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FUSION CAPABILITY OF RAT EMBRYONIC EXTRA-ORAL TISSUE *IN VITRO*

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Summary—Epithelial mesenchymal interaction in the fusion process was studied by the means of fusing different extremities of a 15 day 16 hr rat foetus. The extremities used were the hands, feet, and tails, which at this stage of embryologic development appear to have a similar type of epithelial covering but differ markedly in the degree of specialization of the underlying mesenchyme. Two hands from one embryo were placed in palm to palm contact and grown under organ culture conditions for 72 hr with ten pairs of hands making up the experimental group. Ten pairs of feet made up the second experimental group with two feet from the same embryo being placed in sole to sole contact and grown under organ culture conditions. The third experimental group consisted of ten pairs of tails from littermate embryo's and were placed side by side and cultured for 72 hr.

In all three experimental groups, fusion with epithelial breakdown occurred though to different extents. It was noted that the degree of mesenchymal penetration across the interrupted epithelial barrier was related to the extent of specialization of the mesenchymal tissue. The greater the degree of specialization of the mesenchyme the less was the intermingling with the mesenchyme of the opposite member. The most specialized extremity the hand, also showed the lowest incidence of the fusion process progressing as far as the epithelial breakdown stage.

INTRODUCTION

DURING embryologic development, the mammalian embryo undergoes increasing structural complexities as a result of tissue differentiation. This differentiation is characterized by proliferation, migration and aggregation of cells. Some cell layers undergo bending, folding, splitting and/or fusion (AREY, 1966). The present investigation examines the process of fusion of epithelial structures of several different origins in the embryonic rat. Epithelial fusion occurs in development of the closure of the neural folds into the neural tube. Again, fusion is involved in closure of the floor of the nasal pits and the roof of the mouth. Each of these cases of fusion occur in a somewhat different manner such as intermingling of the epithelial cells of the neural folds as they come into contact. In the palate on the other hand, after contact of the shelves, the epithelium breaks down and the mesenchyme intermingles with that of the opposing shelf. The sequence of events in the process has been described as (1) contact of epithelial cells, (2) delamination, (3) degeneration of the cells and rupture of the basement membranes and (4) intermingling of the connective tissue (GOSS, BODNER and AVERY, 1970. In press). Gross failure of fusion may result in a non-viable embryo if the area involved is critical to development such as spina bifida of the neural tube. Failure of fusion of the palate however, results in a deformity compatible with life.

Clefts of the lip and hypospadias are other clinical examples of less severe failures of fusion of embryonic processes.

Recently *in vitro* techniques have been developed to study the process of fusion, in particular that of palatal shelf fusion. These studies show that the fusion *in vitro* of palatal shelves is sufficiently similar to the fusion *in vivo* process for the technique to be used as a valid model of the normal process (MORIARTY, WEINSTEIN and GIBSON, 1963; KONEGNI *et al.*, 1965; REEVE, PORTER and LEFKOWITZ, 1966). Detailed descriptions of the *in-vitro* fusion of palatal shelves have been published (POURTOIS, 1966; POURTOIS, 1968; MYERS, PETRAKIS and LEE, 1968). Further, the fusion of palatal shelves with eyelids (VARGAS, 1968) has been reported. In a previous series of experiments the present investigators studied the *in-vitro* capability of several oral tissues to fuse. Combinations of oral embryonic rat palatal shelves, tongues and palatal shelves to tongues were found to have the capability to completely fuse.

The present experiment was designed to investigate the *in-vitro* capability of selected non-oral epithelial covered mesenchymal tissues to fuse together. The tissues were explanted and cultured from embryonic rats of the same age and using a similar technique to those used to study the fusion capability of oral tissues. The tissues selected were: extremities, the upper and lower limbs and the tail. These tissues were chosen as the experimental model as first they are not normally involved in *in vivo* fusion. Second, they have a similar gross morphology and structural arrangement of epithelium and mesenchyme to palatal shelves. Third, the three chosen extremities each are at different stages of specialization in a single embryo. The upper limb is more advanced developmentally than the lower limb and the tail in 15 day rat embryo.

METHODS AND MATERIALS

Experimental animals

Mature Sprague–Dawley rats were kept on a diet of stock rat pellets and water *ad libitum* under conditions of controlled light and temperature. Each breeding night, two female rats were placed in a cage with one male rat at midnight and separated again at 8 a.m. the next day. The assumed time of conception was taken as halfway through the breeding period, consequently the age of each litter was known within a maximum of $\pm$ 4 hr. The day of separation was called day zero. On day fifteen all rats were tested for pregnancy by abdominal palpation. Pregnant rats were killed by decapitation at 8 p.m. on the fifteenth day. Hence, all material used in this study was aged 15 days 16 hr $\pm$ 4 hr at time of explantation.

The abdomen was shaved, swabbed with alcohol and the peritoneal cavity opened surgically by sterile technique. The uterus was removed and placed in a large sterile petri dish. Each individual embryo in its amniotic sac was removed from the uterus and placed in separate small sterile petri dishes containing 0·5 ml of sterile Hank's Buffered Salt Solution.

In vitro *culture technique*

After dissection all preparations were grown under the same simple standarized conditions. The technique, which uses L-15 media in free gaseous exchange with the

environment has been previously described (MYERS, PETRAKIS and LEE, 1967). The dissected tissues were placed on a millipore filter which in turn rested on a metal grid in a plastic organ culture dish.

The cultures were maintained at $37{\cdot}0 \pm 0{\cdot}5$°C in free exchange with the atmosphere for 72 hr. The media was replaced after 36 hr. After incubation the preparations still resting on the millipore filter was placed in 4 per cent glutaraldehyde for 24 hr. The tissues were then embedded in paraffin, serially sectioned at right angles to the axial plane at 8μ and stained with haemotoxylin and eosin. The only departure from standard histological technique being that all tissues were placed in eosin for 2 min between the 70 and 80 per cent alcohols during dehydration after fixation. This superficial staining greatly facilitated the subsequent orientation of the tissues for sectioning. It did not interfere with subsequent staining.

DISSECTIONS

Three different preparations were used and all were obtained under strict sterile conditions using microdissection instruments under a dissecting microscope.

The purpose of the first group was to study the potentiality of fusion between upper limbs. These tissues were obtained by excising both forearms at the level of the elbow joints. One was placed dorsal surface down on the millipore filter in the culture chamber and the other in the pair being placed on top in palm to palm contact (Fig. 1).

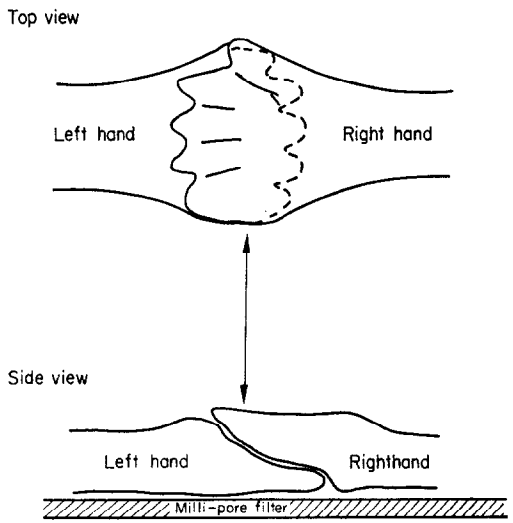

FIG. 1. Hand pair in palm to palm contact. Top and side view.

The second group of tissues was used to study fusion between lower limbs and was obtained by excising both forelegs at the level of the knee joints. One was placed dorsal surface down on the millipore filter in the culture chamber and the other of the pair being placed on top in sole to sole contact (Fig. 2).

The purpose of the third group was to study fusion between tails of litter-mates and these were obtained by excising the tail near its base. A pair of tails thus obtained were placed in side by side contact on the millipore filter in the culture chamber (Fig. 3). All the tissues were obtained from two litters.

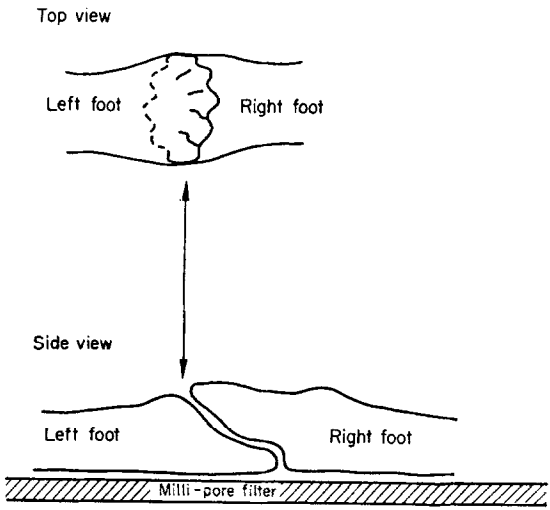

FIG. 2. Foot pair in sole to sole contact. Top and side view.

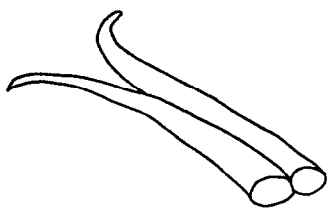

FIG. 3. Tail pair in contact.

RESULTS

On examination of the first group of tissues which consisted of ten pairs of hands placed in palm to palm contact, it was found that they were all fused together. Six pairs exhibited fusion with mesenchymal penetration and four fusion with the epithelial

barrier intact between the hands. Figure 4 reveals a case of epithelial lamination between two hands. In this *in vitro* series of experiments, fused is the term used to describe the situation where the epithelial barrier remains intact between the mesenchymal tissues or where this barrier has been penetrated by the mesenchymal tissue. Hence, the term fused is subclassified into either laminated epithelium or penetrated. Each preparation was recorded according to the most advanced stage of fusion noted, penetration of the lamina being the most advanced.

Fusion with mesenchymal penetration occurred in all ten of the second group which consisted of pairs of feet placed in sole to sole contact. This is shown in Fig. 2 which reveals the absence of epithelial cells between the two embryonic feet. Similarly fusion with mesenchymal penetration occurred in all ten of the third group consisting of pairs of tails placed in side by side contact. This is shown in Fig. 3 in which epithelium is seen to be intact covering the tails although none remains between them.

The results of these experiments are tabulated in Table 1. The difference in the extent and type of fusion between the tissue groups, in particular between the hand pairs as compared to the foot and tail pairs, is more significant than the tabulated results suggest. It has been observed that there is a marked difference in degree of development between the upper and lower limbs in a 15 day old rat embryo. Each of the digits of the hands appears larger and separated about half way whereas the digits of the feet are still webbed together. Microscopically some of the hands show areas of distinct cartilage development and some muscle differentiation in the digits whereas in the feet the cartilaginous tissue is just beginning to form and muscle differentiation is not yet evident. The tails appeared similar to the lower limbs in that the organization of the undifferentiated mesenchyme appeared to be in the early phases. The extent of histodifferentiation in the fore and hind limb is not as great in the *in vitro* grown tissues as can be seen in Figs. 4, 5 and 6.

TABLE 1

Experimental group	Number	Fused	Laminated	Penetrated
Hand/Hand	10	10	4	6
Foot/Foot	10	10	—	10
Tail/Tail	10	10	—	10

With the *in-vitro* handpairs, penetration of the epithelial barrier occurred in some cases although there appeared to be only minimal intermingling of the mesenchymal tissue. The mesenchyme cells and fibres remained organized in a concentric pattern about the organizing central cartilaginous core of the digits, with only a few cells passing through the disrupted epithelial barrier. With the pairs of feet and tails, fusion with penetration was much more complete. The mesenchymal cells intermingled

freely between the two tissue masses, it being impossible in most cases to determine the original member from which any mesenchymal cell originated. (Figs. 5 and 6).

No dissimilarity was noted between the epithelial covering of the hands, feet and tails involved in the area of fusion. In all instances there was first a stage of lamination in which two epithelial components were fused together but individually distinct. This type of union is however strong enough to withstand the rigours of histlogical preparation without separation. Subsequently the two epithelial layers lost their individual identity and became a single, irregular epithelial barrier between the two masses of mesenchyme. The epithelial barrier was then disrupted and the previously described mesenchymal penetration occurred.

DISCUSSION

The process of fusion is of fundamental importance in the early stages of embryological development. Failure of fusion will either be incompatible with life or result in congenital cleft deformities. It is often difficult to determine the primary site and cause of a developmental cleft for it may be either from a failure of fusion *per se*, or from prior deviations in development which prevents tissue process from apposing and thus fusing.

The *in vitro* technique has been used extensively to study the morphology and mechanism of palatal shelf fusion. MORIARTY, WEINSTEIN and GIBSON, 1963; POURTOIS, 1966; MYERS, PETRAKIS and LEE, 1968). These studies have shown that the *in vitro* process of fusion is quite similar to the *in vivo* fusion process. The sequence of events described for *in vitro* fusion (1) contact of epithelial cells, (2) delamination, (3) degeneration of the cells and rupture of basement membranes and (4) intermingling of connective tissue (GOSS, *et al.* 1970, in press) is the same as that described as the *in vivo* sequence of fusion (HOLMSTEDT, 1968). However, it is important to determine the behavior of other embryonic epithelial covered mesenchymal tissues placed in contact under *in vitro* conditions. This provides information as to whether the ability to fuse is confined to those tissues which are normally involved in this process of fusion or whether it is a more generalized property of all embryonic epithelial covered mesenchyme.

Previous studies demonstrate the ability of embryonic oral tissues not normally involved in *in vivo* fusion to fuse under *in vitro* conditions (GOSS, *et al.* 1970, in press). This more generalized property of fusion of embryonic epithelial covered mesenchyme is further demonstrated by this study. Histologically the process of fusion between the tail and foot pairs appears similar to that observed between palatal shelf pairs, tongue pairs and tongue palate combinations (GOSS, *et al.* 1970, in press). In none of these combinations was any epithelial specialization such as thickening, noted in the area of fusion. The process of fusion in hand pairs was different in that although epithelial breakdown occurred mesenchymal penetration was only minimal. No specialization of the mesenchyme involved in the fusion process of either hands, feet or tails was noted by the histologic techniques used in the experiment. Further studies are required to document the enzymatic and subcellular processes involved in the fusion process.

A number of specific conditions are required for *in vitro* fusion to occur. The epithelial covered mesenchyme must be placed with their surfaces in contact. Under the conditions of *in vitro* organ culture the tissues are in quiescent contact. This differs from the *in vivo* situation in which epithalial covered mesenchymal tissues may be in contact but not in quiescent contact. The relationship of the tongue to the palatal shelves is an example of this (GOSS, *et al.* 1970, in press). It is also apparent that the developmental age of the tissues influences the ability of fusion. It has been shown that rat palatal shelves only develop the capability to fuse at about 36 hr (age 15/0) prior to the normal *in vivo* age at which they fuse (16/12). Shelves explanted and cultured prior to this time (15/0) fail to fuse. (POURTOIS, 1966).

Similarly rat palatal shelves cultured in vitro from cleft animals have the capability to fuse at a developmental age of 17/16 which is 24 hr after the normal age of *in vivo* palatal fusion, but shelves from cleft animals cultured at age 20/16 which is about 96 hr after the normal *in vivo* age of palatal fusion fail to fuse. (GOSS, *et al.* 1970, in press).

In the present experiment there was a marked difference in the type and degree of fusion between the upper extremity pairs and lower extremity pairs, although the tissues were from littermates. The behaviour of the epithelium involved in fusion appeared similar, the difference in behavior being with the mesenchymal tissue. It is of interest that the mesenchyme of the hands which showed the greatest degree of differentiation revealed the least degree of intermingling.

It is probable thus, since fusion does not occur in palatal shelves prior to 15 days nor after 20/16 that the fusion potential is limited to a particular transient stage of mesenchyme development. It also seems apparent that the *in-vitro* capability of embryonic epithelial covered mesenchymal tissues to fuse is a more general property of these tissues and not one confined to those tissues which fuse normally *in vivo*.

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Résumé—Les interactions épithélio-mésenchymateuses, au cours du processus de fusion palatine, ont été étudiées en faisant fusionner diverses portions de foetus de rat de 15 jours et 16 heures. Les portions suivantes furent utilisées : les pattes antérieures et postérieures et les queues qui, à ce stade de développement, présentent le même type de recouvrement épithélial, mais se distinguent par le degré de spécialisation du mésenchyme sous-jacent. Deux pattes antérieures d'un embryon furent placées, paume contre paume, et mises en culture d'organe pendant 72 heures, avec dix paires de pattes antérieures, constituant le premier groupe expérimental. Dix paires de pieds constituent le second groupe expérimental, avec deux pieds du même embryon, placés plante contre plante, et mis en culture. Le troisième groupe expérimental comprend dix paires de queues, d'embryons de la même portée, placées côte à côte et mises en cultures pendant 72 heures.

Dans les trois groupes, on observe une fusion avec désintégration épithéliale, d'intensité variable. Il apparait que le degré de pénétration mésenchymateuse, à travers la barrière épithéliale rompue, est fonction du degré de spécialisation du tissu mésenchymateux. Plus cette spécialisation est élevée et moins l'interpénétration mésenchymateuse est élevée. Le membre le plus spécialisé, la patte antérieure, présente la plus faible fréquence de fusion, allant jusqu'à la désintégration épithéliale.

Zusammenfassung—Die Wechselwirkung zwischen Epithel und Mesenchym beim Fusionsprozess wurde durch Zusammenwachsen verschiedener Extremitäten eines 15 Tg. 16 Std. alten Rattenfoetus untersucht. Die hierfür benützten Extremitäten waren die Vorder und Hinterpfoten sowie die Schwänze, die in diesem Stadium ihrer embryonalen Entwicklung dieselbe Art epithelialer Bedeckung besitzen, sich jedoch deutlich bezüglich des Spezialisationsgrades des darunterliegenden Mesenchyms unterscheiden. Zwei Vorderpfoten eines Embryo wurden in palmarem Kontakt 72 Stunden lang unter den Bedingungen einer Organkultur gehalten; diese Versuchsgruppe umfaßte insgesamt 10 Paare solcher Vorderpfoten. 10 Paare Hinterpfoten bildeten die zweite Versuchsgruppe, wobei jeweils die Hinterpfoten desselben Embryo mit den Fußsohlen gegeneinander plaziert und kultiviert wurden. Die dritte Versuchsgruppe bestand aus 10 Schwanzpaaren von Embryonen gleicher Würfe; sie wurden 72 Stunden lang in seitlichem Kontakt kultiviert.

Bei allen 3 Versuchsgruppen trat eine Fusion mit Abbau des Epithels ein, wenngleich das Ausmaß unterschiedlich war. Es wurde festgestellt, daß der Grad mesenchymaler Verbindung über die unterbrochene Epithelgrenze mit dem Ausmaß der Spezialisierung des mesenchymalen Gewebes zusammenhängt. Je größer der Spezialisierungsgrad des Mesenchyms, desto geringer war die Vermischung mit dem Mesenchym der Gegenseite. Die Vorderpfoten als am meisten spezialisierte Extremitäten zeigten auch die geringste Häufigkeit des Fusionsprozesses, der bis zum Stadium des Epithelabbaues fortschritt.

REFERENCES

AREY, L. B. 1966. *Developmental Anatomy.* (3rd ed.). p. 24, Saunders, Philadelphia.

GOSS, A. N., BODNER, J. W. and AVERY, J. K. Fusion capability of rat embryonic oral tissues. *In vitro. Archs oral Biol.* To be published.

GOSS, A. N., BODNER, J. W. and AVERY, J. K. 1970. *In vitro* fusion of cleft shelves. *Cleft Palate J.* In press.

GRÜNEBERG, H. 1943. The development of some external features in mouse embryos. *J. Hered.* **34,** 89–92.

HOLMSTEDT, J. O. V. 1968. A study of the process of fusion of the secondary palate in A/Jax mice. Master's Thesis, The University of Michigan.

KONEGNI, J. S., CHAN, B. C., MORIARTY, T. M., WEINSTEIN, S. and GIBSON, R. D. 1965. A comparison of standard organ culture and standard transplant techniques in the fusion of the palatal processes of rat embryo's. *Cleft Palate J.* **2,** 219–228.

MORIARTY, T. M., WEINSTEIN, S. and GIBSON, R. D. 1963. The development *in vitro* and *in vivo* of fusion of the palatal processes of rat embryos. *J. Embryol. exp. Morph.* **11,** 605–619.

MYERA, G. S., PETRAKIS, N. L. and LEE, M. 1967. Cultivation of embryonic rat palates in defined and semi-defined media. *Archs oral Biol.* **12,** 565–567.

MYERS. G. S. PETRAKIS, N. L. and LEE, M. 1968. Factors influencing fusion of rat palates grown *in vitro. Anat. Rec.* **162,** 71–81.

POURTOIS, M. 1966. Onset of squired potentiality for fusion in the palatal shelves of rats. *J. Embryol. exp. Morph.* **16,** 171–182.

POURTOIS, M. 1968. La fusion des crêtes palatines et son alteration par quelque agents tératogènes. *Archs Biol.* (Liege) 79.

REEVE, W. L., PORTER, D. and LEFKOWITZ, W. 1966. *In vitro* closure of the rat palate. *J. Dent. Res.* **45,** 1375–1380.

SICHER, H. 1966. *Orban's Oral Histology and Embryology.* (Ed. by SICHER, H.) (6th ed.) pp. 6–12. Mosby, St. Louis.

VARGAS, V. I. 1968. Fusion of the palatine shelves with heterotypic explants in the mouse. *Archs oral Biol.* **13,** 845–848.

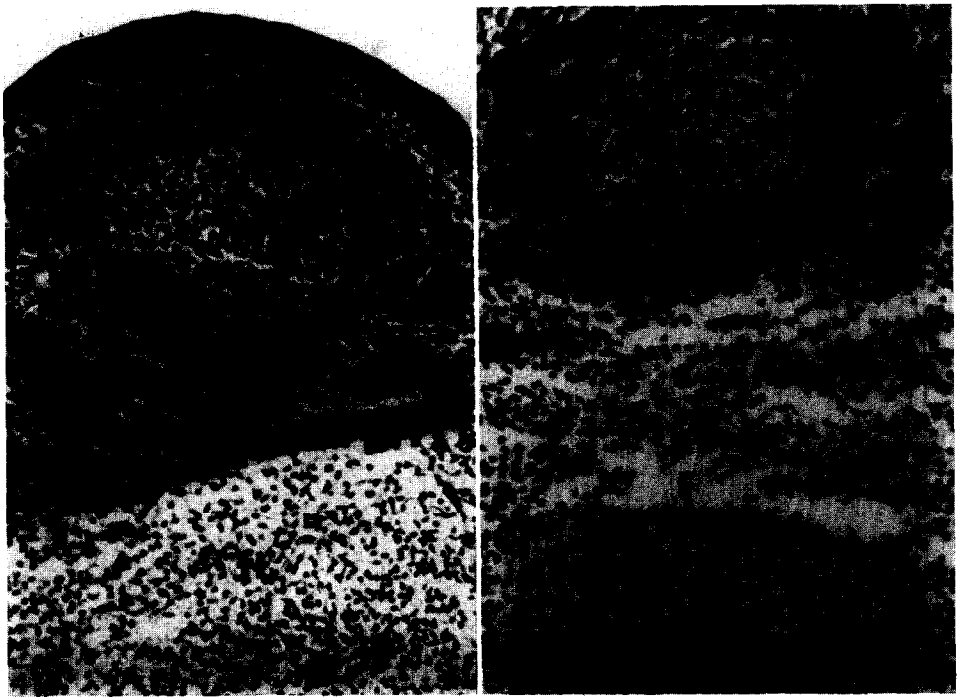

FIG. 4. Hand to hand culture, showing the usual laminated fusion F, between one digit D, and the palm of the other hand H. ×80

FIG. 5. Foot to foot culture, showing complete fusion between one toe T, and the sole of the other foot F. ×80

PLATE 1

A.O.B. f.p. 868

FIG. 6. Complete fusion between two tails. ×80