An Adenoid Squamous Carcinoma-Forming Cell Line Established From an Oral Keratinizing Squamous Cell Carcinoma Expressing Carcinoembryonic Antigen

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A neoplastic epithelial cell line, TYS, was isolated from a well-differentiated squamous cell carcinoma expressing carcinoembryonic antigen (CEA) that arose in human oral mucosa. Expressions of CEA and amylase as well as ample tonofilaments were detected in cultured TYS cells. Transplantation of the cells into athymic nude mice resulted in production of adenoid squamous cell carcinoma containing CEA and amylase. Cultivation of TYS cells in the presence of sodium butyrate resulted in suppression of cell growth and production of secretory granules with amylase in the cytoplasm of the cells. When the sodium butyrate-treated cells were transplanted into nude mice, a small mass developed transiently at the inoculation site and then disappeared. This mass was histopathologically interpreted as acinic cell carcinoma with squamoid lesion. These findings suggest that we have established a human adenoid squamous carcinoma cell line presumably derived from a minor salivary gland present in oral mucosa. (Am J Pathol 1986, 124:496–509)

ADENOID squamous cell carcinoma, also known as adenoacanthoma or pseudoglandular squamous cell carcinoma, is an uncommon variant of squamous cell carcinoma that is characterized by the admixture of adenocarcinomatous elements in combination with a squamous component of the tumor and is now recognized as a definite entity.[1–4] This tumor, which has been often encountered on the skin of the head and neck, also occurs in the oral cavity, with considerable frequency on the lips.[5–9]

Although it has been suggested that adenoid squamous cell carcinoma of the skin in the sun-exposed area originates from the pilosebaceous structures,[1–4] there has been controversy concerning the histogenesis of the tumor that arises in an unexposed location without the pilosebaceous apparatus, such as oral mucosa or the vulva.[5–10] One interpretation is that the tumor occurs as a consequence of squamous metaplasia of neoplastic glandular cells[5,11,12]; in another interpretation, this tumor is considered to be a variant of squamous cell carcinoma with an origin in nonglandular structures, in which the tubular and alveolar spaces represent pseudoglandular spaces arising from acantholysis of solid nests of squamous cell carcinoma.[2,3,13–15]

On the other hand, the presence of carcinoembryonic antigen (CEA) in certain squamous cell carcinomas of the uterine cervix and bronchus has been reported.[16,17] To understand the significance of CEA present in squamous cell carcinoma, we have attempted to isolate human oral squamous carcinoma cell lines expressing CEA *in vitro* and to characterize their biologic nature. During the course of this study, we have found that the transplantation into athymic nude mice of a neoplastic epithelial clonal cell line expressing CEA, which was

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isolated from well-differentiated squamous cell carcinoma arising in the oral mucosa, results in the production of adenoid squamous cell carcinoma. In the current commmunication, we report the isolation of a human adenoid squamous carcinoma cell line derived from an oral keratinizing squamous cell carcinoma and the effects of sodium butyrate on phenotype and proliferation of the cell. In addition, we discuss herein the possible histogenesis of adenoid squamous cell carcinoma arising from the oral mucosa.

Materials and Methods

Primary Culture and Cell Cloning

The neoplastic tissue used for this study was obtained at the time of biopsy of primary cancer arising in oral mucosa of an 81-year-old Japanese woman whose tumor mass measured 4 × 4 × 2 cm in size. Part of the biopsy material was immersed in Eagle's minimal essential medium (MEM) containing gentamicin (25 μg/ml; Schering, Kenilsworth, NJ) for 15 minutes at room temperature and was then minced with a sharp pair of scissors into about 2–3-cu mm fragments. They were placed into a 60-mm plastic Petri dish (Falcon Labware, Oxnard, Calif) and left for 1 hour at room temperature. Thereafter, the fragments were cultured in MEM containing 1000 U penicillin/ml and supplemented with 10% newborn calf serum and 2 mM L-glutamine in the presence of 5% CO_2 in an incubator at 37 C. The growth medium was changed twice weekly. After about 20 days of cultivation, the cell propagation was observed in the periphery of some explants. Almost all of the cells grown from explants were morphologically epithelioid cells. In some instances, these cells consisted of a mixture of epithelioid and mesenchymal cells. Thus, the explants propagating the mesenchymal cells were removed from the surface of the Petri dish with a rubber scraper as completely as possible, and the explants with outgrowths of epithelioid cells alone were left to be cultured. When enough monolayer of epithelioid cells was formed after 60 days of incubation, the cells were harvested by treatment with 0.08% trypsin and 1.4% ethylenediamine tetraacetic acid in calcium- and magnesium-free phosphate-buffered saline (pH 7.2). These cells, dispersed in a single-cell suspension, were washed twice by MEM and then seeded into a 100-mm plastic Petri dish at a density of 3×10^6 cells in 15 ml of growth medium in a 5% CO_2 incubator at 37 C. The colony formation of the second passage level cells in semisolid agar was performed according to the modified method of Macpherson and Montagnier.[18] Briefly, suspensions of 10^2 or 10^3 cells in the growth medium containing 0.3% Special Agar Noble (Difco Laboratories, Detroit, Mich) were poured into the agar medium, which had been hardened by the addition of 0.6% agar to the growth medium. After about 20 days' incubation at 37 C, the colonies were isolated with Pasteur's pipets, and the cells were cultured in 3 ml of growth medium in 30-mm plastic Petri dishes until confluent cell monolayers were formed. About 11% of these cultured cells at this passaged level formed the colonies in semisolid agar. The growth medium was changed twice a week during that period. Of 10 colonies isolated at random, one clone that showed the most stable growth was designated TYS and used for the current study. This TYS clone was found to express CEA extensively when assessed by immunohistochemical method and radioimmunoassay as described below. Subculture was made at 4- or 5-day intervals. The TYS cells grew without interruption for 12 months, and 75 serial passages were successively carried out (cell doubling time, 42.5–46.8 hours). The TYS cells between passages 35 and 42 were used for this study. At the time of the initiation of these experiments, the cell culture was free of *Mycoplasma* contamination when tested in *Mycoplasma* medium (GIBCO, Grand Island, NY).

Cell-Doubling Time

Cell-doubling time was determined by counting the number of viable cells from freshly trypsinized monolayers. Twenty-eight 30-mm Petri dishes each receiving 2×10^5 cells were used, and counting was performed at 12- to 24-hour intervals for 7 days. Throughout the entire procedure, cell viability was determined by means of the trypan blue exclusion method.

Tumorigenicity

Six-week-old female Balb/c nude mice (Clea Japan, Inc., Tokyo, Japan) were housed 5 mice/cage in a pathogen-free environment in a vinyl isolator (Clea Japan) and fed autoclaved food *ad libitum*.

Mice received a total number of 10^7 cells which were injected subcutaneously through an injection needle in the anterior aspect of the lateral thoracic region. Tumor growth was observed weekly, and animals were sacrificed by cervical dislocation from 1 to 3 months after the inoculation of cells. Tumors were fixed in phosphate-buffered 10% formalin and sectioned for histologic staining with hematoxylin and eosin (H&E) or for immunoperoxidase staining.

Immunoperoxidase Staining

The biologic markers of tumor cells were essentially investigated by the peroxidase–antiperoxidase (PAP)

method described by Sternberger et al.[19] The specific antigens chosen were CEA and amylase. Rabbit antiserum to highly purified human CEA isolated from hepatic metastasis of colon adenocarcinoma was purchased from Dakopatts, Copenhagen, Denmark, and rabbit antiserum to highly purified amylase from whole human saliva was purchased from Nordic Immunological Laboratories, Tilburg, The Netherlands. According to the product specification sheets supplied by manufacturers, the antibodies used are characterized as follows. Trace amounts of contaminating antibodies to plasma protein present in the anti-CEA have been removed by absorption with insolubilized human plasma. Absorption was also done with blood group antigens A_2 and B until agglutination of erythrocytes was abolished. The anti-CEA reacts with substances like nonspecific cross-reacting antigen (NCA) which share antigenic sites with CEA. The anti- salivary amylase reacts with amylase present in other exocrine secretions because of the presence of common antigenic determinants. In the present experiments, anti-CEA serum was used at a dilution of 1:1000, and anti-amylase serum was used at a dilution of 1:200.

The cultured cells were washed three times with phosphate-buffered saline (PBS, pH 7.2) and fixed with 95% ethanol/acetone (4:6) for 10 minutes at 4 C. The paraffin-embedded specimens were cut in serial sections approximately 4 μ thick. The paraffin sections were deparaffinized with xylene and rehydrated step by step with descending concentrations of ethanol. These preparations were incubated at 37 C with 0.3% H_2O_2 in absolute methanol for 10 minutes to block endogenous peroxidase. After being washed three times with PBS, they were incubated with an appropriate dilution of rabbit antiserum to each of the above-described antigens for 30 minutes at room temperature, then rinsed with PBS for 30 minutes. Thereafter, goat anti-rabbit IgG (Medical and Biological Laboratory Ltd., Nagoya, Japan) was applied in a dilution of 1:40 in PBS for 30 minutes at room temperature. After being washed with PBS for 30 minutes, these samples were incubated with rabbit horseradish PAP complex (Miles Laboratories, Inc., Elkhart, Ind) diluted 1:50 in PBS for 30 minutes and rinsed with PBS for 20 minutes. Finally, the peroxidase was localized by treatment of the samples with a fresh mixture of 0.05% 3,3'-diaminobenzidine and 0.01% H_2O_2 in 10 mM Tris-HCl buffer (pH 7.6) for 5 minutes; after being washed with distilled water, these samples were counterstained with hematoxylin.

The following controls were used. The rabbit antiserum to CEA was absorbed with normal human spleen extract to remove NCA as follows. Antibody sample (0.2 ml) was mixed with 100 μg of acetone powder prepared from normal human spleen and was incubated at room temperature for 2 hours. Thereafter, this mixture was added with 0.8 ml of PBS; and the supernatant, obtained by centrifuging at 500*g* for 15 minutes at 4 C, was filtered through a type HA Millipore membrane (Millipore Co., San Francisco, Calif) and used as absorbed antiserum for the present study. The acetone spleen powder was made as follows. Washed, diced spleen was ground with acetone in a mortar and filtered or squeezed onto coarse grade filter paper. After the deposit was washed five times with acetone, it was dried overnight at room temperature and used as the acetone spleen powder in the present study. A blocking test in the use of anti-CEA serum was provided by incubating parallel samples with the anti-CEA serum absorbed with CEA extract of human colon cancer tissues. The negative control was provided by incubations of parallel samples with a relevant dilution of normal rabbit serum instead of the rabbit-specific antiserum of the initial incubation. To test the specificity of antiserum used for detection of amylase, specific antiserum was replaced by PBS, normal rabbit serum, or the specific antiserum absorbed with amylase from human saliva (Sigma Chemical Co., Saint Louis, Mo). Antibody solution diluted fivefold in PBS (1 ml) was mixed with 200 μg of specific antigen and incubated at room temperature for 1 hour. Thereafter, this mixture was centrifuged at 500*g* for 15 minutes at 4 C and then filtered through a type HA Millipore membrane for removal of microaggregates. The supernatant was used as absorbed antiserum for the present study. This antibody sample gave a negative result.

Morphologic Observation

The paraffin sections of biopsy material and nude mouse tumors were stained with H&E. The monolayer cell culture was fixed in methanol and stained with 0.1 M phosphate-buffered Giemsa (pH 6.8).

For transmission electron microscopy, the cultured cells harvested by scraping with a rubber policeman were fixed in 2.5% glutaraldehyde buffered with 0.1 M phosphate (pH 7.4) containing 0.005 M calcium chloride for 2 hours and then dissected into small pieces of 2 cu mm. After washing in the phosphate buffer, these materials were postfixed with 1% osmium tetraoxide, dehydrated step by step with ethanol, and embedded in Epon 812. The ultrathin sections were cut on an LKB ultramicrotome and stained with uranyl acetate and lead hydroxide. The finished preparations were observed under Hitachi Model H-500 electron microscope.

For immunoelectron microscopy, the cell monolayers were washed in PBS and fixed with peroxidase–lysine–paraformaldehyde as described by McLean and Nakane[20] for 2 hours at 4 C and then washed for 1 hour

in five changes of PBS containing 10% (wt/vol) sucrose. Thereafter, cells were incubated overnight with anti-CEA serum or anti-amylase serum at 4 C. After being washed in PBS for 30 minutes, cells were reacted with horseradish peroxidase-labeled Fab' fragment of anti-rabbit IgG (Cappel Laboratories, Cochranville, Pa) for 5 hours at room temperature. After being washed in PBS for 30 minutes, these preparations were fixed in 2% glutaraldehyde for 30 minutes at 4 C and then washed three times in PBS. Then they were incubated for 5 minutes in Karnovsky's solution containing 0.1% dimethylaminoazobenzene (Wako Pure Chemical Co., Osaka, Japan) and 0.03% hydrogen peroxide (Wako) in 50 mM Tris-HCl buffer (pH 7.6) at room temperature and then washed in PBS. After being incubated in 2% osmium tetroxide for 1 hour at 4 C, they were dehydrated step by step with ethanol and embedded in Epon 812 resin. After polymerization, the Epon sheet was detached from the dishes. Small blocks were excised and attached to Epon blocks with adhesives. Ultrathin sections were cut on an LKB ultramicrotome and examined without staining under a Hitachi electron microscope. A control experiment was done with normal rabbit serum.

CEA Determination

CEA activity was measured by the use of ^{125}I-radioimmunoassay sandwich method as described by Nishi and Hirai.[21] A commercially available CEA-RIA kit (Hoffman-LaRoche Inc., Nutley, NJ) was used for this study.

The cell preparation for CEA assay was essentially done according to the extraction method of Egan and Todd.[22] Briefly, the cells were harvested by rubber policeman and washed once with PBS. They were resuspended in three volumes of distilled water and treated with an equal volume of 2 M perchloric acid for 20 minutes at room temperature with occasional shaking. The precipitate was removed by centrifugation at 8000*g* for 20 minutes. The supernatant was dialyzed against distilled water until neutral and assayed for CEA activity. The detection limit of this RIA method was 1.0 ng/ml.

Amylase Assay and Isoamylase Analysis

The cells and culture media were used as materials for the current experiments. The cells were washed three times with PBS and were homogenized at 4 C in three volumes of 0.25 M sucrose solution containing 20 mM $CaCl_2$ in Potter-Elvenhjem homogenizer with a Teflon pestle. The homogenates were centrifuged at 105,000*g* for 60 minutes at 4 C, and the supernatants were used for the determination of amylase activity.

Amylase activity was assayed by a chromogenic method[23] using blue-dyed starch polymer (Pharmacia Fine Chemical Co., Uppsala, Sweden). Briefly, 24 μl of culture media or supernatants from the cell homogenates were incubated with 1 ml of a 10 mg/ml suspension of blue starch polymer in PBS (20 mM sodium phosphate; 50 mM NaCl, pH 7.0). Incubation was performed at 37 C for 30 minutes, and the reaction was terminated by the addition of 0.25 ml of 0.5 M NaOH. A standard curve was prepared with the use of standard serum from Wako Pure Chemical Co. and the blue starch polymer. From this curve, the concentration, in international units per liter, was determined for the testing samples.

Amylase isoenzyme separation was performed by horizontal sheet electrophresis with 5% polyacrylamide gel of 1-mm thickness. The discontinuous buffer system was used, the gel buffer being 0.19 M Tris [tris(hydroxymethyl)aminomethane], pH 8.8, and electrophoresis was carried out at 4 C for 2.5 hours at 0.8 mA/cm gel plate. After electrophoresis, the amylase isoenzyme was identified with the use of the starch-iodine reaction.

Results and Discussion

The histopathologic appearance of serial tissue sections that were prepared extensively from the original tumor used for the present study was interpreted as well-differentiated squamous cell carcinoma (Figure 1A). Repeated determinations of serum CEA level before the onset of cancer treatment ranged from 7.0 to 19.5 ng/ml. This level of CEA is high enough to be characteristic of a disseminated tumor, because the mean plasma CEA level of 36 patients with oral squamous cell carcinoma, who were in various clinical stages, was 2.0 ± 1.7 ng/ml.

The tissue sections from this tumor showed extensively positive staining for CEA (Figure 1B). Figure 1C and D show representative samples of the morphologic appearance and CEA expression of a cultured TYS clone, which was isolated from the original tumor. The cells were polygonal in shape, with nuclei with prominent nucleoli, and grew in a pavementlike fashion with formation of occasional multilayered foci. Almost all of the TYS cell population showed strongly positive staining for CEA in the cytoplasm. Figure 2A–C shows the ultrastructure of cultured TYS clone. Junctional complexes between neighboring cells represent distinct desmosomes, occasional tight junctions, and ample micro-villi. Tonofilaments were markedly well developed. Mitochondria, rough endoplasmic reticulum, and Golgi complexes were moderately developed. In addition, secretory granules were sparsely observed in the cytoplasm.

Because it has been reported that the treatment of some human colorectal tumor cells expressing CEA

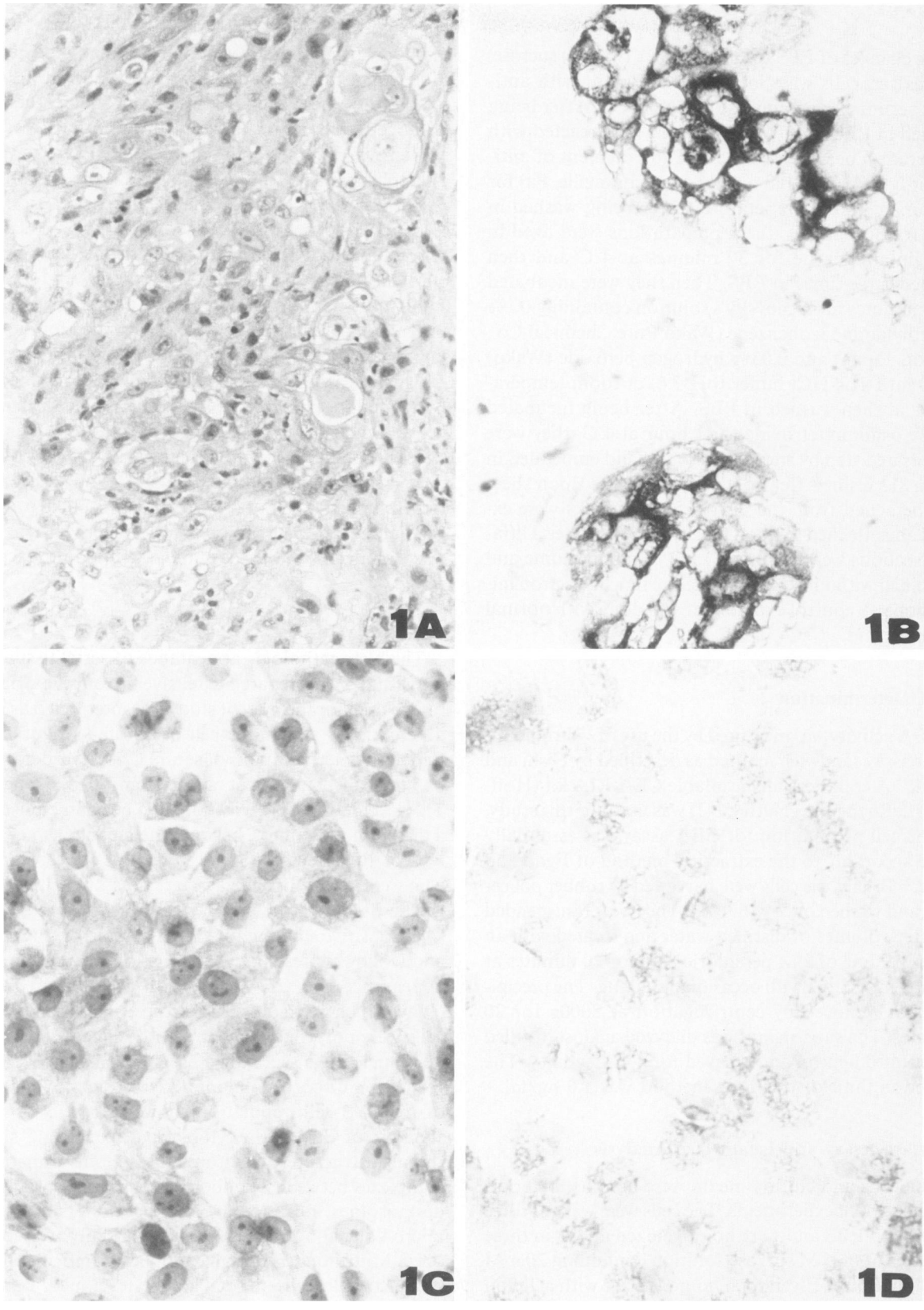


Figure 1—Morphologic appearance and CEA expression of the original tumor and its cultured cell line TYS, which were used for this study. **A**—Original tumor. This is interpreted as well-differentiated squamous cell carcinoma. **B**—Immunohistochemical detection of CEA in the original tumor. **C**—Cultured TYS cells derived from the original tumor. Cells grow in a pavementlike fashion. **D**—Immunohistochemical detection of CEA in the cultured TYS cells. (**A**, H&E, ×240; **B**, hematoxylin counterstain, ×480; **C**, Giemsa, ×360; **D** hematoxylin counterstain, ×720)

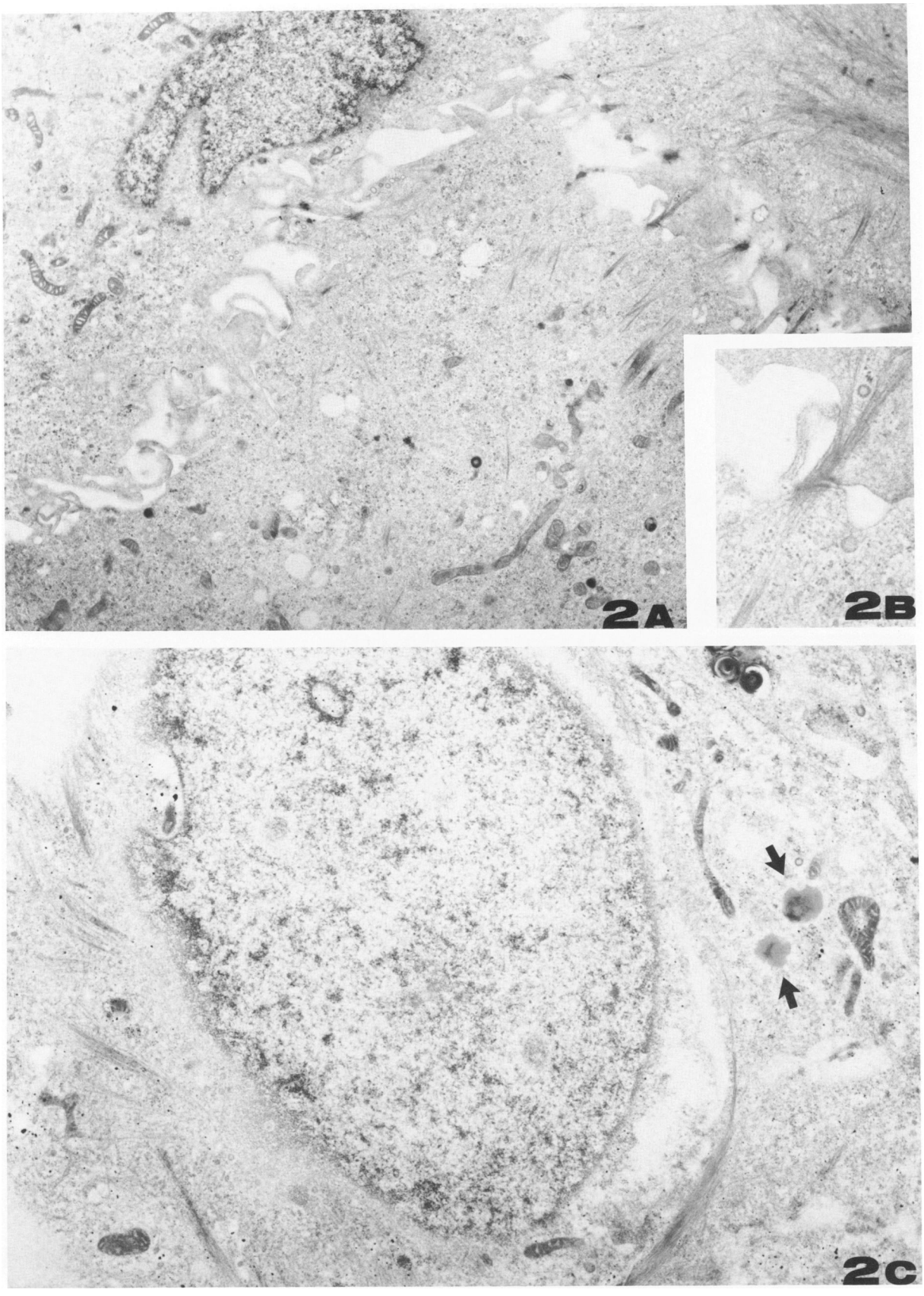


Figure 2—Ultrastructure of the cultured TYS cells. **A**—Junctional complexes between neighboring cells represent distinct desmosomes, occasional tight junctions, and ample microvilli. Tonofilaments are markedly well developed. **B**—High-power view of **A**, showing distinct desmosome and tonofilaments. **C**—In addition to the presence of ample tonofilaments, secretory granules (*arrows*) are sparsely observed in the cytoplasm. (**A**, ×12,000; **B**, ×36,000; **C**, ×28,000)

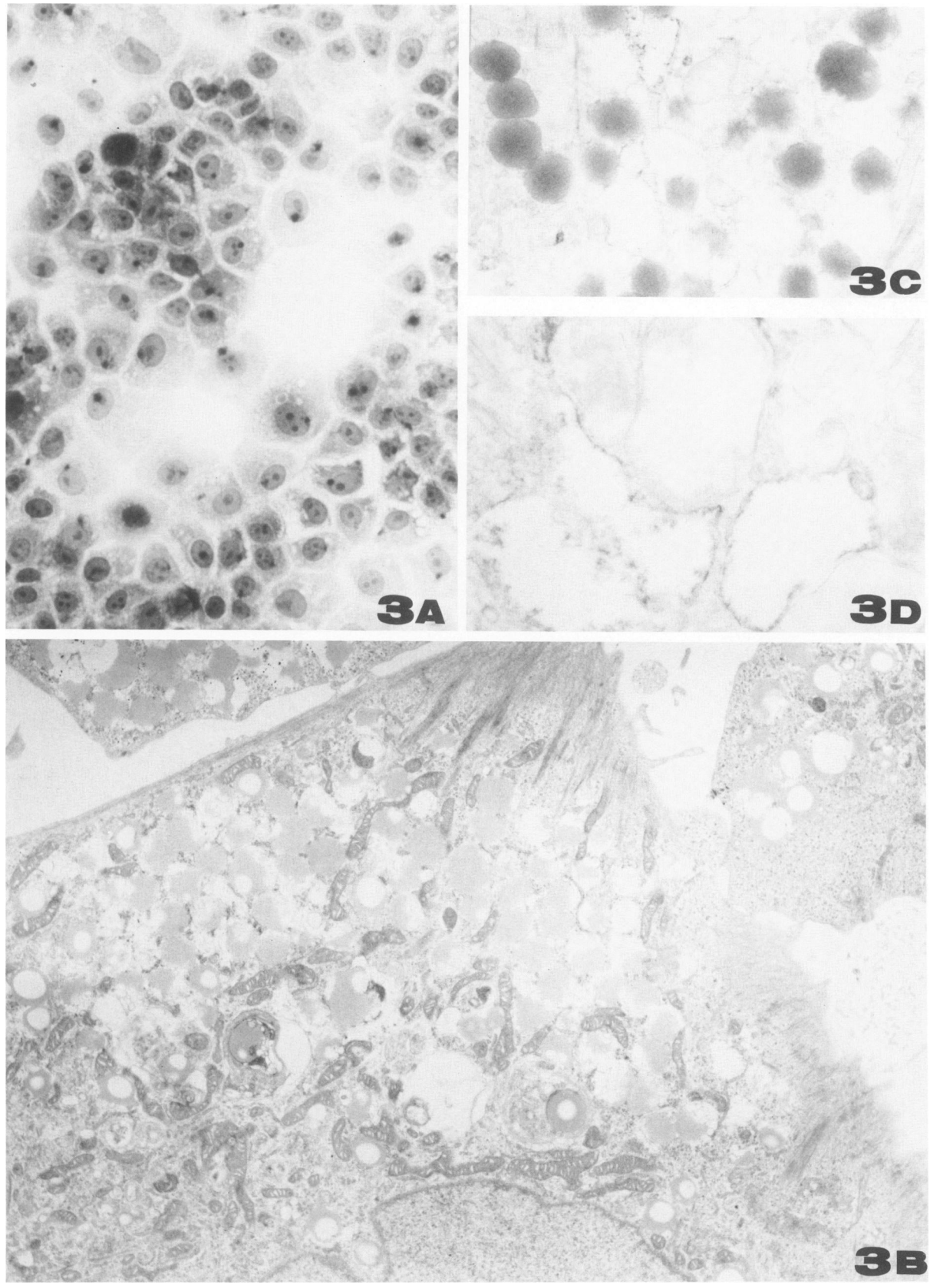


Figure 3—Morphologic appearance of cultured TYS cells treated with sodium butyrate. **A**—Monolayer culture of TYS cells in the presence of sodium butyrate. Numerous vacuoles are observed in the cytoplasms. **B**—Ultrastructure of the cultured TYS cells treated with sodium butyrate. In addition to the presence of ample tonofilaments, numerous secretory granules or vacuoles are observed in the cytoplasm. **C** and **D**—Immunoelectron micrographs demonstrating the presence of secretory granules reactive to anti-amylase serum (**C**) and the presence of the substance reactive to anti-CEA serum (**D**) in the rims of vacuoles. (**A**, Giemsa, ×420; **B–D**, ×28,000) TYS cells were cultured in the presence of 5 mM sodium butyrate for 5 days at 37 C and were examined for their morphologic appearance.

with sodium butyrate causes an elevation in CEA production[24] and that sodium butyrate produces reversible changes in morphologic features, growth rate, and enzyme activities of several cell types in culture,[25] we examined the effects of sodium butyrate on CEA expression, phenotype, and proliferation of the cultured TYS clone. Treatment of the cultured TYS clone with sodium butyrate resulted in some morphologic alteration of the cells. Figure 3A shows a representative sample of the morphologic appearance of the TYS clone treated with sodium butyrate. Twenty-four hours after the treatment, the cells having vacuoles in the cytoplasm began to ap-

Table 1—Summary of the Immunohistochemical Characteristics of TYS Cells Treated With Sodium Butyrate and Untreated Cells

Specific antigens	% Positive cells*	
	TYS cells treated with sodium butyrate	Untreated TYS cells
Amylase	97.5	20.5
CEA	98.5	95

* The percentage of positive cells was microscopically computed. The number indicated in this table is the average of two experiments. TYS cells were cultured in the presence and absence of 5 mM sodium butyrate for 5 days at 37 C and were examined by the PAP method for the above antigens.

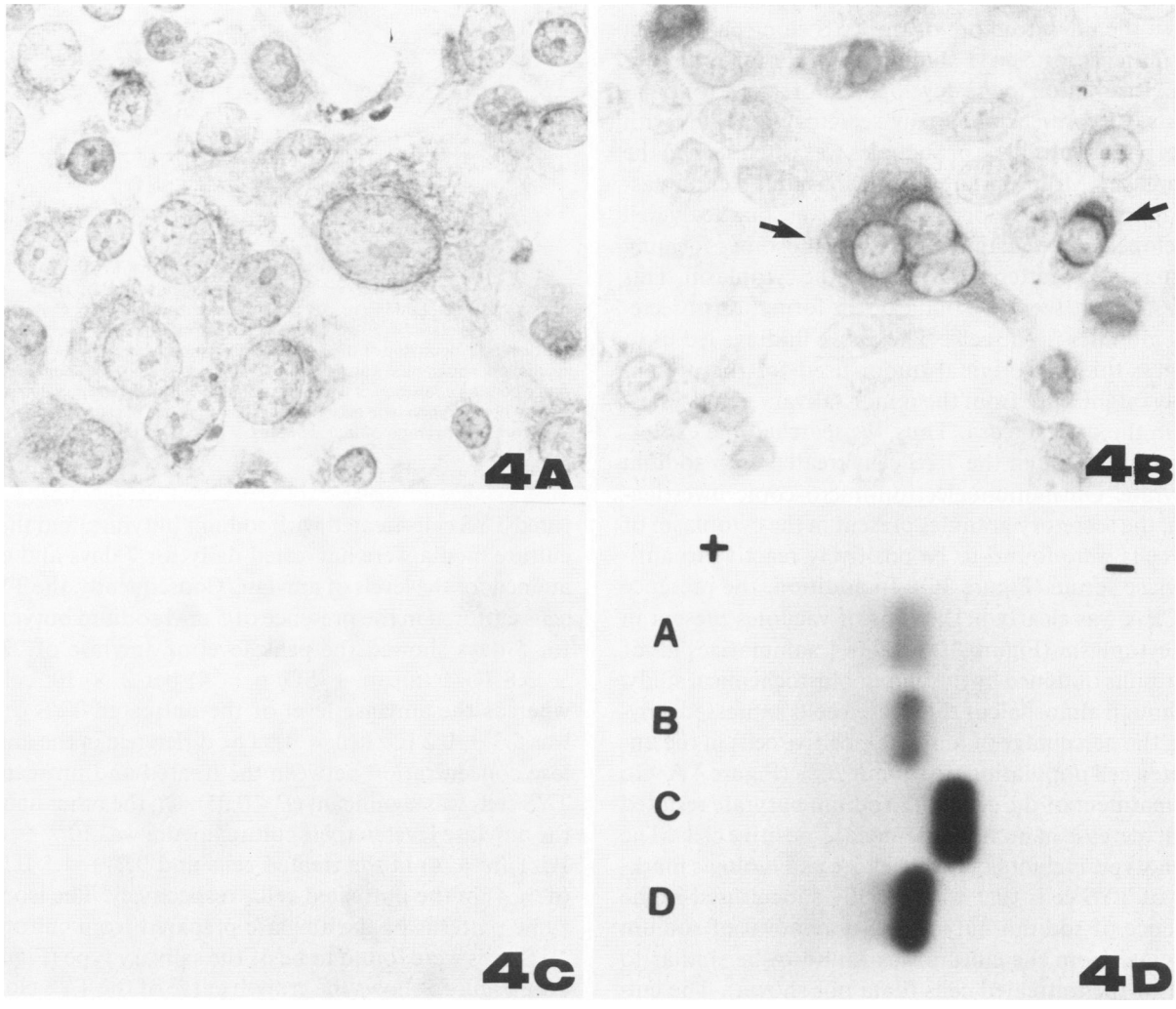


Figure 4—Immunohistochemical detection of amylase in TYS cells with and without sodium butyrate and amylase isoenzymes of TYS cell extract. **A**—Cells treated for 5 days with 5 mM sodium butyrate. Almost all of the cells show positive staining. **B**—Untreated cells. *Arrows* indicate positive staining. **C**—The treated cells. Cells were examined for amylase by the PAP method, with anti-amylase serum that was absorbed with amylase from human saliva (Sigma). All of the cells indicating negative staining. (**A–C**, hematoxylin counterstain, × 640). **D**—Isoamylase analysis. Lane A, amylase preparation (200 IU/1) from TYS cells (2×10^8) cultured in the presence of 5 mM sodium butyrate for 5 days at 37 C. Lane B, amylase (200 IU/1) highly purified from human saliva (Sigma). Lane C, Amylase preparation (600 IU/1) from human pancreas tissue. Lane D, Amylase preparation (600 IU/1) from human submandibular salivary gland tissue. The human pancreas and submandibular salivary gland tissues used for the current experiments were taken by surgical operation from patients with pancreas cancer or with submandibular salivary gland cancer. Parts of the tissues showing no tumor lesion histopathologically were subjected to amylase assay and isoamylase analysis as described for TYS cells in Materials and Methods.

pear in the cultured TYS cells in the presence of sodium butyrate (2 or 5 mM). At Day 5, almost 100% of the cells cultured in the growth medium with 5 mM sodium butyrate contained many vacuoles in the cytoplasm. At Day 7, the cells were trypsinized and subcultured serially in the growth medium without sodium butyrate. Consequently, the cells with the vacuoles gradually disappeared from the subculture, and the morphologic features of the third passage level cells were similar to those of the untreated cells. The TYS cells cultured in growth medium with sodium butyrate at a concentration of 5 mM were harvested daily for 7 days and examined for the level of CEA. Consequently, the TYS cells cultured for 5 days showed the peak level of CEA of 59.0 ng/10^6 cells, whereas the CEA level of the untreated TYS cells was 15.4 ng/10^6 cells. Figure 3B shows the ultrastructure of the TYS clone cultured in the presence of 5 mM sodium butyrate for 5 days at 37 C. In addition to the development of ample tonofilaments, the presence of many secretory granules with amorphous substance or vacuoles was often seen in the cytoplasm. Moreover, mitochondria, rough endoplasmic reticulum, and Golgi complexes were markedly well developed. A large number of free ribosomes forming clusters were scattered throughout the cytoplasm. This ultrastructure seemed to indicate the formation of secretory granules in the cells. The above findings led us to suggest that the original tumor used for the present study might arise from the minor salivary glands present in the oral mucosa. Thus, we searched the expression of amylase in the TYS cells treated with sodium butyrate. On immunoelectron-microscopic investigation, the secretory granules present in the cytoplasm of the cells were found to be positively reactive to anti-amylase serum (Figure 3C). In addition, the presence of CEA was clearly in the rims of vacuoles present in the cytoplasm (Figure 3D). Table 1 summarizes all of the results obtained by the immunohistochemical study. Although almost all of the treated cells expressed amylase, the percentage of amylase-positive cells in the untreated cell population was about 20% (Figure 4 A-C); ie, treatment of the cells with sodium butyrate resulted in an increase in number of amylase-positive cells. The phenotype, including ultrastructure and biologic markers, of TYS cells that were serially subcultured in the absence of sodium butyrate after removal of sodium butyrate from the culture was found to be similar to that of the untreated cells (data not shown). The cultured TYS cells treated with sodium butyrate, and their culture media were harvested daily for 7 days and examined for the levels of amylase. Consequently, the TYS cells cultured in the presence of 5 mM sodium butyrate for 5 days showed the peak level of amylase of 22.3 ± 3.8 IU/1 (mean ± SD, n = 4) per 2 × 10^7 cells, whereas the amylase level of the untreated TYS cells was 5.3 ± 0.2 IU/1 (n = 4). The difference in the amylase concentration between the treated and untreated TYS cells was significant ($P < 0.05$). On the other hand, the amylase level in their culture media was 10.7 ± 1.0 IU/1 (n = 4) in the treated cells and 9.2 ± 1.5 IU/1 (n = 4) in the untreated cells, respectively. The isoenzyme patterns of the amylase prepared from cultured TYS cells were found to be of the salivary type (Figure 4D). Figure 5 shows the growth curve of the TYS clone

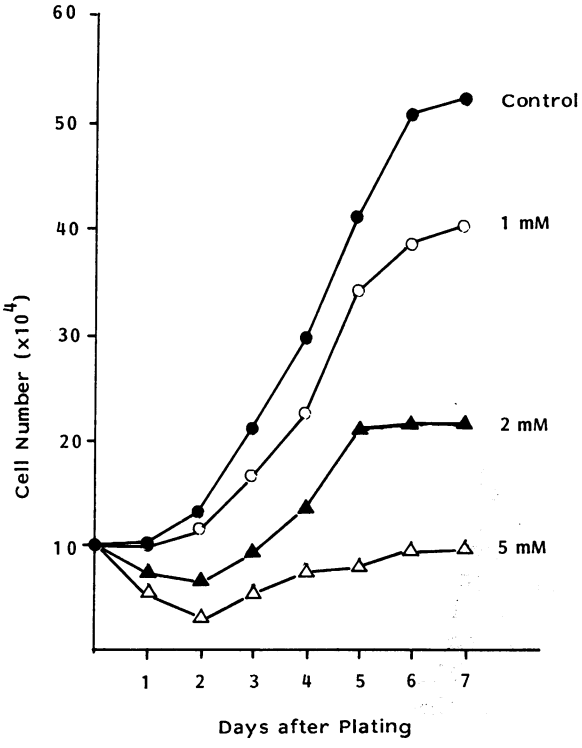
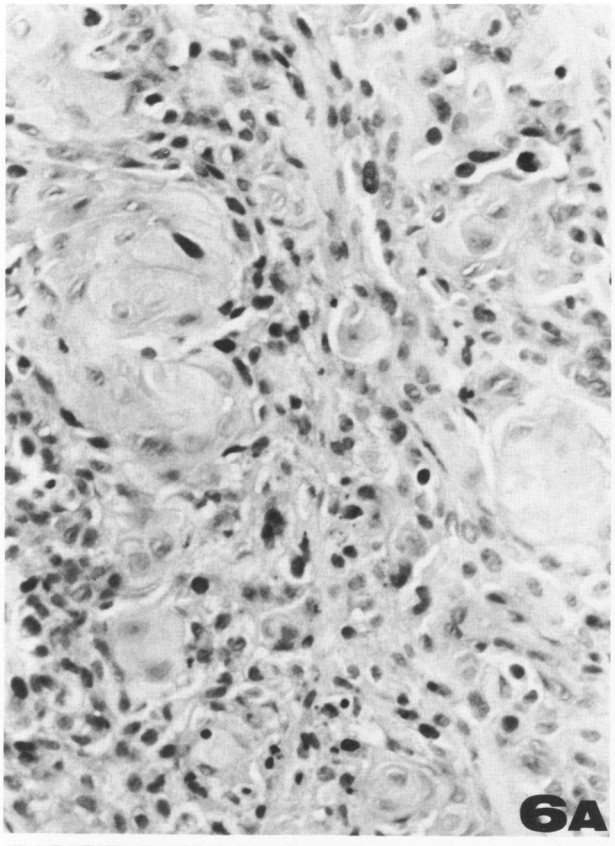


Figure 5—Effect of sodium butyrate on the growth of TYS clone in culture. ●——●, control; ○——○, sodium butyrate (1 mM); ▲——▲, sodium butyrate (2 mM); △——△, sodium butyrate (5 mM). Cells (Passage 40) were plated in 30-mm plastic Petri dishes at a density of 10^5 cells per dish and incubated at 37 C in a 5% CO_2 incubator. After 24 hours of incubation, sodium butyrate (1, 2, or 5 mM) was added. The numbers of viable cells as a function of time were counted with a hemocytometer with the use of the trypan blue exclusion test. For the sake of clarity, each point represents an average of four samples.

Figure 6—Histologic appearance of the tumors produced after transplantation of cultured TYS cells into nude mice. **A**—This area in a tissue section from a nude mouse tumor shows keratinizing squamous cell carcinoma similar to that of the original tumor. **B**—This area shows a characteristic adenoid pattern that consists of numerous glandular formations. **C**—Photomicrograph show the histologic appearance similar to adenocarcinoma (*lower portion*), in addition to the presence of a squamoid area (*upper portion*). **D**—High-power view of the squamoid area of **C**. (**A** and **B**, H&E, ×360; **C**, H&E, ×120; **D**, H&E, ×420)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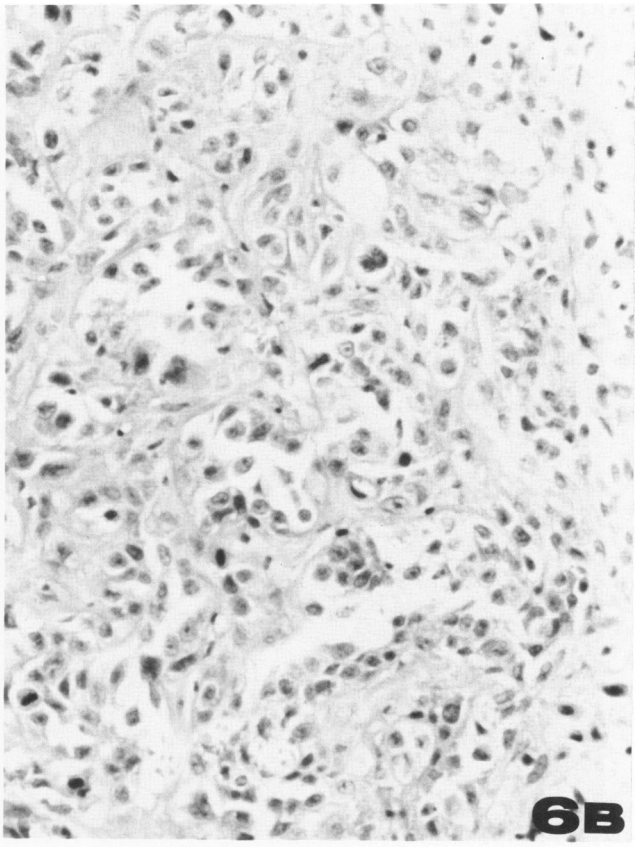
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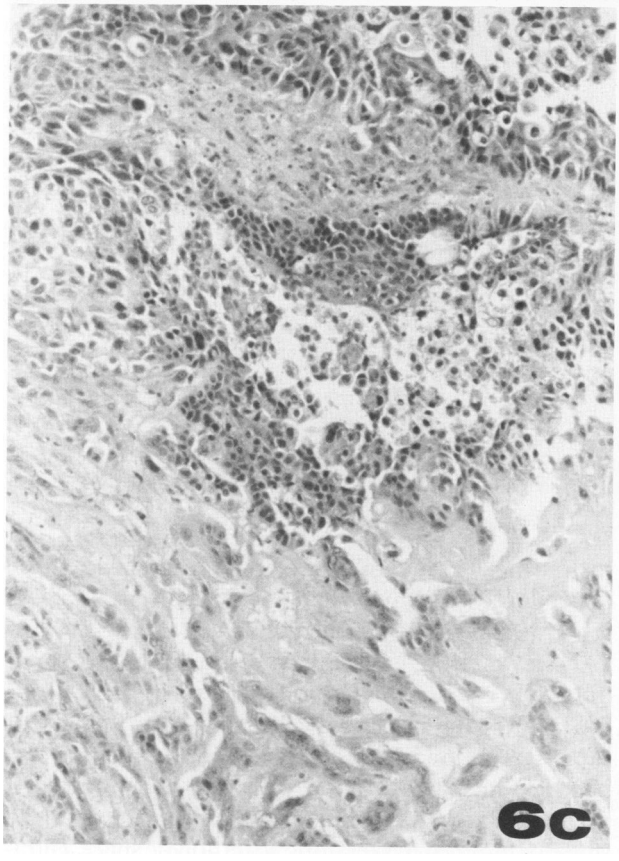
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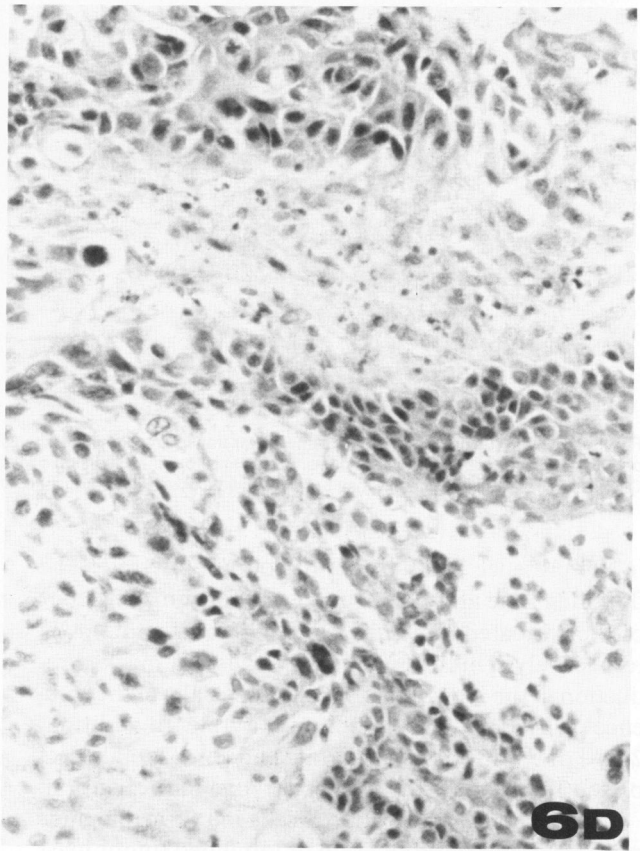
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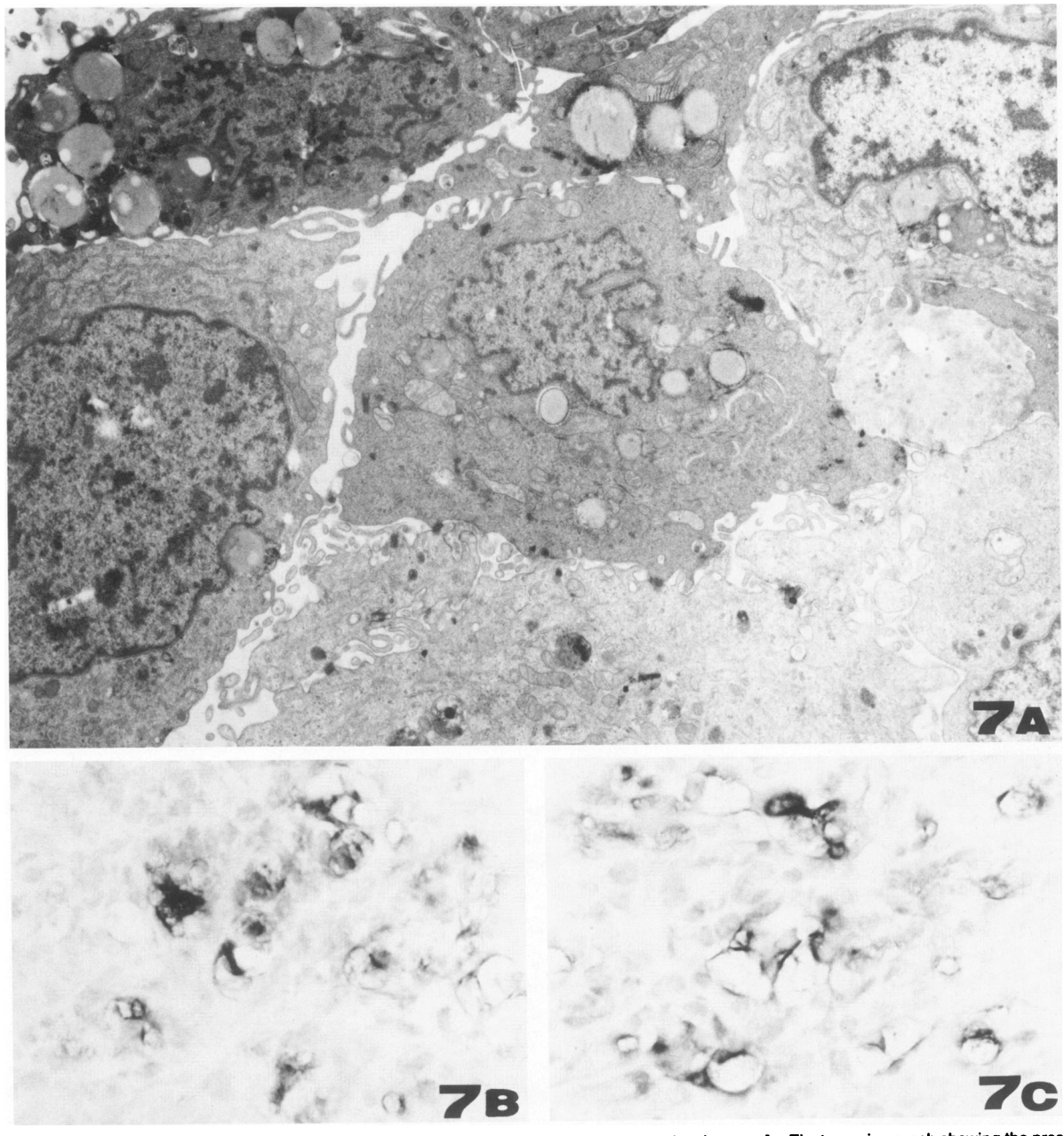
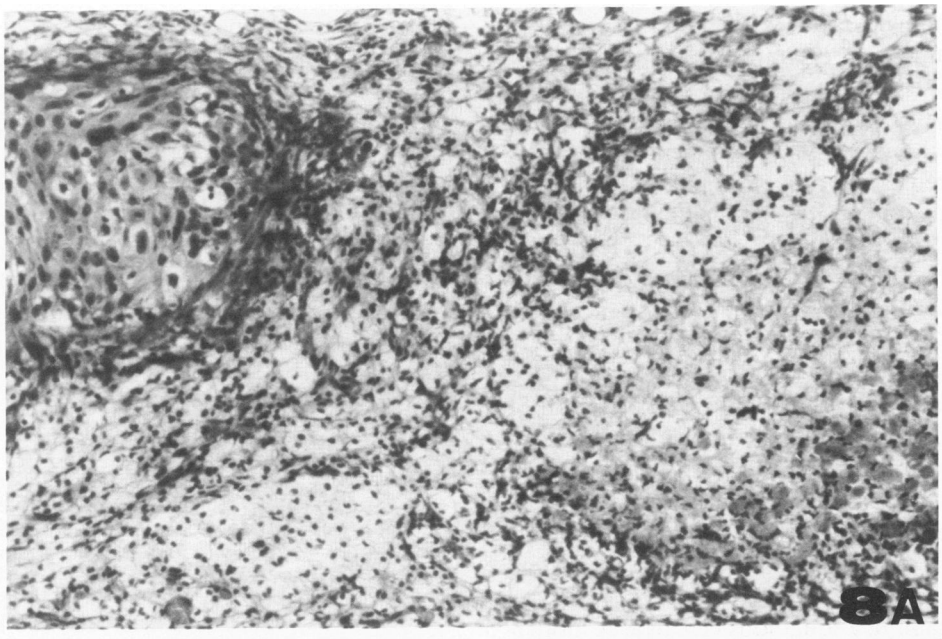


Figure 7—Ultrastructure of the tumor formed after transplantation of cultured TYS cells into nude mice. **A**—Electron micrograph showing the presence of both dark and clear cells. Secretory granules and rough endoplasmic reticulum are often seen, particularly in the cytoplasm of dark cells. **B** and **C**—Tissue sections from nude mouse tumors showing positive staining for amylase (**B**) and CEA (**C**). (**A**, ×3400; **B** and **C**, hematoxylin counterstain, ×480)

in the presence and absence of sodium butyrate. The doubling time and soft agar colony-forming efficiency of the untreated TYS cells were 44.4 hours and 21.8%, respectively. This chemical agent caused a marked reduction in the growth rate of cells, especially in the presence of 2 mM or 5 mM sodium butyrate. In addition, 2 mM or 5 mM sodium butyrate had some lethal effect on the TYS clone, as assessed by trypan blue exclusion.

Transplantation of 10^7 TYS cells into athymic nude mice resulted in a production of lobulated tumor mass measuring 7–11 mm in diameter in the inoculation site about 30 days after transplantation. Figure 6A–D shows the histologic appearance of the nude mouse tumors. This was interpreted as adenoid squamous cell carcinoma, eg, in addition to the presence of basal cells of the keratinizing squamous cell type similar to those of the original tumor, the adenoid structure being composed principally of one cell thickness formed a rounded space with a definite wall and lumen contained single or grouped dyskeratotic acantholytic space. Ultra-



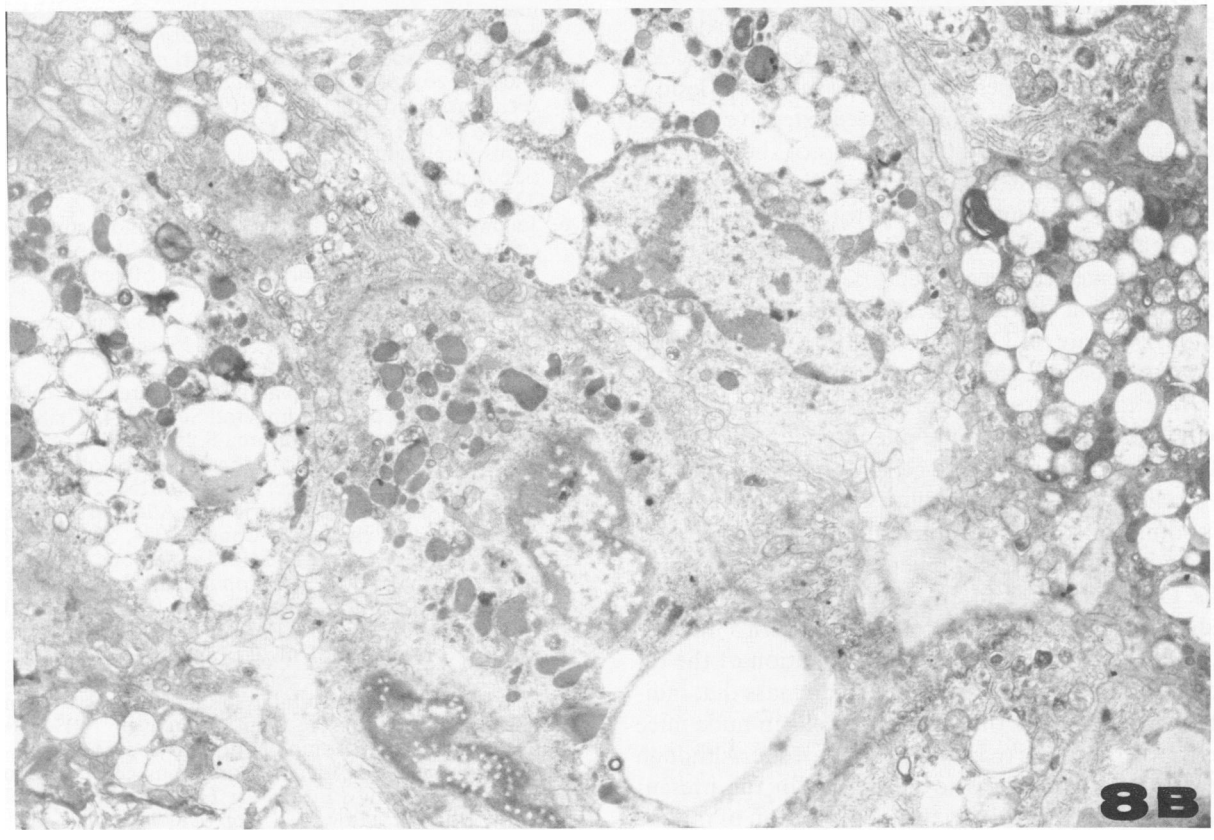


Figure 8—Histologic appearance and electron micrograph of the small mass formed after transplantation of cultured TYS cells treated with sodium butyrate. **A**—This mass is histopathologically interpreted as acinic cell carcinoma with squamoid area. **B**—Electron micrograph revealing the presence of numerous secretory granules and well-developed rough endoplasmic reticulum and Golgi complexes in the cytoplasm of the cells. (**A**, H&E, ×160; **B**, ×3600) A total of 10^7 TYS cells that were cultured at 37 C for 5 days in the growth medium containing 5 mM sodium butyrate were transplanted into a nude mouse. A small mass that developed transiently at the inoculation site was extripated and examined for morphologic study.

structurally, the tumor tissue was composed of both dark and clear cells (Figure 7A). Two neighboring tumor cells were attached, with distinct desmosomes, occasional tight junctions, or ample villous protrusions. The presence of secretory granules and rough endoplasmic reticulum was often observed in the cytoplasm of dark cells. Mitochondria and tonofilaments were poorly developed. Moreover, the tissue sections from the nude mouse tumors showed positive staining for amylase and CEA (Figure 7B and C).

When a total of 10^7 TYS cells incubated in the presence of 5 mM sodium butyrate in a 5% CO_2 incubator for 5 days at 37C was transplanted into 5 nude mice each, a small mass measuring about 3–5 mm in diameter developed transiently at the inoculation site in all of the nude mice and then disappeared within 3 months after inoculation of the cells. The small masses formed after transplantation of the treated cells into other nude mice were extirpated and examined for the morphologic study. As shown in Figure 8, the histopathologic features and ultrastructure were interpreted as acinic cell carcinoma containing a squamous cell area. A moderate lymphocyte infiltration, which might be expected for active rejection of the sodium butyrate-treated cells, was observed in the periphery of the tumorous lesion. Moreover, the tissue sections showed extensive positive staining for amylase and CEA (data not shown). This indicates that the altered phenotype that appeared after treatment of cultured TYS cells with sodium butyrate is maintained in nude mice and that the tumorigenicity of the treated cells is markedly decreased when compared with that of the untreated cells. The action of sodium butyrate on TYS cells in culture was reversible, although a few cell passages in the absence of sodium butyrate after treatment with sodium butyrate were needed for the return of the phenotype of the treated cells to that of the untreated cells. This implies that cell division in the absence of sodium butyrate after removal of sodium butyrate from the culture is necessary for the return to the cells with phenotype of the untreated cells. On the other hand, transplantation into athymic nude mice of cultured TYS cells treated with 5 mM sodium butyrate did not result in a formation of the tumor, although the formation of a small mass that indicates cell division of the inoculated cells in nude mice was transiently observed at the inoculated site. Although the mechanism remains to be proven in the present study, it is more than likely that the injection of mitotically altered TYS cells fail to grow *in vivo* as a result of the state of the cells, rather than an animals response to the tumor. The fact that TYS cells express amylase and inoculation of the cells treated with sodium butyrate into athymic nude mice results in a production of a mass with the histologic feature of acinic cell carcinoma strongly suggests that the TYS cell line presumably originates in salivary gland cells. It is well known that squamous metaplasia frequently occurs in glandular tissue, including the salivary gland.[26,27] Therefore, it can probably be presumed that the squamous components present in adenoid squamous cell carcinoma arising from the oral mucosa are closely associated with squamous metaplasia of the neoplastic duct cells arising in the minor salivary gland present in the oral mucosa and that the cells composing the adenoid structure represent the remnants of the glandular cells. We report here a new type of neoplastic epithelial cell line probably derived from a human minor salivary gland.

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