ELECTRON MICROSCOPY OF ORAL CELLS IN VITRO

II. Subsurface and Intracytoplasmic Confronting Cisternae in Strain KB Cells

M. KUMEGAWA, M. CATTONI, and GEORGE G. ROSE

From the Department of Medicine (Periodontics), The University of Texas Dental Branch at Houston; the Department of Biology, The University of Texas M.D. Anderson Hospital Tumor Institute at Houston; and the Tissue Culture Laboratory, Hermann Hospital, Houston, Texas 77025

ABSTRACT

Two special areas involving membranous components in strain KB cells were studied by electron microscopy. The first area described is that of the subsurface regions of two apposing cells in which flattened cisternae (one cisternae in each subsurface region) with membranes spaced 110–230 A apart were found in a confrontation alignment. The long dimension of the profiles of these cisternae ranges from 0.5 to 2 μ. At these intercellular contact areas, each cisterna is closely applied to the adjacent plasma membrane; the intervening space is 60–100 A. We have named the cisternae in these roughly symmetrical areas of cell contact the *subsurface confronting cisternae*. Communications between these cisternae and those of the rough-surfaced endoplasmic reticulum also were observed. The second area described is that of the *intracytoplasmic confronting cisternae*. These cisternae were observed as oval or round images about 0.3–1.4 μ in diameter, each image being composed of a pair of concentrically arranged confronting cisternae with membranes spaced 200–400 A apart. The apposing membranes of the two confronting cisternae are electron opaque, smooth, and free of ribosomes, whereas the unapposed membranes are less dense, scalloped, and associated with ribosomes. The spacing between the two intracytoplasmic confronting cisternae is 70–110 A.

INTRODUCTION

KB cells were established as an in vitro strain from a human epidermoid carcinoma by Eagle (6) in 1955. Since then, they have been used in various types of investigations (3, 16–18, 22, 23, 26–36).

As with most strains, the KB cells form colonies on the glass walls of culture vessels. At their centers, the colonies become thick and are full of degenerating cells, while toward their peripheries the thickness diminishes and the cells become flatter. If a colony is considered to consist of three concentric areas, the cells described in this electron microscope study would be found most frequently in the intermediate zone. Cells cultivated in multipurpose culture chambers under sheets of dialysis cellophane assume a rather uniform flattening, so that there is no pronouncedly thickened central area in the cell colonies.

The purpose of this report is to give a detailed description of the structure of two types of membranous cisternae occurring in some of these cells: (1) subsurface confronting cisternae (SCC) which appeared where cells made contacts and which formed, with the two apposing plasma mem-

branes, a rather symmetrical image of six relatively parallel membranes; and (2) intracytoplasmic confronting cisternae (ICC) which, in all instances, appeared in the sections as four membranes in ringlike structures. Both of these types of confronting cisternae were found only in the KB cells cultivated on the glass walls of tubes or chambers where they were in direct contact with the fluid nutrient; they were not present in the KB cells cultivated under the cellophane in microenvironments containing a dialysate of the fluid nutrient.

MATERIALS AND METHODS

The KB Cell Strain

Cells obtained from a commercial source (Microbiological Associates, Bethesda, Maryland) were maintained in our roller tubes revolving at 4 rpm and in our nutrient formula at 37°C for 3 months before study.

Nutrient Medium

Two kinds of nutrients were used: (*a*) an equal mixture of Medium 109 and Medium V-614 (Difco Laboratories, Detroit), and (*b*) Waymouth's Medium 752/1 (Hyland Laboratories, Los Angeles). Both were supplemented with calf serum (20%), whole egg ultrafiltrate (5%), phenol red (0.001%) as a pH indicator, and penicillin G (1,000 units/ml).

Specimen Preparation of Roller Tube Cultures for Electron Microscopy

At 4, 8, 30, and 45 days of cultivation, the KB cells were fixed either in: (1) phosphate-buffered osmium tetroxide solution (1%, 21) for 1 hr at 4°C; (2) phosphate-buffered glutaraldehyde (2.5%, 39) for 1 hr prior to fixation in the osmium tetroxide; or (3) 0.5% unbuffered potassium permaganate for 0.5 hr. After fixation, washing, and dehydration, the cells were embedded in Araldite resin (20) either in situ (13) or after removal from the glass walls of the tubes.

For the in situ embedding, the KB cells were treated with alcohol and propylene oxide and infiltrated with a 50:50 mixture of the Araldite resin and propylene oxide for 0.5 hr prior to their infiltration with the undiluted resin for 12 hr. We then replaced this resin with the embedding resin and, after polymerization, plunged the hot tube into an ice-water bath to break the tube. With a scalpel, the colonies embedded in the polymerized resin were cut from the long mass of hard resin into 2-mm cubes. These cubes were oriented and cemented onto the ends of the resin rods so that the cells could be sectioned in a plane which coincided with that of the wall of the test tube in which they were cultivated.

After dehydration in 95% alcohol, the KB cells in other tubes were scraped with a rubber policeman and the dislodged cells were centrifuged into pellets. The pellets were treated with alcohol (100%) and propylene oxide prior to embedding in the Araldite resin as denoted above.

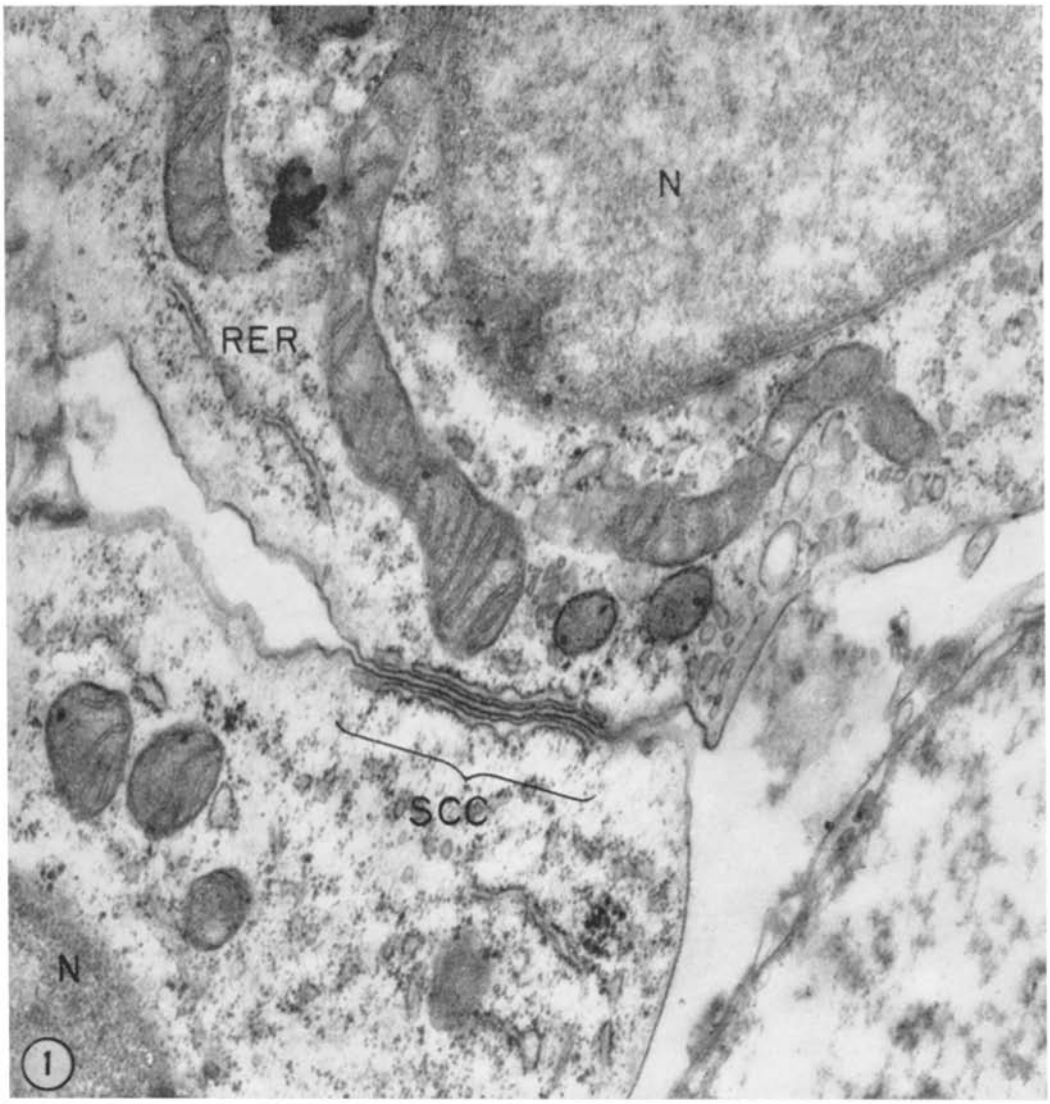
The embedded specimens were sectioned on a

Abbreviations Used on Micrographs

ICC, Intracytoplasmic confronting cisternae
IM, Inner membranes of ICC
Int M, Intermediate membranes of ICC
OM, Outer membranes of ICC
L, Lipid droplet
N, Nucleus
PM, Plasma membranes
RER, Rough-surfaced endoplasmic reticulum
SCC, Subsurface confronting cisternae
Di M, Distal membranes of SCC
Pr M, Proximal membranes of SCC
M, mitochondrion

FIGURE 1 KB cells in contact with each other at a limited area. Subsurface confronting cisternae (*SCC*) are observed at the intercellular contact. Fixed in OsO_4. Stained in lead citrate. × 45,000.

FIGURE 2 High magnification of Fig. 1. Micrograph shows the region of the two subsurface confronting cisternae. Two plasma membranes (*PM*) are closely apposed, the intervening space being about 150 A. The membranes of each cisterna are about 110–230 A apart. Each cisterna is tightly applied to the adjacent plasma membrane (*PM*), the light intervening zone being 60–100 A. Ribosomes are associated only with the proximal membranes (*Pr M*). Fixed in OsO_4. Stained in lead citrate. × 101,000.


N
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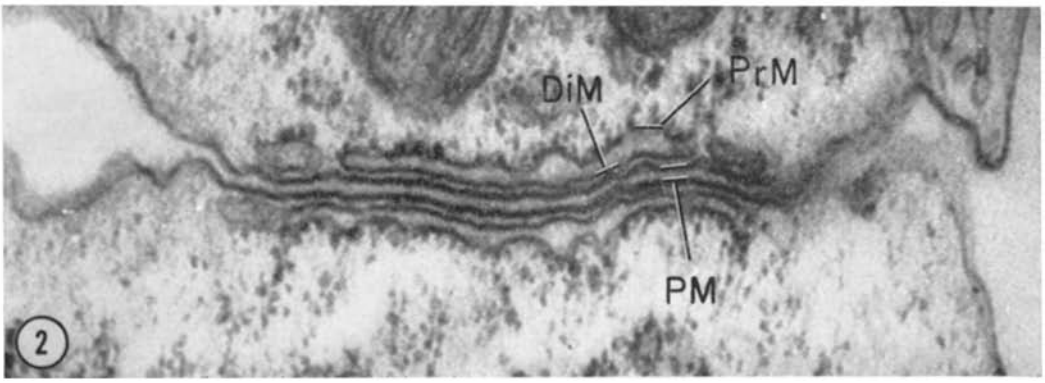
1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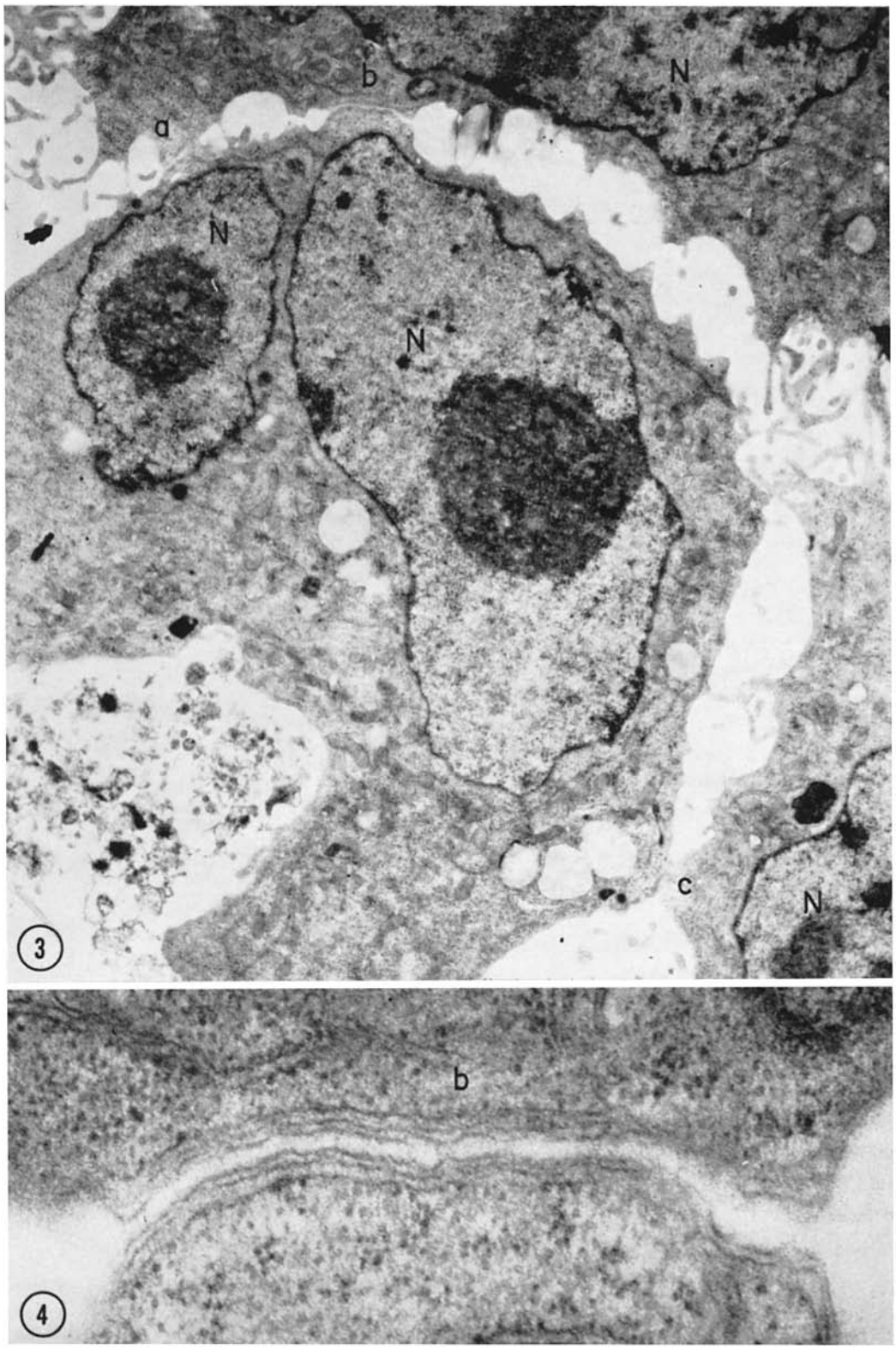

FIGURE 3 Low-power micrograph showing a KB cell surrounded by two other cells. The cells are in contact with each other at three areas (*a–c*). At each contracting area, there is a unit of subsurface confronting cisternae. Fixed in glutaraldehyde-OsO_4. Stained in uranyl acetate and lead citrate. × 9,000.

FIGURE 4 High magnification of the region of the subsurface confronting cisternae shown at *b* in Fig. 3. × 109,000.

Porter-Blum II microtome with a glass knife, and the sections were stained either in 0.2–0.5% lead citrate (42) or in 2% uranyl acetate, followed by a second staining in the lead citrate. The preparations were examined in an RCA EMU-III electron microscope.

OBSERVATIONS

Subsurface Confronting Cisternae (SCC)

The KB cells cultured in roller tubes are typically pleomorphic. At their periphery, finger-like cytoplasmic protrusions and microvilli of various sizes occur. Ordinarily, the KB cells are in contact at limited cell areas which, in profile, are about 0.5–2.0 μ in length (Figs. 1–4). In contact zones, the closely apposed plasma membranes are separated by an intercellular space of 130–220 A containing a gray "fuzzy" material. The most remarkable feature of many of these intercellular contact areas is the mirror-image appearance of the confronting cisternae (one cisterna in the subsurface region of each cell) in the two apposing cells. These cisternae, which will be called the "subsurface confronting cisternae," together with the two apposing plasma membranes form a six-membrane unit (SCC unit). The frequency of these structures varied among the cells in a colony; a preponderance of them occurred in cells in the intermediate zones of the colonies.

In sectioned specimens, the SCC appeared as flattened sacs ranging up to 2 μ in their long dimension. The pair of cisternae, i.e., one cisterna in each of the apposing cells, generally is symmetrical. In Figs. 1 and 2, the six membranes of the SCC unit may be seen to be composed of four roughly parallel inner membranes and two scalloped and ribosome-studded outer membranes. In these figures, the two distal membranes (*DiM*) of the subsurface cisternae and the two interposed plasma membranes (*PM*) were sectioned normal to their flattened surface through most of this profile so that their interrelationships are clear.

At high magnification, the distance between the plasma membrane and the distal membrane of each subsurface cisterna was measured and found to be 60–100 A, whereas the depth of each cisternal space is 110–230 A (Fig. 5). The two apposing plasma membranes are spaced 130–220 A apart. The two plasma membranes and the two distal membranes of the subsurface cisternae produce a relatively smooth unit of four parallel, though slightly wavy, lines; the proximal membranes of the cisternae have the same appearance as the rough-surfaced endoplasmic reticulum (RER), though the concentration of ribosomes on them is not abundant. In cells fixed in phosphate-buffered osmium tetroxide, the plasma membranes and distal cisternal membranes are more electron opaque than the proximal cisternal membranes and most portions of other cytomembranes (Figs. 1 and 2). Between the two plasma membranes of an SCC unit, there is no delineated structure such as the intercellular contact line of desmosomes (9) or the "septate attachment" observed in hydra (43), although there is in most images a gray "fuzzy" intercellular material. In cells fixed in glutaraldehyde, the density of the four intermediate membranes in the SCC unit is not so marked.

In situations in which a cell was apposed to a mitotic cell, a subsurface confronting cisterna was observed only in the nonmitotic cell, although in the mitotic cell the cisternae of the endoplasmic reticulum were detected in the deeper areas and parallel to the plasma membrane.

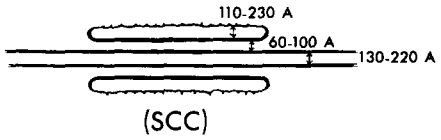

FIGURE 5 Diagram of the region of the subsurface confronting cisternae (*SCC*) showing measurements of membrane spacing.

Direct communications between the SCC and the cisternae of the RER are prominent (Figs. 6 and 7). Connections between the SCC and the more deeply embedded endoplasmic reticulum occur either at the ends of the SCC (Fig. 6) or at more central portions of the SCC (Fig. 7). In places in which SCC become detached from their close association with the plasma membrane, their distal membrane also is studded with ribosomes, as it is for other elements of the endoplasmic reticulum.

Intracytoplasmic Confronting Cisternae (ICC)

Pairs of intracytoplasmic cisternae arranged in oval, round, or distorted ring forms were found in 1 or 2% of the sectioned KB cells (Figs. 8 and 9). The diameter of the units of ICC ranged from 0.3 to 1.4 μ, and one to four units were detected in a KB cell. Normal constituents of the cytoplasm

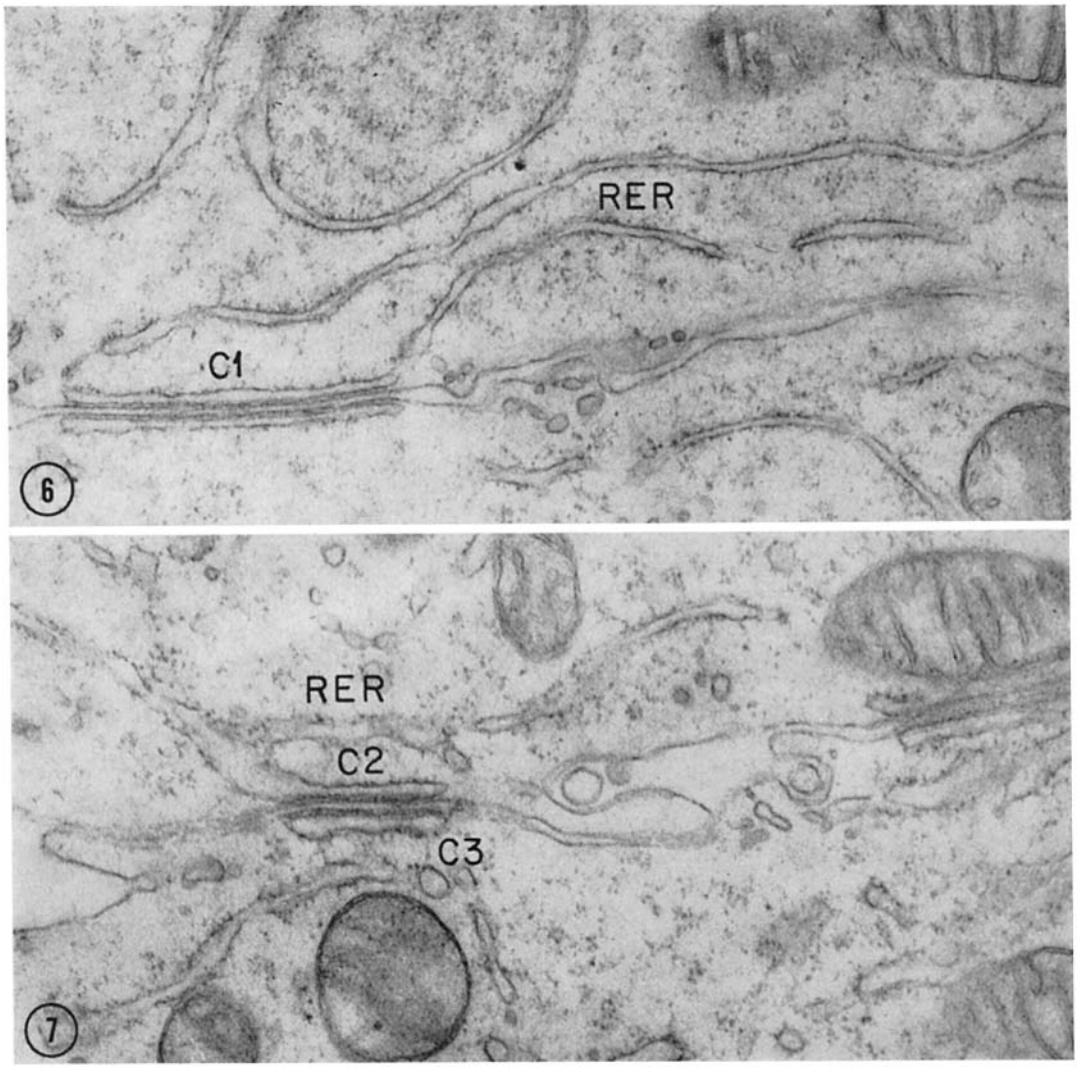

FIGURE 6 Micrograph showing a communication between a subsurface confronting cisternae (C1) and the *RER*. Fixed in OsO_4. Stained in lead citrate. × 45,000.

FIGURE 7 Two communications between the *RER* and the subsurface confronting cisternae are shown in this micrograph. The *RER* communicates with one end of a cistern at *C2* and possibly with the central part of a cistern at *C3*. Fixed in OsO_4. Stained in lead citrate. × 45,000.

(lipid, RER, mitochondria, and glycogen) were observed within the limiting membranes of the ICC unit.

At high magnification, the two closely apposed cisternae were found to be spaced 70–110 A apart, whereas the membranes of a single cisterna were spaced 200–400 A apart (Fig. 10). The closely applied intermediate membranes were more electron opaque and smoother than the inner and outer membranes which were studded with ribosomes.

DISCUSSION

The subsurface confronting cisternae observed in the KB cells cultivated in roller tubes bear some resemblance to the "subsurface cisterns" described

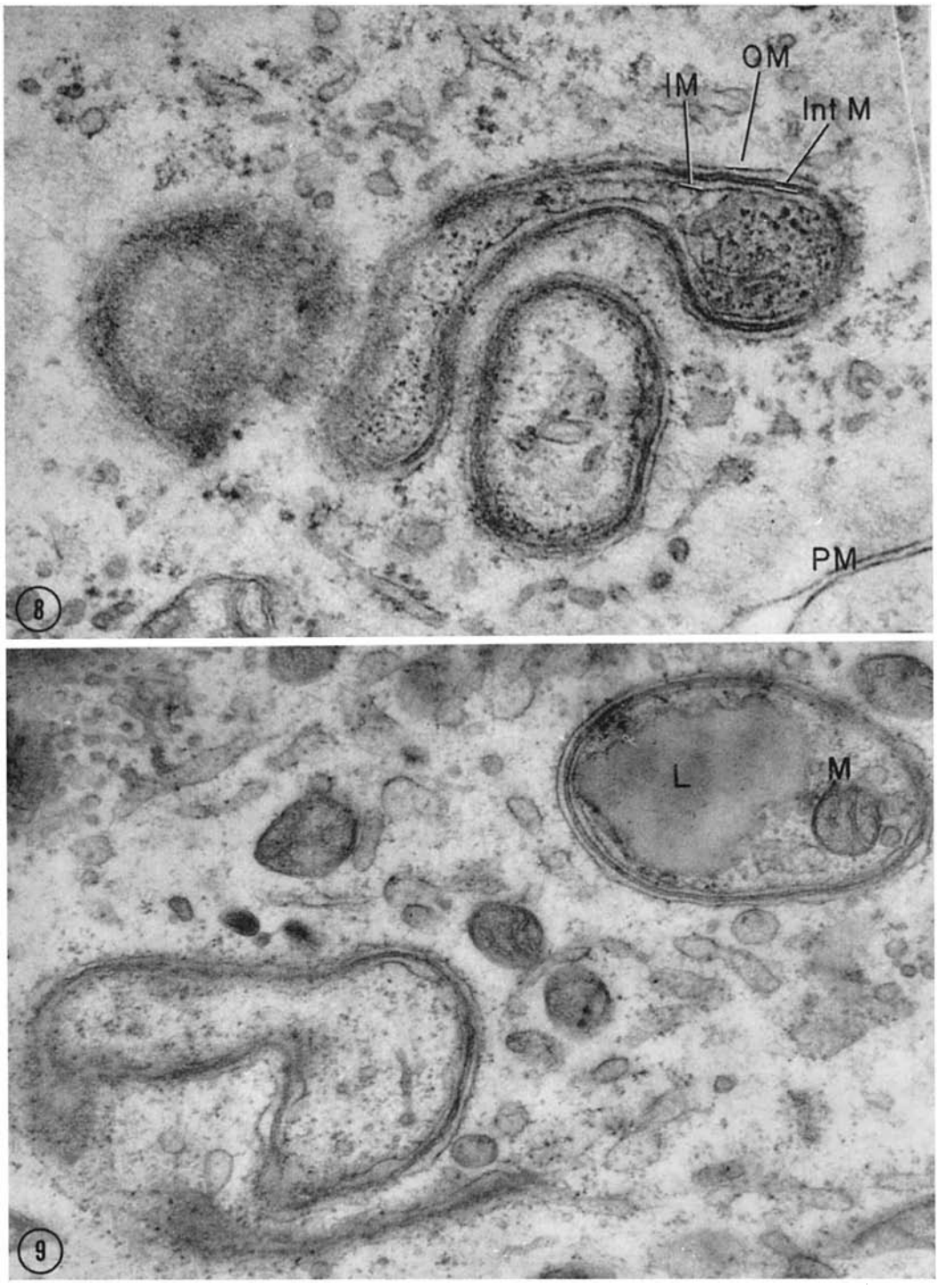

FIGURE 8 Three units of intracytoplasmic confronting cisternae are shown. The membranes of each of the two cisternae in a unit are spaced 200–400 A apart. The spacing between the two closely applied cisternae is 70–110 A. Fixed in OsO_4. Stained in lead citrate. × 69,000.

FIGURE 9 An area containing two units of intracytoplasmic confronting cisternae. A lipid droplet (*L*) and a mitochondrion (*M*) are contained in the unit at the right. Fixed in OsO_4. Stained in lead citrate. × 47,000.

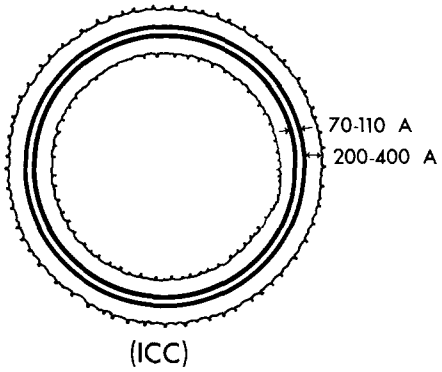

FIGURE 10 Diagram of unit of intracytoplasmic confronting cisternae (*ICC*) showing measurements of membrane spacing.

in sensory receptors and nerve cells (2, 4, 5, 12, 15, 37, 40), to dyads and triads of the sarcoplasmic reticulum in various muscle cells (10, 24, 25, 38), and to the cisternae in Sertoli cells of white mice (11). Paramembranous cisternae in mouse hapatocytes (41) and the paramembranous tubular system in proximal tubule cells of the rat kidney (8) appear to be nearly identical with the SCC of the KB cells. The only report of subsurface cisterns in cultured cells is that by Bunge et al. (5) who found them in cultured spinal cord; however, those subsurface cisterns are not the confronting type described in this report.

Although the functional significance of these subsurface cisternae is not yet clear, several suggestions concerning their function have been proposed. In nerve cells, these cisternae may be related to the transmission of neuronal impulses (12) or to the distinctive physiological properties of neuronal surfaces (37). In muscle cells, these membranous components may be involved in excitation-contraction coupling (24). In Sertoli cells, they have been likened to desmosomes (11).

Our observations suggest, in addition, that the SCC play a possible role in the process of cellular interaction. KB cells cultured in roller tubes are actively proliferative and exhibit a loss of contact inhibition. In sections of such cells prepared for electron microscopy, the cells were found to be closely apposed to one another at limited regions, and it was in these areas of contact that the two subsurface cisternae of a pair were observed in confrontation. However, cells which were in serum-free nutrient under cellophane appeared, by time-lapse cinemicrography, to demonstrate contact inhibition. When these cells were sectioned for electron microscopy, they were found not to contain the SCC. It is possible that the narrowing of the intercellular cleft at the site of SCC may indicate their desmosome-like function as suggested by Flickinger and Fawcett (11) for these membranous components in the Sertoli cell. In fact, desmosomes occasionally were found near SCC in KB cells.

The intracytoplasmic confronting cisternae described in this report resemble the membranous structure composed of closely applied pairs of narrow cisternae observed by Epstein (7) in HeLa cells and by Hruban et al. (14) in the Novikoff and Morris 3683 hepatoma cells. However, the membranous structures they described extended over an irregular course and did not form ring-shaped bodies like the ICC in the KB cells. More recently, Leak et al. (19), in an electron microscopic study of a fibromyxosarcoma, have observed paracisternae which are associated with ribosomes and often are continuous with the RER, but which are not circular or ring-shaped.

The two types of confronting cisternae found in the rapidly growing KB cells cultivated in roller tubes are morphologically similar. Since these cisternae were found only in the cells of the rapidly growing culture systems, viz. in roller tubes and in culture chambers unrestricted by cellophane, it is reasonable to suspect that they are related to the growth and proliferative properties of this cell line. The structural similarities between the SCC and ICC and their presence together in the cells of roller-tube cultures suggest their potential relationship to each other; perhaps one is derived from the other. Since both the SCC and ICC are structures related to the RER, it is quite likely that they are involved in the process of protein synthesis.

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REFERENCES

1. ABERCROMBIE, M., and E. J. AMBROSE. 1958. Interference microscopic studies of cell contacts in tissue culture. *Exptl. Cell Res.* **15**:332.
2. BERGER, E. R. 1967. Subsurface membranes in paired cone photoceptor inner segments of adult and neonatal *Lebistes* retinae. *J. Ultrastruct. Res.* **17**:220.
3. BERNARDE, M. A., and R. J. KUCKLER. 1963. Quantitative effects of alkyl benzene sulfonate on KB cells in tissue culture, *Proc. Soc. Exptl. Biol. Med.* **116**:781.
4. BRZIN, B., V. M. TENNYSON, and P. E. DUFFY. 1966. Acetylcholinesterase in frog sympathetic and dorsal root ganglia. *J. Cell Biol.* **31**:215.
5. BUNGE, R. P., M. B. BUNGE, and E. R. PETERSON. 1965. An electron microscope study of cultured rat spinal cord. *J. Cell Biol.* **24**:163.
6. EAGLE, H. 1955. Propagation in a fluid medium of a human epidermoid carcinoma, strain KB. *Proc. Soc. Exptl. Biol. Med.* **89**:362.
7. EPSTEIN, M. A. 1961. Some unusual features of fine structure observed in HeLa cells. *J. Biophys. Biochem. Cytol.* **10**:153.
8. ERICSSON, J. L. E. 1964. Absorption and decomposition of homologous hemoglobin in renal proximal tubular cells, an experimental light and electron microscopic study. *Acta Pathol. Microbiol. Scand. Suppl.* **168**:1.
9. FARQUHAR, M. G., and G. E. PALADE. 1963. Junctional complexes in various epithelia. *J. Cell Biol.* **17**:375.
10. FAWCETT, D. W., and J. P. REVEL. 1961. The sarcoplasmic reticulum of a fast-acting fish muscle. *J. Biophys. Biochem. Cytol.* **10**(4, Suppl.):89.
11. FLICKINGER, C., and D. W. FAWCETT. 1967. The junctional specializations of Sertoli cells in the seminiferous epithelium. *Anat. Record.* **158**:207.
12. HAMA, K. 1965. Some observations on the fine structure of the lateral line organ of the Japanese seal eel *Lyncozymba nystromi*. *J. Cell Biol.* **23**:193.
13. HEYNER, S., and M. S. ISRAEL. 1962. The examination of roller-tube cultures by electron microscopy. *Exptl. Cell Res.* **30**:236.
14. HRUBAN, Z., H. SWIFT, and M. RECHIGLM, JR. 1965. Fine structure of transplantable hepatoma of the rat. *J. Natl. Cancer Inst.* **35**: 459.
15. JANDE, S. S. 1966. Fine structure of lateral-line organs of frog tadpoles. *J. Ultrastruct. Res.* **15**: 496.
16. KOHN, A., and D. YASSKY. 1962. Growth of measles virus in KB cells. *Virology*. **17**:157.
17. LANE, N. J., and A. B. NOVIKOFF. 1965. Effects of arginine deprivation, ultraviolet radiation, and x-radiation on cultured KB cells. *J. Cell Biol.* **27**:603.
18. LAPIS, K., and W. BERNHARD. 1965. The effect of mitomycin C on the nucleolar fine structure of cells in cell culture. *Cancer Res.* **25**:628.
19. LEAK, L. V., J. B. CAULFIELD, J. F. BURKE, and C. F. MCKAHANN. 1967. Electron microscopy studies on human fibromyxosarcoma. *Cancer Res.* **27**:261.
20. LUFT, J. H. 1961. Improvement in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409.
21. MILLONIG, G. 1962. Further observations on a phosphate buffer for osmium solution in fixation, electron microscopy. *In* Electron Microscopy. Fifth International Congress on Electron Microscopy. Held in Philadelphia, Pa., Aug. 29th–Sept. 5th, 1962. Sydney S. Breese, Jr. editor. Academic Press Inc., N. Y., **2**:8.
22. MOGABGAB, W. J. 1962. 2060 virus (ECHO 28) in KB cell culture. *Am. J. Hyg.* **76**:15.
23. NEWMAN, R. E., and A. A. TYTELL. 1962. Inhibitory effects of hadacidin on KB cultures. *Proc. Soc. Exptl. Biol. Med.* **112**:57.
24. PORTER, K. R., and G. E. PALADE. 1957. Studies on the endoplasmic reticulum. III. Its form and distribution in striated muscle cells. *J. Biophys. Biochem. Cytol.* **3**:269.
25. REVEL, J. P. 1962. The sarcoplasmic reticulum of the bat cricothyroid muscle. *J. Cell Biol.* **12**: 571.
26. ROSE, G. G. 1954. A separable and multipurpose tissue culture chamber. *Texas Rep. Biol. Med.* **12**:1074.
27. ROSE, G. G. 1957. Special uses of the multipurpose tissue culture chamber. *Texas Rep. Biol. Med.* **15**:310.
28. ROSE, G. G. 1964. Phase-contrast microscopy in living cells. *J. Roy. Microscop. Soc.* **83**:97.
29. ROSE, G. G. 1964. Nuclear folds versus intranuclear inclusions in tissue cultures. *J. Roy. Microscop. Soc.* **83**:377.
30. ROSE, G. G. 1965. Time-lapse cinemicrography of cells in tissue culture. *Bull. Johns Hopkins Hosp.* **116**:33.
31. ROSE, G. G. 1966. Observations on oral tumors in vitro 3. Morphological properties, longevity of nonviral intranuclear inclusions. *J. Natl. Cancer Inst.* **36**:747.
32. ROSE, G. G. 1966. Zeiosis. 1. Ejection of cell nuclei into zeiotic blebs. *J. Roy Microscop. Soc.* **86**:87.
33. ROSE, G. G. 1966. Cytopathophysiology of tissue cultures growing under cellophane membranes. *In* International Review of Experi-

mental Pathology. G. W. Richter and M. A. Epstein, editors. Academic Press Inc., N. Y.

34. ROSE, G. G., M. CATTONI, and C. M. POMERAT. 1963. Observations on oral tumors in vitro. 1. Zeiosis versus the nucleus and endoplasmic reticulum in strain KB. *J. Dental Res.* **42**:38.
35. ROSE, G. G., M. CATTONI, and C. M. POMERAT. 1964. Observations on oral tumors in vitro. 2. Karyobiosis in strain KB cells. *J. Dental Res.* **43**:580.
36. ROSE, G. G., C. M. POMERAT, T. O. SHINDLER, and J. B. TRUNNELL. 1958. A cellophane-strip technique for culturing tissue in multipurpose culture chambers. *J. Biophys. Biochem. Cytol.* **4**: 761.
37. ROSENELUTH, J. 1962. Subsurface cisterns and their relationship to the neuronal plasma membrane. *J. Cell Biol.* **13**:405.
38. ROSENBLUTH, J. 1965. Ultrastructural organization of obliquely striated muscle fibers in *Ascaris lumbricoides*. *J. Cell Biol.* **25**:495.
39. SABATINI, D. D., K. G. BENSCH, and R. J. BARRNETT. 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.* **17**:19.
40. SMITH, C. A., and F. S. SJÖSTRAND. 1961. Structure of the nerve endings on the external hair cells of guinea pig cochlea as studied by serial sections. *J. Ultrastruct. Res.* **5**:523.
41. TRUMP, B. F., R. J. GOLDBLATT, and R. E. STOWELL. 1965. Studies of necrosis in vitro of mouse hepatic parenchymal cells. Ultrastructural alterations in endoplasmic reticulum, Golgi apparatus, plasma membrane, and lipid droplets. *Lab. Invest.* **14**:2000.
42. VENABLE, J. H., and R. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25**:407.
43. WOOD, R. L. 1959. Intercellular attachment in the epithelium of *Hydra* as revealed by electron microscopy. *J. Biophys. Biochem. Cytol.* **6**: 343.