FURTHER OBSERVATIONS ON THE OCCURRENCE OF NEXUSES IN BENIGN AND MALIGNANT HUMAN CERVICAL EPITHELIUM

N. SCOTT McNUTT, ROBERT A. HERSHBERG, and RONALD S. WEINSTEIN

From the James Homer Wright Pathology Laboratories, Department of Pathology, and the Mixter Laboratory for Electron Microscopy, Massachusetts General Hospital 02114, and the Department of Pathology, Harvard Medical School, Boston Massachusetts 02114

ABSTRACT

An estimate is made of the frequency of occurrence of nexuses ("gap junctions") in a spectrum of human cervical epithelia, ranging from normal to malignant, since a deficiency of nexuses may be important in abnormal cell-to-cell communication in malignant tissues. The normal cervical epithelium has approximately ten nexuses per cell in the basal layer of proliferating cells and 200 nexuses per cell in the more differentiated intermediate zone. Nexuses are rare between invasive malignant epithelial cells (carcinoma cells). In many areas of cell proliferation near the edge of the tumor mass, fewer than one nexus per cell is present. However, up to four nexuses per cell can be found in some well differentiated regions of invasive carcinoma. Preinvasive malignant epithelia (severe dysplasia and carcinoma–*in situ*) have as few nexuses as invasive carcinoma. In abnormal but benign epithelia (squamous metaplasia and mild dysplasia), nexuses are abundant. The data indicate that a decrease in number of nexuses correlates with the severity of the morphological alteration in the dysplastic epithelium. Also the deficiency of nexuses in groups of carcinoma cells can occur many cell generations before the development of invasion of the malignant epithelium into the connective tissue. The diminution of nexuses before invasion suggests that a deficiency of nexuses may be one of the important factors in eventually permitting the development of the diffusely infiltrating type of invasion which is characteristic of highly malignant tumors such as squamous carcinomas.

INTRODUCTION

An abnormality in cell-to-cell communication may account for some of the abnormal behavior of malignant cells (1, 2, 3, 4, 37, 57, 71, 72, 105, 106). In normal tissues, intercellular communication is partially mediated by specific ultrastructural modifications in the plasma membranes of adjacent cells. These modifications are called cell-to-cell junctions (38, 39, 40, 41). In normal heart and smooth muscle, the nexus (or "gap" junction) provides direct electrical coupling of adjacent cells, a feature which coordinates muscular contraction in these tissues (10, 11, 15, 34, 35, 36, 109; cf. 24, 84). Nexuses can also be found in a variety of normal epithelia such as that of liver (12, 92) and the human cervix; however their physiological role in intercellular communication in these tissues is unknown.

A controversy has arisen over whether cell-to-

cell communication via direct small ion interchange is abnormal in malignant tissues (19, 57, 71, 72; cf. 44, 89, 100). This direct ionic coupling is often measured as low resistance electrical coupling of adjacent cells. Loewenstein et al. (57, 71, 72) have described an absence of electrical coupling between adjacent cells in malignant epithelia (carcinomas) in vivo whereas the normal epithelial counterparts contain cells which are electrically coupled. In apparent contradiction to this finding, Sheridan (100) has demonstrated examples of electrically coupled malignant cells in vivo. Under certain in vitro conditions, malignant epithelial cells (carcinoma) lack electrical coupling whereas malignant connective tissue cells (sarcoma) can be electrically coupled (19). The ultrastructural basis of this presence or absence of electrical coupling has not been fully investigated, although most studies reported to date indicate that the nexus probably is the type of cell junction responsible for electrical coupling (10, 11, 85). The data presented in this study may eventually shed further light on the controversial electrophysiological data (19, 44, 57, 71, 72, 89, 100).

We have reported elsewhere that invasive squamous carcinoma of the human cervix is markedly deficient in nexuses whereas nexuses are abundant in normal cervical epithelium (78). As emphasized earlier (78), quantitation of the number of nexuses in specimens requires the use of the lanthanum hydroxide tracer technique (92) to allow identification of all of the nexuses present in thin sections. Without this tracer, only transversely sectioned nexuses are clearly recognizable. When tissue blocks are exposed to an aqueous colloidal dispersion of lanthanum hydroxide at pH 7.4, electron-opaque lanthanum fills the extracellular space and, in addition, impregnates thin channels within the central region of the nexus, where these channels outline a closely packed subunit array (77, 85, 92). The lanthanum in the channels gives the nexus a characteristic appearance in transverse, oblique, and *en face* view in thin sections. Therefore, in favorable specimens, the number of nexuses per cell can be estimated.

The present study is an attempt to more accurately correlate the observed deficiency of nexuses in malignant epithelia (37, 78) with the abnormal properties of malignant cells. Fully malignant cervical epithelial cells often appear to arise from epithelia which have undergone a partial alteration in appearance and function (reviewed by 58). This partial alteration shows some of the features of malignant transformation. Therefore the frequency of occurrence of nexuses is compared in specimens from a spectrum of normal to abnormal benign to premalignant and malignant conditions of the human cervix.

The six abnormal members of this spectrum are given the names squamous metaplasia, mild dysplasia, moderate dysplasia, severe dysplasia, carcinoma–*in situ*, and invasive carcinoma. The first two states are benign and the last three are commonly considered premalignant or malignant. Whether moderate dysplasia is to be considered benign or premalignant is still a matter of some dispute which can only be settled in the future by careful histopathological study correlated with epidemiological study to determine the fate of the various conditions (58, 104, 110). Statistically, the conditions called squamous metaplasia and mild dysplasia appear to be reversible in most cases (60, 104). Also, statistical evidence is accumulating for a significant number of cases of progression from severe dysplasia to carcinoma–*in situ* to invasive carcinoma over a period of years, although not all cases show this progression (60, 104). Such evidence clearly indicates the inadequacy of a simple comparison of normal benign states versus invasive malignant states and points to the complexity of events which may occur in a malignant transformation. The availability of biopsy material from such a spectrum of abnormality allows the opportunity to observe whether a particular ultrastructural or physiological alteration of the epithelium can be correlated with a specific change in the behavioral properties of that epithelium.

MATERIALS AND METHODS

3–5 mm punch biopsies were obtained over a three year period from the cervices of a total of thirty-two carefully selected human female patients of reproductive age. 24 females had benign conditions: 12 normal except for mild chronic inflammation, five with squamous metaplasia of the endocervix (including early squamous metaplasia or "reserve" cell hyperplasia), and seven with mild to moderate dysplasia of the cervix. Eight females had malignant conditions: three with severe dysplasia or carcinoma–*in situ* and five with early invasive squamous carcinoma. All diagnoses were determined by light microscope examination of tissue biopsies. 1 μ sec-

tions of plastic-embedded tissue blocks were matched with the lesions in the diagnostic biopsy material to assure that material examined in thin sections was representative of the lesion of interest. All biopsies for electron microscopy were obtained before surgical, hormone, or radiation therapy from tissue sites which were not biopsied or painted with noxious preparative solutions (e.g. Schiller's iodine reagent) for at least 1 month before sampling for electron microscopy. All invasive carcinomas were biopsied near the edge of the tumor.

Standard Thin Section Technique

Each biopsy was hemisected and one half was prepared as in group A and the other half as in group B. Group A specimens were fixed in one of three fixative solutions: (*a*) 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4; (*b*) 2% paraformaldehyde–2% glutaraldehyde in 0.1 M cacodylate buffer; or (*c*) 1% paraformaldehyde–1% glutaraldehyde in 0.1 M cacodylate buffer. Fixatives (*b*) and (*c*) represent modifications of the mixture of Karnovsky (62). Group B specimens were similarly fixed but also impregnated with colloidal lanthanum hydroxide (92) to aid in identification of nexuses in thin sections. The specimens were postfixed in 1% osmic acid in 0.1 M cacodylate buffer either with 1% lanthanum hydroxide (group B) or without it (group A). Some of the tissues in group A were stained en bloc with 1% uranyl acetate in Veronal buffer, pH 5.0, for 1 hr (40). After dehydration in a graded ethanol-water series, the tissue blocks were infiltrated with Epon 812 (73), oriented with a dissecting microscope, and cured in a 65°C oven. Thin sections were cut with glass or diamond knives and were examined at 80 kv in a Siemens Elmiskop I electron microscope.

Estimation of Nexus Frequency

To compare the frequency of occurrence of nexuses in the specimens, a simple method for estimating the number of nexuses per cell was derived which is adequate for the purposes of this study since we are interested in gross deficiencies of nexuses. The method gives an approximation of nexus frequency rather than exact determination of number of nexuses per individual cell. An exact nexus frequency and distribution would require a high resolution three dimensional analysis of many cells from hundreds of serial sections. This has not been possible with standard microscopy for the specimens in this study. In the simple method for estimated nexus frequency (ENF), the primary data are: (*1*) a count of the number of nexuses visualized per section (n); (*2*) the number of cell profiles examined per section (c); (*3*) measurement of average cell diameter; and (*4*) estimate of the section thickness used.

At least two to four tissue blocks from each diagnostic category were selected in which the cells were well preserved and the lanthanum hydroxide dispersed in the extracellular space. 1 μ sections of these blocks were stained with toluidine blue and examined at 400 × in a light microscope with a calibrated ocular micrometer for measurement of maximum and minimum cell diameters. These data on cell diameters are needed for a rough estimation of cell size from which the average fraction of cell surface included in a given thin section (f), can be estimated. For example, since a cell 9 μ (or 90,000 A) in diameter can be cut into 100 slices each 900 A in thickness, an estimate of (f) can be obtained from the section thickness divided by the average cell diameter, if the section includes many cells. Thus in a given 900 A section, if 100 randomly oriented cell profiles are included, then the section can be said to include a total amount of surface belonging to one "typical" or average cell if the cells are approximately 9 μ in diameter. When the orientation of the cells appeared random in the direction of sectioning, the maximum and minimum diameters were averaged for the calculation. Since the normal basal single layer of cells had a distinct orientation to the section plane, the average transverse diameter was used for calculation of (f).

Thin sections for nexus counts were cut from the tissue blocks so that they would be adjacent to the 1 μ sections used for light microscopy. For each specimen, the section thickness used was the same, which had a bright silver interference color, estimated to be approximately 750–900 A according to the scale published by Peachey (86). The sections were understained with lead citrate to prevent the lead stain from obscuring the lanthanum distribution. The nexuses in several nonserial sections were counted and the results calculated separately for each thin section to prevent duplication in counting. At least 20 sections were taken from each tissue block under study.

The number of nexuses visualized and the number of cell profiles within the open area of the grid were recorded. Very small processes of cells were not included in the counts of number of cell profiles. Any questionable nexuses were photographed for precise identification. The number of nexuses was counted in the basal zone (two to three layers of cells in thickness) and in the intermediate zone of each of the epithelia. In invasive carcinoma, the counts of number of nexuses were pooled for all of the malignant squamous epithelial cells. This analysis included at least 60–100 cell profiles where the nexus frequency seemed high and 400–1000 cell profiles where the frequency was low.

From these data, the ENF was estimated by dividing the number of nexuses per section (n) by the product of the number of cell profiles per section (c) multiplied by the fraction of cell surface included per section (f).

$$\text{ENF} = \frac{n}{(c)\ (f)}$$

$$f = \frac{\text{section thickness in A}}{\text{average cell diameter in A}}$$

For example, if one nexus was found after examining a section 900 A in thickness containing 100 cell profiles averaging 9 μ in diameter, then this area was said to average one nexus per cell or have an ENF = 1.

A greater degree of accuracy is possible but is probably not warranted for these data since the cell shape is irregular, the size of the nexuses encountered may vary, and the section thickness is only known to within 15–20%. Some method of estimation of nexus frequency must be used if the diagnostic categories of specimens are to be meaningfully compared to one another. The method of estimation used in this study does not have the sensitivity to allow a statement that no nexuses exist at all for a tissue in which many random sections have failed to reveal nexuses. Such a result by this method must be considered an ENF less than 1, even though other methods might indeed show the nexus frequency to be 0. Examination of thick serial sections by high voltage electron microscopy may be able to provide more accurate quantitation.

Serial Section Technique

Serial sections were cut from several selected tissue blocks using a diamond knife and Porter-Blum MT-2 microtome. The sections were mounted on 0.5% Formvar films held by 1 × 2 mm slot grids, thereby allowing examination of the entire section. It was possible to check the estimated nexus frequency of a few specimens by examining all of the surface of the cells that was present in a given thin section mounted on these grids. Serial sections also allowed a check on the consistency of the ENF data obtained from section to section on a given small region of a specimen but complete reconstruction of cell-to-cell contacts was not possible.

Freeze-Cleave Technique

The freeze-cleave protocol of Bullivant (22, 23), as modified by Weinstein (112), was employed for the examination of plasma membranes of several normal squamous epithelia and invasive squamous carcinomas. Small tissue blocks were taken from the biopsy specimens and glycerinated to 20–40% glycerol in 0.9% sodium chloride without the use of chemical fixation. The tissues were rapidly frozen in Freon 22 at −150°C and cleaved under the surface of a bath of liquid nitrogen. Replicas were cast of the cleaved topography by shadow-casting with platinum-carbon pellets (Ladd Research Industries, Inc., Burlington, Vt.) and backing with pure carbon deposition in a Kinney PW 400 evaporator (Kinney Vacuum Co., Boston, Mass.). Heat etching was employed for some specimens as described elsewhere (115). The replicas were retrieved from the tissues with Chlorox®, washed with distilled water, picked up on uncoated 400-mesh or 200-mesh grids coated with 0.1% Formvar, and examined in a Siemens Elmiskop I electron microscope. Electron micrographs of replicas were printed as positives so that accumulations of platinum appear dark and shadow regions, devoid of platinum, appear white.

TERMINOLOGY AND GENERAL BACKGROUND

Cell Junctions

Considerable confusion exists in the literature with respect to the terminology used to refer to the type of cell-to-cell junction called the nexus. The junction originally named as a nexus by Dewey and Barr (34) was later confused with the macula occludens type of junction because of their apparent ultrastructural similarities when viewed with the thin section techniques then available. We wish to update the original definition of the term nexus so that it applies to that biological structure consisting of the apposition of two plasma membranes to form a junction 150–190 A in over-all thickness with a closely packed globular substructure that can appear in transverse view to have either three, five, or seven layers depending on the chemicals used in specimen preparation for thin sections. The nexus often is shown as: (a) three layers with osmic acid fixation and lead citrate staining; (b) five layers with permanganate fixation, or glutaraldehyde–osmic acid fixation and staining of sections with uranyl acetate and/or lead citrate; and (c) seven layers with glutaraldehyde and/or osmic acid fixation and staining before dehydration with aqueous uranyl acetate at pH 5.0 (21, 42, 65, 77, 78, 96). The nexus characteristically exhibits a closely packed globular subunit array with lanthanum hydroxide (92), negative staining (12, 46, 47), and freeze-cleave and etch techniques (68, 79). Some authors have

preferred the term "gap" junction for the same biological structure (21, 46, 47). The Mauthner cell synaptic disk described by Robertson (96) would also be included by this definition of a nexus (see also 21).

The nexus is not the same as the "tight" junction, or zonula occludens, terms suggested by Farquhar and Palade (38) to apply to the belt-like permeability seal at the apical border of many epithelia (38; cf. 63, 64,114). The tight junction has been shown to correspond to a network of ridges of considerable length running in the plane of the membrane which are most distinctly shown in freeze-cleave replicas (47, 68, 102, 116). Lanthanum hydroxide does not permeate the normal tight junction. The relative thickness of each junction and its permeability to lanthanum as well as the presence of a ridge network substructure to the tight junction and a globular substructure to the nexus provide definitive means to distinguish tight junctions and nexuses from one another.

Histology and Biology of Normal and Abnormal Cervical Epithelia

The cervix of the human uterus has two histological types of epithelia (18). A nonkeratinized stratified squamous epithelium covers most of the intravaginal surface of the cervix (portio vaginalis). A simple columnar epithelium lines the endocervical canal. A transition zone between these two epithelia is usually located near the external orifice of the endocervical canal. Abnormal epithelium usually arises near or within the endocervical canal (58, 59). This localization has raised the question of whether it arises from the cells responsible for the regeneration of the endocervical epithelium. The origin of these cells has not been fully investigated (see 58). Studies of other columnar epithelia indicate that mitosis of differentiated cells may account for regeneration (reviewed by 91), but, in the cervix, a small number of less differentiated cells ("reserve cells") near the basement membrane may be responsible (59, 60).

The term "squamous metaplasia of the cervix" indicates the replacement of the simple columnar endocervical epithelium by stratified squamous epithelium, usually as a result of chronic irritation. The origin of the cells forming the metaplastic epithelium is a matter of dispute (58) but the mature metaplastic epithelium is very similar to the stratified squamous epithelium of the exocervix.

It is commonly believed that the earliest form of recognizable epithelial malignancy is characterized by malignant transformation of the epithelial cells without invasion into the underlying connective tissue (reviewed by 58, 95). This preinvasive malignant state is called carcinoma–*in situ* and is characterized by the same marked variation in nuclear size, shape, and chromatin pattern that is observed in the epithelial cells of invasive carcinoma (6, 7, 8, 16, 49; reviewed by 50). These features, called nuclear atypicalities, have been associated with chromosomal abnormalities, (9, 61, 67, 87) such as an aneuploid chromosome number which is also found in invasive carcinoma (61).

Another condition called dysplasia of the cervical epithelium is intermediate between squamous metaplasia and carcinoma–*in situ* in morphology (49, 95) and in growth properties in vitro (reviewed by 117). Carcinoma–*in situ* has been shown to arise frequently within a previously dysplastic epithelium. In dysplasia, the cells characteristically have undergone only a partial alteration of the nature and degree of cell differentiation (52, 59, 60, 95 104). Mild, moderate, and severe degrees of dysplasia are recognized depending on the amount of surface maturation and the degree of nuclear atypicality present in the epithelium. For example, those epithelia with nuclear atypicality but exhibiting distinct differentiation of a flattened superficial layer which is free of mitoses are usually classified as carcinoma–*in situ* (50, 56, 61). Because of the difficulty in making a sharp distinction between dysplasia and carcinoma–*in situ*, we have classified our precancerous specimens into two groups: (*a*) mild and moderate dysplasia, which are usually clinically benign, reversible conditions (43, 52, 95) and (*b*) severe dysplasia and carcinoma–*in situ* which are considered premalignant or early malignant conditions and are frequently clinically irreversible (59, 60, 95). The grouping together of severe dysplasia and carcinoma–*in situ* is for convenience since a purely histological distinction between severe dysplasia and carcinoma–*in situ* cannot be precisely defined in many cases (54, 95, 110).

Freeze-Cleave Interpretation

For the purposes of this study, the interpretation of freeze-cleave images can be briefly sum-

marized. Although the exact path of the cleavage through membranes is somewhat controversial (see 79), frozen plasma membranes appear to be split down a central plane thereby cleaving them into two lamellae as originally suggested by Branton (20) and demonstrated experimentally by Branton (28, 29, 31) and others (107, 111, 113). This process of membrane splitting reveals surfaces called "faces" generated from within the interior of membranes. These faces have an associated population of small 60–100 A particles which have a distribution often characteristic for a given membrane. For descriptive purposes the faces of the membrane are labeled face A and face B. Face A is the outwardly directed face of the inner half membrane and it often displays many 60–100 A particles in a rather random distribution. Face B is the inwardly directed aspect of the outer half membrane and usually has fewer associated particles. Face A and face B of the membrane are apposed to one another within the membrane before the fracturing process (111, 113). At the nexus, membranes also appear to be split (79). Nexus face A is covered with many small particles which are closely packed, often in a hexagonal array with a 90-100 A center-to-center spacing. Nexus face B has a complementary array of small pits or depressions which probably accommodate the particles before membrane splitting. These two faces of the nexus are quite distinctive, as first noted by Kreutziger (68). Nexuses can be easily distinguished from desmosomes in the cervical epithelium because the adjacent plasma membranes appear to be in contact at nexuses and the membranes are separated by approximately 300–350 A of extracellular space at desmosomes. This difference is also observed in thin sections (66, 77). The detailed substructure of the desmosome will be considered separately (McNutt and Weinstein, data in preparation), but it can be noted that both membrane faces A and B at desmosomes often have a distinctive associated fibrillar component which can be easily distinguished from the components of the nexus.

RESULTS

Thin-Section Studies

NORMAL EPITHELIUM: The stratified squamous epithelium of the exocervix can be subdivided into three zones: a basal zone, two to three cell layers in thickness, composed of cuboidal to columnar cells; an intermediate zone of large roughly spherical cells with numerous "short" projections of their surfaces (Fig. 1); and a superficial zone of flattened cells. Within each zone, the nuclei are similar in chromatin distribution, size of nucleoli, and configuration of the nuclear envelope (Fig. 1). The general electron microscope appearance of this stratified squamous epithelium in the human cervix has been previously reported (6, 7, 8, 30, 51).

Emphasis in this study is placed on the cell junctions. Three types of contact relationships are frequently observed (Fig. 2): (*a*) Close apposition, where the plasma membranes of adjacent cells are separated by a 200–300 A interspace with little apparent specialization; (*b*) Desmosomes (maculae adherentes), where the plasma membranes are separated by a 300 A interspace which contains dense proteinaceous material frequently with a central dense stratum; and (*c*) Nexuses (Fig. 3), or "gap" junctions, where the adjacent plasma membranes are in direct contact by specialized subunits which bridge a 20 A interspace. Detailed descriptions of the substructure of the nexus have recently been published (79). Occasionally a fourth type of spotlike cell-to-cell contact is found which corresponds in dimensions to a "tight" junction or macula occludens (~140 A in thickness) and which excludes lanthanum hydroxide.

Nexuses are very abundant in the intermediate zone of the normal epithelium (Figs. 2, 6). The ENF in this region is 200 ± 50 nexuses per cell expressed as an average ± the observed range. These nexuses are generally round, 0.1–0.5 μ in diameter, and occur as a form of side-to-side apposition of the finger-like processes of adjacent cell membranes. Often nexuses occur immediately adjacent to desmosomes. In the intermediate zone, the frequency of occurrence of nexuses is less than the desmosome frequency; for example, groups of cell processes may be attached by as many as four to ten times as many desmosomes as nexuses in a given section (Fig. 2).

In the basal zone of the normal epithelium, there are fewer nexuses than in the normal intermediate zone. If one considers the basal zone as two to three cells in thickness, the ENF is quite high, e.g. approximately 40 nexuses per cell (Fig. 6, I*a*). If only the lowest basal layer of cells is considered, an estimated nexus frequency of

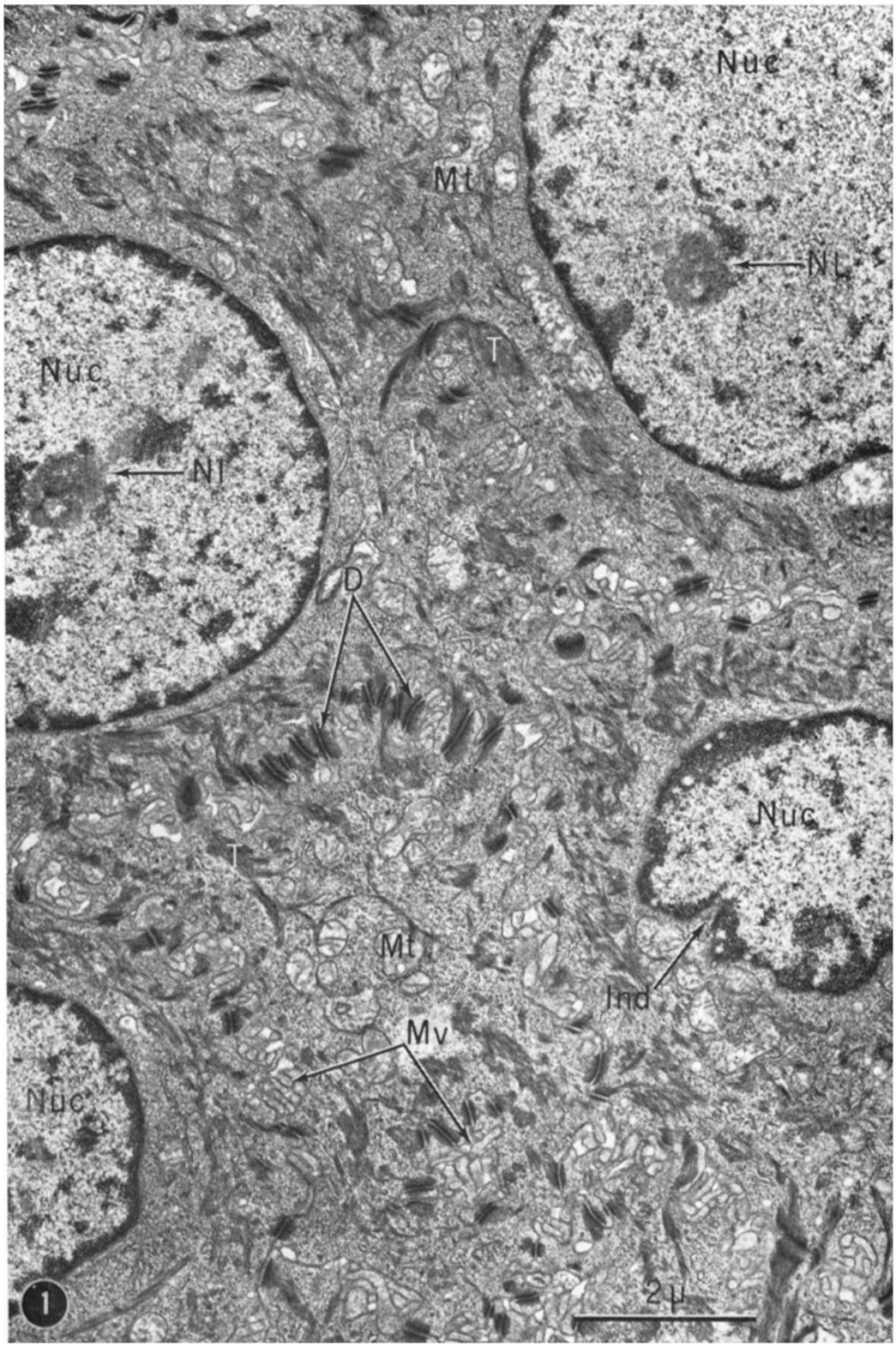


FIGURE 1 Normal human exocervix, intermediate zone at low magnification. This section includes portions of the cytoplasm of at least six cells and portions of the nucleus of four of the cells. The nuclei (*Nuc*) are quite similar in chromatin distribution. The nuclear envelope is generally smooth in outline with only an occasional indentation (*Ind*). Nucleoli (*Nl*) are small. The borders of the cells have numerous microvilli (*Mv*) which are closely apposed so that only close inspection allows distinction of approximate cytoplasmic boundaries. Many desmosomes (*D*) are present which appear here as small dense bodies. Nexuses are frequent but are not visualized at this magnification (see Fig. 2). Tonofilaments (*T*) and mitochondria (*Mt*) are abundant in these cells. × 14,000.

approximately ten nexuses per cell is calculated (Fig. 6, *Ib*). From examination of serial sections and multiple nonserial sections, the nexuses appear to be evenly distributed suggesting that all of the cells are connected to each other by nexuses. Cells in mitosis are encountered too infrequently to determine whether nexuses connect mitotic cells to their interphase neighbors, as has been observed in some other proliferating epithelia (80).

The number of nexuses is not quantitated for the superficial zone of flattened cells since this zone is lost in some abnormal epithelia such as carcinoma–*in situ* and is a nonproliferative region in normal epithelia. Nexuses are frequently observed in this region except at the desquamating surface.

The normal human endocervical epithelium is a simple columnar epithelium containing two cell types, one mucus secreting and the other ciliated. These cells are attached to each other near their apical ends by typical columnar epithelial junctional complexes as described by Farquhar and Palade (38). Nexuses are rarely observed.

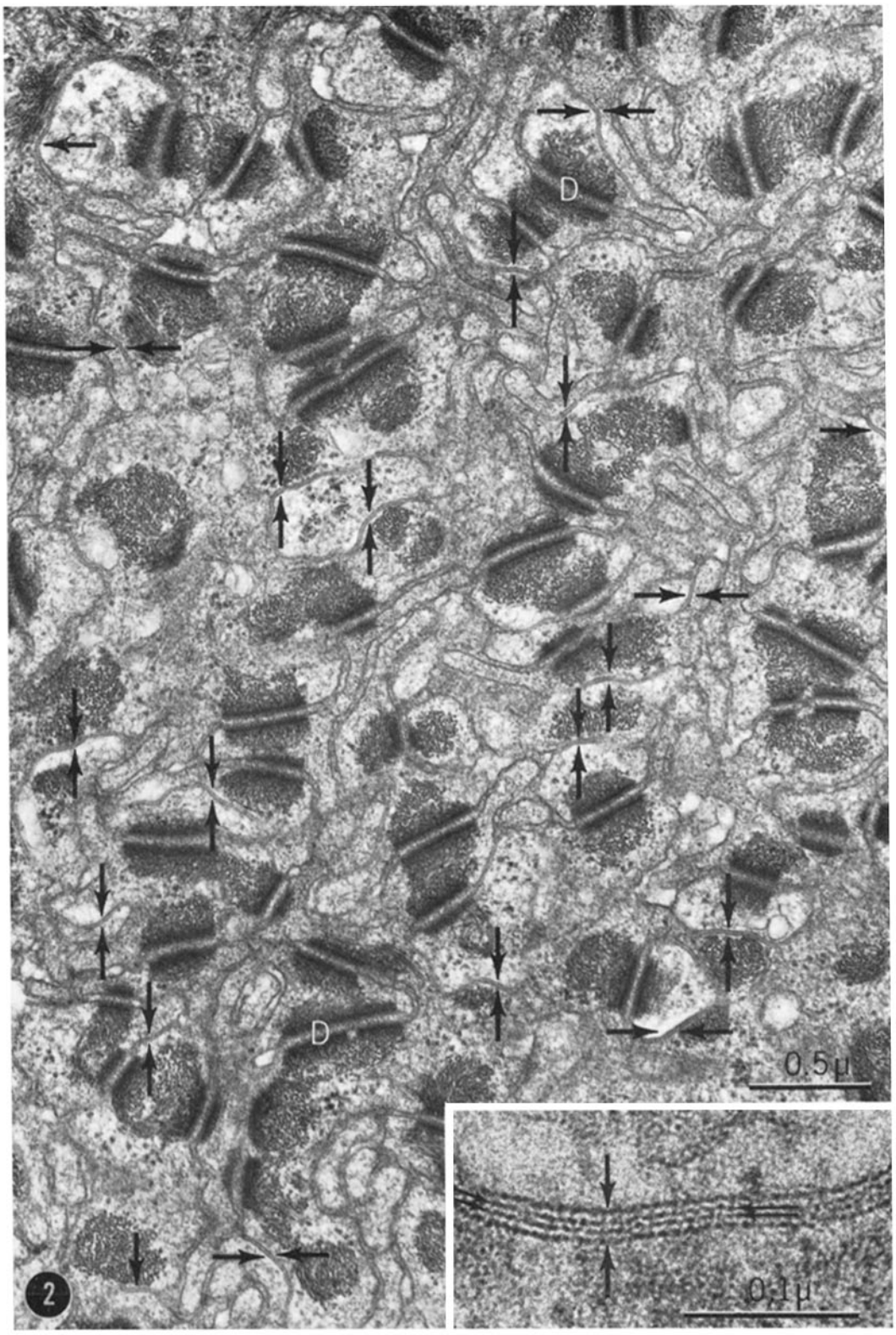
SQUAMOUS METAPLASIA: A few differences are noted between metaplastic and normal stratified squamous epithelia. The nuclear envelope in metaplastic cells has more shallow infoldings of the nuclear membranes and the nucleoli are larger than in normal cells (Fig. 4). The ENF is slightly less than in the normal epithelium but nexuses are still very abundant in metaplastic epithelium (Fig. 6). The basal zone of two to three cells in thickness has an ENF equivalent to that of the basal layer of cells in the normal epithelium. The intermediate zone of metaplastic epithelium contains as many nexuses as the normal basal zone, but contains slightly less than in the normal intermediate zone.

With respect to the origin of metaplastic cervical epithelium, it may be noteworthy that in one specimen of very early squamous metaplasia, we have observed the surface layer of cells to be composed of thin flattened cells containing a few mucus droplets and having a few blunt microvilli projecting into the lumen. While these surface cells bear a certain resemblance to columnar mucus cells, the underlying region of six to ten cells in thickness is indistinguishable from the basal and intermediate zone cells of mature squamous metaplasia. The ENF is similar (Figs. 4, 6) in early and mature squamous metaplasia.

DYSPLASIA AND CARCINOMA–IN SITU: A general description of the ultrastructure of human cervical dysplasia and carcinoma–*in situ* has recently been published (101, 118). In these epithelia, the nuclei exhibit striking variation in size, shape, and chromatin distribution (Fig. 5). The cell surface has numerous microvillous processes. Fewer desmosomes are present than in the corresponding zone of either normal or metaplastic epithelia. Close cell-to-cell apposition is variably preserved in these abnormal epithelia but areas of the specimen where close apposition is preserved often have the best cytoplasmic fixation. In some specimens, the separation of the cells is accompanied by an accumulation of flocculent proteinaceous material in the intercellular space. This material is easily extracted particularly near the surface of tissue blocks with the method of specimen preparation used for this study.

Counts of nexuses (Fig. 6) reveal a decrease in nexuses in the intermediate zone of all the dysplastic epithelia and carcinoma–*in situ* compared to the intermediate zone of normal or squamous

FIGURE 2 Normal exocervix, intermediate zone. This thin section passes just through the zone of contact between the microvilli of several cells so that the interdigitating processes appear approximately in transverse section and thus each process is represented by an insular profile. The microvilli are closely apposed with 200–300 A of intervening extracellular space except at the desmosomes (*D*) and at nexuses (single and apposed arrows). The dense plaques and associated cytoplasmic filaments make the desmosomes prominent. However nexuses are also very frequent although less conspicuous, as indicated by 20 nexuses in transverse section or near transverse section in this area corresponding to $20\mu^2$. × 38,000. *Insert,* High magnification of a nexus fixed and stained to reveal the seven layered appearance sometimes called a gap junction. The over-all thickness, i.e. approximately the distance between the apposed arrows, is 190 A and the central lucent zone (marked by double small arrows) is 20–30 A in width. These features serve to distinguish the nexus from the tight junction or macula occludens. (see text). × 275,000.


D
D
0.5μ
0.1μ
2

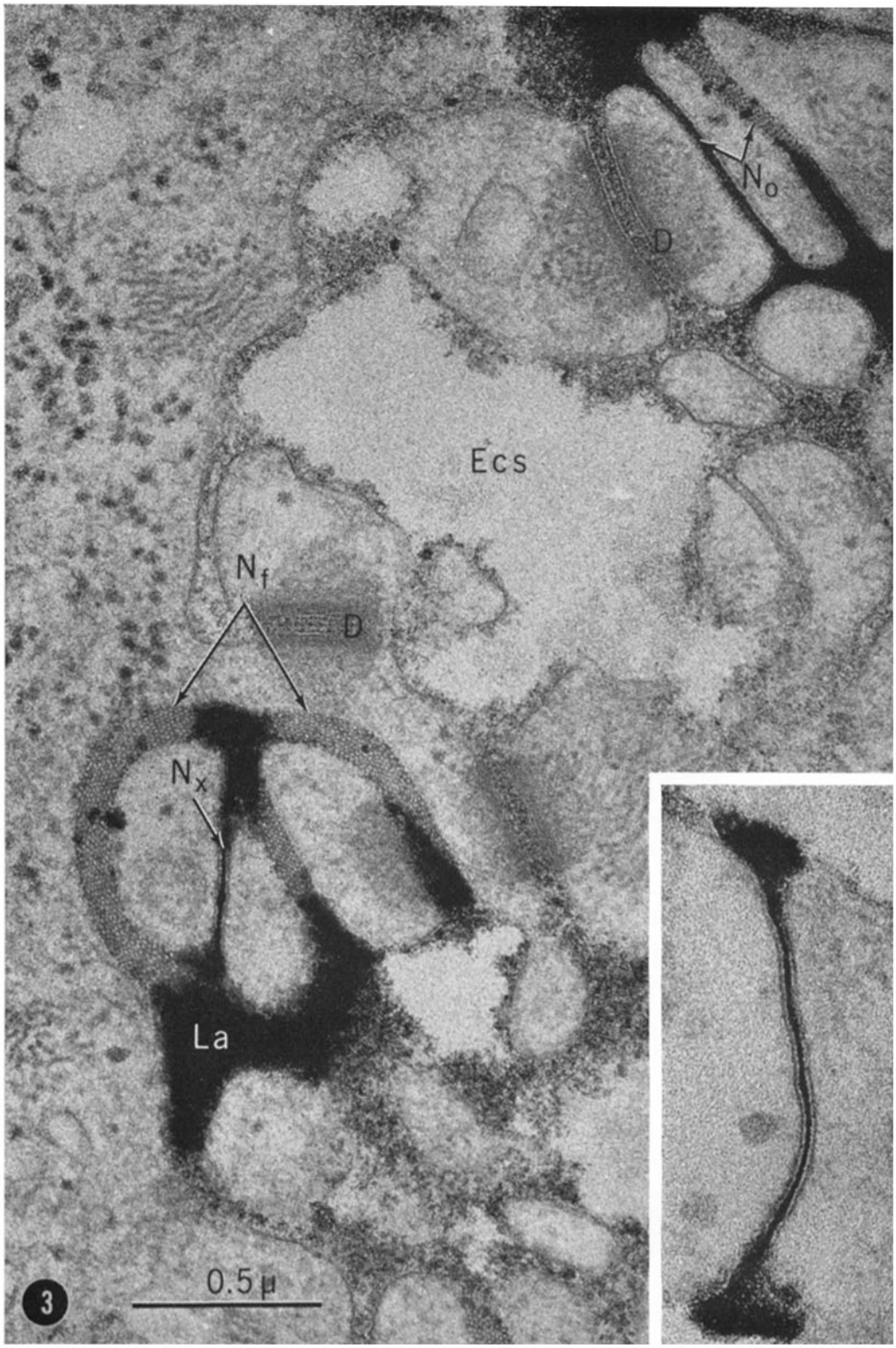


FIGURE 3 Normal exocervix, intermediate zone. Colloidal lanthanum hydroxide (*La*) has produced the very electron-opaque precipitate in the extracellular space (*Ecs*) between the processes of several epithelial cells. The lanthanum precipitate tends to be loosely adherent to the surfaces of the cells and is trapped in the central region of the nexus. Several nexuses are visible in transverse (*Nx*), oblique (*No*), and *en face* (*Nf*) views. Precipitate is also loosely trapped in desmosomes (*D*). × 59,000. *Insert*, High magnification of a rare nexus in invasive squamous carcinoma showing the lanthanum penetration of the central region of the nexus. × 137,000.

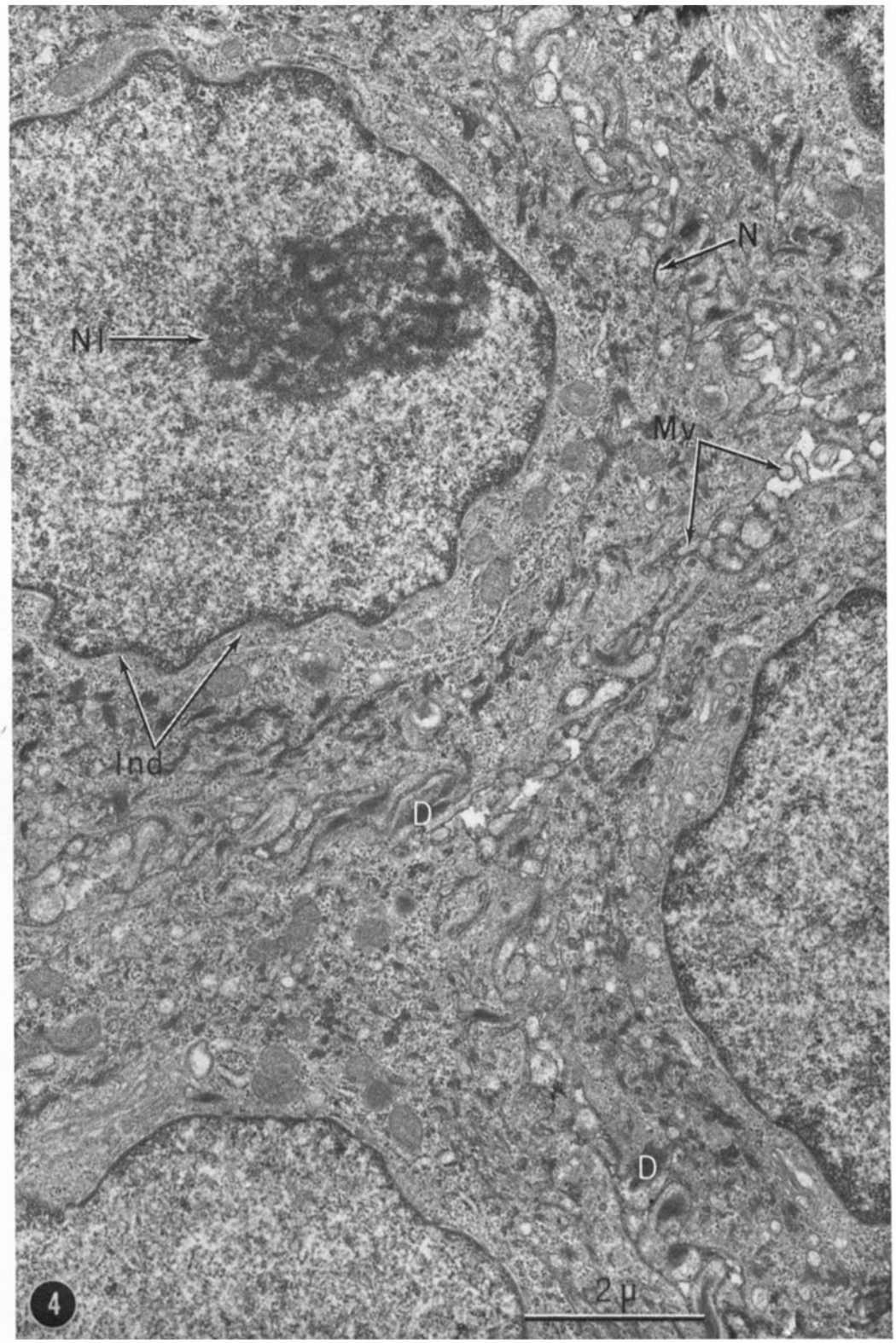


FIGURE 4 Squamous metaplasia, intermediate zone at low magnification. As in the normal epithelium, the distribution of chromatin is similar in the nuclei of adjacent cells; but, in contrast to the normal, shallow indentations (*Ind*) are present in the nuclear envelope and the nucleoli (*Nl*) are prominent. At the cell periphery, microvilli (*Mv*) are numerous and are usually closely apposed to one another. There are fewer desmosomes (*D*) and nexuses (*N*) than in the normal epithelium. Lanthanum hydroxide trapped in a nexus (*N*) can be seen at this low magnification × 14,000.

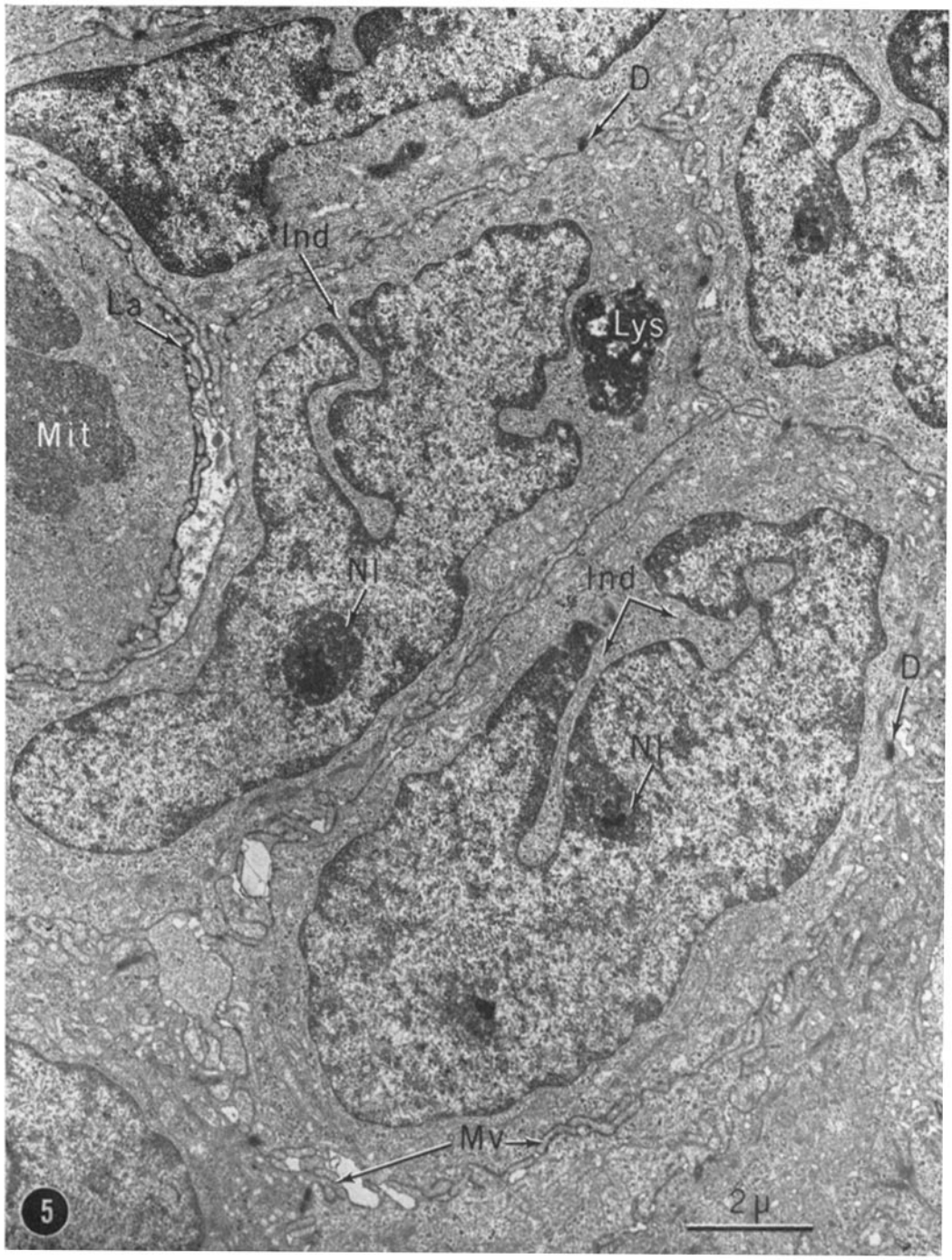


FIGURE 5 Severe dysplasia to carcinoma–*in situ*, intermediate region at low magnification. Although the chromatin distribution within the nuclei appears similar in the adjacent cells, the nuclear envelope is deeply indented (*Ind*) and the nucleoli (*Nl*) are prominent. Mitoses (*Mit*) are frequent, occasionally abnormal, and present in the upper region of the epithelium. Microvilli (*Mv*) are not strikingly reduced in number and close apposition of the cells is maintained in this specimen. Desmosomes (*D*) are small and infrequent. No nexuses have been found in this particular specimen although others in the same category had regions of up to four nexuses per cell (see text). Electron-opaque lanthanum (*La*) is present in the extracellular space and is particularly dense near the mitotic figure. A large lysosome (*Lys*) is also present. × 9100.

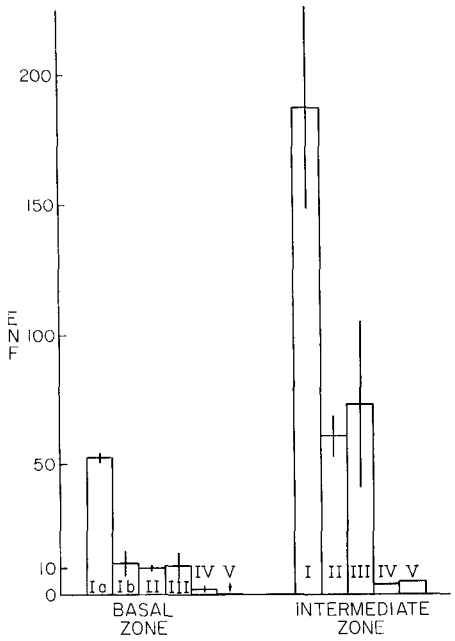


FIGURE 6 Estimated nexus frequency in the basal and intermediate zones of each of the categories studied. Roman numeral I indicates normal epithelium, II squamous metaplasia, III mild and moderate dysplasia, IV severe dysplasia and carcinoma–*in situ*, and V invasive squamous carcinoma. Single lines indicate the range of one standard deviation above and below the charted value. The basal zone is considered to be two to three cell layers in thickness in each of the epithelia. The value for the normal basal zone is given as I*a* and the value for the most basal single layer of cells is given as I*b*. The representative ENF values of IV and V are given under the category of basal zone. In the intermediate zone, the values given for I, II, and III are representative; but, for the IV and V categories, the maximum observed ENF values are used since there is no cytological zonation in these latter epithelia. From these data it can be seen that nexuses are very frequent in the normal intermediate zone, less frequent in the normal basal zone (I*a*), and even less frequent in the normal basal single layer of cells (I*b*). There is no significant difference between the nexus frequency (ENF) in the region of most active proliferation in the normal (I*b*), squamous metaplasia (II), and mild and moderate dysplasia (III). Nexus frequency drops in the basal or proliferative region of the severe dysplasia and carcinoma–*in situ* and is frequently less than one nexus per cell in the invasive carcinoma. In the intermediate zone, the nexus frequency is diminished in squamous metaplasia (II) and mild to moderate dysplasia (III), which might be explained by a retention of proliferative activity into higher layers of the epithelium. The maximum nexus frequency found in the malignant epithelia (IV and V) is similar to the nexus frequency observed in the normal basal layer of cells (I*b*). The nexus frequency in the basal or proliferative region is decreased in the malignant epithelia (IV and V) and in the intermediate or differentiated zone as well. The most important comparison is between the regions of benign rapid growth (I*b*, II, and III, basal zones) and the regions of malignant rapid growth (IV and V, basal zones).

metaplasia. In the basal zone of mild and moderate dysplasia, the ENF is approximately the same as in the normal basal layer of cells, i.e. approximately ten nexuses per cell (Fig. 6). In severe dysplasia and carcinoma–*in situ*, the number of nexuses is decreased throughout the epithelium averaging approximately two nexuses per cell. In one specimen of carcinoma–*in situ* (Fig. 5), the regions studied have no detectable nexuses. Serial sections of 150 cells of this specimen reveal no nexuses in 40 sections, each 1000 A in thickness with an average cell diameter of 13 μ. Another specimen in the severe dysplasia–carcinoma–*in situ* category has regions with up to four nexuses per cell.

INVASIVE SQUAMOUS CARCINOMA: The striking decrease in the ENF in invasive squamous carcinoma of the human cervix has been briefly reported previously (78). The results of that study are summarized here for the sake of comparison to the preinvasive malignant conditions of the cervical epithelium. The majority of the tumor cells resemble cells seen in the basal and intermediate zones of the severe dysplasia and carcinoma–*in situ* (Fig. 5). There is often marked variation in the configuration of the nuclear envelope and in the chromatin pattern within the nuclei, (6, 7, 8, 16, 97), but not all cells within the tumor clearly exhibit these very abnormal nuclei. Areas of close apposition between tumor cell surfaces are rare and are mainly in the well differentiated regions of the tumor. Frequently the intercellular spaces are 0.2–0.5 μ in width and contain a flocculent proteinaceous material which is readily extracted by the preparative procedures used. Due to the presence of dying cells in the population of carcinoma cells, some of the flocculent intercellular material may be derived both from the remnants of dead cells and the connective tissue invaded by the carcinoma. The size of the extracellular compartment may be somewhat artefactually enlarged during

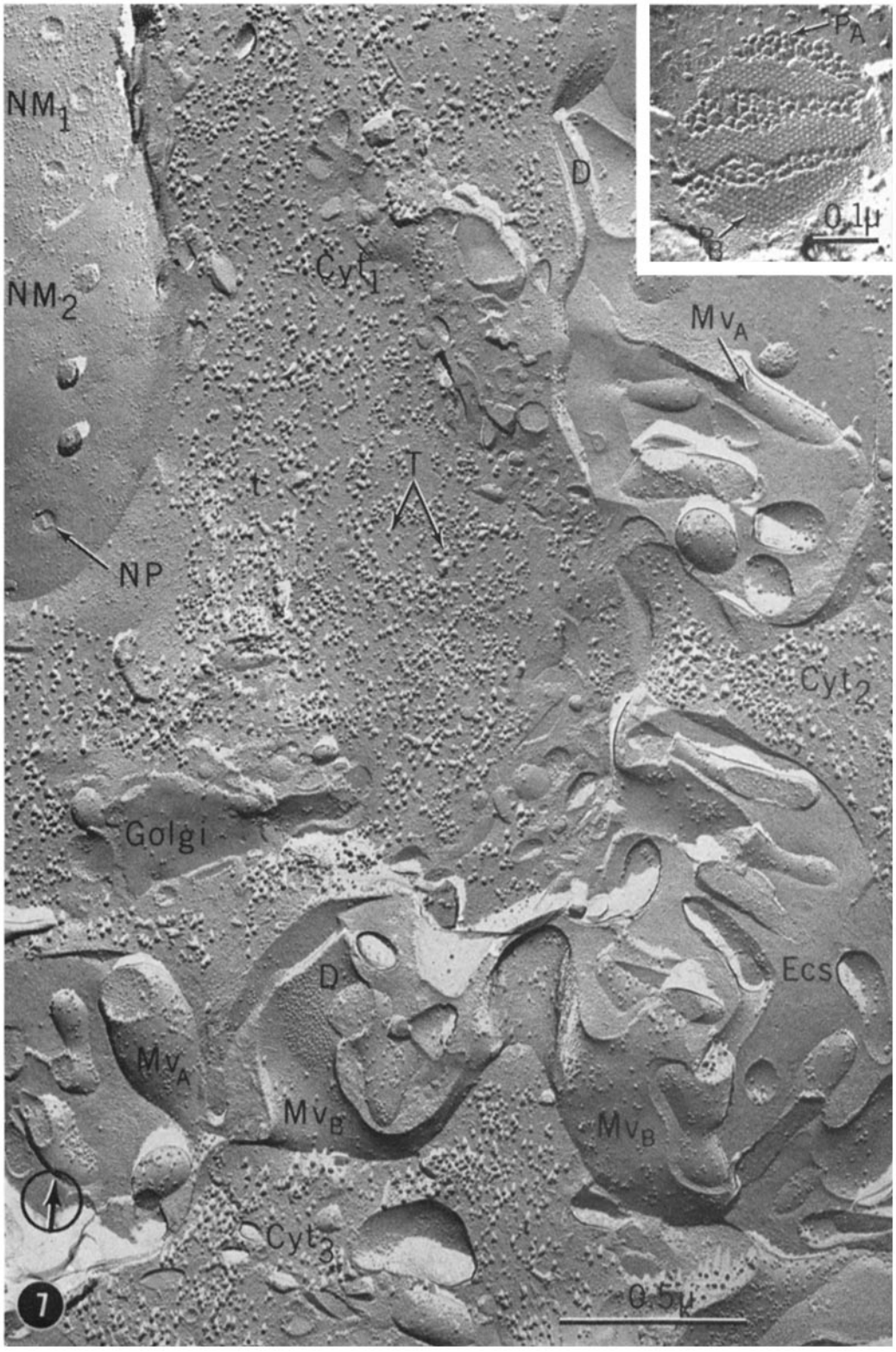
specimen preparation because of the decreased interadhesivity of the cells (25, 26). Desmosomes are generally decreased in number between invasive carcinoma cells but, in the best differentiated regions, many desmosomes with a normal substructure can be found (see also 101).

Counts of the number of nexuses in poorly differentiated regions of carcinoma frequently reveal areas with less than one nexus per cell. A few nexuses are present in well differentiated areas of tumor (Fig. 6) and up to four nexuses per cell are found in some areas. The tumor nexuses contain a subunit array similar to that in normal nexuses (78). No true tight junctions (zonulae or maculae occludentes) are observed between the squamous carcinoma cells.

COMPARISON OF ENF DATA: The ENF values for selected zones in each of the types of epithelia are compared in Fig. 6. The ENF counts for the basal zones are separated from those of the intermediate zones since basal zone values clearly represent the ENF for a proliferating portion of the epithelium, whereas intermediate zone values usually represent a region of greater differentiation. For the normal epithelium, basal zone ENF is expressed as the basal two to three cell layers (Fig. 6, I*a*). Since there is a controversy about how many layers of basal cells are capable of proliferation in the normal epithelium (reviewed by 69, 48), the contribution of the basal single layer of cells to this ENF is noted (Fig. 6, I*b*). The basal zone of squamous metaplasia (Fig. 6, II) and mild to moderate dysplasia (Fig. 6, III) are also considered as two to three cells in thickness since it is known that proliferative activity extends higher in these epithelia than in the normal (93). Therefore the nexus frequency in the most rapidly proliferating regions of all these benign epithelia (Fig. 6, I*b* versus II and III in basal zones) averages approximately ten nexuses per cell with some specimens having a range from five to 15 nexuses per cell. In the category of severe dysplasia—carcinoma-*in situ*, proliferative activity has been shown to occur throughout the epithelium (93). Consequently, the representative nexus frequency for the whole premalignant epithelium is grouped with the basal zone values of the benign epithelia. The representative premalignant ENF averages approximately two nexuses per cell, with a range from less than one to as many as four nexuses per cell (Fig. 6, IV basal zone). In invasive carcinoma, the representative ENF less than one is based on the observation that many poorly differentiated areas appear to have less than one nexus per cell (Fig. 6, V, basal zone). Only in the premalignant and malignant epithelia does the ENF fall below five per cell in the proliferating region (Fig. 6, IV, V, basal zones). It is also important to note that the same deficiency of nexuses observed in invasive carcinoma can be found in preinvasive malignancy as well.

By charting the ENF in the intermediate zones, it is possible to compare the ENF in a region of greater cellular differentiation than occurs in the basal zone for some of the epithelia (Fig. 6). There is a marked decrease in ENF from the normal intermediate zone value of approxi-

FIGURE 7 Normal human cervical epithelium, low intermediate to basal zone as seen in a freeze-cleave replica of unfixed tissue. At upper left, the cleavage plane has split portions of each membrane (NM_1 and NM_2) of the nuclear envelope, revealing nuclear pores (*NP*). In the other areas, the cleavage reveals portions of the cytoplasm of three cells (Cyt_1, Cyt_2, Cyt_3), their finger-like processes, and intervening extracellular space (*Ecs*). In the cytoplasm, cross-fractured tonofilaments (*T*) are abundant. Portions of several membranes of a Golgi complex are also shown. At the cell periphery, numerous finger-like and blunt microvilli are exposed. The plasma membrane fracture face directed toward the cytoplasm (i.e. face B labeled Mv_B) has a clustering of particles at a desmosome (*D*). The other aspect of split plasma membrane is directed toward the exterior (i.e. face A, labeled Mv_A) and it has a few scattered particles but none clustered at desmosomes in these unfixed preparations. In chemically-fixed preparations, desmosomal fracture faces contain fine fibrils. The membranes at desmosomes are separated by a 300–350 A interspace. × 58,000. *Insert,* Nexus in normal cervical epithelium at higher magnification. The cleavage plane has revealed two faces: face A covered with closely packed particles (P_A); and face B having a similar array of small pits or depressions (P_B). In this unfixed preparation, the nexus membranes have split in an irregular steplike fashion. A nexus can be distinguished from a desmosome in such unfixed preparations by at least two criteria: at nexuses, the membranes appear in contact and it is membrane face A which has particles; at desmosomes, the membranes are separated by a 300 A interspace and it is membrane face B which has particles. Encircled arrow indicates direction of platinum shadowing. × 99,000.


NM1
NM2
NP
Cyt1
D
MvA
Cyt2
Golgi
D
Ecs
MvA
MvB
MvB
Cyt3
0.5μ
7
PA
PB
0.1μ

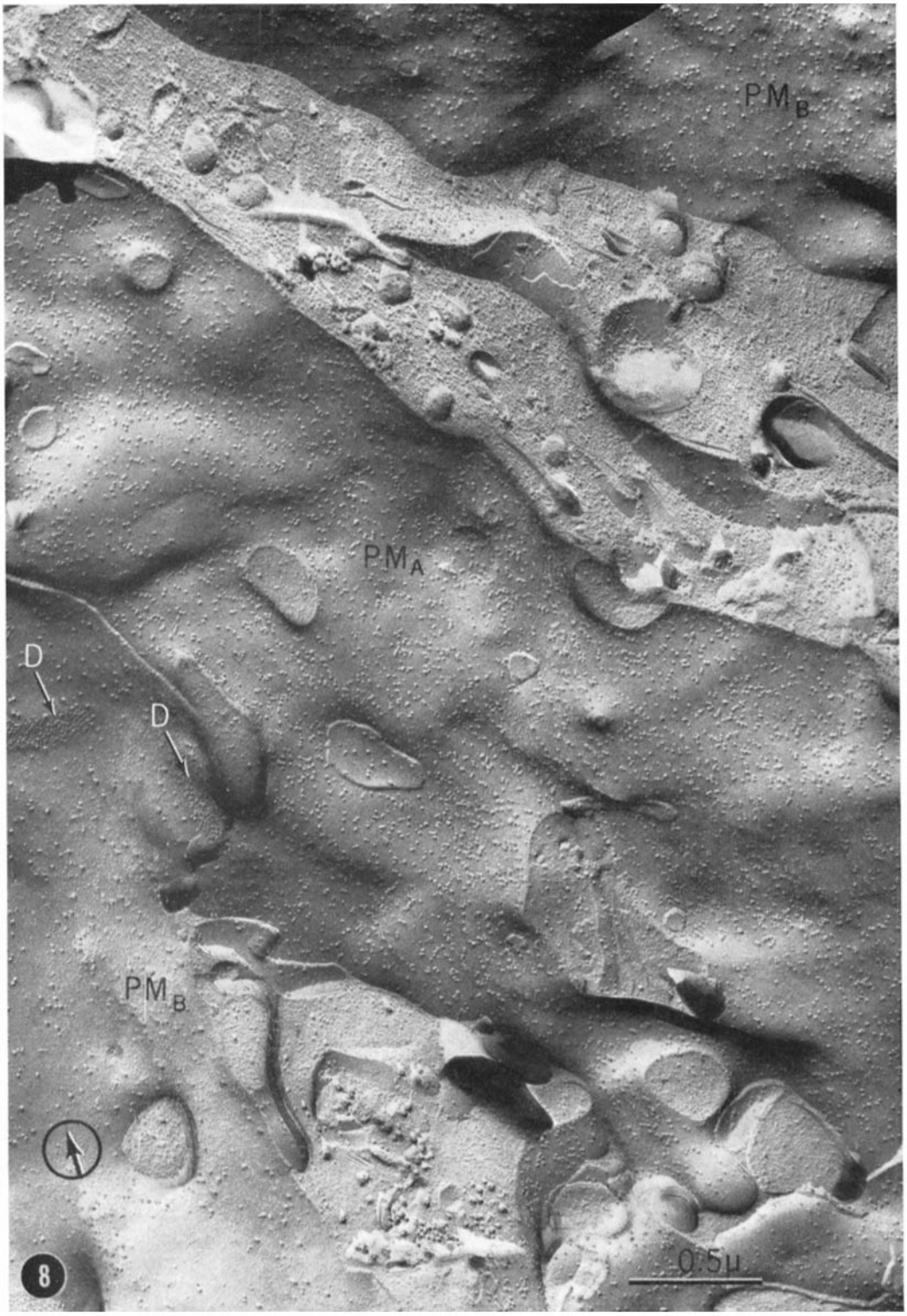


FIGURE 8 Invasive squamous carcinoma of cervix, in freeze-cleave replica of unfixed tissue. Large areas of plasma membrane face A (PM_A) and face B (PM_B) belonging to malignant cells are exposed in this cleavage. Regions suggestive of desmosome (D) membrane are present. The small 60–120 A particle component is not distributed in a strikingly different way than in the normal epithelium. However, no regions characteristic of cleaved nexuses are identified in the replicas, indicating that large nexuses are rare. Very small nexuses composed of only one or several subunits cannot be excluded by this technique. Encircled arrow indicates direction of platinum shadowing. × 41,000.

mately 180 nexuses per cell down to the ENF for squamous metaplasia and mild and moderate dysplasia, approximately 60–70 nexuses per cell (Fig. 6, I, II, III, intermediate zones). Such a diminution may be explained on the retention of proliferative activity into the higher regions of the abnormal benign epithelia (93); but nexuses in all of these benign epithelia are very abundant. Concerning the intermediate zone values for severe dysplasia—carcinoma-*in situ* and for invasive carcinoma (Fig. 6, IV and V for intermediate zone), the charted values are the ENF in those regions which had the maximum number of nexuses observed. There is no significant difference in the maximum number of nexuses in premalignant and malignant epithelia averaging approximately five nexuses per cell compared to the minimum number observed in the normal basal single layer of cells (Fig. 6, I*b*). This comparison should not obscure the fact that the malignant and premalignant epithelia have a severely decreased over-all number of nexuses compared to the benign epithelia and only in the malignant and premalignant epithelia are there regions with less than one nexus per cell.

FREEZE-CLEAVE STUDIES: Replicas of freeze-cleaved specimens of normal exocervix stratified squamous epithelium corroborate many of the features observed in standard thin sections (Fig. 7). When the tissue is prepared without heat-etching, the two main features revealed in normal cervical epithelial cells are cross fractured tonofilaments and membranous structures. The plasma membranes of cells are often revealed in dramatic three dimensional relief (Figs. 7, 8). The split membranes at the nexus are easily recognized since they exhibit either closely packed arrays of particles on membrane face A or else similar arrays of pits on membrane face B. Often the arrays are hexagonal with a 90–100 A center-to-center spacing. A detailed correlation of thin section and freeze-cleave images of plasma membranes at the nexus has been previously published (79). General close apposition of the cell membranes with a 200 A interspace is variably preserved with the method of specimen preparation used.

Although nexuses are easily demonstrated in normal freeze-cleaved cervical epithelium (Fig. 7), replicas of invasive squamous carcinoma cells confirm that nexuses are quite rare in these specimens, prepared by a technique very different from thin sections. No nexuses have yet been observed in replicas of freeze-cleaved carcinoma cell membranes despite the fact that large areas of split plasma membranes are revealed by cleaving (Fig. 8). The freeze-cleave technique cannot exclude the possibility that extremely small nexuses, composed of only several subunits, might be present. However, the deficiency of nexuses in freeze-cleaved squamous carcinoma cell membranes does eliminate the possibility that the deficiency of nexuses observed in thin sections is the result of nexuses being "pulled apart" during preparation for thin sections. When two apposed membranes forming a nexus are pulled apart by hypertonic, Ca^{++} containing solutions e.g. in cardiac muscle held at a slightly stretched length, (10, 11), the distinctive substructure within each individual membrane at the nexus can still be identified in replicas, although the individual nexus membranes resemble nonjunctional membranes in standard thin sections (McNutt, unpublished observations).

The freeze-cleave technique is a less sensitive method for estimating the frequency of occurrence of nexuses compared to thin sections because of the inability to accurately control the cleavage plane, the inability to perform serial cleavages on the same specimen, and the difficulty in visualizing nexus cross-fractures without heat etching (see 79).

DISCUSSION

This study demonstrates that the deficiency of nexuses in invasive squamous carcinomas of the cervix (78) also can occur in noninvasive malignant conditions of the cervix. It is possibly important that, when the whole epithelium is considered, the decrease in nexuses correlates with the severity of the morphological alteration of the dysplastic epithelium. This decrease in nexuses might be related to an increase in the proportion of proliferating cells in the epithelium. For example, an apparent diminution from 200 nexuses per cell down to ten nexuses per cell can occur as the result of a comparison of an area of differentiation (i.e. the normal intermediate zone) to an area of proliferation (i.e. the normal basal layer of cells). An increase in number of proliferating cells is the most likely explanation for the observed decrease in nexuses in the intermediate zone of mild and moderate dysplasia (ENF = 50)

compared to the number of nexuses in the normal intermediate zone (ENF = 200). This explanation is supported by the fact that cells in the intermediate zone of dysplastic epithelia have been shown to retain proliferative activity as shown by tritiated thymidine uptake in vitro (93). However, the loss of nexuses (ENF < 1) in preinvasive and invasive malignant epithelia is not easily explained away on the basis of proliferative activity alone because in vitro studies suggest that carcinoma-*in situ* and dysplasia do not have a growth rate significantly different from normal squamous epithelium (81, 94, 117) and this loss does not appear in the basal zone of benign epithelia. Any hypothetical relationship between the number of nexuses and cell proliferation must take into account the fact that some rapidly proliferating normal epithelia (e.g. ovarian granulosa cells) do not necessarily lose their nexuses (80; Merk et al., in preparation). Therefore there is no simple constant relation between a deficiency of nexuses and proliferative activity which can be applied to all types of epithelia in benign states.

The data in this study on severe dysplasia and carcinoma-*in situ* also show that a focal loss of nexuses (ENF < 1) is not necessarily associated with invasion, because preinvasive malignant epithelia may precede, by many cell generations, the development of invasive carcinoma (95). Difficulty is encountered in relating any given surface property of a malignant cell to invasion of the tumor mass into the adjacent tissue. This difficulty arises because it is not entirely clear whether invasion is the result of a generalized abnormality of malignant cell surfaces or the result of a focal recurrent abnormality in the population of proliferating cells at the edge of a tumor mass (70). It is important to realize that in a malignant tumor in vivo, not all of the malignant cells are growing (14, 53) or are capable of growth on transplantation (88). Therefore emphasis should be placed on abnormalities found in the population of growing tumor cells compared to the population of growing normal cells. The observation of a focal severe deficiency of nexuses in the tumors in this study appears to represent such an abnormality found near the margins of the tumor where many growing tumor cells are present. Despite this observation, it must be recognized that some cells within a malignant epithelium can produce nexuses (74, 78; cf. 5). Also some types of minimally invasive and very slowly growing tumors, e.g. basal cell carcinomas of the skin, have abundant nexuses (McNutt et al. in preparation). Since malignant tumors vary widely in their pattern and degree of invasion, there is no a priori reason to expect that all tumor invasion in vivo will be easily ascribed to a single property of malignant cells. Consequently, a demonstration that some types of minimally invasive malignant tumors contain abundant nexuses does not exclude the possibility that a deficiency of nexuses may be important in eventually permitting the diffusely infiltrating type of invasion (27) often found in squamous carcinoma of the cervix.

Another important question is whether the observed diminution in number of nexuses simply represents the loss of a highly differentiated cellular function which bears no direct relationship to any regulatory process. At present, no answer is available for this question because the functions of the nexus in normal epithelia are not clearly defined. Several observations point to the possibility that nexuses are important structures. Nexuses occur early in embryogenesis when many morphological patterns are being established (98, 99, 108). In the developing mammalian heart, other specialized intercellular junctions form in spatial proximity to nexuses (77). Since nexuses can pass small electrolytes and substances up to a molecular weight of 500 (13, 85) directly from cell to cell without significant leakage into the extracellular space, it is also reasonable that nexuses may pass low molecular weight substances which could potently affect reactions at the cell surface responsible for the differentiation of the cell surface. There is also the possibility that the passage of low molecular weight substances may be important in growth regulation since cytoplasmic factors seem to be important in the timing and regulation of mitosis (reviewed by 33, 90).

This study provides some evidence for a focal abnormality in nexus formation in preinvasive and invasive malignant squamous epithelia since, at the edge of the growing malignant epithelial tissue, the ENF is less than one. Because nexuses electrically couple adjacent cells by allowing small ion passage from cell to cell, our results suggest that it may be useful for electrophysiologists to examine the electrical properties of the epithelial cells in benign abnormalities versus

preinvasive and invasive malignant conditions of the human cervix. Data do exist on the electrical properties of other types of epithelial malignancies compared to their normal epithelial counterparts. A deficiency of electrical coupling has been reported in several carcinomas in vivo and between carcinoma cells in vitro (19, 57, 71, 72). In contrast, electrical coupling has been reported to be present between connective tissue tumor cells (sarcomas) in vivo (100) and in vitro (44, 89). Of particular interest is the study recently published by Sheridan (100) who found that both malignant epithelial and connective tissue cells grown in vivo can exhibit electrical coupling between component malignant cells. However, he also notes that he found cells within the tumors which were not electrically coupled to each other although he attributed this finding to the possibility that mechanical trauma during impalement with microelectrodes caused loss of coupling. Alternatively, Sheridan's evidence of both electrical coupling and lack of electrical coupling within solid tumors possibly could be correlated with local variations in the number of nexuses (78, 100). However such a conclusion cannot be reached directly on the basis of available evidence since: (*a*) the functional competence of nexuses cannot yet be judged with certainty on the basis of their electron microscope appearance; (*b*) the same tumor material has not been examined both morphologically and electrophysiologically; (*c*) the nexus has not been shown to be the only type of intercellular junction which can produce electrical coupling (55, 98, 108); and (*d*) there is no information on the minimum size of a nexus which can produce electrical coupling. A few extremely small nexuses composed of only one or several subunits may escape morphological detection but may be capable of providing for electrical coupling.

Finally, the nexus is only one of several types of cell-to-cell junctions responsible for cell-to-cell adhesion (82, 83). In the cervix, both desmosomes and nexuses appear important for strong cell-to-cell adhesion. It is possible that the sum of strengths of attachment by nexuses and desmosomes may produce an important quantitative balance between cohesion of like cells and adhesion of cells to surrounding tissues. Such a balance has been postulated by Steinberg (103) to have a profound influence on the spatial relationship of tissues (103). The deficiency of nexuses observed in this study, as well as decreased numbers of desmosomes, appear to be factors responsible for the observed decrease in adhesion between carcinoma cells (2, 17, 25, 76, 119; see also 32, 45, 75). A loss of cell-to-cell "connections" was postulated by Cowdry in 1940 (27) to be a "necessary prelude" for the diffusely infiltrating type of invasion frequently exhibited by squamous carcinomas.

The authors gratefully acknowledge the cooperation of Dr. Howard Ulfelder and the help of Dr. Melvin Platt, and the resident staff of the Department of Gynecology, Massachusetts General Hospital, in providing specimens for this study. We also thank Dr. Lorna D. Johnson and Dr. Charles L. Easterday for donating several important specimens from the Boston Hospital for Women. The technical assistance of Mrs. Leonida Ivanetich, Miss Joyce Jakobsen, and Mr. Frederick B. Merk is appreciated.

This work was supported in part by grant No. CA-07368 from the National Cancer Institute of the National Institutes of Health, United States Public Health Service, as well as by a grant from the King Trust Foundation.

Received for publication 7 April 1971, and in revised form 27 July 1971.

REFERENCES

1. Abercrombie, M., and E. J. Ambrose. 1958. *Exp. Cell Res.* **15**:332.
2. Abercrombie, M., and E. J. Ambrose. 1962. *Cancer Res.* **22**:525.
3. Abercrombie, M., and E. M. Heaysman. 1954. *Exp. Cell Res.* **6**:293.
4. Abercrombie, M., E. M. Heaysman, and H. H. Karthauser. 1957. *Exp. Cell Res.* **13**:276.
5. Anderson, H. C., B. Kim, and S. Minkowitz. 1969. *Cancer.* **24**:585.
6. Ashworth, C. T., F. J. Luibel, and E. Sanders. 1960. *Amer. J. Clin. Pathol.* **34**:9.
7. Ashworth, C. T., F. J. Luibel, and E. Sanders. 1960. *Amer. J. Obstet. Gynecol.* **79**:1149.
8. Ashworth, C. T., V. A. Stembridge, and F. J. Luibel. 1961. *Acta Cytol.* **5**:369.
9. Atkin, N. B. 1969. *Obstet. Gynecol. Survey.* **24**:794.
10. Barr, L., M. M. Dewey, and W. Berger. 1965. *J. Gen. Physiol.* **48**:797.
11. Barr, L., W. Berger, and M. M. Dewey. 1968. *J. Gen. Physiol.* **51**:347.
12. Benedetti, E. L., and P. Emmelot. 1968. *J. Cell Biol.* **38**:15.
13. Bennett, M. V. L., and P. B. Dunham. 1970. *Biophys. Soc. Annu. Meet. Abstr.* **10**:114 *a.*

14. BENNINGTON, J. L. 1969. *Cancer Res.* **29**:1082.
15. BERGMAN, R. A. 1968. *J. Cell Biol.* **36**:639 .
16. BERNHARD, W. 1963. *Progr. Exp. Tumor Res.* **3**:1.
17. BERWICK, L., and D. R. COMAN. 1962. *Cancer Res.* **22**:982.
18. BLOOM, W., and D. W. FAWCETT. 1968. A Textbook of Histology. W. B. Saunders Company, Philadelphia. 9th edition. 728.
19. BOREK, C., S. HIGASHINO, and W. R. LOEWENSTEIN. 1969. *J. Membrane Biol.* **1**:274.
20. BRANTON, D. 1966. *Proc. Nat. Acad. Sci.* U.S.A. **55**:1048.
21. BRIGHTMAN, M. W., and T. S. REESE. 1969. *J. Cell Biol.* **40**:648.
22. BULLIVANT, S. 1969. *Micron.* **1**:46.
23. BULLIVANT, S., and A. AMES, III. 1966. *J. Cell Biol.* **29**:435.
24. COBB, J. L. S., and T. BENNETT. 1969. *J. Cell Biol.* **41**:287.
25. COMAN, D. P. 1944. *Cancer Res.* **4**:625.
26. COMAN, D. R. 1961. *Cancer Res.* **21**:1436.
27. COWDRY, E. V. 1940. *Arch. Pathol.* **30**:1245.
28. DA SILVA, P. P., and D. BRANTON. 1969. XI. Int. Bot. Congr. Abstr. 171.
29. DA SILVA, P. P., and D. BRANTON. 1970. *J. Cell Biol.* **45**:598.
30. DAVIES, J., and R. B. WOOLF. 1963. *Clin. Obstet. Gynecol.* **6**:265.
31. DEAMER, D. W., and D. BRANTON. 1967. *Science (Washington).* **158**:655.
32. DEFENDI, V., and G. GASIC. 1963. *J. Cell Comp. Physiol.* **62**:23.
33. DE TERRA, N. 1969. *Int. Rev. Cytol.* **25**:1.
34. DEWEY, M. M., and L. BARR. 1962. *Science (Washington).* **137**:670.
35. DEWEY, M. M., and L. BARR. 1964. 1964. *J. Cell Biol.* **23**:553.
36. DREIFUSS, J. J., L. GIRARDIER, and W. G. FORSSMANN. 1966. *Pflugers Arch. Gesamte Physiol. Menschen Tiere.* **291**:13.
37. EMMELOTT, P., and E. L. BENEDETTI. 1967. Carcinogenesis: A Broad Critique. M. D. Anderson Hospital Symposium. R. W. Comley, editor. The Williams & Wilkins Co., Baltimore, Md. 471.
38. FARQUHAR, M. G., and G. E. PALADE. 1963. *J. Cell Biol.* **17**:375.
39. FARQUHAR, M. G., and G. E. PALADE. 1964. *Proc. Nat. Acad. Sci. U.S.A.* **51**:569.
40. FARQUHAR, M. G., and G. E. PALADE. 1965. *J. Cell Biol.* **26**:263.
41. FAWCETT, D. W. 1966. The Cell: its organelles and inclusions. W. B. Saunders Company, Philadelphia, Pa. 365.
42. FAWCETT, D. W., and N. S. MCNUTT. 1969. *J. Cell Biol.* **42**:1.
43. FOX, C. H. 1967. *Amer. J. Obstet. Gynecol.* **99**:960.
44. FURSHPAN, E. J., and D. D. POTTER. 1968. *Curr. Top. Develop. Biol.* **3**:95.
45. GASIC, C., and GASIC, T. 1962. *Nature (London).* **196**:170.
46. GOODENOUGH, D. A., and J-P. REVEL. 1969. *J. Cell Biol.* **43** (2, Pt. 2):44 *a.* (Abstr.)
47. GOODENOUGH, D. A., and J-P. Revel. 1970. *J. Cell Biol.* **45**:272.
48. GOSS, R. J. 1970. Advances in Cell Biology. D. M. Prescott, L. Goldstein, and E. McConkey, editors. Appleton-Century-Crofts, New York. **1**:233.
49. GOVAN, A. D. T., R. M. HAINES, F. A. LANGLEY, C. W. TAYLOR, and A. S. WOODCOCK. 1969. *J. Clin. Pathol.* **22**:383.
50. GRUBB, C., and I. JANOTA. 1967. *J. Clin. Pathol.* **20**:7.
51. HACKEMANN, M., C. GRUBB, and K. R. HILL. 1968. *J. Ultrasturct. Res.* **22**:443.
52. HALL, J. E., and L. WALTON. 1968. *Amer. J. Obstet. Gynecol.* **100**:662.
53. HARRIS, J. W., F. MEYSKENS, and H. M. PATT. 1970. *Cancer Res.* **30**:1937.
54. HOLMQUIST, N. D., C. A. MCMAHON, and O. D. WILLIAMS. 1967. *Arch. Pathol.* **84**:334.
55. HYDE, A., B. BLONDEL, A. MATTER, J. P. CHENEVAL, B. FILLOUX, and L. GIRARDIER. 1969. *Progr. Brain Res.* 283.
56. International committee on histological terminology for lesions of the uterine cervix. *Proc. Int. Congr. Exfoliative Cytol.* 283.
57. JAMAKOSMANOVIC, A., and W. R. LOEWENSTEIN. 1968. *J. Cell Biol.* **38**:556.
58. JOHNSON, L. D. 1969. *Obstet. Gynecol. Survey.* **24**: 735.
59. JOHNSON, L. D., C. L. EASTERDAY, H. GORE, and A. T. HERTIG. 1964. *Cancer.* **17**:213.
60. JOHNSON, L. D., R. J. NICKERSON, C. L. EASTERDAY, R. S. STUART, and A. T. HERTIG. 1968. *Cancer.* **22**:901.
61. JONES, H. W., H. J. DAVIS, J. K. FROST, I. J. PARK, R. SALIMI, P. Y. TSENG, and J. D. WOODRUFF. 1968. *Amer. J. Obstet. Gynecol.* **102**:624.
62. KARNOVSKY, M. J. 1965. *J. Cell Biol.* **27**:137A.
63. KARNOVSKY, M. J. 1967. *J. Cell Biol.* **35**:213.
64. KARNOVKSY, M. J. 1968. Biological Interfaces: Flows and Exchanges. N. Y. Heart Association Symposium. Little, Brown & Co., Inc., Mass. 64.
65. KARRER, H. E. 1960. *J. Biophys. Biochem. Cytol.* **7**:181.
66. KELLY, D. E. 1966. *J. Cell Biol.* **28**:51.
67. KIRKLAND, J. A. 1969. *Obstet. Gynecol. Survey.* **24**:784.
68. KREUTZIGER, G. O. 1968. Proceedings of the 26th Meeting of the Electron Microscopy Society of America. Claitor's Publishing Division, Baton Rouge, La. 234.

69. LEBLOND, C. P., R. C. GREULICH, J. P. M. PEREIRA. 1964. Advances in Biology of Skin. W. Montagna and R. E. Billingham, editors. Pergamon Press, Inc., New York. **5**:39.
70. LEIGHTON, J. 1967. The Spread of Cancer. Academic Press Inc., New York. 33.
71. LOWENSTEIN, W. R. 1966. *Ann. N. Y. Acad. Sci.* **137**:441.
72. LOEWENSTEIN, W. R., and Y. KANNO. 1967. *J. Cell Biol.* **33**:225.
73. LUFT, J. H. 1961. *J. Biophys. Biochem. Cytol.* **9**:409.
74. MARTINEZ-PALOMO, A. 1970. *Lab. Invest.* **22**:605.
75. MARTINEZ-PALOMO, A., C. BRAISLOVSKY, and W. BERNHARD. 1969. *Cancer Res.* **29**:925.
76. MCCUTCHEON, M., D. R. COMAN, and F. B. MOORE. 1948. *Cancer.* **1**:460.
77. MCNUTT, N. S. 1970. *Amer. J. Cardiol.* **25**:169.
78. MCNUTT, N. S., and R. S. WEINSTEIN. 1969. *Science (Washington).* **165**:597.
79. MCNUTT, N. S., and R. S. WEINSTEIN. 1970. *J. Cell Biol.* **47**:666.
80. MERK, F. B., N. S. MCNUTT, and C. R. BOTTICELLI. 1969. *J. Cell Biol.* **43**: (2, Pt. 2): 90 *a*. (Abstr.)
81. MELLGREN, J., B. BOERYD, and M. HAGMAN. 1962. *Cancer Res.* **22**:139.
82. MOSCONA, A. A. 1963. *Proc. Nat. Acad. Sci. U. S. A.* **9**:742.
83. MUIR, A. R. 1967. *J. Anat.* **101**:239.
84. NISHIHARA, H. 1970. *J. Anat.* **107**:101.
85. PAYTON, B. W., M. V. L. BENNETT, and G. D. PAPPAS. 1969. *Science (Washington).* **166**:1641.
86. PEACHEY, L. D. 1958. *J. Biophys. Biochem. Cytol.* **4**:233.
87. PECKHAM, B. M. 1969. *Obstet. Gynecol. Survey.* **24**:837.
88. PIERCE, G. B., C. WALLACE, and P. K. NAKANE. 1969. *J. Cell Biol.* **43**:105 *a*. (Abstr.)
89. POTTER, D. D., E. J. FURSHPAN, and E. T. LENNOX. 1966. *Proc. Nat. Acad. Sci. U. S. A.* **55**:328.
90. PRESCOTT, D. M. 1970. Advances in Cell Biology. D. M. Prescott, L. Goldstein, and E. McConkey, editors. Appleton-Century-Crofts, New York. **1**:57.
91. REDMAN, R. S., and L. M. SREEBNY. 1970. *J. Cell Biol.* **46**:81.
92. REVEL, J-P., and M. J. KARNOVKSY. 1967. *J. Cell Biol.* **33**:C7.
93. RICHART, R. M. 1963. *Amer. J. Obstet. Gynecol.* **86**:925.
94. RICHART, R. M. 1964. *Cancer Res.* **24**:662.
95. RICHART, R. M., and B. A. BARRON. 1969. *Amer. J. Obstet. Gynecol.* **105**:386.
96. ROBERTSON, J. D. 1963. *J. Cell Biol.* **19**:201.
97. SCHRODT, G. R., and C. D. FOREMAN. 1965. *Cancer Res.* **25**:802.
98. SHERIDAN, J. D. 1966. *J. Cell Biol.* **31**:C1.
99. SHERIDAN, J. D. 1968. *J. Cell Biol.* **37**:550.
100. SHERIDAN, J. D. 1970. *J. Cell Biol.* **45**:91.
101. SHINGLETON, H. M., R. M. RICHART, J. WIENER, and D. SPIRO. 1968. *Cancer Res.* **28**:695.
102. STAEHELIN, L. A., T. M. MUKHERJEE, and A. W. WILLIAMS. 1969. *Protoplasma.* **67**:165.
103. STEINBERG, M. S. 1963. *Science (Washington).* **141**:401.
104. STERN, E. 1969. *Obstet. Gynecol. Survey.* **24**:711.
105. STOKER, M. 1967. *Curr. Top. Develop. Biol.* **2**:107.
106. STOKER, M. G. P., and H. RUBIN. 1967. *Nature (London).* **215**:171.
107. TILLACK, T. W., and V. T. MARCHESI. 1970. *J. Cell Biol.* **45**:649.
108. TRELSTAD, R. L., J. P. REVEL, and E. D. HAY. 1966. *J. Cell Biol.* **31**:C6.
109. UEHARA, Y., and G. BURNSTOCK. 1970. *J. Cell Biol.* **44**:215.
110. VON HAAM, E. 1969. *Obstet. Gynecol. Survey.* **24**:879.
111. WEHRLI, E., K. MUHLETHALER, and H. MOOR. 1970. *Exp. Cell Res.* **59**:336.
112. WEINSTEIN, R. S. 1969. Red Cell Membrane Structure and Function. G. A. Jamieson and T. J. Greenwalt, editors. J. B. Lippincott Co., Philadelphia, Pa. 36.
113. WEINSTEIN, R. S., A. W. CLOWES, and N. S. MCNUTT. 1970. *Proc. Soc. Exp. Biol. Med.* **134**:1195.
114. WEINSTEIN, R. S., and N. S. MCNUTT. 1970. Microcirculation, Perfusion, and Transplantation of Organs. T. I. Malinin, B. S. Linn, A. B. Callahan, and W. D. Warren, editors. Academic Press Inc., New York. 23.
115. WEINSTEIN, R. S., and N. S. MCNUTT. 1970. Proceedings of the 28th Meeting of the Electron Microscopy Society of America. Claitor's Publishing Division, Baton Rouge, La. 106.
116. WEINSTEIN, R. S., N. S. MCNUTT, S. L. NIELSEN, and V. W. PINN. 1970. Proceedings of the 28th Meeting of The Electron Microscopy Society of America, Claitor's Publishing Division, Baton Rouge, La. 108.
117. WILBANKS, G. D. 1969. *Obstet. Gynecol. Survey.* **24**:804.
118. YOUNES, M. S. 1969. *Obstet. Gynecol. Survey.* **24**: 768.
119. ZEIDMAN, I. 1947. *Cancer Res.* **7**:386.