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CORRELATION BETWEEN AUDITORY BRAINSTEM RECORDINGS AND MORPHOLOGY AS SEEN THROUGH THE SCANNING ELECTRON MICROSCOPE

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Abstract

Pregnant CBA/CBA mice were exposed to 0.5, 1 and 2 Grey (Gy), (1 Gy = 100 rad) in single doses with whole body gamma-irradiation on the 12th, 13th and 16th gestational days, respectively. The animals were tested at an age of one month for vestibular and cochlear function. Thereafter the inner ears were analyzed with scanning electron microscopy. A morphological analysis with cytocochleograms was performed.

Morphological changes in the vestibular part showed gross malformations in the cristae ampullares. Hair cells of type I seemed to be more severely changed than hair cells type II. The macula utriculi also showed malformations of the otoconia. All these changes were more pronounced when the irradiation was given early during pregnancy and with the highest doses used, except the otoconia which were more injured when irradiated day 16 of gestation. No disturbances of the equilibrium reflexes were noted.

In the cochlea a dose-dependent, time- related damage pattern was demonstrated with pathological changes of outer (OHC) and inner (IHC) hair cells.When tested electrophysiologically for auditory function with auditory brainstem recordings (ABR), elevated thresholds were revealed different in shape depending on when during pregnancy irradiation took place.

A good correlation existed between the morphological changes as seen in the cytocochleograms and the functional changes documented with the ABR.

Key words: Inner ear,morphology embryology, irradiation, hair cells, otoconia, aging, auditory brainstem recording.

Introduction

The normal surface morphology of the vestibular and cochlear parts of the inner ear is well known. With scanning electron microscopy (SEM) the examination is limited to findings in the surface structures only. The most difficult areas to evaluate are the extracellular specialized structures like the tectorial membrane and the cupula of the crista ampullaris which are easily destroyed by preparation for morphological examination such as fixation, etc.(31). The surface morphology of the hair cells (HC), however, is usually well preserved by standard methods.

The development of the inner ear of the mouse has been thoroughly described. The organogenesis and cytodifferentiation takes place on the 12th - 13th and 16th gestational days, respectively (2,35, 43,45). The cells are, during these periods, more often in an active cell cycle and are therefore considered to be more vulnerable to irradiation.

The influence of radiation given to rodents during embryonic and fetal life was first documented to induce organ malformations by Job et al, in 1935 (32) and critical periods in embryonic life for each tissue type was described by Russel in 1950 (44). Hicks confirmed these findings in 1953 (22) and produced a timetable for the mouse, that has a 21 day long gestational period. During the first week of pregnancy, the preimplantation period, the embryo is quite radioresistant. High doses can, however, be lethal. Various organs start to form at the end of the first week and day 9-15 represent the organogenetic period when irradiation has its greatest effect in producing congenital malformations.

The inner ear develops at days 10-13 gestational age. Inner ear structures and function may show different changes depending on when irradiation was given (25).

Exposure to irradiation later during the fetal (cytodifferentiational) period leads to few abnormalities.

Clinical aspects of irradiation and hearing are still under debate. Knowledge is still scarce concerning the effect on human embryos because we are limited to studies of persons exposed to nuclear catastrophes and accidental X-ray irradiation during pregnancy. The dangerous consequences of irradiation have been studied following mass exposure after the Hiroshima and Nagasaki nuclear bombs. Reports are, however, lacking concerning the effects on hearing and balance of these victims. A close relationship is documented between the incidence of malformations, gestational age and distance to the hypocenter of the bomb (39).

Clinical irradiation treatment of cancer and its consequences in the adult inner ear are still confusing. Many reports, however, do not show any alteration of hearing (16,17), while others do (33,40,49). Bohne (14) showed delayed irradiation effects in chinchillas, which increased after increasing doses of irradiation. These findings are difficult to transfer to man since LD_{50}-doses indicate that man is more radiosensitive than most mammalian species tested.

Low-dose gamma-irradiation has recently been shown to induce malformation in the gross shape of the crista ampullaris (27). Gamma irradiation is also known to alter the otoconia in the vestibular organs, creating large conglomerates (29).

Cochlear HC damage leading to alterations in hearing thresholds has been described (30). Both inner and outer HC show extensive damage with formation of giant hairs and/or fusion of stereocilia. The sensory hairs seem to be the target of primary damage and malformations also in other ototraumatic lesions, e.g., after exposure to ototoxic drugs and in genetic inner ear diseases.

Purely age related changes in the inner ear HC have been described: The number of HC appears commonly reduced and the cuticular plate is affected (15,18). So far no comprehensive descriptions exist on how an already damaged HC reacts to normal aging, and if so, if irradiation acts as a sensitizer.

The SEM-technique has been used in the present study to analyze the total damage to the adult and aging inner ear following prenatal gamma-irradiation during the embryonic inner ear development.

Material and Methods

Material

The material consisted of 75 CBA/CBA mice from which 27 cristae ampullares, 19 maculae utriculi and 40 cochleas were investigated. Another 30 animals were allowed to reach an age of between 24 and 54 months (which is considered to be a high age for mice) before sacrifice. The normal non-irradiated material consisted of 30 animals, 10 in each group (the vestibular part, the cochlear part and in the group of old animals).

Methods

Pregnant mice were kept in a small box when exposed to whole body single doses of gamma-irradiation using a Siemens Gammatron 3, emitting Co gamma rays. The irradiation field area was 15 x 15 cm at a distance of 100 cm. The fetuses were irradiated in utero with single doses of 0.5, 1 and 2 Gy. The irradiation took place on the 12th, 13th and 16th gestational days respectively, i.e., prior to the organogenesis and when the cytodifferentiation process is in progress (3,10).

The gestational age was determined by the vaginal plug technique and the litters were born at normal time, i.e., gestational day 21. At the age of one month the animals were sacrificed and the inner ears were processed for SEM analysis, after clinical testing of the vestibular function and determination of thresholds by the auditory brainstem response (ABR).

The vestibular function was tested according to the method described by Lyon (36) with 1) Air righting reflex, tested by high jump to assess the ability of the animal to right itself in the air and land on its feet 2) Position change reflex, tested by holding the animal by the tail looking for a normal response with raised head, flexed spine and extended and protracted forelimbs 3) Linear acceleration, tested when the animal was held by the tail and suddenly lowered. Protraction and extension of the forelimbs are the normal response 4) Posture tested by swimming and for the ability to orientate in water 5) Observations of disturbed balance like head tilting and circling behaviour in the cages.

The cochlear part was tested at the age of one month, with the ABR technique using a band pass filtered single full cycle sine wave at a rate of 30 cycles/s. Eight different frequencies ranging from 2 - 31.5 kHz were after calibration, delivered into the ear by a TDH 39 telephone supplied with a funnel

and a 10 cm long rubber tube fitted into the ear canal by rubber plaster. The signals were picked up by 3 subcutaneous needle electrodes, filtered 50-4700 Hz, amplified 10,000 times and fed into an averager (Datalab 4000). 2048 responses were stored and presented on a storage oscilloscope, where response latencies could be measured.

Hearing loss was calculated with the normal material as reference. Only one ear was tested, and the eardrum was inspected before and after the test. The thresholds were defined as the lowest value at 5 dB attenuator step giving reproducible responses (Fig. 1).

After sacrifice of the animals by spinal dislocation, the temporal bone from one side was removed and the bulla tympanica opened to reveal the inner ear structures (8). The specimens were fixed in 3% glutaraldehyde, dissected in 70% alcohol and thereafter dehydrated in alcohol by the critical point method (Baltzer´s critical point dryer 010) for SEM. The inner ear organs were attached to the specimen holder by silver glue and sputtercoated with gold according to standard methods. A Philips 505 scanning electron microscope was used for the morphological evaluation.

Animals allowed to reach a senile age, were once again tested with ABR before being sacrificed.

Cytocochleograms were performed counting a row of hair cells in different levels of the cochlea and classifying them into a four graded scale:

- ○ Normal hair cell.
- ⊕ Mild damage: Loss of stiffness of sensory hairs, or more than approximately fifty per cent normal sensory hairs.
- ⊙ Pronounced damage: Fusion of stereocilia, giant cilia or less than approximately fifty per cent normal sensory hairs.
- ● Missing hair cell.

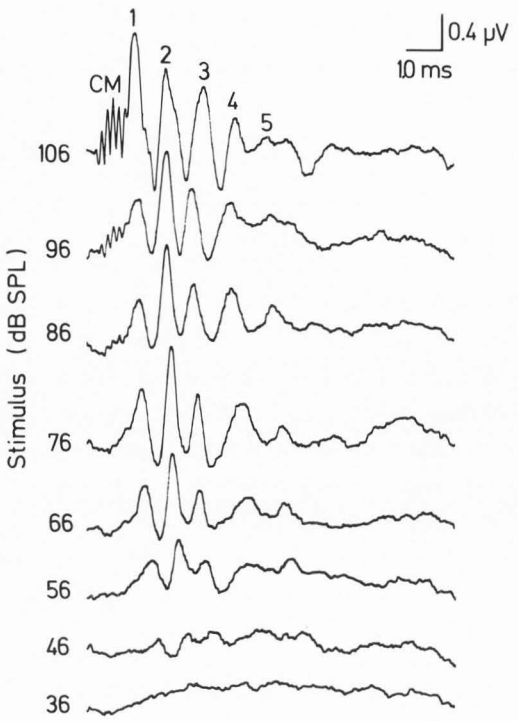


Fig. 1. Auditory brainstem response to a 6.3 kHz 1/3 octave sine wave in a normal unirradiated mouse. The threshold is at 46 dB SPL (peak-to-peak equivalent level before filtering), below which no reproducible wave can be traced.

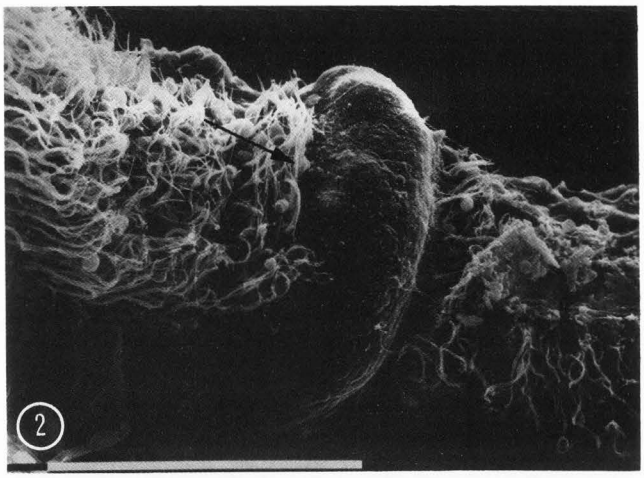
Results

Vestibular organs

No disturbance of equilibrium reflexes were noted in any irradiated or control animal. A normal position righting reflex and air righting reflex were thus found. In the tests for swimming, a normal orientation ability was registered. Also, the behaviour in the cages seemed to be completely normal. In the irradiated animals the gross anatomy of the vestibular end organs showed malformations of either the entire crista or of individual regions of the crista (Figs. 2,3). The gross appearance of the crista was uneven and it was covered by HC and supporting cells as in the normal case, but it also showed irregularities with wider and narrower parts intermingled with normal areas. Abnormal structures seemed to be superimposed on the summit of the crista or on the sides of the crista (Fig. 4). Deformation of the septum cruciatum with ballooning herniations was a common feature.

The most common effect of irradiation was gross derangement of the sensory hair cells. The HC covering the cristae were sometimes totally or in part missing. In other parts of the vestibular end organ, the HC showed bulging of the cuticular plate, extensive fusion of stereocilia and formation of giant hairs (Figs. 5,6). Centrally located HC type I appeared most vulnerable but damaged HC could be found all over the surface.

The gross morphology of the macula utriculi remained unaltered, but individual HC showed similar types of morphological changes after irradiation as seen in the cristae ampullares. The changes were present all over the surface but more pronounced in the striolar area (Fig. 7).



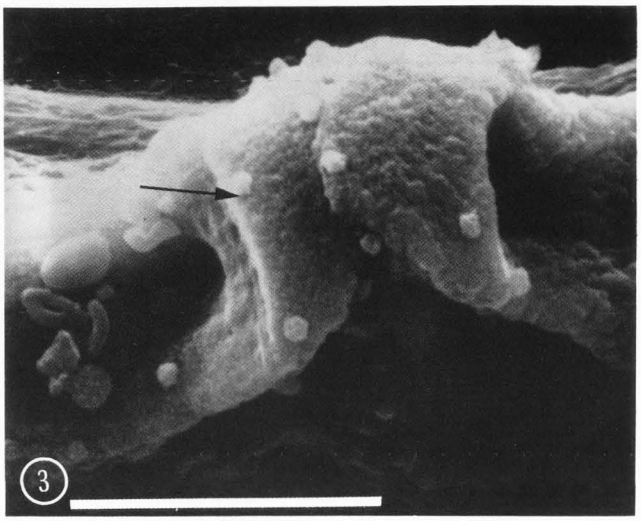


Fig. 2. Scanning electron microscopy (SEM). A normal shaped crista ampullaris from a non-irradiated mouse. Arrow pointing at the septum cruciatum. Bar= 100 µm.

Fig. 3. SEM. Crista ampullaris irradiated in utero on the 13th gestational day with 2 Gy showing an in toto malformed shape. The anatomical location for the septum cruciatum is indicated with an arrow. Bar= 100 µm.

The otoconia overlying the macula utriculi and sacculi revealed bizarre configurations. The outer contour was uneven and the normal hexagonal shape was gone, leaving an irregular surface and defect shape. Extensive fusion between adjacent otoconia forming big conglomerates were common compared to the non-irradiated animals (Figs. 8,9). Irradiation with the highest doses on the 16th gestational day injured more than 50% of the total amount of otoconia and 30 to 50% when exposed day 12 or 13 of gestation as compared with the non exposed animals.

The irradiation damage to the vestibular end organs was more pronounced when irradiation was given on gestational days 12 and 13 than on gestational day 16 and as could be expected after the higher dose of 2 Gy as compared with 1 and 0.5 Gy. The qualitative appearance of the morphological changes was, however, comparable throughout the entire material.

These findings were confirmed by the LM and TEM analyses (5,27,30). TEM showed also remaining fetal forms of otoconia in the adult animal together with disarrayed internal arrangement of the otoconial matrix (29).

Cochlea

Testing for auditory function revealed elevated thresholds more pronounced in the groups exposed to the highest doses of irradation on each day tested. The shape of the threshold curves tended to differ depending on when, during gestation, the exposure took place. Animals exposed on gestational day 12 showed a flat hearing loss curve and so did animals irradiated on the 16th gestational day, but less

Fig. 4. SEM. Crista ampullaris irradiated on the 16th gestational day with 2 Gy. On the side located against the dark cell region an irregular outgrowth can be distinguished (star). Bar= 100 µm.

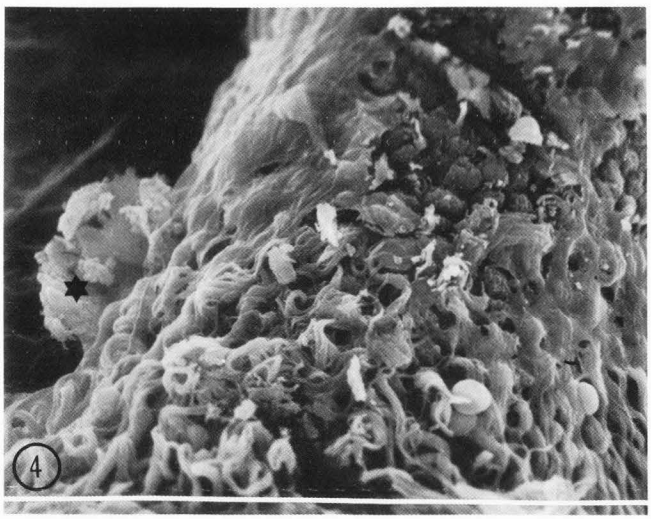
Fig. 5. SEM. Close up view from a non-irradiated mouse crista ampullaris showing normal sensory hair-bundles (star). Bar= 10 µm.

Fig. 6. SEM. Fusing of sensory hair bundles (arrowheads) and bulging of the cuticula on a crista ampullaris irradiated in utero with 2 Gy on the 16th gestational day. Bar= 10 µm.

Fig. 7. SEM. Macula utriculi after having been irradiated on the 16th gestational day with 1 Gy showing missing hair bundles (asterisk) and hair bundles in fusion (arrow). Bar= 10 µm.

Fig. 8. SEM. The otoconial layer of adult mouse, following prenatal irradiation of 1 Gy on the 16th gestational day, showing minor changes with defect shape of otoconia. Bar= 10 µm.

Fig. 9. SEM. Irregular shaped otoconia in fusion, forming a big conglomerate (star). This feature was found all over the macula and very frequent compared to controls. Adult mouse irradiated with 2 Gy on the 16th gestational day. Bar= 10 µm.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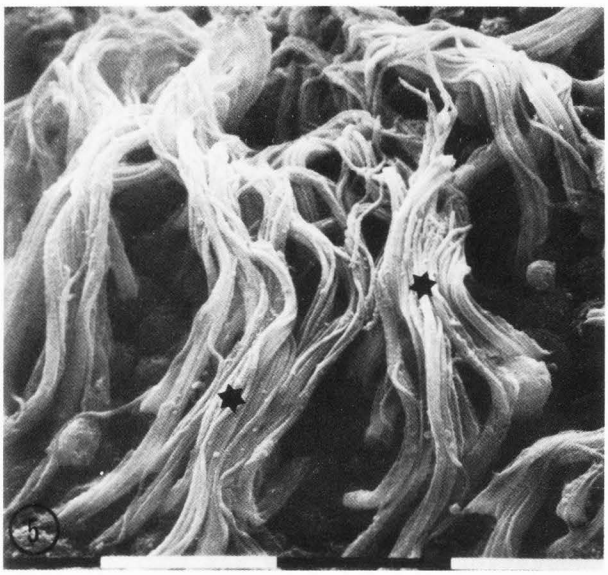
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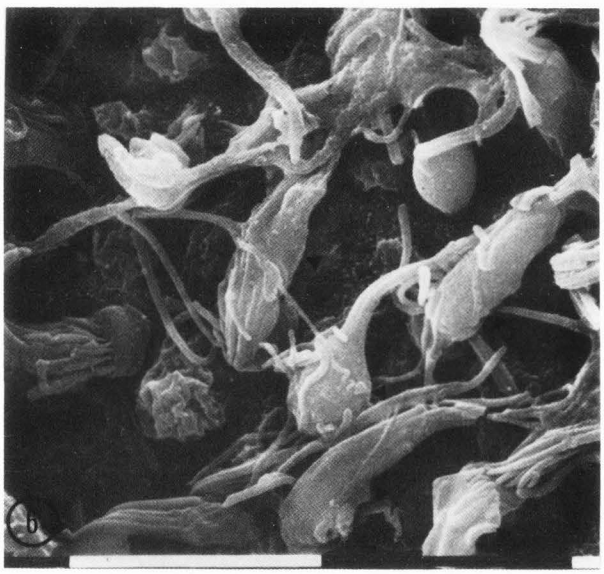
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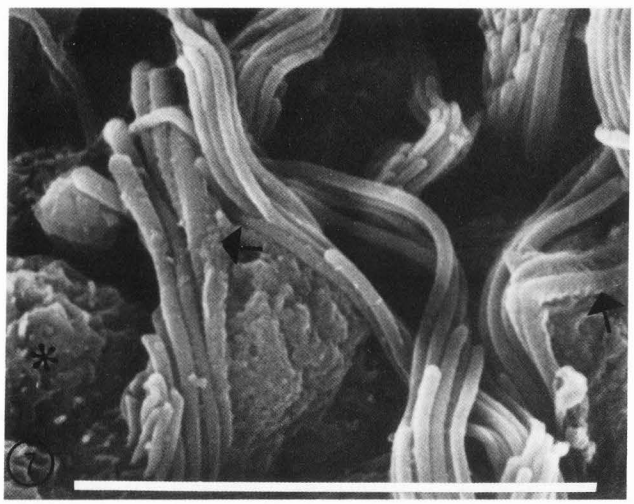
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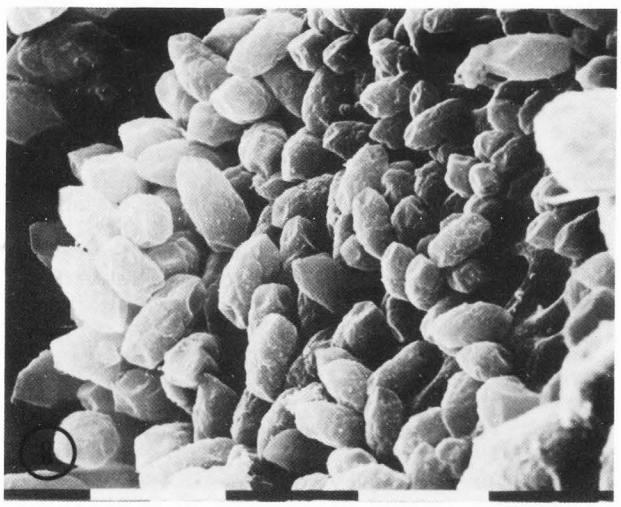
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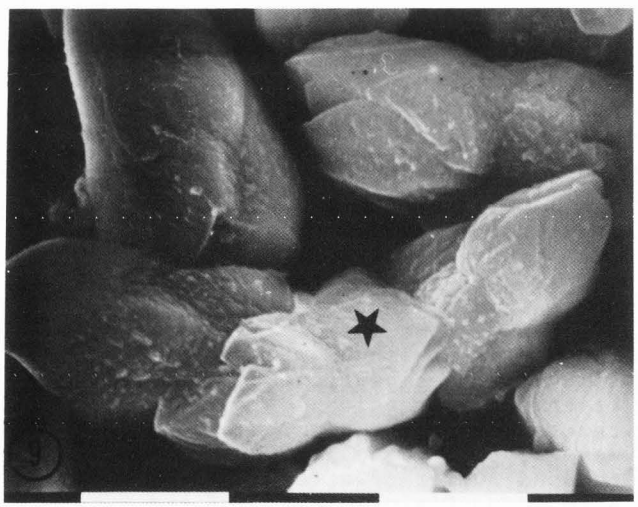
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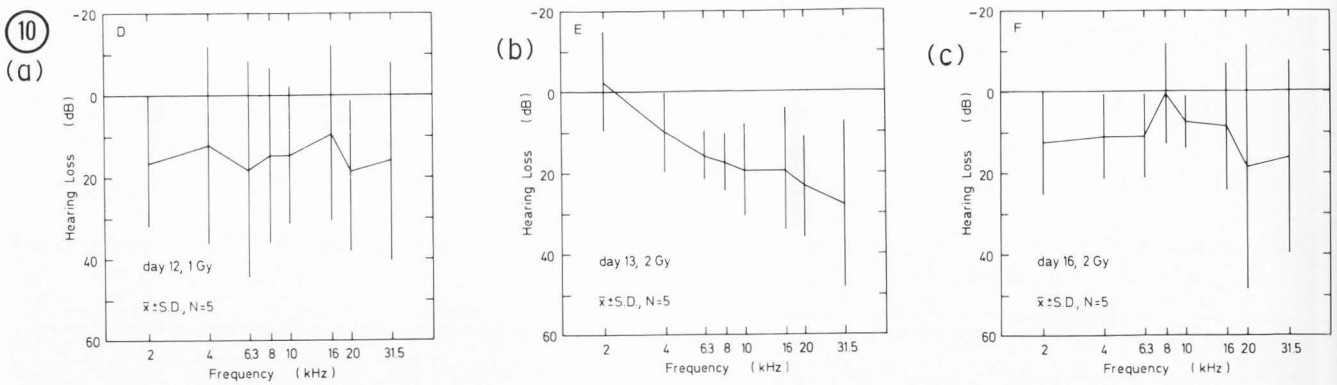


Fig. 10. Diagrams showing the summarized audiograms in the different gestational groups after irradiation with the highest tolerable dose ± SD. The hearing loss following irradiation has a different appearance depending on which day of gestation the exposure was performed. Animals exposed day 12 of gestation (a) showed a flat hearing loss curve, as did the animals exposed on the 16th gestational day(c) though less pronounced. Animals exposed on the 13th gestational day(b) had a decreasing threshold from the lower frequencies where no hearing loss could be traced, but with an inclination towards the higher frequencies where a loss of about 40 dB was seen. Here a dose-response pattern was evident. The different shapes of the threshold curves probably reflect the sequential developmental pattern of the cochlea.

pronounced. The group of animals irradiated with 2 Gy on the 13th gestational day had a sloping curve towards the higher frequencies (Fig. 10).

Irradiation with 2 Gy produced more extensive pathological changes in the stereociliary hair bundles than lower doses. An obvious dose-response relationship was noted. There were no considerable qualitative or quantitative differences in stereociliary pathology between outer (OHC) and inner (IHC) hair cells at the same level in the cochlea except that giant hairs occurred only in the IHC. In the OHC fused or missing cilia were frequent (Figs. 11,12). The stereocilia showed varying degrees of injury such as loss of rigidity, abnormal shape of individual stereocilia, different stages of fusion or partial total loss of sensory hairs. The cuticular plate showed similar alterations as in the vestibular HC with disintegration, poor delineation and sometimes protrusion. Occasionally, scarring of the tissue was found. These findings were revealed also in TEM where a reduced number of afferent nerve endings were seen. Disintegration of the cuticular plate and poorly developed hair rootlets were found. In the supporting cells disarrayed microtubules were a common feature.

The morphological HC changes were correlated to the functional changes documented in the cytocochleograms. A dip or elevated hearing threshold in the threshold curve could be correlated to more serious altered sensory hair changes or even totally missing hairs at corresponding levels of the basilar membrane (Fig. 13).

Aging animals

According to calculated threshold curves, extremely elevated thresholds were obtained in all animals that had been irradiated. No hearing at all could be detected in the lowest and highest frequencies tested in any irradiated animal and only minimal hearing could be detected at and around 10 kHz, which is the frequency of optimal hearing in the young CBA mouse. The non-irradiated animals also showed elevated hearing thresholds but the calculated mean value never exceeded 27 dB, and in almost all animals thresholds could be detected in the highest and lowest frequencies tested (Fig. 14). When adding the mean value of the non irradiated old animals to any mean value from the irradiated-groups of young animals there seems to be a clear difference with poorer hearing compared to the mean value of the old irradiated animals.

Compared to non-irradiated animals where signs of degeneration were found, the aged in utero irradiated mice showed far more extensive inner ear pathology. In the exposed groups, besides the irradiation injury, HC were often devoid of stereocilia in large areas of the cochlea. This ciliary deficiency was more pronounced in the OHC. The IHC also showed the same pattern but were not missing to the same extent as the OHC. The remaining HC had only a few cilia that seemed to have lost their rigidity pointing in all directions. Some of the completely nude areas showed scarring.

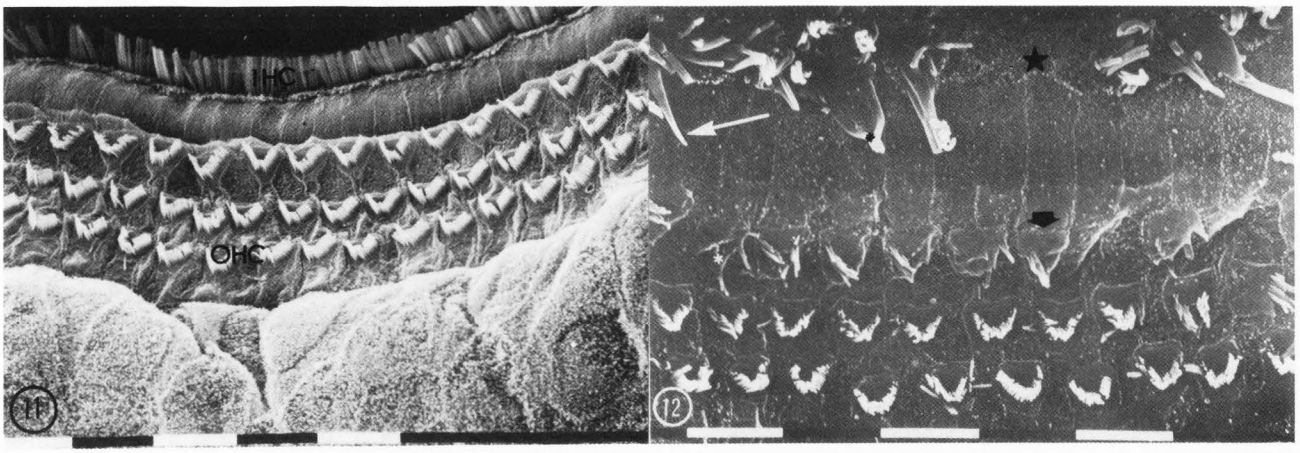


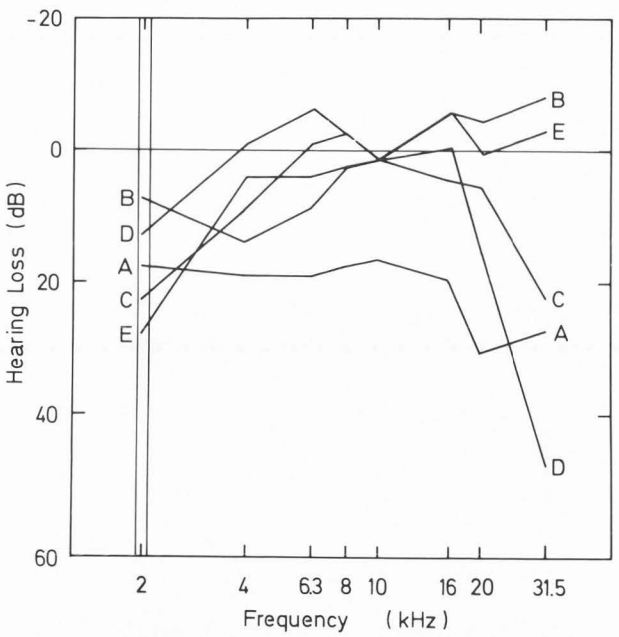
Fig. 11. SEM. Cochlea irradiated in utero on the 16th gestational day with 1 Gy. An almost normal visualization of IHC and OHC is seen, with proper stiffness and normal appearance of the HC. Bar= 10 µm.

Fig. 12. SEM. Cochlea from an adult mouse irradiated in utero on the 16th gestational day with 2 Gy. Giant hair (white arrow), fusion beginning at the top of the sensory hairs (asterisk) and totally missing HC (star) are seen among the IHC. The OHC show bulging of the cuticular plate (black arrow) and clumping of the sensory hairs (white asterisk). Bar= 10 µm.

(13)

		N	○ %	⊕ %	⊙ %	● %
A	IHC	99	2.1	80.9	17.0	-
	OHC I	115	1.7	5.2	91.3	1.7
	OHC II	114	3.5	29.8	63.2	3.5
	OHC III	115	-	26.9	68.6	4.5
B	IHC	53	-	-	100	-
	OHC I	63	98.4	1.6	-	-
	OHC II	63	100	-	-	-
	OHC III	63	85.7	9.5	1.6	3.2
C	IHC	52	-	100	-	-
	OHC I	64	14.1	35.9	50.0	-
	OHC II	62	11.3	35.5	53.2	-
	OHC III	65	-	29.2	63.1	7.7
D	IHC	35	2.8	91.4	-	5.8
	OHC I	42	59.5	28.6	11.9	-
	OHC II	42	61.9	38.1	-	-
	OHC III	42	69.0	7.2	21.5	2.3
E	IHC	78	93.6	5.1	1.3	-
	OHC I	95	47.4	28.4	22.1	2.1
	OHC II	94	43.6	34.0	17.0	5.4
	OHC III	95	53.7	25.3	10.5	10.5

Fig. 13. Cytocochleogram from mice analyzed 1/4 turn from apex, corresponding to the 2 kHz frequency region, A, B and C from animals irradiated on the 16th gestational day with 2 Gy. D and E from animals irradiated on the 16th gestational day with 1 Gy. The individual audiograms are shown to the right. HC classified according to a four graded scale of increasing damage. ○ Normal hair cell, ⊕ Mild damage, ⊙ Pronounced damage, ● Missing hair cell. (See methods).



Unusual configurations of the pillar cells were a common finding. Signs of hypoxia with small protrusions could also be seen (Fig. 15).The basal and apical turns of the cochlea were most affected but injury was also found in the middle turn. These findings seem to correlate well with the threshold curves with the most pronounced hearing loss in the lower and higher frequencies.

Within the group of animals irradiated in utero, that were allowed to age, the dose of irradiation did not appear to be relevant.

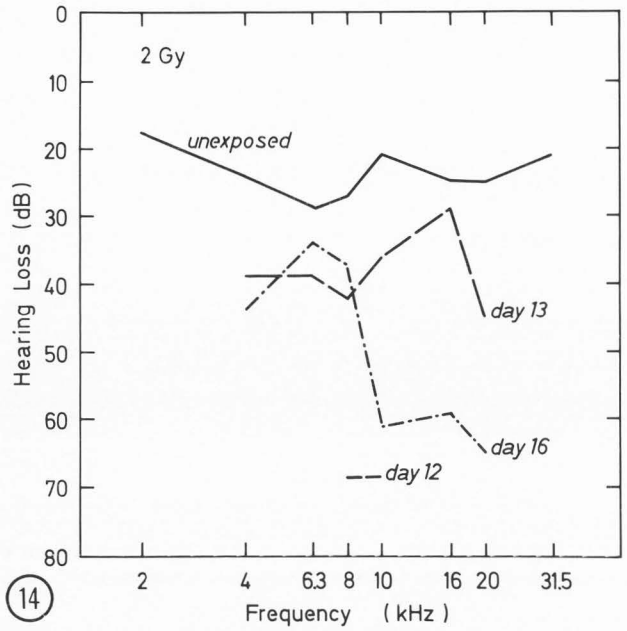


Fig. 14. Representative individual audiograms from animals exposed at 12, 13 and 16 days of gestation tested 2 1/2 years following the birth. Animals exposed early during inner ear organogenesis, i.e., day 12 of gestation, show the most pronounced hearing loss with no hearing in the higher or lower frequencies compared to the non-irradiated aged control.

Fig. 15. SEM. Aging animal (2.5 years) irradiated in utero with 0.5 Gy on the 12th gestational day from the upper middle turn of the cochlea. The IHC shows fusion (arrow) and many OHC are missing (asterisk). Remaining OHC shows bulging of the cuticular plate (unfilled arrow). Small protrusions of the basilar membrane might indicate hypoxia (white arrow). Bar= 10 µm.

Discussion

Irradiation effects on developing fetuses have been known for more than 60 years (32). Hicks and Russel (22,44) showed about thirty years ago that irradiation in higher doses can alter developing organs and result in malformations at a dose-dependent rate. Irradiation was considered to be one of the most potent agents causing malformations.

The morphological development of the mouse ear is well known (3,9,35,45, 47). The organogenesis of individual inner ear end organs occurs on the 13th-14th gestational days and shortly thereafter. The HC pass their phase of terminal mitosis which starts day 14 of gestation and lasts over a five day period and restricts the size of the cell population. The vestibular part precedes the cochlear one.

The cytodifferentiation occurs in a direction from the HC surface to its base. Maturation of the HC surface starts as a regulatory arrangement of microvilli that subsequently are transformed into stereocilia. This period is followed by a polarization of the stereocilia and ends with the formation of the cuticular plate. The formation of the general features of HC takes only 1-2 days (15-16th day of gestation in the vestibular part and around day 18 in the cochlea) but the maturation of the sensory hairs is a long process which proceeds postnatally.

In the vestibular end organs most sensory hairs were mature at the 20th gestational day and all immature sensory hairs had disappeared 5 days after birth. Maturation takes place starting from the striolar area in the utricle and in the crista ampullaris from the top, toward the periphery. The maturation of the innervation mainly takes place after birth (2).

The cochlea in the mouse matures postnatally and resembles, at birth, that of a 15 week old human fetus. The cytological differentiation of the developing organ of Corti proceeds from the base towards the apex. By the end of day 13 of gestation the coiling starts and on the 16th day comprises one and one half turns. On the 17th day of

gestation IHC and OHC are identified. At birth most of the HC have reached a mature state and nerve endings are identified. Action potentials first appear on the 10th to 12th day postnatally emphasized to correspond to the maturation of OHC (38). Maturation of the efferent innervation is the last event to occur before the organ of Corti starts to function and can be detected day 10 postnatally. ABR responses were observed in the frequency range of greatest sensitivity in the adult mouse at 10-15 kHz (42).

In general, cells are most radiosensitive during development due to their rapid proliferation exposing their genetic material to the irradiation injury which can change normal development and cause malformations. According to general radiobiology, the more differentiated the cells are, the less sensitive are they to irradiation injury. It is known that a developing neuroblast tolerates only 0.4 Gy, while the embryonic ectoderm tolerates 4 Gy and the adult neurons 400 Gy (34).

When exposed at day 16 of gestation a flat loss curve in the threshold curve was obtained but not as extensive as in animals exposed at day 12. The HC have then passed their terminal mitosis and reached a higher degree of maturation.

During irradiation all stages of developing hair cells were exposed at all anatomical sites in the cochlea. The present study shows that the HC damage is found in all turns although normal cells are also present. Flat loss threshold curves are obtained from the groups of animals irradiated on the 12th and 16th gestational days with the highest doses used. On the other hand animals irradiated day 13 of gestation show an increasing threshold loss towards the higher frequencies, which are located in the basal region of the cochlea. At this age the cochlea is just about to start its development with an outpouching from the otocyst. ABR measurements indicate that at this stage the basal part was most vulnerable. According to Ruben (43) the cochlear development proceeds from the base. The apically located cells pass their terminal mitosis first but are the last ones to complete cytodifferentiation. This could indicate that a longer time passes between time for exposure and onset of cytodifferentiation in the apical part, which consequently has the longest time to repair irradiation induced damage.

The vestibular part which develops earlier shows after irradiation more pronounced damage with sometimes malformation of the whole end organ. Despite these serious changes, no functional loss could be demonstrated in the vestibular part, possibly due to the existence of compensatory mechanisms.

According to the present study, development of inner ear HC also occurs after exposure to gamma irradiation. Audition, though altered, has developed and mature cristae and maculae can be found. The morphology depends on the stage of differentiation of the developing sensory cell at the time for exposure.

The vulnerability of the HC surface seems to be much the same in a given tissue independent of the type of noxious agent, although comparable injuries must be interpreted carefully because changes like this have also been shown after osmotic shock. Changes of the sensory hairs with protrusion of the HC surface have been described in experimental animals treated with aminoglycoside antibiotics (52,54), chlorhexidine (13), in degenerating HC in waltzing guinea pigs (20,46), in Shaker 2 mice (11) and in the vestibular epithelia of adult guinea pigs exposed to single high doses of irradiation (48,53). The exact anatomical localization, however, for the primary changes and further progress of HC alterations varies.

Recently, Michel and Fritz-Niggli (37) showed that irradiation during development combined with iodacetamin, lucanthone and tetracyclines led to a significant sensitization to irradiation effects regarding the development of fetal anomalies and damage to the sensory epithelia. The progression of surface pathology is comparable to that seen after treatment with aminoclycosides and in genetic inner ear diseases (20,51,52). The latter, however, starts with a protrusion of the cuticular plate. Prenatal irradiation has also been shown to follow this pattern (5). The pathology as seen with TEM is confined to the highly specialized surface structures. HC stereocilia exposed to ototoxic drugs first appear disarrayed. The attachment points of stereociliary plasma membranes become altered. Adjacent stereocilia create giant hairs through formation of protoplasmic bridges. In the early stages of stereociliary fusion several individual stereocilia can still be identified, but later the sensory hair rootlets in the cuticular plate and the stereociliar cytoskeleton disintegrates, the cuticular plate bulges out and the hair cells herniate out into the endolymphatic space (7). In a strain of genetically deaf mice, the first sign of stereociliary alteration is the bulging of the cuticular plate resulting in a final fusion of stereocilia with the formation of giant hairs. In the cristae ampullares the central part shows more exten-

sive change in HC morphology than the peripheral part. The same condition prevails in the central, striolar area of the maculae. HC type I show more extensive damage than HC typ II. This is true also after irradiaton but here the changes can be found all over the organ surface albeit somewhat concentrated to the central areas (5,26).

In the cochlea the OHC after irradiation seems to be more vulnerable than the IHC with a localization favoring the basal turns. This is also the fact after exposure to ototoxic antibiotics (54), cisplatin (41) and loop diuretics (12). A different pattern of drug-induced cochlear HC degeneration has been described following the administration of atoxyl (1). In the case of noise induced HC damage, Bredberg et al. described stereociliary changes using SEM later corroborated by Fermin and Cohen (15,21) and in 1981 Ward described critical intensity concept in noise damage (50). In a recent study, Engström documented in detail the onset and further progress of the typical sensory hair fusion and formation of giant hairs on IHC, while the OHC appeared less damaged (19).

The effect of irradiation during embryonic development shows a number of morphological changes in the HC. However, the localization of irradiation induced HC damage is usually not the same as in earlier descriptions. The mechanism behind inner ear degeneration is different in ototoxic antibiotics, genetic disorders and irradiation but the primary target seems to be the HC leaving adjacent supporting cells quite unaffected except for the formation of scars in the cochlea. The morphological changes suggest that the normal stability of the cell surface becomes altered. The causes of such changes are not fully understood but probably include derangement of the anchoring mechanism between the sensory hairs, the plasma membrane and the cytoskeleton (24).

Irradiation during prenatal life leads to a dose-dependent, time-related type of HC injury. The pattern is more pronounced if irradiation is carried out earlier during the inner ear development, i.e., during organogenesis, before the cells have passed their terminal mitosis and are in an active vulnerable phase. The stereociliary pathology increases in a linear way with increasing doses of irradiation given. The otoconia show most derangements later during pregnancy, i.e., day 16 of gestation but this correlates well with the time for their development which is later than that of the HC.

The study of irradiation induced hearing loss presents a complex problem because of the in other types of injuries well known poor relation between pure tone thresholds and HC injuries. The guinea pig for instance, does not show extensive changes in the surface structures only, but shows hearing loss combined with considerable loss of OHC after noise exposure (19). It is not possible to establish a detailed correlation between structure and function in the present material but there is a principal pattern of extension of sensory hair injuries at various locations in the cochlea. Most of the irradiation damage must be considered to alter the peripheral end organ. A direct irradiation effect could be confirmed as the same morphological cell injuries could be found after irradiation of an organ culture of inner ear anlage (28). Some component in the hearing loss can be correlated to increased latencies in the ABR (6).

The aging hair cell in non-irradiated controls showed the common signs of normal aging (4) which affects the specific stereociliary arrangement and their individual anchoring to the cuticular plate. The OHC were more vulnerable than the IHC. These animals showed a flat threshold shift curve with the calculated mean value of the hearing loss never exceeding 27 dB. At times, however, no thresholds could be obtained in the extreme low and high frequency regions, i.e., 2 and 31.5 kHz.

After exposure to irradiation elevated thresholds were detected in all groups of animals independent of the dose of irradiation given. No measurable hearing at all was found in the higher and lower frequencies tested. A minimal hearing residue could be traced in the 10 kHz region in most of the exposed animals. In SEM the same irradiation changes as in the young exposed animals were found together with the common signs of aging.

Areas of totally missing HC, replaced by a thin layer of expanded Deiter´s cells were seen. Also signs of hypoxia were noted. These areas were most pronounced in the basal and apical turns, corresponding to the frequency regions with undetectable thresholds in the ABR measurements, but such areas could also be found in the middle part of the cochlea. Aging rats with hypertension exposed to noise, with a calculated reduced blood flow through the cochlea, show similar pathology. The morphological changes were localized to the upper basal turn, corresponding to hearing loss in the high frequency region (23).

In the present study the hearing loss was more pronounced in the aging, irradiated animals than in aging control animals. The hearing loss, however, ex-

ceeded the additive effects of hearing loss in young irradiated animals plus the hearing loss in a normal aging population. This can indicate that irradiation might have a sensitizing effect of the aging HC, resulting in an accelerative effect of degeneration and hearing loss in the cochlea.

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Discussion with Reviewers

R.V. Harrison: The author uses frequencies up to 31.5 kilohertz delivered by a TDH 39 telephone. The frequency response of this type of transducer is such that there is very little acoustic energy produced at high frequencies such as 31.5 kilohertz. I wonder if the author made any

calibrations of this sound system to verify that such high frequency stimuli were indeed being delivered to the experimental animal.
Author: Yes, the system was calibrated with a 1/4 inch Brüel and Kjaer condensator microphone type 4135.

H. Rask-Andersen: Figure 10 shows the diagram of the summarized audiograms in the different gestational groups after irradiation. In my opinion the diagram should also include a normal control group with the same number of animals.
Author: It is impossible to include a control group in Figure 10 because the zero line is equivalent to the control material and the irradiated audiograms show the difference from the normal material.

Reviewer IV: The experimental methodology as described in the manuscript does not allow drawing of significant conclusions from the results provided. The pregnant mothers were whole-body irradiated, yet the systemic or localized effects on them or on the litters were not investigated. Information on the rate of survival of neonates and on the percent of animals which reached maturity should be provided. Without this information as well as data on the condition of non-sensory elements in the inner ear, any conclusions on direct sensitivity of inner ear hair cells to irradiation cannot be valid.
Author: Pregnant mothers were whole-body irradiated, but systemic and localized gross effects were not investigated, apart from noting the fact that the mothers ate the litters that were not completely normal. This experimental set-up and the resulting changes in the inner ear were also compared to *in vitro* cultured inner ears after exposure to radiation (see ref. 28). This comparison showed that the same type of injury was found in both experimental situations. From this it is concluded that the effect of irradiation on the inner ear is direct.

Reviewer IV: In a morphological study reporting on experimental conditions, using results from SEM only is insufficient.
Author: This review is limited to SEM data. Transmission electron micrographs and light micrographs can be found in other papers published by our laboratory (see ref. 5, 26, 27, 29, and 30).

Reviewer IV: If cells are more susceptible to irradiation at their proliferative stages, why was the damage after treatment on day 16 equivalent to day 12?
Author: Statistically the damage was not equivalent on day 12 and 16, but the animals were more susceptible on day 12, even though the shape of the curves is similar.