A Monolayer Culture of Human Gastric Epithelial Cells

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Our aim was to develop a fibroblast-free monolayer culture of human gastric mucosal cells, using the specimens obtained by routine endoscopic biopsy. Human gastric mucosa obtained from normal volunteers by endoscopic biopsy was dissociated from collagenase and hyaluronidase. Dissociated cells were cultured in supplemented Coon's modified Ham's F-12 medium. Within 24 hr of inoculation, the cells were attached to the culture dishes. This was followed by cellular outgrowth. On phase-contrast microscopy, all cells had epithelial characteristics and fibroblasts were not observed. Ninety percent of cells contained periodic acid Schiff reaction-positive mucous granules after diastase digestion consistent with mucous epithelial cells. Two percent of the cells gave a strong reaction for succinic dehydrogenase activity (parietal cells). Immunohistochemical staining for pepsinogen in cultured cells was negative. On EM, microvilli-like projections, junctional complexes, Golgi apparatus, and mucous granules were apparent in the majority of cells. Mitotic figures were observed by day 3 with Giemsa staining. Autoradiographically, these cells were able to incorporate [^{3}H]TdR into the nuclei. Cells were capable of synthesizing DNA, and this function was inhibited by cycloheximide. Cells could be cultured for up to two weeks without fibroblast contamination. A method of primary monolayer culture of human gastric mucosa obtained by a routine endoscopic biopsy has been successfully developed.

Tissue culture of human gastric mucosal cells offers a convenient tool for the study of the effects of hormones and drugs on these cells in conditions independent of the neural, hormonal, and blood flow factors. Only a few studies of cell culture of gastric mucosa have been reported in animals (1, 2). The only study of human gastric mucosa was published by Miller et al (3), who cultured gastric oxyntic cells obtained from surgical specimens. In their culture, however, fibroblast overgrowth occurred unless pentagastrin was used. The aim of the present study was to develop a monolayer culture of human gastric mucosal cells, based on specimens obtained by routine endoscopic procedures and without use of pentagastrin. The morphological characteristics of these cells and their ability to proliferate and to synthesize DNA was then studied.

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MATERIALS AND METHODS

Materials. Medium I: Coon's modified Ham's F-12 medium (4) (F-12 medium, KC Biological Inc., Lenexa, Kansas) containing 0.1% collagenase (120 units/mg, GIBCO, Grand Island, New York) and 0.05% hyaluroni-

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dase (type I-S, 270 units/mg, Sigma Chemical Co., St. Louis, Missouri), 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamycin (these antibiotics were obtained from Sigma). Medium II: F-12 medium supplemented with heat-inactivated (at 56° C for 30 min) 10% fetal bovine serum (GIBCO), 10 mM HEPES buffer (Sigma), fibronectin (2 μg/ml, Sigma) and antibiotics described above. Hanks' balanced salt solution (BSS) was also supplemented with antibiotics. Trypsin, colchicine, and cyclohexamide were purchased from Sigma. [^{3}H]Thymidine ([^{3}H]TdR, specific activity 20 Ci/mmol) was obtained from New England Nuclear, Boston, Massachussets. Antipepsinogen II used for immunofluorescence testing was kindly supplied by Dr. I.M. Samloff. Tissue culture dishes (35 × 10 mm) were purchased from Falcon, Oxnard, California. Nuclear Track Emulsion (NTB 2), D 19 and Rapid Fix were obtained from Eastman-Kodak, Rochester, New York.

Cell Preparation. The study was approved by the institutional Human Studies Subcommittee. Specimens of human gastric body mucosa were obtained from normal volunteers aged 22–34 years by routine endoscopic procedure (Olympus GIF Q, FB-25K biopsy forceps, 6–12 biopsies per person). Additional biopsies were stained with hematoxylin and eosin; mucosal histology of all subjects studied was normal. Specimens obtained were immediately transferred to ice-cold medium II, then rinsed several times with BSS and minced into 1- to 2-mm^3 pieces using fine curved scissors. The minced tissue was suspended in medium I and incubated at 37° C in the atmosphere of 5% CO_2 and 95% O_2 for 60 min. Then the tissue was pipetted several times to complete dispersion of cells and centrifuged at 600 rpm for 5 min. The sediment was washed twice in F-12 medium and the final pellet was resuspended in medium II.

Culture. Suspended cells in medium II were inoculated into tissue culture dishes. The cultures were maintained in an incubator at 37° C in a humidified atmosphere of 5% CO_2 in air. After 24 and 36 hr, cultures were washed five times, respectively, and fresh medium II was added to the cultures. The medium was changed every day.

Morphological Studies. Cultures were examined daily with a phase-contrast photomicroscope. The trypan blue exclusion test for viability of isolated cells was performed by the method of Phillips (5). For histochemical identification of cultured cells, periodic acid–Schiff (PAS) reaction with and without 1.0% diastase digestion (for mucus-producing cells) and succinic dehydrogenase activity (for parietal cells) determined by the method of Nachlas et al (6) were employed. Indirect immunofluorescence test for pepsinogen was also performed according to the method of Samloff and Liebman (7). For electron microscopy, cells were dislodged by incubation in 0.05% trypsin for 10 min at 37° C, fixed with 3% glutaraldehyde for an hour, and postfixed with 1% OsO_4 for an hour. The cells were dehydrated with graded alcohol and embedded in Araldite 502 and polymerized at 60°C overnight. Thin sections were stained with uranyl acetate followed by lead citrate and examined in a RCA EMU-4 transmission electron microscope operated at 75kV.

Cell Growth. To determine cell growth, the nuclear counting method was utilized (8). The cultured cells were dislodged by incubation of the cells in 0.25% trypsin for 10 min. The cells then were incubated in 0.1 M citric acid for an hour at 37° C and pipetted vigorously. The nuclei were separated from the supernatant by centrifugation at 1000 rpm for 20 min and suspended in the 0.1% crystal violet solution. The nuclei in the suspension were counted using a hemacytometer.

Mitosis of Cells. The cells on the culture dishes were washed with BSS and fixed in methanol–acetic acid (3:1). After washing the cells with absolute methanol, the cells were stained with Giemsa solution. The mitotic index was expressed as a percentage of mitotic cells in approximately 1000 cells. To study the effect of colchicine on mitosis, the cells were incubated in the culture medium containing 10 μg/ml of colchicine for 8 hr and processed as described above.

DNA Synthesis. To determine the rate of DNA synthesis in the cultured cells, cells were pulsed in the culture medium with 1 μCi/ml [^{3}H]TdR for 6 hr each day. To study the effect of cyclohexamide on DNA synthesis, cells were incubated in the culture medium containing 1 μCi/ml [^{3}H]TdR for up to 24 hr with or without cyclohexamide (100 μg/ml). Excess [^{3}H]TdR was removed by washing the cells twice with BSS. TCA soluble [^{3}H]TdR remaining in the cells was extracted in 5% TCA at 4°C for

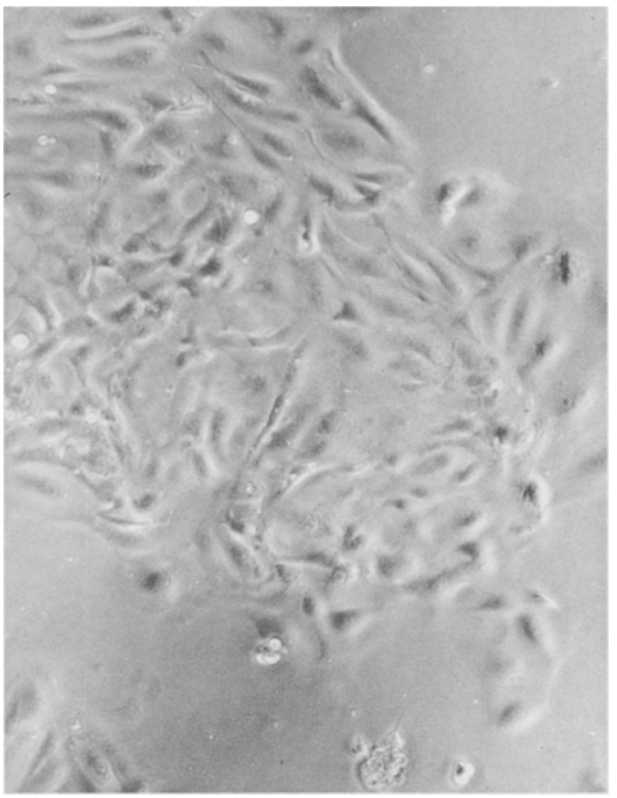

Fig 1. Phase-contrast photomicrograph of a primary monolayer culture derived from human fundic mucosa, 3-day-old culture (×200).

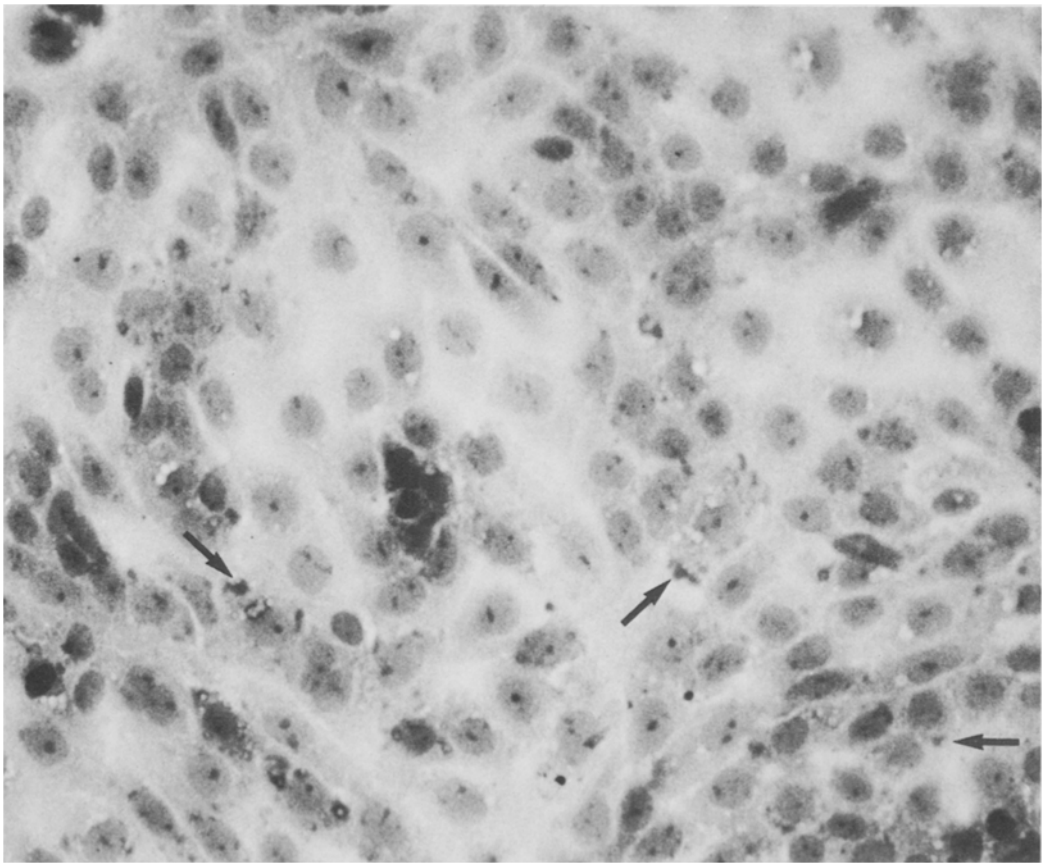

Fig 2. PAS-stained human gastric epithelial cells in tissue culture after 1.0% diastase treatment, 5-day-old culture (×200). Dark-staining material in the cytoplasm (arrows) is PAS-positive material.

6 hr. The TCA insoluble material in the cells was dissolved in 1 N NaOH and neutralized with 1 N HCl. The radioactivity of [^{3}H]TdR in the cells was counted with a liquid scintillation counter. Cell protein was determined according to the method of Bradford (9).

Autoradiography. The cells were pulsed in the culture medium with 1.0 μCi/ml [^{3}H]TdR for 6 hr each day. After washing the cells with BSS, the cells were fixed in methanol–acetic acid (3:1). The cells were then washed twice in absolute methanol and air dried. NTB 2 emulsion was added to the cells on the culture dishes and dried. The culture dishes were stored in the dark at 4° C for 1 week. They were developed in Kodak D19 for 6 min and fixed in Kodak Rapid Fix. The cells were poststained with hematoxylin. Labeling index was expressed as percentage of the labeled cells (containing more than 10 silver grains) in approximately 1000 cells.

RESULTS

Light Microscopy. Using the collagenase dissociation procedure, cell clumps with good viability (94 ± 1%) were obtained. Dissociated single cells showed lower viability (56 ± 3%). Within 24 hr after inoculation, the cell clumps attached to the surface of the dishes and cells began to spread out and grow (Figure 1). There was no bacterial contamination for periods up to two weeks.

Phase-contrast microscopy showed that this culture consisted of a homogeneous population of epithelial cells with large, oval nuclei, polyhedral shape, and organized sheetlike growth pattern (Figure 1). Throughout the culture periods, the cultures were free of fibroblast overgrowth. While the cytoplasm of these cells was clear at early stages of culture, numerous cytoplasmic perinuclear granules were observed in most of the cells after day three by phase-contrast microscopy.

These characteristics of the cells were maintained for up to two weeks. After two weeks, however, cells became irregularly separated from each other and consequently degenerated. This phenomenon occurred even if insulin, transferrin, corticosteroid,

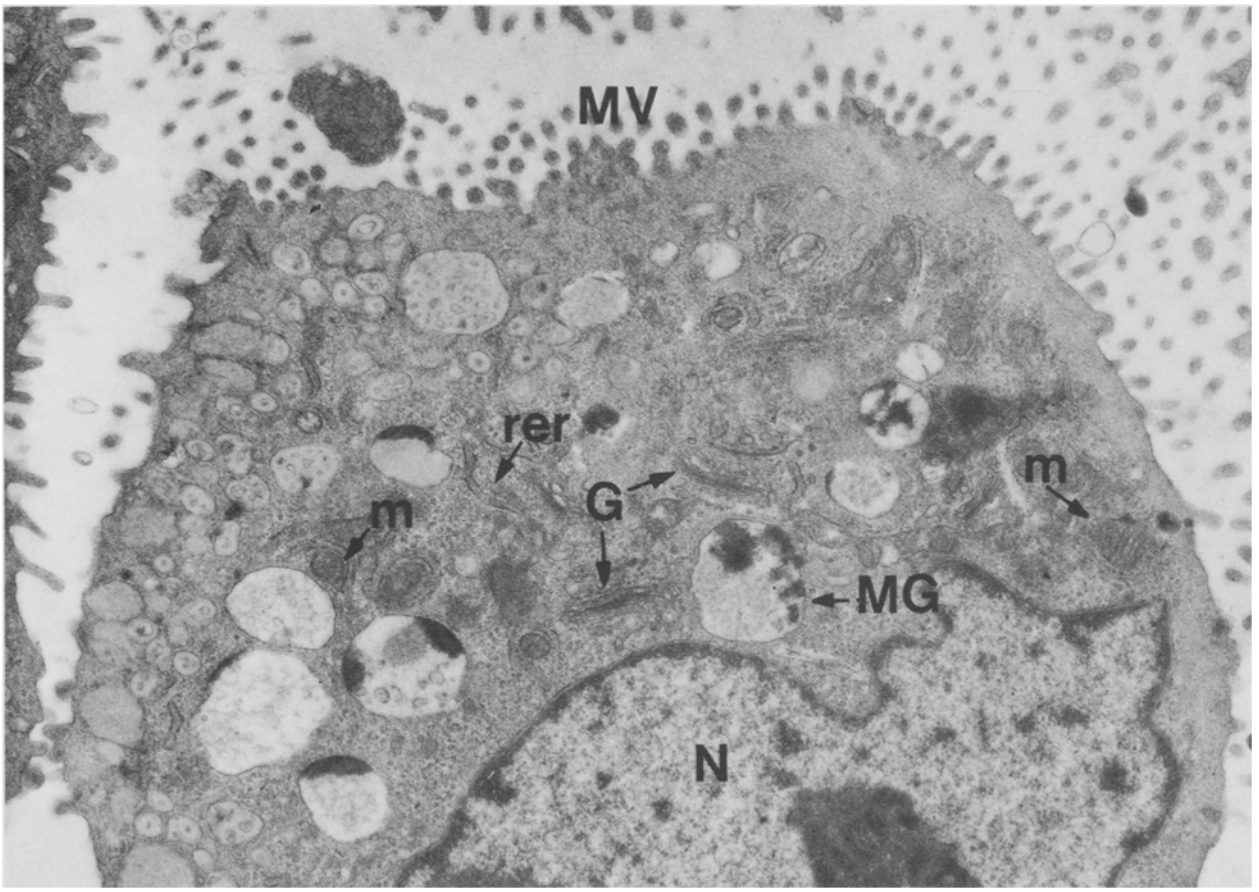

Fig 3. Transmission electron micrograph of human gastric epithelial cells in tissue culture, 3-day-old culture (×12,000). N, nucleus; MV, microvilli; G, Golgi complex; m, mitochondria; rer, rough endoplasmic reticulum; MG, mucous granules.

a higher concentration (25 mM) of HEPES buffer or lower concentration of serum (2.5%) were employed for culture (10).

Histochemical Study. Ninety-five percent of the cells in 5- to 7-day-old cultures contained PAS-positive material in the cytoplasm (Figure 2); 67% contained single to numerous strongly PAS-positive granules, while 28% stained weakly, ie, contained tiny and coarse PAS-positive material, which was resistant to diastase digestion. Two percent of the cultured cells showed a strong positive reaction for succinic dehydrogenase. Immunohistochemical localization of pepsinogen by indirect immunofluorescence was negative.

Transmission Electron Microscopy. Ultrastructurally, cultured cells showed the characteristics of epithelial cells (Figure 3). Numerous microvillus-like structures were present on the surface of the cells. At the site of cell–cell contact, junctional complexes, ie, tight junctions and desmosomes, as well as interdigitations of cell membrane were observed (Figure 4). Mitochondria scattered throughout the cytoplasm had well-defined cristae. Free ribosomes were abundant in the cytoplasm, and rough endoplasmic reticulum was evident. Well-developed Golgi complexes were prominent, and some secretory granules were located close to Golgi apparatus (Figure 3). Intracellular canaliculi were absent. A number of membrane-limited granules were distributed in the cytoplasm. The majority of granules contained some electron-dense substances presumed to be mucus in nature (Figure 3). According to Ito (11), mucous cells contain electron-dense substances, while zymogen granules of chief cells show moderate density. These electron-dense substances corresponded to PAS-positive granules on

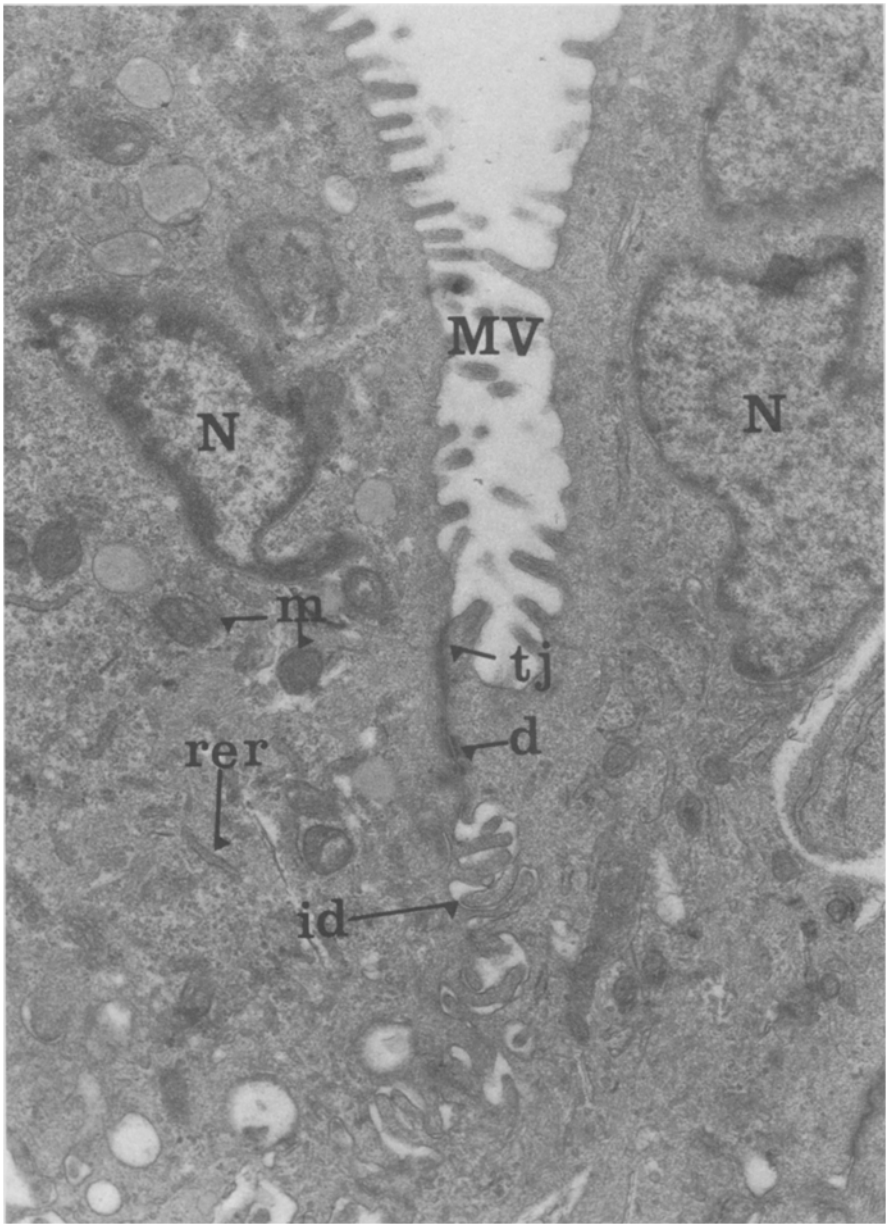

Fig 4. Transmission electron micrograph of human gastric cultured cells, showing junctional complex (×12,000). N, nucleus; MV, microvilli; m, mitochondria; rer, rough endoplasmic reticulum; tj, tight junction; d, desmosome; id, interdigitation.

histochemical study; also in our study, immunohistochemical localization of pepsinogen was negative thus ruling out pepsinogen granules.

Cell Proliferation. Population doubling time for the cultured cells was 40 hr (Figure 5). Mitotic figures were prominent (Figure 6) and the mitotic index was maximum on day 3 (1.5%). This value was increased to 4.2% by colchicine.

DNA Synthesis. Autoradiography of [^{3}H]TdR in the cultured cells demonstrated the ability of the cells to synthesize DNA (Figure 7). The labeling index was maximum on day 2 (4.8%), which paral-

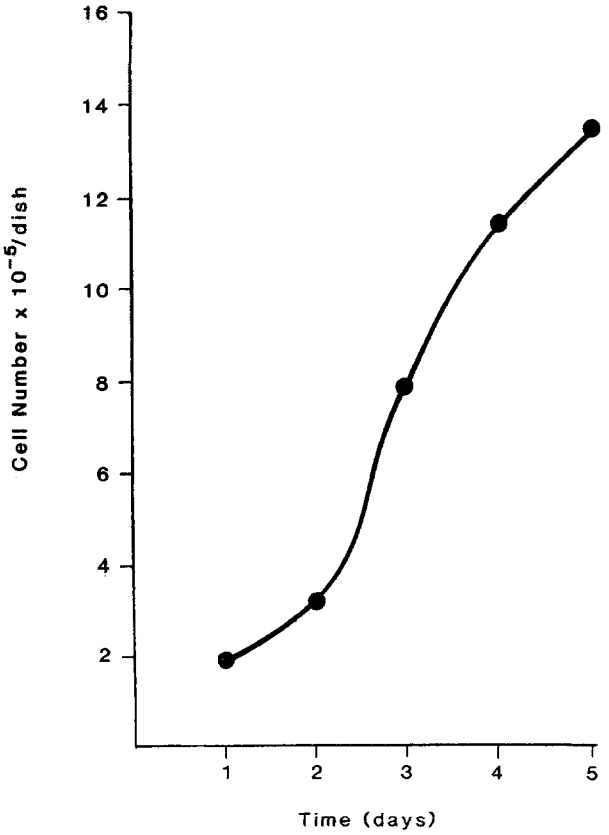

Fig 5. Growth curve of human gastric cultured cells. Each point is the mean of four cultures. The differences are statistically significant ($P < 0.05$).

leled the result of [^{3}H]TdR incorporation determined biochemically. Cyclohexamide (100 μg/ml) inhibited the capability of the cells to synthesize DNA.

DISCUSSION

The present study shows that human gastric mucosal cells obtained through routine endoscopic biopsy can be cultured for two weeks. The cultured cells were predominantly mucus-producing cells. Miller et al (3) reported cell culture of human gastric mucosa from surgical specimens. In their study, however, fibroblasts were the predominant cells unless pentagastrin was used and the identification of the cultured cells was not as complete as in the present study. Further, our inoculated cells started to grow within 24 hr after incubation, whereas Miller's culture had a latent period of 7 days.

In our study, specimens of human gastric oxyntic mucosa were obtained from normal volunteers through routine endoscopic biopsy procedure. Endoscopic biopsy specimens are much easier to obtain than surgical specimens and material obtained is more likely to be normal. Although a potential disadvantage of our method is bacterial contamination of tissues during the endoscopic procedures, this problem was overcome by washing the biopsy specimens and the cultures extensively as well as by adding antibiotics to the medium.

An additional advantage of this culture technique was that it did not need any hormonal substances, for example, insulin or pentagastrin, which could affect studies of the effects of gastrointestinal hormones on the cells.

Fibroblast overgrowth has been one of the major problems of the epithelial cell culture and various attempts to eliminate fibroblastic contamination have been reported (12–16). Our tissue culture of human gastric mucosa was free of fibroblasts for the whole study period of two weeks. One reason for this is that collagenase was employed for the dissociation of gastric tissues in this study. Collagenase was first used by Lasfargues and Moore (17) and since then, it has been applied to the dissociation of several kinds of tissue to eliminate the fibroblast contamination (14–16, 18). However, in the cell culture of rat gastric mucosa, which we reported previously (2), although the fibroblast overgrowth was prevented by collagenase dissociation for three to four days, the predominant cells were fibroblasts after two weeks. Therefore, another reason may be that biopsy specimens contain only a small amount of lamina proprial connective tissue.

The evidence that cultured cells were epithelial was obtained by light microscopy, histochemistry, and ultrastructure of the cells. In phase-contrast microscopy, the cultured cells possessed a rounded, polyhedral shape and a sheetlike organized growth pattern, whereas fibroblasts have a slender, spindle shape and a disorganized growth pattern (19). Ultrastructurally, numerous microvillus-like projections were present on the surface of the cells as well as junctional complexes on the borders of the cells. These findings are characteristic of epithelial cells (13). Contamination with a considerable amount of endothelial cells can be excluded, because the majority of the cultured cells contained mucous granules.

PAS staining demonstrated that over 90% of the cultured cells contained mucous granules. Differentiated parietal cells are characterized by numerous mitochondria (11). In this culture, only 2% of cells

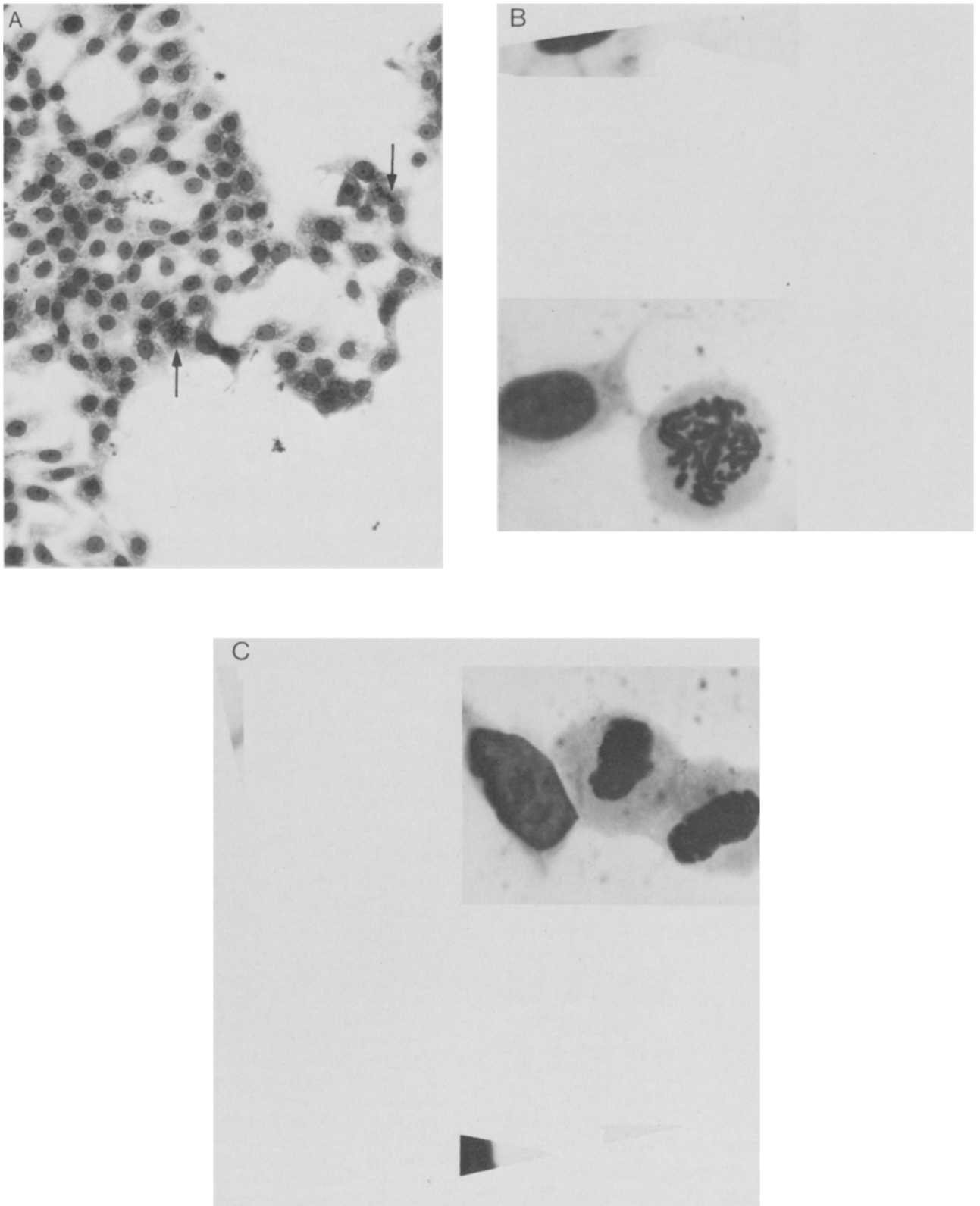

Fig 6. Giemsa-stained human gastric epithelial cells in tissue, 3-day-old culture. (A) Mitotic figures are shown by arrows (×200); (B) a cell in metaphase of mitosis (×1000); (C) a cell in telophase of mitosis, showing cytoplasmic division (×1000).

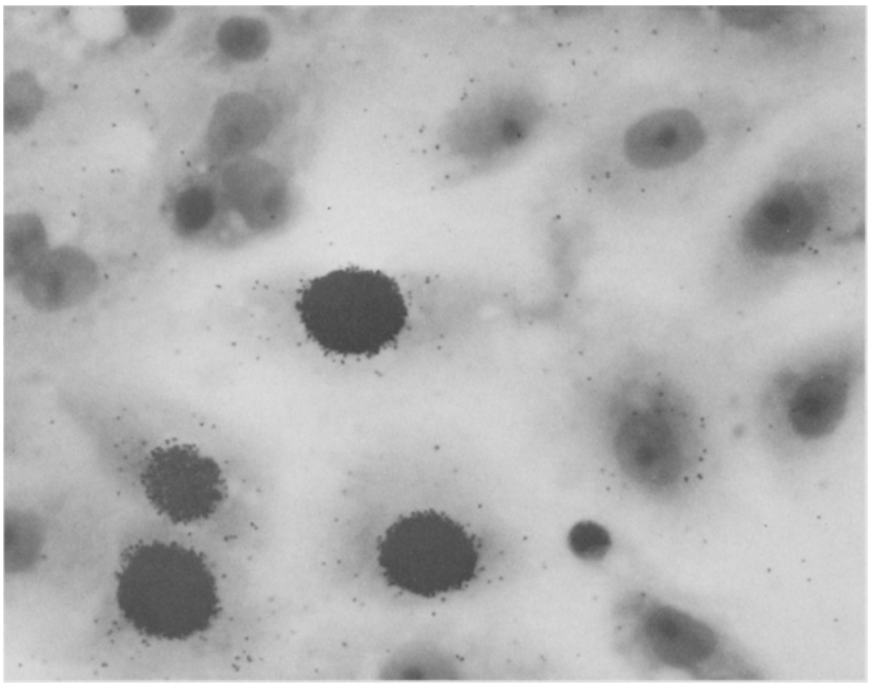

Fig 7. [^{3}H]Thymidine autoradiograph of human gastric cultured cells (hematoxylin stain, ×400). The cells which contained more than ten silver grains in the nucleus are considered labeled cells.

gave a strong reaction for succinic dehydrogenase, which is located in mitochondria. Immunohistochemical study for pepsinogen revealed that none of the cells in the culture exhibited specific fluorescence.

On transmission electron microscopy, the majority of the cells studied possessed a large number of membrane-limited secretory granules with electron-dense substance which correspond to PAS-positive granules and were considered to be mucous material (11). Intracellular canalicular-like structures or zymogen granules in the cytoplasm were not present.

These findings indicate that the majority of the cultured cells were composed of mucus-producing cells, although a small number could represent poorly differentiated parietal cells. It is well known that cultured cells are generally not fully differentiated and could represent stem cells with multipotent differentiation (19). Similar characteristics of gastric glandular epithelium, as found in this structure system, were described by Helander during development of rat fetal gastric mucosa (20, 21).

The ability of the cultured cells to proliferate and to synthesize DNA was demonstrated by the studies of mitosis, incorporation of [^{3}H]TdR into cellular nuclei, and cell growth. Such data provide a good basis for future studies of ulcerogenesis or carcinogenesis in human gastric mucosa *in vitro*.

In summary, we have described a method for monolayer culture of human gastric mucosal cells obtained by routine endoscopic biopsy from the body of the stomach. This culture was free of fibroblasts, and the majority of the cells were identified as mucus-producing cells. This culture system offers a valuable tool for the investigation of cellular function in human gastric mucosa.

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