Human Palatal Development. In Vitro

ALASTAIR N. GOSS, B.D.S. (Otago)., F.R.A.C.D.S.
Adelaide, South Australia

Organ culture of palatal processes from experimental animals has resulted in significant advances in knowledge of the events associated with normal and abnormal palatal fusion. Theoretically this technique could also be applied to the study of post-fusion separation as a mechanism of cleft formation (10). With one exception (5) all published studies on *in vitro* palatal fusion have utilized rodents as the tissue source. Although the development of the secondary palate in rodents bears general similarity to humans (7) there are some important differences, particularly in regard to palate formation and stage of gestation, the length of time for fusion to occur and the persistence of the epithelial seam. This paper presents a method of human palatal culture which investigates palatal fusion, post-fusion rupture and the fate of midline epithelial remnants. The problems and future applications of this technique are discussed.

Method and materials

TISSUE. Human foetuses were obtained during therapeutic abortion by hysterotomy or hysterectomy in specialist obstetric units in the major teaching hospitals in Adelaide, South Australia. These abortions were performed in accordance with the South Australian legal code (1) and the author was in no way involved in the patient's medical management. Foetuses were kept in the intact amniotic membranes or if these were ruptured, in sterile nutrient media containing antibiotics for transport to the laboratory. On arrival in the laboratory the foetuses were examined for evidence of gross congenital malformation and the crown rump length recorded. Variation of up to 4 weeks was noted between the clinical age and crown rump length but there was good correlation between crown rump length and facial development as compared to published standards (*11*, *17*).

DISSECTIONS. All dissections were performed under sterile conditions using a stereo dissecting microscope. The tissues were bathed in nutrient media during dissection.

Four types of dissection were used.

a. Palatal preparation (Case II). The maxilla and cranium was separated from the mandible by an horizontal incision through the corners of the mouth. The upper face and cranium was removed by a parallel incision

Dr. Goss is Lecturer in Oral Surgery, Department of Oral Pathology and Oral Surgery, The University of Adelaide, Adelaide, South Australia

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below the level of the eyes and the pharyngeal tissue posterior to the palate was removed. The lip and remaining cheek was removed by an incision along the buccal sulcus. Hence the tissue for culture consisted of the palate and alveolus. This dissection closely follows that previously described and illustrated for rodent tissue (8).

b. Palatal shelf preparation (Case I). The palate and alveolus was dissection as for dissection a/. A midline incision was then made between the still unfused palatal shelves taking care not to damage the medial edges of the shelves. The individual palatal shelves and alveolus was then dissected free from maxilla. This dissection closely follows that previously described and illustrated for rodent tissue (9).

c. Paramedian surgical 'cleft' (Cases III, IV). The palate and alveolus was dissected as for dissection a/. A paramedian incision extending two thirds of the length of the secondary palate was then made from the oral surface through to the nasal surface (Figure 1).

d. Midline seam preparation. (Case V). The palate and alveolus was dissected as for dissection a/. The median palatal seam was excised by two parallel incisions, each parallel to the seam and 2 mm from it. The long strip of seam was then cut in four pieces, one being primarily soft palate, two being hard palate and one primary palate. The technique was developed for older foetuses where the palate was too large to culture intact.

For all specimens the mandible was removed and fixed immediately.

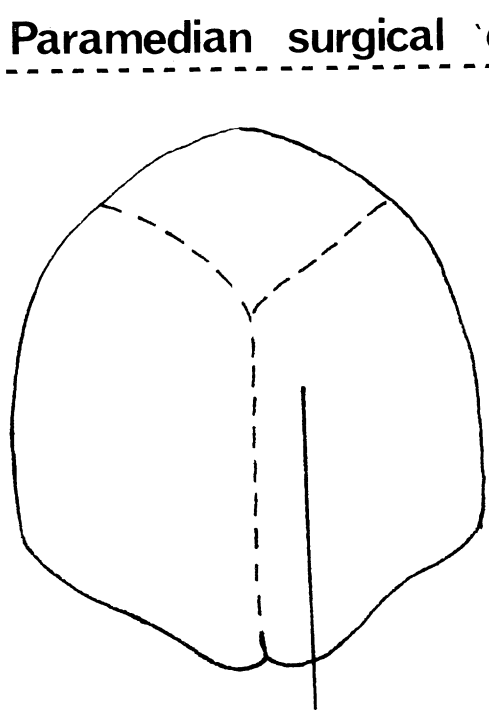

FIGURE 1. Paramedian Surgical 'Cleft.'

The mandible was then processed histologically at the same time as the cultured palatal tissue from the same foetus.

In Vitro Culture. The dissected tissues were cultured under standardized conditions similar to those used for rodent tissues. The dissected tissues were placed with the nasal surface on a Millipore filter which in turn rested on a wire grid in a plastic organ culture dish.* The cultures were maintained for periods ranging from 48 hours to 240 hours at 38°C in a closed gaseous humidified environment of 5% CO_2 and air. The following media† was used for all cultures.

NCTC 199	10 ml (× 10 strength)
Bovine serum	10 ml
Sodium bicarbonate	2 ml
Solution D.G.P.	0.1 ml
Distilled water	78 ml
Penicillin (100,000 I.U.)	0.2 ml
Streptomycin (20,000 I.U.)	0.5 ml
Nystatin (50,000 I.U.)	0.1 ml

In all cases the gaseous environment and nutrient media was changed every 24–36 hours.

Histology

After incubation the tissues were fixed in Bouin's fixative for a minimum of 24 hours and embedded in paraffin. Serial sections 8μ thick in the frontal plane were taken from the uncultured mandible and the cultured palate. Four stains were applied to sequential slides; haemotoxylin and eosin, periodic acid schiffs, modified mallory's (6) and papanicolaou.

Results

No gross malformations were detected prior to dissection in any of the five foetuses.

Case 1—C.R. 30 mm.
Palate unfused.
Palatal shelf preparation type b/.

The palatal processes were widely separated posteriorly with a small area of contact at the anterior one third of the secondary palate, fusion had not commenced at this site. The individual palatal shelves were placed in contact and culture was commenced within two hours of termination.

At 84 hours of culture the anterior half appeared fused but there was some separation posteriorly. Under sterile conditions the specimen was

* Organ Culture Dish Number 3011, Falcon Plastics, 5500 West 83rd St, Los Angeles, California 90045, U.S.A.

† Commonwealth Serum Laboratories, 45 Poplar Road, Parkville, Victoria 3052, Australia.

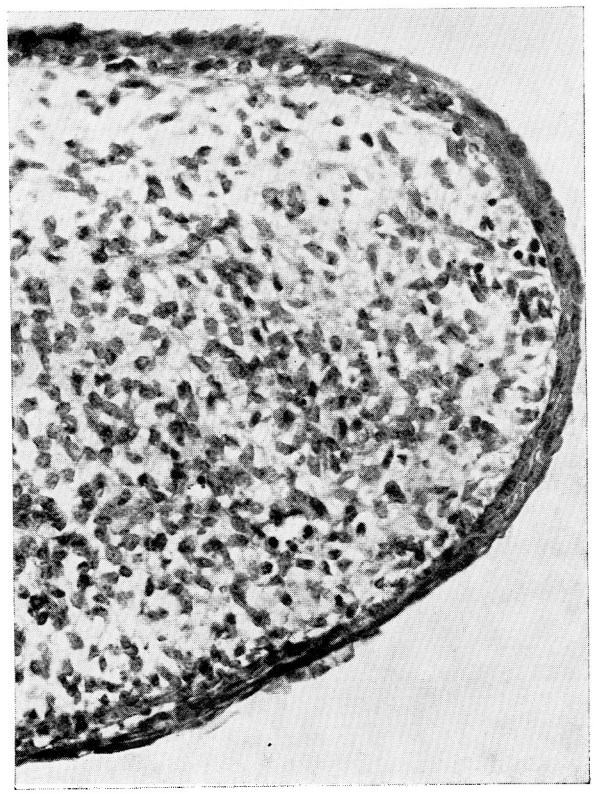

FIGURE 2. Case I, part A. Medial edge of single palatal process. H&E × 100.

bisected and the posterior half fixed (part A). Culture was continued for a further 48 hours on a total of 132 hours of the anterior part (part B).

Histologically the tissues of the uncultured mandible, cultured posterior palate (part A) and cultured anterior palate (part B) were identical.

Case 1 part A. Histologically this consisted of a single palatal shelf with a clearly identifiable nasal and oral epithelial surface. The medial edge of the shelf was covered by a 2 cell layer epithelium (Figure 2). There was no evidence of epithelial thinning, invagination, separation or breakdown and no underlying mesenchymal condensation. On the lateral oral side of the palate a developing tooth was at cap stage of development.

Case 1 part B. Histologically this consisted of a single piece of tissue with an epithelial seam extending one quarter of the way through the tissues on the oral side only (Figure 3). Epithelial pearls were present near the end of the seam, below this there being complete mesenchymal penetration across the area of fusion. The amount of epithelium present after six days culture of the 50 day (approximate) palate was similar to that of a palate from a 63 to 70 day foetus (11). At one end of the specimen near the area of bisection there was an epithelial lined cyst.

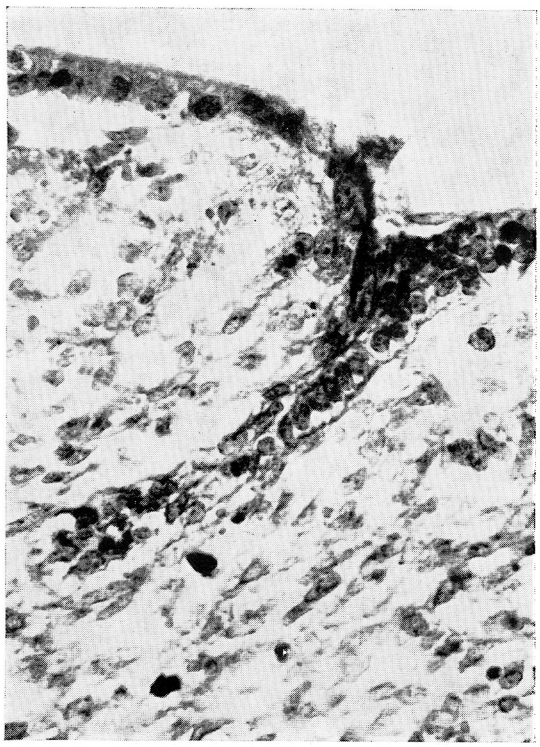

FIGURE 3. Case I, part B. Epithelial seam at site of fusion. Modified Mallory's stain × 400.

Case II—C.R.—40 mm.
Palate fused.
Palatal preparation, type a/.

The palate was intact although the palatal seam was translucent. Culture was commenced within 2 hours of termination and maintained for 72 hours.

There was no change in size of the specimen during culture but the palatal seam lost its translucency. The palatal capillaries were visible only for the first 48 hours of culture.

Histologically the tissues were in excellent condition. A midpalatal epithelial seam was present on the oral side only throughout the length of the secondary palate (Figure 4). Toward the nasal side the epithelial seam was discontinuous leaving small round epithelial pearls. On the nasal side there was no epithelial seam or pearls between the nasal septum and the palate.

Bone was present in the alveolar margins and about the developing teeth. The bone extended approximately half the distance between the alveolus and midline.

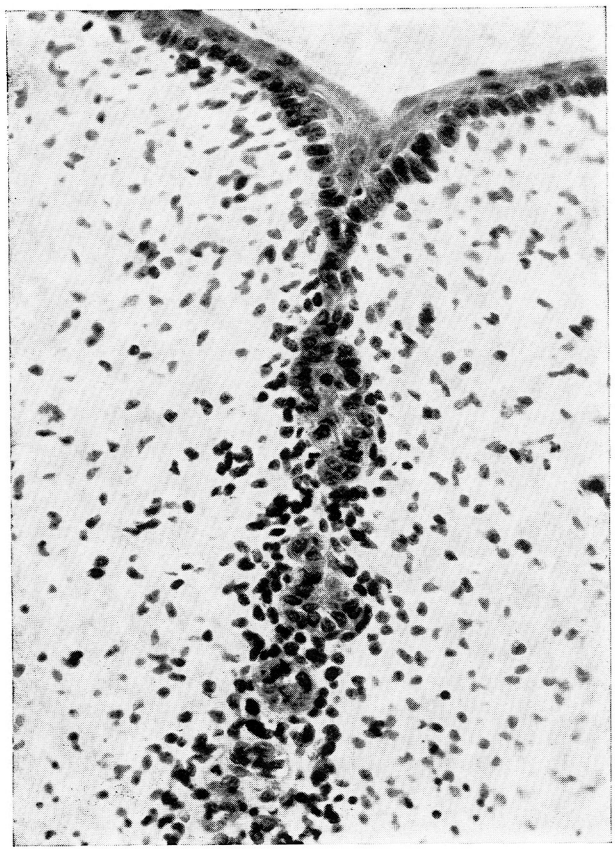

FIGURE 4. Case II. Midpalatal epithelial seam. H&E × 250.

Case III—C.R. 60 mm.
Palate fused.
Paramedian 'cleft', dissection c/.

The foetus was kept in the intact amniotic sac for 3 hours after termination and then transferred to nutrient media and stored at 4°C for a further 3 hours. The palate was intact and the midline seam could be seen as a fine white line. Culture was commenced 6 hours after termination and maintained for 72 hours.

There was no change in gross morphology during culture and the 'cleft' was visible at completion of culture. Histologically the tissues were in excellent condition. The epithelial seam was present throughout the length of the palate although it was more evident in the anterior region. Posteriorly the seam was very short although epithelial pearls extended deeper into the tissue. The incision in the posterior two thirds of the palate was evident and a thin layer of epithelium had migrated from both nasal and oral sur-

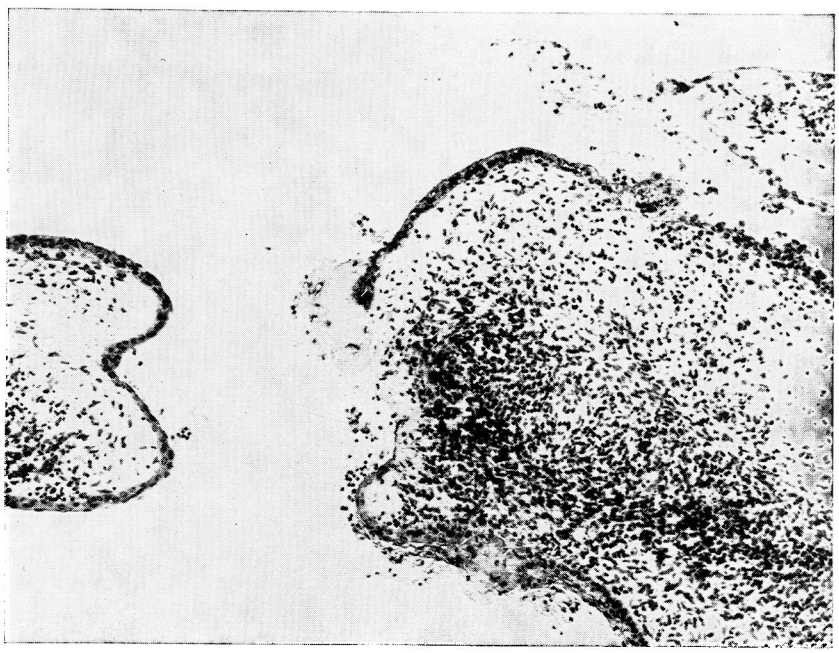

FIGURE 5. Case III. Area of surgical 'cleft.' On right side nasal (superior) and oral (inferior) epithelium in process of migrating over exposed mesenchyme. On left side epithelial migration is complete.

faces over the exposed mesenchyme (Figure 5). In some sections areas of exposed connective tissue were present but in others there was complete coverage so that it resembled a palatal process.

Case IV—C.R. 45 mm.
Palate fused.
Paramedian 'cleft', dissection c/.

The palate was intact although the mid-palatal seam was translucent. Culture was commenced within 2 hours of termination and maintained for 7 days.

There was no change in gross morphology during culture and the 'cleft' was evident throughout. Histologically the tissues were in excellent condition. The mid-palatal epithelial seam and some pearls were evident throughout the secondary palate. The paramedian incision was evident in the posterior two thirds of the palate and a thin layer of epithelium covered the cut surfaces. In one area these two epithelial surfaces contacted and had undergone lamination fusion leaving a small cystic area between them (Figure 6). This area was midway between the oral surface and the nasal surface which was in contact with the filter.

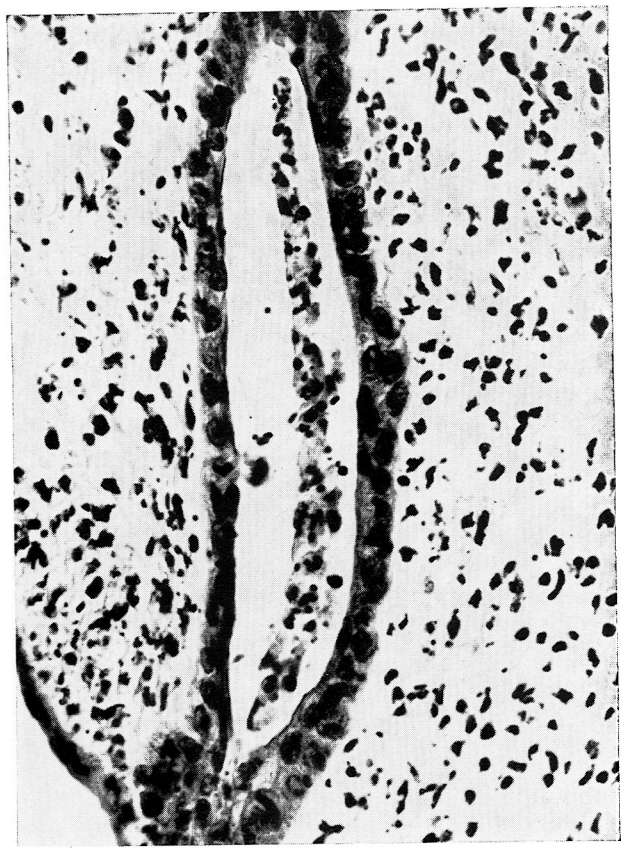

FIGURE 6. Case IV. Area of surgical 'cleft.' The epithelium had completely migrated over the exposed mesenchyme. In the area illustrated the two epithelial surfaces were in contact and had undergone epithelial fusion at two points leaving a cystic space between them. Papanicoulaou stain × 400.

Case V—C.R. 120 mm.
Palate Fused.
Midline seam preparation, type d/.

The midpalatal seam was dissected into four pieces.

Piece A—soft palate
Piece B—posterior hard palate
Piece C—hard palate
Piece D—primary palate and lip

Culture was commenced within 2 hours of termination. Piece D was fixed at 48 hours, C at 96 hours and A & B at 144 hours.

Histologically all four pieces appeared similar to each other and to the mandible which was fixed at the time palatal culture commenced. It was possible to match neighbouring pieces to each other so that a serial pattern of the whole palate was obtained. Piece D consisted of lip muscle and hair follicles, posterior to this the nasopalatine duct. Piece B & C were similar consisting of palatal bone with midline suture and a midline epithelial rest under the oral mucosa.

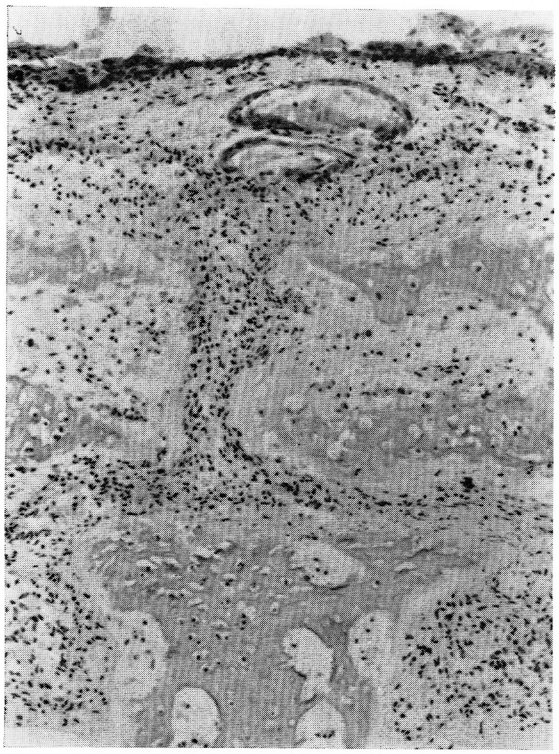

FIGURE 7. Case V. Median epithelial rests on the oral side of the midpalatal suture. H&E × 100.

Two major epithelial rests were seen and they were virtually continuous throughout all sections obtained from piece B & C. Hence they were of a cylindrical shape rather than a number of isolated spheres. All stages of epithelial rest formation from a solid epithelial cell clump to a keratin filled cyst were seen in the same epithelial rest (Figure 7). The keratin filled parts appeared larger than those seen in human palates obtained from foetuses of similar age (11). This was the only apparent difference between this tissue cultured specimen and uncultured palates.

Piece A consisted of soft palate muscle with a respiratory and oral epithelial lining. There was a short epithelial seam from the oral surface with no pearl formation.

Discussion

The main technical problem is one of supply of suitable foetuses. Only foetuses obtained by open surgical methods are suitable for palatal culture so the available number of suitable foetuses is small. Closed surgical methods such as suction evacuation or dilation and curettage are used for terminations up to age 12 weeks *in utero* and by these methods the abortus is grossly fragmented. Personal review of about fifty such specimens failed to find an identifiable face although the thorax and limbs were sometimes

recognizable. Digits were obtained from some specimens for evaluation of different tissue culture techniques because they are similar in size and contain similar tissues to palates. Hormonal methods of termination are being used with increasing frequency and they result in foetal death *in utero* approximately 1 day before expulsion. By then the tissues are severely necrotic and even cell culture is difficult (16). Open surgical methods e.g. hysterotomy and hysterectomy are used primarily on gynaecological grounds and for termination of older foetuses. The complications of these procedures are high and the medical indications limited and decreasing (4).

If the foetus is obtained immediately following operation and still within the intact amniotic sac the risk of bacterial contamination is small. This risk can be decreased by use of media containing antibiotics both for washing during dissection and for culture. Once bacterial colonization has commenced however these antibiotic solutions fail to control infection. A further hazard is that the staff of surgical units are trained to promptly fix all pathologic specimens in formalin and a number of suitable specimens were lost for tissue culture purposes by this means.

For the short time periods used for culture in this study methods of culture which have been developed for rodent tissues were very satisfactory. Presumably for longer periods of culture supplements of Vit C could be advantageous (13).

Besides the five cases reported in this paper two other cultures have been attempted by the author. These were unsuccessful in maintaining a viable explant but resulted in important technical modifications. The first failed attempt resulted in improved liaison with the obstetric staff; increased preparedness to perform cultures at short notice and the realization that uncultured tissue should be fixed at the time culture commenced to act as a control as to the degree of post termination degeneration of the foetus. Hence the mandible was fixed and compared to the cultured palate in the reported cases. The second unsuccessful explant was an attempt to culture a complete palate from a foetus of C.R. 70 m.m. A subsequent large palate (Case V) was thus dissected into 4 smaller explants to allow adequate nutrition to the tissues.

The five successful cultures indicated that in broad terms it is possible to stimulate *in vivo* fusion, epithelial pearl formation *in vitro*, and investigate some aspects of post fusion rupture.

Case I demonstrated that *in vitro* fusion of human palates does occur. The fused area showed rapid mesenchymal penetration, this is contrary to the finding in rodents. The epithelial seam is lost in 12 hours in normal rat development (3) whereas it persists for at least 24 hours *in vitro* (9). In normal human development the epithelial seam persists for many weeks (11). The unfused part A of Case II did not show epithelial degeneration on the medial edge of the shelf as has been reported to occur in mice (15).

Case II demonstrated that the general morphology of the palate was retained. The amount of epithelial seen present however was somewhat

less than 'normal' for a C.R. 40 mm embryo and was consistent with a C.R. 50 mm embryo (11).

Case III and IV demonstrate that if the intact palate is ruptured then normal healing by epithelial covering of the exposed mesenchyme occurs. This finding is relevant to palatal clefts which occur by rupture of the intact palate. Rupture of the previously fused palate has been postulated as a mechanism of cleft palate in humans (10) but has received scant experimental animal investigation. Studies currently in progress by author demonstrate that cleft palate can be induced *in vivo* in the rat by rupture of the intact palate. With some types of palatal rupture continued growth of the face distracts the ruptured palate thus increasing the width of the cleft. Other sites and sizes of palatal rupture heal with time so that the palate has reformed at birth.

In vitro techniques have a role in investigation of clefts caused by post fusion rupture as the site and size of the 'cleft' is more easily controlled. Further, it is important to know if the 'cleft' so produced has the potential to re-fuse if given the opportunity. *In vitro* studies of normal fusion have demonstrated that the capability to fuse is gained just prior to the time of normal *in vivo* fusion (12) and is subsequently lost (9). Initial results from surgical para median 'clefts' in intact palates all demonstrate epithelialization over the exposed mesenchyme. When these reepithelialization surfaces are placed in contact the degree of fusion which occurs varies with age of the foetus at time of explantation.

Case V demonstrated that it is possible to study median epithelial rests *in vitro*. This is not possible in rodent studies as the epithelium disappears within hours of fusion.

It has been postulated that cleft palate in humans may result from rupture through large median palatal cysts (14). In this single case although the epithelial cysts were larger than normal (11) they were still confined to the oral side of the palatal bones and had not deformed then. The cyst in Case I was much larger relatively and in a palate containing only connective tissue. Hence this cyst constituted an area of weakness, which could easily rupture. It is probable however that this cyst arose from traumatic implantation of epithelium at the time of bisection rather than being a true median palatine cyst.

A short epithelial seam was seen on the oral side of the soft palate explant. This is contrary to the suggestion that the soft palate forms by a merging rather than a fusion process (2). However in *in vitro* studies explants which consist of both hard and soft palate components fuse without merging (*9*, *12*).

From a series of five cases it is not possible to draw firm conclusions and it is unlikely that it would be possible to accumulate sufficient cases to repeat all the rodent experiments. However it is possible to spot check extrapolations of rodent experiments to humans by such a small series. In broad terms there was agreement between the human and rodent experi-

ments. There were some differences, primarily related to differences in behaviour of the epithelium in the area of fusion.

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Reprints: *A. N. Goss, B.D.S.*
Dept. of Oral Pathology and Oral Surgery
University of Adelaide
Adelaide, South Australia 5001

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