In Vitro Cell. Dev. Biol. 26:791-802, August 1990

0883-8364/90 $01.50+0.00

PRIMARY CULTURE OF NORMAL RAT MAMMARY EPITHELIAL CELLS WITHIN A BASEMENT MEMBRANE MATRIX. I. REGULATION OF PROLIFERATION BY HORMONES AND GROWTH FACTORS

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(Received 19 December 1989; accepted 5 April 1990)

SUMMARY

A serum-free primary culture system has been developed which allows for three-dimensional growth and differentiation of normal rat mammary epithelial cells (RMECs) within an extracellular matrix preparation. RMECs were isolated from mammary glands of immature 50- to 60-d-old rats and the organoids embedded within a reconstituted basement membrane matrix prepared from the Engelbreth-Holm-Swarm sarcoma. Cells grown in a serum-free media consisting of phenol red-free Dulbecco's modified Eagle's medium-F12 culture medium containing 10 μg/ml insulin, 1 μg/ml prolactin, 1 μg/ml progesterone, 1 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor (EGF), 1 mg/ml fatty-acid-free bovine serum albumin (BSA), 5 μg/ml transferrin, and 5 μ*M* ascorbic acid proliferated extensively (15- to 20-fold increase in cell number as quantitated using the MTT dye assay) over a 2- to 3-wk culture period and remained viable for months in culture. Several types of colonies were observed including the alveolarlike budding cluster which predominates at later times in culture, units with no or various degrees of ductal-like projections, stellate colonies, and two-and three-dimensional web units. Optimal proliferation required insulin, prolactin, progesterone, EGF, and bovine serum albumin. Hydrocortisone was not required for proliferation, but the colonies developing in its absence were morphologically altered, with a high frequency of colonies that formed an extensively branched network with many fine projections. Cell proliferation was also dependent on substratum, with significantly less growth and development occurring in RMECs grown within a type I collagen gel matrix compared to RMECs grown within the reconstituted basement membrane. In conjunction with other studies demonstrating extensive differentiation as well as proliferation, it is concluded that this model should prove to be an important tool to study the hormonal regulation of the growth and development of rat mammary cells.

Key words: mammary epithelial cells; model system; extracellular matrix; rat; primary culture.

INTRODUCTION

Breast cancer is the second leading cause of cancer deaths among women. In spite of an increased recognition of some of the differences between normal and breast cancer cells, there is little understanding of the biochemical, cellular, or molecular mechanisms by which normal mammary epithelial cells (MECs) progress to the malignant state and the alterations in the cell that are responsible for or are associated with this progression. This paucity of knowledge derives in part from the lack, until recently, of a suitable model system in which these events could be manipulated and monitored in vitro. An ideal model would be one which mimics the normal functioning of the mammary gland in vivo, i.e. a model capable of both proliferation and differentiation in response to the appropriate hormonal stimuli. Additionally, the model system must permit growth of isolated mammary epithelial organoids under defined serum-free conditions so that the hormonal and growth regulation of proliferation and differentiation can be assessed.

In vitro differentiation of the mammary gland was elegantly demonstrated in studies using isolated whole mammary gland in organ culture [reviewed by Banerjee and Antoniou (1)]. This model system does not permit direct study of epithelial cells, however, in that adipose and stromal elements are present along with the epithelial cells, and proliferation cannot be directly quantitated except through [^{3}H]thymidine incorporation. Moreover, there is a major problem with carry over of hormones and growth factors. Primary culture systems offer the distinct advantage of being able to precisely define the hormonal, growth factor, and substratum conditions required for both proliferation and differentiation of a defined cell population. They also eliminate the cell-to-cell interactions that may occur in the organ culture model and allow

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the direct enumeration of cell number. Considerable effort has been devoted to defining these requirements in the mouse mammary epithelial cell (MMEC) system. However, with few exceptions, this work has been limited to studies of either proliferation or differentiation. For example, while MMECs grown on tissue culture plastic are able to proliferate to a limited extent (9,31), they cannot differentiate (40), and cells taken from pregnant or lactating mice dedifferentiate on plastic (9). Plating the cells on a fixed collagen gel improves their ability to proliferate (31), but differentiation is limited (9,24,35,40). In contrast, cells plated on floating collagen (8,9) or on a reconstituted basement membrane (26) can undergo both functional and morphologic differentiation, although in the former case at least (the latter has not been tested) there is no proliferation (9). The optimal system for growth of MMECs seems to be that described extensively by Nandi's group. In this model, MECs from virgin mice are plated within collagen gels in a completely defined serum-free media. This system allows extensive three-dimensional growth and proliferation (17) of the cells as well as their morphologic differentiation (13). Functional differentiation can also occur under these serum-free conditions when the collagen gel is released to float in the medium (25). Using serum, Tonelli and Sorof (40) and Durban et al. (5) were able to demonstrate casein production by cells embedded within fixed collagen gels.

It has been the interest of our laboratory to develop a suitable model system for growth of rat mammary epithelial cells (RMECs). The selection of the rat as a model system was made so that we could pursue our long-term interest in rat mammary carcinogenesis at the cellular level. At the time these studies were initiated, available model systems in the rat were quite limited. For example, the pioneering studies of Kidwell and colleagues (20) defined the growth factor requirements for proliferation of RMECs on plastic or collagen substrata. However, although this group also carried out extensive studies on basement membrane synthesis in this RMEC model, no reports of the ability of the model to differentiate were made and only short-term proliferation was possible. The serum-free systems of Ethier (10,11), and of Nandi's group (4,27) allow extensive proliferation of RMEC on or within fixed collagen gels, respectively; however, differentiation of the cells has not been reported. Wicha et al. have achieved marked functional differentiation by plating RMEC on a basement membrane reconstituted from mammary gland (44) or the Engelbreth-Holm-Swarm (EHS) tumor (2); however, to date these studies have been limited in that RMECs were taken from perphenazine-stimulated rats (to increase prolactin release and thus mammary development), the cultures were not serum-free, and proliferation could only be assessed by [^{3}H]thymidine incorporation.

In view of the above, we undertook the development of a model system that would allow us to investigate hormonal regulation of RMEC proliferation and differentiation in a defined serum-free medium and subsequently to study transformation. Using a reconstituted basement membrane gel in which RMECs from immature virgin rats are embedded, we have designed and characterized a system which allows both extensive proliferation as well as morphologic and functional differentiation of the RMEC equivalent to that of the lactating rat. The proliferation studies are reported herein, whereas functional and morphologic differentiation are reported in Hahm et al. in the accompanying paper (12) and Darcy et al. (manuscript in preparation).

Materials and Methods

Materials. Collagenase class III was a product of Worthington Biochemical (Freehold, NJ); dispase of Boehringer-Mannheim Biochemicals (Indianapolis, IN); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), insulin, progesterone, hydrocortisone, transferrin, ascorbic acid, and fatty-acid-free bovine serum albumin (BSA) of Sigma (St. Louis, MO); fetal bovine serum (FBS) of Hyclone (Logan, UT); and Ham's nutrient mixture F12, Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, Earle's balanced salt solution (EBSS), and gentamicin of GIBCO (Grand Island, NY). The Engelbreth-Holm-Swarm sarcoma was a generous gift of Drs. George Martin and Hynda Kleinman, Laboratory of Developmental Biology and Anomalies, National Institute of Dental Research, National Institutes of Health, Bethesda, MD. [^{3}H]-Thymidine (6.7 Ci/mmol) was purchased from Dupont/New England Nuclear (Boston, MA). Ovine prolactin (NIDDK-oPRL-17) was a gift of the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases. Bovine pituitary extract (BPE) and epidermal growth factor (EGF) were products of Collaborative Research (Bedford, MA).

Animals. Female Sprague-Dawley CD rats (Crl:CDBR) purchased from Charles River (Wilmington, MA) were used as the source of the mammary gland in all the experiments reported herein. Female CDF1 mice, bred in our laboratory from BALB/cAnNCrlBR female (Charles River, Raleigh NC) and DBA/2NCrlBR (Charles River, Raleigh NC) were used to carry the EHS sarcoma. Animals were fed rat or mouse chow diets (Teklad, Madison, WI), respectively, ad libitum, and had free access to water. Animal rooms were air-conditioned and humidity-controlled, with light cycles of 14 h on 10 h off (rats) or 12 h on 12 h off (mice).

Preparation of rat mammary epithelial cells. Excised mammary glands from 50- to 60-d-old rats were minced finely and placed in digestion solution (10 ml/g wet wt) consisting of 0.2% (wt/vol) collagenase class III and 0.2% (wt/vol) dispase in EBSS containing 5% FBS and 50 μg/ml gentamicin, and incubated at 37° C for 16 to 20 h. The digested tissue was then pelleted, washed twice with EBSS, resuspended in EBSS, and filtered initially through a 530-micron nitex filter and then through a 60-micron nitex filter to trap the epithelial organoids but allow passage of single cells. The organoids were washed off the filter with a 1:1 mixture of DMEM-F12 (phenol red-free) containing 12 m*M* HEPES, 5% FBS, and 50 μg/ml gentamicin, placed in a plastic tissue culture flask

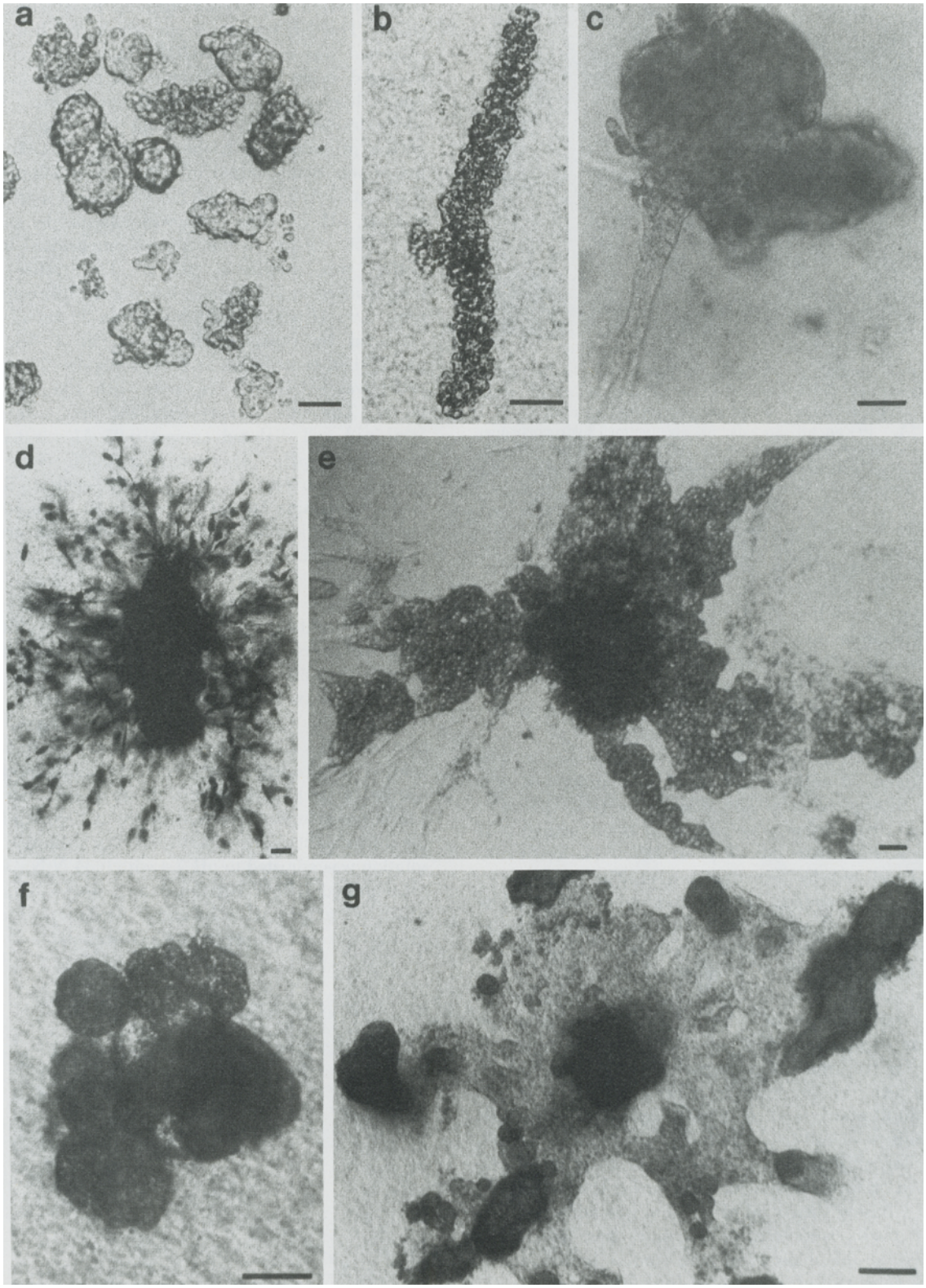

FIG. 1. Morphology of colonies of RMEC growing within a reconstituted basement membrane under the serum-free conditions described in Materials and Methods. *a*, Organoid suspension used for plating into the reconstituted basement membrane; *b*, viable but nonproliferating organoid at Day 14 in culture; *c*, unit demonstrating ductule formation 24 h after plating; *d*, branched unit with ductulelike projections; *e*, stellate-type colony with lipid in the ducts; *f*, budding cluster. This unit increases with time in culture. It is highly differentiated, has a lobuloalveolar appearance, and the cells actively synthesize and secrete casein. *g*, Three-dimensional web unit showing a combination of ducts and budding clusters. Bars: 1 cm = 25 microns.

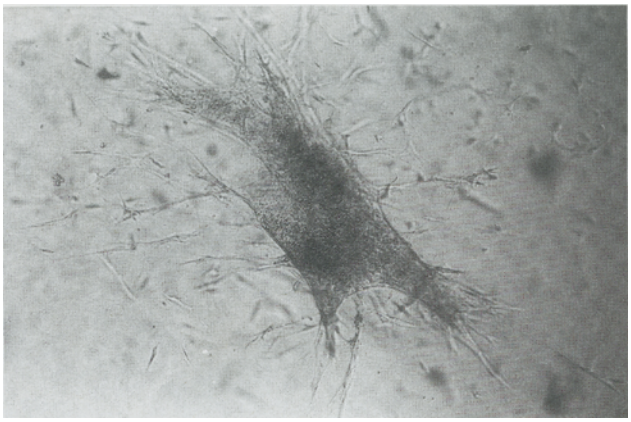

FIG. 2. Rat mammary epithelial cell colony growing within a type I collagen gel. Culture medium was F12 containing 10 μg/ml insulin, 50 μg/ml BPE, 0.1 μg/ml progesterone, 10 ng/ml EGF, 1 mg/ml BSA, 10 μg/ml transferrin, 5 μg/ml hydrocortisone, 10^{-8} *M* sodium selenite, and 5 μ*M* ethanolamine. In an experiment done concurrently with RMECs growing within the basement membrane matrix, highly differentiated structures similar to those shown in Fig. 1 were observed with the same tissue culture medium described for the collagen gel experiment. ×190.

and incubated for 4 h at 37° C to facilitate the attachment of stromal contaminants. The nonadherent organoids were counted as described below and then resuspended into the reconstituted basement membrane gel. Cell yield from this procedure was ~7 × 10^6 cells/g mammary gland.

At this step, cell enumeration was carried out by isolation and counting of nuclei according to the procedure of Sanford et al. (34). Briefly, a 1-ml aliquot of the organoid suspension was diluted to 8 ml with 0.1 *M* citric acid and incubated at 37° C for 1.5 h. The cell nuclei were then pelleted by centrifugation at 2560 ×*g* for 10 min and resuspended in phosphate-buffered saline (PBS). One hundred microliters of nuclear suspension was then combined with 10 μl of 0.2% trypan blue in PBS and the stained nuclei counted in a hemacytometer.

Cell culture procedure. After the 4-h incubation to facilitate attachment of stromal contaminants, the nonadherent organoids were pelleted by centrifugation at 500 ×*g* for 10 min, then resuspended in reconstituted basement membrane matrix at a concentration of 2 × 10^5 cells per 50 μl matrix and maintained on ice. One hundred fifty microliters of cell-free matrix was then placed in each well of a 24-well plate and the 50 μl matrix containing the cells layered on top. The matrix was allowed to gel at 37° C for 0.5 to 1 h, and then 1 ml of medium was added to each well. The media were changed 3 times per week. The media used in the majority of the studies (unless stated otherwise) consisted of phenol red-free DMEM-F12 (1:1) containing 10 μg/ml insulin, 1 μg/ml progesterone, 1 μg/ml hydrocortisone, 5 μg/ml transferrin, 10 ng/ml EGF, 1 μg/ml prolactin, 5 μ*M* ascorbic acid, 1 mg/ml fatty-acid-free BSA, and 50 μg/ml gentamicin. Photographs of cell cultures were taken with a Nikon FX-35A camera mounted on a Biostar phase contrast inverted microscope.

Preparation of reconstituted basement membrane (BM). The EHS tumor from which the BM matrix was extracted was carried in CDF1 male or female mice and passaged every 3 wk. Tumors were frozen in liquid N_2 upon harvesting, and stored at −70° C. Matrix was extracted from 200 g tumor by a slight modification of the procedure of Kleinman et al. (22). Briefly, 200-g tumor was homogenized in 400 ml of a solution of 3.4 *M* NaCl, 0.05 *M* tris, 4 m*M* EDTA, 2 m*M* *n*-ethylmaleimide (NEM), pH 7.4. The pellet was collected by centrifugation at 11 950 ×*g* for 15 min and rehomogenized in the above buffer two additional times. The pellet was then homogenized in 200 ml of a buffer (pH 7.4) containing 2 *M* urea, 0.05 *M* tris, 0.15 *M* NaCl, and 50 μg/ml gentamicin, extracted by overnight incubation at 4° C, rehomogenized, then centrifuged at 23 420 ×*g* for 20 min, and the pellet was extracted once more with 100 ml of the urea buffer. The supernatants from the urea extractions were combined and dialyzed against 0.05 *M* tris-0.15 *M* NaCl (tris-saline) (pH 7.4) containing 0.5% chloroform, two changes of tris-saline, and finally against RPMI 1640 media containing 50 μg/ml gentamicin. The resulting BM matrix was aliquoted into sterile 50-ml tubes and stored at −20° C. Each batch was checked for sterility before use.

Preparation of collagen gel. Collagen gels were prepared as described by Richards et al. (30). In brief, collagen fibers were extracted from rat tails by first placing the fibers in a capped sterile flask with 70% (vol/vol) ethanol (sufficient volume to cover the fibers) overnight at 4° C, removing the ethanol, and then dissolving the fibers by the addition of 200 ml of acetic acid [1:1000 dilution (vol/vol) of glacial acetic acid in distilled water] per g fiber with stirring for 48 h at 4° C. The suspension was then centrifuged at 1900 ×*g* for 2 h, and the supernatant used as the stock collagen solution. This stock solution was adjusted to pH 7.4 with 0.34 *N* NaOH and 10× concentrated RPMI 1640 medium, using volumes of each which brought the pH to 7.4 and allowed for the formation of a firm gel at room temperature which was stable at 37° C. The

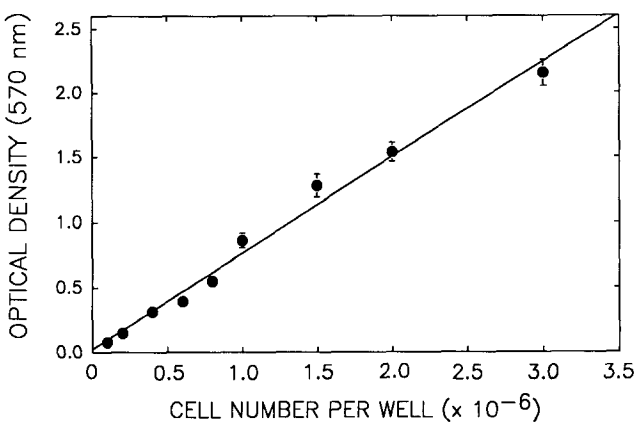

FIG. 3. MTT standard curve showing the linear relation between cell number and the absorbance of the formazan crystals after their solubilization in DMSO. Each point is the mean ± SEM from nine separate experiments. In some cases the SEM is smaller than the data point.

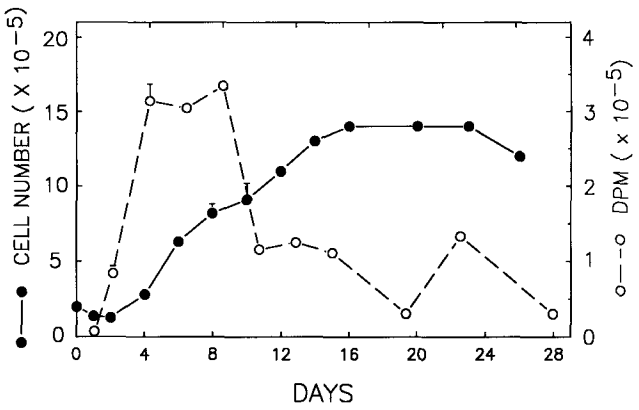

FIG. 4. Proliferation of RMECs in the reconstituted basement membrane in the serum-free medium described in Materials and Methods. *Solid circles* = cell number per well; *open circles* = [^{3}H]thymidine incorporation per well. *Each point* is the mean ± SEM of triplicate wells.

RMEC organoids were resuspended in this collagen solution at 4° C, pipetted into tissue culture plates, and the samples allowed to gel at room temperature for 30 min. The medium was then added to the cultures and they were placed into a CO_2-air incubator at 37° C.

Quantitation of Cell Growth

MTT Assay. Cell number was quantitated by modification of the MTT assay (28,42). In this assay, viable cells convert the soluble tetrazolium MTT dye to insoluble (in aqueous media) blue formazan crystals. Briefly, 1 mg MTT (200 μl of a stock 5 mg/ml solution in PBS) was added per ml of medium and the cultures allowed to incubate at 37° C for 4 h. The media were then removed and 1 ml of 1% (wt/vol) dispase in EBSS added to each well and the matrix dissociated with a Pasteur pipette. The matrix was allowed to digest for 2 h at 37° C. The digested material was then transferred to tubes, the well rinsed with PBS, and the combined fractions centrifuged at 4552 ×*g* for 15 min. The supernatant was discarded, the pellet dissolved in dimethyl sulfoxide (DMSO) and the color read in a Bausch and Lomb Spectronic 1001 spectrophotometer at 570 nm. A standard curve was set up utilizing the newly isolated RMECs before each experiment. Production of formazan crystals and therefore color formation after dissolution of the crystals is directly related to viable cell number.

[^{3}H]Thymidine incorporation. DNA synthesis was evaluated using a modification of a standard radiolabeled thymidine incorporation technique (32). Five microcuries of [^{3}H]thymidine was added to each well, and the cells were incubated at 37° C for 24 h. The media was then removed from each well, 1 ml of 1% (wt/vol in EBSS) dispase was added, and the basement membrane matrix was digested as described above. The digested matrix and cells were transferred to centrifuge tubes and then centrifuged at 500 ×*g* for 10 min. The cell pellet was washed once with cold PBS, recentrifuged at 1100 ×*g* for 10 min. and the pellet resuspended in 1 ml of 5% trichloroacetic acid and allowed to incubate overnight at 4° C. The precipitate was collected by centrifugation at 1100 ×*g* for 10 min and the pellet was dissolved in 1 ml of 0.1 *N* NaOH containing 0.1% Triton X-100. The contents of the tube were transferred to a scintillation vial, 100 μl of 1*N* HCl was added, and the vials were counted after the addition of 15 ml of scintillation fluid.

RESULTS

Morphology of Rat Mammary Epithelial Cells Grown Within the Reconstituted Basement Membrane Matrix

Figure 1 *a* shows an example of the mammary epithelial organoid suspension used in plating cells into culture. In general, well-organized cellular structures are preserved within the organoids. These units can be spherical as shown in Fig. 1 *a*, or elongated as demonstrated in Fig. 1 *b*. The majority of these units proliferate into the different types of colonies shown in Fig. 1 *c–g*, while others, although nonproliferative, remain viable (Fig. 1 *b*). Ductal projections are initiated as early as 24 h (Fig. 1 *c*) and become very extensive (Fig. 1 *d*). These ducts are often filled with lipid as shown in the stellatelike colony of Fig. 1 *e* and by oil O red staining (12). The colonies containing lobularlike budding clusters (Fig. 1 *f*) increase in frequency with time in culture. This type of colony has been extensively characterized at the electron microscopic level (12, Darcy et al. manuscript in preparation) and shown to consist of polarized secretory epithelial cells that are organized into a lobuloalveolar arrangement and which actively secrete both casein and lipid (12). Three-dimensional web units (Fig. 1 *g*), composed of both ducts and budding clusters are also seen at high frequency in this culture system.

Initially, attempts were made to grow RMECs within a collagen type I gel matrix which has been used by other investigators for the growth of mammary epithelial cells (5,17,40). However, under comparable growth conditions, it was clear that growth in the reconstituted basement membrane was superior to that in collagen, both in terms of cell proliferation (estimated in terms of colony size) as

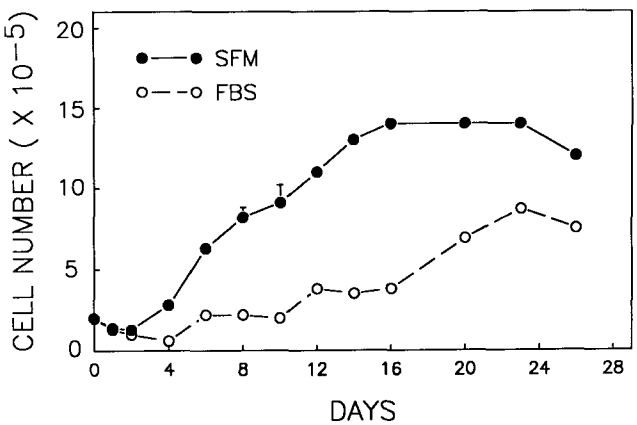

FIG. 5. Comparison of growth of RMECs in the serum-free medium described in Materials and Methods, with growth in DMEM-F12 containing 5% FBS. *Each point* is the mean ± SEM of triplicate wells.

well as morphologic development. Figure 2 demonstrates the limited morphogenesis seen after 21 d of growth within a collagen gel (compare to the extensive branching, budding, and web unit formation seen in Fig. 1 for RMECs grown within a basement membrane matrix).

Quantitation of Cell Growth Within the Reconstituted Basement Membrane

A modification of the MTT dye assay was developed to quantitate growth of the MECs within the reconstituted basement membrane. As can be seen in Fig. 3, there is a linear relationship between cell number and MTT dye conversion (measurement by color development after dissolution of the formazan crystals). A number of other methods were also examined in an attempt to quantitate growth of the MECs within the reconstituted basement membrane. These included a fluorometric method to quantitate DNA (16) as well as direct counting of cells or nuclei. The former, although useful in the collagen gel system (31), was found to be unsuccessful with the basement membrane matrix due to interference from one or more components of the matrix in the assay. Additionally, direct cell or nuclei counting was found not to be feasible. Enzymatic digestion of the matrix results in the release of cell clusters rather than single cells, and therefore a second step involving further enzymatic treatment or nuclei isolation would be required for cell quantitation by Coulter counter or by hemacytometer. Attempts with nuclei counts were further confounded by debris present in the sample. Although this could be circumvented with additional washes or by counting in a hemacytometer, both alternatives would be tedious and time-consuming. Thus, the MTT assay for cell enumeration was adapted for this model system. This assay has several advantages in that it is relatively easy to perform, gives reliable and reproducible results, and quantitates only viable cells. A possible limitation is that MTT metabolism may differ between undifferentiated and differentiated cells. However, as noted below, assessment of [^{3}H]thymidine incorporation as a second measure of cell growth in this system suggests that the MTT assay is a reliable estimate of cell number.

Hormone and Growth Factor Requirements for Proliferation

Proliferation of the cells in serum-free DMEM-F12 media containing insulin, hydrocortisone, EGF, prolactin, progesterone, BSA, transferrin, and ascorbate (concentrations as described in Materials and Methods) is shown in Fig. 4. There is an initial decrease in cell number during the first 2 d after plating of the mammary epithelial organoids; however, cell number then increases for at least 16 d (Fig. 4) and frequently longer (data not shown). In the example shown, cell number increased approximately 14-fold from the 1×10^5 cell number at Day 2. This is probably an underestimation of the extent of growth because the percent of the original organoids plated that went on to proliferate was not quantitated. In fact, the decreased cell number during the first few days in culture apparently reflects the inability of some of the plated organoids to proliferate (they were viable at Day 0 based on their ability to reduce MTT) because there was a steady rise in [^{3}H]thymidine incorporation during the first 4 d in culture, which peaked between Days 4 and 8, falling to a lower level thereafter (Fig. 4). Concurrent with this, the slope of the cell number curve was increased between Days 4 and 8, but decreased after Day 8, demonstrating that although cell number was still increasing, the rate of growth had slowed. This is consistent with the [^{3}H]thymidine incorporation data, which indicates that proliferation is still occurring but at a decreased rate; at Day 11, in fact, DNA synthesis is still approximately one-third of the maximum rate seen between Days 4 and 10. At later times in culture, both the cell number curve and the [^{3}H]thymidine incorporation curve plateau. This could suggest a balance between proliferation, differentiation, and cell death. Alternatively, it could suggest that highly differentiated cells metabolize MTT to a lesser extent. In this culture system, cells remain viable for at least 3 mo. (longer times have not yet been tested), although they are no longer actively proliferating. Figure 5 demonstrates that growth of cells in this serum-free medium (SFM) is superior to that in medium containing 5% FBS.

The hormonal requirements for optimal proliferation in the DMEM-F12 culture medium are shown in Fig. 6. In this experiment, each of the growth factors and hormones described in Materials and Methods was deleted, one at a time, for the entire culture period, and growth in the absence of this factor but in the presence of all the other factors was determined. Results are shown at Day 21 in culture. Insulin is required for long-term maintenance of cell viability; in the experiment shown, cell number fell below the detectability of the assay. In more recent studies where we have improved the sensitivity of the MTT assay, cell number was shown to decline with time

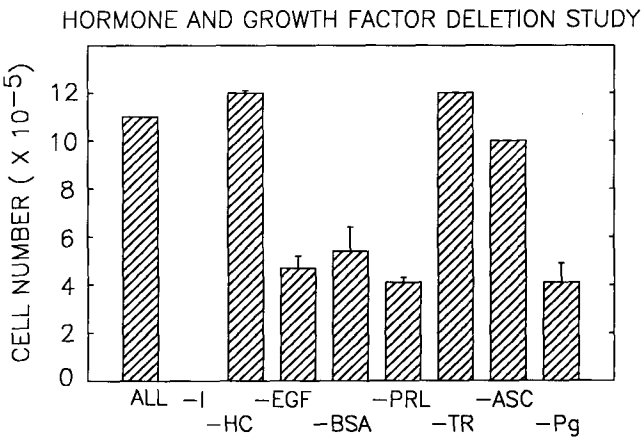

FIG. 6. Effect of depletion of individual hormones or growth factors from the serum-free medium described in Materials and Methods on proliferation of RMECs within the reconstituted basement membrane matrix. Cell number is expressed per well at Day 21 in culture. *Each point* is the mean ± SEM of triplicate wells.

when insulin was omitted from the culture (data not shown). Systematic deletion of other components of the system also demonstrated that EGF, BSA, prolactin, and progesterone are required for optimal cell growth. The omission of hydrocortisone, transferrin, or ascorbic acid had no appreciable effect on cell proliferation, although cells grown in the absence of hydrocortisone were morphologically altered, with a high frequency of extensively branched colonies with many fine projections. The optimal concentrations for insulin, EGF, BSA, and progesterone are shown in Fig. 7. From this and other experiments, these concentrations were determined to be 10 μg/ml for insulin, 10 ng/ml for EGF, 1 mg/ml for BSA, and 1 μg/ml for progesterone. Hydrocortisone did not

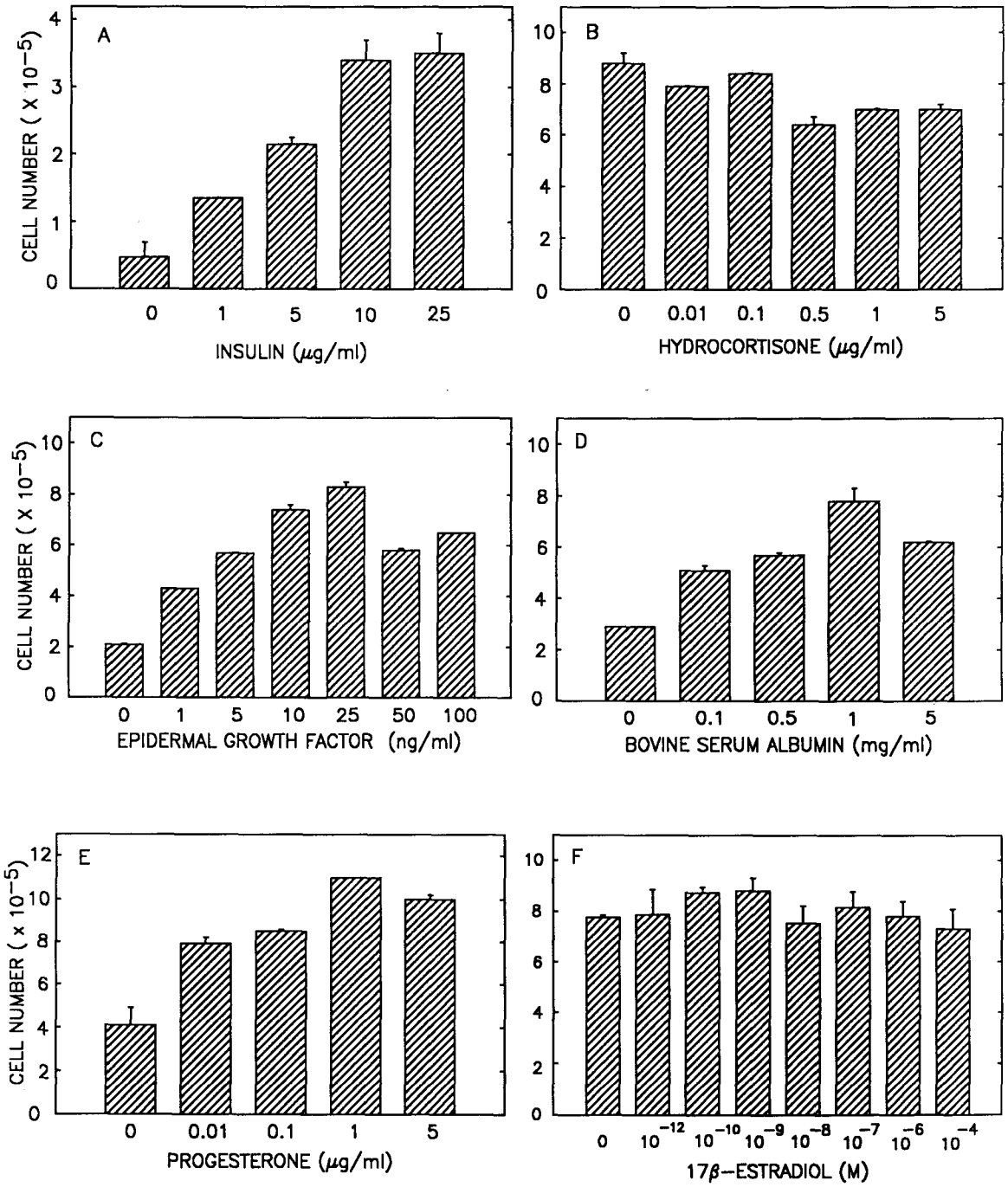

FIG. 7. Dose-dependent effect of various hormones and growth factors on the proliferation of RMECs within the basement membrane matrix. Tissue culture media were as described in Materials and Methods with the exception that the concentration of the hormone or growth factor under investigation was altered as indicated in the figure. Cell numbers are expressed as mean ± SEM per well ($n = 3$) at Day 21 in culture. *A*, effect of insulin on RMEC proliferation; *B*, effect of hydrocortisone on RMEC proliferation; *C*, effect of EGF on RMEC proliferation; *D*, effect of BSA on RMEC proliferation; *E*, effect of progesterone on RMEC proliferation; *F*, effect of 17β-estradiol on RMEC proliferation.

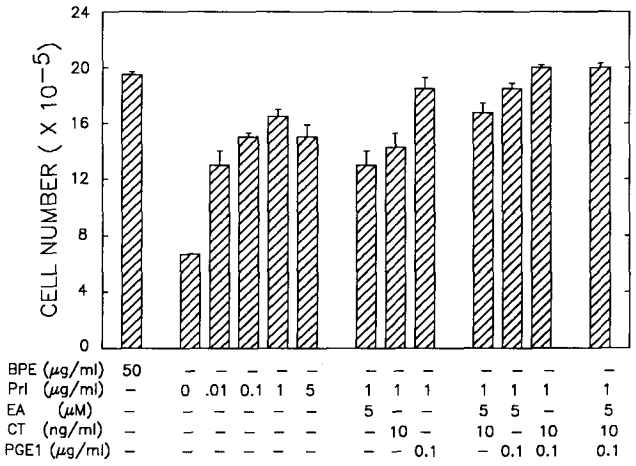

FIG. 8. Comparison of effects of BPE with various combinations of prolactin (*Prl*), ethanolamine (*EA*), cholera toxin (*CT*), and PGE_1 on proliferation of RMECs within the reconstituted basement membrane. Basal media was DMEM-F12 with 10 μg/ml insulin, 1 μg/ml progesterone, 10 ng/ml EGF, 1 mg/ml BSA, 10 μg/ml transferrin, 1 μg/ml hydrocortisone, and 5 μ*M* ascorbic acid. *Each point* is the mean ± SEM of triplicate wells at Day 21 in culture.

affect cell proliferation at low concentrations, but had a very modest inhibitory effect at higher concentrations (Fig. 7 *B*). Interestingly, 17-β-estradiol at concentrations ranging from 10^{-12} to 10^{-4} *M* also did not have any effect on cell proliferation (Fig. 7 *F*).

Figure 6 demonstrates that prolactin is an important mitogen for the RMECs growing within the basement membrane matrix. In an attempt to detemine the optimal concentration of prolactin for proliferation, as well as to determine if proliferation could be increased to an even greater extent by other pituitary hormones and growth factors, the mitogenic effect of prolactin was compared with that of BPE in a basal medium consisting of insulin, progesterone, transferrin, BSA, EGF, and hydrocortisone. As can be seen in Fig. 8, BPE stimulated proliferation to a greater extent than did the optimal concentration of prolactin, 1 μg/ml. In this experiment, cell number increased at least 20-fold, from a measured cell number of 1×10^5 cells at Day 2. The combination of ethanolamine (EA) or cholera toxin (CT) with prolactin did not increase proliferation beyond that seen with prolactin alone; however, the combination of all three agents did stimulate proliferation significantly. Interestingly, when 1 μg/ml PGE_1 was used in combination with prolactin, proliferation was similar to that seen with BPE; the addition of cholera toxin or ethanolamine or both to the combination of prolactin and PGE_1 did not stimulate proliferation further. When administered individually, both PGE_1 and EA stimulated cell growth; unexpectedly, however, CT alone inhibited proliferation (data not shown). Taken together, these data demonstrate that the combination of prolactin and PGE_1 effectively replaces BPE in terms of proliferation of the RMEC within the basement membrane matrix.

In addition to hormones and growth factors, the tissue culture medium also had a marked effect on growth of the RMECs within the basement membrane matrix. A number of our early studies were done using F12 medium. As shown in Fig. 9, cell proliferation was lower in F12 when compared to the DMEM-F12 combination or to DMEM alone. Moreover, cell proliferation in F12 was quite variable from one experiment to the next, in contrast to the results using DMEM-F12, which are extremely consistent. We conclude, therefore, that for optimal proliferation, cells should be grown in the DMEM-F12 combination. Nevertheless, the studies done with F12 were useful in helping us develop the optimal proliferation medium. Additionally, growth factor responsiveness was generally greater in the suboptimal F12 medium. For example, in F12, both transferrin and ascorbate stimulated proliferation (Fig. 10 *A* and *B*), whereas no stimulation was observed in the more optimal DMEM-F12 medium (Fig. 6).

DISCUSSION

This report, as well as other studies from our laboratory (12; Darcy et al. manuscript in preparation), details the development of a primary culture model system in which RMECs obtained from immature virgin rats are grown within a reconstituted basement membrane matrix in a serum-free defined medium. This model system is unique in that it allows for both extensive proliferation of the MECs (at least 15- to 20-fold within 14 to 21 d) as well as functional and morphologic differentiation equivalent to that of the lactating mammary gland. It therefore has an advantage over many other model systems in which only proliferation or differentiation can be studied.

Proliferation of RMECs was shown to be sensitive to a number of hormones and growth factors as well as to the tissue culture medium and to the reconstituted basement membrane. Mitogenic hormones include EGF, prolactin, insulin, and progesterone. In addition, BSA and PGE_1 were shown to stimulate proliferation. A major difference from other RMEC models (4,11) is that extensive proliferation does not require CT; rather, naturally

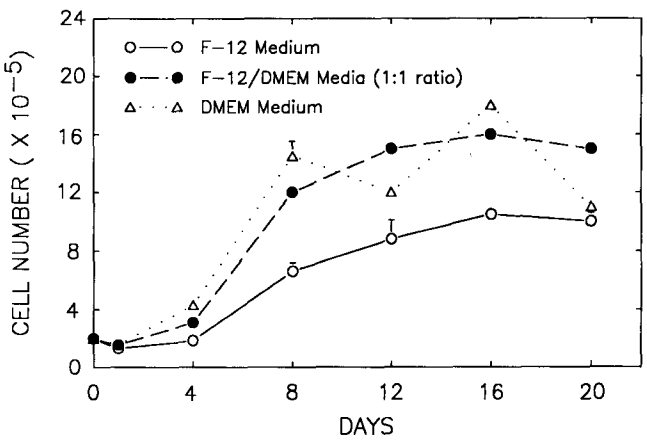

FIG. 9. Comparison of proliferation of RMECs in F12, DMEM, and DMEM-F12 tissue culture media containing the supplements described in Materials and Methods. *Each point* is the mean ± SEM of triplicate wells.

occurring hormones and growth factors are sufficient. In this system, the RMEC undergo an initial 2- to 3- wk active cell growth period and then remain viable for months in culture.

Many of these hormones have also been shown to be mitogenic in other mammary model systems. EGF, for example, has been shown to stimulate proliferation of mammary gland both in vivo (3,36,43) as well as in primary cultures of RMECs (4,11,33), MMECs (17,37,38), human mammary epithelial cells (HMEC) (14), and explants in vitro (39). When applied directly to the mammary gland of ovariectomized mice, EGF stimulated ductal development by the formation of new end buds; moreover, DNA synthesis was stimulated in the cap cells of the end buds, which were also shown to have very high levels of the EGF receptor (3). EGF has also been shown to directly stimulate lobuloalveolar development (43).

In our model system, insulin has been shown to be required for long-term maintenance of cell viability as well as for cell proliferation. On a more short-term basis (< 14 d), however, based on the ability of MTT to stain the nongrowing colonies, viability is maintained in the absence of insulin. The nonphysiologic levels of insulin required in our studies as well as in the experiments of others (4,11,20) are thought to be because insulin is acting through the IGF-1 receptor rather than its own receptor, and in fact Deeks et al. (4) have reported that physiologic levels of IGF-1 can substitute for insulin in stimulating the proliferation of RMECs.

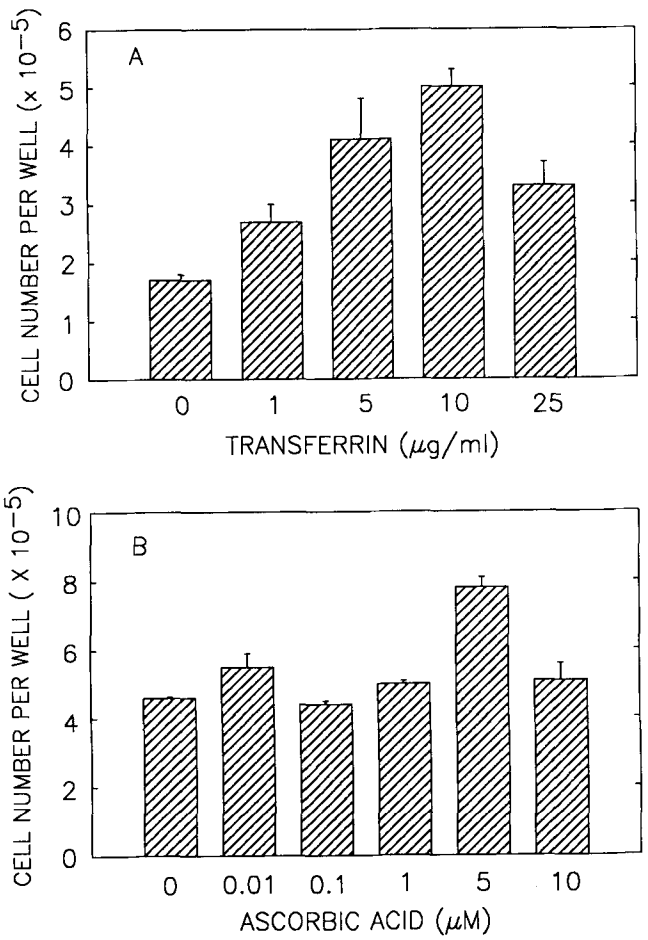

FIG. 10. Effect of transferrin and ascorbic acid on proliferation of RMECs in Ham's F12 tissue culture media containing 10 μg/ml insulin, 1 μg/ml progesterone, 1 μg/ml hydrocortisone, 10 ng/ml EGF, 50 μg/ml BPE, 1 mg/ml BSA, (5 μg/ml transferrin), 50 μg/ml gentamicin, and 1.2 μg/ml phenol red. *A*, effect of transferrin on RMEC proliferation (no ascorbate present); *B*, effect of ascorbic acid on RMEC proliferation (transferrin present at 5 μg/ml).

In addition to insulin and EGF, pituitary factors have also been shown to stimulate proliferation of MECs. In the studies reported herein, BPE stimulated proliferation of RMECs approximately threefold. Hammond et al. (14) reported a similar stimulatory effect in HMECs growing on plastic. In their system, BPE could be replaced by PGE_1, ethanolamine and phosphoethanolamine, but no requirement for prolactin was demonstrated. In contrast, we demonstrate that in our model system, in which RMECs are growing three dimensionally within a reconstituted basement membrane matrix, prolactin has significant mitogenic activity, almost as great as that induced by BPE. The combination of prolactin and PGE_1 completely replaced the requirement of the cells for BPE. Significantly, the mitogenic effect of prolactin did not require and was not further increased by CT. This differs from the collagen gel culture of RMECs where prolactin induced maximal proliferation only in the presence of CT (4) or under suboptimal conditions (27), and the two-dimensional culture of RMECs where prolactin was mitogenic only in the absence of progesterone (11). Prolactin does stimulate proliferation in the three-dimensional collagen culture of MMECs (17). In vivo, prolactin is a lactogenic hormone required for both ductal and alveolar growth (41).

The steroid hormones, 17 β-estradiol, progesterone, and hydrocortisone, have been shown to be required for the development of the mammary gland in vivo. A requirement for estrogen has been difficult to demonstrate in cultured MECs, however, and our data showing a lack of effect of estradiol on RMEC proliferation in phenol red-free tissue culture media confirms that of Richards et al. (29) in the three-dimensional MMEC, RMEC, and HMEC culture models. The reason for this lack of effect of estradiol has not been established. It may be that estrogen effects in vivo are mediated by an estrogen-stimulated growth factor secreted by the mammary stroma, and indeed this possibility is suggested by the observation of Haslam (15) that estrogen can stimulate MMEC proliferation only in the presence of fibroblasts. The lack of effect of estrogen would seem to be restricted to proliferation inasmuch as estradiol was shown to induce the progesterone receptor in cultured RMECs (7) and MMECs (6). We reported such a dissociation between estrogen-stimulated proliferation and progesterone receptor induction several years ago (19).

As has been demonstrated in other MEC models, progesterone is mitogenic for the RMECs growing within the reconstituted basement membrane. This steroid hormone stimulated proliferation beyond that seen with prolactin, EGF, and the other growth factors in our medium. Similar results were reported by Imagawa et al.

(17) in the three-dimensional MMEC model, but in RMECs, progesterone stimulated proliferation in the two-dimensional model only in the absence of prolactin (11), and in the three-dimensional model only under suboptimal conditions (27) or in the presence of cholera toxin (4). In vivo, progesterone has been shown to be required for lobuloalveolar growth, although not for ductal growth (41).

An effect of glucocorticoids on MEC proliferation in primary culture may be species specific or depend on the substratum on which the cells are grown or both. In the experiments reported herein, where the RMECs were grown within a reconstituted basement membrane, omission of hydrocortisone from the culture medium slightly enhanced cell proliferation. Of more interest, however, was the observation that morphology was considerably altered, with an increase in ductal formation resulting in colonies with an extensive branching network, and a decreased number of the colonies with lobularlike budding clusters. This would be consistent with the in vivo observation that extensive ductal growth does not require glucocorticoids, but that lobular growth and lactogenesis do require this steroid hormone (41). In this regard, we have found in preliminary studies that casein production is decreased when RMECs are grown in the absence of hydrocortisone, suggesting that the lobuloalveolarlike cells are the major source of casein in this system. This is currently being examined in other studies in the laboratory. The role of glucocorticoids in proliferation of MECs in other models is not clear-cut. Kidwell et al. (21) demonstrated a requirement for glucocorticoids for proliferation of RMECs grown on plastic or type I collagen; however, this requirement was significantly reduced in RMECs growing on type IV collagen. No requirement for glucocorticoid was reported for proliferation of RMECs growing within a type I collagen gel (27); it was required, however, for MMECs grown under the same conditions (17). Glucocorticoids were also shown to be required for HMECs growing on plastic (14) or within collagen gels (45).

A major growth promoter in ours, as well as other model systems (10,18) is BSA. This protein could act to stabilize peptide growth factors such as prolactin or EGF, or as a carrier protein to transport other molecules into the cell. The iron-binding protein transferrin was shown to stimulate proliferation in the suboptimal F12 medium (Fig. 10 *A*); however it was not required in the more optimal DMEM-F12. This apparent lack of requirement for cells undergoing active proliferation may be because MECs have the capacity to synthesize transferrin; this has been demonstrated for RMECs growing on basement membrane (2) as well as for MMECs growing on floating collagen gels (23). The synthesis of transferrin by the mammary cells in culture is not absolutely dependent on lactogenic hormones, yet in vivo there are very low levels of transferrin in virgin mammary tissue whereas the level increases significantly during pregnancy and this high level is maintained during the lactational period. Finally, the requirement for ascorbic acid under the suboptimal F12 conditions probably reflects its role as an antioxidant, and that for PGE_1 may reflect its ability to stimulate cyclic AMP levels, although the inability of CT to stimulate proliferation under the same conditions may argue against this.

In summary, we believe that the system reported here as well as in Hahm et al. (12) and Darcy et al. (manuscript in preparation) is unique among the existing models and should prove to be an important tool to study the regulation of the growth and development of rat mammary cells, as well as to carry out in-depth studies of the process of neoplastic transformation. There are several advantages to this model system. First, the most significant advantage is the ability of RMECs isolated from immature virgin rats to differentiate to an extent comparable to that of the lactating mammary gland. To our knowledge, this extent of differentiation has not been reported in any other model system, and may reflect the influence of the reconstituted basement membrane. Second, in addition to differentiation, the RMECs proliferate extensively, and this proliferation can readily be quantitated using the MTT assay as well as by [^{3}H]thymidine incorporation. This is in contrast to other reconstituted basement membrane systems where proliferation could only be assessed by [^{3}H]thymidine incorporation. Third, it is also possible to quantitate the various types of colonies present at each time point, and in response to hormone and growth factor modulation. Studies of this nature should lead to a greater understanding of the regulation of ductal and alveolar morphogenesis, as well as of proliferation and functional differentiation, by specific hormones and growth factors. Fourth, growth of cells in this model is three dimensional, just as it is in vivo, possibly accounting for the ability to undergo extensive ductal and alveolar morphogenesis. Finally, growth of the RMECs seems to be substratum-dependent, based on the less extensive morphogenesis and proliferation in RMECs grown within type I collagen (Fig. 2 and data not shown) compared to that within the basement membrane. We recognize that the reconstituted basement membrane is an undefined component of this otherwise completely defined model system. In spite of this, it has been possible to define the essential role of the lactogenic hormones prolactin and insulin for proliferation, in addition to the requirements for EGF and progesterone. Ongoing studies in the laboratory are focused on the hormonal and growth factor regulation of morphologic and functional differentiation of RMECs growing in this model system.

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We are grateful to Kathleen Darcy for most of the photomicrographs in Figure 1 and for preparing the composite, and to Drs. Hynda Kleinman and George Martin for providing us with the Engelbreth-Holm-Swarm sarcoma for transplant into mice in our laboratory. This work was supported by grants CA 33240 and CA 35641 and by core grant CA 24538 from the National Institutes of Health, Bethesda, MD.