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ELECTRON MICROSCOPIC OBSERVATION ON THE CYTOLOGIC SMEARS

BY

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ABSTRACT

Interpretation of the fine structures of cytologic smears is needed for further understanding of morphological and physiological properties of malignant cells in the smears. However, application of electron microscopic study usually has been limited to histologic tissues affected with diseases, taken by either biopsy or autopsy materials. Several methods have been devised for sampling the cultured or smeared cells for electron microscopy but it is inconvenient to apply these methods for routine examination of cytologic smears because of requiring skillful technique and expensive instruments. The present method makes it easy to obtain electron microscopic samples from the cytologic smears and to observe the same cells in the smears with both light and electron microscopes.

The results of application of this technique are shown in the electron micrographs of vaginal and cervical smears of the carcinoma of portio cervix or chronic endocervicitis. There are not any distinctive differences in the details of the remaining ultrastructures, such as endoplasmic reticulum, mitochondria, free ribosomes, tonofibrils, cytoplasmic membrane, nuclear chromatin granules, desmosomes, etc., as compared with those of usual block tissues.

INTRODUCTION

Exfoliative cytologic examination is now being widely used as one of the main tools in the diagnosis of malignant lesions and the determination of sex hormonal conditions in females, since the cellular make-up of human normal vaginal smears was observed by Pouchet in 1847, and the desquamated cells for the purpose of diagnosing cancer were studied by Beal in 1860, Hampeln in 1876, Menetrier in 1886, Bestscardt in 1895, and others[1]. Adequate comprehension of the suitable cytologic sample has not only disclosed the presence of malignant lesions but also helped to differentiate the type of lesions. Although such noteworthy progresses have been made in the study of cytologic characteristics of cancer cells, the substantial knowledge of cytologic smears is still rather fragmentary because the immediate

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correlation between cytologic findings and biopsy for the confirmation of positive smear features is not apparent, and applicability of the biopsy method is limited for some organs.

In recent years, many valuable informations[2] have frequently been given for difficult diagnostic cases in the surgical and autopsy pathology, according to the electron microscopic examination for changes in the cytoplasmic organelles in the cells and tissues affected with diseases. In the same way, observation on the fine structures of cytologic smears is especially needed for further understanding of the morphological and physiological properties of the malignant cells in smears.

Various methods[3-10] have been devised for sampling the single cell or the cultured cells for electron microscopy. Howatson and Almeida[5], and Heyer[9] tried to embed cultured cells growing on slides. Nebel and Minick[4], and Nishiura and Ranyan[6] tried the embedding of tissue culture cells on coverslips or slide glass. Open-face embedding technique, by which cells from tissue cultures or smears on the flat surface of slides are prepared for electron microscopy, has been described by Sparvoli, Gay, and Kaufman[10]. These methods can supply electron micrographs of the particular cells selected under a light microscope, but they are limited only to the observation of a few, unstained cells previously orientated and occasionally fail to detach the cells from the smears. Therefore, it is impossible to use these methods for routine examination of cytologic smears.

It is possible to make electron microscopic sample skillfully from cytologic smears and to observe the same cells in smears with both light and electron microscopes, through the present method obtained by the modification of "Electron microscopic technique of paraffin-embedded sections"[11]. Because of the good preservation of cellular ultrastructure of smears and simplicity of the procedure, it will be very useful for electron microscopic examination of cytologic smears.

Material and Method

Materials (Vaginal and cervical smears):

Materials were collected from the posterior fornix of vagina or the ectocervix with a glass pippette, or with a cotton swab or with spatula, from women with squamous cell carcinoma of portio cervix or chronic endocervicitis with epidermization.

Procedure:

1. Preparation of epoxy resin slides.
2. Collected materials are smeared on epoxy resin slides.

3. Slides immediately immersed in fixatives.
4. Smears are stained with various stains for light microscopy.
5. Dehydration, embedding with epoxy resin, and polymerization.
6. Locating the chosen cells under light microscope and photography.
7. Making the electron microscopic block and ultrathin sectioning.
8. Staining with electron stains and observation with an electron microscope.

Preparation of resin slides:

The epoxy resin slides instead of glass slides can be obtained by using the epoxy resin embedding fluid of Luft[12]. The practical method has already been described in detail[11].

Obtaining smears, fixation, and staining:

The collected materials are spread on the surface of clean epoxy resin slides coated previously with albumin solution. The moist smears on the epoxy resin slides are immersed immediately in 2% glutaraldehyde solution buffered to pH 7.2 with phosphate buffer and left for a minimum of 15 min to a maximum of 30 min, and then it is post-fixed in 0.5% osmium tetroxide solution buffered to pH 7.2 with phosphate buffer for 5 to 15 min. Over fixation for a long period of time may result in an alteration in the staining reaction of the cells. The methods of staining which are most commonly used, are as follows:

Papanicolaou's stain:

1. After removing the fixative, the smears are stained in Harris' Hematoxylin for 5 min, and then washed in running tap water.
2. The smears are differentiated in ammoniated ethanol for 30 sec and then washed in distilled water.
3. The smears are rinsed in 50%, 70%, 80%, 90%, and 95% ethanols for 2–3 min each.
4. The smears are stained in OG-6 for 2 min and rinsed in 95% ethanol, three changes.
5. The smears are stained in EA-36 for 1-2 min and rinsed in 95% ethanol, three changes.
6. The smears are dehydrated and cleared by running through absolute ethanol and acetone.
7. To mount, the smears are covered with Epon 812 embedding mixture[12], through a mixture of an equal part of absolute acetone and Epon 812 embedding mixture, and only Epon 812 embedding mixture, for 15–30 min each.

8. The smeared slide is incubated in 60°C oven for 48 hr for polymerization.

Harris' Hematoxylin-Eosin stain:

1. After fixation, the smears are rinsed in distilled water for 2 min and left for 2 min in Harris' Hematoxylin solution.
2. The smears are washed in running water and then left for 30 sec in ammoniated ethanol or lithium carbonate solution.
3. The smears are washed in distilled water and left for 1 min in Eosin solution.
4. The smears are then dehydrated in progressive concentrations of ethanol (50, 70, 90, 95% and mounted as in Pananicolaou's stain.

Trimming the block and ultrathin sectioning:

The methods of trimming and ultrathin sectioning are made in the same manner as in "Electron microscopic technique of paraffin embedding sections"[11]. The precise area on the epoxy resin slide, including the selected cells for electron microscopic analysis under a light microscope, is attached to the usual electron microscopic resin block, with an adhesive. The ultrathin sections and electron staining[13,14] are made in the usual manner.

Result and Discussion

The results of the application of this technique are shown in Figs. 1–6. There are certain qualitative differences in the staining reaction of the smear cells in the present method compared with the cells smeared on a glass slide, although any staining can be to applied to them, at the level of light microscopy, but there has been not any outstanding differences or diagnostic trouble in the definition of nuclear details and differentiation of cells. The main reason for the difference seems to be the difference in the kinds of fixatives used. Papanicolaou's fixative is not recommended for the poor preservation of membranous ultrastructures. The smears must not be allowed to dry, during the whole period particularly before fixation.

There are no distinctive differences in the details of the ultrastructures between the electron micrograph of the smears (Fig. 6), made by this method and that of the usual method from blocks of tissue. According to the findings of light microscopy (Figs. 1 and 3, insert), the electron microscopic study of the cancer cells on the smears showed enlargement and marked irregularity in the shape of nuclei due to deep clefts and infolding of the nuclear membrane, with hyperchromatic granular materials and prominent-

ly larger nucleoli, and narrow extension of cytoplasm including fewer subcellular organelles. In other cells, mitochondria were increased in number and appeared deformed, swollen, vacuolated, and less cristae. Poorly formed endoplasmic reticulum was noted in the cytoplasm (Fig. 2). A considerable modification of cell surface forming villous cytoplasmic projections was encountered in the cancer cells. On the other hand, a typical squamous metaplastic cells included tonofibrils and scattered free ribosomes in the cytoplasm (Fig. 6). A cluster of exfoliated squamous cells demonstrated apparent desmosomes at the intercellular villous junctions (Fig. 5). Marked vacuolation of the cytoplasm was also noted as the secretion granules and vacuolar degeneration of the intracytoplasmic organelles (Fig. 3). Neutrophil in the smear had well preserved intracytoplasmic organelles and azurophilic granules (Fig. 3). The cytoplasmic changes of squamous cells in the smears, reflected in the staining reaction exhibiting pronounced basophilia or acidophilia under a light microscope were proved to be the result of proliferation of organelles and ribosomes or keratinization. In accordance with the above findings, the light microscopic features in the smears seem to correspond with the electron microscopic pictures, similar to that in blocks of tissue reported by Luibel *et al.*[15], and by Sirtori and Morano[16].

The structural cellular abnormalities, proved to be related to malignancy in smears, are considered to be due not only to the malignant neoplasms but to biological variations and inflammatory lesions[17]. Therefore, it has sometimes been much more difficult to determine the presence or absence of maignancy, based on changes in the structural of individual cells and subdivided into three main groups: (i) Structural modification of cells and their nuclei, (ii) changes in the interrelationship of cells forming cell clusters and tissue fragments, and (iii) indirect criteria, by Papanicolaou[1]. The diagnosis of malignant lesions in exfoliated cells seems to rely chiefly on the intuitive identification of nuclear changes in the cells, such as nuclear enlargement, multinucleation, anisokaryosis, and hyperchromasia. The cytoplasmic changes of the smeared exfolliated cells appear at present to be so little interested in the recognition of various malignant tumors, because it is rather difficult to obtain general characteristics of the cytoplasmic changes as malignant cytological criteria by a light microscopy, except for particular characteristics like melanin granules, phagocytic vacuoles, or mucous droplets (signet ring cell) in the cytoplasms. The cytological criteria of malignancy in the smears may probably be made by the objective recognition of the fine structures of cytoplasmic and nuclear changes. Gradual accumulation of observations on the ultrastructures of

cytologic smears will contribute to a new interest in the study of cytologic smears.

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Explanation of Figures

Plate 1

Fig. 1. Cervical smear: A cluster of exfoliated cancer cells, showing sharp and well preserved chromatin clumping in the nucleus and scant cytoplasm with poor borders. The arrow indicates the cell located in the central portion of Fig. 2. Hematoxylin-Eosin stain. ×400.

Fig. 2. Electron micrograph of Fig. 1. The cells are loosely joined together by cytoplasmic microvilli, showing enlarged and pleomorphic nuclei (N) with prominent nucleoli (Nn). ×9,600.

Plate 2

Fig. 3. Cervical smear: Light micrograph in the right upper insert, illustrates degenerative changes with intracytoplasmic vacuoles and nuclear pyknosis in the exfoliative squamous cells. Papanicolaou stain ×400.
Electron micrograph of the portion surrounded by the arrows in the insert, presents a group of exfoliative squamous cells. One of them has scattered vacuoles (Va) and nucleus (Nl) dislocated peripherally in the cytoplasm. Others show slightly degenerated mitochondria (Mt), and irregularly clumped chromatin (N_2) or usual chromatin pattern (N) in the nuclei. ×11,000.

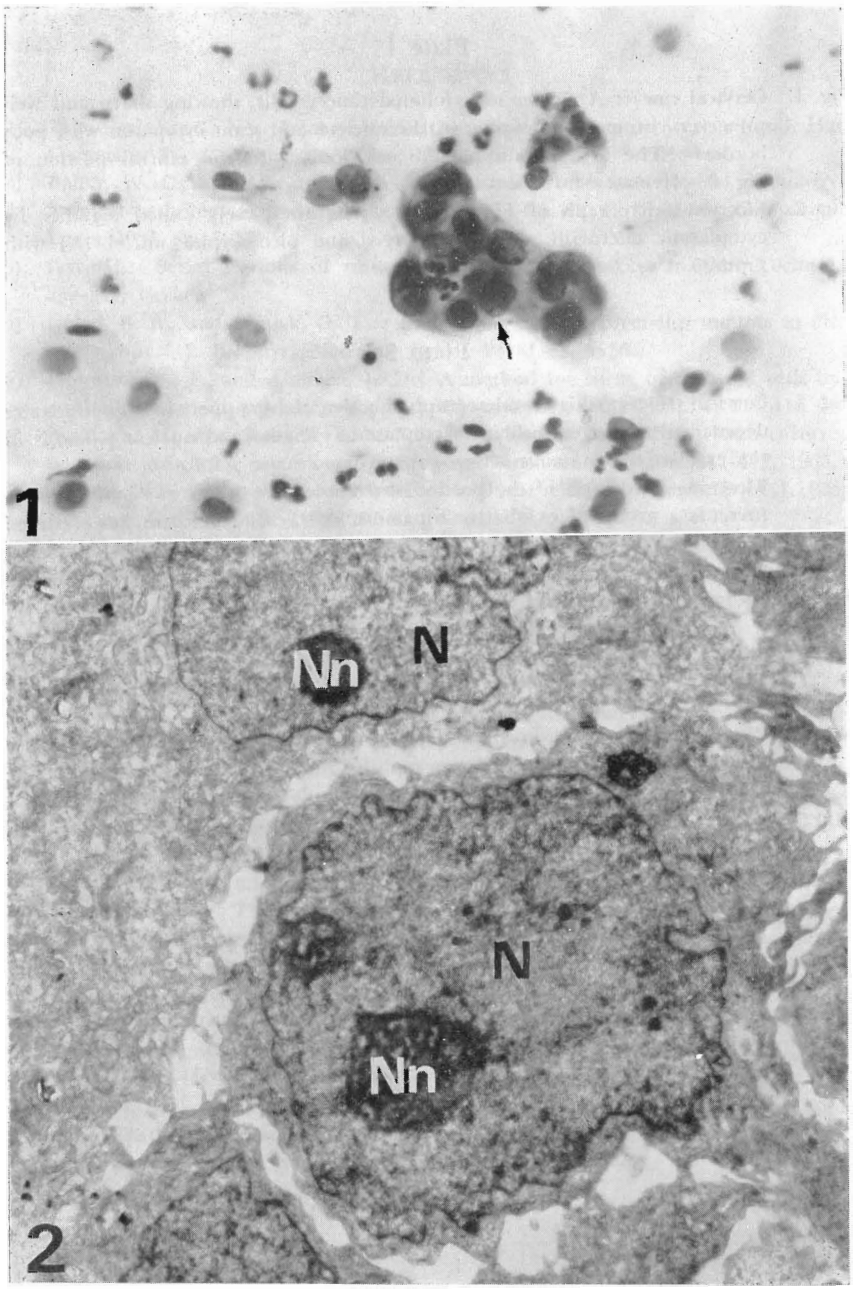
Plate 3

Fig. 4. Vaginal smear: Electron micrograph of neutrophil. In this cell the nucleus is multilobulated. A few large azurophilic granules (Gr) are dispersed. The cell membrane is irregular and extends pseudopodia. ×11,400.

Fig. 5. Vaginal smear: Electron micrograph of clustered exfoliative squamous cells. Desmosomes (arrows) connecting the cell processes of each cell are noted. ×11,000.

Plate 4

Fig. 6. Cervical smear: Electron micrograph of the exfoliative squamous metaplastic cell. The cell contains deformed mitochondria (Mt) in the perinuclear cytoplasm, with tonofibrils (Tf), rough-surfaced endoplasmic reticulum (Er), and free ribosomes (Ri). ×38,000.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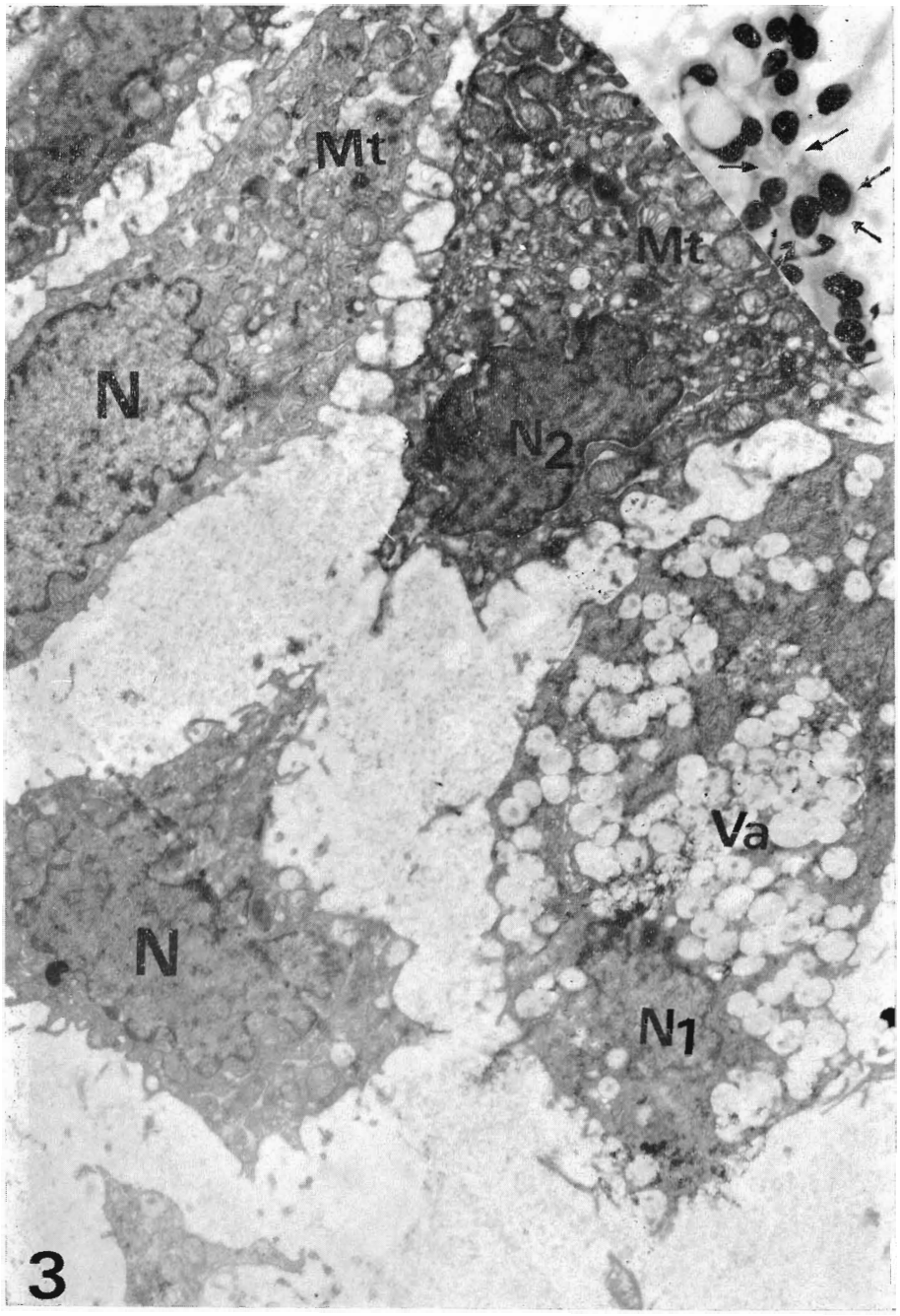
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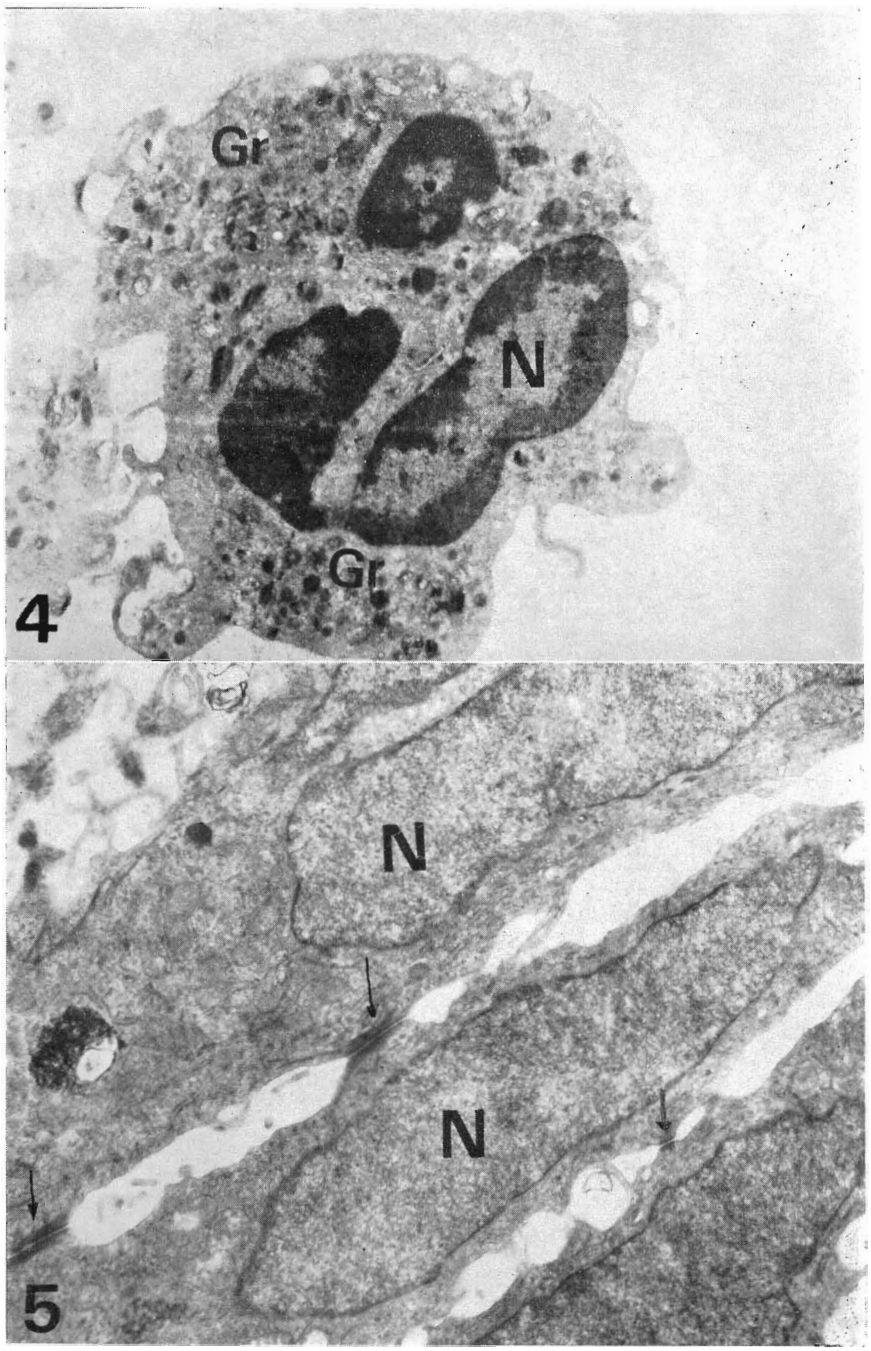
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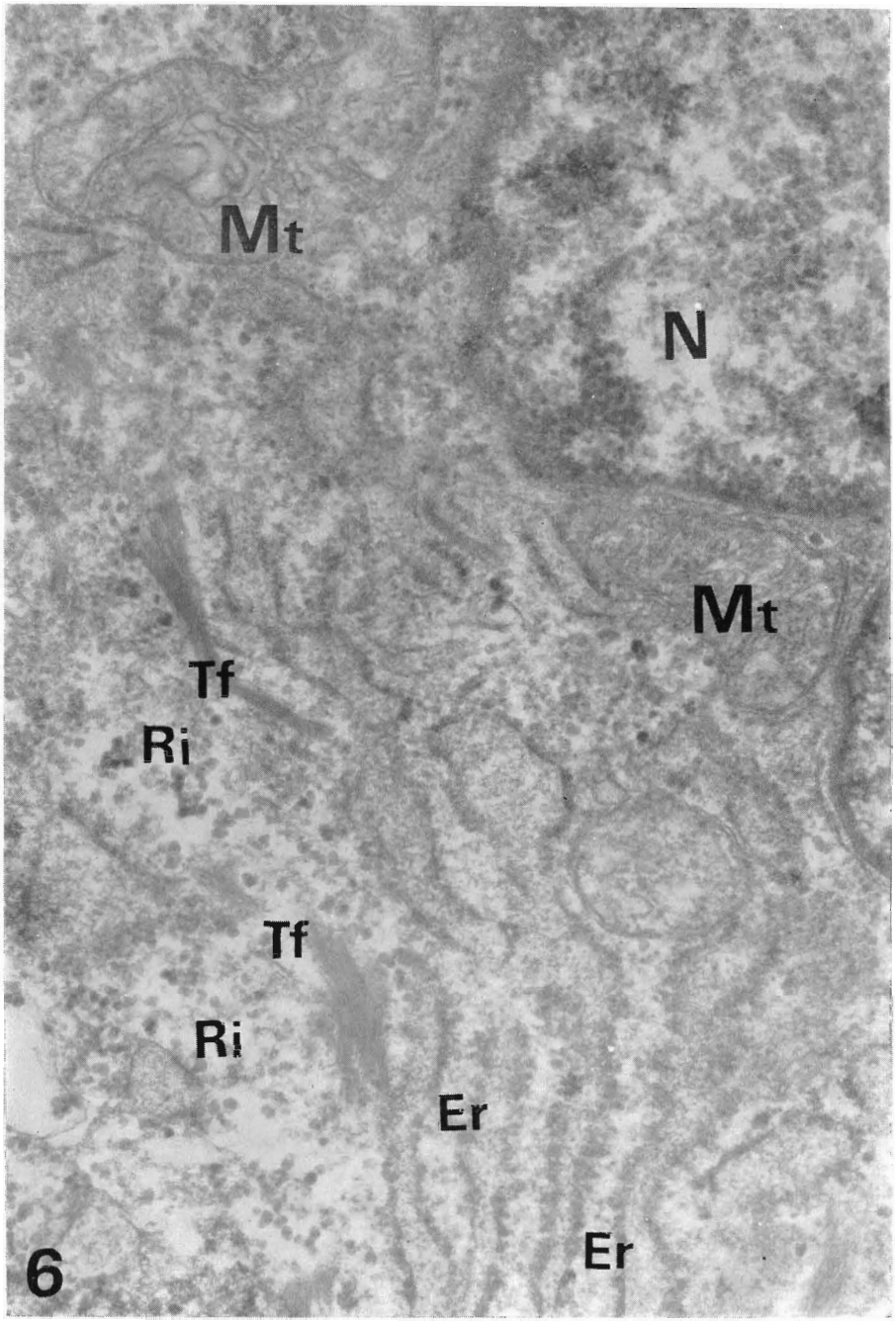
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