

BOVINE COLOSTRUM SUPPORTS THE SERUM-FREE PROLIFERATION OF EPITHELIAL CELLS BUT NOT OF FIBROBLASTS IN LONG-TERM CULTURE

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ABSTRACT

Medium lacking serum but supplemented with milk will support the growth of sparse cells in culture. Milk obtained within 8 h after the birth of a calf (day 1 colostrum) is the most effective in supporting proliferation. In mixed cultures of early-passage bovine embryonic kidney (BEK) or early-passage calf kidney (CK) cells, both epithelial cells and fibroblasts grow in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with serum. However, only cells that appear to be epithelial-like grow in DMEM supplemented with colostrum. Sparse cultures of early-passage human and rat fibroblasts that grow readily in DMEM supplemented with serum do not grow in DMEM supplemented with colostrum. Canine kidney epithelial cells (MDCK), when plated sparsely, grow exponentially in DMEM supplemented with day 1 bovine colostrum. The generation time is 26 h, the same growth rate as in DMEM supplemented with calf serum. The MDCK cells can be subcultured and regrown to confluence repeatedly in colostrum-supplemented DMEM. Growth in DMEM supplemented with colostrum does not alter the morphological characteristics of the MDCK cells, which are polygonal, contain microvilli at the apical surface, and are connected by tight junctions and desmosomes. MDCK cells do not proliferate in DMEM supplemented with milk obtained 1 wk after the birth of a calf.

KEY WORDS colostrum · milk · serum · epithelial cells · fibroblasts · cellular proliferation

Conventionally, cells in culture are grown in nutrient medium supplemented with serum, a fraction derived from the clotting of blood. The importance of serum in cell culture can be ascribed partially to the presence of growth-promoting factors. The mitogens in serum are polypeptides that in whole blood are associated with plasma and platelets and are released into the serum fraction during clotting (10, 16, 23). In a previous report from this laboratory, it was demonstrated that human milk also contains growth-promoting mitogens (9). The growth-promoting factors in milk differ biochemically from those that have been isolated from platelets (9, 10, 16, 23).

Milk is a medium that can maintain viable cells. Both human and bovine milk and, in particular, colostrum, contain up to 4×10^6 cells/ml, mostly macrophages, T lymphocytes, and B lymphocytes that synthesize secretory IgA (7, 15). The presence of mitogens and viable cells in milk raises the possibility that milk can replace serum for long-term growth of cells in culture. This report demonstrates that bovine milk can indeed be used as a medium supplement in the absence of serum. However, the growth of cells in milk is most successful when colostrum obtained within the first 8 h after birth of a calf is used. Bovine milk obtained from the same cow 1 wk after birth and later is inactive as a medium supplement. In addition, colostrum is selective in supporting proliferation and appears to be particularly well suited for the growth of epithelial cells. Both established

epithelial cell lines and early-passage epithelial-like cells proliferate in colostrum-supplemented medium while early-passage fibroblasts do not.

MATERIALS AND METHODS

Source of Milk

Bovine milk was obtained at various times after birth of a calf and provided by Dr. Edward Kingsbury (Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, Mass.). The milk was obtained from Holstein and Jersey cows and frozen immediately after milking. In this report, day 1 colostrum is obtained 8 h after the birth of a calf and day 8 milk is obtained 176 h after birth of the same calf.

Preparation of Milk for Cell Culture

Frozen samples of milk were thawed and then centrifuged in a RC-5 superspeed Sorvall centrifuge (DuPont Instrument Co., Sorvall Biomedical Div., Wilmington, Del.) at 12,000 *g* for 30 min. The fat floating at the top of the centrifuge tube was removed and discarded. Cellular debris and other sediment at the bottom of the centrifuge tube were also discarded. The milk was sterilized by filtration through Nalgene filter units (Nalge Co., Nalgene Labware Div., Rochester, N.Y.). The presence in milk of casein micelles and other particles makes it difficult to filter milk at a concentration >10% (vol/vol). Therefore the milk was diluted into Dulbecco's Modified Eagle's Medium (DMEM, Grand Island Biological Co., Grand Island, N.Y.) at concentrations of $\leq 10\%$ (vol/vol), prefiltered with an 0.80-micron filter, and subsequently filtered with an 0.45- μm filter. The sterile milk samples were kept frozen at -20°C , with no apparent loss in activity up to at least 3 mo.

Cell Culture

Madin-Darby canine kidney epithelial cells (MDCK), rabbit kidney epithelial cells (RK13), primary calf kidney cells (CK), and primary bovine embryonic kidney cells (BEK) were purchased from Flow Laboratories (Rockville, Md.). Human skin fibroblasts were prepared by explant culture of human foreskin. Human skin fibroblasts between passages 10 and 18 were used. Rat embryo fibroblasts of passage 4 were obtained from Dr. K. Steimer (Harvard Medical School, Boston, Mass.) and prepared as described by Steimer and Boettinger (20). All cells were grown at 37°C in DMEM containing glucose (4.5 g/liter), penicillin (50 U/ml), and streptomycin (50 $\mu\text{g}/\text{ml}$), and supplemented with either calf serum (Colorado Serum Co., Denver, Colo.) or bovine milk prepared as described above. Both the cells grown in DMEM supplemented with serum and those grown in DMEM supplemented with milk were subcultured with 0.1% (vol/vol) trypsin (Grand Island) and 0.02% (wt/vol) EDTA in phosphate-buffered saline lacking calcium and magnesium.

Cell Proliferation

The following protocol was used to measure cell proliferation. Cells were detached by incubation with 0.1% (vol/vol) trypsin and 0.02% (wt/vol) EDTA made up in phosphate-buffered saline lacking calcium and magnesium. The cells were resuspended in unsupplemented DMEM at a concentration of $\sim 10^4$ cells/ml, and 1 ml of cells was plated into each well of a 24-well microtiter plate (16-mm diameter, Costar, Data Packaging, Cambridge,

Mass.). Between 2 and 6 h after plating, the DMEM containing unattached cells was removed and the attached cells were detached with trypsin and counted in a Coulter model ZF electronic particle counter (Coulter Electronics Inc., Hialeah, Fla.). The plating efficiency under these conditions was $\sim 25\text{--}50\%$. The attached cells in replicate wells were then fed either with unsupplemented DMEM or DMEM supplemented with milk or serum. On every 3rd or 4th d, duplicate wells were counted in the Coulter counter and the rest of the cells were refed with the appropriate fresh medium.

Electron Microscopy

Cells were plated onto sterile Millipore filters (25 mm, type HA, 0.45 micron, Millipore Corp., Bedford, Mass.), as described by Misfeldt et al. (13). The cells were grown either in DMEM supplemented with milk or in DMEM supplemented with serum. After reaching confluence, the cells on the filter were fixed with 2.5% glutaraldehyde. The fixed cells were cut into thin sections and analyzed by electron microscopy, as described by Ausprunk et al. (1).

Photography

Cells were photographed under phase using a Nikon model MS inverted-phase microscope with a Wild Heerbrugg MK4 camera attachment (Wild Heerbrugg Instruments Inc., Farmingdale, N.Y.).

RESULTS

Cultures of primary bovine embryonic kidney cells (BEK) and primary calf kidney cells (CK) contain both epithelial cells and fibroblasts when grown in DMEM supplemented with serum. The epithelial cells are eventually overgrown by the more rapidly dividing fibroblasts (Fig. 1a and 1b). A colony of CK epithelial cells surrounded by CK fibroblasts is clearly seen in Fig. 1b. The pattern of growth of mixed kidney cell cultures is different when the growth medium is DMEM supplemented with day 1 colostrum. First, the cell density of BEK and CK cells grown in DMEM supplemented with day 1 colostrum is $\sim 10\text{--}15\%$ of those grown in DMEM supplemented with serum. Second, the cells that grow in colostrum are cuboidal and resemble epithelial cells; there is apparently no growth of fibroblasts (Fig. 1c and 1d). The shape of the epithelial-like cells changes little when colostrum is replaced by serum. The apparent lack of fibroblast growth in DMEM supplemented with colostrum was verified in experiments with early-passage human and rat fibroblasts. Sparse cultures of human fibroblasts (Fig. 2) and rat fibroblasts (Table I) were unable to grow in DMEM supplemented with colostrum. However, the sparse cultures did grow readily in DMEM supplemented with serum. The colostrum is not toxic to the fibroblasts; the growth of these cells resumes when

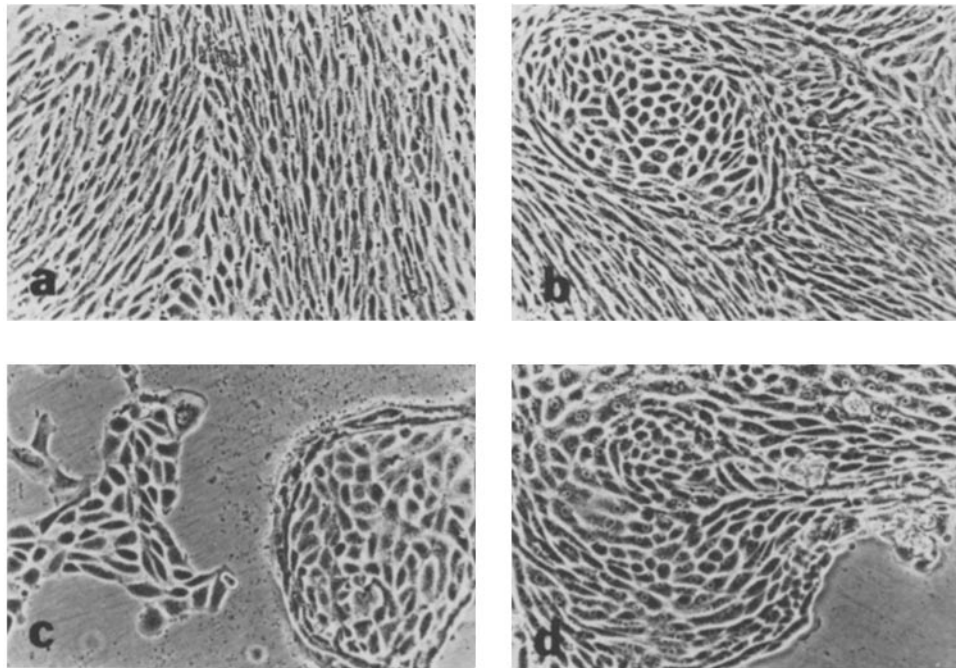


FIGURE 1 Photomicrographs of BEK and CK cells grown in colostrum and in serum. Primary cultures of BEK and CK cells were trypsinized, resuspended in DMEM, and plated sparsely (10^4 cells/well, 5×10^3 cells/cm²). The DMEM containing unattached cells was removed 6 h after plating, and the cultures were fed either with DMEM supplemented with 2.5% (vol/vol) serum or DMEM supplemented with 2.5% (vol/vol) day 1 colostrum. The cultures were refed every 3rd d. After 2 wk of growth, the cultures were photographed under phase: (a) BEK in serum; (b) CK in serum; (c) BEK in day 1 colostrum; (d) CK in day 1 colostrum. $\times 100$.

day 1 colostrum is replaced with serum (Fig. 3).

The ability of epithelial cells to grow in medium supplemented with colostrum was investigated using MDCK epithelial cells. MDCK is an established canine kidney epithelial cell line that preserves the structure and function of kidney epithelium (13, 18). The growth of MDCK cells in DMEM supplemented with various concentrations of milk and serum is shown in Fig. 4. The milk used was either day 1 colostrum or milk obtained 1 wk later (day 8 milk). The optimal concentration of day 1 colostrum for the growth of MDCK is 2.5% (vol/vol), whereas the optimal concentration of calf serum is 5% (vol/vol). At these concentrations, there are similar 90- to 100-fold increases in the number of cells in a 10-d period. At higher concentrations of colostrum, adhesion of MDCK cells to the culture flask is poor and results in a lowered final saturation density. DMEM supplemented with day 8 milk is completely inactive in supporting MDCK proliferation at any concentration tested.

Growth curves for the proliferation of MDCK cells in either unsupplemented DMEM or DMEM supplemented with day 1 colostrum or day 8 milk are shown in Fig. 5. In 2.5% (vol/vol) day 1 colostrum, sparse MDCK cells grow exponentially over a 10-d period with a generation time of ~ 26 h. A similar growth rate is observed with 5% (vol/vol) serum (not shown). There is no proliferation of MDCK cells at any time in unsupplemented DMEM or in DMEM supplemented with day 8 milk. Similar results are found when the established rabbit kidney epithelial cell line, RK13, is used.

The failure of MDCK cells to proliferate in day 8 milk or in milk obtained at any subsequent date in the lactation period is probably not due to the presence of inhibitors. After 9 d of growth in a mixture of day 1 colostrum and day 8 milk, MDCK cells attain approximately the same saturation density as when grown in day 1 colostrum alone (Table II).

Because MDCK cells grown to confluence in

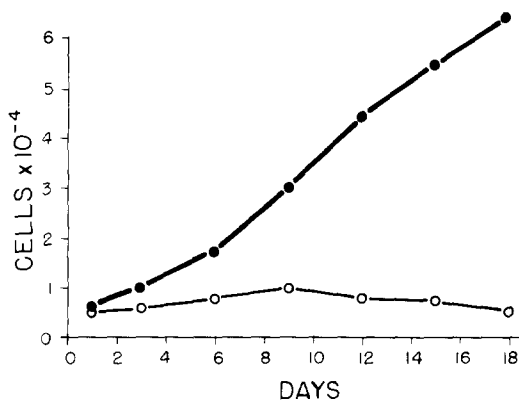


FIGURE 2 Time-course of fibroblast growth in colostrum and in serum. Human foreskin fibroblasts (passage 12) were trypsinized, resuspended in DMEM, and plated sparsely (10^4 cells/well, 5×10^3 cells/cm²). At 6 h after plating, the cultures were fed with either DMEM supplemented with 2.5% (vol/vol) serum (●) or DMEM supplemented with 2.5% (vol/vol) day 1 colostrum (○). Every 3rd d, cells in duplicate were trypsinized and counted, and the rest of the cells were refed with fresh medium.

TABLE I
Growth of Rat Fibroblasts in Day 1 Colostrum
and in Serum

Medium	Final number of cells ($\times 10^{-3}$)
DMEM	1.5
DMEM + 1% colostrum	2.7
DMEM + 5% colostrum	4.0
DMEM + 10% colostrum	4.2
DMEM + 15% colostrum	4.0
DMEM + 20% colostrum	5.9
DMEM + 5% serum	81.0

LR1 cells were plated sparsely (10^4 cells in DMEM/well) into 24-well microtiter plates. The DMEM was removed 2 h after plating, and attached cells were fed with either DMEM supplemented with colostrum or DMEM supplemented with serum. The cells were refed 4 and 8 d after plating and were counted in a Coulter counter 11 d after plating.

DMEM supplemented with day 1 colostrum can be subcultured repeatedly, bovine colostrum can be used to supplement medium for long-term cell culture. In one series of experiments, MDCK cells were repeatedly grown to confluence (4×10^5 cells/cm²) in DMEM supplemented with 2.5% (vol/vol) day 1 colostrum, subcultured by trypsin-

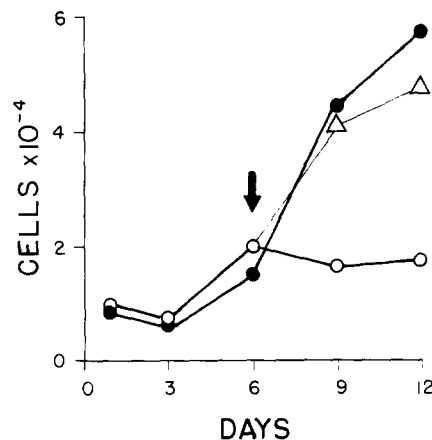


FIGURE 3 The growth of fibroblasts after replacement of colostrum with serum. Human foreskin fibroblasts were plated and grown in DMEM supplemented with either serum or day 1 colostrum, as described in Fig. 2. At 6 d after plating (arrow), some of the replicate cell cultures maintained up to that point in DMEM supplemented with day 1 colostrum were refed with DMEM supplemented with serum. Fibroblasts maintained for 12 d in DMEM supplemented with 2.5% (vol/vol) day 1 colostrum (○); fibroblasts maintained for 12 d in DMEM supplemented with 2.5% (vol/vol) serum (●); fibroblasts maintained first for 6 d in DMEM supplemented with 2.5% (vol/vol) day 1 colostrum and then for 6 d more in DMEM supplemented with 2.5% (vol/vol) serum (△).

ization with 0.1% (wt/vol) trypsin and 0.02% (wt/vol) EDTA, plated at a density of 1×10^4 cells/cm², and grown to confluence again. Over a period of 5 mo, these MDCK cells were subcultured 12× and underwent about 80 doublings before the termination of the experiment. The generation time in the subsequent passages was about 26 h, the same as in the original passage.

Observations with both light and electron microscopes indicate that there are no morphological differences between MDCK cells grown in serum and MDCK cells grown in colostrum. In either medium, MDCK cells grow and form a confluent monolayer of polygonal cells. The morphology is characteristic of epithelial cells. Fig. 6a shows an electron micrograph of a thin section cut perpendicular to the plane of MDCK cells grown in DMEM supplemented with 2.5% (vol/vol) day 1 colostrum. The apical surfaces of the MDCK cells contain microvilli projecting into the medium above. The cells are connected to each other on the lateral side by an abundance of interdigitating processes. At a higher magnification, tight junctions are seen at the apical surface and several

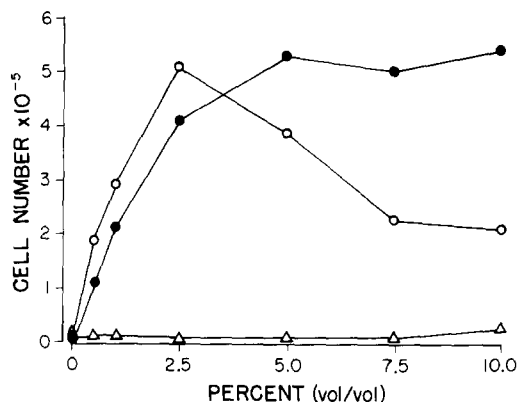


FIGURE 4 Dose response of MDCK cell growth in colostrum and in serum. MDCK cells were seeded sparsely (10^4 cells (in DMEM)/well, 5×10^3 cells/cm²) into 24-well microtiter plates. The DMEM was removed 6 h after plating, and the attached cells were fed with DMEM supplemented with various concentrations of day 1 colostrum (○), day 8 milk (△), and calf serum (●). The medium was changed every 3rd d, and after 12 d the cells were trypsinized and counted in a Coulter counter.

well-developed desmosomes are seen connecting the two MDCK cells (Fig. 6b). There is no evidence that growth of MDCK cells in colostrum leads to any adverse effects on epithelial morphology.

DISCUSSION

Bovine milk obtained within 8 h after birth of a calf, i.e., colostrum, can replace serum for the growth of sparse cells in culture. However, colostrum acts selectively in supporting the growth of cells in culture. For example, although epithelial cells proliferate, human, rat, and bovine early-passage fibroblasts do not. The lack of fibroblast growth is apparently not due to the presence in colostrum of toxic or inhibitory factors. Possibly colostrum contains specific factors necessary for epithelial growth but lacks those needed for fibroblast growth.

Fibroblast overgrowth is still a major problem in the culture of uncloned epithelial cells. There have been numerous but inconclusive attempts to solve this problem by the use of selective media that inhibit fibroblast growth. These include media in which L-valine is replaced by D-valine (4), media in which arginine is replaced by citrulline (21), and media lacking tyrosine (2) and glutamine (11). Because preliminary experiments indicate

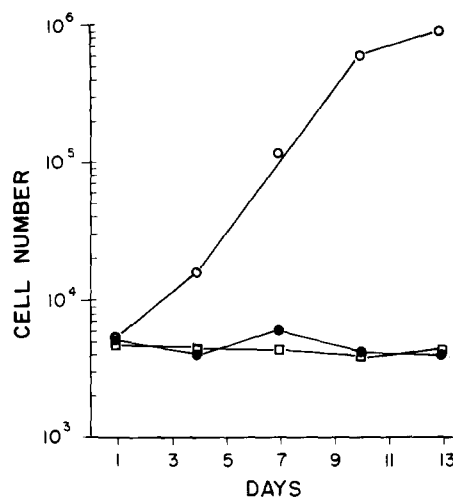


FIGURE 5 Time-course of MDCK cell growth in colostrum. MDCK cells were plated sparsely (10^4 cells (in DMEM)/well, 5×10^3 cells/cm²) into 24-well microtiter plates. The DMEM was removed 6 h after plating, and the cells were fed with unsupplemented DMEM (□), DMEM supplemented with 2.5% (vol/vol) day 1 colostrum (○), or DMEM supplemented with 2.5% (vol/vol) day 8 milk (●). Every 3rd d, cells in duplicate were counted and the rest of the cells were refed with fresh medium.

TABLE II
Growth of MDCK Cells in a Mixture of Day 1
Colostrum and Day 8 Milk

Milk sample	Milk vol/vol in DMEM	Final number of cells ($\times 10^{-3}$)
	%	
Day 1 colostrum	1.25	360.0
Day 8 milk	1.25	3.8
Day 1 colostrum + day 8 milk	1.25*	297.0
Day 1 colostrum	2.5	440.0
Day 8 milk	2.5	4.2
Day 1 colostrum + day 8 milk	2.5*	507.0

MDCK cells were plated sparsely (10^4 cells in DMEM/well, 5×10^3 cells/cm²) into 24-well microtiter plates. The DMEM was removed 3 h after plating, and attached cells were fed with either day 1 colostrum, day 8 milk, or a mixture of both. MDCK cells were fed every 3rd d with the appropriate fresh medium and counted 9 d after plating.

* Final percentage after mixing colostrum and milk.

that colostrum supports the selective growth of kidney and gastrointestinal epithelial cells in mixed primary cultures, the use of colostrum in culture medium may be a promising general ap-

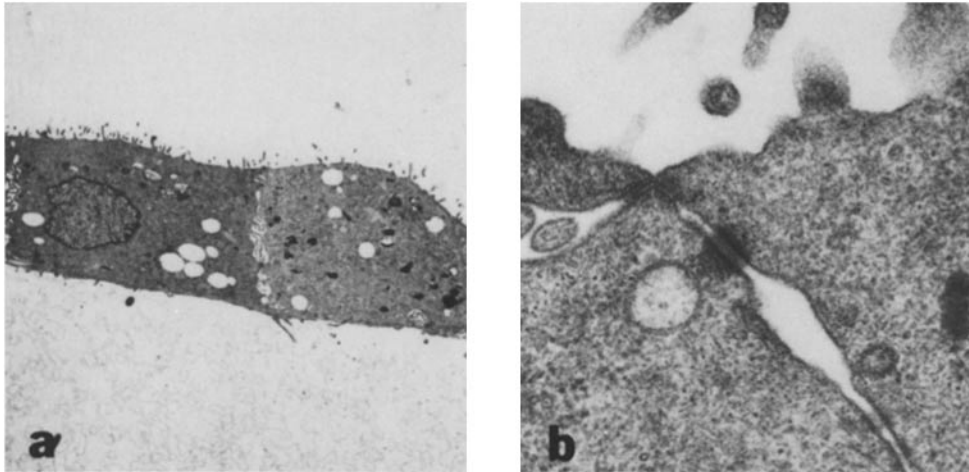


FIGURE 6 Electron micrographs of MDCK cells grown in colostrum; MDCK cells were plated sparsely, as in Fig. 5, onto sterile Millipore filters as described in Materials and Methods. The cells were grown over a 10-d period to confluence in DMEM supplemented with 2.5% (vol/vol) day 1 colostrum, thin sectioned, and analyzed by electron microscopy. (a) $\times 300$. (b) $\times 36,000$.

proach to overcoming the problem of fibroblast overgrowth.

Although colostrum, in the absence of serum, supports the growth of such epithelial cells as the MDCK cell line, milk obtained as early as a week after the birth of a calf is completely inactive. The failure of MDCK cells to proliferate in milk obtained a week after birth or later does not appear to be the result of inhibitors in milk. Recently, we found that MDCK will grow in milk obtained 1 wk after birth, if the milk is supplemented with human transferrin.¹ In fact, milk supplemented with transferrin is as active as colostrum in stimulating MDCK growth. Sato and his colleagues (6, 8, 12, 19, 22) have demonstrated that the serum requirement for the growth of GH₃ cloned rat-pituitary cells, HeLa cells, melanoma cells, MDCK cells, and embryonic carcinoma cells can be replaced by mixtures of hormones, mitogens, and other factors. Transferrin is the only component whose presence is required in each mixture and therefore may be essential to the growth of cells in culture. Transferrin is also found in bovine milk (5); perhaps the levels of transferrin decrease as the lactation period proceeds. It is known that the number of cells (15) and the levels of immunoglobulins (14) and lactoferrin (3, 17) decline rapidly in the postpartum period. Colostrum may be

¹ M. Klagsbrun and R. Packard. Manuscript in preparation.

an exceptionally rich nutrient medium that is fortified with growth factors, hormones, and immunoglobulins. These factors may be highly important for the growth and development of cells and tissue in the newborn during the first few days of life. As the newborn matures, the need for such factors declines. The decline in these factors may be the reason that effective use of milk in cell culture is limited to milk obtained immediately after birth, the colostrum.

I would like to thank Dr. E. Kingsbury, University of Massachusetts, Amherst, Mass., for kindly providing samples of milk; Dr. D. Ausprunk, Harvard Medical School, Boston, Mass., for kindly providing the electron micrographs; Dr. B. Zetter and Dr. K. Steimer, Harvard Medical School, Boston, Mass., for their insightful comments; and Robert Packard for assistance.

This work was supported by grant R01-CA21763-02 from the National Cancer Institute.

Received for publication 26 November 1979.

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