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CHANGES IN THE TRACHEAL CILIATED CELLS IN RABBITS TREATED BY CIS-DIAMMINEDICHLOROPLATINUM (II) AS STUDIED BY ELECTRON MICROSCOPY

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Abstract

The ciliated epithelium of the rabbit's trachea was investigated after a single 5 mg dose of cis-diamminedichloroplatinum (cis-DDP). Specimens were taken for scanning electron microscopy, transmission electron microscopy and light microscopy. Examination was performed daily for 20 consecutive days. A cytotoxic effect of the drug on the ciliated epithelium was observed with bent ciliary tips, swollen tips and broken cilia. Finally the cilia were lost and large areas of the surface were covered with microvilli. However, 20 days after the drug injection, the restitution of the ciliary carpet was almost complete.

Key words: Cilia, tracheal epithelium, cis-diamminedichloroplatinum, scanning electron microscopy, transmission electron microscopy.

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Introduction

The use of cis-diamminedichloroplatinum (cis-DDP) in combination with radiation has been shown to increase the survival and to give better local control of the disease in patients with squamous cell carcinoma in the head and neck region (Wendt et al. 1987, Murthy et al. 1987). This type of treatment has also been used for pulmonary carcinoma (Shank et al. 1985) and esophageal carcinoma (Richmond et al. 1987, Seydel et al. 1988, Keane et al. 1985), and it gives fair expectations of a better outcome in these diseases. However, the optimal dose and the time between administration and irradiation, as well as the dose of irradiation, are parameters which may be crucial and have to be examined carefully. The importance of this is further underlined by the results of von der Maase (1984a, b), which showed that different tissues react to the treatment in unequal ways. Earlier reports (Albertsson et al. 1986) regarding concomitant irradiation and cis-DDP in experiments on rabbits have shown a cytotoxic effect on ciliated cells in the trachea. This finding calls for further examination of the effect of the drug when used alone. In this paper, by use of scanning (SEM) and transmission (TEM) electron microscopy, the tracheal mucosa of the rabbit has been investigated daily for 20 consecutive days after an intraperitoneal administration of 5 mg cis-DDP to the animals.

Materials and Methods

Animals

Twenty full-grown rabbits weighing between 1.8 - 2.3 kg were selected for this study.

Drug

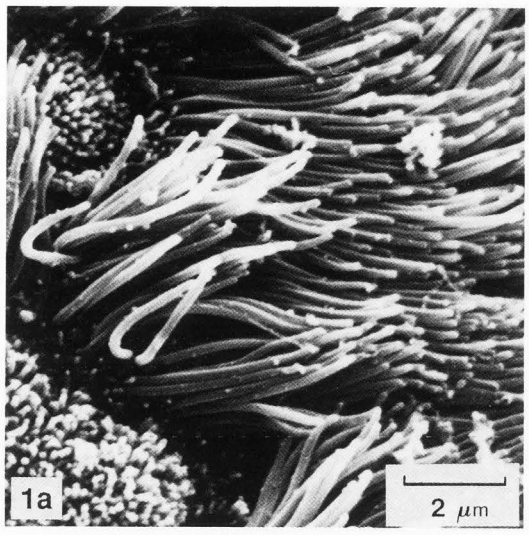
Cis-diamminedichloroplatinum (II), (cis-DDP, Cis-platinum), (Platinol, Bristol Myers Company), was dissolved in normal saline at a concentration of 0.5 mg/ml.

Experiments

The animals received 5 mg cis-DDP intraperitoneally. One rabbit per day was examined from day 1 to day 20 after the injection. Each animal was sacrificed by a blow on the skull in order to avoid pharmacological side-effects. The trachea was dissected out in its entire length (7 - 9 cm). Samples for SEM and TEM were taken from two separate sites: the upper and lower parts of the trachea.

Preparation for SEM

The specimens for SEM-examination were not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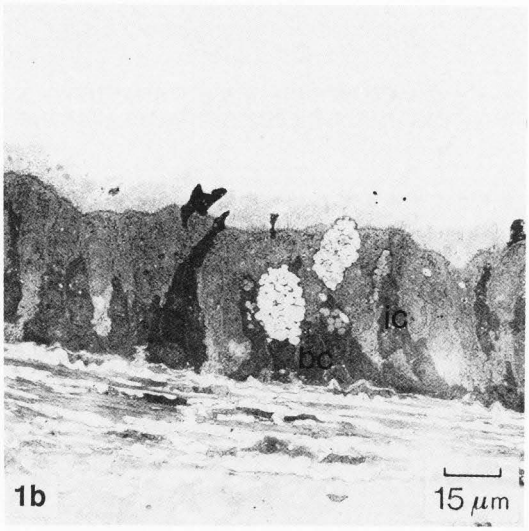
2 μm


ic
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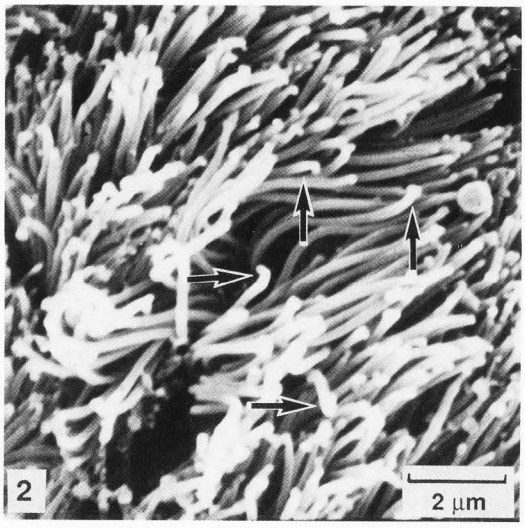
1b
15 μm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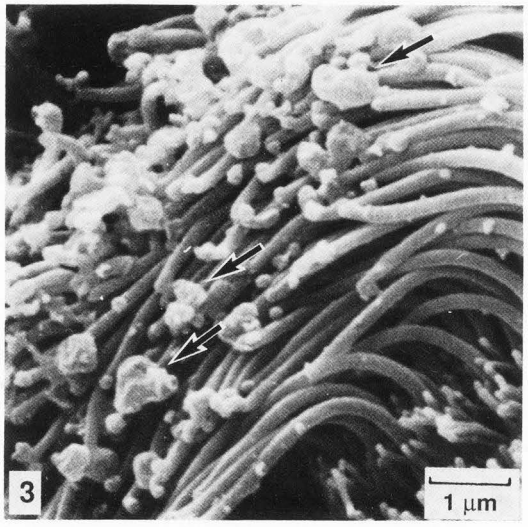
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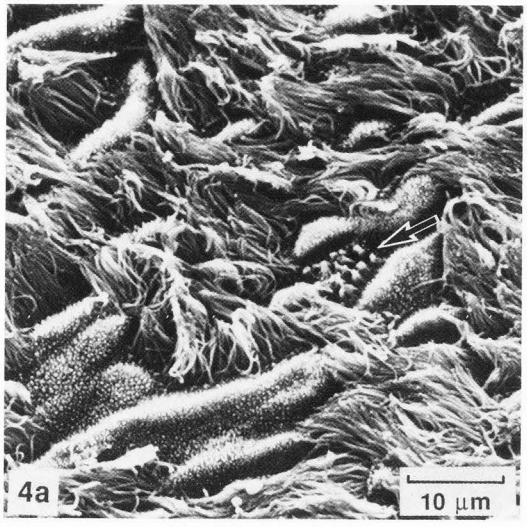
1 μm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10 μm

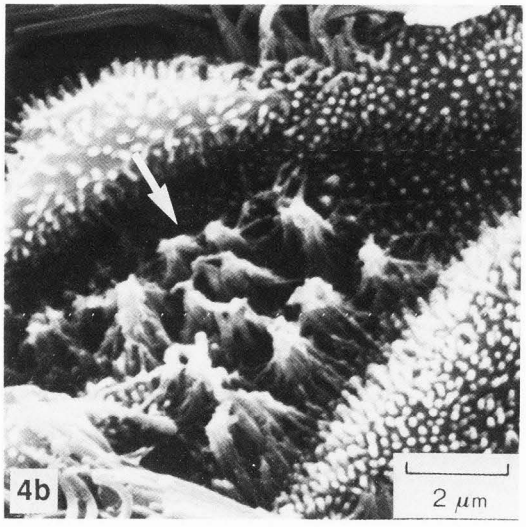
4b
2 μm

Fig. 1. SEM-micrograph (a) and TEM-micrograph (b) of normal ciliary epithelium. Bc = basal cell. Ic = intermediate cells.
Fig. 2. SEM-micrograph of ciliary epithelium. Bent ciliary tips (arrows). Score 1. Day 2 after drug injection.
Fig. 3. SEM-micrograph illustrating swollen, blow-up ciliary tips (arrows). Score 2. Day 4 after drug injection.
Fig. 4. SEM-micrographs (b at higher magnification of a) illustrating cilia broken off near the base and clustering together (arrow). Score 3. Day 10 after drug injection.

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rinsed. They were fixed in 2.5% glutaraldehyde in 0.15 M cacodylate buffer (pH = 7.3) for 12 hours. They were then transferred into the same buffer, and were later osmium-fixed in 1% osmium tetroxide in 0.15 M cacodylate buffer for two hours.

Dehydration took place with a graded series of ethanol, after which the preparations were transferred to Freon TF 618. The specimens were dried according to the critical point method in a Balzers-000 Critical Point Dryer. They were finally sputter-coated with gold plus palladium in a Polaron SEM coating unit E 5000. The prepared specimens were examined in a Philips 515 SEM. The microscope was operated at 20 kV.

Preparation for TEM

The samples were fixed as for the SEM preparations. In addition, they were fixed in 1% osmium tetroxide in 0.15 M cacodylate buffer (pH = 7.3) for two hours, washed in 0.15 M cacodylate buffer, dehydrated in ethanol, and embedded in Vestopal W or Epon. Ultra-thin sections were cut and contrasted with lead citrate and uranyl acetate. For TEM examination, a JEOL 200 CX TEM was used both within the low and high range of magnification.

Scoring System

From SEM micrographs of six different areas selected at random from each rabbit trachea, the ultrastructural changes were scored on a scale from 0 - 4, in the following way:

0 = normal ciliary carpet (Fig. 1a).

1 = loss of ciliary tonicity, bent ciliary tips, increased amount of blebs (Fig. 2).

2 = Swollen tips of the cilia (Fig. 3).

3 = broken ciliary tips, reduced amount of cilia (Figs. 4a, b).

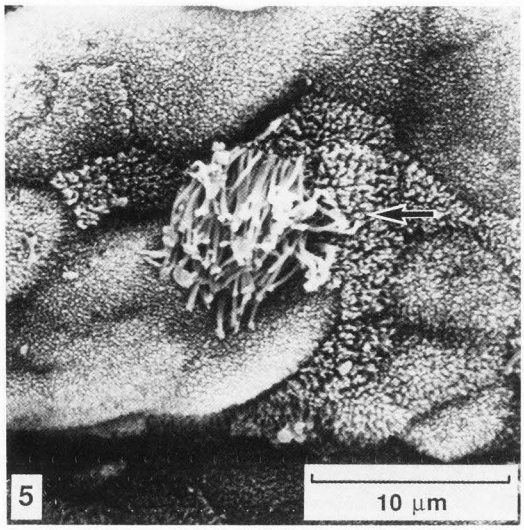
4 = areas where the normal ciliated carpet was denuded and the surface was covered with microvilli (Fig. 5).

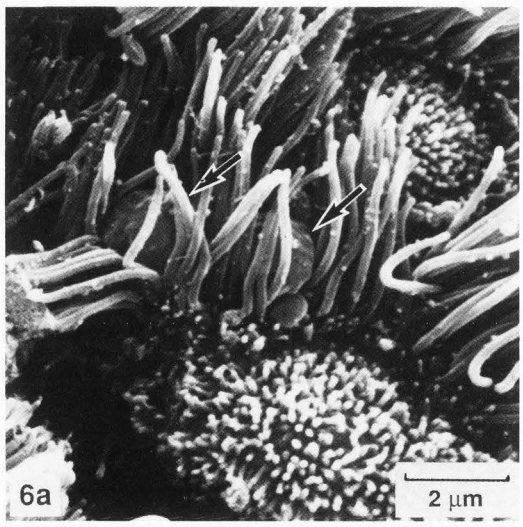
Results

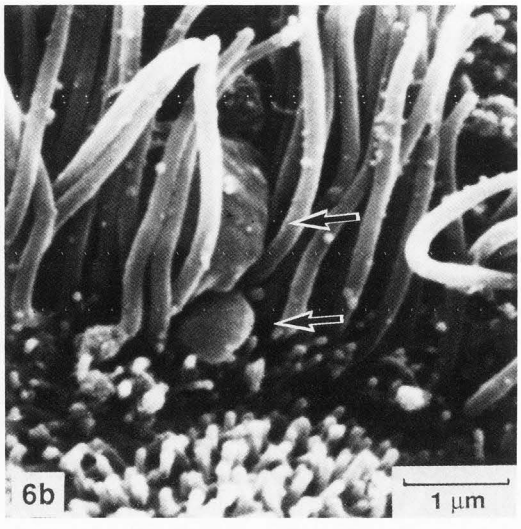
Scanning Electron Microscopy

The SEM-observations showed a characteristic time-dependent pattern of damage after the cis-DDP injection. Scoring of the SEM-micrographs was carried out according to the criteria listed above. The normal ultrastructure of the tracheal epithelium is shown in Fig. 1a.

Fig. 5. SEM-micrograph illustrating score 4. Huge areas covered by microvilli. Solitary ciliary remnants (arrow). Day 12 after drug injection.
Fig. 6. SEM-micrographs (b at higher magnification of a) illustrating cytoplasmic extrusions between the cilia (arrows). Day 8 after drug injection.







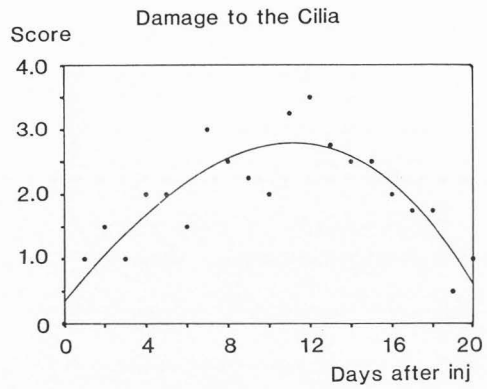


Fig. 7. The development of ciliary damage over time as scored from SEM-micrographs.

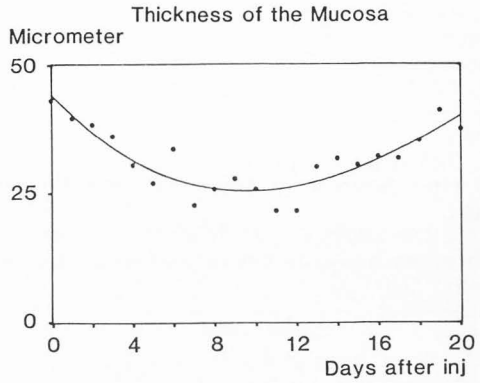


Fig. 8. Height of the ciliary epithelium as measured each day after injection of cis-DDP.

The development of ciliary damage seems to follow a pattern which starts with bent ciliary tips (Fig. 2). Next the ciliary tips appear swollen, sometimes multi-lobulated (Fig. 3). A break in the upper part of the cilia seems to be the next phase of the chronological degradation. It seems logical that intracellular substances consequently gush out from the cilia, sometimes adhering them to each other (Figs. 4a, b). In that stage of degradation, the amount of blebs on the side of the cilium is reduced. Cytoplasmic extrusions are seen between the cilia (Figs. 6a, b). The ultimate degree of damage after cis-DDP injection in this series is a denudation, which appears as large convex, confluent cell areas lacking cilia and occupied by microvilli (Fig. 5). Still, ciliary remnants can be seen. Parallel with progressive damage, blebs on the side of the cilia appear.

For the above designed model, the ciliary damage can be summarized in the schedule shown in Fig. 7 derived from SEM micrograph scores. It illustrates how the ciliary damage reaches a maximum on day 10-12 after a single injection of 5 mg cis-DDP. The damage already starts on day 1 after the injection and is possible to detect even at day 20.

Transmission Electron Microscopy

During the period directly following the injection of cis-DDP a reduction in the height of the ciliary mucosa was noted. The mucosa consists of three to four cell layers which have been measured in several untreated animals (Albertsson et al. 1985) (Fig. 1b). The normal height of the ciliated epithelium is about 43 microns. The reduction in height following cis-DDP treatment occurred rather quickly and reached a low value seven days after injection (Fig. 8). On TEM, the epithelium at that time consisted of 1-2 cell layers. Also, the maximal reduction in height was noted to be earlier than the maximal damage to the cilia (Fig. 7). The time delay between the minimum of the one curve (Fig. 8) and the maximum of the other (Fig. 7) was estimated to be about two days. The curve illustrating the height of the ciliary epithelium has its minimum at about day 9 (Fig. 8). After this time, the height of the ciliary mucosa increases again, and by 20 days after injection it had nearly regained its normal height. The reduction in height reflects a gradual disappearance of the intermediate cells a phenomenon earlier observed after treatment with misonidazole and fractionated irradiation (Albertsson et al. 1985).

On days 1-3, examination of the cilia revealed the ciliary tension to be normal, phases of their strokes could be seen. A minimal number of blebs were observed on the tips or the sides of the cilia. The ciliary crown seemed normal, and the ciliary membrane was intact. On days 4-7, blebs occurred at the sides and tips of the cilia. The ciliary crown was usually damaged. The ciliary membrane was wrinkled, and an increased distance to the tubules inside was seen. The number of cilia on each cell was somewhat reduced; however, they had not completely disappeared on day 7 (Fig. 9). The tonicity of the cilia gradually declined. During the damage phase, the number of basal bodies was reduced. Signs of damage were also seen on microvilli, which in some cases were blown up in balloon-like formation (Fig. 10). Within the apical part of the ciliated cells, vacuolization of the cytoplasm was seen, as were cytoplasmic extrusions on the surface (Fig. 11). Goblet cells or goblet-like cells were relatively increased in number.

During the last few days of the observation period (days 15-20), the height of the ciliary epithelium was nearly restored. The microvilli and cilia seemed normalized, and were seen in large numbers. The basal bodies were regularly arranged. The apical ciliated cells and basal cells looked normal, and newly-formed intermediate cells were found. A relative increase in goblet cells was still observed.

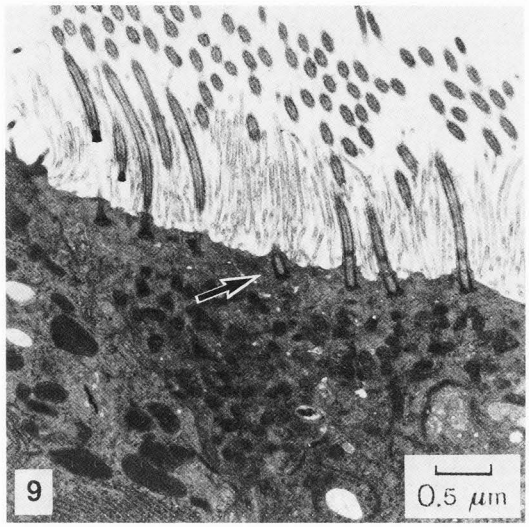
Discussion

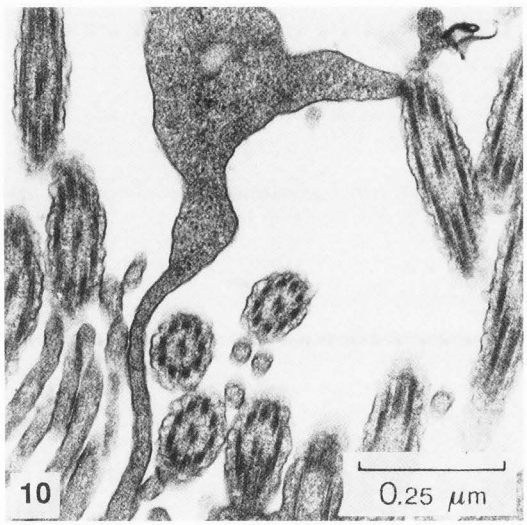
Cis-DDP, given as a single injection intraperitoneally to rabbits in a dose corresponding to the one given to humans, results in damage to the ciliary epithelium. The damaging effects diverge to some degree from those observed with the addition of fractionated irradiation (Albertsson et al. 1986). For instance, in the latter, a rich amount of remnant bodies could be defined on the ciliary carpet. Ultrastructurally, the degradation of the cilia was considered to be identical in the two experiments. Therefore, the same score system as described previously (Albertsson et al. 1986) has been used for evaluation of the cilia in the present work. However, some modifications have been made since new phenomena appeared, which made it difficult to adhere to earlier definitions. Regarding the cilia, the score system is focused on degradation of the cilia and the surface

of the ciliated cells. Membrane-equipped protuberances (blebs) on the cilia are seen in the normal physiological state (Dalen 1982), and they have also been described as a damage to the ciliary membrane induced by ionizing radiation (Albertsson et al. 1983). The blebs on the side of the cilium may be situated in sensitive, weak regions (Dalen 1982); however, external stimulus may cause an increase in their number. The ensuing degradation of the cilia seems to start with loss of the normal tonicity of the cilia. A further stage in the development of damage seems to be swollen, multi-lobulated tips. Finally, the cilia seem to break, particularly at the base, and to perish. The damage is most pronounced on days 9-14 after the drug injection (Fig. 7). Parallel to the degradation of the cilia, a cytotoxic effect on the apical ciliated cells was seen, most prominently on days 7-13 (Fig. 8). During this time period, the ciliated epithelium loses the intermediate cell layer and exhibits damage with vacuolization and extrusions of the cytoplasm. The time delay, illustrated in Figs. 7 and 8, may depend on a toxic effect of the drug which starts at the base of the mucosal epithelium.

It seems logical that the cytotoxic drug from the blood vessels initially reaches the basal cells. Influenced by the drug, they are reduced in number. This phenomenon seems to start early, probably by the first day after injection, since the height of the ciliary mucosa seems to be already reduced at that time (Fig. 8). The effects visible on goblet cells and ciliated cells are further delayed, which may depend on the fact that the drug reaches these cells somewhat later - perhaps by slow diffusion. Even though the half-life of the drug in plasma is about 25-50 minutes, the plasma clearance of total platinum following single doses of cisplatin has generally been reported as biphasic with a terminal phase and a half-life on the order of days (Lange et al. 1973). During this time, cis-DDP is bound to plasma proteins (Litterst et al. 1976).

The ciliary toxicity could be compared to the nephrotoxicity of cis-DDP which manifests itself as an acute effect mainly localized to the proximal convoluted tubules. The damage depends on the drug concentration and can be partly overcome by hydration and forced diuresis (Comis et al. 1980). Ultrastructural investigations of rat kidney after drug administration revealed severe mitochondrial degeneration (Aggarwal et al. 1980). Effects of the drug on the mitochondria were not observed in this experiment; however, the dose administered to the rats was higher. The spontaneous reversibility of the damage in the trachea is seen in Figs. 7 and 8 . Whether a complete normalization has occurred 19 days after injection is difficult to say. The trachea has a very good regeneration capacity. Hilding and Hilding (1966) showed that after removing the ciliary epithelium on the rabbit trachea and leaving the basal cell layer intact, a new ciliary mucosa formed after three days.





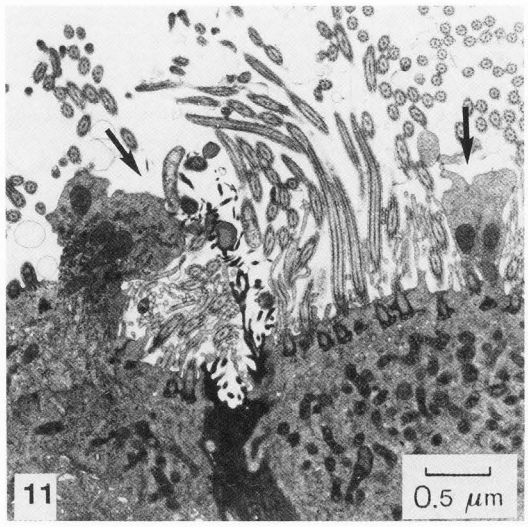


Fig. 9. TEM-micrograph illustrating loss of basal bodies and cilia (arrow). Day 7 after drug injection.

Fig. 10. TEM-micrograph. Blown-up, balloon-like microvillus. Day 8 after drug injection.

Fig. 11. TEM-micrograph. Cytoplasmic extrusions (arrows). Day 11 after drug injection.

For comparison, the hematological toxicity of cis-DDP is also mentioned. The maximal damage in the peripheral blood and bone marrow after single drug injection in female Wistar Rats is seen on days 2-7 with a restitution by day 14 (Harrap et al. 1980). Other side effects observed in the clinic are peripheral neuropathy (11%), ototoxicity (5%) and mucositis which occur in about 20% of the cases (Albertsson et al. 1987). Cis-DDP is used nowadays world-wide for its known cytotoxic effect aganist a variety of tumors, although, initially, its side effects on normal tissues limited its use.

In this study it appears that the ciliary carpet seems to be sensitive as a biological instrument, in so far as it reacts on stimuli, either external (smoking, irritant gases, heat, radiation) or internal (sensitizers, cytostatic drugs).

Conclusion

Cis-DDP exerts a cytotoxic effect on the ciliated epithelium of the rabbit trachea. The ciliary damage is observed a few days after injection. The degeneration of the cilia seems to follow a sequential pattern of bent ciliary tips, swollen tips, broken cilia and areas of denudation. Finally, the cilia are lost and large areas of the surface are covered with microvilli. A few days after injection of cis-DDP death and loss of the intermediate cells of the ciliated cell layer is observed.

Acknowledgements

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Discussion with Reviewers

J. Reitan: From a non-expert point of view both Fig. 4 and pronounced on Fig. 5 show different types of cell surfaces. The denudation may be either loss of cilia or loss of ciliated cells. Some of the cells could represent intermediate cells in the process of differentiation, consistent with the reported reduction in intermediate cells. The intermediate cells could also be recruited from the basal cells which also seem reduced in numbers. The goblet cells may tolerate the drug whereas the other cells are killed, resulting in thickness reduction and relative increase in goblet cells.

Do you have other data indicating that drug penetration kinetics can explain the changes of the different cell compartments? Why does this not represent toxic cell death, reactive proliferation changes in differentiation (Hilding and Hilding 1966)? Have you done cell kinetic experiments, e.g., thymidine incorporation at the time of drug administration?

Authors: Unfortunately cell kinetic experiments have not been done. However, our interpretation is exactly as you describe it, namely that the reduction in number of intermediate cells is caused by the fact that the basal cells from which the intermediate cells derive also are reduced in number.

B. Afzelius: You claim that the presence of bent ciliary tips represents a (mild) state of damage of cilia. Is this really so? Others (e.g., Sturgess in Scanning Electron Microscopy 1978;II:1083) have shown that all cilia in normal tissues have bent tips and some (Veerman, in Ultramicroscopy 4, 133, 1979) have even taken this as a criterion to see whether cilia are oriented or not. The tips of the cilia are bent in the direction of the ciliary beat. Also, you have no controls that show cilia with no damage. Any comments?

Authors: When we first noticed the phenomenon of bent tips we used it as a parameter for the evaluation of the damage. The definition is vague, however. Bent tips as we judge them are aggravation of what you normally see as bent tips. In addition, the bent tips are occurring in different directions even if they are located in a group of synchronously working cilia.