Immediate and delayed effects of repeated doxorubicin injections on rat incisor mesenchymal cells

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Effects of multiple injections of the cytotoxic drug doxorubicin hydrochloride (Adriamycin®) on incisor mesenchymal cells were studied by light microscopy. Rats were killed 1 day after two and three injections, and 1 day and 5 days after four injections. Doses of 5 and 10 mg/kg were used and given on consecutive days. Necrotic alterations expanded in preodontoblast and basal pulp regions when the total dose was increased and finally embraced the entire progenitive part of the incisor. The necrotic area became encapsulated by cellular predentin produced by differentiated pulp cells to prevent further tissue damage, and commencing regeneration was observed after 5 days. When the total dose was split up, the cytotoxicity of doxorubicin increased. □ *Antineoplastic agents; dental pulp; dentinogenesis; histology; necrosis*

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The mesenchymal cell line of the continuously growing rat incisor has been found useful as an experimental model in toxicologic testing, especially of substances interfering with cell division (1). The effects of a single dose of the antineoplastic drug doxorubicin hydrochloride (Adriamycin®) has previously been studied in this model (2). One day after the injection, odontoblast precursors—that is, preodontoblasts—were destroyed, and single necrotic cells and islands of necrosis were found in the basal (progenitive) pulp tissue. The most apically positioned odontoblasts, the young odontoblasts, appeared irregular and partly necrotic. The disarrangements found were dose dependent, since an increasing dose potentiated the cytotoxic effect. Doxorubicin affects cycling cells and is regarded most lethal to cells during the synthesis phase (3). Intercalation into nuclear DNA and blockade of the transcription mechanism, leading to inhibition of DNA and RNA synthesis, are believed to mediate the cytotoxic effect (4, 5).

The aims of the present study were to investigate the immediate and delayed effects of multiple injections of doxorubicin on rat incisor mesenchymal cells and to compare them with the effects of a single dose. It was thus interesting to ascertain whether the mode of dosage—that is, the total dose given as one bulk injection or in several injections—could influence the response. Further, the sequence of morphologic alterations produced by an increasing number of injections was to be studied.

Materials and methods

Of 56 female rats (Wistar, Möllegaards Breeding Centre Ltd, Skensved, Denmark) with a mean weight of 200 g, 40 served as experimental animals and 16 as controls. All experimental animals were, under light ether anesthesia, given intravenous injections of doxorubicin hydrochloride (Adriamycin®; Farmitalia, Montedison Farmaceutica, Italy) dissolved in sterile water. The volume of each injection was 0.25 ml/200 g body weight. Injections of 0.25 ml saline solutions/200 g body weight were administered to control animals. The animals were divided into three groups and dosed in accordance with the regimen shown in Table 1. The injections were made in the tail vein during the morning at 24-h intervals. All animals dosed two and three times and half of the animals dosed for four times were decapitated under ether

Table 1. The administration regimen of doxorubicin and saline solution

	Group I			Group II			Controls		
Single injection	5 mg doxorubicin/kg			10 mg doxorubicin/kg			Saline		
No. of animals	5	5	10	5	5	10	4	4	10
No. of injections	2	3	4	2	3	4	2	3	4
Total dose (mg/kg)	10	15	20	20	30	40			

anesthesia 1 day after the last injection. The remaining animals were killed after 5 days. The heads were divided by a median line incision, the maxillae freed from soft tissue, and the lateral nasal wall removed. After fixation in 2% Sörensen-buffered glutaraldehyde the maxillae were demineralized in an aqueous solution of 44% formic acid and 20% sodium citrate. The incisors were divided into pieces of 3 mm apicoincisal length, postfixed in 1% Milonig-buffered osmium for 2 h, and embedded in Vestopal W (Chemische Werke, Hüls AG, Mari, FRG) (6). Approximately 60 longitudinal sections 1–2 µm thick were made from each piece and stained with methylene blue–azure II–basic fuchsin (7). The sections were evaluated by light microscopy.

Results

In control animals the mesenchymal cells of the incisor were divided into the following regions in accordance with morphology and function: progenitive (basal) and non-progenitive pulp cells, early and late preodontoblasts, and young and mature odontoblasts (2, 8) (Fig. 1).

The alterations found in group I animals 1 day after injection are schematically presented in Fig. 2. In rats given 5 mg doxorubicin/kg body weight twice, the early preodontoblast zone was cell-free and liquefacted, and normally appearing late preodontoblasts were separated by edema. The most apical part of the progenitive pulp was necrotic, whereas scattered cells with a pyknotic nucleus and small, stamped-out areas containing two to six fragmented nuclei were seen among the remaining basal pulp cells (Fig. 3). In animals given 5 mg/kg three times, the entire preodontoblast region was necrotic, and the adjacent part of the basal pulp showed liquefaction and cells with karyorrhexis. The young odontoblasts had a pseudostratified appearance (Fig. 4). The mature odontoblasts of rats given 5 mg doxorubicin/kg two and three times were like those of the controls. Necrosis of the preodontoblasts and of the basal pulp extending into the non-proliferative region was found in the group I rats dosed four times. The young odontoblasts and the most basal mature odontoblasts were shortened, with depolarized nuclei. A few odontoblast-like cells and a small zone of predentin were observed in the pulp towards the necrotic area (Fig. 5).

In all animals in group II, doxorubicin induced alterations in young odontoblasts and in progenitive cell regions 1 day after injection (Fig. 2). The early and late preodontoblasts were necrotic. In rats dosed twice, normal-appearing basal pulp cells were intermingled with liquefacted areas and cells with pyknotic and karyorrhetic nuclei. Some non-proliferative pulp cells and the entire basal pulp of the remaining group II animals (dosed three and four times) were necrotic. Depolarization and, with increasing number of injections, shortening of the young odontoblasts and the apical mature odontoblasts were also observed. Several cells in the pulp which exhibited odontoblast-like morphology (Fig. 6) were embedded in growing amounts of predentin with increasing total dose of doxorubicin.

In group I animals observed 5 days after the four doses of 5 mg/kg, the early and late preodontoblast regions remained cell-free and liquefacted. The young odontoblasts and the apical cells of the mature odontoblast zone appeared irregular and shortened, with

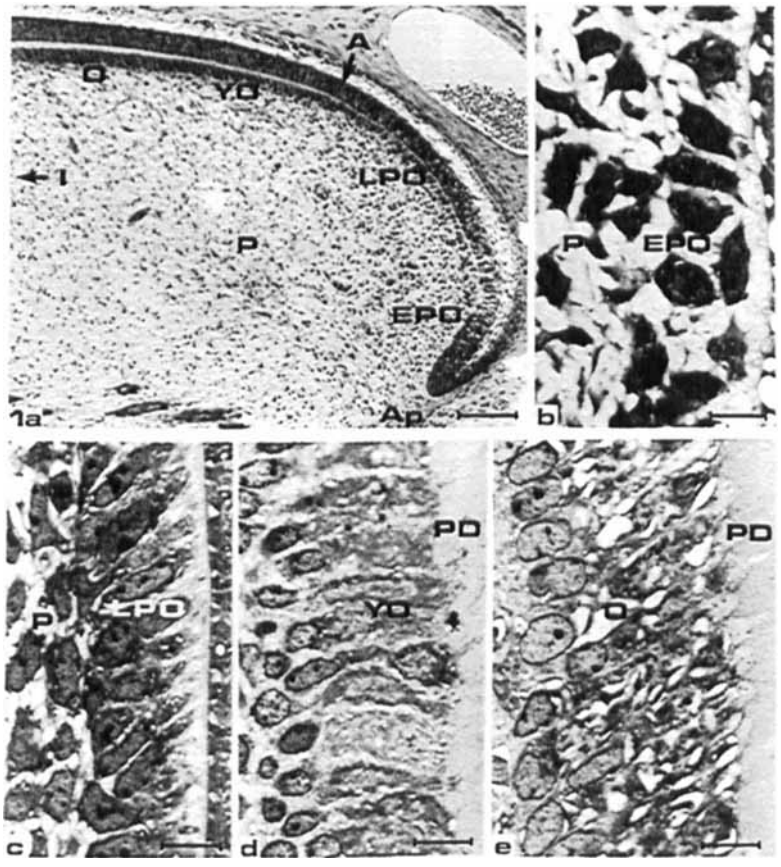

Fig. 1. Photomicrographs of longitudinal Vestopal W-embedded sections from the apical part of the maxillary rat incisor of control animals. *1a*. Low-magnification survey. *1b-e*. High-power details of (b) stellate pulp cells (P) and randomly arranged cubodial early preodontoblasts (EPO), (c) late preodontoblasts (LPO) appearing more elongated and closely packed, (d) columnar, mantle dentin-producing young odontoblasts (YO) and (e) circumpulpal dentin-depositing, mature odontoblasts (O) with pseudostratified morphology. The intercellular vacuolization of the odontoblasts is likely an artifact of fixation. A = preameloblasts and ameloblasts; Ap = apically; I = incisally. *1a*. Bar, 100 µm. *1b-e*. Bar, 10 µm. Methylene blue–azure II–basic fuchsin stain.

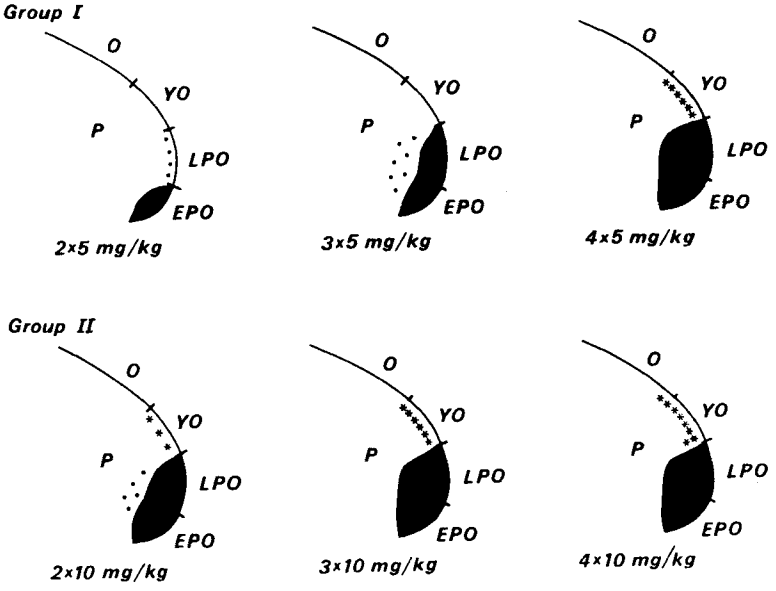

Fig. 2. Schematic presentation of the alterations found in the apical part of the maxillary incisor of doxorubicin-injected rats 1 day after injection. Black areas represent regions of liquefaction necrosis. Dots display single necrotic cells. * = Depositions of irregular predentin in the pulp; O = odontoblasts; YO = young odontoblasts; LPO = late preodontoblasts; EPO = early preodontoblasts; P = pulp.

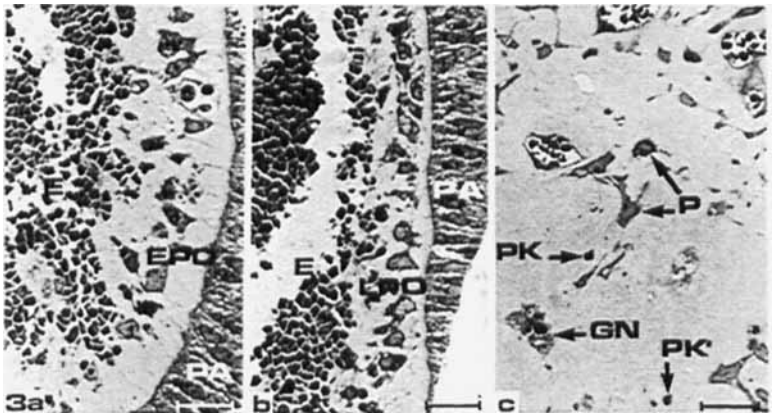

Fig. 3. Photomicrographs of longitudinal Vestopal W-embedded sections of maxillary incisor of a rat given 5 mg/kg doxorubicin twice. *3a*. Necrosis of the early preodontoblast (EPO) region. *3b*. Reduced number of late preodontoblasts (LPO). *3c*. In the basal pulp tissue, a group of necrotic cells (GN) and pyknotic nuclei (PK) intermingle with the normally appearing pulp cells (P). PA = preameloblasts; E = erythrocytes. Bar, 25 μm.

depolarized nuclei. A marked reduction of mantle and circumpulpal dentin production was noted, corresponding to the transformed cells (Figs. 7a and b). Irregular predentin was deposited pulpally to these cells (Fig. 7b). Embedded in the apical part of the predentin were numerous elongated cells with asymmetrically located nuclei and granular cytoplasm, resembling odontoblasts (Fig. 7c). Most of the pulpal predentin was observed at the young odontoblast level delimiting the normal-appearing pulp tissue. A few thin-walled blood vessels and several stellate mesenchymal cells in the fibrillar pulp matrix were situated apically to the irregular predentin (Fig. 8). The remaining basal pulp area resembled structureless necrotic tissue.

Most of the basal pulp cells and the entire preodontoblast zone remained destroyed in the group II animals (dosage, 4 × 10 mg/kg) (Fig. 9). The alterations of odontoblast morphology and the diminished mantle and circumpulpal dentin deposition described were also observed in group II, which also showed an extensive bulk of irregular predentin with cellular inclusions in the pulp. The irregular predentin separated a liquefacted basal pulp from the remaining non-progenitive pulp tissue. The basal pulp

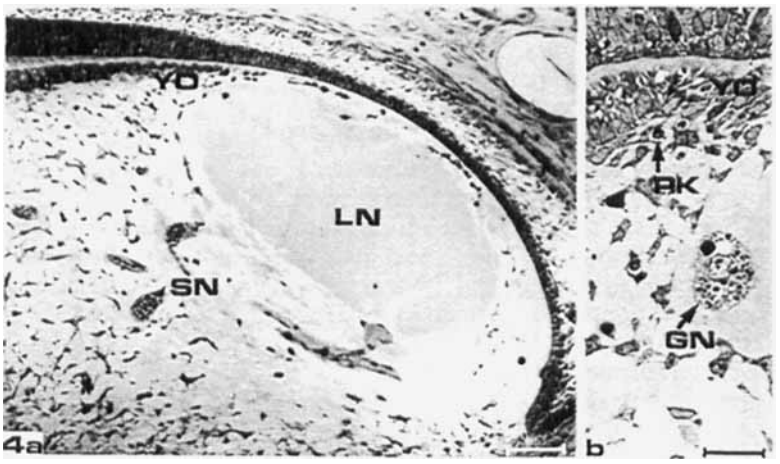

Fig. 4. Photomicrographs of longitudinal Vestopal W-embedded sections of rat maxillary incisor after 5 mg/kg doxorubicin three times. *4a*. Liquefaction necrosis and pyknotic nuclei (LN) in the preodontoblast regions and in adjacent pulp cells. SN = areas of scattered necrotic cells; YO = young odontoblasts. Bar, 100 μm. *4b*. Higher magnification of irregular young odontoblast (YO) and the nearby pulp showing necrosis of single cells (PK) and of a group of cells (GN). Bar, 25 μm.

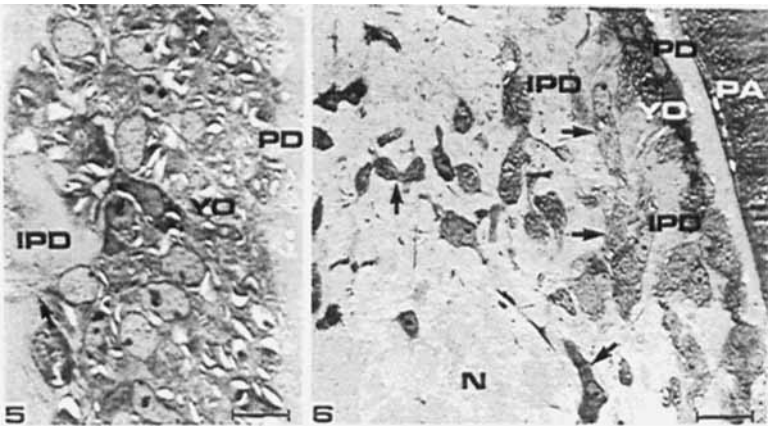

Fig. 5. Photomicrograph of altered young maxillary incisor odontoblasts (YO) of a rat given 5 mg/kg doxorubicin four times. Minor island of irregular predentin (IPD) and odontoblast-like cells (arrow) can be seen in the pulp. PD = predentin. Bar, 10 µm.

Fig. 6. Photomicrograph of shortened young odontoblasts (YO) with displaced nucleus and the adjacent pulp showing odontoblast-like cells with long cytoplasmic processes (arrows) embedded in irregular predentin (IPD) towards the necrotic region (N). PD = predentin; PA = preameloblasts. Bar, 25 µm.

region embodied a few thin-walled blood vessels and stellate mesenchymal cells, especially along the irregular predentin (Fig. 9). In both groups the morphology of the odontoblasts incisally to the zone of reduced dentinogenesis concurred with that of the controls.

No animal presented symptoms of immediate doxorubicin intoxication, but on the 5th day of observation, some of the animals, especially in group II, developed diarrhea and signs of fatigue.

Discussion

Necrotic nuclear changes and a marked loss

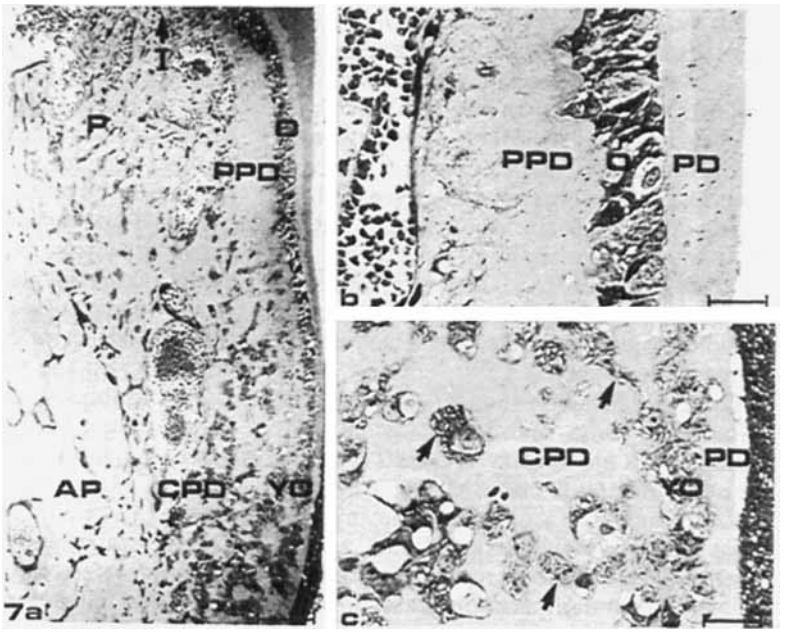

Fig. 7. Photomicrographs of longitudinal sections from the apical part of maxillary incisor of a rat given 5 mg doxorubicin/kg four times, 5 days after the last injection. *7a*. Low magnification exposes deposition of irregular predentin tissue (PPD/CPD) in the pulp (P) and a reduced mantle and circumpulpal dentin deposition. Ingrowth of blood vessels and cells in the basal pulp area is observed. O = odontoblasts; YO = young odontoblasts; I = incisally. Bar, 100 µm. *7b*. Higher magnification of depolarized odontoblasts (O) and of irregular predentin (PD) in the pulp (PPD). Bar = 25 µm. *7c*. Higher magnification of irregular predentin (CPD) with odontoblast-like cells (arrows) pulpally to the young odontoblast (YO) region. Bar, 25 µm.

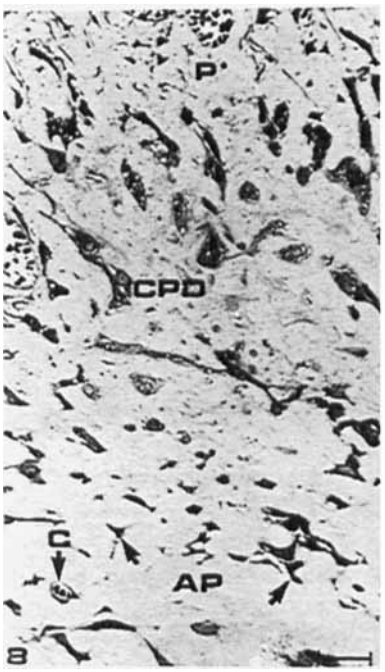

Fig. 8. Photomicrograph of longitudinal section of maxillary incisor of a rat given 5 mg doxorubicin/kg four times after 5 days. Irregular predentin with cellular inclusions (CPD) demarcates normal pulp (P) from altered pulp tissue (AP) with ingrowing capillary (C) and stellate mesenchymal cells (arrows). Bar, 40 μm.

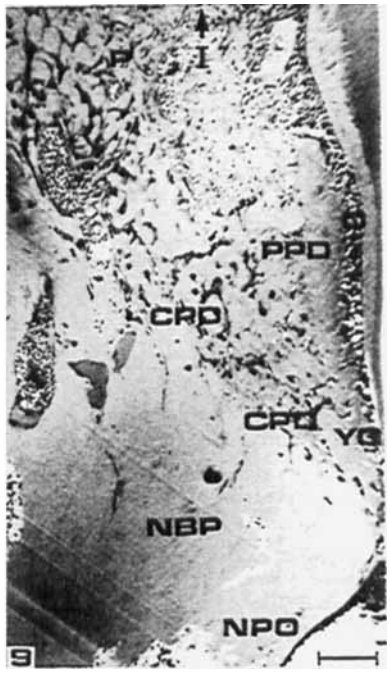

Fig. 9. Photomicrograph of longitudinal section of the apical part of maxillary incisor of a rat given 10 mg doxorubicin/kg four times, 5 days after the last injection. Reduced thickness of mantle and circumpulpal dentin can be seen, together with deposition of pulpal irregular predentin (PPD) at the odontoblast level and of predentin entrapping odontoblast-like cells (CPD) towards necrotic preodontoblast (NPO) and basal pulp (NBP) regions. O = odontoblasts; YO = young odontoblasts; P = pulp. Bar, 25 μm.

of cells were taken as evidence of the cytotoxic effects of doxorubicin on dividing cells. At all dose levels except the lowest one, 2 × 5 mg/kg, all preodontoblasts were necrotic (Figs. 2–4). After a single dose of doxorubicin of 10 mg/kg or more, the entire preodontoblast region was destroyed (2). This indicates that a fractionation of the total dose into two smaller portions might diminish the effect. It is, however, infeasible to establish dose-effect relations, since the complete preodontoblasts zone was necrotic at most dose levels.

Doxorubicin also produced necrosis of the basal pulp cells manifested as cell loss, stamped-out areas of several cells with fragmented nucleus, and single cells with pyknotic nucleus 1 day after the injections (Figs. 2–4). Neighboring cells are frequently in the same period of the cell cycle (8), and the stamped-out areas constitute groups of synchronous cells undergoing necrosis (2). The medley of single and grouped necrotic cells in the pulp tissue was found in regions that were cell-free at higher dosage, and these cell-free areas increased with the number of injections in both experimental groups (Fig. 2). A comparison of this finding with those after a single injection (2) showed that fractionation of the total dose magnified the cytotoxicity. The broader zone of necrosis observed after 5 mg/kg four times compared with that after 10 mg/kg twice further corroborates this fact. This is similar to the observation made concerning the cardiotoxic effect of doxorubicin (9) but contradictory to the relationship suggested for preodontoblasts. However, pulp necrosis seems a more likely factor in this respect, since the findings are explicable. Doxorubicin is most lethal to cells of the synthesis phase (3), and by subdividing the total dose into several smaller doses with 24-h intervals, the time period of doxorubicin influence expands. Thus, more of the continuously cycling cells will enter the synthesis phase and become sensitive to the drug.

The dissemination of necrosis in the apical

part of the incisor, evaluated by longitudinal observation (Fig. 2), shows the early preodontoblasts followed by the late preodontoblasts as the cells most sensitive to doxorubicin, in agreement with previously published results (2). The basal pulp cells are regarded more resistant to doxorubicin (2), but with increasing number of injections necrosis was found primarily in cells bordering the preodontoblasts, followed by cells deeper into the pulp (Figs. 2–4). After 5 mg/kg four times, 10 mg/kg three times, and 10 mg/kg four times, necrosis seemed to extend into the region of the pulp defined as non-proliferative (8). Cell death of the entire proliferative part of the pulp probably initiates the re-entry of the stable pulp cells into the cell cycle, and they thus become susceptible to doxorubicin.

The widespread destruction of progenitive mesenchymal cells of the incisor observed immediately after multiple doxorubicin injections persisted also 5 days after the last injection (Figs. 7a and 9). In the basal pulp region the ingrowth of blood vessels and stellate cells (Fig. 8) might represent an early phase of a healing process. Renewal of basal pulp cells by cell multiplication was seen in a partly necrotic pulp after a single injection of doxorubicin (2, 10). It has also been reported that indifferentiated perivascular mesenchymal cells are capable of differentiating into odontoblasts and pulp cells after injury (11), and this might well be the situation in the present study, in which the entire basal pulp seemed destroyed shortly after the injections (Fig. 4). The perivascular cells have been shown in vitro to be the mitotically active cells in cultured explant from the non-proliferative pulp (12). No apparent difference in the degree of regeneration was found among the two experimental groups. This could be expected, since the immediate cellular destruction after 5 and 10 mg/kg four times was equal (Fig. 2). The capability of the apical pulp area to regenerate totally could not be investigated, however, because the reduced condition of the animals made prolonged observation unwarranted.

Irregular predentin was deposited in the pulp adjacent to altered odontoblasts of group II animals and of animals dosed 5 mg/kg four times (Figs. 2, 5, 7a, and 7b). In group II, the bulk of predentin increased with the number of days after the first injection (Fig. 2). Similar enlargement of the pulpal predentin was observed during the 5 days after the 5 mg/kg four times regimen (Fig. 7). Pulpal predentin was found 5 days after a single doxorubicin dose (2) and regarded a reparative phenomenon induced by cell necrosis (13). However, a direct non-lethal odontoblast injury, which depolarized the cells enabling them to secrete predentin into the pulp (14), must also be considered an explanation. In addition, the nearby tissue necrosis may initiate a differentiation of non-proliferative pulp cells into predentin-secreting cells (Figs. 6 and 7). The cellular predentin may function as a barrier against the necrotic tissue (Figs. 8 and 9), preventing further damage, and thus ensure the maintenance of the circumpulpal dentinogenesis. However, the dentin production in the pulp-distant part of the altered odontoblasts was reduced (Figs. 7 and 9), as seen after single-dose doxorubicin and cyclophosphamide injection (2, 15), most probably due to depolarization of the odontoblast nucleus (14).

In conclusion, by splitting up the total dose of doxorubicin into several smaller portions, the degree of tissue destruction was increased, protective predentin deposition expanded, and regeneration greatly delayed compared with single-dose administration.

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