1972

An Ultrastructural Study of Palatal Fusion in Rat Embryos

William Michael Kelly
Loyola University Chicago

AN ULTRASTRUCTURAL STUDY

OF

PALATAL FUSION IN RAT EMBRYOS

by

William Michael Kelly

A Thesis Submitted to the Faculty of the Graduate School

of Loyola University in Partial Fulfillment of

the Requirement for the Degree of

Master of Science

June

1972

ACKNOWLEDGEMENTS

I wish to express sincere appreciation to all those who contributed so much to the success of this thesis.

Gratitude is expressed to Dr. Ravindra Nanda, whose unselfish contribution of time and knowledge to design and advise this study will always be remembered.

Thanks is given to Dr. Donald C. Hilgers for the opportunity to receive an education in orthodontics, and to Dr. Gustav Rapp and Dr. John Madonia for making graduate study in the field of oral biology possible.

Warmest appreciation is given to my parents, for without their devotion, support, and guidance my education would not have been possible, and to my wife, whose understanding and encouragement made possible my graduate training.

Special thanks is given to Mrs. B. Prapuolenis for her unselfish assistance with the electron microscopic portion of this thesis.

AUTOBIOGRAPHY

I was born on September 16, 1945, in St. Louis, Missouri. My family moved to Taylorville, Illinois, when I was two years old. I received my elementary and high school education there, graduating from Taylorville High School in May, 1963.

For my pre-dental education I attended the University of Illinois at Urbana, Illinois, until June, 1966. I then attended Loyola University School of Dentistry, Maywood, Illinois, receiving my D.D.S. degree in June, 1970.

I started my graduate work in orthodontics and oral biology in June, 1970. After receiving a certificate of speciality in orthodontics and a Master of Science degree in oral biology, I will spend a two year tour of duty in the United States Army.

TABLE OF CONTENTS

INTRODUCTION 1

I. REVIEW OF THE LITERATURE 2

II. MATERIALS AND METHODS. 14

III. OBSERVATIONS 16

A. Light microscopic findings 16

B. Electron microscopic findings 28

1. Pre-fusion stage 28

2. Fusion stage 30

3. Breakdown of epithelium with mesenchymal penetration 31

4. Electron micrographs 33

IV. DISCUSSION 66

V. CONCLUSION 70

VI. SUMMARY 71

BIBLIOGRAPHY 72

INTRODUCTION

The normal fusion of the palatal shelves of the secondary palate is of interest for several reasons. An understanding of normal development of the palate is essential to investigate developmental defects such as cleft palate. Furthermore, studies concerning the degeneration of the palatal epithelium during the fusion process are important as an essential part of normal morphogenesis.

An investigation of the sequence of epithelial-mesenchymal fusion of the embryonic palatal shelves at the ultrastructural level would shed a refined light upon the development of the secondary palate. This study was conducted with the above considerations in mind.

REVIEW OF THE LITERATURE

Studies concerning the fusion of the secondary palate have encompassed many different approaches. Before fusion or closure begins, the palatal shelves are in a vertical position, hanging down from the maxilla, lateral to the tongue. The mechanism of closure, or reorientation of the shelves, has been explained in many ways. Walker and Fraser (1956) first proposed that there was an "internal force" in the palatal shelves which gradually increased in strength until it drove them to a horizontal position. When the tongue was experimentally displaced in living embryos at the appropriate state of development the palatal shelves moved from vertical to horizontal in a few seconds. This movement could be reversed by merely pressing the mouth closed again. In fact the shelves could be induced to move from the vertical to the horizontal position repeatedly, but they did not assume and maintain an intermediate position. Histological examination by routine methods revealed no unusual structural specialization of the palatal epithelium or mesenchyme that could account for the rapid change of position observed. They also tried numerous histochemical stains. It was reported, on the basis of metachromatic staining with toluidine blue, that acid mucopolysaccharides were present throughout the connective tissue of the palate. It was suggested that this might reside in a network of elastic fibers when the aldehyde fuchsin-positive

material was found in the same area.

The gradual buildup of acid mucopolysaccharides in the ground substance of the palatal shelves just before closure was shown by Larsson et al. (1959), Larsson (1960), and Walker (1961). They studied the developing palate with various histochemical and autoradiographic techniques. Following their report that acid mucopolysaccharides were present in the palatal shelves at the time of closure, interest focused on the synthesis and metabolism of these materials in the ground substance of the shelves, and their possible role in the expression of an internal force.

Larsson (1962) showed a diminished content of acid mucopolysaccharides in palatal tissues of embryos with cortisone induced cleft palate. However, Nanda (1970, 1971) stated that the ^{35}S-sulfate uptake in the different groups of clefts induced by cortisone, vitamin A, and a combination showed no positive co-relation between the occurrences of cleft palate and disturbed mucopolysaccharide metabolism. It was assumed that reduced uptake of ^{35}S-sulfate leads to cleft palate; but if this was so, it was not clear how increased uptake of ^{35}S-sulfate also leads to cleft palate. The relation of disturbed acid mucopolysaccharide metabolism and cleft palate was further confused by the finding that ^{35}S-sulfate uptake in the palatal processes of the offspring treated with vitamin A and cortisone was significantly greater than that in controls.

Interestingly enough, Nasjleti et al. (1969), when comparing cortisone induced clefts with normal fusion, found no change in mice chromosomes

to perpetuate the hereditary theory for clefts. The chromosomal patterns in cells from the palatal mucosa of the secondary palate of normals did not differ from the pattern seen in cells derived from the epitheliums of the cortisone-induced cleft palate shelves. The only variations seen were those attributed generally to murine-type cells and to artifacts arising during chromosome preparations.

The correlation between the amount of acid mucopolysaccharide present in the palatal shelves and their ability to build up an internal shelf force, and effect closure, is tentatively accepted as causal by some investigators. Others hold reservations based mainly on experiments done by Walker (1956, 1961, 1967) on shelf movement in embryos that had been incubated for up to two hours in distilled water, hyaluronidase, 70% alcohol, or weak solutions of acid or base. None of these agents inhibited shelf movement in a specific way, yet some of the treatments should have had an effect on the mucopolysaccharides present in the palatal shelves. This suggests that some other mechanism, possibly a mechanical one, is responsible for providing the internal shelf force.

Harris (1964) reported that the cranial base of mouse and rat embryos was flexed at the onset of palate closure, and that this flexure was gradually reduced as morphogenesis of the palate progresses, until finally, when the palatal shelves were fused, the cranial base was straight. He felt that the process of palate closure involved a change in spatial relation between the palatal shelves. He suggested that the range of movement possessed by an embryo might determine its susceptibility to

cortisone-induced cleft palate, since cortisone reduces the amount of amniotic fluid and causes constriction of the embryo. Fraser (1967) confirmed the work by Harris that maternal treatment with cortisone reduces the volume of amniotic fluid. But by using a dose of cortisone that causes cleft palate in about half the embryos in a litter and comparing the amount of amniotic fluid in normal and cleft-palate embryos they were able to demonstrate that decreased amniotic fluid volume was not related to cleft palate, since the amount of decrease was the same in embryos with and without clefts.

Verrusio (1970) created a mechanical model to illustrate the phenomena that the change of flexure of the cranial base provides the internal force. The model not only imitated the morphogenetic movements of the palatal shelves very closely, but also suggested why none of the noxious agents used by Walker (1961) to interfere with shelf movement had any effect. If the internal shelf force was the result of a mechanical stress placed on the shelves by stretching their attached edges, nothing, short of fixing the tissue, or otherwise removing its pliability, should affect shelf movement. The mucopolysaccharides present in the shelves at the time of palate closure was suggested to serve as a flexible structural support within the shelves on which the tension created by the straightening of the cranial base may act.

Lazzaro (1940) proposed three possible methods for shelf elevation: external force by the tongue, a rapid rotation of the shelves due to some intrinsic force, or a regression of the vertical and an outgrowth of the horizontal portions.

Although the tongue has been discussed as a possible force for providing elevation of the palatal shelves, evidence has not been presented to support the theory. Moriarty et al. (1963) have shown that elevation may occur with the tongue absent. Orban (1957) suggests the rotation theory, occurring due to a rapid proliferation of cells on the lateral surface of the vertical palatal process. Coleman (1965) has described a medial process that projected from the vertical palatal shelf of the 16-day rat fetus and further reported that the processes of the right and left palatal shelves had approximated and fused by the 17th day.

Veau (1938), Steinger (1939), and Tondury (1961), all relate the etiology of cleft palate to the imperfect fusion or degeneration of the midpalatal lamina. They consider that the process of formation and involution of the epithelial sheet plays a vital role in the correct effectuation of the process of fusion of the secondary palate. Only its formation and subsequent involution, characterized first by its thinning and fragmentation and then by disappearance of the epithelial rests, can result in direct continuity of the mesenchyme of the two opposing palatal shelves. Incomplete epithelial fusion or abnormal persistence of the epithelial sheet might, according to these researchers, be among the causes of cleft palate.

Hughes et al. (1967) found no evidence to support any of the above theories concerning shelf fusion. In the 16-day rat fetus, the palatal shelves were suspended in a vertical direction. There was no evidence of either a rapid proliferation of cells or a projection of a medial

process from the palatal shelf. They state that the rat might possibly be reevaluated as an experimental animal for cleft palate studies due to the apparent different formation and destruction of the midpalatal epithelial lamina as compared to humans.

Whatever the method for closure, the fact still remains that the secondary palate must progress through two events, namely the convergence of the shelves and their fusion.

Recent investigators have been concerned with the fusion, more than the mechanism of shelf movement. This fusion has been viewed as a sequence of four interdependent events by Pourtois (1968) who showed that the formation of a "zone of stickiness" by the cell layers differentiating at the edge of the shelves; fusion of these differentiated epithelial cells leading to the formation of a laminated wall of epithelium between the shelves; rupture permitting mesenchymal intervention; and degeneration of all remaining epithelial cells marking the completion of the fusion process. Hughes et al. (1967) showed all of the epithelial remains are rapidly resorbed in rats. They state that the degeneration may be related to the apparent absence of a basement membrane adjacent to the epithelial lamina. Concurrent with this lack of a definite basement membrane, the mesenchymal cells were aligned parallel to the lamina. This was followed by both cytoplasmic and nuclear degeneration of the epithelial cells. Thus, with the lack of a basement membrane, the epithelial lamina was susceptible to the undetermined influences of the mesenchymal cells leading to their breakdown and disappearance.

Anderson and Matthiessen (1967) showed that in humans after the fusion between the palatal processes and between the palatal processes and the nasal septum, the junctional epithelium is disintegrated by histiocytes. Bergengrum (1909) and Peter (1924) have reported the persistence of epithelial pearls in human palates. Peter discussed the origin and significance of these epithelial remnants which appear in the embryonic connective tissue.

Wood and Kraus (1962) also demonstrated these epithelial pearl remnants in human palates. An interesting phase of differentiation in the hard palate was the development of cornified epithelial pearls, formed originally by inclusion of epithelium in the connective tissue of the midline at the time of shelf fusion. Specimens of progressively older ages showed increasingly greater size of these structures.

Orban (1957) has reported that epithelial pearls found in the midline were the result of persistence of epithelial remnants in the line of fusion of the palatal processes. These pearls in human palates are evidence of incomplete destruction of the epithelial lamina. Sicher (1966) attributed the epithelial remnants responsible for developmental cysts found in the palatal midline in man.

Farbman (1968) observed by electron microscopy signs of autolysis in the epithelial wall before the basement membrane ruptures. These were electron dense bodies. Such a phenomena would lead to a breakdown of the epithelial wall and subsequent palatal fusion. Angelici and Pourtois (1968) observed the electron dense bodies with customary histological

staining procedures. The same were found by Mato et al. (1966), Smiley and Dixon (1967), and Farbman (1968). Angelici and Pourtois (1968) also demonstrated the presence of a hydrolytic enzyme, and acid phosphatase activity, in the same epithelial layers. This probably indicated the presence of secondary lysosomes as shown by de Duve (1966). In the secondary palate the association of secondary lysosomes and acid phosphatase took place at first in the epithelial wall, even before the wall disintegrated. This was an indication that the enzymatic agents of epithelial breakdown were located in the cells of the seam and that one was observing a process of autophagy (de Duve, 1966). As for the action of the histiocytes from the mesenchyme, it appeared to be a secondary one, involved in the elimination of the debris of the previously disintegrated epithelial wall. Autolysis was thus observed to be contemporaneous with epithelial fusion in the case of normal palatal fusion.

Angelici (1966) stated that the absense of acid phosphatase activity in the epithelial wall should be considered as a pathological situation which eventually leads to the reopening of the seam and finally resulting in the formation of a cleft. Angelici and Pourtois (1968) demonstrated in the case of incomplete and temporary fusion, such as the eyelid, the lack of acid phosphatase or autolytic activity. In the fusion of the eyelids, the peridermal cells trapped in the epithelial wall did not immediately degenerate. Since they remained in place, the epithelial seam could persist during the prenatal period.

Mato et al. (1965) utilizing the electron microscope, found various

types of lysosomes appearing in the epithelium covering the tips of the lateral palatine shelves at the stage of preparation of attachment and fusion during a secondary palate formation. They stated that lysosomes eliminated useless products, cytoplasmic organelles, and cells. They indicated that the nucleus takes a significant role in the formation of a lysosome. Also the lysosome was found to be an autophagic vacuole and a residual body, as compared to two additional functional forms stated by de Duve (1963), that of a storage granule and digestive vacuole. In a later study, Mato et al. (1968) again showed electron bodies, appearing before the attachment of the palatine shelves, some of which were identified as lysosomes. The degenerated cells in the fused regions were ingested by mesenchymal cells in situ or excluded into newly formed oral and nasal cavities.

Mato et al. (1967) demonstrated the cell reaction of the nasal epithelium with palatal fusion. The cell reaction began at the early stage of approach to palatine shelves and had a tendency to expand to the whole surface of the presumptive contact regions. Further, the reaction was mainly composed of epithelial cells and partially by migration of mesenchymal cells, and characterized by the appearance of cytolysosomes in the epithelial cells.

Shapiro and Sweeney (1969) investigated the programmed cell death theory of the epithelial cells during fusion. They found a decrease in respiration in these cells as indicated by degradation of mitochondria and by reduction in staining for SDH, an enzyme of the citric acid cycle.

Also the basement membrane, perhaps in altered form, persisted until and during autolysis of the cells. (The name for the basement membrane most currently used is basement or basal lamina, as shown by Fawcett, 1962, 1966.) Cytochemical changes were noted in the ectodermal and neighboring cells about the time that key events in their differentiation have been said to occur. It was hypothesized that the transitory appearance of alkaline phosphatase in subjacent mesenchyme and decreased respiration in the ectoderm were crucial events in the differentiation of the cell type.

Farbman (1968) found the basal lamina not to be a continuous sheet prior to, during, or immediately after palatal fusion. If the basal lamina was fully intact, and the fusion did occur, epithelial cells adjacent to the intact basal lamina might not die but be retained by the organism as many epithelial islands or remnants, as also reported by Barry (1961) and Scott and Symons (1964).

According to Farbman (1968), at the time of fusion no membrane specializations were found on contacting surface cells of opposing palatal processes. There was evidence, however, of true adhesions between epithelia of opposing processes, but no extracellular sticky substance was demonstratable. Certain epithelial cells die, the surrounding epithelial cells engulf them, and they are destroyed by lytic enzymes within the vacuole, being replaced by amorphous material. In a later study, Farbman (1969) demonstrated again with electron microscopy the presence of large, dense acid phosphatase bodies.

Hayward (1969) found that the epithelium of unfused processes of

rats showed typical features of immature cells with many free ribosomes but little membrane in the cytoplasm. The cells gave a positive acid phosphatase reaction in the Golgi apparatus and in small basal bodies believed to be primary lysosomes. When the two palatal processes came into contact, desmosomes formed between the touching plasma membranes. Epithelial breakdown involved the formation of cytolysosomes, some of which contain acid phosphatase. The cytolysomes were believed to arise in part by ingestion of neighboring epithelial cells. The final destruction of the cells was by the action of macrophages.

De Angelis and Nalbandian (1968) found that the palatal shelves of mice and rats exhibited an irregular outer epithelial plasmalemma which became more regular just prior to contact. Desmosomes formed at the contacting shelf surfaces and appeared to bind the two new processes together until mesenchymal union was ultimately made. The basal lamina separating the epithelium from the mesenchyme remained intact and the underlying mesenchymal cells were unchanged until final epithelial cell disintegration occurred.

Smiley and Dixon (1968) found in the midline of the palatal fusion of mice the presence of different morphological types of relatively large dense electron dense granules. The epithelial seam was delineated on both sides by a typical basal lamina which was interrupted. Later palatal development showed epithelial breakdown and fibroblastic migration across the midline.

Brusati (1969) indicated a disparity in growth rates between the

mesenchymal and epithelial sheet. He showed the formation of desmosomes between the two opposed epithelia was fundamental to the process of epithelial fusion. The involution of the epithelial sheet was found to be due to both an autolytic process and mechanical factors. Brusati and Possenti (1969) have shown a significant reduction of mitotic activity in the epithelial sheet during its formation and reduction. Brusati and Miani (1968) described the spindle shaped rest cells of the epithelial sheet surrounded by mesenchyme. Intense phagocytosis surrounded the rest cells, often involving capillary vessels close to the clusters. The elimination phenomena of the epithelial cells was by the involution phenomena accompanied by the appearance of macrophages and culminating in the disappearance of all cell residue in the mesenchymal framework.

The present study will be conducted to investigate the normal fusion of the secondary palate of the rat at the ultrastructural level. An attempt will be made to demonstrate the epithelial pre-fusion stage, its fusion, the epithelial breakdown, and the consequent mesenchymal fusion of the two palatal shelves.

MATERIALS AND METHODS

Fifteen pregnant rats of the Sprague-Dawley strain were used in this study. The pregnant rats were obtained from Abram's Supply House, Chicago, Illinois. The rats were kept in a standard animal research laboratory environment and were fed with normal diets. Three pregnant rats were sacrificed on each day from the 14th through the 18th day. Day zero started when the vaginal plug was found. Prior to sacrificing the rats were anesthetised by ether inhalation, the abdominal cavity was opened and fetuses were obtained. The fetuses were given a careful visual examination for any possible malformation. The number of fetuses obtained from each mother ranged from eight to twelve. Very few resorbed fetuses were observed.

Three fetuses from each mother were immediately decapitated and the palatal area was carefully dissected by removing the mandible, floor of mouth, tongue, part of the top of the skull and brain. The palatal area was kept to a minimum volume for electron microscopic study. The dissection microscope was used for the younger fetuses. Fixing fluid was dripped on the specimens while these operations were being carried out.

An average of four fetus heads from each mother were fixed in 10% neutral formalin. Only the head including mandible and part of the neck was preserved and the rest of the body was discarded.

The electron-microscopic specimens were immersed in glutaraldehyde

2.5% in .1M cacodylate buffer with 4% dextran at pH 7.3-7.4. They were kept at 4°C for a minimum of two hours. They were then post-fixed in 2% osmium textroxide (OsO_4) solution buffered to pH 7.4 with Veronal acetate for 30-90 minutes. The tissue was washed in distilled water and then dehydrated with a series of increasing concentrations of alcohol rinses. After rinsing with propylene oxide, the tissue was embedded in Epon 812, mixed in the routine procedure with DDSA, NMA, and DMP 30. The embedded capsules were then placed at 60°C for approximately twenty-four hours.

Thin sections, approximately .075 microns, were cut on a Porter Blum MT-1 Ultra-Microtome. They were then mounted on copper grids and stained with uranyl acetate and lead citrate. The ultrastructure was then examined using a Zeiss EM 9-52 electron microscope.

Throughout the study the electron micrographs were correlated with light microscopy for orientation procedures. Some whole fetus heads which were randomly selected were embedded in paraffin, and stained with hematoxylin and eosin. Serial frontal sections were cut approximately six microns thick. Also semi-thin sections were cut with the microtome and stained with toluidine blue for help in orientation using light microscopy.

OBSERVATIONS

LIGHT MICROSCOPIC FINDINGS:

The frontal sections of 14 to 18 day old fetuses were studied under the light microscope to examine the sequence of fusion of the palatal shelves. The findings were also used for basic orientation of the specimens used for electron microscopic observations.

On day 14 the palatal shelves were found to be still vertically orientated at the lateral sides of the tongue which almost touched the nasal septum area. In 15 day old fetuses the shelves were slightly medially directed at their free ends and the tongue was slightly retracted inferiorly. On day 16 both shelves were horizontally positioned and faced each other. A varying amount of space was observed separating the two shelves on the 16th day. The tongue appeared completely ventral to the horizontally oriented shelves. On day 17 in all the embryos the palatal shelves were fused in the midline or were found to be in the process of fusion. In several fusion areas epithelial rests and strands were observed. Complete mesenchymal fusion was observed on the 18th day.

Various stages of the process of fusion and epithelial degeneration could be observed in several 17 day old fetuses.

A detailed description of light microscopic findings will be avoided here as it was intended only to study the stage of development of fetus palatal shelves related to the fusion sequence at an ultrastructural level.

Figures 1, 2, 3 and 4 show the different stages of palatal fusion in a single 17 day old fetus. Figures 5 and 6 show the area of fusion with epithelial cells undergoing degeneration with subsequent mesenchymal penetration.

FIGURE 1

Frontal section through the anterior one-third of the secondary palate of a 17 day old fetus. The palatal shelves (PS) are lying horizontal. Epithelial fusion with the nasal septum (NS) at the mesiodorsal side of the approaching palatal shelves could be observed. The tongue (T) is lying retracted on the ventral side of the palatal shelves. H & E, x40.

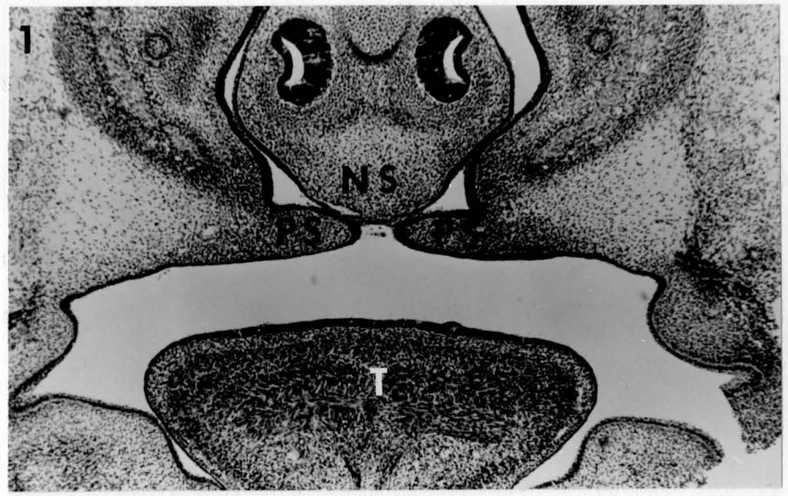


FIGURE 2

Frontal section through the middle region of the secondary palate of a 17 day old fetus. Note the epithelial fusion of the two palatal shelves (PS) and their fusion with the nasal septum (NS). H & E, x40.

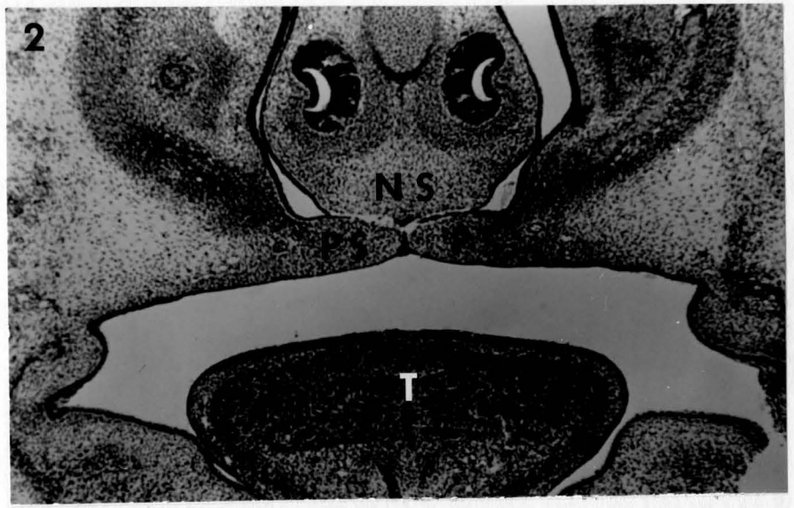


FIGURE 3

A higher magnification of the fusion area of the frontal section shown in Figure 2. The epithelial cells of the palatal shelves (PS) are in contact with each other. The epithelial fusion of the palatal shelves and the nasal septum (NS) can also be seen broken at two places indicating a start of mesenchymal fusion. H & E, x100.

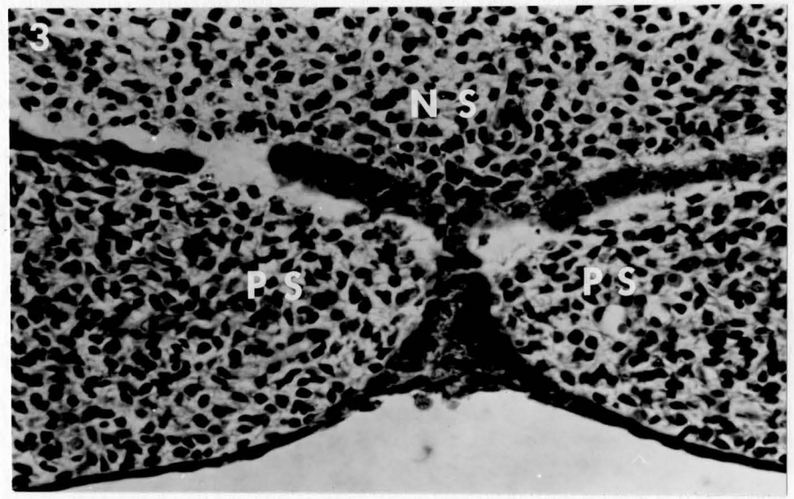


FIGURE 4

Frontal section through the posterior one-third of the secondary palate of a 17 day old fetus. Note the fusion of the palatal shelves (PS) and the presence of a one to two cell thick epithelial strand at the fusion site. A disruption of the epithelial strand at its dorsal end can be observed. H & E, x40.

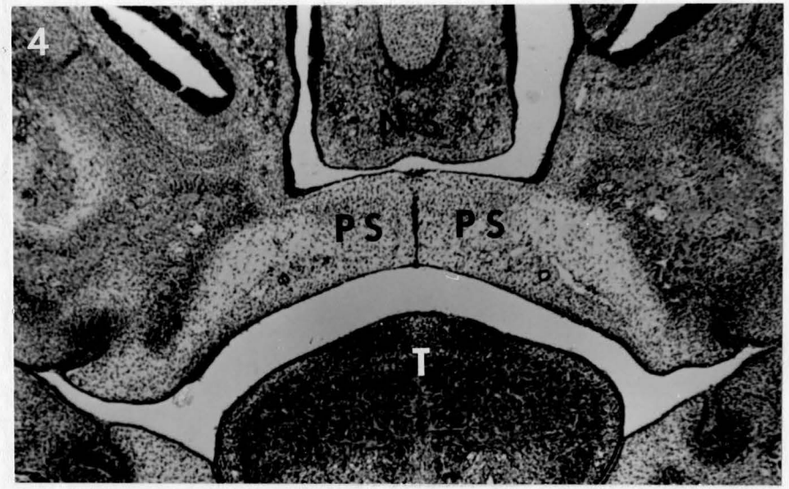
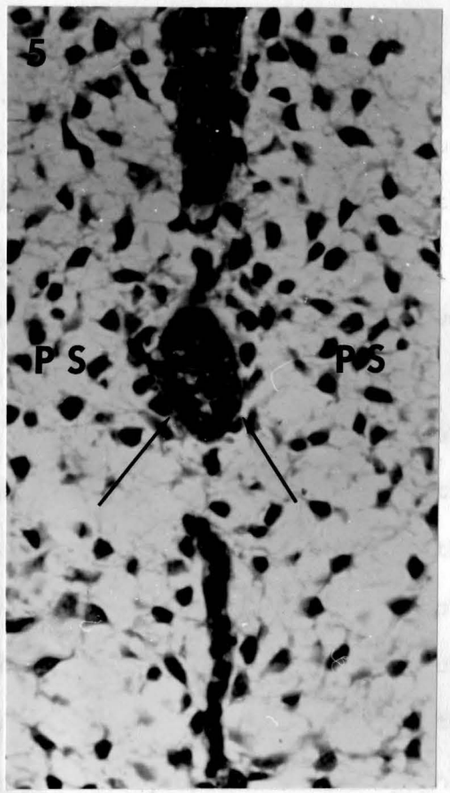


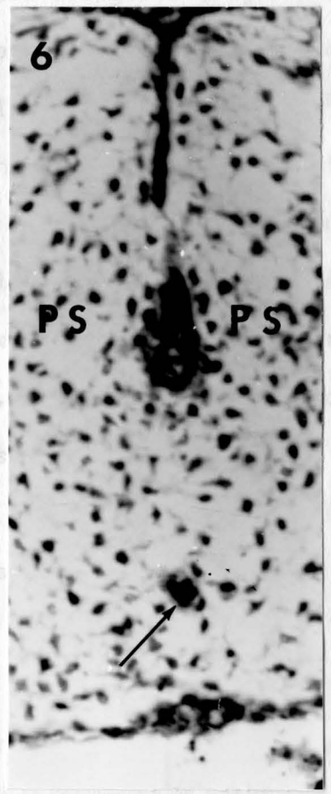
FIGURE 5

A higher magnification of the fusion area of the palatal shelves (PS). Note the thickness of the epithelial strand and degeneration of the epithelial cells (arrows). H & E, x250.

FIGURE 6

A higher magnification of the fusion area of the palatal shelves (PS). Note an epithelial strand on the dorsal side, a drop-like arrangement of the degenerating epithelial cells in the middle, and an epithelial rest on the ventral side of the fusion (arrow). In several places mesenchymal fusion can be observed. H & E, x150.





ELECTRON MICROSCOPIC FINDINGS:

Pre-fusion stage - The epithelium lining the medial aspect of the palatal shelves before fusion was supported by an underlying basal lamina and mesenchyme (Figure 7). The epithelium ranged from a thickness of 2-4 cells. These cells appeared to be short columnar, cuboidal, and squamous. The covering epithelium varied in its thickness in different areas.

The superficial layer of epithelium appeared to be flat as compared to the basal layer. The nuclei of the epithelial cells were found in different shapes. The surfaces of the nuclei were irregular (Figure 8). On the surface of the superficial epithelium the plasma membrane had small cytoplasmic extensions in the form of microvilli projections (Figure 9). These projections extended towards the direction of the approaching epithelium. The cytoplasm of the epithelial cells contained few mitochondria. Several of the present mitochondria were slightly distended and were partially devoid of cristae (Figures 8 and 9). Few granular ribosomes associated with the endoplasmic reticulum were seen. There was an abundance of free ribosomes. Also small deposits were visible which could be glycogen. The chromatin content of the nucleus and shape of the nucleolus showed no specific disturbance. Many desmosomes were apparent between the neighboring epithelial cells. These seemed to be connecting the epithelial cells together (Figures 7 and 8).

The basal layer of the epithelial cells was lined with a thin basal

lamina (Figures 10 and 11). These cells appeared to be of short columnar and irregular cuboidal shape. Their nuclei were of irregular shape, ranging from oval to round, and frequently were notched to some degree. Several of these nuclei were eccentrically placed. A pronounced nucleolus was evident. The chromatin content seemed variable, mostly occuring as clumps (Figures 10 and 11).

The cytoplasm contained an abundance of free ribosomes. Their association with endoplasmic reticulum was more frequent here than in the superficial layer. The mitochondria seemed more numerous. An accumulation of mitochondria bordering the basal lamina was an interesting observation (Figure 11). There were basically rod shaped. Golgi apparatus were small and rarely seen. Glycogen deposits were evident. Dense bodies, probably lysosomes, were observed in small number. These were spherical in shape, with a definite limiting membrane and an electron dense matrix. Desmosomes and hemi-desmosomes were evident between the basal cells (Figures 10 and 11).

The neighboring mesenchyme was composed of irregular cells with loosely arranged ground substance. These cells had long cytoplasmic processes. The nuclei were round, and contained fairly evenly distributed chromatin contents with one or more nucleoli. Cisterns of endoplasmic reticulum were present, along with free ribosomes. The mitochondria were rod shaped. Small electron dense bodies were infrequent. Glycogen deposits, appearing as electron transparent areas, were present. The regular nuclear membrane, combined with the irregular plasma membrane,

gave the mesenchymal cells a star-shaped appearance (Figure 12).

Fusion stage - With the palatal shelves approaching each other, the superficial layer of epithelial cells seems to become more and more degenerated until the superficial layer disappears. The epithelial cell layer was 1-2 cells thick. Pyknotic nuclei appear, with fragments of granular endoplasmic reticulum, Golgi apparatus, and mitochondria. The microvilli still persist, possibly being a key factor in fusion. However, with the advent of apposition, these microvilli seem to flatten into the cell surface (Figure 13).

The space between the opposing epithelia was variable. The contact along the cell surface was not uniform. In some areas the contact was made initially in less than one-third of the cell surface (Figures 14, 15 and 16). The basal lamina seems to be thin but intact with the fusion (Figure 14). Very few desmosomes were observed between the two fusing epithelia.

The actual epithelial fusion site was composed of 2-3 cells, mainly the basal epithelia. A rapid thinning of this seam or sheet was noticeable. An increase in the distance between the epithelial cells appear as the fusion progresses. Some of the cells were connected loosely with each other projecting their cytoplasmic processes into the expanded intercellular spaces (Figures 14, 15 and 16).

The nuclei of the cells were irregular in shape and had several

indented portions. They had some marginal condensation of chromatin in the nucleus (Figure 14).

The cytoplasm was dark, containing several inclusion bodies. These had different electron opacity. These dense bodies were well defined with a dense matrix in the central portion containing vesicles of high electron opacity (Figures 15 and 16). Some cells without inclusion bodies were seen indicating that degeneration or involution of the epithelium had not yet occurred (Figure 16).

Several spherical and elongated mitochondria were present with partial or complete absence of cristae. Elongated cisterns of endoplasmic reticulum and Golgi apparatus were found. The glycogen content was not clear (Figures 14 and 15).

Breakdown of epithelium with mesenchymal penetration - The epithelial cells gradually loose their continuity with the basal lamina and become isolated islands of closely packed cells (Figure 17). Both epithelial cells of normal structure and those having highly irregular outline and elongated processes were found together, sometimes connected by desmosomes. The degenerating epithelial cells exhibited striking morphological changes. (Figure 18)

At the fusion area cells of increased cytoplasmic density were noticed. The nuclei were highly irregular in shape with large invaginations and projections with clumped chromatin (Figures 18 and 19). The degenerating cells had dense areas in both the nucleus and the cytoplasm. The perinuclear space seemed to be enlarged (Figure 19). The vacuolated areas

and several dense bodies were observed throughout the entire degenerating epithelial cell area (Figure 18). The plasma membrane of the epithelial cells was frequently open and exposed to the neighboring cell area.

The cells undergoing degeneration had swollen mitochondria with decreased numbers of cristae which were irregular and broken. The cells contained a certain amount of glycogen-like substance distributed in the cytoplasm and also had several ingested dense bodies (Figure 19).

At the fusion site several macrophage-like cells were observed. They seemed to enclose the dense degenerated cells partially or completely (Figure 20). The cytoplasmic extensions of these cells give the impression of an attempt to phagocytize or engulf the degenerating cell parts. These were difficult to differentiate from connective tissue macrophages.

In the several fusion areas of terminal epithelial degeneration epithelial rest cells were found (Figure 21). The typical pattern of underlying mesenchyme of the palatal shelves remained unaltered.

ELECTRON MICROGRAPHS

FIGURE 7

The bordering epithelium of a palatal shelf with supporting basal lamina (BL) and underlying mesenchyme (Me) is shown. Microvilli (Mv) are present extending towards the opposing shelf. Desmosomes (D) appear to be joining the epithelial cells. x6,650.

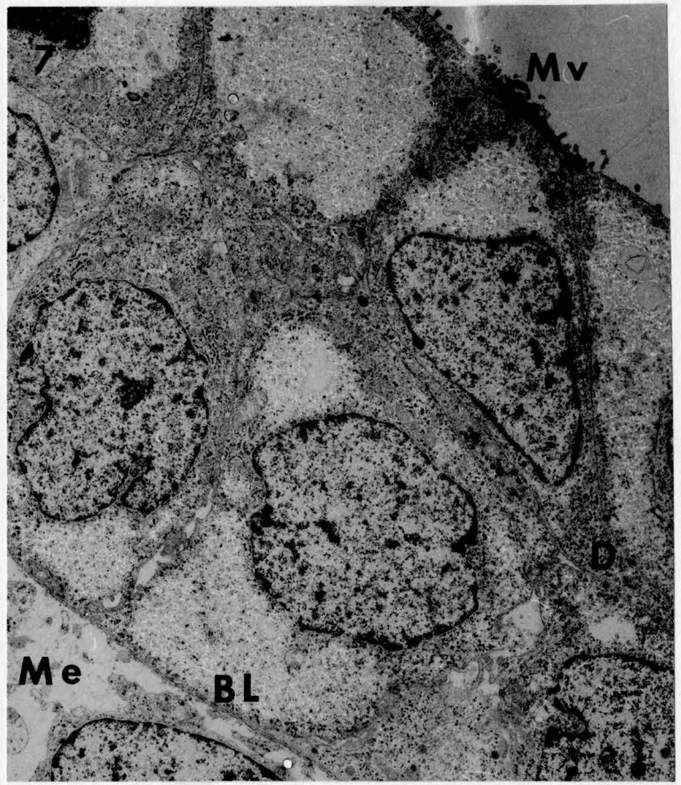


FIGURE 8

The bordering epithelium is shown at a higher magnification. Microvilli (Mv) and Desmosomes (D) are present. Mitochondria (M) and Golgi apparatus (Go) are also visible. Note the irregular nucleus (N). Many free ribosomes (R) are present. An electron dense body (DB) is seen near the nucleus. x16,800.

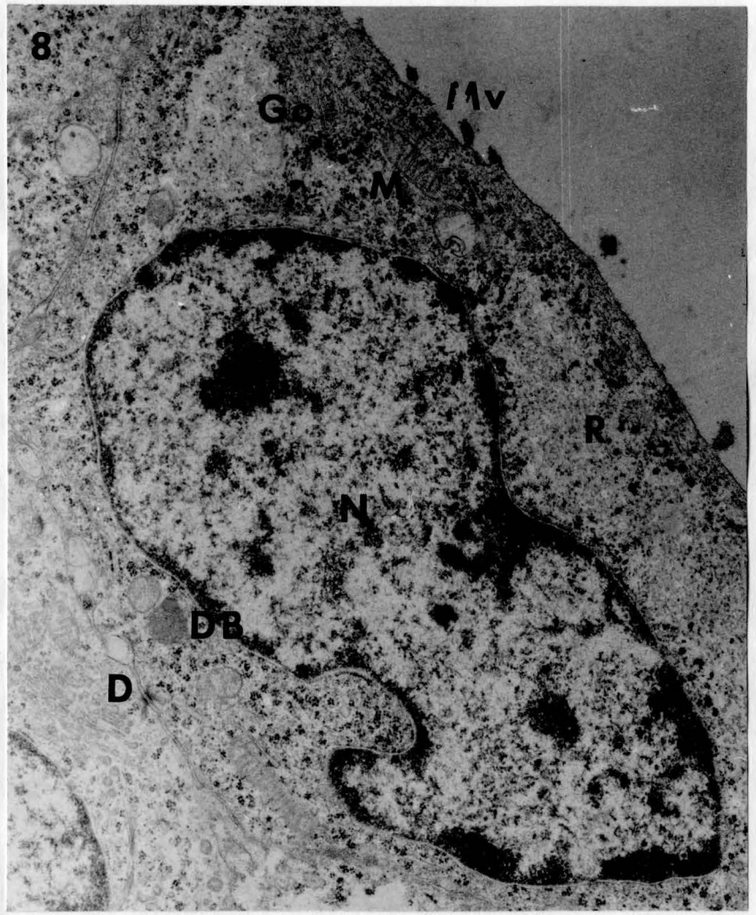


FIGURE 9

Another view of the superficial layer of epithelium is shown. The microvilli (Mv) are apparent, along with desmosomes (D). The mitochondria (M) appear distended. A Golgi apparatus (Go) is visible along with many free ribosomes (R). x16,800.

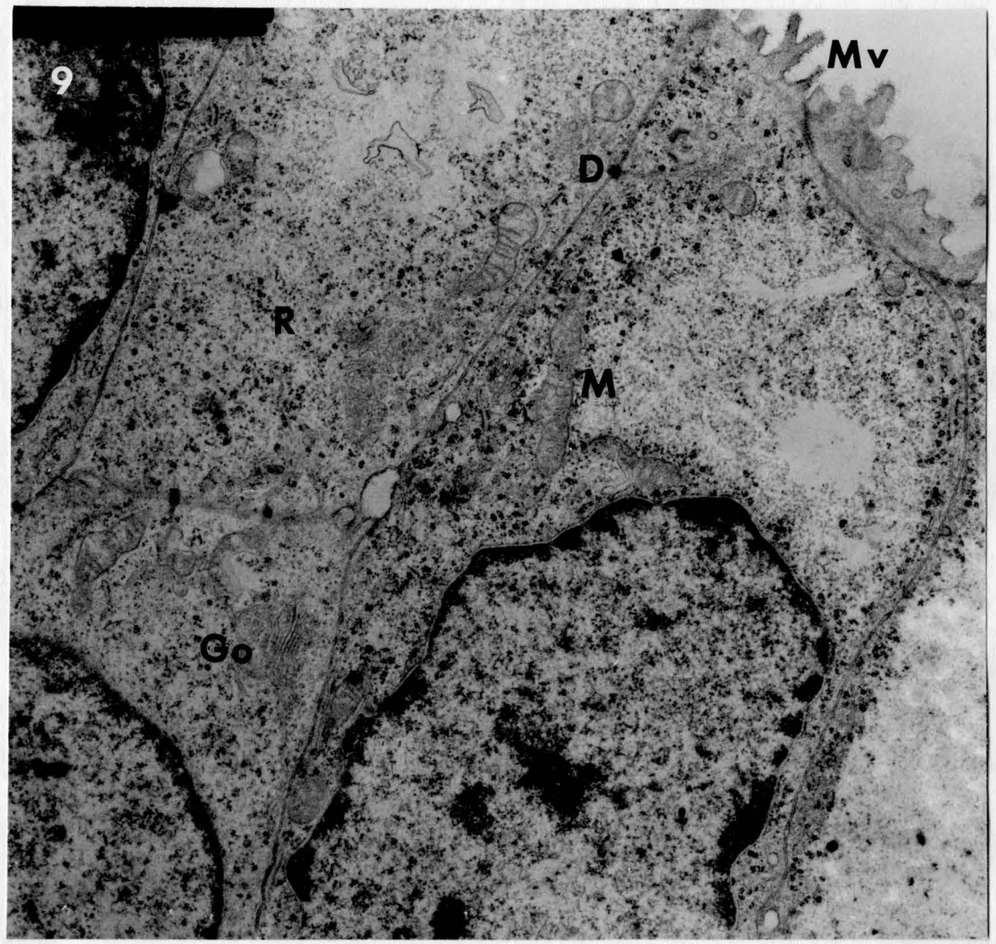


FIGURE 10

Note the basal lamina (BL) separating the epithelial and mesenchymal cells. Cisterns of endoplasmic reticulum (CER), mitochondria (M), and free ribosomes (R) are apparent in both epithelium and mesenchyme. An irregular nucleus (N) is present. x16,800.

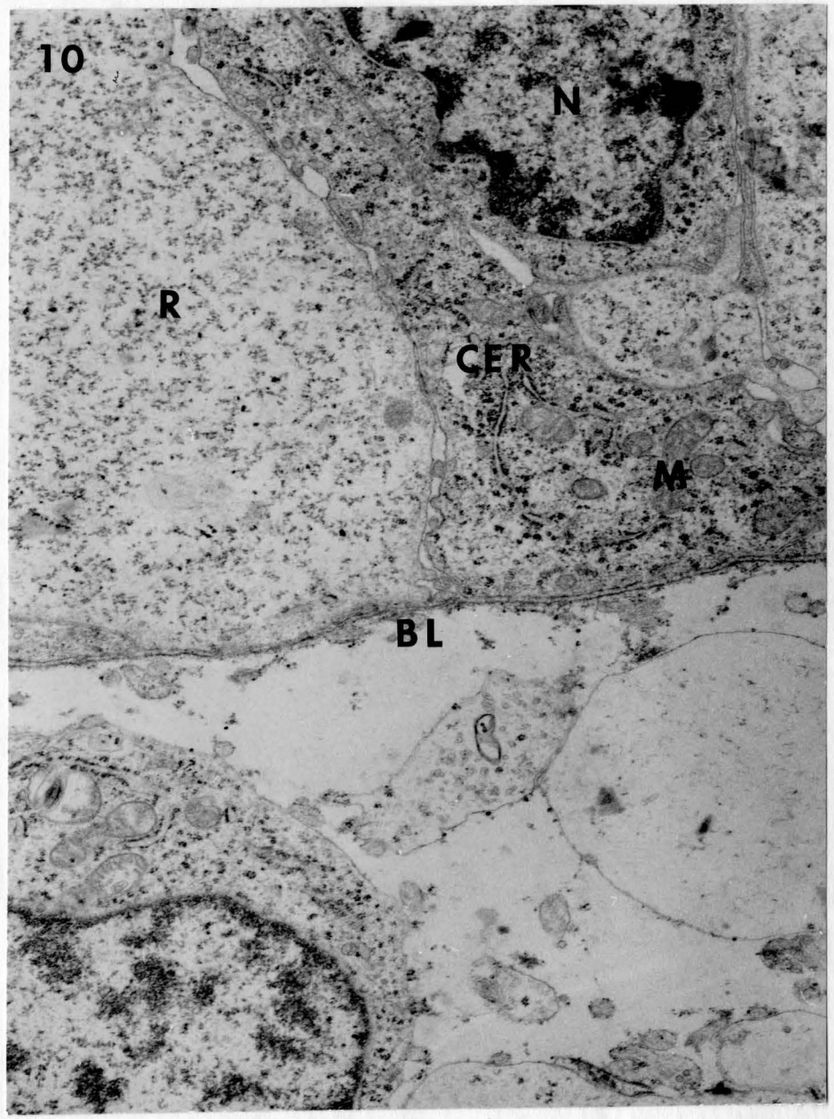


FIGURE 11

The basal lamina (BL) is again shown, demonstrating a peculiar accumulation of mitochondria (M) at its border. Lysosomal-like bodies (L) are present near the basal lamina. x16,800.

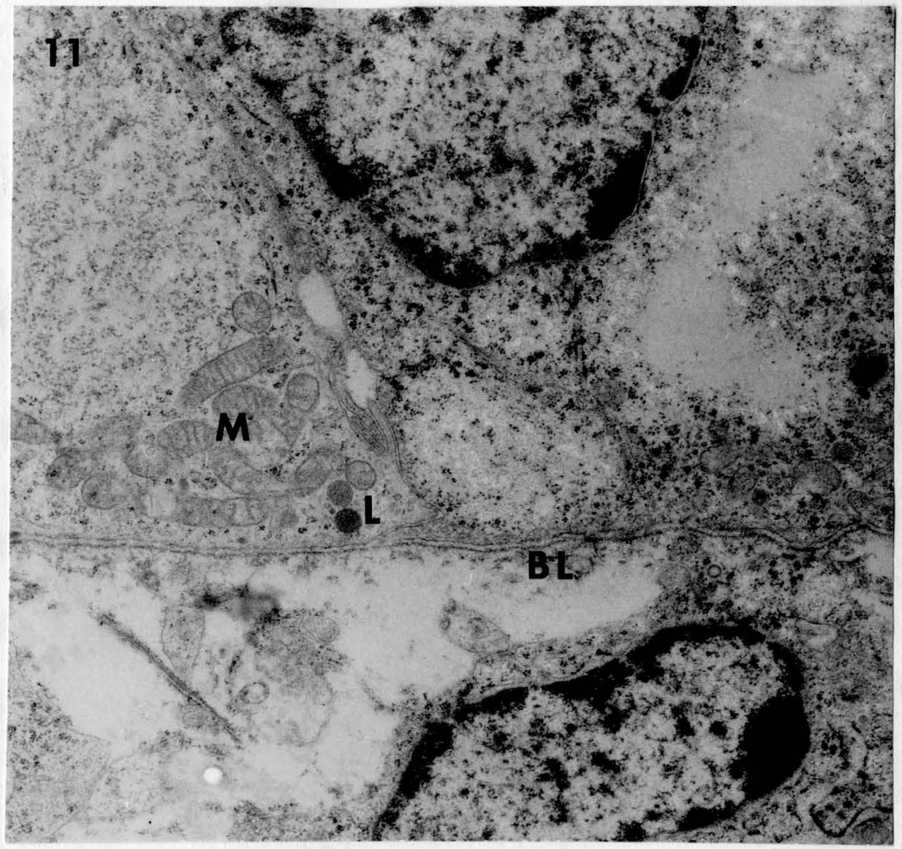


FIGURE 12

The loosely arranged irregular mesenchymal cells are shown. Note the lysosomal-like (L) dense bodies. The nuclei appear rounded (N). Mitochondira (M), cisterns of endoplasmic reticulum (CER), as well as free ribosomes (R) are present. x6,650.

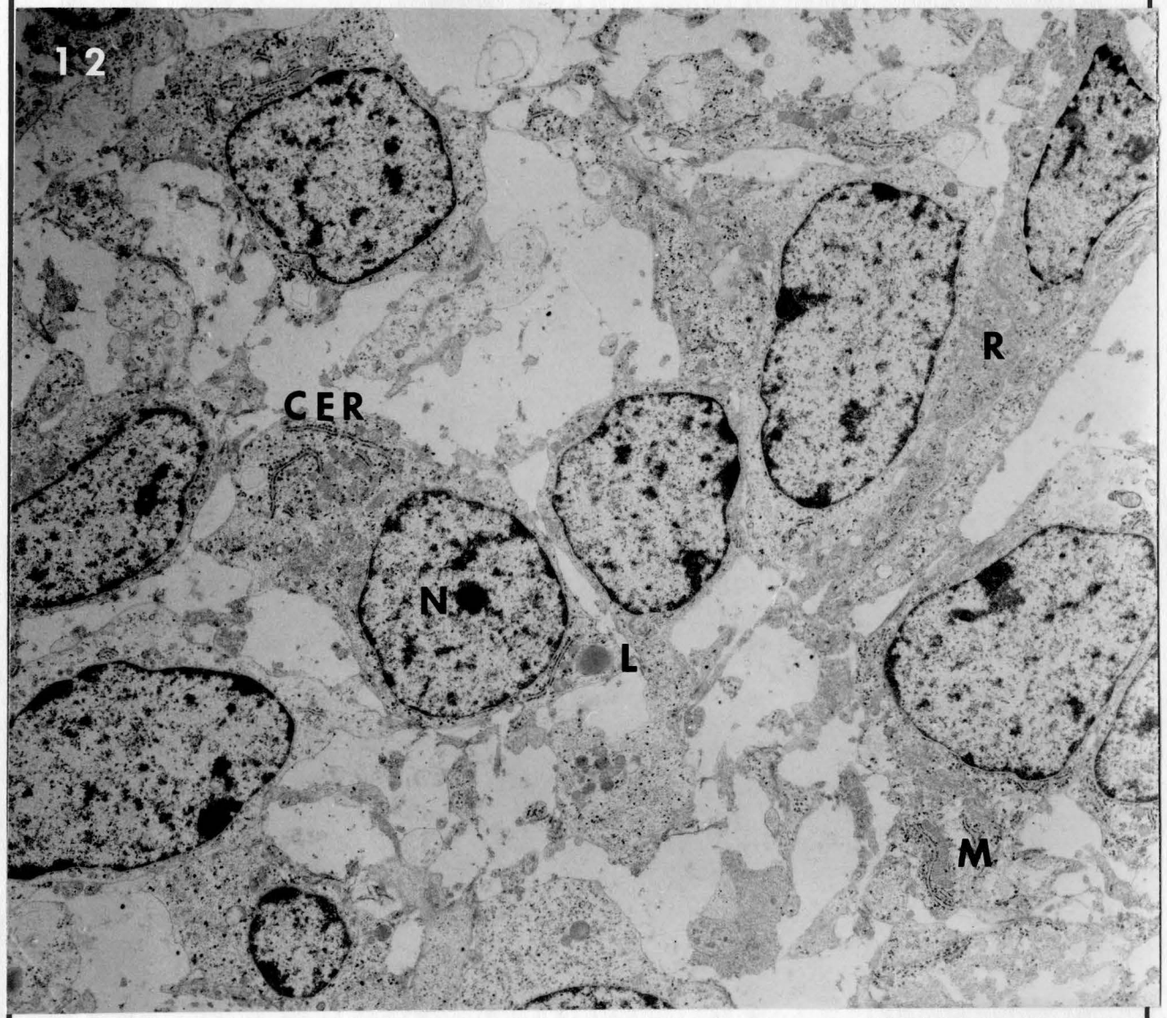


FIGURE 13

The superficial layer of epithelium with a flattening of the microvilli (Mv) and a pyknotic nucleus (N) is shown. Present are Golgi apparatus (Go), cisterns of endoplasmic reticulum (CER), and mitochondria (M). x16,800.

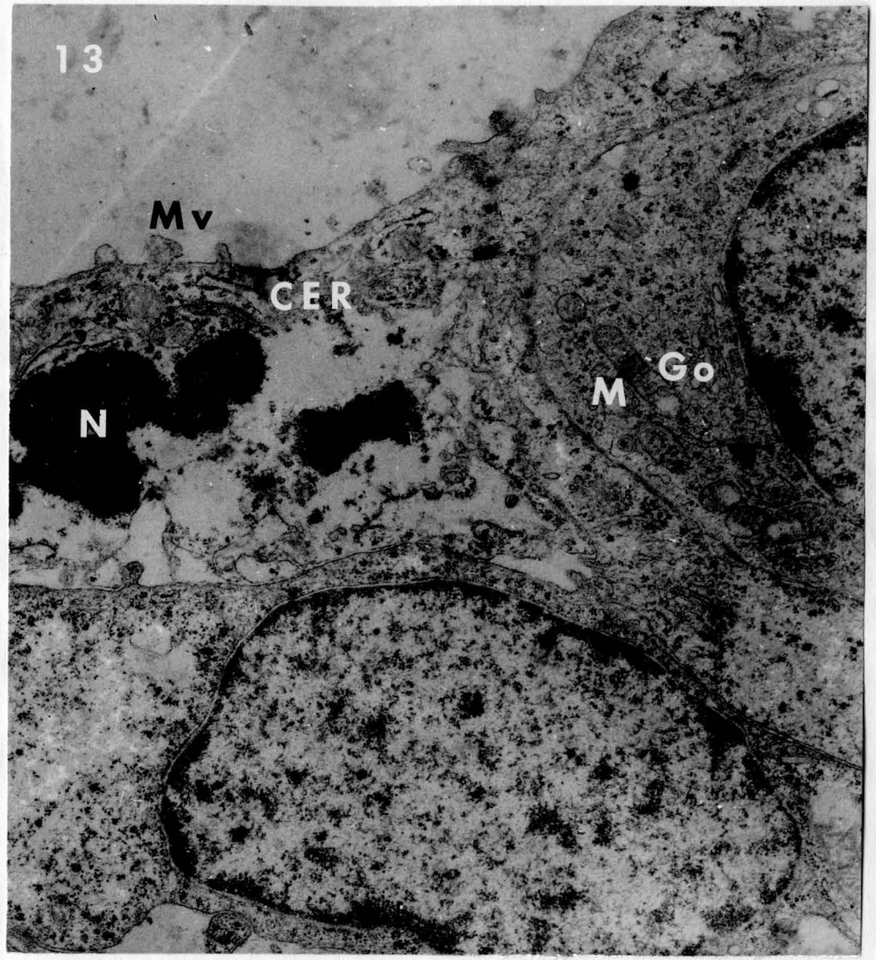


FIGURE 14

The fusing epithelium is shown with a supporting basal lamina, (BL) intact but narrowing. The nucleus (N) is starting to degenerate, as well as the mitochondria (M). x16,800.

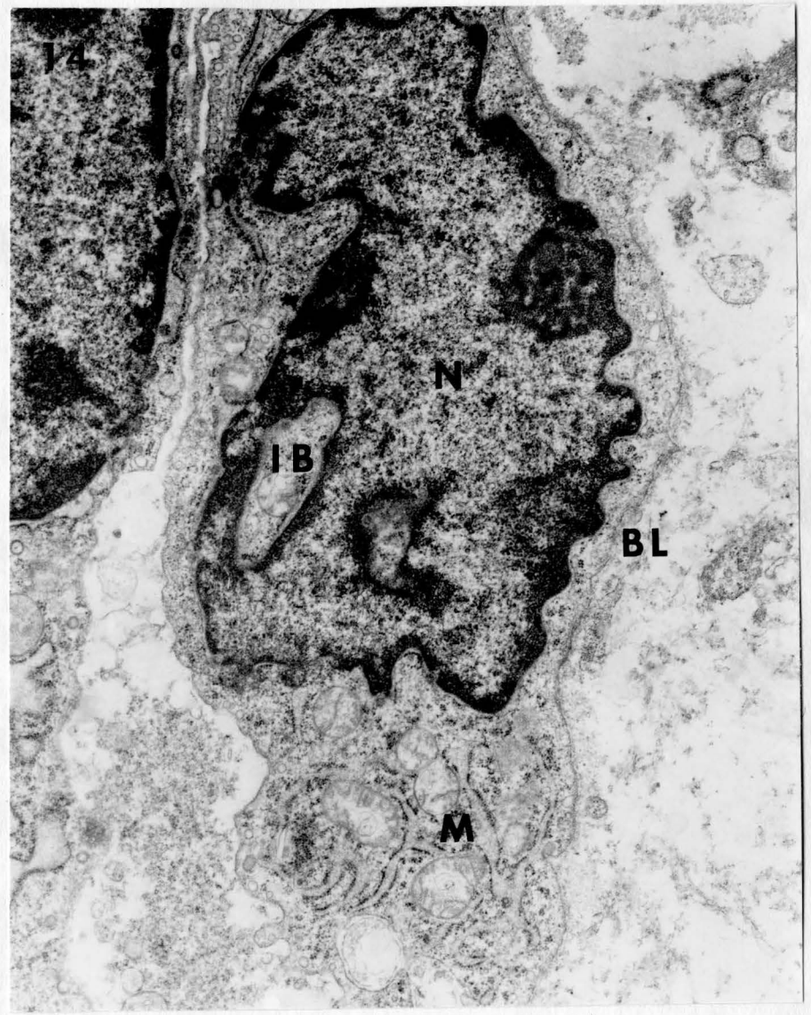


FIGURE 15

Shown are fusing epithelial cells with a residue of the superficial cells between them (arrow). Note the enlarged cisterns of endoplasmic reticulum (CER) as well as free ribosomes (R) and degenerating mitochondria (M). x16,800.

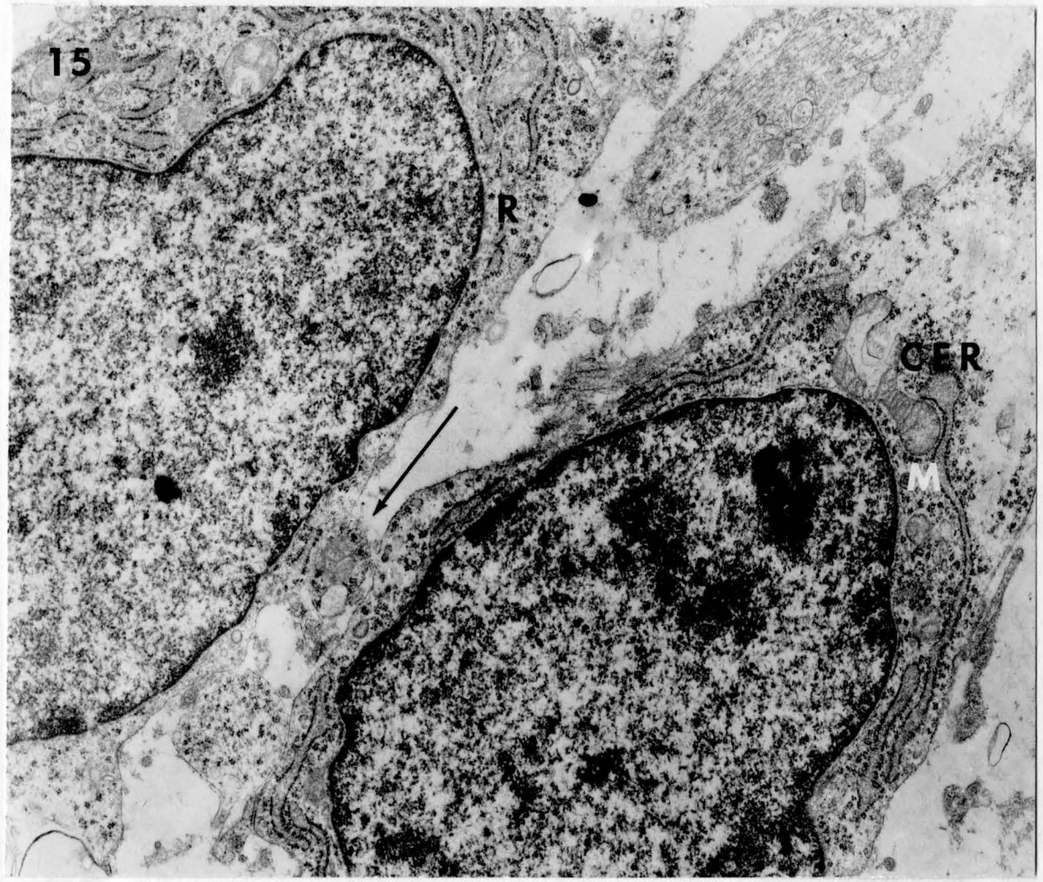


FIGURE 16

Note the epithelial cells in the process of fusion. Electron dense bodies (DB) and desmosomes (D) are present. x16,800.

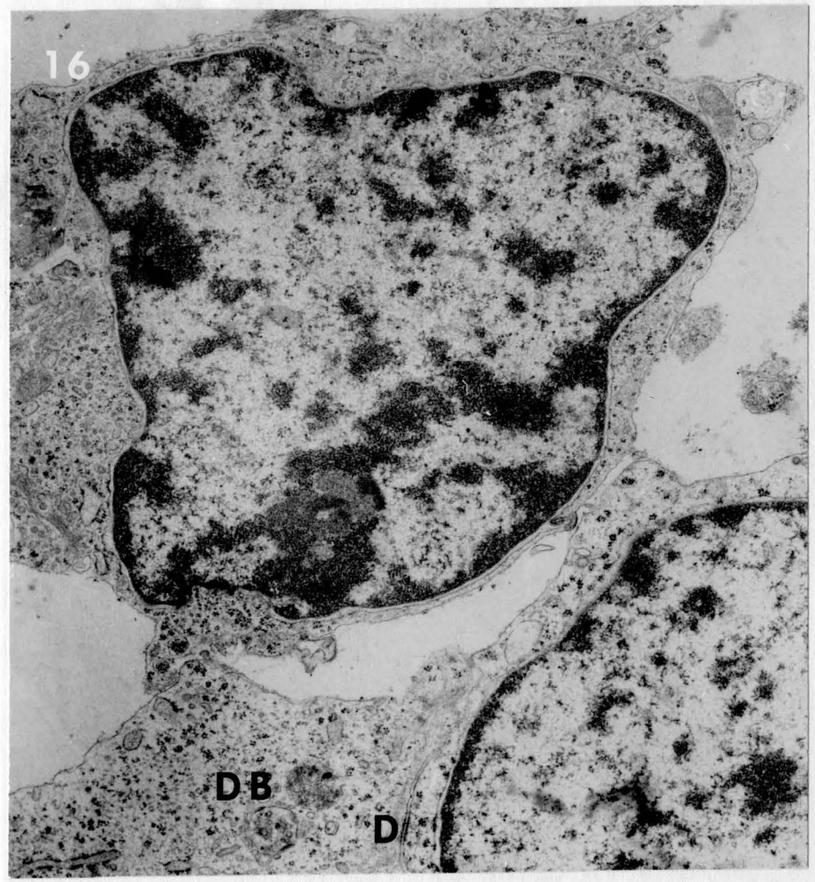


FIGURE 17

Degenerating epithelial cells are shown. Lysosomal-like bodies (L) are present, with mitochondira (M) having a reduced number of cristae. x16,800.

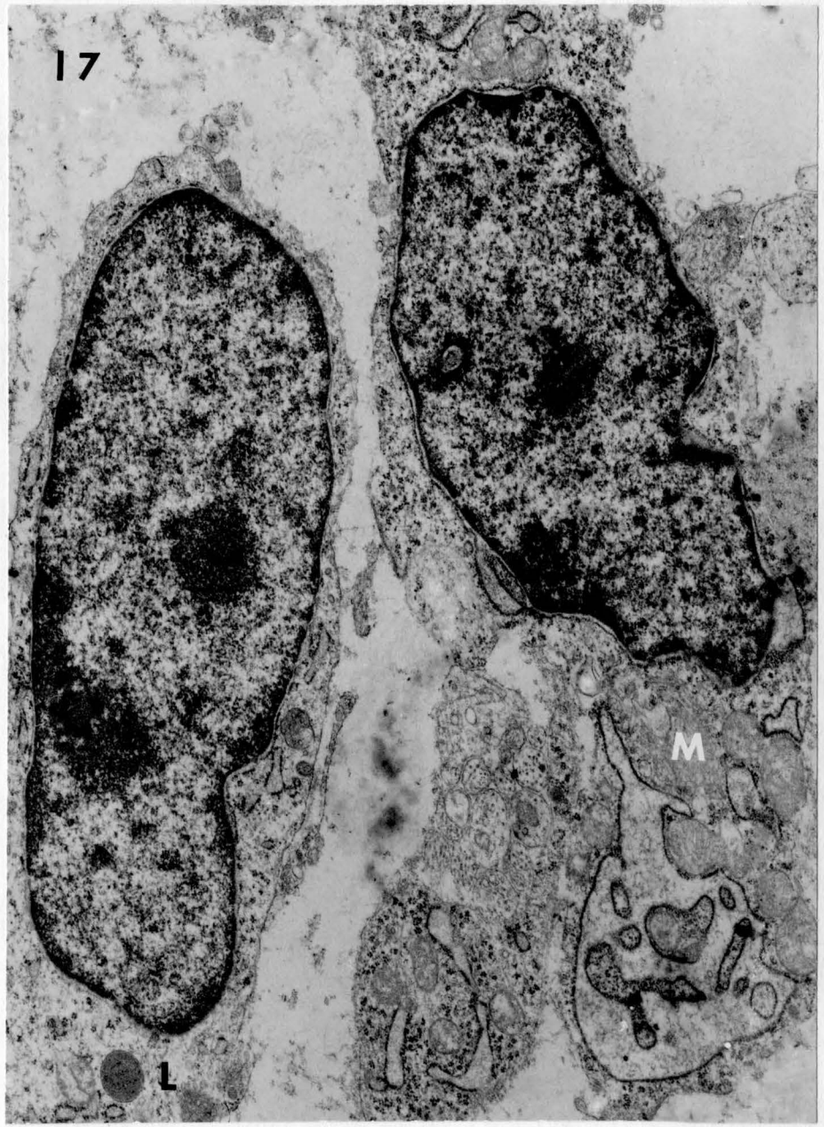


FIGURE 18

Shown are degenerating epithelial cells with an accumulation of lysosomal-like (L) bodies. Note a degenerative vacuole (DV) in the cytoplasm. x16,800.

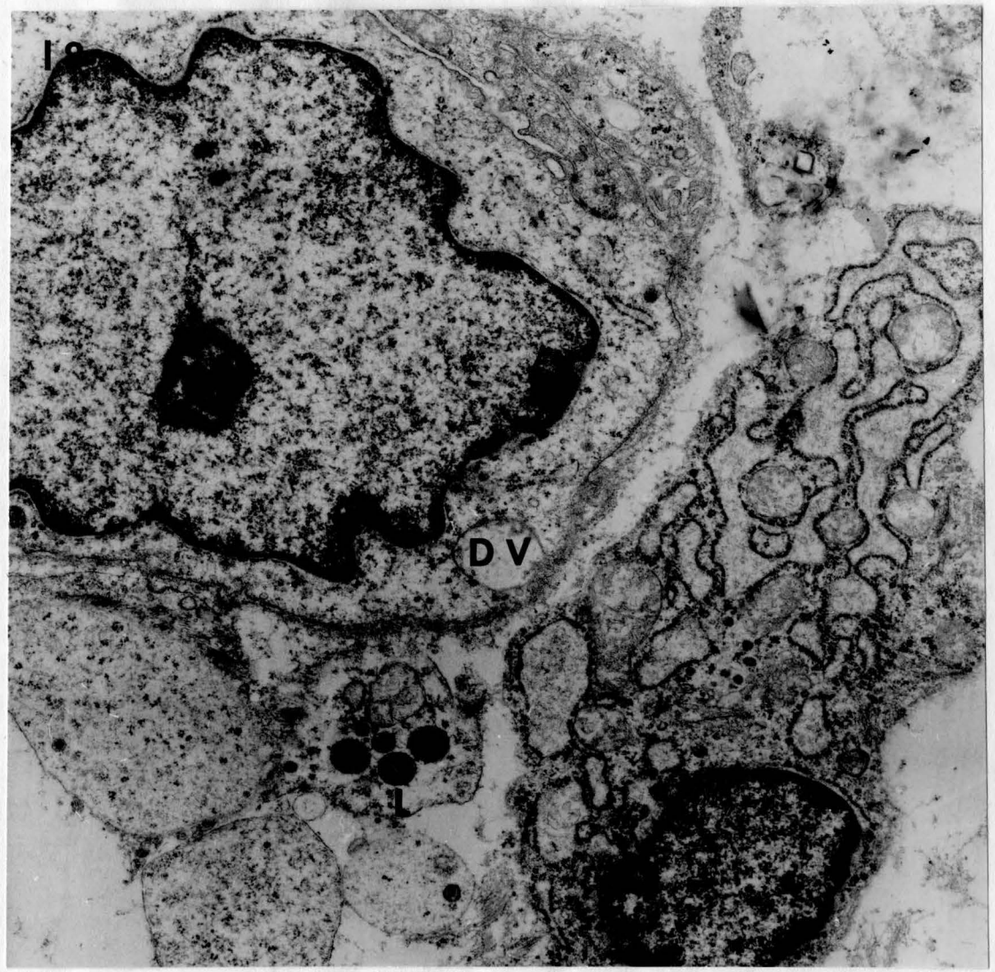


FIGURE 19

Note the enlarged perinuclear space (arrow) in the degenerating epithelial cells. x16,800.

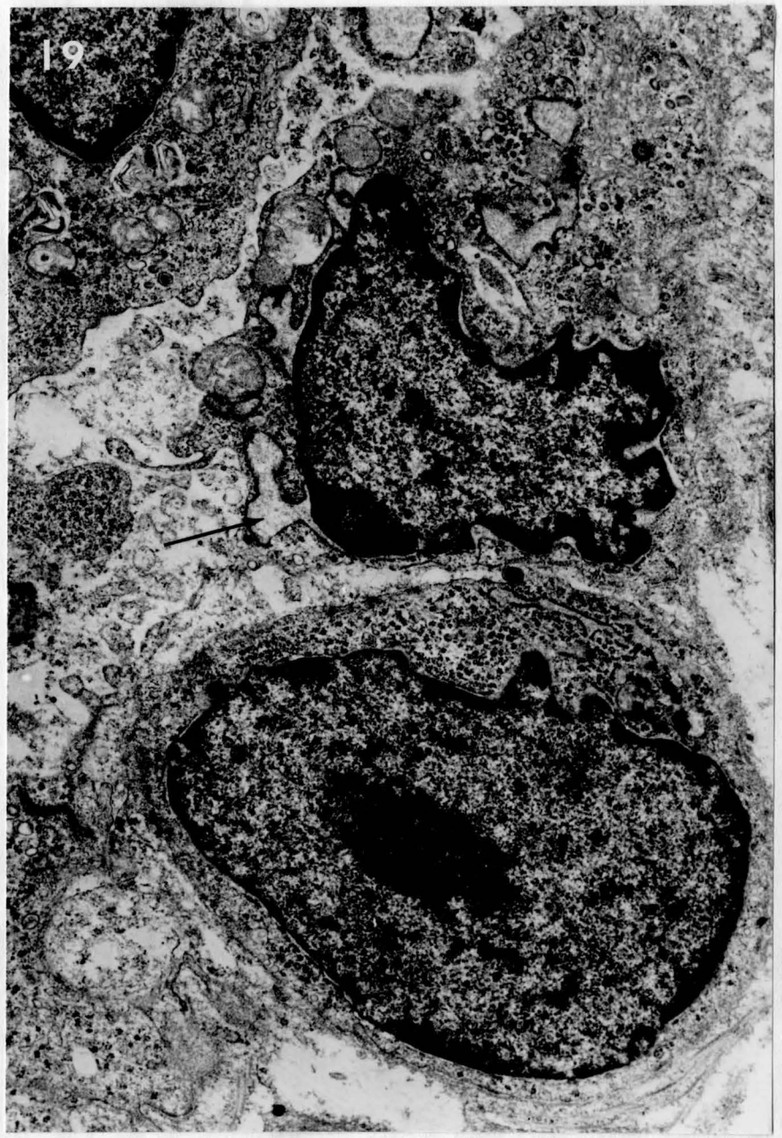


FIGURE 20

A macrophage (MA) is shown conforming to a degenerative vacuole (DV). x16,800.

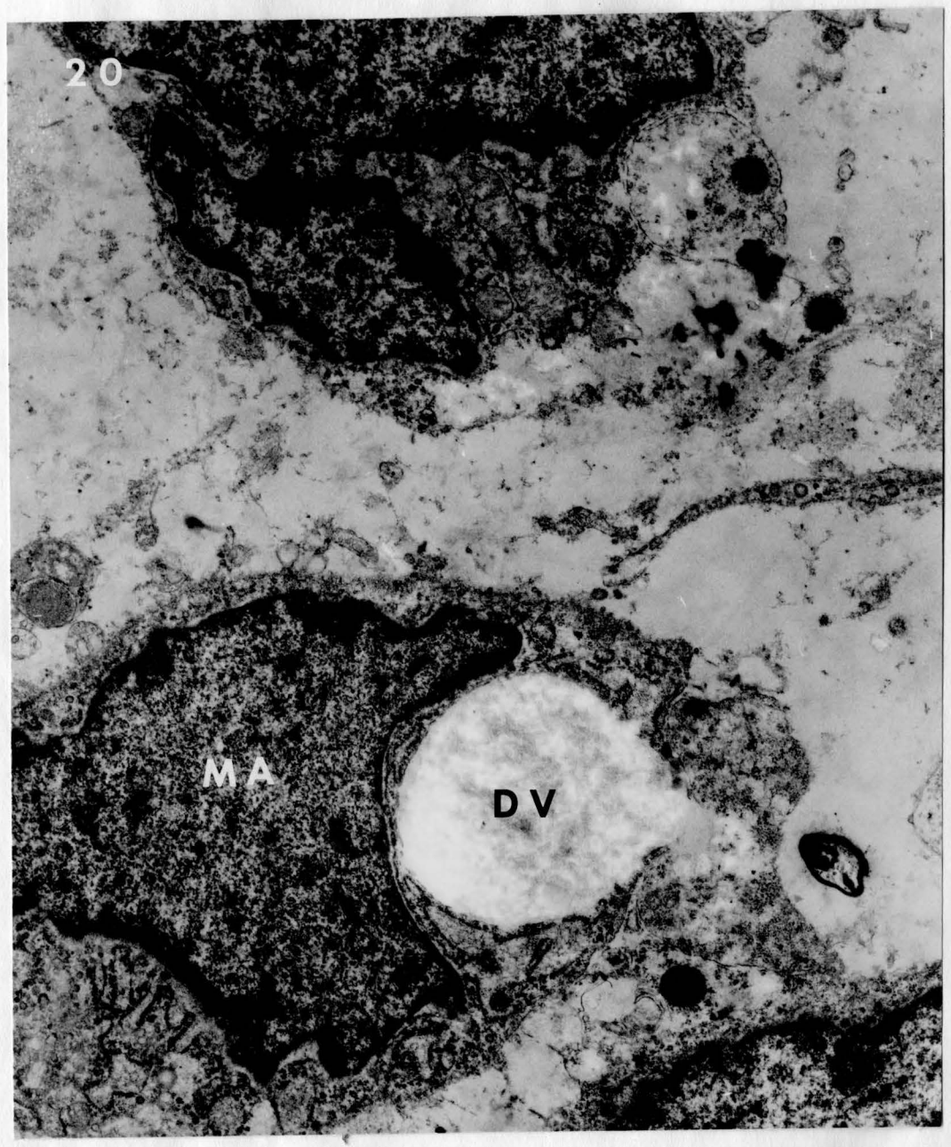
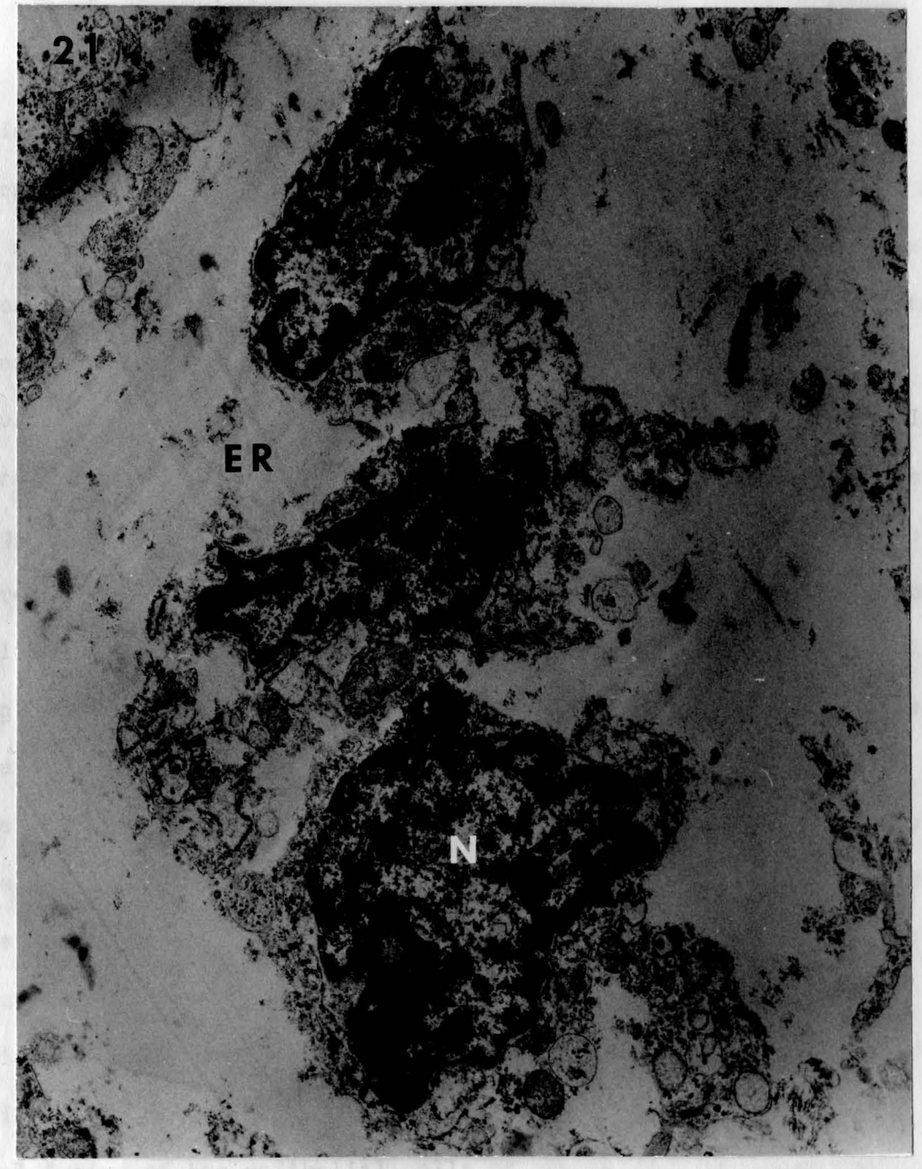


FIGURE 21

Shown are epithelial rest cells (ER). Note the multilobular nuclei (N). x16,800.



DISCUSSION

In the present study certain interesting observations were made concerning the fusion of the epithelial cells of the palatal shelves and their subsequent breakdown. Small microvilli, or tiny cytoplasmic extensions, bordering the medial surface of the epithelium of the palatal shelves were found in the present study. It has been suggested that microvilli secrete certain substances. (Bloom and Fawcett, 1968). In the present study the microvilli were pronounced and abundant, suggesting a large amount of secretory activity going on in the epithelial cells in the pre-fusion stage. Mato et al. (1967) in a similar study as the present one also observed these microvilli projecting from the epithelial cells lining the medial walls of the palatal shelves. They called these cells "adhesive cells", indicating their direct potential in the fusion of the palatal shelves. Pourtois (1966) stated that the medial walls of the epithelium of the palatal shelves had a "zone of stickiness" which helps in fusion of the palate. The microvilli found in the present study at the future fusion site of the epithelium could be related to their possible role in holding the palatal shelves together during their initial contact.

It was observed that the microvilli seemed to flatten with the initial fusion of the epithelial cells of the palatal shelves. This can be related to the cell stretching or morphological changes of the epithelium during the fusion process. This finding is in accord with

DeAngelici and Nalbandian (1968) who mentioned flattening of the microvilli after the epithelial fusion.

In several areas an epithelial contact between the opposing cells of the two palatal shelves was observed. However, few desmosomes were observed connecting the two epithelial cells of the opposing palatal shelves. This finding does not support the observation of Brusati (1969) who found an abundance of desmosomes in the fusing epithelial cells of the palatal processes. Desmosomes were observed more between the individual epithelial cells in this study.

Another factor which should be considered in the fusion of the palatal shelves is the internal force within the shelves pushing them towards each other. This internal force has been described by Walker and Fraser (1956) as elastic fibers, by Nanda (1969) as blood vessels, by Larsson (1962) as the mucopolysaccharides, and by Nanda (1970) as the osteogenic maxillary growth at the lateral ends of the developing palatal shelves. Since the fusion of the palatal shelves of the secondary palate is a dynamic process, the pressure effect of the growing palatal shelves might also act as an inducer for the breakdown of the epithelial contact of the palatal shelves after their initial fusion. Before and during the fusion a disparity of the mitotic rate of the epithelial and mesenchymal cells has also been reported by Brusati and Possenti (1969), indicating that the growth of the epithelial cells is negligible before their fusion.

In the present study the basal lamina was found to function as a

separation of the epithelium of the palatal shelves from the underlying mesenchyme. The basal lamina contained a medium electron transparent lamina lucida, as found by Stern (1965), with a connective tissue side lamina densa, and an epithelial wall membrane. Investigators have shown that it can act as a selectively permeable filter for substances passing through between the epithelium and connective tissue (Cohen, 1961, and Farquher, 1964). The present study showed a few perforated areas of the basal lamina by epithelial cell cytoplasm. However, not enough of these epithelial cytoplasmic extensions were demonstrated to substantiate the claims of Farbman (1969) that these processes are instrumental in the destruction of the epithelium.

The continuity of the basal lamina was not demonstrated in this study after fusion. This is in agreement with Hughes et al. (1967) and Farbman (1968). However, these findings do not concur with those of Smiley and Dixon (1966) and DeAngelis and Nalbandian (1968), who showed that the basal lamina remains intact until mesenchymal penetration takes place. The present findings concur with Brusati (1969), demonstrating only a few remnants of a basal lamina at the end stage of fusion.

As the morphological degenerative changes occur to the epithelial cells during the fusion process, the presence of large, dense bodies were evident. These dense bodies were also shown by Angelici and Pourtois (1968) who found them acid phosphatase positive. The presence of these bodies indicates their possible role in the lysis of the epithelial cells. Many of the spherical looking dense bodies demonstrated a definite

limiting membrane and electron dense matrix. These bodies have been identified as lysosomes (Mato et al., 1966, 1968). de Duve and Wattiaux (1966) have demonstrated that lysosomes are autophagic in nature. Contrary to this Hayward (1969) claimed them to be heterophagic in nature. The presence of these lysosomes in the epithelial cells of the palatal shelves indicates their possible active role in the breakdown of the epithelial sheet separating mesenchyme of the two palatal shelves.

As the epithelial degeneration continued in this study, certain macrophage-like cells were observed. These cells seemed to originate from the epithelial cells and conformed to the degenerating parts of neighboring cells. This phenomena was also shown by Farbman (1969). The organelles of the engulfed cells loose their structure and are replaced by amorphous material. These morphological evidence of these macrophagic-like cells seemingly originating from epithelium in this study possibly confirm the findings of Singer and Saltpeter (1961) that the epithelial cell can phagocytize.

Light microscopic results showed the presence of epithelial rests at the fusion site in several 17 day old fetuses of the present study. These were confirmed at the ultrastructural level. It has been indicated in the literature that the persistence of these epithelial rests might be a potent cause of the cleft palate in human beings (Veau, 1938 and Tondury, 1961). According to Sicher (1966) these epithelial remnants are held responsible for the occasional clinical appearance of so-called developmental cysts found in the palatal midline.

CONCLUSIONS

The observation at the light microscopic and ultrastructural levels of the stages of fusion of the secondary palate in the rat fetus demonstrated several phenomena. As the lining epithelium proceeds medially for the fusion process, small microvilli are present as cytoplasmic extensions of the superficial epithelium.

The basal lamina was evident as a continuous membrane separating the epithelium from mesenchyme prior to the fusion of the palatal shelves. As the epithelial sheet or seam was established a break of continuity was observed. The basal lamina was finally totally obliterated.

Desmosomes were evident joining cells of the superficial layer and the basal layer of epithelium. At the fusion site, however, an electron transparent area or space seemed to overshadow the desmosome role in holding the epithelium together.

Gradual cell degeneration of the palatal epithelial cells at the site of fusion linked with possible lysosomal and autophagic activity along with a phagocytic action of neighboring epithelial cells was observed.

A gradual penetration of mesenchyme was finally found with few remaining epithelial islands at the site of the fusion.

SUMMARY

An electron microscopic study to investigate normal palatal fusion was conducted utilizing 14 to 18 day old rat fetuses of the Sprague-Dawley strain. The epithelial and mesenchymal fusion was observed before, during, and after fusion.

The pre-fusion stage demonstrated an intact basal lamina, tiny microvilli projections from the superficial layer of epithelial cells, and desmosomes between the epithelial cells.

Evidence of a true adhesion during epithelial fusion did not seem to be due directly to the action of desmosomes.

The disappearance of the epithelium and the mesenchyme intervention seemed to be due to an autolytic process as well as epithelial phagocytosis.

BIBLIOGRAPHY

Anderson, H. and Matthiessen, M.E.: "Histochemistry of the early development of the human central face and nasal cavity with special reference to the movements and fusion of the palatal processes." Acta Anat., 68: 473-508, 1967.

Angelici, D.R.: "A histological and histochemical study of palatal shelf movement in the mouse and the embryogenesis of the hydrocortisone-induced cleft palate." Thesis, Harvard School of Dental Medicine, Forsyth Dental Center, 1966.

Angelici, D.R. and Pourtois, M.: "The role of acid phosphatase in the fusion of the secondary palate." J. Embryol. Exp. Morph., 20: 15-23, 1968.

Barry, A.: "Development of the bronchial region in human embryos with special reference to the fate of epithelia." In: Congenital Anomalities of the Face and Associated Structures. Thomas, ed. Pruzanski, S., Springfield, Illinois, pp. 46-62, 1961.

Bergengrum, P.: "Epithelperlen und Epithelstrange in der Raphe des harten Goumens." Roux Arch. Entio. Mech., 28: 277-326, 1909.

Bloom, W. and Fawcett, D.W.: A Textbook of Histology. W.B. Saunders, Co., Philadelphia, Chapt. 3, p. 90, 1968.

Brusati, R.: "Ultrastructural study of the process of formation and involution of the epithelial sheet of the secondary palate in the rat." J. Submicr. Cytol., 1: 215-234, 1969.

Brusati, R. and Miani, C.: "Ultrastructural features of the involution of the epithelial sheet of the secondary palate in rats." Acta bio-medica, 39: 583-593, 1968.

Brusati, R. and Possenti, P.: "L'attivita mitotica del setto epitheliale del palato secondario del ratto nel corso della sua formaziare ed involuziare." R.I.S., 24: 101-107, 1969.

Cohen, A.I.: "Electron microscopic observations of the developing mouse eye. I. Basement membranes during early development and lens formation." Develop. Biol., 3: 297-316, 1961.

Coleman, R.D.: "Development of the rat palate." Anat. Rec., 151: 107-112, 1965.

De Angelis, V. and Nalbandian, J.: "Ultrastructure of mouse and rat palatal processes prior to and during secondary palate formation." Archs. Oral Biol., 13: 601-608, 1968.

Duve, de C.: "The lysosome concept." In: Ciba Foundation Symposium Lysosomes. J. and A. Churchill, London, 1963.

Farbman, A.I.: "Electron microscope study of palate fusion in mouse embryos. Develop. Biol., 18: 93-116, 1968.

Farbman, A.I.: "The epithelial-connective tissue interface during closure of the secondary palate in rodent embryos." J. Dent. Res., 48: 617-624, 1969.

Farquhar, M.G. and Palade, G.E.: "Segregation of ferritin in glomerular protein absorption droplets." J. Biophys. Biochem. Cytol., 7: 297-304, 1960.

Farquhar, M.G. and Palade, G.E.: "Functional evidence for the extence of a third cell type in the renal glomerulus. Phagocytosis of filtration residues by a distinctive 'third cell'." J. Cell. Biol., 13: 55-87, 1962.

Fawcett, D.W.: "Physiologically significant specializations of the cell surface." Circulation, 26: 1105-1125, 1958.

Fawcett, D.W.: An Atlas of Fine Structure. The Cell. Saunders, Philadelphia, p. 353, 1966.

Glucksmann, A.: "Cell death in normal vertebrate ontogeny." Biol. Rev. Cambridge Phil. Soc., 26: 59-86, 1951.

Harris, J.W.: "Oligohydramios and cortisone-induced cleft palate." Nature, 203: 533-534, 1964.

Hayward, A.F.: "Ultrastructural changes in the epithelium during fusion of the palatal processes in rats." Arch. Oral Biol., 14: 661-670, 1969.

Hughes, L.V., Furstman, L., and Bernick, S.: "Prenatal development of the rat palate." J. Dent. Res., 46: 373-379, 1967.

Larsson, K.S.: "Studies on the closure of the secondary palate. II. Occurrence of sulpho-mucopolysaccharides in the palatine processes of the normal mouse embryo." Exp. Cell Res., 21: 498-503, 1960.

Larsson, K.S.: "Closure of the secondary palate and its relation to sulphomucopolysaccharides." Acta Odontol. Scand., 20. Suppl 31: 1-35, 1962.

Larrson, K.S.: "Studies on the closure of the secondary palate. III. Autoradiographic and histochemical studies of mouse embryos from cortisone-treated mothers." Acta Morph. Neerl. Scand., 4: 369-386, 1962 b.

Larsson, K.S., Bostron, H., and Carlsoo, S.: "Studies on the closure of the secondary palate. I. Autoradiographic study in the normal mouse embryo." Exp. Cell Res., 16: 379-383, 1959.

Lazzaro, C.: "Sul meccanismo de chiusura del palato secondario." Monit. Zool. Ital., 51: 249-265, 1940.

Majno, G., and Palade, G.E.: "Studies on inflammation. I. The effect of histamine and serotonin on vascular permeability; an electron microscopic study." J. Biophy. Biochem. Cytol., 11: 571-605, 1961.

Mato, M., Aikawa, E., and Katahira, M.: "Appearance of various types of lysosomes in epithelium covering lateral palatine shelves during secondary palate formation." Gunma J. Med. Sci., 15: 46-56, 1966.

Mato, M., Aikawa, E., and Katahira, M.: "Alteration of fine structure of the epithelium on the lateral palatine shelf during the secondary palate formation." Gunma J. Med. Sci., 16: 79-99, 1967.

Mato, M., Aikawa, E., and Katahira, M.: "Further studies on 'cell reaction' at the lower surface of the nasal septum of human embryos during the fusion to the palate." Acta Anat., 71: 154-160, 1968.

Moriarty, T.M., Weinstein, S., and Gibson, R.D.: "The development in vitro and in vivo of fusion of the palatal processes of rat embryos." J. Embryol. Exp. Morph., 11: 605-619, 1963.

Nanda, R.: "The normal palate and induced cleft palate in rat embryos." PhD Thesis, Gebr. Janssen, Publishers, Nymegen, p. 120, 1969.

Nanda, R.: "The role of sulfated mucopolysaccharides in cleft palate production." Terat., 3: 237-244, 1970.

Nanda, R.: "Tritiated thymidine labelling of the palatal processes of rat embryos with cleft palate induced by hypervitaminosis A." Archs. Oral Biol., 16: 435-444, 1971.

Nasjleti, C.E., Spenier, H.H., Blankenship, J., and Walden, J.M.: "Cytogenetic studies of normal and cleft-palate epithelium in mice." J. Dent. Res., 48: 590-594, 1969.

Orban, B.J.: Oral Histology and Embryology. Mosby, St. Louis, Chapt. 1, p. 23.

Peter, K.: "Die Entwickling des Saugetiergaumens." Ergebn. Anat. Entwickl-Gesch., 25: 448-564, 1924.

Pourtois, M.: "Onset of the acquired potentially for fusion in the palatal shelves of rats." J. Embryol. Exptl. Morphol., 16: 171-182, 1966.

Pourtois, M.: "La fusion des cretes palatine et son alteration par quelques agents teratogenes." Archs. Biol., 78: 1-75, 1968.

Ross, L.M. and Walker, B.E.: "Movement of palatine shelves in untreated and tetratogen treated mouse embryos." Am. J. Anat., 121: 509-522, 1967.

Scott, J.H. and Symons, N.B.: Introduction to Dental Anatomy. 4th ed., Livingstone, Edinburgh, p. 51, 1964.

Shapiro, B.L. and Sweney, L.: "Electron microscopic and histochemical examination of oral epithelial-mesenchyme interaction (programmed cell death)." J. Dent. Res., 48: 652-660, 1969.

Singer, M. and Salpeter, M.M.: "Regeneration in Vertebrates: The role of the wound epithelium." Growth in Living Systems, Zarrow, M.X., ed., New York, Basic Books, Inc., pp. 277-311, 1961.

Smiley, G.R. and Dixon, A.D.: "Fine structure of embryonic midline palatal epithelium." I.A.D.R., 45th General Meeting abstract no. 258, 1967.

Smiley, G.R. and Dixon, A.D.: "Fine structure of midline epithelium in the developing palate of the mouse." Anat. Rec., 161: 293-310, 1968.

Stark, R.B. and Ehrmann, B.S.: "The development of the center of the face with particular reference to surgical correction of bilateral cleft lip." Plast. Reconstr. Surg., 21: 177-191, 1958.

Steinger, F.: "Neue Beobachtungen an der erblichen Hasenscharte der Maus." Z. Menschl. Vereb. Konstit. Lehre., 23: 369-404, 1939.

Stern, I.B.: "Electron microscopic observations of oral epithelium. I. Basal cells and the basement membrane." Periodontics, 3: 224-238, 1965.

Tondury, G.: "On the mechanism of cleft formation." In: Congenital anomalies of the face and associated structures. Thomas, ed. Pruzansky, S., Springfield, pp. 85-100, 1961.

Veau, V.: "Hasenscharten menschlicher Keimlinge auf der Stufe 21-23 mm SSL." Zeitschr. f. Anat. Entwick. Geschicht., 108: 460-493, 1938.

Verrusio, C.A.: "A mechanism for closure of the secondary palate." Terat., 3: 17-20, 1970.

Walker, B.E.: "The associaton of mucopolysaccharides with morphogenesis of the palate and other structures in mouse embryos." J. Embryol. Exp. Morph., 9: 22-31, 1961.

Walker, B.E. and Fraser, F.C.: "Closure of the secondary palate in three strains of mice." J. Embryol. Exp. Morph., 4: 176-189, 1956.

Wood, P.J. and Kraus, B.S.: "Prenatal development of the human palate." Arch. Oral Biol., 7: 137-150, 1962.

APPROVAL SHEET

The thesis submitted by Dr. William M. Kelly has been read and approved by members of the Department of Oral Biology.

The final copy has been examined by the director of the thesis and the signature which appears below verifies the fact that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted as partial fulfillment of the requirements for the degree of Master of Science.

May 15, 1972.

Ravindra Nanda