The role of acid phosphatase in the fusion of the secondary palate

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Complete closure of the secondary palate must progress through two consecutive events: the converging movement of the palatal shelves and their subsequent fusion at the line of contact. Each step is indispensable in normal palatal development since, theoretically, a palatal cleft might be the consequence of a failure of either.

Until recently, the mechanisms of shelf movement received most attention (Peter, 1924; Lazarro, 1940; Walker & Fraser, 1956; Larsson, 1960). However, recent investigations have focused on the subsequent step, properly referred to as fusion. These studies, based on organ culture methods (Pourtois, 1966) and electron microscopy (Mato, Aikawa & Katahira, 1966; Farbman, 1967; Smiley & Dixon, 1967), have emphasized the complexity of the fusion process. This process may be viewed as a sequence of four interdependent events: (1) differentiation of the cell layers at the edge of the shelves resulting in the formation of a 'zone of stickiness' (Pourtois, 1968); (2) fusion of these differentiated epithelial cells leading to the formation of a laminated wall of epithelium between the shelves; (3) rupture of that partition permitting contact between the elements of the mesenchyme from either side; and (4) finally, degeneration of the epithelial remains of the seam marking the completion of the fusion process. In rodents all of these epithelial clusters are rapidly resorbed (Hughes, Furstman & Bernick, 1967). In humans, however, some of these isolated fragments undergo a Malpighian-like differentiation and produce 'pearls' which persist until after birth (Bergengrün, 1909; Kitamura, 1966).

Several attempts have been made to elucidate the mechanisms responsible for the fragmentation and the eventual resorption of the epithelial wall (stages 3 and 4 above). Some observations indicated that histiocytes from the mesenchyme could phagocytose remnants of the epithelial wall (Andersen & Matthiessen, 1966). But this should be considered as a consequence rather than as a cause of the epithelial disaggregation. Indeed, signs of autolysis, namely the presence of electron-dense bodies, were observed by electron microscopy in the epithelial

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wall before its basement membrane ruptures (Farbman, 1967). Analogous inclusions were also noticed, using light microscopy, in the so-called fin of the primary palate. These inclusions were found to be highly basophilic and to show acid phosphatase activity (Pourtois, 1967a; Lejour, 1967).

Such phenomena suggest the existence, in the epithelial cells, of an autolytic activity which would lead to the breakdown of the wall and, consequently, to complete palatal fusion. This last hypothesis was tested in the present investigation by comparing palatal closure with a process of incomplete and temporary coalescence, namely, the fusion of the eyelids. One knows that the fusion of the eyelids is limited to the stages 1 and 2 above. Afterwards, the peridermal cells, which remain enclosed in the unscathed wall, progress through a Malpighian-like differentiation, which ultimately leads to a reopening of the eyelids (Koyama, 1960). On the other hand, normal palatal fusion is characterized by the quick degeneration of the epithelial wall.

The comparison between fusion of the palate and that of the eyelids was by histological as well as histochemical methods. The occurrence of cell lysis and of acid phosphatase activity was investigated in cell layers involved in either type of fusion in mouse and rat embryos. Moreover, the same techniques were applied to palatal shelves which failed to fuse, either in pathological situations or under artificial conditions. These last sequences involved the examination of spontaneous cleft palates (consecutive to cleft lips) in A/Jax mice embryos as well as mouse and rat palatal shelves which were cultivated separately in vitro to prevent eventual contact and subsequent fusion.

**METHODS AND MATERIALS**

The material used to study palatal fusion was obtained from A/Jax mouse foetuses from 14½–16½ days of gestation and from Sprague–Dawley rat foetuses from 15½–17½ days. The fusion of the eyelids was observed from 16½ days in the mouse and 17½ days in the rat up to the end of the gestation period. Pregnant females were killed by cervical dislocation and all viable foetuses were quickly removed and decapitated.

Series of heads, encompassing each critical stage, were fixed immediately after removal. In addition, palatal processes were dissected from newly collected foetuses and cultivated in vitro before being in turn fixed and prepared for histochemical and histological examination.

The palatal shelves cultivated in vitro prior to histochemical or histological investigations were dissected from 14½-day mouse foetuses and from 15½-day rat foetuses. The explants were then laid down, either in homologous pairs or separately, on the surface of a gel (Pourtois, 1966). This gel contained the following constituents: (1) medium 109 (Evans et al. 1956), (2) Plasma Albumin (Mann Research Laboratory), 1 g/100 cc; (3) ‘Ionagar’ grade no. 2 (Consolidated Laboratories), 0-8 g/100 cc. The explants were incubated for either 48 h or 72 h at 37 °C in an atmosphere containing 5 % CO₂ and 95 % air.
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Both whole heads and temporarily explanted shelves used in the histochemical investigation were fixed overnight in a cold (4 °C) 4 % formaldehyde and 1 % calcium chloride solution whose pH had been recently adjusted to 7.2. Following fixation all specimens were maintained (usually for 2–5 days) in a cold 1 % gum-acacia and 30 % (0.88 M) sucrose solution (Holt, 1959) until sectioned. They were then quickly frozen on solid CO₂ and attached to a pedestal with distilled water. The entire length of the palatal processes was sectioned in the coronal plane at 16 μ on a freezing microtome whose temperature was maintained between −18 ° and −20 °C. A majority of these sections were obtained serially.

The acid phosphatase method of Barka & Anderson (1962) was used throughout the experiment: substrate, naphthol AS-TR phosphate; reagent, hexazotized pararosanilin; buffer, Michaelis veronal-acetate stock solution, pH 5.0. Cryostat sections were air-dried at room temperature for 2 h and incubated at 37 °C for 1 h in the freshly prepared incubation mixture. After incubation the sections were rinsed twice in distilled water and counterstained for 30 s in a 1 % methyl green–phosphate buffer solution at pH 4.0. Dehydration for a total time of 2 min was accomplished with 70 %, 95 % and 100 % ethanol. Sections were then cleared in xylol and mounted in Permount. Control sections incubated in substrate-free media (pH 5.0) were negative throughout the course of the experiment.

The histological sections were obtained either from whole heads of normal or cleft palate foetuses, fixed after removal, or from palatal shelves of normal foetuses, previously incubated in vitro following the procedure described above. The cultured shelves were fixed in Zenker’s fluid and embedded in paraffin. Staining of these sections was either in hematoxylin-eosin or by Masson’s trichrome.

Observations

No consistent differences were found between rat and mice foetuses. It is therefore possible to provide a general description applying to both species. The developmental stages in the mouse foetus took place approximately 1 day ahead of equivalent events in the rat. For this reason, the data are best described referring to the developmental stage rather than to the actual age of the foetuses.

(a) Histological observations with hematoxylin-eosin and Masson’s trichrome

The histological changes which accompany the differentiation of a zone of adhesiveness in the palatal processes were described in a previous paper. They consist essentially of a slight thickening of the marginal epithelium as it approaches the other side (Pourtois, 1968).

As soon as fusion begins (stage 5, Walker & Fraser, 1956), one observes signs of pyknosis in the epithelial cells which form the seam. With the light microscope, this alteration appears as a degeneration of the nuclear material which condensed into one or several dense and highly basophilic spherules. These
signs of degeneration are first visible in the cells of peridermal origin actually trapped in the median part of the wall. At later stages, however, they are found also in the cells originating from the basal layer or epidermis proper. Finally, basophilic granules persist in the epithelial cell clusters isolated in the mesenchyme after the breakdown of the wall as well as in the macrophages which surround these regions.

Since the fusions obtained in vitro by combining homologous palatal shelves had the advantage of evolving at a slower pace, sections at different stages of formation of the epithelial wall were obtained with relative ease. In these circumstances basophilic granules were observed also in the peridermal cells and in the cells which originated from the epidermis proper (Plate 1, fig. 1). Identical pyknosis was also observed in the palatal processes of A/Jax cleft-palate embryos at 15½ days (Plate 1, fig. 2) as well as in the shelves cultivated in vitro separately for 48 or 72 h. The phenomenon took place selectively in the marginal areas though fusion was, of course, impossible in these conditions because of the absence of a symmetrical shelf.

To summarize, pyknotic degeneration was a striking event which occurred in vivo as well as in vitro in the zones of contact. Normally this change was contemporaneous with the meeting of the shelves, but it was also seen in isolated palatal processes provided that these were grown for a period of time which exceeded the actual time of fusion in rat or mouse foetuses.

(b) Localization of acid phosphatase activity

The following description applies only to the marginal areas involved in the process of fusion of palatal shelves, nasal septum and eyelids. A general study of other surrounding structures including the body anlages as well as the cartilages of the nasal septum was previously reported (Angelici, 1966).

(1) Palatal fusion

In the foetuses fixed immediately after removal, a few sites of enzymic activity were detected in stages preceding fusion. At these stages (shelves still in the vertical position or already horizontal but unfused) the method used did not reveal acid phosphatase activity in the epithelium. In the marginal mesenchyme a positive reaction was shown only by the endothelia of the capillaries (Plate 2, fig. 3). In the septum, however, the epithelium lining the ridge gave a slight reaction at the stage immediately preceding the meeting with the shelves.

The epithelia in contact displayed a distinct reaction as soon as fusion was observable. At the earlier stages of fusion, acid phosphatase was consistently found in the peridermal cells. This was especially obvious in the most anterior part of the palate where many of these cells filled the rather wide space between the ridges (Plate 2, fig. 4). In the central part of the palate, where the epithelial seam consisted of two or three cell layers, all types of epithelial cells showed acid phosphatase activity at an early stage. After the epithelial breakdown, the
Fig. 1. Coronal section across an area of fusion obtained *in vitro* by combining the two palatal shelves of a 15½-day-old rat foetus after 48 h incubation. Phagosomes (arrows) were present in the epithelial remnants. Masson's trichrome. × 800.

Fig. 2. Section across the edge of a non-fused palatal shelf in a cleft palate, 15½-day-old A/Jax mice foetus. Phagosomes (arrows) present in the thickened epithelium of this presumptive area of fusion. Masson's trichrome. × 800.
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remnants of the wall—that is, the isolated clusters of epithelial cells as well as the pyknotic cells being eliminated at the border of the seam—remained positive (Plate 2, fig. 5). Finally, some of the cells in the underlying mesenchyme also displayed acid phosphatase activity in numerous minute cytoplasmic granules. Most of these ‘histiocytes’ surrounded the areas still occupied by epithelial remnants.

Three cleft-palate A/Jax embryos were also observed using the same technique. Among them, one was unilateral and two were bilateral clefts. All three originated from 15½ day-old litters; that is, presumably, a short time after the normal onset of palatal closure in that strain. In the specimens with bilateral clefts, acid phosphatase activity was found at the edge of both unfused shelves. In the unilateral cleft embryo, an intense reaction took place on the ridge of the unfused palatal process and the nasal septum, principally in the anterior part.

The epithelial walls formed in vitro by the combination of homologous processes also displayed acid phosphatase activity. The enzyme was also found in the peridermal cells which were eventually trapped in the wall, and was also present in some other epithelial cells which originated from the basal layer, especially in those places where the peridermal cells had already been eliminated.

The palatal shelves which were not permitted to fuse because of prolonged

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Plate 2

Fig. 3. Coronal section across the head of a 14½-day-old mouse foetus passing between the anterior third and the posterior two-thirds of the palatal shelves. Reaction of Barka & Anderson (1962). The secondary palate was not yet closed. No demonstrable acid phosphatase activity in the epithelium of the palatal shelves nor in their mesenchyme; enzyme present in the endothelia of the blood vessels and in the rudiments of the vomer bone, the maxilla and the mandible. × 40.

Fig. 4. Coronal section across the most frontal part of the secondary palate of a 15½-day-old mouse foetus, showing both the inter-palatine and the stepto-palatine sutures. Acid phosphatase activity conspicuous in the peridermal cells of the epithelial seam. Method of Barka & Anderson. × 280.

Fig. 5. Coronal section across the posterior part of the secondary palate in a 15½-day-old mouse embryo. The epithelial remnants of the ruptured wall display a conspicuous acid phosphatase activity, as do the pyknotic cells actually eliminated from the upper part of the seam. Reaction of Barka & Anderson. × 280.

Fig. 6. Section across a palatal shelf dissected from a 14½-day-old mouse foetus and cultivated separately in vitro for 48 h. The reaction of Barka & Anderson was highly positive in the area which is naturally aimed at the abutment with the absent symmetrical shelf. Enzyme activity is conspicuous not only in the marginal epithelium but also in the underlying mesenchyme. In addition, slight background reaction could be seen in the deeper mesenchyme; this is typical of all palatal shelves explanted and maintained in culture for 2 days or more. × 280.

Fig. 7. Coronal section across a palpebral suture in 17½-day-old mouse foetus. Reaction of Barka & Anderson. No positive enzymic reaction in the epithelial seam nor in the cutaneous epithelium at this stage, but a yellowish metachromatic staining of these layers. The only obvious localization of acid phosphatase activity is in the condensed mesenchyme adjacent to the buds of an eyelash and of a Meibom's gland (arrows). × 280.
isolation in vitro nevertheless displayed acid phosphatase activity in their marginal cells after 2 or 3 days of culture. The reaction was found in the epithelium at the edge of the shelves as well as in the underlying mesenchyme (Plate 2, fig. 6). However, in addition to the intense reaction characterizing the areas of fusion, the explants cultivated in vitro also displayed a slight but uniform acid phosphatase activity in most mesenchyme cells as well as in their epithelium.

All enzyme reactions were localized in the cytoplasm. Pyknotic cells, loaded with large basophilic granules (slightly stained with methyl-green), were always noticed in the regions of acid phosphatase activity.

(2) Eyelid fusion

From the 17th day in the mouse and the 18th day in the rat, both the mesenchyme and the epithelium of the eyelids display a slight acid phosphatase reaction uniformly observable in most of the cells. Apart from this mild activity characteristic of many ageing tissues, the only sharp reactions were found around the buds of Meibom’s glands and follicles of the eyelashes as well as in the cytoplasmic granules of the histiocytes frequently encountered at the external surface of the eyelids. However, the method used did not disclose other fields of activity in the marginal areas of the eyelids at the intra-uterine stages. In particular, there was no noticeable acid phosphatase activity in the epithelial seam (Plate 2, fig. 7). This situation persisted up to 2 days after birth; that is, up to the stage when the peridermal cells trapped in the suture finally began to keratinize, thereby initiating the process of reopening. From that time, the upper spinous layer cells exhibited a slight acid phosphatase activity like all cells of the stratum spinosum in the mature skin (Susi, 1965).

DISCUSSION

The electron-dense bodies observed with the electron microscope by Mato et al. (1966), Farbman (1967) and Smiley & Dixon (1967) in the epithelial wall of the secondary palate were found again in the present investigation, using customary histological staining procedures. Moreover, we have demonstrated the presence of acid phosphatase activity in the same epithelial layers. This association of hydrolytic enzyme with pyknotic cells probably indicates the presence of an intensely active lysosome system. More precisely, the basophilic bodies described here represent vacuoles containing cell constituents in the process of being digested under the action of a number of lytic enzymes released by the primary lysosomes. Such intracytoplasmic vacuoles should be called secondary lysosomes (de Duve, 1966). In the secondary palate the association of secondary lysosomes and acid phosphatase takes place at first in the epithelial wall, even before this wall disintegrates. This is an indication that the enzymic agents of epithelial breakdown are located in the cells of the seam and that one
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is observing here a process of autophagy (de Duve, 1966). As for the action of the histiocytes from the mesenchyme, it appears 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 closure. This is an indication that the strong adhesiveness which develops in presumptive areas of fusion can be maintained in spite of a progressive disintegration of their cells, at least at the beginning of the process of fusion. It seems, however, that the forthcoming death of these epithelial cells might finally put an end to their peculiar stickiness. Indeed, a number of pyknotic cells at the border of the seam scattered to the oral or nasal cavities (Plate 2, fig. 5). Obviously these cells had lost their former tendency to stick together. This could be indicative of the fate of all necrotic cells of the seam, including those which were trapped in the epithelial remnants. It is therefore possible that these remnants might constitute weak points in the palatal suture before a complete mesenchymal penetration could be achieved.

The meaning of cell autolysis in the palatal seam could be indirectly deduced by comparing this process of complete fusion with a process of incomplete and temporary fusion. In the fusion of the eyelids, the peridermal cells trapped in the epithelial wall do not immediately degenerate. Since they remain in place, the epithelial seam can persist during the perinatal period. Our observations indicate that this type of evolution is linked to the absence of acid phosphatase activity in the peridermal cells of the eyelid seam. Even in the secondary palate, the absence of acid phosphatase activity in the epithelial wall was occasionally encountered (Angelici, 1968). However, this should be considered as a pathological situation which eventually leads to the reopening of the seam and finally results in the formation of a cleft.

The synthesis of lytic enzymes thus appears to be a prerequisite for the breakdown and the disintegration of the wall leading to mesenchymal penetration. On the contrary, the Malpighian-like differentiation, observed at the level of the eyelid suture, requires a period of time during which synthesis of acid phosphatase apparently must be delayed.

Finally, the observations made on cleft-palate foetuses or on palatal processes cultivated alone in vitro demonstrate that the enzymic lysis and the production of secondary lysosomes in the marginal epithelium of the palatal shelves is not necessarily dependent upon the actual contact of the palatal processes and the nasal septum. On the contrary, these events take place in defined areas and at a defined time, whatever the fate of the converging movement of palatal closure (which seems to be prevented in the A/Jax cleft lip embryos merely by an abnormal position of the tongue: Trasler & Fraser, 1963; Pourtois, 1967b). Epithelial autolysis therefore appears to be a necessary consequence of the differentiation which takes place on the edges of the palatal shelves immediately before the actual process of fusion, as previously reported (Pourtois, 1968).
SUMMARY

The disintegration of the epithelial wall, a prerequisite of the complete fusion of the secondary palate, was studied in mouse and rat embryos. Histological examination combined with histochemical observation of acid phosphatase activity confirmed that phagosomes were present in the epithelial cells of the seam from the very beginning of the fusion process. This sign of early epithelial autolysis was not encountered in a case of incomplete and temporary fusion, that of the eyelids. However, it could be observed in the marginal epithelium of palatal shelves which were prevented from meeting each other. This was demonstrated both in cleft palate A/Jax mouse foetuses and, after artificial isolation, in palatal shelves cultivated in vitro.

REFERENCES


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