Glucocorticoid Inhibition of RNA Synthesis Responsible for Cleft Palate in Mice: A Model*

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Abstract. A study was undertaken to elucidate the molecular mechanisms by which glucocorticoids induce cleft palate in mice. It was hypothesized that a compound such as triamcinolone acetonide inhibits m-RNA synthesis; that this results later in depressed protein synthesis; and that the latter is ultimately responsible for slowed palate formation and cleft palate. Support for the model derives from the fact that the palatine shelves rise and fuse 3-4 days after the most sensitive time of administration of steroid; RNA synthesis was markedly inhibited 6-24 hr after its administration; and coadministration of cycloheximide partially reversed the tendency toward cleft palate formation.

Prenatally administered cortisone produces cleft palate in mice1,2 and has been reported to inhibit mucopolysaccharide synthesis in the fetal mouse palate.3,4 Larsson3 postulated that the cleft palate was due to the block in the synthesis of mucopolysaccharides (supposedly responsible for the "internal shelf force" that raises the secondary palatine shelf from the horizontal position to the vertical). Alternative explanations are oligohydramnios,5 excessive hydration of palatine tissue,6 and protein catabolism.7

To try to clarify the mechanism by which glucocorticoids cause cleft palate in mice, an alternative approach to the problem was taken. The model proposed was predicated on the known inhibition of RNA synthesis by glucocorticoids, responsible for growth suppression in certain target cells in vivo8 and in vitro.9 It was postulated that such an inhibition of mRNA synthesis in the fetal palate would lead to a later inhibition of protein synthesis and hence cleft palate; i.e., protein and RNA synthesis would be out of phase due to the sequential synthesis of mRNA molecules during the 3-4 day period of time that the glucocorticoid was administered and the time that the palatine shelves rise.

Experiments were undertaken to test the model with triamcinolone acetonide,10 since a single dose of extremely small quantities of this glucocorticoid causes cleft palate in mice.11 It was shown that triamcinolone acetonide (hereafter referred to as triamcinolone) inhibits RNA synthesis in mouse embryos within 6 hr of administration, and furthermore that cycloheximide partly prevents induction of the cleft palate.

Materials and Methods. The inbred mouse strain C3H/An (Cumberland View Farms, Clinton, Tenn.) was used. Vaginal plugs on the morning following overnight
mating were considered evidence for conception and the time was called day 0.5 of gestation. Triamcinolone acetone (Kenalog, Squibb, N.J.) and cycloheximide were administered between 10 and 11 a.m. on the appropriate day of gestation. Triamcinolone (10 mg/ml) in an aqueous microsuspension of the manufacturer's vehicle, was injected intramuscularly, while cycloheximide (2.5 mg/ml) dissolved in 0.85% saline was injected subcutaneously. In this study, vehicle was not used as a control for triamcinolone; but the vehicle does not cause cleft palate in C3H mice, a strain in which this malformation does not occur spontaneously.

In studies of the teratogenic and lethal effects of the drugs, pregnant mice were killed by cervical dislocation at day 17.5 or 18.5. The number of viable fetuses (moving spontaneously) and resorptions were recorded and the number of cleft palates was scored by examination of live fetuses under a dissecting microscope.

In other studies the effect of triamcinolone on the rate of RNA and protein synthesis in whole embryos was measured by the incorporation of [14C]orotic acid and [3H]leucine into RNA and protein, respectively. Pregnant mice were killed at the indicated time (Fig. 3), 2 hr after intraperitoneal injection of 25 μCi of [6-14C]orotic acid (38.5 Ci/mol) and 0.5 hr after similar injection of 25 μCi of [4,5-3H]leucine (55,000 Ci/mol). The uterine horns were removed and placed in a Petri dish in ice, and with the aid of a dissecting microscope each embryo was separated from placenta and membranes. Non-resorbed embryos from each pregnant mouse were pooled and weighed. Cold distilled water was added to a final concentration of 5% (w/v) and homogenized in a motor-driven Thomas homogenizer. Four replicate samples of 1.5 ml each were removed, made to 5% trichloroacetic acid (cold), and centrifuged in the cold for 20,000 g-min. Pellets were washed twice with 5 ml of cold 5% trichloroacetic acid and dissolved in 1 ml of Soluene 100 (Packard) at 37°C with shaking overnight. 15 ml of a toluene-2,5-diphenyloxazole-1,4[bis-(5-phenyloxazolyl-2)]benzene mixture was added and 3H and 14C were assayed simultaneously in a scintillation counter with appropriate quench correction with an automatic external standard.

Results. Latent period: One of the most striking phenomena observed in drug-induced teratogenesis is the fact that teratogens have to be administered some time before the teratogenic effect first becomes visible. This time is especially long in glucocorticoid-induced cleft palate. In mice the palatine shelves move to a horizontal position and fuse between 14.5 and 15.5 days, and cortisone has to be administered several days before these events in order to prevent closure of the palate. When single 10 mg/kg doses of triamcinolone were administered in gestation days 10.5-14.5, we observed that the most susceptible time (critical period) in C3H mice was day 11.5 (Fig. 1): 83%
cleft palate resulted. No malformations were produced when the drug was given at day 14.5. Thus the effects of triamecinolone appear similar to that of cortisone\textsuperscript{18} or cortisol\textsuperscript{16} except that the fluorinated analogue is much more potent (32-fold greater in C3H mice than cortisol\textsuperscript{17}).

**Model.** In order to explain the long latent period between the time of the critical period (day 11.5) to the time of palate shelf elevation (day 14.5–15.5), we postulate that during this interval there occurs a series of molecular steps in the differentiation of the palate, one or more of which is blocked by triamecinolone, thus causing cleft palate: (1) differentiation and morphogenesis of embryonic tissue are characterized by a sequential synthesis of different mRNA molecules in echinoderms,\textsuperscript{17} amphibians,\textsuperscript{18} and mammals,\textsuperscript{19} which are stored as long-lived cytoplasmic ribonucleoprotein particles and are responsible for the translation of specific proteins at later times in development\textsuperscript{20}; (2) actinomycin D, an inhibitor of RNA synthesis, causes developmental anomalies in mammals\textsuperscript{21}; (3) cortisol inhibits RNA synthesis in rat thymus.\textsuperscript{8} In view of the above, a tentative working hypothesis has been formulated (Fig. 2). In this model, mRNA\textsubscript{1} is synthesized at 11.5 days and translation of protein\textsubscript{1}, coded by this messenger RNA, occurs between 11.5 and 15.5 days. Proteins synthesized at 11.5 days, *e.g.*, protein\textsubscript{a}, have their information encoded as mRNA some time before, *e.g.*, mRNA\textsubscript{a}. If triamecinolone is administered at 11.5 days, palate formation at 14.5–15.5 days is blocked. This might be explained if triamecinolone at the time of its administration blocked the synthesis of mRNA\textsubscript{1} and ultimately blocked the synthesis of protein\textsubscript{1} by 14.5–15.5 days. Note that if triamecinolone was administered on day 14.5, no malformations occurred (Fig. 1). If this hypothesis is correct, then the simultaneous administration of the teratogen (triamecinolone) and an inhibitor of protein synthesis, such as cycloheximide, would inhibit both mRNA and protein synthesis (cycloheximide blocks the translation of mRNA at time 11.5). Then after both drugs are eliminated from the embryo either through metabolism or maternal renal excretion, mRNA and protein synthesis might resume in phase and no abnormality would express itself. In other words, development might be delayed a short time, but the proper relationship of specific mRNA and proteins synthesized would be maintained.

**Effect on RNA synthesis:** In order to test the assumption that triamecinolone inhibits RNA synthesis soon after its administration, triamecinolone was administered at day 11.5 and at subsequent times (6–96 hr) [\textsuperscript{14}C]lorotic acid and [\textsuperscript{3}H]leucine were simultaneously administered to experimental and control mice to measure RNA and protein synthesis in total embryo homogenates. The results (Fig. 3) are plotted as percentage of control at the same time of pregnancy. Triamecinolone inhibited RNA synthesis within 6 hr (60% of con-
Effect of triamcinolone (10 mg/kg) administration during development on triamcinolone-induced cleft palates and resorptions. Number of litters: triamcinolone alone, 14; triamcinolone plus cycloheximide, 5; except day 11.5, 11.

**Table 1.** Effect of cycloheximide on triamcinolone-induced cleft palate in mice.

<table>
<thead>
<tr>
<th>Triamcinolone (mg/kg)</th>
<th>Cycloheximide (mg/kg)</th>
<th>No. of litters</th>
<th>No. resorbed</th>
<th>No. of implants</th>
<th>Per cent resorption</th>
<th>Per cent cleft palate</th>
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Procedure as described in Fig. 1 except that resorptions were also scored and triamcinolone and/or cycloheximide was administered on day 11.5 of gestation.
other experiments it was found that protein synthesis was inhibited by 80% in the embryo and 50% in the maternal liver within 1 hr after administration of triamcinolone at day 11.5, as measured by \(^{1}H\)leucine incorporation. The results of triamcinolone administered alone or plus cycloheximide at day 11.5 are also seen in Table 1. Triamcinolone (10 mg/kg), by itself, caused 83% cleft palate and 34% resorption, while simultaneous administration of both drugs (10 mg/kg each) caused 30% cleft palate and 75% resorption. Triamcinolone plus a lower dosage of cycloheximide (5 mg/kg) produced 22% cleft palate and 45% resorption. It therefore appears that cycloheximide was not differentially killing embryos that would have developed cleft palate. Further evidence supporting this contention is shown in Fig. 4. Cycloheximide (5 mg/kg) administered at different days of pregnancy produced fairly constant resorption rates, but only when cycloheximide was administered at the same time as triamcinolone (day 11.5) was cleft palate significantly reversed.

**Discussion.** Much of the past work on glucocorticoid-induced cleft palate in mice has focused on an antiinflammatory action of these drugs, i.e., the suppression of mucopolysaccharide synthesis.\(^5\)\(^6\) However, the growth-inhibitory action of these steroids has been largely ignored as a possible mechanism for cleft palate. One of the most profound growth-inhibitory effects of such compounds, the inhibition of RNA synthesis,\(^9\) was the basis for postulating our model. The results obtained in this investigation indicated that a glucocorticoid, triamcinolone, indeed depressed the synthesis of RNA in mouse embryos shortly after the time of its administration (within 6 hr), which agrees with the time course of inhibition in thymocytes \textit{in vitro}.\(^22\) The recovery of RNA synthesis in the embryo by day 13.5 is not surprising since triamcinolone is no longer found in the embryo at this time.\(^24\) Presumably the early inhibition of RNA synthesis could be responsible for a later block in the translation of protein, causing cleft palate (see Fig. 2).

It would be expected that the marked inhibition of RNA synthesis would mean that all species of RNA (mRNA, rRNA, and tRNA) are inhibited, and that localized RNA synthesis (e.g., in the palate) is also inhibited, both of which have been verified.\(^22\)

The results also indicated that protein synthesis was inhibited, although to a lesser extent than synthesis of RNA (Fig. 3). However it is not likely that the immediate inhibition of protein synthesis by triamcinolone, 6–24 hr after its administration, would be responsible for cleft palate, because (1) cycloheximide did not produce cleft palate (Table 1), (2) puromycin, another inhibitor of protein synthesis, produced no cleft palate\(^25\)\(^26\) in rats, (3) simultaneous administration of cycloheximide and triamcinolone, which presumably additively inhibited protein synthesis, did not increase the frequency of cleft palate. In fact, the opposite result was obtained.

One of the assumptions of the model is that the inhibition of RNA synthesis by triamcinolone causes RNA and protein synthesis to go out of phase during development. In fact it was observed that the simultaneous administration of triamcinolone and cycloheximide at day 11.5 produced a lower frequency of cleft palate than triamcinolone alone, which supports the assumption. Conversely,
if cycloheximide were given either days before or after triamcinolone, RNA and protein synthesis should not resume in phase, and no reversal of cleft palate should occur. Fig. 4 indicates that this is so: no reversal took place.

It is well documented that glucocorticoids stimulate protein synthesis in rat liver, owing to the rapid increase in synthesis of the enzymes involved in gluconeogenesis. Evidence has also been presented that the synthesis of mRNA molecules coding for these proteins (e.g., tyrosine aminotransferase) has been stimulated. Our results did not show that triamcinolone stimulated RNA and protein synthesis in murine embryos. This is not surprising, since a steroid can elicit different responses in different target tissues. For example, glucocorticoids depress RNA synthesis in the thymus and cause involution of the gland, as well as cells growing in culture. These growth inhibitory effects of glucocorticoids, in fact, represent the basis for their use in cancer chemotherapy.

Since glucocorticoids also inhibit DNA synthesis in growing cells as well as in cells undergoing development and differentiation (e.g., sea urchin), it is possible that they cause cleft palate by their effect on DNA synthesis. However, cytosine arabinoside, which inhibits DNA synthesis in rat embryos, produces a large range of malformations in these embryos. Yet the glucocorticoids are rather specific in producing cleft palate; the incidence of other anomalies in mice and of cleft palate in rats is very low.

The reason for this specificity in producing cleft palate in mice is unclear. It may be that mouse embryos, after the rate of RNA synthesis returns to normal, can resume differentiation in each organ without malformation except for the palate. The palatine shelves, as a result of the block in synthesis of mRNA molecules which later causes impaired translation of their proteins, would thus be delayed in moving to the horizontal position. Since the cranium is larger when the shelves finally move up, the distance would be too great to allow fusion of the separated shelves.

It is possible in this model to link the early inhibition of RNA synthesis by triamcinolone (12.5 days) with the observed later inhibition of mucopolysaccharide synthesis in the palate (14.5 days). Thus mRNA synthesized at day 11.5 could presumably code for protein involved in mucopolysaccharide synthesis. That this is unlikely and that glucocorticoids cause cleft palate by a mechanism other than an effect on mucopolysaccharide synthesis comes from the following considerations. (1) Although Larsson showed that cortisone inhibited mucopolysaccharide synthesis in a sensitive mouse strain (A/J), it was equally inhibited in the CBA strain, which produced cleft palate in a very low frequency. (2) Nanda reported that cortisone inhibited mucopolysaccharide synthesis in the embryonic palate of the rat, which shows a low frequency of cleft palate defects. (3) In our laboratory we have observed that 10 mg/kg of triamcinolone administered on day 11.5 has only a small effect on mucopolysaccharide synthesis on day 14.5 (26% inhibition), about equal to 64 mg/kg of cortisol, which was not teratogenic at this dose. Cortisol at the higher dose (320 mg/kg) produced both a high frequency of cleft palate (70%) and a marked (65%) inhibition of mucopolysaccharide synthesis.
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† Requests for reprints may be addressed to Dr. E. F. Zimmerman, Children’s Hospital Research Foundation, Elland and Bethesda Avenues, Cincinnati, Ohio 45229.
10 Triamcinolone acetonide is 9α-fluoro-11β,16α,17,21-tetrahydroxypregnan-1,4-diene-3,20-dione-16,17-acetonide.
22 Zimmerman, E. F., D. Gustine, and F. Andrew, unpublished observations.