THE ULTRASTRUCTURAL LOCALIZATION OF ADENOSINE TRIPHOSPHATASE-LIKE ACTIVITY IN PRELACTATING MAMMARY GLAND, HYPERPLASTIC ALVEOLAR NODULE, AND MAMMARY ADENOCARCINOMA OF THE C3H FEMALE MOUSE

by

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TO MY TEACHERS

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INTRODUCTION

STATEMENT OF THE PROBLEM

In the female C3H mouse mammary gland, at least three histologically similar, but biologically different states exist: the prelactating gland, the preneoplastic hyperplastic alveolar nodule, and the adenocarcinoma. Since individual nodules may differ in preneoplastic potency, hyperplastic nodule outgrowths were compared to tumors which arose from these outgrowths. The specific aims of this work are:

1. To determine if any electron cytochemical difference exists in the above tissues with regard to the localization of nucleoside phosphatase, utilizing the substrate adenosine triphosphatase.

2. To decide whether any differences in distribution are related to any of the essential characteristics of carcinoma in the mouse mammary gland.

3. To establish insofar as possible the enzymatic nature and specificity of the cytochemical reaction involved.

4. To gain by electron cytochemistry information on the formation and release of the virus-like particles found abundantly in the hyperplastic alveolar nodule and adenocarcinoma.

SURVEY OF THE LITERATURE

Cancer is not a single disease entity, but rather "an ill-defined heterogeneous group of diseases" (1) characterized by the autonomous growth of a group (clone) of cells. Direct study of a single animal tumor system in which as many variables as can be known or held constant

offers the best opportunity for understanding carcinogenesis and, through biological analogy, an understanding of human cancer.

Prior to the investigations of the Imperial Cancer Research Fund Laboratory (established about 1900), the nature and extent of cancer in lower animals was not known. Tumorous specimens of domestic and wild animals either captive or in a natural state were collected and analyzed. Cancer was present throughout the vertebrate phylum and there was no reason to assume its absence in invertebrates. The histologic patterns of animal tumors were identical to those of man. This initial comparative investigation (2) supported the relevance of cancer research of animals to human cancer.

In 1906, Apolant reported the first detailed study of mouse mammary tumors, describing 276 such tumors occurring in 221 mice at the Ehrlich Institute. In addition to offering a histological classification, he considered the transformation of hyperplastic adenomas into adenocarcinomas (3). Another early study of mouse mammary tumors by J. A. Murray (4) with the Imperial Cancer Research Fund Laboratory reviewed previous reports and collected 142 spontaneous mammary tumors among 119 mice. Histologically, he distinguished between a solid, alveolar type and an acinar, glandular type of tumor. (The many histologic differences were concluded to be variations of the general acinar pattern, which is not unlike the normal mammary gland.) The course of tumor growth was progressive, and 13 percent had pulmonary metastases at autopsy, frequently in the pulmonary artery.

Continuing Murray's work, Haaland (5) reported on 300 mice developing spontaneous cancers. After exclusion of lymphomas, pulmonary

adenomas, and sebaceous adenomas, 288 mice remained with 353 primary tumors and of these all but 17 were considered mammary tumors. There was a relative lack of correlation between the histological pattern and biological properties. He, too, called attention to a localized proliferation of acini, the hyperplastic nodules, noting that all histological gradations between hyperplastic nodules and tumors exist. Even the delineation between incipient nodules and normal gland acini was difficult. The association of hyperplastic nodules in glands where tumors have already developed suggested they were either preneoplastic or metastatic satellites from another tumor; however, serial histological sections of the nodules demonstrated their connection with normal mammary ducts.

Little has been added to the early morphological studies other than modern histological nomenclature. When studying mammary tumors induced by methylcholanthrene, workers at the National Cancer Institute outlined a morphological classification (6) distinguishing among adenocarcinoma, adenoacanthoma, carcinosarcoma, sarcoma, and unclassified miscellaneous tumors. Dunn (7) further subdivided the adenocarcinoma group into types A, B, and C. Whereas the Type A tumor is predominantly a uniform acinar pattern with the remainder a glandular epithelium, Type B is clearly of glandular epithelial origin, but lacks the predominant acinar pattern of Type A or the multiple, single cell layer-lined cysts of Type C. There is in Type C also a layer of spindle cells (possibly myoepithelial) surrounding the cysts.

The need for inbred mouse strains was recognized early as a necessity in the genetics of such complex phenotypes as the susceptibility

to cancer. On the development of inbred strains, Heston (8) has remarked, "Their final appearance marks one of the greatest contributions that have been made toward the advancement of experimental oncology."

Murray (9), using the mouse colony of the Imperial Cancer Research Fund, statistically analyzed the relation of cancer incidence to cancerous ancestry. Of 340 females, when the mother and one or both grandmothers had cancer, 18 percent contracted tumors. With no incidence in the mother or grandmother, cancer affected only 8.6 percent of 222 females. Proof of the hereditary influence was important for understanding at that time since it moderated the thinking of cancer as strictly an infectious disease.

Before development of inbred strains, Lathrop and Loeb (10), reporting on the incidence of mammary tumors in hybrid females of high and low cancerous strains, implicated a maternal influence since the incidence of mammary tumors in hybrids was more dependent on the maternal than the paternal tumor incidence. Although Syle did a prodigious amount of work on the genetics of mammary cancer susceptibility, she failed to establish the isogenicity of her strains (11). However, with the advent of isogenic strains, some of which were bred for high and low mammary tumor incidence, the staff of the Roscoe B. Jackson Memorial Laboratory (12) compared the incidence of mammary tumors in female hybrids of reciprocal crosses between distinct high and low tumor incidence strains. Assuming no mutations, the female hybrids of reciprocal crosses have identical genomes. Any significant difference in tumor incidence between the hybrids of a reciprocal cross would be extrachromosomal in origin. The tumor incidence in the F_1 females was

significantly higher when the cross was between a high-tumor strain female and low-tumor strain male. This definitely extrachromosomal maternal influence could be transmitted by one of three possible mechanisms: in the cytoplasm of the ovum, by an intrauterine influence, or in the mother's milk.

A preliminary report by Bittner (13) described the foster nursing of high-tumor strain A newborn females on low-tumor strain CBA and the subsequent incidence of tumors in those foster-nursed and their female progeny. The foster-nursed tumor incidence decreased from 90 percent in maternal nursed strain A to 30 percent in the foster nursed; hence, the Bittner milk agent. Further extended studies (14) demonstrated that some agent was transferred in the milk and that a female of the cancerous strain did not have to have a tumor to do so. If the progeny of the foster females are nursed by females of high tumor strain, the tumor incidence approaches that seen in the high cancerous stock.

With the demonstration of the mammary tumor milk agent, investigators had to now consider its interrelation with inherited susceptibility. Bittner (15) collected data from a large number of reciprocal crosses between cancerous strain A and low cancer strain C57BL and the incidence in F_1 hybrids nursed by A females did not significantly differ from the incidence in strain A. Pooling the data from all hybrid generations supported the theory that susceptibility to mammary tumors in this cross was transmitted as a single dominant gene. However, detailed analysis of the F_1 and F_2 generations revealed the inherited susceptibility for mammary cancer, as transmitted by high-incidence strain A, probably depends on multiple genes, one of which may be linked

with the gene for brown coat color. Similarly, Heston and Deringer (16) reported the relationship of the gene for agouti coat color to the development of mammary tumors.

In addition to genetic control of tumor susceptibility, Heston *et al.* (17) suggested a separate genetic influence over the propagation of mammary tumor agent. Susceptible strain C3H females with the mammary tumor agent were crossed with resistant strain C57BL males without the agent. The F_1 females (MTA positive) were then backcrossed with C3H males and C57BL males. This provided two groups of backcross females which differed genetically due to paternal differences, but were alike with respect to the agent they received from the genetically uniform F_1 females. More tumors occurred in the females foster nursed by the C3H backcross females than those nursed by the C57BL backcross females, indicating the backcross groups differed in their ability to transmit the agent, presumably because of their different genotypes. Successive backcrosses to resistant-strain males led to complete elimination of the agent by the third backcross generation (18).

Questioning the possible intrauterine influence, Fekete and Little (19) transferred zygotes from low-tumor C57BL to the uterus of high-tumor DBA mice and *vice versa*. Those born from the transferred zygotes were nursed by the mother that gave birth to them. The increase and decrease in mammary tumor incidence was greater than had been noted by foster nursing alone, suggesting a possible intrauterine influence.

The mammary tumor agent may be transmitted through the milk, but it is distributed throughout the body of the mouse (20). Bittner (21) demonstrated that the mammary tumor agent remained in the filtrate of

lactating mammary tissue by injecting the filtrate into susceptible agent-free mice and inducing mammary tumors. By ultracentrifugation, the agent was sedimented from cell free extracts (22). These experiments, coupled with the knowledge that the agent must propagate since it could be transmitted through many generations mice, are all characteristics of viruses. An electron microscopic study (23) of mammary tumors revealed the presence of two types of virus-like particles: a cytoplasmic A particle, a symmetrically doughnut-like structure, 70 mμ in diameter, not found free in the gland acini or ducts; and a larger, extracellular B particle characterized by a dense, eccentric nucleoid. Not infrequently the A particles are found surrounding a cytoplasmic vacuole occasionally containing B particles, and A particles are often reported budding from the apical cell surface into the acinar spaces. It is thought that these budding A particles mature into the extracellular B particles.

The low-cancer sublines were established by foster nursing a high-cancer strain on mothers not carrying the mammary tumor agent. Bioassays of tissues and secretions from these low cancer sublines have been negative (24). However, in the C3Hf substrain in which repeated bioassays of milk and tumor extracts have been negative, viral particles seen were indistinguishable in abundance, morphology, and distribution from those in C3H tumors and hyperplastic nodules (25). For convenience, MTV has come to indicate the biologically active infectious tumor agent and EMV to indicate the typical B particles observed by electron microscopy in tissues from which biologically active MTV cannot be recovered, although they may represent active, inactive, variant MTV,

or all three.

Conventional biological assay of MTV infectivity and tumorigenic potential involved the injection of MTV-containing extracts into susceptible virus-free mice and the observation of mammary tumor development. One to two years was required. Nandi (26), relating the MTV activity to the development of hyperplastic alveolar nodules, devised a noduligenic test. Exposure to MTV followed by exogenous estradiol and DOCA reduces the period required for the development of hyperplastic alveolar nodules to 13 weeks. However, Blair (27), by an immunodiffusion assay, can correlate a precipitate line appearing in four to five days with the presence of MTV and/or the results of neutralization studies. She has also been able to demonstrate murine antisera directed against MTV. Since small inocula of MTV antigen may not evoke a measurable antibody titer in the mouse unless multiplication of MTV occurs, a quantitative distinction between infectious (multiplying) and noninfectious (non-multiplying) MTV may be possible (28).

The elucidation of the molecular nature of the MTV was only recently accomplished. In 1965 Lyons and Moore (29) reported that MTV contains 30 percent lipid and its nucleic acid is RNA. The isolation and characterization of MTV-RNA described by Duesberg and Blair (30) used MTV from the milk of lactating females of A and Balb/c strains, fostered on C3H. They showed that MTV, a high molecular weight, single-stranded RNA, is comparable in size and structure to the RNA of others of the Thylaxouridae family of RNA oncogenic viruses (31).

That mammary tumors were limited to females implied the influence of female sex hormones on the hormonally responsive mammary tissue.

Consequently, Lathrop and Loeb (32), investigating the influence of pregnancies on the incidence of mammary cancer, noted that the breeding female mice had a higher incidence of mammary tumors occurring at an earlier age than the non-breeding females. There was a definite variation in tumor incidence among strains, and the incidence of mammary tumors was higher in the virgin females of a high-incidence strain than in the virgin of low-incidence strains. A further extension of the observations by the same investigators (33) involved the influence of ovariectomy. Castration of females less than 6 months old led to a marked delay and decrease in mammary tumor incidence. Ovariectomy after 6 months of age had no effect.

If indeed the ovary was responsible for mammary tumors in females, ovarian grafts in males should have produced mammary tumors. After unsuccessful attempts by Loeb (34) and Cori (35) to transplant ovaries into males, possibly due to lack of isogenic strains, W. S. Murray (36), using Little's DBA strain which had been inbred since 1909, subcutaneously grafted ovarian tissue to castrated males and obtained a 7 percent incidence. By 1930, several inbred mice strains were available. Numerous experiments injecting estrogenic compounds in both sexes paralleled refinement of chemical extraction of chemically pure hormones. An extensive eight year study (37), using a large number of mice from seven strains of both high and low tumor incidences, substantiated numerous other reports. The incidence of mammary tumors in breeding mice of various strains generally paralleled the increase in tumor incidence in these strains in the virgin females or the tumor incidence induced in males. The greater the dose of estrogen in a

high-tumor incidence strain, the earlier the mammary tumors appeared. The incidence was similar in the males and virgin females belonging to the same strain, injected with large doses of estrogen. Conclusions drawn were that mammary carcinogenesis through endogenous or exogenous estrogens was by the same mechanism, the strain differences reflecting variation in the inherited responsiveness of the mammary gland.

It had long been known that the breeding females acquired a greater incidence of tumors earlier than their virgin litter mates. Even after copulation with vasectomized males, pseudopregnant strain A females developed a 22 percent incidence of mammary cancer. These observations suggest that progesterone enhances mammary carcinogenesis. Reports attempting to prove this have conflicted (38, 39). Gardner (40) has criticized that low dosages given at relatively long intervals would have little effect since progesterone is rapidly removed from the plasma. When young, susceptible, hybrid (C3HxA) virgin females were administered a subcutaneous pellet of progesterone every 28 days until death or a period of two years, the treated mice had a higher incidence and earlier appearance of tumors.

The dominant role of ovarian estrogens in mammary carcinogenesis was further emphasized by Loeb and Kirtz (41), who subcutaneously implanted adenohypophyses into virgin, ovariectomized females of strain A. Virgins normally have a low (2 to 5%) tumor incidence, but with the grafts the incidence increased to 44 percent, with the tumors appearing at an earlier age. No tumors were found in the ovariectomized females or males. It was reasoned that the pituitary influence was mediated through the ovary. However, hypophysectomized C3H mice with longterm

estrogen administration produced no tumors (42).

A series of endocrine experiments by the Cancer Research Genetics Laboratory of the University of California at Berkeley established the hormonal requirements of lobuloalveolar mammogenesis, lactogenesis, as well as noduligenesis and tumorigenesis in hypophysectomized-ovariectomized-adrenalectomized young C3H females (43, 44, 45, 46, 47). The following hormones were used for replacement therapy: estradiol-17β (E), progesterone (P), deoxycorticosterone-acetate (DOCA), cortisol (F), bovine somatotropin (STH), and ovine mammotropin (MH). The results can be summarized as follows:

1. The minimal hormonal requirement for lobuloalveolar mammogenesis and noduligenesis was E + P + STH and/or MH. The time required for noduligenesis was considerably longer than that for lobuloalveolar development.

2. With lobuloalveolar or nodule development, lactation could be stimulated by the injection of F + MH and/or STH.

3. Although hyperplastic alveolar nodules were heterogeneous in respect to their hormonal requirements, most were dependent on MH and/or STH + F for their maintenance, not requiring estrogen as in their formation.

4. The nodules transplanted into cleared fat pads needed pituitary hormones (MH and/or STH) and either adrenal (F or DOCA) or ovarian (E or P) steroids for neoplastic transformation. Estrogen, though not necessary, enhanced tumorigenesis.

In summary, in the C3H strain adrenal corticoids were interchangeable with progesterone in lobuloalveolar development and in nodule

formation and maintenance. The pituitary hormones STH and MH were interchangeable in all phases of mammary growth including tumor development.

Since it is the endocrine stimulation primarily involved in mammary growth and development, genetic and viral influences must have their effect by modifying the production or metabolism of hormones and/or the responsiveness of the mammary tissue to hormonal stimulation. Nandi and Bern (48) compared the hormonal requirements of the C3H strain to those of other strains. Mammotropin-containing combinations were capable of inducing lobuloalveolar development in all the strains studied: C3H, C3Hf, RIII, DBA, A, and A/3. However, mammary responsiveness to somatotropin-containing hormone combinations was observed in those strains (C3H, RIII, and DBA) which have a high incidence of hyperplastic alveolar nodules and tumors in virgin mice, but not in those strains (A or A/3) which have a low incidence of nodules and tumors in virgin mice with a high incidence in breeding females. The sensitivity to STH was not related to the presence or absence of MTV, since mammary glands of C3H and C3Hf mice responded in a similar way to STH.

By transplanting C3Hf and C3H mammary tissues into C3H hosts and A and C3H mammary tissues into F_1 hybrids of reciprocal A and C3H crosses, it was demonstrated that the hormonal responsiveness of the transplanted tissues was unaltered, indicating that the genetic differences are not due to the hormonal metabolism of the host, but were at the tissue level (49, 50).

Progress in understanding the interaction among hormonal, viral, and genetic factors followed detailed studies of the hyperplastic alveolar nodules in relation to normal and neoplastic tissues. Comparing

the histology of the high mammary cancer DBA and the low C57BL strains, Fekete (51) described persistent alveolar cell multiplication throughout pregnancy in DBA strain, while in the C57BL cell multiplication ceased with the advent of milk secretion in late pregnancy. In the post-lactation state, cessation of secretion is followed by regression of the alveolar epithelium in the C57BL; however, in DBA small lobules of alveoli persisted. She concluded that the persistent alveolar cell multiplication during late pregnancy seemed to lead to adenocarcinoma in the DBA strain. Histologically, these abnormal groups of alveoli corresponded to the hyperplastic nodules described by Haaland (5). Gardner, Strong, and Smith (52) studied these hyperplastic alveolar nodules in the mammary glands of multiparous mice of five strains with varying incidence of mammary carcinoma. Nodule occurrence was greatest in those strains with a high incidence of mammary tumors and rarely encountered in the low tumor strain. As the age of the animals increased the number of nodules also increased. Their presence was independent of the stage of the estrous cycle and the duration of time after cessation of lactation.

Detailed study of the hyperplastic alveolar nodules, one of a family of hyperplastic entities, was not pursued until 1956 at the Cancer Research Genetics Laboratory, when research was begun attempting to define their biological characteristics. As a marker of general metabolic activity the uptake of radioactive phosphate was measured in the normal mammary gland, the hyperplastic alveolar nodule, and tumor under varying endocrine influences. The hyperplastic alveolar nodule showed a radioactive phosphate uptake intermediate between that of

tumor, which was greatest, and normal mammary tissue, which was least. Endocrine manipulations of ovariectomy, and androgen, estrogen, or cortisol treatment had no appreciable effect. With the second half of pregnancy the uptake by both normal and hyperplastic gland increased (53). Further cytochemical studies of hyperplastic alveolar nodules from the C3H females compared them to prelactating gland and adenocarcinoma. The adenocarcinoma differed from the non-neoplastic tissues by: the absence of alkaline phosphatase-positive myoepithelium; intense apical alkaline phosphatase activity; a more intense reaction for protein-bound sulfhydryl groups; and the presence of increased amounts of DNA by microspectrophotometric measurements on Feulgen-stained sections indicated a higher mitotic rate. The amounts of DNA were consistent with tetraploidy and polyploidy. There were no morphological or cytochemical features that would distinguish prelactating gland from hyperplastic alveolar nodule (54).

The comparisons were extended by electron microscopy (55) where it was observed that nodules differ from physiologically similar normal gland, but resemble the tumor with respect to the increased number of both intracellular and extracellular virus-like particles present. Since its earliest descriptions, the hyperplastic alveolar nodule has been considered preneoplastic, based on only indirect and inferential evidence. Direct evidence that hyperplastic alveolar nodules were especially prone to undergo neoplastic transformation was possible by the technique of transplantation into gland-free mammary fat pads. In the three week old female mouse, the duct system is rudimentary and can be dissected from its origin at the nipple, leaving the major portion of

the mammary fat pad gland-free (56). Transplantation of normal mammary gland and hyperplastic alveolar nodule tissue into gland-free (cleared) fat pads demonstrated that the hyperplastic alveolar nodules of virus-infected, multiparous female C3H mice gave rise to tumors more frequently and in less time than did normal gland. Nearly 50 percent of the nodules transplanted into cleared fat pads gave rise to tumor; whereas, one out of 67 transplanted into the dorsal subcutis and observed as long as 80 weeks developed a tumor. Although both normal and nodule tissue survived in the subcutaneous site they did not produce outgrowth. It is also of interest that two of 11 outgrowths from nodules in the cleared fat pads were indistinguishable from normal gland outgrowths (57). A nodule outgrowth is not neoplastic in that the lobuloalveolar growth remains within the fat pad; the outgrowth from normal tissue is characteristically the growth and arborization of a duct system without lobuloalveolar development.

Through the use of gland-free fat pads, Faulkin and DeOme (58) designed experiments demonstrating the presence of a growth-regulating mechanism. In the growth pattern of the normal mammary gland the ductal elements extend throughout the fat pad, allowing a 0.25 mm unoccupied zone between ducts and between the edge of the fat pad and the ducts. Under hormonal stimulation, lobuloalveolar development occupies this space. Outgrowths from normal or nodule transplants, but not from tumors, are inhibited by the presence of an intact mammary gland in the fat pad. The possibility that the rate of mammary duct growth is systemically controlled by a self-regulatory mechanism was investigated by comparing the growth rates of the mammary ducts remaining after subtotal

mastectomy in three week old females with that of sham-operated and intact controls (59). These experiments provided no evidence for a systemically mediated humoral factor responsible for autoregulation of mammary duct growth. The ability to override the growth-regulating system appears to be the essential characteristic of carcinogenesis in the mouse mammary gland.

The hyperplastic alveolar nodules are not a homogeneous group of biologically similar entities. Even though elderly virgin and multiparous C3H females may have many nodules they rarely have more than three primary adenocarcinomas. Not all outgrowth from nodules transplanted into gland-free fat pads gave rise to tumors, and the outgrowth of some resembled normal duct outgrowth (57), nor are the hormonal requirements for maintenance of nodules uniform (46). By the method of transplantation into gland-free mammary fat pads it has been possible to follow the fate of different portions of individual nodules from multiparous tumor-bearing female C3H/Crgl mice and maintain them in isologous hosts as hyperactive outgrowths through many generations of serial transplantations (60). The characteristics of growth rate, secretory activity and tumor-producing capability of nodule outgrowth lines were compared. The outgrowths from transplanted portions of a single nodule differed in their outgrowth characteristics and during serial transplant generations, the characteristics of each line remained remarkably stable. Also, each cell line possessed a characteristic pattern of responsiveness in male and endocrine-manipulated females. Of eight different outgrowth lines derived from only two nodules, three had strong tumor-producing capabilities. The hormone dependence of the

nodule outgrowth lines varied among the lines and in different hosts with respect to growth rate, secretory state, and tumor-producing capability. There was no correlation among growth rate, secretory pattern, and tumor producing capabilities of the lines tested.

In the mouse mammary gland the neoplastic cell variants possess one essential characteristic, an unresponsiveness to duct growth regulator; whereas, nodule cells which exhibit a hormonal independence remain responsive. Investigations into the apparent stepwise progression in tumorigenesis have proceeded to elucidate the various *etiologic* factors involved. The *mechanisms* of nodule and tumor formation remain unknown. The growth pattern within the gland or in an isologous host's gland-free fat pad is presently the only way to distinguish among neoplastic, nodule and normal mammary cells. A cytochemical marker capable of distinguishing among those histologically similar, but biologically different states would be a useful research tool. In the present experiments the prelactating gland, the preneoplastic hyperplastic alveolar nodule, and the adenocarcinoma are compared by electron cytochemistry, specifically, ATPase-like activity.

Preneoplastic potency appears to be inherent in certain nodule cell populations. Tumors arise from the transplanted nodule tissue outgrowth and this tumor-producing capability of a given outgrowth remains constant throughout serial transplants. The present experiments, therefore, also compare hyperplastic nodule outgrowths to tumors which arise from them, since in this circumstance other variant characteristics such as tissue organization, hormone responsiveness, enzymatic patterns of biochemical pathways, *etc.*, that may represent nonessential variants,

would less likely be construed as indicators of the essential characteristic of tumorigenesis (61).

MATERIALS AND METHODS

WHOLE MOUNT PREPARATIONS

The skin was incised in the central midline from the arch of the mandible to the anus and down the ventral aspect of the extremities from the midline. By blunt dissection the entire skin was separated from the corpus (excepting the snout), stretched, and pinned fur side down. The mammary glands in their subcutaneous location remained with the skin. After fixation in 10 percent formaldehyde, the mammary glands were dissected from the skin and placed in 100 percent acetone. They were then dehydrated in graded ethanols prior to staining by hematoxylin (pH 1.0), after which they were washed in water, re-dehydrated through ethanols, cleared in toluene, and stored in methyl salicylate (62). The whole mount preparations were studied with a dissecting microscope.

TISSUES AND FIXATION

Prelactating mammary tissue was gathered from 4 to 8 month old female C3H/Crgl or C3H/HeJ mice thirteen days following 48 hour exposure to C3H/Crgl males. Hyperplastic alveolar nodules and 0.5 to 1 cm adenocarcinomas were dissected from 8 to 14 month old non-pregnant multiparous mice of the same substrains. Only morphologically typical nodules were selected; these were opaque, slightly orange (52) lobuloalveolar formations located in the periphery of the fat pad. All mice were anesthetized with intraperitoneal pentobarbital (63).

Samples of tissue were cut into 1 mm cubes and treated as follows: some were left 1 to 2 hours in cold (4° C) 1 percent OsO_4, buffered with

veronal acetate (pH 7.1) (64); others were fixed for 5 hours in cold (4° C) glutaraldehyde buffered at pH 7.2 with sodium cacodylate (65). The osmicated tissues were then rapidly dehydrated in a graded series of ethanols at 4° C, and embedded in araldite (66). The glutaraldehyde-fixed tissues were washed in several changes of 0.1 M sodium cacodylate and 0.2 M sucrose solution (pH 7.2) for at least one week before histochemical experiments were performed.

PREPARATION OF AND TRANSPLANTATION INTO GLAND-FREE MAMMARY FAT PADS

At 3 weeks of age the female mouse mammary gland is a small group of short, branching duct elements arising from the nipple and extending a short distance into the mammary fat pad. The gland anlage of the inguinal (#4) mammary fat pad can be easily excised, leaving a gland-free fat pad for a transplantation site. For this purpose, an inverted Y-shaped incision was made in the abdominal midline and extending laterally between the fourth and fifth nipples and midway down the ventral aspect of each hind extremity. The skin containing the inguinal mammary gland was reflected laterally by blunt dissection and the free edge pinned down. The large vessels to the area ventral to the lymph node between the fourth and fifth fat pads and the nipple area were cauterized. The fat pad ventral to the lymph node (proximal to the nipple) containing the gland elements was then excised, leaving the remainder of the fat pad without any glandular elements. Small (1 mm) cubes of tissue were then transplanted into the fat pad, by means of watchmakers forceps. The same procedures were carried out on the opposite side. Skin was then approximated and closed with metal clips.

Hyperplastic alveolar nodules were dissected from 10 to 14 month old C3H retired breeder females, and transplanted into a gland-free fat pad. When a palpable tumor appeared in four months (67), the tumor and a sample of the hyperplastic nodule outgrowth were taken for electron cytochemistry.

HISTOCHEMISTRY

The glutaraldehyde-fixed tissue was washed by several changes of 0.1 M cacodylate and 0.2 M sucrose solution (pH 7.2) for at least one week. The blocks were frozen and sectioned by means of a cryostat at 50 to 70 μ for electron cytochemistry, and at 10 μ for light histochemistry. In order to obtain satisfactory specimens of prelactating gland for light histochemistry, however, it was necessary to embed the tissue in 7 percent agar and chop it with a S & F tissue chopper (68), which prevented disruption while handling. The sections were transferred by Gooch porcelain filter crucibles and incubated at 37° C in glass stain dishes. The duration of incubation varied from 20 to 40 minutes.

After incubation, the sections for light histochemistry were rinsed in distilled water, exposed for 2 to 3 seconds to an aqueous solution of 1 percent $(NH_4)_2S$ and mounted from a water bath onto glass slides. The slides were then coated with colloidin and counterstained in hematoxylin.

The sections for electron cytochemistry were washed 15 minutes in the cold cacodylate-sucrose solution after incubation and then post-fixed in cold 1 percent osmium tetroxide and processed as described

above.

Sections, 1 μ thick, were cut with glass knives on an LKB ultrotome, mounted on glass slides, and stained with toluidine blue. Other sections, 500 to 700 Å thick, were collected on copper grids with a carbonized parlodion film, and examined in an RCA EMU-3G and Phillips 200 electron microscope, unstained, doubly stained with lead citrate (69) and saturated uranyl acetate (70), and triply stained with lead citrate, uranyl acetate, and lead citrate.

INCUBATION

The 10 μ sections for light histochemistry were incubated for 20 to 45 minutes at 37° C in one of the following media:

A. Alkaline phosphatase, method of Gomori (71).

B. Nonspecific phosphomonoesterase, method of Wachstein and Meisel, 8 mM β-glycerol phosphate as the substrate at pH 7.2 (72).

C. Nucleoside phosphatase, method of Wachstein and Meisel, using 2 mM adenosine triphosphate (ATP) as a substrate at pH 7.2 (73).

The 50 to 70 μ sections for electron cytochemistry were incubated 20 to 45 minutes in media C with 2 mM ATP as the substrate. Control sections were as follows:

(1) Incubation in medium C without ATP.

(2) Incubation in medium C without $Pb(NO_3)_2$ capture agent.

(3) Incubation in medium C without ATP for 20 minutes followed by the addition of 0.5 cc of 0.1 M Na_2HPO_4 and incubation for another 20 minutes. The Na_2HPO_4 clouds the medium with a fine white precipitate.

LIGHT HISTOCHEMISTRY ENZYME INHIBITION STUDIES

To determine the specificity of the reaction, 10 μ sections of pre-lactating gland and adenocarcinoma were immersed for 20 minutes in 10 mM L-cysteine. Subsequently, the same sections were incubated 30 minutes in media A, B, and C with L-cysteine added (final concentration 1 mM).

RESULTS

WHOLE MAMMARY GLAND MOUNTS

Whole mounts of prelactating mammary gland, stained with hematoxylin, demonstrate a well developed, arborizing duct system which fills the mammary fat pad (Figure 1). From the ends of the ducts, and along the tertiary duct branches, lobules of alveoli protrude (Figure 2). There still remains a substantial amount of adipose stroma, which is known to virtually disappear as the fat pad is filled with lobuloalveolar formations. In the glands of 8 to 14-month-old, non-pregnant, multiparous females (Figure 3) the duct system is fully developed, and possesses scattered isolated alveoli. Irregularly localized throughout the gland are lobuloalveolar formations, the classical *hyperplastic alveolar nodules* (Figure 4). They are 1 ± 0.5 mm in diameter, of a light orange color *in situ*, arising from a single tertiary duct, and generally located near the periphery of the fat pad.

LIGHT MICROSCOPY

Prelactating mammary glands, hyperplastic alveolar nodules, spontaneous adenocarcinomas, nodule outgrowths, and adenocarcinomas arising from nodule outgrowths are similar in that all are composed of alveoli, lined by cuboidal epithelium (Figures 5 through 9). Adipose stroma is observed within the prelactating tissue and hyperplastic nodule outgrowths; whereas, adipose stroma is found only at the edge of hyperplastic alveolar nodules, and may or may not be seen at the unencapsulated margins of tumors, depending on whether that portion of the tumor lies

within the mammary fat pad.

Histochemical studies were performed on mammary adenocarcinomas. Alkaline phosphatase and adenosine triphosphatase histochemical experiments with and without exposure to L-cysteine were carried out on 15 separate tumor samples in six separate experiments, and in a similar experiment on two separate samples of prelactating gland tissue. The basal and luminal borders and vascular stroma were positive for alkaline phosphatase and adenosine triphosphatase in the tumor (Figures 10 and 12); however, prior exposure and concurrent incubation in L-cysteine eliminated or decreased the activity of alkaline phosphatase (Figure 11), while having no effect on the activity of adenosine triphosphatase (Figure 13). Alkaline phosphatase activity in prelactating gland was localized to all cell membranes, and was inhibited by L-cysteine. ATPase activity in prelactating gland was absent from the luminal, but present on the lateral and basal cell membranes, and not inhibited by L-cysteine.

ELECTRON CYTOCHEMISTRY

The reaction product is seen as a granular, electron opaque precipitate distributed in a beaded pattern along positive cell membranes. Nine separate samples in as many experiments demonstrated no reaction when either ATP or lead nitrate was absent from the incubation media. The cloudy control (incubation in the medium with a fine lead phosphate precipitate) demonstrated a diffuse, electron opaque precipitate of similar character along the edge of the specimen, scattered randomly throughout the gland tissue, and showing no preferential adsorption

(substantivity) to the cell membrane or intracellular organelles.

Reaction product in the prelactating mammary gland (Figure 14) and hyperplastic alveolar nodule (Figure 16) was deposited along the external surface of the basal and lateral cell membranes with no deposit within the lumina, on the apical microvillar surface, or intracellularly. The region of the subapical tight junction was devoid of reaction product (Figures 15 and 17). In the prelactating tissue the basement membrane and stromal tissues had reaction product scattered throughout.

In contrast to the prelactating and hyperplastic nodule mammary epithelium, reaction product was deposited within lumina and on the external surface of the apical microvilli of the spontaneous mammary adenocarcinomas arising in normal gland or in hyperplastic nodule outgrowths (Figures 18 and 19). The luminal virus-like particles (presumed mammary tumor virus "B" particles), which are thought to gain their outer membrane from the apical cell membrane, exhibited inconstant deposition of reaction product on their outer surfaces with a distribution pattern suggesting adsorbed reaction product (Figures 18, 19, and 20).

Unlike the hyperplastic alveolar nodules examined, the preneoplastic nodular outgrowth demonstrated reaction product located on the external apical surface and within lumina (Figure 21). Myoepithelial cells of nodule outgrowths possessed reaction product scattered throughout the cytoplasm and nucleoplasm without consistent external cell membrane localization (Figure 21). Tumor arising from the outgrowths demonstrated apical surface reaction product (Figures 22 and 23).

DISCUSSION

With the advent of fixatives that preserve both ultrastructure and enzymatic activity, classical methods of light histochemistry can be applied to electron microscopy. One of the most readily adapted is the metal salt technique for phosphatase histochemistry. The histochemical product of lead phosphate is electron dense; whereas, for light microscopy further reaction of the lead phosphate with ammonium sulfide results in the formation of visible brown-black lead sulfide. Comparison between light and electron microscopy is valid assuming the relative insolubility of those lead salts. Electron microscopy extends the resolution available by light microscopy.

The specificity of membrane-associated nucleoside phosphatase and nonspecific phosphatase activity is frequently overlapping (74). Cardiff (75) demonstrated the identical distribution of glutaraldehyde-resistant enzymes hydrolyzing ATP, adenosine diphosphate (ADP), adenosine monophosphate (AMP), and sodium β-glycerophosphate; the addition of L-cysteine to the incubation media inhibited the enzymatic hydrolysis of sodium β-glycerophosphatase while preserving that of ATP. This would serve to distinguish the enzymatic hydrolysis of ATP from the activity of a nonspecific phosphatase. The lack of reaction product when the tissues were incubated with lead phosphate, in the absence of ATP, mitigates against nonspecific binding of the reaction product. Therefore, the reaction product observed in the present experiments is formed by enzymatic hydrolysis of ATP substrate. This hydrolysis could be due to one or more related glutaraldehyde-resistant ATPase-like enzymes.

Although as yet histochemical studies have not added appreciably to an understanding of carcinogenesis, cytochemical markers for malignant cells have been described in some tumor systems (76, 77, 75). There have been several alkaline phosphatase studies (53, 78, 79, 80, 81) of mouse mammary tumors. Comparing prelactating mammary gland, hyperplastic alveolar nodule, and tumor, Harkness *et al.* (53) described luminal alkaline phosphatase in prelactating gland and hyperplastic nodules, and a marked increase of luminal reaction in the tumor. Pakdaman and Stein (82) with human surgical breast specimens showed myoepithelial ATPase activity in the normal, but no increase in activity in specimens of fibrocystic disease or lobular hyperplasia, and complete absence in adenocarcinoma. Also, ATPase has been demonstrated in the lactating mammary gland of the rat (83) and rabbit (84). Novikoff (85) has demonstrated ATPase activity in the mouse mammary tumor along the lateral and basal cell wall as well as at the luminal surface.

Electron cytochemical phosphatase studies in the mouse mammary tumor system are limited to acid phosphatase localized to lateral cell wall, Golgi apparatus, and lysosomes in Cytoxan and vitamin A treated mouse mammary tumors (86), and thiamine pyrophosphatase, acid phosphatase, and nucleoside phosphatase in the lactating mammary gland and mammary tumors of cortisone-treated female C3H mice (75). Using the same techniques as the present study, Cardiff demonstrated NPase reaction product along the lateral and basal cell membranes in both benign and malignant cells; however, the reaction product on the apical and microvillous surface of the cortisone-treated adenocarcinoma cells was absent from the microvilli of the lactating epithelial cells. In

addition, nucleoside phosphatase reaction product was present in some of the cytoplasmic vacuoles in the treated tumors. de-Thé (87) has reported the inability to demonstrate luminal ATPase in C3H mouse mammary tumors which is contrary to the results of the present study and of others (85, 75).

In relation to previous studies, the present results support the localization of ATPase-like activity to the entire adenocarcinoma cell surface including the apical-microvillous surface. In the prelactating and lactating gland and hyperplastic alveolar nodule the luminal surface is negative in those specimens examined; however, a tumorigenic hyperplastic outgrowth is positive, as is the tumor which arises from it.

Electron cytochemistry indicated that budding avian myeloblastosis and herpes virus particles probably derived their outer membranes from the ATPase-active plasma membrane of avian thymocytes (88) and HeLa cell membranes, respectively (89). In the present studies the luminal virus-like particles (presumed mammary tumor virus B particles), which are thought to gain their outer membrane from the apical cell membrane, exhibited inconstant ATPase-like activity with a distribution pattern of adsorbed reaction product perhaps originating by diffusion from the apical cell surface. If indeed the B particles do gain their envelopes from the ATPase-like positive apical cell surface, but fail to retain this activity during extracytoplasmic maturation, the host plasma membrane of the viral envelope must be modified with regard to this cytochemical characteristic.

If there are any functional inferences to be made from these observations other than the apparent marker of apical ATPase-like activity

to neoplastic and preneoplastic potency, they must rest on the similarity, if not identity, of the ATPase-like enzyme activity to ATPase investigated by standard enzymological techniques. The histochemical assay (the presence or absence of reaction product) does not allow quantification and what might appear to be a qualitative difference may be merely a reflection of the lack of sensitivity. Recent interest in ATPase stems from the demonstration of an ATP-hydrolyzing enzyme system in the transport of sodium and potassium ions across cell membranes against an electrical gradient (90). The occurrence of this so-called "transport ATPase" was studied in 21 tissues from ten different species (91). It was present in all tissues studied, but varied in activity according to the amount of cation transport, from the actively pumping electric organs of fish to sluggish mammalian erythrocytes. By differential centrifugation (92), ATPase has been localized in the subcellular fraction that contains the broken cell membranes and likewise in the ghosts of erythrocytes (93). The loss of ATPase activity and digestion of the erythrocyte ghost membrane by trypsin is prevented by the addition of ATP with Mg^{++} (94). Such evidence suggests that ATPase may be an integrated structural component of the red cell membrane. Although characteristic of transport ATPase, activation of ATPase by Na^{+}, K^{+}, or Mg^{++} ions, or inhibition by oubain was not demonstrated by histochemistry in glutaraldehyde-fixed tissue (95).

Changes in the cancer cell surface may be closely related to such basic malignant properties as invasiveness and the ability to metastasize. Ambrose (96) offers evidence for altered adhesiveness, surface charge, and possible biochemical changes in malignant cell membranes.

The loss of contact inhibition is considered to be a tissue culture equivalent of tumor development. Contact inhibition is composed of both inhibition of mitosis and inhibition of cellular locomotion. It was thought that contact inhibition was mediated by actual contact of cell surfaces (97); however, Rubin (98) has isolated a substance released into the tissue culture medium which has a growth inhibitory effect. S. B. Carter (99) has demonstrated in tissue culture that cell to cell relationships are directed by the relative strength of cellular adhesions and it is possible to completely change growth patterns by changing the culture surfaces so as to allow different degrees of cellular adhesion. The development of apical surface ATPase-like activity in the preneoplastic and neoplastic mammary tissue may relate to cell growth and mobility in tissue culture which affects, or is mediated by, the cell surface.

It follows that tumor specific antigens of tumor cell membranes might reasonably be expected and that those surface antigens are likely to play a significant role in host-tumor cell immunologic interactions. Weiss (100) found that immunization of mice with mouse mammary tumor cell-membrane fraction followed by a challenge of tumor cells elicited an immunologically mediated response of enhanced tumor growth.

The association of apical surface ATPase-like activity with the mammary adenocarcinoma and the tumor-producing hyperplastic nodule outgrowth suggests its origin in the process of tumorigenesis. Study of the origin of this apparently tumor-specific distribution of the ATPase-like enzyme may afford some insight into mouse mammary tumorigenesis. It is not possible to know for certain whether the appearance

of apical ATPase-like activity in the tumor and tumorigenic hyperplastic nodule outgrowth is due to the appearance of a new enzyme or an increase in activity to a level of recognition. If one can assume that the enzyme has arisen *de novo*, then the question presents itself as to its origin. The genetic information for the enzyme can either be part of the host genome or in the nucleic acid of the infecting virus. Virus-induced tumor specific antigens have been observed in the polyoma system (101), and this antigenic specificity is not influenced by the histologic type of tumor, or the strain, or even the species of host animal involved. In this instance, the viral genome is believed to give rise to the specific neoplastic marker. Tumor-inducing virus could also affect the host genome by altering the activity of regulator genes, in which circumstance the host genome itself would give rise to the neoplastic marker, as a result of the viral infection.

The presence of ATPase-like activity in the outgrowth from a hyperplastic alveolar nodule, and its absence from *in situ* nodules in the present study may reflect the specialized metabolic characteristics of the explant outgrowth, or the absence of a functioning duct drainage system in the outgrowth. To more fully establish that apical ATPase-like activity in tumorigenic nodule outgrowths is a marker of preneoplasia, it would be necessary to examine outgrowths from normal duct with end-buds and non-tumorigenic nodule outgrowths.

Since hyperplastic alveolar nodules have been conclusively demonstrated to be preneoplastic (56), the results of the present experiments indicate that apical cell surfaces of some hyperplastic nodule cells might have ATPase-like activity, even though none was observed. This

might be expected considering the cell population of a single nodule is heterogeneous with regard to preneoplastic potency (59), and it has been estimated in the C3H female mouse that one nodule in sixty develops into a tumor (102). Also, only a relatively few cells from a limited number of nodules could be sampled by electron cytochemistry; therefore, the infrequent positive cells might not have been represented in the samples.

SUMMARY AND CONCLUSIONS

It appears that the prelactating mammary gland and hyperplastic alveolar nodule differ from the mammary adenocarcinoma by their lack of apical surface ATPase-like activity. However, the present experiments offer no explanation for the correlation between apical surface localization of ATPase-like activity and mammary adenocarcinoma. That hyperplastic nodule outgrowths which give rise to adenocarcinoma demonstrate ATPase-like activity localized to the apical cell surface suggests such localization may also correlate with preneoplastic potency of hyperplastic alveolar nodule cells. It can be concluded from the present experiments that the reaction product is formed by the enzymatic hydrolysis of ATP, which could be due to one or more related glutaraldehyde-resistant ATPase-like enzymes. The inconstant ATPase-like activity of the luminal virus-like B particles neither supports nor disproves that the viral envelope is formed of the cytochemically positive apical cell membrane.

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ILLUSTRATIONS

FIGURE 1: Prelactating gland whole mount preparation demonstrating a well developed, arborizing duct system. (X5)

FIGURE 2: A higher magnification of prelactating gland whole mount preparation showing lobules of alveoli protruding from the ends of the ducts and along the tertiary ducts. (X20)

FIGURE 3: Whole mount mammary gland preparation from a non-pregnant, multiparous, 8 to 14 month old female. The duct system is fully developed with vestiges of alveolar lobules along, and at the ends of, tertiary ducts. Hyperplastic alveolar nodules (HAN) are seen scattered throughout the gland (Ni, nipple). (X5)

FIGURE 4: A hyperplastic alveolar nodule. (X20)

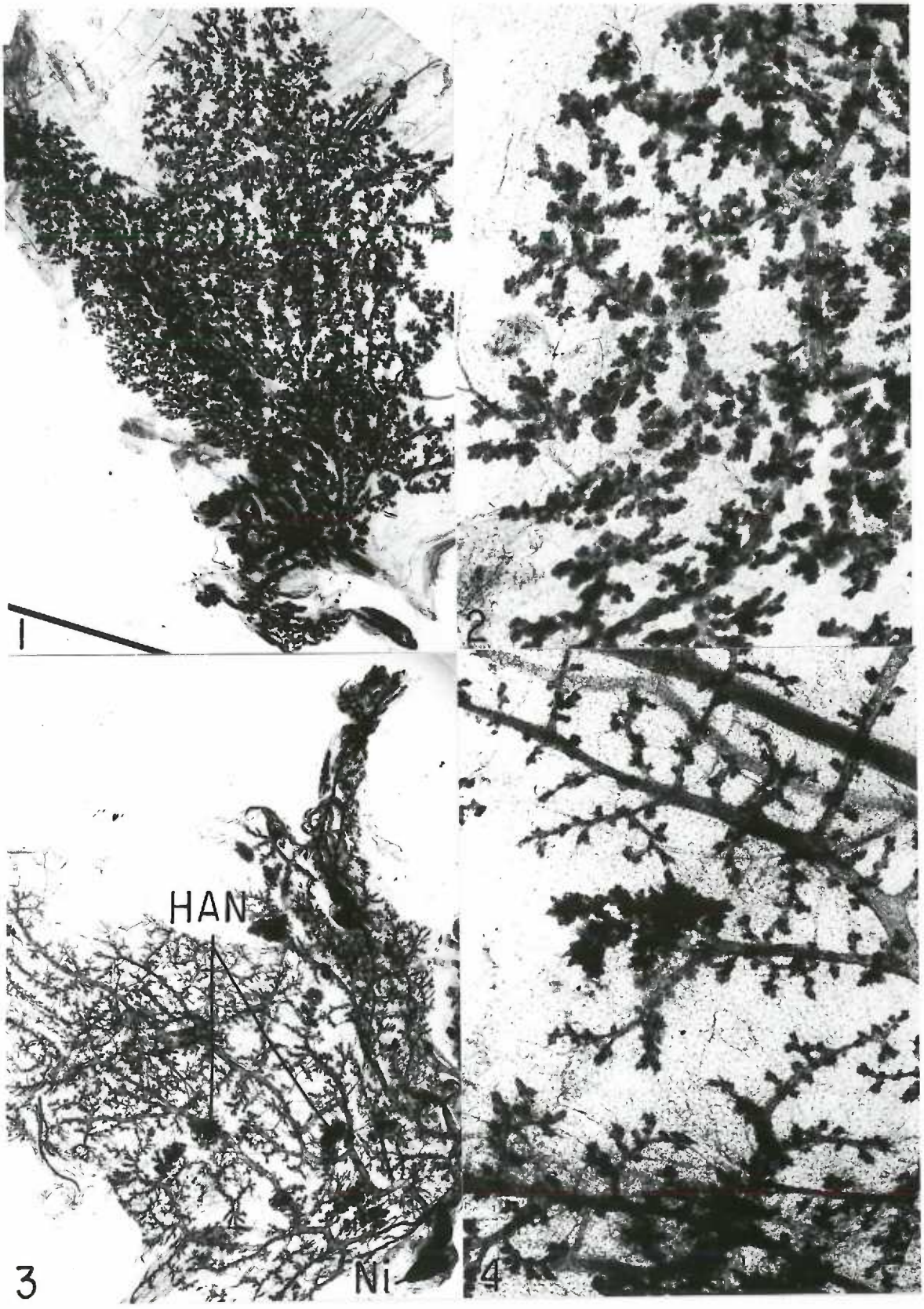
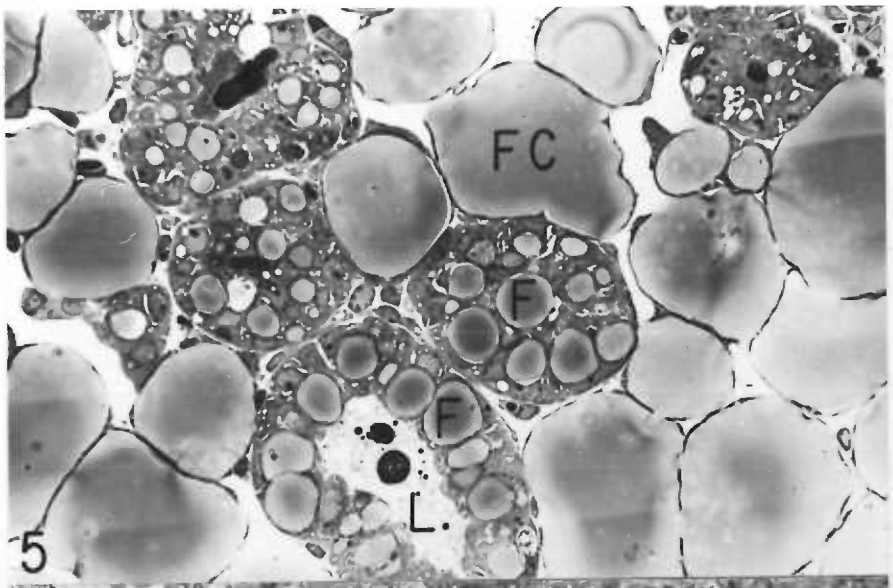

1
2
HAN
3
Ni
4

FIGURE 5: Prelactating mammary gland. The fat cells (FC) are admixed with the mammary gland elements. (F, fat droplet; L, lumen.) (X400)

FIGURE 6: Hyperplastic alveolar nodule. The alveolar lumina (L) are dilated in a secretory state. Fat cells (FC) are seen at the edge of the nodule. (X400)

FIGURE 7: Mammary adenocarcinoma. Although the cells are more closely arranged, there is lumen (L) formation. (C, capillary.) (X400)



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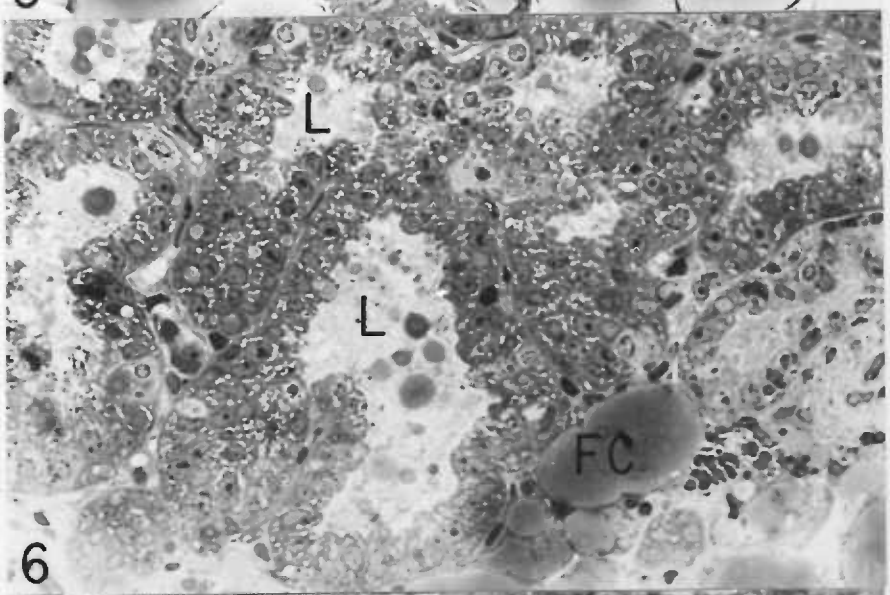

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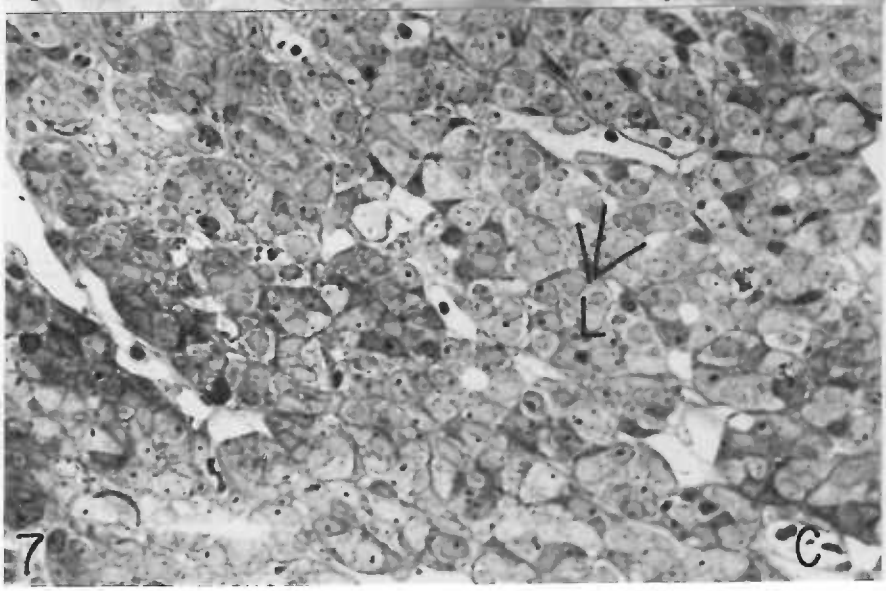
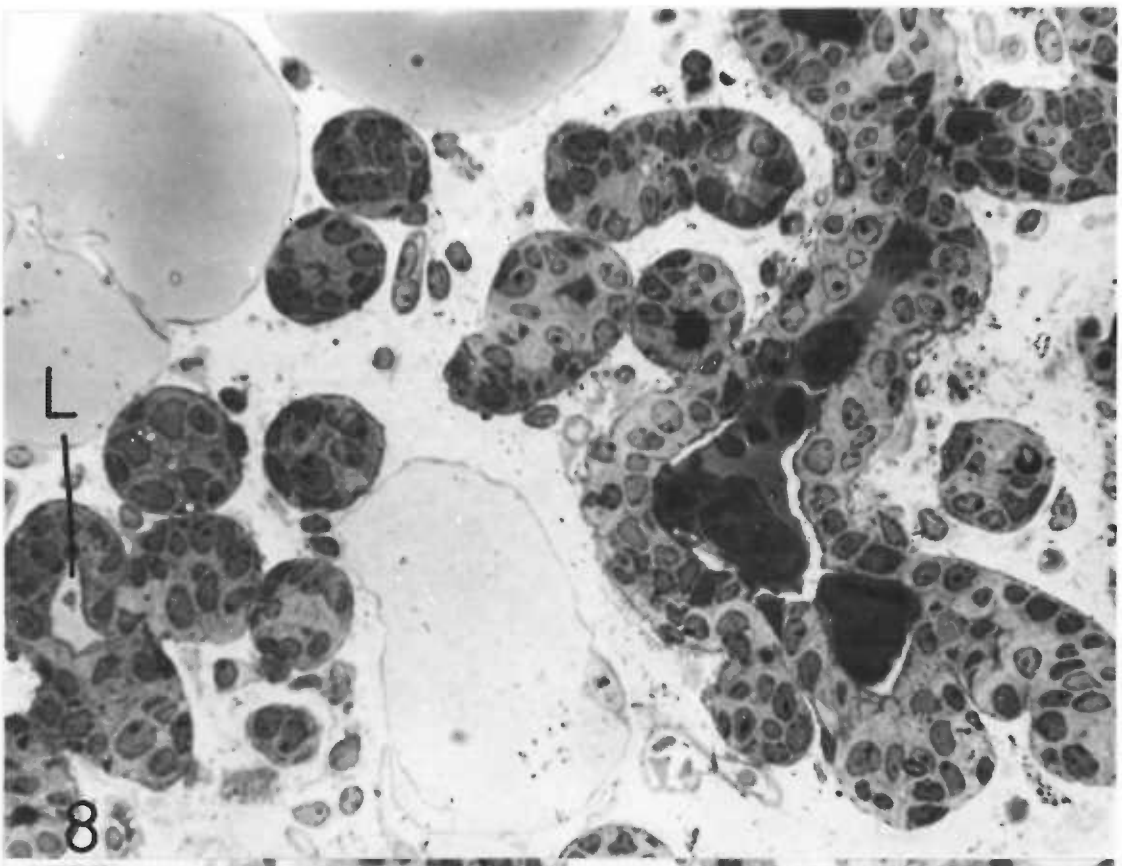


7

FIGURE 8: Nodule outgrowth. Admixed among the fat cells of the mammary fat pad are alveoli, some of which are dilated with inspissated secretory material. (X508)

FIGURE 9: Adenocarcinoma which arose from a nodule outgrowth. Among the tumor cells are numerous abortive alveoli with lumina (L). (X508)


L
8

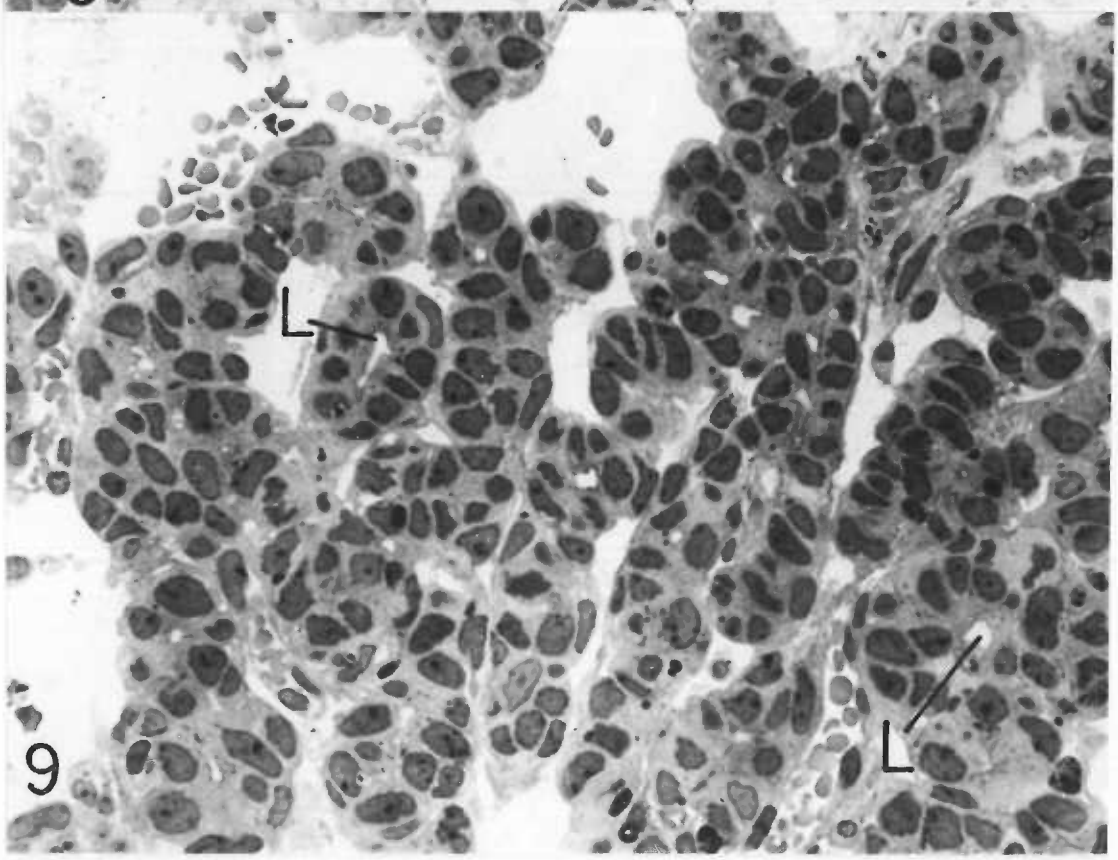

L
L
9

FIGURE 10: Mammary adenocarcinoma with alveolar formation. The reaction product (RP) indicates alkaline phosphatase activity within the lumina and along the basal and lateral external cell membrane. (X400)

FIGURE 11: Mammary adenocarcinoma demonstrating the inhibition of alkaline phosphatase activity by L-cysteine. (X400)

FIGURE 12: Mammary adenocarcinoma with ATPase reaction product within lumina and along the basal and lateral external cell membranes. (X400)

FIGURE 13: Mammary adenocarcinoma exposed to L-cysteine as the tissue in Figure 11; however, the L-cysteine had no inhibitory effect on ATPase activity. (X400)

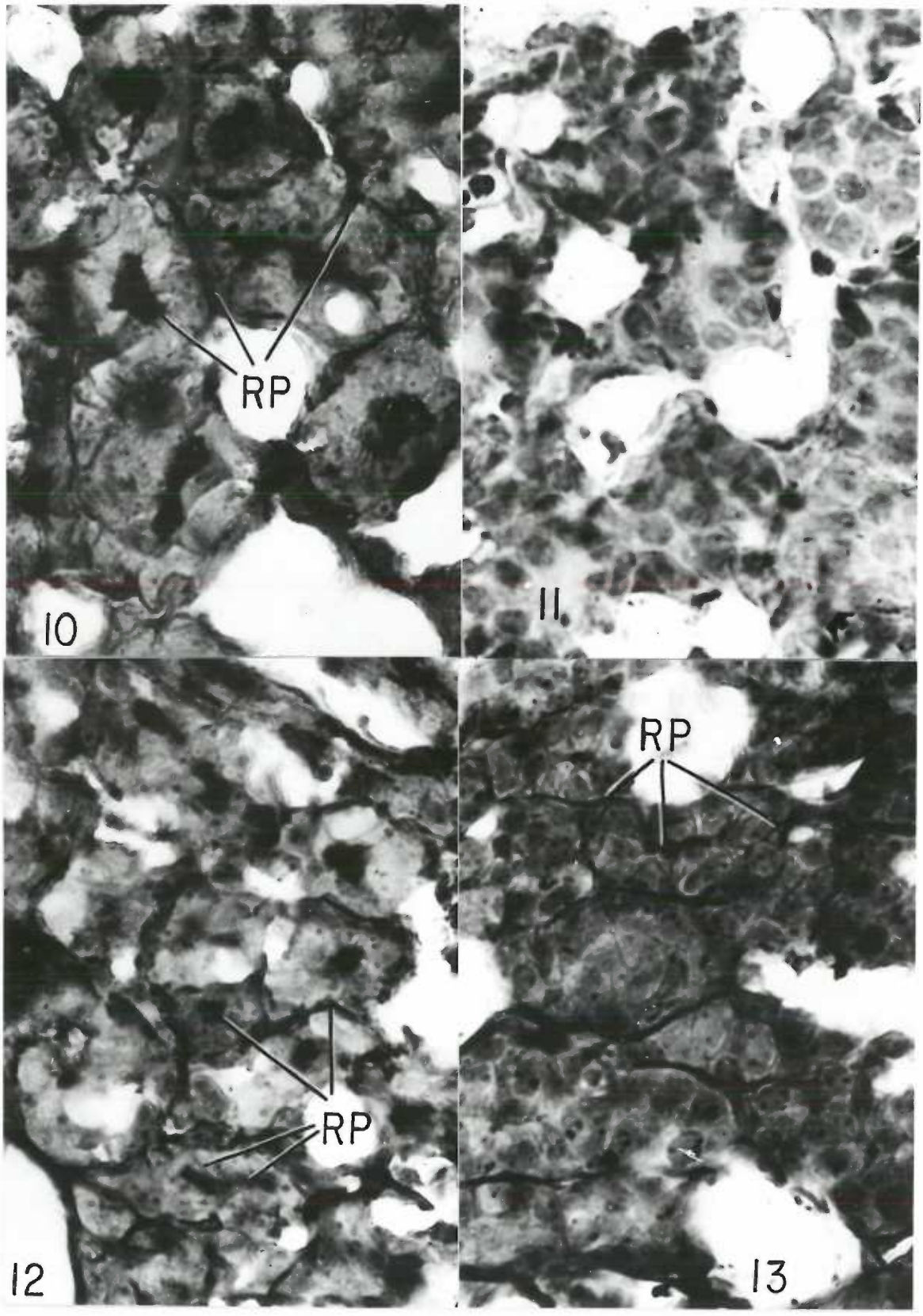

RP
10
11
RP
RP
12
13

FIGURE 14: Prelactating mammary gland with the apical microvilli (MV) lining a central lumen. As is characteristic of prelactating epithelium, milk protein droplets (MPD) in the apical cell region and fat droplets (F) are present. Reaction product is present along the lateral cell membrane and diffusely distributed throughout the basilar stroma. There is no reaction product within the lumen or on the apical cell surface. (X5800)

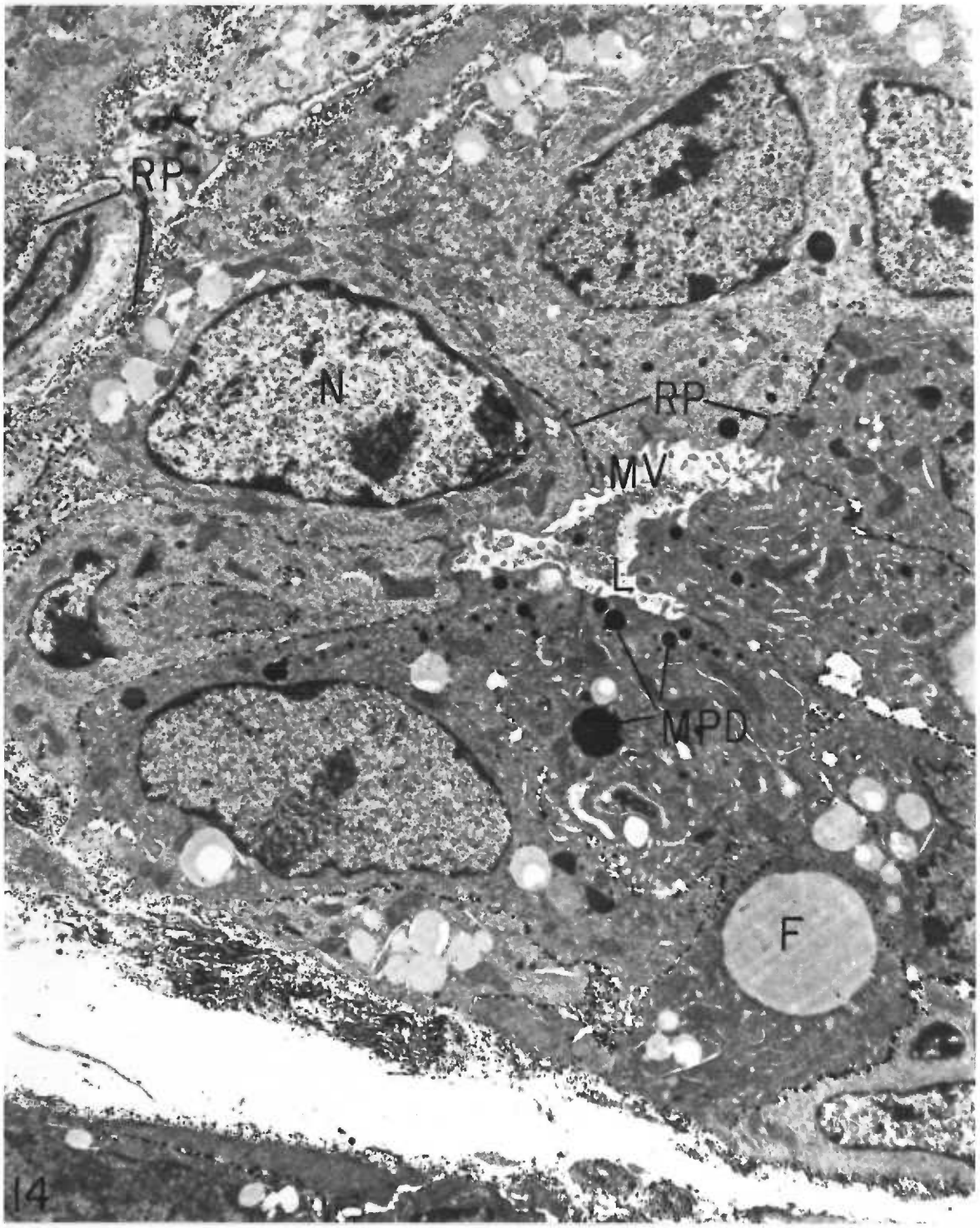

RP
N
RP
MV
L
MPD
F
14

FIGURE 15: Hyperplastic alveolar nodule with radially arranged epithelium about a central lumen (L) containing milk protein droplets (MPD) and lined by microvilli (MV). Reaction product (RP) is along the lateral and basal cell membrane and absent from the lumen and apical cell surface. Numerous artefactual clefts are present. (N, nucleus.) (X11,600)

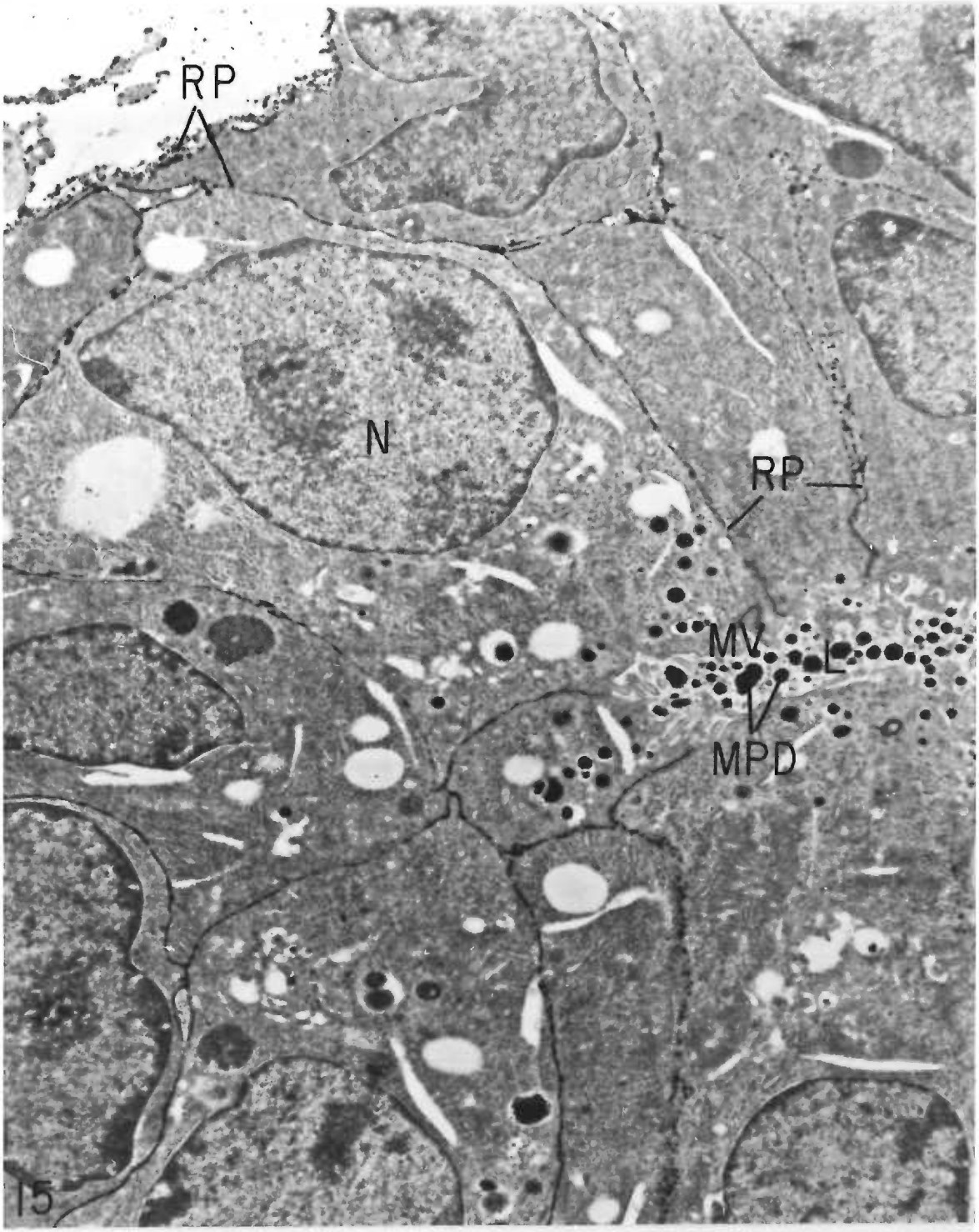

RP
N
RP
MV
L
MPD
15

FIGURE 16: The lumen (L) of a hyperplastic alveolar nodule at higher magnification. Note that reaction product (RP) is absent in this region of the tight junction (TJ) as well as absent from the apical microvilli (MV) and lumen (L). (X31,600)

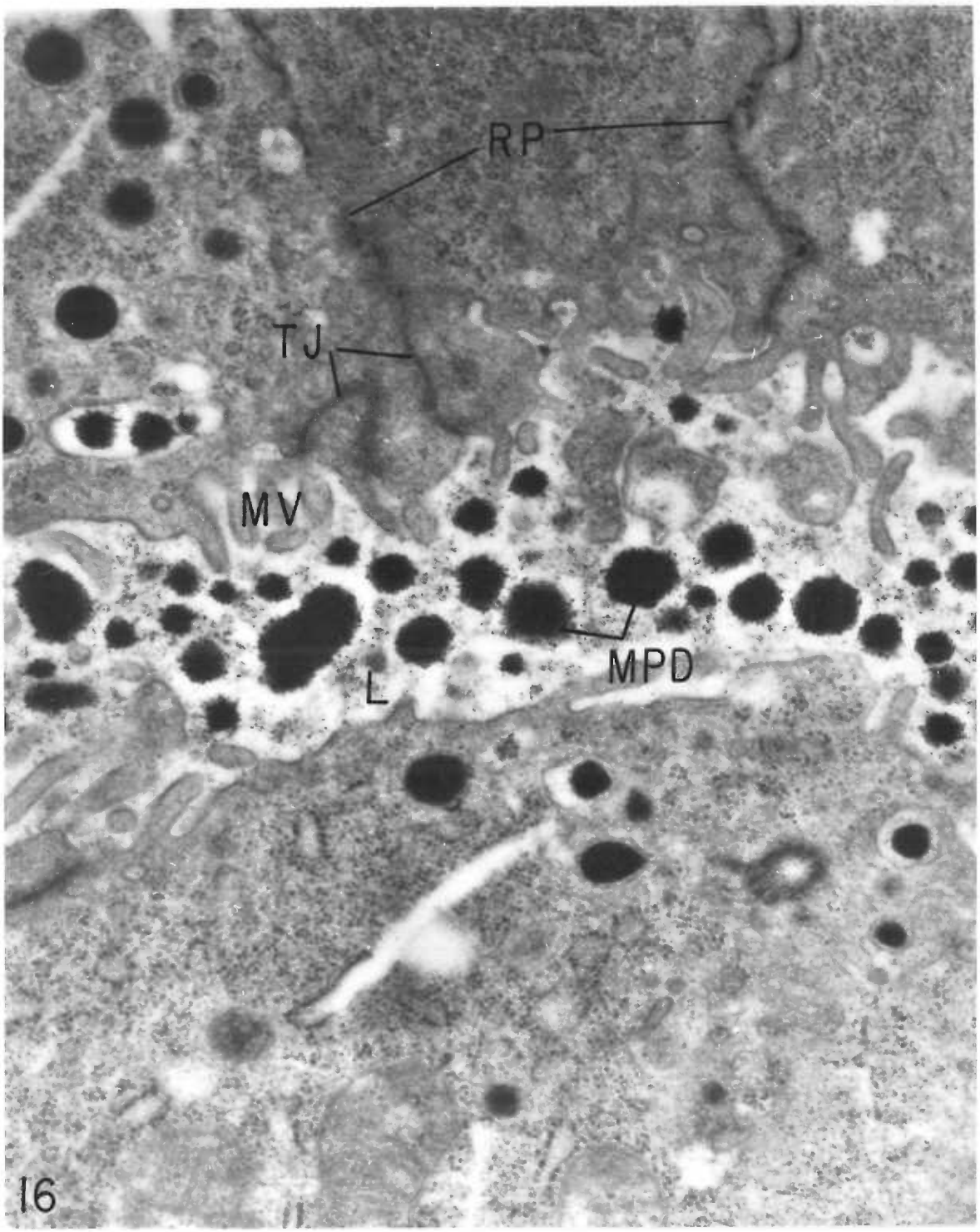

RP
TJ
MV
MPD
L
16

FIGURE 17: A spontaneously arising adenocarcinoma with reaction product (RP) within the lumen and associated with the microvilli (MV). Reaction product (RP) is present along the lateral cell membrane. Note the presence of virus-like particles (VLP). (N, nucleus.) (X16,400)

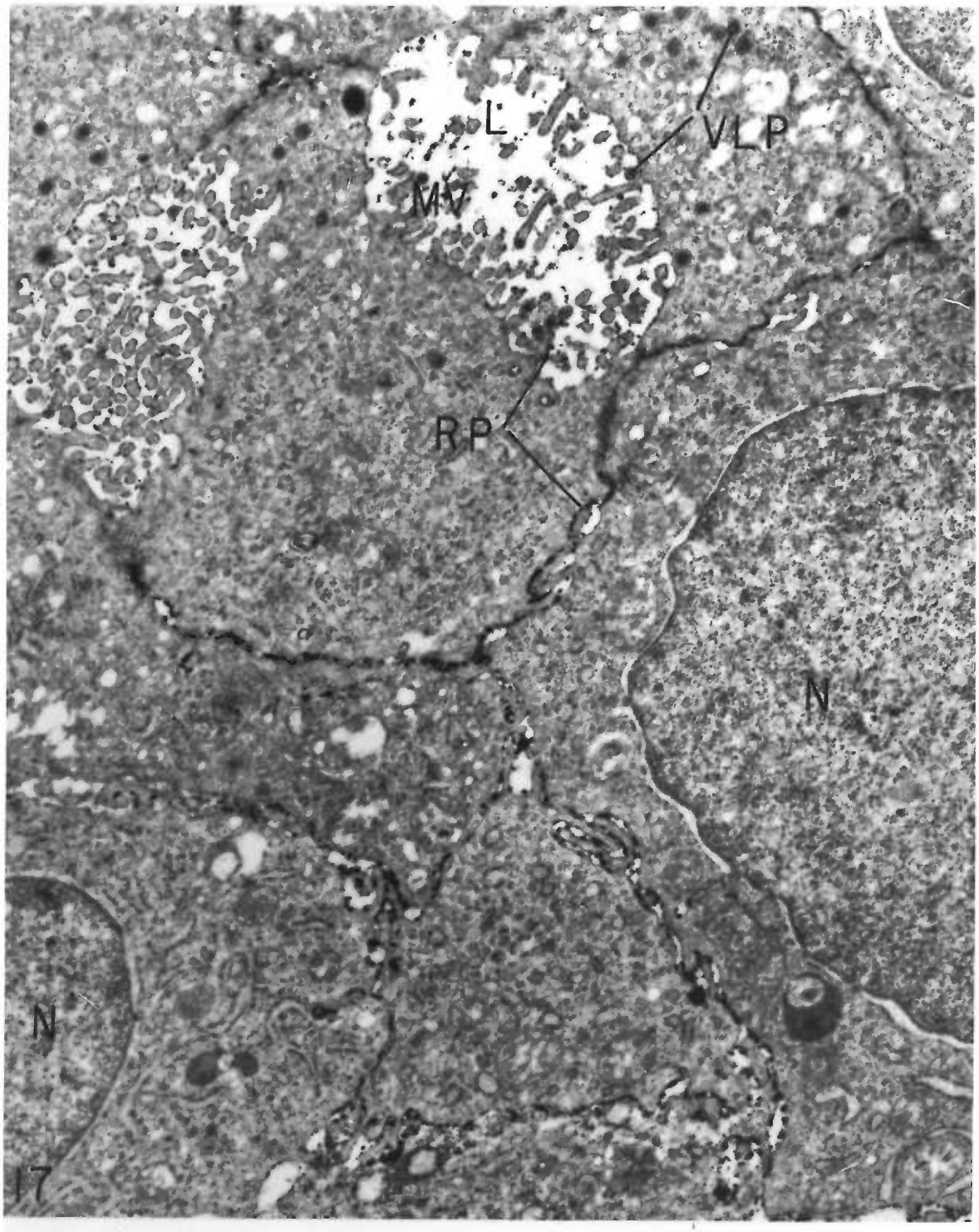

L
VLP
MV
RP
N
N

FIGURE 18: A spontaneously arising adenocarcinoma with reaction product (RP) on the microvilli (MV), within the lumen (L), and along the lateral cell membrane. Virus-like particles are circularly arranged or present singly in the cytoplasm. (N, nucleus; NC, nucleolus.) (X22,800)

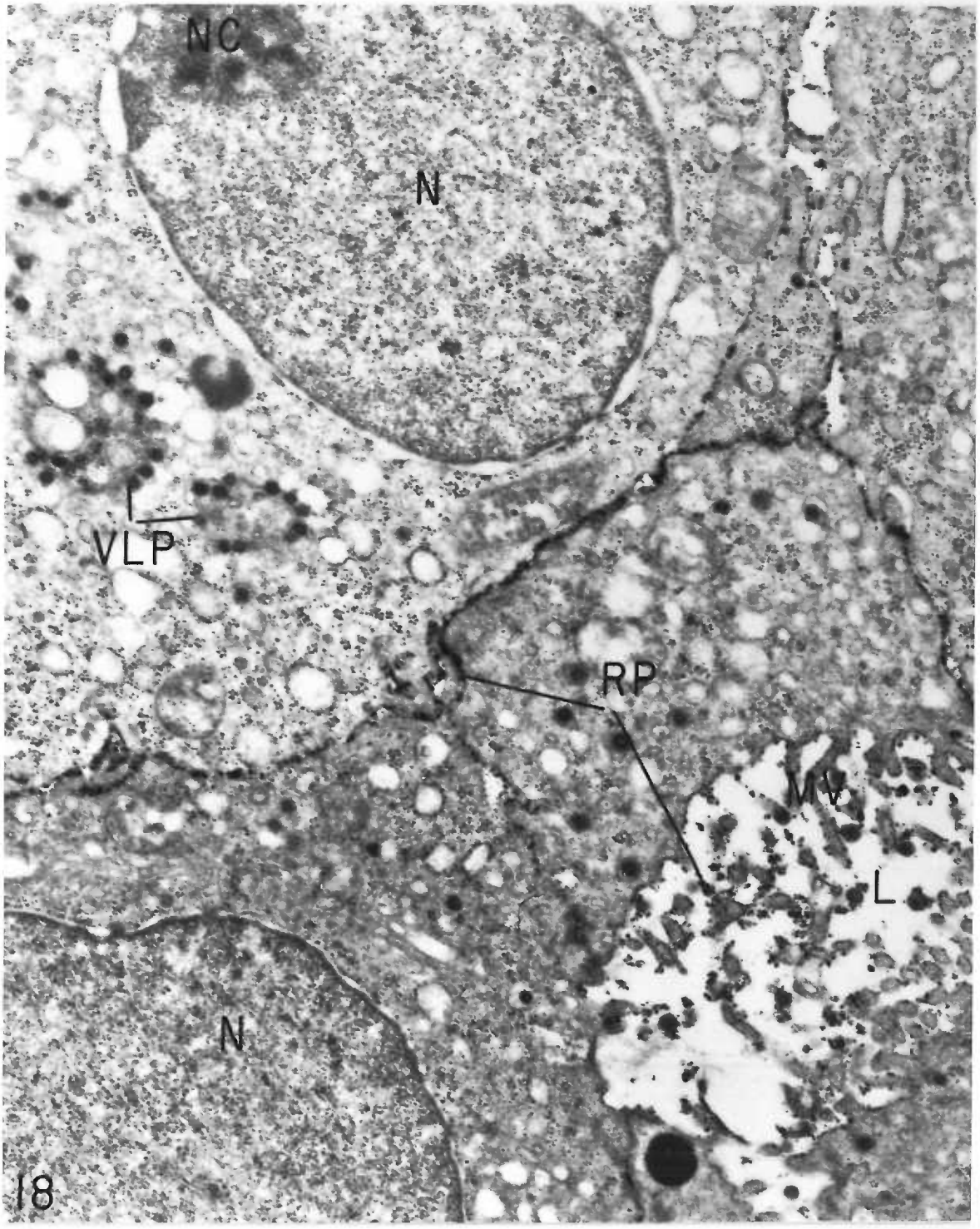

NC
N
VLP
RP
MV
L
N
18

FIGURE 19: A higher magnification of an adenocarcinoma lumen with reaction product (RP) scattered randomly along the microvillous surface. Virus-like particles (VLP) are both intracellular in microvilli and extracellular in the lumen (L). (X46,800)

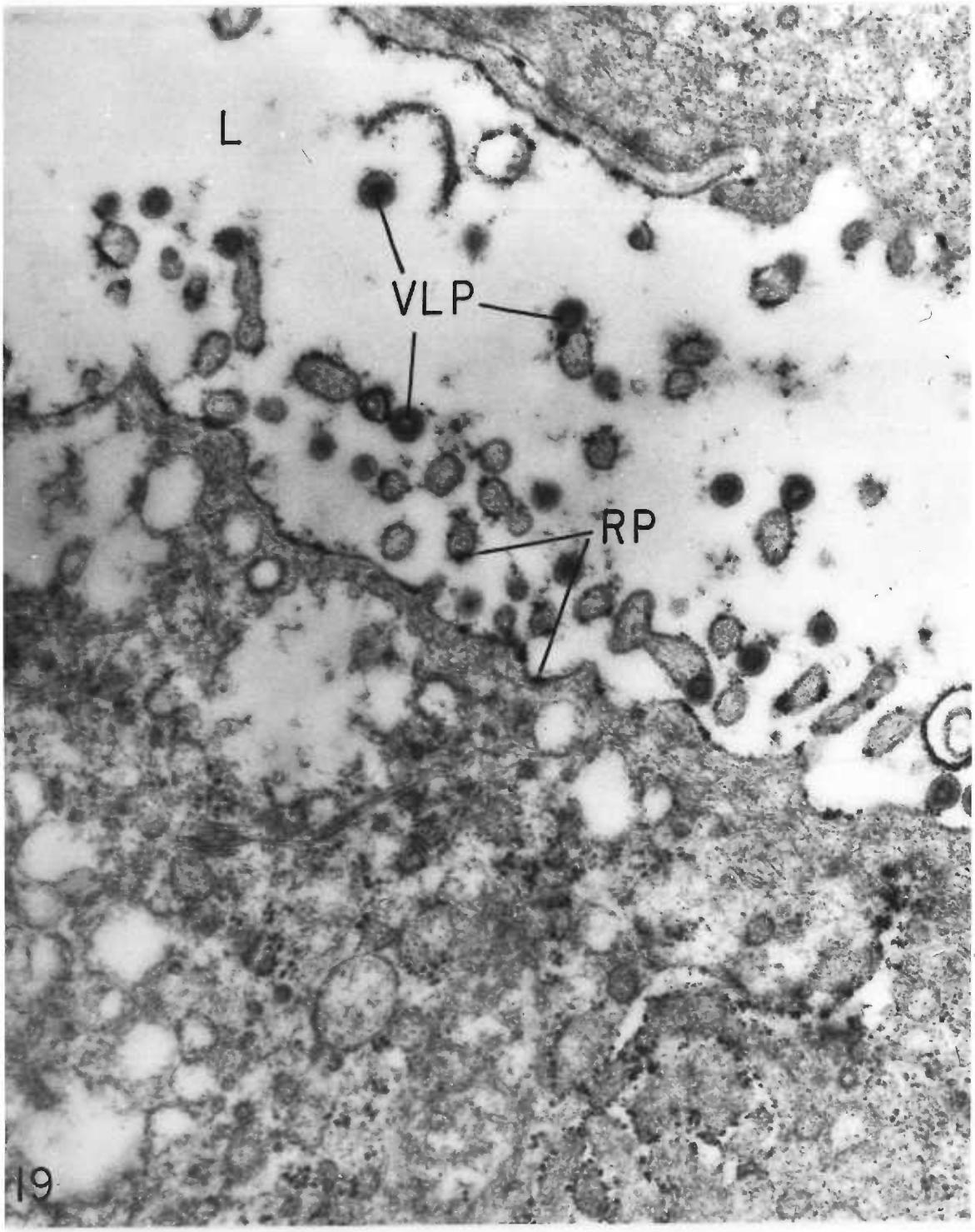
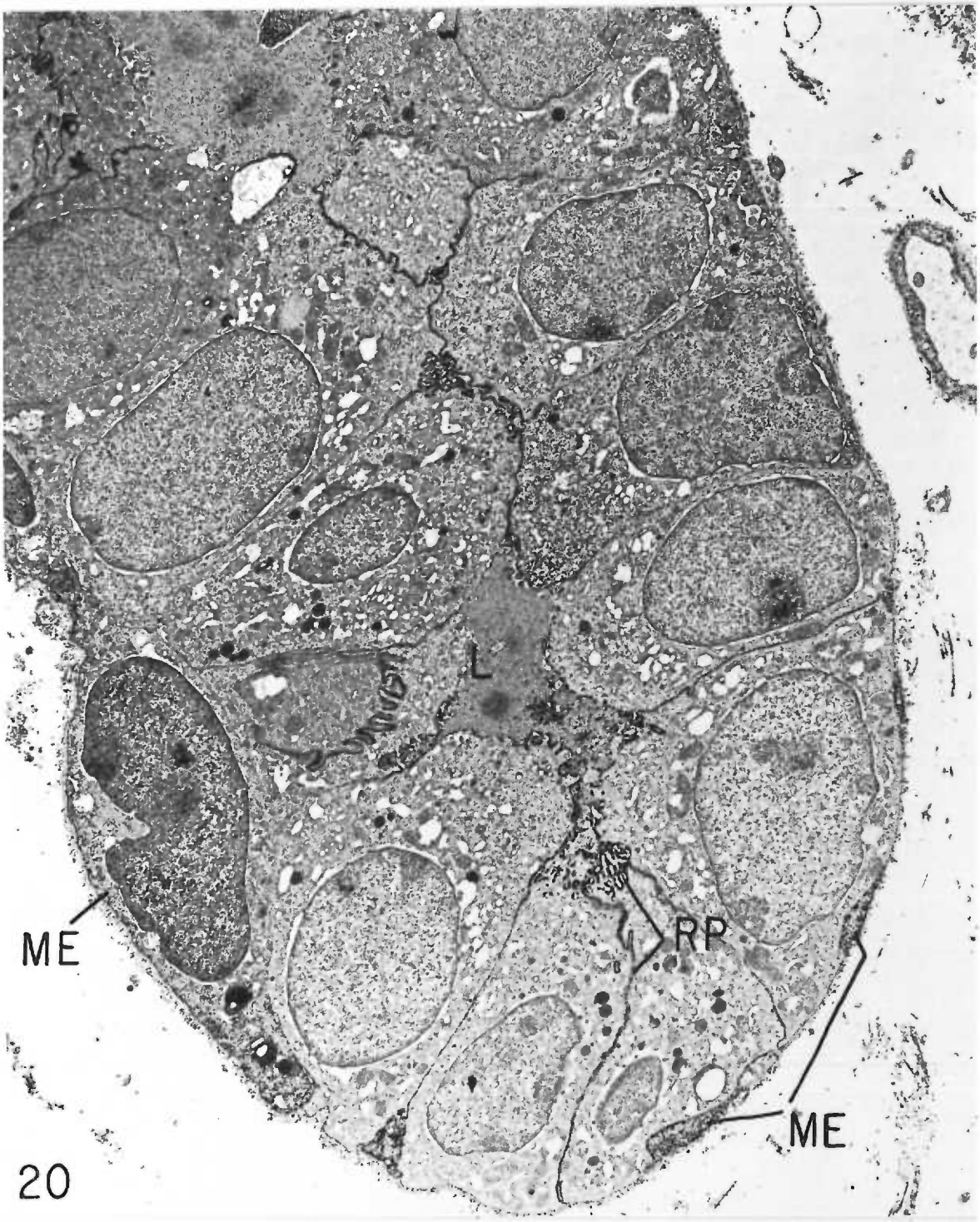

L
VLP
RP
19

FIGURE 20: A preneoplastic hyperplastic nodule outgrowth with reaction product (RP) on the external apical surface and within the lumen in contrast to the hyperplastic alveolar nodules examined. Myoepithelial cells (ME) have reaction product throughout the cytoplasm and nucleoplasm. The basket-like cytoplasmic processes (ME) of the myoepithelial cells are inconspicuous at the edge of the nodule outgrowth due to reaction product (RP) localization. (X6000)



20

FIGURE 21: A higher magnification of the lumen (L) of a preneoplastic hyperplastic nodule outgrowth. Note luminal virus-like particles with inconstant reaction product association. (X38,750)

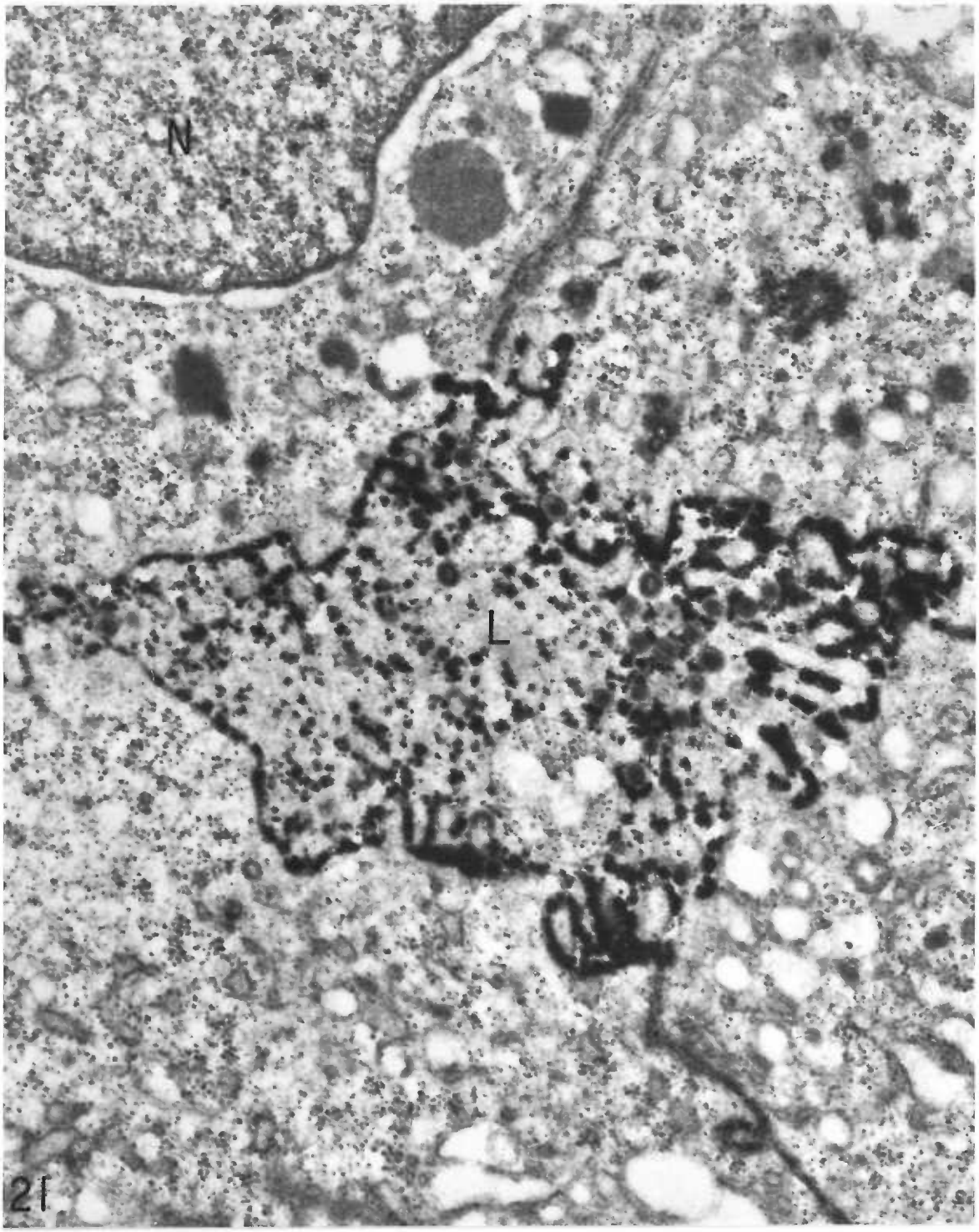

N
L
21

FIGURE 22: Adenocarcinoma arising from a hyperplastic nodule outgrowth. Reaction product (RP) is localized to external cell membrane including the microvilli. There are large collections of intracellular virus-like particles (VLP). (N, nucleus; NC, nucleolus; M, mitochondrion.) (X17,375)

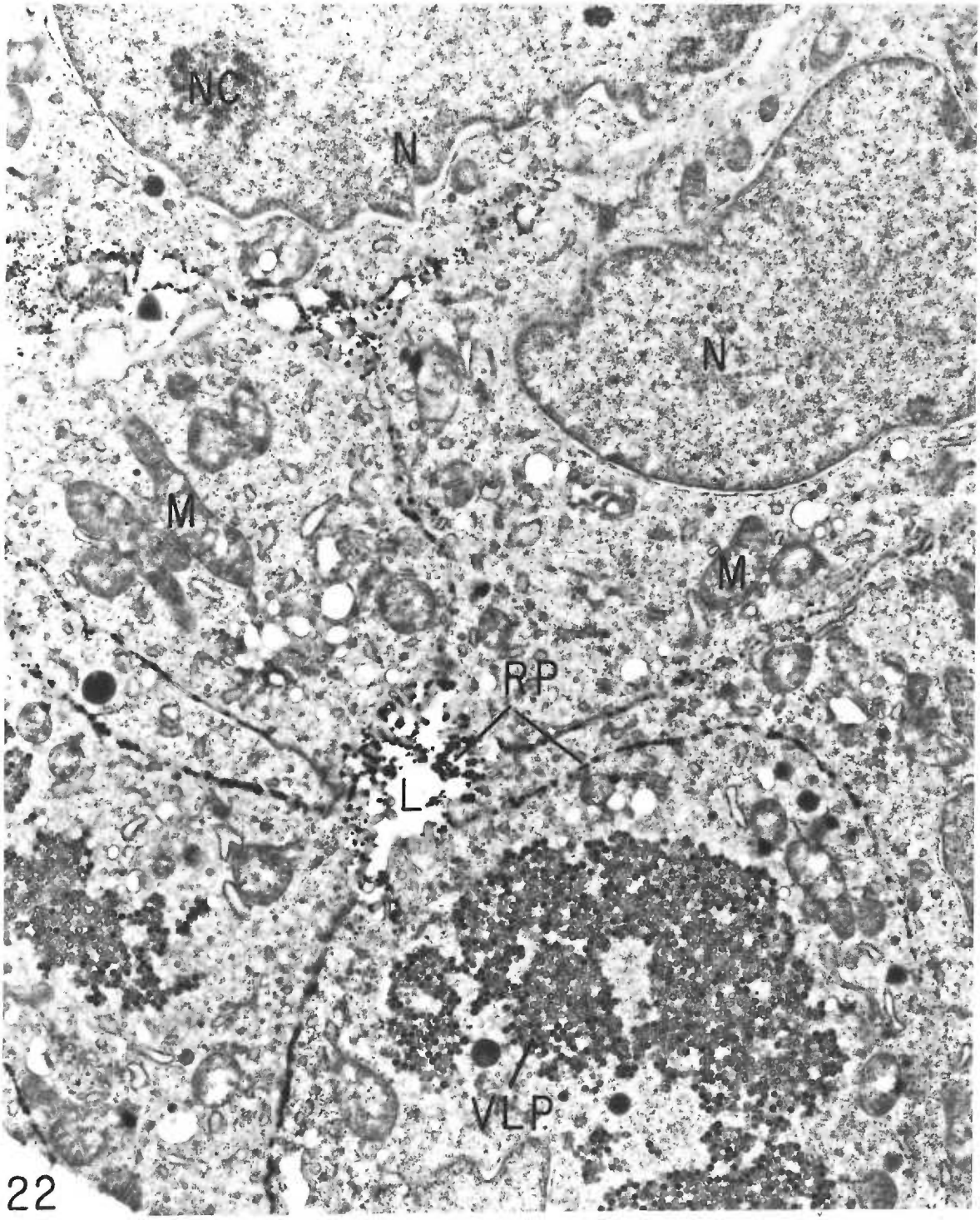


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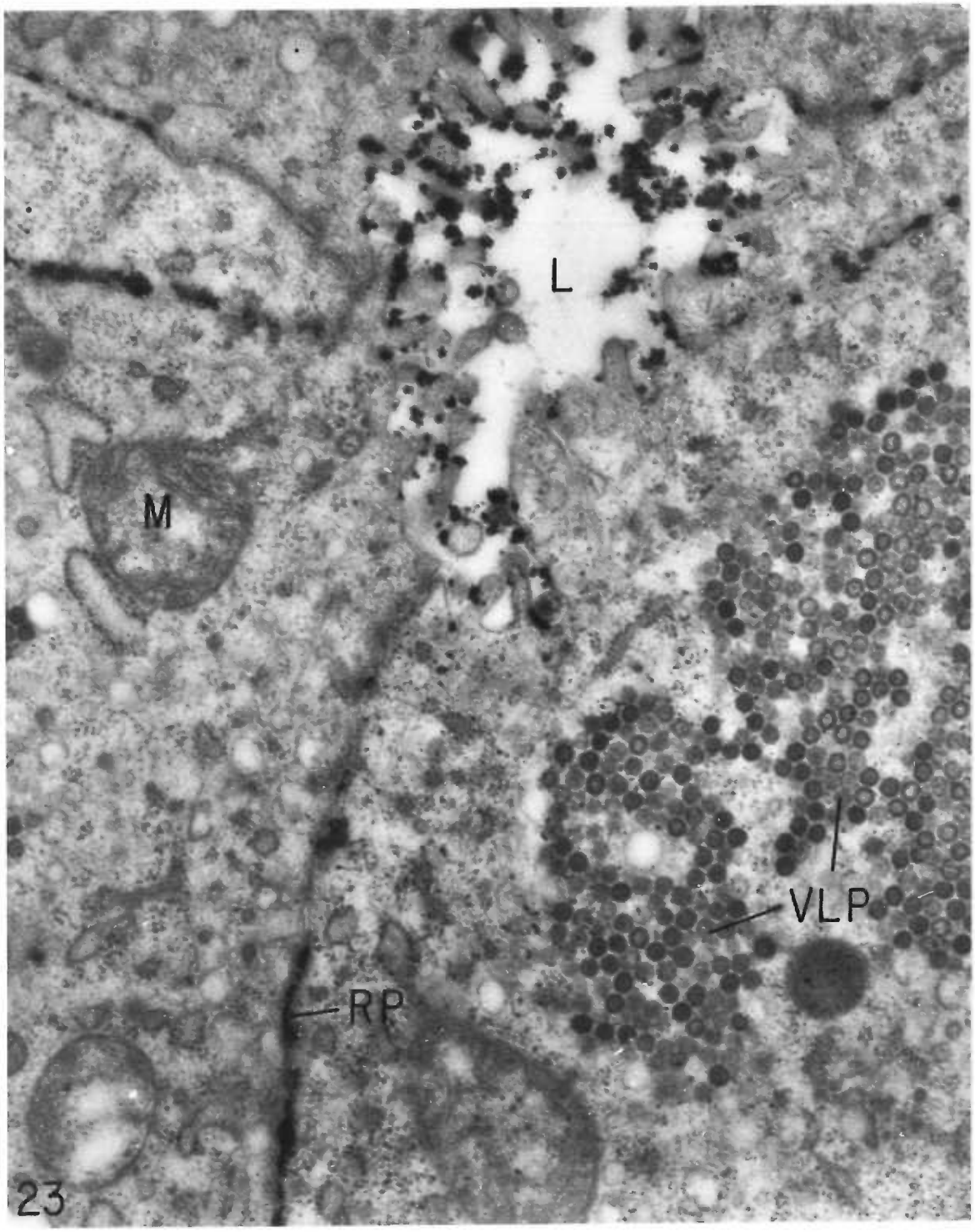
FIGURE 23: A higher magnification of an adenocarcinoma lumen (L) demonstrating abundant reaction product (RP) which is also along the lateral cell membrane. (M, mitochondrion; VLP, virus-like particles.) (X31,600)


L
M
VLP
RP
23