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The Effect of Vitamin A on Fusion of Mouse Palates II. Retinyl Palmitate, Retinol, and Retinoic Acid In Vitro

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ABSTRACT A fixed-exposure culture technique is described using palatal shelves removed from mouse fetuses on day 14 of pregnancy. In this technique growth is eliminated as a possible variable. The addition of retinyl palmitate, retinol, or retinoic acid to culture medium at concentrations similar to those found associated with cleft palates produced in vivo after dosing dams with vitamin A on day 14 prevented fusion of explanted shelves in vitro after a 24-hour exposure period. Retinoic acid prevented fusion of explants after as little as 4 hours exposure. Retinoic acid was more active in vitro than retinol, which was itself eight to ten times more active than retinyl palmitate. An explanation of the effect of vitamin A on the fusing mouse palate is given based on altered glycoprotein synthesis.

Newall and Edwards ('81) have shown that failure of fusion of mouse palates in vivo on day 14 of pregnancy after dosing the dam on day 11 of pregnancy with retinyl palmitate or retinoic acid is not a result of the presence of residual vitamin A in the fetal environment. However, after three oral doses of retinyl palmitate or retinoic acid on day 14, failure of palatal fusion occurs, which can be associated with elevated levels of vitamin A.

Fetal retinyl palmitate, retinol, and retinoic acid levels found to cause clefts in vivo may therefore be directly compared with concentrations of the same found to prevent fusion in vitro.

Rodent palates fused in culture both morphologically and histologically resemble palates fused in vivo (Moriarty et al., '63; Smiley and Koch, '71). Myers et al. ('67) suggested that the main effect of vitamin A on cultured rat palatal shelves was retardation of growth rather than inhibition of fusion. Shelves removed en bloc and placed in culture in such a way that some growth of the palatal shelves was necessary before contact of the medial epithelium could occur failed to fuse in the presence of vitamin A, whereas shelves placed in contact at the commencement of culture fused successfully. Nanda ('74) repeated the first of the above experiments, and found that 10 IU/ml (~ 10 μM/liter) retinyl palmitate added to culture medium prevented fusion of rat palates in vitro, whereas palatal shelves from fetuses of vitamin A-tested dams showed some degree of fusion after prolonged incubation. He too suggested that the major effect of vitamin A was probably on growth.

There is, however, some circumstantial evidence that vitamin A can affect the fusion potential of the expected fusion zone (EFZ), as well as retard growth (Newall and Edwards, '81).

Myers et al. ('67) assumed that if vitamin A were to cause loss of fusion potential, shelves in contact from the commencement of culture would lose it as easily as shelves allowed to grow together for an undefined period of time. However, duration of exposure of vitamin A may well be as important as its concentration. Furthermore, less than 50% of the explants taken by Nanda ('74) from fetuses of vitamin A-treated dams showed mesenchymal fusion, even after twice the control incubation period; and the validity of comparing cleft palates in rat fetuses from dams treated with vitamin A in vivo no later than day 13 with fusion failure in vitro on day 16 has already been questioned (Myers et al., '67; Newall and Edwards, '81).

In view of the above, we feel justified in repeating this work specifically to investigate the effect of vitamin A on the EFZ, although this should not be taken to imply that we rule out retardation of growth as a cause of cleft

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palate. Myers et al. ('67) have pointed out that explanting the palate from the fetal head into culture almost certainly disrupts the normal growth pattern of the shelves. Therefore, we have removed growth as a variable and used the fixed exposure technique described below.

MATERIALS AND METHODS

Female C57B1 mice were mated overnight and checked each morning for the presence of a vaginal plug. Sperm-positive females (day 0 of pregnancy) were housed individually and fed tap water and Mouse Breeding Diet No. 3 (B. P. Nutrition) ad libitum.

In vitro technique

Palatal shelves removed from day 14 mouse embryos just prior to fusion in vivo were exposed in vitro to: 5, 10, 25 and 50 μM/liter retinyl palmitate, or 0.5, 1.0, 2.5 and 5.0 μM/liter retinol, or 0.5, 1.0, 2.5, and 5.0 μM/liter retinoic acid for 24 hours, before fusion was allowed to begin.

Forty-two pregnant females were used. All were sacrificed by cervical dislocation between 1000 and 1200 hours on day 14 of gestation, and the embryos removed aseptically in a laminar flow cabinet. The wet weight of each embryo was measured on a 500-mg torsion balance, and embryos weighing within the range of 200 to 240 mg were used. These embryos had palates at stages 2–4 (Walker and Crain, '60).

The method was essentially that used for culturing human fetal palates (Newall and Edwards, '76). The head was detached from the body and the tongue and mandible removed. An incision was then made parallel to the roof of the mouth, just below the level of the orbits. The nasal septum and any excess tissue posterior to the palatal shelves was trimmed away. Dissection was performed in Hanks' balanced salt solution; explants were then washed in culture medium. Three to five explants were placed, oral surface up, on a millipore filter supported on an expanded metal grid in a 3-cm borosilicate glass culture dish, containing 1.5 or 2 ml of culture medium. Two dishes, an experiment and a control, were placed in a 9-cm glass petri dish containing filter paper saturated with 0.9% sodium chloride. Each two-dish unit represented the contents of the uterus of a single female.

The cultures were maintained at 37 ± 0.5°C in an atmosphere of 5% CO_2 in air. Explants were placed in culture with both shelves well apart (Fig. 1a). After about 1 hour the medium was changed, and in each petri dish one culture received medium containing vitamin A, the other medium containing the vehicle (10 μl absolute ethanol). When the exposure time had elapsed, the explants were divided in the midline and placed with their medial edges touching (Fig. 1b). Culture was continued for 48 hours. Culture medium was BGJ-Original (Gibco Bio-Cult) supplemented with 15% fetal calf serum and 150 mg/liter ascorbic acid; no antibiotics were used. Medium was renewed every 24 hours.

Explants were fixed in Zenker's solution, embedded in paraffin wax, and serial sections cut at 10 μm. Sections were stained in Delafield's haematoxylin and chromotrope 2R.

Analysis of vitamin A content of culture medium

Vitamin A content of culture medium was measured at regular intervals during the pe-

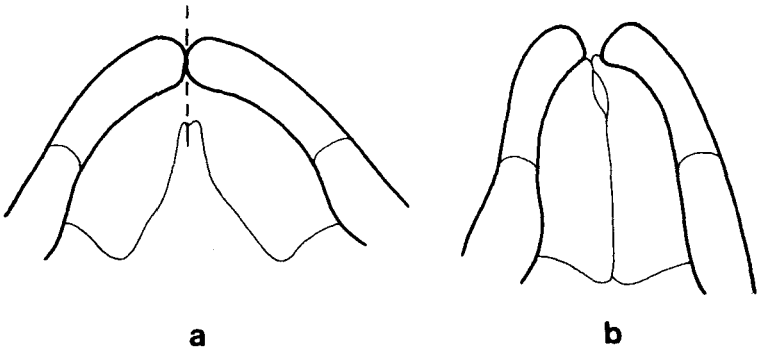

Fig. 1. Diagrammatic representation of palatal explants in culture. a. Exposure period. b. Fusion period.

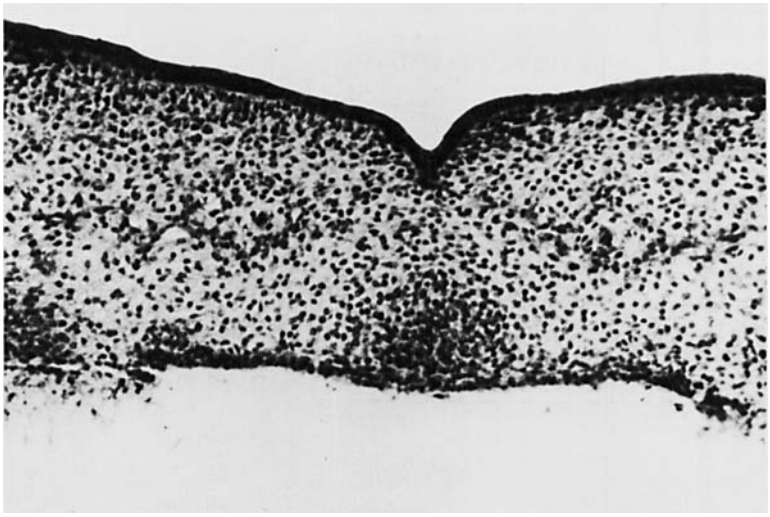

Fig. 2. Fusion of day 14 control explant after a 24-hour exposure period and 48-hour fusion period. Section stained with Delafield's haematoxylin and chromotrope 2R.

riod of exposure by the method of Neeld and Pearson ('63). Samples of culture medium were also analyzed by thin-layer chromatography. Details of both these methods are given in Newall and Edwards ('81).

Miscellaneous techniques

Statistical analysis compared treatment groups with controls using Student's t-test.

Each dish represented a single experimental unit; samples consisted of three or four units. In all cases the level of significance chosen was $P < 0.05$.

RESULTS

After a 24-hour exposure to vitamin A a palate was considered to have fused if there was evidence of breakdown at any point along the epithelial seam.

Of 127 control palates only 11 failed to fuse (Fig. 2), whereas 78 of 147 palates exposed to vitamin A for 48 hours failed to fuse (Fig. 3). A significant increase in rate of fusion failure over control level was observed in concentrations of retinyl palmitate above 25 μM/liter, and of retinol above 2.5 μM/liter, the effect of retinoic acid was significant at a concentration as low as 1 μM/liter (Fig. 4a).

Retinyl palmitate, retinol, and retinoic acid all fell to about 70% of their original concentration after 3 hours of culture. After 24 hours, a further decrease of no more than 1% was

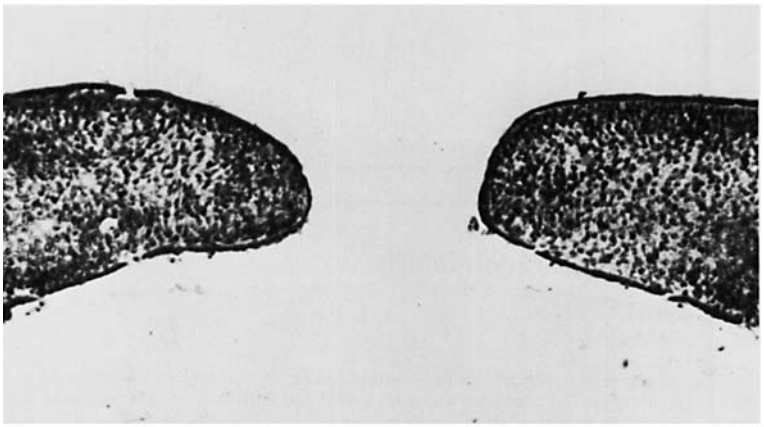

Fig. 3. Failure of fusion of day 14 explant in 5 μM/liter retinoic acid after a 4-hour exposure period and 48-hour fusion period. Section stained with Delafield's haematoxylin and chromotrope 2R.

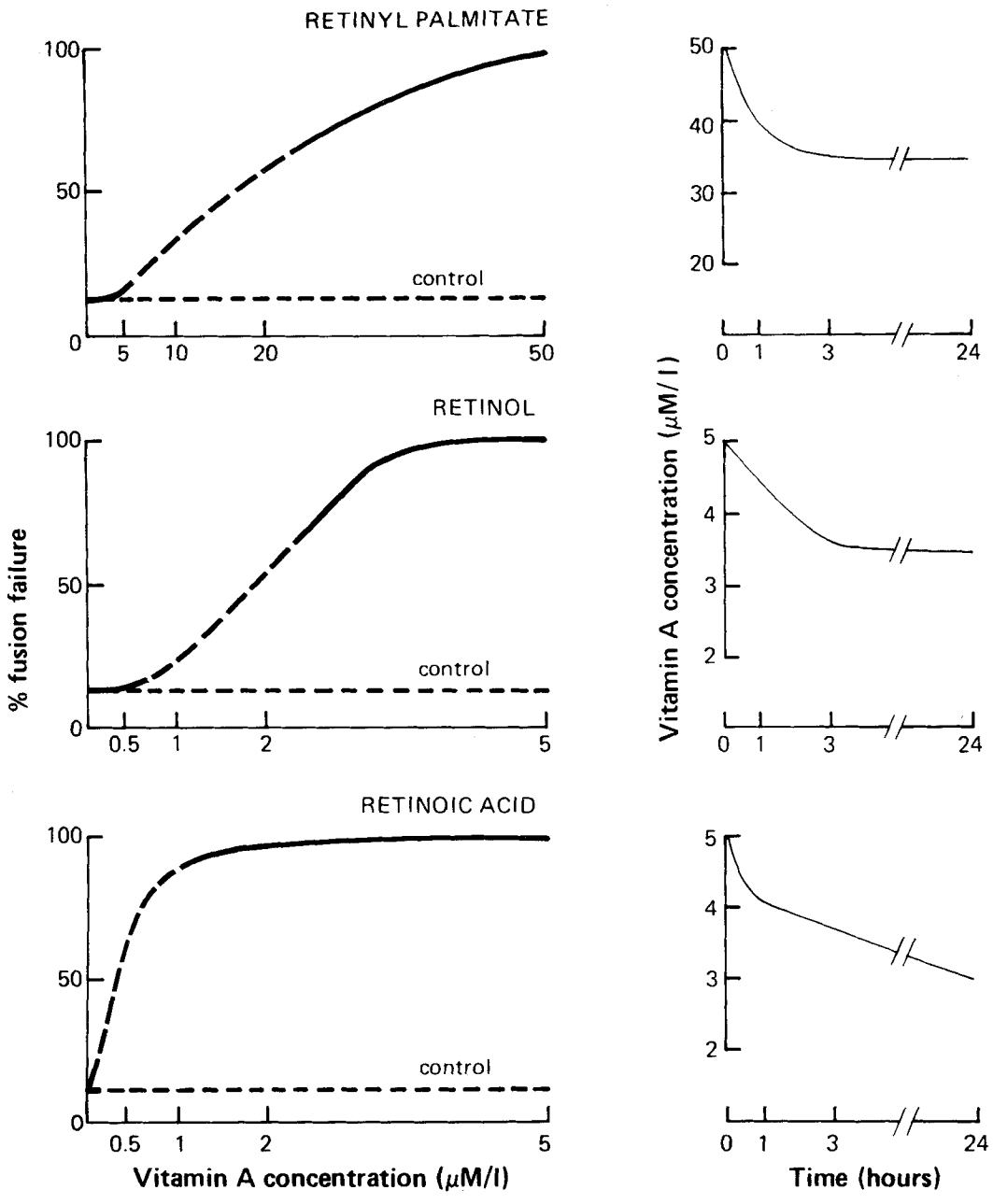

Fig. 4. a. The effect of increasing concentrations of retinyl palmitate, retinol, and retinoic acid on the ability of day 14 palates to fuse in vitro, after a 24-hour exposure period and 48-hour fusion period. Bold line indicates results significantly different from control. Each curve represents data from 40–50 palates. b. Levels of retinyl palmitate, retinol, and retinoic acid in culture medium during 24-hour exposure period. Data are shown for maximum concentration used.

recorded (Fig. 4b). Control culture medium contained 0.2 μM/liter vitamin A (retinol and retinyl ester) and 0.1 μM/liter retinoic acid. After 24 hours' incubation, there was no significant increase in retinol levels in medium containing retinyl palmitate of retinoic acid, or in retinoic acid levels in medium containing retinyl palmitate or retinol.

DISCUSSION

In vitro retinyl palmitate, retinol, and retinoic acid were all found to prevent fusion of palatal shelves removed from mouse fetuses on day 14 of pregnancy. Furthermore, as growth was not involved it may be assumed that the fusion potential of the EFZ was affected.

For exposure periods of 24 hours the actual level of vitamin A to which the explants were exposed was some 60–70% of the nominal value. Significant failure rates were therefore recorded at the following minimum concentrations: retinyl palmitate, 15 μM/liter; retinol, 1.5 μM/liter; and retinoic acid, 0.6 μM/liter. It appears that retinoic acid is considerably more active in vitro than retinol, which is itself ten times more active than retinyl palmitate. Morriss and Steele ('77) have suggested that retinol may be stored intracellularly in an inactive form, and this may also be true of retinyl palmitate. The maximum levels of vitamin A measured in vivo after giving three doses of retinyl palmitate or retinoic acid to C57B1 dams on day 14 of pregnancy were: retinyl ester and retinol, 9.3 μM/liter; retinol, 1.4 μM/liter; and retinoic acid, 7.3 μM/liter (Newall and Edwards, '81). Direct in vivo/in vitro comparison of concentration of vitamin A is not possible for the following reasons: first, the comparison would not take into account the differences in activity of vitamin A derivatives in culture medium and fetal serum; second, retinyl ester, retinol, and retinoic acid are all present together in fetal serum after dosing dams with retinyl palmitate; and third, duration of fetal exposure to vitamin A in vivo may be as important to the formation of clefts as its concentration in fetal serum. Nevertheless, concentrations of vitamin A associated with the occurrence of clefts in vivo are not unlike those found to prevent fusion in vitro.

The concentration of retinoic acid at which a significant failure rate occurred with exposure periods of 4 or 8 hours was about 4 μM/liter. These values of concentration and exposure time more closely resemble those found after retinoic acid treatment in vivo.

Generally, the above concentrations of vitamin A are of the same order as those found to be active in other in vitro systems (retinyl ester: Myers et al., '67; Nanda, '74; Yuspa and Harris, '74; retinol: Morriss and Steele, '77; retinoic acid: Kochhar, '76; Morriss and Steele, '77).

As all three forms of vitamin A can directly prevent fusion from taking place in vivo, it is likely that the mechanism is the same in each case and it seems reasonable that it will somehow be related to its normal physiological action. Retinol and its phosphoryl and glycosylphosphoryl derivatives appear to be ideally suited structurally to act as carriers of glycosyl residues in the biosynthesis of glycoproteins (De Luca et al., '70). Recently Frot-Coutaz et al. ('76) have shown that synthetic and biologically isolated retinyl phosphate (RP) are more active than retinol in reversing keratinization of hamster tracheal epithelium in organ culture, and it has now been established (De Luca, '77) that retinol is involved in the formation of mannosyl RP (MRP) and galactosyl RP. The mannosyl residues transferred by RP appear to be associated with short oligosaccharide chains of glycoprotein molecules; however, whether the acceptor molecules are constituents of the membrane or secretory, or even both, is not known. Retinoic acid which can replace retinol in its growth function is metabolized by epidermal cells in culture to products which resemble RP and MRP.

Breakdown of the midline is probably a result of programmed cell death of the EFZ (Shapiro and Sweeney, '69; Hudson and Shapiro, '73; Pratt and Martin, '75); such a mechanism may require the synthesis of highly specific glycoproteins.

De Luca and Yuspa ('74) have demonstrated a twofold increase in incorporation of fucose and glucosamine in mouse epidermal cells treated with retinyl acetate in vitro; furthermore, the glycopeptide isolated from treated cells had a higher molecular weight than glycopeptides from control cells.

The evidence, although circumstantial, would therefore seem to suggest that elevated fetal retinol, retinyl palmitate, and retinoic acid levels on day 14 may all interfere with the normal biosynthesis of glycoprotein in the cells of the EFZ.

Clearly further work will be necessary to establish the true nature of the relationship between altered glycoprotein synthesis and failure of palatal fusion.

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