Influence of the antineoplastic agent cyclophosphamide on dental development in rat molars

Margareta Näsman and Lars Hammarström

Department of Orthodontics and Pediatric Dentistry and Center for Oral Biology, Karolinska Institutet, Stockholm, Sweden

Näsman M, Hammarström L. Influence of the antineoplastic agent cyclophosphamide on dental development in rat molars. Acta Odontol Scand 1996;54:287–294. Oslo. ISSN 0001-6357.

The effect of cyclophosphamide (Cy) on tooth development was studied in molars of 18 young Sprague–Dawley rats. Doses of 30 mg/kg body weight of Cy dissolved in 1 ml 0.9% NaCl were administered to 18 experimental rats and 1 ml 0.9% NaCl to 18 control rats at 10 and 13 days of age. The most obvious changes in the experimental teeth could be seen in the developing pulp of the third molar and developing roots of the first and second molars. Wide cell-free areas appeared in the third molar 2 days after the last injection; later these areas turned into mineralized osteodentin. Similar areas could be observed also in the roots of the first and second molars. These changes were related to the developmental stage of the area. ☐ *Dental pulp; dentin; developmental disturbances; osteodentin*

Margareta Näsman, Department of Orthodontics and Pediatric Dentistry, Karolinska Institutet, School of Dentistry, P.O. Box 4064, S-141 04 Huddinge, Sweden

Advances in the treatment of childhood malignancies have led to a dramatic improvement in survival. Almost 60% of the children treated for a malignant disease can now be expected to be long-term survivors (1). This progress has directed interest to the late sequelae of both the disease and the therapy given (2). One of these late sequelae is a disturbed tooth formation. Children treated with chemotherapy in combination with irradiation of the head and neck at an early age show severe disturbances in dental development, whereas those who were treated with chemotherapy alone before reaching 10 years of age had less advanced disturbances (3–8).

One of the most commonly used chemotherapeutic agents against cancer in children is cyclophosphamide (Cy). It acts as an alkylating agent that cross-links the guanine bases in double-stranded DNA and thereby inhibits cell division and causes mutations (9). The effects of Cy on the developing dental tissues have been examined by various methods, using the continuously growing incisor of the rat as the model organ (10–13). The extent of injury in odontogenesis has proved to be related to the dose of the drug. It mainly involves the undifferentiated mesenchymal cells in the proliferating zone of the tooth pulp, resulting in acellularity of the basal part of the pulp, whereas the differentiated ameloblasts and odontoblasts appear to be unaffected (14). When high doses of Cy (75 mg/kg body weight) were injected, the malformations included extra long, malformed, and supernumerary incisors (15). Koppang (16) showed that a single injection (40 mg/kg b.w.) of Cy to rats on the 20th day of pregnancy caused a reduction in the growth rate of the mandibular incisors of the offspring. A single injection of Cy reduced the eruption rate in unimpeded rat incisors for a long time (17). Karim et al. investigated the effect of adriamycin on developing hamster molar tooth germs in culture, and these in vitro results confirmed earlier in vivo studies on the effect of adriamycin on the rat incisor tooth (18–20). Other antineoplastic drugs such as adriamycin, vinblastine, vincristine, and colchicine were also shown to cause osteodentin formation in rat incisors (21–23). Since all experimental studies in vivo on developing teeth so far have been carried out on continuously growing rodent incisors, it was of interest to study the effects of Cy on rooted teeth such as the rat molars, which in this respect are more similar to the teeth in the human dentition. Thus the purpose of this study was to examine the effects of two injections of Cy on the different stages of dental development in rat molars, and to find which dental disturbances and changes in dental growth it causes.

Materials and methods

Six litters of Sprague–Dawley rats of both sexes with six siblings in each litter were used for the study. At 10 days of age 18 rats from 3 litters (mean weight, 26.3 g) received an intraperitoneal injection of 30 mg/kg b.w. of Cy (Cyclofosfamid®, Lääkefarmos/Farmos, Åbo, Finland) dissolved in 1 ml 0.9% NaCl. Before the injection the rats were anesthetized with a subcutaneous injection of 0.05 ml fentanylfluanisone (Hypnorm, Vet. Leo, Helsingborg, Sweden). Eighteen control rats (mean weight, 25.7 g) were injected with 1 ml 0.9% NaCl. Before the injection the rats were anesthetized with a subcutaneous injection of 0.05 ml fentanylfluanisone. A second injection of 30 mg/kg b.w. Cy was given to the experimental groups of rats at 13 days of age. The control rats were injected with 1 ml 0.9% NaCl at the

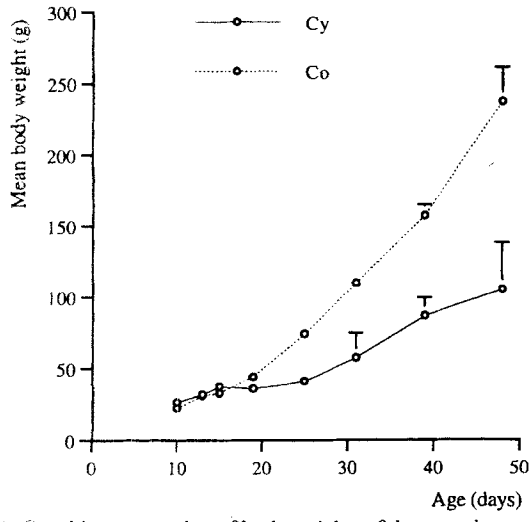
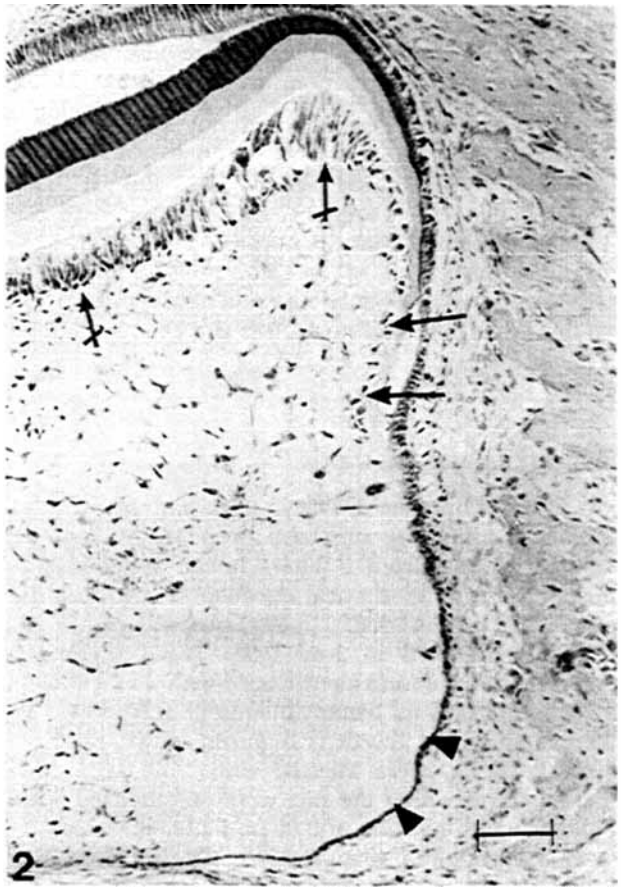


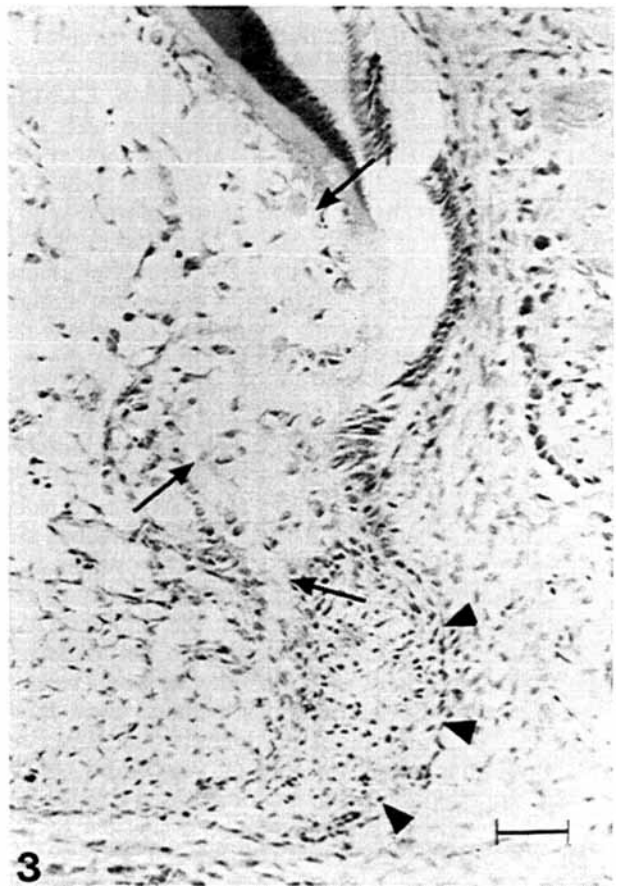
Fig. 1. Graphic presentation of body weights of the experimental rats (Cy) and the control rats (Co) at 2, 6, 12, 18, 26, and 35 days after the last injection.

same age. All rats were weighed 2, 6, 12, 18, 26, and 35 days after the last injection, and mean weight for the experimental groups and control groups were noted. The condition of the furs was recorded, as was the behavior of the animals. At 2, 6, 12, 18, 26, and 35 days after the last injection three experimental rats and three control rats were killed by decapitation after CO_2 asphyxiation (4 min). The heads were then fixed in Histofix (buffered 10% formaldehyde), demineralized in 20% formic acid, and embedded in paraffin. Step-serial sections, 4–5 µm thick and 4–5 µm apart, were taken sagittally through the central part of the molars and stained with hematoxylin and eosin. All the sections from the experimental rats and the control rats were examined and read by both authors. The division into three regions in the description was based on the obvious changes in those regions.

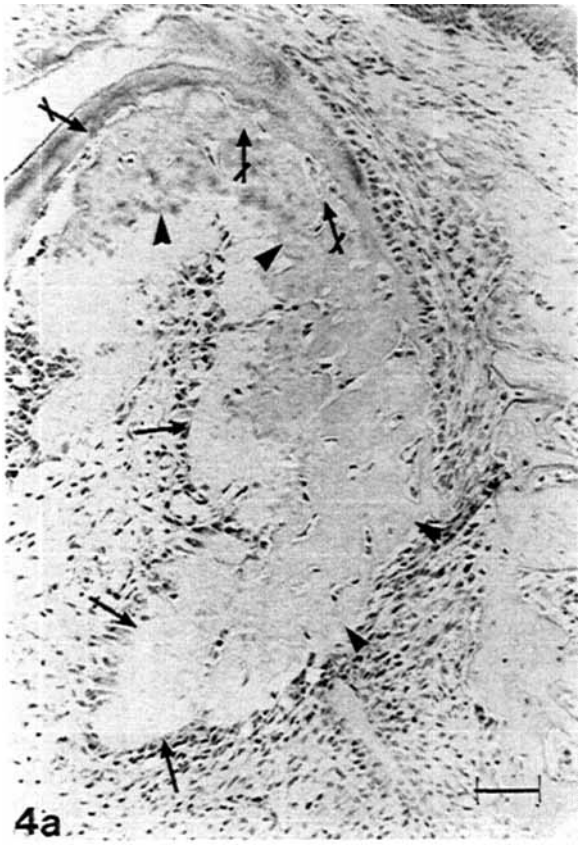
Results

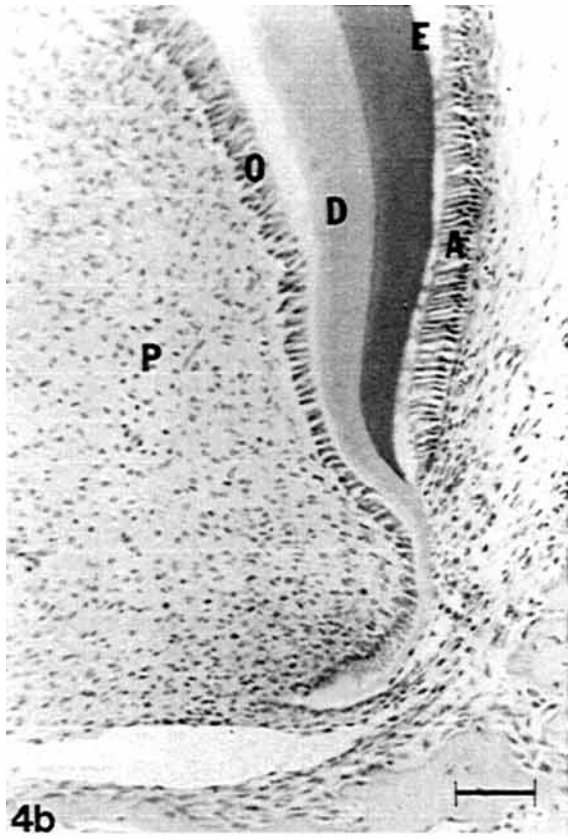
The rats that received Cy did not gain weight in the same manner as the control rats during the first 2 weeks after the second and last injection (Fig. 1). After this period the experimental rats began to gain weight





Figs. 2-5. Photomicrographs of the distal parts of maxillary third molars of 15- to 31-day-old rats injected with cyclophosphamide (Cy) at 10 and 13 days of age. (Hematoxylin and eosin.)





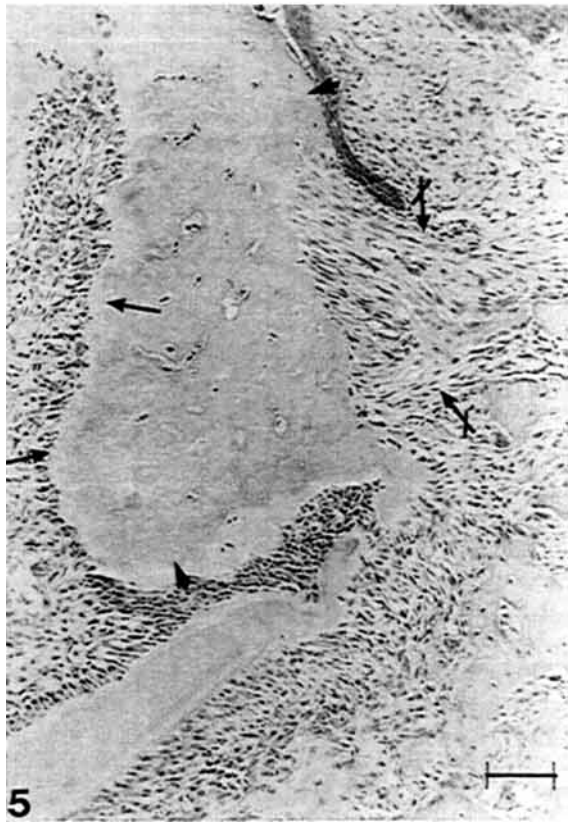


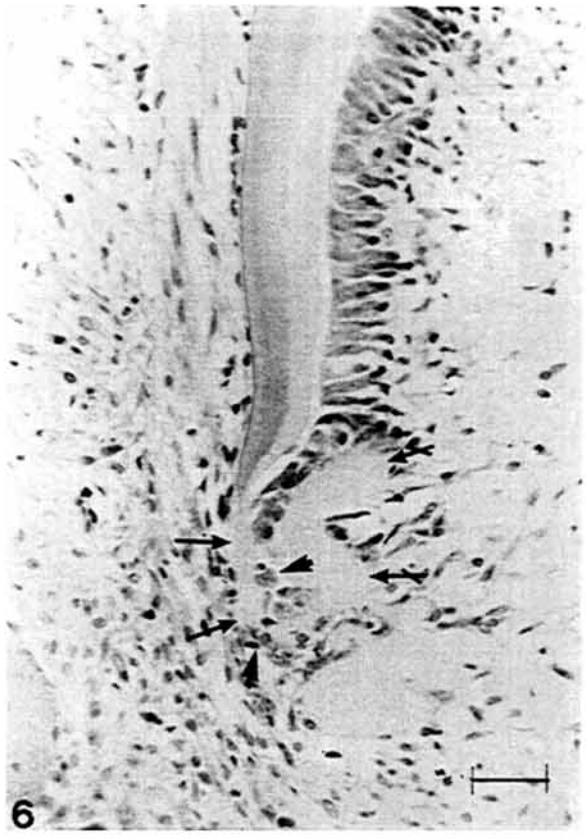
Fig. 2. Fifteen-day-old rat, 2 days after the last injection. Note the cell-free area in the cervical region of the pulp close to the epithelium (arrowheads). The young odontoblasts adjacent to the non-mineralized mantle dentin (arrows) and the mature odontoblasts (crossed arrows) close to the first thin layer of circumpulpal dentin are located in vacuoles or in a narrow space between the dentin and a rather structureless area in the pulp (bar = 100 μm).

Fig. 3. Nineteen-day-old-rat, 6 days after the last injection. There is a high cell density (arrowheads) cervically to the structureless area (arrows), suggesting that cell growth has recommenced (bar = 100 μm).

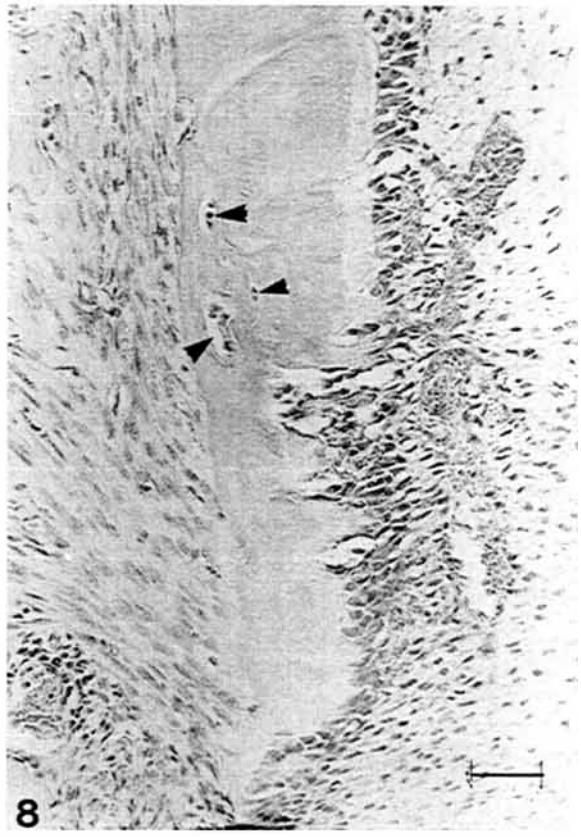
Fig. 4a. Twenty-five-day-old-rat, 12 days after the last injection. The subodontoblastic cell-free, structureless area is partly mineralized (arrowheads). The mineralization seems to spread from the mineralized dentin via numerous bridges (crossed arrows) from the dentin. There is a wide non-mineralized zone close to the pulp (arrows). Mineralization may be conceived to start in the first formed matrix (bar = 100 μm).

Fig. 4b. Twenty-five-day-old control rat. Pulp tissue (P), odontoblasts (O), ameloblasts (A), dentin and predentin (D), and enamel (E) (bar = 100 μm).

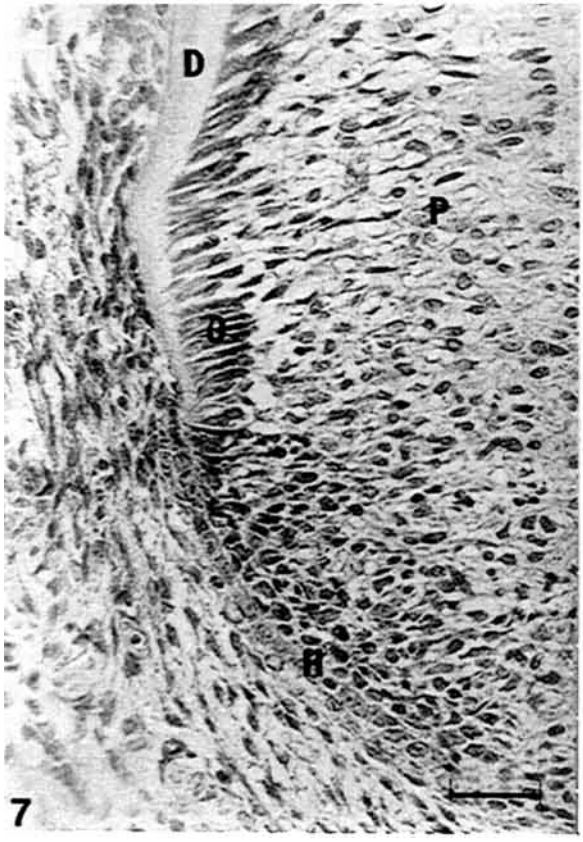
Fig. 5. Thirty-one-day-old-rat, 18 days after the last injection. Almost all of the pulpal hard tissue, the osteodentin, is mineralized (arrowheads). There is a non-mineralized predentin zone close to the pulp (arrows). Collagenous fibers (crossed arrows) extend from the osteodentin to the neighboring bone (bar = 100 μm).



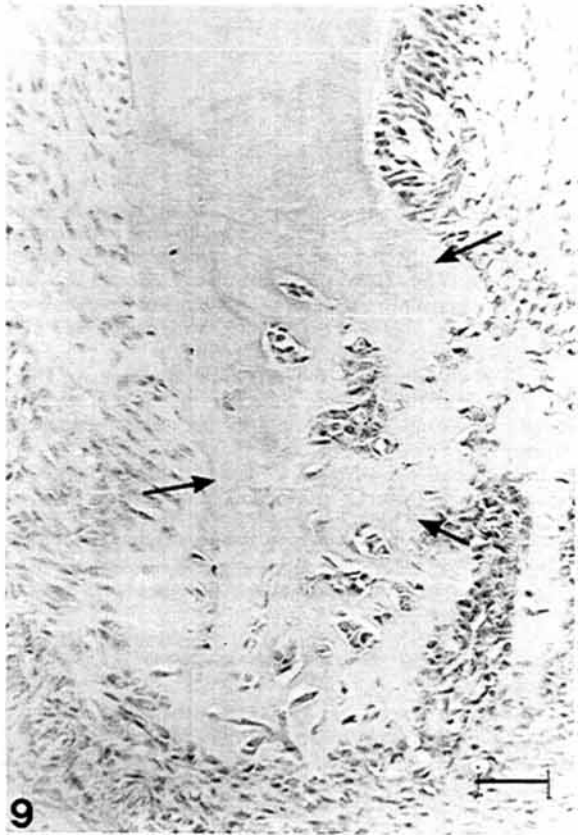
6



8



7



9

Figs. 6–9. Microphotographs of the apical ends of the mesial roots of the maxillary first molars of 15- to 31-day-old rats injected with cyclophosphamide (Cy) at 10 and 13 days of age. (Hematoxylin and eosin.)
Fig. 6. Fifteen-day-old-rat, 2 days after the last injection. Dentin formation has ceased in the apical end. There are some young odontoblasts located in a narrow space (arrowheads) between the non-mineralized mantle dentin (arrows) and a pulpal structureless area (crossed arrows) (bar = 200 μm).
Fig. 7. Nineteen-day-old control rat. Pulp tissue (P), odontoblasts (O), dentin and predentin (D), and epithelial root sheath of Hertwig (H) (bar = 200 μm).
Fig. 8. Twenty-five-day-old-rat, 12 days after the last injection. The apical end of the mesial root consists of partially mineralized osteodentin with some enclosed cells (arrowheads) (bar = 100 μm).
Fig. 9. Thirty-one-day-old-rat, 18 days after the last injection. The apical end consists of partially mineralized osteodentin (arrows) (bar = 100 μm).

again, but during the experimental period (38 days) the experimental rats did not reach the size and weight of the control rats. The experimental animals began to lose their fur 2 days after the last injection, and after another 4 days they had lost all of their fur, but the animals seemed lively and active, and the mothers accepted them well. After another week the experimental animals gradually got their normal fur back. The control animals did not show these changes.

The most obvious changes in the teeth could be seen in the developing pulp of the third molar and in the apical regions of the developing roots of the first and second molars. Wide cell-free areas appeared in the third molar a few days after the injections. With increasing time these areas turned into mineralized osteodentin. Similar but less widespread areas could be observed also in the roots of the first and second molars. There were regional differences in the changes, and these differences were related to the developmental stage of the area. Three regions were distinguished, and the changes in these regions at different intervals after the injections were as follows.

Region I

The area adjacent to the inner enamel epithelium of the crown of the third molar and the free end of the epithelial root sheath of Hertwig at the apical ends of the roots of the first and second molars constituted region I.

Third molar. Two days after the last injection the normal pulp tissue in this region of the third molar was replaced by a structureless necrotic area without cells (Fig. 2). Four days later this area was more dense than before and contained some randomly distributed cells. A limited area with granulation tissue was found in the most apical part (Fig. 3). Twelve days after the last injection the previously structureless area was partly mineralized (Fig. 4a). The mineralization had a globular appearance, and it extended from the periphery towards the center of the pulp. There was a rather

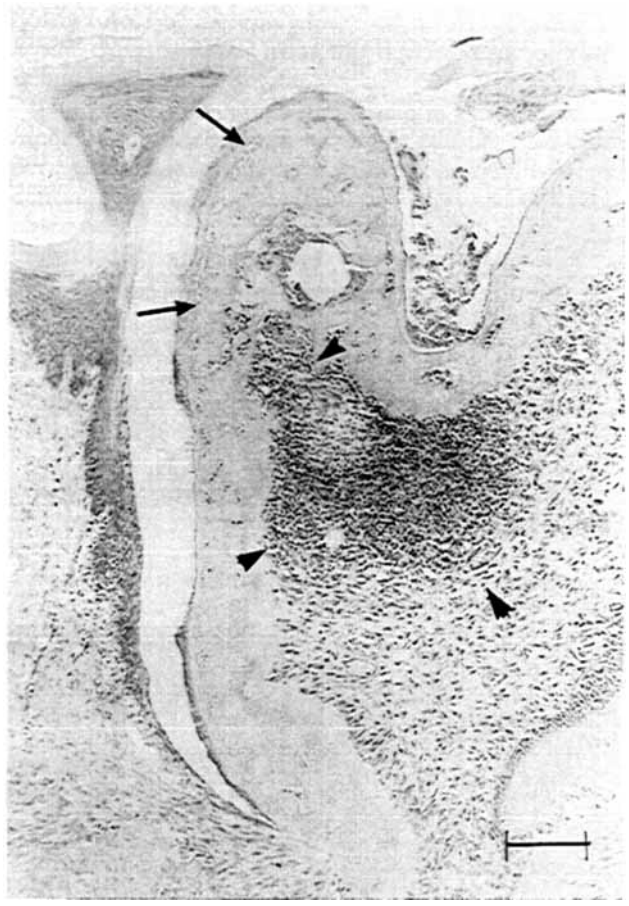


Fig. 10. The maxillary third molar of a 31-day-old-rat, 18 days after the last injection. The peripheral layer of mantle dentin is separated from the osteodentin by a narrow space or row of vacuoles occupied by young odontoblasts (arrows) at the time of injection. The tooth is partly erupted, and bacteria from the oral cavity have invaded the inhomogeneous osteodentin and caused a pulpal pustule (arrowheads) (bar = 100 μm).

wide non-mineralized area towards the pulp. Close to the epithelial root sheath in the bottom of the pulpal cavity there was an increased number of cells, indicating recovery and a continuation of the dental development in this area. A thin dentinal wall in the distocervical area was the first sign of the formation of a distal root of the third molar (Fig. 4a). No root formation was found in the mesial part of the tooth.

Eighteen days after the last injection all of the previously structureless area was mineralized and showed the features of osteodentin (Fig. 5). The cervical two-thirds of the distal side of the crown were not covered by epithelium, and in this area collagenous fibers extended from the osteodentin to the neighboring alveolar bone. Twenty-six and thirty-five days after the last injection the structures in the pulpal cavity were similar to the ones seen after 18 days. In addition, a distal root and normal dentin forming the bottom of the pulpal cavity could be seen.

Second and first molars. At the apical ends of the roots of the first and second molars the epithelial root sheath was markedly shorter than in the controls, and the normal development of the roots seemed to have stopped. The shortness of the epithelial root sheath made it difficult to distinguish clearly the effects of the cyclophosphamide treatment on the pulpal tissue adjacent to the root sheath.

Two days after the last injection there was a reduction in the cell number in the apical region, but no large cell-free areas like the ones found in the third molar could be distinguished (Fig. 6). Six days and more after the last injection an increasing amount of osteodentin was found in the apical region (Figs. 8 and 9). Large collagenous bundles connected this osteodentin with the surrounding alveolar bone.

Control groups. No abnormality was seen in the control groups (Figs. 4b and 7).

Region II

The area adjacent to the non-mineralized and mineralized mantle dentin in the crown of the third molar and at the apical ends of the first and second molars constituted region II.

Third molar. The disturbances in the third molar were as follows. Two days after the last injection the young odontoblasts in contact with the non-mineralized or mineralized mantle dentin were edematous or disrupted and located in small vacuoles or a narrow space formed between the mantle dentin and a neighboring structureless, almost cell-free, pulpal tissue (Fig. 2). A few pulpal cells were unevenly distributed in this otherwise structureless area. The pulpal, almost cell-free, area was slightly eosinophilic and appeared more dense close to the odontoblasts. Small bridges of dentin of various widths connected the mantle dentin with the subodontoblastic area in the pulp. Six days and more after the last injection the third molar had tilted, and no good sagittal sections were obtained (Fig. 3). The histology of the tissue including the 'odontoblastic space' was very similar to that seen after 2 days. After 12 days the non-mineralized mantle dentin and the peripheral part of the subodontoblastic, structureless areas were mineralized (Fig. 4a). The mineralization seemed to originate from the mineralized mantle dentin and then spread gradually to the adjacent tissues via the bridges over the odontoblastic space. After 18 days no cells were seen in the odontoblastic space, and the space seemed narrower than before (Fig. 5). All of the structureless area was now mineralized. One of the crowns was partly erupted, and the mesial cusp was exposed to the oral cavity (Fig. 10). Bacteria had entered the narrow space between the mantle dentin and osteodentin and also some irregular defects in the osteodentin and had reached the pulp, where colonies of bacteria and a pulpal pustule could be seen. Twenty-six and 35 days after the last injection the histologic picture was very similar to the one seen after 18 days. In one of the teeth bacteria were found in irregular spaces in the osteodentin. The pulp was necrotic, and there was a periapical inflammation.

Second and first molars. In the apical region of the roots of the first and second molars disturbances similar to the ones seen in the third molar could be observed adjacent to the mantle dentin. Two days after the last injection odontoblasts were seen enclosed in a space between the mantle dentin and a cell-free, structureless area in the pulp (Fig. 6). Four days later this subodontoblastic area started to mineralize. With increasing time after the injections this osteodentin increased in size, resulting in early closure of the apical foramina (Figs. 8 and 9). It was always separated from the mantle dentin by a narrow space or row of vacuoles initially occupied by odontoblasts.

Control groups. No abnormality was seen in the control groups (Figs. 4b and 7).

Region III

The area adjacent to circumpulpal dentin in the crowns and roots of the first and second molars constituted the third region.

This region seemed to be unaffected by the Cy treatment both in the crowns and in the roots of the first and second molars.

Control groups. No abnormality was seen in the control groups.

Discussion

The development of the hard tissues of the rat molars takes place during the first few weeks of life. During this period most organs and tissues of the body develop very rapidly, making them sensitive to cytostatic drugs. This became obvious when the doses used in previous experiments on adult rats proved to be lethal for the young rats used in the present study (11–17). The dose used in the present study appeared to be the highest non-lethal dose in the young animals.

The present study shows that Cy alone irrespective of other drugs or disease can cause disturbances of the developing teeth. In most reports concerning the effects of cytostatic drugs on developing teeth in humans several different drugs, often in combination with irradiation, have been given. This has made it difficult to distinguish the harmful effects of each component of the treatment on tooth development. Experimental studies on the effect of Cy on rat incisors have shown that the toxic effects of the drug were restricted to the undifferentiated epithelial and mesenchymal cells, and the extent of the injury to the odontogenesis was related to the dose of the drug. The advantage of studying developing rat molars is that it is possible to examine the effects of the cytostatic agent on crown and root formation similar to the situation in humans, and the

effects can be followed for a long period. A disadvantage may be the limitation of the dose that can be given to young growing animals, but this study shows the same type of effects in the developing molars as has previously been found to occur in the incisors after markedly higher doses to adult rats (10–15).

At the age of 10 days, when our rats received their first injection, the crowns of the first molars are almost fully formed, and the enamel is undergoing final mineralization. Root formation has just started. In the second molar enamel matrix secretion is going on in the cervical two-thirds of the crown, and root formation has not yet started. In the third molar formation of dentin and enamel has just begun in the occlusal part (24).

In studies of the effects of Cy on the continuously growing rat incisor, the major changes have been described as interrupted odontogenesis, constrictions, niches, and apical cysts. The niches are associated with formation of pulpal osteodentin (11). In principle, the same types of change seem to occur in the molars with the exception of the apical cysts. The cessation of mantle dentin formation in the distal part of the crown of the third molar and of root formation of the first and second molars noted in the present study is probably the same type of disturbance as the interrupted odontogenesis previously noted in the continuously growing incisors. In the rat incisor, mantle and circumpulpal dentin production has been shown to continue after Cy administration, but at a lower speed (25). The reduction in size of the third molar has probably the same pathogenesis as the constrictions in the incisors—that is, a reduction in the number of ameloblasts and odontoblasts caused by the cytostatic agent. The niches occurred in areas where the young odontoblasts associated with the formation of the mantle dentin were to differentiate into mature odontoblasts forming circumpulpal dentin. The mechanism behind this interference with differentiation by Cy seems to be unknown.

The osteodentin developing in this region may at least to some extent be the result of an ectopic deposition of predentin by probably newly differentiated mesenchymal cells from the pulpal mesenchyme (11). The fact that the mineralization of the pulpal osteodentin spreads gradually from the mineralized mantle dentin via numerous small bridges suggests that the mineralization of the osteodentin requires a mineralized focus from where it can progress. When the mineralization had occurred in most of the osteodentin, apparently normal odontoblasts bordered the osteodentin, and tubular predentin and dentin were formed. The mechanism behind this late differentiation of odontoblasts remains unknown.

The reduction of the root length and the early closure of the apical foramina of the first and second molars agree with clinical experience with chemotherapy treatment (2–8). The present results indicate that this reduction in root length is caused by cessation of the downgrowth of the epithelial root sheath and that the apical closure results from the development of osteodentin in the niche region.

The ease with which the bacteria penetrated the mantle dentin and the osteodentin, causing pulpal and periapical pustules, illustrates the poor quality of the dentin. The clinical conclusion of this should be careful observation of teeth whose crowns were under development during the chemotherapy treatment, when the teeth finally erupt. The poor quality of the dental hard tissues may warrant an oral care program to prevent dental caries.

There are numerous potential causes of disturbances in the tooth development of long-term survivors of malignant diseases that may alter growth. These include the severity of the illness and its initial presentation, generalized growth suppression, hormone suppression, and intensity of chemotherapy, which all can affect tooth development. Our findings with regard to developing rat molars appeared to be very much the same as those found in developing teeth of children receiving anti-cancer drugs.

Acknowledgements.—This study was supported by a grant from the Swedish Medical Research Council (Project 06001) and the School of Dentistry, Karolinska Institutet, Stockholm, Sweden.

References

1. Gustafsson G, Garwicz S, Hertz H. A population-based study of childhood acute lymphoblastic leukemia diagnosed from July 1981 through June 1985 in the five Nordic countries. Acta Paediatr Scand 1987;76:781–7.
2. Byrd R. Late effects of treatment of cancer in children. Pediatr Clin North Am 1985;32:840–56.
3. Jaffe N, Toth BB, Haer RE, Ried HL, Sullivan MP, McNeese MD. Dental and maxillofacial abnormalities in long-term survivors of childhood cancer. Effects of treatment with chemotherapy and radiation of the head and neck. Pediatrics 1984;73:816–23.
4. Maguire A, Murray JJ, Craft AW, Kernahan J, Welbury R. Radiological features of the long-term effects from treatment of malignant disease in childhood. Br Dent J 1987;162:99–102.
5. Koppang HS. Dental damage after radiotherapy for malignant lesions in the head and neck region. Nor Tannlaegeforen Tid 1975;85:159–65.
6. Dahllöf G, Barr M, Bolme P. Disturbances in dental development after total body irradiation in bone marrow transplant recipients. Oral Surg Oral Med Oral Pathol 1988;65:41–4.
7. Rosenberg SW, Kolodney II, Wong GY. Altered dental root development in long-term survivors of pediatric acute lymphoblastic leukemia. A review of 17 cases. Cancer 1987;59:1640–8.
8. Näsman M, Björk O, Söderhäll S, Ringdén O, Dahllöf G. Disturbances in the oral cavity in pediatric long-term survivors after different forms of antineoplastic therapy. Pediatr Dent 1994;16:217–23.
9. Calabresi P, Parks RE. Alkylating agents, antimetabolites, hormones and other antiproliferative agents. In: Goodman LS, Gilman A, editors. The pharmacologic basis of therapeutics, 4th ed. New York: Macmillan 1962:1348–95.
10. Nordlinder H. Malformations in newborn rats treated with a single dose of cyclophosphamide. Acta Soc Med Upsal 1971; 76:90–7.

11. Koppang HS. Histomorphologic investigations on the effect of cyclophosphamide on dentinogenesis of the rat incisors. Scand J Dent Res 1973;81:383–96.
12. Adatia AK. The effects of cyclophosphamide on odontogenesis in the rat. Arch Oral Biol 1975;20:141–4.
13. Vahlsing HL, Sun-Kee K, Feringa ER. Cyclophosphamide-induced abnormalities in the incisors of the rat. J Dent Res 1977;56:809–16.
14. Adatia AK. Cytotoxity of cyclophosphamide in the rat incisor. Br J Cancer 1975;32:208–18.
15. Vahlsing HL, Ferringa ER, Britten AG, Kinning WK. Dental abnormalities in rats after a single large dose of cyclophosphamide. Cancer 1975;35:2199–202.
16. Koppang HS. Histomorphologic investigations of dentinogenesis in incisors of off-spring of cyclophosphamide-treated pregnant rats. Scand J Dent Res 1978;86:444–58.
17. Adatia AK, Berkovitz BKB. The effects of cyclophosphamide on eruption of the continuously growing mandibular incisor of the rat. Arch Oral Biol 1981;26:607–13.
18. Karim AC, Pylypas SP. Osteodentin formation in rat incisors as visualized by radioautography after 3H-proline administration. Anat Rec 1986;216:19–26.
19. Karim AC, Eddy EL. A light and electron microscopic study of osteodentin formation in the rat incisor after adriamycin administration. Am J Anat 1984;169:207–19.
20. Karim AC, Woltgens JHM, Bervoets TH, Lyaruu DM, Bronckers AL. Effect of adriamycin on hamster molar tooth development in vitro. I. Morphological changes. Anat Rec 1989; 225:318–28.
21. Mikkelsen HB. Acute and protracted effects of vinblastine on odontoblasts and dentinogenesis in rat incisors. Scand J Dent Res 1978;86:313–24.
22. Stene T. Vincristine's effect on dentinogenesis in rat incisor. J Dent Res 1979;87:34–9.
23. Noguiera TD, Stene T, Koppang HS. Colchicine's effect on rat incisor odontoblasts and dentinogenesis. Scand J Dent Res 1981; 89:48–58.
24. Kurahashi Y, Nagai N, Wertanabe K, Watanabe H, Yama K. Chronological observation of the odontogenesis of rat molars. Bull Tokyo Med Dent Coll 1968;4:147–59.
25. Koppang H. Effect of cyclophosphamide on dentinogenesis in the rat incisor: fluorescence microscopic and microradiographic investigations. Scand J Dent Res 1981;89:59–70.

Received for publication 6 November 1995
Accepted 3 January 1996