Light and Electron Microscopic Observations on Hydrocortisone-induced Cleft Palate in Hamsters [1]

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ABSTRACT Palatal histogenesis in hydrocortisone-treated hamster fetuses was studied by both light and electron microscopy. At an early stage in the hydrocortisone-affected fetuses, when the palatal shelves hung vertically on either side of the tongue, necrotic changes could be seen in some of the basal epithelial cells which lay adjacent to the fragmented basal lamina. The normal looking cells lay on an intact basal lamina and were attached to the contiguous necrotic cells by desmosomes. With horizontal reorientation of the palatal shelves and their approach to the midline, cellular necrosis and fragmentation of the basal lamina increased. When compared with normal cells, the hydrocortisone-affected ones were seen to be lighter, to contain fewer ribosomes and no lysosomes. At a later stage, when midline palatal fusion was lacking, the epithelium underwent stratification and keratinization while the necrotic debris was removed by mesenchymal macrophages. It appears that the normal process of protein synthesis is inhibited following hydrocortisone administration and that this, in turn, during palatogenesis, disrupts normal cellular differentiation and the integrity of the basal lamina, which are associated with the production of a cleft palate.

In 1950, Baxter and Fraser reported that administration of cortisone to pregnant mice resulted in offspring with cleft palates. Since then glucocorticoid-induced cleft palates have been reported in a variety of species, e.g., different strains of mice (Fraser and Fainstat, '51; Kalter, '54; Ingalls and Curley, '57; Chaudhry et al., '66; Dostal and Jelinek, '70), rabbit (Fainstat, '54), rat (Dostal and Jelinek, '71), human (Harris and Ross, '56; Doig and Coltman, '56; Bongiovanni and McFadden, '60; Popert, '62; Warrell and Taylor, '68) and hamster (Shah and Chaudhry, '73; Chaudhry and Shah, '73b). Numerous attempts were made in the past to explore the mechanism by which glucocorticoid treatment induces cleft palate (Walker and Fraser, '57; Larsson, '62a,b; Loevy, '62; Jacobs, '64a,b; Ross and Walker, '67, Zimmerman et al., '70; Andrew and Zimmerman, '71; Shapira and Shoshan, '72; Dostal and Jelinek, '72; Green and Kochhar, '73; Andrew et al., '73; Holst and Mills, '75; Shah and Travill, '76). These studies used light microscopic, histochemical, biochemical and autoradiographic techniques. With the exception of an abstract by Shah ('75), a comprehensive ultrastructural study on the development of glucocorticoid-induced cleft palate in any species has hitherto been unreported.

In their study, Shah and Chaudhry ('73) showed that hydrocortisone is a potent teratogen when administered to pregnant hamsters and produces cleft palate in the fetuses. Subsequently, on the basis of morphologic study, Shah and Travill ('76) observed that hydrocortisone administration prevented fusion between opposing palatal shelves in hamster fetuses. We, therefore, studied the subcellular events during the formation of cleft palate in hamster fetuses following hydrocortisone administration.

The results of light and electron microscopic investigations of normal palatal de-

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velopment in hamsters have been reported earlier (Chaudhry and Shah, '73a; Shah and Chaudhry, '74a,b). It was observed that reorientation of the palatal shelves from a vertical to a hortizonal plane, the fusion of their opposing epithelia to form a seam, and its subsequent fragmentation and disappearance were essential sequential events prior to its replacement by mesenchyme during palatogenesis. Furthermore, it was suggested that the timing of appearance of lysosomes was of great significance and that the combined process of autophagy and heterophagy played an important role in the elimination of intervening epithelia prior to, during, and after normal palatal closure. The detailed observation and documentation of normal palatal development in hamsters, therefore, will not be repeated in this report.

MATERIALS AND METHODS

The environmental controls and breeding procedures are described elsewhere (Shah and Travill, '76). Pregnant hamsters were given either a single intramuscular injection of an aqueous suspension of 35 mg hydrocortisone or a comparable amount of water on day 11 of gestation. Both control and hydrocortisone-treated fetuses were obtained at hourly intervals between day 12 and 12:12 (12 days, 12 hours), then at 2-hour intervals from day 12:12 to day 13 and finally at 12-hour intervals until day 15 of gestation. Fetuses for light microscopy were fixed in Bouin's solution and processed for serial sectioning.

For electron microscopic study, decapitated fetal heads were fixed in cold (0°–4°C) 3% glutaraldehyde buffered with 0.1 M Sorenson's phosphate buffer at pH 7.2 for two hours (Sabatini et al., '63). Subsequently the mandible, the tongue and the extraneous tissues were removed from each head and the palates were fixed further in fresh glutaraldehyde for a total of 12 hours. The tissues were then rinsed every half hour in cold Sorenson's phosphate buffer (pH 7.2) four times and subsequently post-fixed in 1% osmium tetroxide in 0.2 M phosphate buffer (pH 7.2) for 90–120 minutes at 0°–4°C. They were then rinsed in cold phosphate buffer (pH 7.2) followed by cold distilled water. The palates were then dehydrated through ascending concentrations of ethyl alcohol for one hour each, starting from 30% alcohol. This procedure was followed by two 10-minute changes of propylene oxide. The dehydrated tissue was then dipped for two hours each in 1:3 and 1:1 mixtures of Epon-Araldite and propylene oxide. Prior to their final embedding, the palates were transferred to aluminum cups containing fresh Epon-Araldite mixture (Mollenbauer, '64) and placed in a vacuum for approximately two to four hours in order to remove air bubbles and to promote infiltration of the embedding material into the tissues. For final embedding in the capsule, an accelerator, dimethyl aminoethyl phenol (DMP-30) was added to the embedding medium and thoroughly mixed in amounts to achieve 2% concentration of the accelerator. Each palate was cut into anterior, middle and posterior thirds and oriented in the capsule to obtain coronal sections. The capsules were placed in an oven maintained at 60°C for 72 hours. One-micron thick sections were obtained from the blocks for identification and orientation of tissues. Then, from appropriate blocks, thin sections approximately 60 $m\mu$ in thickness were obtained and transferred to 200-mesh copper grids. The grids were stained with methanolic uranyl acetate (Stempak and Ward, '64) for ten minutes, followed by lead citrate (Reynolds, '63) for one minute. They were examined on an Hitachi HU-11E-1 electron microscope at an accelerating potential of 80 KV.

OBSERVATION

The vertical palatal shelf in hydrocortisone-treated fetuses was covered with an epithelium one to three cells thick (fig. 1). Its superficial cells were flat and attached to subjacent cells by desmosomes (fig. 2). The cells contained flattened nuclei surrounded by polyribosomes, a few cisternae of rough endoplasmic reticulum and mitochondria. Basal epithelial cells, on the other hand, were roughly cuboidal and varied in their electron lucency, some being dark and others light. The former (fig. 2) contained large spherical or oval nuclei surrounded by polyribosomes, a small Golgi complex, a few strands of

rough endoplasmic reticulum and sparse small bundles of tonofilaments; both intact and swollen mitochondria were also present in the cytoplasm. These cells in hydrocortisone-treated palatal shelves resembled the basal epithelial cells of the normal vertical palatal shelf and were separated from the subjacent mesenchyme by a continuous, intact basal lamina.

The lighter cells were not seen in the epithelium at a comparable stage during normal palatal development. They contained spherical or oval nuclei surrounded by relatively electron-lucent cytoplasm (fig. 3). The chromatin was condensed at the periphery of the nucleus, while the nuclear membrane was swollen. The cytoplasm had reduced polyribosomes and mitochondria as compared with the darker cells. Cisternae of rough endoplasmic reticulum were swollen and bundles of tonofilaments were scattered in the cytoplasm of light cells. Trump et al. ('74) have interpreted such intracellular alterations as necrotic changes. Both the dark and the necrotic light cells were attached to one another by desmosomes. The basal lamina supporting the lighter cells was discontinuous and often fragmented.

The mesenchymal cells within the palatal shelves were stellate and resembled their normal counterparts, though they contained fewer cytoplasmic organelles than the dark basal epithelial cells.

During reorientation of the hydrocortisone-treated palatal shelf, there was an increase in the number of light necrotic cells (fig. 4). The morphology and content of both the light necrotic and the dark cells remained unchanged.

In contrast to normal development, the midline epithelium of the hydrocortisone-treated horizontal shelf showed two distinct layers of cells (fig. 5). The normal-looking superficial cells and the necrotic basal cells were both attached to one another by desmosomes. The morphology of the superficial cells remained unchanged though there was an increase in the number of cytoplasmic organelles (fig. 6). The basal necrotic cells varied in electron-lucency primarily due to reduction in the number of polyribosomes. At this stage the basal lamina supporting the light cells was either fragmented or completely destroyed (fig. 7).

The mesenchymal cells (fig. 7) of the hydrocortisone-treated palatal shelf were irregular and contained polyribosomes, numerous cisternae of rough endoplasmic reticulum, extensive Golgi complexes, small spherical or oval mitochondria and many lipid droplets. In addition, a few of these cells contained lysosomes and appeared to be differentiating into macrophages; however, at the corresponding stage in the development of the normal palate no such cells were evident.

On the approximating hydrocortisone-treated palatal shelves, the epithelium was two to three cells thick (fig. 8). The necrotic basal cells, seen in the epithelium of the earlier reoriented shelf, were absent at this stage. Instead the epithelial cells were flat and contained oval or irregular nuclei (fig. 8). The cytoplasm contained polyribosomes, rough endoplasmic reticulum and tonofilaments; some mitochondria were swollen, others intact. An occasional superficial cell was darker and attenuated. The attenuated cells contained flat nuclei surrounded by polyribosomes and swollen mitochondria (fig. 8). In contrast, the superficial cells of the untreated approximated midline epithelia were seen degenerating through intracellular lysosomal autophagy. Also unlike normal development, basal laminae separating the midline epithelium from the subjacent mesenchyme in hydrocortisone-treated shelves were fragmented near some epithelial cells and completely absent at other sites. The mesenchymal cells in the treated shelves resembled those of the untreated ones.

Subsequently, the hydrocortisone-treated palatal shelves remained in the horizontal or approximated position. Fusion of the epithelia observed during normal development did not occur following hydrocortisone administration. Instead, the midline epithelia of the opposing palatal shelves differentiated to become characteristically stratified squamous. Following the stage of approximation, the basal epithelial cells showed an increase in the number of cytoplasmic organelles (fig. 9). Also, new basal laminae appeared though they were still discontinuous.

As development progressed, midline epithelia became three to five cells thick (fig. 10). Intact basal laminae separated epithelial cells from underlying mesenchyme. Spaces intervened between the epithelial cells. The epithelial cells were attached to one another by well-developed desmosomes, though many spaces were seen between contiguous cells. Numerous hemidesmosomes were seen on the plasma membranes of the basal cells opposing the basal laminae. These basal cells were cuboidal to irregular and contained numerous organelles. The cells in the superficial two or three layers were squamous and contained relatively fewer organelles than basal cells. In addition, groups of electron-dense granules, which were interpreted as glycogen particles, could be seen dispersed through the cytoplasm.

DISCUSSION

Early necrosis of epithelial cells with subsequent replacement, alterations in the basal laminae and the absence of lysosomes characterize palatal epithelium during palatogenesis in hydrocortisone-treated fetuses. These are the main cellular and subcellular features that distinguish abnormal from normal palatal development.

In the treated fetuses cellular necrosis was first recognized in the epithelium of the vertical shelf. During and after reorientation of the palatal shelf, the number of electron-lucent necrotic cells increased as opposing epithelia approached each other in the midline. Significantly, the necrosis remained limited to the basal cells in the epithelium. At a later stage, when the hydrocortisone-treated palatal shelves remained in the horizontal or approximated position, the number of cells undergoing necrosis decreased and eventually they all disappeared as the midline epithelia became stratified.

At no time during palatal development did the superficial epithelial cells undergo necrosis in those fetuses subjected to the influence of hydrocortisone. This is in contrast to normal palatal development in which the degeneration of superficial cells is observed when the shelves lie horizontal or approximated (Chaudhry and Shah, '73a; Shah and Chaudhry, '74b). It is possible that degeneration of superficial cells provides the "potentiality for fusion" between the epithelia of opposing palatal shelves(Pourtois, '66; Vargas, '67). Persistence of attenuated superficial cells following hydrocortisone administration suggests that epithelial integrity inhibits cellular fusion and thus plays an important role in formation of the observed cleft.

In order to reach the epithelial cells and exert its influence, hydrocortisone or its metabolites must cross the basal laminae. It has already been shown that during normal development, an intact basal lamina persists as long as the epithelial cells are present in situ (DeAngelis and Nalbandian, '68; Brusati, '69; Shapiro and Sweney, '69; Morgan, '69; Sweney and Shapiro, '70; Smiley, '70; Smiley and Dixon, '68; Smiley and Koch, '71, '72; Mato et al., '72; Chaudhry and Shah, '73a; Shah and Chaudhry, '74b; Hinrichsen and Stevens, '74). Basal laminae are lost only after disappearance of the epithelial cells. Following treatment with hydrocortisone, however, the basal laminae fragment and become discontinuous below the epithelial cells that are undergoing necrosis. This implies that either (1) the permeability of the basal laminae is altered due to hydrocortisone treatment, which in turn allows premature necrotic changes to occur in the overlying basal epithelial cells, or (2) the hydrocortisone crosses the basal laminae without changing the latter's permeability. The teratogen then interferes with the synthetic activity of the cells and causes necrotic changes. It has been suggested that the dead epithelial cells cannot synthesize the basal laminae, which therefore fragment and become discontinuous (Pierce, '70; Briggaman et al., '71; Hay and Dodson, '73). We observed that unlike normal development, necrosis of the basal epithelial cells, following hydrocortisone administration, started when the palatal shelves were vertical. The basal lamina was fragmented only below the necrotic basal cells. As the number of light necrotic cells increased progressively, fragmentation of the basal lamina also extended further. If the integrity of the basal laminae is dependent upon the synthetic activity of the epithelial cells, as shown by Pierce ('70), Kafalides ('73) and Hay and Dodson ('73), then one may reason-

ably assume that hydrocortisone inhibits protein synthesis in the epithelial cells. The retardation of the synthetic process, in turn, may prevent the production of molecules necessary for maintaining the basal lamina's integrity. Observation during normal development shows that disintegration of basal laminae occurs only after the loss of support from the epithelial cells (Chaudhry and Shah, '73a; Shah and Chaudhry, '74b). This observation further strengthens the hypothesis that any alteration in the integrity of basal laminae will be preceded by changes in the epithelial cells (Pierce, '70).

The precise mechanism by which hydrocortisone causes cleft palate is not yet fully understood. It is believed that at the cellular level hydrocortisone influences many metabolic processes (Tapperman, '68; Kornel, '73). It has been said to stabilize lipoprotein membranes in particular (Weissman and Dingle, '61; Weissman and Thomas, '64). Hydrocortisone has been thought to be implicated in the stimulation of synthesis of those enzymes which deaminate amino acids, thus probably delaying protein synthesis (Kenny et al., '65). It has also been suggested that the catabolic effect of glucocorticoid is mediated through inhibition of glucose uptake (Munck, '71; Feldman et al., '72). The hormone-induced decrease in glucose uptake promotes catabolism in some cases to the point of cytolysis (Feldman et al., '72). On the other hand, in the liver, hydrocortisone stimulates synthesis of ribonucleo protein (Beato et al., '70; Agarwal, '72). On the basis of studies in vitro, Reynolds ('66) suggested that hydrocortisone inhibits desoxyribonucleic acid synthesis and thereby depresses cellular proliferation in a growing organ. At the tissue level Jurand ("68) recently showed that hydrocortisone exerts a toxic effect on the mesenchymal cells of limb buds but not on the epithelial cells. In our study, following hydrocortisone treatment, there was a striking absence of lysosomes and decrease in amount of polyribosomes in the light necrotic cells of the palatal epithelium. On the basis of their biochemical studies, Zimmerman and associates ('70) showed a close correlation between inhibition of ribonucleic acid synthesis in the whole fetus and the production of cleft palate following glucocorticoid treatment. It is possible that hydrocortisone delays or retards the synthesis of protein in the cells of midline palatal epithelium, thereby causing early necrosis of cells. Since the timing of cell death is a necessary prerequisite to removal of the midline epithelia (Shapiro and Sweney, '69; Shah and Chaudhry, '74a), it may be expected that an early necrosis would alter the subsequent differentiation of cells and thus facilitate the production of palatal clefts.

It has been repeatedly shown that intracellular lysosomal autophagy plays an important role in removal of midline epithelia during normal palatogenesis (Mato et al., '66; Angelici and Pourtois, '68; DeAngelis and Nalbandian, '68; Farbman, '68, '69; Brusati, '69; Morgan, '69; Hayward, '69; Shapiro and Sweney, '69; Koziol and Steffek, '69; Sweney and Shapiro, '70; Smiley, '70; Vargas et al., '72; Chaudhry and Shah, '73a; Shah and Chaudhry, '74a,b). However, following hydrocortisone treatment, lysosomes were not observed in the cells of the palatal epithelium at any stage, not even in the light necrotic cells (fig. 3). Kerr ('71) has suggested that lysosomes are not necessarily involved in the genesis of cellular necrosis, and Schweichel and Merker ('73) have observed non-lysosomal death of cells following administration of numerous teratogens. These authors inferred that inhibition of protein synthesis probably causes subsequent retardation in the synthesis of hydrolytic enzymes. It is, therefore, possible that in hydrocortisone-treated palates, protein synthesis in epithelial cells is disturbed prior to the appearance of lysosomes. It would appear that inhibition of protein synthesis occurs as a fundamental aberration in hydrocortisone-affected palatogenesis, because the reduction of protein in the light cells at an early stage may prevent the subsequent formation of lysosomal enzymes; and so the controlled process of cellular degeneration and death is inevitably deflected and results in cleft palate.

Veau ('38) proposed that the palatal clefts are produced because mesenchymal tissue fails to invade and destroy the surrounding epithelium. The failure to in-

vade, he suggested, may be due to the poor quality of the mesenchymal tissue itself. Stark ('58) supported Veau's theory and attributed cleft formation to mesenchymal inadequacy. Subsequent studies on experimentally induced cleft palates have tended to support the theory that the cause of abnormal palatal development lies primarily in the mesenchymal tissues (Larsson, '62a,b) though this has been disputed recently (Andrew and Zimmerman, '71). During the present investigation we found no evidence that mesenchyme "invades" or breaches the overlying epithelium at any stage of development. Furthermore, the differentiation of mesenchymal cells in hydrocortisone-treated palates parallels that of the normal. At a later stage of development, however, some of the mesenchymal cells in the treated shelf assume a phagocytic function and remove the necrotic epithelial debris. Mesenchymal cells, therefore, appear to have no formative role in cleft palatogenesis, apart from "mopping up" excess debris.

The results of our study do not support the theory that cleft palates result from a rupture of fused epithelia (Kitamura, '66). At no stage following hydrocortisone treatment were we able to recognize a fusion of the opposing epithelia.

From the foregoing discussion it appears obvious that following hydrocortisone administration, the fusion of epithelia between the opposing shelves is prevented, and this in turn thereby produces palatal clefts. The underlying mechanism appears to be inhibition of protein synthesis in the epithelial cells, leading to membrane inadequacy and consequent attenuation of the superficial layers, coupled with premature non-lysosomal necrosis of the basal epithelial cells.

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PLATES

Abbreviations

BL, Basal lamina	Ly Lysosome
D, Desmosome	M, Mitochondria
GC, Golgi complex	N, Nucleus
Gly, Glycogen	RER, Rough endoplasmic reticulum
HD, Hemi-desmosome	Sup, Superficial cell
L, Lipid	Tf, Tonofilament

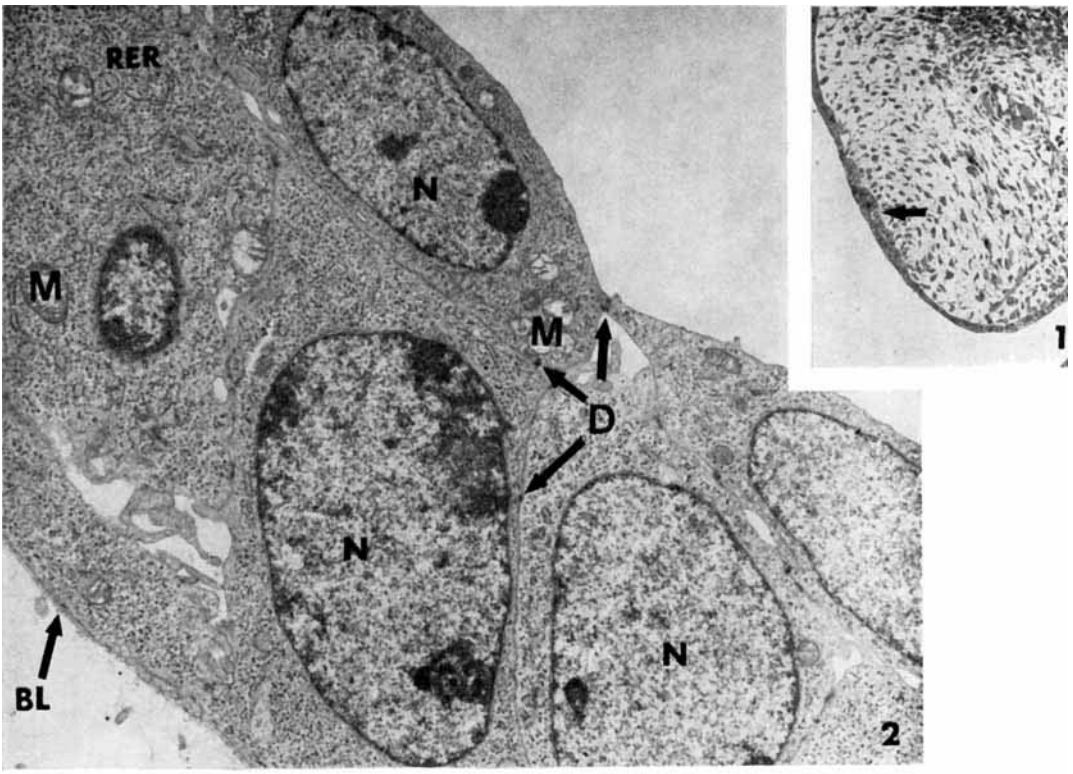
PLATE 1

EXPLANATION OF FIGURES

1 Methylene blue-stained thick epon section through a vertical palatal shelf of a hamster fetus at day 12:04 of gestation, following hydrocortisone administration, showing a necrotic epithelial cell (arrow). × 32.

2 Electron micrograph of a vertical palatal shelf at day 12:04 of gestation, following hydrocortisone administration. An intact basal lamina is present between the epithelium and the mesenchyme. Desmosomes are present between the cells in the epithelium. Note that both the superficial and the deeper cells contain few cytoplasmic organelles. × 8,800.

3 Electron micrograph of a vertical palatal shelf at day 12:00 of gestation, following hydrocortisone administration, showing a necrotic light cell. The cytoplasm is relatively electron-lucent, due to reduced polyribosomes, and contains swollen rough endoplasmic reticulum, bundles of tonofilaments and mitochondria. The light cell is attached to the relatively darker cell by desmosomes. The basal lamina below the light cell is not continuous (arrowheads). × 6,000.


RER
N
M
M
D
N
N
BL
2
1

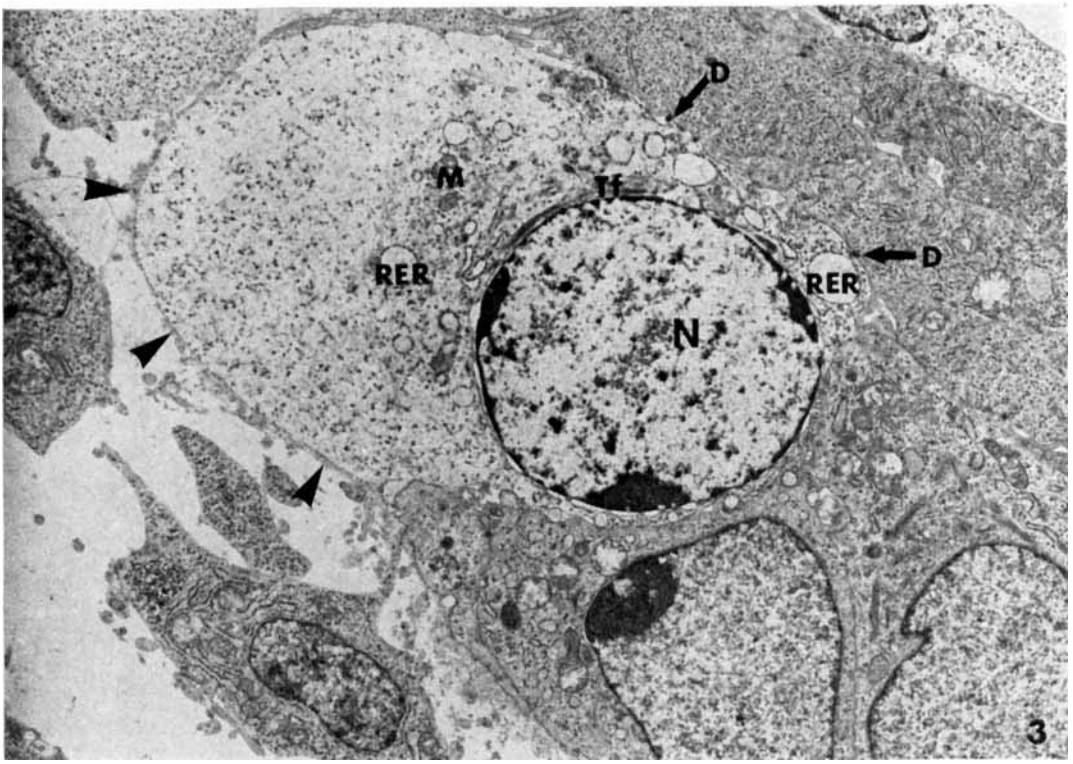

D
M
Tf
RER
D
RER
N
3

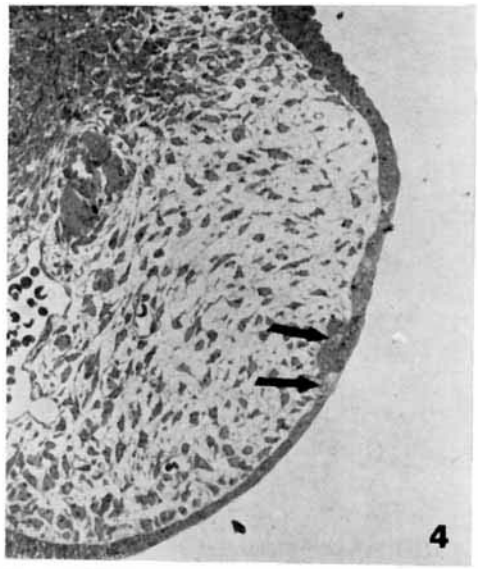
PLATE 2

EXPLANATION OF FIGURES

4 Methylene blue-stained thick epon section through a reorienting palatal shelf of a hamster fetus at day 12:04 of gestation, following hydrocortisone administration. Note the presence of light necrotic cells (arrows). × 32.

5 Methylene blue-stained thick epon section through a horizontal palatal shelf of a hamster fetus at day 12:08 of gestation, following hydrocortisone administration. The basal cells are undergoing necrosis. The superficial cells are intact (arrows). × 32.

6 Electron micrograph of a horizontal palatal shelf at day 12:08 of gestation, following hydrocortisone administration. The epithelium has two distinct layers of cells — necrotic basal cells and darker superficial cells. These cells are attached to one another by desmosomes. × 8,800.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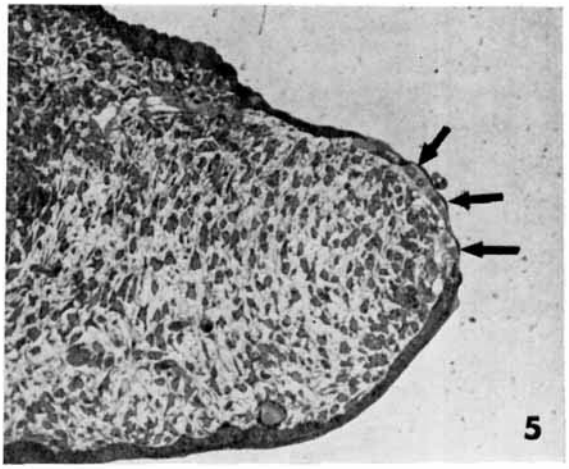
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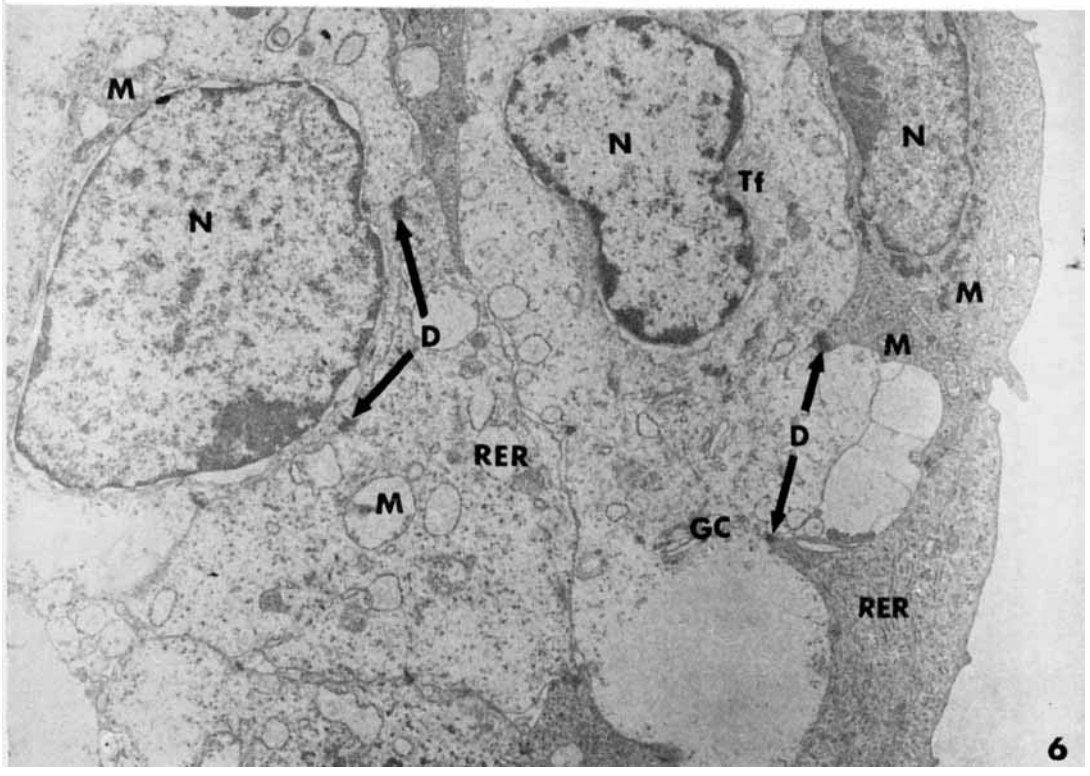
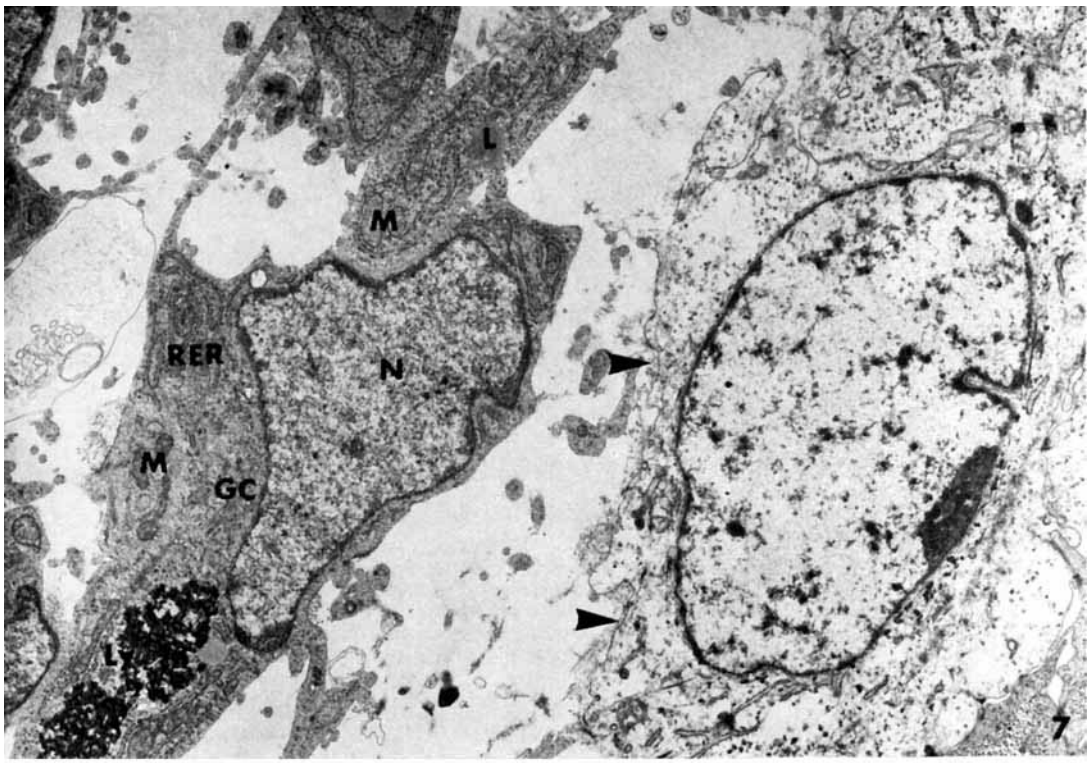

M
N
D
N
Tf
N
M
M
D
RER
M
GC
RER
6

PLATE 3

EXPLANATION OF FIGURES

7 Electron micrograph of a horizontal palatal shelf at day 12:08 of gestation, following hydrocortisone administration. The basal lamina (arrowheads) supporting the necrotic cell is fragmented. A mesenchymal cell appears to be differentiating into a macrophage. It contains, among other organelles, lysosomes. × 8,800.

8 Electron micrograph of the approximating palatal shelves at day 12:12, following hydrocortisone administration. The opposing epithelia of the shelf are very close to one another. The necrotic light cells seen at the previous stage (cf. figs. 6 and 7) are absent at this stage. The superficial cells are attenuated. The basal lamina is fragmented in one shelf (arrowheads) and absent in the other. × 6,000.


L
M
RER
N
M
GC
L
7

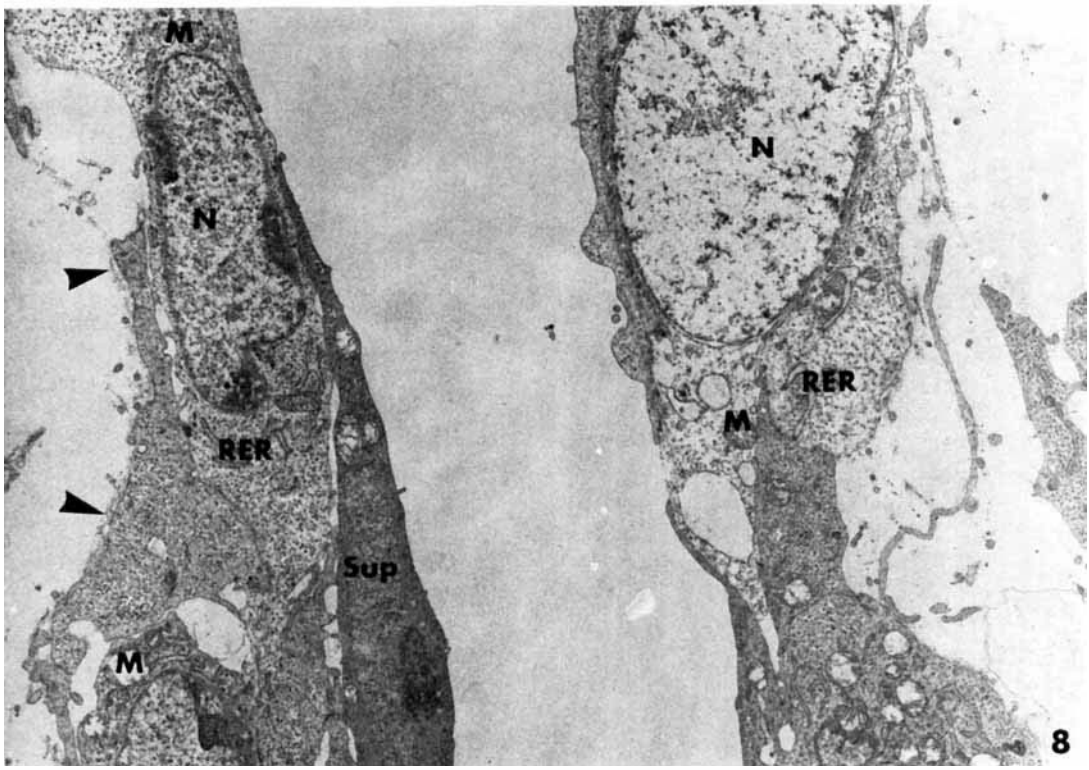
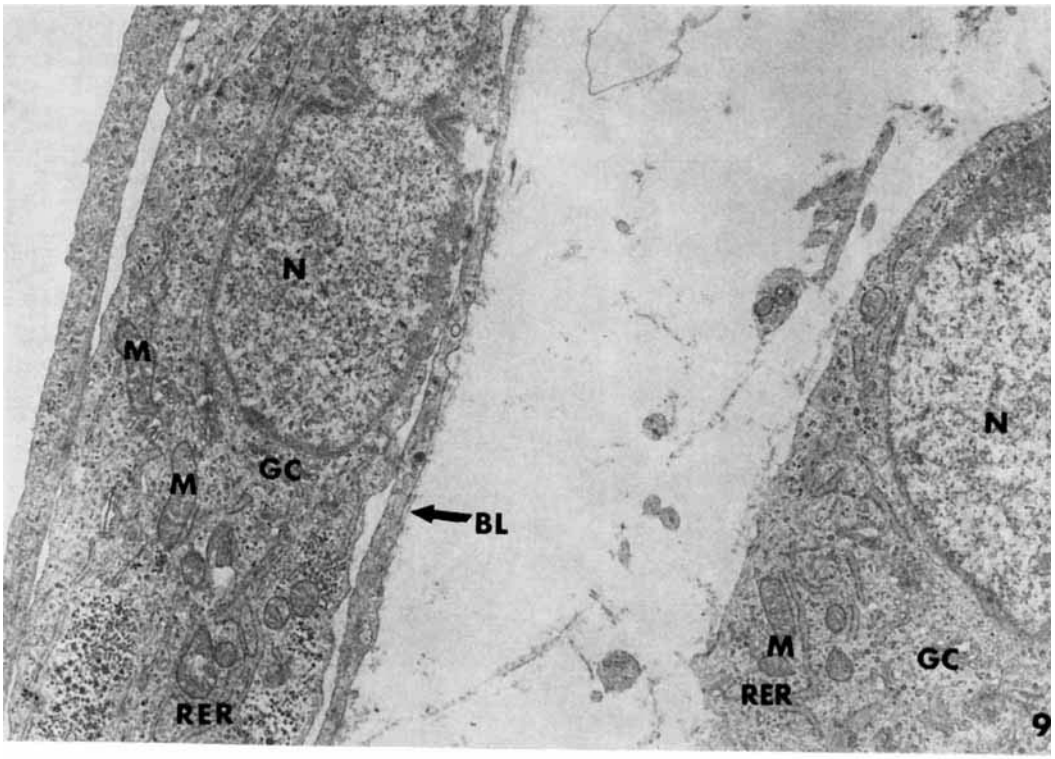

M
N
RER
Sup
M
N
RER
M
8

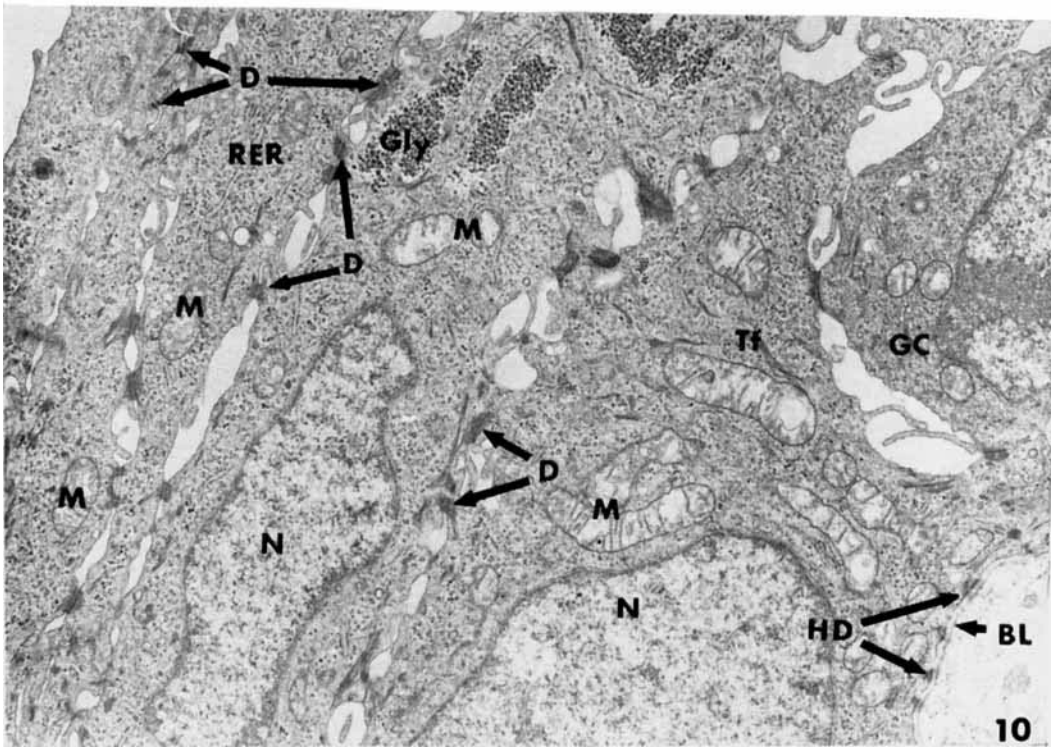
PLATE 4

EXPLANATION OF FIGURES

9 Electron micrograph of a horizontal palatal shelf at day 12:16 of gestation, following hydrocortisone administration. Both the epithelial and mesenchymal cells are more differentiated than in the previous stage (cf. fig. 8). The basal lamina is still discontinuous. The necrotic cells seen in early horizontal shelf (cf. figs. 6 and 7) are not seen at this stage. × 13,200.

10 Electron micrograph of a horizontal palatal shelf at day 13:00 of gestation, following hydrocortisone administration. The basal lamina is continuous. Numerous hemi-desmosomes are present along the basal lamina. The epithelial cells are attached to one another by numerous desmosomes. The cells of the stratified squamous epithelium are well differentiated and contain numerous cytoplasmic organelles. × 13,200.


N
M
M
GC
BL
N
M
GC
RER
RER
9


D
RER
Gly
M
D
M
Tf
GC
D
M
M
N
N
HD
BL
10