## Ultrastructural Observation of Lysosomal Activation as a Possible Cause of Incisor Malformation in Cyclophosphamide-Treated Rat

In a pilot ultrastructural study of the acute effects of cyclophosphamide on the incisor primordium in rat, an appreciable amount of autophagic vacuoles were found in the dental pulp and in the odontoblasts. Autophagic vacuoles are generally considered to be related to the lysosomal system, and it is possible that lysosomes were increased in amount.

Interestingly, a survey of the literature showed that perhaps the most constant feature in treatment of experimental tumours with cyclophosphamide was the appearance of autophagic vacuoles and activation of the lysosomal system. The possible significance of this observation is discussed.

One of the late effects of a single sublethal dose of cyclophosphamide in rats is irreversible damage to the incisors[1,2], apparently due to effects on the dental pulp[3]. It was therefore considered to be of interest to examine the initial ultrastructural changes of the incisor primordium in rat following cyclophosphamide treatment.

*Experiments.* The experiments were performed with new-born and 3-week-old Sprague-Dawley rats. 2 rats, weighing 55 g each, were given a single i.p. injection of 110 mg cyclophosphamide/kg body weight, and 2 newborn rats were given 40 mg/kg body weight s.c. They were killed 7 or 24 h later. Control rats were given the same volume of distilled water. Cyclophosphamide ('Sendoxan') was kindly supplied by AB Pharmacia, Sweden.

At sacrifice, pieces of the dental pulp were fixed in 1.7% glutaraldehyde in 0.1 *M* phosphate buffer, pH 7.4, for 3 h, rinsed in 0.1 *M* phosphate buffer with 7.5% sucrose overnight, postfixed in 1% $OsO_4$ in 0.1 *M* phosphate buffer for 5 h, and embedded in Epon. Sections were cut with an LKB ultramicrotome, contrasted with uranyl acetate-lead citrate and examined in a Zeiss electron microscope.

*Observations.* In all cyclophosphamide-treated rats, especially those killed after 24 h, autophagic vacuoles and an apparently increased amount of lysosomes were found in the pulp cells and in the odontoblasts (Figure). The Golgi system was prominent. Lamellar bodies were often seen, but they occurred also in the controls. (It is a common finding that myelin figures occur in increased amount in aldehyde-fixed material.) The mitochondria were sometimes slightly swollen with derangement of the cristae, but that was also seen in several of the controls. Therefore this observation cannot be ascribed to the cyclophosphamide treatment, but may be a fixation artefact. Autophagic vacuoles were not found in the controls. The rough-surfaced endoplasmic reticulum, nuclei and nucleoli showed no changes. The enamel organ showed less pronounced changes than the dental pulp.

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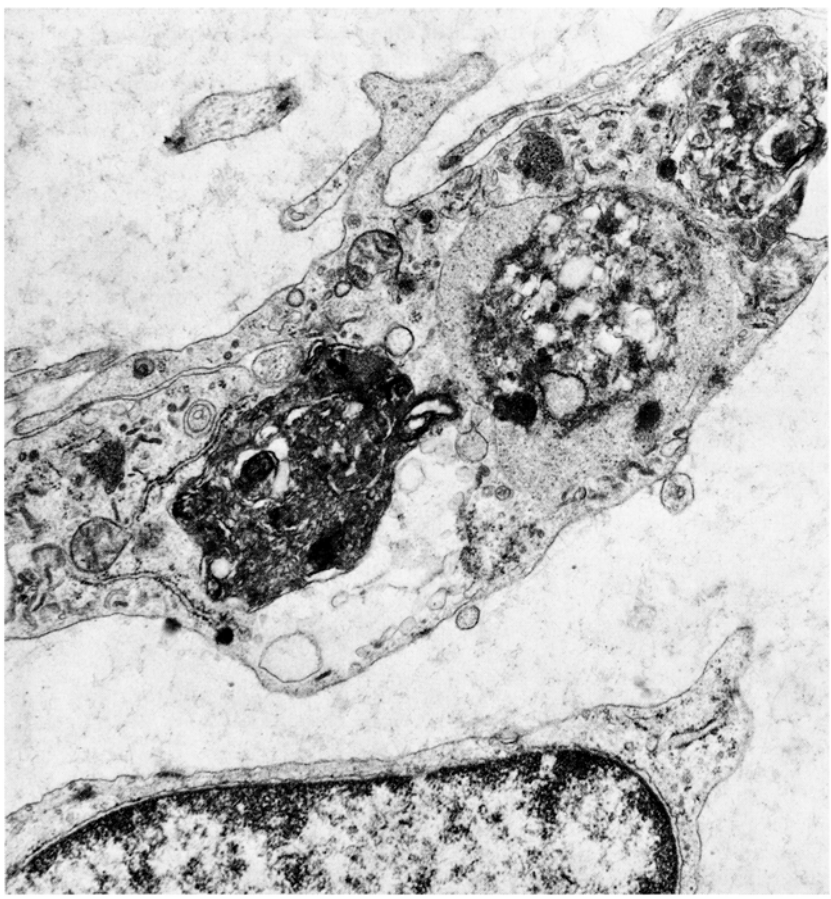

Dental pulp cell of adult rat given 110 mg cyclophosphamide/kg body weight 7 h prior to death. Autophagic vacuoles, lysosomes and lamellar figures are seen. Mitochondria, rough-surfaced endoplasmic reticulum, cellular membrane and nucleus are essentially normal. Uranyl acetate-lead citrate. × 18,000.

*Discussion.* Though the animals studied are few, it is quite evident that autophagic vacuoles appear following cyclophosphamide treatment, and there may be a hypertrophy of the Golgi system. There thus appear to be changes in the lysosomal system, which is generally considered to be responsible for the autophagic vacuoles. Further studies, including lysosomal enzymes, are desirable. Early changes were not observed in the rough-surfaced endoplasmic reticulum, free ribosomes and polysomes and the nuclei including nucleoli.

It is interesting to note that lysosomes have been found in odontoblasts in large numbers and have been considered to take part in the formation of dentine[4].

The literature on the ultrastructural effects of cyclophosphamide is scant and mainly consists of a few studies on tumour cells. Most of these papers[5-8] describe a hypertrophy of the lysosomal system and/or appearance of autophagic vacuoles, though the relationship between these alterations is not always stressed. Vitamin A, a lysosomal labilizor, potentiated this effect[6,7]. Interestingly, hypervitaminosis A has been described as decreasing the appositional growth of the rat incisor dentine[9], but this change was later considered to be due to decreased food intake[10].

These observations suggest that the effects of cyclophosphamide, or some of them, may be exerted via the lysosomal system. Mitoses are found during treatment, and it may be the interphasic cells that are sensitive[6]. The questions arise, primarily, whether tumour cells rich in lysosomes are especially sensitive to cyclophosphamide, and if that is of importance in the treatment of human malignancies; and secondly, if the use of lysosomal labilizors, for example in perfusion treatment, may potentiate the therapeutic effect of cyclophosphamide. A third question is to what extent the deleterious effects of cyclophosphamide on normal cells (cf. [2]) are mediated via the lysosomal system, and if the effects can be counteracted by lysosomal stabilizors. Finally, it should be emphasized that even if lysosomes are involved early by cyclophosphamide, they are not necessearily the primary target.

*Zusammenfassung.* In vorläufigen Untersuchungen akuter Effekte von Cyklophosphamid wurden in der Rattenzahnpulpa elektronenoptisch autophagische Vakuolen gefunden, was auf eine Aktivierung der Lysosomen hinweist.

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## The Effect of Methyltestosterone and 17β-Estradiol on DNA Synthesis of Mesonephric Blastemae of Frog Larvae

Previous studies have indicated that frog larvae can be masculinized or feminized by hormonal treatments[1]. In *Rana pipiens* administration of methyltesterosterone (0.1 µg to 5 mg/l of aquarium water) masculinizes the larvae. Estradiol (20 µg to 500 µg/l of aquarium water) feminizes, but above 500 µg/l of aquarium water, estradiol masculinizes the larvae and in addition causes adrenal hyperplasia[2]. It is also known that adrenal hyperplasia and sex reversal are independent responses to estradiol administration. According to Witschi[1], the mesonephric tubules, medullary and efferent parts of the sex glands and the cortical part of the adrenal have a common origin from the mesonephric blastemae. Mittwoch[3] has suggested that sex differentiation may be determined by a quantitative difference in number of cell divisions of the undetermined gonads. The present experiments were designed to learn if hormonal treatments that cause sex reversal in *Rana pipiens* affect DNA synthesis of the mesonephric blastemae.

The methyltestosterone and 17β-estradiol were prepared as stock solutions in absolute alcohol (5 mg and 2.5 mg per ml of absolute alcohol, respectively). Approximately 90 larvae (stage 25 of Shumway[4]) were raised for a period of 2 weeks in bubbled tap water, methyltestosterone (1 mg/l) and 2 concentrations of estradiol (500 µg and 250 µg/l). The solutions were changed daily and the animals fed each day with precooked high protein cereal. Concentrations of estradiol above 500 µg/l were not used since the growth of the larvae was reduced.

The larvae were stage 27 at the conclusion of 2 weeks. At this stage they were washed 3 times with boiled tap water and incubated for 24 h at 20 °C in 2.5 µg/ml of $H^3$-thymidine with the respective hormones present in the tap water. The larvae were washed 3 times in boiled tap

DNA synthesis in mesonephric blastemae of hormone-treated larvae

| Treatments | Biological effect (Chang and Witschi[2]) | Total cpm DNA/Total cpm acid-soluble pool ÷ Total µg DNA |
|---|---|---|
| Controls | ---------- | $9.92 \times 10^{-2} \pm 1.84 \times 10^{-2}$ |
| Methyltestosterone (1 mg/l) | Complete masculinization | $14.51 \times 10^{-2} \pm 1.52 \times 10^{-2}$ |
| 17β-Estradiol (500 µg/l) | Partial masculinization | $9.05 \times 10^{-2} \pm 3.47 \times 10^{-2}$ |
| 17β-Estradiol (250 µg/l) | Complete feminization | $8.81 \times 10^{-2} \pm 2.33 \times 10^{-2}$ |

3 separate experiments were performed and the average values are reported.