ISOLATION OF THE EPITHELIAL SUBCOMPONENTS OF THE MOUSE MAMMARY GLAND FOR TISSUE-LEVEL CULTURE STUDIES

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SUMMARY: We have developed a tissue dissociation procedure for the mouse mammary gland whereby it is possible to isolate the parenchyma as intact structural subcomponents essentially free of mesenchyme. Whole mammary fat pads are coarsely minced and subjected to a limited collagenase digestion, with mechanical dissociation for 90 min at 37° C. The parenchyma is released as a mixture of multicellular organoids and monodispersed cells. Use of a graded series of filters (400, 250, 150, 95, and 51 μm pore size) allows the separation of parenchyma from non-parenchymal material, with a further enrichment of the former into ductal, ductal-lobular, and terminal end-bud or alveolar populations. Yield is variable and dependent upon the nature of the starting tissue, e.g., mouse strain, age, and parity. These organoid fractions may be established separately in culture. This procedure allows for the study of the ductal and terminal regions of the mouse mammary gland in culture at the tissue level.

Key words: mouse mammary gland; tissue dissociation; ducts; end-buds.

I. INTRODUCTION

The unique physiology and pathobiology of the terminal epithelial region of the mouse mammary gland have been poorly studied at the tissue level in culture, due partly to inadequate tissue isolation procedures. We describe a simple and efficient method for the isolation of the epithelial subcomponents of the mouse mammary gland for tissue-level culture studies. Preservation of the structural integrity of the glandular epithelium aids in the maintenance of its differentiated function in culture. The ability to provide discrete ductal and end-bud (or alveolar) populations for tissue-level culture studies is important in our efforts to understand the nature of the differences in growth pattern and function between the two regions of the gland during adolescence, pregnancy, senescence, and disease.

II. MATERIALS

A. Animals

Female mice of choice, at any stage beyond 12 wk (e.g., BALB/c or Swiss Webster; virgin, hormone-primed, midpregnant, or lactating).

B. Dissection equipment

Sterile dissecting area or culture hood

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Dissecting instruments
One pair small scissors
One pair iris scissors
One pair small forceps for dissecting
Pins
Dissecting board
Instrument sterilizer
Pre-weighed sterile 35-mm tissue culture dish, Cat. no. 3001, Falcon[1]

C. Dissecting materials

95% Ethanol
Sterile glass-distilled water
Culture medium of choice (e.g., Dulbecco's Modified Eagle Medium, supplied in powder form to make 1 liter, Cat. no. 430-1600, GIBCO[2]

D. Tissue dissociation equipment

Incubator at 37° C
Multipurpose rotator, Model no. 151, Scientific Industries Inc.[3]
Universal specimen tubes, 30-ml capacity, with conical bottoms, Cat. no. 10500, Superior Plastic Products[4]
"Shortie" pipettes, Bellco Glass Inc.[5]
25-ml Capacity, Cat. no. 1228-25050
10-ml Capacity, Cat. no. 1226-10020
5-ml Capacity, Cat. no. 1226-05010
Inverted microscope with ×4 objective
Benchtop centrifuge, Model HN, International Equipment Co.[6]
Acrodisc sterilizing filters, 0.2 μm, Cat. no. 4192, Gelman[7]
ACRO 50 sterile filter unit, 0.2 μm, Cat. no. 4251[7]
Nuclepore filter holder unit with 0.8-μm pore-size presterilizing filter, Cat. no. 420410 Nuclepore[8]

E. Tissue dissociation materials

Collagenase Type I-A, Cat. no. C-9891, Sigma[9]
Bovine serum albumin, Fraction V, Cat. no. A-8022[9]
Deoxyribonuclease I from beef pancreas, Cat. no. DN-25[9]
Medium of choice
Sodium hydrogen carbonate, Analar grade, Cat. no. 1-3506, Baker[10]
Penicillin
Streptomycin
1.0 *N* Hydrochloric acid

F. Filtration equipment

Aluminum plates 9 cm × 9 cm × 2 mm, with a 3-cm diam hole placed centrally (5 pairs required)
Steel clips
Swiss polyester monofilament screen fabric ("Pecap"), Tetko Inc.[11]
400-μm Pore size, Cat. no. HC7-400
250-μm Pore size, Cat. no. HC7-250
Swiss nylon monofilament screen fabric ("Nitex")[11]
150-μm Pore size, Cat. no. HC3-150
95-μm Pore size, Cat. no. HC3-95
51-μm Pore size, Cat. no. HC3-51
250-ml Pyrex brand beakers, Cat. no. 1003-250, Corning[12]
"Shortie" pipettes, 10-ml capacity
Universal specimen tubes, 30-ml capacity, with conical bottoms
Incubator at 37° C
Multipurpose rotator
Inverted microscope with ×4 objective
Pipet-Aid automatic pipettor, Cat. no. 82, Drummond[13]
Disposable hypodermic syringe, 30-ml capacity, Cat. no. 5662, Plastipak[14]
Hypodermic needles, 23 g 3/4 gauge, Cat. no. BD5143[14]

G. Filtration materials

Culture medium of choice
Bovine serum albumin

H. Solutions

Culture medium

1. For 1 liter of culture medium, dissolve one 1-liter package of powdered DME medium in 1 liter of sterile glass-distilled water.
2. Add penicillin (50 mg/l) and streptomycin (100 mg/l).
3. Add sodium hydrogen carbonate as directed on the package (3.7 g/l).
4. Adjust pH to 7.4 by dropwise addition of sterile 1.0 *N* hydrochloric acid.
5. Filter-sterilize through a 0.2-μm ACRO 50 filter unit.
6. Store refrigerated in bottles, at 4° C.

Digestion mixture

To prepare 30 ml of digestion mixture, proceed as follows:

1. Weigh out 300 mg BSA in a 30-ml Universal specimen tube to give a final concentration of 10 mg/ml of digestion mixture.
2. Weigh out collagenase to give a final enzyme activity of 250 U/ml of digestion mixture and transfer to above tube containing BSA.
3. Add 30 ml culture medium slowly to tube, close cap securely, and allow contents to solubilize by placing tube to mix gently (30 rpm) on a rotary mixer at 37° C, for 20 min.

4. Pass mixture through a 0.8-μm Nuclepore membrane filter.
5. Sterilize mixture through a 0.2-μm Acrodisc sterilizing filter.
6. Maintain digestion mixture at 37° C.

DNase solution

1. Dissolve DNase at 1 mg/ml in glass-distilled water.
2. Filter sterilize through a 0.2-μm Acrodisc sterilizing filter.
3. Store frozen until required for use.

Wash medium

1. Add BSA to culture medium at 10 mg/ml.
2. Filter-sterilize wash medium through 0.2 μm ACRO 50 filter unit.
3. Use at 37° C.

Preparation of filters

1. Cut out 6-cm squares of the whole range of meshes from 400-μm pore size to 51-μm pore size.
2. Wash each filter square sequentially in absolute ethanol and acetone, then rinse well in distilled water, in order to remove all traces of loom oil; allow filter squares to dry.
3. Place each mesh between two aluminum holders and secure filter assembly together with two clips.
4. Autoclave filter assemblies prior to use.

III. PROCEDURE

A. Dissection

Carry out all procedures under sterile conditions.

1. Kill mice by cervical dislocation.
2. Pin each of the animals to the dissecting surface with ventral side upwards and sterilize exposed surface with 95% ethanol.
3. Make an incision along the ventral midline of the body from the base of the abdomen to the thoracic region.
4. Cut from base of abdomen along both thighs as far as the knee joint.
5. Draw skin flaps back and pin down.
6. Cut away muscle band terminating in nipple region of no. 4 inguinal mammary gland on each side.
7. Dissect out pair of no. 4 inguinal mammary glands as whole structures.
8. Transfer glands to preweighed culture dish containing 2 ml medium of choice.
9. Repeat Steps 1 to 8 for all mice.
10. Rinse instruments in distilled water and then in 95% ethanol between mice.
11. Weigh dish and contents and calculate total amount of tissue obtained.
12. Transfer tissue onto a flat glass surface and cut up tissue into small fragments, approximately 2 mm^3, using a pair of crossed scalpel blades.
13. Place tissue in 30-ml Universal specimen tube containing 30 ml warm (37° C) wash medium.
14. Place contents of tube to wash for 30 min on a rotary mixer set at 60 rpm, at 37° C.

B. Tissue dissociation (Fig. 1).

1. Allow tube to stand for 5 min. Using a pair of iris forceps, transfer floating mammary tissue into collagenase mixture up to a maximum of 2 g of tissue/30 ml of digestion mixture. When adding additional material add extra digestion mixture.
2. Set up mixture for digestion at 37° C on a rotary mixer for a total of 90 min. Set rotation speed at approximately 50 rpm.
3. Take tube off mixer at 30 min of digestion. Break up tissue fragments using a 25-ml "Shortie" pipette with a large orifice with the aid of Pipet-Aid automatic pipettor. Terminate pipetting once the tissue has broken down and passes through the pipette easily. Return tube to mixer for further digestion.
4. Repeat Step 3 at 60 min of digestion, but this time use a 10-ml "Shortie" pipette. Ensure good breakdown of tissue and a totally disrupted fat component. Check on progress of tissue disruption under an inverted microscope. If the fat forms an emulsion that makes microscopic visualization difficult, you may obtain a rough check on the progress of the tissue dissociation by merely placing the tube to rest on its side for 2 min and observing the tube from below. At this stage, it should be possible to find much tissue material at the bottom of the tube. On close inspection, it should be possible to identify large ductal structures. When this stage is reached, continue with the next phase of the digestion.
5. Repeat tissue disruption again at 90 min, using a 5-ml "Shortie" pipette. The disruption at this stage serves to break the epithelial component down into its separate glandular subcomponents, i.e. ducts, and end buds/lobules or alveoli.

6. Allow tube to stand for 3 min.
   a. If fat layer at the top of the tube has a clean milky-white appearance then proceed with the next stage.
   b. If the fat layer still has distinct globular and particulate material in it, then this suggests incomplete breakdown of the adipose component of the gland and incomplete release of the parenchyma. If this is the case, break up the fat layer with a 5-ml "Shortie" pipette and return material for further digestion for 15 to 30 min at 37° C on mixer. Repeat pipetting with 5-ml pipette at the end of the additional period of digestion.
7. Spin down contents of tube at 200 ×*g* for 5 min while tube is still warm (37° C).
8. Use a hand held Pasteur pipette to remove fat layer and two-thirds of supernatant fluid.
9. Add wash medium up to 30 ml and resuspend tissue pellet in wash medium for 10 min at 37° C.

TISSUE DISSOCIATION PROCEDURE

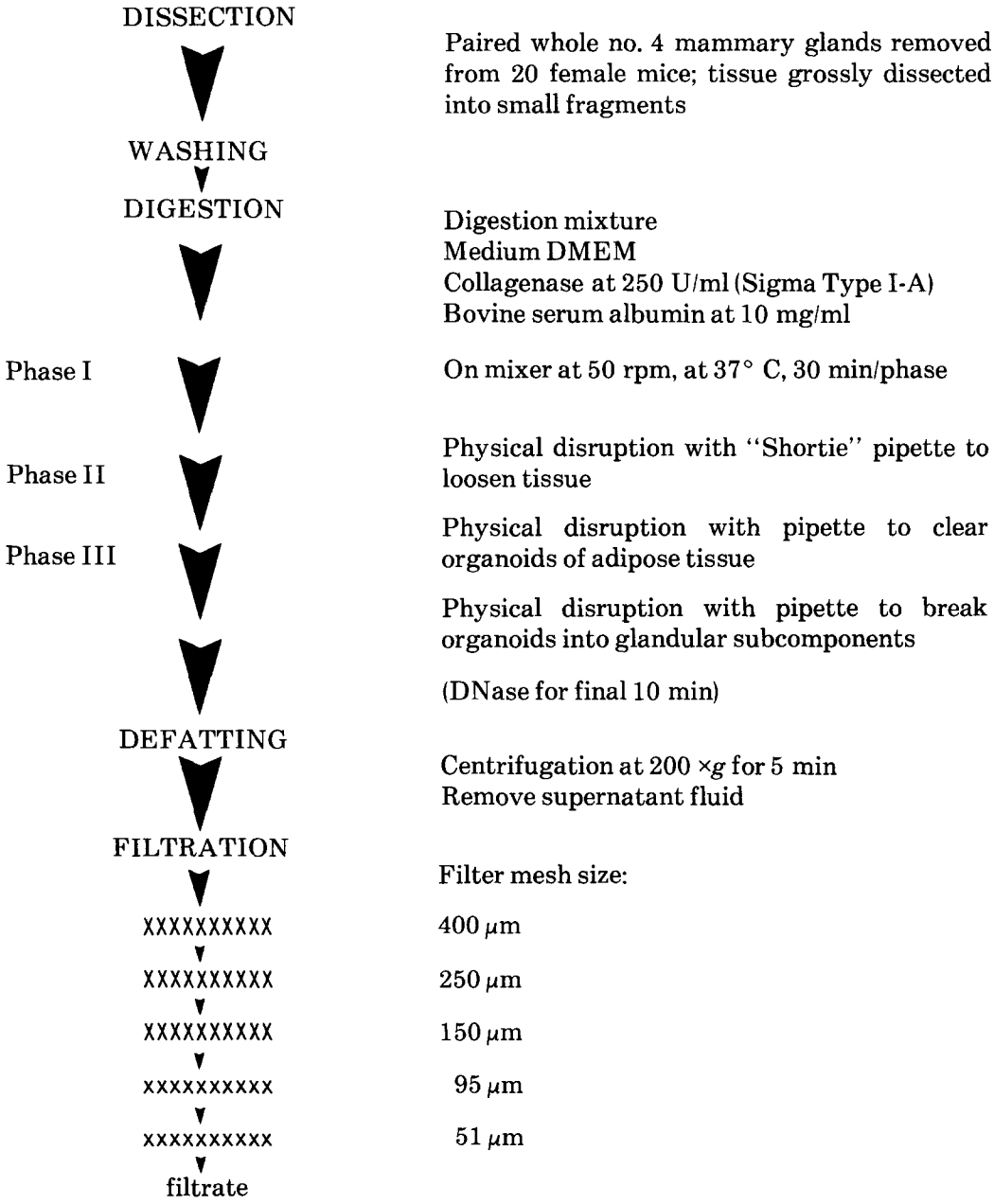

FIG. 1. Tissue dissociation procedure.

10. Repeat washing.
11. If the parenchymal tissue appears to be sticking together as a mass, then add DNase to the medium to 0.01% and return material for incubation at 37° C on a mixer for 10 min. Stickiness of the tissue material is usually due to release of DNA as a result of cellular damage.

C. Filtration procedure (Fig. 2).

1. Place 400-μm filter assembly over an open 250-ml beaker. Wet the mesh on the top surface, remove excess fluid, and then invert filter assembly. This procedure serves to break down surface tension effects on the filter.
2. Pass the tissue released by the dissociation procedure through the filter, using a 10-ml "Shortie" pipette. Material whose cross-sectional dimension is smaller than the diagonal of the filter pores will pass through the filter into the collecting beaker below.
3. Wash the material retained upon the filter with 20 ml of wash medium in order to rinse through any attached smaller organoid material.
4. Drain the filter before inverting over another 250-ml beaker and wash the collected material off into the beaker below by forcing medium through a hypodermic syringe with needle attached. Transfer this material into a 30-ml Universal specimen tube, make up the volume to 30 ml with warm wash medium, and place the tube on a rotary mixer set at 30 rpm for washing at 37° C. This aids in rinsing away the collagenase from the organoids. Mark the tube with details of its contents.
5. Repeat Steps C1-4 for all the other filters in the series (filters of 250-, 150-, 95-, and 51-μm pore size, respectively). Collect each of the filter-retained tissue fractions separately and wash. The 51-μm filtrate fraction may be collected by centrifugation at 1000 ×*g* for 5 min, followed by suspension of the cell pellet in warm wash medium. This fraction contains mainly small epithelial cell clusters and stromal cells. Cell clusters may be separated from single cells by Percoll density centrifugation (4).
6. After all the filter collected fractions have been obtained, introduce them into fresh wash medium and place them on a mixer set at 30 rpm for 30 min at 37° C.

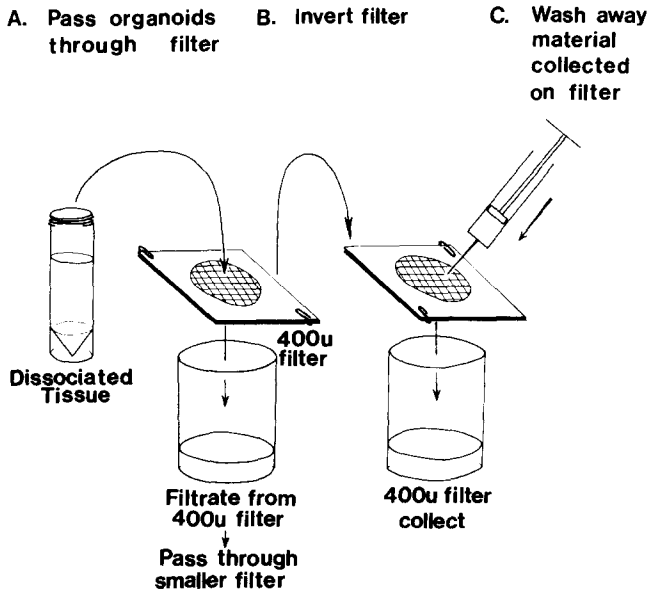

FIG. 2. Filtration procedure.

D. Subsequent utilization of the filter-collected tissue fractions.

Figure 3 demonstrates a typical parenchymal yield obtained on each of the filters used in the filtration procedure.

1. Each of the filter-collected fractions may be established in culture as separate populations. Ductal organoids collect upon the largest of the filters in the series, i.e. the 400- and 250-μm filters. Nerve fibers also collect upon these two filters, with intact lymph nodes collecting upon the 400-μm filter. The fractions collected upon these two filters may require some purification by removal of unwanted material by micromanipulation under a dissecting microscope. The parenchymal tissue collecting upon the smaller filters displays a trend towards smaller ductal structures with attached end buds or lobules/alveoli (dependent upon the starting tissue). The 51-μm fraction is enriched for the terminal epithelial population. If the filtration procedure fails to provide an adequate fractionation and separation of the glandular subcomponents, then each fraction may be enriched further by separating the organoids mechanically with a micropipette under a dissecting microscope.
2. The organoid populations may be disrupted by conventional cell dispersion techniques using trypsin or pronase (1) to provide an enriched monodispersed mammary epithelial cell suspension.

E. Characterization of the parenchymal component released by the dissociation procedure.

The dissociation procedure releases the parenchymal component of the mammary gland as intact structural units essentially free of stroma (organoids). Ducts and lobular units were clearly recognizable as such and their description correlated well with the histology of each fraction. Figure 4 shows representative sections of ductal, lobular, and end-bud fractions obtained from mammary tissue from 12-wk-old virgin BALB/c mice. The organoids are relatively free of stromal cells and matrix. Cell-to-cell relationships of the parenchymal fraction are also preserved, with the organoids displaying well-preserved structural integrity and microarchitecture.

There is occasionally a small population of adherent stromal fibroblasts that proliferate rapidly in culture. Such contamination may be further reduced by establishing the organoids in culture within a collagen gel matrix (5) for 3 d in a simple maintenance medium consisting of DMEM with insulin (10 μg/ml) and BSA (5 μg/ml). During this "preliminary culture period" contaminating fibroblasts migrate into

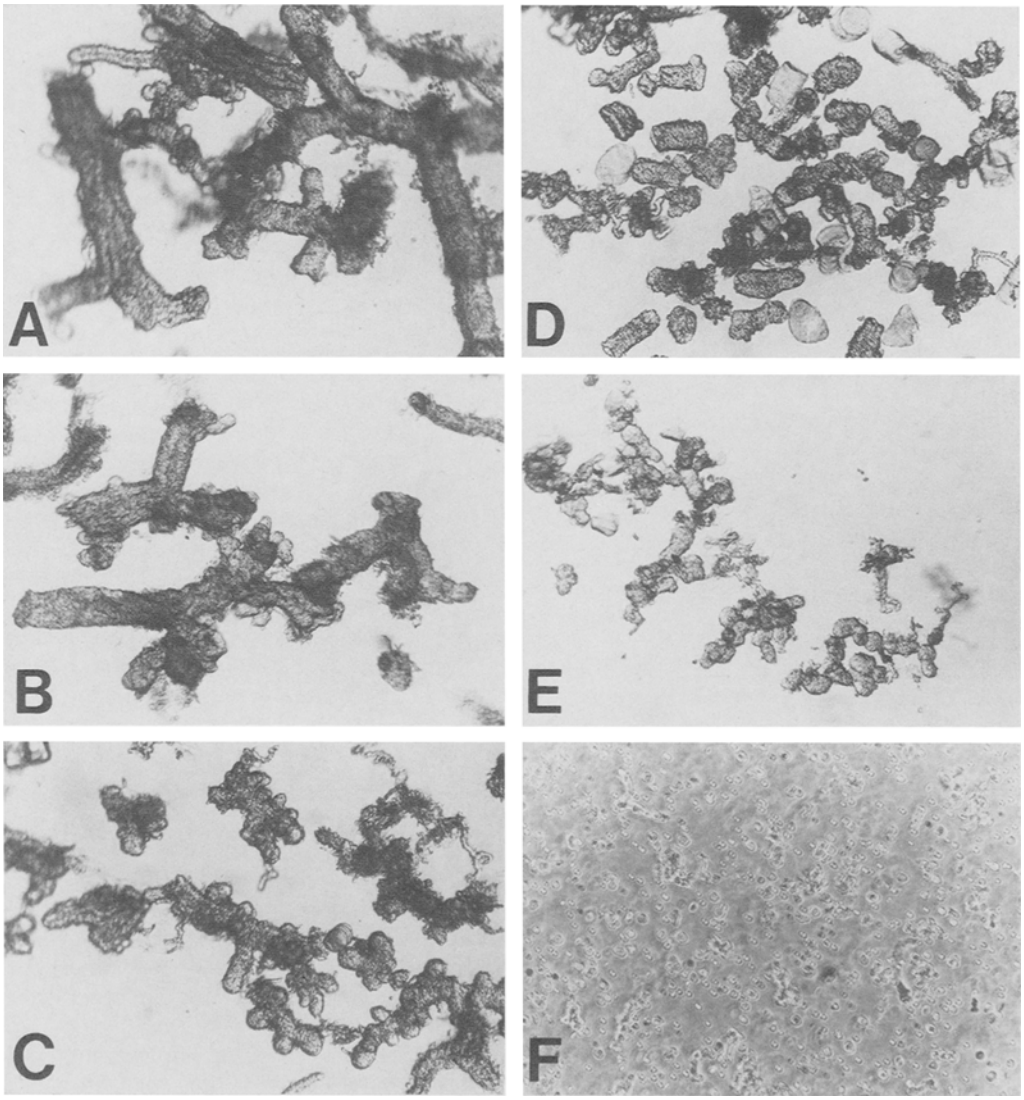

FIG. 3. Filter-collected fractions of tissue derived from mammary glands of 12-wk-old virgin BALB/c mice. *A*, 400-μm Filter-collected material, consisting of large ducts. ×38; *B*, 250-μm filter-collected material, consisting of ducts. ×38; *C*, 150-μm filter-collected material, consisting of ductal-lobular structures and lobular units. ×38; *D*, 95-μm filter-collected material, consisting of small ducts and lobular units. ×38; *E*, 51-μm filter-collected material, consisting of lobular units and terminal end buds. ×38; *F*, filtrate fraction from 51-μm filter, consisting of epithelial clusters, epithelial cells, and stromal cells. ×95.

the surrounding gel. Digestion of the gel with collagenase (Sigma Type I-A, 50 U/ml in medium of choice) releases the fibroblasts as a suspension of monodispersed cells readily separated from the organoid fraction either by settling under unit gravity or a further filtration step.

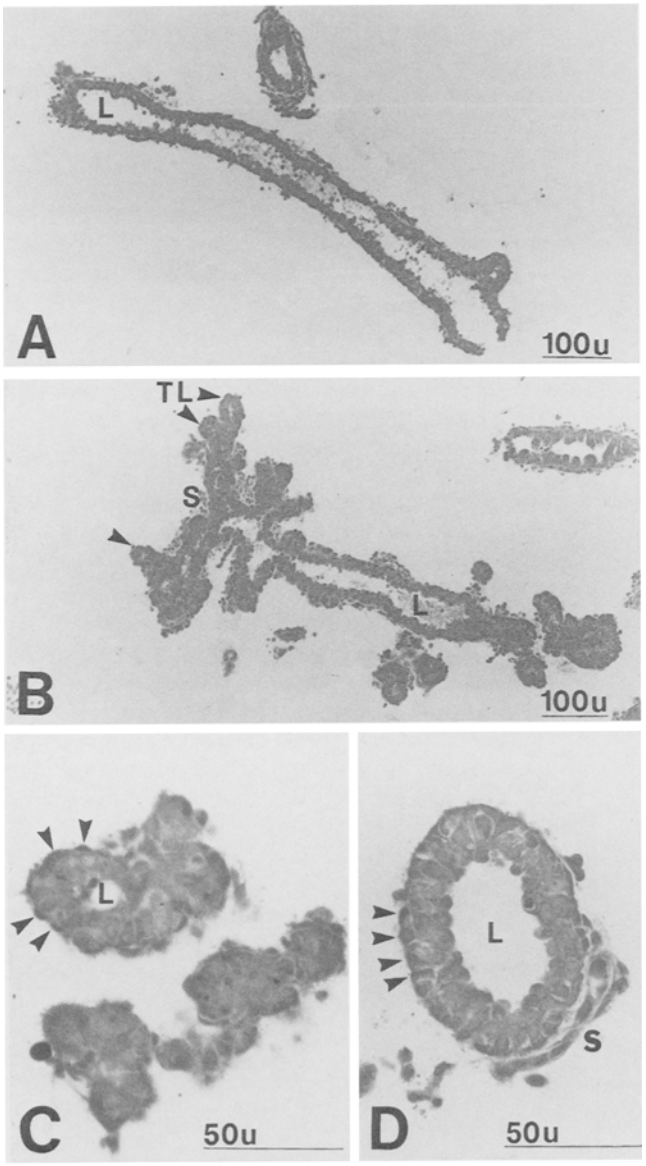

FIG. 4. Histology of organoid structures released by the tissue-dissociation procedure. *A*, Ducts in longitudinal and transverse sections. Note prominent lumen (*L*) running whole length of duct. Serial-section reconstruction revealed duct open at both ends. Stained with toluidine blue. ×90. *B*, Terminal-ductal-lobular unit (TDLU) in longitudinal section, showing prominent luminal cavity (*L*) extending into the terminal lobules (*TL*) (*arrows*). Some contamination of organoid with stromal cells (*S*). Serial-section reconstruction confirmed lobular nature of the TDLU. Stained with toluidine blue. ×90. *C*, Terminal lobular units collected upon 95-μm filter, showing small luminal cavity (*L*). Note dark-staining outer sheath of flattened cells (*arrows*) and cuboidal-columnar lining epithelium. Serial-section reconstruction confirmed lobular nature of these units. Stained with toluidine blue. ×355. *D*, Cross-section of ductal unit. Note prominent luminal cavity (*L*) lined by cuboidal-columnar epithelium surrounded by a dark-staining outer sheath of flattened cells (*arrows*). Some stromal contamination (*S*). Stained with toluidine blue. ×355.

We have observed two distinct cell populations in cultures of stroma-free organoids on collagen gel matrix. In their morphologies the basal and cuboidal cell populations closely resemble those described by Stampfer et al. (7) for the human breast. The basal cell type is elongate in shape and flattened, resting directly upon the collagen substratum. The cuboidal cell population forms a tight sheet of cells having rather small surface areas, making it difficult to distinguish them individually. They rest upon the basal cells, with their luminal aspects facing the medium (Fig. 5). It seems therefore that the tissue dissociation procedure described efficiently releases the mammary parenchyma with its two cell types (basal and cuboidal cells) and their microarchitecture and cell-to-cell relationships are preserved, allowing for more meaningful studies of the mouse mammary gland at the tissue level in culture.

DISCUSSION

A. Source of starting material

The present paper describes an efficient tissue dissociation procedure that releases mammary parenchymal structures from the mammary glands of mice. The method was successful in isolating the parenchymal component of the mammary glands from BALB/c, Swiss Webster, and C3H mice.

The parenchymal yield varies according to the nature of the starting mammary tissue. Mammary glands from young virgin mice at 12 wk of age provide a limited supply of parenchyma, mostly ducts, with a small end-bud population. Parous females give larger parenchymal yields with a larger proportion of terminal lobular organoid material, similarly with the yield from the glands of midpregnant mice. Glands of lactating mice provide a large amount of lobular-alveolar material, collecting upon all the filter fractions.

B. Dissection

The dissection method is based on the method of Foster and Feldman (2). The mammary glands are dissected out intact, inclusive of the lymph nodes. The filtration procedure efficiently separates these on the 400-μm filter.

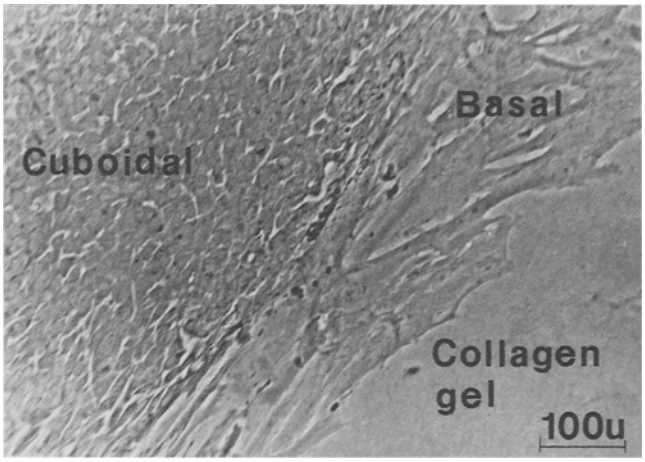

FIG. 5. Morphology of a parenchymal sheet established from an organoid cultured upon a collagen gel substratum. Organoids attach to collagen substratum and, by means of migration and growth, establish bilayered sheets of cells with two distinctive morphologies. A flattened *basal* cell population rests upon the gel surface, with a compact layer of *cuboidal* epithelium resting upon the basal cell layer. Both morphological cell types are obtained from organoids of ductal and lobular form. Phase optics. ×120.

The mammary glands are cut up into pieces approximately 2 mm^3 in size using a pair of crossed scalpel blades to minimize traumatic damage.

If larger organoid structures are required for use, then the tissue may be cut into larger pieces. The only problem with this approach is that it will be more difficult to achieve tissue disruption with a pipette. Nevertheless, this has been attempted and provides ductal parenchymal structures that are clearly visible with the naked eye, with some structures up to 0.3 cm in length. The washing step that follows cutting serves an important function in that it results in the removal of any contaminating muscle, hair, or connective tissue from the mammary glands.

For quantitative culture purposes we find that mammary tissue from approximately twenty virgin mice at 12 wk is necessary. A pair of mammary glands from such animals weighs approximately 0.15 g, giving an epithelial yield of 2×10^6 cells. Although the weight of the glands of midpregnant and lactating animals is much more (up to 0.5 g/pair of mammary glands), with the parenchymal yield correspondingly greater, the actual increase in cell numbers may not be that great, since the epithelial cells show secretory activity and swell up to many times the volume of the resting epithelial cells. Comparison of cell numbers of each of the organoid fractions collected upon the filters of each of these starting sources may be impractical for this reason.

C. Tissue dissociation procedure

This method is adapted from the method of Foster and Feldman (2). It is summarized diagrammatically in Fig. 1. The tissue dissociation is conducted in a sealed tube since it is much easier to observe its progress microscopically with minimal manipulation of the contents. The Universal Specimen tubes described in the Materials section are used from preference. First, the tubes have good optical properties for observation of the dissociating tissue, and, second, the released tissue collects easily as a pellet in the conical bottom of the tubes. The unit activity of collagenase digestion mixture is similar to that mentioned in many other mammary tissue dissociation procedures, except that in these studies use was made of Sigma collagenase Type I. Other studies may require use of alternative collagenase preparations, or concentrations. The use of BSA instead of serum in the digestion mixture aids in defining the system further for those studies that involve culture under defined media conditions (3). It may however be necessary to include serum as a constituent of the digestion mixture to protect the tissue from the adverse effects of the nonspecific proteases present in some collagenase preparations. Again, for some analyses (e.g., membrane receptors) it may be necessary to digest at a temperature lower than 37° C, such as 30° C (4). The important point to be made here is that the pattern of the parenchymal yield depends upon what happens during the dissociation phase, and, because of this and the biological variation of the starting tissue, it is recommended that the progression of the tissue dissociation be monitored carefully throughout the dissociation period, particularly before and after the physical disruption of the tissue with pipetting at 30, 60, and 90 min of digestion.

The aim of the first pipetting is to loosen the tissue, making it more accessible to the collagenase. An extensive breakdown of the fatty component and the collagenous stroma is usually found by the end of the 60-min digestion period. At this stage it is possible to observe large ducts settling to the bottom of the tube on standing, but when viewed under the inverted microscope they still appear surrounded with much contaminating stromal material. If there is only poor disruption of the tissue and release of glandular structures at this stage, then the tissue should be introduced into fresh collagenase digestion mixture. During the next phase of digestion the parenchymal structures are cleaned of most of the

contaminating adherent stromal material. The final pipetting serves to fragment the parenchymal structures into smaller pieces, with a tendency for the terminal parenchymal structures to break off from their ductal portions.

The pattern of parenchymal yield can be controlled to a certain degree by the extent of the pipette disruption of the tissue, as well as by how long the tissue is subjected to the enzyme dissociation.

D. Filtration procedure

This method is adapted from that used by Hallowes et al. (5) for separation of the parenchymal structures of reduction mammoplasty material from the human breast. The procedure is illustrated in Fig. 2. The difference in the physical size of the structural components of the mouse and human mammary glands requires the use of differently graded series of filters for the collection of the organoid material.

The filtration procedure works on the principle of separation according to size. The tissue dissociation procedure described here conveniently releases the parenchyma as multicellular organoids of different sizes, with the larger organoid population being mainly of a ductal nature and with the smaller fractions tending to be more of a terminal glandular population (Fig. 3). The filter system takes advantage of this size difference to separate the parenchymal subpopulations. The procedure does not allow for an exclusive separation of ductal versus terminal glandular material, but is capable of providing consistently enriched parenchymal populations for tissue culture purposes. If a greater separation of organoid material is required than that obtained by filtration, it is possible to enrich each fraction further by use of a micromanipulation technique under a dissecting microscope, to separate mechanically the units of particular interest.

It should be noted that a more elaborate one-piece filtration apparatus has been described recently by Wigle et al. (6). This apparatus has not been tested in our laboratory but it may be a feasible alternative to the simplified procedure described here. The 51-μm filtrate fraction contains epithelial cell clusters and an abundance of stromal cells, particularly fibroblasts. This may be a useful fraction for the study of the mesenchymal component of the mouse mammary gland, but the epithelial and mesenchymal populations should be further separated.

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