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Short Title of Thesis:

Face Shape & Mitotic Index in Cleft Lip Mice

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Morphologie Faciale et Index Mitotique chez des Souris avèc Bec-de-lièvre causé par des Agents Tératogènes et Héréditaire

RESUME

Le dévelopement facial au moment de la formation de la lèvre éte étudié chez (1) des embryons C57Bl/6 ayant reçu au neuvième jour et demi ($9\frac{1}{2}$) de gestation du 6-aminonicotinamide (6AN) ce qui amené 18% de bec-de-lièvre médian; (2) des embryons C57Bl/6 traités eux au dixième jour et un tiers (10 1/3) avec comme résultat 22% de bec-de-lièvre latéral; (3) des embryons avec 20% de bec-de-lièvre latéral causé par le gène danseur (Dc) et(4) dex embryons de la souche Cl/Fr avec 26% de bec-de-lièvre dû à une prédisposition héréditaire-multifactorielle. Le developement gènèral et facial est retardé par le 6AN et le gène danseur. Ceci est mis en évidence par des différences dans le taux morphologique, l'index mitotique, l'histologie et le mesurement de la morphologie faciale.

Les embryons avec bec-de-lièvre médian causé par un agent tératogène ont dex processus médians petits. Les embryons avec bec-de-lièvre causé par un agent tératogène et ceux avec bec-de-lièvre dû à l'héredité ont dex processus médian et latéraux petits. Ceci suggère que le lien entre le bec-de-lièvre causé par un agent tératogène et celui causé par le gène danseur réside dans une réduction du bolume mésenchymateux possiblement amené par une migration anormale de cellules de la crête nerveuse.

Face Shape and Mitotic Index in Mice with Teratogen-induced and Inherited Cleft Lip

by

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ABSTRACT

Face development at lip formation time was studied in (1) C57Bl/6 embryos treated on gestation day 9 1/2 with 6-aminonicotinamide (6AN) which later show 18% median cleft lip, (2) C57Bl/6 embryos treated day 10 1/3 with 22% lateral cleft lip, (3) embryos with 20% lateral cleft lip induced by the mutant gene dancer (Dc), and (4) embryos of the Cl/Fr strain with a multifactorially inherited predisposition to 26% lateral cleft. The general and facial development was retarded by 6AN and the dancer gene. Evidence for this was provided by differences found in morphological rating, mitotic index, histology and the face shape measurements.

Treated embryos with median cleft lip had small medial processes and both treated and dancer embryos with lateral cleft lip tendency had small medial and lateral nasal processes. This suggested that the common link between teratogen-induced and dancer-induced cleft lip was a reduction in volume of the mesenchyme possibly due to abnormal migration of neural crest cells.

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Table of Contents

INTRODUCTION

Malformations both inherited and induced may be roughly subdivided as being due to one of two possible types of mechanism. Malformations may be caused by cell death or by a decrease in the proliferative rate of the tissue. In the normal course of development, there is normal physiological or programmed cell death. Programmed cell death is clearly illustrated in the formation of the digits or the formation of the palate. If the cell death gets out of hand it may lead to malformation thus an increase in programmed cell death may be one of the ways of bringing about a cleft lip and or a cleft palate. A 'split face' with or without cyclopia may result from a deficiency of mesoderm beneath the neural plate because there is a failure of migration of the neural mesenchyme which in turn is a result of an under-developed forebrain. This is an example of a decrease in proliferative rate of the forebrain tissue.

Another general mechanism for producing malformations may be attributed to defects in fluid regulation. In higher vertebrates, vascular defects such as haemmorhages may stop or disturb development. Another disturbance in fluid regulation may be in the spinal fluid. Accumulation of excessive fluid may for example cause rupture of the

neural tube (exencephaly), or formation of blebs in various parts of the body. A third type of disruption of fluid regulation comes from the amniotic fluid. Lack of amniotic fluid may produce compression that ultimately produces cleft palate.

The present thesis is concerned mainly with decreased cell proliferation and/or increased cell death as mechanisms of malformation.

LITERATURE REVIEW

6 Aminonicotinamide (6AN) is an antimetabolite which, when introduced in large amounts, produces deleterious effects in animals. Wolf (1959) showed that 6AN when given intraperitoneally at 8 mg per kg body weight to rats produced neurological impairments and lesions in their spinal cords within twenty-four hours. These toxic effects were not only seen in experimental animals, as Hertel et al (1961) used 6AN to treat tumors in humans and found that the drug also caused neurological disturbances and symptoms of vitamin B complex deficiencies.

Dietrich and Friedland (1958a) found that 6AN caused regression of 755 tumors in mice and Ofori et al (1972) found that it also inhibits glycolysis in ascite tumor cells. The toxic effects of nicotinamide depletion have been noted in cell cultures. Gardner and Sato (1972) working with adrenal tumor cells found that in the absence of nicotinamide, inhibition of growth occurred after the second subculture. These nicotinamide depleted cultures were found to be susceptible to 3AP (3-acetyl pyridine) and the susceptibility was inversely proportional to the pyridine nucleotide content of the cell.

6AN as a teratogen -- 6AN when given to pregnant female animals often produces malformations in the young. It has been found that 6AN when given early in pregnancy to rats may prevent or delay implantation and retard early embryonic development (Chamberlain 1963). When given to various experimental animals such as rats, mice, chicken, hamsters and rabbits at appropriate gestational time, malformations such as micromelia, cleft lip, cleft palate, hydrocephalus, spina bifida and other skeletal and or neural malformations were found. (Pinsky and Fraser 1960, Ingalls 1964, Overman et al 1971, Schardein 1967, Turbow et al 1969)

Ingalls et al (1964) found that 6AN causes chromosomal anomalies and he suggested that malformations caused by a teratogen may be a result of the chromosomal anomalies. Any teratogen could also cause decrease in protein synthesis, RNA and DNA synthesis, reduction in cell division, disturbance of any of the numerous metabolic pathways and or cell death.

Biochemistry of 6AN

6-aminonicotinamide (6AN) is a nicotinamide analogue and also an antimetabolite possessing antagonistic properties to nicotinamide.

In a normal biochemical reaction usually there is formation of an enzyme-metabolite complex, however in the presence of an antimetabolite, an enzyme-antimetabolite complex will be formed. When 6AN is administered to an animal, 6AN competes with nicotinamide inhibiting the NAD dependent reactions.

Coper and Neubert (1964) suggested that 6AN replaces the nicotinamide moeity of NAD by giving rise to an abnormal nicotinamide molecule and that phosphorylation at the sixth position of nicotinamide ring is involved.

Dietrich, Freidland and Kaplan (1958 a& b) found that 6AN when administered to mice caused a decrease in β-hydroxybutyrate and α-ketoglutarate dehydrogenase activity. Activities of these enzymes were assayed spectrophotometrically. Both these enzymes are mitochondrial restricted systems coupled with oxidative phosphorylation. The 6AN analogue of NAD is postulated to become bound to the apo-dehydrogenase producing unusual and ineffective holoenzymes resulting in blockage of oxidative phosphorylation and impaired synthesis of ATP, lowering the efficiency of high energy bond synthesis. Pullman et al (1960) using quantum mechanics calculations showed that

the lowest empty molecular electronic orbital is placed much higher in the 6AN analogues of NAD than in NAD. The 6AN analogue of NAD which now acts as the coenzyme does not have the same electron acceptor abilities of the true coenzyme resulting in a loss of electron accepting capacity.

When 6AN was used in the presence of NAD and succinate, the phosphorylated product NADH~P was found to be markedly reduced. But when the 6AN was tested for the transphosphorylation reactions of NADH~P $\longrightarrow$ ATP no decrease was found in the amount of ATP formed.

6AN was also found to cause accumulation of 6-phosphogluconate. This indicates that 6-phosphogluconate dehydrogenase is inhibited, but the rate of glucose metabolism via the pentose phosphate cycle as well as the rate of fatty acid synthesis was not affected. (Kather and Rivera 1972)

The above evidence indicates that the electron transport system is somehow being disturbed and that perhaps there is interference with the NAD and NADP (coenzyme I and coenzyme II) that take part in the oxidative-reduction system.

If the synthesis of high energy bonds has been reduced, one would expect that the amount of ATP synthesized would also be reduced. Coper and Neubert (1964) were

unable to show any significant decrease in ATP formation when they added 6-ANAD in concentrations as high as 1mM to their reaction mixtures. But other workers (Dietrich and Friedland 1958a & b) and Ritter et al (1973) have found that 6AN causes a decrease in the amount of ATP.

Dietrich et al (1958a) working with mice have found that there was a decreased amount of ATP and ADP and an increased amount of AMP. Working with rat embryos, Ritter et al (1973) also found that 6AN causes a fifty per cent decrease in ATP synthesis when compared to untreated embryos.

Replacing ATP in the embryos however, does not seem to alleviate the effect of 6AN. Chamberlain (1970) gave an intraamniotic injection of ATP as countertherapy for 6AN but with no positive effects.

Since 6AN acts as a competitive inhibitor to nicotinamide and acts by lowering the efficiency of the electron transport system making energy unavailable, it is logical to assume that flooding the system with nicotinamide or other high energy intermediates may serve as a source of protection to the 6AN treated animal.

Pinsky and Fraser (1960) found that a single dose of 6AN given simultaneously with nicotinamide on D9/12, D10/12 and D11/12 of gestation in mice respectively, caused no increase in the frequency of resorptions or malformations. But the effectiveness of nicotinamide in protecting the embryo against 6AN decreases with time.

Overman et al (1972) found that by preloading chick embryos with nicotinamide prior to treatment with 6AN (100μg of nicotinamide to 10 μg of 6AN), no gross malformations were found. Simultaneous administration of nicotinamide with 6AN produced the same results. But the same level of nicotinamide had little protective value following previous treatment with 6AN. When nicotinamide was given after 6AN treatment, the number of embryos that were protected decreased rapidly with time. When nicotinamide was given 24-48 hours after administration of 6AN, it was found to be ineffective in protecting the embryo against the teratogen.

Verrusio (McGill thesis 1966) found that the amount of nicotinamide required for protection against 6AN also varies with the strain of mice used. He found that C57Bl/6 mice needed less nicotinamide than A/J mice to counteract the effect of 6AN. A/J mice needed one and a half times as much nicotinamide as C57Bl/6. Perhaps C57Bl/6 is able to metabolize nicotinamide in a more efficient manner.

Landauer and Sopher (1970) found that high energy intermediates such as succinate, glycerophosphate, and ascorbate greatly reduced the teratogenic effects produced by 6AN. They concluded that these compounds counteract 6AN by counteracting the interference with cellular energy production in tissues in which the teratogens are likely to produce their specific effects, although Chamberlain found ATP, which is a high energy intermediate, produced no such effect.

Effects of 6AN on Mucopolysaccharides

It has been shown that the mesoderm in the region of the head is mostly derived from neural crest cells. In the head of the chick these neural crest cells seem to migrate through a matrix largely composed of mucopolysaccharides (Johnston, personal). If any disturbance of the matrix occurs, it seems logical that the migration of neural crest cells may also be disturbed. Failure of neural crest cells to reach the facial region of an embryo may result in malformations of the face. Smith and Monie (1969) have found that cleft lip in rats may be caused by mesenchymal deficiencies. This may be suggestive of failure of neural crest migration.

Kochhar and Larsson (1968) suspected that some teratogenic agents act at least partly by altering the mesodermal tissue via biochemical changes in mucopolysaccharide synthesis. In order to test their hypothesis they administered S^{35} in the form of sodium sulphate to mice at the time when the teratogen was introduced and found that cortisone caused a decrease in the uptake of S^{35} whereas vitamin A resulted in a higher level of uptake of S^{35}. Thus congenital malformations may be a result of morphological disturbances in the embryos caused by alterations in their metabolism of acid mucopolysaccharides.

Overman and Beaudoin (1971) were also impressed that mucopolysaccharide synthesis was affected in teratogenesis. They measured the amount of S^{35}-sulphate in heart tissue after treating mice with 6AN and found that there was inhibition in the rate of sulphate uptake in vivo.

Seegmiller et al (1972) working with chick embryos found that 6AN produced micromelia when introduced into the embryos on day 4 of incubation. When sections of these embryonic limbs were stained for mucopolysaccharide with toluidine blue, the extracellular matrix around the chondrogenic cells showed a reduction in metachromatic staining. Futher investigation (Overman 1972) using S^{35} in the form of sodium sulphate and S^{35} incorporation as an indication of the amount of mucopolysaccharide present, demonstrated a selective inhibition of S^{35} incorporation by 6AN. Also they found protein synthesis, measured by incorporation of ^{3}H-leucine was unchanged in the treated embryos.

The above evidence implicates alterations in mucopolysaccharides as playing a role in teratogenesis.

Embryology of the Primary Palate

The primary palate (comprised of the lip and the gum behind it) is formed from the lateral and the medial nasal processes, their adjacent mesenchyme and the maxillary processes. In the mouse, the nasal placodes are apparent at D9/2 hours of gestation, and appear slightly before the lens placode formation as a thickening of the epithelium. At this time the neural tube is still open. The ectodermal thickening is at first adjacent to the neural tissue in the forebrain area, and this thickening is pushed away from the neural tissue by invading mesenchyme. The mesenchyme appears to have arisen from ventrolateral sectors of the cephalic epithelium induced by the prosencephalon (Pourtois 1972). The nasal placodes, however are induced by parts of the forebrain that will eventually become the olfactory lobes (Jacobson 1963 a & b).

The mesenchyme that surrounds the nasal placode area is an integral part of the facial swellings or processes. This mesenchyme originates from neural crest cells. Johnston (1966) who did a study on the migration of cranial neural crest cells in chick embryos using transplanted segments of radioactive neural tube to unlabelled hosts found that the labelled neural crest cells migrate anteriorly and posteriorly around the developing eye in sheets to the face region. These migrating neural

crest cells diverge less as they approach the olfactory placodes, they then proliferate, giving rise to the medial, lateral and maxillary processes. He did another study that involved the removal of segments of mid or forebrain neural crest cells prior to migration (Johnston 1964) in the chick and found that these embryos ultimately had absence of or deficient fronto-nasal mandibular processes. If he removed the neural crest cells from the forebrain region, he often found clefts of the primary palate and removal of one side of the forebrain neural crest gave rise to unilateral clefts of the primary palate on the same side.

Pourtois (1972) suggested that the next step that followed was an increase in mitosis causing an increase in the size of the lateral, medial and maxillary processes. Concurrent with this, there is a localized increase in intercellular adhesiveness with localized increase of volume and the number of cells. As a result, the base starts invaginating and this depression gets more pronounced by the raising of the edges of the placode and by the moving in of the adjacent mesenchyme. This invagination eventually leads to the formation of a nasal groove. The nasal groove deepens due to the elevation of its borders by the lateral and medial nasal folds. These start to fuse because the folds converge, and fusion

takes place in an postero-anterior direction starting from the isthmus (Pourtois 1972, Lejour and Jeanty 1965).

Fusion involves the formation of an epithelial seam and its dissolution (Tondury 1961). This may involve cellular autolysis (Pourtois 1972), straining, alteration of basement membrane and phagocytosis (Pourtois 1970).

If all the above steps progress in a coordinated manner, in the correct spatial relation at the critical time, a normal primary palate is formed.

Malformations of the Primary Palate

The common malformation involving the primary palate is a lateral cleft lip that occurs either unilaterally or bilaterally. Numerous authors have concluded that a cleft of the face is formed because of the failure of fusion of the lateral and medial nasal processes. This failure of fusion may be due to failure of the processes to approach each other resulting from an underdevelopment. Another possible explanation is that fusion takes place but the fusion cannot be maintained, or an incomplete fusion is present.

Reed (1933) supported the theory that fusion did not occur because of retarded growth in the facial processes. This may be caused by failure of neural crest to migrate, therefore reducing the volume of mesoderm (Johnston 1964). Stark (1954) also felt that mesodermal deficiency may ultimately result in a cleft.

Underdevelopment of lateral and medial nasal processes may also be due to cell necrosis. Lejour (1970) working with rats found that Hadacidin given to rats causes cells to die in the olfactory placode underlying mesoderm that results in a delay or failure of the nasal processes to fuse.

Both Pourtois (1972) and Lejour (1970) felt that even if fusion did occur, there could be a partial or a complete reopening of the nasal groove resulting in a

partial or whole cleft.

This failure to fuse may also result from differences in direction of growth of the processes, that is these processes may fail to maintain a certain degree of approximation (Fraser 1971), or in mice that are predisposed to having cleft lip, there is a lack of divergence of the medial processes towards the lateral processes (Trasler 1968).

Another theory of formation of a cleft suggested by Töndury in 1961 is that the epithelial wall fails to develop. He regarded a primary cleft as "having arisen in consequence of the discontinued development of the epithelial wall" and the cause of this faulty development to be from "defective growth of the lateral nasal swelling."

Veau (1938), however, felt that a cleft is formed by incomplete substitution of the epithelial wall by mesenchyme. Yet another author, Steiniger (1939) felt that it was a rupture due to cysts in the epithelial wall that produced cleft lips.

Not much is known about the formation of a median cleft lip. It has been suggested that a lack of tissue in the centre of the face (medial processes) may result in a median cleft lip (Fraser 1971). DeMyer (1963 a & b) suggested that median clefts of the face may be a result

of maldevelopment of the prosencephalon or forebrain. This theory can be supported by the fact that forebrain formation is important in inducing nose formation (Jacobson 1963 a & b). Gruneberg (1960) found that median clefts may be formed because of mechanical obstruction between the two medial processes (by a liquid filled bleb) so that fusion cannot occur.

The Role of Face Shape in the development of the Lip

In the formation of a normal face, normal development depends on the activity of growth centres in the early embryo as described by Stark (1954). In the older embryo, the formation of a normal lip depends on the merging of the posterior ends of the medial nasal and lateral nasal processes with the medial end of the maxillary processes.

It has been postulated that the topography and growth of the facial processes is somehow related to the formation of a cleft lip.

Trasler (1968) working with mice with a predisposition towards cleft lip — (A/J strain that has 12% spontaneous cleft lip), found that these mice had a different embryonic facial topography than a strain of mice (C57Bl/6) that virtually never has spontaneous cleft lip. She examined the embryonic faces just before and during lip formation. When the embryonic heads were viewed from the front, the A/J embryos were found to have less divergence of the medial nasal processes from the midline when compared to the C57Bl/6 embryos. This lack of divergence may result in partial or complete failure of the epithelium around the isthmus to fuse, subsequently causing break-down in the isthmus. The breaking down or lack of consolidation of the isthmus leads to the formation of a cleft lip in the A/J embryo. She also found that the

C57Bl/6 embryos had a wider proportioned face, that is the anterior limits of the nasal pit were placed further apart on the face when compared to that of the A/J strain.

Hamly (1971) produced two lines of mice through several generations of selection — one line responded to the teratogen 6AN by producing lateral cleft lips and the other line responded by producing median cleft lips. The idea was that the embryonic face shape of these two lines of mice would differ from one another, and hopefully there would be a correlation between the shape of the face and the kind of cleft obtained. She suggested that by selecting for a difference in the type of teratogen-induced cleft lip response she was also selecting for a difference in face shape. She was able to show that the nasal placodes of the embryos from the line that responded with a median cleft had more widely spaced placodes than the embryos from the line that produced lateral cleft lips with 6AN. But the sample size was extremely small (two in each case) and the results were not conclusive.

The selection experiment was continued by Rajchgot (1971). He continued to select for a line of mice that responded with lateral cleft lips to 6AN and a line that responded with median cleft lips. He examined the faces of untreated embryos from females who had reacted

positively in previous pregnancies with the appropriate type of cleft and found no difference in the two lines (Rajchgot 1971).

Rajchgot (1971) also examined embryos from C57Bl/6 females that were treated with 6AN on D9/12 of gestation (this treatment produces median cleft lips). He had found that the embryonic face shape was unchanged in the treated embryos, but the size of these embryonic heads were reduced and the nasal placodes were thinner when compared to the untreated embryos.

Smith and Monie (1969) also looked at the face area of Long Evans rat embryos after treatment with a teratogen to see if the face had changed. They found that the embryos that had lateral cleft lips had underdeveloped maxillary processes and abnormal subdivision of the maxillary tissue. In embryos that had median cleft lips, they found the naso-medial processes had failed to merge.

The role of the shape of the face in cleft lip formation has also been investigated in human subjects. Aduss et al (1971) looked at the interorbital distance of cleft lip patients and a control population. The interorbital distance was measured on longitudinal postero-anterior roentgencephalograms. They found that patients with cleft lip and palate had a greater interorbital distance than patients with only cleft palate and they

felt that this increase in interorbital distance may be a result of abnormal development in the naso-frontal process which will also affect the lip. This same group of workers also found that the gonial angle was larger and the anterior cranial fossa was more elevated in the cleft lip population than the non-cleft population.

Another approach to the method to examine the role of face shape in cleft lip is to examine the parents of cleft lip patients and compare them to parents of a non-cleft population. Pashyan and Fraser (1969) carried out a study to try to find if there were any relationship between parents who had children with cleft lip and parents that had normal children. They studied various measurements and contours on the face by using a physioprint, and found that the intraocular-chin measurement and the dyzygomatic measurement was larger in parents with cleft lip children. There was also a tendency for the anterior surface of the maxilla to be flatter in the experimental group. They also found that the parents of cleft lip children had facial profiles that tended to be receeding or vertical and the non-cleft lip group to be more of the convex shape. There was also a higher proportion of rectangular and trapezoid faces in the parents of cleft lip children, and the symmetry of the nostril did not seem to be involved. They concluded that face shape is a predisposing factor for being susceptible to cleft lip.

It has been found that mice that are homozygous for the dancer gene (Dc/Dc) have a unilateral or bilateral cleft lip as well as cleft palate, and therefore die (Deol 1966a). On examination of embryos heterozygous for this gene, it was found that most of them possess abnormalities of the inner ear which were confined to the vestibular part. These animals exhibit abnormal behaviour such as jerking movement of the head, a tendency to run in circles and hyperactivity. Undoubtedly, their abnormality in behaviour is connected to their malformed inner ear. These dancer mice usually have a white spot on the middle of the head.

It has been suggested by Deol that the neural tube and neural crest have a definite role the differentiation of the inner ear (Deol 1966b). Evidence supporting this theory ha been produced by transplantation experiments in birds and amphibians.

Role of the Neural Tube in Inner Ear Malformations

A study made of mice with the kreisler gene showed that they had abnormalities of the inner ear. Deol (1964a) showed that besides abnormalities of the inner ear, there also exist abnormalities of the neural tube in these kreisler embryos. The abnormal neural tube was spread over at least three rhombomeres and covers the floor as well

as the roof of the tube, and the fifth and sixth rhombomere were also missing. This could result in the unavailability of the normal place of invagination of the otic vesicle. Deol (1964b) also showed that in another strain of inner ear mutants (dreher dr/dr), abnormalities in the neural tube were observed at least a day before the abnormalities of the inner ear. He postulated that the primary effect of the dr gene was on the neural tube accompanied by an impairment of its inductive function.

Deol (1966b) also examined mutant mice where the neural tubes are known to be abnormal but with no known defects of the inner ear. The mutants examined were the splotch (Sp) mutants and the loop tail (Lp) mutants, the homozygotes in both these mutants are lethal and have extensive abnormalites of the neural tube. On examination of the inner ears of Sp/Sp and Lp/Lp embryos, the majority of the embryos showed abnormal differentiation of the otic vesicle.

When the above evidence was considered simultaneously with the knowledge available on the kriesler and dreher mutants, it seemed very likely that the differentaition of the inner ear is controlled by the neural tube.

The Acoustic Ganglion and the Inner Ear Mutants

In some of the inner ear mutants of the mice, it was found that the acoustic ganglion is affected. In the kriesler mutants (kr) the acoustic ganglion is greatly reduced and it seldom divides into its usual constituents. In the dancer mutants, the abnormalities of the inner ear could be traced back to the ten day stage. In the affected embryos, the acoustic ganglion appears to be smaller and has inadequate coverage over the otic vesicle. In a normal embryo the acoustic ganglion is divided into its cochlear and vestibular ganglions on day eleven of gestation, the mutants at this stage showed a reduced or complete absence of the vestibular ganglion. There have been suggestions that the acoustic ganglion is of neural crest origin (Bartelmez 1922, Adelmann 1925). The presence of a white spot on the head of the dancer mutant (hypopigmentation due to lack of melanocytes) and a deficiency of cells in the acoustic ganglion suggest that the neural crest cells may be the site of action of these dancer genes.

One of the derivatives of the neural crest cells are melanoblasts. Deol has suggested that pigmentation and inner ear defects which involve the acoustic ganglion are related via the neural crest cell. I have already mentioned previously, dancer embryos with inner ear defects also have a white spot on the middle of the head. Three other genes affecting the inner ear, which also affects

the pigmentation of the mice,have been found. The pallid gene (Lyon 1955 a & b), a recessive gene that causes dilution of the coat colour also affects the inner ear of the mouse. These inner ears lack otoliths in the sacculus and utriculus. The otoliths are secreted by the macula which may be formed as a result of interaction between the acoustic ganglion and the otic epithelium. The hypo-pigmentation and lack of otoliths (malfunction of the acoustic ganglion) have a common link — they both may be derived from the neural crest cells.

Cleft Lip, Cleft Palate and Inner Ear Mutants

The mutant gene twirler was first discovered by Lyon in 1958. The heterozygotes showed abnormal behaviour such as jerking of the head, circling and absence of postural reflexes. This abnormal behaviour was attributed to abnormal inner ears. The homozygotes soon die after birth and a large percentage of them exhibit cleft lip together with cleft palate (13/30) or cleft palate only (17/30). These homozygotes have all found to have defective inner ears.

Another gene that produced similar effects was the dancer gene (Deol 1966a), the homozygotes of this gene also had cleft lip and cleft palate as well as inner ear defects and lack of pigmentation on the top of the head. It may be reasonable to associate these malformations with abnormality of the nervous system which ultimately leads

to the suspicion that the neural crest is also involved.

EXPERIMENTAL HYPOTHESIS

In order to find out whether there is any relationship between and or common mechanism in the formation of cleft lips caused by : a single mutant gene — the dancer gene, by a multifactorially inherited predisposition in the inbred Cl/Fr strain, by a teratogen — 6-aminonicotinamide, answers to the following questions were sought:

A. Are the general development of (C57Bl/6) embryos treated with 6AN and embryos carrying the dancer gene on C57Bl/6 background the same as that of C57Bl/6 embryos which are used as controls? This may be checked by observing morphological development of the teratogen treated embryos and embryos carrying the mutant gene.

B. Are there any differences in growth of the face area in particular in the 6AN treated embryos and mutant gene embryos that may lead to the formation of a cleft lip?
The development of the nasal placode, mitotic index and histological studies will provide the answer to whether 6AN retards growth, inhibits cell proliferation, or cause cell death in the face area.

C. Are there differences in the embryonic face shape of 6AN treated embryos, dancer gene embryos and Cl/Fr embryos from the control (C57Bl/6) embryos and if so, what kind of differences?

It has been shown (Trasler 1968) that embryos predisposed to forming cleft lips have a different embryonic face shape then embryos that are resistant to forming cleft lip. , Face shape measurements done on embryos treated with 6AN and embryos carrying the dancer gene plus embryos that have a multifactorially inherited predisposition towards cleft lip may provide the clue to the role of face shape in cleft lip formation.

D. Are there any differences between embryos treated with 6AN on D9 and producing median cleft lips and embryos treated with 6AN on D10 and producing lateral cleft lips?

Comparisons of development of the face, mitotic index of the head, shape of the face may yield clues as to the mechanism of formation of either a lateral cleft lip or the formation of a median cleft lip.

MATERIALS AND METHODS

Maintenance

The mice were kept at a room temperature of 71 ± 2°F with a light cycle of sixteen hours of light and eight hours of darkness. They were kept in plastic cages, five or less to a cage and were maintained on Purina Laboratory Chow and water, available to the animals ad libitum. Whole wheat bread soaked in whole milk with lettuce was given to the mice once a week.

A male was placed in a cage containing four to five females late in the afternoon, and the females were checked for vaginal plugs the following morning.

Animals

Five experimental groups of animals were used:

1. Control group

C57Bl/6 females were used as the control group of animals. Females with a vaginal plug were considered day zero of gestation with the assumption that fertilization had occured at around 2AM the same morning (Snell et al 1940). These females were then sacrificed and their embryos collected at different gestational times, starting from D9/12 to D12/12 of gestation. This period was chosen because it is the time just prior to and up to completion

of lip formation. This strain of mice has shown virtually no spontaneous cleft lips from a long period of observation in our laboratory.

2. Teratogen-induced Median cleft lips

C57Bl/6 females were mated to C57Bl/6 males, the resulting pregnant females were given a standard dose of 6AN intraperitoneally (19mg/kg body weight) at D9/12 of gestation with a protecting dose of nicotinamide given three hours later (7.3 mg/kg body weight). 6AN was made up in a solution of distilled water, the concentration being 45 mg per 20cc while nicotinamide had a concentration of 17 mg per 20cc of distilled water. The frequency of cleft lips was determined by sacrificing the treated females between D18-D19 of gestation and the embryos were examined for cleft lip and other abnormalities. This part of the experiment was first carried out by Trasler, then by Rajchgot in our laboratory. The frequency of median cleft lips in the 6AN treated embryos was found to be 18%. (Frequency quoted is that obtained by both of the above authors.) After the cleft lip frequency had been determined, pregnant females were treated with 6AN and embryos were subsequently collected from mothers at different gestational stages starting from D9/18 (six hours after treatment) to D12 when the development of the lip has been completed.

3. Teratogen-induced Lateral cleft lips

C57Bl/6 females were mated to C57Bl/6 males and the pregnant females were given a standard dose of 6AN intraperitoneally on D10/8 of gestation followed by a standard protective dose of nicotinamide three hours later. The incidence of lateral cleft lips and other abnormalities were determined by sacrificing the mothers around D19 of gestation and examining the embryos. The embryos were found to have a lateral cleft lip frequency of around 22%. (Eight litters of 43 viable embryos were examined and nine of which had lateral cleft lips.) Teratogen treated embryos were collected at different gestational times starting at D10/14 (eight hours after treatment) to D12 of gestation.

4. Gene-Induced Lateral cleft lips

The C57Bl/6 strain of mice is usually very resistant to the formation of spontaneous cleft lip. When mice of the dancer stock carrying the dancer (Dc) gene were outcrossed to C57Bl/6, it was found that animals carrying the gene in a single dose had cleft lips. (On dancer stock background only homozygous Dc/Dc have cleft lip.) In the first outcross of C57Bl/6 to a Dc/+, 0% cleft lip was found where the mother was a C57Bl/6, and 3.9% lateral cleft lips were found when the mother was a Dc/+. In the third backcross to C57Bl/6, the frequency of cleft lip has increased to 26.4% (C mother) and 37.5% (Dc/+C BC2) mother. So we now have a gene that induces cleft lip

when present on a predominantly C57Bl/6 background (upper limit Of 87.5% C57Bl/6 at the third backcross with 5% variation due to the fact that we select for Dc every time). The frequency of cleft lips of the original outcross and subsequent backcrosses can be seen in Table 1. This data was collected from January 1967 to June of 1973. Embryos of the cross C57Bl/6 X Dc/+C (BC2) and its reciprocal backcross were collected.

5. Spontaneous cleft lip producing strain

Cl/Fr is a strain of inbred mice maintained by Fraser in this laboratory that has a spontaneous frequency of cleft lips of around 26% in viable D17 foetuses. Embryos of this strain were collected from D10/20 to D11/8 of gestation for the study of embryonic face shape.

Table 1 The cleft lip frequency of the original outcross of C57Bl/6 to Dc/+ and their subsequent backcrosses to C57Bl/6

Female	Male	Cleft lip Frequency	%CL	Max. % of C background
C57Bl/6	Dc/+	0/91	0	50.0
Dc/+	C57Bl/6	2/51	3.9	50.0
C57Bl/6	Dc/+C(F1)	59/434	13.6	75.0
Dc/+C(F1)	C57Bl/6	21/164	12.8	75.0
C57Bl/6	Dc/+C(BC1)	65/342	19.0	87.5
Dc/+C(BC1)	C57Bl/6	17/76	22.3	87.5
C57Bl/6	Dc/+C(BC2)	71/269	26.4	93.75
Dc/+C(BC2)	C57Bl/6	9/24	37.5	93.75

Morphological Rating

All embryos were collected by removing the uterus and fixing the whole uterus with its contents in Bouin's solution for a minimum of 24 hours. They were then transferred to 70% alcohol and stored until ready for examination. The embryos were then dissected out from the uterus and were rated morphologically for the number of body somites, fore and hind limb stages, stages of development of the optic vesicle the auditory vesicle and the stage of development of the nasal placodes. A numerical scale assigned to the various stages of nasal placode development is described in Table 2.

Mitotic Index

The heads of the embryos were removed and kept in 70% alcohol until they were ready for histological sectioning. These heads were then dehydrated through an alcohol series, embedded in Tissue Prep, and serial sections cut at a thickness of six microns. They were stained with Erlich's haemotoxylin and counter-stained with Eosin Y.

The number of mitoses were counted within the area of a 1 sq cm grid in the eye piece for every fifth section in both the neural and the nasal placode area at a magnification of 1,000X (oil immersion). Only metaphases, anaphases and telophases were counted as mitosis

as prophases was not well defined. The number of cells in the grid area were also counted and the mitotic index was established as the number of mitosis per hundred cells.

Example:

Mitotic index of the nasal placode area of one embryonic head

Σx = sum of mitoses of nasal placode of all sections counted per unit area

n = number of sections counted

x/n = number of mitosis per unit area

$$\text{Mitotic index} = \frac{\text{Number of mitosis per unit area}}{\text{Number of nuclei per unit area}} \times 100$$

Histological Observations

Photomicrographs of the sections were taken using panatomic X film. The shapes of cells, density of cells, cell death and the overall outlines of the nasal processes were observed.

Table 2 Nasal Placode Stages

Stage 0	Bulge or indentation
Stage 1	Oval
Stage 2	Oblong
Stage 3	Crescent
Stage 4	Comma

These are the same nasal placode stages described by Trasler (1968) and Rajchgot (1971).

Face Shape Measurement

The heads of the embryos were removed when measurements of the face were made. The lower jaw was also removed to give a better view of the inferior aspect of the face. These heads were photographed with a polaroid camera that was connected to a dissecting microscope, the resulting photograph had a magnification of 28X. Each embryonic head was photographed in two different positions. First in the inferior position showing the nasal placodes (Figure 1A) and then in an anterior position showing the angle formed by the medial nasal processes. A series of measurements were made on these photographs. Distances were measured with a pair of calipers, usually at least twice and areas were measured by a planimeter. (Linear measurements in mm and areas in sq in.)

Measurements made on the inferior view (Figure 1A)

1. The distance between the anterior limits of the nasal placode —— Db
2. The widest distance of the head at the level of the nasal placode —— Dba
3. Area of the inferior view enclosed in the black heavy line — Ab
4. The sum of the width of the two lateral processes was calculated as the difference between the widest distance of the head at the level of the nasal placode and the distance between the anterior pits (Dba - Db).

Measurements made on the front view of the embryonic face (Figure 1B):

1. The jut measurement as indicated by J in Figure 1B.
2. Area of the front view enclosed by the black heavy line — Af.

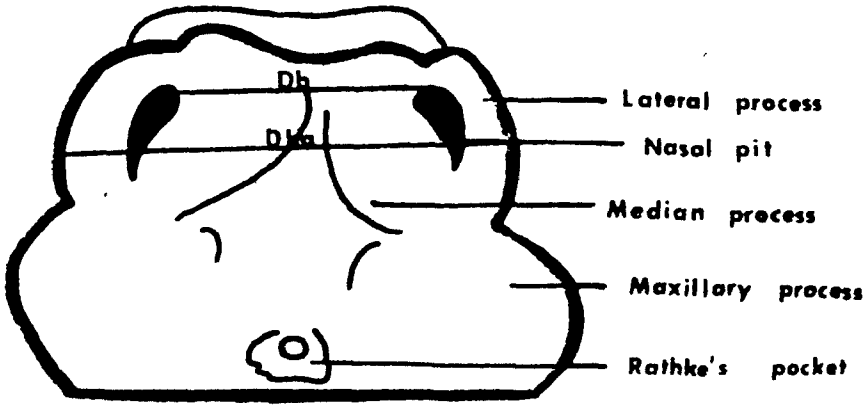


FIGURE 1A INFERIOR VIEW OF THE EMBRYONIC HEAD

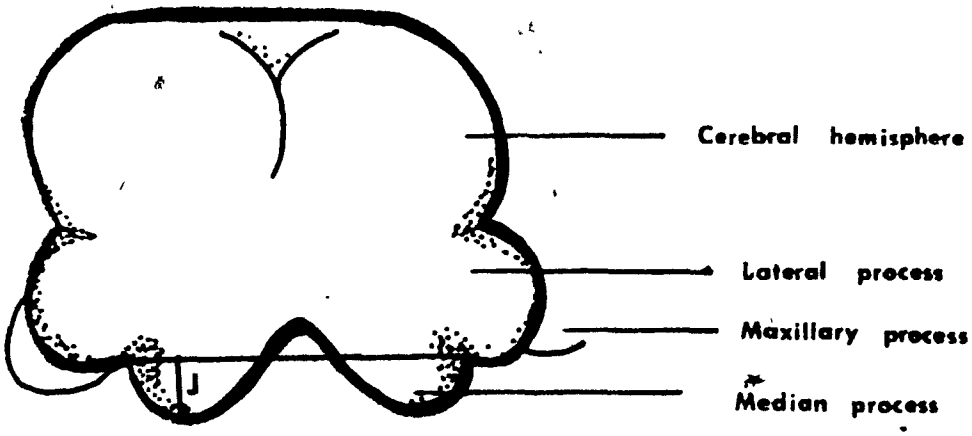


FIGURE 1B FRONT VIEW OF THE EMBRYONIC HEAD

Statistical Analysis

A two tail t test for difference between the means was used when comparison of two sets of measurements or values had to be analyzed. This analysis involved the testing of the null hypothesis, that is

H_o : $_{1-2} = 0$ against the alternative of

H_1 : $_{1-2} \neq 0$

If H_o is rejected, then a difference exists between the two means. This is a two tail t test because it is a non-directional test, that is two tails or two sides of the distribution are employed in estimating the probabilities. The formula used to obtain the t value is extracted from "Statistical Analysis in Psychology and Education" by G. A.=Ferguson.

It is used to test the difference between means when population variances are unequal.

$$t = \frac{\bar{X} - \bar{Y}}{\sqrt{SX^2 + SY^2}} \qquad \bar{X} = \frac{\Sigma X}{n_1} \quad \bar{Y} = \frac{\Sigma Y}{n_2}$$

$$S_{\bar{x}-\bar{y}} = \frac{\Sigma (X - \bar{X})^2}{n_1(n_1 - 1)} + \frac{\Sigma (Y - \bar{Y})^2}{n_2(n_2 - 1)} = \sqrt{SX^2 + SY^2}$$

RESULTS

Normal Development of the Nasal Placodes

In the C57Bl/6 inbred embryos, the nasal placode appears as thickenings on the surface of the epithelium around D 9/12 hours of gestation. Some of the embryos have placodes that may appear as slight indentations and others have shallow ridges already formed around the indentation. As the embryo continued to develop, the thickened ectoderm in the placode area grows in thickness and starts to invaginate resulting in a definite indentation on the surface of the embryo in the future nasal pit area. Invagination begins at around D10/8 of gestation. At this time, the embryo can be seen to have distinctive lateral nasal and median nasal processes.

When these embryos were examined on D10/12 of gestation, the nasal pits were invaginated further. On the surface they appear to resemble the shape of an oval, with some of the more advanced faces having nasal pits resembling an oblong in shape. By D10/20 of gestation, that is eight hours later, the lateral and medial nasal processes begin to approach one another and externally they surround an oblong shaped pit and the more advanced embryos have nasal pits that resemble a crescent in shape.

The lateral nasal and medial nasal processes start to fuse by D11/12 and where fusion has occurs, epithelial breakdown is observed histologically. Externally these

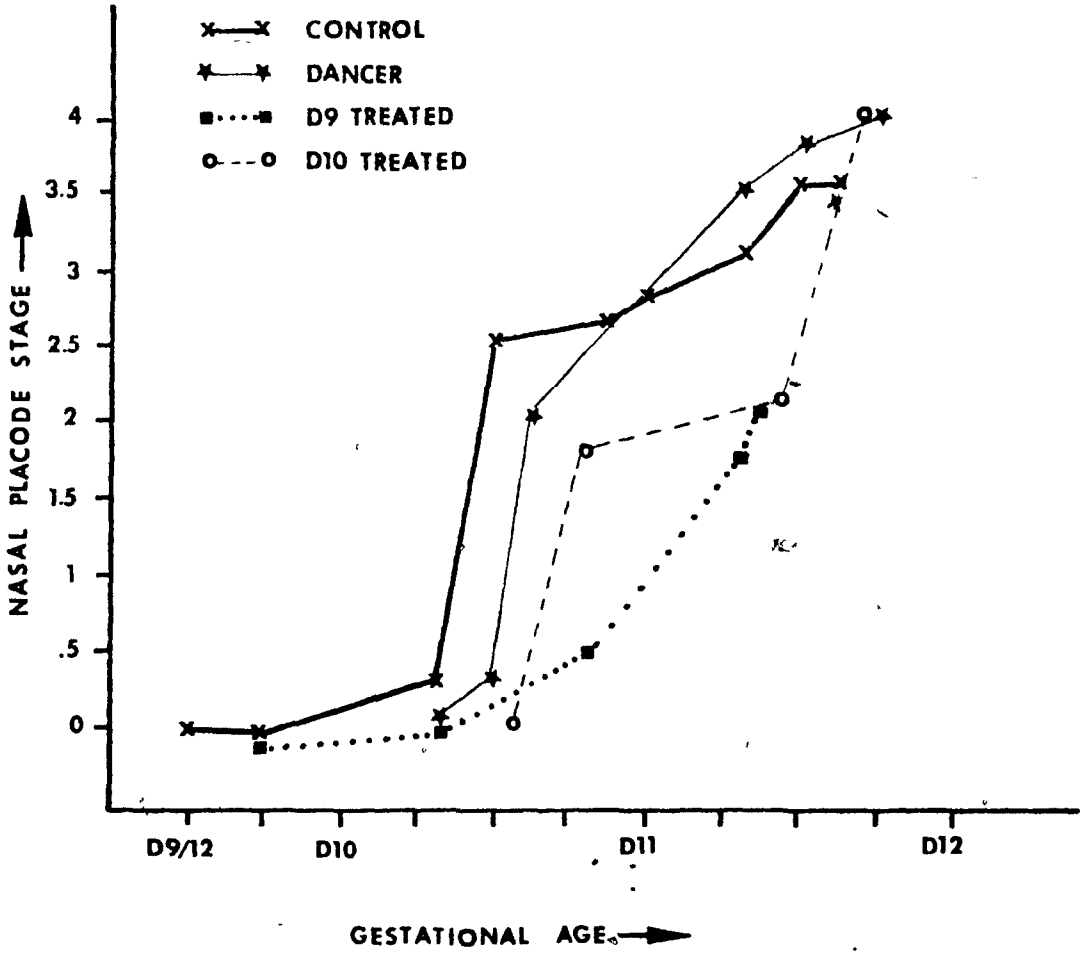
pits look like commas, with fusion starting at the posterior end. Nasal pits may be found to have half fused processes while others may be completely fused.

By D12, fusion of the nasal processes over the pits has been completed and one would be able to identify cleft lip embryos, if any, by the lack of fusion between the lateral and medial nasal processes.

Each nasal placode stage was assigned a numerical value starting with zero for the least developed nasal placode and progress to a value of four for the complete fusion of the nasal pit. (See Table 2 in Materials and Methods)

The nasal placode stages for all groups are tabulated in Table 3 and are plotted against gestational time in Figure 2. A linear increase or advancement in nasal placode stage is observed with increase in gestational age.

FIG. 2 AGE VERSUS NASAL PLACODE STAGE



The Development of Nasal Placodes in Embryos treated on D9/12 of gestation

Embryos were first examined on D9/18 of gestation, externally the nasal placodes appear as slight bulges or thickenings with no indentations. In histological sections, the ectoderm of the nasal placode in the region of the slight thickenings, was found to be two to three cells thick. At six hours after treatment, the nasal placodes did not seem to be different outwardly or histologically from the control embryos.

By D10/8, the nasal placodes still remained as bulges with hardly any indentation on the surface. Histologically they showed thickenings of the ectoderm. They look retarded compare to the controls which already showed distinct invagination and the appearance of lateral and medial nasal processes. (Control nasal placode mean stage of 0.29 versus treated nasal placode mean stage of 0.0) The nasal placode mean stages of the controls and treated are tabulated in Table 3a & 3b.

The nasal placodes still remained retarded on D10/20 of gestation. The treated embryos showed a wide range of developmental stages, some of the placodes were beginning to invaginate , while others had progressed as far as the oval stage. They seemed to be around twelve hours behind in development when compared to the

controls. (Control 2.71 vs 0.5 treated mean nasal placode stage)

The most retarded embryos may be the ones that have less ability to recuperate from the teratogen and may eventually form a cleft lip.

By D11/8 of gestation, the nasal processes around the pits of these embryos were seen to approach each other and the majority of them assume a crescent shape. The developmental stages of the nasal placode still covered a wide range, placode stages ranged from shallow indentations to comma stages with their mean at late oblong stages. (Control 3.23 vs. treated 2.14) The D11/8 placodes were also retarded compared to the controls.

Only two litters with a total of seventeen embryos were examined on D11/14 hours. They were found to be severely retarded and it was concluded that these were either late resorptions or the mating time had occured much later than normal. Some of these embryos had open neural tubes and incomplete turning of the bodies and these have not been included in any comparisons.

The nasal placode stages were tabulated in Table 3b and they were plotted against the gestational age in Figure 2. A definite retardation in development could be seen starting around the middle of D10.

Histological observations were made on embryos matched for their developmental age at the crescent

nasal placode stage rather than chronological age.

Control embryonic head sections showed a well formed lateral nasal process and a well developed medial process. When the control embryos were compared to embryonic head sections treated on D9, these embryos appeared to have a markedly reduced medial process, the reduction in size was probably due to a reduced volume of mesenchyme. Figure 3 illustrates the reduction of the medial process. The lateral process is also seen to be reduced, but the reduction was not as prominent as that of the medial process. It is appropriate to note that 6AN when given to the pregnant female on D9/12 produces median cleft lips in the offspring.

The nasal placodes were retarded in development because the time of treatment — D9/12 hours may be a critical time for the formation of the nasal placode, 6AN therefore had a very large effect on the development of the nasal placodes when adminstered at that time.

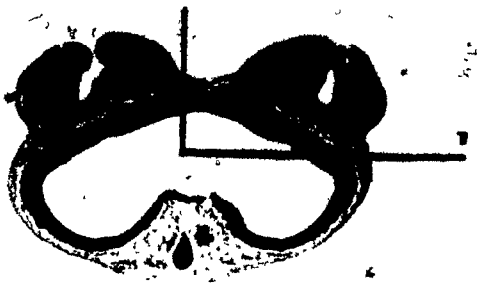
Figure 3A Transverse section through the embryonic head of a Control embryo (C57Bl/6) showing well formed lateral and median nasal processes at crescent nasal placode stage (D10/20).
Magnification : 64X

Figure 3B A higher magnification of the nasal placode that is shown in the inset of 3A.
Magnification : 126X

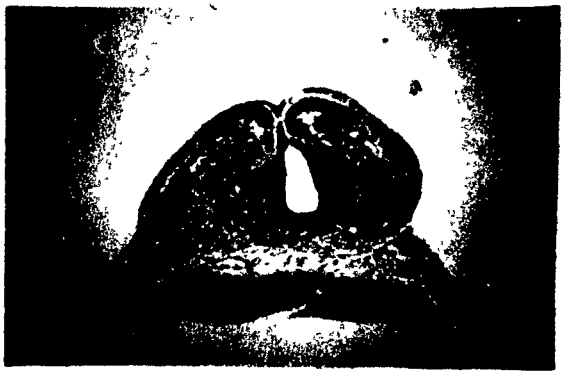
Figure 3C Transverse section through the head of an embryo that received 6AN on D9/12. Note the reduced medial processes and the smaller lateral processes at crescent nasal placode stage (D11/8).
Magnification : 64X

Figure 3D A higher magnification of the nasal placode that is shown in the inset of 3C.
Magnification : 126X

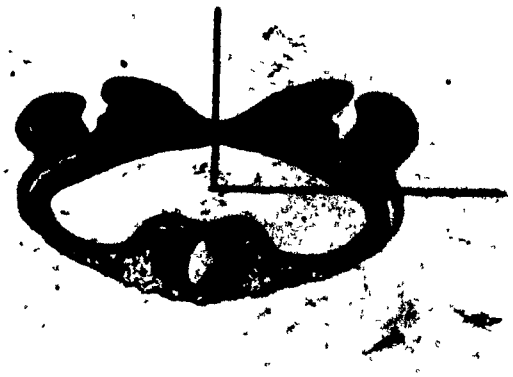
A



B



C



D

Development of Nasal placodes in Embryos treated with 6AN on D10/8 of gestation

The pregnant females in this series were treated on D10/8 of gestation. The first group of treated embryos examined were on D10/14, six hours after treatment with 6AN.

At D10/14, the nasal placodes appear either in the shape of an oval or were still at the shallow indentation stage with the majority at shallow indentation stage. When compared with controls whose nasal placodes had assumed an oval or oblong shape (1.21 vs. 0.14 nasal placode mean stage); they were retarded significantly. This is demonstrated in Figure 2 where the nasal placode stages are plotted against gestational age together with the controls.

When these treated embryos were examined on D10/20, they were found to range from a bulge to a crescent stage, with the majority of the embryos at oblong or crescent stages. These were not found to be different from the control animals, although a larger range of placode stages were observed when compared to the controls. This may be due to the fact that not all embryos were affected by 6AN to the same degree.

By D11/8, these treated embryos had reached late

crescent or early comma stages with some only slightly behind in development compared to the controls (Control 3.23 vs. treated 2.29 nasal placode mean stage). The processes were seen to approach each other and fusion began.

By D11/14, nasal placodes ranged from late crescent stages to comma stages with some of the nasal placodes three quarters fused, and they did not differ from the controls. (Refer to Table 3A & 3C)

By D12/12, outwardly embryos were very well developed, but on examination of some of the histological sections, fusion of the two nasal processes were incomplete, this may lead to formation of incomplete lateral clefts.

In summary, nasal placode development of embryos treated on D10/8 was retarded at six hours after treatment, but the nasal placode appeared to recuperate from the effect of 6AN soon afterwards. This can be seen from Table 3C and Figure 2 where the nasal placode stages of the D10 treated embryos were tabulated and plotted against their gestational age.

On histological sections, embryos that were given 6AN on D10/8 were found to have small processes. They were also found to have small medial processes. This can be demonstrated from the photomicrographs shown in Figure 4. 6AN when administered to the pregnant mother

Figure 4A Transverse section through the embryonic head of a control embryo (C57Bl/6) showing well formed lateral and medial nasal processes at crescent nasal placode stage (D10/20)
Magnification : 64X

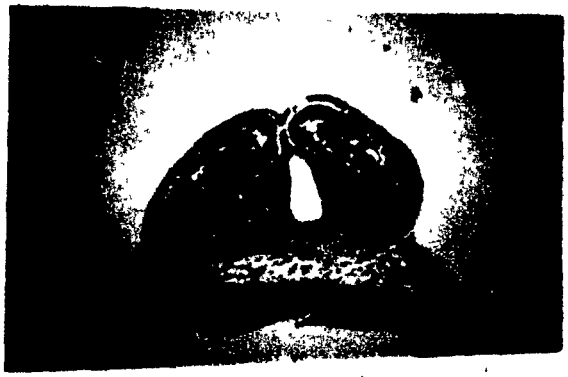
Figure 4B A higher magnification of the nasal placode that is shown in the inset of Figure 4A.
Magnification : 162X

Figure 4C Transverse section through the head of an embryo that has received a standard dose of 6AN on D10/8. Note the reduced lateral processes and smaller medial processes at crescent nasal placode stage (D11/8).
Magnification : 64X

Figure 4D A higher magnification of the nasal placode that is shown in the inset of 4C.
Magnification : 162X

A

B



C

D

on D10/8 produces lateral cleft lips in the offspring.

Nasal placode development of dancer embryos

Dancer embryos were examined starting from D10/8 of gestation. No retardation in development of the nasal placodes was apparent at first, but when the morphological rating data for nasal development was lumped into two stages — D10 and D11, the nasal placode development was found to be retarded in the dancer embryos (Control 1.695 vs. dancer 1.02 mean nasal placode stage) on D10 but was not retarded on D11 (Control 3.47 vs. dancer 3.50). Nasal placode stages of dancer embryos are tabulated and plotted against their gestational age in Table 3D and Figure 2.

Histological observations of matched serial sections show that dancer embryos that eventually get a cleft lip have both reduced lateral and medial processes, and this is illustrated in the photomicrographs shown in Figure 5.

Figure 5A Transverse section through the embryonic head of a control embryo (C57Bl/6) showing well formed lateral and medial nasal processes at crescent nasal placode stage (D10/20). Magnification : 64X

Figure 5B A higher magnification of the nasal placode that is shown in the inset of 5A. Magnification : 162X

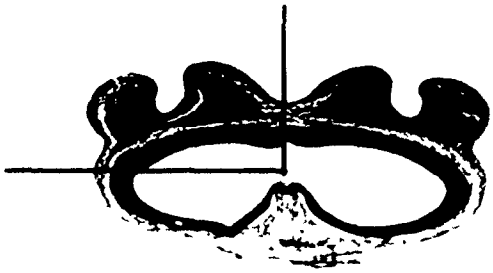
Figure 5C Transverse section through the head of an embryo that carries a dancer (Dc) gene with C57Bl/6 background at crescent nasal placode stage (D11). Note the reduction of both lateral and medial processes.

Figure 5D A higher magnification of the nasal placode that is shown in the inset of 5C. Magnification : 162X

A

B

C



D

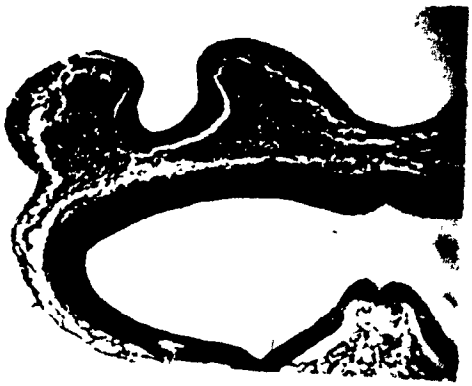


Table 3 Nasal Placode Stages

3A Control

Age	Mean ± S.E. Nasal Placode stage	N
D9/12	0 ± 0	16
D9/18	0 ± 0	13
D10/8	0.29 ± 0.17	7
D10/12	2.57 ± 0.2	14
D10/14	1.21 ± 0.17	14
D10/20	2.71 ± 0.17	21
D11/8	3.23 ± 0.17	35
D11/12	3.6 ± 0.2	5
D11/14	3.57 ± 0.28	14

3B D9 Treated

Age	Mean ± S.E. Nasal Placode stage	N
D9/18	0 ± 0	8
D10/8	0 ± 0	6
D10/14	0.273 ± 0.14	11
D10/20	0.5 ± 0.14	12
D11/8	2.14 ± 0.2	43

Table 3 Nasal Placode Stages (Continued)

3C D10 Treated

Age	Mean ± S.E. Nasal Placode stage	N
D10/14	0.14 ± 0.14	7
D10/20	1.91 ± 0.28	11
D11/8	2.29 ± 0.52	7
D11/14	4.0 ± 0.0	8
D12/12	4.0 ± 0.0	5

3D Dancer

Age	Mean ± S.E. Nasal Placode stage	N
D10/8	0 ± 0	20
D10/12	0.5 ± 0.15	32
D10/15	2.0 ± 0.28	18
D10/20	1.57 ± 0.25	14
D11	2.8 ± 0.25	17
D11/8	3.4 ± 0.14	40
D11/12	3.6 ± 0.15	31
D11/18	4.0 ± 0.0	19

External malformations of 6AN treated embryos

The majority of the malformations noted were seen in the embryos treated on D9/12. In some embryos crowding of body somites was noted and splitting of somites was also found. In one embryo the posterior end of the body had an unusual twist and these may be early signs that the embryos are affected by 6AN.

In some embryos that have been treated with 6AN, ridges were observed on the top of the head between the two nasal placodes. In the histological sections, these were seen to be the prosencephalon neural tissue to be pushing its way between the two medial processes. Since this phenomenon was found in the D9 treated embryos that react to the teratogen by forming a median cleft lip, it may be responsible for the failure of the medial processes to merge.

The general development of embryos

The gross morphological development of an embryo could be estimated by the number of somites present, stages of the fore and hind limbs, the state of invagination of the optic vesicles and the stage of the otic vesicles. All the above characteristics were examined, but only the body somite number will be presented here as an indication of general development.

Control embryos

In the inbred C57Bl/6 embryos, the number of somites were seen to increase with time. At the age of D9/12, the mean somite number was 17, and it increased to a mean number of 39 by D11/12. The increase seemed to be in a linear fashion. (Figure 6) The number of somites were tabulated and plotted against gestational age in Table 4 and Figure 6.

D9/12 treated embryos

On D9/18 when they were first examined, the mean number of somites was found to be 19. The t test for significance between the means of body somites was not different. At this stage, it may be too soon for the toxic effects of 6AN to be apparent externally (Table 4).

By D10/8, twenty hours after treatment, a definite retardation can be seen, the treated embryos have a mean somite number of 21 whereas the controls have progressed to a mean number of 29. T tests of significance showed

them to be significantly different from each other at the .001 level. This was also true at all subsequent times examined. The mean number of body somites of the D9 treated embryos were plotted against their gestational age in Figure 6 together with the controls and it is quite obvious that the D9 treated embryos were retarded in somite formation.

D10/8 treated embryos

These embryos were found to be retarded in the beginning at D10/14, at six hours after treatment, but by D11/14, the general body development did not differ from that of the controls. The development of the body correlated fairly well with the nasal placode development in that both seem to be recovering from the effects of 6AN by D11/14. T tests are presented in Table 4. The body somites were plotted against their gestational age in Figure 6.

Dancer embryos

Dancer embryos on the whole did not seem to be significantly different except at D10/12 and D11/12 in development from the control animals (Table 4 and Figure 6).

Table 4 T tests of significance between Control group and D9/12 treated group for somite number at different gestational ages

Age	Control	Nc	D9 treated	Nt	P
D9/18	18.5 ± 1.14	13	19.13 ± 1.37	8	.8-.7
D10/8	29.43 ± 1.04	7	21.12 ± 0.79	6	>.001***
D10/13	34.93 ± 0.56	14	26.64 ± 0.79	11	>.001***
D10/20	34.67 ± 0.47	21	27.92 ± 0.97	12	>.001***
D11/8	37.4 ± 0.6	35	33.91 ± 0.6	47	>.001***
D11/14	39.43 ± 1.36	14	21.18 ± 1.22	17	>.001***

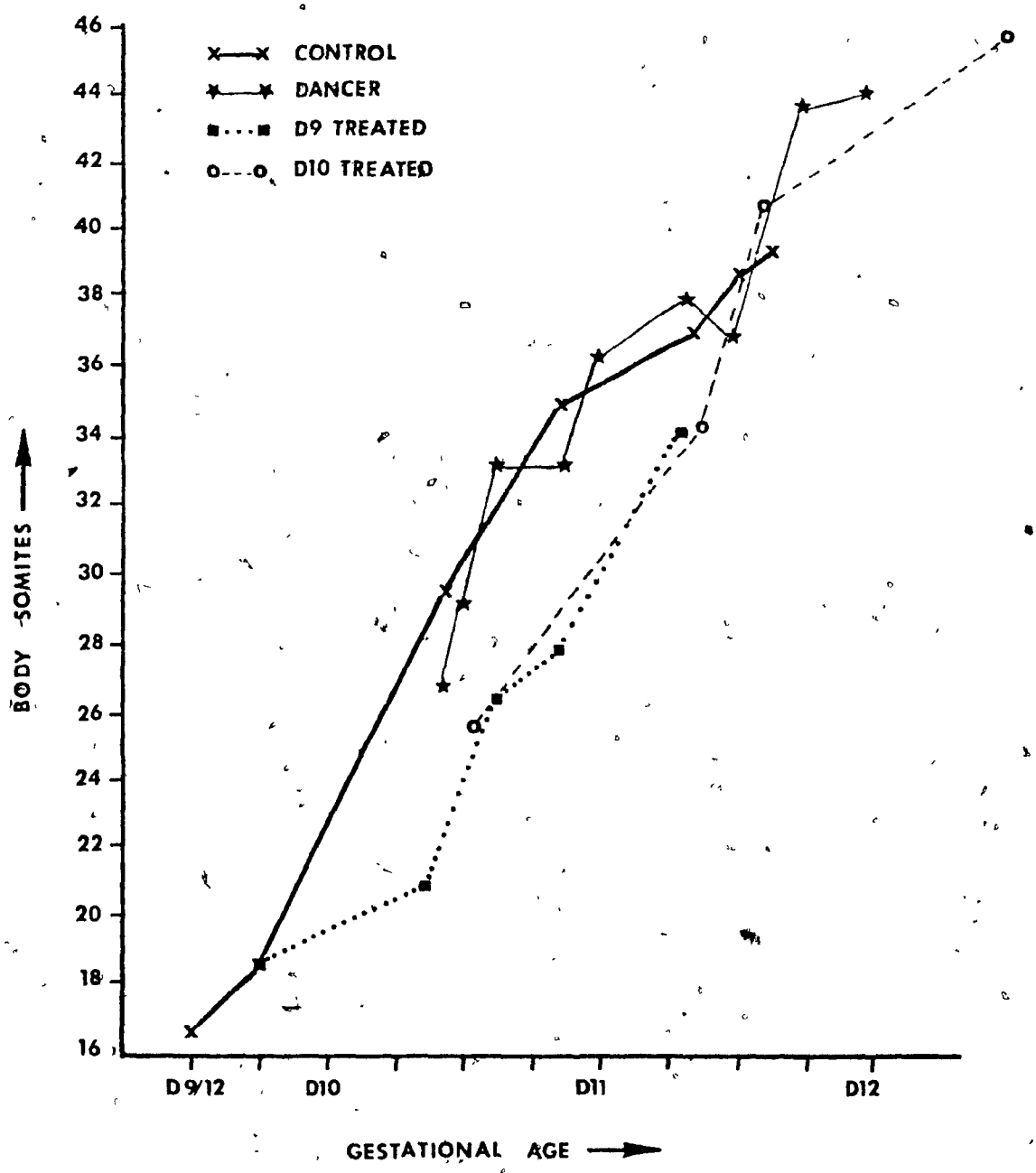
T tests of significance between Control group and D10/8 treated group for somite number at different gestational age

Age	Control	Nc	D10 treated	Nt	P
D10/14	30.86 ± 1.15	14	25.57 ± 0.75	7	.001***
D10/20	34.67 ± 0.47	21	33.18 ± 0.57	11	.1-.05
D11/8	37.4 ± 0.6	35	34.29 ± 1.32	22	.001***
D11/14	39.43 ± 1.36	14	40.88 ± 0.52	8	.5-.3

T tests of significance between Control group and Dancer group for somite number at different gestational age

Age	Control	Nc	Dancer	Nd	P
D10/8	29.43 ± 1.04	7	26.65 ± 0.342	20	.02-.01*
D10/12	34.93 ± 0.56	14	28.8 ± 0.62	31	>.001***
D10/15	30.96 ± 1.15	14	33.05 ± 0.79	18	.2-.1
D10/20	34.67 ± 0.47	21	33.2 ± 0.57	14	.1-.05
D11/8	37.4 ± 0.6	35	37.72 ± 0.5	40	.7-.5
D11/12	38.8 ± 0.73	5	36.74 ± 0.49	31	.05-.02*

FIG. 6 AGE VERSUS BODY SOMITES



Nasal ectoderm

In the control embryos, the nasal ectoderm had a psuedo-stratified appearance, the cells were elongated, and prominent nucleoli could be s een. Cells were also seen to be dividing. This is illustrated in Figure 7, a photomicrograph taken under oil immersion of the nasal ectoderm of a control embryonic head.

In the embryos treated on D9/12, and examined on D11/8 of gestation, the nasal ectoderm looked normal, it seemed to have fewer cells but necrosis was not noticeable. Figure 8 is a photomicrograph of the nasal ectoderm of a D9/12 treated embryo and when compared to the control section it was found to have fewer cells for a constant area.

In the D10 treated embryos examined on D11/8 of gestation, cells were densely packed, and did not seem to be psuedo-stratified and were generally rounder in shape. It looked as if the cells in the nasal ectoderm had lost their organization after treatment. This is demonstrated in Figure 9 which is a photomicrograph of the nasal ectoderm of a D10 treated embryo.

Dander embryos had nasal ectoderm that looked quite normal except for the fact that they seemed to have a higher number of cells for a given area. This can be seen from the photomicrograph shown in Figure 10.

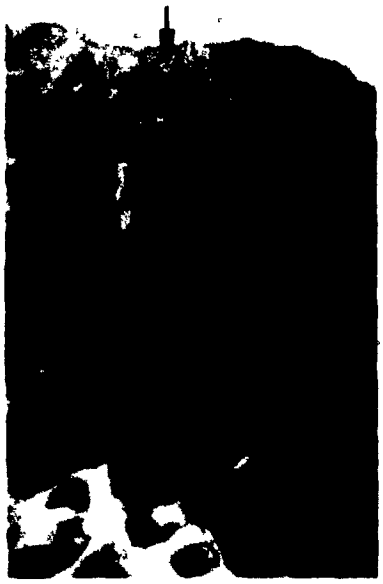
Figure 7 Nasal ectoderm of a control embryo (D10/20 hours, late crescent stage). The arrow is pointing at a dividing cell. (magnification : 2,000X)

Figure 8 Nasal ectoderm of a D11/8 embryo at crescent stage that was treated with 6AN on D9/12. They do not seemed to be too different from controls. (Magnification : 2,000X)

Figure 9 Nasal ectoderm of an embryo that has been given a standard dose of 6AN on D10/8, killed on D11/8. Note that the cells have rounded up, lost their psuedostratified appearance and are generally closely packed. (Magnification : 2,000X)

Figure 10 Nasal Ectoderm of an embryo that carries a dancer gene, killed on D11 at crescent nasal placode stage. Nasal ectoderm looks normal, but the cells are more densely packed. (Magnification : 2,000X)

7



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Mesenchyme

On examination of the mesenchyme adjacent to the nasal ectoderm of embryos at crescent stage, 'dense bodies' of condensed chromatin were found.

These dense bodies were also found in the control embryos, if they are pycnotic or dead cells, then these dense bodies may represent physiological cell death in the normal process of development. Figure 11 shows the mesenchyme of a control embryo, a dividing cell can be seen in the lower right quadrant of the photomicrograph and a few dense bodies can also be seen scattered in the picture. Figure 15 is a higher magnification of the mesenchyme and in a couple of cells the condensed chromatin can be seen.

In both the D9 treated and the D10 treated embryos examined at crescent stage around D11/8, a relatively larger number of dense bodies were seen when compared with the controls. Because the sections were cut at six microns they were too thick to tell whether the dense bodies were inside the nucleus or in the cytoplasm of the cell. Figure 12 is a photomicrograph of the mesenchyme adjacent to the nasal placode in a D10 treated embryo, note the increase number of dense bodies. Figure 13 is a photomicrograph of a D9 treated embryo's mesenchyme where an increase in the number of dense bodies can

also be seen.

Figure 16 is a higher magnification of the mesenchyme of a D9 treated embryo, the dense bodies are indicated by arrows. It will only be possible to determine the location of the dense bodies in the cell by electron microscopy.

These dense bodies are also present in embryos carrying the dancer gene, in approximately the same number as the controls. This is illustrated in Figure 14 and Figure 17.

Figure 11 Mesenchyme adjacent to the nasal placode in a control embryo at D10/20 crescent stage. The arrow is pointing at a dividing cell. One of the dense bodies is encircled by a dark line. (800X)

Figure 12 Mesenchyme adjacent to the nasal placode of an embryo at D11/8 that has received a standard dose of 6AN on D10/8 of gestation. Notice the numerous dense bodies scattered around in the mesenchyme. (800X)

Figure 13 Mesenchyme adjacent to the nasal placode of an embryo on D11/8 that has received a standard dose of 6AN on D9/12 of gestation. There are numerous dense bodies scattered around in the mesenchyme. (800X)

Figure 14 Mesenchyme adjacent to the nasal placode area in a dancer embryo on D11 of gestation. They do not have an increase number of dense bodies that were shown in the treated embryos. (800X)

11

12

13

14

Figure 15 A higher magnification of the mesenchyme adjacent to the nasal placode in the control embryo. In the lower part of the photomicrograph there are cells with condensed chromatin. (2,000X)

Figure 16 A higher magnification of the mesenchyme of an embryo treated with 6AN on D9/12, the arrows are pointing to 'dense bodies' which may be pycnotic cells. (2,000X)

Figure 17 Mesenchyme of an embryo carrying a dancer gene at high magnification. Arrows are pointing at abnormal looking granules or dense bodies. (2,000X)

15

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17

Cell Population Density

The number of cells for a given area will be called 'cell density' in this study. Each section was observed under the microscope at a magnification of 1,000X. An ocular grid of one square centimeter was inserted into one of the eye pieces and the number of cells within the ocular grid was counted.

For each of the control and experimental groups of embryos, the cell density of the nasal ectoderm in the nasal placode and the neural ectoderm of the telencephalon was calculated for each of the gestational times under study.

The control embryos (C57Bl/6) had a cell density that increased as the embryo developed. This is illustrated in Figure 18 and Figure 19 which are graphs of the cell density versus the gestational age for nasal or neural ectoderm. Table 5 gives the averages for cell density for both neural and nasal ectoderm at different ages.

When the D10 treated embryos were examined they were found to be more dense, this agreed with what Seegmiller (1972) had found with his chick embryos that were treated with 6AN causing micromelia. (Refer to Figure 18 and 19 and Table 5). Figure 20 is a photomicrograph of the neural ectoderm of a control

embryo, and Figure 21 is one of a D10 treated embryo, it is obvious that the cells in the D10 treated embryo are more densely packed and rounded in shape.

Dancer embryos also had a higher density of cells in both the neural and nasal tissue. This is illustrated in Figure 18 and 19 and tabulated in Table 5. Figure 22 is a photomicrograph of the neural ectoderm of a dancer embryo, when compared to the control in Figure 20 it is more dense.

Cell densities of the D9 treated embryos however were less dense than those of the control. (Figure 18 and 19, Table 5) Figure 23 is a photomicrograph of neural ectoderm of a D9 treated embryo, when compared to the control in figure 20 it appears that the cells are not packed as tightly as those of the control embryo.

Table 5 Cell Densities (Mean number of cells per $1cm^2$ grid)

Control

Age	Nasal	Neural	N
D10/8	192	208	6
D10/12	216	254	6
D10/20	231	274	5
D11/8	253	289	7
D11/12	268	314	5
D11/14	271	339	5

D9 Treated

Age	Nasal	Neural	N
D10/8	191	176	4
D10/14	186	199	10
D10/20	224	225	6
D11/8	233	253	12
D11/14	208	253	4

D10 Treated

Age	Nasal	Neural	N
D10/14	274	286	4
D10/20	273	345	10
D11/9	273	336	9
D11/14	304	410	7
D12/12	375	436	6

Dancer

Age	Nasal	Neural	N
D10/8	276	246	9
D10/15	281	342	6
D11	270	324	I5
D11/8	322	404	13
D11/12	347	429	9

FIG. 18 CELL DENSITIES OF NASAL TISSUE

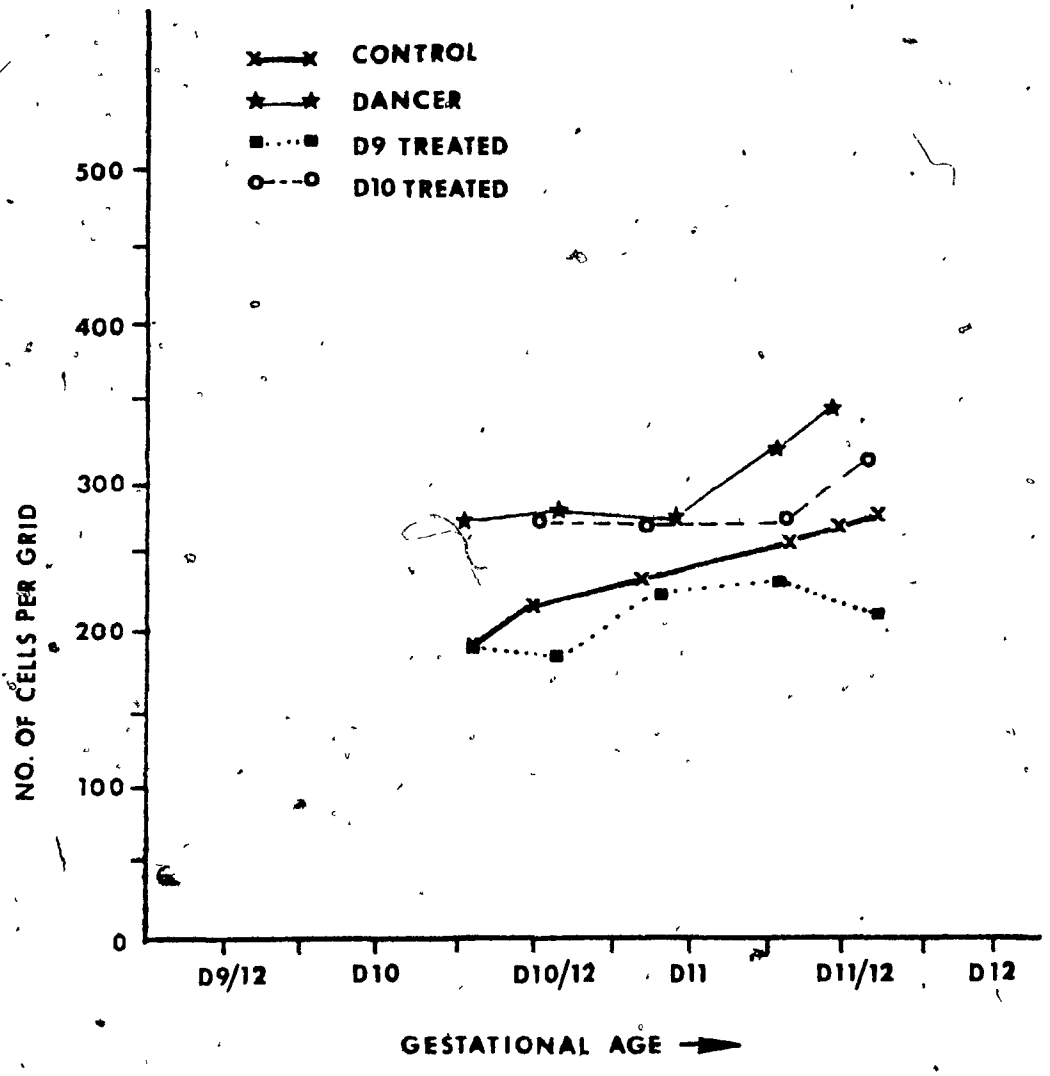

FIG. 19 CELL DENSITIES OF NEURAL TISSUE

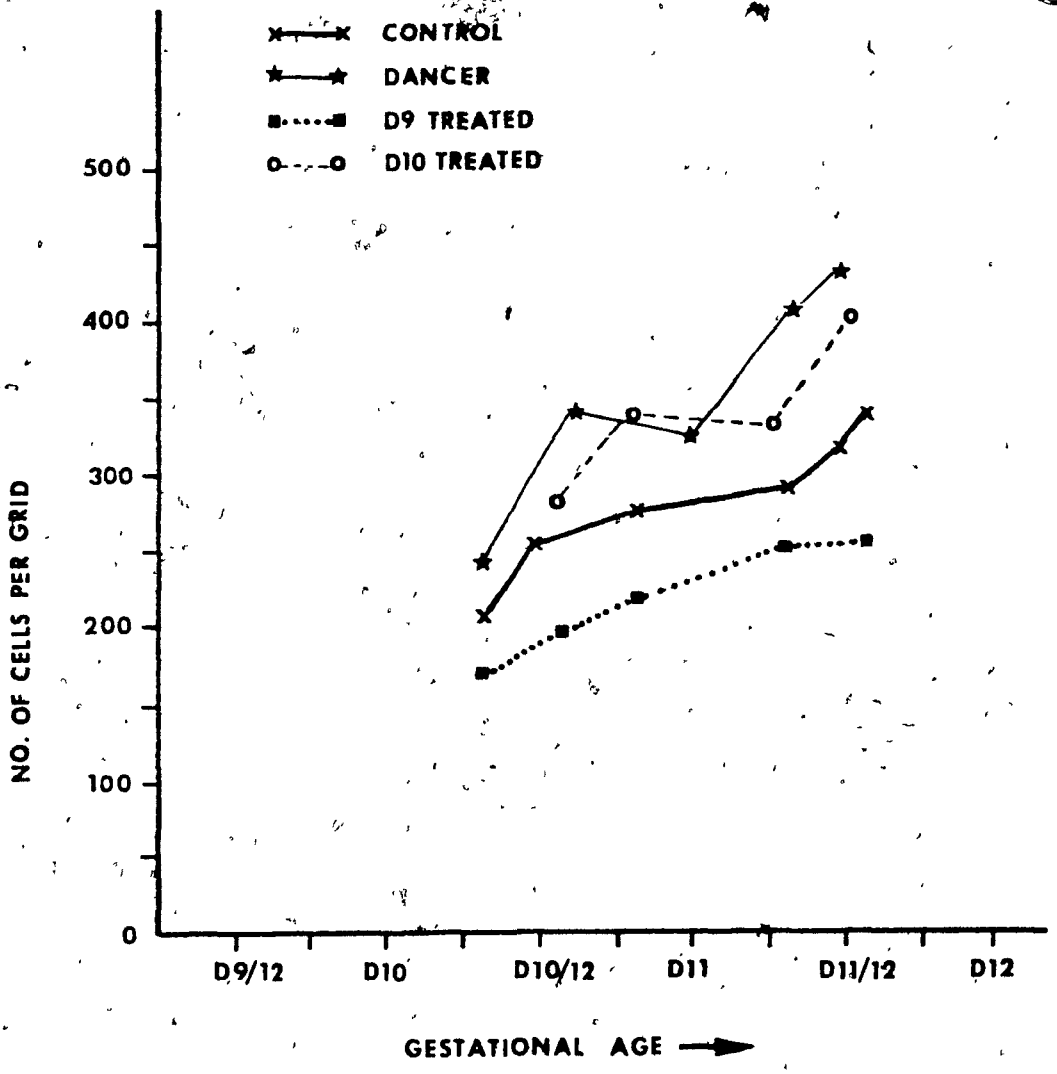

Figure 20 Neural ectoderm of a D10/20 embryo at cresecnt stage. (2,000X)

Figure 21 Neural ectoderm of a D11/8 embryo that was treated with 6AN on D10/8 (crescent stage). The cells are rounded up and are densely packed. (2,000X)

Figure 22 Neural ectoderm of a dancer embryo on D11 at crescent stage. Cells are relatively densely packed when compared to controls. (2,000X)

Figure 23 Neural ectoderm of a D9/12 treated embryo on D11/8 at crescent stage. Cells are relatively loosely packed when compared to controls. (2000X)

20

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23

Mitotic Index

The number of mitoses per one hundred cells is defined as the mitotic index.

$$\text{Mitotic Index} = \frac{\text{Mean No. of mitoses/grid } (1\text{cm}^2)}{\text{Mean no. of cells/grid } (1\text{cm}^2)} \times 100$$

The mitotic index was calculated for two different areas on the histological section of the embryonic head.

(i) An area around the nasal placode

(ii) An area around the neural ectoderm (part of the telencephalon)

Every fifth section was counted for number of mitoses in one grid from the appearance of and right through the nasal placode area. The average number of mitoses per grid calculated for the nasal area and neural area for each head. The number of cells per grid was found by counting the number of cells per grid.

In the neural area of the control embryos, mitosis increased with age, with a large spurt around early D11. In the nasal area mitosis increase sharply around early D10 and levelled off afterwards. (Figure 24 and Figure 25)

When the treated embryos were studied, it was found that mitosis in both the neural and nasal tissue was generally retarded. In the embryos that received a standard dose of 6AN on D9/12, mitosis was not retarded throughout the nasal placode development in the neural tissue (Table 6A) but was found to be depressed at all

times in the nasal ectoderm. (Table 6B) When the mitotic index of the D9 treated embryos was plotted against gestational age on the same graph as that of the control, it can be seen that the neural tissue was affected early (D10/8) and recovered while the nasal tissue was affected later on D10/12 and did not recover. (Figure 24 and Figure 25)

Embryos treated with 6AN on D10 showed a similar picture, with mitosis depressed initially in the neural tissue and recovering by D11/14 and mitosis depressed throughout the period examined of nasal placode development in the nasal ectoderm. This is tabulated in Table 7A and 7B where t test for the differences between means are given for the controls and the D10 treated embryos. The mitotic index is plotted against gestational age on the same graph as the controls and illustrates the above points in Figures 24 and 25.

Mitotic indices of dancer embryos were calculated for both neural and nasal tissue throughout the gestational time when the nasal placode was forming. Changes were not expected in this group of embryos because they were hybrid animals and even if mitosis was reduced it would only be expected to be found in embryos carrying the dancer gene (50%), that is only half of the embryos

would be affected. If the above was true, a bimodal distribution would be expected or a large variability within the group resulting in large standard errors would be found, but neither was the case.

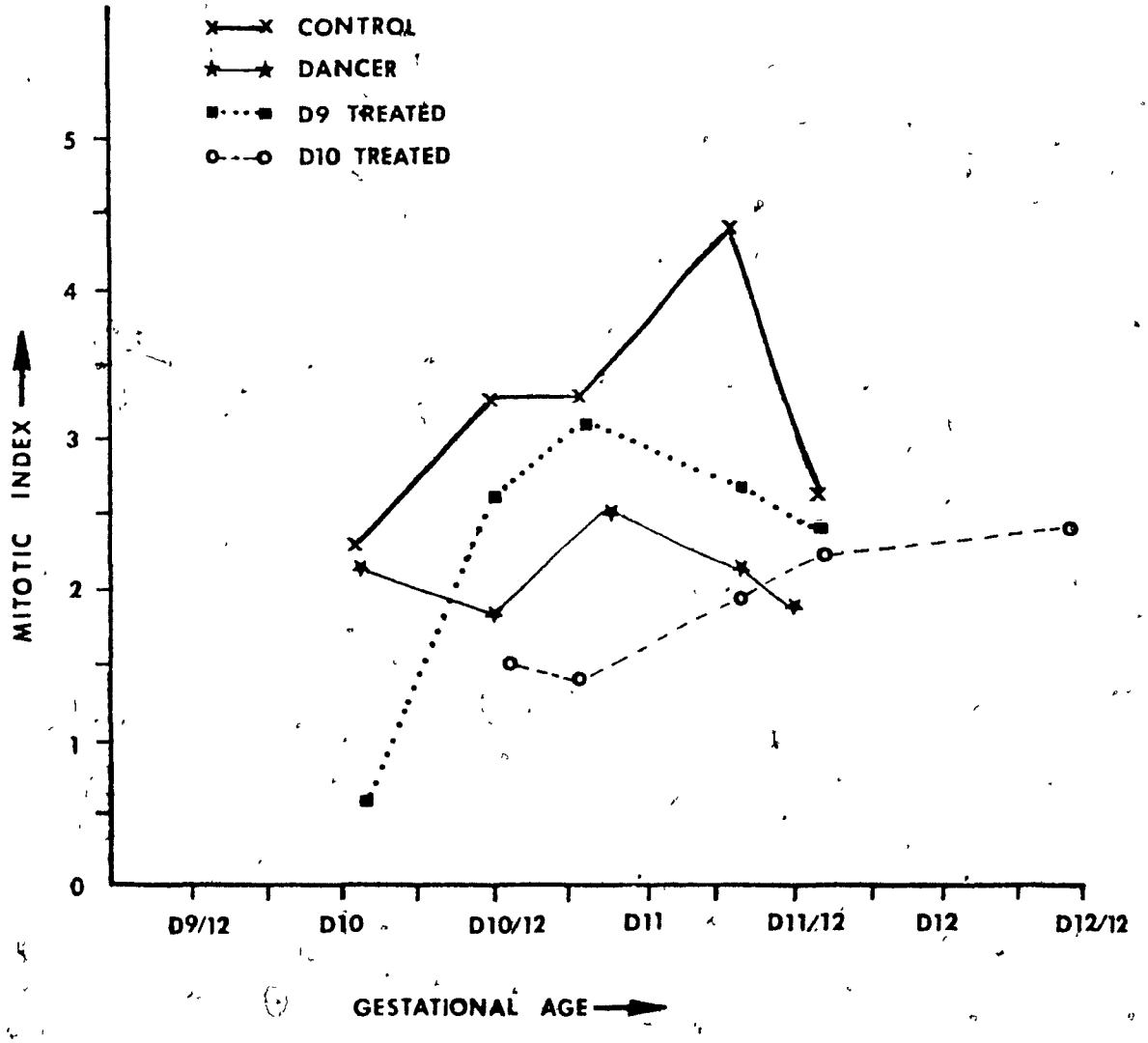
Mitotic index was found to be reduced in both the neural and nasal tissue from D10/12 onwards. Table 8A and 8B showed t tests for difference in the means between the dancer group and the controls. These results were not in agreement with the results obtained from the morphological rating data where the dancer embryos were found to be no different from the controls in morphological development. The area of the face, however was found to be smaller. (This will be presented in the next section). This decrease in mitotic index may account for the decreased area of the face. The dancer embryos may therefore be equivalent to a control C57Bl/6 in morphological development but with the size of the head reduced.

Looking back on previous raw data on mitotic counts per grid -- that is the number of mitosis for a given area, the mitotic counts from a dancer embryo in both the neural and nasal tissue was equal to the control embryos. T tests have been performed, and even on D11/8 when they had shown a reduced trend in mitosis, the P values were barely significant. Figure 26 and 27 are graphs of the mitotic counts versus gestational time of the control embryos and dancer embryos.

When the mitotic index was calculated taking into account the density of cells in the tissue, then the mitotic index was found to be lower in the dancer embryos as mentioned earlier.

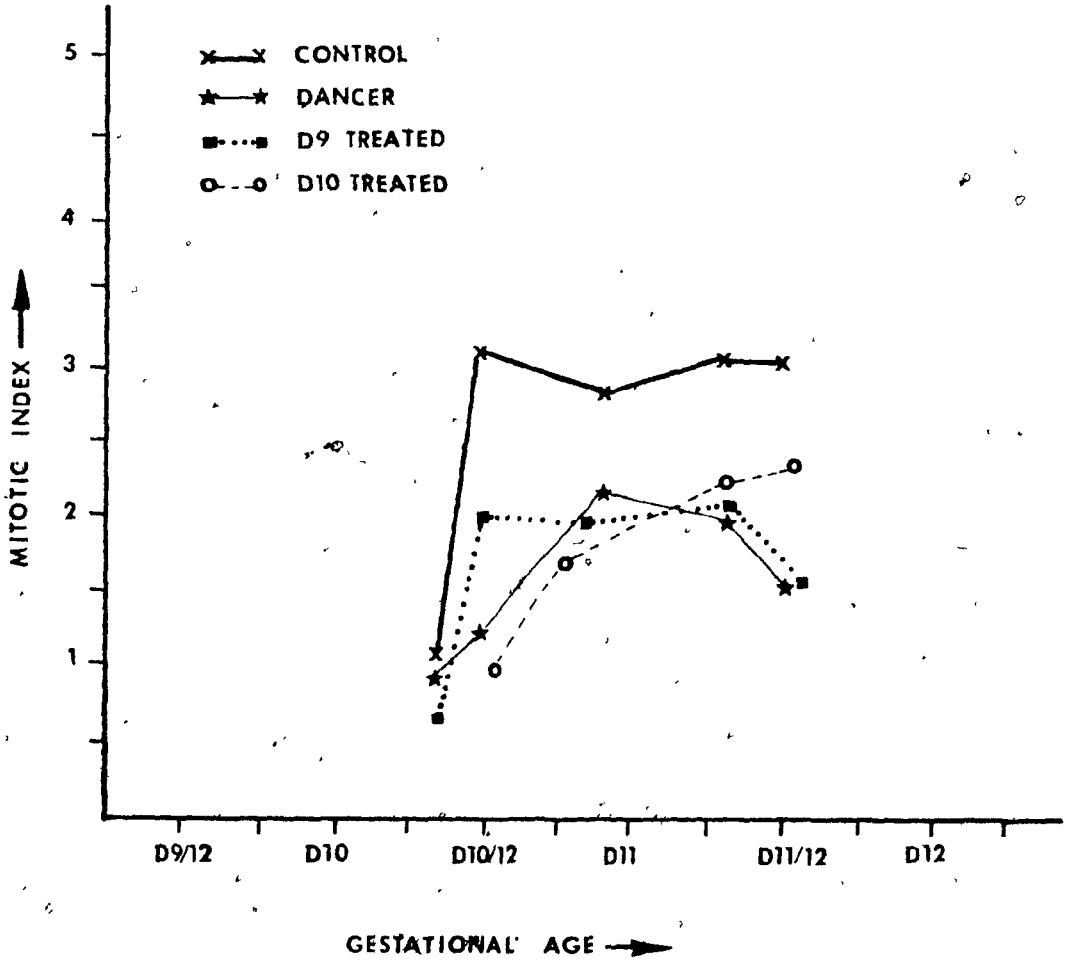
It is concluded that these embryonic heads were smaller in size although they are morphologically well developed because of the cells being more tightly packed. In order to test this theory, the DNA content of the areas where mitotic counts were done should be estimated. This however involves microsurgery to isolate the nasal and neural ectoderm and has yet to be done.

FIG. 24 ☆MITOTIC INDEX OF NEURAL AREA VS. AGE



☆MITOTIC INDEX = NO. OF MITOSIS/100 CELLS

FIG. 25 ☆MITOTIC INDEX OF NASAL AREA VS. AGE



☆MITOTIC INDEX= NO. OF MITOSIS/100 CELLS

Table 6 T tests of significance between Control group and D9 treated group for mitotic index at different gestational times

A Neural tissue

Age	Mean ± S.E. Control	Nc	Mean ± S.E. D9 treated	Nt	P
D10/8	2.35 ± 0.35	6	0.505 ± 0.23	2	.01-.001***
D10/12	3.37 ± 0.26	11	2.64 ± 0.41	9	.2-.1
D10/20	3.27 ± 0.24	16	3.148 ± 0.15	8	.7-.5
D11/8	4.44 ± 0.34	7	2.64 ± 0.37	9	.01-.001***
D11/14	2.64 ± 0.24	6	2.41 ± 0.25	10	.5-.3

B Nasal placode tissue

Age	Mean ± S.E. Control	Nc	Mean ± S.E. D9 treated	Nt	P
D10/8	1.16 ± 0.29	6	0.73 ± 0.0	2	.1-.05
D10/12	3.12 ± 0.23	11	2.0 ± 0.28	9	.01-.001***
D10/20	2.82 ± 0.197	16	1.92 ± 0.13	8	>.001***
D11/8	3.123 ± 0.17	6	1.5 ± 0.12	10	>.001***
D11/14	3.015 ± 0.17	6	1.5 ± 0.12	10	>.001***

Table 7 T tests of significance between Control group and D10 treated group for mitotic index at different gestational times.

A Neural tissue

Age	Mean ± S.E. Control	Nc	Mean ± S.E. D10 treated	Nt	P
D10/14	3.36 ± 0.26	11	1.57 ± 0.162	3	>.001***
D10/20	3.274 ± 0.234	16	1.46 ± 0.3	8	>.001***
D11/8	4.44 ± 0.34	7	1.89 ± 0.07	7	>.001***
D11/14	2.64 ± 0.24	6	2.2 ± 0.13	6	.2-.1

B Nasal placode tissue

Age	Mean ± S.E. Control	Nc	Mean ± S.E. D10 treated	Nt	P
D10/14	3.123 ± 0.234	11	0.867 ± 0.3	3	>.001***
D10/20	2.819 ± 0.197	16	1.765 ± 0.24	8	.01-.001***
D11/8	3.123 ± 0.254	7	2.213 ± 0.12	7	.01-.001***
D11/14	3.015 ± 0.175	6	2.315 ± 0.105	6	.01-.001***

Table 8 T tests of significance between Control group and dancer group for mitotic index at different gestational times.

A Neural tissue

Age	Mean ± S.E. Control	Nc	Mean ± S.E. Dancer	Nd	P
D10/8	2.35 ± 0.35	6	2.11 ± 0.26	8	.7-.5
D10/12	3.37 ± 0.264	11	1.795 ± 0.23	2	.001***
D10/22	3.27 ± 0.24	11	2.56 ± 0.15	16	.02-.01**
D11/8	4.44 ± 0.351	7	2.22 ± 0.006	12	.001***
D11/12	3.55 ± 0.11	4	1.86 ± 0.14	9	>.001***

B Nasal placode tissue

Age	Mean ± S.E. Control	Nc	Mean ± S.E. Dancer	Nd	P
D10/8	1.16 ± 0.29	6	0.86 ± 0.1	8	.5-.3
D10/12	3.12 ± 0.23	11	1.2 ± 0.04	2	>.001***
D10/22	2.82 ± 0.2	11	2.17 ± 0.15	16	.02-.01**
D11/8	3.12 ± 0.25	7	1.89 ± 0.11	12	>.001***
D11/12	2.66 ± 0.28	4	1.52 ± 0.05	9	.01-.001***

FIG. 26 MITOTIC COUNTS PER GRID OF NEURAL AREA VERSUS GESTATIONAL AGE

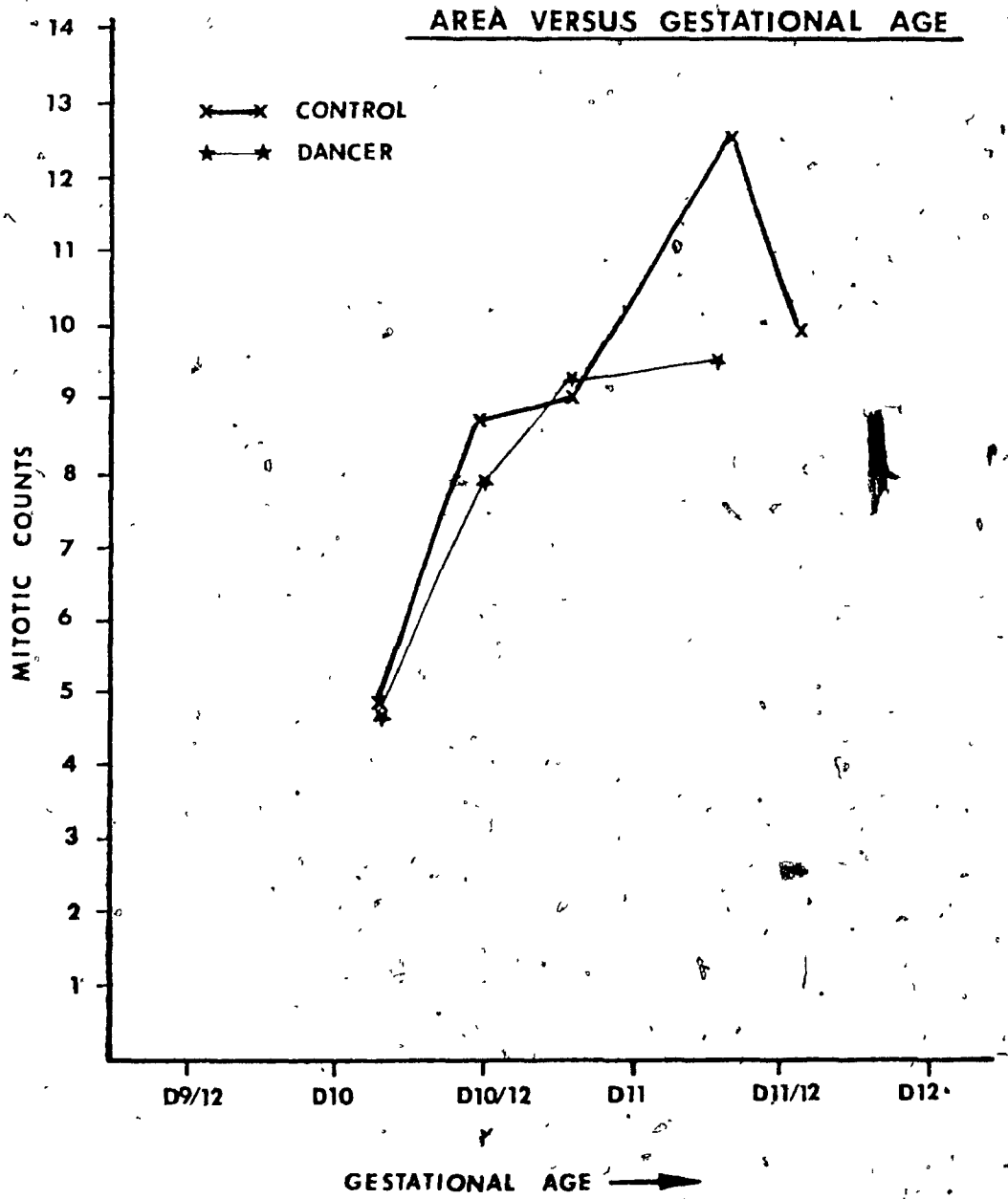
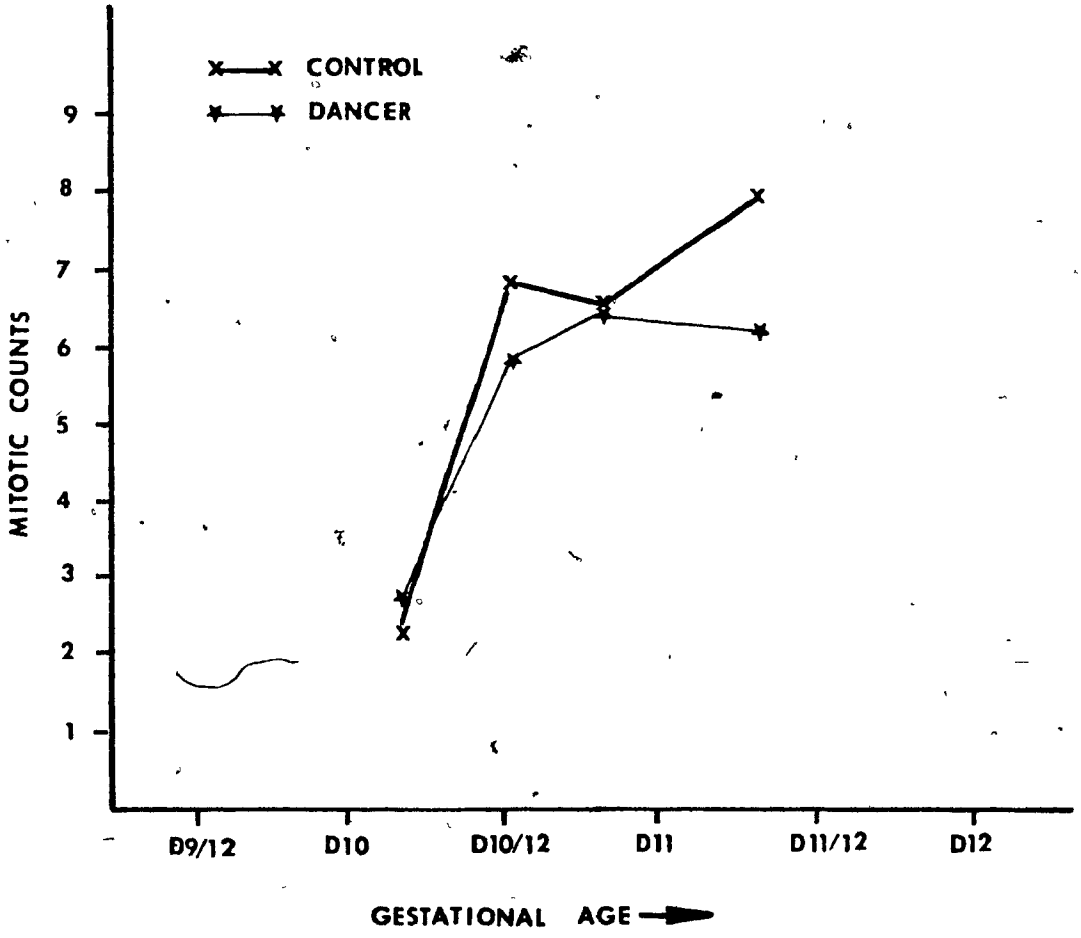


FIG. 27 MITOTIC COUNTS PER GRID OF NASAL AREA VERSUS GESTATIONAL AGE



Embryonic face shape

As already mentioned in Materials and Methods, polaroid pictures of 28X magnification of the inferior and superior view of the embryonic head were taken and measurements were made directly on these photographs. Each measurement was done at least twice on the photograph and a mean value was obtained. All embryos measured were at the crescent or early comma nasal placode stage.

The first measurement made was the widest distance of the nasal placodes in the inferior view, — Dba as shown in Figure 28.

T tests were performed on the mean of Dba distance of each of the experimental groups versus the control group. The C57Bl/6 control had a significantly wider face distance (Dba) at the level of the nasal placode than embryos who had received a dose of 6AN on D9/12 Table 9, P = .005) and embryos that carried a dancer gene (Table 11, P .001). The control embryos tended to have a larger Dba measurement than the embryos that received a dose of 6AN on D10/8 but the difference between the means was not found to be significant (Table 10).

The Db distance (shown in Figure 28), the distance between the anterior limits of the nasal pit was next analyzed. The control group was found to be significantly bigger in the Db value than embryos treated on D9/12 (Table 9). This can be illustrated by photographs of the inferior aspect of the face as shown in Figure 30A

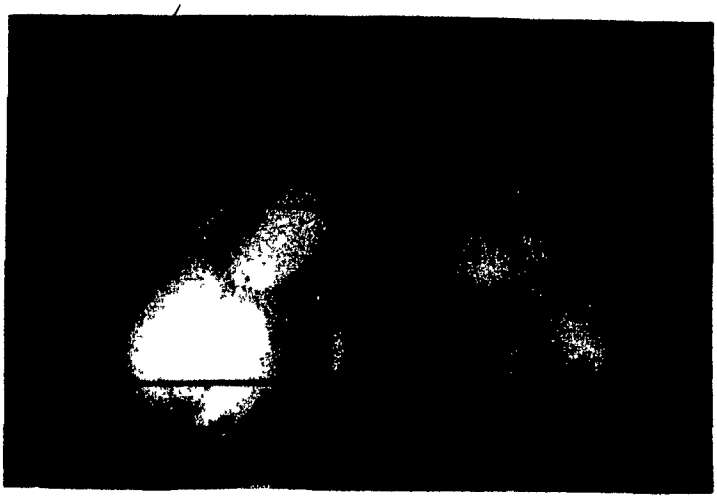


Figure 28 Inferior view of the embryonic face (30X)

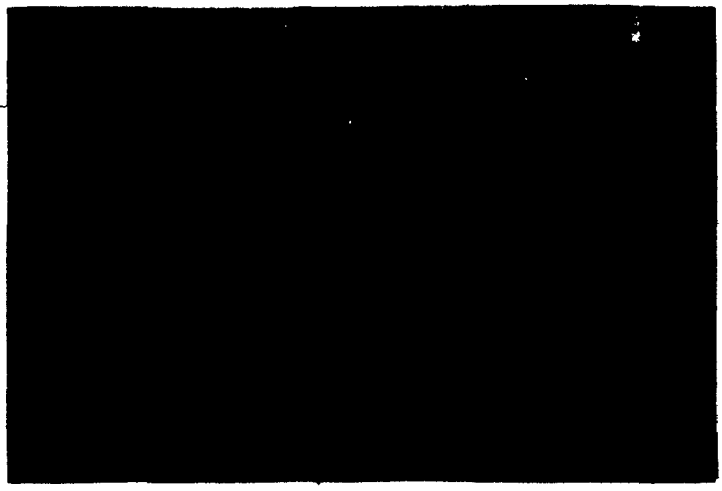


Figure 29 Front view of the embryonic face (30X)

and 31 A. The control Db distance was also significantly bigger than that of embryos from dancer backcrosses (Table 11), but it was not bigger than that of embryos treated with 6AN on D10/8.

A ratio — Dba/Db was set up with the above measurements. This was used as an indication of the type of face (narrow or wide). The embryos treated with 6AN on D9/12 and which reacted with median cleft lips had the tendency to have a narrower face than that of the control group, that is they had a larger Dba/Db ratio. (Table 9) This is contradictory to what was expected since it was thought that a median cleft lip may be formed because the median nasal processes were placed too far apart for fusion between them to take place. The embryos treated with 6AN on D10/8 destined to have median clefts had a wider distance between pits (p .001) than the controls (Table 10). Neither Dba nor Db was found to be significantly different from the controls previously (Table 10), the Dba had a tendency to be smaller while the Db had a tendency to be bigger in the D10 treated embryos. This resulted in the face having the nasal placodes placed further apart in the D10 treated embryos.

So far it appears that the mechanism for a teratogen induced cleft lip may be different from that of a spontaneous cleft lip since the results did not agree with predictions that were formulated taking into account Trasler's theory

on embryonic face shape.

other parameters were measured hoping to shed more light on the problem The jut which was measured from a front view photogrpah of the face is an indication of the amount of tissue in the medial nasal process (See Figure 29).

There was less amount of jut in the dancer embryos and in embryos that received 6AN on D9/12 (Table 11, P = .05 - .02 in both comparisons.) The amount of jut was also found to be less in the embryos treated on D10/8 but this was found to be only borderline significant (Table 10, P = .1-.05). The reduction of medial process tissue in the D9 treated embryos can be demonstrated in Figure 30A and 31A. These are inferior views of the embryonic face, arrows on Figure 31A indicate that there is a lesser amount of tissue in the medial process. Figure 30B and 31B compares the jut of the control to that of a D9 treated embryo, the arrow on the D9 treated embryo indicates the reduction of the jut.

The amount of lateral process tissue available was calculated by subtracting Db from Dba. The difference being the sum of the two lateral nasal processes. On examining the data for this measurement it was found that the dancer embryos and embryos treated on D10/8 had smaller lateral processes (Table 10, P =.05-.02and

P = .01 - .001 respectively) when compared to controls. Figure 32A and B and 33A and B are photographs of control embryo and D10 treated embryo respectively. On both the inferior view and front view the lateral process is seen to be reduced when compared to controls. The embryos treated on D9 were found to have a similar size of lateral processes to the control (Table 9).

The divergence ($\angle^{O}$) of the medial nasal processes from each other was measured as an angle between the two medial processes. In the two 6AN treated groups, there was a tendency for the medial nasal processes to be less divergent than the controls (Table 9 and 10). In the dancer embryos, the medial processes diverged significantly less than the controls (Table 11), this lack of divergence may result in a decrease in fusion between the medial and lateral processes.

Area measurements of the face were next carried out using a planimeter, Af being the area of the face viewed from the front and Ab the area of the face in the inferior view.

The dancer group of embryos were found to be significantly smaller than the control group of embryos in both front and inferior view (Table 11). The D9 treated group of embryos and D10 treated group of embryos were found to be similar in size in both front and

inferior view of the face when compared to the controls (Table 9 and 10). This may be due to the fact that the nasal processes of the animal are selectively more retarded by 6AN than the rest of the face, so by the time the nasal placode has reached crescent stage, the rest of the head has had time to catch up to the control embryos, the recovery of mitosis in the neural tissue supports this idea. Therefore the jut or the lateral process may be smaller while the total face area is not reduced.

Summary

	Dba	Db	Dba/Db	J	Dba→Db	∠°	Af	Ab
Control	0	0	0	0	0	0	0	0
D9 treated	-	---	⊞	-	⊟	⊟	⊟	⊞
D10 treated	⊟	⊞	---	-	--	⊟	⊟	⊟
Dancer	---	---	⊞	-	-	-	---	--,
Cl/Fr	0	---	+	+++	⊞	-7-	0	+

Control is considered as the normal — designated by 0 value, if a value is greater than control, it is indicated by a + (Pvalue of .05-.02 indicated by +, .02-.01 by ++, .01-.001 or less by +++). If the value is less than the control value, it will be indicated by a -, (P value of .05-.02 by -, .02-.01 by -- and .01-.001 or less by ---).

⊟ not significantly different but less than control

⊞ not significantly different but more than control

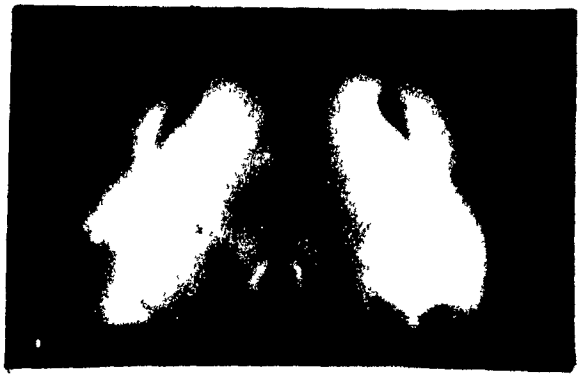
Figure 30A Inferior view of the face of a control embryo at early comma stage (D10/20, 7 tail somites). Magnification : 25X

Figure 30B Front view of the face of a control embryo at early comma stage. (25X)

Figure 31A Inferior view of the face of an embryo treated with 6AN on D9/12. Arrows indicate lack of tissue in the medial nasal process when compared to controls. (D11/20, 7 tail somites) Magnification : 25X

Figure 31B Front view of the face of a D9/12 treated embryo. The arrow is pointing at the reduced jut. (25X)

30A



30B

31A

31B

Figure 32A Inferior view of the face of a control embryo at crescent stage. (D10/20, 7 tail somites) Magnification 25X

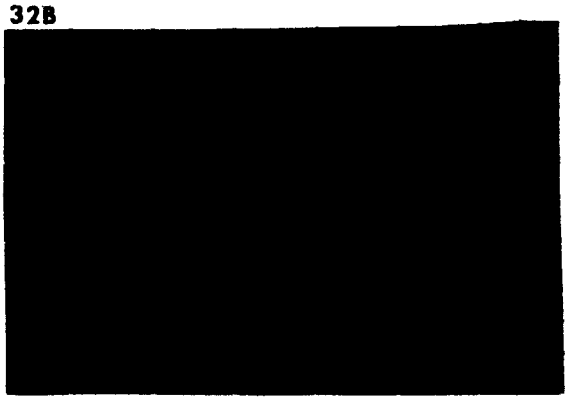
Figure 32B Front view of the face of a control embryo at crescent stage. (25X)

Figure 33A Inferior view of the face of an embryo treated with 6AN on D10/8. Arrow indicates thin lateral processes. (D11/8, 7 tail somites, early crescent stage) Magnification : 25X

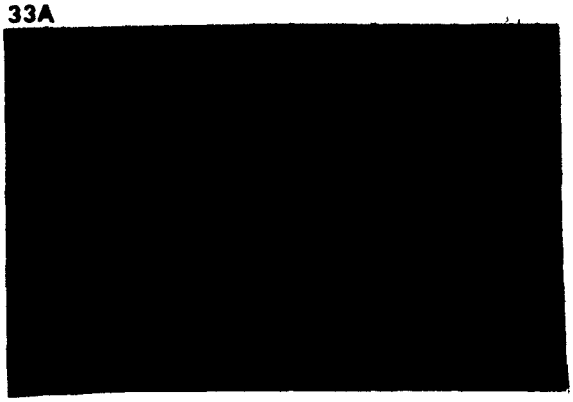
Figure 33B Front view of the face of an embryo treated with 6AN on D10/8. The arrow indicate reduced lateral process. (25X)

32A

32B



33A



33B

Face shape and strain difference

Trasler has already found a difference in embryonic face shape when working with A/J and C57Bl/6 embryos, A/J having a narrow Db and a large jut and being predisposed towards cleft lip. In the present study Cl/Fr embryos were studied as another group with multifactorially inherited cleft lip. Cl/Fr animals have a spontaneous cleft lip frequency of 26%.

The Cl/Fr embryos at crescent stage were found to have a smaller distance between the anterior limits of the nasal pit (Db) (Table 12, P .001), therefore making their faces narrower than that of the C57Bl/6 embryos. The juts of the Cl/Fr embryos were found to be significantly bigger than that of the C57Bl/6 group but the lateral processes were found to be of the same size (Table 12). This can also be deomonstrated in photomicrographs of C57Bl/6 and Cl/Fr embryonic head sections at crescent nasal placode stage in Figure 34. The angle of divergence of the medial processes was found to be significantly less than that of C57Bl/6 (Table 12). The above findings agree with Trasler's theory that a cleft lip is formed because of lack of divergence of the medial processes causing difficulty in fusion between the lateral and medial processes. The face shape of the Cl/Fr is similar to the A/J which is also predisposed towards cleft lip (12%).

It appears that we cannot easily extrapolate from a teratogen-induced malformation to one that occurs spontaneously.

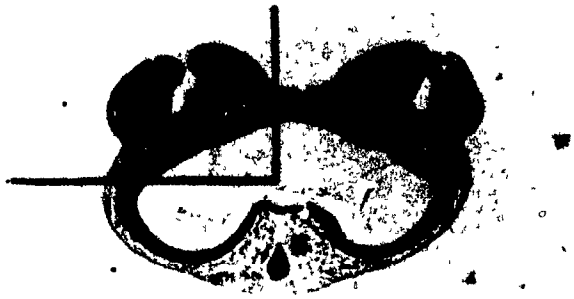
Figure 34A Transverse section through the embryonic head of a control embryo (C57Bl/6) showing well formed lateral and medial nasal processes at crescent stage. (64X)

Figure 34B A higher magnification of the nasal placode that is shown in the inset of 34A. (162X)

Figure 34C Transverse section through the head of an embryo that belongs to the Cl/Fr strain at crescent stage. Note the increase in size of the medial processes. (64X)

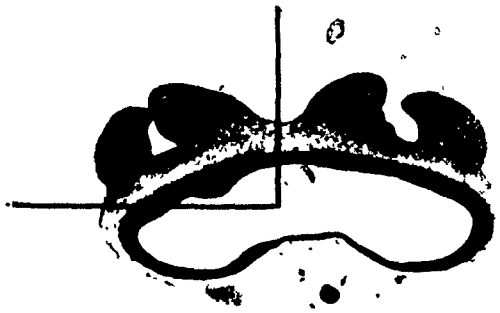
Figure 34D A higher magnification of the nasal placode that is shown in the inset of Figure 34C. (162X)

A



B

C



D

Table 9 Mean and P values of face shape measurements of control and D9/12 treated embryos at crescent nasal placode stage.

	N	Dba	Db	Dba/Db	J	Dba-Db	∠°	Af	Ab
Control	12	5.53	3.268	1.69	0.46	2.25	120	0.28	0.27
D9 treated	8	5.19	2.99	1.74	0.37	2.2	106	0.26	0.27
P values		.05-.02*	.001***	.3-.2	.05-.02*	.7-.5	.01*	.2-.1	.9-.8

Table 10 Mean and P values of face shape measurements of Control and D10/8 treated embryos at crescent nasal placode stage

	N	Dba	Db	Dba/Db	J	Dba-Db	∠°	Af	Ab
Control	12	5.53	3.268	1.69	0.46	2.25	120	0.28	0.27
D10 treated	12	5.28	3.37	1.56	0.40	1.83	117	0.27	0.26
P values		.1	.1	.001***	.1-.05	.001***	.4	.9	.7-.5

Table 11 Mean and P values of face shape measurements of control and dancer embryos at crescent nasal placode stage

	N	Dba	Db	Dba/Db	J	Dba-Db	∠°	Af	Ab
Control	12	5.53	3.268	1.69	0.46	2.25	120	0.28	0.27
Dancer	11	4.93	2.95	1.698	0.37	1.98	109	0.22	0.23
P values		.001***	.001***	0.9	.05-.02*	.035*	.035*	.001***	.001***

Table 12 Mean and P values of face shape measurements of control and Cl/Fr embryos at crescent nasal placode stage.

	N	Dba	Db	Dba/Db	J	Dba-Db	∠°	Af	Ab
Control	12	5.53	3.268	1.69	0.46	2.25	120	0.28	0.27
Cl/Fr	17	5.42	3.04	1.78	0.58	2.383	98	0.27	0.29
P values		.5-.3	.001***	.02-.01**	.001***	.2-.1	.001**	.4	.02-.01

DISCUSSION

Teratogenicity of 6AN

6AN is an antimetabolite, when administered to a pregnant female, 6AN becomes a noxious enviromental agent to the embryos. When 6AN was administered to C57Bl/6 pregnant females on either D9/12 or D10/8 of gestation, cleft lips together with other malformations were produced. In an animal where development is controlled by a polygenic system such as is the case for the formation of the lip or palate, there normally exists some kind of 'buffering system' in the development of the embryo protecting it against these noxious enviromental influences.

6AN may cause disturbances in the electron transport system (Dietrich et al 1958 a & b), reduce ATP (Ritter et al 1973), reduce acid muco-polysaccharides (Overman et al 1972), induce chromosomal anomalies (Ingalls 1964), inhibit DNA synthesis (Ritter et al 1972) and probably many other biochemical disturbances related to the above as well. All these disturbances contribute to the 'noxious environment' of the embryo. Adams et al (1967) suggested that when the buffering activity against these noxious agents becomes too low, increased fluctuation in development occurs, that may ultimately result in a malformation

such as failure of fusion of a normal lip. Thus when 6AN is introduced into a pregnant female it may reduce the developmental stability, buffering action may be inadequate, therefore a malformation is produced.

Effect of 6AN on the general development of the embryo

6AN given either on D9/12 or D10/8 of gestation retards the rate of development of both the body somites and the nasal placodes in the embryo. Although both embryos treated on D9 and D10 are affected by 6AN, they are not affected to the same degree. Embryos that were treated on D9 did not show any signs of retardation in both the body somites and nasal placodes until almost twenty-four hours after treatment, but the effect of the drug persisted for a long period of time — for at least the next forty-eight hours, that is throughout the period of lip formation. Embryos that were treated on D10 were affected by the drug immediately but the noxious effects of the teratogen did not remain as long as in the embryos that were treated with 6AN on D9. This may be because younger embryos (D9 treated) have cells that are less differentiated, therefore when these cells are affected, the prospective tissues derived from these undifferentiated cells will be more affected — the effect will be more widespread. But these younger embryos' undifferentiated cells may also have more 'regulatory potential' in the genome, therefore they may initially have been more resistant to the teratogen but once the teratogen overcomes this resistance, the consequences may be more severe than the effect of 6AN on an older embryo.

In the older embryos, where cells are in a more differentiated state, they have less 'regulatory potential', and because of that they may succumbed more easily to a teratogen. However by that time, the nasal placode is further along in development and the teratogen can only disrupt continued differentiation and growth, but it cannot 'unform' differentiated tissue.

Effect of 6AN on the nasal placode area

When histological sections of the treated embryonic heads were examined, the volume of mesenchyme of the nasal processes was found to be reduced. In the embryos treated on D9 with 6AN and reacting with a median cleft lip, the volume of mesenchyme in the median nasal processes was markedly reduced. Smith and Monie (1969) suggested that rat embryos examined at the end of the pregnancy with median cleft lips induced by 9-methyl-PGA had underdevelopment of the maxillary process and mesenchymal deficiency in the nasomedial processes resulting in the failure of the nasomedial processes to merge.

In embryos treated with 6AN on D10 and reacting with a lateral cleft lip, there was a reduction in the volume of mesenchyme in both the median and lateral nasal processes. This reduction of mesenchyme may cause the processes to be too wide apart to approach each other for fusion to occur. Smith and Monie (1969) also found that in 9-methyl-PGA induced lateral clefts in rats, there was a reduction of mesenchyme in the nasal processes. Reed (1933) and Stark (1954) also related lack of mesenchyme to cleft lips.

On close examination of the nasal placode area at crescent stage, no localized areas of cell death were found in the nasal ectoderm of the treated embryos (both types of treatment), but an increase in

the number of dense bodies which may be pycnotic cells was found in the mesoderm adjacent to the nasal ectoderm. Lejour-Jeanty (1966) also found dead cells in the mesenchyme adjacent to the nasal ectoderm when she treated rats with Hadacidin - a penicillin derivative. These hadacidin-treated rat embryos eventually have cleft lips.

From the above observations, it appears that the mesenchyme plays an important role in lip formation. The mesenchyme has been postulated to arise from the neural crest (Horstadius 1950) and Johnston (1964) demonstrated that removal of fore-brain neural crest in chicks often results in clefts of the primary palate. Rajchgot (1971) using a modified alkaline phosphatase technique on whole embryos found that 6AN appears to cause retardation in neural crest cell migration. One can speculate that one of the primary targets of 6AN may be the neural crest cells. 6AN may delay or inhibit migration of neural crest cells to specific face areas that may therefore finally have a reduction of mesenchyme volume in that area.

Effect of 6AN on the mitotic index

Mitotic index counts at the time of lip formation were done on the neural ectoderm and the nasal ectoderm including some adjacent mesoderm where cells are actively dividing. 6AN affected the mitosis both in the neural and nasal ectoderm on both days of treatment. In embryos that received a standard dose of 6AN on D9/12 or D10/8 the mitoses in the nasal placode tissue was severely depressed. The mitotic index in the treated neural ectoderm was seen to be depressed but in an irregular manner. Mitosis was only depressed at certain gestational times, and the mitotic index did not follow the same general pattern as the control when plotted against gestational time. Frank (1925) observed that there is an increase in mitosis before a local thickening of the neural tube and Takaya and Watanabe (1961) observed that mitotic index varies for different parts of the neural tube with the highest percentage of mitosis in the parts where the wall is thickest and lowest where the wall is thinnest. Since mitotic counts in the neural ectoderm in the telencephalon of the embryo were done at random with no reguard to the thickness of the neural tube wall, the results obtained from the present mitotic counts are difficult to interpret. If the treated embryos' development is retarded morphologically, the spurts of mitosis in the neural tube may not correspond

chronologically to that of the control since these spurts accompany the thickening or development of the neural tube.

This may account for the observation that the mitotic index for the neural ectoderm of the treated embryos was depressed only at intervals and did not follow a similar pattern to the control. The only interpretation that I could give to this data is that 6AN probably affects mitosis in the neural ectoderm of the treated embryos. Overall it appears that neural tissue was affected immediately after treatment for both treatments and recovered within twentyfour hours.

When the mitotic index of the nasal placode of treated embryos was examined (Both D9 treated and D10 treated), it was found that mitosis was depressed throughout lip formation. Treated embryos were depressed and follow a similar pattern of mitosis as that of the control (i.e. an increase in mitosis in early D10 of gestation, then a levelling off for the rest of the period when the lip is forming.) It appeared that nasal tissue was not affected until twenty to twenty-four hours after treatment on D9/12 while it was immediately (four hours) affected after treatment on D10/8.

The embryos treated on D10 had begun to show slight signs of recuperation by the end of D11/14, although a t test between the means of D10 treated and control still indicated that the mitotic index of the treated embryos was significantly reduced. The same reasoning used for the general development of the treated embryos may also apply here, that when 6AN is given to an older embryo, it may have less 'regulatory potential', therefore be more susceptible initially but 6AN cannot undifferentiate any tissue that is already differentiated, it can only retard its growth. Ritter and Scott (1972) showed that 6AN depressed DNA synthesis in rat embryos and Franz (1971) showed that there were quantitative changes in mitosis of whole mouse embryos when these embryos were treated with teratogens. Disturbance in DNA synthesis may disturb the S period in the cell cycle leading to disturbances in mitosis. Disturbances in the metabolic pathways by 6AN may cause changes in the G period of the cell cycle which also may cause delay or changes in mitosis. Although the specific site of the action of 6AN is unclear, from mitotic index studies of the head region of 6AN treated mouse embryos, it can be concluded that 6AN does affect the rate at which cells enter mitosis in the head and especially the nasal placode region when it is administered to the embryo at the time of lip formation.

Effect of 6AN on cell density

6AN tended to cause an increase in the number of cells per unit area in embryos treated on D10 of gestation. Cells were also smaller and the tissue had a disorganized appearance. The effect of 6AN resulting in compactly arranged cells has also been observed by Seegmiller and Overman (1972) in limbs of chick embryos that has been treated with 6AN.

6AN did not cause an increase in cell density when given to mice on D9 of gestation. In fact it tends to reduce the density of the cells instead.

Seegmiller and Overman (1972) suggested that the appearance of compact cells is an indication of subnormal production of protein-polysaccharides. It can be speculated in the present study that 6AN may affect synthesis of mucopolysaccharides in the head region when given to mouse embryos on D10 but this explanation does not account for the reduction in cell density on D9.

One can only conclude that 6AN may act in a different manner in the mouse embryo at different gestational times.

Effect of 6AN on face shape

The most interesting effects of 6AN when given to mice at two different gestational times resulting in two different types of clefts came from the comparisons of face shape.

Embryos that were given a dose of 6AN on D10 produced lateral clefts. At crescent stage of the nasal placode the embryonic face had a wider distance between pits than the control embryos. The embryos that were treated on D9 had median cleft lips had a narrower pit distance than the controls but not significantly so.

Trasler (1968) had found that A/J mice which are predisposed to forming lateral cleft lips had a narrow proportioned face (small pit distance) compared to C57Bl/6. This was also true of the Cl/Fr strain (26% spontaneous cleft lip), which also have a predisposition towards forming lateral cleft lips. The embryos that will form lateral cleft lips when treated with 6AN had a wider distance between nasal pits, just the opposite of what was found in the spontaneous cleft lip embryos.

If one expects that a median cleft lip is formed because of the failure of the medial processes to merge then embryos that react to 6AN by forming median cleft lips should have a wide distance between nasal pits,

but the opposite tendency was found.

Thus it would appear that in teratogen-induced cleft lip, the association of a particular face shape with a cleft lip may not be the same face shape as that which was associated with spontaneously formed cleft lip. 6AN may be selectively affecting certain areas of the face resulting in the type of face shape noted in the treated embryo.

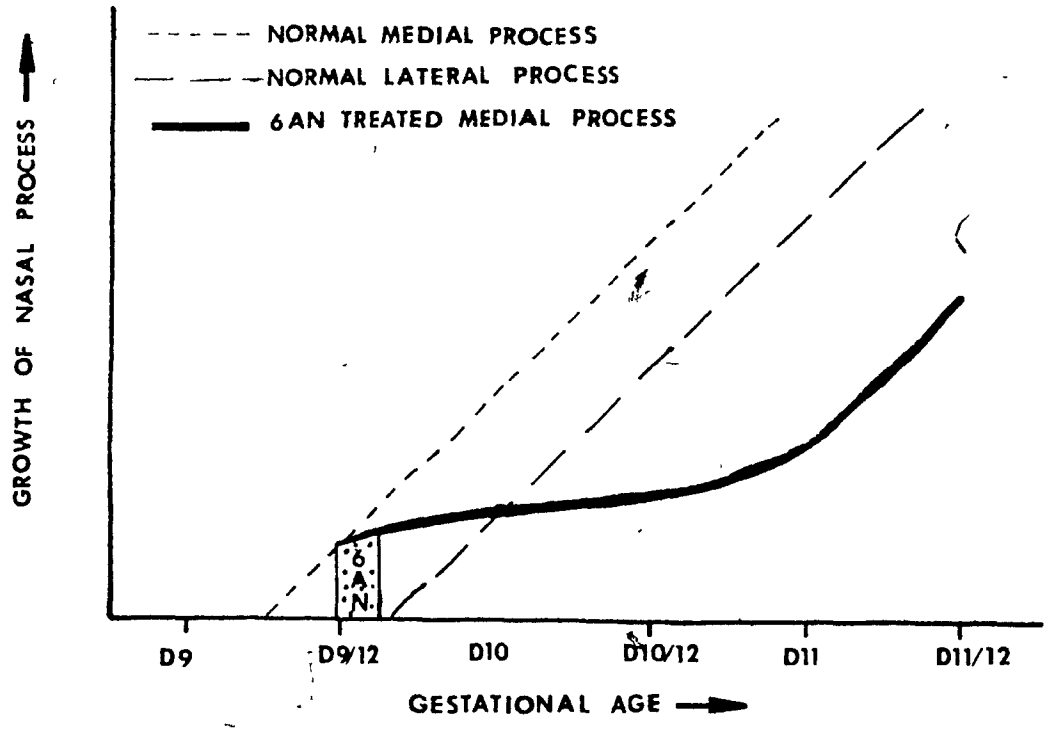
The amount of lateral nasal process and medial nasal process was measured in these treated embryos. In the D9 treated embryos that result in a median cleft lip, the nasal pit distance had a tendency to be narrower, however the jut or the amount of medial nasal process was found to be significantly reduced. The pit distance may have a tendency to be narrow because there is lack of tissue in the medial nasal process.

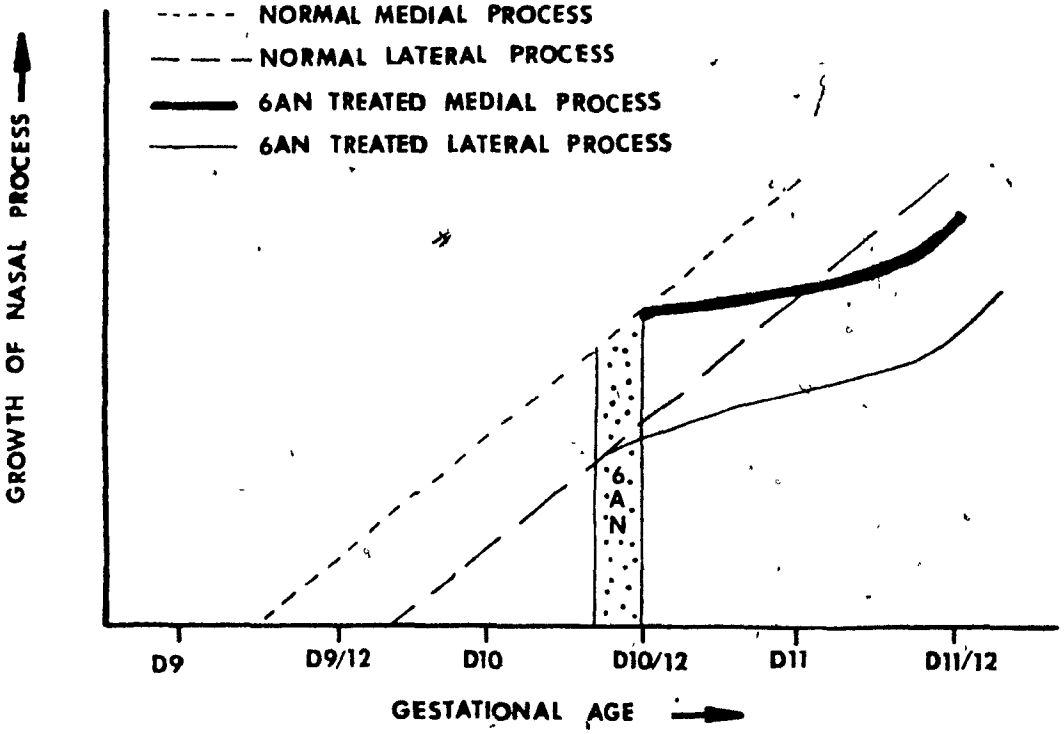
The D10 treated embryos had a smaller Dba/Db ratio (indicating wider pit distance) however the distance between the anterior limits of the nasal pit was not found to be wider, and the width of the head across the nasal placodes was narrower, therefore giving an impression that the nasal placodes were widely placed on the head.

On examination of the medial and lateral nasal processes of the D10 treated embryos, the amount of lateral processes was found to be greatly reduced, therefore reducing the maximum width of the head across the nasal placode giving a false impression that the nasal placodes were widely spaced. The medial nasal processes were also found to be reduced in the D10 treated embryos. 6AN reduces mitosis and decreases volume of mesenchyme in both treatment times, but the face shape data show us that 6AN does not affect the whole face, it is in fact selective in its action. It may be that the medial process was actively dividing on D9 when 6AN was given to the embryo on D9, 6AN will likely affect tissue that is more actively dividing, therefore it results in a small medial process (causing median cleft lip). On D10 when 6AN is given to the embryo it may be that the medial nasal placode is still actively proliferating and the lateral process has just begun its spurt of growth, so 6AN affects both lateral and medial processes (causing lateral cleft lips). This hypothesis is illustrated graphically in Figure 34.

Figure 34A 6An administered on D9/12 will affect only the medial nasal process if medial process starts actively proliferating before the lateral process.

Figure 34B 6An administered on D10/8 will affect both medial and lateral processes if both are actively proliferating on D10/8.


NORMAL MEDIAL PROCESS
NORMAL LATERAL PROCESS
6AN TREATED MEDIAL PROCESS
GROWTH OF NASAL PROCESS
6 A N
D9
D9/12
D10
D10/12
D11
D11/12
GESTATIONAL AGE


NORMAL MEDIAL PROCESS
NORMAL LATERAL PROCESS
6AN TREATED MEDIAL PROCESS
6AN TREATED LATERAL PROCESS
GROWTH OF NASAL PROCESS
6 A N
D9
D9/12
D10
D10/12
D11
D11/12
GESTATIONAL AGE

Effect of the dancer gene on the development of the embryo

When a C57Bl/6 female is crossed to a Dc/+C(BCl) male (or its reciprocal backcross), 50% of the offspring will carry the dancer gene. Of these embryos carrying the dancer gene, a maximum of 87.5% of their background will be from the C57Bl/6. Embryos in fact will contain not only the dancer gene from the dancer stock, but genes that are closely linked to the dancer gene as well. One also notes that all of the offspring from the second backcross will be hybrid animals.

Generally the dancer embryo was found to be slightly slower in development than the C57Bl/6 embryos. The number of body somites at any particular age appears to be slightly less in the dancer and at times even significantly so. The development of the nasal placode was slower during D10 of gestation, but was equivalent or ahead in development during the later stages of lip formation.

If the dancer gene was to affect the general development of the embryo, one would expect only 50% of the embryos to be affected, and the ones not carrying the dancer gene should be at least equivalent if not faster in development than the C57Bl/6 embryos, since they are hybrid embryos. If the above was true one would expect a high degree of variability

within the offspring of the second backcross, but on examination of the standard errors and the range, they were found to be no bigger than the standard errors or range of the control group (C57Bl/6). Thus because of the small standard error it was not deemed necessary to test the offspring of the second backcross for bimodal distribution. The best comparison for development of these second backcross embryos is to compare them to embryos that result from a backcross to C57Bl/6 background but having the normal gene at the dancer locus. This part of the investigation is presently being carried out.

The conclusion form this data is that the offspring from the second backcross embryos of the dancer gene to C57Bl/6 are slightly retarded in development when compared to the C57Bl/6 embryos.

Effect of the dancer gene on the nasal placode area

Embryos that were suspected of having cleft lip (induced by the presence of dancer gene) showing failure of the lateral and medial nasal processes to merge or fuse at the posterior end of the nasal pit were examined. In the histological sections, the volume of mesenchyme in both the medial and lateral processes was reduced. Homozygotes for the dancer gene in the dancer stock have cleft lip and cleft palate as well as inner ear defects and heterozygotes also have inner ear defects and lack of pigmentation on the top of the head (Deol 1966) leading to the suspicion that neural crest cells are involved. As already mentioned previously, it has been postulated that facial mesenchyme arises from neural crest cells (Horstadius 1950), if dancer gene affects neural crest cells or their migration, it is only logical that the mesenchyme of the face region would be reduced because some of the neural crest cells failed to get there. Lack of mesenchyme causes a reduction in size of the nasal processes which may result in failure of the nasal processes to approach each other enough to fuse. Failure of approximation and fusion results in a cleft lip.

Effect of dancer gene on the mitotic index

As shown in the results, the mitotic index of both the nasal and neural area of dancer embryos was reduced when compared to C57Bl/6 controls. The number of cells undergoing mitosis per unit area was equivalent to that of C57Bl/6 in both neural area and nasal area. The reduced mitotic index may in some way be associated with the fact that the ectodermal cells of the nasal and neural ectoderm were denser than those of the control, that is these cells were tightly packed. This phenomenon may result from a lack of matrix in the face tissue which in turn may be due to a reduced amount of acid mucopolysaccharide. Neural crest cells migrate through mucopolysaccharide (Johnston personal 1970), and a disturbance in mucopolysaccharide synthesis may cause disturbances in neural crest cell migration.

Effect of dancer gene on the face shape

The embryonic face of the dancer embryos was found to be proportioned in the same manner as that of the C57Bl/6 embryos, that is the relative position and the spatial relationship of the nasal placode on the face is similar to that of a C57Bl/6 embryo. The distance between the anterior limits of the nasal pit and the width of the head across the nasal placode area was smaller. The lateral nasal and medial nasal processes as well as the area of the face was also found to be smaller. The dancer embryonic head is therefore morphologically well developed but smaller in size. This smallness in size may again be due to lack of matrix resulting in closely packed cells.

Relationship between a teratogen-induced and gene-induced cleft lip

The common abnormal findings among the teratogen-induced (both days of treatment) and gene-induced cleft lip is a reduction in the volume of mesenchyme in the nasal processes. The mechanism for reduction of volume of mesenchyme in the nasal processes by the teratogen and gene have differences and similarities. 6AN reduces the amount of mesenchyme by inhibition of growth, cell death (results of the present investigation) and retardation of migration of neural crest cells (Rajchgot 1971). Dancer gene may reduce the amount of matrix in the nasal processes therefore hindering the migration of the neural crest cells, resulting in reduced mesenchyme in the face region. Lack of mesenchyme in the formation of a cleft lip has already been suggested by other workers (Smith and Monie 1969, Stark 1954, Reed 1933). In the case of the lateral cleft lip, lack of mesenchyme in both the lateral and medial processes results in the lack of fusion between the lateral and medial process. In the median cleft lip, lack of mesenchyme in the medial process causes a failure of fusion between the two medial processes.

This is in contrast to the relative lack of divergence of medial processes (i.e. their positioning at a threshold) towards the lateral processes postulated as the mechanism leading to cleft lip in the A/J and C1/Fr inbred strains, in both of which there does not appear to be a lack of mesenchyme in the processes.

CONCLUSION

Formation of a normal lip involves the normal growth of the nasal and maxillary processes and their fusion in a coordinated manner. Many things could go wrong during lip formation that may result in a cleft of the primary palate.

Formation of a lateral cleft lip

1. Lack of induction of the placode by the anterior endoderm, prechordal plate or the forebrain. DeMyer (1964) has found bilateral clefts of the lip in humans being associated with maldevelopment of the prosencephalon or holoprosencephalon which is the result of failure of induction of the rostral neural ectoderm by the prechordal mesoderm.
2. Failure of lateral and medial nasal processes to approach each other and fuse.
   a. Positioning of the nasal processes
      The C1/Fr strain, with a spontaneous cleft lip frequency of 26% has a narrower face and little divergence of the medial processes when compared to a strain (C57B1/6) with virtually 0% cleft lips.
   b. Lack of mesenchyme in both the lateral and medial processes
      This is shown in histological sections and by

face shape measurements in the present study in 6AN treated embryos that form lateral cleft lips. This is also true of dancer embryos that produces lateral cleft lips.

c. Lack of proliferation of the nasal processes

Reduction of mitosis in the nasal placode area of the 6AN treated embryos was found. Decreased in mitosis in dancer embryos was also apparent.

d. Epithelial adhesiveness

This lack of epithelial adhesiveness has not been observed in the present study but has been observed by Pourtois (1972) and Lejour (1970).

Formation of a medial cleft lip

1. Lack of induction by the forebrain

DeMyer in 1964 in his human studies showed median clefts to be associated with holoprosencephalon.

2. Lack of merging between the two medial nasal processes

a. Lack of mesenchyme in the medial processes

This is found in the D9 treated embryos that reacted to 6AN with median cleft lips. Evidence was produced from histological studies and face shape measurements.

b. Lack of cell proliferation in the nasal placodes

Reduction of mitosis in the nasal placode area of D9 treated embryos that react to 6AN with a median cleft lip.

c. Lack of epithelial adhesiveness

(See previous section)

d. Mechanical obstruction

Gruneberg (1960) working with the patch gene found that the homozygote Ph/Ph had median cleft lips. Histological sections showed a fluid filled bleb between the two medial nasal processes hindering the movement of the two medial processes towards the midline. Therefore a failure of fusion between the medial processes occurs, and results in a median cleft.

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