Onset of the acquired potentiality for fusion in the palatal shelves of rats

By M. POURTOIS

From the Cleft Palate Research Center, University of Pittsburgh

This paper is concerned with that phase of palate development in rats leading to fusion of the shelves in the midline. Previous experimentation in palate development in mammals has encompassed both the earlier phase of assumption of the horizontal position of the palatal shelves, and the subsequent approximation and fusion of the shelves. Since the two processes do not occur simultaneously and can theoretically be studied separately, it was possible and feasible to confine the experiment to the later fusion phase. The present research was designed to eliminate the possible confounding effects of palate rotation in vitro on the fusion of the shelves by approximation of the explanted palatal shelves in the same horizontal plane, irrespective of their original positions in the oral cavity.

Current theories of cleft palate pathogenesis hold that either the palatal shelves fail to assume (rotate to) the horizontal position, or, that having done so, they fail to fuse. If some clefts of the palate are indeed the result of failure of the shelves to fuse once they have come into close approximation in the midline, then it is of great importance to understand the biological processes underlying the phenomenon of fusion. Histological description of the fusion process in human fetuses has already been provided by Barry (1961) and by Kraus, Kitamura & Latham (1966). In the rat, the histology of the fusion process is not significantly different from that of man (e.g. Coleman, 1965). In order to probe beyond the descriptive level into the more fundamental biological processes involved in fusion, it was necessary first to develop a technique whereby it would be possible to determine if cellular differentiation plays a critical role in the process. This paper presents a preliminary report on such a technique and attempts to determine the time of onset of the acquired potentiality for fusion. The behavior of the shelves in vitro at various stages of their maturation is described.

METHODOLOGY

Eighty rat embryos, selected on the basis of four different stages of development, were utilized in this investigation. Their palatal processes were dissected

1 Author's address: Cleft Palate Research Center, University of Pittsburgh, Pittsburgh, Pennsylvania 15213, U.S.A.
and cultivated in vitro by pairs, in partially defined nutrient media. This portion of the paper will describe the method of age determination, as well as the conditions of culture.

(a) Mating and age determination

Sprague–Dawley rats were mated nightly from 6 p.m. to 8 a.m. The dropped vaginal plugs were sought each morning on a paper band under the screened bottom of their cages. Each vaginal plug was indicative of a copulation as well as a presumptive conception. The day each plug was found was arbitrarily recorded as day zero, and the following days were respectively noted as 1, 2, 3, etc. The pregnant females were killed either at 6 a.m., at noon, or at midnight, so that the litters fell into the following stages: 14 d 12 h (14/12), 15 d (15/0), 15 d 12 h (15/12), and 16 d 6 h (16/6). The last stage preceded by about 2 h the presumed onset of palatal fusion in the Sprague–Dawley rat embryo, which is approximately 16 d 8 h, according to Zeiler, Weinstein & Gibson (1964).

The method used for recording mating did not, however, provide the exact time of copulation. Theoretically, copulation can occur at any time during the 14 h period in which the couples were left together. Therefore, in extreme cases, there could be a 14 h difference in the ages of two litters conceived on the same night, yet both could be incorrectly designated as the same. The following method was used in an attempt to avoid this 14 h span of uncertainty.

An unoperated fetus (control) was selected at random from each litter at the time of killing. After being fixed in Helly’s solution, the controls were prepared for histological study to see if there was any overlapping in maturational or metrical criteria between litters recorded as 14/12, 15/0, 15/12 and 16/6.

The distance between the most lateral parts of the ocular globes was measured on sections for each control embryo. They varied as follows:

- Stage 14/12: 3.1–3.4 mm
- Stage 15/0: 3.4–3.7 mm
- Stage 15/12: 4.0–4.2 mm
- Stage 16/6: 4.6–4.8 mm

In addition, the dental buds, the submaxillary glandular rudiments, Meckel’s cartilage, and the parachordal plate, were examined in each case.

At 14/12, the molar rudiments still consisted of a band of pluristratified epithelium not yet pediculated. The rudiments of submaxillary glands had two divergent acini. The primordia of Meckel’s cartilage and of the parachordal plate were still formed by condensed mesenchyme. The palatal processes were in a vertical position on both sides of the tongue. At 15/0, the picture remained the same except that the cells of Meckel’s cartilage were encircled by their alveolar secretions of precartilaginous substance. At 15/12, the first molar buds were pediculated. The submaxillary glands were composed of three or four acini. Meckel’s cartilage, as well as the parachordial plate, contained precartilaginous ground substance. The palatal processes were still in a vertical position...
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on both sides of the tongue. At 16/6, the enamel organs of the first molars were at the so-called ‘bell stage’. The palatal processes were now lying above the tongue in a horizontal position.

These preliminary observations thus suggest a characteristic degree of development at each stage. The measurements indicate a possibility of overlapping only between stages 14/12 and 15/0.

(b) Culture techniques

The pregnant females were killed by cervical decapitation after ether anesthesia. The embryos were then aseptically collected, and dissected in Hanks’s B.S.S. From a number of embryos the palatal processes were dissected as shown in Text-fig. 1. These explants contained the rudiments of both bony and soft palates. In addition, the incision was done in such a way that both the dental lamina and a fragment of the nasal capsule were included in the explant. These elements were used to determine in histological sections if the palatal processes had been correctly approximated in vitro.

The two palatal processes from each embryo were cultivated in one of the following two media.

(1) Medium NCTC 109 plus fetal bovine serum

The technique of Chen (1954) was slightly modified as follows: a circle of lens paper (Clay-Adams) was glued between two glass rings (diameter 15 mm; height 3 mm) by using a silicone adhesive (Silastic), then autoclaved. The rings were placed in Petri dishes containing enough nutrient liquid to reach the level
of the lens paper and to keep it wet. The explants were then laid down on the surface of the paper. The nutrient medium was composed of the following: (a) NCTC 109 (Evans et al. 1956), 8.8 vol.; (b) fetal bovine serum, 1 vol.; (c) penicillin-streptomycin mixture, 100 units/ml of the final solution. The incubation proceeded at 37 °C, within an atmosphere containing 5% CO₂ and saturated with humidity. The components of the culture medium were purchased from Microbiological Associated.

(2) Medium 199 plus fetal bovine serum

In this ‘two-phase’ device, a thin meniscus of nutrient liquid covered the surface of a gel on which the explants lay. The gel contained the following constituents: (a) medium 199 (Parker & Morgan, 1950), 88 vol.; (b) fetal bovine serum, 10 vol.; (c) penicillin-streptomycin mixture, 2 vol. (=10000 units/100 ml of the final solution); (d) agar (grade 10 N, no. 2), 0.5 g for 100 ml of the final solution.

The solution was allowed to cool in Petri dishes. Absorbent pads (thickness 1 mm) with punched holes (diameter 6 mm) were then placed on the agar-gel surface and soaked with a nutrient solution composed of medium 199, bovine fetal serum, and penicillin-streptomycin in the same proportions as in the agar-gel. The quantity of liquid was adjusted so as to obtain a thin meniscus in the pad holes.

The cultures were incubated at 37 °C under a humid atmosphere containing 5% CO₂ and 95% air.

In both techniques, the edges of the homologous palatal processes were lying in the same plane. In this position, they showed a sinusoid outline so that the distance between them varied between each corresponding point. In our experiments, the explants were so placed that two points of contact were established between them. In some of the older explants (those from embryos at stage 15/12 and 16/6), a short space which never exceeded 0.2 mm at the closest point was allowed to separate the palatal processes (Plate 1, fig. A). Incubation time varied from 24 to 72 h. The nutrient liquids were changed daily. All the explants were fixed in Helly’s solution, sectioned, and stained with hematoxylin-eosin.

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**PLATE 1**

Microphotographs of two palatal shelves in culture on lens paper, This explant was removed at stage 15/12. It is shown at three different stages of its differentiation. The presumptive area of the bony palate is in the lower part, the rudiment of the soft palate is in the upper part of each picture.

Fig. A. Palatal processes just explanted and still separated.

Fig. B. Same explant after 24 h; fusion at the anterior and posterior levels leaving an unfused area in the middle part.

Fig. C. Same explant after 48 h; fusion complete. Magn. × 30.
RESULTS

When cultivated in medium NCTC 109 supplemented with fetal bovine serum, the explants maintained their general configuration. The mesenchyme remained surrounded by epithelium everywhere but at the places of incision. Mitotic figures could be observed throughout. Small necrotic areas, however, often occurred in the lateral parts of the explants.

If the processes were initially separated (Plate 1, fig. A) they joined each other after a few hours. This joining was achieved equally well if a small piece of glass replaced one of the palatal processes, and may thus be attributed to surface tension forces acting on the explants.

In any case, both anterior and posterior parts of the processes came into close contact before the central part (Plate 1, fig. B). This differs from the natural sequence, which proceeds in general from front to back.

A fusion between processes (Plate 1, fig. C) was easily confirmed by histological section. In this study fusion was observed to consist of two stages: (1) epithelial fusion, (Plate 2, fig. D), and (2) mesenchymal transmigration following the rupture of the epithelial lamina between the two shelves (Plate 2, fig. E and Plate 3, fig. F). All phases of mesenchymal transmigration were classified as one stage.

If epithelial fusion did not take place, the shelves invariably separated during fixation. Histological examination then showed no visible change in the areas which were in contact with one another; the epithelium was still continuous and bistratified; no mesenchyme crossed through the epithelial barrier (Plate 3, fig. G).

The results of the experiments conducted in medium NCTC 109 are summarized in Table 1, which enumerates the number of experiments in which various statuses of fusion are achieved according to age of embryo and time of incubation. If one discounts the obvious environmental differences between

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PLATE 2

Fig. D. Section of a pair of palatal processes removed at stage 15/12 and cultivated on lens paper in NCTC 109 plus fetal bovine serum for a period of 24 h. This case shows epithelial fusion between the two shelves.

(a) View of the whole cross-section. Epithelium surrounds the mesenchyme of the explant everywhere but at the places of incisions. The two symmetrical condensations of mesenchyme represent the presumptive areas of the maxillary bones. Magn. × 75.

(b) Detail of (a), showing a continuous epithelial lamina between the two palatal processes. Magn. × 270.

Fig. E. Section of a pair of palatal processes removed at stage 15/12 and cultivated on lens paper in NCTC 109 plus fetal bovine serum for a period of 24 h.

(a) View of the whole cross-section. A dental germ at the cap stage is visible in the mesenchymal condensation at the right. Magn. × 75.

(b) Close up of (a), showing the discontinuity of the epithelia on the mid-line, with transmigration of mesenchyme. Note remnants of blood vessels in the mesenchyme. Magn. × 270.
development *in vivo* and development *in vitro*, these results can be depicted in terms of *total duration* of growth, that is, estimated age of embryo *plus* duration of incubation. In Text-fig. 2 these total durations can be compared to the approximate time of completed palatal fusion *in vivo*, 16/12. The percentage of fusions and/or lack of fusion is presented alongside the horizontal bar representing each series.

Table 1. *Results of in vitro experiments in medium NCTC 109*

<table>
<thead>
<tr>
<th>Age of embryo (d/h) at time of removal</th>
<th>Series no.</th>
<th>Litter</th>
<th>No. of paired explants</th>
<th>Incubation time (h)</th>
<th>No. of fusions</th>
<th>Epithelial fusion</th>
<th>Mesenchymal transmigration</th>
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<td>14/12</td>
<td>1</td>
<td>I, II</td>
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<td>0</td>
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<tr>
<td></td>
<td>2</td>
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<td>72</td>
<td>7</td>
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<td>1</td>
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<td>150</td>
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<td>III, IV</td>
<td>16</td>
<td>60</td>
<td>5</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>15/12</td>
<td>4</td>
<td>V</td>
<td>4</td>
<td>24</td>
<td>0</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>V, VI</td>
<td>10*</td>
<td>48</td>
<td>1</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>VII</td>
<td>2*</td>
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<td>2</td>
</tr>
<tr>
<td>16/6</td>
<td>7</td>
<td>VIII</td>
<td>6</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>XI</td>
<td>10*</td>
<td>54</td>
<td>0</td>
<td>0</td>
<td>10</td>
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</table>

* Cases in which the two processes were placed in culture between 0·1 and 0·2 mm apart.

The following observations can be made from examination of Text-fig. 2:

1. In those series of experiments with equal *total durations* (1 and 4; 2, 3, 5 and 7; and 6 and 8), the percentage of fusions obtained increased in proportion to the ages of the embryos at dissection time until, in series 6, 7 and 8, 100 % fusion was achieved.

2. In those series of the same pre-incubation age (1 and 2; 4, 5 and 6; 7 and 8), the percentage of complete fusion with mesenchymal transmigration

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Fig. F. Section of a pair of palatal processes removed at stage 15/12 and cultivated on lens paper in NCTC 109 for a period of 48 h.

(a) View of whole the cross-section. Magn. × 75.

(b) Close up of (a). The epithelium has disappeared from the zone of fusion and is now completely replaced by mesenchyme. Magn. × 270.

Fig. G. Section of a non-fused pair of palatal processes removed at stage 14/12 and cultivated on lens paper in NCTC 109 for a period of 72 h.

(a) View of the whole cross-section. A dental germ at the bud stage is visible in the mesenchyme at the right. The lacunae in the mesenchyme on both sides are remnants of capillaries. Magn. × 75.

(b) Close up of (a). The epithelia of both sides are continuous in the region where they have been in close contact *in vitro* for 72 h. Magn. × 270.
increased relative to the length of incubation time until 100 % fusion was achieved.

(3) The type of fusion may vary according to: (a) the age of the embryos at dissection time and (b) the length of incubation time. In series 3, dissected at stage 15/0, the length of incubation was 60 h for each explant. In this series, 5 explants out of 16 (31.25 %) did not achieve a mesenchymal transmigration although they underwent an epithelial fusion. In the series (4, 5 and 6) dissected at stage 15/12, epithelial fusion occurred in those explants which were cultivated for only a short period of time (24 h), but most of the explants (91.6 %) underwent a complete fusion after 48 or 72 h of incubation.

In the four series 2, 3, 5 and 7, each differs in age at dissection and in incubation time. The incubation time was adjusted to achieve the same total durations for each series. Thus, for series 2, 3, 5 and 7, the dissection ages were 14/12, 15/0, 15/12 and 16/6, respectively, and their respective incubation periods were 72, 60, 48 and 30 h. For each series, however, the total duration was 17/12. This exceeded the normal time of complete palatal fusion in vivo by 24 h. The different incubation periods were called ‘compensatory’ because they allowed the tissues in the palatal shelves to stay alive for the same period of time regardless of the time of dissection. The results obtained with such incubations are illustrated in Text-fig. 3 which depicts the gradual increase of complete palatal fusion in vitro according to the age of the embryos at dissection. Among the experiments recorded in that graph, incomplete fusions only occurred in the series removed at stage 15/0.
Culture on agar, in the medium 199 supplemented with fetal bovine serum, provided good retention of the form as well as of the vitality of the explants. Necrotic areas seldom occurred, and were smaller than those observed with the previous technique. The processes which were originally separated came into

![Graph showing fusion percentages.](image)

Text-fig. 3. Graphic representation of the results, in percentage of fusions, in four different experimental series with 'compensatory' incubation times.

**Table 2. Results in medium 199**

<table>
<thead>
<tr>
<th>Age of embryos (d/h) at time of removal</th>
<th>Series no.</th>
<th>Litter</th>
<th>No. of paired explants</th>
<th>Incubation time (h)</th>
<th>No fusion</th>
<th>Epithelial fusion</th>
<th>Mesenchymal transmigration</th>
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</thead>
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<td>X</td>
<td>8</td>
<td>72</td>
<td>7</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>15/12</td>
<td>10</td>
<td>XI</td>
<td>4</td>
<td>48</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>15/12</td>
<td>XII</td>
<td></td>
<td>4*</td>
<td>48</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

* Cases in which the two processes were placed in culture between 0.1 and 0.2 mm apart.
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contact after a few hours, probably because of the action of surface tension forces. In any case the contact was established in the front and in the back before it occurred in the center.

Epithelial fusion as well as mesenchymal transmigrations were obtained. In the cases where fusion did not occur, the processes came apart during fixation, and histological section showed no changes in the epithelial layers on both sides.

Table 2 details the results obtained with this technique. It shows that among the eight explants dissected at stage 14/12, only one underwent epithelial fusion after 72 h of incubation. The latter was fused only in one-tenth of its length. No one explant of this series achieved a complete fusion, and seven failed to fuse. On the other hand, all the explants removed at stage 15/12 underwent complete fusion after being cultivated for 48 h.

**DISCUSSION**

In comparing the results of those experimental series having either equal or compensatory incubation periods, it can be seen that the frequency of fusions *in vitro* varies with the differences in age of the embryos at the time of dissection. This contrast is especially obvious in the results of cultures dissected at stages 14/12 and 15/12. In most of the cases, this 24 h difference between the ages of the experimental embryos was sufficient to lead to different results *in vitro*. As one can see by comparing series 2 and 5 (Table 1) or 9 and 10 (Table 2), most of the explants dissected at stage 14/12 failed to fuse while those removed at 15/12 fused in most of the cases.

New potentiality was thus acquired by the palatal shelves *in vivo* between stages 14/12 and 15/12. This potentiality may represent a process of cellular differentiation required for the fusion of the epithelia, their subsequent resorption, and finally the transmigration of mesenchyme. This differentiation occurs *in vivo* before the 15/12 stage. It does not take place *in vitro* in the two media utilized here, since the explants dissected at stage 14/12 generally failed to fuse even though the period of incubation compensated for their early removal (series 2, Table 1, and series 9, Table 2).

Theoretically, for embryos of exactly the same age, the process of cellular differentiation leading to fusion of the shelves must be initiated at approximately the same time. In the present experiment, since age could not be exactly determined, embryos of the same designated age may actually differ by several hours. In addition, embryos of the same litter may also differ in age. Therefore, age differences within and between litters could easily account for divergent results in a single experimental series. These divergences were small among the results of series starting at the stage 14/12 (1 palate fused, 7 non-fused in series 2). They were equally small, but in the opposite sense, in the case of experiments starting at stage 15/12 (1 palate not fused, 9 fused in series 5). At stage 16/6, the individual variations no longer affected the results of cultures (all 6 palates
fused in series 7). On the contrary, a higher discrepancy between experimental results was found at stage 15/0 (5 failures, 5 epithelial fusions and 6 complete fusions in series 3). It appears, therefore, that the series removed at stage 15/0 was more heterogeneous as far as the ability to fuse in vitro is concerned. For this reason, 15/0 may be considered as the mean time at which the palatal processes of a given embryo acquire the potentiality for fusing in vitro. Until now, the only sign of differentiation detected prior to palatal fusion was an increased synthesis of sulfated mucopolysaccharides in the mesenchyme of the palatal shelves in mice embryos at equivalent stages (e.g. Larsson, 1960; Walker, 1961; Jacobs, 1964). This histochemical change was interpreted by Walker (1961), as an element in the build-up of the 'internal force' which, according to Walker & Fraser (1956), leads to the rotation of the shelves. There are no indications, however, that it plays any immediate role in the process of fusion itself. For this reason, the idea of cellular differentiation as a prerequisite for fusion of the shelves still remains a concept deduced from the experimental data recorded in the present paper.

The increase in percentage of palatal fusions in vitro with increase in age of donor embryos was already pointed out in the papers of Moriarty, Weinstein & Gibson (1963), and Konegni et al. (1965). Our results, however, differ especially in the case of the explants taken from embryos at stage 15/12. Using 15½-day-old Sprague–Dawley rat embryos, the above-mentioned authors obtained 26·3 % of complete and incomplete fusions. In the present investigation, 95·8 % of the explants removed at this stage fused. This divergence might be explained by differences in the respective techniques. In the experiments of our predecessors, the palatal shelves were not taken out of the maxillary arches and were cultivated in plasma clots. Under these conditions, the palatal shelves had to assume a horizontal position before fusion could take place. The first step could have hindered the second, and hence have resulted in the low percentage of palatal closures.

In the present investigation, fusion was not affected by shelf rotation. The factor of palatal rotation was artificially eliminated by separating the shelves from the maxillary processes and placing them on a single plane. The surface tension of the nutrient medium rapidly eliminated the space of 0·1–0·2 mm imposed between palatal processes. It thus appears that the techniques utilized here are more accurate for observing specifically the process of palatal fusion. In addition, the partially defined nutrient media utilized in this investigation proved to be effective in allowing the palatal shelves to fuse in vitro. There is, therefore, a new means at our disposal for controlling the environmental conditions during the last steps of palatal closure.
SUMMARY

1. Palatal processes were removed from rat embryos at four different stages of development and were explanted as homologous pairs in vitro in partially defined synthetic media.

2. The incidence as well as the type of palatal fusions obtained varied according to (1) the age of the embryo at dissection, and (2) the length of the incubation period.

3. Analysis of the results leads to the conclusion that a process of cellular differentiation, prerequisite to fusion potentiality, occurs in the palatal shelves of the rat embryo on average before the age of 15 d.

RESUME

Acquisition de la potentialité de fusion dans les processus palatins du rat

1. On a prélevé les processus palatins d'embryons de rat à quatre stades différents de développement et on les a explantés par paires homologues in vitro, dans des milieux synthétiques partiellement définis.

2. La réalisation aussi bien que le type de fusions palatines obtenues ont varié selon (1) l'âge de l'embryon à la dissection, et (2) la longueur de la période d'incubation.

3. L'analyse des résultats conduit à la conclusion qu'un processus de différenciation cellulaire préliminaire, nécessaire au pouvoir de fusion, est réalisé dans les procès palatins de l'embryon de rat, en moyenne avant l'âge de 15 jours.

The author is extremely appreciative of the advice and assistance of Professor Bertram S. Kraus in helping to interpret the results, as well as for the wording of the manuscript. This research was supported in part by Grant no. DE-01697 from the National Institute of Dental Research.

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*(Manuscript received 10 January 1966)*