MONOLAYER CULTURE OF RAT PAROTID ACINAR CELLS WITHOUT BASEMENT MEMBRANE SUBSTRATES

CHERYLS. KISER, FIROZ RAHEMTULLA, AND BRITTA MÅNSSON-RAHEMTULLA¹

Department of Community and Public Health Dentistry (C. S. K., B. M-R.) and Department of Oral Biology (F. R.), University of Alabama School of Dentistry, Birmingham, Alabama 35294

(Received 28 March 1990; accepted 11 May 1990)

SUMMARY

Acinar cells have been difficult to maintain in primary or secondary cultures over extended periods of time. The most successful monolayer culture system reported to date requires basement membrane substrates. We report here a technique for culture of rat parotid acinar cells which does not rely upon basement membrane supports for maintenance and growth. The procedure involves gland excision, treatment to chelate metal ions, enzymatic digestion with collagenases and hyaluronidase, removal of fat and red blood cells by gravimetric separation, and nylon mesh filtration to yield a homogeneous suspension of small aggregates and single cells. The cells were examined for: a) morphology, identity, and growth; b) macromolecular synthesis; and c) secretory output. They were healthy, peroxidase positive, and growing for up to 10 d. Protein synthesis increased from the point of cell layer formation at 3 to 4 d, through 10 d, while DNA synthesis decreased. As in other studies, amylase secretion fell sharply between 2 and 4 d in culture and remained low. Although previous studies indicated that the initial isolation protocol left these acinar cells unable to thrive in monolayer culture except in the presence of basement membrane substrates, the modified technique reported herein allows these cells to attach, spread, and grow on a wide variety of commercially available plasticware. This method lends itself readily to long-term analysis of rat parotid acinar cell metabolism without the complications of dedifferentiation, cell loss through culture manipulation common in suspension cultures, or complex interactions between bioactive supports and cell surfaces.

Key words: acinar cell culture; parotid gland; basement membrane.

INTRODUCTION

Saliva is a complex fluid composed of a wide variety of organic and inorganic constituents which collectively act to modulate the oral environment. One of the principal functions of the salivary glands is the synthesis of macromolecules and their secretion. Up to ~90% of the glandular volume is constituted by acinar cells, which are responsible for the synthesis and secretion of salivary components (41). Although extensive information about the morphology of the salivary glands, composition of saliva, and events leading to secretion of saliva is available, very little information can be found regarding the temporal and topographic aspects of the synthesis of salivary macromolecules. This lack of information is partly due to the difficulty in maintaining acinar cells in primary or secondary cultures over extended time periods. A variety of in vitro methods for examination of salivary gland function have been devised which include gland slices (1,21) and isolated cells in dispersed suspensions (23,29,30). These preparations are suitable

only for a number of hours before metabolic deterioration and cell death occurs. One obvious alternative is long-term culture of acinar cells. A collagen-laminin substrate has been used to maintain rat submandibular acinar-intercalated duct complexes up to 22 d in culture (35). Parotid acinar cells have been maintained as aggregates in suspension for up to 4 wk (27). They have also been cultured as monolayers on a collagen substrate for up to 5 d (15) and on a reconstituted basement membrane gel for up to 21 d (28). Culturing lends itself readily to a host of analytical methods for extended periods of time with one important caveat: the mature, terminally differentiated cellular phenotype must not be sacrificed for the sake of proliferation and maintenance. In vitro studies are of value only so long as they provide useful information about the metabolic processes of whole organisms.

In our laboratory we are interested in acinar cells from the perspective of salivary peroxidase biosynthesis; this enzyme is an important member of the non-immunoglobulin defense factors normally present in saliva (39). For our purposes we wished to culture these cells using a simple, reproducible routine while maintaining the normal secretory apparatus found in

¹ To whom correspondence should be addressed.

mature acini of the adult rat parotid salivary gland. With this in mind we sought to extrapolate from the isolation and culture protocol of Oliver et al. (28) a method for establishment of rat parotid acinar cells in monolayer culture which would demonstrate secretory output comparable to that in adult animals. In this study a system for isolation and culture of rat parotid acinar cells as monolayers, which requires culture on a special substrate of reconstituted basement membrane gel prepared from Engelbreth-Holm-Swarm tumor extracts and coated onto Falcon Primaria plasticware, was reported. This support allows cells to be maintained for periods up to 21 d, during which they undergo growth and division. Identical results have been obtained with a commercially available basement membrane preparation, Matrigel, which contains laminin, collagen type IV, heparan sulfate proteoglycan, and entactin (Constance Oliver, personal communication).

A variety of extracellular matrix-cell surface interactions are known. However, the transduction of these events across the plasma membrane is still poorly understood. Evidence suggests these interactions affect a wide range of functions, including cell motility, cell shape, cell adhesion, cell-to-cell communication, and cell growth (33). Several of the main constituent molecules of basement membranes have also been demonstrated to modulate protein synthesis and secretion. Laminin-rich gel supports are required for the sustained secretion of albumin by rat hepatocytes (4), and collagen gels are necessary for casein synthesis and secretion in mouse mammary epithelial cells (13). The glycosaminoglycan portions of proteoglycans act as stimulators of secretion; heparin, heparan sulfate, and dermatan sulfate selectively increase secretion of proteins up to fivefold over control levels in vascular smooth muscle cells (22).

Because of the potential for such a number of effects generated by basement membrane, further complicated by variation in outcome due to batch-to-batch differences, age of preparation, et cetera, we undertook the task of isolating and culturing rat parotid acinar cells without reconstituted basement membrane substrates. Our approach has permitted us to establish and maintain rat parotid acinar cells as layers on plasticware, without basement membrane component substrates, for up to 10 d.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats, certified free of sialodacroadenitis virus and rat corona virus, were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Typically, single lots of six to seven animals were obtained within 1 wk before experiments and housed in an isolation cubicle of the pathogen-free animal facility maintained by the Animal Resources Program, University of Alabama at Birmingham. Adults rats of at least 30 d of age were used. These animals weighed approximately 160 g each at the time they were killed.

Acinar cell isolation. The protocol for rat parotid acinar cell isolation was modified from an existing procedure published by Oliver et al. (28). Rats were

euthanized by exposure to a CO₂ atmosphere. Using sterile techniques, the parotid glands were removed. A midline incision, extending from below the rib cage up to the chin, was made. The parotid gland was excised, taking care to clean the tissue of fat and fascia, and placed in Ham's F12 medium (GIBCO, Grand Island, NY) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma, St. Louis, MO), and the pH stabilized at 7.4 with 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Eastman Kodak Co., Rochester, NY). The entire procedure for each rat was completed in under 6 min.

Each pair of glands was rinsed in fresh penicillin-streptomycin supplemented Ham's F12 medium (Ham's F12/PS), then minced and placed in a sterile spinner flask containing 25 ml Ham's F12/PS inside a 5% CO₂ and ambient O₂ atmosphere, 100% humidified incubator at 35° C by a second investigator, while subsequent surgeries proceeded, until all glands had been equally treated. The combined tissue was rinsed twice with 25 ml vol of Ca⁺⁺-, Mg⁺⁺-free Hanks' balanced salt solution (Sigma). The tissue was resuspended in 25 ml of this same solution containing 2 mM disodium ethylenediaminetetraacetic acid (EDTA), stirred within the spinner flask in the 35° C incubator for 10 min, and rinsed twice with 25 ml vol of Ham's F12/PS. After rinsing, the tissue was dissociated enzymatically by digestion in 50 ml Ham's F12 medium containing 0.5 mg/ml collagenase type II, 0.5 mg/ml collagenase type IV (each containing less than 0.6 U/ml clostripain activity; Sigma), and 150 U/ml chromatographically purified bovine testicular hyaluronidase (Sigma) for 45 min, with stirring, in the 35° C incubator. The cell dispersion was adjusted to 20% (vol/vol) heat inactivated bovine calf serum (BCS; GIBCO) and centrifuged at 1000 rpm for 4 min in a model TJ-6 centrifuge (Beckman Spinco Division, Palo Alto, CA). The pellet was resuspended in 25 ml Ham's F12/PS, passed through a sterile Micro-Syringe 25-mm filter holder (Millipore, Bedford, MA) containing a Nitex 100-µm nylon mesh screen (Tetko, Lancaster, NY), and slowly layered onto 20 ml Ham's F12/PS/20% (vol/vol) heat inactivated BCS. The cells were allowed to settle for 10 min at room temperature and centrifuged as above for 3 min. Cells were resuspended in 25 ml Ham's F12/PS, centrifuged as before for 4 min, then passed through a second sterile Micro-Syringe 25-mm filter holder containing a Nitex 25-µm nylon mesh screen. The filtrate was centrifuged for 3 min as above and the pellet resuspended in 10 ml of the Ham's F12/PS further supplemented with 10% (vol/vol) heat inactivated rat serum (GIBCO), 10 µg/ml reduced glutathione (Sigma), 1 mM putrescine (Sigma), 5 µg/ml transferrin (Sigma), 5 µg/ml insulin (Collaborative Research Inc., Bedford, MA), 5 ng/ml selenous acid (Collaborative Research), and 10 ng/ml epidermal growth factor (GIBCO). Hereafter, this mixture is referred to as complete medium.

All cultures started from a given isolation were inoculated from a common cell suspension. The substrates tested were: Linbro (Flow Laboratories McLean, VA), Falcon Primaria (Becton Dickinson and

Co., Lincoln Park, NJ) coated with Matrigel (Collaborative Research), bare Falcon Primaria, Falcon (Becton Dickinson), Corning (Corning Glass Works, Corning, NY), Costar (Costar Corp., Cambridge, MA), Gibco (GIBCO), Plastek M (Tetmak Corp., Ashland, MA) Plastek C (Tetmak Corp.), and Sumilon (Sumitomo Bakelite Co., Ltd., Japan). Matrigel was diluted 1:4 with Ham's F12/PS at 4° C, coated onto Falcon Primaria dishes, and allowed to polymerize at 35° C in a humidified, 5% CO₂ and ambient O₂ atmosphere incubator.

For culture, cell suspension aliquots were inoculated into complete medium supplemented with 10 μ M isoproterenol (IP; Sigma) freshly prepared from the solid. With two exceptions, cultures were established in 35-mm dishes or wells with 4 ml complete medium/IP and 10⁶ viable cells. The Plastek dishes were supplied as 60-mm-diameter plates and were inoculated at the same cell density to surface area ratio as the 35-mm dishes. Cultures were provided with fresh complete medium/IP every 2 d by replacement of 3.5 ml of the 4 ml total medium using very gentle aspiration such that loose cells and aggregates were not removed in observable amounts from the plates.

Cell quantitation and viability. After isolation and periodically throughout one set of experiments, cells in duplicate cultures were placed in suspension using a Teflon cell scraper and gentle agitation. Cells were counted using a hemacytometer with improved Neubauer rulings and their viability was assessed by exclusion of 0.05% trypan blue in 0.02 M potassium phosphate/0.15 M NaCl, pH 7.4 (PBS). Total viable cells per culture were quantitated by two investigators under independent examination and the values obtained were averaged. The viability of these cultures ranged between 90.1 and 97.9% over the course of the experiments.

Protein. Total protein was measured after the method of Bradford (5), as modified by Read and Northcote (32), using bovine serum albumin (Fraction V, Sigma) as the standard. Medium was discarded and cell layers washed 3 times with cold PBS. Cell layers were solubilized in 0.1 N KOH for 1 h at room temperature on a rotary stirrer. Cell extracts were neutralized with 10 N acetic acid and aliquots assayed in duplicate for every culture. Medium samples were diluted with PBS and also assayed in duplicate.

Acute secretagogue stimulation. On Days 2, 4, 6, 8, and 10 of incubation, duplicate cultures were treated with 100 μ M isoproterenol or 100 μ M epinephrine or left untreated. After 30 min at 37° C in a 5% CO₂ and ambient O₂ atmosphere incubator, the cultures were processed as follows. Cells were suspended into the media with Teflon scrapers; the suspension was then centrifuged for 5 min at room temperature at 3000 rpm in a model HN-S centrifuge (International Equipment Co., Needham Heights, MA). The supernatants were removed and frozen until assayed for both amylase and salivary peroxidase activities. Cell homogenates were prepared from these cultures by suspending the cell pellets from each duplicate well in 250 μ l PBS followed by six cycles of

freezing and thawing before any analysis. Isoproterenol and epinephrine stocks were prepared fresh from dry powders into medium just before culture incubations.

Amylase. Amylase activity in the medium was measured using the Phadebas Amylase Test (Pharmacia Diagnostics, Fairfield, NJ), which is based on the method developed by Ceska and co-workers (10-12), following the manufacturer's instructions. Reagent blanks were complete medium/IP that had never been incubated with cells. Absorbance measurements were made in a Cary model 219 spectrophotometer (Varian Instrument Division, Palo Alto, CA). Enzyme activities were derived from a standard curve based on absorbance values for known activities in Phadebas Humylase Control standard (Pharmacia Diagnostics), which is manufactured expressly for use with this amylase test.

Salivary peroxidase. Salivary peroxidase activity was determined by two methods. In the Nbs/SCN⁻ assay system, peroxidase activities were determined by measuring the rate of SCN⁻ oxidation to OSCN⁻ as previously described (24). Peroxidase activity was also measured according to the method of Shindler et al. (37), as modified by Månsson-Rahemtulla et al. (24), using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), ABTS, as a substrate. For both methods, the standard for activity was human salivary peroxidase, purified as previously described (25). Samples were dialyzed extensively against distilled H₂O, lyophilized, then resolubilized at their starting volumes or concentrated twofold, before assay.

Peroxidase staining. Seven-day cultures of rat parotid acinar cells were rinsed 3 times with cold PBS, after removal of medium. The cell layers were fixed with 10% (wt/vol) trichloroacetic acid (TCA) at room temperature for 15 min. The fixed cell layers were rinsed twice with PBS and stored overnight at 4° C in PBS. Cell layers were stained for peroxidase after the method of Fahimi (14) by rinsing 3 times with 0.1 M Tris-HCl, pH 8.5, and incubating the plates in the same buffer containing 0.5 mg/ml 3,3'-diaminobenzidine (DAB), free base, and 0.02% (vol/vol) H₂O₂ for 3.5 h in the dark at 37° C. Plates were rinsed with substrate-free buffer and viewed for photomicrography. As controls, parallel cultures exposed to incubation buffer without H₂O₂ were examined as well as cultures of human gingival fibroblasts, grown to confluency under standard culture conditions (31) and treated with incubation buffer with or without H₂O₂. A similar experiment using the method as given by Kaplow (18) and modified by Liem et al. (20) for peroxidase staining, with 3,3',5,5'-tetramethylbenzidine (TMBZ) and H₂O₂ as substrates for the enzyme, was also performed.

Light microscopy. Acinar cells were examined at various times during culture as well as after fixation and staining, in either phase contrast or bright field, using a Nikon Diaphot-TMD inverted microscope (Nippon Kogaku K. K., Tokyo, Japan). Photographs were taken with a Nikon FM2 35mm camera (Nippon Kogaku, K. K.).

Electron microscopy. 10⁷ viable cells were cultured 4 d as suspended aggregates in 80 ml complete medium/IP with medium replacement on Day 2. The aggregates were

TABLE 1
RAT PAROTID ACINAR CELL ISOLATION
PROTOCOL SUMMARY

Step	Procedure	Approximate Time
1	Gland removal	< 6 min/pair
2	Washing, mincing	5 min/pair
3	EDTA chelation	15 min
4	Enzymatic tissue dissociation	1 h
5	100 μ m filtration, centrifugation	25 min
6	Gravimetric cell separation, centrifugation	25 min
7	25 μ m filtration, centrifugation	25 min
8	Viability check, cell number quantitation	10 min

centrifuged, medium removed, resuspended in 4 ml cold PBS, and recentrifuged at 1200 rpm in a model TJ-6 centrifuge for 5 min. Rinsing with cold PBS was repeated twice using 1 ml vol of buffer. The cell pellet was fixed in 3% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate/7% (wt/vol) sucrose for 4 h at room temperature. The pellet was washed 3 times with cacodylate-sucrose buffer and stored in this solution overnight at 4° C. The pellet was washed 5 times with cacodylate-sucrose buffer, postfixed for 90 min in 2% (wt/vol) OsO₄/1.5% (wt/vol) KFeCN in cacodylate-sucrose buffer, and stored overnight at 4° C in the same buffer. A second postfixation was done with 10% buffered formalin for 1 h, followed by 3 rinses in cacodylate-sucrose buffer. The pellet was dehydrated through a graded series of solutions containing ethanol from 25 to 100%, infiltrated with 1:1 Epon:ethanol, then embedded in 100% Epon (Ladd Research Industries Inc., Burlington, VT). After curing,

the pellet was sectioned on a Sorvall MT-2B with a diamond knife (E. I. Du Pont De Nemours & Company, Inc., Wilmington, DE), and put on bare copper grids. The sections were counterstained with 2% (wt/vol) uranyl acetate/50% (vol/vol) ethanol for 5 min and Reynold's lead citrate reagent (36) for 10 min. Grids were viewed on a Philips 300 transmission electron microscope.

[³H]Methyl thymidine incorporation. Cultures were labeled with 5 μ Ci/ml [³H]methyl thymidine (specific activity = 80 Ci/mmol; Amersham, Arlington Heights, IL) for 24 h. The medium was discarded, whereas the cells were rinsed 3 times with cold PBS and solubilized in 0.1 N KOH for 1 h on a rotary stirrer at room temperature. Aliquots of 1 ml were placed in scintillation vials, neutralized with equal volumes of 0.1 N HCl, and diluted 3:1 with Safety-Solve aqueous scintillation cocktail (RPI Corp., Mount Prospect, IL). Radioactivity was quantitated in a LS 1801 liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA).

DNA quantitation. DNA was separated from RNA by HClO₄ precipitation (16) and assayed according to Cerriotti (9) with calf thymus DNA (Sigma) as the standard. Standard DNA was carried through the HClO₄ precipitation procedure with the unknown culture samples.

[³⁵S]Amino acid labeling studies. Cultures were initiated in complete medium/IP with duplicate wells for each time point. On Days 1, 3, 5, 7, and 9 the medium was replaced with cysteine-, methionine-, sulfate-free complete medium/IP (prepared from individual components supplied by Sigma, Collaborative Research, and GIBCO) and incubated for 4 h, after which was added 25 μ Ci/ml Trans ³⁵S-Label (specific activity = 1209 Ci/mmol; ICN Biomedicals, Inc., Irvine, CA). This metabolic labeling

FIG. 1. Electron microscopy of parotid acinar cells. Representative micrograph of healthy acinar cells. $\times 7500$. Secretory granules (SG) occupy most of the cytoplasm surrounding the nucleus (N). Close examination reveals other well-defined cellular features including the nuclear membrane with lamins (arrowheads) and a nucleolus (n).

reagent contains a mixture of compounds, primarily [^{35}S]L-methionine and [^{35}S]L-cysteine, which is stabilized with 10 mM 2-mercaptoethanol. After 24 h labeling, cultures were processed as follows: Medium from each culture was centrifuged for 5 min at room temperature at 3000 rpm in a model HN-S centrifuge to remove loose cells, and transferred to clean tubes. The media were then made 10% (wt/vol) with solid TCA and incubated for 1 h at 4° C. These tubes were centrifuged at 1000 rpm for 10 min at 4° C in a model TJ-6 centrifuge. The supernatants were discarded and the precipitated protein pellets were washed twice with 5-ml portions of 2% TCA. The washed pellets were suspended in 2 ml of distilled H₂O and solubilized with 50 μl 5 N NaOH. After neutralization of the protein solutions with 50 μl 2.5 N HCl, the total volume of each sample was adjusted to 6 ml total with distilled H₂O. Samples were mixed with 4.8 ml of 1% (wt/vol) Nonidet P-40/1% sodium dodecyl sulfate (SDS)/0.5% sodium deoxycholate/PBS. Total protein was assayed as given above and radioactivity was quantitated in a LS 1801 liquid scintillation counter. Aliquots of each sample, containing 100 μg protein with approximately equal volumes and specific activities, and [^{14}C]methylated protein molecular weight markers (Amersham) were subjected to SDS-polyacrylic gel electrophoresis (PAGE) on 5 to 15% polyacrylamide gels by the method of Laemmli (19) as modified by Butler et al. (8). The gels were treated with Fluoro-Hance (RPI Corp.) as given in the product instructions, dried, and exposed to Kodak X-OMAT AR Diagnostic Film (Eastman Kodak Co.) for 14 d before development. Band intensities of the autoradiograms were then compared using a model GS 300 transmittance/reflectance densitometer (Hoefer Scientific Instruments, San Francisco, CA).

RESULTS AND DISCUSSION

Oliver et al. (28) have reported a system for isolation and culture of rat parotid acinar cells as monolayers. The method requires culture of the cells on a special substrate, either reconstituted basement membrane from EHS tumor extracts or Matrigel, coated onto Falcon Primaria plasticware. This support allows cells to be maintained for periods up to 21 d, during which the cells undergo growth and division.

Isolation. Based on previously published methodologies (27,28), the isolation protocol summarized in Table 1 was developed. Several major modifications from the starting procedure should be noted. Only rats of at least 30 d of age were used, to ensure that the acini of their parotid glands had matured to adulthood (34). Euthanasia by asphyxiation in CO₂ vs. cardiac puncture, in combination with the particular surgical field presentation we use, results in minimal connective tissue, fat, and red blood cell contamination in our preparation. This modification also allows us to eliminate a second gravimetric cell separation step required by the starting protocol, so long as the subsequent centrifugation's time does not exceed 3 min.

The enzyme cocktail for tissue dissociation was changed compositionally, to divide the collagenase activity between type II and type IV enzymes, with the sum of their concentrations equal to that of the type II concentration used previously. This combination has been found to improve tissue dissociation (Constance Oliver, personal communication). In addition, we reduced the hyaluronidase concentration from 300 to 150 U/ml. Purity of both the collagenases and the hyaluronidase is critical to maintaining high cell viability along with structural and functional integrity (27,40). The high cost of the particular type of hyaluronidase we employed prompted the decision to lower the concentration used. This had no adverse affects on the results obtained. However, when hyaluronidase was omitted entirely from the enzyme solution, cell viability was reduced from 83.7% in an otherwise identical experiment to 63.8% without hyaluronidase. The number of viable cells fell from 9.64×10^7 to 6.81×10^7 between the two isolations. Reduction of the incubation period in this mixture from 1 h to 45 min produced a cell dispersion that permitted us to omit the required 500- μm nylon mesh filtration of the initial method. Lack of the hyaluronidase necessitated reinstitution of the 500- μm nylon mesh filtration step and introduced a need for greatly increased mechanical shearing forces during all three filtrations. The digestion solution was adjusted to 20% (vol/vol) heat inactivated BSC before centrifugation and rinsing, to limit further enzyme action on the cell suspension. No decrease in cell

TABLE 2
GROWTH OF RAT PAROTID ACINAR CELLS ON
SELECTED CULTURE SUBSTRATES

Substrate	Anchorage Rate and Extent ^a	Spreading Rate and Extent ^b
Falcon Primaria/Matrigel	++++	++++
Linbro	++++	++++
Gibcoware	+++	+++
Corning	+++	+++
Costar	+++	++
Plastek M	++	+++
Plastek C	++	++
Sumilon	+	++
Falcon Primaria	+	++
Falcon	+	+

^a++++ = Maximal aggregate attachment is completed between isolation and Day 1; +++ = maximal aggregate attachment is completed between Days 1 and 2; ++ = maximal aggregate attachment is completed between Days 2 and 3; + = maximal aggregate attachment is completed between Days 3 and 4. In all cases, maximal attachment represents the anchorage of $\geq 90\%$ of the total aggregates present in a given culture. Attachment process is nonreversible under normal culture conditions. Observations were recorded from duplicate plates for each substrate tested and the average results presented above.

^b++++ = Diameter of average spread aggregate is between 4 and 6 cm by Day 4; +++ = diameter of average spread aggregate is equal to 4 cm by Day 4; ++ = diameter of average spread aggregate is between 3 and 4 cm by Day 4; + = diameter of average spread aggregate is between 2 and 3 cm by Day 4. Measurements were taken from 92X prints of the micrographs.

count or viability was observed in response to this alteration.

Elimination of a second EDTA chelation, formerly done between the 100- μ m mesh filtration and the 25- μ m mesh filtration, caused an increase in viability of 15.6% between two otherwise identical isolations. Processing to the point of a cell suspension in complete medium ready for culture inoculation requires about 3.5 h; with

experimental set up, the entire passage of the cells from whole animals to tissue culture occurs in about 4 h. Our protocol yields a preparation of mostly single cells and some small (<10 cells) aggregates that are essentially homogeneous in appearance. Six or seven rats provide, on average, about 10^8 viable cells per isolation from a population of suspended cells that are typically 80% viable or higher.

FIG. 2. Rat parotid acinar cell attachment and proliferation. Phase contrast microscopy. $\times 84$. Cells were cultured for 4 d in complete medium/IP on an assortment of commercially available substrates. Shown are spread aggregates representative of entire plates. A, Linbro; B, Falcon Primaria coated with Matrigel; C, Plastek C; D, Falcon cultureware.

When cells are placed in culture with complete medium/IP, they immediately form large (>30 cells), stable aggregates that persist as long as periodic exposure to fresh IP occurs. We omitted dexamethasone from the complete medium. Redman et al. (35) used dexamethasone as one of several medium supplements to extend the survival of rat submandibular acinar-intercalated duct complexes on collagen-laminin supports from 7 to 22 d,

noting changes in cell morphology in response to the hormone. Similar observations were reported by Oliver et al. (28) for rat parotid acinar cells, but the growth enhancement seemed to be at the expense of the differentiated phenotype. Our cultures thrived in changes of complete medium made $10\text{ }\mu\text{M}$ in freshly prepared isoproterenol every 48 h. When deprived of either fresh medium or fresh IP after 2 d, cells became rounded and began

FIG. 3. Peroxidase staining of rat parotid acinar cells. *A, B*, rat parotid acinar cells, $\times 332$. *C, D*, human gingival fibroblasts, $\times 165$. Cells were cultured for 7 d in complete medium/IP, fixed as described in Materials and Methods, then stained with 3,3'-diaminobenzidine. Staining buffer in *A* and *C* contained H_2O_2 ; buffer in *B* and *D* did not contain H_2O_2 .

detaching within 8 h, even if only one of these two components was withheld. Deprived cultures reintroduced to both fresh components recovered within a day insofar as respreading of rounded but still attached cells occurred. Floating cells were presumed dead and removed with the spent medium.

Cultures were maintained at ambient O_2 conditions without apparent deleterious effect. Oxygen levels have been demonstrated critical to cell viability, especially for parotid tissue as compared to explants of lacrimal and submandibular glands (21). However, organ culture presents difficulty with anoxia in central regions of the tissue mass that is not relevant to cultures inoculated from dispersed cells or small aggregate suspensions.

Morphology, identity, and growth. Cells were examined by electron microscopy for assessing the effects of isolation on their structural integrity. The cells have a healthy appearance and do not display the membrane irregularities, vacuoles, or lipid droplets typical of cells in distress (Fig. 1). In addition, the cell population is almost entirely composed of large cells filled with many secretory granules, consistent with previously published micrographs of rat parotid acinar cells (27,28).

Cultures of these parotid cells were tested for growth on a large number of different substrates: Linbro, Falcon Primaria coated with Matrigel, Falcon Primaria, Falcon, Corning, Costar, Gibco, Plaskon, Plaskon C, and Sumilon. Characteristics assessed in these cultures included rate and extent of aggregate anchorage as well as rate and degree of spreading of attached aggregates (Table 2).

Maximal attachment and spreading occurred on Linbro plasticware and Falcon Primaria coated with Matrigel (Table 2 and Fig. 2). We find that, on these two substrates, large aggregates are loosely anchored to the substrate on Day 1, and are firmly attached by Day 2, with scattered single cells already well spread on the surface. In the period between 75 and 90 h in culture, most aggregates spread profusely across the growing surface to establish cell layers, with some overlap that diminishes with distance from the aggregate anchorage site (Fig. 2 A,B). The differences between substrates were not large, as demonstrated by the mid-range performance of Plaskon M (Fig. 2 C); even in the case of poorest performance, Falcon, a significant number of aggregates attached and spread (Fig. 2 D), particularly when compared to cells cultured on this substrate after isolation by the method of Oliver et al. (28). Interestingly, cells established in Linbro plasticware that had been isolated without benefit of hyaluronidase in the dissociation solution performed identically, once placed in culture, except that the aggregate attachment and cell spreading were delayed by approximately 1 d.

To further address the question of cell identity, layer cultures were tested for peroxidase activity by reaction with DAB and H_2O_2 , which gives a characteristic dark brown stain (14) (Fig. 3). In the presence of both substrates, our rat parotid cell cultures stained heavily for peroxidase (Fig. 3 A), with a concentration in the

cytoplasmic granules common to exocrine cells. Deprivation of H_2O_2 resulted in no staining (Fig. 3 B), supporting the specificity of the color development as a result of peroxidase activity. The areas at the bottoms of Fig. 3 A, B are darkest because of proximity to the aggregate anchorage sites where cells invariably overlap. Furthermore, cultures of an unrelated, peroxidase negative cell type, human gingival fibroblasts (Fig. 3 C,D), gave similar results to H_2O_2 -deprived rat parotid cells. A related series of experiments using TMBZ and H_2O_2 did not give sufficient staining intensity vs. control cultures for photographic reproduction. In the rat parotid salivary gland, only the acinar cells are peroxidase positive and present in numbers such as isolated herein. Taken together with the knowledge that the culture conditions employed are biased against fibroblast outgrowth and duct cell proliferation (28), we have successfully isolated and established cultures of rat parotid acinar cells.

To follow the condition of the cells and measure growth in the course of maintenance, cultures were tested every 2 d, for 10 d total, for cell number and viability (Fig. 4). As shown, there was a sharp increase in the number of viable cells up to Day 4, coincident with cell layer formation. Thereafter, the number of viable cells remained nearly constant, on average, out to 10 d in culture. The percentage of viable cells in the total culture populations ranged between 90.1 and 97.9% over the course of the experiments.

Macromolecular synthesis. Cultures were evaluated by quantitation of [3H]methyl thymidine incorporation into DNA and of cell-associated protein synthesis (Fig. 5).

FIG. 4. Growth of rat parotid acinar cells. On indicated days, cultures were examined for growth and viability as described in Materials and Methods. Viability of these cultures ranged between 90.1 and 97.9% over the course of the experiments.

FIG. 5. Macromolecular synthesis in rat parotid acinar cells. [^3H]Methyl thymidine incorporation into DNA (open squares) and synthesis of cell-associated protein (solid squares) were measured over a 10-d period as described in Materials and Methods. For each point, assays of duplicate cultures were performed.

DNA synthesis, initially low in the large aggregates, peaked at a mean of 1.14×10^4 cpm/ μg DNA on Day 4, then dropped to between 55 and 63% of this maximum as culture continued to Day 10. In contrast, protein content steadily increased from the onset of cell layer formation on Day 4 to the end of the experiments with a final mean value of $138 \mu\text{g}$ total cellular protein per culture.

Secretory output. For our research needs, the rat parotid acinar cells must continue to express the same pattern of exocrine activity found *in vivo* in mature acini, particularly with regard to salivary peroxidase production. To begin, amylase activity was measured, as this enzyme accounts for about 30% of the total parotid saliva protein content (26) and is, therefore, easily quantitated. Amylase activity fell sharply with the transition from cell aggregates to layers (Table 3). Values at Days 4 and 6 dropped to 15 and 6% of the activity measured in Day 2 culture medium, respectively. By Days 8 through 10, activity had leveled off to only 4% of that seen in cultures of freshly isolated cells.

Exocrine secretion in salivary gland cells is stimulated by alpha- and beta-adrenergic receptor activation (2,7). Amylase secretion by rat parotid acinar cells in response to treatment with a beta-agonist, isoproterenol, and epinephrine, a mixed alpha- and beta-agonist was investigated. Acute stimulation of acinar cell cultures with both $100 \mu\text{M}$ isoproterenol and $100 \mu\text{M}$ epinephrine for 30-min periods, concentrations and time frames found optimal for secretory amylase output by parotid gland slices (1), resulted in increased amylase secretion throughout the incubation period of 10 d (Table 3). As expected, stimulation was minimal on Day 2, when amylase secretion is already near maximal. For the

remaining culture period, isoproterenol stimulated amylase secretion by $306 \pm 19.3\%$ over that of untreated cultures, whereas epinephrine stimulation increased amylase output by $233 \pm 11.5\%$. In both instances, susceptibility to secretagogue stimulation remained at consistent levels throughout the experiments.

Assays for salivary peroxidase activity in the acinar cell culture medium by both the ABTS and Nbs/SCN $^-$ methods revealed no detectable amounts of this enzyme (data not shown) when cultures were assayed with and without prior acute secretagogue stimulation of secretion by $100 \mu\text{M}$ isoproterenol or $100 \mu\text{M}$ epinephrine. Because these cells do stain for peroxidase with DAB, we tested the possibility that peroxide concentrations were below the detection limits of the assays. Medium from overnight suspension cultures with 3.62×10^6 cells/ml was assayed by the ABTS method and found to contain 484 mU/ml of peroxidase activity. This would mean that the 10^6 cells of a standard 35-mm dish culture could be expected to secrete a total of about 134 mU of peroxidase into 4 ml of medium, yielding a final concentration of $\sim 33.5 \text{ mU/ml}$. This value lies at the lower limit of reliable detection by either method (24) and is an indication that the failure of the assays with medium from monolayer cultures is due to extreme dilution of the peroxidase. The inability to detect enzyme activity with acute secretagogue stimulation suggests that salivary peroxidase is not sufficiently responsive to the agonists tested under the culture conditions employed in our studies for detection by the Nbs/SCN $^-$ method. In addition, analysis of cell homoge-

TABLE 3

ACUTE SECRETAGOGUE STIMULATION OF AMYLASE ACTIVITY IN RAT PAROTID ACINAR CELL CULTURE MEDIUM

Culture Period, Days	No Acute Secretagogue	Amylase Activity, ^a Units/mliter of conditioned medium,	
		$100 \mu\text{M}$ Isoproterenol	$100 \mu\text{M}$ Epinephrine
2	22.8	23.9	22.7
4	3.41	10.5	7.66
6	1.34	3.77	3.30
8	0.981	3.21	2.32
10	0.986	3.01	2.21

^aAmylase activity in the medium was measured using the Phadebas Amylase Test (Pharmacia Diagnostics, Fairfield, NJ), which is based on the method developed by Ceska and co-workers (10–12), following the manufacturer's instructions. Reagent blanks were complete medium/IP that had never been incubated with cells. Absorbance measurements were made in a Cary model 219 spectrophotometer (Varian Instrument Division, Palo Alto, CA). Enzyme activities were derived from a standard curve based on absorbance values for known activities in Phadebas Humylase Control standard (Pharmacia Diagnostics), which is manufactured expressly for use with this amylase test. Where there was no acute secretagogue treatment, aliquots of conditioned medium were measured after removal of loose cells. Assays after acute exposure to secretagogues were measured in medium of cultures given 30 min of $100 \mu\text{M}$ isoproterenol treatment or $100 \mu\text{M}$ epinephrine treatment. For each point, assays of duplicate cultures were performed.

nates from cultures given acute secretagogue stimulation with isoproterenol and epinephrine as described above did not have detectable peroxidase activity, indicative of no intracellular accumulation of enzyme to a measurable level.

Previous studies (28) indicate that the sharp drop in amylase secretion is unique; other secretory proteins maintain consistent levels in acinar cell cultures when grown on Falcon Primaria Matrigel-coated dishes. Rat pancreatic acinar cells behave likewise in culture (6). Both the parotid glands and saliva of rats undergoing chronic isoproterenol administration display a similar reduction in amylase activity with a concomitant increase in proline-rich protein synthesis (17). To test if this is the case for cell layers grown on Linbro plastic, cultures were established as described and placed in ^{35}S -amino acid labeling medium (methionine-, cysteine-, sulfate-free complete medium/IP) 28 h before analysis. The choice of this labeling agent was made because the majority of all protein secreted by the parotid gland is in the form of proline-rich proteins (around 70% of the total), sequencing studies of which have indicated an absence of sulfur-containing amino acids (3). Cultures were incubated in this medium for 4 h before radioisotope addition to permit clearance of unlabeled proteins in secretory granules already formed in the cells. After 24 h of continuous labeling, protein isolation, and quantitation, 100 μg of protein from Days 2, 4, 6, 8, and 10 culture medium was applied to an SDS-PAGE gel. Analysis of autoradiograms by scanning densitometry revealed that amylase levels do drop over the experiment's course (Fig. 6), in the same pattern as the enzyme activity measurements in Fig. 4. However, other bands displayed significant changes, including the consistent appearance of bands of molecular weight greater than 200 kDa, possibly mucinlike proteins, on Day 6 but not thereafter. The incorporation of radiolabeled amino acids into secreted proteins dropped sharply at Day 10, an indication of diminishing metabolic activity. Under the same conditions of this experiment, salivary peroxidase was not detected as a discrete band on the gels, not unexpected given that this protein is a minor component of saliva, present in the concentration range of 1 to 10 $\mu\text{g}/\text{ml}$ (38). The total protein concentration in whole saliva equals 2.8 to 3.2 mg/ml (26). The results obtained in Fig. 4 with regard to both protein and amylase levels in the cultures, taken together with the ^{35}S -amino acid labeling study suggests this method disrupts only amylase secretion, leaving export of other proteins intact.

In summary, we have refined a system for monolayer culture of rat parotid acinar cells that causes minimal perturbation of normal protein secretion which dispenses with the need for basement membrane substrates to support growth and maintenance of differentiation. This method minimizes cell loss through sequential feedings and permits easy separation of cells from their secretory products. We are now in a position to address the issue of determining what specific effects upon rat parotid acinar culture are exerted by individual basement membrane component molecules both alone and in different combinations. Further, culture of highly attachment positive rat parotid acinar cells as we have described may prove useful in pursuit of immortal strains from these

primary cells with all possible avenues of study typical of such cell lines.

FIG. 6. Scanning densitometry of secreted radiolabeled proteins. Secretory proteins were radiolabeled, isolated, and subjected to SDS-PAGE as described in Materials and Methods. Relative reflectance of signal intensities produced by these proteins on an autoradiogram was compared for cultures on Day 2 (A), Day 4 (B), Day 6 (C), and Day 10 (D) postinoculation. Molecular weight values are indicated for a standard protein mixture subjected to SDS-PAGE simultaneously with the medium protein samples.

REFERENCES

1. Batzri, S.; Selinger, Z. Enzyme secretion mediated by epinephrine beta-receptor in rat parotid slices. Factors governing efficiency of the process. *J. Biol. Chem.* 248:356-360; 1973.
2. Baum, B. J.; Ito, H.; Roth, G. S. Adrenoreceptors and the regulation of salivary gland physiology. In: Kunos, G., ed. *Adrenoreceptors and catecholamine action-part B*, New York, NY: Wiley & Sons; 1983:265-294.
3. Bennick, A. Salivary proline-rich proteins. *Mol. Cell. Biochem.* 45:83-99; 1983.
4. Bissell, D. M.; Arenson, D. M.; Maher, J. J., et al. Support of cultured hepatocytes by a laminin-rich gel. Evidence for a functionally significant subendothelial matrix in normal rat liver. *J. Clin. Invest.* 79:801-812; 1987.
5. Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254; 1976.
6. Brannon, P. M.; Orrison, B. M.; Kretchmer, N. Primary cultures of rat pancreatic acinar cells in serum-free medium. *In Vitro Cell. Dev. Biol.* 21:6-14; 1985.
7. Butcher, F. R.; Putney, J. W. Regulation of parotid gland function by cyclic nucleotides and calcium. *Adv. Cyclic Nucleotide Res.* 13:215-249; 1980.
8. Butler, W. T.; Bhowm, M.; DiMuzio, M. T., et al. Non-collagenous proteins of dentin. Isolation and partial characterization of rat dentin proteins and proteoglycans using a three-step preparative method. *Collagen Res.* 1:187-199; 1981.
9. Cerriotti, G. A. Microchemical determination of desoxyribonucleic acid. *J. Biol. Chem.* 198:297-303; 1952.
10. Ceska, M.; Brown, B.; Birath, K. Ranges of α -amylase activities in human serum and urine and correlations with some other α -amylase methods. *Clin. Chim. Acta* 26:445-453; 1969.
11. Ceska, M.; Hultman, E.; Ingelman, B. G-A. A new method for determination of α -amylase. *Experientia* 25:555-556; 1969.
12. Ceska, M.; Kirath, K.; Brown, B. A new and rapid method for the clinical determination of α -amylase activities in human serum and urine. Optimal conditions. *Clin. Chim. Acta* 26:437-444; 1969.
13. Emerman, J. T.; Enami, J.; Pitelka, D. R., et al. Hormonal effects on intracellular and secreted casein in cultures of mouse mammary epithelial cells on floating collagen membranes. *Proc. Natl. Acad. Sci. USA* 74:4466-4470; 1977.
14. Fahimi, H. D. Cytochemical localization of peroxidatic activity of catalase in rat hepatic microbodies (peroxisomes). *J. Cell Biol.* 43:275-288; 1969.
15. Fritz, M. E.; LaVeau, P.; Nahmias, A. J., et al. Primary cultures of feline acinar cells: dissociation, culturing, and viral infection. *Am. J. Physiol.* 239(2):G288-G294; 1980.
16. Halliburton, I. W.; Thomson, R. Y. Chemical aspects of compensatory renal hypertrophy. *Cancer Res.* 25:1882-1887; 1965.
17. Johnson, D. A. Changes in rat parotid salivary proteins associated with liquid diet-induced gland atrophy and isoproterenol-induced gland enlargement. *Archs. Oral Biol.* 29:215-221; 1984.
18. Kaplow, L. S. Simplified myeloperoxidase stain using benzidine dihydrochloride. *Blood* 26:215-219; 1965.
19. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685; 1970.
20. Liem, H. H.; Cardenas, F.; Tavassoli, M., et al. Quantitative determination of hemoglobin and cytochemical staining for peroxidase using 3,3',5,5'-tetramethylbenzidine dihydrochloride, a safe substitute for benzidine. *Anal. Biochem.* 98:388-393; 1979.
21. Lucas, D. R. The effect of hydrocortisone, oxygen tension and other factors on the survival of the submandibular, sublingual, parotid and exorbital lacrimal glands in organ culture. *Exp. Cell Res.* 55:229-242; 1969.
22. Majack, R. A.; Bornstein, P. Heparin and related glycosaminoglycans modulate the secretory phenotype of vascular smooth muscle cells. *J. Cell Biol.* 99:1688-1695; 1984.
23. Mangos, J. A.; McSherry, N. R.; Butcher, R., et al. Dispersed rat parotid acinar cells. I. Morphological and functional characterization. *Am. J. Physiol.* 229:553-559; 1975.
24. Månsson-Rahemtulla, B.; Baldone, D. C.; Pruitt, K. M., et al. Specific assays for peroxidases in human saliva. *Archs. Oral Biol.* 31:661-668; 1986.
25. Månsson-Rahemtulla, B.; Rahemtulla, F.; Baldone, D. C., et al. Purification and characterization of human salivary peroxidase. *Biochemistry* 27:233-239; 1988.
26. Nikiforuk, G. Saliva and dental caries. In: Nikiforuk, G., ed. *Understanding dental caries 1. Etiology and mechanisms. Basic and clinical aspects*. Basel: Karger; 1985:236-260.
27. Oliver, C. Isolation and maintenance of differentiated exocrine gland acinar cells *in vitro*. *In Vitro* 16:297-305; 1980.
28. Oliver, C.; Waters, J. F.; Tolbert, C. L., et al. Growth of exocrine acinar cells on a reconstituted basement membrane gel. *In Vitro Cell. Dev. Biol.* 23:465-473; 1987.
29. Putney, J. W.; Van de Walle, C. M.; Wheeler, C. S. Binding of 125 I-phylloerythrin to rat parotid cells. *J. Physiol. (Lond)* 301:205-219; 1980.
30. Putney, J. W.; Van de Walle, C. M. The relationship between muscarinic receptor binding and ion movements in rat parotid cells. *J. Physiol. (Lond)* 299:521-531; 1980.
31. Rahemtulla, F.; Moorer, C. M.; Wille, J. J. Biochemical and immunological studies of proteoglycans from human gingival fibroblasts. *J. Dent. Res.* 68:362; 1989.
32. Read, S. M.; Northcote, D. H. Minimization of variation in the response to different proteins of the Coomassie blue G dye-binding assay for protein. *Anal. Biochem.* 116:53-64; 1981.
33. Reddi, A. H. Extracellular matrix and development. In: Piez, K. A.; Reddi, A. H., eds. *Extracellular matrix biochemistry*. New York: Elsevier; 1984:375-412.
34. Redman, R. S. The development of the salivary glands. In: Sreebny, L. M., ed. *The salivary system*. Boca Raton, FL: CRC Press; 1987:1-20.
35. Redman, R. S.; Quissell, D. O.; Barzen, K. A. Effects of dexamethasone, epidermal growth factor, and retinoic acid on rat submandibular acinar-intercalated duct complexes in primary culture. *In Vitro Cell. Dev. Biol.* 24:734-742; 1988.
36. Reynolds, E. S. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* 17:208-212; 1963.
37. Shindler, J. S.; Childs, R. E.; Bardsley, W. G. Peroxidase from human cervical mucus. *Eur. J. Biochem.* 65:325-331; 1976.
38. Tenovuo, J. O. The peroxidase system in human secretions. In: Pruitt, K. M.; Tenovuo, J. O., eds. *The lactoperoxidase system: chemistry and biological significance*. New York: Marcel Dekker; 1985:101-122.
39. Tenovuo, J.; Pruitt, K. M. Relationship of the human salivary peroxidase system to oral health. *J. Oral Pathol.* 13:573-584; 1984.
40. Williams, J. A.; Korc, M.; Dorner, R. L. Action of secretagogues on a new preparation of functionally intact, isolated pancreatic acini. *Am. J. Physiol.* 235:E517-E524; 1978.
41. Williams, M. A.; Cope, G. H.; Pattern, M. K. A system for the study of zymogen granule genesis and discharge in rabbit parotid gland tissue *in vitro*. *J. Anat.* 115:141-142; 1973.

The authors extend their gratitude to Dr. Constance Oliver for her constructive discussions regarding her culture methods. Our gratitude also goes to Ms. Nancy McGee for assistance in preparation of electron micrographs and Ms. Dianne Lovoy in preparation of the manuscript. This work was supported by grants 7RR 05300-25 and DE 07076 from The National Institute of Dental Research, Bethesda, MD.