APPLICATIONS OF ORGAN CULTURE OF THE MOUSE INNER EAR

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In the present thesis work, otic anlagen from gd 12, 13, 13.5, 15 and 16 were used. As a rule the explants were kept in culture until a time point equivalent to the 21st gd.

Analyses using freeze-fracture technique and transmission electron microscopy showed that in cultured 13th gd otocysts the development of junctional complexes followed the same principal pattern as *in vivo*. Tight junctions develop into many strands lying parallel to the apical surface of all epithelial cells. Uncoupling of the hair cells occurs with loss of gap junctions. Some tight junctions had an aberrant appearence, with in part very thick strands and strands running at right angles to the apical surface.

All aminoglycosides are potentially ototoxic. In the inner ear, outer hair cells of the organ of Corti and vestibular type I hair cells are affected by these antibiotics. The access route to the hair cells and the sites and mechanisms of action of aminoglycosides are not precisely defined.

The uptake of tritiated tobramycin in 16th gd inner ears was studied. An initial rapid uptake of the drug, within 10 min, was followed by a slower accumulation, reaching a steady state after 60 min. Most of the tobramycin was bound reversibly, at least after a short period of incubation (2 h). The irreversibly bound fraction was of the same magnitude as the uptake within 10 min. Uptake took place against a concentration gradient.

The otocyst can differentiate even without the statoacoustic ganglion. The interaction of the sensory epithelium with the ganglion was investigated by explanting the statoacoustic ganglion without target tissue. Twenty-five percent of the ganglions survived and had outgrowth of neurites but there was no differentiation into either the cochlear or vestibular type of neuron cells.

Exposure of cultured otocysts (13 or 13.5 gd) to l-azetidine-2-carboxylic acid, a l-proline analog that disrupts formation of collagen, resulted in retarded morphogenesis of the labyrinth and a dose-dependent derangement of the basal lamina.

The expression of intermediate filaments (IFs) was analysed using monoclonal antibodies. The same IF pattern was found in cultured inner ears as *in vivo*. Explants were taken on 13th, 15th or 16th gd. Exposure to gentamicin, ethacrynic acid or cisplatin did not alter the IF composition. Cytokeratins (CKs) 8 and 18 were identified in all inner ear epithelia. In addition CKs 7 and 19 were visualized in the epithelia involved in maintaining endolymph homeostasis. The ganglion cells showed co-expression of CK, vimentin and neurofilaments.

The elemental composition of the endolymph compartment of 16th gd inner ears cultured for 5 days was studied using energy-dispersive X-ray microanalysis. Na to K ratios characteristic of endolymph were found.

Key words: organ culture, embryonic inner ear, intercellular junctions, aminoglycosides, statoacoustic ganglion, intermediate filaments, collagen, endolymph

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Cover illustration:
Schematic drawing of the 13th gestational day mouse fetus
and the explanted otocyst from that age, to the left,
and the same explant after 8 days in vitro, to the right.
Diana Berggren/May Thörn

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We are like dwarfs seated on the shoulders of giants.
We see more things than the ancients and things more distant;
but this is due neither to the sharpness of our own sight,
nor to the greatness of our own stature,
but because we are raised and carried aloft on that giant mass.

Bernard of Chartres, 12th Century

To Per, Ina, Anna and Viktoria

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REPORTS COMPRISING THIS THESIS

This thesis is based on the following reports which will be referred to by their Roman numerals:

I Berggren D, Bagger-Sjöbäck D and Anniko M. Formation of junctional complexes in otocysts developed *in vitro*. A freeze-fracture study. Acta Otolaryngol (Stockh) 104: 146-152, 1987.

II Anniko M, Berggren D and Holm S. Tissue binding kinetics of tobramycin. An experimental study in the mouse inner ear *in vitro*. Acta Otolaryngol (Stockh) 105: 120-125, 1988.

III Berggren D and Anniko M. Lack of differentiation of the isolated murine statoacoustic ganglion during organ culture. ORL 51: 124-129, 1989.

IV Berggren D, Van De Water TR and Anniko M. Age-dependent disruption of basal lamina and extracellular matrix formation in l-proline analog treated otic explants. (Submitted)

V Berggren D, Anniko M, Thornell L-E, Ramaekers FCS and Virtanen I. Intermediate filament proteins in the embryonic inner ear of mice under normal conditions and after exposure to ototoxic drugs. Acta Otolaryngol (Stockh) 109: 57-65, 1990.

VI Berggren D, Klein E, Wróblewski R and Anniko M. Characteristic ionic composition of endolymph is maintained in cultured inner ear. (In manuscript)

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ABBREVIATIONS

BL	basal lamina
CK	cytokeratin
gd	gestational day
IF	intermediate filament
HC	hair cell
LACA	l-azetidine-2-carboxylic acid
LM	light microscopy
mAbs	monoclonal antibodies
OHC	outer hair cell
NF	neurofilament
NGF	nerve growth factor
PAP	peroxidase-antiperoxidase
PBS	phosphate-buffered saline
RT	room temperature
SAG	statoacoustic ganglion
TEM	transmission electron microscopy

INTRODUCTION

In present-day research there are many systems in which cells, tissues and organ rudiments are cultured *in vitro*. A characteristic of organ culture is that an explanted embryonic anlage will grow and differentiate into a complete organ, or as for the inner ear, to many mature organs. The membranous labyrinth, which is represented by an ovoid sac on the eleventh day of gestation, has all the major components of the adult inner ear when the mouse is born.

Organ culture of the avian otocyst

Studies on the isolated embryonic otocyst in tissue culture began with the pioneering work of Dame Honor Fell in 1928. She utilized the otocyst of 3-day fowl embryos. Dissected otic vesicles were placed upon a clot of plasma and embryo extract, in centrifuge tubes placed in a water-bath kept at 38° C. The explants were transferred to new clots every 48 h. After 14 days *in vitro* the explanted otocysts had all the epithelial constituents of the fully formed inner ear labyrinth, but showed only slight indications of gross anatomical differentiation and tended to retain the primitive vesicular form. The culture technique was improved (Fell & Robison, 1929) by placing clots and explants in a watch-glass laid on a layer of moistened cotton woll at the bottom of a Petri dish. The slightly modified watch-glass technique was then succesfully employed in the study of differentiation of the neuro-epithelial elements of the inner ear (Friedmann, 1959; Friedmann & Bird, 1967) and in the evaluation of various ototoxic agents (Friedmann & Bird, 1961). These studies were extended to the ultrastructural level.

Organ culture of the mammalian inner ear

Maximow (1925) was the first to report an attempt at *in vitro* culture of mammalian otocysts. In a study by Lawrence and Merchant (1953) of the culture of the 9-day rat embryo otocyst on a plasma clot, it was reported that after 8 days in culture there was no formation of semicircular canals and no coiling of the cochlear duct. The cells of the organ of Corti resisted in early stages of development.

The first successful cultures of the mammalian otocyst were achieved in 1971 by Van De Water and Ruben. They chose the mouse as experimental animal because many types of heritable deafness had been identified in the mouse. Many of the hearing disturbances found in different mouse strains can be correlated to congenital hearing impairment in humans. An organ culture technique that was 90% effective was established. It routinely allowed the growth and development of the 12th gestational day (gd) inner ear. The otocyst with associated mesenchyme and neural tissue was explanted into Falcon plastic organ culture dishes containing pre-warmed medium (a balanced salt-solution supplemented with extracts of mouse embryos and fetal calf serum). An ambient air athmosphere of 90% relative humidity proved to be the best system. The objective with this new method was to study genetically induced inner ear disturbances, but the main use of the technique has so far been in ototoxic studies, and in studies on interactions between different parts of the otocyst during normal embryonic development.

Ototoxicity

In 1944 Waksman (Schatz *et al.*, 1944; Waksman *et al.*, 1944) isolated streptomycin, the first aminoglycoside antibiotic. This new antibiotic was almost immediately tried out at the Mayo Clinic by Hinshaw and Feldman (1945), who soon reported its sucessful use in the treatment of tuberculosis. At the same time they made known their wholly unexpected observation of both vestibular and cochlear toxicity in a substantial number of their patients.

Aminoglycoside antibiotics are today universally used in the treatment of severe bacterial infections. All aminoglycosides are potentially both ototoxic and nephrotoxic (Walter & Heilmeyer, 1975). The individual types of aminoglycosides cause different patterns of ototoxicity (Lerner *et al.*, 1981) as

well as of nephrotoxicity (Tulkens & Laurenti, 1984). For example, streptomycin is highly toxic to the vestibular part of the inner ear, but less toxic to the cochlear part and to the kidney. Tobramycin is equally toxic to both parts of the inner ear and the kidney (Anniko, 1985*a*). In the inner ear, aminoglycoside antibiotic primarily affects the outer hair cells of the organ of Corti and the type I hair cells of the vestibular sensory epithelia (Hawkins, 1976). Ototoxic side effects can be largely avoided by adhering to carefully controlled serum concentrations of the antibiotic (Lerner & Matz, 1979), and by the development of new semisynthetic aminoglycosides, e.g. netilmicin which has less ototoxic properties (Wersäll & Björkroth, 1978; Anniko *et al.*, 1982).

But still, the access route to the hair cells, the regional specificity and the sites and mechanisms of action of these molecules are not precisely defined. It has long been discussed whether serum, perilymph, endolymph or, in the cochlea, cortilymph is the preferred route of drug access to the labyrinthine tissues, and pharmacokinetic studies on drug distribution in the inner ear have been focused mainly on drug concentrations in these fluids (Stupp, 1970). There is no active accumulation of drugs in inner ear fluids. Using gentamicin, Tran Ba Huy *et al.* (1981) showed that the drug concentrations never exceed the serum level in either perilymph or endolymph, but aminoglycosides have a slow elimination rate from the perilymph and are retained in the endolymph for weeks after administration has ceased (Tran Ba Huy *et al.*, 1983). This seems to be a general rule and has been shown also for other ototoxic substances (Anniko & Plantin, 1977). When a variety of aminoglycosides were applied directly into the cochlea by perilymphatic perfusion, the resulting toxicity was an expression of the intrinsic properties of the drugs rather than being related to their concentration in inner ear fluids (Lodhi *et al.*, 1980, Tachibana *et al.*, 1983).

Recently research concerning questions of whether the aminoglycosides enter the cells passively along or actively against a concentration gradient has been initiated (Schacht & Van De Water, 1986). Immunomorphological studies have documented two-step sequences in the cellular uptake of aminoglycoside antibiotics (Hayashida *et al.*, 1985). At first the drug attacks receptors on the cell surface, and thereafter the interior of the hair cells. This mechanism resembles the nephrotoxic events that occur in the proximal tubular cells in the kidney, where aminoglycosides are taken up by absorptive pinocytosis and stored in the lysosomes (De Broe *et al.*, 1984). Both clinically (Fee, 1980,) and experimentally (Wersäll & Flock, 1964) it is well known that the initially obtained electrophysiological effect of aminoglycosides is reversible. This also implies a two-step mechanism for the aminoglycoside ototoxicity, or two (or more) different routes for the ototoxic effects. To determine parameters of uptake, the drug concentrations in both the surrounding fluid and the tissue have to be known, and this is hard to accomplish in the inner ear *in vivo*.

The use of organ culture of the late embryonic inner ear, from the 16th gd, for ototoxicity studies has been introduced by Nordemar and Anniko (1983).

Tissue interactions

The differentiation of the otic placode to the otocyst seems to be independent of neighbouring tissues (Lewis, 1907). But the morphogenesis of the inner ear requires a sequential interaction between the different tissues within the otocyst and its surrounding mesenchyme (Anniko & Schacht, 1984; McPhee & Van De Water, 1986).

Neuro-epithelial interaction

The otocyst of the mouse is innervated by fibres of the VIII nerve on the 12th gd (Sher, 1971) and innervation of the sensory areas of the inner ear precedes differentiation of the epithelium (Ruben, 1967). These observations supported the hypothesis that innervation of the sensory epithelia is a prerequisite for differentiation of hair cells. But in 1976 Van De Water showed that differentiation of inner ear sensory structures was not dependent upon neuronal elements. In otocysts developed *in vitro*, explanted with or without their statoacoustic ganglia, there was no difference in between the two groups regarding the differentiation of sensory structures. This was later verified at the ultrastructural level concerning the maculae (Van De Water *et al.*, 1977;) and the cristae ampullares (Anniko *et al.*, 1979).

The developing labyrinthine sensory receptors attract ingrowing neurites by chemotaxis, probably during a limited period of time (Van De Water & Ruben, 1984). Ard

et al. (1985) presented data from *in vitro* studies on chick embryo inner ear tissue showing that neuron-target cell interaction supports neuron survival in the staoacoustic ganglion and that both the central and peripheral targets are involved in trophic interactions with these sensory neurons, but presence of brainstem target tissue had no significant effect on the survival of ganglion neurons when the peripheral target tissue was present. Results in agreement whith those of Ard *et al.* were later obtained in studies of the mouse inner ear in organ culture (Zhou & Van De Water, 1987).

Mesenchymal-epithelial interaction

It is the continued reciprocal interaction between the otocyst and its surrounding periotic mesenchyme that eventually leads to the proper development of both the otic capsule and the inner ear labyrinth, with its sensory structures.

The otocyst induces the surrounding mesenchyme to chondrify. The epithelium of the inner ear anlage has this inducing effect also when the otocyst is transplanted to other regions of embryos of the same species, and in the amphibians otocysts transferred from one species to another induce chondrogenesis of the mesenchyme of the host (Lewis, 1907).

When 12.5 - 13th gd otocysts were explanted without their surrounding mesenchyme, they failed to develop (Anniko & Schacht 1984). But when a medium precultured with mesenchyme was added, a development of the gross morphology took place. The same effect was also achieved when the medium was conditioned with rhombencephalon, fetal forebrain or fetal limb. However, in this later experiment dysmorphogenesis was evident. Although morphogenesis occurred in "stripped" otocysts cultured in mesenchyme-conditioned medium, only very few hair cells were identified compared with the number of hair cells in otocysts explanted with their mesenchyme intact (Anniko, 1985*b*). A soluble and diffusible induction factor is apparently produced by mesenchyme and also by other organs, but a normal tissue relationship seems essential for cytodifferentiation of a normal number of hair cells. The epithelium of the otocyst probably also needs the mechanical support of the surrounding chondrifying mesenchyme.

L-azetidine-2-carboxylic acid (LACA), is a vegetable imino acid that can be incorporated into mammalian proteins instead of l-proline (Fowden & Richmond, 1963). LACA disrupts collagen secretion by cells and prevents normal morphogenesis of *in vitro* developing lung and salivary gland (Spooner & Faubion, 1980). LACA disrupted normal *in vitro* otic morphogenesis in inner ear explanted from 10.5 - 13 gd embryos (Van De Water & Galinovic-Schwartz, 1986). Disrupted collagen secretion alters otic extracellular matrix production, which in turn affects the tissue interactions that regulate the progressive expression of otic morphogenesis and differentiation.

The cytoskeleton

The cytoskeleton of higher eukaryotic cells consists of microfilaments (actin filaments; 4-6 nm in diameter), intermediate filaments (8-10 nm), microtubules (22-25 nm) and various interconnecting proteins (Borisy *et al.*, 1984).

The intermediate filament (IF) composition of a cell may reflect both its embryonic origin and its function. The IF proteins are grouped into five major classes: cytokeratins (CKs) which are identified in epithelia, desmin in muscle, vimentin in cells of mesenchymal origin, glial fibrillary acidic protein in astroglia, and neurofilament (NFs) in neuronal cells (Lazarides, 1982). In human epithelial cells there are at least 19 different CK sub-types, each of which probably has a corresponding group among other mammals (Moll *et al.*, 1982). Cytokeratins are expressed in different combinations, and a given epithelial cell can be characterized by the pattern of its CKs. Intermediate filaments are important for the three-dimensional orientation of the cell, and CKs, vimentin and desmin are closely related to the desmosomes. The large number of IF combinations, and the specific patterns of IF proteins that exist in different cell types, imply that this part of the cytoskeleton may have other functions, so far unknown, besides that of the mechanical support.

Immunohistochemical techniques have been applied in inner ear research for the identification of cytoskeletal components (Ramaekers *et al.*, 1987). Intermediate filaments in the human embryonic inner ear are described by Anniko *et al.* (1987 a) and in the embryonic mouse by Wikström *et al.* (1988).

Actin filaments and microtubules both consist of globular protein subunits that can assemble and disperse rapidly in the cell. In contrast, IFs consist of fibrous protein components which are much more stable. No drugs are known to specifically disrupt the IF network.

The cytoskeleton of cultured inner ear anlagen has not previously been investigated. In cultured cell lines, changes in the expression of IFs can be induced by altering the growth conditions so as to modify cell shape or cell-cell contacts (Ben-Zeev, 1985). When a whole organ such as the inner ear is cultured, these types of disturbances are not likely to occur.

Intercellular junctions

In epithelial cells there are three types of intercellular junction: tight junctions, desmosomes and gap junctions (Geneser, 1985). Tight junctions line the entire luminal surface and act as a permeability barrier to fluids and ions. Macula adherens or desmosomes are of great importance for cell to cell adhesion. Hemi-desmosomes fix the epithelium to the basal lamina. Gap junctions are believed to act as electronic couplers, allowing the passage of ions and/or electrical currents between neighbouring cells. The freeze-fracturing technique has been applied to the study of maturation of intercellular junctions in the developing inner ear of mouse (Anniko & Bagger-Sjöbäck, 1982; Bagger-Sjöbäck & Anniko, 1984). Tight junctions, comprised of a few immature sealing strands, and gap junctions occur on the cell surface of all epithelial cells in the otocyst. During differentiation of hair cells an uncoupling occurs, so that morphologically mature sensory cells have no gap junctions, only tight junctions. At birth the tight junctions of inner ear epithelial cells have five to eight regularly arranged sealing strands.

When 16th gd mouse inner ears are explanted and grown *in vitro* for 5 days, i.e. corresponding to birth, *in vitro* development of intercellular junctions parallels *in vivo* maturation (Bagger-Sjöbäck & Anniko, 1986). On the 16th gd, organogenesis has taken place and most cells of the inner ear have passed their terminal mitoses.

AIMS OF THE INVESTIGATION

Against the described background, the specific aims of the present thesis work were as follows:

To determine to what extent tight and gap junctions occur under *in vitro* conditions, when the inner ear anlage is explanted at the otocyst stage, on the 13th gd, and the explants pass their terminal mitoses and reach a high deegree of morphologic specialization in culture.

To analyse the uptake of an aminoglycoside, radioactively labelled tobramycin, separately in the cochlear and vestibular parts of the developing inner ear.

To investigate the developmental potential of the 12th gd statoacoustic ganglion, when explanted without its peripheral (the otocyst) and central (the brainstem) target tissues.

To ultrastructurally characterize disturbances of otic morphogenesis induced by l-azetidine-2-carboxylic acid (LACA). Interest has been focused on the formation of perilymphatic spaces, the matrix of the otic capsule and the basal lamina.

To compare the expression of intermediate filaments in the inner ear developed *in vivo* and *in vitro*, and to ascertain whether the IF pattern changes when the embryonic inner ear is exposed to ototoxic drugs during morphogenesis and cytodifferentiation.

To characterize the elemental composition of the endolymph in cultured inner ear anlagen.

MATERIAL AND METHODS

Animals

Inner ears from CBA/CBA (paper IV, CBA/C57 Bl-6) mouse fetuses were used. Gestational age was estimated by the vaginal plug technique, considering day 1 as the day when the mucoid plug was oberved. Pregnant mice were killed by rapid cervical dislocation on the desired gestational day. The actual age of the embryos was determined by somite counting, plus external features (Theiler, 1972).

For the *in vivo* controls, fetuses removed from uteri on gd 20, and newborn mice, were quickly decapitated with scissors. The calvarium was opened and the brain tissue removed. The exposed labyrinthine capsule was removed with a fine lancet.

Organ culture technique

Following intra-uterine removal of the embryos, the inner ear anlagen were dissected free in Hanks' balanced salt solution. Dissection was done with sharpened watch-makers forceps under a Zeiss dissecting microscope. Inner ears from the 12th, 13th, 15th and 16th gd have been explanted. Altogether 145 explants were used in this thesis work.

The otocysts were dissected from the lateral external surface. Inner ears from the 16th gd were divided at the level of the ductus reuniens into a cochlear and a vestibular part before explantation. The statoacoustic ganglia of the 12th gd otocyst were dissected free by first removing the whole otocyst with its ganglia and surrounding mesenchyme and then using a sharp lancet to detach the ganglia with some mesenchyme from the wall of the otocyst.

Dissection and *in vitro* culturing were performed under sterile conditions. Explanted otocysts were placed in double-chambered organ culture dishes (Falcon). A prerequisite for the development of the younger explants (12 and 13 gd old) is adherence to the underlying surface. This was achieved by making a scratch in the bottom of the dish on which the explant was placed, and then withdrawing medium until the tension of the water surface held the explant in place. After one day, fibroblasts had grown in the roughness of the scratch and the anlage was firmly anchored. In paper IV the otocysts were placed on 0.45 μm ADP Nucleopore filters. Neuman and Tytell's serumless medium supplemented with 15% fetal calf serum (20% in paper IV) and 1% l-glutamine was used. Incubation was performed at 35 ± 0.2^{o} C in atmospheric air supplemented with 5% CO_2. The nutritient solution was renewed every second day. The explants were kept in culture to an age corresponding to the time of birth *in vivo* (21st gd). In paper II the inner ears were kept *in vitro* only for a short period.

Freeze-fracturing (I)

Nineteen inner ear anlagen from the 13th gd were kept *in vitro* for 8 days and therafter taken for morphology studies.

The specimens were fixed for TEM, with one exception. Osmium tetroxide fixatives are not suitable as they denature the biomembranes in such a way that freeze-cleaving becomes impossible. After fixation with 3% glutaraldehyde in 0.133 M sodium phosphate buffer, the inner ears were transferred to a buffered 30% glycerol solution. The specimens were then mounted on gold holders and rapidly frozen in liquid freon. Freezing in the glycerol solution minimizes the formation of ice crystals, which would be prominent if the specimens were frozen in water alone. While still frozen the inner ears were introduced into the high-vacuum chamber of the Balzers 360 M freeze-fracturing unit. Under high vacuum (approximately $2x10^{-6}$ torr) the specimens were fractured. Still frozen and within the high-vacuum chamber, the surface of the fractured specimens was "etched", i.e. water was briefly evapourated from the surface. From an angle of about 45^{o} to the specimen, a mixture of platinum and carbon was evaporated, covering the specimens with a replica about 2 nm thick. To reinforce this very thin replica a layer of 20 nm carbon was evaporated on top of it. The specimens were subsequently removed from the vacuum

chamber and treated in series of corrossive baths. After the removal of all biologic material, the resultant replicas can be transferred to coppermesh grids and examined under a transmission electron microscope. During the fracturing process the cleavage plane runs preferentially within intra- and inter-cellular membranes, splitting the hydrophobic bonds between the bilayer phospholipids, rather than along one side of the membrane (Branton, 1966). Thus, either the internal half of the membrane (the so-called protoplasmic leaflet or P-face) or the external half of the membrane (the explasmic leaflet or E-face) is exposed. Tight and gap junctions (but not desmosomes) are visualized with this method.

Tissue-binding kinetics of tobramycin (II)

In the first series of experiments, 25 inner ears from the 16th gd, divided into cochlear and vestibular parts, were incubated for varying periods (1 - 180 min) in culturing medium containing 2.34 µg tritium-labelled tobramycin per ml of medium. In the second series of experiments, 8 divided inner ears were incubated for 2 h with tritiated tobramycin (2.34 µg/ml) whereafter the tobramycin-containing medium was replaced by normal medium and organ culture continued for an additional 12 - 72 h. Radioactivity in inner ears, culture media and rinse fluids was analysed with the liquid scintillation technique, which method the specimens are destroyed. In order to correlate uptake of tobramycin to protein content, the protein content of another 21 inner ears was measured ad modum Lowry (Lowry *et al.*, 1951). The values of tobramycin content in vestibular versus cochlear halves of the labyrinth were statistically analysed using Wilcoxon signed rank test.

Organ culture of statoacoustic ganglion (III)

Altogether 36 SAGs were explanted and kept in culture for 4 days; of these, 10 ganglions were cultured for 2 more days and 4 were cultured for altogether 8 days. The whole material was sectioned serially on the light microscopic level (LM). Sections for LM were stained with toluidine blue. Based on these findings, adjacent sections were taken for ultrastructural analysis.

Incubation with LACA (IV)

Otic anlagen from 13- or 13.5-day-old fetuses were exposed to LACA throughout the culture period, at a concentration of either 150 or 300 µg/ml of medium (2 in each subgroup). For control purposes 2 inner ears from each age group were cultured without LACA. The nutritient medium contained l-proline at a concentration of 12 µg/ml. The *in vivo* specimens were collected on the 13th gd (n=4) and on the 21st gd, i.e. newborn animals (n=3). The specimens were sectioned serially and analysed with LM and TEM.

Immunohistochemistry (V)

In vivo specimens were collected on the 20th gd. For organ cultures, inner ears were dissected on the 13th, 15th or 16th gd. The control material comprised 6 specimens from gd 13 and 7 from gd 15. Twenty-six explants were exposed to ototoxic drugs (Table I, paper V).

At the end of culturing, the specimens were snap-frozen in liquid isopentane cooled with liquid nitrogen, and serially sectioned at -30° C. Each inner ear yielded 100 - 300 sections approximately 4 µm thick. Every 5th section was stained with haematoxylin-eosin to facilitate orientation. Based on these findings, adjacent sections were stained for immunomorphology using monoclonal antibodies (mAbs) against neurofilaments, vimentin and the cytokeratins 5, 7, 8, 18 and 19, in terms of the current numbering system for human cytokeratins (Moll *et al.*, 1982) (Table II, paper V). Each section was incubated with one type of mAb using the peroxidase-antiperoxidase (PAP) technique (Fig. 1). The following protocol was used. The sections were incubated with preimmune rabbit serum in PBS for 20 min at room temperature (RT) and then with the different mAbs, at appropriate dilution, for 90 min in RT in a moist chamber. After washing in PBS, rabbit antimouse Ig link antibody was added for 1 h of incubation at RT. Rinsing in PBS then followed and incubation with the mouse PAP complex for 1 h. After washing again the diaminobenzidine substrate plus 0.03% H_2O_2 was added for 10 min. After 5 min wash in water the sections were dehydrated, cleaned and mounted in DPX mountant (BDH Chemicals Ltd, Poole, Dorset, England) for viewing with a Zeiss

Axiophot light photomicroscope including phase contrast.

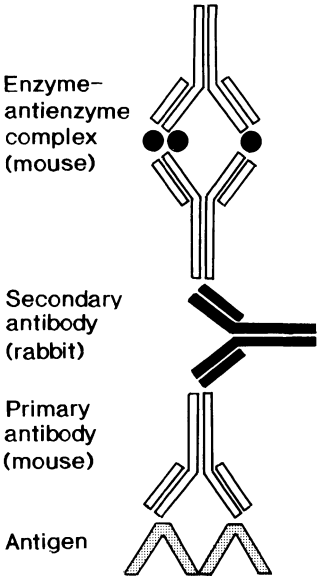

Fig. 1.

Energy-dispersive X-ray microanalysis (VI)

Six inner ears from the 16th gd, divided prior to explantation and cultured for 5 days, were analysed.

The specimens were snap-frozen in liquid propane cooled to -190° C with liquid nitrogen. Until sectioning, the inner ears were stored at -80° C. The whole material was cryosectioned serially with a section thickness of 6 μm. The sections were transferred in the frozen state to special carbon specimen holders with drilled holes covered with carbon coated Formvar film, and freeze dried in the cryostat.

Analytic electron microscopy/electron probe microanalyses were performed with an EDAX 9800 energy dispersive X-ray spectrometer in combination with a Philips 515 scanning electron microscope. The specimens were examined at 20 kV using a take-off angle of 30°. The counting time was 20-300 live seconds. During microscopy a cold trap was used. Simultaneous observations of haematoxylin-eosin-stained adjacent sections faciliated the identification of cell and tissue types.

Analysis of the medium was done after the medium had been dripped onto carbon specimen holders and dried.

Calculations are based on the *P* and *B* values for each element at each measurement. Peak, *P*, is the characteristic intensity and *B* is the background intensity in the same region.

Transmission electron microscopy (I, III, IV)

The replicas obtained by the freeze-fracture technique were examined using a Philips 400 electron microscope (I).

In paper III, fixation was performed in 3% glutaraldehyde in 0.1 M phosphate buffer, and in paper IV in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M Na-cacodylate (Karnovsky, 1965).

After postfixation in osmium tetroxide, and dehydration in increasing concentrations of alcohol, the specimens were embedded in epoxy resin. Ultrathin sections (20-100 nm) were stained with uranyl acetate and lead citrate. Examination was performed with a JEOL 1200 EX electron microscope.

RESULTS

Formation of junctional complexes (I)

After *in vitro* growth for 8 days the explanted 13th gd inner ear anlagen were found to have fully differentiated epithelial cells with regularly arranged stereocilia on the hair cells. The development of tight and gap junctions followed the same pattern as *in vivo*, i.e. tight junctions were developed in many strands apically in all epithelial cells, while gap junctions disappeared from the sensory cells (uncoupling).

There were few gap junctions in the *in vitro* specimens. The tight junctions did not reach the same degree of regularity and maturation as they did *in vivo*. Several tight junctions had an odd appearence, with loss of the normally punctate structure of the strands and areas with considerable thickening.

Tissue-binding kinetics of tobramycin (II)

A rapid binding of tritiated tobramycin occurred within 10 min, in the range of 100 µg tobramycin, per gram protein, in the vestibular half of the labyrinth and about 75 µg in the cochlear half. A steady state in uptake of the drug was reached within 60 min. The uptake reached 390 µg tobramycin, per gram protein, in the vestibular part and 270 µg in the cochlear part of the 16 gd inner ear. A minor fraction of the aminoglycoside, of the same magnitude as initially bound, became irreversibly bound (Fig. 2).

The isolated statoacoustic ganglion (III)

After 4 days in culture, 9 of 36 (25%), of the isolated statoacoustic ganglions (SAGs) had survived. Four explants, which under the operating microscope were considered to be succesfully cultured, were kept *in vitro* for another 4 days. Three of these SAGs showed well preserved morphology on the 8th day. After 4-8 days in culture the explants formed a central bulk surrounded by a thinner peripheral zone. Clusters of ganglion cells bulged out from the surface of the SAG. In the surviving SAGs there were numerous neurites, especially in the outer margin of the explant. The nerve fibres contained neurofilaments and mitochondria.

No differentiation into a cochlear and vestibular part occurred. Mitoses were only sparsely observed. Among the ganglion cells, a rosette-like structure (frequently including mitotic cells) was observed, consisting of numerous nerve cells surrounding nerve fibre outgrowths towards a common center.

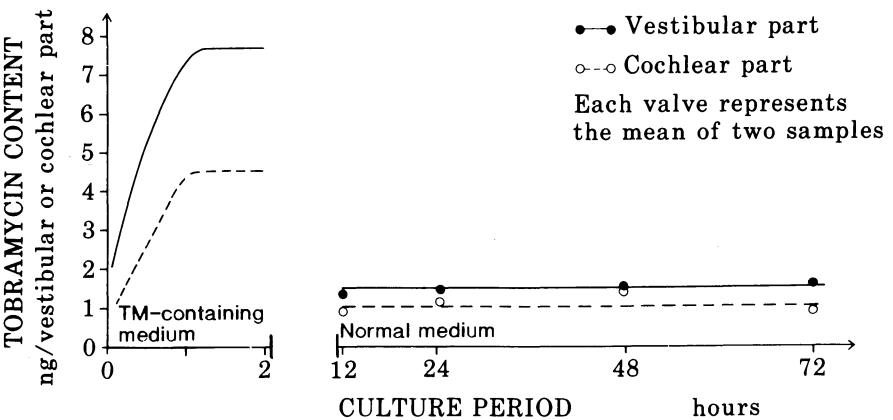

Fig. 2

LACA-exposed otic explants (IV)

When embryonic mouse inner ear explants, taken from 13- or 13.5- gd-old fetuses, were exposed to the proline analoge LACA, this caused dysmorphogenesis, retarded differentiation, a reduced number of collagen fibrils in the perilymphatic spaces and in the otic capsule, and a dose-dependent derangement of the basal lamina (BL). In control specimens, *in vivo* from gd 13 (otocyst) and gd 21 (new born) and in 13- and 13.5- gd explants cultured without LACA, the inner ear epithelium had a dense contiguous BL, and in the older inner ears a well developed network of collagen fibrils beneath it. The electron microscopic appearence of the BL differred between different regions of the inner ear. In the otocyst the BL of the endolymphatic sac was very dense and had a more mature appearence than in the remainder of the otocyst. The BL beneath the epithelum comprising the future cochlear hair cell region had a special structure with a central, thin, electron-dense line, but no distinct inner and outer electron-lucent layers. Also under Kölliker's organ, in the 21 gd specimens, there was only a slight difference in electron density between the layers of the lamina.

When the inner ears were exposed to LACA at a concentration of 150 µg per ml of medium there was a loss of the collagen network and some gaps in the BL. At exposure to 300 µg LACA/ml, scarcely any collagen fibrils were present and the basal lamina was disrupted in many areas, especially beneath the sensory epithelium. In general, the BL of the non-sensory epithelium was more dense and also less affected by LACA than the BL under sensory organs. There was a direct correlation between the embryonic age of explanted inner ears when exposed to LACA and the severity of dysmorphogenesis.

In the LACA-treated explants the numbers of collagen fibres were markedly diminished in the perilymphatic spaces and in the matrix of the capsules, and those explants exposed to the higher concentration of LACA showed hardly any collagen.

Intermediate filaments (V)

A similar principal pattern of IF expression ocurred *in vivo* and *in vitro* developed inner ears as well as in drug-exposed labyrinths.

Cytokeratins

CKs 8 (detected with mAbs RPN 1164 and RPN 1166) and 18 (detected with mAbs 1160 and RGE 53) were identified in all inner ear epithelia. In the maculae and the cristae, the immunostaining of the sensory epithelia was restricted mainly to the basal parts and to the apical surface. In the undifferentiated apical cochlear epithelium, all cell types stained with these mAbs. The innervated and more mature turns of the cochlea showed a selective staining with a positive immunoreaction restricted to the stria vascularis, the cells of Deiter, Hensen and Claudius and to the apical surface of the hair cells. The supporting cells of the vestibular organs, the epithelium of the semicircular canals and the dark cells were consistently positive to CKs 8 and 18 with our set of mAbs, as was the endolymphatic duct and especially the sac, which displayed the most intense immunostaining.

Cytokeratin 7 (mAb RCK 105) and 19 (mAb RPN 1165) were identified in the dark cells, the stria vascularis, Reissner's membrane and the endolymphatic duct and sac. In the stria vascularis the immunostaining was intense, whereas there was no staining in the dark cells of the *in vivo* specimens, and only a weak staining of the dark cells in the *in vitro* cultured inner ears. Parts of the epithelial lining of the semicircular canals showed a slight positivity. The endolymphatic duct and sac showed a strong immunoreaction to these mAbs. The mAb RCK 102 (CKs 5 and 8) had the same distribution as was found for CKs 8 and 18. In additon, an immunostaining of Reissner's membrane (epithelial cell layer), outer sulcus cells and ganglion cells was observed.

Positivity to the mAb RPN 1163 (CK not defined) had the same distribution as for mAb RPN 1165 (CK 19). The mAbs RCK 106 (CK 18) and RPN 1161 (CK not defined) did not stain inner ear epithelium.

A general finding was that well-differentiated epithelial cells had a more distinct and restricted positivity to cytokeratin antibodies than the less mature ones.

Vimentin

Monoclonal antibodies detecting vimentin showed a strong positivity to cartilage and to the mesechymal cells comprising the perilymphatic space. In the cochlear

epithelium, only a few basal cells in the stria vascularis and some cells of the spiral limbus displayed weak positivity to vimentin. A few supporting cells in the vestibular parts of the epithelium lining the endolymphatic space also had an immunoreactivity to vimentin. A distinct immunoreactivity occurred in the vestibular ganglia, whereas the spiral ganglia showed a weaker staining reaction.

Neurofilaments

Most vestibular ganglion cells displayed strong positivity to NF in the cytoplasm, whereas the spiral ganglion cells were mostly weakly stained.

Nerve fibres and nerve chalyces surrounding the vestibular type I HCs were strongly positive. In the inner ears cultured from the 15th or 16th gd and in the *in vivo* preparations, nerve chalyces were regularly stained also in the basal parts of the cochlea.

The ganglion cells showed co-expression of three subclasses of IFs: NF, vimentin and CK. The different types of IF all had a characteristic distribution in the neurons. NF positivity could be observed throughout the cytoplasm, but one pole of the cell was always more intensely stained. Vimentin was found in the mesenchymal cells of the ganglion and in the periphery of the nerve, but there was also a weak staining reaction within the neurons. MAb RCK 102 (detecting CKs 5 and 8) gave a distinct speckled staining of the cytoplasm of all ganglion cells.

Elemental composition of the endolymph (VI)

The elemental composition of the endolymph compartment of 16th gd divided inner ears cultured for 5 days showed Na to K ratios comparable to *in vivo* endolymph of 16th gd fetuses. The elemental spectrum of the endolymph in the cultured labyrinths differed from that of the intracellular environment of the epithelium lining the endolymphatic space, and the Na to K ratio of the endolymph differed significantly from that of the culturing medium.

DISCUSSION

Although the explanted otocyst shows a remarkable development during organ culture, *in vitro* development is not identical with the corresponding growth *in vivo*. During culture, when the labyrinth develops from the otic vesicle, there is an increase in epithelial area, but little growth with regard to mass, i.e. total protein content (Anniko *et al.*, 1981). In contrast, protein content in the inner ear increases three to fourfold during the corresponding period *in vivo*.

Inner ear anlagen explanted early, on the 13th gd, are checked (a temporary shock) *in vitro* by 1/2 - 1 day, compared with *in vivo* conditions, as regards gross development. Full maturation of the gross morphology does not take place *in vitro*, while individual cells develop and differentiate similarily *in vitro* and *in vivo*, concerning both the time of first appearence and morphologic maturation at the time of birth or a corresponding age obtained *in vitro* (Anniko and Wersäll, 1979; own unpublished observations). For exampel, the semicircular canals are shorter and do not have the same exact spatial orientation *in vitro* as *in vivo*, the *in vitro* developed cristae ampullaris contains fewer hair cells, but they are well differentiated, and the cupula develops but often fills out a disproportionate part of the ampullar space.

The 16th gd explants have passed their most dramatic morphogenesis before culturing (Anniko *et al.*, 1978). The only grossly observable changes are lenghtening and increased arcing of the semicircular ducts.

Ototoxicity

The sequences of both the bactericidal and the nephrotoxic actions of the aminoglycoside antibiotics include an energy- dependent uptake into the affected cells (Hancock, 1981; De Broe *et al.*, 1984). Whether an analogous step is also part of the ototoxic mechanism is not known. The first suggestion that tissues of the inner ear may actively transport aminoglycoside antibiotics by a high-affinity uptake system was presented by Schacht and Van De Water (1986). In cultured 16th gd inner ears exposed to gentamicin in concentrations of approximately 0.1 to 17 µg/ml for 1, 3 or 5 days, gentamicin concentrations were up to 16-fold higher in the otic explants than in the culture medium. The uptake of gentamicin into the inner ear was dependent on both time and the drug concentration in the medium.

In our study of tobramycin uptake, explants of 16th gd mouse inner ears were also used. But, prior to explantation we had divided the inner ears into their cochlear and vestibular parts, and analysed the early uptake (up to 2 h of drug exposition) separetely in the two halves of the labyrinth. A rapid binding of tobramycin occurred during the first 10 min, followed by a slower accumulation until a steady state was reached after about 60 min of incubation. In inner ears which were incubated with tobramycin for 2 h followed by continued culture in normal medium, the drug concentration in specimens collected during the subsequent 12-72 h was at a constant level, which was the same as that found after incubation with the drug for the initial 10 min. The binding of the drug to the inner ear was extremely stable and did not decrease during 3 days of culture, irrespective of how many times the culturing medium was renewed.

As we did not weigh the explants, we cannot make an exact calculation of uptake ratios. A rough estimate based on protein content of similar explants and the initial concentrations in the medium, shows that during the first uptake phase, within 10 min, tobramycin concentrations in the otic explants were about half of those in the medium. Under steady-state conditions the tobramycin concentration was higher in inner ears than in medium, with a ratio of 1.5 - 1.7. Some measurements of the tobramycin content in the medium at the end of culturing clearly indicate that the medium surrounding the explants had a lower concentration of tobramycin than had the freshly mixed medium, due to the binding of tobramycin to the plastics used. The implication on the above calculations is that the ratios for uptake are at least the calculated values, and that the ratios are probably much higher. Thus, our experiments imply an active

uptake against a concentration gradient also for tobramycin. The aminoglycoside concentrations in inner ear tissue found in the study by Schacht and Van De Water (up to about 2 µg gentmicin/mg protein) and our results (0.075 to 0.390 ug tobramycin/mg protein) are comparable to those reported for gentamicin in inner ear tissues of the adult rat after *in vivo* treatment with aminoglycosides (Tran Ba Huy *et al.*, 1986)

It is conceivable that the rapid phase (first 10 min) reflects the binding of the drug to specific binding sites on the plasma membrane rather than a cellular internalization. The tobramycin concentration in inner ear tissues above the initial level seems reversibly bound, at least during a short period of exposure.

Throughout our experimental series, tobramycin uptake was significantly higher in the vestibular part of the inner ear than in the cochlea. This could reflect either a greater number of cells with affinity for tobramycin, or the presence of cells with a greater binding capacity for tobramycin than that of cochlear cells.

All hair cells containing tissues possess polyphospho-inositides, and there is a high turnover of these lipids (Tachibana *et al.*, 1983; Anniko & Schacht, 1981). Tissue specificity of aminoglycoside toxicity is correlated to active metabolism of polyphospho-inositides in ear and kidney, in contrast to tissues such as liver and lung, which are less active. It has been suggested that the susceptibility of cells to aminoglycosides is determined by a combination of at least two factors: the presence of an active uptake system and the presence of a physiologically important population of phospho-inositides (Schacht, 1986). Anniko and Schacht (1981) showed that the phospholipid content and turnover are basically the same in cultured embryonic inner ears as in the adult mouse inner ear tissue. The various stages and biochemical mechanisms for aminoglycoside ototoxicity are therefore likely to be the same *in vitro* as *in vivo* for the suggested uptake linked to polyphospho-inositides. In comparative studies it has been shown that the effects of aminoglycosides produce similar patterns of damage in the mouse inner ear in organ culture as in the standard *in vivo* animal model (guinea pig) (Anniko *et al.*, 1982).

Aminoglycosides exert their ototoxic effects *in vivo* and in organ culture, but recently it was demonstrated that when isolated cochlear outer hair cells were exposed to gentamicin, no cytotoxicity resulted (Schacht, 1990). However, gentamicin that had been metabolized by incubation with a subcellular fraction of liver cells showed significantly decreased viability of isolated OHCs. When oxidative metabolism was inhibited, gentamicin was not converted to a toxic product. These results suggest a role for oxidative metabolism in aminoglycoside-induced ototoxicity. But the fact that *in vivo* aminoglycosides are excreted unmetabolized by glomerular filtration contradicts this theory. Another argument against a metabolite beeing the toxic molecule is that the degree of ototoxicity of different aminoglycosides is not proportionate to the drug concentration reached in the perilymph, or to how strong the affinity of different aminoglycosides is to the binding sites, but seems to be related to intrinsic factors of each aminoglycoside molecule per se (Lodhi *et al.*, 1980; Tachibana *et al.*, 1983).

The reversibility of early toxic effects, as well as toxic effects that can develop after drug administration has ceased, indicates that the ototoxic events of aminoglycosides take place in two or more stages, separated by an interval.

Tissue interactions

Neuro-epithelial interactions

Since Hamburger and Levi-Montalcini (1949) showed that sensory neurons of the chick embryo could be eliminated by the removal of their target tissue in the limbs, the possibility that developing neurons may be dependent on interaction with their synaptic targets has been recognized. Such a dependence is the basis of the theory that competition between axons for connections to peripheral synapse formation determines neuronal survival during the period of normal cell death during development (Landmesser & Pilar 1978). Nearly one-quarter of all the neurons in the cochlear and vestibular ganglia in the chick die naturally during the period while the sensory fibres are engaged in differentiation of their synaptic endings (Ard & Morest, 1984). No such investigation has been made concerning the ganglions of the mammalian inner ear.

We can state that only a fraction of early explanted statoacoustic ganglions survive, and that no differentiation takes place

without the presence of target tissue. But we do not know whether the trophic effect exerted by targets on the ganglion depends on a diffusible substance, or whether it requires direct contact betweeen the sensory neurons and the sensory epithelium. The mechanism of trophic effect of target organs on the survival of the developing ganglion and their direct outgrowth in many organs is thought to be regulated by nerve growth factor (NGF) (Hamburger *et al.*, 1981), though the earliest sensory nerve fibres are guided to peripheral targets by so far unidentified attractants other than NGF. (Lumsden & Davies, 1984). Exogenous application of NGF has been shown to prevent the death of ganglion cells during normal development (Hendry & Campbell, 1978). It has also proved possible to prevent some of the naturally occurring cell death by increasing the size of the peripheral tissue (Landmesser & Pilar, 1978).

Formation of synaptic connections to the hair cells of the inner ear in mouse occurs from the 18th gd and continues postnatally (Anniko, 1983*a*; Anniko 1983*b*). Considerable morphologic rebuilding of the cell-nerve ending complex of the hair cells takes place during this time. Established synaptic contacts can be removed and re-established and the initial innervation of type I vestibular hair cells, which resembles that of the type II hair cells, is replaced by the specific mononeural innervation pattern of the type I cells (Favre & Sans, 1979). The early afferent innervation of OHCs is replaced by the predominantly efferent type of mature cell (Pujol, 1985). At birth, synaptic bodies are numerous in the vestibular hair cells, but there are fewer synaptic bodies in the adult stage. There is firm evidence that specificity of peripheral sensory and motor neurons is mediated from their peripheral connections (Jacobsson, 1978), though there is little evidence of absolute specificity in the formation of synaptic connections between different receptor types, since a wide variety of heterologous connections can occur (Privat & Driaw, 1976; Berry *et al.*, 1981).

In our investigation of explanted isolated statoacoustic ganglions, those ganglions that were viable after 4 days in culture were also viable after another 4 days. Thus, whether the explanted ganglion is to survive or not seems to be determined early after explantation on the 12th gd. The cell death of the explanted ganglions did not coincide with the time when synaptogenesis occurs *in vivo*.

Are the neurons surviving in culture a special subgroup of ganglion cells, or are they a cohort of neurons or neuroblasts in one particular stage of maturation at the time of explantation? *In vitro*, the neurons did not differentiate to either the cochlear or vestibular type of cell. Might even a dedifferentiation take place in the ganglion cells during culture? Studies using immunohistochemical methods on cultured isolated ganglion cells may answer some of these questions.

Mesenchymal-epithelial interaction

An important dimension of development is the timing of developmental events. In tissue interactions, during a given period, one tissue is belived to be inducing another tissue. The responding cells are not "undifferentiated", but mostly predifferentiated to some degree. Cells which are predifferentiated would be expected to respond differently to the same inducer molecule, whether that molecule is collagen, nerve growth factor, or some other substance. Such an inducer substance will reach many cells, but will only influence the differentiation of some cells and only during a limited period of time. As for the interaction between the epithelium of the otocyst and its surrounding mesenchyme, it is possible that one substance, secreted from both tissues but at different periods of development, acts as an inducer on both tissues. Collagen, or the precursor of collagen, tropocollagen, could be such a substance. It has been shown that collagens, either alone or in combination with other components of the extracellular matrix, influence both cell growth and differentiation of epithelial tissues (Hay, 1981; Kleinman *et al.*, 1982).

The normal development of the otocyst is dependent on diffusible substances from the surrounding mesenchyme (Anniko & Schacht, 1984), but also needs the mechanical support of the otic capsule. When young inner ear anlagen (12-13 gd) are explanted, *in vitro* growth fails if the explants do not become firmly anchored by fibroblasts to the bottom of the culture dish. The attachment sometimes causes a distortion of the gross morphology of the labyrinth. This might alter the mechanical stress within the epithelia and could be one explanation for the in part very thick tight junctions seen in early explanted inner ear

anlagen. Later, from the 15th gd, when a cartilaginous capsule encloses the membraneous labyrinth, explanted inner ears do develop without beeing attached to the underlying surface. *In vitro* cultured inner ears show the same extent of chondrification as do corresponding *in vivo* specimens, but ossification, which starts *in vivo* on the 17th gd, does not take place *in vitro* (Anniko *et al.*, 1987*b*).

An intact basal lamina is believed to be necessary for normal epithelio-mesenchymal interactions (Saver & Van De Water, 1984) but how this influence is mediated is not known. Early in development when the otic pit has become closed and the otocyst is formed, the basal membrane of the otocyst and that of the rhombencephalon are in contact over an appreciable area (O'Rahilly, 1963). We have observed that during differentiation the basal lamina beneath the sensory epithelium of the cochlea has a structure that differs from other parts of the embryonic inner ear. However, the functional implication of this remains obscure. Since the LACA-induced disturbances of the extracellular matrix and BL occured simultaneously, one cannot ascertain the effect each interference may have on the differentiating otic epithelium.

Intermediate filaments

When cells are extracted with salt solutions or with non-ionic detergents, intermediate filaments remain behind while most of the rest of the cytoskeleton is lost. In fact the term "cytoskeleton" was originally coined to describe these unusually stable and insoluble fibres. Only a part of each IF protein molecule is involved in the formation of filaments. The remaining portion of the molecule varies considerable, and is sometimes a large polypeptide, up to 100,000 daltons (Alberts *et al.*, 1989). In epithelium IFs extend from the nuclear envelope to the cell periphery and are linked from cell to cell at desmosomal junctions and to the basal lamina by hemidesmosomes. This is the basis of the tensile strength of epithelial sheets. But if the function of IFs is merely to resist tension, why are there so many types of subunit proteins? And what is the function of the variable parts of the molecule, which is not involved in formation of the filaments?

The expression of IFs during embryogenesis is very stable. When inner ears were explanted at the otocyst stage and passed both morphogenesis and cytodifferentiation in the presence of an ototoxic environment they still expressed their normal IF pattern. Also mutant mice with progressive degeneration of inner ear sensory epithelium (Anniko *et al.*, 1989*a*) and those with morphogenetic labyrinthine abnormalities (Anniko *et al.*, 1989*b*) show in principle the same pattern of IF as do normal mice. Combinations of CK pairs become more selective and show more distinct positivity to mAbs the higher the degree of specialization the various epithelia reach during embryonic development. In our investigations it was found that at birth, or for the *in vitro* specimens at time corresponding to birth, the hair cells lacked IFs except at their apical surface. At birth, mouse hair cells do not yet have a mature function. In the adult inner ear of humans, no IFs at all are visualized in hair cells (Anniko & Arnold, 1990). The loss of gap junctions and IFs seems to give the hair cells a morphological basis for being electrochemically isolated.

Although the different IFs had a characteristic distribution in the neuron cells it was not possible to correlate these variations to different types of ganglion cells, viz. type I and type II cells (Romand & Romand, 1987; Spoendlin & Schrott, 1988). The weak staining of vimentin and the speckled staining of CK was visualized in all ganglion cells. Some neuron cells showed a very strong positivity to NFs, other cells were only stained at the axon hillock, and spiral neurons stained less intensely than the vestibular neurons. The same co-expression of three different subclasses as found in the ganglion cells of the embryonic mouse inner ear have since been visualized also in the human spiral ganglion (Anniko & Arnold, 1990).

The epithelium involved in maintaining endolymph homeostasis generally expressed a stronger positivity to the CKs 8 and 18 than did the remainder of the inner ear epithelium, and in addition these secretory/reabsorptive cells were positive to CKs 7 and 19. The three different cell layers of the stria vascularis and the delineation of this organ towards the underlying spiral lamina are hard to distinguish in routinely stained light micrographs, and at time just prior to birth the stria vascularis is often described as consisting of only a single epithelial layer (Sher, 1971). By specific staining of the IFs,

the future three layers of the stria vascularis and the border towards the spiral ligament were clearly demarcated. The strongest immunostaining of CKs was found in the endolymphatic sac. This could represent either immaturity, or a high potential to adapt to environmental influences.

Biochemical analysis

By using X-ray microanalysis, the elemental composition of frozen sections of tissues can be analysed even on the subcellular level. This method has made possible studies on the endo- and perilymphatic compartments of the embryonic inner ear, investigations which were difficult to perform with earlier techniques requering withdrawal of the fluids prior to analysis. The unique composition of the endolymph with its high potassium and low sodium content is necessary for the electrophysiological function of the hair cells (Smith *et al.*, 1954). The elemental content of the endolymph is regulated by the stria vascularis in the cochlea and the dark cell epithelium around the vestibular organs (Kimura *et al.*, 1982). The sources, circulation and turnover of inner ear fluids are not fully clarified.

In our study, the 16th gd inner ears were divided prior to explantation. Thus during culture the membraneous labyrinth had one end open to communication with the medium. Still, an endolymphatic type of fluid was maintained in the inner ears after 5 days in culture. It seems unlikely that this could be due to a passive maintenance of the conditions existing at the time of explantation. Rather it seems plausible that the secretory/reabsorptive otic tissues were active and that compartmentalization of the endolymphatic space existed. The tight junctions of the inner ear epithelium show in principle the same pattern *in vitro* as *in vivo*. At partus the tight junctions comprise many strands at the apical surface of the epithelial cells, but not all strands are parallel to the apical surface and there are gaps in individual strands, i.e. the tight junctions seem to be more leaky than in the epithelium of the adult animal (Jahnke, 1975). A fluid of the endolymphatic type within the membraneous labyrinth in cultured inner ears, lacking blood circulation, favours hypothesis (Konishi & Hamrick, 1978) that the source of fluid to the endolymph is the perilymph. Potassium is then excreted and sodium absorbed by the strial and dark cells. Sterkers *et al.* (1982) elucidated the origin of endolymph by kinetic studies on the entry of water and electrolytes into endolymph and perilymph following intravenous administration of radioactive tracers in rats. It was indicated that perilymph rather than plasma was the precursor of endolymph and that the cochlear epithelium was freely permeable to water. In the stria vascularis the concentration of both K and Na is higher in the marginal cells than in the intermediate and basal cells (Anniko *et al.*, 1984). This is consistent with the concept that K is secreted into the endolymph, wheras Na is removed.

While the morphological development of the mammalian inner ear *in vitro* is well documented, biochemical studies have been scarce. The activity of adenylate cyclase develops parallel *in vivo* and *in vitro* until the 19th gd wherafter the specific activity of the enzyme *in vitro* exceeds that of the enzyme *in vivo*, suggesting a lack of control mechanism in organ culture (Anniko *et al.*,1981). Adenylate cyclase is an enzyme which may be important in the regulation of cochlear fluid and electrolyte balance, e.g. in the formation of endolymph. In contrast to adenylate cyclase activity, metabolism of phospholipids shows a similar quantitative relationship *in vivo* and *in vitro* (Anniko & Schacht, 1981).

It is possible to measure energy metabolism in cultured inner ears by using radioactively labeled glucose or deoxyglucose (own unpublished investigation). Deoxyglucose follows the same uptake route into the cells and is phosphorylated in the same way as glucose, but the product deoxyglucose-6-phosphate is essentially trapped in the tissue and not further metabolized (Sokoloff *et al.*, 1977; Canlon & Schacht, 1983). The accumulation rate of deoxyglucose-6-phosphate will be proportional to the utilization of glucose and the absolute rate of utilization can be calculated.

CONCLUSIONS

I The development of tight and gap junctions followed the same pattern *in vitro* as *in vivo*, but there were fewer gap junctions *in vitro* and several strands of the tight junctions showed areas with considerable thickening.

II An active uptake of tobramycin against a concentration gradient took place in the inner ears. The uptake showed an initial rapid phase followed by a slower accumulation.The bulk of the tobramycin uptake was reversibly bound. More tobramycin was accumulated in the vestibular portion of the inner ear anlage than in the cochlea.

III A fourth of the isolated statoacoustic ganglia survived and showed outgrowth of neurites in culture, but no differentiation into cochlear and vestibular parts occurred.

IV Exposure of cultured otocysts to LACA resulted in retarded morphogenensis, loss of collagen and derangement of the basal lamina. The degree of disturbance increased in tact with the dose of LACA and when the otocyst was young at explantation.

V The IF pattern was the same in cultured inner ears as *in vivo*. Exposure to gentamicin, ethacrynic acid or cisplatin did not affect the IF composition. Cytokeratins 8 and 18 were identified in all inner ear epithelia, and in addition CKs 7 and 19 were visualized in the epithelium maintaining endolymphatic homeostasis. The ganglion cells showed co-expression of CK, vimentin and neurofilaments.

VI A characteristic ionic composition of the endolymphatic compartment, comparable to the *in vivo* conditions of the 16th gd, was maintained in cultured inner ears.

GENERAL SUMMARY

The present model of organ culture of the embryonic mouse inner ear has proved highly consistent with the corresponding development *in vivo*, and the method provides a high degree of reproducibility, both when the inner ear anlage is explanted before organogenesis (13th gd) and afterwards (16th gd). The *in vitro* cultured inner ear is very suitable for morphologic studies concerning tissue interactions and ototoxicity. Ototoxic drugs can be studied in well defined concentrations and during exact periods of time. Comparative studies have shown that ototoxic inner ear damage caused by aminoglycosides in adult mammalians is in accordance with the morphological changes found after *in vitro* exposure to aminoglycosides of the 16th gd mouse inner ear.

This model is also well suited to biochemical studies, and studies on enzymatic reactions, metabolism and drug kinetics. So far only a limited number of investigations concerning biochemistry have been performed, and the knowledge of the *in vitro* cultured inner ear in this respect is small. The turnover of phospholipids seems to be essentialy the same *in vitro* as *in vivo*, wherease the adenylate cyclase activity differs. Biochemical studies on the *in vitro* cultured inner ear labyrinth will require careful comparison of the corresponding *in vivo* conditions. If this prerequisit is fullfilled, the model ought to provide just as good possibilities to study the biochemistry of the inner ear as it has provided for morphological studies.

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