Journal of Supramolecular Structure 11:349–359 (1979)
Tumor Cell Surfaces and Malignancy 229–239

The Serum-Free Growth of Balb/c 3T3 Cells in Medium Supplemented With Bovine Colostrum

M. Klagsbrun and J. Neumann

Departments of Surgery and Biological Chemistry, Children's Hospital Medical Center and Harvard Medical School, Boston, Massachusetts 02115

Bovine milk contains growth promoting factors that stimulate DNA synthesis and cell division in confluent monolayers of quiescent Balb/c 3T3 cells. The growth factor activity was highest in colostrum obtained within 24 hours after birth of a calf. Samples of milk obtained 32 hours and 60 hours after birth were 20% and 1% as active respectively as was a sample obtained 8 hours after birth in stimulating DNA synthesis. No activity was detectable 3 days after birth or thereafter. A similar temporal dependence was found in sheep's milk. Bovine colostrum obtained on the day of a calf's birth can be substituted for serum and will support the growth of sparse Balb/c 3T3 cells to confluence. In Dulbecco's modified Eagle's medium (DMEM) supplemented with 2.5% (vol/vol) bovine colostrum, the number of Balb/c 3T3 cells in a dish increased 35-fold, from 2.0×10^4 cells to 7×10^5 cells. The generation time was approximately 38 hours. Proliferation of cells was characterized by formation of clusters of confluent Balb/c 3T3 cells which were smaller in size and more tightly packed than were Balb/c 3T3 cells grown to confluence in serum. No proliferation was detected in DMEM supplemented with milk obtained 10 days after birth of a calf or in DMEM supplemented with bovine serum albumen.

Key words: colostrum, milk, serum, growth factors, mitogens, DNA synthesis, proliferation, 3T3 cells, serum-free growth

In a previous report, we have demonstrated that human breast milk stimulates DNA synthesis and cell division in confluent monolayers of quiescent Balb/c 3T3 cells [1]. The mitogenic activity of the human milk is due to the presence of growth promoting factors that are polypeptides with molecular weights between 14,000 and 18,000 and isoelectric points between 4.4 and 4.7.

Received April 27, 1979; accepted July 2, 1979.

0091-7419/79/1103-0349$02.30

Balb/c 3T3 cells were established as a cell culture line by Aaronson and Todaro [2]. Since then, these cells have been used extensively to study growth control in cell culture [3–5]. Balb/c 3T3 cells are routinely grown in medium supplemented with serum. It is believed that serum contains growth promoting factors that are necessary in order for these cells to proliferate. The factors in serum necessary for the growth of 3T3 cells are thought to be potent mitogenic substances that are derived from platelets [6–8]. The presence of growth factors in milk raises the possibility that the serum requirement of Balb/c 3T3 cells may be replaced with milk. Initial attempts in this laboratory to grow these cells in human milk were unsuccessful. However, it was observed that human colostrum was a better source of growth factor activity than was human milk obtained from the same woman later in her lactation period. Since human colostrum was not readily available for large scale cell culture experiments, bovine colostrum was tried instead.

In this report, we demonstrate that bovine colostrum will stimulate DNA synthesis and cell division in confluent Balb/c 3T3 cells. In addition, these cells, when plated sparsely, will grow to confluence in a medium in which serum has been replaced by colostrum. Successful growth in the absence of serum is only possible using colostrum obtained within 24 hours after birth of a calf. Milk obtained later in the lactation period is inactive.

MATERIALS AND METHODS

Source of Milk

Bovine milk was kindly provided by Dr. Edward Kingsbury of the Department of Veterinary and Animal Sciences at the University of Massachusetts (Amherst, Massachusetts). The milk was obtained from Holstein and Jersey cows and was frozen immediately after milking. The concentration of protein in milk was determined by the method of Lowry [9].

Preparation of Milk for Cell Culture

Frozen milk samples were thawed and spun in a RC-5 superspeed Sorvall centrifuge at 12,000 × g for 30 minutes. The fat which floated on top of the spun milk was removed and discarded. Cellular debris and other sediment at the bottom of the centrifuge tube was also discarded. Milk samples were sterilized by filtration through Nalgene filter units. The presence in milk of casein micelles [10] and other particles makes filtration of milk at concentrations of 10% (vol/vol) or greater difficult. Samples of milk at concentrations of 10% (vol/vol) or less were sterilized by diluting milk into medium, prefiltering with 0.80 micron Nalgene filter units and then by filtering with 0.45 micron Nalgene filter units.

Cells and Cell Culture

Mouse Balb/c 3T3 embryo cells (clone A31) were obtained from Dr. C. D. Scher (Sidney Farber Cancer Center, Boston, Massachusetts). The cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, New York) containing 4.5 gm of glucose per liter, penicillin (50 U/ml) and streptomycin (50 μg/ml), and supplemented with either calf serum (Colorado Serum Co., Denver, Colorado) or bovine milk prepared as described above.

Proliferation of Sparse Cells

The kinetics of Balb/c 3T3 cell proliferation were measured using the following protocol; Balb/c 3T3 cells were detached by incubation with 0.1% (vol/vol) trypsin and 0.02% EDTA (Gibco) in phosphate buffered saline lacking calcium and magnesium. The cells were resuspended in DMEM at a concentration of 10^4 cells/ml and 1 ml of cells was plated into each well of a 24-well microtiter plate (16 mm diameter, Costar, Cambridge, Massachusetts). Approximately 4 hours after plating, the DMEM containing unattached cells was removed and the number of attached cells was counted. The plating efficiency under these conditions was about 20 to 40%. The attached cells were then fed with unsupplemented DMEM or with DMEM supplemented with either milk or with serum. On every third or fourth day, duplicate wells were counted and the rest of the cells were refed with fresh medium. The number of cells in a well was measured by detachment of cells with trypsin and counting in a Coulter Model ZF electronic particle counter (Coulter Electronics).

DNA Synthesis and Cell Division in Confluent Cells

The preparation of confluent Balb/c 3T3 cells in a 96-well microtiter plate (Falcon); the measurement of DNA synthesis by scintillation counting and by autoradiography, and the measurement of cell division by detachment with trypsin followed by counting in a Coulter counter, have been described previously [1, 11].

Photography

Balb/c 3T3 cells were photographed under phase and after fixation and staining. Cells were fixed and stained using the following steps; washing with 0.15 M NaCl, addition of cold methanol for 5 minutes, addition of 10% buffered formalin phosphate for 5 minutes, a rinse with cold H_2O and addition of toluidene blue (0.1% in H_2O) for 1 minute. Photographs were taken using a Nikon Model MS inverted phase microscope with a Wild Heerbrugg Model MK4 camera attachment.

RESULTS

Stimulation of DNA Synthesis by Milk

Samples of milk were obtained from cows within 24 hours after birth of a calf (day 1) and at regular intervals thereafter up to a period of 2 weeks. The milk samples were tested for the ability to stimulate DNA synthesis in quiescent confluent Balb/c 3T3 cells. The stimulatory activity in relation to the time elapsed since birth of a calf, of milk samples obtained from 3 cows is shown in Figure 1. In each case, milk obtained on the day of birth, that is the colostrum, was the most active in stimulating DNA synthesis in the Balb/c 3T3 cells. A concentration of 1% (vol/vol) bovine colostrum was sufficient to label every nucleus in the Balb/c 3T3 population. The stimulatory activity of the bovine milk declined very rapidly after the day of birth. A milk sample obtained 32 hours after birth was only 20% as active in stimulating DNA synthesis as was a sample obtained 8 hours after birth when measured at a final concentration of 1% (vol/vol). On the third day postpartum (60 hours) and thereafter no stimulatory activity could be detected. The decline in stimulatory activity was not due solely to the drop in the protein concentration of milk. While the protein concentration of milk obtained 60 hours after

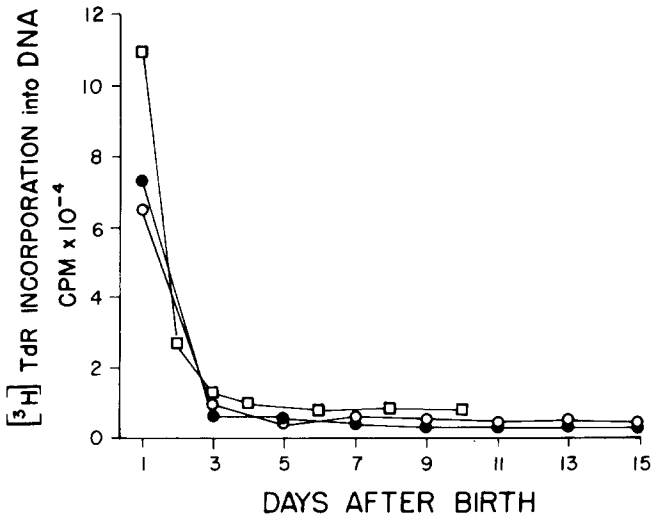

Fig. 1. Stimulation of DNA synthesis in Balb/c 3T3 cells by bovine milk obtained at various times after birth of a calf. Quiescent monolayers of confluent Balb/c 3T3 cells were prepared by plating approximately 10^4 cells into each 0.3 cm^2 well of a 96-place microtiter plate. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum for 7 to 10 days in order to deplete the serum of growth promoting factors. Milk was obtained from 3 different cows on the day a calf was born (day 1) and at regular intervals thereafter. The milk samples were added to the confluent monolayers of quiescent Balb/c 3T3 cells without any medium change along with (^{3}H) TdR (6.7 Ci/mmol, 4 μCi/ml) and incubated for a period of 40–48 hours. The cells were fixed with methanol and TCA and DNA synthesis was measured by scintillation counting. The final concentration of milk in each microtiter well was 1.0% (vol/vol). The background incorporation of the quiescent cells was 3,000 cpm while 20% (vol/vol) calf serum stimulated the incorporation of approximately 80,000 cpm.

birth was about 20% of that obtained 8 hours after birth, the stimulatory activity of the former was less than 1% that of the latter. A similar pattern was observed with sheep's milk. Sheep colostrum obtained on the day of a lamb's birth stimulated DNA synthesis but milk obtained 2 days later did not (Fig. 2).

Confluent monolayers of Balb/c 3T3 cells can be stimulated to synthesize DNA by addition of fresh bovine serum [12]. The stimulatory activity of bovine colostrum and bovine serum were compared (Fig. 3). Bovine colostrum at a concentration of 0.25% (vol/vol) was as active as bovine serum at a concentration of 2.5% (vol/vol). Maximum stimulation of Balb/c 3T3 cells was obtained with 1% (vol/vol) colostrum and 10% (vol/vol) serum. Therefore colostrum was about ten times as active as serum on a per volume basis in stimulating DNA synthesis in confluent Balb/c 3T3 cells. The protein concentration of bovine colostrum is about 200–250 mg/ml and that of bovine serum is about 70–100 mg/ml. Thus the specific activity of colostrum is 3–5 times greater than that of serum.

Stimulation of Cell Division by Milk

Colostrum obtained on the day of birth of a calf induced cell division in confluent quiescent Balb/c 3T3 cells as well as DNA synthesis (Fig. 4). The stimulation of cell division by the colostrum was concentration-dependent. A concentration of 0.8% (vol/

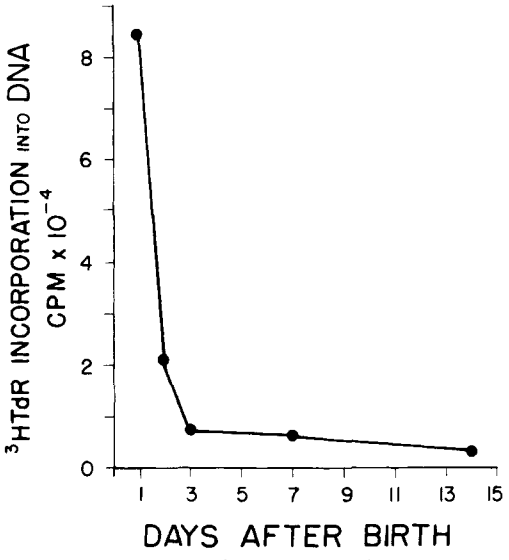

Fig. 2. Stimulation of DNA synthesis by sheep's milk obtained at various times after birth of a lamb. Milk was obtained from a sheep on the day of a lamb's birth and at regular intervals thereafter. Samples of milk at a final concentration of 1.0% (vol/vol) were added along with (^{3}H) TdR to confluent monolayers of quiescent Balb/c 3T3 cells and DNA synthesis was measured as described in Figure 1.

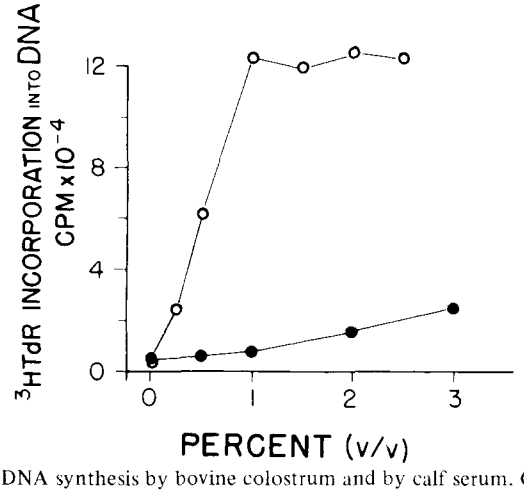

Fig. 3. Stimulation of DNA synthesis by bovine colostrum and by calf serum. Confluent monolayers of Balb/c 3T3 cells were incubated with various concentrations of bovine colostrum obtained on the day of the birth of a calf (○–○) and with various cocentrations of calf serum (●–●) along with (^{3}H) TdR. DNA synthesis was measured as described in Figure 1.

vol) milk induced an approximate 6-fold increase in cell number in a period of 6 days. By comparison, milk obtained from the same cow 10 days after birth of the calf was inactive in stimulating cell division.

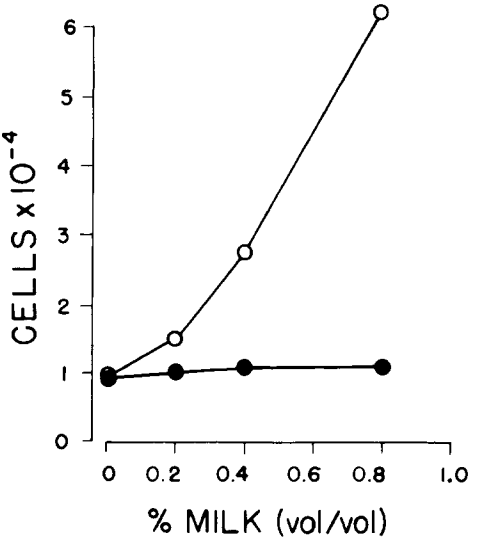

Fig. 4. Stimulation of cell division by bovine milk. Confluent monolayers of quiescent Balb/c 3T3 cells were incubated for 6 days with various concentrations of colostrum obtained on the day of the birth of a calf (○–○) and milk obtained 10 days after birth of a calf (●–●). The final concentration of milk used were 0, 0.2, 0.4, and 0.8% (vol/vol). The cells were refed on day 3 of the experiment. On day 6 of the experiment, the cells were detached from the microtiter wells by incubation with 0.1% (wt/vol) trypsin and counted in a Coulter counter.

The Proliferation of 3T3 Cells in Medium Supplemented With Milk Instead of Serum

Bovine colostrum, like serum, contains factors that stimulate DNA synthesis and cell division in Balb/c 3T3 cells. The presence of these factors suggests that Balb/c 3T3 cells may proliferate in a medium which has been supplemented with bovine colostrum instead of serum. To test this possibility, Balb/c 3T3 cells were trypsinized, plated sparsely in unsupplemented DMEM and, after attachement, were grown in DMEM supplemented with either bovine colostrum obtained on the day of birth of a calf, or in DMEM supplemented with bovine serum. The optimal concentration of bovine colostrum needed for growth of Balb/c 3T3 cells was found to be between 1.0 and 2.5% (vol/vol). At higher concentrations, the cells, while remaining viable, became less adhesive and detached from the dish. The optimal serum concentration was 10% (vol/vol). There was no growth of Balb/c 3T3 cells in DMEM supplemented with milk obtained 10 days after birth, in DMEM supplemented with bovine serum albumen (5 mg/ml), or in unsupplemented DMEM. The growth curves of Balb/c 3T3 cells grown in DMEM supplemented with 2.5% (vol/vol) colostrum, 2.5% (vol/vol) serum and 10% (vol/vol) serum are shown in Figure 5. In 10% (vol/vol) serum, there was immediate exponential growth with a generation time of about 28 hours. The cell number increased 100-fold before a maximum saturation density was obtained 8 days after plating. In 2.5% (vol/vol) serum, after a lag period of about 3 days, the cells grew with a generation time of about 28 hours, increased in number by 50-fold, and reached a maximum saturation density in 10 days which was about one half that of cells grown in 10% (vol/vol) serum. It has been shown previously that the final saturation density of Balb/c 3T3 cells is directly proportional to the amount of serum in the culture medium [13]. In 2.5% (vol/vol) colostrum, after a 3-day lag period,

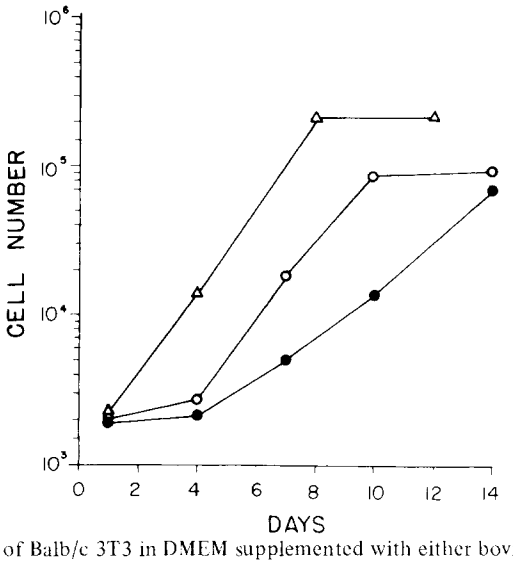

Fig. 5. The growth of Balb/c 3T3 in DMEM supplemented with either bovine colostrum or calf serum. Balb/c 3T3 cells were plated sparsely in DMEM at a density of approximately 5×10^3 cells/cm^2 and those cells that attached were grown in the appropriate medium according to the protocol described in Materials and Methods. Every third or fourth day cells in duplicate wells were trypsinized and counted while all remaining cells were refed with the approproate medium. DMEM + 10% serum (Δ–Δ); DMEM + 2.5% serum (○–○); DMEM + 2.5% bovine colostrum (●–●).

the cells proliferated, but grew more slowly than was the case in serum. The generation time in colostrum was about 38 hours and the increase in cell number was about 35-fold in 14 days. The density of Balb/c 3T3 cells in a well the day after plating is shown in Figure 6A. The density of cells after 12 days' growth in 2.5% (vol/vol) colostrum and in 10% (vol/vol) serum is shown in Figure 6B and Figure 6C, respectively.

Patterns of Cell Growth in Colostrum and in Serum

The pattern of Balb/c 3T3 cell growth in colostrum was different than in serum. Cells in serum grew uniformly throughout the dish, and eventually formed a monolayer that covered the total available surface. On the other hand, the cells in colostrum grew in clusters leaving gaps in the dish. The confluent cells in the clusters were smaller and more densely packed (Fig. 6B) than were the confluent cells grown in 10% (vol/vol) serum (Fig. 6C). After reaching a high cell density, the clusters often lost their ability to adhere to the plastic substrate, and rolled up to form aggregates which detached from the dish. Figure 7 shows a mass of aggregated cells after detachment. The lower saturation density and apparent longer generation time of Balb/c 3T3 cells grown in colostrum was due in part to the loss of cells into the medium during growth. In a few experiments when cell loss due to lack of adhesion was small, Balb/c 3T3 cells grew in colostrum with a generation time equivalent to that found in serum, approximately 28 hours.

Characterization of Bovine Milk

Initial characterization of the factors in bovine milk necessary for the growth of cells in the absence of serum indicated the following: 1) the factors are resistant to the conditions of pasteurization. Milk heated to 61°C for 2 hours and then cooled quickly

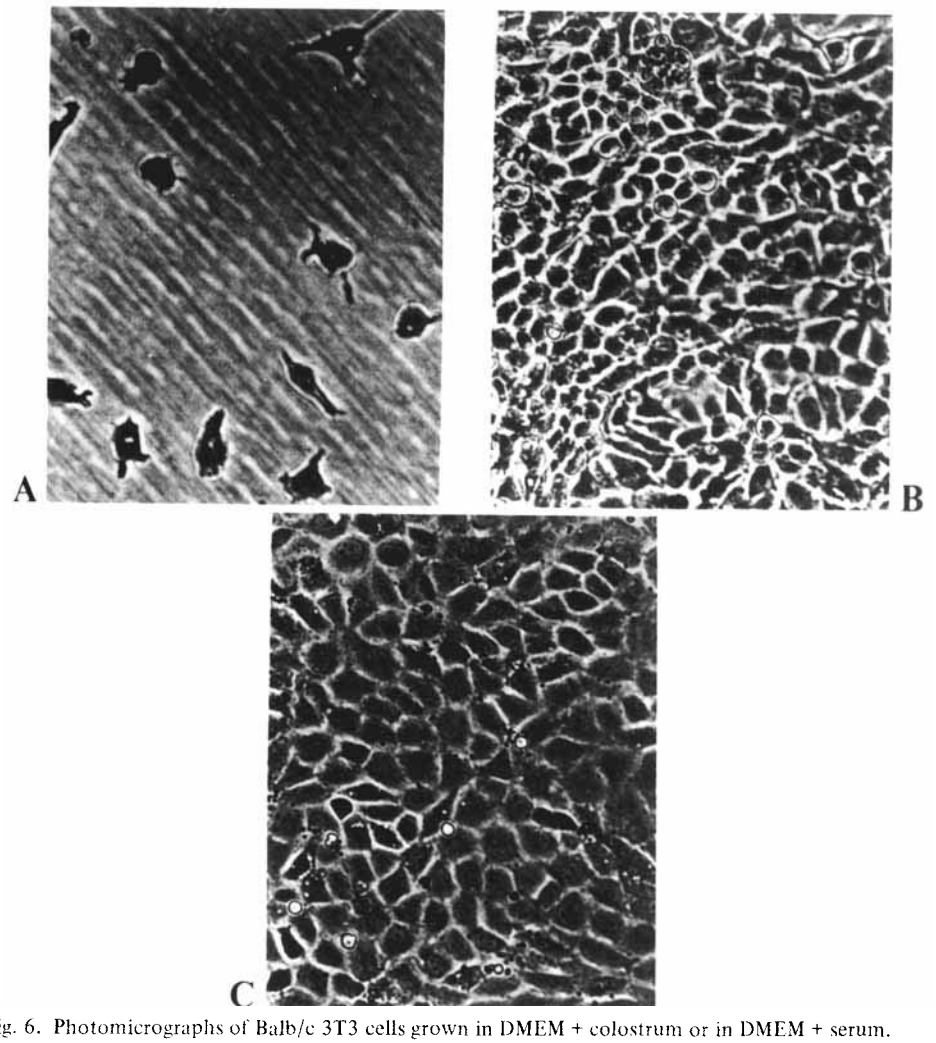

Fig. 6. Photomicrographs of Balb/c 3T3 cells grown in DMEM + colostrum or in DMEM + serum. The Balb/c 3T3 cells grown as described in Figure 5 were photographed at 1 day and 12 days after plating. A, DMEM + 2.5% colostrum 1 day after plating. B, DMEM + 2.5% colostrum 12 days after plating. C, DMEM + 10% serum 12 days after plating. The cells in 6A were photographed after fixation and staining. The cells in 6B and 6C were photographed under phase.

on ice was fully active in supporting cell growth; 2) defatted, skimmed milk is as active as whole milk, suggesting that the growth stimulating activity of bovine colostrum resides in milk protein rather than in milk fat; 3) adjustment of the pH of milk to 4.6 by addition of HCl results in the precipitation of casein. The supernatant fraction remaining after removal of precipitated casein by centrifugation retained the ability to stimulate DNA synthesis in quiescent Balb/c 3T3 cells. This fraction, often referred to as whey, also contained the immunoglobulins and albumens of milk.

DISCUSSION

Bovine milk can be substituted for serum in order to grow sparse Balb/c 3T3 cells to confluence. However, only milk obtained within 24 hours after birth of a calf, that is the colostrum, will support cell growth. Milk obtained later in the lactation period is in-

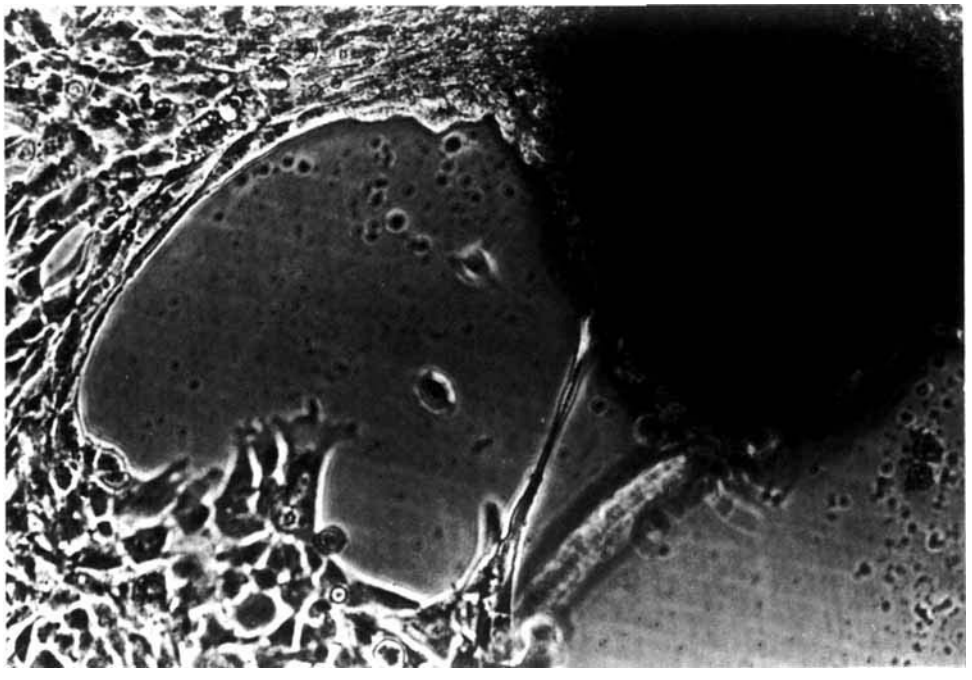

Fig. 7. Photomicrograph of an aggregate of Balb/c 3T3 cells formed during growth in colostrum. Balb/c 3T3 cells were grown in DMEM + 2.5% colostrum. After 15 days in culture, some areas of the dish were found to contain cellular aggregates. An aggregate of cells (top right) is shown coming off the dish leaving a gap in the center. The cells were photographed under phase.

active despite the presence of substantial amounts of protein, lipid, carbohydrate and other nutrients. It is not clear why the milk obtained later is inactive. Perhaps the cow synthesizes mitogenic substances just prior to, or at the time of birth. The mitogens would appear in the first milk but would be diluted out in milk obtained subsequently. Alternatively, the growth promoting factors may be inactivated, or inhibitors of growth factors may appear as the lactation period progresses.

A temporal dependence of growth factor activity is also found in sheep and human milk. The growth factor activity of milk is determined by measuring the stimulation of DNA synthesis in quiescent Balb/c 3T3 cells [1]. In both the cow and the sheep, the level of DNA synthesis that is stimulated by milk obtained 1 day and 2 days after birth is only 20% and 1% respectively of the level stimulated by milk obtained on the day of birth. The decline in growth factor activity with the passage of time is not as dramatic in human milk as it is in animal milk. Preliminary results obtained with the milk of 5 women indicates that a loss of growth factor activity of up to 80% occurs in the first 20 days after birth. However, the growth factor activity of human milk never disappears completely and is detectable even 3 months after birth of the infant.

The growth patterns of Balb/c 3T3 cells in serum and in colostrum are different. Cells at the optimal serum concentration of 10%, grow uniformly throughout the dish, become confluent and adhere very tightly to the plastic substratum. In contrast, at the optimal colostrum concentration of 2.5%, cell growth is characterized by the formation of clusters of cells and of empty gaps on the dish. The cells in the clusters are smaller and packed more densely than are cells grown in 10% serum (Fig. 6). In addition, the cells grown in colostrum are less adhesive than those grown in serum. Eventually clusters of cells

roll up to form large aggregates that detach from the dish (Fig. 7). The lowered adhesion to the plastic substratum of colostrum-grown cells compared to serum-grown cells might be related to different levels of adhesion promoting factors such as fibronectin [14]. The loss of cells from the dish makes it difficult to obtain an accurate generation time for the growth of Balb/c 3T3 cells in colostrum. However, under optimal conditions the generation time of cells grown in colostrum is equivalent to that of cells grown in serum, approximately 28 hours.

Both colostrum and serum will support the growth of Balb/c 3T3 cells. However, the growth promoting factors in colostrum and in serum are probably not the same. The growth factors in serum required to make Balb/c 3T3 competent for growth are derived from platelets [6–8]. The human platelet-derived growth factors are cationic polypeptides with isoelectric points between 9.7 and 10,2 [15, 16]. In contrast, the growth factors in human and bovine milk that stimulate the growth of Balb/c 3T3 cells are anionic polypepties with isolelectric points between 4.4 and 5.0. We have found no evidence to date for the presence of cationic growth factors in milk. In addition, we have shown that the activity of the human milk growth factor is resistant to disulfide bond reduction by sulfhydryl reducing agents such as 2-mercaptoethanol and dithiothreitol [1]. These reagents inactivate the human platelet-derived growth factor irreversibly [15, 16].

The use of colostrum to supplement culture medium provides a new approach to cell culture. Previously, it has been thought that Balb/c 3T3 cells require some blood-derived fraction such as serum or a combination of platelet-poor plasma and platelet-derived growth factor for proliferation. However, it is now apparent that other sources of growth factor activity besides blood can be used for the growth of these cells. We have found that other cell types such as epithelial cells and myoblasts will proliferate in colostrum-supplemented medium. Thus, the growth of cells in colostrum may be a general phenomenon. The use of colostrum in cell culture may provide several benefits. For example, it may be possible to grow cells in colostrum that are difficult to grow in serum. In addition, serum often contains virus, hormones and other molecules which may be undesirable in certain cultures such as those used to prepare vaccines. The use of colostrum would be a feasible alternative that might minimize some of the problems that are associated with growth in serum.

ACKNOWLEDGMENTS

I would like to thank Dr. J. Kadish, Dr. B. Zetter, Dr. D Tapper, S. Smith, G. Emanuel and R. Rybach for their contributions to this study. This work was supported by grant RO1-CA21763 from the National Cancer Institute.

REFERENCES

1. Klagsbrun M: Proc Natl Acad Sci USA 75:5057, 1978.
2. Aaronson SA, Todaro GJ: Science 162:1024, 1968.
3. Holley RW, Kiernan JA: Proc Natl Acad Sci USA 60:300, 1968.
4. Dulbecco R: Nature 227:802, 1970.
5. Holley RW: Nature 258:487, 1975.
6. Kohler N, Lipton A: Exp Cell Res 87:297, 1974.
7. Pledger WJ, Stiles CD, Antoniades HN, Scher CD: Proc Natl Acad Sci USA 74:4481, 1977.
8. Vogel A, Raines E, Kariya B, Rivest J, Ross R: Proc Natl Acad Sci USA 75:2810, 1978.
9. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265, 1951.

10, Jenness R: In Larson B, Smith VR (eds): "Lactation." New York: Academic Press, 1974, Vol III, pp 3–107.
11. Klagsbrun M, Langer R, Levenson R, Smith S, Lillehei C: Exp Cell Res 105:99, 1977.
12. Todaro GJ, Lazar GK, Green H: J Cell Comp Physiol 66:325, 1965.
13. Todaro GJ, Matsuya Y, Bloom S, Robbins A, Green H: In Defendi V, Stoker M (eds): "Growth Regulating Substances for Animal Cells in Culture." Philadelphia: Wistar Institute Press, 1967, pp 87–98.
14. Hynes RO, Ali IU, Destree AT, Mutner V, Perkins ME, Singer DR, Wagner DD, Smith KK: Ann NY Acad Sci 312:317, 1978.
15. Ross R, Vogel A: Cell 14:203, 1978.
16. Antoniades HN, Scher CD, Stiles CD: Proc Natl Acad Sci USA, In press, 1979.