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Original Article

Co-culture of contiguous developmental fields in a serumless, chemically-defined medium: an *in vitro* model permissive for coordinate development of the mouse ear

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ABSTRACT Pattern formation is intrinsically hierarchical, increasing in complexity from the first early embryonic inductive tissue interactions to the eventual integration of multiple organ systems. Viewed as a problem in pattern formation, the vertebrate ear is an exceedingly complex organ system in which normal morphogenesis requires multiple inductive interactions between a variety of adjacent tissues. In order to model the process of higher level pattern formation, we have developed a method for organ culture of the embryonic murine ear. E10.5 mouse embryos (38 to 42 somite pairs) were microdissected into explants that consist of the first and second branchial arches, the otocyst, and the adjacent neural tube. The growth of these explants in a serumless, chemically-defined medium was compared to medium supplemented with 10% fetal calf serum. After 6 days in culture using serumless medium, we observed that this environment was permissive for the formation of pinnae, rudimentary semicircular canals and cochlear ducts, chondrogenesis of the otic capsule and elongation of the endolymphatic ducts. Posterior elements of Meckel's and Reichert's cartilages were identified as ossicular anlagen. All of these structures maintained appropriate anatomic interrelationships during *in vitro* development. Furthermore, no significant differences were observed in explants grown in serum-supplemented medium. We conclude that during ear development several histogenetic and morphogenetic processes, including aspects of higher level pattern formation, are mediated primarily by paracrine and/or autocrine factors. The development of an organ culture model using serumless medium should facilitate the discovery of intrinsic factors which regulate the coordinate development of inner, middle and external ear structures.

KEY WORDS: *organ culture, mouse, ear, serumless*

Introduction

Organogenesis results from sequential inductive interactions between laterally apposed tissue layers (e.g. Kollar and Baird, 1970; Hall, 1980; McPhee and Van De Water, 1986). Such apposed tissue layers are, in turn, the fundamental anatomic units of developmental fields. Developmental fields are geographically discrete regions of the embryo that have a specific morphogenetic potential; within a developmental field, cytodifferentiation, histogenesis and morphogenesis are controlled by the combinatorial expression of a finite set of genes (Opitz and Gilbert, 1993). In the early developmental stages of many organs, the developmental field and the apposed tissue layer is conceptually equivalent (e.g. the optic vesicle and lens placode together constitute the entire developmental field of the eye). In other cases, multiple tissue-tissue interactions are implicated even at the incipient stages of development. Vertebrate ear development, for example, requires numerous pairwise interactions between branchial arch surface ectoderm, otic epithelium, neural crest, neuroectoderm, and pharyngeal endoderm (Reagan, 1917; Waddington, 1937; Zwilling, 1941; Detwiler and Van Dyke, 1950).

An examination of the spectrum of congenital ear anomalies in humans (Gorlin *et al.*, 1990) suggests that it may be useful to consider the mammalian ear as a sum of three developmental fields which correspond anatomically to the inner, middle and external ears (see Discussion). Despite this simplification, each

Abbreviations used in this paper: CNC, cranial neural crest; E, gestational day; FCS, fetal calf serum; LDH, lactate dehydrogenase; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin 3.

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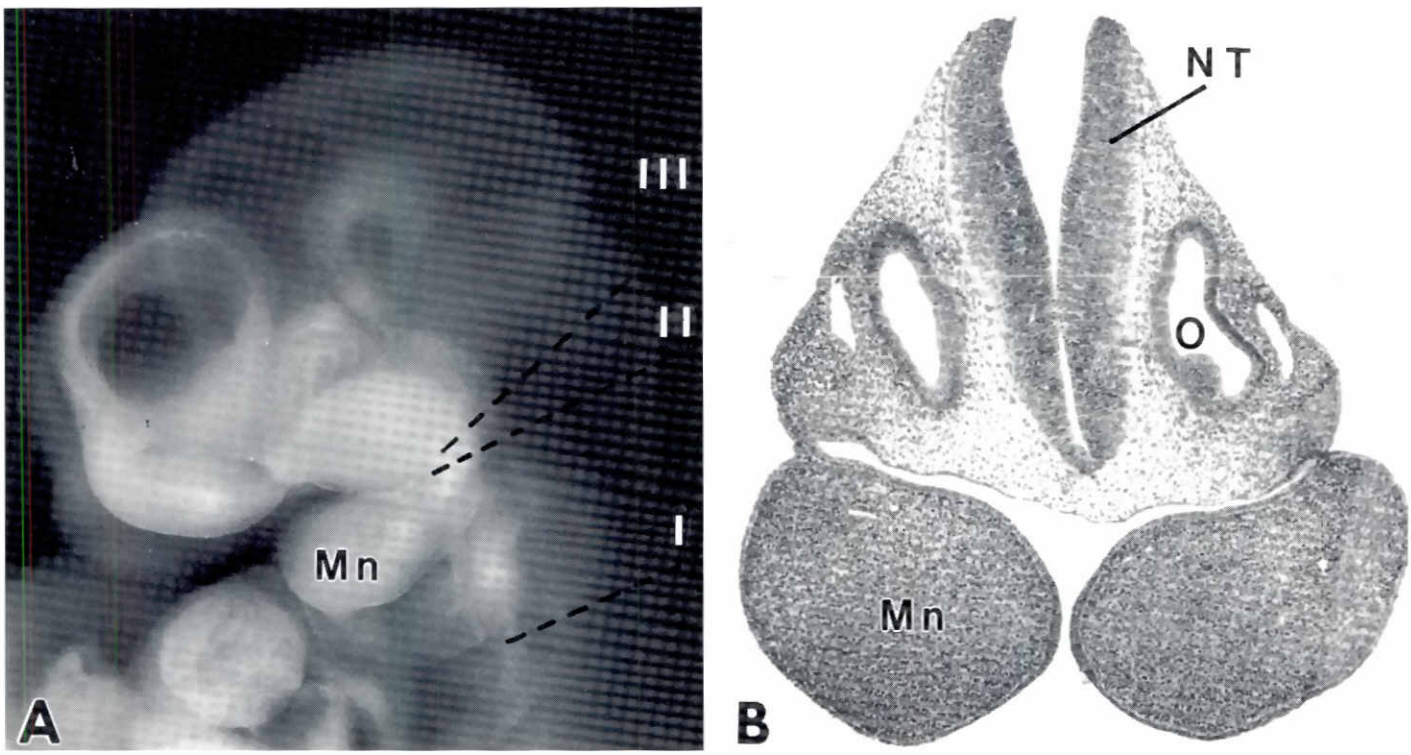


Fig. 1. Microdissection strategy for the first/second arch explant. *Embryos were dissected free from their decidual membranes and then staged by somite count and external features, by the method of Theiler (1972).* **(A)** *Light micrograph of a 38 somite pair embryo. Magnification, 40x. The initial cut (I) is made below the second branchial arch and the second cut (II) is made above the mandibular process of the first arch (Mn). III: "flared" cut described in text.* **(B)** *Montage photomicrograph of hematoxylin and eosin stained section of the explant at the level of the second branchial arch. NT, neural tube; O, otocyst. Magnification, 65x.*

field still encompasses multiple epithelial-mesenchymal interactions. In making the conceptual leap from idealized apposed tissue layers to the assembly of a complex organ, two complementary questions need to be addressed: (i) how are multiple tissue-tissue interactions coordinately regulated (spatially and temporally) within a developmental field, and (ii) how do adjacent fields interact so as to yield an organ with the correct form and function?

Epithelial-mesenchymal interactions have been examined by manipulation of embryos (reviewed by Spemann, 1938), recombination of tissues *in vitro* (Yntema, 1950; Grobstein and Dalton, 1957; Hall, 1978; Thesleff and Hurmerinta, 1981) and explantation of organ anlagen (Fell and Robison, 1930; Tyler and Hall, 1977; Jaskoll *et al.*, 1988). Several groups have recently developed techniques for studying the *in vitro* development of explanted anlagen in serumless, chemically-defined medium (e.g. Hodges and Melcher, 1976; Slavkin *et al.*, 1989; Minkin *et al.*, 1991), thus eliminating the confounding variables implicit in the use of serum or embryonic extracts. This approach facilitates studies designed to define paracrine and autocrine factors which mediate organogenesis (e.g. Shum *et al.*, 1993; Chai *et al.*, 1994).

In this report, we designed studies to test the hypothesis that paracrine and autocrine interactions are prerequisite for the histogenesis and morphogenesis of the inner, middle and external ears. A microdissection strategy was designed to yield an explant containing all tissues classically described as contributing to ear morphogenesis. Such explants were cultured using serumless, chemically-defined medium, and also using serum-supplemented medium. Both culture conditions were permissive for several morphogenetic processes: (1) formation of the pinna; (2) condensation of mesenchyme leading to formation of Meckel's cartilage, Reichert's cartilage, and the ossicular anlagen; (3) condensation of periotic mesenchyme leading to chondrogenesis of the otic capsule; (4) elongation of the endolymphatic duct; (5) formation and elongation of the cochlear duct; and (6) semicircular canal formation. These results are interpreted to suggest that paracrine and/or autocrine factors mediate the cytodifferentiation, histogenesis and morphogenesis of inner, middle and external ear structures. Furthermore, the observation that these structures develop in the correct sequence and anatomic positions suggests that paracrine factors also mediate interactions between contiguous developmental fields.

Results

Mandibular processes, tongue, otic capsules and pinnae develop in serumless, chemically-defined medium

We previously described the microdissection and culture of mouse mandibular processes from E10.5 (42-44 somite pairs) embryos; after 9 days of growth in a serumless, chemically-defined medium, explants show evidence of chondrogenesis, osteogenesis, and formation of tooth, lip and tongue (Shum *et al.*, 1993). By extending the limits of the microdissection, we sought to determine if the external, middle and inner ear could develop under comparable conditions. The microdissection strategy is shown in Figure 1A. Parallel cuts were made below the second branchial arch (I)

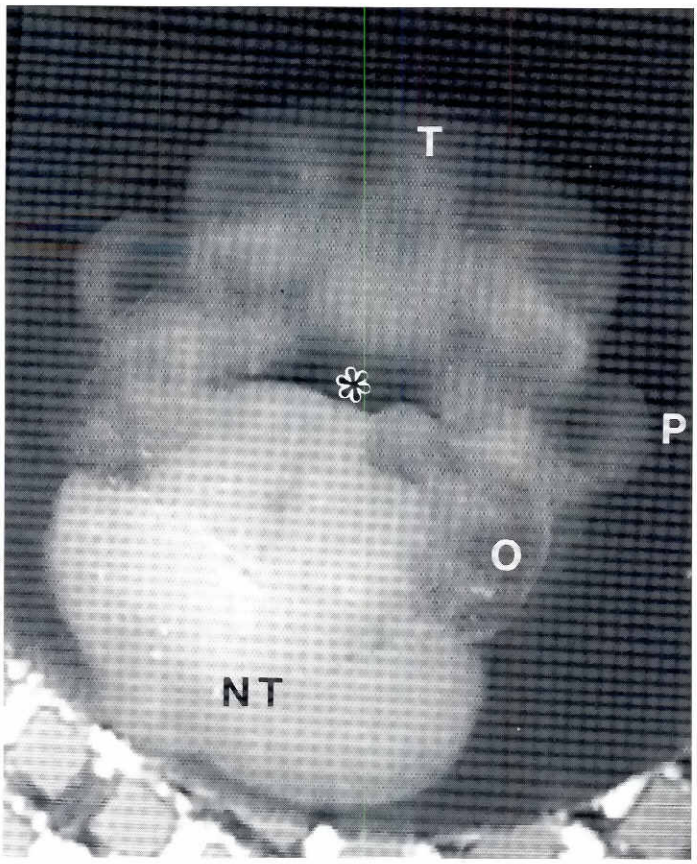


Fig. 2. *In vitro* development of first and second branchial arch structures. *Photograph of explant after 6 days in serumless medium. The explant is shown with the oral surface upwards. NT, neural tube; T, tongue; O, otic capsule; P, pinna; asterisk, pharynx. Magnification, 30x. Each grid division is 0.5 mm.*

and above the mandibular process of the first branchial arch (II). In the early phases of development of this method, the second cut was flared to include a greater portion of neural tube (III). This invariably resulted in medial overgrowth of the neural tube with obliteration of the oropharyngeal space, and only rarely did such explants develop cochlear ducts (data not shown). E10.5 embryos were harvested as described in Materials and Methods and were staged by the method of Theiler (1972); the external features of the embryos corresponded to Theiler stage 17.

In the Theiler stage 17 embryo, the otocyst has formed and a short diverticulum, the endolymphatic duct, arises from the medial surface of the otocyst. At this stage, the periotic mesenchyme is not dense. Immediately after microdissection, the explant consists primarily of the paired mandibular processes, the neural tube, and the otocyst (Fig. 1B).

After 6 days in culture, explants formed a mandible, tongue, otic capsules and pinnae (Fig. 2). Growth of the neural tube is evident relative to the initial explant. The pinnae are positioned immediately ventral to the otic capsules, and are not fused medially to the epithelium overlying the mandible. This mimics normal murine pinna development, in that the pinnae initially form as laterally-directed outgrowths which subsequently grow ventrally to cover the external auditory canal (Theiler, 1972). The explant pinnae most closely resemble pinnae of Theiler stage 23 embryos (E15).

Chondrogenesis of the otic capsule and Meckel's cartilage occurs in serumless and serum-supplemented medium

Otic capsule formation requires the aggregation and subsequent chondrification of the periotic mesenchyme (McPhee and Van De Water, 1985). Otic capsules were grossly evident in all explants by 4 days of culture. No statistically significant trends were noted between serumless and serum-supplemented culture conditions as regards the time of otic capsule development (data not shown). Cartilaginous otic capsules were detected in all explants (Tables 1 and 2). Cross-sectional area measurements indicate that otic capsule growth was not significantly different between the two culture conditions (Fig. 3). Whole-mount analyses demonstrated that in all explants, Alcian blue staining was most prominent along the lateral surface of the capsule; in all cases the medial surface of the capsule stained weakly (Fig. 4). *In vivo*, there is a normal dehiscence of the medial surface of the otic capsule which corresponds to the internal auditory canal.

The first branchial arch cartilage (Meckel's cartilage) is thought to induce osteogenesis of the murine mandible (Frommer and Margolies, 1971); the posterior extensions represent anlagen for the superior portions of the malleus and incus (Hanson *et al.*, 1962). For the purposes of this study, Meckel's cartilage was divided into "posterior" and "anterior" elements, with the posterior inflection point (asterisk in Fig. 4) constituting the anterior/posterior boundary. The anterior and posterior elements of Meckel's cartilage were present not as condensed mesenchyme, but as cartilage in nearly all explants; this was assessed both histologically and by Alcian Blue-stained whole mounts (Tables 1 and 2). By Alcian Blue

TABLE 1

DEVELOPMENT OF THE 1st/2nd ARCH EXPLANT AFTER 6 DAYS IN ORGAN CULTURE

Developmental features	Culture medium	
	Serum-free	10% FCS
MC post	5/7	4/6
MC ant	7/7	6/6
RC med	4/7	1/7
RC lat	1/7	0/7
OC	7/7	6/6
SAG	6/7	6/6
ELD	7/7	6/6
CD	6/7	3/6
SCC	2/7	6/6
P	7/7	5/6
NT	3/7	3/6

Hematoxylin and eosin stained serial sections of explants cultured in serum-free and serum-supplemented media were scored for the presence or absence of a number of developmental features. MC post: chondrification of the posterior moieties of Meckel's cartilage. MC ant: chondrification of the anterior moieties of Meckel's cartilage. RC med: chondrification of the medial moieties of Reichert's cartilage. RC lat: chondrification of the lateral moieties of Reichert's cartilage. OC: chondrification of the otic capsule. SAG: presence of statoacoustic ganglion cells (in all cases, these cells were diffuse and not organized within a discrete region). ELD: elongation of the endolymphatic duct. CD: presence of a cochlear duct. SCC: presence of a semicircular canal. P: presence of a laminar pinna. NT: medial overgrowth of the neural tube. Denominators represent the total number of explants examined from each type of culture medium.

staining, chondrification of the anterior element was less frequent in explants cultured in 10% FCS, but this difference was not statistically significant (p= 0.099). The typical morphology of Meckel's cartilage is illustrated in Figure 4.

The second branchial arch cartilage (Reichert's cartilage) contributes to the hyoid bone, stylohyoid ligament, stapes superstructure, manubrium of the malleus and long crus of the incus (Hanson *et al.*, 1962). In the explants, Reichert's cartilage was observed as separate islands of condensed mesenchyme anterior to the pharynx ("medial elements" of Reichert's cartilage), and/or anterior and medial to the otic capsule ("lateral elements" of Reichert's cartilage). Only occasionally were these regions chondrified (Tables 1 and 2). Chondrification of the medial elements of Reichert's cartilage was occasionally noted in the serumless explants, but not in explants cultured in 10% FCS. This difference was not statistically significant (p= 0.099).

The explants developed ossicular anlagen which were in continuity with the posterior portion of Meckel's cartilage and the lateral portion of Reichert's cartilage (Fig. 5A). Comparison with histologic sections from a Theiler stage 22 embryo (gestational day 14) makes possible the identification of incus and stapes anlagen shown in Figure 5A. While the structures appear "compressed" relative to the stage 22 embryo (Fig. 5B), interrelationships between adjacent structures are maintained.

Pinna development in vitro is similar to pinna development in vivo

Similar to pinna development *in vivo*, the first indication of pinna formation *in vitro* was a flange of tissue jutting at right angles from the explant. This flange appeared in all serumless explants by 4 days *in vitro* (data not shown). The flange turned ventrally as it grew, but did not fuse to the mandibular epithelium. The size and histologic appearance of the pinna resembles a Theiler stage 23 embryo (compare Fig. 6A and B). Pinnae developed in all serumless explants and in 5 of 7 serum-supplemented explants; this difference was not significant (Table 1). Cross-sectional area measurements indicate that pinna growth was not significantly different between the two culture conditions (Fig. 3).

The pinna consists of an epithelium, a less cellular subepithelial layer, and a condensed mesenchymal core (Fig. 6A, asterisk).

TABLE 2

CARTILAGE FORMATION AS ASSESSED IN ALCIAN BLUE WHOLE-MOUNTS

Developmental features	Culture medium	
	Serum-free	10% FCS
MC post	8/8	7/8
MC ant	8/8	5/8
RC med	3/8	0/8
RC lat	1/8	0/8
OC	8/8	8/8

Eight explants from each culture medium were prepared as Alcian Blue-stained whole mounts (described in Materials and Methods). The explants were then scored for staining by Alcian Blue in the indicated anatomical regions. Abbreviations are given in Table 1.

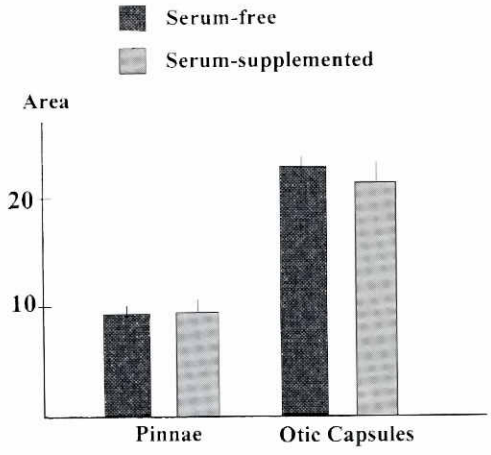


Fig. 3. Comparison of pinna and otic capsule cross-sectional areas. *Comparison of the means of the maximal cross-sectional areas derived as described in Materials and Methods. Error bars represent s.e.m. Area: each 10 units represents 0.12 mm².*

Pinna cartilage formation was not detected in cultured explants examined either histologically or by whole-mount.

In vitro, pinnae formed ventral and lateral to the pars superior (region of the utricle and semicircular canals), and lateral to the pars inferior (region of the cochlear duct). This pattern of morphogenesis is similar to pinna development *in vivo*, although explant pinnae were more ventrally placed. No external auditory canals were observed in any of the explants examined histologically.

The first/second arch organ culture model is also permissive for histogenesis and morphogenesis of the membranous labyrinth

At the time of microdissection, the membranous labyrinth consists of a pear-shaped otocyst with a short, medially-directed endolymphatic duct. The otic epithelium is pseudostratified columnar, with two or three layers of nuclei (Fig. 1B). After 6 days in culture, all explants showed a marked increase in the topologic complexity of the otocyst. The endolymphatic duct turned dorsally and elongated (Fig. 7A), a semicircular canal formed from the posterolateral surface of the otocyst (Fig. 7C), and a cochlear duct formed from the ventromedial portion of the otocyst (Fig. 7E). Comparable structures which developed *in situ* are shown in Figure 7B, 7D and 7F. The cochlear duct is recognizable both by its ventromedial position and its characteristic histology. The lateral and medial surfaces of the duct are formed by a pseudostratified columnar epithelium; the lateral surface has two or three layers of nuclei, while the medial surface (ultimately the site of origin of the sensory epithelium) has about six layers of nuclei (Sher, 1971). The cochlear duct from a Theiler stage 21 embryo (gestational day 13) is shown for comparison in Figure 7F. The cochlear duct formed in organ culture had a thinner epithelium, but the overall pattern was clearly maintained. One notable difference between cultured explants and *in situ* development was the marked diminution of the cochleovestibular ganglion in all explants. Cochleovestibular ganglia were present in the initial E10.5-derived explants, but few cells remained after six days in culture (both serumless and serum-supplemented conditions). This may be due to a lack of appropriate trophic factors, or to physical limitations of the model (see Discussion).

Histologic analysis of the explants demonstrated few statistically significant differences in inner ear development between the two culture conditions (Table 1). Elongation of the endolymphatic duct occurred in all explants. Cochlear duct formation was consistently observed under serumless conditions; the smaller number noted for the serum-supplemented group was not significant. Semicircular canal development was significantly more frequent in the serum-supplemented group ($p = .016$).

An interesting trend was noted when cochlear duct formation was correlated with medial overgrowth of the neural tube. In our initial microdissection efforts, the superior cut was made not parallel to the inferior cut but flared so as to include more of the neural tube (Fig. 1A). This extra lip of tissue tended to be pulled anteriorly by surface tension, and it would grow to obliterate the pharyngeal cavity. Invariably, cochlear ducts did not develop in these explants (data not shown). Subsequently, the microdissection was modified so that the superior cut was parallel to the inferior cut, yielding an explant which was thinner in the rostral-caudal dimension. All of the explants subjected to statistical analysis in this report were microdissected by the latter technique. Cochlear ducts were observed with greater frequency when explants were microdissected in this manner (data not shown). Still, possibly because of minor variations in microdissection, or possibly because of true differences between culture conditions, medial overgrowth of the neural tube was observed in several explants (compare Fig. 8A and B). Among serumless explants, medial overgrowth was observed in 3 of 7 explants, including the only explant that did not possess cochlear ducts. Among serum-supplemented explants, medial overgrowth occurred in 3 of 6 explants, and cochlear ducts formed only in the 3 explants lacking medial overgrowth.

Explant viability is similar between serumless and serum-supplemented culture conditions

To assess gross differences in explant survival between the two culture conditions, medium was removed from each dish after 48 hours of culture and assayed for lactate dehydrogenase (LDH). LDH activity correlates with gross cell death. A comparison of mean LDH activity at each change of medium (i.e., every other day) from the two culture conditions is shown in Figure 9. Mean LDH activity was not significantly different after 48 h or 96 h *in vitro*. After 6 days, LDH activity was significantly elevated in serum-supplemented culture medium ($p<.05$). To control for differences in explant mass and/or cellularity, after 6 days in culture one dish from each culture condition was used for the determination of maximal LDH activity (see Methods). When the mean LDH activity for the serum-supplemented explants was adjusted in this manner, the difference in the means was no longer significant. Thus, at the termination of this experiment, there were no statistically significant differences in explant viability.

Discussion

Paracrine and/or autocrine factors mediate the histogenesis and morphogenesis of inner, middle and external ear structures

In vitro development of first/second arch explants in a serumless, chemically defined medium supports the hypothesis that paracrine and/or autocrine factors are sufficient for a number of histogenetic and morphogenetic processes. Serumless conditions were permissive for the formation of the otic capsule (Fig. 4), ossicular anlagen (Fig. 5A), pinnae (Fig. 6A), semicircular canals, cochlear duct, and an elongated endolymphatic duct (Fig. 7). The only

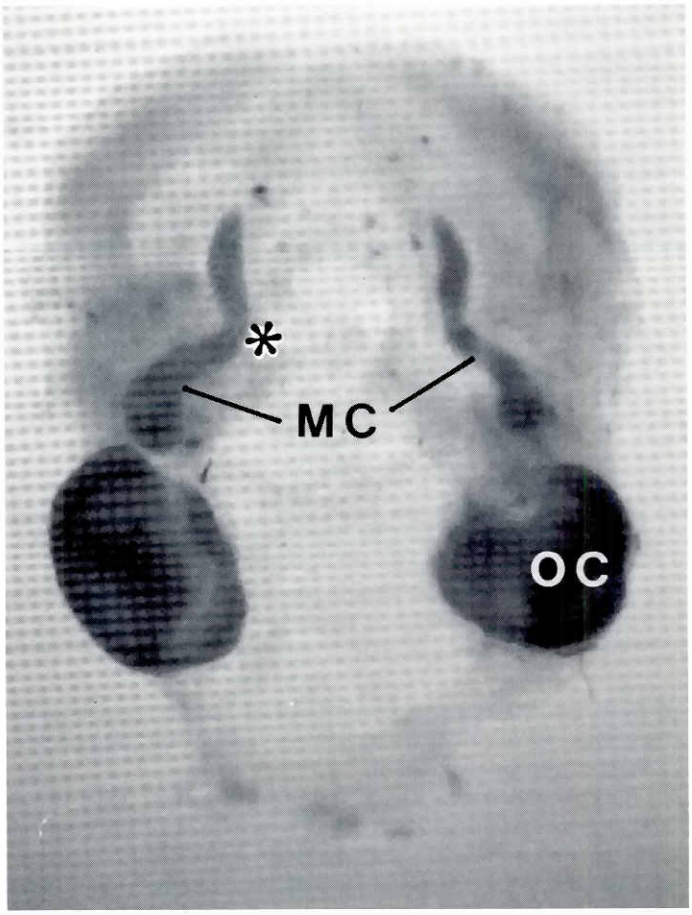


Fig. 4. Alcian Blue whole-mount staining of cartilage. *After 6 days in serumless medium, this explant was processed for Alcian Blue whole mount staining as described in Materials and Methods. MC, Meckel's cartilage; OC, otic capsule; asterisk, inflection point chosen as the arbitrary division between the posterior and anterior portions of Meckel's cartilage (see text). Magnification, 30x.*

significant difference between serumless and serum-supplemented explants was the higher frequency of semicircular canal formation in the latter group. The absence of other significant differences between these two culture conditions suggests, but does not prove, that extrinsic (endocrine) factors are relatively less important during ear development.

In contrast to our findings, attempts at culturing the chick otic vesicle in serumless media have revealed a requirement for serum or other growth factors (Represa *et al.*, 1988; Represa and Bernd, 1989). These authors reported that otic vesicle explants derived from stage 18 to 22 chick embryos undergo growth arrest when cultured in serumless medium; the arrest could be alleviated by adding serum or bombesin, or to a lesser extent, EGF or PDGF (Represa *et al.*, 1988).

The chick embryo explant differs significantly from the mouse embryo explant described in this report. Aside from the difference in species, the developmental stage of the chick embryo explant is earlier (stage 22 corresponds approximately to an E10 mouse embryo: Theiler, 1972; and Hamburger and Hamilton, 1951). It is possible that the younger chick explant is more dependent on extrinsic mitogenic factors than the slightly older mouse explant. In this regard, we note that older chick explants are less fastidious in

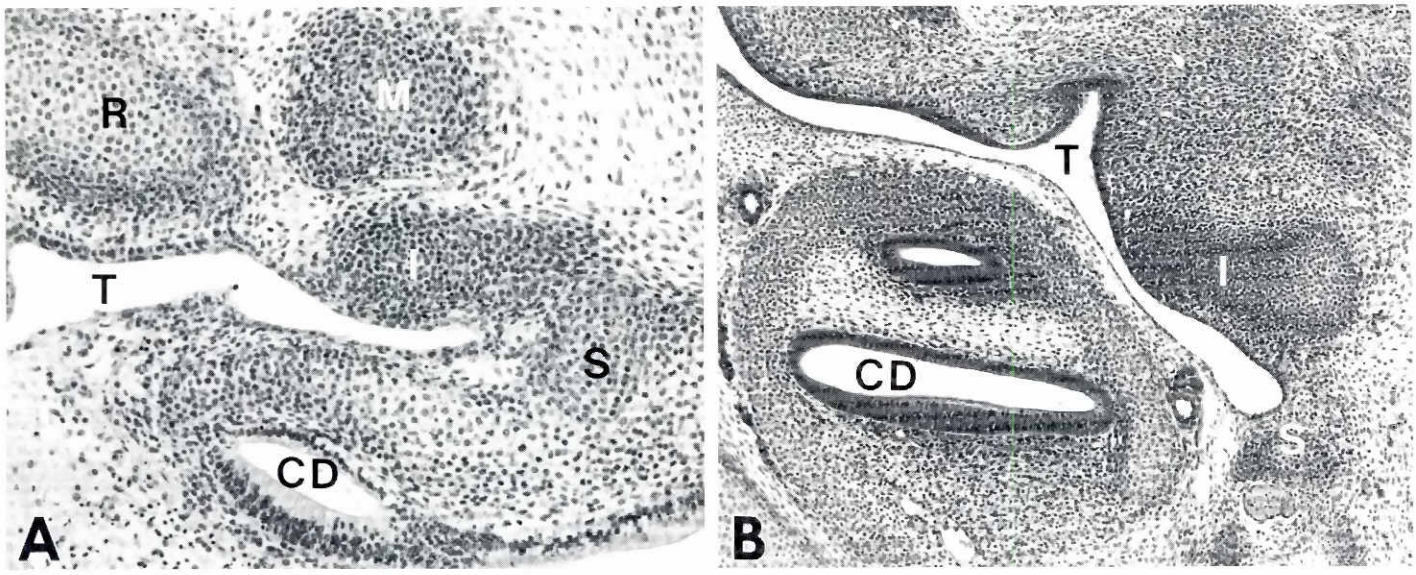


Fig. 5. Development of ossicular anlagen in the first/second arch explant. (A) *Hematoxylin and eosin stained section of an explant maintained for 6 days in serumless medium. T, tubotympanic recess. CD, cochlear duct; R, medial portion of Reichert's cartilage; M, posterior portion of Meckel's cartilage; I, incus anlagen; S, stapes anlagen. Magnification, 170x.* **(B)** *Axial section of a Theiler stage 22 embryo (gestational day 14). Magnification, 85x.*

their serum requirements: explants of the saccules and utricles of hatchling chicks can grow and differentiate in serumless medium (Warchol and Corwin, 1993).

Another difference between the mouse and chick embryo explants is that the latter is a much smaller explant, consisting only of the otic vesicle and periotic mesenchyme. Serum- or growth factor-supplementation may be necessary for development of the chick otocyst because it substitutes for the paracrine factors of the neighboring tissues from which the explant was separated.

Advantages and caveats of the first/second arch organ culture model

In vitro culture of the inner ear was first pioneered by Fell (1928). More recent methods have involved explantation of the otocyst and periotic mesenchyme (Van De Water and Ruben, 1974; McPhee and Van De Water, 1986; Represa *et al.*, 1988), or the organ of Corti (Yamashita and Vosteen, 1975; Kelley *et al.*, 1993; Lefebvre *et al.*, 1993). To our knowledge, the present study is the first model which is permissive for the development of the external ear and ossicular anlagen, in addition to the otic capsule and labyrinth. Since the microdissection strategy includes the entire first and second branchial arch, histogenesis and morphogenesis of the mandible, tooth buds, and tongue (anterior 2/3 as well as posterior 1/3) can be studied in tandem with the ear. The model should also be helpful for the investigation of teratogens (e.g., retinoic acid) known to effect the development of first and second branchial arch structures, and may prove useful in the study of human first and second arch anomalies such as Goldenhar or Treacher Collins syndrome.

By E10.5, the age at which embryos were microdissected in this study, the migration of cranial neural crest (CNC) cells into the branchial arches is complete (Selleck *et al.*, 1993). The CNC provides most of the mesenchymal components of the inner, middle and external ear (Noden, 1983, 1988; Morriss-Kay and Tucket, 1991; Serbedzija *et al.*, 1992; Le Douarin *et al.*, 1993). Murine CNC migration begins at E7.5 (Lumsden, 1988). The mandibular process of the first arch is populated by neural crest derived from rhombomeres 1, 2 and 3, while the second arch is populated by the CNC of rhombomeres 4 and 5 (Lumsden *et al.*, 1991; Sechrist *et al.*, 1993). Quail-chick transplantation studies have provided evidence that the fate of these cells is specified prior to their migration (Noden, 1983, 1988), and indirect evidence suggests that the same is true of murine CNC populations (Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993). Similarly, cells of the dorsal and ventral halves of the otocyst are already fated to develop into vestibular or cochlear elements, respectively (Li *et al.*, 1976). Thus, the first/second arch explant likely contains cell populations whose fates are already specified at the time of explantation. The organ culture model we have developed can serve as a model system for the study of morphogenetic interactions among these cell populations.

Davidson (1993) emphasized that inductive tissue interactions typically involve laterally apposed tissue layers (e.g., otocyst epithelium and periotic mesenchyme) which are engaged in a volley of paracrine factors, resulting in biochemical, histogenetic and morphologic changes in both layers. From this simple model of organogenesis, it is evident that a central challenge is to determine the identity and function of such morphoregulatory molecules. Organ culture in a serumless, chemically-defined medium facilitates a number of experimental strategies which would be difficult or impossible to apply *in vivo*. Addition of exogenous growth factors can be used to analyze gain of function (e.g. Dixon and Ferguson, 1992). In contrast, blocking a putative regulatory gene product by a specific pharmacologic agent or antibody, or antisense abrogation of its mRNA, would provide data pertaining to the loss of function of the morphoregulatory molecule (e.g. Shum *et al.*, 1993; Sugi *et al.*, 1993; Chai *et al.*, 1994). In the study of lung morphogenesis, such strategies have been employed to elucidate the function of EGF (Warburton *et al.*, 1992), TGF 1 (Serra *et al.*, 1994) and PDGF-B (Souza *et al.*, 1994). The simple ear organ culture model reported here should provide novel experimental

opportunities to determine the function of specific paracrine factors in ear morphogenesis.

A major caveat in using explants prepared from E10.5 embryos is that the model is not applicable for studies of the specification of cell fates or lineage restrictions. Furthermore, otic placode invagination, otocyst formation, and endolymphatic duct formation all occur prior to E10.5. Another qualification is that the developmental potential of the explant is limited to 6 days in culture; no new features were observed when explants were cultured for 7.5 days, and additional necrosis was evident (results not shown). Thus, several aspects of ear development cannot be modelled by this system. In particular, the following features of ear development can not as yet be analyzed in this simple culture model: spiral morphogenesis of the cochlea and maturation of the organ of Corti; morphogenesis of the ossicles; development of a middle ear cavity, tympanic membrane, and external auditory canal; maturation of the VIIth and VIIIth cranial nerve ganglia; and establishment of neural connections to the developing labyrinth.

These limitations could be due to the absence of one or more factors not present in fetal calf serum. The failure of the cochleovestibular ganglion cells to proliferate and establish connections with the labyrinthine sensory epithelia is an informative example. The trophic requirements of cochleovestibular ganglion cells have been investigated in organ culture (Represa and Bernd, 1989; Lefebvre *et al.*, 1990, 1991; Pirvola *et al.*, 1992, 1994; Bianchi and Cohan, 1993; Vazquez *et al.*, 1994) and in transgenic null mutations of neurotrophin genes or their receptors (Klein *et al.*, 1993; Ernfors *et al.*, 1994; Farinas *et al.*, 1994; Fritzsch *et al.*, 1995). Collectively, these studies suggest that brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) are required for continued survival of vestibular and cochlear ganglion cells, and that the source of these neurotrophins is the vestibular and cochlear sensory epithelia. Appropriate expression of the high affinity receptors for these neurotrophins, TrkB (BDNF) and TrkC (NT-3), is necessary for continued survival of the cochlear and vestibular ganglion cells (Ernfors *et al.*, 1994; Farinas *et al.*, 1994; Fritzsch *et al.*, 1995).

One possible explanation for the failure of ganglion cells to thrive in the first/second arch explant is that the neurotrophins and/or their receptors are not expressed at the time of explantation (E10.5), and the signals to initiate appropriate expression are not present in culture. By *in situ* hybridization, BDNF and NT-3 mRNAs are present in the mouse cochlea at E14.5, but not at E11.5 or E12.5; BDNF mRNA is present in the vestibular sensory epithelia at E12.5 but not at E11.5 (Schecterson and Bothwell, 1992). In contrast, Pirvola *et al.* (1992, 1994) found that NT-3 mRNA was strongly expressed in the rat E11 otic vesicle, particularly in the region adjacent to the cochleovestibular ganglion; they also found low levels of BDNF mRNA in the E11 otic vesicle. TrkB immunoreactivity is present in the mouse cochleovestibular ganglion at E12 but not E10 (Vazquez *et al.*, 1994). In the rat, *trk*B and *trk*C mRNAs are present in the cochleovestibular ganglion by early E12 (Pirvola *et al.*, 1994).

Underexpression of a neurotrophin or its receptor could explain our findings regarding explant ganglion cell survival. In addition to BDNF or NT-3, NGF or some other otic epithelium-derived factor could be necessary (Lefebvre *et al.*, 1991; Von Bartheld *et al.*, 1991; Bianchi and Cohan, 1993). Expression studies of the neurotrophins and their receptors in the first/second arch explant would be needed to evaluate this hypothesis. Supplementation with specific neurotrophins could also be informative.

Aside from growth factor limitations, *in vitro* development may also be limited by a combination of physical and chemical factors. Chemical factors are limiting as a result of the explant's lack of a circulatory system: nutrients and oxygen must reach all cells by diffusion. Inadequate oxygenation or nutrition could cause growth retardation and subsequent dysmorphogenesis. Physical factors include forces deforming the explant which would not be acting *in vivo*: the uniformly caudal pull of gravity, the frictional forces which cause the explant to adhere to the Millipore filter disc, and surface tension at the air-fluid interface. Such forces could tend to deform the explant by bringing tissues into contact which would not otherwise be in contact, or by preventing tissues from contacting one another.

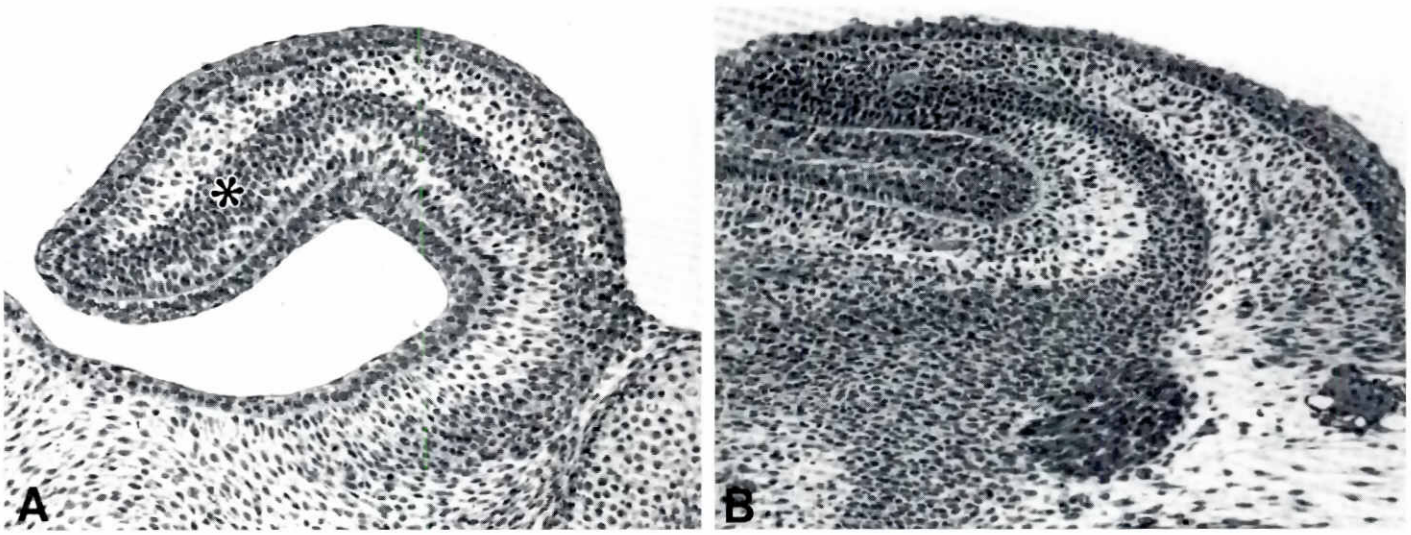
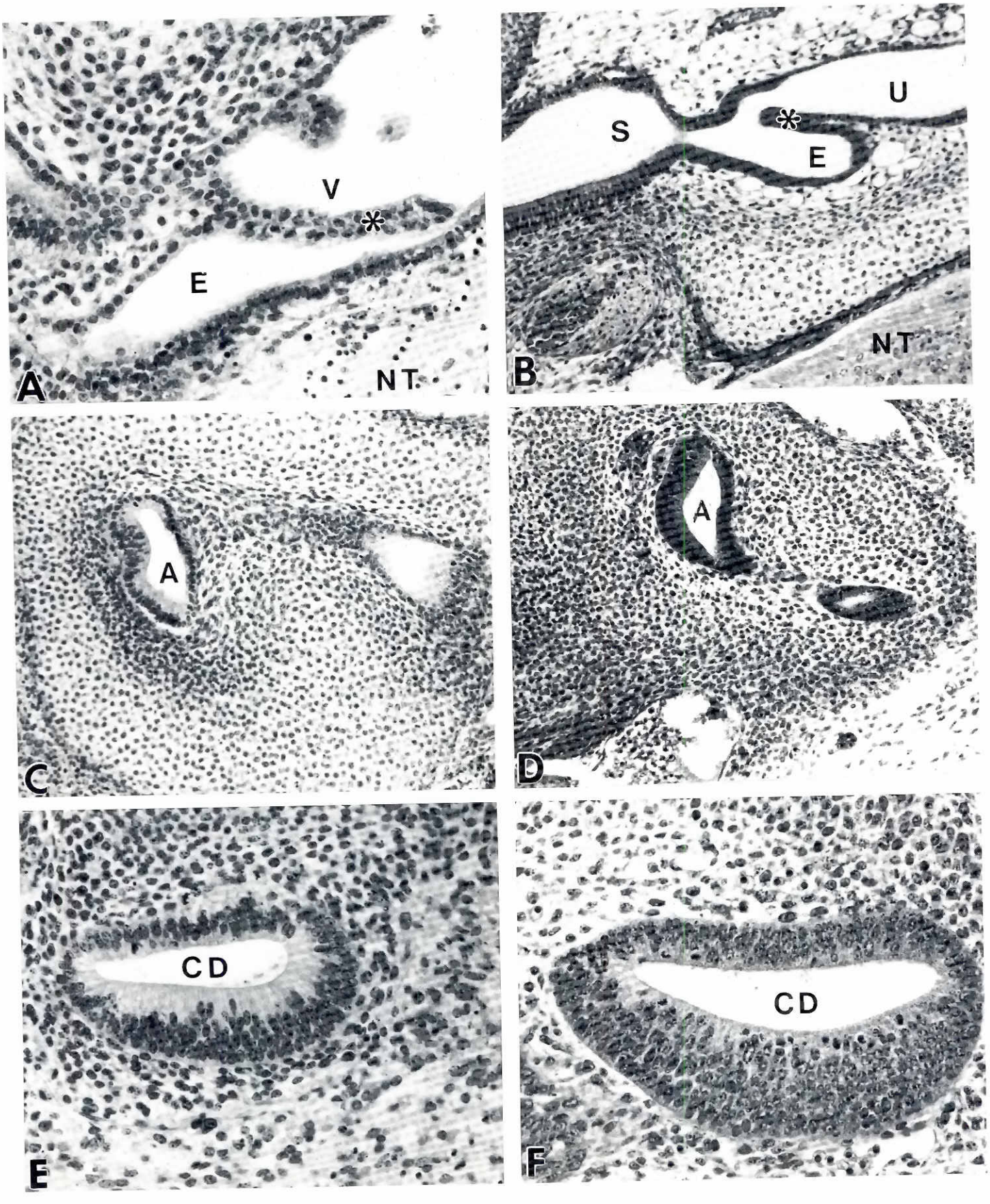


Fig. 6. Pinna development in the first/second arch explant. (A) *Hematoxylin and eosin stained section of pinna from an explant maintained for 6 days in serumless medium. Note mesenchymal core (asterisk). Magnification, 160x.* **(B)** *Horizontal section of pinna from a Theiler stage 23 embryo (gestational day 15). Magnification, 160x.*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These forces could drastically alter morphogenesis. As a possible example, we observed that the neural tube of explants tends to tip anteriorly, leading to medial overgrowth of the neural tissue. Cochlear duct formation is uncommon in such explants (Fig. 8). Whether this is due to inappropriate trophic influences from the adjacent neural tissues, the lack of interaction with the contralateral otocyst, or some other factor, is not known. Finally, we cannot exclude the possibility that these forces act at the cellular level to alter cytoskeletal architecture, leading to cell shape changes and even alterations in gene expression (Ingber, 1993).

Co-culture of contiguous developmental fields in a serumless, chemically-defined medium

In the spectrum of congenital syndromes of the head and neck, ear malformations are often narrow rather than global; examples include stapes fixation in Apert syndrome (Gorlin *et al.*, 1990), or Mondini malformation of the cochlea in Pendred syndrome (Schuknecht, 1993). In surveys of children born with atresia of the external auditory canal, inner ear malformations occur at a rate higher than the general population, but the labyrinths are more commonly radiologically and functionally normal (13 of 24 patients in Potter, 1969, and 58 of 66 patients in Naunton and Valvassori, 1968). Given that the inner, middle and external ear developmental programs are physically and temporally separate during embryogenesis (Langman, 1981), it may be useful to view the ear as an organ which arises from three corresponding developmental fields. If so, then the first/second arch explant can be viewed as a conglomerate of developmental fields leading to formation of the ear, mandible, teeth and tongue.

In all of our explants, the ossicular anlagen were noted to be appropriately positioned with respect to the otic capsule, as were the pinnae. One explanation for this observation is that the cell populations giving rise to each structure were already specified and appropriately-positioned within the first and second branchial arches at the time of explantation. However, it is additionally possible that these populations continue to signal to one another, so that appropriate anatomic relationships are maintained during development. The first/second arch explant could prove to be a useful model for studying the inductive tissue interactions required for morphogenesis of a functionally-integrated inner, middle and external ear.

Materials and Methods

Mouse embryos

Virgin female Swiss-Webster mice were purchased from Simonsen Labs (Gilroy, CA, USA) and were housed and bred as previously described (Slavkin *et al.*, 1989). Appearance of a vaginal plug was taken to indicate Day 0 of embryogenesis. At post-coital day 10.5 (E10.5), gravid females were sacrificed by cervical dislocation. The embryos were dissected free of the decidua and were then washed in 4°C Hanks' Buffered Saline (HBS). All embryos were staged by external features and by somite count (Theiler, 1972). Only embryos with 38 to 42 somites were used for microdissection. Although this range would suggest that some Theiler stage 18 embryos were included in the microdissection (i.e., the 40 to 42 somite embryos), in fact, the external features of our embryos were all consistent with Theiler stage 17.

Care and handling of pregnant mice was conducted in accordance with the standards of the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act (7 U.S.C. et seq.) The protocols used were approved by the Institutional Animal Care and Use Committee of the University of Southern California.

Microdissection and organ culture

The embryo was maintained in 4°C HBS during the microdissection. A single cut was made immediately caudal and parallel to the second branchial arch and a second cut was made parallel to the first cut, cephalad to the mandibular process of the first branchial arch (Fig. 1A). Early in our experience with this technique we flared the second cut so that more cephalad neural tube would be included in the explant. This invariably resulted in medial overgrowth of the neural tube with obliteration of the oropharyngeal space, and only rarely did such explants develop cochlear ducts (see text).

The first/second arch explants were cultured using a modified Trowell method as previously described for the culture of isolated mandibular processes (Slavkin *et al.*, 1989). In brief, explants were transferred to 4 mm diameter Millipore type AABP filters, 0.8 micron pore size, with the oral surface facing upwards. The filters were placed on stainless steel mesh triangles, which were in turn placed in Grobstein Falcon dishes under maximal humidity conditions at 37°C, in an atmosphere containing 5% CO_2 and 95% air. The medium consisted of 1 ml of BGJb (GIBCO-BRL, Grand Island, New York, USA) supplemented with 0.1 mg/ml ascorbic acid, 100 u/ml penicillin and 0.1 mg/ml erythromycin (streptomycin was omitted, so as to avoid any possible ototoxic agents). Four explants were cultured in each dish. Five dishes (20 explants) were used for two different culture conditions: "Plain" (no serum or growth factor supplements) and "serum- supplemented", which included 10% heat-inactivated fetal calf serum (Gibco-BRL). The medium was changed every 48 h; conditioned media was immediately assayed for LDH activity (see below).

Light microscopy

After 6 days in culture, 7 explants from each culture condition were randomly selected for histologic analyses. Explants were fixed in Bouin's solution for 4 h. Explants adherent to their filters were washed, dehydrated in a graded series of ethanol, cleared in xylene, infiltrated and embedded in paraffin. Paraffin blocks were serially-sectioned at 5 micron thickness and sections were then stained with hematoxylin and eosin.

The sections were examined for the presence or absence of the following features:

1. Meckel's cartilage: presence of anterior and/or posterior elements (as mesenchymal condensations or as cartilage).
2. Reichert's cartilage: presence of medial and/or lateral elements (as mesenchymal condensations or as cartilage).
3. Cartilaginous otic capsule.
4. Statoacoustic ganglion cells.
5. Endolymphatic duct: elongation, relative to the initial explant.
6. Pinna: a laminar structure consisting of an epithelium, a loose subepithelial layer, and a dense mesenchymal core.
7. Cochlear duct.

Fig. 7. Inner ear development in the first/second arch explant. *Comparison of inner ear structures in explants maintained for 6 days in serumless medium* **(A,C and E)** *and equivalent structures from normal embryos* **(B,D and F)**. **(A)** *Explant, 6 days. Endolymphatic duct at its point of origin (E) from the vestibule (V). NT, neural tube. Magnification, 350x.* **(B)** *Comparable section from a Theiler stage 23 embryo (gestational day 15). S, saccule; U, utricle. Magnification, 175x.* **(C)** *Explant, 6 days. Semicircular canal and otic capsule. Note ampulla of semicircular canal (A). Magnification, 175x.* **(D)** *Comparable section from a Theiler stage 23 embryo. Magnification, 180x.* **(E)** *Explant, 6 days. Cochlear duct (CD). Magnification, 350x.* **(F)** *Comparable section from a Theiler stage 21 embryo (gestational day 13). Magnification, 475x.*

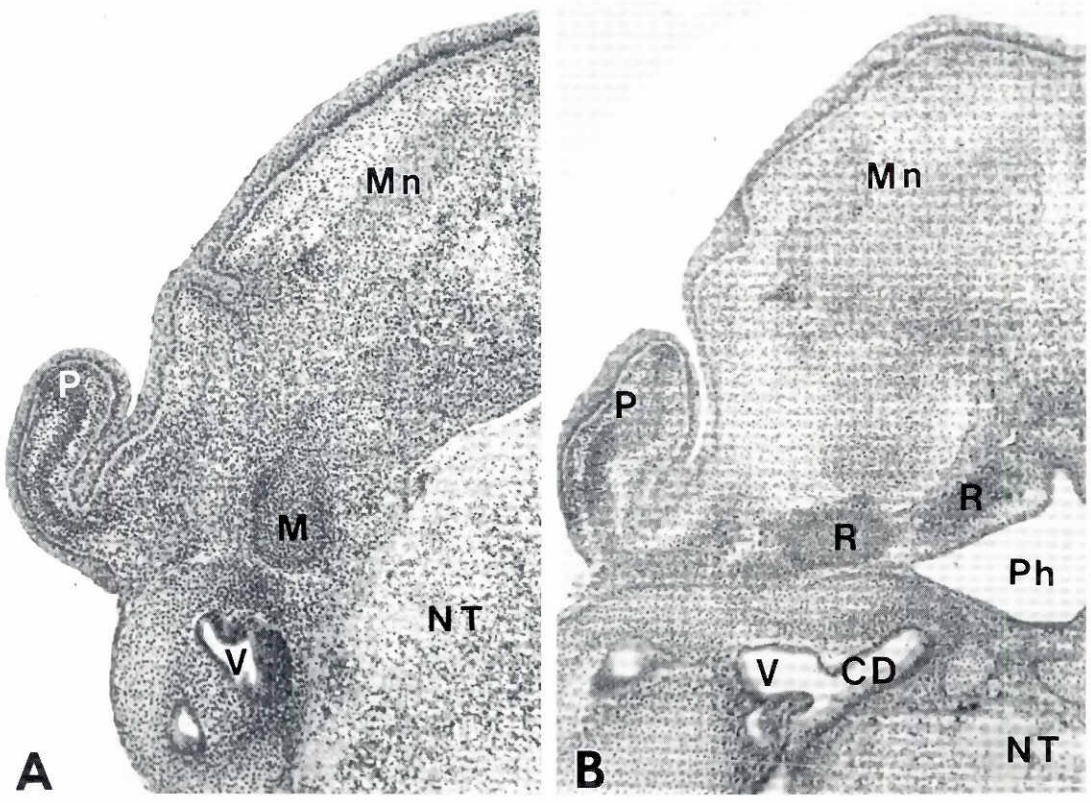

Fig. 8. Correlation of failed cochlear duct development and neural tube overgrowth. **(A)** *Explant maintained for six days in serumless medium. Note medial overgrowth of neural tube nearly obliterating the pharynx; also note lack of cochlear duct. NT, neural tube; V, vestibule; P, pinna; M, Meckel's cartilage; Mn, mandible.* **(B)** *Explant grown six days in serumless medium, no supplements. Note limited growth of neural tube (NT), intact pharynx (Ph), and cochlear duct (CD). R, Reichert's cartilage. Magnification, 90x.*

8. Semicircular canal(s).
9. Medial overgrowth of the neural tube, with or without obliteration of the pharynx. This was noted because of our preliminary observations that neural tube overgrowth correlated with cochlear duct agenesis or hypogenesis.

Whole-mount staining

Upon termination of the experiment, 8 explants from each culture condition were processed for Alcian Blue whole-mount staining to examine the gross morphology of Meckel's cartilage, Reichert's cartilage (when present), and the otic capsule. The details of this method have been previously described (Shum *et al.*, 1993). After completion of whole-mount staining, all specimens were photographed. Specimens were examined individually for the following features:

1. Meckel's cartilage: posterior and anterior elements
2. Reichert's cartilage: medial and lateral elements
3. Otic capsule: gross morphology

Morphometry

For otic capsule and pinna area measurements, hematoxylin and eosin-stained serial sections were examined and traced using a Bausch and Lomb projecting microscope. As a metric to estimate the overall growth of a particular structure, the section demonstrating the greatest cross-sectional area was traced onto a standard weight filter paper and weighed on a Mettler balance. Errors accrued from tracing variation and paper weight variation were negligible relative to intersample variation (data not shown).

Scanning electron microscopy

Explants selected for scanning electron microscopy were fixed in glutaraldehyde/phosphate buffered saline, then dehydrated in acetone/alcohol. Critical point drying was performed using amyl acetate as the miscible solvent. The explants were coated with platinum-palladium alloy and imaged using the Cambridge 360 scanning electron microscope.

Cytotoxicity

Medium removed at each of three changes was analyzed for lactate dehydrogenase (LDH) activity as an assay for gross cell death (Wroblewski and La Due, 1955). LDH activity was measured using the Sigma LD-L 20 kit (Sigma Co.) LDH activity was typically 20 to 30 units/L, and in no case exceeded 40 units/L. At termination of the experiment, one dish from each culture condition was used for the determination of maximal LDH activity. First, medium from these dishes was assayed for LDH activity. Second, four explants were removed from their filters and placed in the medium. Third,

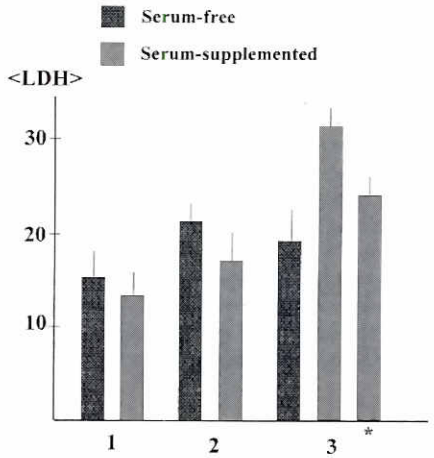

Fig. 9. Gross explant viability as determined from LDH assay. *Mean LDH concentrations (units/litre) at each of three culture medium changes during the 6 day culture period. Error bars represent s.e.m. 3*: LDH activity adjusted for differences in explant mass and/or cellularity, as described in Results.*

the medium and explants were frozen at -20°C, thawed to room temperature, and then lysed by sheering through a 25 gauge needle. Finally, solid material was then pelleted by centrifugation and the supernatant was used for LDH assay.

Data analysis

Observed characteristics of the explants were regarded as dichotomous variables (e.g. presence or absence of a cochlear duct). These were tabulated for each culture condition and the differences were examined for significance using Fisher's exact test, taking the confidence level at $P<0.05$. The Epistat Statistics Program was used for these calculations.

For comparison of serumless and serum-supplemented explants with respect to mean LDH activity, mean maximal pinna cross-sectional area and mean maximal otic capsule cross-sectional area, an unpaired t test was used, taking the confidence level at $P<.05$. The Instat Statistics Program was used for these calculations.

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