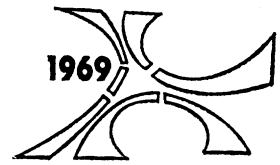
Is There an Optimal Stage for the Induction of Cleft Palate in the Mouse?



D. O. E. GEBHARDT Ph.D.
G. J. SCHADE, D.D.S.
Amsterdam, Holland

It is well-known that each organ of the embryo passes through a period of development during which it is susceptible to the action of a teratogen. Since a certain malformation can be induced by various means, the question arises whether each teratogen has its own critical period for the production of such a defect. In the case of cleft palate, there is some evidence for the existence of different critical induction periods for different agents (*5*). For instance, Murakami (*12*) showed that irradiation of CF I mice on the eleventh day of pregnancy gave a higher incidence of embryos with cleft palate than when irradiation took place on any other day. On the other hand, Curley and co-workers (*2*), who induced cleft palate with 6-aminonicotinamide, found that the embryos were most sensitive to the action of the teratogen on the thirteenth day of pregnancy. Unfortunately the latter authors did not use the same strain of mice as Murakami. It is therefore difficult to compare their results, especially since Fraser (*6*) has shown that, in the A/Jax strain, the palate closes later than in the C57BL strain. This consideration made it of interest to repeat the experiments of Murakami and of Curley and his associates using one strain of mice only. It was also decided to extend the work of these authors by determining the period in which dexamethasone and cyclophosphamide could induce cleft palate. The teratogenic properties of these two drugs have been described respectively by Pinsky and diGeorge (*13*) and by Gibson and Becker (*7*).

The data collected during the investigation made it also possible to determine the sex ratio of embryos with cleft palate. According to Fogh-Andersen (*4*) and also Meskin and associates (*10*), the human female is more frequently born with a cleft than the male. Dagg and co-workers (*3*) found in mice more female than male fetuses with a cleft when the mothers had been treated with 5-fluorouracil. The question was

The authors are affiliated with the National Institute of Public Health, Utrecht, and the Department of Oral Surgery, University Hospital, Amsterdam.

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therefore asked whether X rays, 6-aminonicotinamide, or dexamethasone would produce significantly more cleft palates in female than in male fetuses.

Methods

All experiments were done with a strain of Swiss albino mice which has been randomly bred in our institute, as a closed colony, since 1962. The spontaneous frequency of cleft palate in these mice, based on an examination of 1633 control embryos from 159 dams, was 0.2%. The mice were mated by caging two nine-weeks-old nullipara, with an average weight of 28 g., with one male. The females were examined daily at 8:30 a.m. for vaginal plugs. When these were found, the day was called the first day of pregnancy. This definition of timing corresponds to the nomenclature of Kalter (*9*). On the assumption made by Walker and Fraser (*16*) that fertilization takes place at 2 a.m., it was estimated that the palate of control embryos closed at the end of the fifteenth day. This estimate was based on the finding that all of the palates of a sample of 100 15½-day-old control embryos were still open, whereas almost all palates from the same number of 16⅓-day-old embryos were completely fused.

The mice had free access to Muracon food pellets and to water. On different days of pregnancy, about twenty mice were treated once with one of the teratogenic agents. In general, three dose levels were used: one yielding a high, one a medium, and one a low percentage of cleft palates. The low dose series was considered of especial importance since Wilson (*17*) has pointed out that the most susceptible period can only be found if a minimal dose of a teratogenic agent is administered.

The mice were killed on the eighteenth day of pregnancy and the embryos were then removed from the uterus. A cut was made through the mouth of each embryo to facilitate the macroscopic examination of the palate. In agreement with Dagg and co-workers (*3*), the sex was only determined in litters in which at least one, but not all fetuses, had a cleft palate. The anogenital distance served as the criterion for differentiating between the sexes. At birth this distance is about twice as long in the male as in the female mouse.

The dexamethasone (Decadron) was purchased from Merck and Co., Rahway, New Jersey, the 6-aminonicotinamide came from Sigma, Saint Louis, Missouri, and the cyclophosphamide (Endoxan) was obtained from Asta-Werke, Brackwede, Germany. In the irradiation experiments, the exposure rate was 35 R/min. Each mouse was placed in a small perspex container and irradiated separately. The dose absorbed by the embryos was considered to be identical to the air dose. The distance of the gravida from the x-ray tube was 50 cm. The radiation apparatus was run at 250 kV and 4.6 mA with filtration of 1.1 mm Cu and 1 mm Al.

Results

In Figure 1, the percentages of induced cleft palate are given as a function of the day when the pregnant mice were irradiated with either 150 R, 200 R, or 250 R. (In Figures 1–5, each point represents a percentage based in general on the examination of about 150 embryos). The critical period for the induction of cleft palate with X rays is shown to extend from about the tenth to the thirteenth day. The highest response was found on the eleventh and twelfth day of pregnancy. When the mice were irradiated with 250 R on the tenth day or earlier, most embryos were resorbed. In that case, it was not possible to determine the incidence of cleft palate with the same precision as when the sample consisted of 150 embryos. However, it is unlikely that the critical period should commence long before the tenth day because non-embryotoxic doses (200 R or 150 R) were then also incapable of producing more than a few per cent of clefts.

The radiomimetic, cyclophosphamide, showed a somewhat similar critical period (Figure 2). It was impossible to determine the incidence of cleft palate when this agent was injected on the ninth or tenth day because a) at the dose levels of 20 mg/kg or 15 mg/kg none of the embryos survived such treatment; and b) at a lower dose level (10 mg/kg), the incidence of cleft palate was reduced to control values. The critical period for dexamethasone (Figure 3), on the other hand, did not coincide with that found for X rays or cyclophosphamide. It was shifted by twenty-four hours to a later stage of embryonic development (Figure 4).

6-Aminonicotinamide was also capable of inducing cleft palate on the

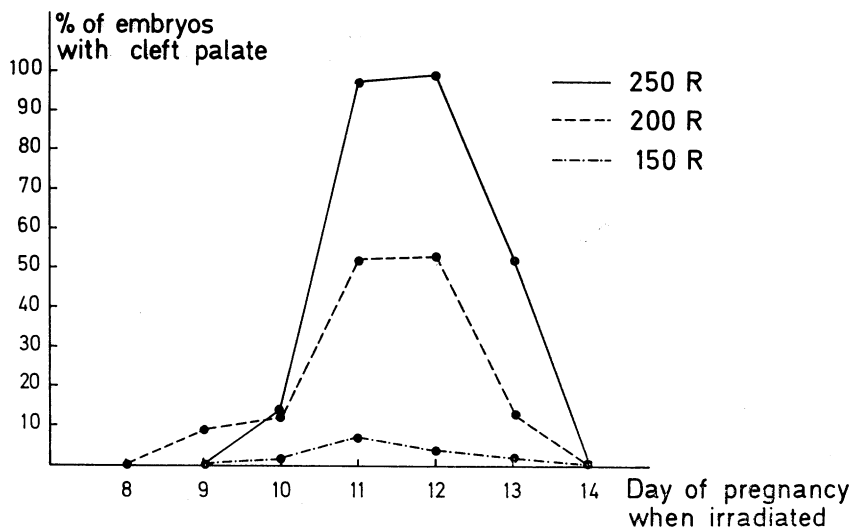

FIGURE 1. Summary of x-ray treatment data.

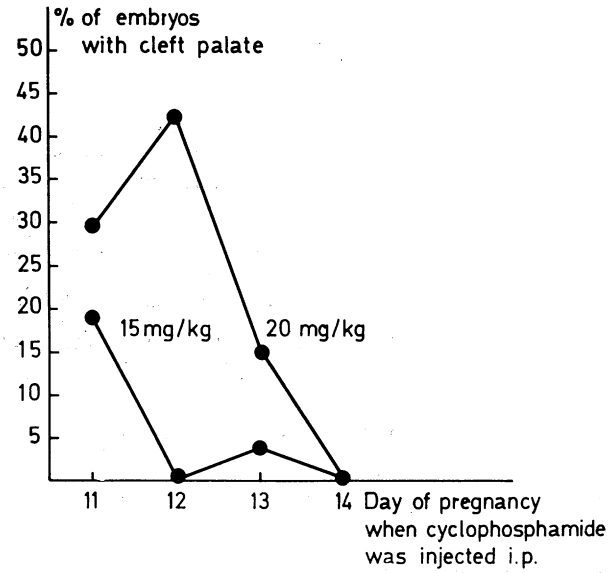

FIGURE 2. Summary of cyclophosphamide treatment data.

fourteenth day (Figure 5). However, the highest response with this agent was obtained earlier than when dexamethasone was injected.

The comparison between the frequency of cleft palate induction in male and female embryos is shown in Table 1. The chi-square test was used to determine whether there was a statistically significant difference between these values at the 5% level. In contradistinction to the

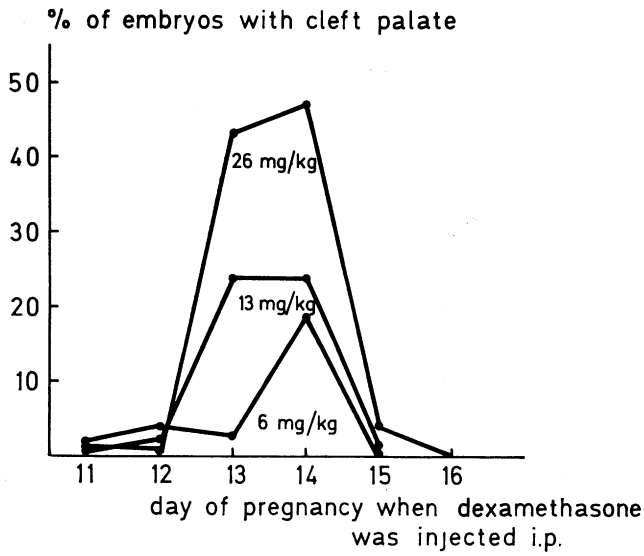

FIGURE 3. Summary of dexamethasone treatment data.

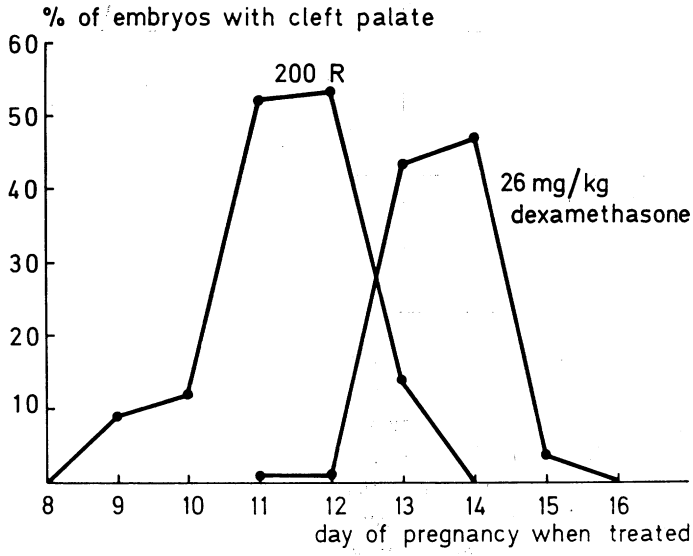

FIGURE 4. X ray and dexamethasone combined.

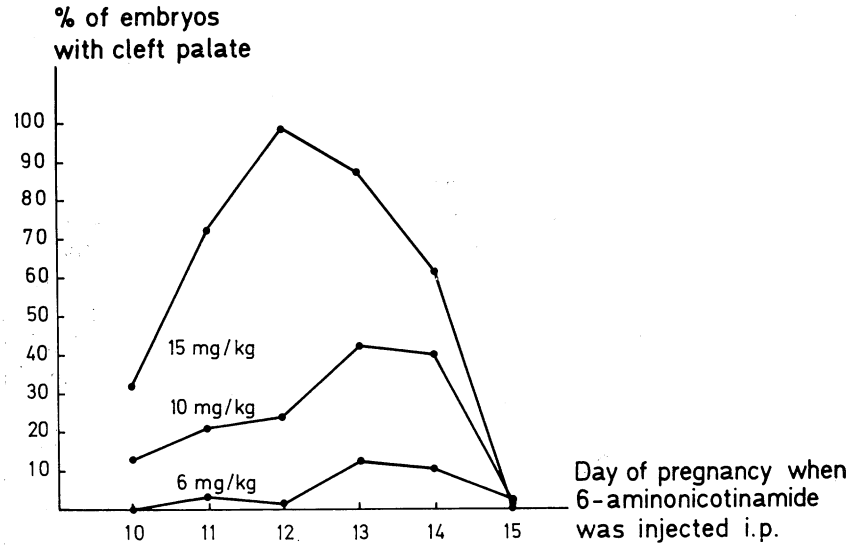

FIGURE 5. Summary of 6-aminonicotinamide treatment data.

results of Dagg (*3*), who induced cleft palate with 5-fluorouracil, it was found that neither X rays, dexamethasone, nor 6-aminonicotinamide could induce more cleft palates in females than in male embryos.

Discussion

The sensitive period for the induction of cleft palate by X rays, as shown in Figure 1, has also been found by Murakami (*12*). It differs

TABLE 1. Summary table of results.

teratogen	*number and (%) of male embryos with cleft palate*	*number and (%) of female embryos with cleft palate*	*significant difference (chi-square test) (.05 level)*
5-fluorouracil (Dagg)	489 (37)	676 (45)	yes
dexamethasone	267 (43)	253 (47)	no
6-aminonicotinamide	157 (38)	126 (34)	no
X rays	94 (39)	98 (42)	no

from the one presented by Russell and Russell (*14*) who described two critical periods: an early one from day 7 to 8 and a later one from day 10 to 14. Neither Murakami nor we have been able to confirm such a biphasic induction of cleft palate. It is of interest that an increase in the dose of irradiation does not cause an extension of the critical period, although it does increase the percentage of clefts. In this respect, our results also differ from those of Russell and Russell.

The critical period of cleft palate induction by cyclophosphamide (Figure 2) has also been described by Gibson and Becker (*7*). It is probably no coincidence that the radiomimetic alkylating agent should induce this malformation during the same period as X rays. However, both Bodenstein (*1*) and Haddow (*8*) have stressed that there are definite differences in their detailed mechanism of action.

As far as we know, this is the first time that the critical period for the induction of cleft palate by dexamethasone has been described (Figure 3). In agreement with Trasler and associates (*15*), we were unable to induce a cleft after the closure of the palate. It is of interest that dexamethasone can still produce clefts twenty-four hours after X rays have become ineffective (Figure 4). Furthermore, it is possible to extend the susceptible period by injecting higher doses (Figure 3). This finding is not restricted to cleft palate formation but is, in fact, quite common in experimental teratology (*17*).

The critical period, found on injecting 6-aminonicotinamide (Figure 5), does not differ much from the one described for dexamethasone. However, in the former case, the optimal response shifts towards younger stages when higher doses are used. At present, this remains unexplained, as does the finding that the optimal response for the induction of clefts with cyclophosphamide shifts towards a later stage on increasing the dose (Figure 2).

There was no preferential induction of cleft palate in male or female embryos when three of the present agents were used. It is therefore unlikely that dexamethasone, 6-aminonicotinamide, or X rays act by way of a mechanism similar to that of 5-fluorouracil (*3*).

It has been shown that cleft palate can be induced during different

periods of embryonic development. These critical periods are defined by the agents causing the malformation. It might therefore be useful to differentiate between critical periods which depend on the teratogen being used, and an overall sensitive period, specific for a certain malformation and encompassing all critical periods.

That different critical periods exist was not unexpected considering the many ways by which one and the same malformation can arise. Morris and Greulich (*11*) showed, for instance, that chlorcyclizine caused cleft palate by inhibiting the rotation of the palatal processes to the horizontal position, while excessive amounts of vitamin A inhibit the development of the processes.

It was a surprise to discover that X rays have to be administered almost three days before the closure of the palate, if a cleft is to be induced. Dexamethasone or 6-aminonicotinamide may still induce the malformation on the fourteenth day, but these agents also lose their teratogenic activity long before the palate has closed. Therefore, an analysis of the early alterations of the developing palate is of primary importance for any determination of the cause of palatoschisis in the mouse.

Summary

An answer was sought for the question whether various teratogenic agents have to be applied during the same or during different periods of pregnancy to induce cleft palate in the mouse. The optimal stage for the induction of cleft palate by X rays or cyclophosphamide was the eleventh and twelfth day and by dexamethasone and 6-aminonicotinamide the thirteenth and fourteenth day of pregnancy. This difference makes it plausible that the critical period for the induction of cleft palate depends on the nature of the teratogenic agent. Neither dexamethasone, 6-aminonicotinamide, nor X rays induced more clefts in male than in female embryos. These agents exerted their teratogenic effect only if they were applied long before the closure of the palate.

reprints: *Dr. D. O. E. Gebhardt*
National Institute of Public Health
Sterrenbos 1
Utrecht, Holland

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