Tumor Res. 3 (1968) 145 - 185

LIVER TUMORS INDUCED IN RATS BY 3'-METHYL-4-DIMETHYL AMINOAZOBENZENE

II. Electron Microscopic Investigation -- Dedifferentiation of Cancer Cells --

Yusuke Fuse, Tomomi Iwasaki and Tamenori Onoé

Department of Pathology, Sapporo Medical College

Introduction

Liver tumors induced by aminoazo dye have been used extensively as experimental models for the study of carcinogenesis. Several authors (1-10) have conducted electron microscopic investigations on the chemical carcinogen induced liver tumors, but a lack of attention on the cancer cells themselves seems to be present. Thus, the cytogenesis of liver tumor is still a matter of dispute.

In this report, ultrastructural features of hepatoma cells induced by 3'-Me-DAB were described and compared with those of the surrounding non-neoplastic hepatocytes. Ultrastructural characteristics of the hepatoma cells were also discussed from a view point of dedifferentiation.

As already described (11), in order to investigate electron microscopically the liver tumors induced by 3'-Me-DAB, it should be understood that the histologic features of the tumors differ considerably in one particular portion or even within a single cancer nodule. The cancer nodules are histologically classified into three patterns; hepatocellular carcinoma, adenocarcinoma and undifferentiated carcinoma

(anaplastic carcinoma). The present report deals with the fine structures of the three different cancer cell types.

Materials and Methods

Cancer nodules and cirrhotic liver parenchyma of 19 rats among 29 animals developed liver tumors (11) were studied under electron microscopic examination. Tissues for light microscopy were processed and examined by conventional methods (11). The remainder of the tissues from the same nodules were cut up into small blocks (1 mm cube), macroscopically necrotic and hemorrhagic areas were removed for electron microscopic examination. These blocks were fixed in veronal acetate buffered 2% osmium tetraoxide et pH 7.4 for 2 hours, and were then processed for electron microscopy according to the method of Luft (12). The epon-embedded blocks were cut, stained with toluidine blue, and examined with a light microscope. Ultrathin sections were cut with a Porter-Blum microtome, stained with uranyl acetate and lead cytrate and examined a Hitachi 11 electron microscope.

Results and Discussion

I. Non-neoplastic liver tissue

Alterations of hepatocytes induced by 3'-Me-DAB in the cancer stage, consisted mainly of a decrease in rough endoplasmic reticulum and an increase in smooth endoplasmic reticulum. Rough endoplasmic reticulum (RER) were diminished in its parallel arrays and

degranulation, fragmentation and dilatation were seen. Smooth endoplasmic reticulum (SER) appeared to form vesicular patterns intermingled with glycogen particles (Fig. 4). Myelin figures which consisted of whorls of concentric, smooth surfaced membranes (partly attached ribosomes) enclosing some cytoplasmic portions were frequently observed (Figs. 1 and 2). Organelles such as peribiliary lysosomes, microbodies and coated vesicles around the Golgi zone were also increased. Golgi zones were usually hypertrophic. Mitochondria were generally normal with some swollen forms. One of the characteristic changes of the non-neoplastic hepatocytes was the appearance of pericanalicular rod shaped bodies (Fig. 3). These bodies were found in the majority of the non-neoplastic hepatocytes. On the other hand, these bodies were not observed in the cancer cells. Hence, the non-neoplastic hepatocytes were readily distinguishable from the cancer cells by electron microscopy.

In the cancer stage, the proliferation of the ductular cells (so-called oval cells) were frequently observed as well as in the precancerous stage. The areas of ductular cell proliferation revealed that acini were occasionally formed partly by ductural cells and partly by hepatocytes. Some of these ductural cells revealed bleb-formation and diminution of the microvilli while containing fat droplets in their cytoplasms. These changes were considered to be degenerative signs. Kupffer cells usually contained numerous phagosomes with swollen cytoplasm. Such changes of the Kupffer cells may have been induced by the degeneration of liver parenchyma.

These alterations of the liver tissue may well be produced by the toxic action of 3'-Me-DAB. Similar changes are not found in the cancer cells which seem not to be affected by azo-dye as previously described by Miller and Miller (13).

II. Hepatocellular carcinoma cells

Hepatocellular cancer cells were not associated with the basement membrane. The intercellular surfaces were separated from each other by irregular microvillous cytoplasmic projections (Fig. 5) in partial adherance in which tight junctions and desmosomes were observed. Bile canaliculi were not frequently observed (Figs. 5 - 7). Cancer cells revealed fewer desmosome connections compared with that of normal hepatocytes and adenocarcinoma cells. Intercelluar spaces considered to represent the space of Disse were widened in some places, in which aggregated fibrin masses were occasionally seen. Cells resembling sinusoidal lining cells were observed to encircle the cords of cancer cells, forming sinusoidal structures. Sinusoidal lining cells were usually vascularized and associated with the basement membrane. But, there were the spaces which represented the space of Disse between the vascularized sinusoidal cells and the cord of cancer cells. No direct attachment of cancer cells to the basement membrane was observed.

The cancer cells were rich in free ribosomes and moderately devloped RER, mitochondria and Golgi apparatus that were dispersed arround the nucleus. However, no microbody or percanalicular lysosomes were observed in the cancer cells, while secondary lysosomes (focal

cytoplasmic degradation) were frequently observed (Fig. 7). SER was extremely scanty and was limited to a few small vesicles in close proximity to the Golgi apparatus. Glycogen particles were not found in cancer cells. Some nuclei revealed highly irregular nuclear outline associated with fat inclusions (Fig. 7) and with hypertrophic nucleoli (Fig. 5). Such atypical cell which possessed ten centrioles in a cell was seen (Fig. 6). The peribiliary locations of Golgi apparatus and primary lysosomes found in non-neoplastic hepatocytes were not observed in the cancer cells. Some cells had numerous dense inclusions which could not be identified as to their nature (Fig. 8)

Table 1 Comparative characteristics of hepatocellular cancer cells and surrounding non-neoplastic hepatocytes

	Non-neoplastic hepatocytes	Hepatocellular cancer cell
1) Proliferation of smooth endoplasmic reticulum	Abundant	Not observed
2) Rough endoplasmic reticulum	Diminution of parallel arrays	Diminution of parallel arrays
3) Mitochondria	Variable configuration	Markedly decreased in number
4) Glycogen	Moderately abundant	Diminution or loss
5) Microbody	Fairly increased in number	Not observed
6) Peribiliary lysosome	Markedly increased	Not observed
7) Focal cytoplasmic degradation	Infrequently observed	Observed in most cases
8) Rod shaped body	Present	Absent
9) Golgi apparatus	Hypertrophic	Rudimentary

Although cancer cells showed highly ultrastructural variations from cell to cell, it was ascertained that each cancer cell resembled hepatocytes and the mass of cancer cells imitating the hepatic architecture. However, there was a marked decrease of cytoplasmic organelles in hepatocellular cancer cells as compared with normal hepatocytes.

Normal hepatocytes have various cytoplasmic organelles reflecting the various functions required of these cells. The cytoplasmic organelles represent the specific carriers of each specifically differentiated function. Therefore, it may be said that the dedifferentiation of cancer cell results in a decrease or disappearance of certain organelles, i.e. SER, microbody glycogen, and pericanalicular lysosomes, which are responsible for the specific functions of hepatocytes.

III. Adenocarcinoma cells

The structural organization of adenocarcinoma cell also varied from cell to cell. Adenocarcinoma cells were always accompanied by basement membranes and projecting microvilli to glandular spaces (Fig. 9). Microvilli in number varied greatly depending on individual cancer cells (Figs. 11 and 12). The neighboring plasma membranes were usually interdigitated with many desmosome connections. Cancer cells possessed richly increased free ribosomes, small mitochondria, Golgi apparatus, RER and matrical fibriles (Fig. 10). It was clearly observed that these cells revealed an irregularity of nuclear outline, enlarged nucleoli, increased nucleo-cytoplasmic ratio, unequally sized and disordered arrangement of mitochondria as well as RER indicating the general

characteristics of cancer cells. Cells with rudimentary microvilli were found adjacent to the cells with highly developed microvilli (Fig. 11). The pyknotic dark cells considered to be undergoing death were frequently found next to the cell in mitosis (Fig. 11). The retention of the mucoid material between cells and basement membrane was also observed frequently (Figs. 11 and 12). This feature was considered to be a disturbance of the secretory polarity of cancer cells. Thus the adenocarcinoma cells showed various appearances as groups of cells, however, the general ultrastructures of cells closely resembled those of bile duct epithelial cells.

Biliary epithelial cells have a simple and poorly developed cytoplasmic organelles reflecting their simple function which serves merely as cells forming the passage way of bile. Adenocarcinoma cells generally possessed more abundant cytoplasmic organelles compared with the bile ductal cells.

Cancer cells have a rapid reproductive capability. Therefore, they must have the certain cytoplasmic organelles which are indispensable for the proliferation of the cell. It is considered that such cytoplasmic organelles may include free ribosomes, mitochondria and certain endoplasmic reticulum. From this point of view, it may be expected that adenocarcinoma cells generally would have larger amounts of free ribosome, mitochondria and RER than those of the bile ductal cells. On the other hand, cytoplasmic fibriles and microvilli which may not be indispensable for proliferation are decreased in the cancer cells.

IV. Undifferentiated carcinoma cells

Undifferentiated carcinoma cells histologically appeared to be composed of extremely monotonous cells. However, electron microscopically the cells were quite variable. The majority of the cells were small and oval shaped resembling proliferated ductular cells in the precancerous stage. The nucleo-cytoplasmic ratio was extremely high (Figs. 13 - 15). Plasma membranes were generally smooth and were connected with each other by tight junctions and desmosomes (Fig. 13). Some of the cells revealed microvilli and basement membrane formation. A group of these cells were usually arranged in a sheet likewise without glandular or sinusoidal structures. There were occasionally relatively wide intercellular spaces among the cancer cells. The cells facing these spaces were seen having a marked microvilli formation. At times the secretion granules of undetermined nature with high electron opacity were found in these cells. Among undifferentiated carcinoma cells were found hepatocellular cancer cells (Figs. 13 - 16). They rarely formed the bile canaliculi like space with neighboring ductular cancer cells. These hepatocellular cancer cells contained a large amount of free ribosomes and poorly developed RER and mitochondria.

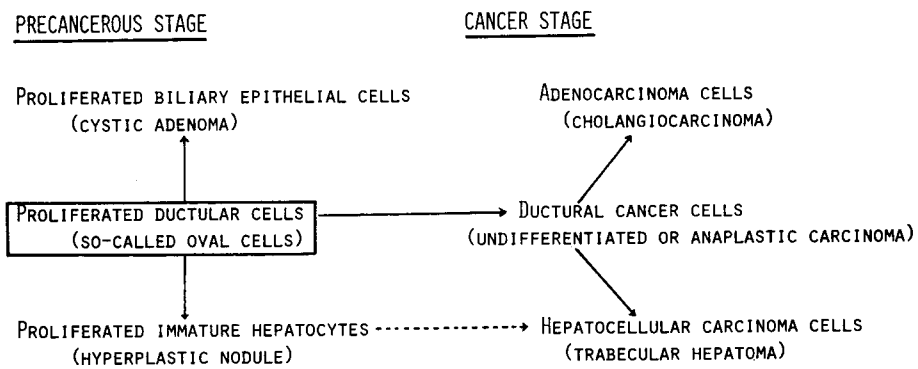
These observations found in the undifferentiated carcinoma obvio··-ly indicate that the ductular cancer cells composed of undifferentiate :arcinoma tissue develop into hepatocellular and also adenocarcinoma c· .s.

Although the amount of these organelles in these cells differed markedly from cell to cell, these cells closely resembled the ductular

cells. However, compared to ductular cells observed in the precancerous stage, these cancer cells has more abundant cytoplasmic organelles such as free ribosomes and mitochondria.

Recently, several investigators (9, 14, 15) have emphasized that hyperplastic nodules or hyperbasophilic foci appear in the precancerous stage which are regarded as sites of origin of chemical carcinogen induced hepatoma. Karasaki (9) reported that the clear cells in the hyperbasophilic foci may be candidates for hepatoma cells. The clear cells are characterized by the abscence of proliferation of SER, microbodies and glycogen granules. He suggested that the clear cells arose from hepatocytes in the foci. However, in our observations, a sheet like proliferation of ductular cells did not always show a formation of the basement membrane and some of the proliferated ductular cells have relatively larger mitochondria.

Table 2



From our observations, a consideration to the cytogenesis of hepatoma induced by 3'-Me-DAB is shown in Table 2.

V. Variability of cancer cells

A mass of cancer cells shows highly varying ultrastructures. Adenocarcinoma cells revealed well-developed microvilli adjoining cancer cells without microvilli formation (Fig. 17). This may indicate that the degree of dedifferentiation differs from cell to cell. Single necrotic cancer cells are seen among the vigorous cancer cells (Figs. 18, 21, 22). At times, a mitotic cancer cell is found adjacent to the necrotic cancer cell (Fig. 23). The appearance of focal cytoplasmic degradation occurs chaotically among cancer cells (Figs. 7, 12, 17 and 24). These findings are interpreted as follows. A mass of cancer cells are composed of postmitotic and intermitotic cells and the degree of degereration differs depending on individual cancer cells. Naturally, the degree of atypism found in cancer cells which affect the morphological appearance of the cytoplasmic organelles differs from cell to cell. Furthermore, a mass of cancer cells consists of cells in various stages and type of disdifferentiation as well as dedifferentiation. The disdifferentiation of cancer cells will be discussed later. These conditions mentioned above, are considered to be the reason for the variability of cancer cells.

VI. Dedifferentiation

One of the basic characteristics of cancer cells is dedifferentiation. The common ultrastructural findings of dedifferentiation are a general simplification and a disappearance of certain organelles seen in the

original mother cell. In this observation, the hepatocellular cancer cells were confirmed by the abscence of proliferation of SER, microbodies and glycogen particles. This is considered to be the ultrastructural manifestation of dedifferentiation of cancer cells.

Ma and Webber (3) found that trabecular carcinoma cells induced by 3'-Me-DAB possessed microbodies. Hepatocellular carcinomas induced by 2-fluorenylacetamide (16) show a surprising resemblance to non-neoplastic hepatocytes investigated in this study. They contain a prominence of SER, microbodies and glycogen stores. Flacks (4, 5) reported that the hepatoma cell induced by 2-acetylaminofluorene contained microbodies, glycogen stores, occurrance of membrane-glycogen structures and even tubular structures which seem to be similar to pericanalicular rod shaped bodies (17). Both membrane-glycogen structures and rod shaped bodies are found only in non-neoplastic hepatocytes and never in hepatoma cells in this study as indicated in Table 1. These discrepancies of the ultrastructures of hepatoma cells are interpreted to depend on the degree of dedifferentiation of cancer cells which produced by using carcinogens and feeding conditions.

Similar circumstances are reported on human hepatomas. Wilis (18) reported that hepatoma cells were almost similar to normal hepatocytes possessing abundant glycogen stores and parallel arrayed RER. However, Ghadially et al (19) reported that hepatoma cells showed an inability to form desmosomes, lysosomes and vascular pole. Toker and Trecino (20) reported two cases of human hepatoma cells which were dissimilar from each other, although both cancer cells showed a lack of glycogen stores

and microbodies. From these reports, the degree of dedifferentiation also may differ in each human hepatoma.

It may be said that hepatoma induced by 3'-Me-DAB vigorously manifested the degree of dedifferentiation. It is generally accepted that a degree of differentiation and a proliferative ability of cells are in inverse proportion. As cancer cells possess a very rapid proliferative ability, it is reasonable to assume that they partly lose specific organelles of the original mother cell which are not important for proliferation.

Morphological manifestation for dedifferentiation of the cancer cell is partially similar to that of embryonal cells with respect to the abundance in free ribosomes and a simplification of cytoplasmic organelles. However, in cancer cells various atypism depending on individual cells, and disturbance of cellular polarity and disdifferentiation which will be discussed later, was observed and these appearances of cancer are definitely different from those of embryonal blast cell aggregates which show a monotonous but well organized appearance (21).

Since ductular cells may differentiate into hepatocytes as well as bile ductal cells, it is easily understood that undifferentiated carcinoma composed of ductular cells are differentiating toward hepatocellular cancer and adenocarcinoma and also there are three histologic pattern in a nodule induced by 3'-Me-DAB.

Summary

On the basis of the ultrastructural findings of hepatoma induced by

3'-Me-DAB, it was demonstated that the three forms of carcinoma (Undifferentiated, hepatocellular and adenocarcinoma) corresponded to ductular cell, hepatocyte and bile ductal cell respectively. Ductular cancer cells seem to develop into hepatocellular and adenocarcinoma cells. Hepatocellular cancer cells and non-neoplastic hepatocytes were compared and variability and dedifferentiation of hepatoma cell were also discussed.

(Recieved December 20, 1968)

References

1. Onoé, T. & Fuse, Y.: Electron microscopic study on azo-dye carcinogenesis. Tumor Res. 1, 143-173 (1966).

2. Inaoka, Y.: Significance of the so-called oval cell proliferation during azo-dye hepatocarcinogenesis. Gann 58, 355-366 (1967).

3. Ma, M.H. & Webber, A. G.: Fine structure of liver tumors in the rat by 3'-Methyl-4-dimethylaminoazobenzene. Cancer Res. 26, 935-946 (1966).

4. Flaks, B.: Unusual aspects of ultrastructural differentiation in rat hepatoma cells. J. Cell Biol. 38, 230-238 (1968).

5. Flaks, B.: Formation of membrane-glycogen arrays in rat hepatoma cells. J. Cell Biol. 36, 410-414 (1968).

6. Furuta, F.: Electron microscopic studies on the fine structure of liver. Japn Arch. Intern. Med. 3, 820-830 (1956).

7. Tujimura, H.: Electron microscopic studies on the DAB induced hepatoma. Med. J. Osaka Univ. 9, 147-162 (1958).

8. Svoboda, D. J.: Fine structure of hepatomas induced in rats with p-dimethyl-aminoazobenzene. J. Natl. Cancer Inst. 33, 315-339 (1964).

9. Karasaki, S.: The fine structure of proliferating cells in pre-neoplastic rat livers during azo-dye carcinogenesis.

J. Cell Biol. 40, 322-335 (1969).

10. Mikata, A. & Luse, S. A.: Ultrastructural changes in the rat liver produced by N-2-fluorenyldiacetamide. Am. J. Path. 44, 455-466 (1964).

11. Fuse, Y., Motoya, M. Iwasaki, T., Dempo, K. and Onoé, T.: Liver tumors induced in rats by 3'-Methyl-4-dimethyl aminoazobenzene. I. Histologic investigation. Tumor Res. 3, 111-(1968).

12. Luft, J. H.: Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9, 409-414 (1961).

13. Miller, E. C. & Miller, J. A.: The presence and significance of bound aminoazo dyes in the livers of rats fed p-Dimethylaminoazobenzene. Cancer Res. 7, 468-480 (1947).

14. MacDonald, R. A.: Experimental carcinoma of the liver. "Regeneration" of liver cells in premalignant stages. Am. J. Path. 39, 209-220 (1961).

15. Molnar, F. & Daoust, R.: Nucleocytoplasmic ratios in rat liver parenchyma during DAB carcinogenesis. Cancer Res. 25, 1213-1218 (1965).

16. Willis, E. G.: Fine structure and surace adenosinetriphosphatase activity of a human hepatoma. Cancer 22, 1046-1052 (1968).

17. Markow, L. P., Epstein, S. M., Farber, E., Pardo, M. and Bartus, B.: Cellular analysis of liver carcinogenesis. Comparison of the ultrastructure of hyperlastic nodules and hepatocellular carcinoma induced in rat liver by 2-fluorenylacetamide. J. Natl. Cancer Inst. 43, 33-49 (1968).

18. Fuse, Y. & Onoé, T.: Pericanalicular rod-shaped bodies. Sapporo Med. J. 35, 135-145 (1969).

19. Ghadially, F. N. & Parry, E. W.: Ultrastructure of a human hepatocellular carcinoma and surrounding non-neoplastic liver. Cancer 19, 1989-2004 (1966).

20. Joker, C. & Jrecino, N.: Ultrastructure of human primary hepatic carcinoma. Cancer 19, 1594-1606 (1966).

21. Onoé, T.: Biological significance of variabilities of the fine structure of cancer cells. Japanese J. Cancer Clinics 12, 414-420 (1966).

Explanation of figures

Fig. 1 Non-neoplastic hepatocyte
Concentric whorl formation and a marked decrease of rough endoplasmic reticulum are seen in the cytoplasm. The closely packed and laminated conglomerate of endoplasmic reticulum in the upper portion of the figure is noted to be compressing the nucleus of the neighboring cell. x 16,000

Fig. 2 Non-neoplastic hepatocyte
A marked increase of vesicular smooth endoplasmic reticulum intermingled with the glycogen particles and the concentric whorl of smooth endoplasmic reticulum are noted. x 16,000

Fig. 3 Non-neoplastic hepatocyte
Numerous dense rod shaped bodies are located in the immediate vicinity of bile canaliculus (BC). The bile canaliculus is moderately dilated and a marked decrease in the number of microvilli is seen. x 23,000

Fig. 4 Non-neoplastic hepatocyte
A great portion of the cytoplasm is occupied by vesicular smooth endoplasmic reticulum intermingled with a small amount of glycogen particles. N: nucleus BC: bile canaliculus x 23,000

Fig. 5 Hepatocellular cancer cell
Note the increase of the nucleo-cytoplasmic ratio of the cancer cells. Nucleoli are dense and hypertrophic. The cytoplasm of cancer cells consists maninly of mitochondria, rough endoplasmic reticulum and free ribosomes. x 6,000

Fig. 6 Hepatocellular cancer cell
Note the irregular outlines of two nuclei which contained several vacuoles. The cell has ten centrisomes (arrows) in the vicinity of bile canaliculus (BC). x 6,000

Fig. 7 Hepatocellular cancer cell
The cell located in the center has a large dense inclusion considered to be a focal cytoplasmic degradation. BC: bile canaliculus x 13,000

Fig. 8 Hepatocellular cancer cell
Note the irregular outlines of nucleus. The rudimentary developed Golgi zones (G) located in the vicinity of nucleus. Small dense particles are seen scattered in the cytoplasm. x 6,000

Fig. 9 Adenocarcinoma cell
Cylindrical cancer cells form glandular space in which cellular debris and mucinous materials are contained. BM: basement membrane N: neutrophil x 5,000

Fig. 10 Adenocarcinoma cell
A cross section of adenocarcinoma cell. Note the increase of the heterochromatinic areas in the nuclei and the abundance of the free ribosomes in the cytoplasm. x 5,000

Fig. 11 Adenocarcinoma cell
The development of microvilli varies from cell to cell. The mitotic cell (M) is seen adjoining the degenerative cell (D). Arrows indicate the accumulation of the mucinous material of low density which contained needle-like crystals. BM: basement membrane
L: glandular lumen N: neutrophil x 4,000

Fig. 12 Adenocarcinoma cell
The accumulations of mucinous materials in variable densities are seen between the cancer cells and the basement membrane (arrows). The cancer cells contain the cytolysosomal inclusions in various sizes.
M: mitotic cell BM: basement membrane
L: glandular lumen x 4,000

Fig. 13 Undifferentiated cancer cell
The small cells (oval cell type) revealing an extremely high nucleo-cytoplasmic ratio are arranged in a sheet form. The smooth cell membranes of the cancer cells are connected by tight junctions and desmosomes. Some of the cells (H) have wider cytoplasms which resemble hepatocytes rather than oval cells. x 4,000

Fig. 14 Undifferentiated cancer cell
Undifferentiated cancer consists of the cells of oval cell type. The cells contain many dense inclusions in variable size. Some cells (H) resembling hepatocytes are also seen. x 4,000

Fig. 15 Undifferentiated cancer cell
The cancer cells (oval cell type) represent a small amount of simple cytoplasmic organelles and extremely high nucleo-cytoplasmic ratio. The cells of oval cell type show a gradual shift to the hepatocellular cancer cell (H). x 4,000

Fig. 16 Undifferentiated cancer cell
The cells observed in this figure generally have a wider cytoplasm compared with the cells in figures 13 to 15. The rough endoplasmic reticulum, mitochondria and free ribosomes in these cells are more abundant than those of the voal cell. x 4,000

Fig. 17 The adenocarcinoma cell revealed well-developed microvilli adjoining the cells without microvilli formation. The latter cell has two focal cytoplasmic inclusions, while the former cell has only minute cytolysosomal inclusions. The glandular lumen (L) contains cellular debris and mucinous material.
BM: basement membrane N: neutrophil x 5,000

Fig. 18 A necrotic cell is seen in the upper corner among the vigorous adenocarcinoma cells. The mitochondria appear to be vacuolated and the cytoplasm is condensed in the necrotic cell. The nucleus reveals pyknosis with a nuclear body. x 5,000

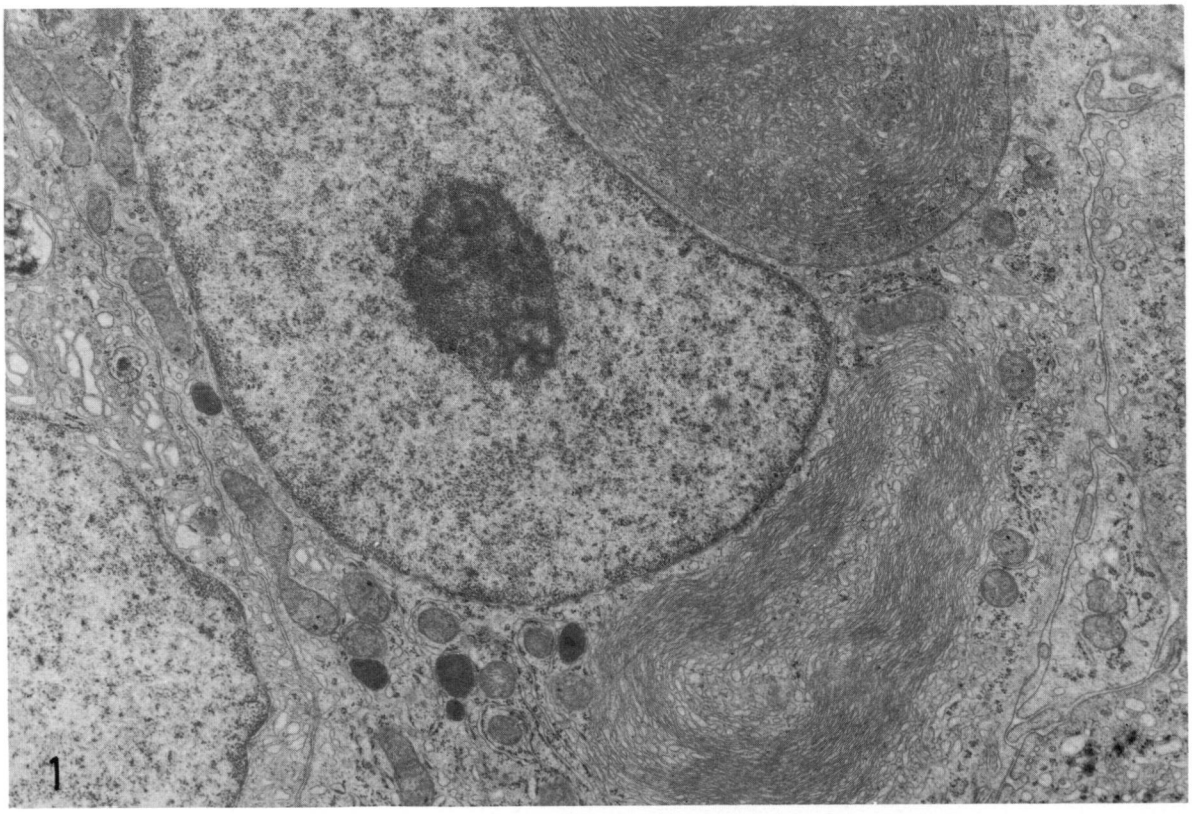
Fig. 19 The intestinal metaplastic cells associated with microvilli and terminal web structures intermingle with the usual adenocarcinoma cells (arrows) and they form a common glandular lumen (L). The lumen is filled with cellular debris. x 12,000

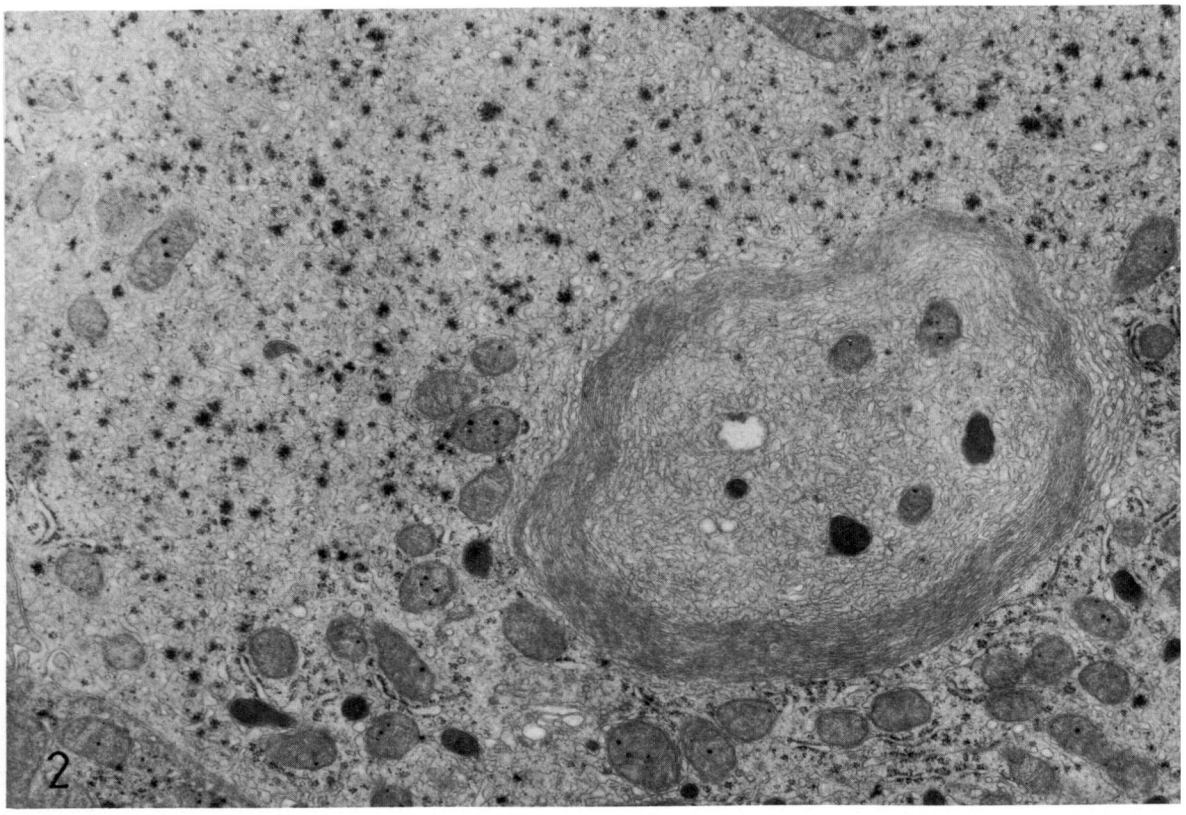
Fig. 20 Two adenocarcinomas forming glandular structures are seen in a back to back arrangement. Most of the cancer cells lose their microvilli except in the areas indicated double-headed arrows. An accumulation of mucin between the basement membrane and the cells are seen (arrows) BM: basement membrane x 4,000

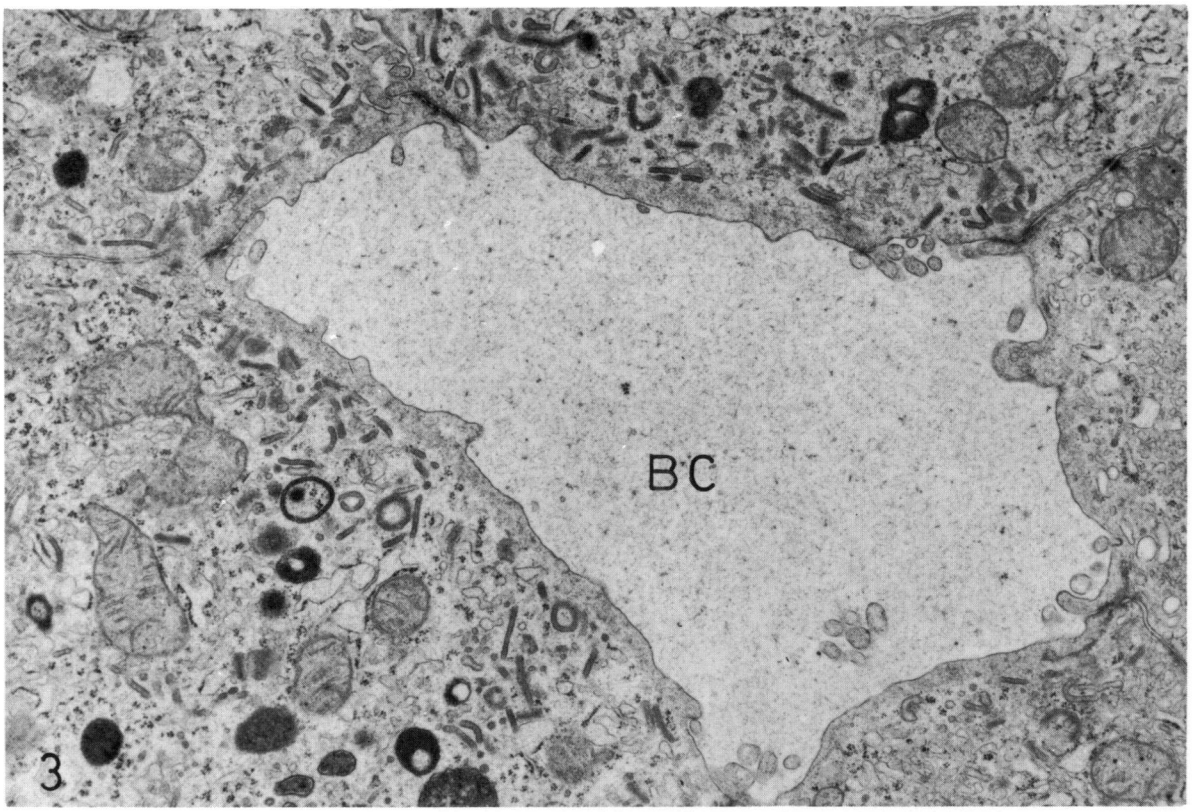
Figs. 21 and 22
A dark pyknotic degenerative cell in contact with a basement membrane is seen. x 8,000

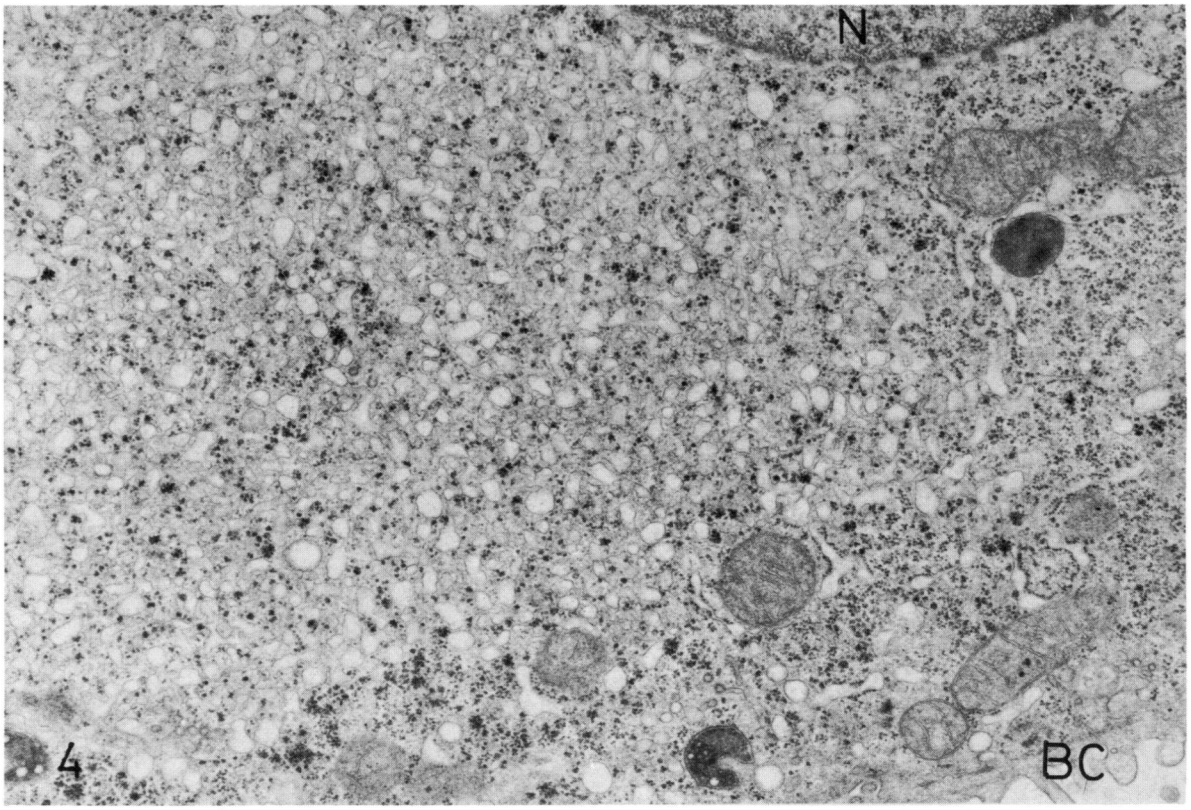
Fig. 23 The mitotic cancer cell (M) is seen adjacent to the necrotic cancer cell (D). Mitochondria in the necrotic cell are vacuolated with dense granular materials. x 12,000

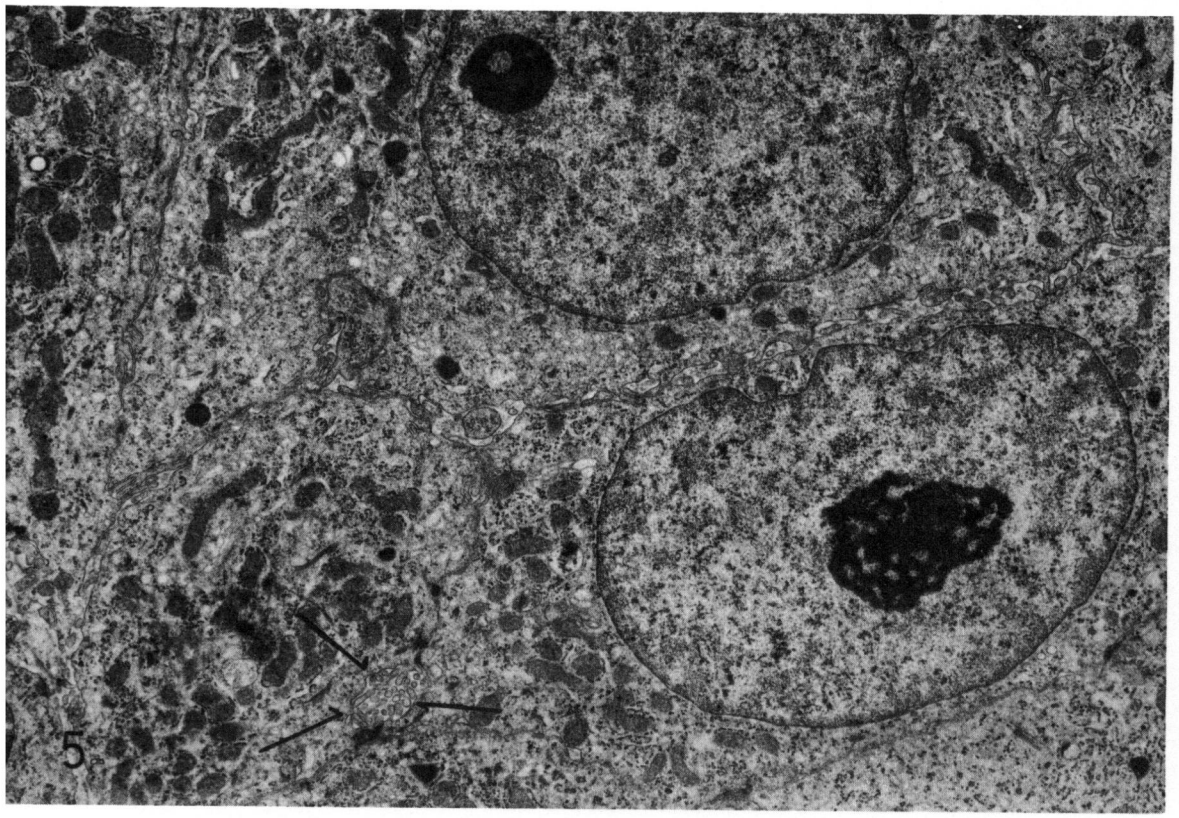
Fig. 24 A cancer cell containing a large focal cytoplasmic degradation (FCD) is seen adjacent to the intestinal metaplastic cancer cell. x 10,000

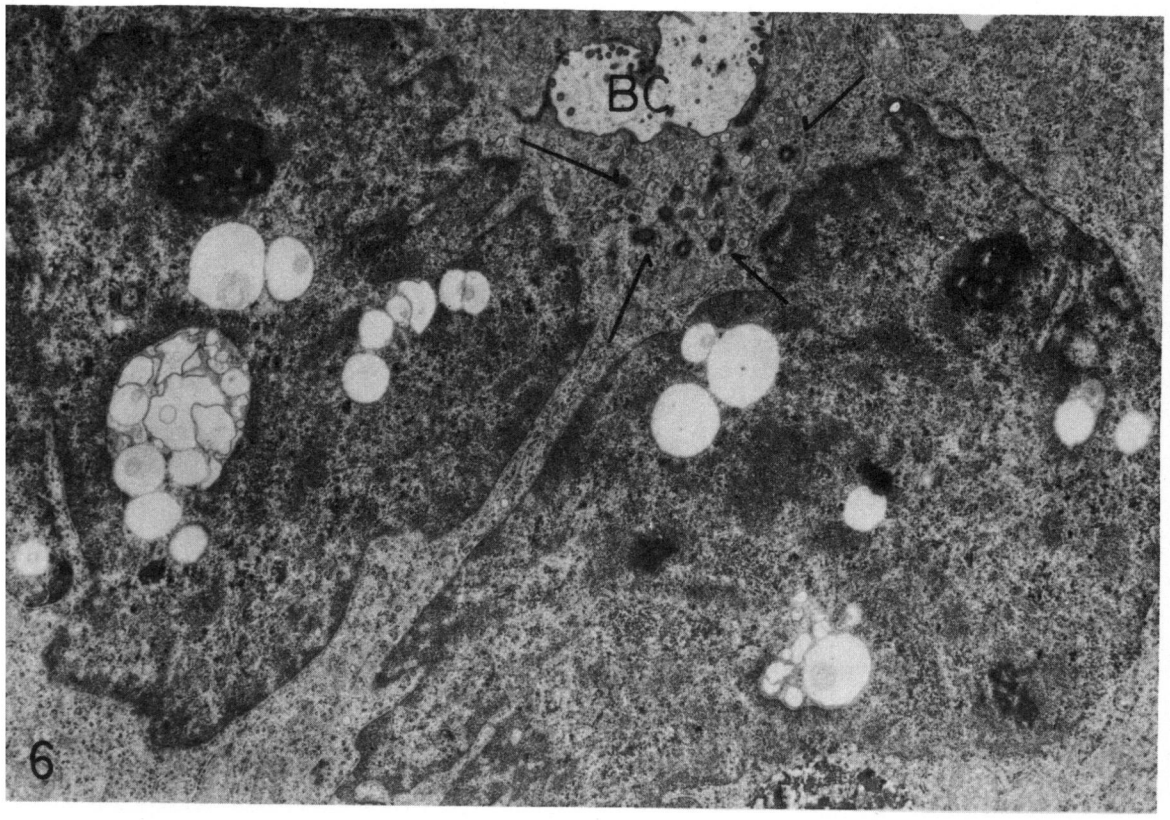

1

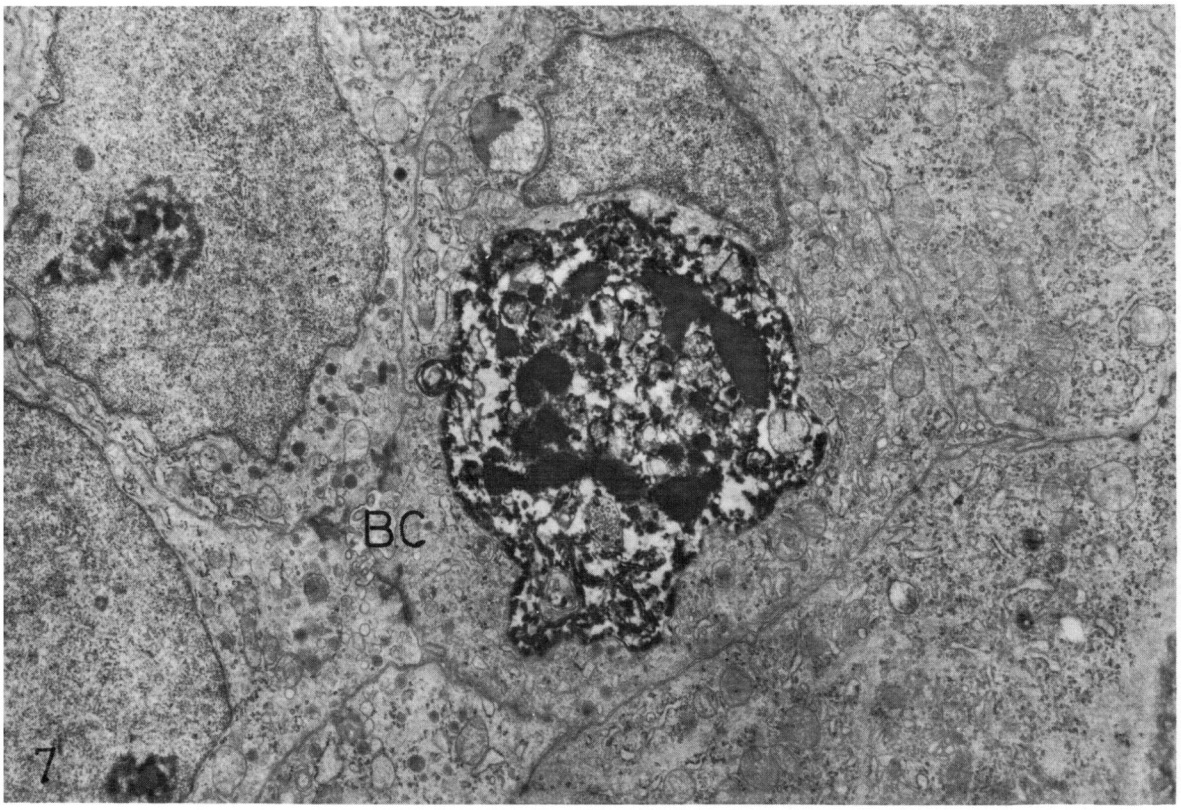

2

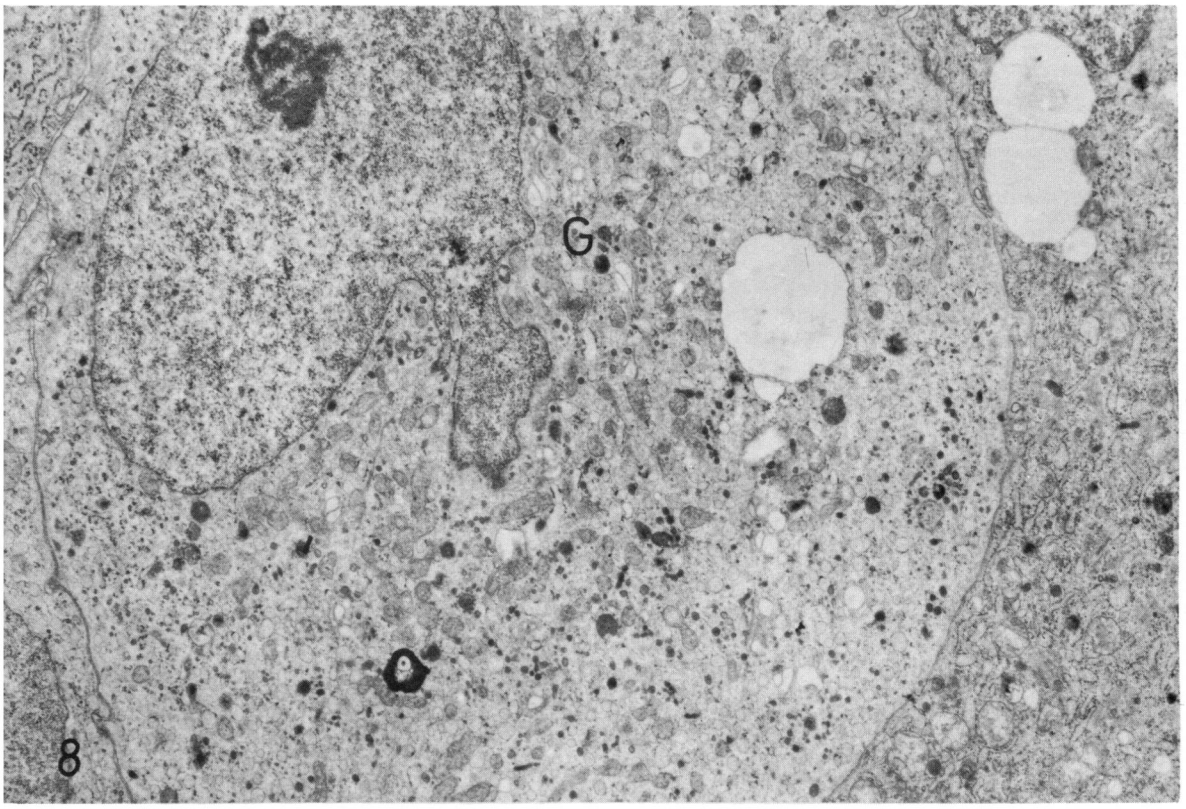

BC
3

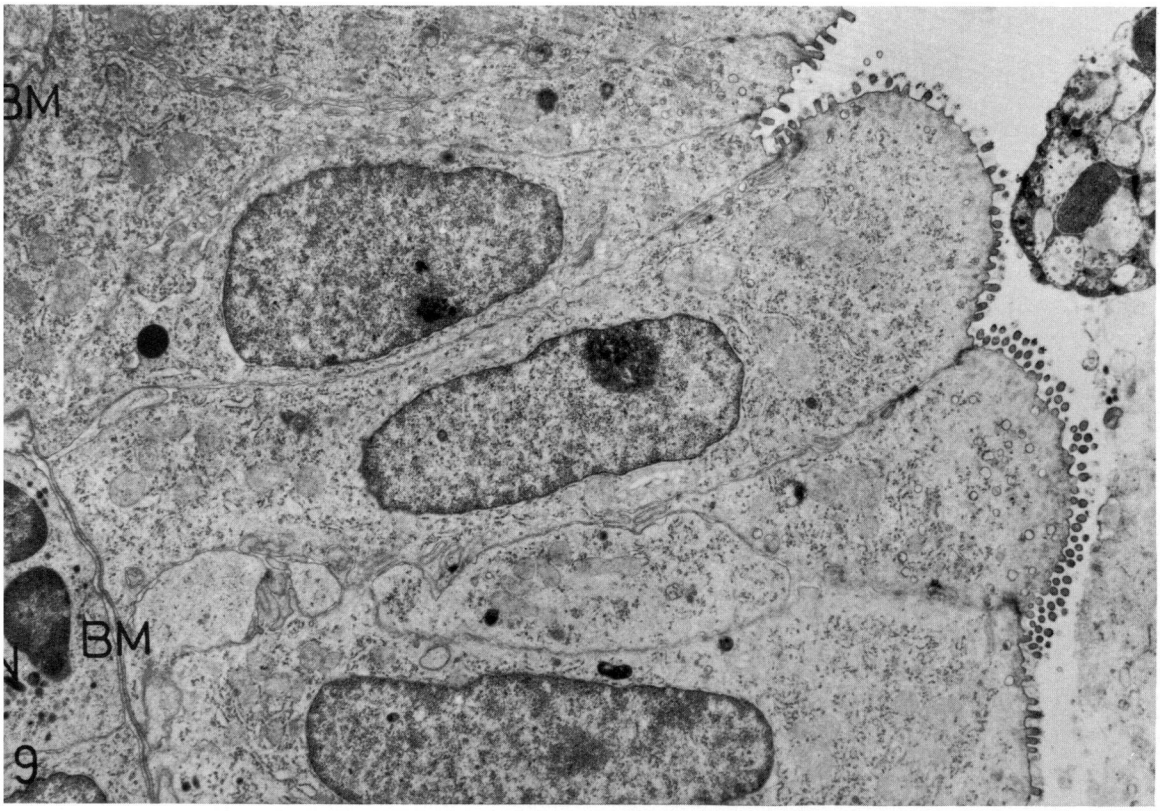

N
4
BC

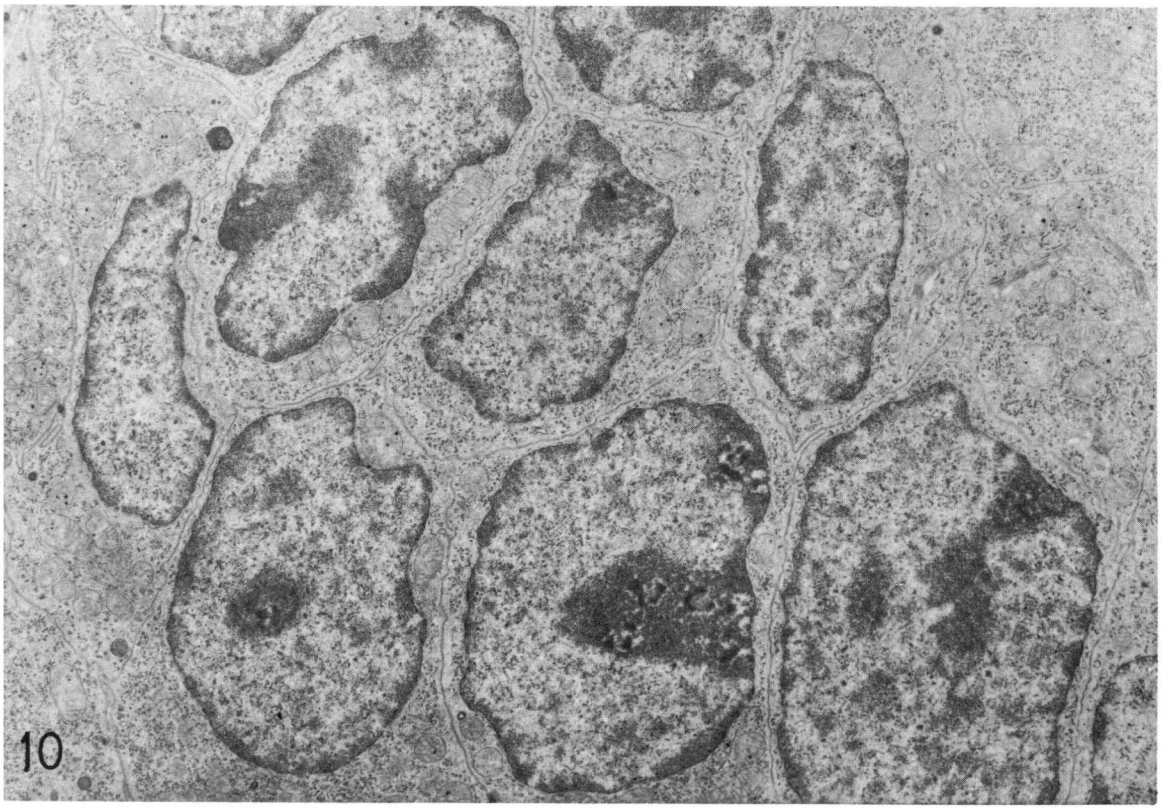

5

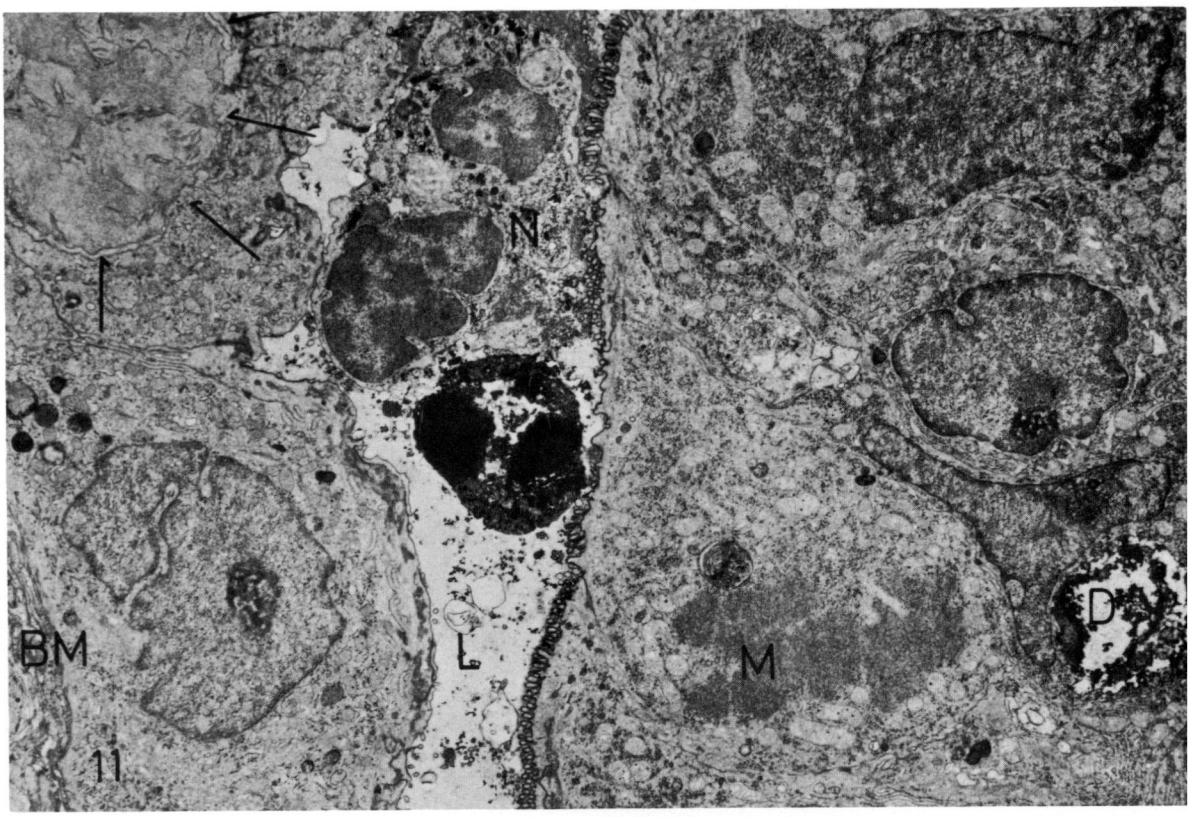

BC
6

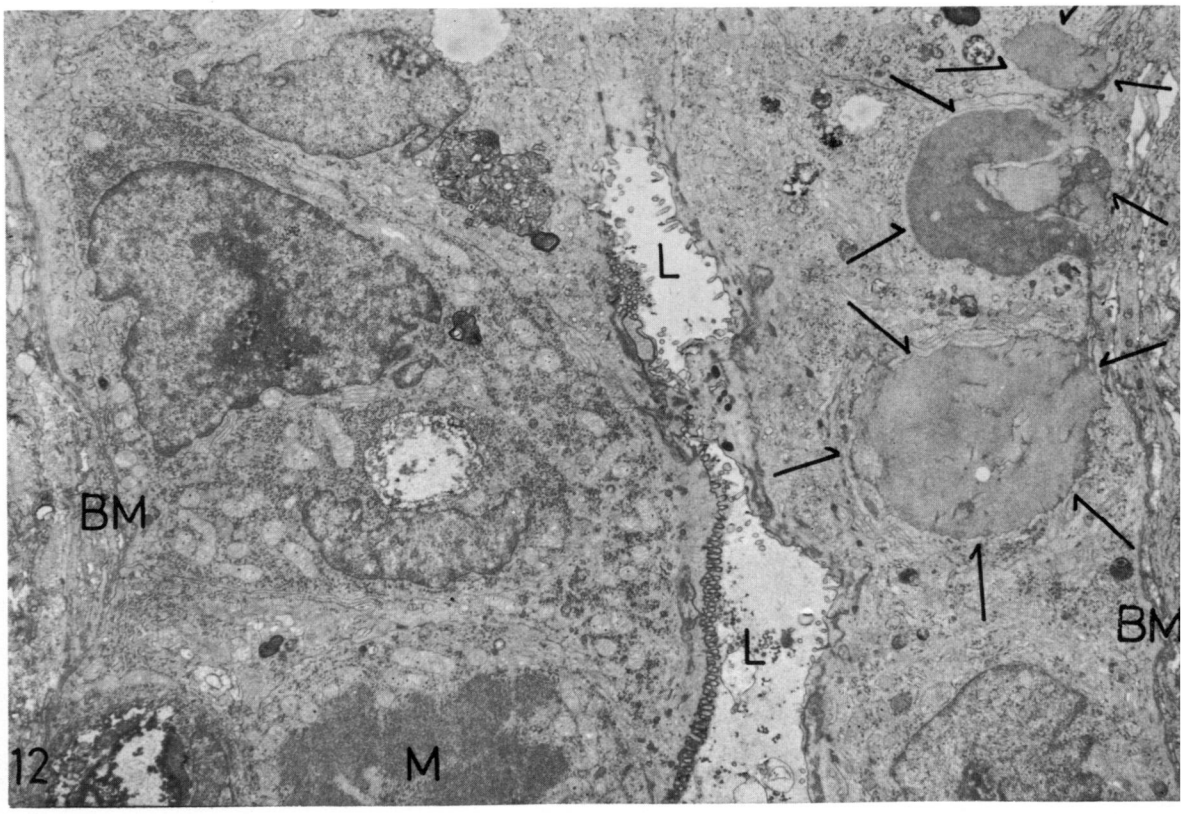

BC
7

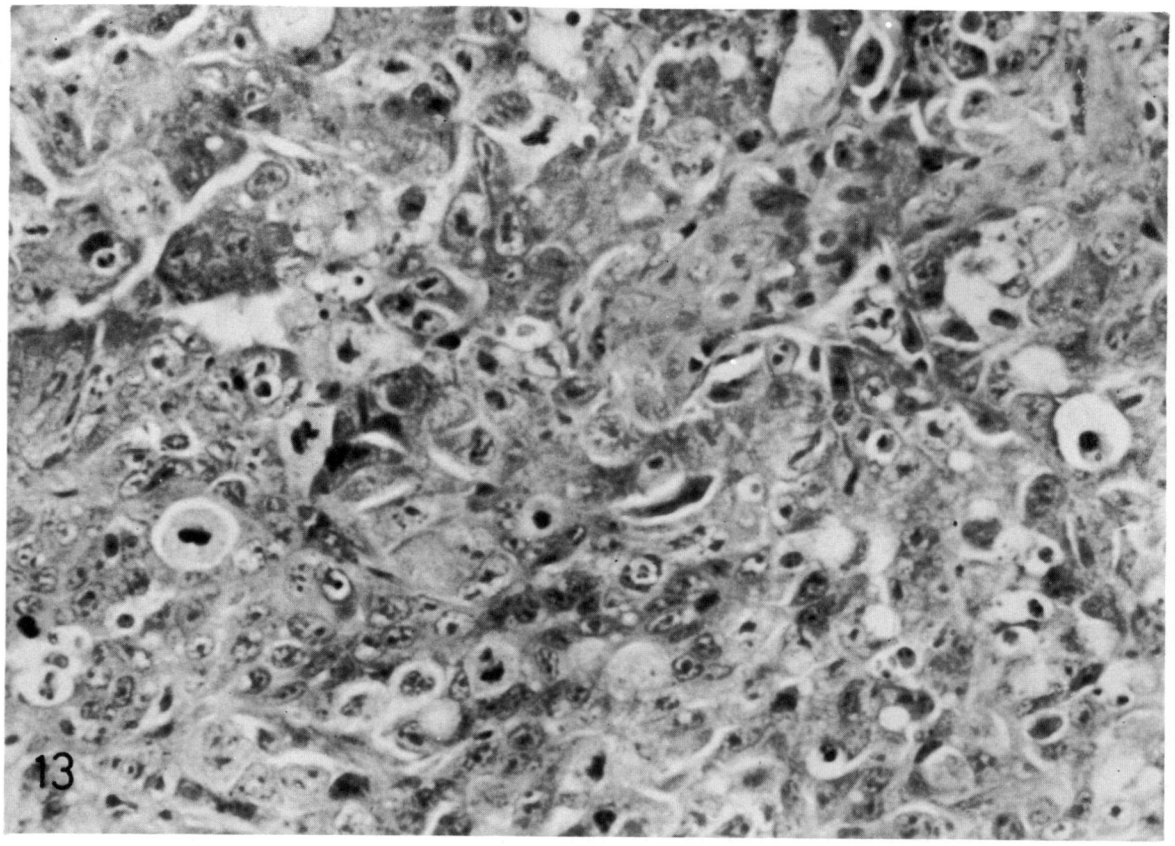

G
8

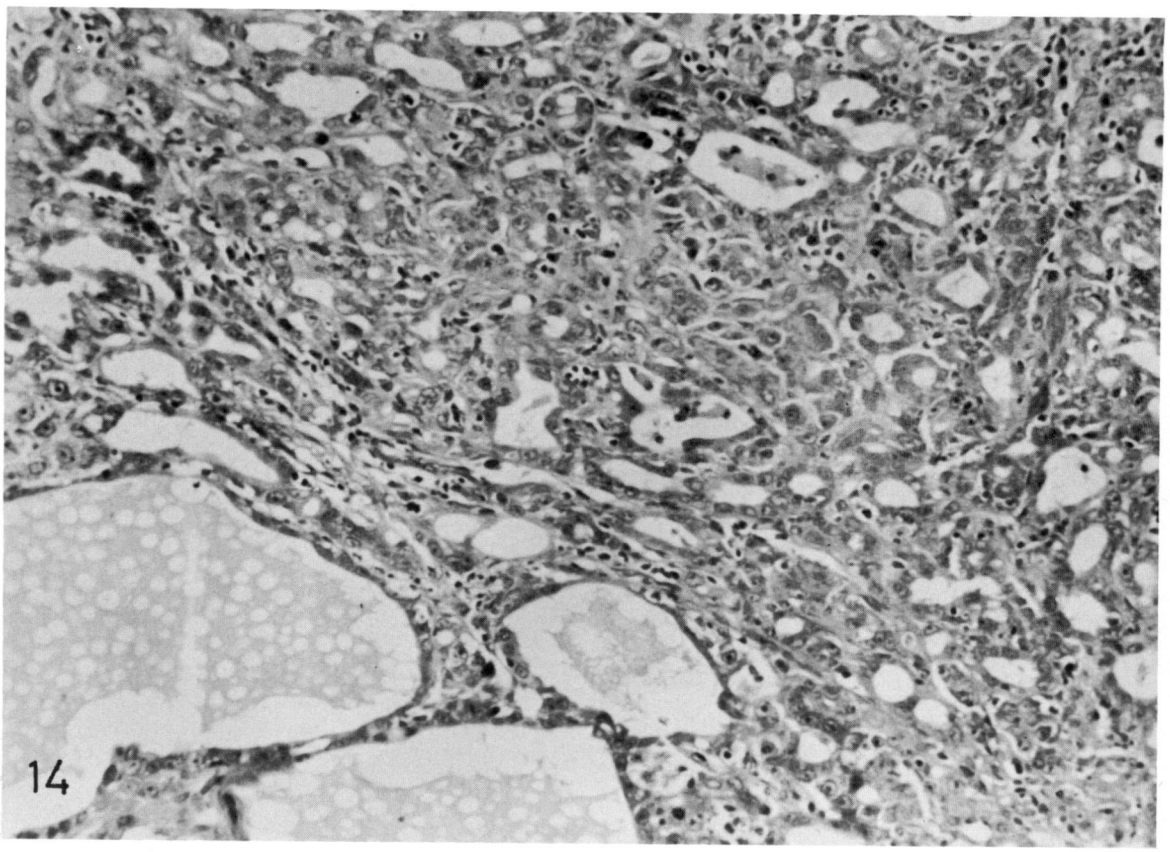

BM
BM
9

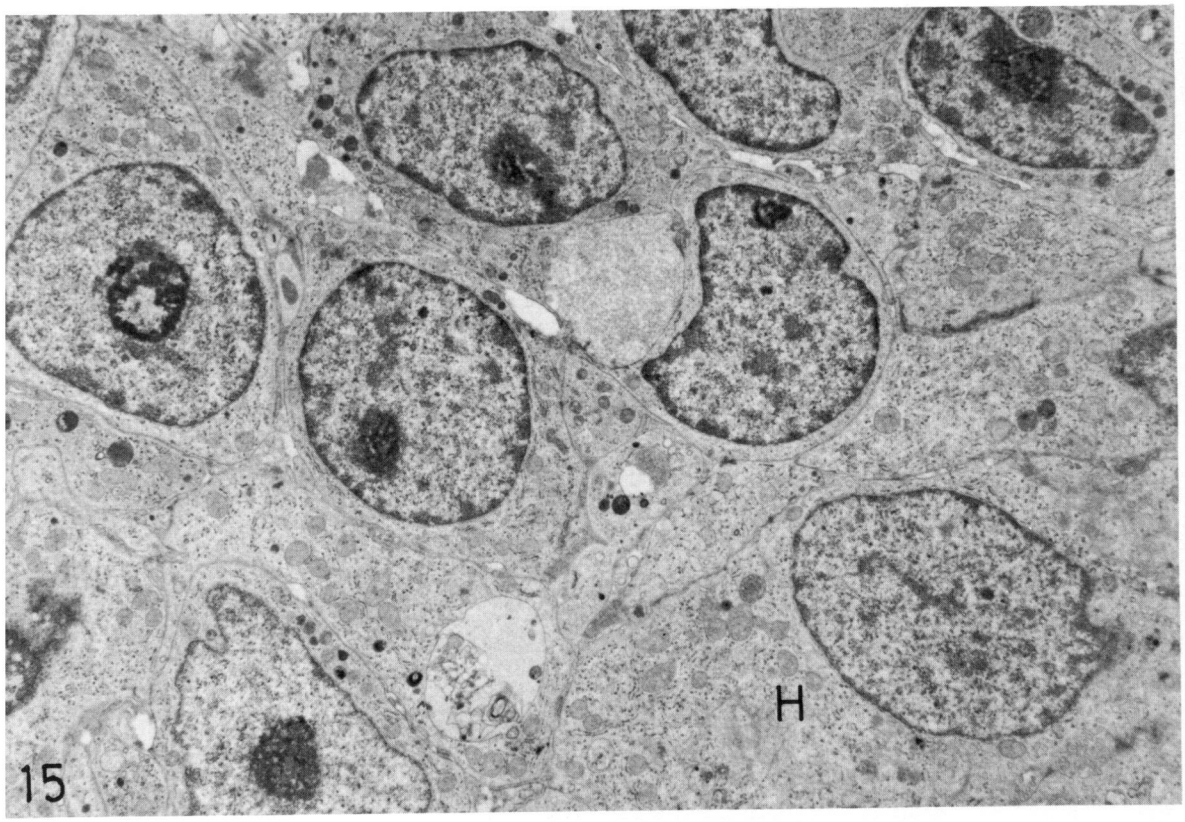

10

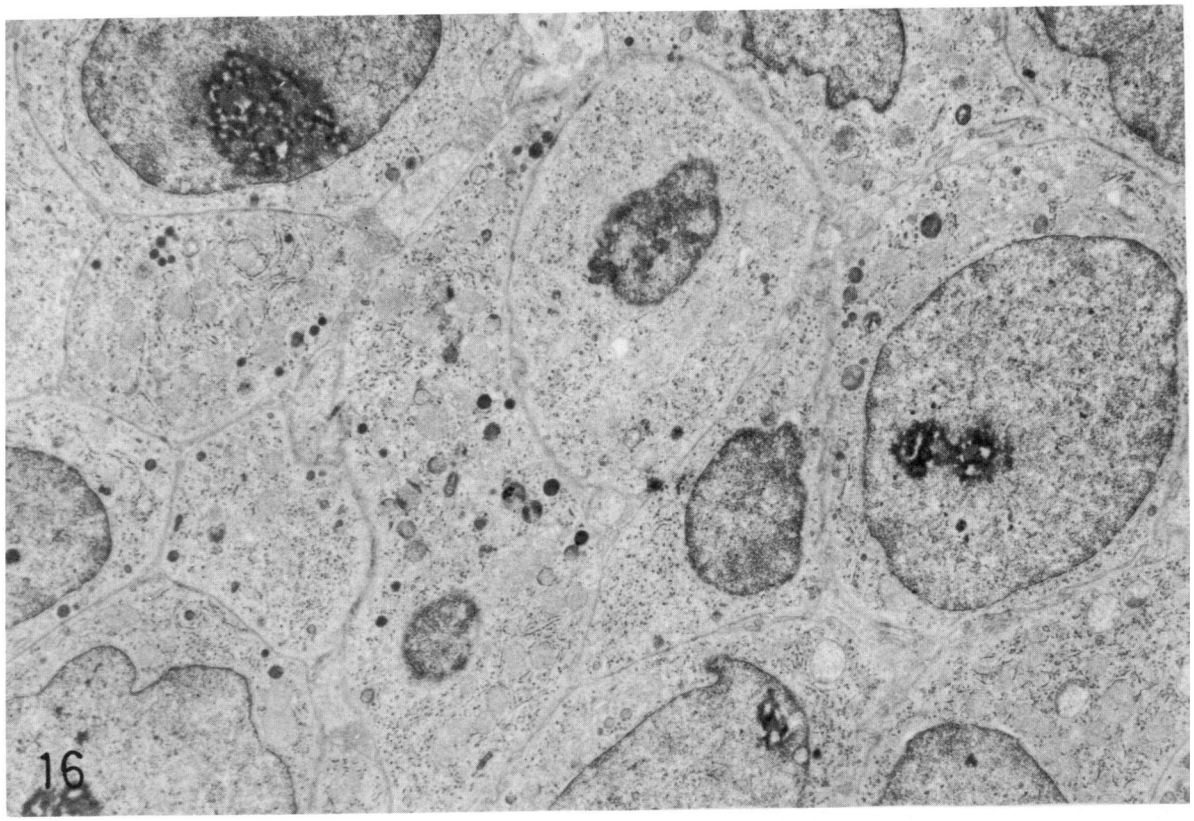

N
BM
L
M
D
11

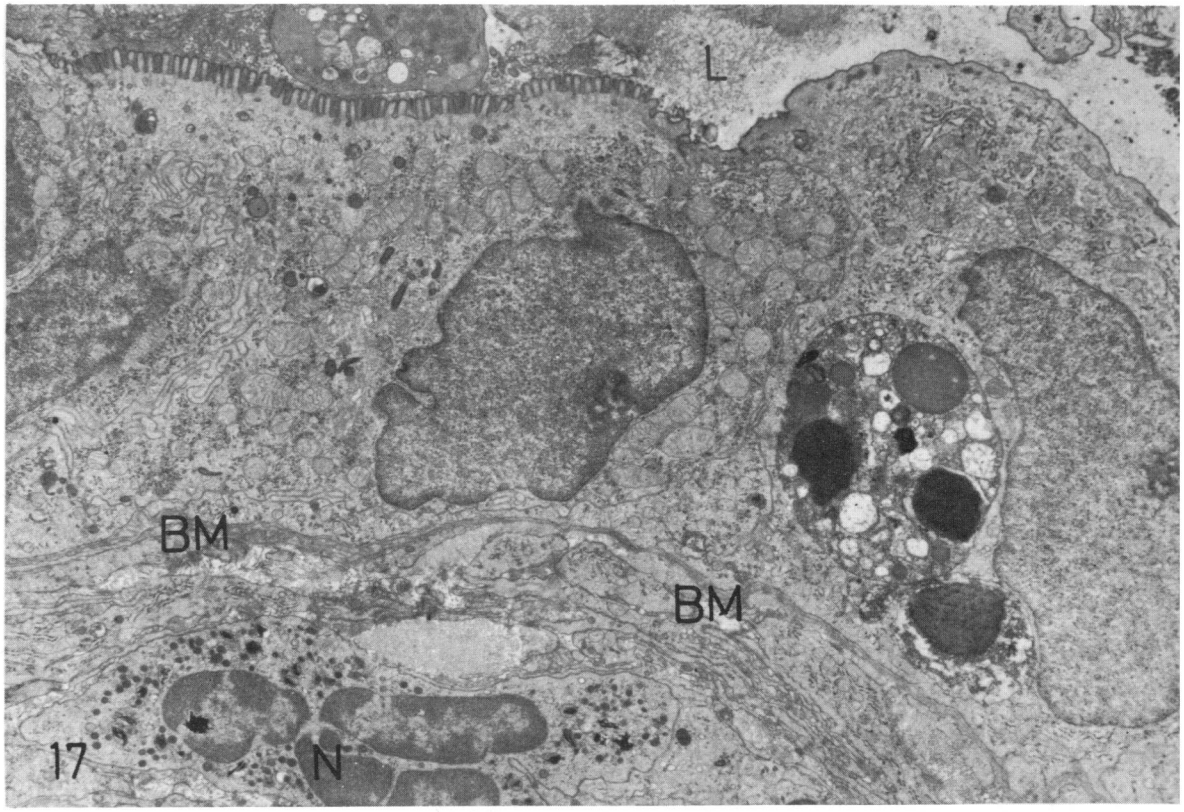

L
BM
L
BM
12
M

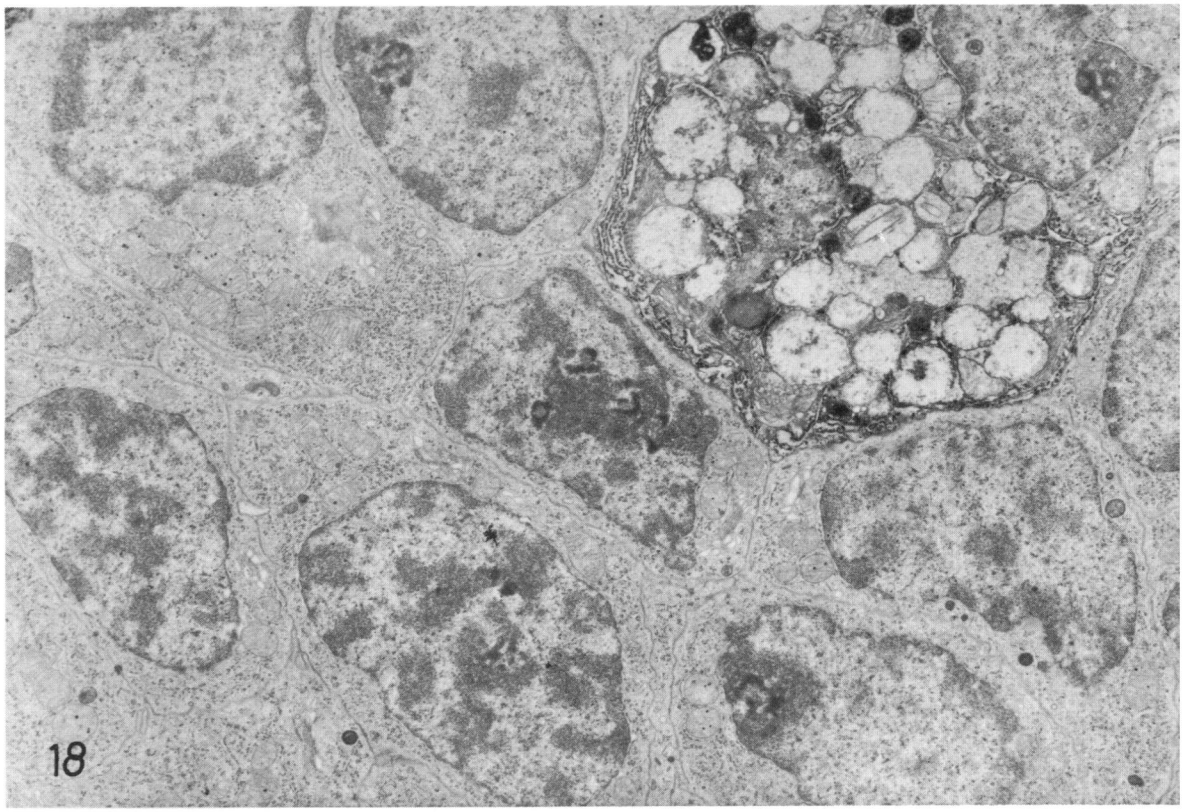

13

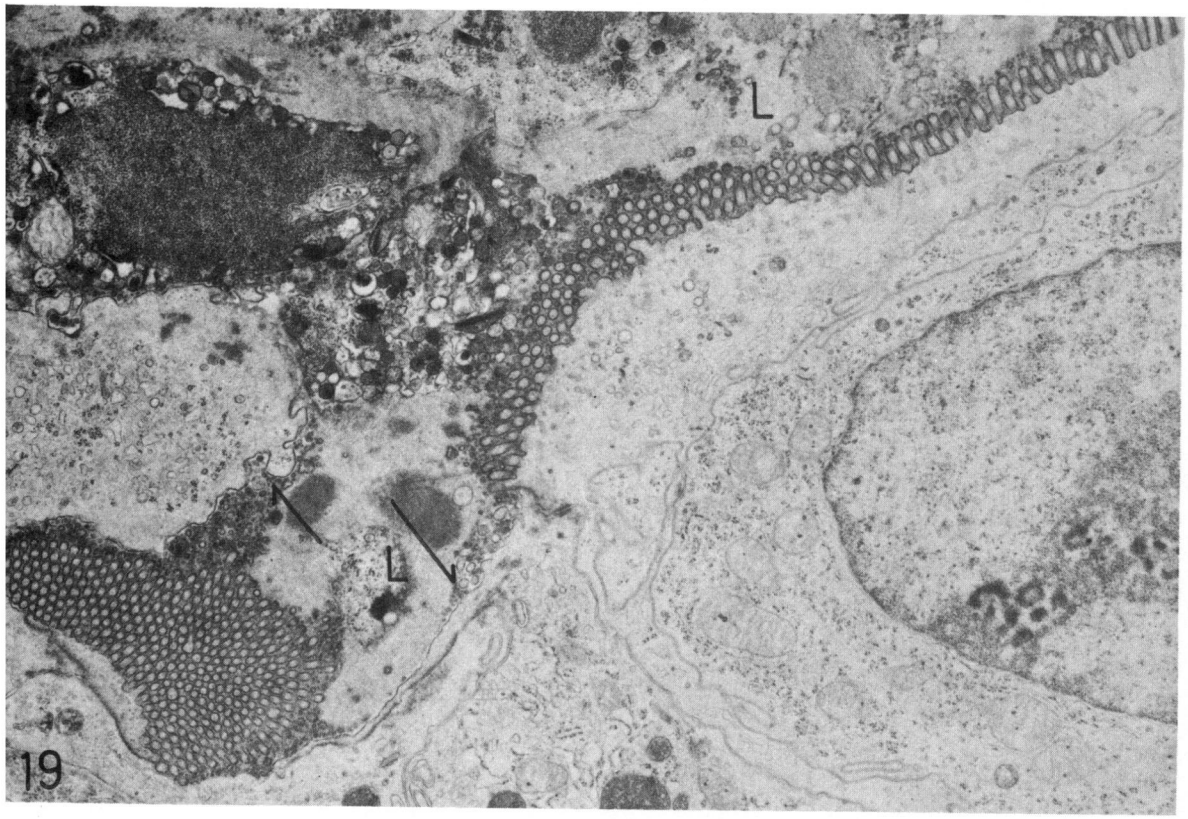

14

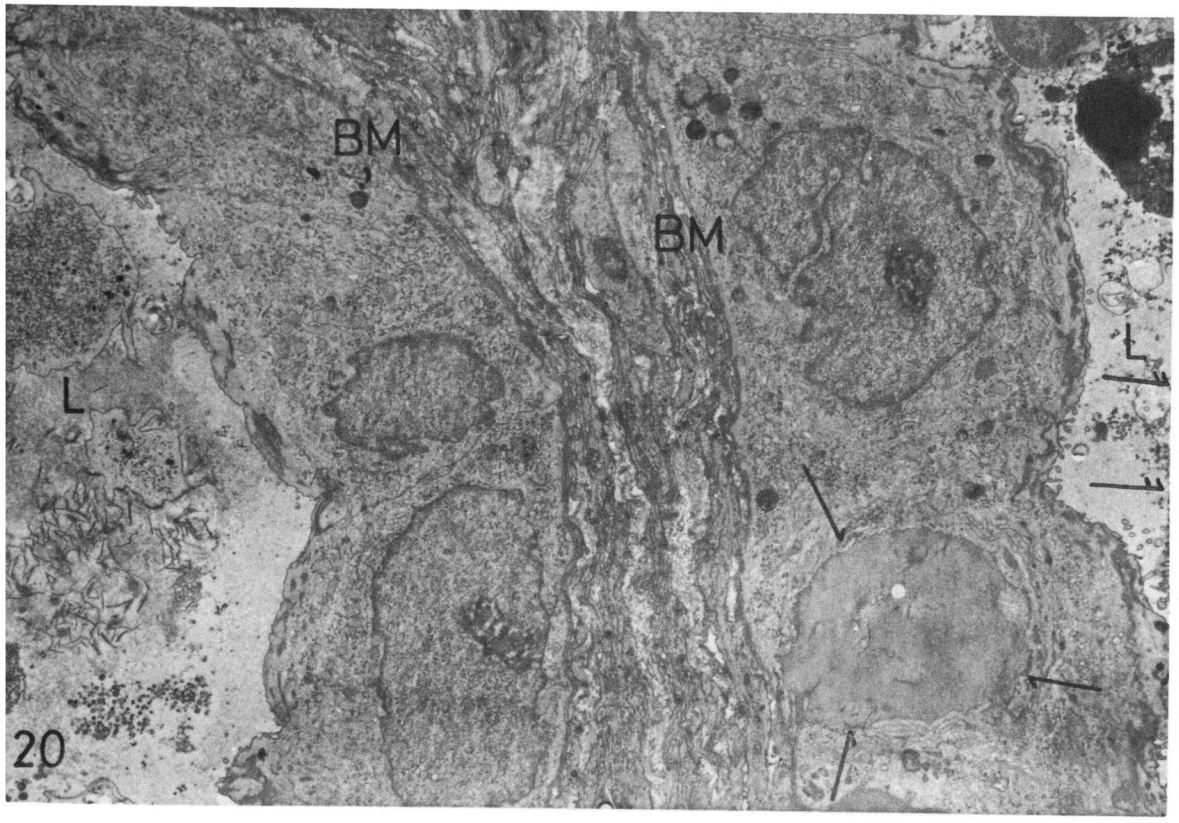

H
15

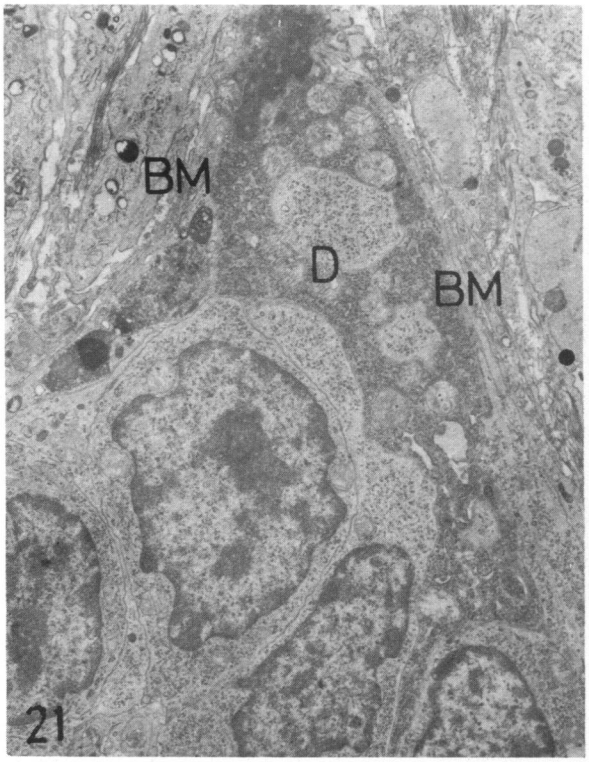

16

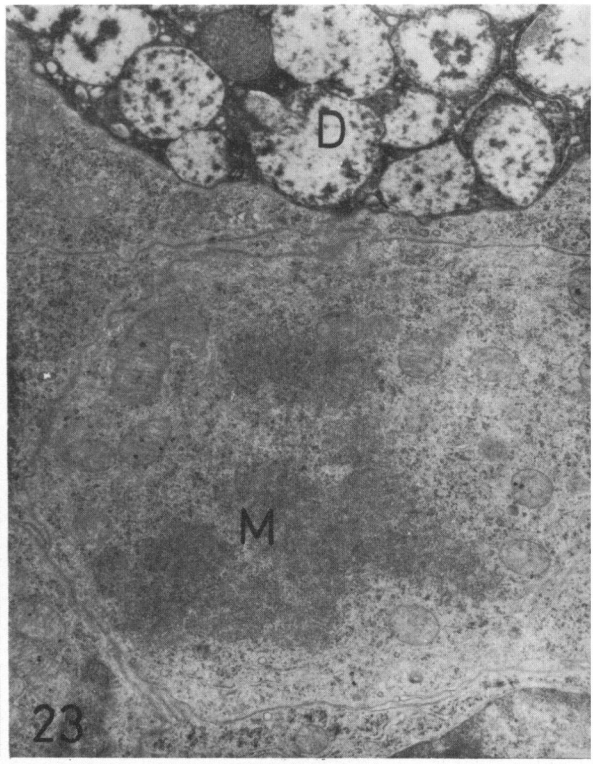

L
BM
BM
N
17

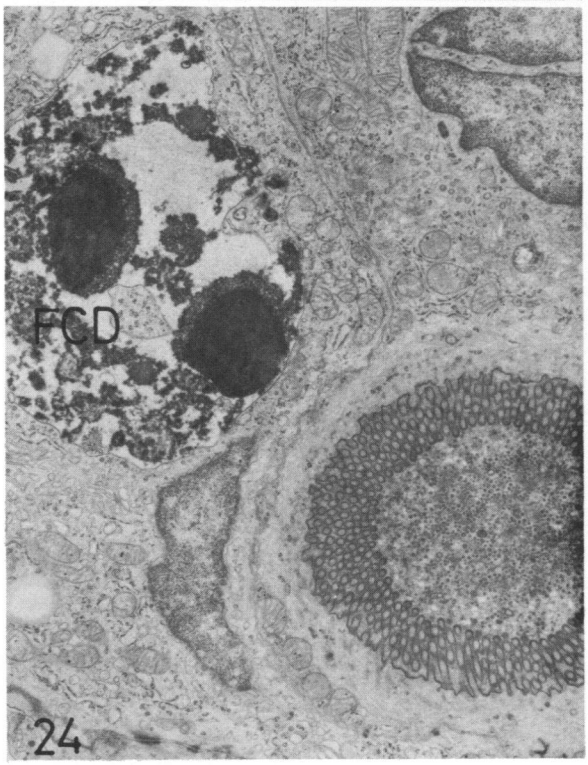

18

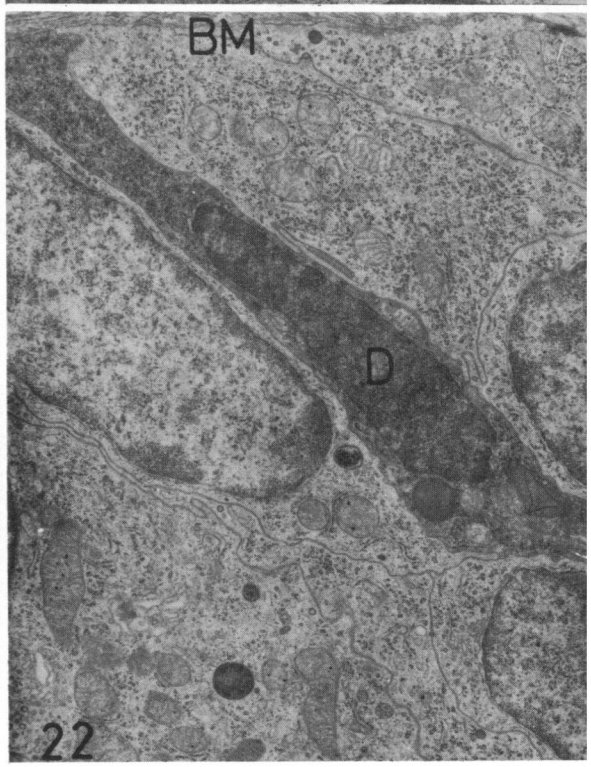

L
L
19


BM
BM
L
L
20


BM
D
BM
21


D
M
23


FCD
24


BM
D
22