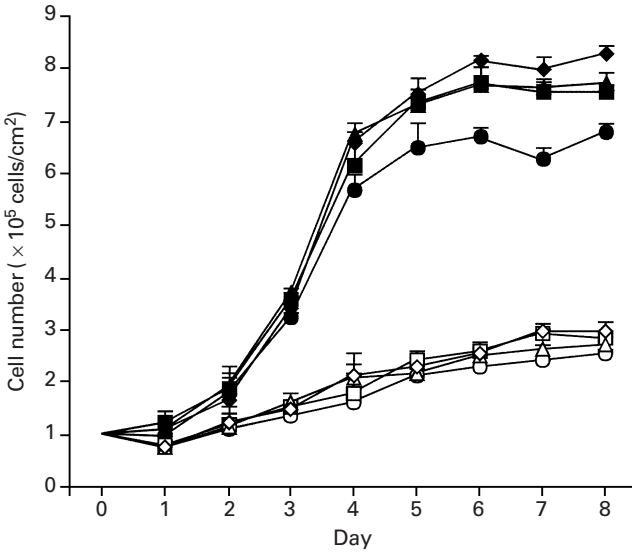

Fig. 2. Number of MEC (+SE) on plastic over 8 d in growth medium with no addition (●○), or containing 100 ng/ml of IGF-1 (■□), 10 ng/ml of EGF (▲△), or both IGF-1 (100 ng/ml) and EGF (10 ng/ml; ◆◇), at a FCS concentration of either 17% (●■▲◆) or 5% (○□△◇).

At an FCS concentration of 17%, the clone grew vigorously until it approached confluence, at ~ 5 d after passage (Fig. 2). At a serum concentration of 5%, growth was significantly slower ($P < 0{\cdot}05$; analysis of variance). When IGF-1 or EGF were included in the 17% FCS GM, the cell number at the end of the period was significantly greater ($P < 0{\cdot}05$). However, this effect was not apparent when the cells were grown in medium containing 5% FCS. As the cells in the 17% FCS control media were confluent by day 6 of culture, it is probable that the IGF-1 and EGF brought about a smaller cell size. By passage 40, laddering of the nuclear material was increasingly apparent and cytoplasmic vacuoles were evident (Fig. 1*b*).

Karyotype

Between passage 15 and 30 the modal karyotype of the cells remained at 60: the average values for passages 15, 20, 25 and 30 were $59{\cdot}8 \pm 1{\cdot}24$, $59{\cdot}2 \pm 1{\cdot}57$, $59{\cdot}8 \pm 1{\cdot}43$ and $59{\cdot}5 \pm 2{\cdot}01$, respectively. By passage 35, the average karyotype was $57{\cdot}1 \pm 2{\cdot}25$ and the modal value was 58, and by passage 40, the average value was $56{\cdot}7 \pm 2{\cdot}67$ and the modal value 58.

Immunofluorescence of cytoskeletal proteins

Confluent monolayers of the cells between passages 20 and 35 were stained for anti-pan-cytokeratin (Fig. 1*c*) and anti-type VII cytokeratin (Fig. 1*d*; a marker for epithelial cell types), vimentin (Fig. 1*e*; an intermediate filament protein characteristic of proliferating mesodermally derived cells) and desmin (Fig. 1*f*; a marker for muscle cells). All cells were strongly stained for both of the cytokeratin antibodies and, to a much lesser extent, all cells were positive for vimentin. However, desmin was not apparent for this MEC clone on plastic.

Morphological changes with Matrigel in vitro

When cells were was plated on a thin layer of Matrigel, lobular, alveoli-like, three-dimensional structures were formed (Fig. 1*g*, *h*). After ~ 24 h, cells began to aggregate into duct-like structures, and after ~ 5 d multicellular structures

https://doi.org/10.1017/S0022029902005551 Published online by Cambridge University Press

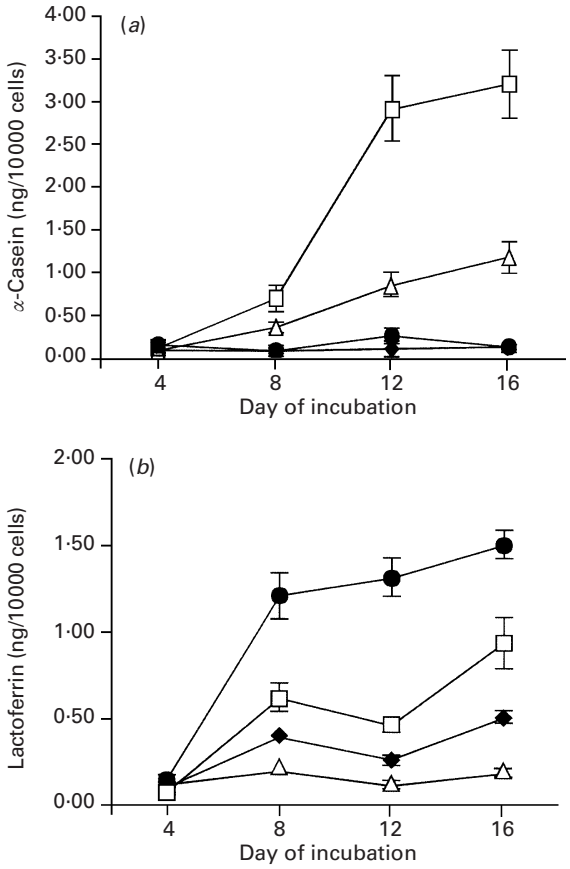

Fig. 3. Expression of (*a*) α-casein and (*b*) lactoferrin in media supernatant (◆△) and lumen extract (●□) from an MEC clone at passage 25 incubated on Matrigel over a 16-d period in either GM (◆●) or DM (△□).

containing clusters of duct-like and alveoli-like structures were formed, entirely or partly surrounded by a sheet of monolayer cells. The aggregation of cells was not apparent when serum was completely excluded from the medium, indicating that morphological re-organization was serum-dependent. The duct-like system was composed of a tubular network with a clearly visible lumen, and end bud structures.

When the clone was plated on thicker layers of Matrigel or collagen type IV, or embedded within Matrigel, or collagen type IV, no lasting organization was apparent. Attachment to thick layers of Matrigel was very poor; after ~ 24 h, some structural reorganization of the cells took place but this detached from the surface of the matrix shortly thereafter. MEC attachment onto type IV collagen was very good, though there was no further morphological development. When the collagen gels were detached, there was an occasional, limited, and unpredictable development of lobule-like structures.

Expression of casein and lactoferrin

When the cells were plated onto a thin layer of Matrigel in DM, detectable quantities of α-casein were produced (Fig. 3*a*; result shown for cells at passage 25). Casein concentrations remained either at or below the limit of detection for the casein ELISA for cells incubated in GM. Lumen α-casein levels using DM were not

https://doi.org/10.1017/S0022029902005551 Published online by Cambridge University Press

significantly different between passages 20, 25 and 30 ($3{\cdot}24 \pm 0{\cdot}32$, $3{\cdot}18 \pm 0{\cdot}37$, $3{\cdot}02 \pm 0{\cdot}26$ ng/10^4 cells per d, respectively; $P > 0{\cdot}05$; Student's t test), while casein production at passage 35 was lower ($0{\cdot}62 \pm 0{\cdot}19$ ng/10^5 cells; $P < 0{\cdot}01$; Student's t test) and attempts to induce α-casein expression for cells at passage 40 of the cells failed completely. Omission of prolactin, insulin or hydrocortisone significantly reduced or inhibited levels of α-casein in the lumen fluid for cells at passage 23: $0{\cdot}09 \pm 0{\cdot}06$, $0{\cdot}19 \pm 0{\cdot}07$ and $0{\cdot}22 \pm 0{\cdot}09$ ng/10^4 cells per d, respectively (all $P < 0{\cdot}01$, relative to $3{\cdot}39 \pm 0{\cdot}26$ ng/10^4 cells/d for full DM; Student's t test).

In contrast to casein, production of lactoferrin was greater for cells treated with GM rather than with DM (Fig. 3*b*; result shown for cells at passage 20). Lumen lactoferrin production levels using DM were not significantly different between passages 15, 20, 25 and 30 ($0{\cdot}72 \pm 0{\cdot}23$, $0{\cdot}94 \pm 0{\cdot}18$, $0{\cdot}62 \pm 0{\cdot}24$ and $0{\cdot}49 \pm 0{\cdot}25$ ng/10^4 cells per d, respectively; $P > 0{\cdot}05$; Student's t test). By passage 40, attempts to induce lactoferrin expression failed. Omission of prolactin, insulin or hydrocortisone did not significantly affect the levels of lactoferrin in the lumen fluid (tested at passage 23): $0{\cdot}68 \pm 0{\cdot}16$, $0{\cdot}62 \pm 0{\cdot}15$ and $0{\cdot}52 \pm 0{\cdot}22$ ng/10^4 cells per d respectively (all $P > 0{\cdot}05$, relative to $0{\cdot}73 \pm 0{\cdot}29$ ng/10^4 cells per d for full DM; Student's t test).

DISCUSSION

This report details the establishment and characterization of an untransformed bovine MEC clone. Cloned cells have the advantage over primary cultures in that they retain reasonably constant characteristics for numerous passages, and therefore over relatively long periods. The cells used in the present study exhibited some important functional and biochemical markers of the *in vivo* state. When growing on plastic, the cobblestone morphology characteristic of epithelial cells was seen, as was a cytoplasmic network of cytokeratins, and responsiveness to the mitogens IGF-1 and EGF. When given appropriate stimulation, the cells produced α-casein and lactoferrin. A fully robust indicator of cellular differentiation might also have included the expression of β-lactoglobulin and α-lactalbumin. Unfortunately, our attempts to make MoAb to these bovine whey proteins were not successful. When grown on plastic, weak positive staining for vimentin may indicate that the cells have some characteristics of myoepithelial cells (Li *et al*. 1999).

The level of casein expression by MEC was comparable to that reported by Talhouk *et al*. (1990) who used a bovine MEC primary culture. However, the present results are $\sim$ 10-fold lower than those of Delabarre *et al*. (1997), who used a primary culture of bovine mammary cells. Talhouk (1990) found lactoferrin expression to be $\sim$ 10-fold higher than in the present experiment. Differences between the latter and the present results are possibly due to the variety of cell types present in primary cultures affecting mammary epithelial cell function that were not present in the culture of this study. A comparison of casein production in the present study with that from immortal bovine MEC lines is not possible because of the inconsistent methods used for presenting casein expression.

Matrigel is extracted from the murine Engelbreth-Holm-Swarm (EHS) sarcoma, and as such is less than ideal as an extracellular matrix for bovine MEC. It contains principally laminin and collagen IV as well as a number of growth factors (McGuire & Seeds, 1989). Other workers have used floating collagen gels extracted from rat or calf tails (Talhouk *et al*. 1990, 1993; Gibson *et al*. 1991; Huynh *et al*. 1991). Using collagen gels, the best results in terms of differentiation have been achieved when the cells are allowed to form a confluent layer, and the collagen is then released from the

bottom of the well causing the collagen to shrink, which in turn stimulates the cells to differentiate (Emerman & Pitelka, 1977). This method was attempted for the cells used in the present study but with poor results relative to the results for Matrigel. The reason for this is not clear, though Ilan *et al.* (1998), using an immortal ovine MEC line, also noted that morphological and functional differentiation with Matrigel was greater than that when floating collagen gels were used.

Formation of the lobule structures was not dependent on the presence of the lactogenic hormones in the DM. Indeed, the lobule structures appeared to be more prevalent with GM than with DM. This is possibly because of the higher concentration of FCS in the GM than in the DM; when FCS was omitted from the GM very limited lobule development was apparent. Morphological reorganization and functionality of the ovine MEC line used by Ilan *et al.* (1998) was also serum-dependent, though others have not found this to be the case (Li *et al.* 1987). Casein secretion by the lobules was dependent on the presence of lactogenic hormones. In particular, omission of prolactin reduced casein levels to below the level of detection. This has generally been the case for other workers using primary cultures (Talhouk *et al.* 1993), though for reports using immortalized ruminant MEC, often, either no milk proteins have been secreted at all, or it has been independent of prolactin (Zavizion *et al.* 1996; Düchler *et al.* 1998; Ilan *et al.* 1998).

Regulation of bovine lactoferrin secretion is completely different from that of casein, in terms of both the secretory vesicle packaging and intracellular routing (Schanbacher & Smith, 1975; Talhouk *et al.* 1993). Furthermore, while *in vivo*, the secretion of casein, and most other milk proteins, is clearly greatest during lactation, that for lactoferrin is greatest prior to lactation (during mammary gland development) and during the involution process (reviewed by Schanbacher *et al.* 1993). Additionally Barcellos-Hoff (1989) noted that for murine MEC in culture, lactoferrin was secreted in both the apical (lumen) and basal directions. In the present experiment, media and lumina concentrations of lactoferrin were greatest when the cells were treated with GM. As stated previously, the greatest lobule development was apparent for cells treated with GM, and accordingly this may be why lactoferrin was higher than that for the DM. Schanbacher *et al.* (1993) using a primary bovine MEC culture also demonstrated that lactoferrin secretion was not dependent on prolactin. This group have demonstrated that *in vitro*, lactoferrin synthesis is dependent upon the nature of the substratum, the presence of serum and the cell plating density. The much greater lactoferrin and α-casein concentrations in the lumen fluid relative to the supernatant concentrations are possibly due to tight junctions between the cells partly preventing leakage from the lumen structures, though it is unlikely that MEC in the present experiment achieved sufficient stability to completely prevent escape of the lumen contents into the surrounding media (Nguyen & Neville, 1998).

In common with our own experience, it has been noted in a number of studies that it is more difficult to establish primary cultures *in vitro* from tissue taken from a lactating mammary gland than it is for tissue taken from a developing mammary gland (MacKenzie *et al.* 1985; McGrath, 1987). That the cells used in the present experiment were from a pregnant animal that was not lactating did not affect the ability of the cells to differentiate; the cells were grown on plastic for several passages, and thus completely de-differentiated, reducing the importance of the differential stage of the initial tissue.

Interpretation of the present results is complicated by the necessity for serum for casein expression to occur, and as such is a drawback to the use of this model for the

study of factors that control mammary differentiation; this is clearly an area that requires further investigation. Additional experiments indicated that the greatest α-casein response occurred at 5% FCS in the DM, leading us to include it at this rate in the DM, though lower levels of α-casein were still found when the DM contained 17% FCS (results not shown). FCS contains a number of growth factors that are thought to inhibit the complete differentiation of MEC (for example, EGF is thought to inhibit the differentiation promoting effects of mammary derived growth inhibitor in mice, Kurtz *et al.* 1998).

In conclusion, this study demonstrates the establishment of a functional bovine MEC clone, which is responsive to mitogenic and lactogenic hormones and an extracellular matrix. Notwithstanding the limitation of the dependency of the clone on FCS, this clone has application to the investigation of bovine lactation *in vitro*.

REFERENCES

Barcellos-Hoff, M. H., Aggeler, J., Ram, T. G. & Bissel, M. J. 1989 Functional differentiation and alveolar morphogenesis of primary mammary cultures on reconstituted basement membrane. *Development* **105** 223–235

Baumrucker, C. R., Deemer, K. P., Walsh, R., Riss, T. L. & Akers, R. M. 1988 Primary culture of bovine mammary acini on a collagen matrix. *Tissue and Cell* **20** 541–554

Baumrucker, C. R. & Stemberger, B. H. 1989 Insulin and insulin-like growth factor-I stimulate DNA synthesis in bovine mammary tissue *in vitro*. *Journal of Animal Science* **67** 3503–3514

Delabarre, S., Claudon, C. & Laurent, F. 1997 Influx of several extracellular matrix components in primary cultures of bovine mammary epithelial cells. *Tissue and Cell* **29** 99–106

Düchler, M., Schmoll, F., Pfneisl, F., Brem, G. & Schellander, K. 1998 OMEC II: a new ovine mammary epithelial cell line. *Biology of the Cell* **90** 199–205

Emerman, J. T. & Pitelka, D. R. 1977 Maintenance and induction of morphological differentiation in dissociated mammary epithelium on floating collagen membranes. *In Vitro* **13** 316–328

Gibson, C. A., Vega, J. R., Baumrucker, C. R., Oakley, C. S. & Welsch, C. W. 1991 Establishment and characterisation of bovine mammary epithelial cell lines. *In Vitro Cell Development Biology* **27A** 585–594

Huynh, H. & Pollak, M. 1995 HH2A, an immortalised bovine mammary epithelial cell line, expresses the gene encoding mammary derived growth inhibitor (MDGI). *In Vitro Cell Development Biology – Animal* **31** 25–29

Huynh, H. T., Robitaille, G. & Turner, J. D. 1991 Establishment of bovine mammary epithelial cells (MAC-T): an *in vitro* model for bovine lactation. *Experimental Cell Research* **197** 191–199

Ilan, N., Barash, I., Gootwine, E. & Shani, M. 1998 Establishment and initial characterisation of the ovine mammary epithelial cell line NISH. *In Vitro Cell Development Biology – Animal* **34** 326–332

Keys, J. E., Fekry, A. E., Wood, D. L. & Capuco, A. V. 1992 The ability of bovine mammary tissue to synthesise lipids for 96-H when co-cultured with liver and adipose tissue. *Biochemistry and Cell Biology* **70** 343–346

Kurtz, A., Spitzer, E., Zschiesche, W., Wellstein, A. & Grosse, R. 1998 Local control of mammary gland differentiation: mammary-derived growth inhibitor and pleiotrophin. *Biochemical Society Symposium* **63** 51–69

Larson, B. L. 1976 Comparative production of beta lactoglobulin and orotic acid with lactose in bovine mammary cell cultures: effects of cell density and constituent inhibition. *Journal of Dairy Science* **59** 1881–1889

Li, M. L., Aggeler, M. D., Farson, D. A., Hatier, C., Hassell, J. & Bissell, M. J. 1987 Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells. *Proceedings of the National Academy of Sciences, USA* **84** 136–140

Li, P., Wilde, C. J., Finch, L. M., Fernig, D. G., Rudland, P. S. 1999 Identification of cell types in the developing goat mammary gland. *Histochemical Journal* **31** 379–383

MacKenzie, D. D. S., Brooker, B. E. & Forsyth, I. 1985 Ultra-structural features of bovine mammary epithelial cells grown on collagen gels. *Tissue and Cell* **17** 39–51

McGrath, M. F. 1987 A novel system for mammary epithelial cell culture. *Journal of Dairy Science* **70** 1967–1980

McGuire, P. G. & Seeds, N. W. 1989 The interaction of plasminogen activator with a reconstituted basement membrane matrix and extracellular macromolecules produced by cultured epithelial cells. *Journal of Cell Biochemistry* **40** 215–227

Nguyen, D. D. & Neville, M. C. 1998 Tight junction regulation in the mammary gland. *Journal of Mammary Gland Biology and Neoplasia* **3** 233–246

Nomura, M., Nakajima, I., Matsuzaki, M., Kimoto, H., Suzuki, I. & Aso, H. 1997 The N-terminal sequence of *Lactococcus lactis* phosphoglucose isomerase purified by affinity chromatography differ from other species. *Archives of Biochemistry and Biophysics* **341** 315–320

Schanbacher, F. L., Goodman, R. E. & Talhouk, R. S. 1993 Bovine mammary lactoferrin: implications from messenger ribonucleic acid (mRNA) sequence and regulation contrary to other milk proteins. *Journal of Dairy Science* **76** 3812–3831

Schanbacher, F. L. & Smith, K. L. 1975 Formation and role of unusual whey proteins and enzymes: relation to mammary cell function. *Journal of Dairy Science* **58** 1048–1062

Schmid, E., Franke, W. W., Grund, C., Schiller, D. L., Kolb, H. & Paweletz, N. 1983 An epithelial cell line with elongated myoid morphology derived from bovine mammary gland. Expression of cytokeratins and desmosomal plaque proteins in unusual arrays. *Experimental Cell Research* **146** 309–328

Sun, N. C., Chu, E. H. Y. & Chang, C. C. 1973 Staining method for the banding patterns of human mitotic chromosomes. *Mammalian Chromosomes Newsletter* **14** 26

Talhouk, R. S., Neiswander, R. L. & Schanbacher, F. L. 1990 *In vitro* culture of cryopreserved bovine mammary cells on collagen gels: synthesis and secretion of casein and lactoferrin. *Tissue and Cell* **22** 583–599

Talhouk, R. S., Neiswander, R. L. & Schanbacher, F. L. 1993 Morphological and functional differentiation of cryopreserved lactating bovine mammary cells cultured on floating collagen gels. *Tissue and Cell* **25** 799–816

Zavizion, B., van Duffelen, M., Schaeffer, W. & Politis, I. 1996 Establishment and characterisation of a bovine mammary epithelial cell line with unique properties. *In Vitro Cell Development Biology – Animal* **32** 138–148