Cyclophosphamide-Induced Abnormalities in the Incisors of the Rat

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A single injection of 75 mg/kg cyclophosphamide caused gross dental abnormalities in rats. Broken, malformed, overgrown, and "extra" incisors developed several weeks after drug treatment. Radioautographic investigations show no unusual features in the morphology or labeling with H^3-thymidine in the odontogenic cells. The results suggest that the cytotoxic effect of the drug is a temporary one which induces a residual alteration of tooth growth.

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Cyclophosphamide is an alkylating agent with an antitumor effect,[1] which is also used as an immunosuppressant. The antitumor effect of the drug is believed to be due to its ability to cross-link the guanine bases in double-stranded DNA, inhibiting cell division.[2]

Rats treated with a single intraperitoneal dose of 75 mg/kg of cyclophosphamide have evidence of acute toxicity but recover in a few weeks.[3,4] However, slow progressive wasting which results in the death of a large percentage of the animals occurs three to four months after treatment with this drug.[5–8] In an earlier publication,[8] we reported that this high delayed mortality after cyclophosphamide treatment was the result of simple starvation caused by bizarre tooth abnormalities and a consequent inability of the animals to eat. In this paper, we will discuss the pathogenesis of these dental abnormalities as determined by radiographic, histologic, and radioautographic evaluations of animals treated with cyclophosphamide.

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Materials and Methods

Animals.—Rats used in this experiment were an inbred strain derived from Wistar albino rats, brother-sister mated for 74 generations to assure that they were isogeneic. Skin transplant experiments also demonstrated that the animals were isogeneic.

Drug treatment.—Five male and five female six-week-old rats received a single intraperitoneal injection of cyclophosphamide, 75 mg/kg, dissolved in sterile water. The rest of our large colony of more than 100 rats served as a control inasmuch as no untreated animals have shown similar dental abnormalities at any time.

The animals were examined every two to five days for the next seven months. Animals which had dental abnormalities and weight loss were placed on powdered food to supplement their normal diet of hard food pellets.

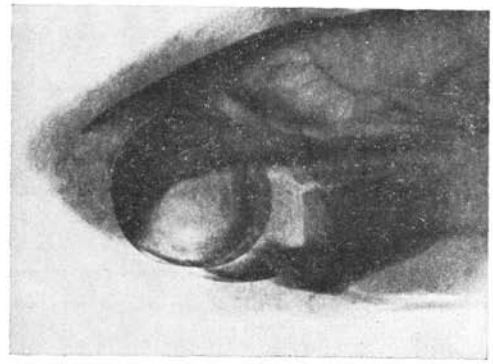

Fig 1.—X-ray film of rat with extra-long maxillary incisors. Incisors have grown so long that they form almost a complete circle with the teeth growing through the palate.

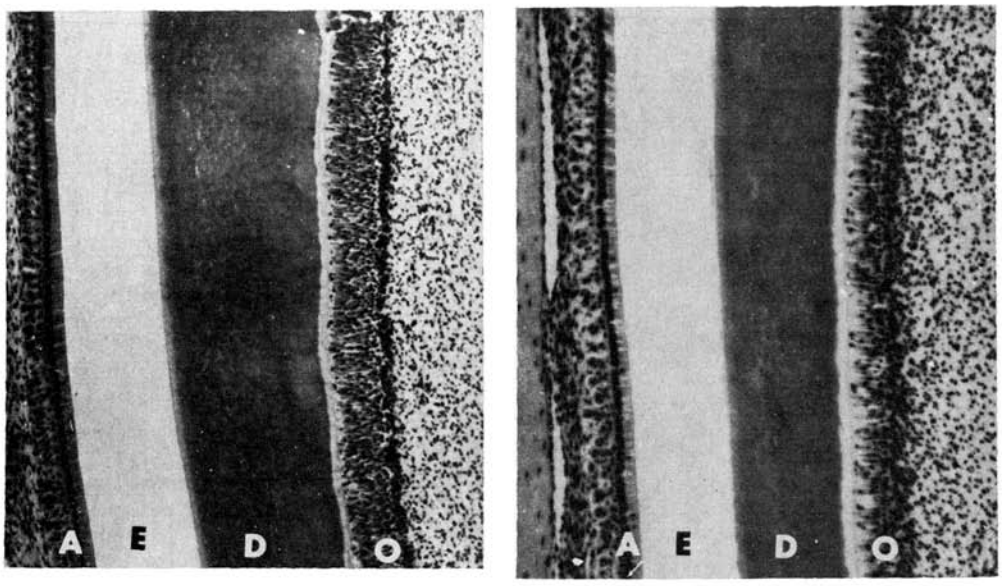

FIG 2.—Small portions of the labial side of an extra-long incisor (left) from a rat injected with cyclophosphamide (75 mg/kg) and a normal incisor (right) from a control rat. Ameloblasts (A), enamel space (E), dentin (D) and odontoblasts (O) of the experimental rat appear normal seven months after the injection. Hematoxylin and eosin stain, orig. × 80.

Other animals received standard Purina Mouse Chow and all animals received water ad libitum.

HISTOLOGY.—For histologic studies, three treated and two normal animals of like age were used. Two treated rats, one male and one female, were selected because they had extra long maxillary incisors, while the third rat, a female, had extra incisors in the mandible. These three animals had been fed powdered food after abnormal teeth prevented normal eating. Rats were sacrificed by perfusion; the heads were removed and immersed in Bouin's fixative. After fixation, the upper and lower jaws were dissected, washed, and suspended for decalcification in 20% EDTA (saturated solution) which was adjusted to pH 7 with NaOH. Decalcification time ranged from 45 to 60 days. After decalcification, the tissues were washed in running water for 24 hours, dehydrated through a graded series of alcohols followed by dioxane, infiltrated and embedded in paraffin. Serial sections were made to cut through the incisors longitudinally so that different regions among the incisors could be examined. Sections were stained with hematoxylin and counterstained with eosin.

RADIOAUTOGRAPHY. — Two treated rats which had both long maxillary and extra mandibular incisors, and one like-age normal control were injected intraperitoneally with 1 μCi H^3-thymidine (specific activity 6.7 Ci/mmole)/gram body weight and sacrificed one hour later. The treated rats had been injected with cyclophosphamide seven months prior to the preparations for radioautography. The animals were anesthetized and perfused with 10% formalin buffered with 1% $CaCl_2$. The heads were removed and further fixed in the same fixative. After fixation, the anterior portions of the maxilla and the mandible were dissected and washed for 24 hours before decalcifying in 20% EDTA as described above for 60 days. Tissues were again washed, dehydrated, infiltrated, and embedded in paraffin, and sectioned.

Glass slides with 7μ sections were dip-coated with nuclear tract emulsion* and exposed for 22 days in the dark in a refrigerator. They were developed for five minutes at 18 C in developer† diluted 1:2 with water, cleared, washed, and stained with Ehrlich's hematoxylin. Alternate slides from serial sections of each specimen were stained with hematoxylin and eosin for histologic evaluation.

RADIOGRAPHY.—X-ray films were made of

* Kodak NTB-3.
† Kodak D-19.

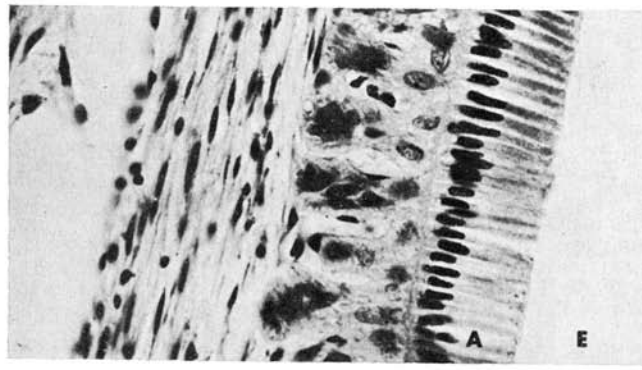

FIG 3.—Small portion of incisor of a treated animal shown in Fig 2 (left) showing cells of the enamel organ and connective tissue. Tall ameloblasts (A) are aligned along the enamel space (E). Cells of the enamel organ are found at the base of the ameloblasts. Hematoxylin and eosin stain, orig. × 700.

the head of one animal with very long incisors, and of the mandible of others with extra incisors.

Results

CLINICAL OBSERVATIONS.—The details of the clinical observations are outlined in the Table and have been reported in our previous publication.[8] Dental abnormalities were of three types: (1) temporarily shortened or completely missing maxillary incisors (all ten rats), (2) extremely long maxillary incisors which frequently were abnormal in color (four rats) (one had chalky white distal surfaces), (3) well-developed extra mandibular incisors (five rats).

Short (broken) teeth first were noted at 65 days after drug treatment, but, on average, they appeared about 100 days after treatment.

Extra long teeth developed several weeks after we first noted the normal incisors were broken or missing. In the following 20 to 30 days, they grew to sizes which were noticeably longer than normal. While normal maxillary incisors of our rats rarely exceeded 3 or 4 mm in length, the long teeth could be from 6 mm to even 25 mm in length. Some even grew around and into the palate (Fig 1).

Supernumerary incisors appeared in the mandible as early as 89 days, but, on the average, they were noted at about 100 days after treatment with cyclophosphamide. These apparent extra teeth were not necessarily preceded by broken or missing teeth. The "normal" mandibular incisors, however, were usually loose and could be easily removed with forceps at the time the extra teeth first appeared. Extra teeth developed in four out of five females, but in only one of five males. In all five animals, the extra incisors were found bilaterally. In most animals, both extra teeth appeared at the same time. Occasionally, an extra tooth erupted between the original incisors, but usually each was found lateral to the existing mandibular incisor.

EXTRA LONG MAXILLARY INCISORS.—Histological observations of serial sections through the extra long incisors of the treated rats (three animals) revealed no detectable abnormalities associated with either hard or soft tissues of these teeth seven months after the injection of cyclophosphamide. The ameloblasts and odontoblasts in different stages of differentiation

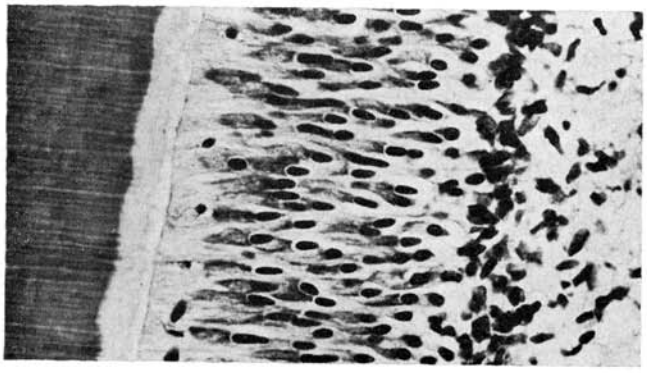

FIG 4.—A small portion of the incisor of a treated animal shown in Fig 2 (left) showing from left to right layers of dentin, predentin matrix, odontoblasts, and connective tissue cells in pulp. Dentinal tubules are clearly visible in dentin and odontoblast layer appears to be made up of numerous cells. Hematoxylin and eosin stain, orig. × 700.

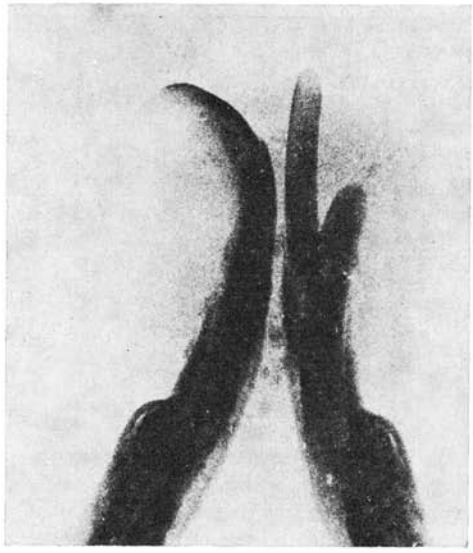

FIG 5.—X-ray radiogram of a cyclophosphamide-injected rat showing an extra incisor. Two teeth are contained in one alveolar socket on the right. On the left, the old (pre-experimental) incisor has been removed leaving only the new "extra" incisor.

were arranged along the continuously growing incisors. Undifferentiated odontogenic cells were localized at the apex of the incisors in the region of the epithelial loop. Cells in more advanced stages of differentiation were found toward the incisal edge of the teeth. The labial sides of the incisors of both treated and untreated rats were capped by enamel, and the sections through this region revealed layers of ameloblasts, enamel, dentin, and odontoblasts as seen in Figures 2 left and 2 right which show the labial sides of the incisors of the treated and untreated rats, respectively. As in the incisors of untreated rats, the ameloblasts of the treated animals were tall columnar cells which formed a single layer localized between the enamel surface and the cells of the stratum intermedium (Fig 3). The dentin layer of treated and untreated rats revealed areas of calcified dentin, globular dentin, and uncalcified predentin matrix. Parallel arrays of dentinal tubules were embedded in the dentin extending from the odontoblasts to the dentinoenamel junction. The odontoblasts also formed a layer along the surface of predentin matrix in the pulp space (Fig 4).

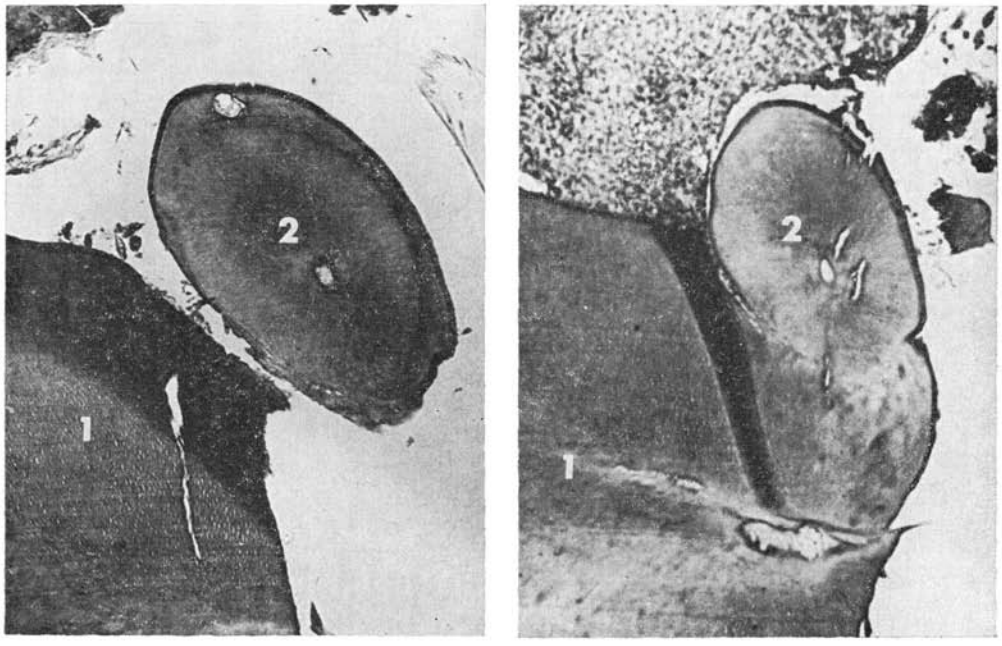

FIG 6.—Two examples of serial sections through apical portion of an apparent extra incisor of the mandible from a cyclophosphamide-injected rat. Left, tooth 1 and 2 are side by side and appear unconnected. Right, a section taken from a more basal portion of the teeth, the two teeth are fused. Tooth 2 seems to be a bud off the incisor 1. Hematoxylin and eosin stain, orig. × 200.

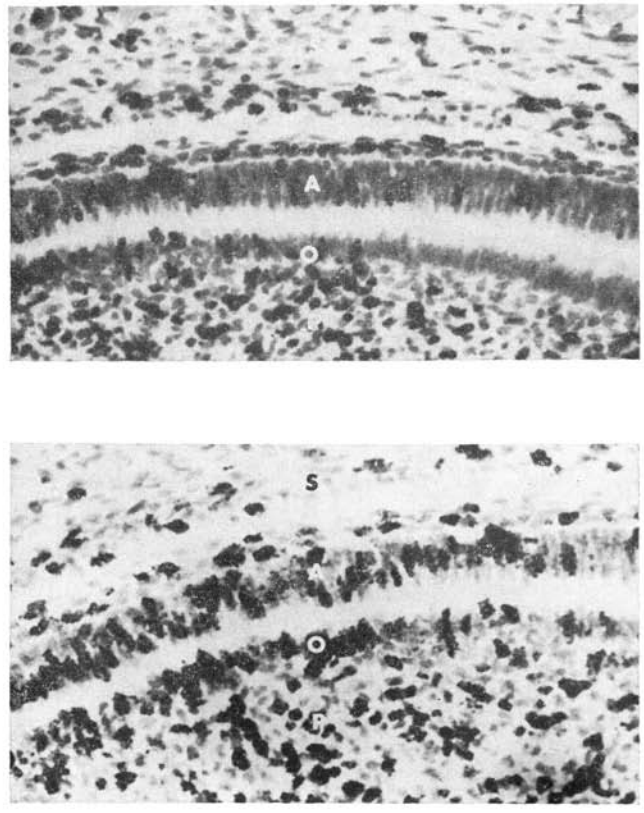

FIG 7.—H^3- thymidine radioautograph of a cyclophosphamide-injected (top) and a control (bottom) rat showing the labeling at the apex of incisors. Precursor cells of ameloblasts (A) and preodontoblasts (O) as well as the cells of pulp (P) and dental sac (S) are labeled in both rats. Hematoxylin stain, orig. × 300.

TABLE

DAYS AT WHICH MALFORMATION WAS FIRST NOTED

Rat	Broken or Absent Maxillary Incisor(s) Right	Broken or Absent Maxillary Incisor(s) Left	Extra Long Maxillary Incisor(s) Right	Extra Long Maxillary Incisor(s) Left	Extra Mandibular Incisors Right	Extra Mandibular Incisors Left	X-ray	Comments
A ♀	124				124	124		
B ♀		141			89	89		
C ♀		65	118	118			X	Histology studied.
D ♀	65	65	84	84	103	110		Tritiated thymidine radioautography.
E ♀		75			124	127	X	Histology studied.
F ♂	110	103						
G ♂		154					X	
H ♂	82	84	99	99	97	118		Tritiated thymidine radioautography.
I ♂	96	117	118	118			X	Histology studied; chalky maxillary teeth.
J ♂	120						X	

For each animal, the number indicates the total days from treatment with one injection of 75 mg/kilo of cyclophosphamide to the first appearance of the specified dental abnormality on that side. Specific animals which were studied by histology; radioautography and roentgenography are noted in the comments section, as are other unusual abnormalities of tooth appearance and/or growth.

STUDIES OF "SUPERNUMERARY" MANDIBULAR INCISORS.—Attempts were made to determine whether the extra "supernumerary" teeth seen in the mandible of these animals were complete teeth. Two approaches were taken. X-ray examinations of animals with "supernumerary" teeth showed that the extra tooth appeared to be contained in the same alveolar socket with a normal mandibular incisor (Fig 5). Therefore, this incisor that appeared to be an extra tooth is, in fact, not a complete tooth.

Histological observations on one animal with two extra teeth confirmed this finding. In serial sections of the mandible of this animal, the extra tooth abruptly appeared at the level of the alveolar bone. On one side, the "supernumerary" incisor was determined to be a branch of one of the complete incisors. This is shown in Figures 6 left and 6 right, serial sections of the mandible at different levels. There is an extra tooth adjacent to the normal incisor in one plane of section (Fig 6 left). However, sections at a different level show that the small tooth fuses with the normal incisor at the level of the gingival mucosa (Fig 6 right).

RADIOGRAPHIC STUDIES. — Radiographic studies of the cyclophosphamide-injected rats indicated that the odontogenic cells in these rats undergo mitosis as do those in the control rat. Most of the cells with labels were localized at the apex of the incisors near the apical loop in both treated and control rats (Fig 7 top and 7 bottom). The labels were present in cells of the pulp, preodontoblasts, preameloblasts, cells of the stratum intermedium, and of the dental sac. The ameloblasts and odontoblasts located toward the incisal edge were not labeled in both treated and control rats.

Discussion

The present examinations of the grossly abnormal incisors, which developed following a single injection of cyclophosphamide, have revealed no unusual microscopic features. These results suggest that the damaging effect of a single dose of the drug on tooth development is temporary.

The histological observations of the extra-long incisors failed to reveal any significant abnormalities involving the teeth or the alveolar sockets. ^{3}H-thymidine radioautography again showed no significant differences in the labeling pattern between treated and control rats. In both animals, cells in the region of the incisors near the apical loop were labeled. This region corresponds to the "presecretory portion" of incisors where mitotic figures are usually concentrated.[9] Thus, it appears that the odontogenic cells of the experimental rats proliferate as do similar cells in untreated rats. However, we cannot rule out the possibility of differences between the treated and untreated rats in the number of cells undergoing mitosis or in the rate of mitosis. The small sample size used for radioautography in this study does not allow statistical analysis of numbers of labeled cells.

"Supernumerary" teeth were shown to be contained in the same alveolar socket with one of the normal incisors. The clinical observation that the original incisor was usually loose and could be easily removed after the development of a "supernumerary" tooth adjacent to it, together with the X-ray and histologic evaluations showing that this "supernumerary" tooth is contained in the same socket, tend to support a pattern of pathogenesis. Apparently at the time of treatment with the large dose of cyclophosphamide, sufficient injury is done to the cells of the developing tooth so that the production of dentin and/or enamel is temporarily arrested.

Koppang [10,11] has reported that the mandibular and maxillary odontogenesis was temporarily but completely interrupted in rats following a single injection of a high dose of cyclophosphamide (40 to 75 mg/kg). At lower doses (25 mg/kg), she encountered other dental abnormalities, such as circular and mesiodistal dental constrictions, nichelike defect in the dentin, and the formation of cystic spaces in the pulp space. The complete interruption in the odontogenesis, as well as the other listed defects, could create a gap between pretreatment and posttreatment growth of the incisors involved. Then, as seen in this study, the pretreatment incisor of the mandible remains in the alveolar socket but without connection with the incisor which developed (formed) posttreatment. For reasons unknown, this newly developing tooth does not push out the remnants of the pretreatment incisor, but rather grows out obliquely from the same socket to appear in the oral cavity as an apparent supernumerary incisor.

Apparently the odontogenic cells can be affected in different ways even in the same animal after cyclophosphamide. In one animal studied, the odontogenesis continued in an asymmetrical way on one side, and an apparent supernumerary tooth developed as a bud

directly continuous with the original incisor. This is most likely the result of the alterations in the alignment of the odontoblasts and ameloblasts during the dentin and enamel formation. This misalignment probably occurs as a consequence of cyclophosphamide affecting the odontoblasts and ameloblasts directly or, as suggested by others[10,12] by affecting the precursors of these cells.

Our clinical observation that maxillary incisors that developed three months after treament were occasionally abnormal in color and had chalky white distal surfaces is probably a reflection of a disturbance in calcification secondary to partial injury to the odontogenic cells.

In the rat, incisors grow constantly and are worn down by the habitual chewing on hard food pellets. The constantly growing teeth of the rat are therefore more similar to the growing teeth of children than they would be to adult human teeth. From this experiment, it would seem reasonable to be alert to possible dental abnormalties in children receiving cyclophosphamide either as treatment for neoplasm or as a method of inducing immunological paralysis. To our knowledge, there is no report of similar dental abnormalities in human incisors following use of this drug. However. Adatia[13,14] has reported that defects occur in developing molars of the children with Burkitt's lymphoma who were treated with cyclophosphamide. Apparently odontogenesis continues in these children, but a total or partial destruction of the functional epithelial diaphragm was observed in developing molars, and defects were noted in the distal root and crowns of the molars.[14] Thus, it seems that dentists and physicians should be alert to such a possibility as cyclophosphamide becomes widely used.

Conclusions

A single injection of cyclophosphamide induced abnormalities in the incisors of rats including short, extra-long, or malformed teeth, and frequently apparent supernumerary teeth. Histological and radioautographic observations made seven months after the injection failed to reveal detectable abnormalities in the morphology or labeling with H^3-thymidine in the odontogenic cells of these incisors. Observations of the apparent supernumerary tooth revealed that a remnant of a pre-experimental incisor was retained in the alveolar socket while the "extra" tooth was a new incisor which grew postexperimentally in the same socket. These observations indicate that the process of odontogenesis is temporarily interrupted by cyclophosphamide and that this interruption leads to a break in the continuously growing incisors. In addition, one mandibular incisor of a treated rat revealed a budlike protrusion on the tooth, suggesting that cyclophosphamide might also have an effect in altering the pattern of odontogenesis without interrupting it completely.

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References

1. Livingston, R.B., and Carter, S.K.: *Single Agent in Cancer Chemotherapy*, New York, Plenum Publishing Corp., 1970.
2. Brookes, P.: Reaction of Alkylating Agents with Nucleic Acids, In Plattner, P.A. (ed.), *Proceedings of International Symposium on Chemotherapy of Cancer, Lugano*, Amsterdam, Elsevier, pp 32–43, 1964.
3. Lane, M.: Preliminary Report of Animal Studies with Cytoxan (Cyclophosphamide), *Cancer Chemother Rep* 3:1–5, 1959.
4. Paterson, P.Y., and Hanson, M.A.: Cyclophosphamide Inhibition of Experimental Allergic Encephalomyelitis and Cellular Transfer of the Disease in Wistar Rats, *J Immunol* 103:795–806, 1969.
5. Feringa, E.R.; Wendt, J.S.; and Johnson, R.D.: Immunosuppressive Treatment to Enhance Spinal Cord Regeneration in Rats, *Neurology* (Minneap) 24:287–293, 1975.
6. Miller, E.E., and Tsou, K.C.: Comparative Study of Lymphoma-Bearing Rats Treated with DAAP, DMAP, Cytoxan, *Proc Am Assoc Cancer Res* 7:50, 1966.
7. Stenram, U., and Nordlinder, H.: Delayed Death in Rats Treated with Cyclophosphamide, *Nature* 219:1154, 1968.
8. Vahlsing, H.L.; Feringa, E.R.; Britten, A.G.; and Kinning, W.K.: Dental Abnormalities in Rats After a Single Large Dose of Cyclophosphamide, *Cancer Research* 35: 2199–2204, 1975.
9. Smith, C.E., and Warshawsky, H.: Cellular Renewal in the Enamel Organ and the Odontoblast Layer of the Rat Incisor as Followed by Radioautography Using ^{3}H-Thymidine, *Anat Rec* 183:523–561, 1975.

10. KOPPANG, H.S.: Histomorphologic Investigations on the Effect of Cyclophosphamide on Dentinogenesis of the Rat Incisor, *Scand J Dent Res* 81: 383–396, 1973.
11. KOPPANG, H.S.: Autoradiographic Investigations on the Effect of Cyclophosphamide on Dentinogenesis of the Rat Incisor, *Scand J Dent Res* 81:397–405, 1973.
12. ADATIA, A.K.: The Effects of Cyclophosphamide on Odontogenesis in the Rat, *Arch Oral Biol* 20:141–144, 1975.
13. ADATIA, A.K.: Burkitt's Tumor in the Jaws, *Brit Dent J* 120:315–326, 1966.
14. ADATIA, A.K.: Response of the Dental Elements to Chemotherapy of Burkitt's Tumor, *Int Dent J* 18:646–653, 1968.