THE FORMATION AND RUPTURE OF OROPHARYNGEAL MEMBRANE IN THE MOUSE EMBRYO. THE ROLE OF CELL DEATH

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SUMMARY

The formation and rupture of the oropharyngeal (buccopharyngel) membrane was examined by light and transmission electron microscopy in the mouse embryo. The oral membrane is first recognizable at 5-7 somite embryos as an approaching area between the pharyngeal and stomodeal epithelium, during this fusion process an extracellular space appears between the ectoderm and endoderm, which contained granule fibrillar material, neither cell processes nor cellular bridges were bound. Tight junctions were observed either among ectodermal or endodermal cells, but not between both of those two cell populations. At 10 to 15 somite embryos, during the period ofo rupture, necrotic cells occur in the oral membrane and these cells contain autophagic vacuoles and phagolysosomes. A topographic study of the appearance of the degenerating cells showed two different areas of cell death, located on the upper and lower slopes of the oral membrane. Pycnotic cells appear also randomly localized within the epithelial sheets of the oral anlage as well as in the dorsal mesenchyme to the pharynx. The process of cell death is related to the detachment of the oral membrane from the stomodeal ectoderm and foregut endoderm, which subsequently perforates and disappears.

Key Words:
Cell death. Oropharyngeal membrane. Mouse embryo.

RESUMEN

Se realiza un estudio histológico y ultraestructural de la formación y desaparición de la membrana orofaríngea (bucofaringea) durante el desarrollo embrionario del ratón. La membrana orofaríngea aparece en embriones de ratón de 5-7 somites, como una zona de aposición entre el ectodermo del estomodeo y el endodermo de la faringe faringeo. Entre ambas capas epiteliales aparece un material extracelular granulofibrilar, como elemento de fusión, pero sin encontrarse puentes o extensiones intercelulares. Pudiendo observarse uniones estrechas entre células de una misma capa epitelial pero nunca conectándolas entre sí. Durante el estadio de 10-15 somites la membrana orofaríngea se rompe, apareciendo necrosis celulares con vacuolas autofágicas y fagolisosomas. Topográficamente las muertes celulares se ubican en los bordes superior e inferior de la membrana, así como entre endodermo y ectodermo. La localización y evolución de la muerte celular programada sugiere su intervención como mecanismo morfogenético en la regresión de la membrana orofaríngea.

Palabras Clave:
Muerte celular. Membrana orofaríngea. Desarrollo embrionario del ratón.

INTRODUCTION

The oral (buccopharyngeal) membrane is formed during early cranial development in vertebrate embryos as a localized epithelial fusion between the endodermal wall of the foregut and the adjacent stomodeal ectoderm. As development proceeds the oral membrane ruptures and degenerates providing an opening of the embryonic mouth into the developing foregut.

The oral membrane has been mentioned in several studies of cranial development which are, however, focused on distinct cephalic structures such as Rathke's pouch or the precordal plate (1, 2, 3, 6, 7, 11, 12, 15). In such studies, detailed examinations of the oral membrane have been rare.

Although the ultrastructure of the oral membrane has been investigated in hamster embryo (24, 25), in chick embryo (28) as well as in amphibia (8, 9, 23), the mechanisms controlling perforation and rupture of the oral membrane remain largely unknown.

Studies of development of this structure in the hamster (24, 25, 28) suggest that the progressive thinning of the oral membrane, as a result of cellular reorganization, is a major mechanism of its breakdown. On the other hand, cell death, as a morphogenetic mechanism, provides a possible explanation for the rupture of the oral membrane in anuran embryo (23). However evidence of cell death has not been observed during rupture and disappearance of the buccopharyngeal membrane in either chick or hamster embryos (24, 25, 28).

The present work was undertaken in order to study the morphological changes leading to the formation and rupture of the oral membrane in the mouse embryo, and to investigate the occurrence of cell death in order to assess its role in the regression of this transient structure.

MATERIALS AND METHODS

The study was carried out on mouse embryos at stages between 3 to 16 somites. The age of pregnancy was calculated by finding of a vaginal plug. At an appropriate stage the pregnant mice were killed by cervical subluxation under mild Ether anesthesia. Immediately afterwards both uterine horns were dissected out of the abdomen and the embryos isolated in Hank's saline solution (pH 7.3) so that they could be eventually staged according to the criteria described by Theiler (20). All of the embryos were dissected free of their membranes and were fixed in 2,5% glutaraldehyde in 0.1 M sodium cacodylate buffer. After 1 h. the material was postfixed in 1% osmium tetroxide for 35 min. then dehydrated and embedded in epoxyresin (19). Semithin sections (thickness about 2 u), taken and stained by the method of Richardson et al (17), were used for light microscopy. Thin sections were cut and double stained with uranyl acetate and lead citrate (16) and viewed in a Zeiss transmision electron microscope.

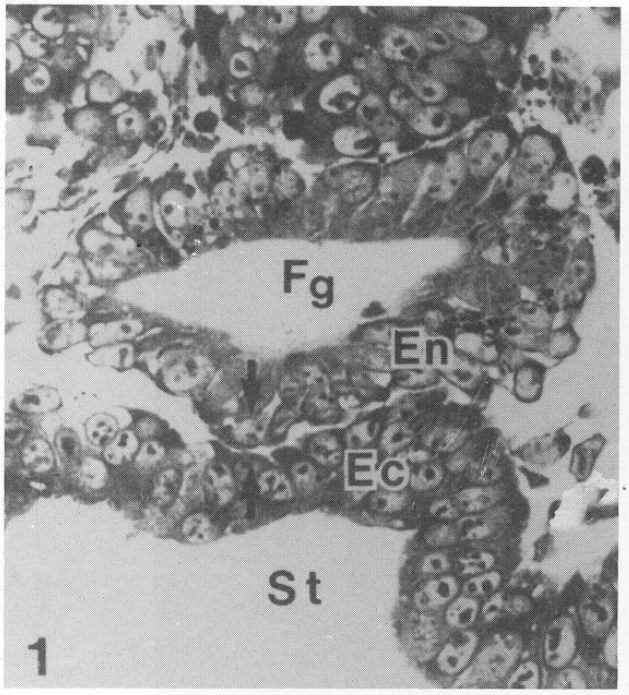
RESULTS

a) Embryos of 3 to 7 somites

During this early stage for foregut appeared as a digitiform formation, placed cranially between the forefront of the neural groove and the ventral ectoderm, which has already started to invaginate to form the stomodeal cavity. This approaching area, between the pharyngeal and stomodeal epithelium, corresponds to the buccopharyngeal membrane. In this early period, there were no ectoendodermic contacts at this level (Fig. 1). Between the two cellular groups there was, however, a narrow gap filled with a granule-fibrillar material that could be observed by EM (Fig. 5). The origin of this material is presumably mesodermal. Cellular components were never observed in this gap. Both the endoderm and ectoderm of this region show the characteristics of a cuboidal monostratified epithelium, and intercellular gaps between the two epithelial layers were never found.

b) Embryos of 7 to 10 somites

During this period the gradual growing up of the embryo and its curvature in the ventral direction considerably increase the size of the stomodeum and, at the same time, the size of the buccal membrane.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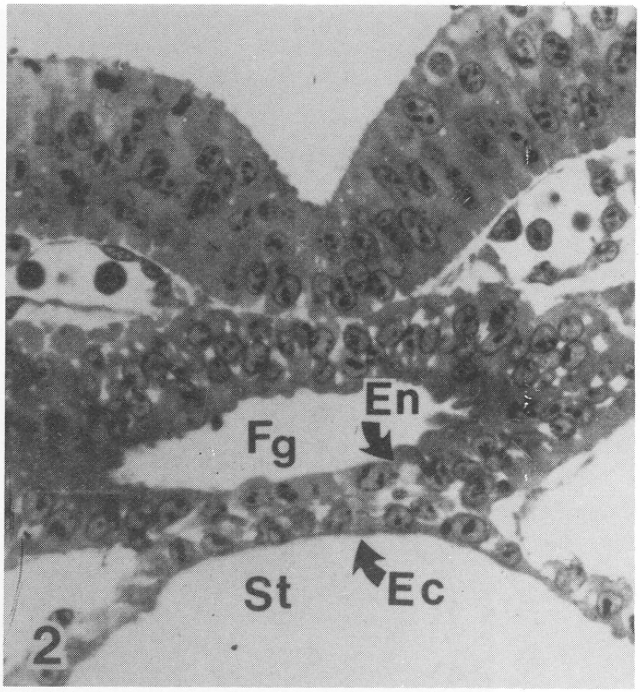
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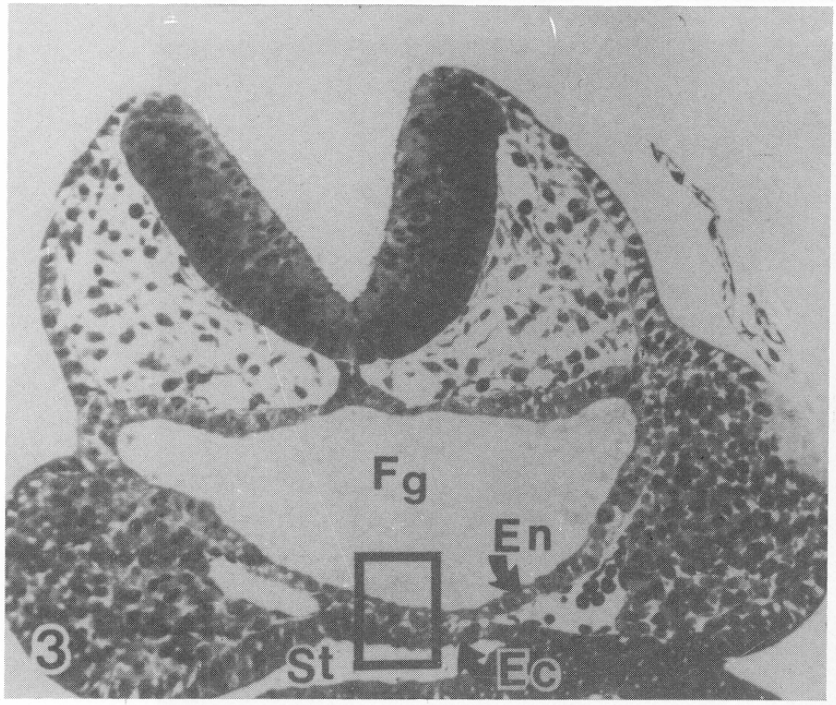
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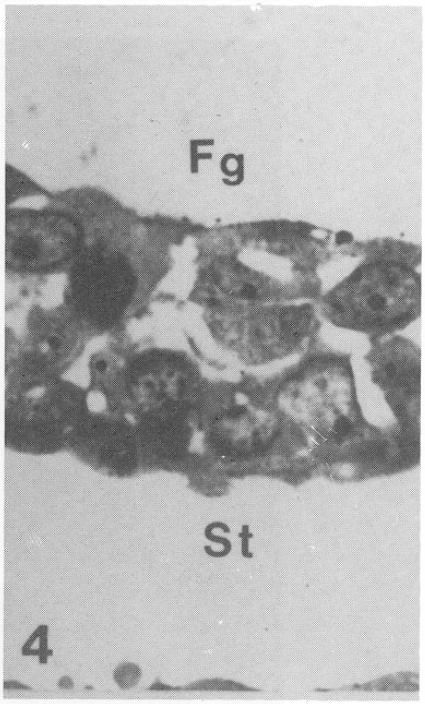
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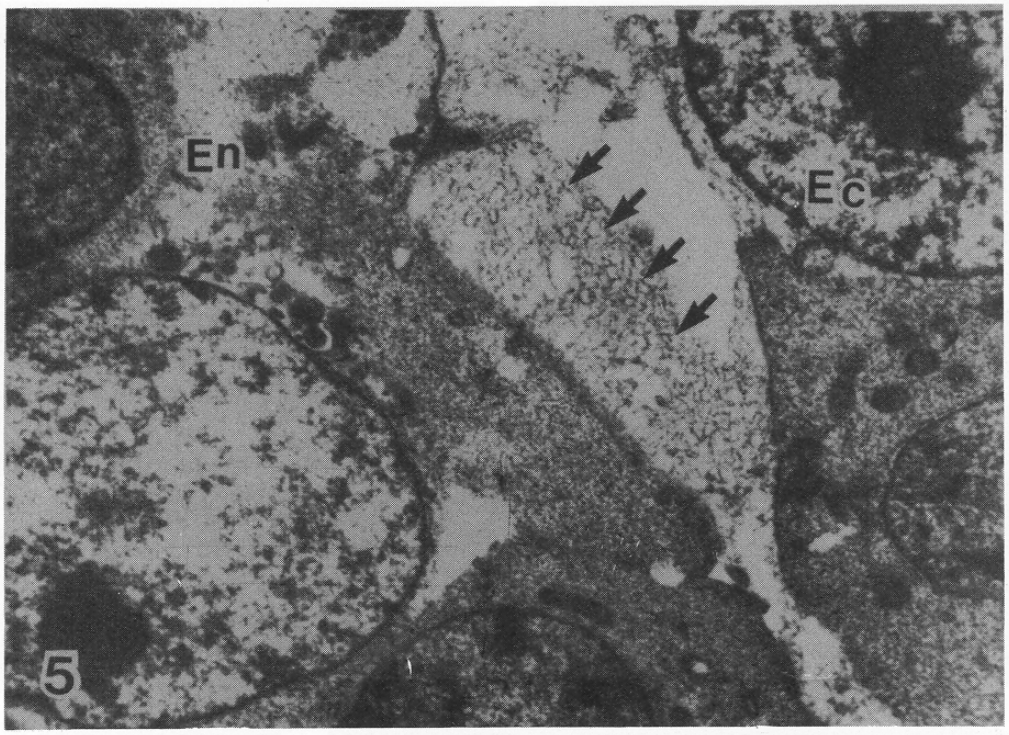
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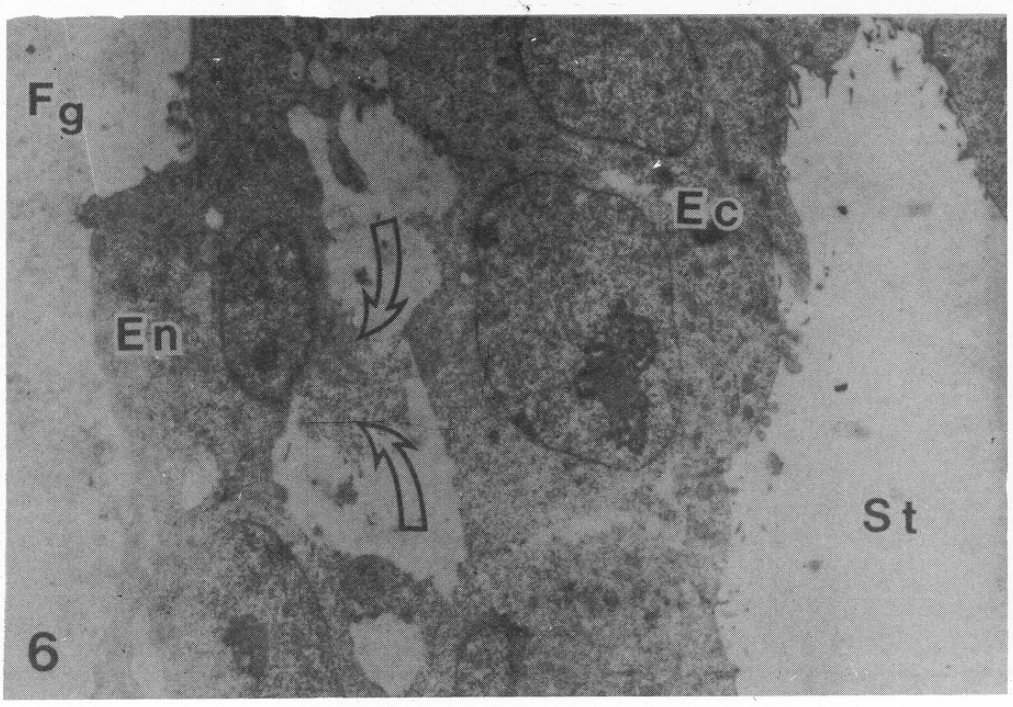
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The buccopharyngeal membrane could be observed by optical microscopy (Fig. 2), and consisted of a two-layer sheet formed by two cellular rows. Despite the fact that the ectodermal and endodermal components had already made contact, it was still possible to discern the constitutive elements from each epithelium.

The epithelial contact was larger on the central area of the oral plaque than on the lateral edges (Fig. 3). We found abundant intercellular gaps that, as mentioned above, were not present in previous stages (Fig. 4). Electron microscopy revealed some granular material that was very similar in appearance to that of the interepithelial gap at the previous stage (Fig. 6). Tight junctions appeared on the buccal and pharyngeal slopes of the buccal membrane, but never between the endodermic and ectodermic colonies.

The pharyngeal span was filled with a material that was in direct contact with the inside surface of the pharyngeal epithelium. This could be discerned both through electron and optical microscopy (Fig. 6).

c) Embryos of 10 to 15 somites

During this period a set of consecutive changes takes place, ending with the final rupture and shedding of the buccopharyngeal membrane.

The epithelial contact that had started in previous stages became more noticeable during stages 10-15. The ectodermal and endodermal cells of the oral sheet intermingled, and it was now impossible to discern one cellular colony from the other (Figs. 7 and 9).

Due to this increase in intercellular contacts the thickness of the buccopharyngeal membrane decreased considerably, reaching the point of being composed in some places only of small cytoplasmic bridges (Fig. 9). The intercellular gaps observed in previous stages were still present. However, the material filling the spaces had disappeared (Fig. 11). At this stage, tight junctions can also be observed on both pharyngeal and buccal slopes of the buccal membrane and the pharyngeal material, found previously, also persisted.

Two areas of cellular necrosis, located on the upper and lower slopes of the buccal membrane, appeared during these stages.

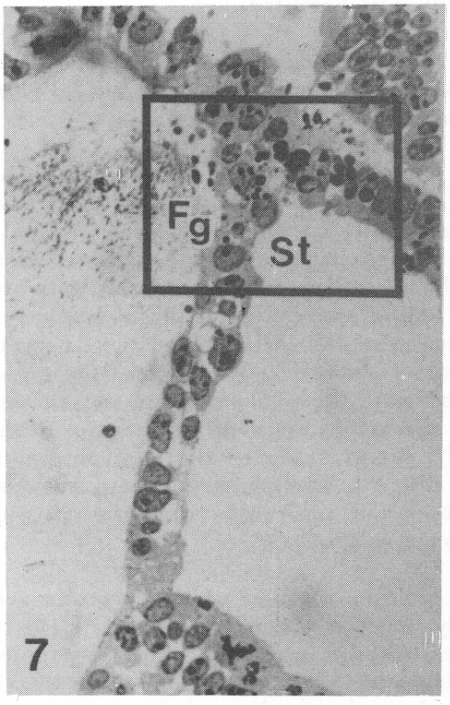
The upper necrotic area appeared in embryos of 13 somites (Figs. 7 and 10). This area was deeply localized and included the ectoderm and endoderm above the buccal membrane (Fig. 8). Some cellular necrosis were also present in the upper edge of the buccopharyngeal membrane and incidentally, some necrotic cells could be notice at the level of the dorsal mesenchyme to the pharynx (data not shown). Optical microscopy showed the existence in this area of pyknotic nuclei with high chromatin density (Fig. 8). Electron microscopy revealed the existence of autophagic vacuoles and phagolysosomes (Fig. 12).

The evolution in time of this necrotic area was very short. It started in embryos of 13 somites and disappeared in those of 15 somites, coinciding with the rupture and shedding of the buccal membrane. Nevertheless, some isolated necrosis could still be observed in the area of transition between the stomodeum and pharynx.

The lower necrotic area appeared later (Fig. 10). It was very clear in embryos of 14 and 15 somites, and was placed on the limit between the thyroid plate and the stomodeum. Some cellular necroses invade the lower slope of the buccopharyngeal membrane. Isolated cellular necroses could be found on the central portion of the buccopharyngeal membrane. The buccal membrane rupture occurred already in 15 somite stage, persisting some remains of the membrane. An established order in the appearance of the rupture points was not apparent, although it was observed that the rupture was preceded by a marked thinning of the oral sheet (Figs. 9 and 10).

DISCUSSION

The development of the buccal membrane is a characteristic example of epithelial joining and interaction. The basic mechanism of this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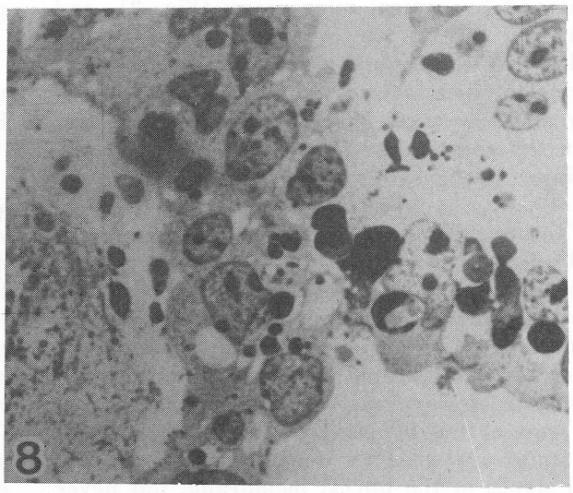
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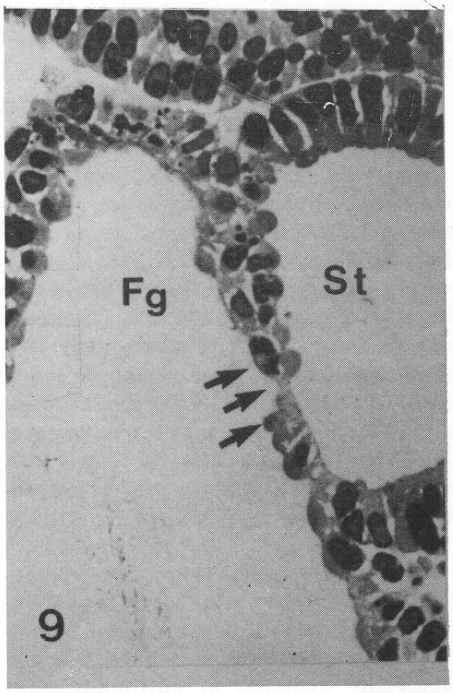
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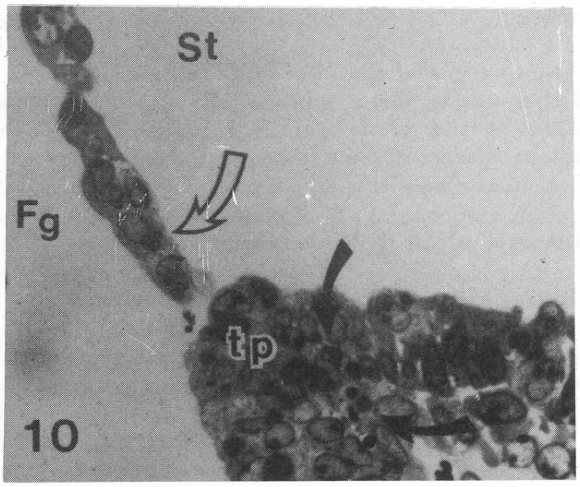
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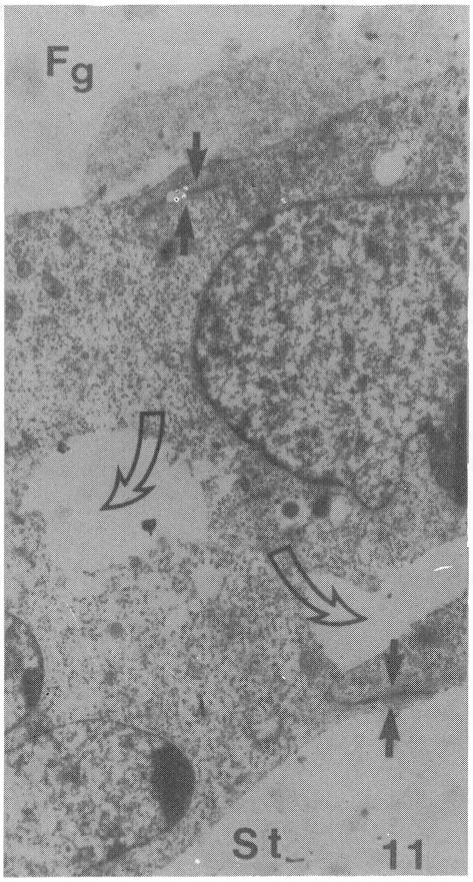
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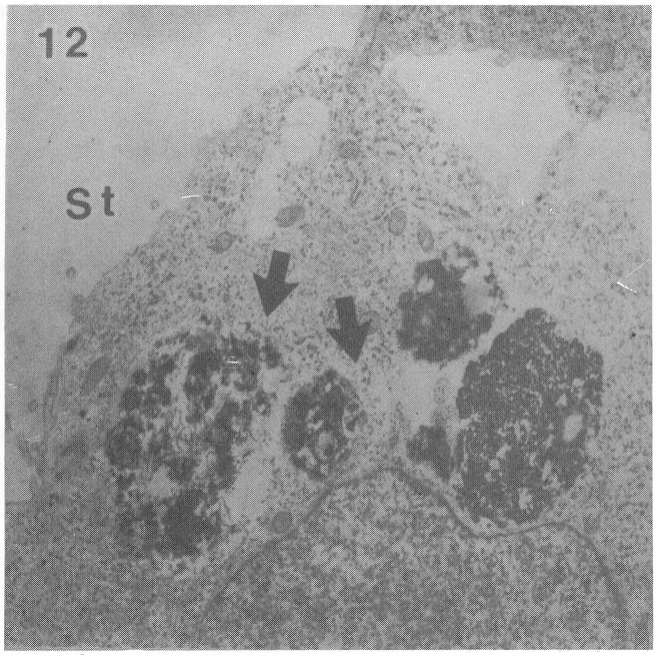
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Figure 1. Light micrograph of transversal section at 3 somites stage. A continuous extracellular space (arrows) is present between foregut endoderm and stomodeal ectoderm in the presumptive region of the oral membrane. x400.

Figure 2. Light micrograph of transversal section through the oral membrane at 7 somite stage. The oral anlage consis of a two-layer sheet, the endodermic (En) and ectodermic (Ec) components which had already made contact, both of them remain clearly defined. x250.

Figure 3. Light micrograph of transversal section through the oral membrane at 9 somite stage. Note that the epithelial contact is larger on the central portion of the oral membrane (squared field). x100.

Figure 4. Light micrograph showing the squared field of fig 3 in a larger detail. Note abundand intercellular gaps. The boundary between the foregut endoderm and stomodeal ectoderm becomes indistinct. x1000.

Figure 5. Transmission electron micrograph through the oral membrane at 5 somite stage. The interval between the basal surfaces of endoderm and ectoderm is narrow and discontinous. Extracellular material (arrows) is found within of the oral membrane but, not cell processes can be noted. x6000.

Figure 6. Transmission electron micrograph through the central portion of the oral membrane at 8 somite stage. Endodermal and Ectodermal epithelia are still distinctive. Intercellular spaces inside the oral membrane and foregut's cavity appear filled with granular material (arrows). x4000.

Figure 7. Light micrograph of midsagittal section at 13 somite stage. The upper slope of the oral membrane appeared in the squared field. x400.

Figure 8. Higher power view of the squared field shown in fig 7. Note many dead cells and cellular debris located in the craneal edge of the oral membrane. x1000.

Figure 9. Light micrograph of midsagital section through the oral membrane at 12 somite stage. The endodermic and ectodermic cells of the oral anlage appear intermingled and the central portion of the membrane becomes markedly thinner (arrowheads). x400.

Figure 10. Light micrograph of midsagital section through the oral membrane at 15 somite stage. Degenerating cells are found either in the thyroid plate or in the oral epithelial layer (arrowhead). x600.

Figure 11. Transmission electron micrograph through the ectodermal cells of the oral membrane at 13 somite stage, showing tigh junctions at stomodeal end of cells (arrowheads). Open arrows are indicating some empty extracellular spaces. x8000.

Figure 12. Transmission electron micrograph of the craneal margin of the oral membrane at 14 somite stage. Degenerating cells and cellular debris are seen within the oral epithelium (arrows). x5000.

joining is still unknown. But it has been proposed that certain factors may play an important role, Watermann and Balian (27) have suggested that the extracellular matrix may participate in the joining process of the buccal membrane in the chicken embryo. In this respect, it should be emphasized that we have observed the existence of a granule fibrillar material located between the ectoderm and the endoderm in stages prior to the joining. Although we have not determined its specific characteristics, this material has a similar appearance to that observed inside the intercellular gaps after the fusion has occurred. We consider that there is a process of catching of the extracellular material during the fusion phase. We have not been able to determine the cause of the disappearance of this material in later stages, but it could be due to a phagocytic process on the part of the buccal membrane cells, as it has been described at the level of the closing plates for the chicken embryo by Watermann (26).

On the other hand, it has also been suggested that the basement membrane can influence the epithelial joining process. A disruption in the basement membrane seems to help the epithelial contact, as showed for the fusion of the palatal processes by Morgan (13), the buccal membrane by Watermann (25), Watermann and Schoenwolff (28) and for the process of fusion between the endoderm of the pharyngeal pouch and the ectoderm from the branchial clefts (26). On the other hand, in the oral membrane, cellular bridges appear between the ectodermic and endodermic populations, after the disruption of the basement membrane in chick embryo (28). As shown here, this does not only at certain points.

The presence of cellular death in the buccopharyngeal membrane, has only been reported by Watanabe et al (23) in Japanese frog embryos, and it has not been documented in any other species.

In the present work we show the presence of distinct areas of cell death in the buccopharyngeal membrane of the mouse embryo. This cellular necrosis tend to settle on the upper and lower edges of the oral sheet. The role that cellular death plays in the processes of reabsorption of structures, and the fusion of sketches, is well-known. We think that the necrotic areas at the buccal membrane level could play the role of detaching this structure from the epithelial wall. This mechanism would be simillar to the one described in the separation of epithelial sketches from their original epithelium (4, 5, 10, 22).

This necrotic process could be also involved in the fusion of different cellular colonies in the ecto-endodermic transition area of buccopharyngeal membrane (10, 14, 18, 21).

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