A morphologically distinct candidate for an epithelial stem cell in mouse mammary gland

GILBERT H. SMITH

Laboratory of Tumor Immunology and Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

and DANIEL MEDINA

Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030, USA

Summary

Transplantation studies demonstrate that an epithelial stem cell component must exist in the mouse mammary gland throughout life. Samples taken from any portion of the mammary gland at any age and at any developmental stage, including full functional differentiation, give rise to mammary epithelial outgrowths with complete developmental capacity. Cytological examination of mouse mammary gland explants revealed the presence of morphologically distinct cells distributed sporadically among the mammary epithelium, whose behaviour *in vivo* and *in vitro* suggested that they might represent a latent epithelial stem cell population. These pale-staining cells possessed large spherical nuclei, a clear cytoplasm and a round smooth-contoured shape. Electron microscopy confirmed their pale-staining characteristics and revealed a cytoplasm sparsely populated with organellar structures, such as mitochondria and endoplasmic reticulum. Their epithelial genealogy was demonstrated by the presence of terminal bars and tight junctions formed with their epithelial cell neighbours. *In vivo*, these cells were found among mammary epithelial cell populations in 16-day-old embryos onward in both ductal or lobular structures during all stages of pregnancy, lactation and involution. In explant cultures, these cells did not undertake a secretory morphology in the presence of lactogenic hormones, although occasionally they became immunologically positive for casein. They did not incorporate [^{3}H]-thymidine into their nuclei under any of the experimental conditions used; however, they appeared to undergo mitosis within 4 h regardless of the presence or absence of hormone(s). At 24 h increased numbers of pale cells were found in pairs or in groups. At 72 h in the presence of IFPrl (medium containing insulin, hydrocortisone and prolactin), the pairs and groups of pale cells observed at 24 h were not found. Instead, individual pale cells were seen among groups of cytologically and functionally differentiated secretory epithelial cells. When lactogenic hormones were not present, groups of pale cells were still present in the explants at 72 h. These findings suggest that the pale cells are arrested at G_2 phase of the cell cycle and that they give rise by mitosis to daughter cells capable of differentiating in the presence of lactogenic stimuli. Inhibition of DNA synthesis in the explants did not alter these cellular events.

Key words: stem cell, mammary gland, epithelium.

Introduction

The mouse mammary gland originates from a thickening of the embryonic ectoderm, which subsequently invades the underlying mesenchyme. During the earliest stages of development, the ectoderm forms a closely packed knob of cells that projects into the underlying mesenchyme. This knob lengthens in the female mouse embryo, beginning at 14 days to form an elongated solid cord of epithelial cells that later branches in a manner characteristic of the adult mammary gland. At birth, the female mouse possesses mammary glands composed of branched epithelial cords that contain lumina, i.e. a system of branching hollow epithelial tubes that open at the nipple (Hogg *et al.* 1983). Little subsequent mammary growth and development occur until the onset of puberty, when the

Journal of Cell Science 89, 173–183 (1988)
Printed in Great Britain © The Company of Biologists Limited 1988

immature gland grows rapidly to produce the tree-like pattern of ducts, upon which secretory alveolar lobules will develop during pregnancy. During this period of growth and invasion of the mammary fat pad by the mammary ductal system, beginning at approximately 3 weeks of age and continuing until approximately 12 weeks of age in virgin females, intense mitotic activity occurs within the mammary end buds (Bresciani, 1965). These bulb-like structures at the ends of the expanding ducts are specialized at their advancing edge to permit rapid penetration of the surrounding fatty stroma (Silberstein & Daniel, 1982). The posterior regions of the end buds provide differentiating parenchymal and myoepithelial cells that contribute to the formation and elongation of the subtending ducts (Williams & Daniel, 1983). The growing end buds branch at regular intervals and generate the characteristic pattern of the mature gland. Local factors appear to regulate the spacing between the growing branches so that competition of extending ducts is avoided. When insufficient gland-free fat pad is available for continued growth, the end buds disappear and the subtending ducts become mitotically quiescent (Faulkin & DeOme, 1960).

Despite the disappearance of the end buds and their specialized structures, including the undifferentiated 'cap cells' (Williams & Daniel, 1983), which appear to give rise to both myoepithelial and parenchymal epithelial cells, portions of mammary gland from virgin female mice of any age are capable of regenerating a complete functional epithelial tree upon transplantation into an epithelium-free mammary fat pad (DeOme *et al.* 1959, 1960; Hoshino, 1962, 1978; Daniel *et al.* 1968; Medina, 1973). These observations argue that mammary epithelial-specific stem cells persist within the mammary gland population throughout their lifetime. Serial transplantation of mammary epithelium demonstrates that normal mouse tissue has a finite proliferative capacity (Daniel *et al.* 1968) and that the rate of senescence is governed by the length of time between transplant generations, suggesting that mitotic events determine the rate of loss of proliferative capacity (Daniel, 1972).

In the present study, we have examined the 'repopulating capacity' of various portions of the mouse mammary gland during various stages of its development and differentiation. In addition, we have attempted to identify the potential mammary epithelial stem cells among these mammary populations *in situ* and within explant cultures by employing autoradiographic, immunocytochemical and morphological criteria. In our concept, the term mammary epithelial stem cell describes an uncommitted mammary cell (i.e. not functioning as myoepithelium or glandular epithelium) that can give rise through mitosis to committed epithelial cell progeny without losing its own uncommitted status. It is implicit in this definition that the mitotic event has been unequal in the sense that the progeny are sensitive to certain mammogenic stimuli whereas the stem cell is not.

Materials and methods

Mice

Balb/c/CRGL, C3H/He and C3H/Sm mice were maintained at 23 °C on a 12 h/12 h, light/dark cycle and given food and water *ad libitum*. Females from each strain were evaluated in explant culture and/or by transplantation and morphological techniques, at all stages of mammary gland function and development, for the presence and fate of potential mammary epithelial stem cells.

Explant culture

The abdominal mammary glands were aseptically removed and cultured as described (Smith & Vonderhaar, 1981; Vonderhaar & Smith, 1982; Smith, 1987). The pooled explants from 6–13 animals were cultured in medium 199 containing insulin (I; 5 μg ml^{-1}), hydrocortisone (F; 1 μg ml^{-1}) and prolactin (Prl; 1 μg ml^{-1}). Where indicated, cytosine arabinoside (araC) was added at 15 μg ml^{-1} to virgin mouse explants and at 30 μg ml^{-1} to explants of pregnant gland. These levels of araC inhibit DNA synthesis by 95 % without toxic side effects (Owens *et al.* 1973; Smith & Vonderhaar, 1981; Vonderhaar & Smith, 1982). The culture medium was changed routinely after 24 and 72 h in each experiment; part of the pooled explants was used for biochemical analyses and the remainder was prepared for morphological studies.

Preparation of epithelial cell pellet

A fraction enriched in mammary epithelial cells was obtained from freshly excised tissue or from explants by modification of methods described previously (Topper *et al.* 1975). Fat cells were removed by incubating up to 600 mg of tissue in 5 ml of medium 199 (pH 7·4) containing 1·5 mg of crude collagenase per ml and 4 % bovine serum albumin (BSA). Incubation was in a shaking 37 °C water bath for 30–45 min. At 5-min intervals, the contents of the incubation vessels were aspirated into Pasteur pipettes; the final two aspirations were done using fine-bore pipettes. The dispersed cells were collected by centrifugation for 5 min at 800 ***g*** at room temperature and washed three times with phosphate-buffered saline (PBS). This gentle disruption procedure allows many ductal and alveolar structures to remain intact (Wicha *et al.* 1979).

Preparation of mammary cells for light and electron microscopy

Epithelial cell pellets or intact mammary explants were fixed overnight at 4 °C in 3 % formaldehyde (freshly prepared from paraformaldehyde) in 0·1 M-sodium cacodylate buffer (pH 7·4). The explants or cell pellets were trimmed, then rinsed thoroughly in 0·1 M-sodium cacodylate prior to postfixation for 1 h in Dalton's (1955) chrome–osmium fixative. The specimens were then stained *en bloc* with 0·05 % uranyl

acetate at pH 4·9, dehydrated through a graded series of ethanol and propylene oxide, infiltrated and subsequently embedded in Epon–Araldite (Mollenhauer, 1964). For each experimental or control group, at least eight blocks were sectioned and studied by light and electron microscopy. Sections (1–2 μm) thick, were cut from the block with glass knives, picked up on glass slides, dried, stained with Toluidine Blue–Azure II and examined under a light microscope. Comparable areas in all groups were selected for electron-microscopic examination. Profiles of at least 300 epithelial cells were evaluated ultrastructurally in each experimental group. Sections with a silver–silvergold interference colour were cut for electron microscopy. The sections were picked up on Formvar–carbon-coated grids and stained with uranyl acetate and lead citrate. The stained sections were viewed in a Philips 400 electron microscope at 60 kV accelerating voltage. Electron image photographs were taken at initial magnification of 1500–20 000 diameters.

Autoradiographic analysis of thymidine-labelling patterns

Explant cultures were labelled with 0·1 μCi ml^{-1} of $[^3H]$thymidine for 72 h and washed free of label as described above. The tissue was fixed and embedded in the same manner as for electron microscopy except that OsO_4 fixation was omitted. Sections (1–2 μm thick) were cut and dried on gelatin-coated glass slides, and the plastic was removed as mentioned above. The slides were dipped in NTB-2 Kodak liquid emulsion, stored in the dark for 10–14 days and then developed in Kodak D-19. Distribution of the autoradiographic grains was determined on haematoxylin-stained sections with a Zeiss photomicroscope.

Vaginal smears from mature virgin female mice were checked daily at 8.00 a.m. to ascertain their oestrous cycle. Mice at various stages in the oestrous cycle were inoculated with 0·1 mCi of $[^3H]$thymidine at 8.30 a.m. and were killed 1 h later. The no. 2 and no. 3 mammary fat pads were excised and fixed as whole mounts on Millipore filters in 4·0 % paraformaldehyde. Subsequently, the glands were dehydrated, embedded, sectioned and stained. Stained sections were dipped in emulsion and autoradiography was performed for 12–14 days. The sections were examined for radionucleotide-labelled nuclei under phase-contrast optics.

Tissue localization of casein

Explants of mammary tissue that had been incubated with various hormone combinations were fixed and embedded in Araldite for immunocytochemical studies as described by Hogan & Smith (1982). Sections (1–2 μm thick) were cut and placed on glass slides. Embedding plastic was removed with a solution of benzene:methanolic KOH:acetone (1:1:1, by vol.). Casein in the sections was localized by an avidin–biotin indirect immunoperoxidase staining technique as described (Smith & Vonderhaar, 1981; Hogan & Smith, 1982). The specificity of the anti-casein antibody was confirmed by absorption with purified mouse caseins and by immunoprecipitation (Smith & Vonderhaar, 1981). Non-specific absorption and background peroxidase activity were assessed by examining the staining of sections after stepwise omission of each individual reagent used in the technique. Sections were cut consecutively, exposed to rabbit preimmune serum and to anti-MMTV and served as controls. The slides were coded and evaluated by two separate viewers.

Transplantation studies

Female Balb/c mice of different ages whose mammary glands were in various stages of development and differentiation were injected with 0·5 % Trypan Blue 16 h before being used as donors so that the gland was more easily visualized in the living fat pad. The donors were anaesthetized and the mammary glands were exposed and examined under a dissecting microscope. In all experiments, the smallest dissectable piece of duct, alveolus or end bud was collected and transplanted into virgin Balb/c mice (3 weeks old) whose inguinal mammary fat pads were surgically exposed and cleared of all epithelial elements as described by DeOme *et al.* (1959) and Medina (1973). In the majority of experiments, the transplant recipients were maintained as virgins for 6 weeks after transplantation. In some experiments, the recipients were subsequently mated and nursed their young for 3–5 days before the glands were collected. Whole mounts were made of all fat pads containing mammary epithelial growth and stained with haematoxylin. The extent of the cleared fat pad filled by growth of the implanted epithelium was estimated according to the method described by Daniel *et al.* (1968). The estimate of the number of epithelial cells per transplant piece was performed as described by Medina *et al.* (1978). Briefly, 40–60 transplant-sized pieces were dissected from the gland, pooled and then digested with collagenase to provide a single cell suspension. For each estimation, three to four pooled samples were evaluated. The collagenase-digested samples were washed in serum-free Dulbecco's minimal essential medium by centrifugation, resuspended, and the number of cells was determined in a Coulter counter. The total number of cells in each pooled sample was divided by the number of pieces placed in the original pool to give the average number of cells per transplant-sized piece.

Results

Transplantation studies

Different segments of the mammary parenchyma were transplanted into the cleared fat pads of young mice to assess their morphogenic potential. The results are shown in Table 1. The portions of the mammary gland taken from primary ducts of mature female virgin mice, tertiary ducts of immature and mature female virgin mice, tertiary ducts from uniparous non-pregnant non-lactating mice, 15-day pregnant mice and 10-day lactating mice, and end buds from 6-week-old mice all gave rise to well-developed ductal outgrowths in the mammary fat pad at 6 weeks after transplantation. In the majority of experiments, the values for percentage of successful transplants and percentage of fat pad filled were greater than 80 %. These two parameters were used to assess growth potential of the mammary parenchyma. The outgrowths from lactating mammary gland provided the major exception to the general pattern of growth and morphogenesis. In experiment 1

Table 1. *Morphogenic potential of different segments of mammary parenchyma of Balb/c female mice*

Type of mammary parenchyma	Donor group	Successful/total transplants (%)	$\bar{X}$ (% Fat pad filled)*	Range
3° duct	6-week virgin	13/14 (93)	100	(100)
3° duct	6-week virgin	19/20† (95)	99	(90–100)
3° duct	16-week virgin	11/12 (92)	99	(90–100)
3° duct	Uniparous regressed	16/20 (80)	85[a]	(50–100)
3° duct	Multiparous regressed	19/20 (95)	80[a]	(10–100)
1° duct	16-week virgin	19/20 (95)	94	(40–100)
Alveoli	15-day pregnant	14/16 (88)	95	(50–100)
Alveoli	10-day lactating	19/20‡ (95)	60[b]	(10–100)§
End bud	6-week virgin	15/20¶ (75)	86[a]	(25–100)

* The groups with letter superscripts (a or b) are significantly different from 3° duct of 6-week virgin. Groups with superscript b are significantly different from groups with superscript a. The data were analysed by one-way analysis variance.
† Approximately 7200 cells per transplant.
‡ Approximately 33 000 cells per transplant.
§ Few branch points were observed in the epithelial ducts compared with the outgrowths derived from mammary implants in adult virgin mice.
¶ Approximately 2200 cells per transplant.

(Table 1), transplants of lactating alveoli were successful in 95% (19 of 20) of the cases, but the mean percentage of fat pad filled, the successful transplants, was only 60%. In addition to the slower growth rate, the outgrowth exhibited fewer branch points compared with outgrowths from either virgin ductal or pregnant alveolar tissue. The slower growth could not be attributable to fewer cells in the original transplant, since there were significantly greater numbers of cells per transplant in the lactating group (33 000/transplant) compared with ducts from virgins (7200/transplant) or end buds (2200/transplant). The slow growth rate and decreased number of branches in outgrowths from lactating gland were observed in three separate experiments (Tables 2 and 3), where the percentage of successful transplants was high (49/60, 82%) but the mean percentage of fat pad filled was low (53%).

In the second set of experiments, mammary tissue fragments containing tertiary ducts from mature virgins or alveoli from lactating mice were transplanted into recipients who subsequently became pregnant and lactating, in order to examine the differentiation potential of the two groups of transplants (Table 2). The extent of growth of mammary epithelial cells derived from lactating mice once again showed a reduced capacity for repopulation of the fat pad in virgin mice. However, mammary cells from either source completely filled the fat pad in recipients who subsequently became pregnant and lactated. Full lactogenic responses were observed in all the outgrowths at 10 days of lactation regardless of the source of the implanted tissue.

To determine whether a further loss in the morphogenic capacity of lactating epithelium was experienced

Table 2. *Differentiation potential of cells derived from virgin and lactating mammary gland*

Donor	Recipient	Time (weeks)	Successful/total transplants (%)	$\bar{X}$ (% Fat pad filled)	Range
3° duct, virgin	Virgin	6	20/20 (100)	98	(60–100)
3° duct, virgin	10-day lactating	12–14	20/20 (100)	100	(100)
10-day lactating	Virgin	6	17/20 (85)	57	(10–100)
10-day lactating	10-day lactating	12–14	20/20 (100)	100	(100)

Table 3. *Morphogenic potential of different segments of lactating glands*

Donor	Group*	Successful/total transplants (%)	$\bar{X}$ (% Fat pad filled)	Range
10-day lactating	Zone 1	19/20 (95)	67	25–100
10-day lactating	Zone 2	14/20 (70)	73	10–100
20-day lactating	Zone 1	13/20 (65)	34	10–75
20-day lactating	Zone 2	13/20 (65)	68	20–95

* Zone 1, most peripheral region of gland; zone 2, intermediate region of gland.

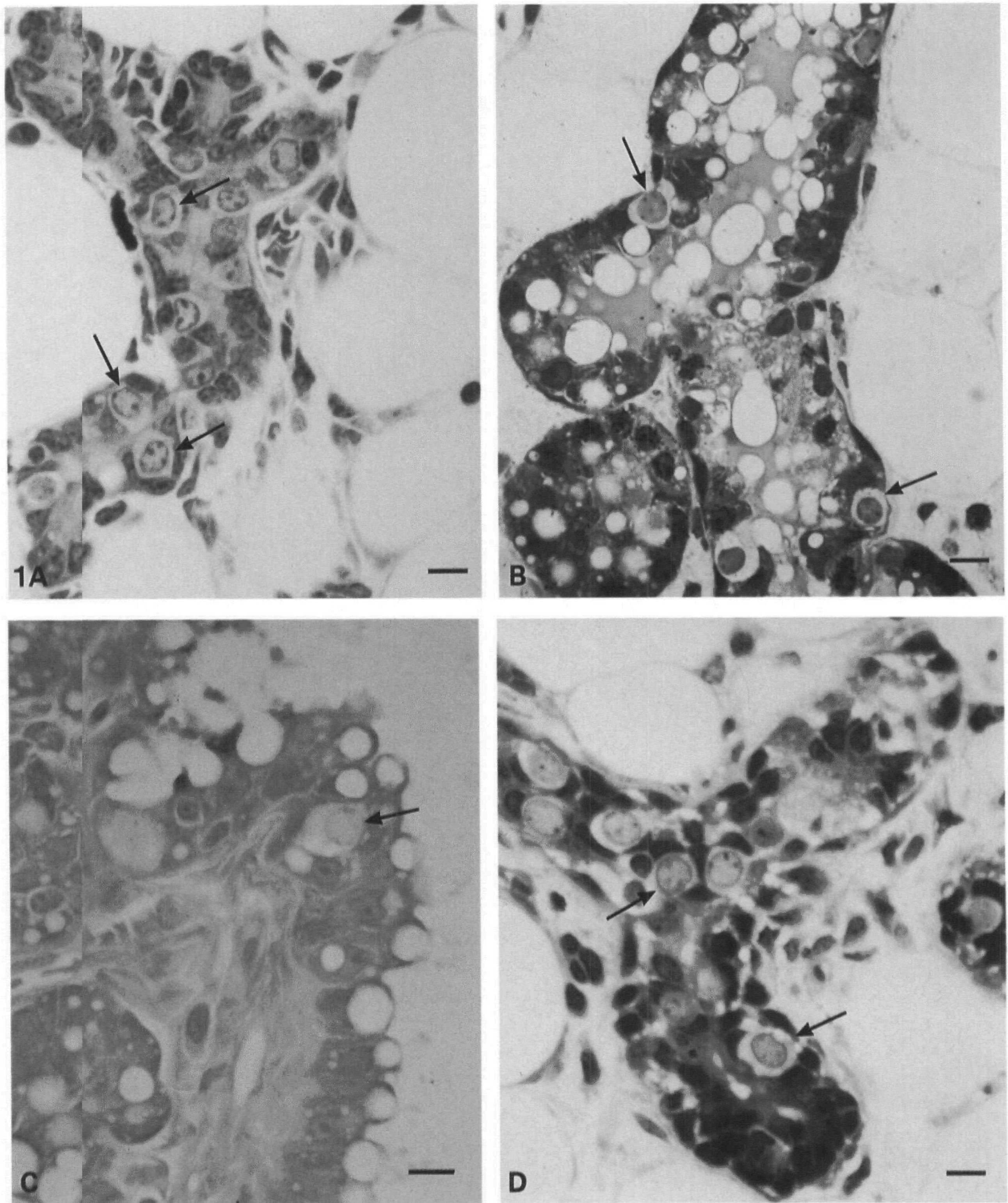
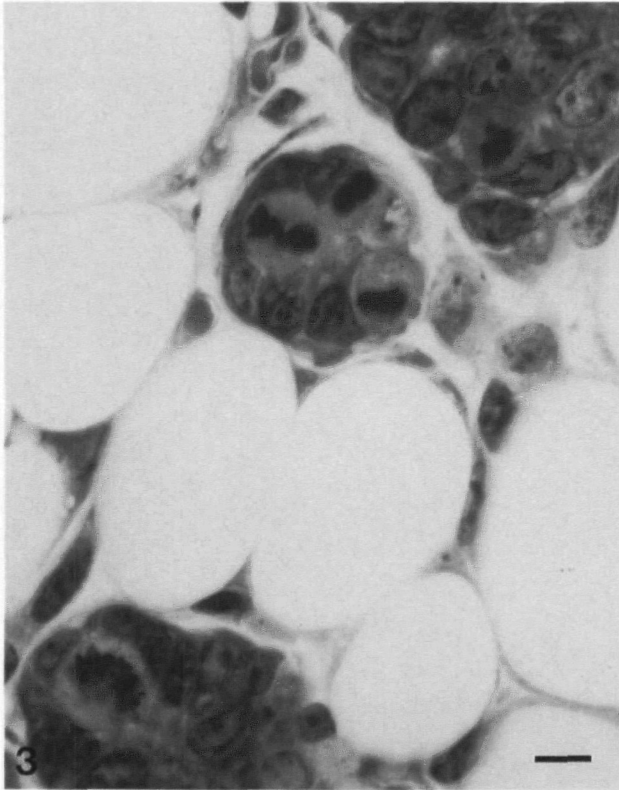
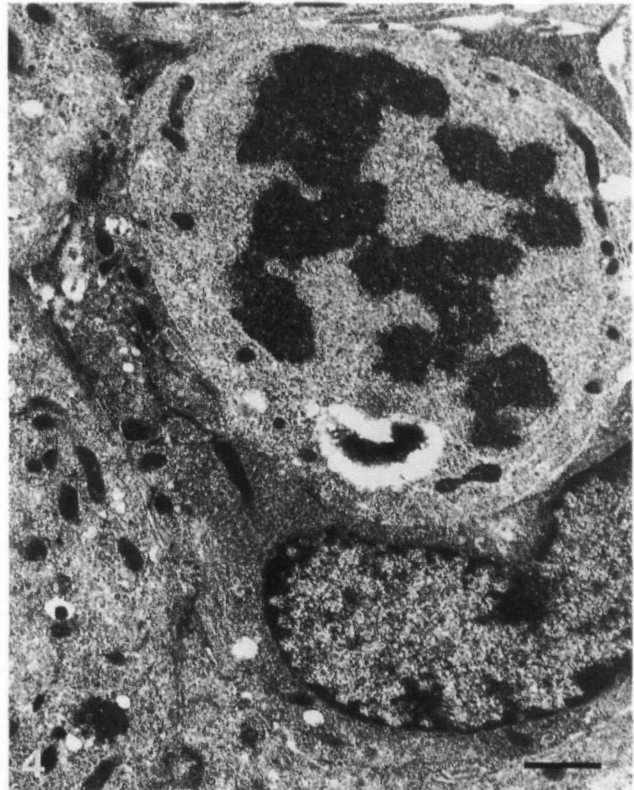
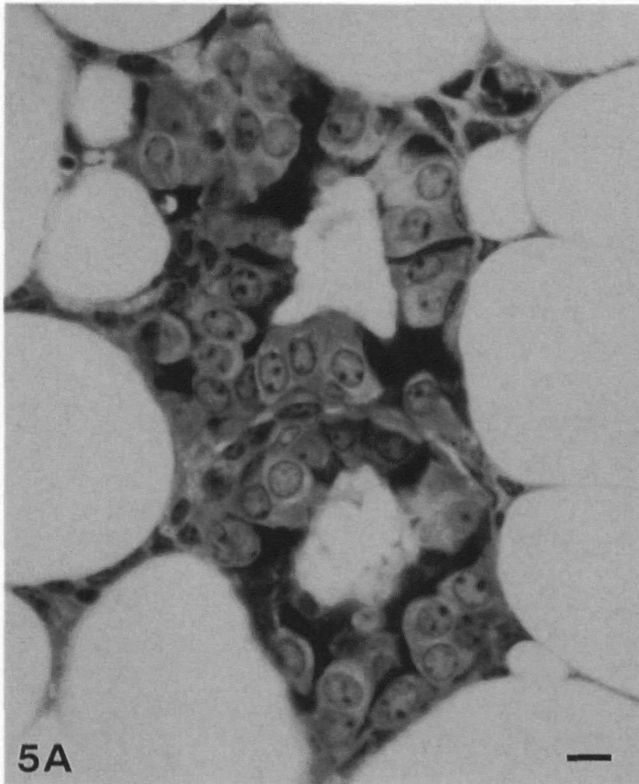


Fig. 1. Large pale-staining epithelial cells (arrows) are readily identified in Toluidine Blue-stained 1 μm thick sections of mouse mammary glands at all stages of development and differentiation: A, in the virgin mouse; B, 12 days pregnant; C, 6-day lactating; and D, in male mammary anlage that have been induced to grow by chronic stimulation with diethylstilboestrol. Bars, 10 μm.







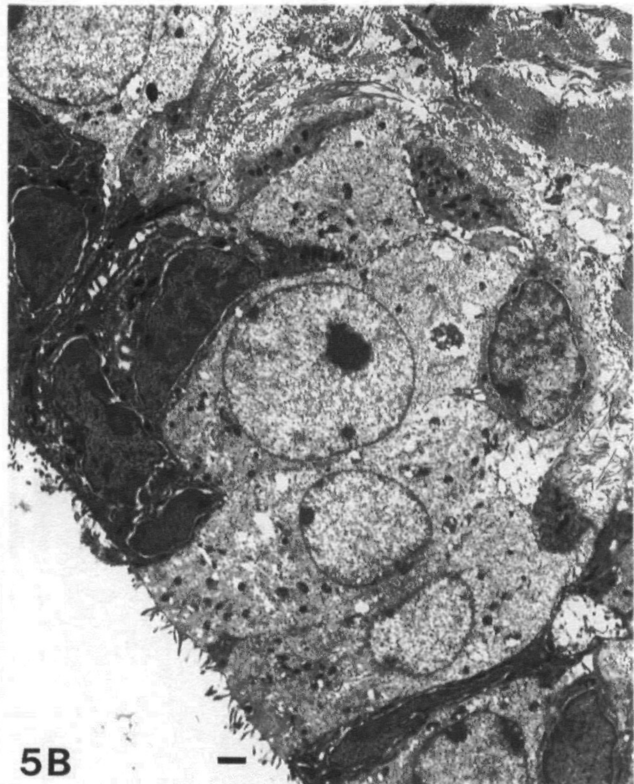


Fig. 3. Mitotic figures are found in the pale-staining mammary epithelial cells, just 4 h after explant culture in the presence or absence of araC. Bar, 10 μm.

Fig. 4. Electron microscopy of the dividing cells in the 4-h cultures confirms that they possess an ultrastructural morphology identical with the large pale-staining epithelial cells shown in Fig. 2A. Bar, 1·0 μm.

Fig. 5. In A, after 24 h in culture, pale cells appear in groups of two or more among the darker staining epithelium, supporting the conclusion that they have divided at least once during this culture period. In B, electron-microscopic analysis of these groups of pale cells suggests that, in the groups of three, a second mitotic event has occurred in one of the pale cell daughters leading to the production of two smaller clear-staining cells. Close examination shows that cellular membranes have not yet been re-established between these newly formed cells. Bars: A, 10 μm; B, 1·0 μm.

at later stages of lactation, fragments from different portions of 10-day and 20-day lactating mammary glands were transplanted (Table 3). A significant variability in repopulation potential was observed when all the lactating transplants were evaluated. However, the poorest repopulation potential was observed in transplants taken from the most peripheral portion (zone 1) of the lactating gland after 20 days of lactation. Histological examination showed that no differences in cellularity were present with respect to zone 1 and zone 2 at either 10 or 20 days of lactation.

Light and electron microscopy

Microscopic examination of the functional and cytological differentiation of hormone-stimulated mouse mammary epithelium in explants cultured in serum-free chemically defined medium revealed the presence of a morphologically distinct mammary epithelial cell. These cells were sporadically distributed among mammary epithelia in both ductal and lobuloalveolar structures (Fig. 1A). They possessed spherical nuclei and a pale-staining cytoplasm in Toluidine Blue-stained sections from plastic-embedded mammary explants. A survey of mouse mammary epithelium at various stages in the development and functional differentiation of the mammary gland indicated that these cells were present in the 16-day foetal gland (Hogg *et al.* 1983) through puberty, pregnancy, lactation (Fig. 1B,C) and involution and in glands of aged non-breeding parous or non-parous females. When the rudimentary mammary anlagen of mature male mice are stimulated to grow and develop under the influence of the synthetic oestrogen, diethylstilboestrol, pale cells identical to those found in the female glands are found distributed prominently within the newly formed mammary epithelium (Fig. 1D).

Ultrastructural examination of these morphologically distinct cells confirms their pale-staining nature and demonstrates that their cytoplasm is sparsely populated with mitochondria, ribosomes and vacuoles (Fig. 2). The mitochondria are in a condensed intracristal matrix configuration, suggesting low levels of intracellular ATP and an inactive oxidative phosphorylation and metabolism (Munn, 1974). The nuclei are large, mainly spherical and have relatively small amounts of condensed heterochromatic regions. The nucleolar region is not defined and is often not apparent. Examination of these cells in functionally differentiated mammary epithelia, such as pregnant or fully lactating glands, reveals little alteration in their ultrastructural appearance under these conditions, although their relative number when compared with the total epithelial composition of the gland is much reduced. Once again, the pale cells are found within functional lobular structures as well as in ducts and ductules. Their epithelial genealogy is demonstrated in the formation of terminal bars, tight junctional complexes and desmosomes with their functionally differentiated epithelial cell neighbours (Fig. 2B, inset). They also formed hemidesmosomes with adjacent myoepithelial cells (Fig. 2A, inset). In addition to the possession of these ultrastructural features characteristic of epithelial cells, pale cells stained positive for epithelial keratins when tested by immunocytochemical technique (not shown). These immunological observations were part of a larger study, which demonstrates that differential expression of epithelial keratins occurs during mammary gland growth and within preneoplastic and malignant mammary epithelial populations (unpublished data).

Explant culture

Explants of mammary tissue from mature virgin female mice were incubated in medium 199 in the presence of various hormone combinations. The explants were collected after culture periods of 4, 24 and 72 h and were examined for cell division, [^{3}H]thymidine incorporation, cytological differentiation and milk protein synthesis. Mitosis was determined by direct observation of semithin sections of plastic embedded explants, stained with Toluidine Blue–Azure II, after incubation in different hormone combinations for various time periods. Cellular DNA synthesis was evaluated by autoradiography of the same sections. In duplicate cultures, araC was added (15 μg ml^{-1}) to block DNA synthesis and [^{3}H]thymidine incorporation.

Prominent mitotic figures were observed among the epithelium of virgin, parous or pregnant mouse mammary gland explants cultured for 4 h in serum-free medium (Figs 3, 4). The numbers of mitotic figures were not influenced to a significant degree by the presence or absence of hormones or the hormone combinations used. However, the duration of mitotic activity or the mitotic event seemed to be prolonged in the presence of IFPrl-containing medium, as judged from the continued observation of mitotic cells in the 24-h cultures containing these hormones compared with those with insulin alone or no hormones. The mitotic cells observed in the 4-h cultures appeared to be derived from the pale cells described in the previous section. This conclusion is based on the distribution of the mitotic cells within the ducts and acini of the explants, the manifestation of pairs and groups of pale cells (presumptive evidence for pale cell division) in duplicate cultures maintained for 24 h under identical conditions (Fig. 5) and electron microscopic analysis of the very same dividing cells observed in semithin sections of 4 h explants (see Fig. 4). This examination was accomplished using ultrathin sections taken from the same areas contained in these 1 μm sections. All of these mitotic events and the subsequent appearance of

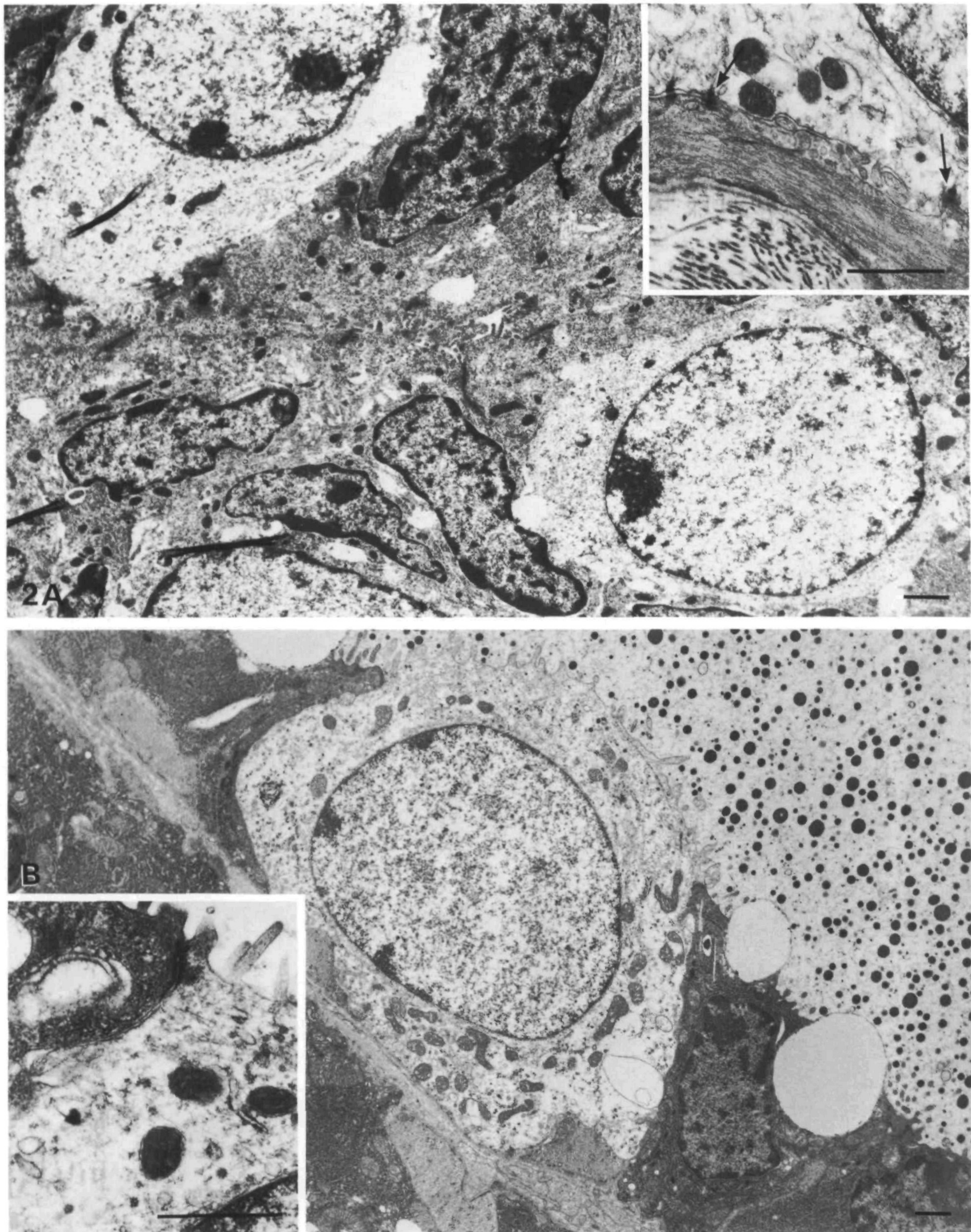


Fig. 2. The ultrastructural morphology of the pale-staining epithelial cells seen in Fig. 1A,C shows that these cells contain very few cytoplasmic organelles, such as mitochondria and ribosomes, compared with their dark-staining neighbours. As can be seen in B, very little change in the pale cell ultrastructure is observed even in the 6-day lactating gland. The epithelial pedigree of these cells is demonstrated by the formation of hemidesmosomes (arrows) with adjacent myoepithelial cells (inset in A) and terminal bars at their luminal surface when in contact with their dark-staining, fully differentiated neighbours (inset in B). Bars, 1 μm.

pairs and small groups of pale cells at 24 h within the epithelial population of the mammary explants occurred to the same extent in cultures where DNA synthesis was inhibited by araC. At 72 h, the pairs and groups of pale cells, conspicuous at 24 h, were not observed any longer in explants incubated in IFPrl-containing medium. Instead, the number of identifiable pale cells was much decreased. This observation implies that at least some of the cells produced from the mitotic events observed at early culture times had become differentiated (Fig. 6). Electron-microscopic examination of the epithelium in IFPrl-treated explants confirmed that nearly all the parenchymal epithelium had taken on a secretory ultrastructural appearance as described (Vonderhaar & Smith, 1982). In those explant cultures incubated with insulin alone or with hydrocortisone, groups of pale-staining epithelial cells remained evident at 72 h (Fig. 6B), no cytological differentiation was apparent.

DNA and RNA synthesis

Autoradiographic study of explants incubated in the presence of [^{3}H]thymidine for the entire 72 h culture period demonstrated that 49·2–56·4 % (Table 4) of the mammary epithelial cells incorporated thymidine into their nuclei in the presence of IFPrl-containing medium (Smith *et al.* 1981). Somewhat smaller numbers of epithelial cells were labelled when explants incubated with either insulin alone or no hormone for 72 h were evaluated. In no case was label discerned in the nuclei of the pale cells, although the epithelium immediately adjacent to these cells was at times heavily labelled (Fig. 7A). However, no matter what hormones were present, labelled nuclei were only rarely observed when cultures were incubated for 72 h with araC (15 μg ml^{-1}). Similar results, i.e. absence of labelling in the pale cell nuclei, were obtained when [^{3}H]thymidine was administered *in vivo* at various stages in the oestrous cycle.

Exposure of similar cultures to a 5-h pulse of [^{3}H]uridine at 48–53 h showed that pale cells incorporated and subsequently retained this radionucleotide during the 72 h culture period, suggesting that RNA-synthetic activity was present. This observation supports the conclusion that these cells are not completely dormant metabolically (Fig. 7B). No difference in the levels of incorporation of uridine into the mammary epithelium could be detected under different hormonal conditions by autoradiography with reference to the comparison of pale- or dark-staining epithelial cells.

Table 4. *Labelling index of epithelial cell populations organ culture*

Mammary tissue source	Hormones present	Cells counted	Cells labelled	%
Virgin	IFPrl	790	422	53·4
	IFPrl + araC	500	–	0
	IFPrl + 0·01 μg ml^{-1} colchine	600	328	54·1
Primiparous	IFPrl	802	400	49·9
	IFPrl + araC	620	–	0
Virgin	I*	701	157	22·3
	IFPrl*	835	422	50·5
	IFPrl + araC	420	–	0

Explants were cultured as described in Materials and methods. [^{3}H]Thymidine was present throughout the 72 h culture period and was chased for 12 h. Tissue was processed for autoradiography as described in Materials and methods.

* Labelled non-epithelial cells in cultures with insulin alone accounted for an additional 456/1651 (27·2 %) positive cells. In IFPrl, only 77/1300 (5·9 %) non-epithelial cells were labelled.

Casein synthesis and cytological differentiation

Functional differentiation in these explant cultures was determined by indirect immunoperoxidase detection of casein. Casein synthesis was detectable by immunoperoxidase as early as 24 h after incubation of virgin mouse mammary explants in IFPrl; additional amounts of casein and an increase in positive cells were found at 72 h. At 72 h, an occasional pale cell was stained positive for intracellular casein (not shown); however, most pale cells remained negative and unaffected by the presence of lactogenic hormone stimulation. Casein was not detected in virgin mouse mammary gland explants incubated in insulin alone, in hydrocortisone or without hormone stimulation. Coincidentally, the epithelial cells in these explants did not take on a secretory cytological and ultrastructural morphology as did those in explants incubated in IFPrl. The conversion to a secretory morphology occurred in the presence of IFPrl even when DNA synthesis was inhibited throughout the duration of the experiment by araC. Thus, most of the cells produced by the mitotic events observed at 4 h of culture and seen as pairs and groups of pale-staining epithelial cells at 24 h were converted to secretory cells in the presence of IFPrl. In the presence of insulin or hydrocortisone, when no casein synthesis or secretory activity was initiated, these pale cells remained non-secretory and were easily distinguished from their dark-staining neighbours (Fig. 6A).

Discussion

Normal mouse mammary epithelium has a finite capacity to regenerate functional mammary parenchyma upon serial transplantation into epithelia-free mammary fat pads (Daniel *et al.* 1968). Further, the rate of senescence (as measured by regenerative capacity) was proportional to the replication frequency imposed upon the regenerating mammary tissue as opposed to the passage of time; e.g. senescence could be reached more quickly by shortening the period between the

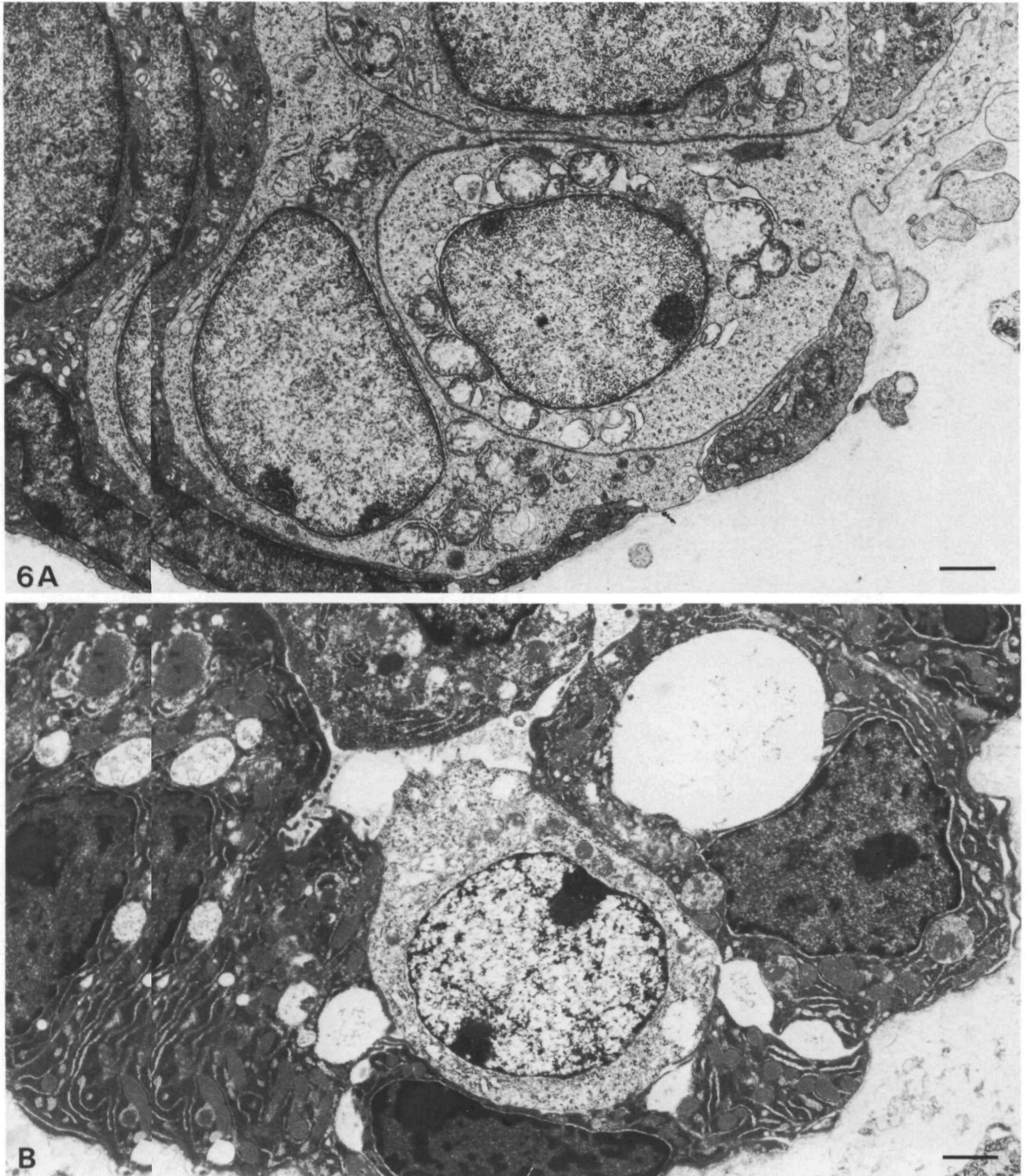
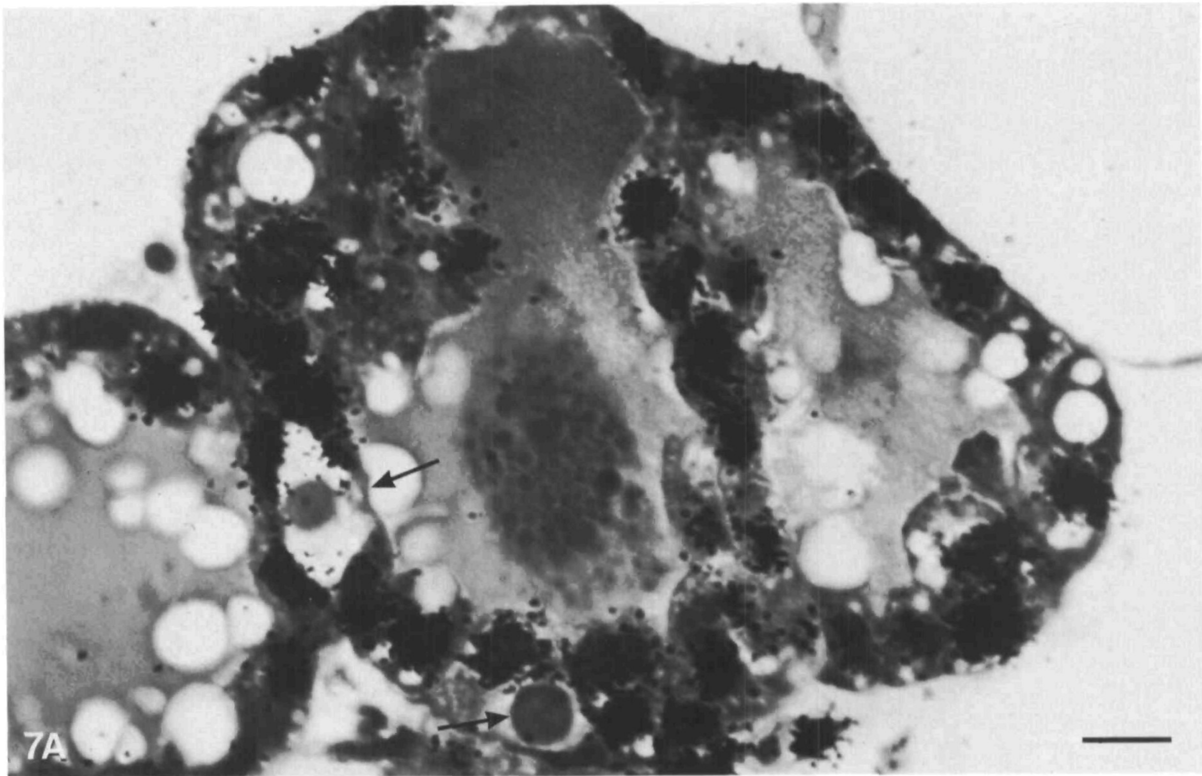


Fig. 6. Electron microscopy of explants cultured for 72 h in IF (A) or in IFPrl (B). In the presence of I and F, the pale-staining cells are still recognizable in groups after 72 h. They do not show any extensive differentiation. In contrast, in the presence of IFPrl, only occasional pale cells are found among neighbouring epithelium that has uniformly undertaken a secretory ultrastructural morphology, which includes a highly developed rough endoplasmic reticulum, apically located intracytoplasmic lipid droplets and casein micelles. Bar, 1·0 μm.



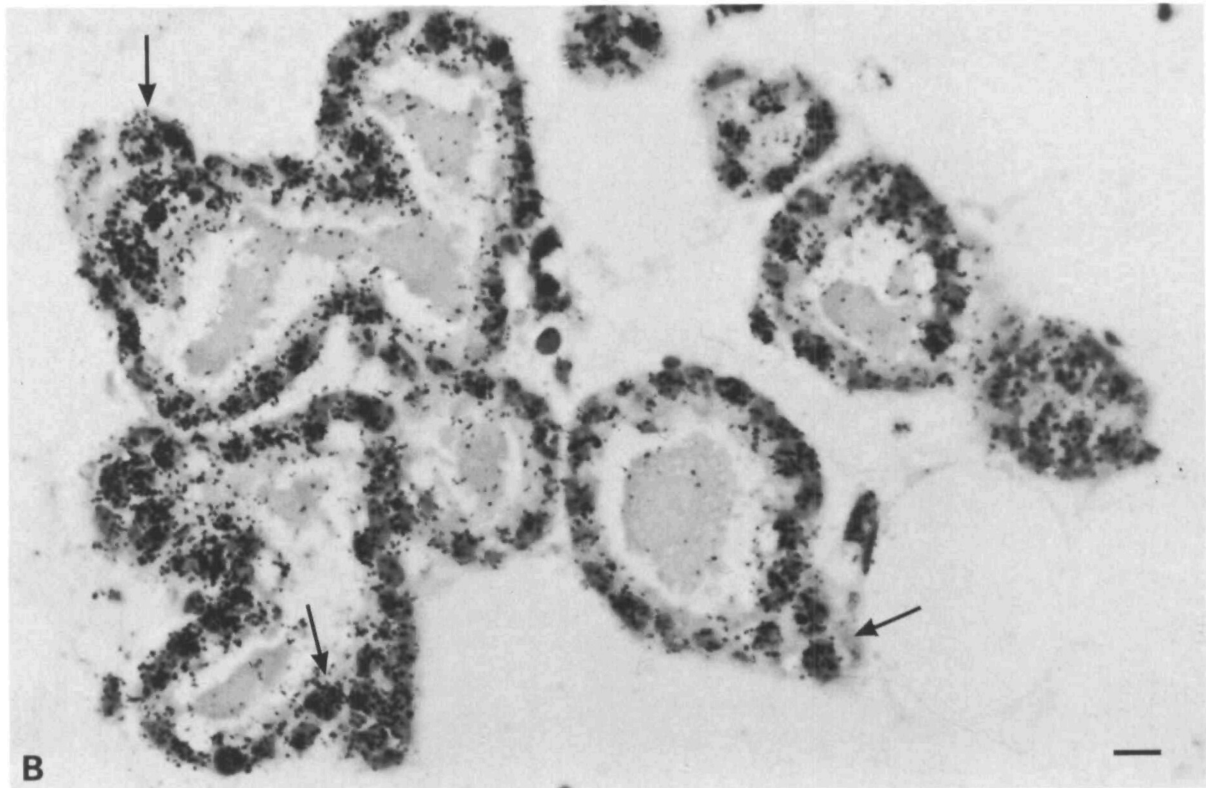


Fig. 7. Explants were cultured in IFPrl and [^{3}H]thymidine for 72 h and then with cold thymidine for an additional 16 h. Autoradiographic grains were found over the nucleus in a high percentage of the dark-staining secretory epithelial cells but never over the pale-staining cells (A). Conversely, when IFPrl-treated cultures were pulsed with [^{3}H]uridine for 5 h (48–53 h) during explant culture and subsequently in cold medium for 20 h, all the epithelial cells, including the pale cells (arrows), contained autoradiographic grains indicative of an active RNA synthetic metabolism. Bars, 10 μm.

transplantation generations (Daniel, 1972, 1975). This fact suggests that the indigenous mammary epithelial stem cells undergo only a finite number of cell divisions before they themselves become replicatively defunct. Very similar results are obtained when normal mammary epithelium is cultured *in vitro*, i.e. the cultured epithelial cells lose their ability to repopulate the cleared mammary fat pads of syngeneic or nude mice as a consequence of the number of subculture passages rather than the duration of their maintenance *in vitro* (Ehmann *et al.* 1987; Larry Young, personal communication).

Examination of mouse mammary epithelium *in situ* and during explant culture *in vitro* reveals the presence of morphologically distinct, pale-staining cells among the mammary epithelium that appear to behave in a manner consistent with the concept that their primary role is to generate mammary epithelial cells by cell division. Our results demonstrate that: (1) these pale cells are not wandering lymphocytes, since they form desmosomes, hemidesmosomes and terminal bars with their neighbours, and they are present at all stages of mammary development and functional differentiation; (2) pale cells undergo cell division shortly after being placed in culture; (3) these cells undertake this mitotic event despite the inhibition of DNA synthesis; they are dormant with regard to DNA synthesis both *in situ* and throughout the explant culture period, even though they incorporate the nucleotide precursors necessary for RNA fabrication; and (4) finally, albeit indirectly, the daughter cells produced from their mitotic events differentiate functionally in explant culture in the presence but not in the absence of lactogenic hormonal combinations.

Pale-staining or clear mammary cells have been described in normal human mammary gland (Toker, 1967), particularly in association with the basal layer in mammary ductules and occasionally extending from the basal lamina to the luminal surface as we have seen in the mouse gland. In addition, pale cells were found in lactating rat mammary gland by Helminen & Ericsson (1968). However, in this study the authors did not distinguish these pale cells from 'wandering lymphocytes' as we have done in this study. Recently, clear epithelial cells that undertake mitosis *in situ* during the functional differentiation of the rat mammary gland were characterized by Chepko (personal communication).

Our transplantation studies suggest that the cells responsible for repopulation of the mammary fat pads are present throughout the gland and are not localized to any given portion, a conclusion similar to that reached by Hoshino (1978). Furthermore, the mammary epithelial stem cells are resident in different stages of mammary gland differentiation (i.e. virgin, pregnant, involuted) and with the exception of lactating gland, there was no significant variation in the ability of various differentiation stages of mammary tissue to repopulate the cleared fat pad. Even the reduced capacity of lactating gland to repopulate the fat pad is eradicated if the recipient is allowed to undergo pregnancy and lactation (Table 2). These observations argue for the existence of dormant mammary epithelial stem cells at each stage of mammary differentiation, which upon transplantation give rise through cell division to daughter cells capable of further replication. Presumably these stem cells are functionally active at the growing ends of the repopulating implant (i.e. end buds) where, like cap cells at the growing ends of the expanding indigenous mammary epithelia of intact fat pads (Williams & Daniel, 1983), they can give rise to both glandular and myoepithelial components of the mammary epithelial population; however, they are also disposed throughout the mammary parenchyma. Senescence or the loss of the ability to repopulate the fat pad is conceivably related to the loss of the dormant stem (pale) cell populations. Conversely, we speculate that immortal mammary cell populations, such as preneoplastic alveolar nodules, continue to maintain their pale cells upon serial transplantation. We are currently testing this hypothesis by serial transplantation of normal mammary ducts and preneoplastic mammary cell populations, and by identification of unique antigenic determinants that may be expressed by these dormant stem cells.

The authors are grateful to Inger Margolis and Lance Liotta for their kind assistance during the electron-microscopic analysis of the mammary explants, and to Frances S. Kittrell for excellent technical assistance during the transplantation experiments.

References

BRESCIANI, F. (1965). Effect of ovarian hormones on duration of DNA synthesis in cells of the C3H mouse mammary gland. *Expl Cell Res.* **38,** 13–82.

DALTON, A. J. (1955). A chrome–osmium fixative for electron microscopy. *Anat. Rec.* **121,** 281.

DANIEL, C. W. (1972). Aging of cells during serial propagation *in vivo*. *Adv. Gerontol. Res.* **4,** 167–199.

DANIEL, C. W. (1975). Regulation of cell division in aging mouse mammary epithelium. *Adv. exp. Med. Biol.* **52,** 1–19.

DANIEL, C. W., DEOME, K. B., YOUNG, L. J. T., BLAIR, P. B. & FAULKIN, L. J. (1968). The *in vivo* life span of normal and preneoplastic mouse mammary glands: a serial transplantation study. *Proc. natn. Acad. Sci. U.S.A.* **61,** 53–60.

DEOME, K. B., FAULKIN, L. J., BERN, H. A. & BLAIR, P. B. (1959). Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free

mammary fat pads of female C3H mice. *Cancer Res.* **19,** 515–520.
EHMANN, U. K., GUZMAN, R. C., OSBORN, R. C., YOUNG, L. J. T., CARDIFF, R. D. & NANDI, S. (1987). Cultured mouse mammary epithelial cells: normal phenotype after implantation. *J. natn. Cancer Inst.* **78,** 751–757.
FAULKIN, L. J. & DEOME, K. B. (1960). Regulation of growth and spacing of gland elements in the mammary fat pad of the C3H mouse. *J. natn. Cancer Inst.* **24,** 953–963.
HELMINEN, H. J. & ERICSSON, J. L. E. (1968). Studies on mammary gland involution. 1. On the ultrastructure of the lactating mammary gland. *J. Ultrastruct. Res.* **25,** 193–213.
HOGAN, D. L. & SMITH, G. H. (1982). Unconventional application of standard light and electron immunocytochemical analysis to aldehyde-fixed Araldite-embedded tissues. *J. Histochem. Cytochem.* **30,** 1301–1306.
HOGG, N. A. S., HARRISON, C. J. & TICKLE, C. (1983). Lumen formation in the developing mouse mammary gland. *J. Embryol. exp. Morph.* **73,** 39–57.
HOSHINO, K. (1962). Morphogenesis and growth potentiality of mammary glands in mice. I. Transplantability and growth potentiality of mammary tissue of virgin mice. *J. natn. Cancer Inst.* **29,** 835–851.
HOSHINO, K. (1978). Mammary transplantation and its histogenesis in mice. In *Physiology of Mammary Glands* (ed. A. Yokoyama, H. Mizuno & H. Nagasawa), pp. 163–228. Baltimore: University Park Press.
MEDINA, D. (1973). Preneoplastic lesions in mouse mammary tumorigenesis. *Meth. Cancer Res.* **7,** 3–53.
MEDINA, D., SHEPHERD, F. & GROPP, T. (1978). Enhancement of the tumorigenicity of preneoplastic mammary nodule lines by enzymatic dissociation. *J. natn. Cancer Inst.* **60,** 1121–1126.
MOLLENHAUER, H. H. (1964). Plastic embedding mixtures for use in electron microscopy. *Stain Technol.* **39,** 111–114.
MUNN, E. A. (1974). Relationships between mitochondrial structure, metabolic control and ion transport. In *The Structure of Mitochondria* (ed. E. A. Munn), pp. 320–350. London, New York: Academic Press.
OWENS, I., VONDERHAAR, B. K. & TOPPER, Y. J. (1973). Concerning the necessary coupling of development to proliferation of mouse mammary epithelial cells. *J. biol. Chem.* **248,** 472–477.
SILBERSTEIN, G. B. & DANIEL, C. W. (1982). Glycosaminoglycans in the basal lamina and extracellular matrix of the developing mouse mammary duct. *Devl Biol.* **90,** 215–222.
SMITH, G. H. (1987). Functional differentiation of virgin mouse mammary epithelium in explant culture is dependent upon extracellular proline. *J. cell. Physiol.* **131,** 190–199.
SMITH, G. H. & VONDERHAAR, B. K. (1981). Functional differentiation in mouse mammary gland epithelium is attained through DNA synthesis inconsequent of mitosis. *Devl Biol.* **88,** 167–179.
TOKER, C. (1967). Observations on the ultrastructure of a mammary ductule. *J. Ultrastruct. Res.* **21,** 9–25.
TOPPER, Y. J., OKA, T. & VONDERHAAR, B. K. (1975). Techniques for studying development of normal epithelial cells in organ culture. *Meth. Enzym.* **39,** 443–454.
VONDERHAAR, B. K. & SMITH, G. H. (1982). Dissociation of cytological and functional differentiation in virgin mouse mammary gland during inhibition of DNA synthesis. *J. Cell Sci.* **53,** 97–114.
WICHA, M. S., LIOTTA, L. A. & KIDWELL, W. R. (1979). Effects of free fatty acids on the growth of normal and neoplastic rat mammary epithelial cells. *Cancer Res.* **39,** 426–435.
WILLIAMS, J. M. & DANIEL, C. W. (1983). Mammary ductal elongation: differentiation of myoepithelium and basal lamina during branching morphogenesis. *Devl Biol.* **97,** 274–290.

(Received 24 November 1987 – Accepted 15 January 1988)