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Cellular Events Associated With The Teratogenesis Of Retinoic Acid In Mouse Embryos.

Steve Virgil Bynum
University of Alabama at Birmingham

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Bynum, Steve Virgil, Ph.D.

University of Alabama at Birmingham, 1991

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CELLULAR EVENTS ASSOCIATED WITH THE TERATOGENESIS OF RETINOIC ACID IN MOUSE EMBRYOS

by

STEVE VIRGIL BYNUM

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biology in the Graduate School, The University of Alabama at Birmingham

BIRMINGHAM, ALABAMA

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ABSTRACT OF DISSERTATION
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Title Cellular Events Associated with the Teratogenicity of Retinoic Acid in Mouse Embryos

Previous experiments have demonstrated that oral administration of retinoic acid to ICR mice on day 10 of gestation results in preaxial reduction defects and an enhanced expression of postaxial polydactyly of the forelimbs. In the present investigations, the development of the limbs from the time of treatment until the appearance of the defect on day 14 were studied at 12 to 24 hour intervals with histological, morphometric, biochemical, and vital staining techniques. A diffuse cytotoxicity in the central proximal mesoderm was noted with a safranin-toluidine blue stain 12 hours after the treatment which then peaked 24 hours after the treatment. Treated limbs stained with Nile blue sulfate showed a significant increase of necrotic mesodermal cells in the foyer preaxial primaire on day 11.5 and in the foyer marginal I and foyer marginal V on day 12 when compared to the respective control group. The treated postaxial ectoderm was more hyperplastic than the control ectoderm on day 12. The treated group developed a squarish bulge on the postaxial boundary of the fifth digital ray on day 14

that was larger than those of the controls. Most of the controls had bulges that were completely necrotic. The treated group also had some completely necrotic bulges, but a large number of the treated fetuses had bulges which were characterized as being less than 60% necrotic. Electrophoretic analysis of isoforms of acid phosphatase, hexokinase, phosphoglucomutase and phosphohexose isomerase with an isozymic difference index found no significant differences between the control and treatment groups. Though the cellular and molecular events that underlie defect formation are not known, electrophoretic analyses revealed protein differences between the control and treatment groups.

Abstract Approved by: Committee Chairman Charles F. Wagg

Program Director Daniel W. Jones

Date 6/5/91 Dean of Graduate School Kathryn Barnard

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF TABLES

NOTE

A portion of this manuscript has been published as an article in the journal of Teratology (Bynum, '91). The publication was comprised of pages 29-36, 39-59, 79-84, and 92-106.

INTRODUCTION

Since the original report of animal teratogenecity with vitamin A or retinol in 1953 by Cohlan, retinoic acid and a number of other retinoids have been found to be teratogenic in numerous species (Sever and Brent, '86). A wide spectrum of congenital defects has been noted in laboratory animals and humans after maternal ingestion of retinoids. Treating pregnant mice with all-trans retinoic acid has been found to result in cleft palate, anal atresia, eye and ear defects, urogenital anomalies, malformed tails, and limb defects in the offspring (Cusic and Dagg, '84; Kochhar, '73). Cleft palate, ocular defects, central nervous system problems, and hydrocephaly have been associated with maternal use of 13-cis retinoic acid or Accutane in humans (Lammer et al., '85). This diversity of defects may be explained partially by the isomeric form of retinoic acid (RA) given, the different doses administered, the various gestational periods in which the treatment was given (Shenefelt, '72), and in part by the pleiotropic effects of retinoids.

There have been many lines of evidence supporting the contention that several different cellular effects can be attributed to retinol and its derivatives. One major

way in which drugs can result in cellular changes is to interact with the nucleus via transcription or translation. Indeed, retinoids have been shown to affect the cell by bringing about dramatic changes in genomic expression. For example, RA was initially observed to induce the differentiation of feathers where there should be scales on the feet of treated chick embryos (Hardy et al., '81). In certain ectodermal metaplasias, retinoic acid has been shown to transform these particular neoplastic cells into a benign, more differentiated cell type (Sporn and Newton, '79). Thus, retinoids must modify gene expression, since they modify the state of cell differentiation. More direct evidence for the interaction of retinoids with the genome came with further investigations.

It has been reported that retinoids interact with numerous and specific binding sites in the chromatin. Both cellular binding proteins for retinol and retinoic acid react with nuclei <u>in vitro</u> in "a specific, saturable, and temperature-depedent manner" (Blunck, '84) and transfer retinoids to the nuclei. (The number of these nuclear retinoid binding sites was greater than the number of steroid binding sites by an order of magnitude.) Furthermore, cytoplasmic retinoic acid binding proteins were preferentially present in embryonic and undifferentiated tissues. More recently, retinoic acid has been shown to affect genomic expression by activating

and suppressing specific genes. Such action results in the appearance and disappearance of some 40 specific proteins (Chytil and Sherman, '87). These effects are time-dependent, some genes being influenced in a matter of a few hours while effects on other genes require considerable time. It is expected that there would be stage-specific or developmentally related changes in the proteins of retinoic acid-treated fetuses which might be relevant in the context of this investigation.

Other cellular effects have been reported to be due to the presence of retinol derivatives. Observations of an increase of hyaluronidase associated with limb defects (Kwasigroch et al., '85) and interference in glycoprotein synthesis in the formation of cleft palate (Newell and Edwards, '81) after RA treatment pointed to the involvement of the extracellular matrix as a mechanism for the teratogenecity. More recently, a spatial distribution of RA in the digit primordia supported contentions of retinoic acid being a morphogen (Eichele and Thaller, '87). Retinoids were noted in early reports to cause mitochondrial swelling and destabilize lysosomal membranes (Fell et al., '62), cause hemolysis of red blood cells (Dingle and Lucy, '62), and perturb cellular membranes such as isolated mouse liver lysosomes (Wang et al., '76). A degradation of the protein moiety of the polysaccharide-protein complex occurred and resulted in the breakdown of embryonic chick cartilage (Bangham et al., '64) cultured in the presence of retinoic acid.

Cellular necrosis has been documented as another route by which retinoids cause malformations such as neural tube defects in mice (Marin-Padilla and Ferm, '65). These findings may seem contradictory but are not mutually exclusive. There are primary and secondary effects which depend upon, among other things, the experimental design, the species, the cell type, the strain of animal being studied, and the objectives of the investigators. As Sherman ('86) concluded, except for the area of visual systems, "we know little about the mechanisms of action of retinoids" and must understand that their effects are complex.

The above overview of retinoids' activities in a variety of systems demonstrates their wide ranging effects on developmental processes. In order to understand more completely how retinoic acid might be working in this investigation, it is necessary to examine, in detail, the morphogenesis of normal limb bud development in the mouse. After the normal pattern is known, anomalies will be apparent in the treated limb bud by comparison with the control group. It is the intention of this dissertation to concentrate on stages soon after the treatment on day 10 and up through the development of digits and digital defects on day 14. Particular detail will be paid to the areas of physiologically normal cell death in the very early but critically important stages of limb development. Some general information follows to provide background on limb morphogenesis and retinoic acid.

PART I - A REVIEW OF LIMB DEVELOPMENT

The development of the limbs begins in gestation on day 9 in mice (Wilson and Fraser, '77), day 3 in chickens (Oppenheimer and Chao, '84) and at the end of the fourth week in man (Moore, '83). Though there are obvious differences in shape & function of these various adult organisms' limbs, several morphogenetic processes apply to the development of most vertebrate limbs. The general organization of limb development will be reviewed by discussion of processes such as cell proliferation, differentiation, polarity of outgrowth, and cell death.

Early investigations into the development of limbs noted that the first visible sign of limb development is a raised area along the lateral mesoderm of the embryo. This structure was termed the Wolffian ridge. The ridge is the result of the proliferation of a strip of mesodermal cells running the length of the body wall from the pectoral to the pelvic girdle on each side. Hence, the mesoderm initiates formation of the limb bud structure (Hopper and Hart, '85).

Subsequent to the ridge stage, cells in certain limited areas called limb bud fields continue to divide at

a high rate. Most of the ridge loses its high mitotic index and later becomes unrecognizable except as a vestige when the rest of the surrounding mesoderm grows in size. Transplantation experiments have shown the presumptive limb-forming region can be localized prior to the visual appearance of the limb. Grafting this limb bud region to a different site on the body will result in limb formation at the proper time but with an improper location. By using such grafts, it was found that non-limb ectoderm would suffice for proper development and that the mesoderm was a crucial component for limb formation. Thus, at a specific spatial and temporal point, the presumptive fate of the mesoderm is said to be determined or that determination of limb mesoderm has occurred (Saunders, '82).

In these transplantation experiments, interactions between the ectoderm and mesoderm became apparent. Grafts of limb bud mesoderm and limb bud ectoderm developed normally. Grafts of limb bud mesoderm covered with non-limb ectoderm developed normally also, but grafts of limb bud mesoderm without any ectoderm withered and died. It was concluded that the structure ultimately formed was determined by the mesoderm but required induction by the ectoderm.

Cell division is the driving force behind limb outgrowth. Proliferation of cells occurs at the apex of the limb, opposite of the situation in hair follicles. Soon after the limb bud forms, a thickened ridge of

ectoderm at the apex of the limb develops which is known as the apical ectodermal ridge (AER). Mesodermal cells just beneath the AER are actively undergoing mitosis. Surgical removal of the AER covering the apical mesoderm results in the lack of distal structures.

Formation of the limbs proceeds with chronological and spatial gradients. There is a temporal cephalocaudal gradient in that the upper limbs (pectoral) form before the lower limbs (pelvic). This time difference is about one day in mice. A proximodistal (PD) gradient also exists in which differentiation of proximal structures occurs before the distal structures (i. e., the humerus forms prior to the radius and the ulna). The PD time difference is about two days.

Many of the observations from above are considered in the Saunders-Zwilling theory (Zwilling, '61) of limb development. This theory stipulates that the mesoderm promotes the formation of the AER; the AER subsequently stimulates proliferation of the mesoderm, and the mesoderm reciprocates by providing a "maintenance factor" which is essential for AER viability. Such an hypothesis which centers around these sequential interactions is supported by several types of mutants and a number of experimental studies.

Mutations resulting in the same phenotype can result from totally different genetic backgrounds. Examples of this situation are the mutations found in chickens known as "eudiplopodia" and "polydactylous." In eudiplopodia,

the ectoderm is responsible for the presence of extra digits. Combining the mesoderm from a normal embryo with the ectoderm from a eudiplopodia mutant results in polydactyly, whereas a combination of mutant mesoderm and normal ectoderm does not. In the mutant polydactylous (Hinchliffe and Ede, '67), the mesoderm is responsible for the defect. Mixing of mesoderm from a polydactylous mutant with ectoderm from a normal embryo causes excessive digits but the reverse procedure does not. Furthermore, if polydactylous mutant mesoderm and eudiplopodia ectoderm are combined, limbs develop with an even greater excess of digits than is seen with either mutant alone (Ham and Veomett, '80).

As mentioned above, removal of the AER in developing chicks results in the absence of distal structures. When an additional AER is grafted, extra limb parts or extra digits are formed depending on whether the AER is grafted early or late. Replacement of the AER by an earlier or older AER will produce a normal limb. Thus, the information for the proper sequence of limb development resides in the mesoderm itself. Generally speaking, the AER is permissive and non-instructive in that it induces structures which are inherent in the mesoderm (Ebert and Sussex, '70).

The last portion of the Saunders-Zwilling model for limb development maintains the mesoderm functions in the maintenance of a viable AER. Though the putative chemical has not been purified, mutants such as <u>wingless</u> help

support the idea of this theoretical mesodermal-derived "apical ectoderm maintenance factor" (AEMF). Wingless is a recessive mutation in which homozygotes fail to produce wings. When the limb bud is examined, the AER does form and is present early on the third day of development but subsequently regresses. Combining mutant mesoderm with normal ectoderm produces a wingless chick. A deficiency of a factor known as AEMF which is necessary to viability of a functional AER is lacking in this mutation (Swinyard, '69).

From such evidence, three types of interactions are recognized. First, the mesoderm initiates limb outgrowth and subsequent AER formation. Second, the AER stimulates further outgrowth and differentiation of the mesoderm. Third, the mesoderm provides a factor essential to the maintenance of a normal AER.

All vertebrate limbs have the same basic development (see Table 1) but differ in the end product. These differences occur because of spatial patterns of how cell types are organized. Therefore, as the limb of each organism develops, some means of producing positional information must be present. Again, the analysis of the chick allows insight into general developmental mechanisms needed in the process of pattern formation.

Examination of developing limbs reveals that the apical mesoderm is loosely-packed and undifferentiated. These mesenchymal cells are just underneath the AER in an area termed the "progress zone." The more proximal cells

Table 1. Comparative stages of early development in the forelimbs of mouse and chick embryos (from Jurand '64, Theiler '72, and Shepard '86).

mouse			chick		
ectoderm	mesoderm	day	ectoderm	mesoderm	stage
single-layer cuboidal epithelium	initial acculumu-lation	9.5	double-layer cuboidal epithelium	initial acculumu-lation	16
initial appearance of AER	prolifera-tion	10	initial appearance of AER	prolif-eration	18
accumulation of AER cells, mitosis, migration	prolifera-tion	10.5	elongation of AER cells, mitosis, migration	prolif-eration	20
AER develops further, necrotic cells appear	prolifera-tion	11	AER develops further, necrotic cells appear	prolif-eration	22
AER at its maximum	first skeletal blastema	12	AER at its maximum	first skeletal blastema	24
regression of AER	further growth & development	13	AER starts regression	further growth & development	26
AER disappears	further growth	14	further regression	further growth	28
---	-----	--	continued	more growth	30
---	-----	--	AER disap-pears	more growth	31

are denser. They become denser as the processes of migration, aggregation, and differentiation into muscle and cartilage take place. Hypotheses have been proposed to account for this situation.

Wolpert ('79) has proposed that as cells leave the progress zone of limbs in general they differentiate and their developmental options become more restricted. The first cells to emerge form proximal tissues of the upper limb. Cells leaving later form more distal structures. The longer a cell is in the progress zone, the more distal its location will be. In the proximodistal gradient, some time mechanism (perhaps the number of mitotic divisions) imparts positional value in the proximodistal dimension (Browder, '84).

The basis for Wolpert's hypothesis is partially due to work done with X-rays. Limbs exposed to X-rays early in development were similar in appearance to those of thalidomide-children in that they form digits normally but the more proximal tissues were lost or reduced. Wolpert ('79) suggested limb bud cells and other cells are damaged by the harmful X-rays. When the number of dividing cells in the apical mesoderm is low, there is no exodus of cells from the progress zone. A few healthy cells are able to recover and repopulate the progress zone. When enough cell divisions occur, a critical mass is reached and cells are pushed out of the progress zone. The exodus of cells occurs too late for upper tissues, but in time for digit formation. This theory is controversial but evidence

indicates there is some type of a timing mechanism which is functioning in the process of providing positional information for the PD gradient in developing limbs. "Positional information is the process that allows cell-cell signalling and establishment of the correct spatial pattern of cellular differentiation for pattern in even the simplest of organisms" (Kay and Smith, '89).

A totally different situation is believed to be responsible for generating the anterior/posterior gradient of avian limbs. Saunders ('72) found if a small block of posterior mesoderm was grafted to a notch in the anterior portion of the limb, a mirror-image duplication of the digits occurred in a 4-3-2-2-3-4 fashion (with 2-3-4 being normal). This area which duplicated the normal anteroposterior (AP) axis was termed the "zone of polarizing activity" or the ZPA. (The dorso-ventral axis has not been investigated to the degree of AP & PD gradients).

It has been postulated the ZPA is the source of a morphogen which diffuses in such a way as to influence digit formation. According to the model, the area closest to the ZPA receives the most morphogen and forms digit 4. The area next to digit 4 has a lower level of the compound and develops into digit 3. At the lowest level at the anterior side of the limb, digit 2 forms as the most anterior digit. In in vitro studies, a substance has been isolated from ZPA extracts which seems to act similar to the putative morphogen.

Tickle et al. ('85) implanted small beads which slowly released known amounts of all-trans retinoic acid (RA) at the anterior margin of 3.5-day-old chicks. The continuous release of RA was shown to create an AP concentration gradient of RA that was stable with time. By changing the concentration in the range of 1 to 25 nM, the 2-3-4 digit pattern progressively altered to a 2-2-3-4, to a 3-2-2-3-4, and then to a 4-3-2-2-3-4 pattern. When the same beads were grafted to the posterior margin, digit pattern was unaffected. Results such as these support Saunders' hypothesis on a molecular basis.

The signal for the AP axis may be the same in leg and wing buds, since leg ZPA grafted to the wing stimulates proper wing formation. Also, regions of mouse limbs homologous to chick ZPA induces the same pattern as chick ZPA in transplantation experiments (Hopper and Hart, '85). Therefore the AP axis is considered to be the result of a chemical gradient in contrast to the clocklike mechanism of the PD gradient.

In the morphogenetic processes discussed above, the limb acquired outgrowth and positional information. To make the limb the shape which is characteristic for the species, various adhesions and movements of cells are necessary. Muscle cells must move to their proper place (generally peripheral) as chondrogenic cells assume their position near a more central location.

It has been shown experimentally that the spatial arrangement of the precursor condensations for both

both cartilage and muscle tissues are somehow affected by the AER. Central to these movements, adhesions, and condensations are the extracellular matrix components such as the sulfated glycosaminoglycan, chondroitin sulfate. As is the case with other phenomenon, the occurrence of mutants and their analysis provides insight into the mechanisms involved.

In the talpid mutant of chickens, precartilage condensations are poorly defined. This lack of well-defined cartilage rays results in the fusion of proximal bones and the formation of excessive distal digits. The mesenchymal cells of talpid mutants are less mobile and also more adhesive than their normal counterparts (Hopper and Hart, '85).

The pattern of cartilage formation appears to be similar in chickens and mice. The first step in chondrification is the formation of areas of high cell density. After mesodermal cells aggregate, the cells then acquire the ability to form cartilage. It is of interest that the dorsal and ventral mesodermal cells (presumptive muscle) show a decrease in chondroitin sulfate whereas the cartilage cells show an increase in chondroitin sulfate synthesis. Once the cells have migrated, adhered, condensed, and differentiated, chondrocytes eventually undergo ossification.

Studies by Kochhar et al. ('84) showed another extracellular matrix component was also variable. Hyaluronic acid was increased two-fold in cell cultures

treated with RA over that of the normal controls. It was suggested the displacement of hyaluronate from the membrane into the media (or surrounding tissues) may play a role in the disruption of cell differentiation and limb morphogenesis. Thus, in these two cases, the extracellular matrix may have a bearing on the developmental outcome.

The development of limbs also includes a well-defined pattern of cell death (Glucksmann, '51; Goel, '83) leading to several defined necrotic zones. For example, cell death helps delineate the area between the body trunk and the projecting limb. Cell death is also needed for the removal of excess tissue between joints such as between the radius and ulna. There are four more areas of cell death that are associated with the normal development of the distal portion of most vertabrate appendages. For example, cell death is the predominant mechanism for the removal of tissue during digit formation.

One region of cell death in the chick which has been studied extensively is the posterior necrotic zone (PNZ). The PNZ is an area along the margin of mesoderm near the posterior edge of the wing. (The ZPA and PNZ develop in a similar location. They are not considered to be causally connected even though they may be related.) About 2500 cells die within 8 to 10 hours at day 4.5 of gestation. These cells have been shown to be programmed at stage 17 to die when they reach stage 24 of development. The cells of the PNZ die on schedule, even when placed in cell culture. This observation suggests the signal for death is inherent in genes switched on at stage 17 and not due to some

external signal. However, this intrinsic program can be altered and reversed by a diffusable factor from central limb mesodermal cells up to stage 21. Saunders has suggested many cell types contain so-called "death clock" programs and that they may be rescued from programmed death by the influence of signals from their cellular environment (Ham and Veomett, '80).

Chicks also possess an anterior necrotic zone (ANZ). The ANZ precedes the PNZ in that it takes place in stages 21 thru 23. The ANZ assists in shaping the shoulder region. Since rodents possess the maximum number of digits (i. e., 5) and birds usually possess three digits on their upper limbs, it is thought the ANZ and PNZ may function to reduce the amount of mesoderm available for digit formation in birds. In support of this concept is the fact that the talpid mutant mentioned earlier lacks the ANZ/PNZ and develops a preponderance of digits (Hinchliffe and Gumpel-Pinot, '81).

A third zone of necrosis in the chick is the opaque patch (OP). The OP appears between stages 24 and 25 in the central mesoderm of the forearm. Necrosis there serves to separate the condensations giving rise to the radius and ulna (Dawd and Hinchliffe, '71). Unlike the situation in the PNZ, condensed chromatin, an increase of lysosomal enzymes, and a vacuolated cytoplasm give prior indications that cell death will occur in the OP. Also, it has been noted that the cells of the OP cease producing chondroitin sulfate and lose their cell-to-cell adhesion properties as

the OP changes from presumptive chondrocytes to cells programmed to die (Hinchliffe and Thorogood, '74).

The last necrotic zone to develop in chicks and mammals is the interdigital necrotic zone (INZ). Cell death in the INZ is instrumental in shaping the digits out of a paddle-like limb. In ducks and retriever dogs, the INZ is greatly reduced and hence the feet are webbed. When a duck leg mesoderm is covered with chick ectoderm, the cross turns out web-like feet similar to the duck. The reciprocal cross of chick mesoderm and duck ectoderm remains chick-like (Hopper and Hart, '85). Therefore, the pattern specificity is inherent in the mesoderm.

Cell death also occurs in mice (see Fig. 1) but differs from chicks in several ways. It is sometimes hard to compare studies between mice and chicks since the necrotic patterns of mammals are not equivalent to that of birds. Bird studies concentrate almost totally on mesodermal necrosis while mammal studies pay equal attention to ectodermal and mesodermal necrosis. Both groups exhibit cell death in the area termed the interdigital necrotic zone (Milaire and Rooze, '83).

Mammals are unique due to a zone of necrosis on the preaxial portion (thumb side) of the mesoderm known as the foyer primaire preaxial or fpp. It is thought to function in controlling the size and number of preaxial digits. (Scott et al., '80). Later, a limited area of cell death occurs along the margin on both sides of the hand plate. The area on the anterior side is known as the fmI while the

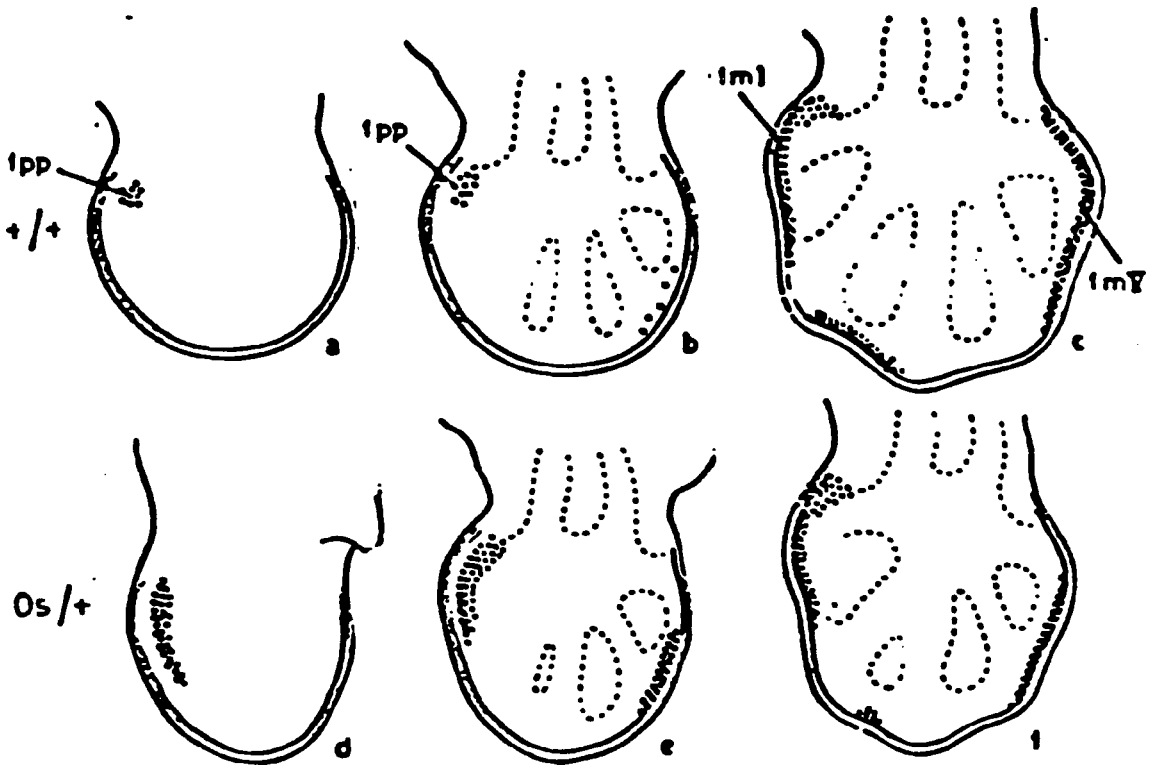


Figure 1. Schematic distribution of dead cells (small and large dots) demonstrated by Nile blue preparations. A) day 11 control B) day 11.5 control C) day 12 control D) day 11 mutant E) day 11.5 mutant F) day 12 mutant (Milaire and Rooze, '83). The Os/+ refers to the heterozygote oligosyndactylous mutant mouse. Interrupted lines delineate the preskeletal mesodermal condensations. (fpp:"foyer preaxial primaire," fmI:"foyer marginal preaxial," fmV:"foyer marginal postaxial.")

area on the posterior side is known as fmV (the foyer marginal I and foyer marginal V, respectively). They are not as extensive or as long-lived as the similar areas on the chick termed the ANZ & PNZ mentioned above. Degeneration of the AER and the mesodermal necrotic areas mentioned are closely related. The OP is missing in mammals.

A number of mutants and experimental treatments alter normal cell death patterns and produce defects. Cell death may be missing, excessive or changed in time. The most intriguing cases are those in which excess digital tissue is the result of some alteration in the normal cell death pattern. As an example, inhibitors of DNA synthesis are known to alter cell death and in certain instances cause limb malformation in mouse and rat embryos.

Treatment with 5-bromodeoxyuridine led to polydactyly subsequent to the fpp not appearing. Other studies indicated extra digits instead of missing digits also formed when 5-FU (5-fluorodeoxyuridine), mercaptopurine riboside, or cytosine arabinoside (ARA-C) is administered. It was interesting that treatment with ARA-C on day 11.5 led to polydactyly whereas treatment on day 12 led to missing digits. The authors thought altered ectodermal-mesodermal relations and the 6 to 12 hours delay in ectodermal cell death might explain the pathogenesis of polydactyly. Both ARA-C and 5-FU did have a cytotoxic effect on limb-bud mesoderm (Scott, '81) as was expected.

Earlier studies (Rooze, '77) described similar events in a mouse strain with hereditary dominant hemimelia (Dh). It was observed that the normal pattern of cell death in the limb bud ectoderm was delayed in association with variable degrees of mesodermal loss in embryos destined to possess extra digital tissue. Therefore, a gene mutation caused extra digit formation in a similar manner noted with cytotoxic agents such as caffeine, aspirin, 5-FU, ARA-C, cyclophosphamide, and 6-mercaptopurine. A similar abnormal extension of the AER was also reported to be present in polydactylous human embryos (Yasuda, '75).

More recently (Rooze, '83), "results obtained after hadacidin or cytosine arabinoside treatment support the idea that the hyperplasia of the AER might be somehow involved in the excessive regulatory phenomena responible for the genesis of polydactyly or hyperphalangy." Heterozygote Pdn mice developed a homozygote-like polydactyly after 5-FU and ARA-C treatment. In these cases, each drug induced necrosis in the mesoderm, and the AER was unusual in that it was free of dead cells (Naruse and Kameyama, '83). A Pdn gene was theorized which exhibited a preferential sensitivity to cytotoxic drugs and combined the effects of the gene with the effects of the teratogen. Hypertrophy of the ectodermal AER was a consistent finding in these studies.

Xenobiotics have variable and confusing effects depending upon when the embryos are exposed to them. Development of the limbs seems to be complicated and also somewhat plastic. A study involving ARA-C (Rooze, '83)

provides a good example. Treatment of mice with 5 mg/kg of ARA-C on day 9 produces fused digits. The same dose on day 10 results in extra digits and 5 mg/kg on day 11 was not teratogenic. No simple hypothesis suffices to account for these results and others from above. It is concluded that the factors influencing cell death and digit formation are complex in nature.

And far from being completely understood, the development of the limb exhibits the classical organ forming processes of growth, sequential inductions, gene control, differention, cell movements, and finally cell death. While retinoic acid has been shown to cause a wide spectrum of limb defects (Kochhar, '73), little is known about the pathogenetic mechanisms behind these deformities. It is important not only to know that these defects occur, but moreover they afford an opportunity to examine aberrations in limb development due to retinoic acid that may provide a means for understanding more about retinoic acid's cellular effects as they relate to the interactions of organ forming processes and retinoic acid teratogenesis.

PART II - RETINOIC ACID

The retinoids (retinol, retinoic acid, retinaldehyde and other analogs) are compounds derived from plant products called carotenoids. Carotenoids, such as beta carotene, are accessory pigments of plants. They give vegetables like carrots their bright orange color. These accessory pigments can serve as alternate receptors of light energy. Carotene is a long polyisoprenoid molecule having conjugated double bonds with each end having an unsaturated substituted cyclohexane ring. One molecule of carotene is split into two molecules of the fat-soluble Vitamin A or retinol during digestion (Lehninger, '75).

Retinoic acid is a derivative of vitamin A which occurs as a natural occurring product available in the diet (Figure 2). It is the oxidized form of retinol and can replace most of the metabolic functions of retinol except for those involved with vision (Shepard, '86). This property is a result of vertebrate systems having enzymes that convert retinol to RA, retinol to retinaldehyde, retinaldehyde to retinol, but not RA to retinol. Retinoids are an important class of compounds derived from beta-carotene that function in maintaining membrane integrity, growth promotion, differentiation and maintenance of epithelial tissue, vision (DeLuca, '79) and reproduction (spermatogenesis). Figure 3 shows a

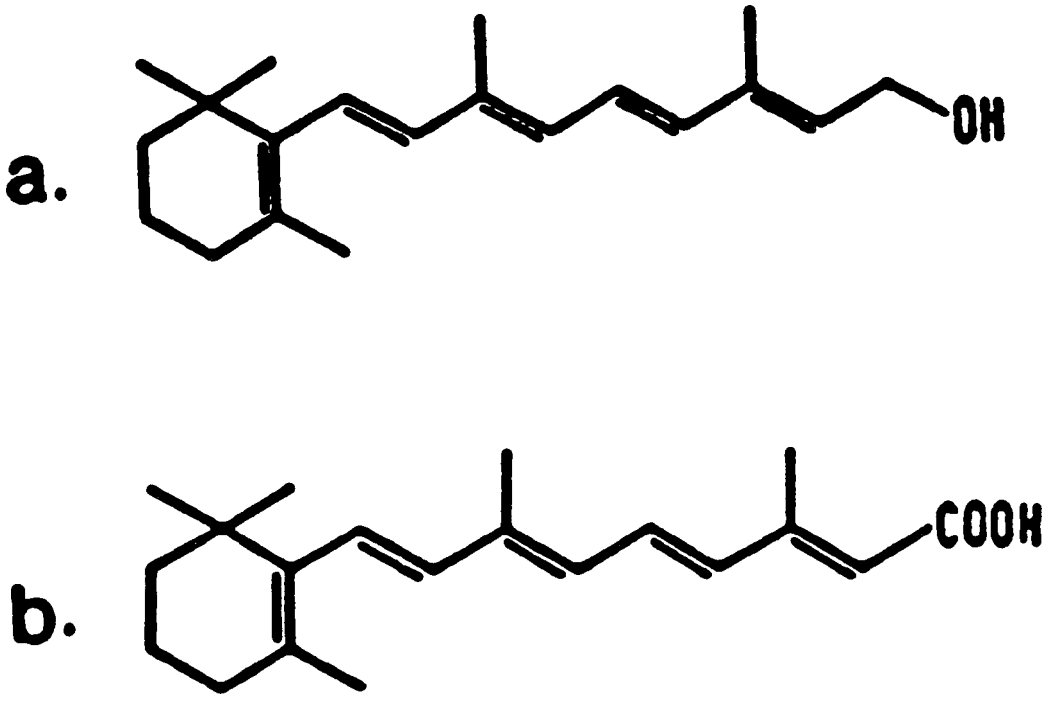


Figure 2. The linear chemical structures of (a) vitamin A or retinol and (b) all-trans retinoic acid. These naturally occurring 20 carbon compounds are characterized by a hydrophobic ring structure with a long chain carbon hydrophilic side group.

diagrammatic representation of the conversion of these retinoids and their respective functions.

Experimentation with retinoids led to the discovery of the therapeutic uses of the isomeric form of RA known as 13-cis RA (isotretinoin or Accutane). This drug found medical use in the treatment of neoplasias (Benke, '84), dermatological disorders such as psoriasis and acne, and a controversial role in wrinkle reduction (Elias, '87). But megadoses of 13-cis RA and other retinoids have side effects such as headaches, irribility, bone pain, and congenital defects. Giroud and Martinet as early as 1956 warned "that it useful to insure sufficient vitmain A intake, but, on the other hand, to avoid big excess." Even though many investigators reported laboratory animals showing a great risk of congenital deformities with maternal use of other retinoids, a significant number of human malformations resulted from oral treatments with 13-cis RA during the first trimester of gestation (FDA Drug Bulletin, '83).

The overall objective of this thesis is to investigate the pathogenesis of retinoic acid-induced defects by focusing on histochemical, histological and biochemical changes that occur in the mouse limb bud(s) when exposed to

retinoic acid. Examination of retinoic acid's effects at the cellular level with enzymatic and electrophoretic methods may help understand the cellular effects of retinoic acid during development. Cusic and Dagg ('85)

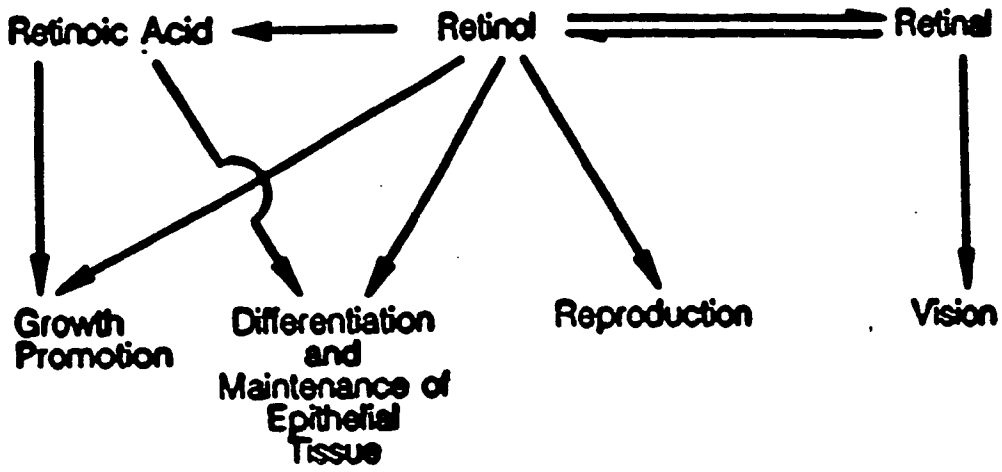


Figure 3. Diagrammatic representation of the conversion of the retinoids and their respective functions (New England Nuclear Product News, '86).

showed that forelimb defects in an inbred ICR mouse strain had a linear dose-defect relationship when all trans-RA was given to the mother in the range of 25 to 100 milligrams per kilogram of body weight on day 10 of gestation. Several types of limb defects occurred with RA treatment. Skeletal defects of the forelimb included reduced scapula/humeri; thickened and/or bent radii and ulnae; missing or shortened metacarpii; and fused digits. But besides these typical reduction-type defects, there was the development of an extra digit in the form of a postaxial polydactyly. (This strain of mice exhibited a low frequency of spontaneous postaxial polydactyly which increased substantially with retinoic acid treatment.) The formation of this supernumerary digit material is an example of "the 'super-developmental' effects of retinoids" (Maden, '85) where more structure than normal develops. This occurrence of a reduction defect and an excess defect resulting from the same treatment is an interesting developmental phenomenon.

Four important phenomena have been recognized (Kochhar, '77) to-date as possible mechanisms of abnormal development in limbs: 1) alterations in the behavior of limb mesenchyme, 2) alterations of molecular events related to chondrogenic differentiation, 3) lack of or deviation of cellular interactions between ectoderm and mesoderm, and, 4) deviations of cell death patterns in either normal or ectopic sites. This investigation uses 100 mg/kg dose of RA on day 10 and follows the cellular

effects caused by retinoic acid, especially the deviations in the ectoderm-mesoderm interactions and cell death patterns by using histological, histochemical, and biochemical techniques, to determine how they are related to these different developmental defects.

Inherent in these studies is the hypothesis of "the maximum sensitivity for inducing structural deviations is when tissues are segregating into their organ primordia" (Wilson, '77). Or as Gruneberg ('63) states in relation to limb defects specifically, "developmental consequences are related to their prechondrogenic states." In his thorough and systematic studies of various deformities in general and limb defects especially, Gruneberg (1963) concluded skeletal defects can be explained by developmental events prior to their appearance. It is with these ideas in mind that analysis of cellular events related to the teratogenencity of retinoic acid is undertaken. It is hypothesized that retinoic acid teratogenecity is related to alterations in cell death, alterations in ectodermal and mesodermal interactions, and/or changes in biochemical events during the early events of limb morphogenesis.

The specific objectives of this dissertation were as follows:

(1) to evaluate the embryotoxicity of RA as measured by embryolethality and growth retardation,

(2) to determine the patterns of cell death in normal and treated embryos from day 10 until day 14,

(3) to examine the mesodermal-ectodermal interactions in the postaxial regions,

(4) to describe the details of the morphogenesis of retinoic acid-induced postaxial polydactyly,

(5) and to investigate the enzyme patterns of acid phosphatase, hexokinase, phosphoglucose isomerase and phosphohexosekinase along with total protein banding 12, 24, 36, 48, 72, and 96 hours post-treatment.

MATERIALS AND METHODS

ICR mice were used throughout these experiments and were obtained originally from Southern Animal Farms (now Harlan Sprague-Dawley, P.O. Box 29170, Indianapolis, Indiana, 46229). They were maintained in a temperature controlled room at 22 degrees Celsius with 12 hour light and 12 hour dark periods. The animals were given commercial diet routinely (Agway, P.O. Box 4933, Syracuse, N.Y. 13221) and switched to a high protein (Agway "Hi-Pro") during pregnancy and nursing. They were given food and water ad libitum. Females were time-mated and checked for vaginal plugs on the following morning. Fertilization was assumed to occur at 2 AM on the day the plug was noted, which was considered to be day zero of gestation.

Treatment protocol

On day 10 of gestation, females were weighed and the treatment group was given retinoic acid orally. The drug was administered in a small volume of solution (0.2 ml) with a 1.0 ml syringe and a curved animal feeding needle. A dose of 100 mg/kg body weight on day 10 was chosen since it caused a relatively low fetal resorption rate and also produced a high rate of limb defects (Cusic and Dagg, '84). The retinoic acid (Sigma No. R-2625) was dissolved in a solution of 8% Cremophor EL (a derivative of ethylene oxide and castor oil-Sigma No. C-5135) and 10% propylene

glycol in distilled water. Precautions against decomposition of the retinoic acid solution were taken by minimizing exposure to light and making the solution only as needed. Females were killed by cervical dislocation on days 10.5, 11, 11.5, 12, 13, and 14. The uteri were removed and the number of resorptions was recorded. Any trace of dead embryo or placental tissue was classified as a resorption. For the statistical analysis of embryolethality, the fetus was considered the experimental unit and the groups were compared with a t-test (Sokal and Rohlf, '81). All litters with less than three fetuses were excluded from the tabulations.

Histology

For histological studies, the uteri were collected and fixed in Lavdowsky's solution (Mossman's modification- 10 ml formaldehyde, 30 ml 95% ethanol, 10 ml glacial acetic acid, 50 ml water) for 24-48 hours (C.P. Dagg-personal communication). The embryos were rinsed and then placed in 70% ethanol for storage. The limbs were embedded in wax and tangential sections were cut in the 7 to 10 micrometer range with a AO microtome. A 0.6% hematoxylin and 1% eosin stain was used for general histology (MCI Biological Stains, Inc.). A 0.5% safranin and 1% toluidine blue stain was used to differentiate between healthy cells and those which were dead or dying on days 10.5, 11, and 11.5. This latter procedure called for fixation in a mixture of 2% glutaraldehyde and 2% formaldehyde in a 0.1 M sodium cacodylate buffer with 2.5 mM calcium chloride for optimal

results. The staining procedure (Martin-Partido et al., 1986) was modified by shortening the time in toluidine blue to five seconds and the time in safranin to 30 seconds. All reagents were from Sigma and analytical grade unless otherwise noted.

Histochemistry

Since physiological necrosis is important in normal development and many teratogens act by killing cells (Scott, '78), vital staining is useful in localizing necrosis in teratological studies. Vital stains such as Nile blue are taken up by dead cells and fetal macrophages responsible for the digestion of dead cells (Pexeider, '75). Macrophages become recognizable as large blue masses while dead cells are small blue spots in comparison (Hinchliffe and Griffiths, '86). Healthy cells are able to exclude most of the dye from entering the cytoplasm because of their intact, functional membranes. That dye which does enter the cell is removed by phagocytosis. Compared to serial histologic analysis, this technique permits a rapid evaluation of superficial cell death patterns in three dimensions. One drawback of the method is that its use is limited to necrosis located in superficial tissues (Ritter and Scott, '78).

For the histochemical studies of cell death by vital staining, the uteri were placed in a cold phosphate-buffered saline and the embryos were quickly and carefully dissected out. The fetal membranes were removed and the embryos were transferred to a solution of 1:20,000

Nile blue sulphate in Dulbecco's phosphate-buffered saline (GIBCO) for twenty minutes. After a cold saline wash that served to remove any nonspecific stain, the embryos were placed back in cold phosphate-buffered saline for observation. The amount of cell death was determined at 40x on a Nikon binocular microscope or with photographs taken at 23x with a Zeiss Photomicroscope III. The number of necrotic foci in the fpp, fmI, and fmV (see introduction), as determined by the Nile blue technique (Hinchliffe and Griffiths, '86), were compared using a t-test (Sokal and Rohlf, '81) at the 95% confidence limits.

Morphometric analysis

The retinoic acid-induced postaxial polydactyly first became apparent as a squarish bulge on day 14 of gestation. A large number of the control embryos possessed a similar protuberance on day 14 but by day 18 almost all of the control embryos were without postaxial polydactyly. It has been suggested that the initial mass of this bulge on day 14 might be a controlling factor in the presence or absence of the defect on day 18 (Cusic and Dagg, '85). To determine whether this size variable did contribute significantly to the persistence of this defect, the bulge sizes on day 14 were compared. The bulge sizes were measured on fresh specimens using a light microscope with an ocular micrometer. The bulge sizes were calculated by multiplying the length by maximum height on day 14. Since there was not a normal Gaussian distribution, the means of the bulge sizes and the amount of cell death in the bulge

were compared with the nonparametric Mann-Whitney test statistic at the 95% confidence limits (Sokal and Rohlf, '81). Similar developmental studies have used morphometric analysis to assess teratological impact on pathogenesis (Lopes et al., '82; Gruneberg, '63).

Biochemical analysis

Genetic differences are reflected by differences in gene products such as tissue proteins. Measurements of perturbations in total protein or certain enzymes can provide useful data if the time period considered is sufficient for cellular perturbations to affect protein levels. For example, a comparison of embryos 12 to 24 hours apart would allow enough time for repression or induction of protein metabolism to occur. In order to circumvent the disadvantages of whole embryos and limb bud pools, determinations of protein and enzyme levels were performed on singular limb buds.

Protein patterns with electrophoresis

Initially, limb buds were squashed between two small pieces of filter paper according to the protocol of Cline et al. ('87). The samples were isoelectrically focused on an ultrathin layer of agarose using the procedure of Gorg et al. ('78). A 0.40 millmeter thick, 1% agarose gel (Pharmacia) was poured on Gel-Bond film and contained 3.5-5% LKB Ampholines in the pH range of 3.5 to 9.5. As many as 20 samples could be focused at one time on a LKB Ultraphor 2117 electrophoresis unit. An electrical field was supplied by a Macrodrive 5 power supply (1000 volts, 10

milliamps, and 10 watts) and was run 40 to 80 minutes until the milliamps decreased and stayed stable. This procedure allowed for assaying total protein and isozyme levels or a duplication of either. A surface electrode was used to measure the pH gradient across the gel.

Subsequently, electrophoretic studies used single forelimbs individually microhomogenized with 100 microliters of sample buffer containing 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10% glycerol (Laemmli, '70) and 1 mM phenylmethylsulfonyl fluoride (protease inhibitor). Homogenization utilized a 1500 microliter plastic mortar with tight Teflon pestle. After homogenization, the specimens were centrifuged in a refrigerated centrifuge for 20 minutes at 16,000X G. The supernatant was carefully drawn off and frozen until needed. Different protein analyses were performed on 20 microliter aliquots upon thawing. Samples were either isoelectrically focused on LKB polyacrylamide Immobiline plates with a pH 4-7 gradient or conventionally electrophoresed with 10% polyacrylamide gels. These gels were either fixed with TCA for protein analysis or used unfixed for enzyme analysis. Coomassie blue and silver stains were used for detection of total proteins. Protein bands were detected by a densitometer after double staining with Coomassie blue and silver nitrate (Heukeshoven and Dernick, '85). Alternatively, these gels were allowed to react with various substrates to visualize specific isozyme systems detailed below. A similar methodology for

invertebrate tissue has been described by Cline et al. ('88).

Isozyme analysis

The enzymes, acid phosphatase , phosphoglucomutase, phosphohexose isomerase and hexokinase were chosen for isozyme analysis. Acid phosphatase (E.C. 3.1.3.2), a lysosomal enzyme (DeDuve et al., '74), should be present in low concentrations and is involved in several metabolic pathways. According to Schweichel and Merker ('73), acid phosphatase was useful to monitor changes in cell metabolism such as autophagy, cell death processes (Ballard and Holt, '68) and cytotoxic effects that may be related to the retinoic acid treatment. The other three enzymes are related to energy production, exhibit polymorphism and are usually present in higher concentrations than degradative enzymes such as acid phosphatase. They were useful to monitor basic metabolism (glycolysis) and provide an assessment of normal energy production. All of these enzymes were detected by the development of various colored reaction-products described in detail by Harris and Hopkinson ('76).

The isozyme acid phophatase cleaves phosphate bonds and is important in energy metabolism and cell death processes. Comparison among days 10.5, 11, 11.5, and 12 were of particular interest since these days were found to have the most cell death. Fifty mg of the substrate beta-naphthol phosphate (Sigma) and 50 mg of Fast blue BB were dissolved in 50 ml of citrate buffer at pH 4.5. At

this pH, there should be adequate enzyme activity and the low pH helps eliminate the nonspecific phosphatases that are active at a higher pH.

Hexokinase (E.C. 3.1.1.1) converts glucose into glucose-6-phosphate. The reaction mixture was dissolved in a 0.1 M Tris/HCl buffer of pH 7.5. The reaction mixture contained 9.0 mg of glucose, 40 mg of ATP, 7.5 mg of NADP in 1.5 ml of water, 5 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in 1.0 ml of water, 2.5 mg of phenazine methosulfate (PMS) in 0.5 ml of water, 40 microliter of glucose-6-phosphate dehydrogenase (140 units/ml), and 10 ml of 0.02 mM magnesium chloride mixed in 40 ml of the Tris/HCl buffer. Four isozymes of hexokinase which migrate anodally have been described in mammalian tissues (Harris and Hopkinson, '76).

Phosphoglucomutase (E.C. 2.8.5.1) converts glucose-1-phosphate into glucose-6-phosphate. The reaction mixture was dissolved in a 0.3 M Tris/HCl buffer of pH 8.0. The reaction mixture contained 2.5 mM EDTA, 5.0 mM histidine-HCl, 0.025 mM magnesium chloride, 1.82 mM glucose-1-phosphate, 0.5 mM NADP, 3 IU of glucose-6-phosphate dehydrogenase, 0.24 mM PMS and 0.18 mM MTT tetrazolium dissolved in 20 ml of buffer. Three autosomal forms of phosphoglucomutase have been described in mammalian tissues. These isozymes migrate anodally also (Harris and Hopkinson, '76).

Phosphohexose isomerase (E.C. 5.3.1.9) converts fructose-6-phosphate into glucose-6-phosphate. The reaction mixture was dissolved in a 0.3 M Tris/HCl buffer of pH 8.0 containing 3.6 g of Tris, 146 mg of EDTA, 154 mg histidine-HCl, and 200 mg magnesium chloride. The reaction mixture contained 10 mg fructose-6-phosphate, 0.4 mg of PMS, 0.4 mg of MTT, and 2.0 mg of NADP. These components were added to 8.0 ml of buffer, kept dark and stored in the cold. Immediately before assaying, 2.0 mg of glucose-6-phosphate dehydrogenase was added to the cocktail carefully to avoid bubbles. This enzyme is dimeric and migrates cathodally (Harris and Hopkinson, 1976).

After an adequate period of incubation (usually 30 to 90 minutes at 35 degrees Celsius), the positions of isozyme bands were noted on the back of the Gel-bond film by marking with an idelible marker. Acetic acid (7%) was used to stop the reaction when the enzymes had produced a visible end product. The filter paper with the Gel-bond and agarose was dried and stapled together for future reference. The dried gels were protected from light in order to reduce the fading of the color. All enzyme studies were done in duplicate on limb bud squashes. Isozyme difference indices were determined from these gels by a computer program analysis discussed below.

The isozyme difference index (IDI) was calculated using a computer program by Angus et al. (1988). This program quantitates the similarity or difference between any two sets of proteins or isozyme patterns. The IDI

values were used to determine if the variation or difference between the treatment and control groups was significant. Gelfand's similarity used the formula:

$$F\ (X,Y) = 1\ /\ 1 + X(t) - Y(t)$$

where (X,Y) is equal to the frequency matrix of populations X,Y. Nei's transformation equates difference as being the negative log of the similarity. The isozyme difference index is equal to one minus Gelfand's similarity value.

RESULTS

Embryotoxic effects

The results of the embryotoxic effects of 100 mg/kg retinoic acid are summarized in Table 2. The number of resorptions versus the number of implantation sites was analyzed as a general indicator of embryotoxicity. Resorptions were compared between individuals in the control group and treatment group and also between litters in the control group and treatment group. The resorption rates from day 11 through 14 in the control fetuses ranged from 1.7% to 8.3% (mean = 5.9%) whereas the treated fetuses resorbed in the range from 5.7% to 15.6% (mean = 12.9%). The per cent resorptions in the control litters ranged from 20% to 43% (mean = 27%) while the resorption rate in the treated litters ranged from 50% to 100% (mean = 66.6%). Therefore, an oral dose of retinoic acid at a concentration of 100 mg/kg of body weight on day 10 was mildly but significantly embryolethal.

A less severe form of embryotoxicity which occurred was growth retardation. Crown-rump length was measured on days 10 through 14 with an ocular micrometer and the fixed wet weight was determined on day 18. The results of these comparisons are presented in Table 3. No significant differences in fetal length were noted until day 12. On

Table 2 - Number and per cent of resorptions occurring in litters and fetuses on days 11, 12, 13, and 14.

	# of feti resorbed	# of feti viable	%	litters with resorption(s)	Total # of litters	%
Day 11 control	1	44	2.2	1	5	20
Day 11 treated	2	35	5.7	2	4	50
Day 12 control	12	142	8.3	3	12	25
Day 12 treated	16	102	15.6	6	10	60
Day 13 control	1	58	1.7	1	5	20
Day 13 treated	3	33	9.1	2	3	66.6
Day 14 control	4	61	6.5	3	7	42.8
Day 14 treated	6	43	13.9	4	4	100
Total control	18	305	5.9	8	29	27.5
Total treated	27	213	12.6	14	21	66.6

Table 3. Measurements of size on days 11, 11.5, 12, 13, 14, and 18.

	CONTROL	TREATED	Rugh's range
day 11	6.30 mm	6.24 mm	3 - 6.5 mm
day 11.5	6.99 mm	6.99 mm	----
day 12	8.55 mm	8.36 mm *	5 - 8.0 mm
day 13	9.84 mm	9.53 mm **	7 - 9.5 mm
day 14	11.30 mm	11.33 mm	9.5-11.0 mm
day 18	1.08 grams	1.14 grams	0.57-1.125 g

* - the value of the treated group was significantly less ($p<.05$) than the control group

** - the value of the treated group was significantly less ($p<.01$) than the control group

The crown-rump length was used as a measure of size on days 11 through 14 and the fixed weight was used as a measure of size on day 18. The ranges given are those reported in R. Rugh's "The mouse: its reproduction and development", Burgess Publishers, Minneapolis, Minnesota, 1968.

day 12, the treated mean of 8.36 millimeters was less than the control mean of 8.55 millimeters. Also, the mean on day 13 of 9.53 millimeters for the treated group was significantly less than the 9.85 millimeters average of the control. On day 14, the mean of the treated group (11.33 mm) was greater than the control mean of 11.30 mm but the difference was not statistically significant. The treated group on day 18 averaged 1.14 grams per fetus whereas the control group was only 1.08 grams per fetus. In this instance, the treated was larger than the control group, but the difference was not statistically significant. (Interlitter and intralitter variability was evident. The variances were compared by the approximate F-max test and found to be not significantly heterogeneous.)

Macroscopic observations

At the time of treatment on day 10, the forelimb was a small, discoid-shaped tissue which was less than 1 mm in width and length. This simple ridge grew rapidly and was projecting further and curving forward by day 10.5. The limb had a paddle shape on day 11. On day 11.5, the handplate or footplate formed in the anterior limb bud. This handplate area was indicated by a definite constriction which divided the forearm from the hand. By day 12, the handplate was no longer roundish and took on a slightly angular contour. The day 13 forelimb had an indentation of the handplate which was characteristic of

this period. The indentation was shallow in younger embryos and very distinct in older embryos. Alcian blue staining revealed the hand portion of the limb remained mesenchymous still while the skeletal elements of the forelimb already contained cartilage.

The forelimb was much larger on day 14. It was several millimeters in length and the handplate was 1.5 to 2.0 mm in width in the control and treatment groups. With magnification, the individual digits were seen to be separated in the handplate. The fingers were divergent and would not become parallel until much later. The digits had become so well-defined by this time that several defects were recognizable. A substantial number of the treated fetuses were noted to possess absent digits (ectrodactyly), shortened digits (brachdactyly) and/or fusion of digits (2-3 syndactyly). Nearly 100% of the treated fetuses exhibited a postaxial bulge compared with only 70% of the control fetuses. The bulge usually was present on both limbs if it was present at all.

Measurements of the width of the handplate taken on day 11 and day 12 revealed the treated group was slightly smaller in size. The difference in size was not significant statistically and was probably related to the general effects of retinoic acid in regard to growth retardation. This detail is mentioned because it rules out the occurrence of polydactyly due to an increased tissue mass resulting from the treatment.

It deserves mention that the postaxial portion of the limb was larger than the preaxial portion of the limb in both the treatment and the control groups. This disproportion occurred early in the development of the limb. This asymmetry, which was probably related to the fact that the extensor muscles are generally larger than their flexor counterparts, has been noted in normal mouse limb development by others (Muneoka et al., '89; Milaire and Rooze, '83).

Histological observations

At the time of treatment on day 10, the bulk of the limb was composed of non-differentiated mesenchymal cells without any aggregations of cells that proceed chondrification. The mesoderm was covered with a two-cell layer thick ectoderm. Rapid mesodermal mitosis occurred at the apex of the limb underneath the ectoderm.

Paraffin sections from day 10.5 were observed to possess the ectodermal structure known as the apical ectoderm ridge (AER). The limb was slightly larger than the day 10 forelimb and was still without precartilageneous condensations. A comparison of the forelimbs stained with safranin/toluidine blue showed an increase in necrotic cells within 12 hours. The number of macrophages and necrotic cells were markedly increased on day 11 (see Figure 4). Evidence from these studies indicated retinoic acid was cytotoxic to mesodermal cells of the forelimb. The cell death resulting from retinoic

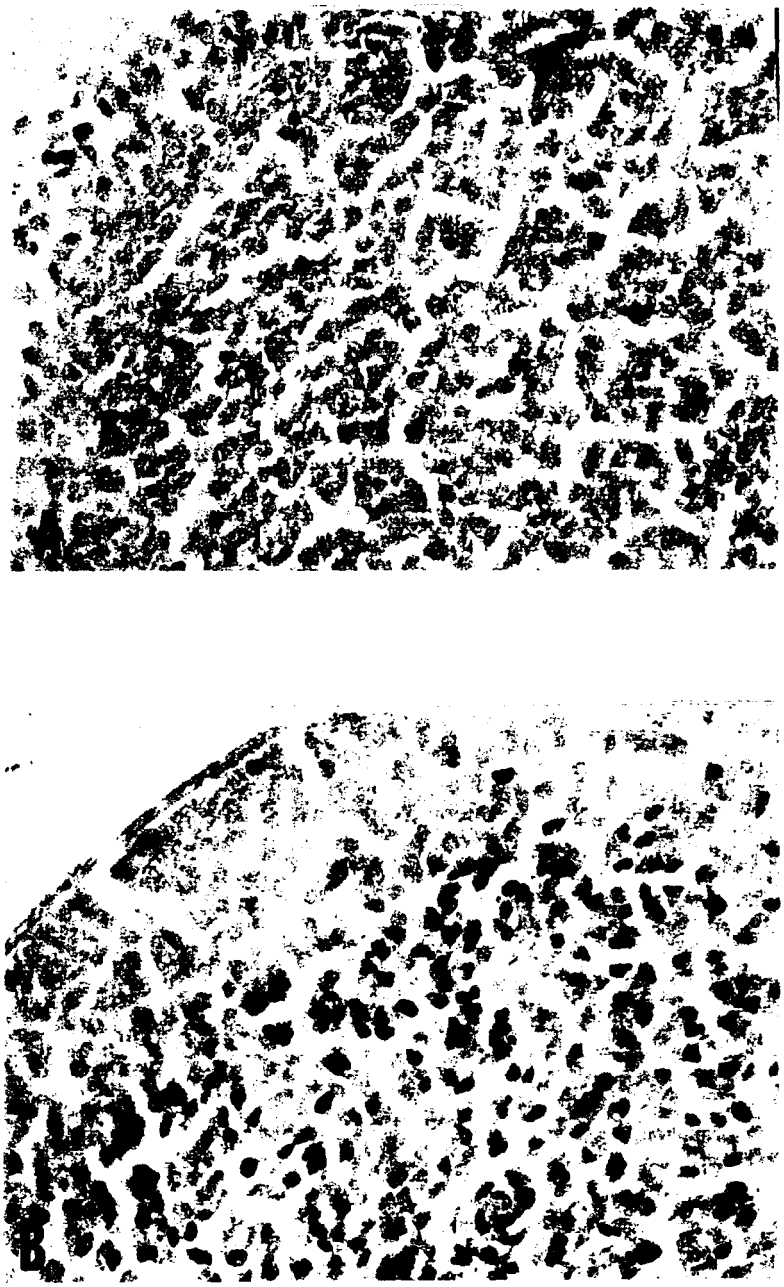


Figure 4. Tangential sections of forelimbs on day 11. The control (a) and treated (b) sections were stained with a safranin toluidine blue solution. Note the presence of dead cells in (b) and the absence of dead cells in (a).

acid treatment could be described as diffuse since necroses were scattered throughout the mesoderm of the central proximal core of the forelimb.

By day 12, the histology of the forelimb had changed dramatically. Condensations representing the humerus, ulna, radius, and the pentagonal handplate were evident in both the control and treatment groups. There were no indications of either excess digital rays or of an increase in the size of the handplate in general as is the case in some types of digital deformities (Naruse and Kameyama, '82). The most postaxial portion of the ectoderm in the control group was normal appearing in that it had a stratified, well-organized, two-cell layer thick ectoderm. This description was consistent with an ectoderm in the process of regression. In contrast, many of the treated forelimbs had ectoderm in this same area which was relatively hyperplastic or metaplastic (see Figure 5). The treated postaxial ectoderm on day 12 was different in that it was more than two cell layers thick, tended to be more cuboidal or pseudo-stratified, and possessed a different organization that was more hyperplastic. This change was temporary since there were no significant differences on day 13 between the control and treated fetuses in the postaxial ectoderm (i.e., both groups possessed a two-cell layer thick postaxial ectoderm).

Figure 5. Tangential sections of forelimbs on day 12. The control (a) and treated (b) limbs were stained with hematoxylin-eosin. The postaxial region of the control embryo possessed a two-cell layer thick, morphologically normal ectoderm whereas the treated embryo's ectoderm was irregular, hyperplastic and multicellular.

A

B

Histochemical staining and patterns of cell death

Four different areas of physiologically normal cell death occurred in the mesoderm of the forelimb between the time of the treatment on day 10 and the initial appearance of defects on day 14. The first mesodermal necrosis was the foyer preaxial primaire or fpp. The fpp is a small, roughly circular patch of dead cells located on the preaxial or thumb side of the footplate area of the forelimb on day 11 (Milaire and Rooze, '83). The foyer marginal I and foyer marginal V (fmI and fmV respectively) both appeared on day 12. They consisted of a row of dead cells extending along the right and left margin of the handplate. They are thought to serve to reduce the amount of digit material for the first and fifth digits (Milaire and Rooze, '83). The last necrotic area to develop was the interdigital necrotic zone or INZ. The INZ was first noted on day 13 and persisted past day 14. The INZ serves to help separate the digits from each other (see introduction for more information).

Little or no Nile blue uptake was obvious 12 hours after the time of treatment in most of the control and treated fetuses. About 20% of the treated day 11 fetuses possessed a small amount of cell death in the area of the necrotic zone on the preaxial side of the forepaw designated as the fpp. The fpp was noted in both the treated and the control groups on day 11.5. The treated fpp appeared to be more intense in the amount of dye uptake

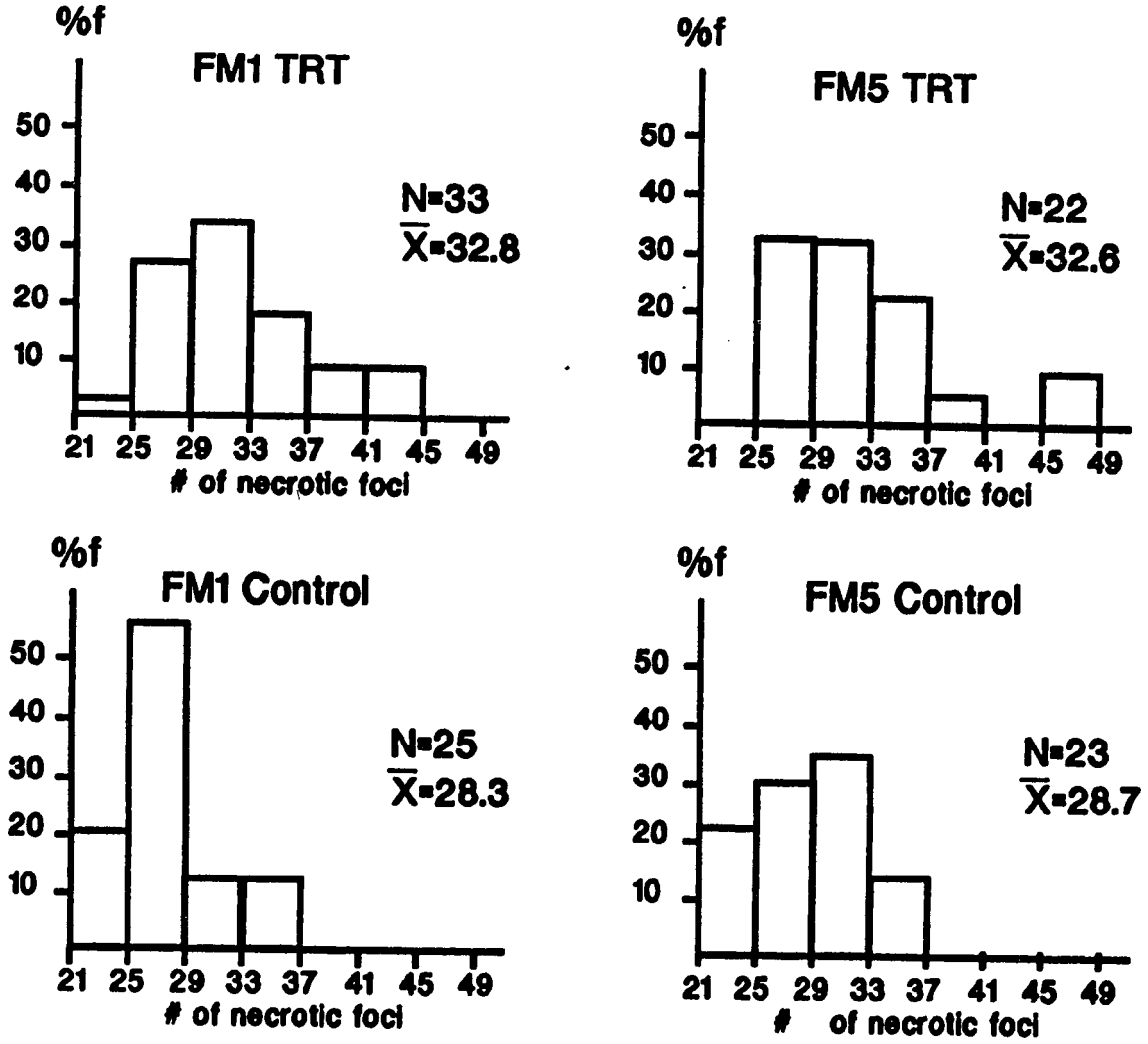
at this time. Counts of the fpp (Bynum and Dagg, '86) showed about 75% more macrophages were present in the treated fpp than the control fpp (Table 4). The fpp had a range of 13, with a minimum and maximum of 15 and 28, a mean of 20.0, SD of 4.16, CV of 20.82%, a s.e. of 1.31 and n of 10. The fpp treated had a range of 53, a minimum and maximum of 19 to 72, a mean of 35.2, SD of 11.85, CV of 33.69%, a s.e. of 2.16 and n of 30.

On day 12, the necrotic zones extending along the preaxial and postaxial margin of the forepaw known as the fmI and the fmV, respectively, were present. The fmI extended from the apex of the second digit all along the subectodermal margin down to the most anterior portion of the first digit. The fmV extended from the apex of the fourth digit along the margin of the posterior portion of the handplate back to the base of the fifth digit. The treated group exhibited an increase in cell death that was greater in both the fmI and the fmV (Figure 6). The relative increase of necrotic cells was greater in the fmI than in the fmV. As can be seen from the above figure, there was considerable overlap in the amount of necrosis, but the treated fetuses tended to have more Nile blue uptake than the control fetuses in both the fmI and the fmV (Bynum and Dagg, '86). There was a fairly normal distribution of necroses in both the control and treatment groups. The fmI control had a mean of 28.32, SD of 3.4, CV of 12.3% and a s.e. of 0.687 while the fmI treated had a mean of 32.8, SD of 5.08, CV of 15.49% and a s.e. of 0.88.

Table 4: Counts of dead cells in the fpp on day 11.5 by histochemical staining.

Control_group		
21	18	n = 10
16	22	mean = 20.0
15	28	SD = 4.16
25	16	se = 1.31
19	20	CV = 20.82%
Treatment_group		
19	24	n = 30
26	27	mean = 35.2
20	26	SD = 11.85
27	24	se = 2.16
33	28	CV = 33.69%
33	29	
40	42	
33	36	
38	45	
53	72	
29	30	
51	24	
58	34	
44	28	
39	41	

Figure 6: Incidence of mesodermal cell death on day 12 as determined by Nile blue staining. Bar graphs show frequency distributions of fm1 control, fm1 treated, fm5 control and fm5 treated. (fm1 = foyer marginal preaxial, fm5 = foyer marginal postaxial, trt = treatment group)

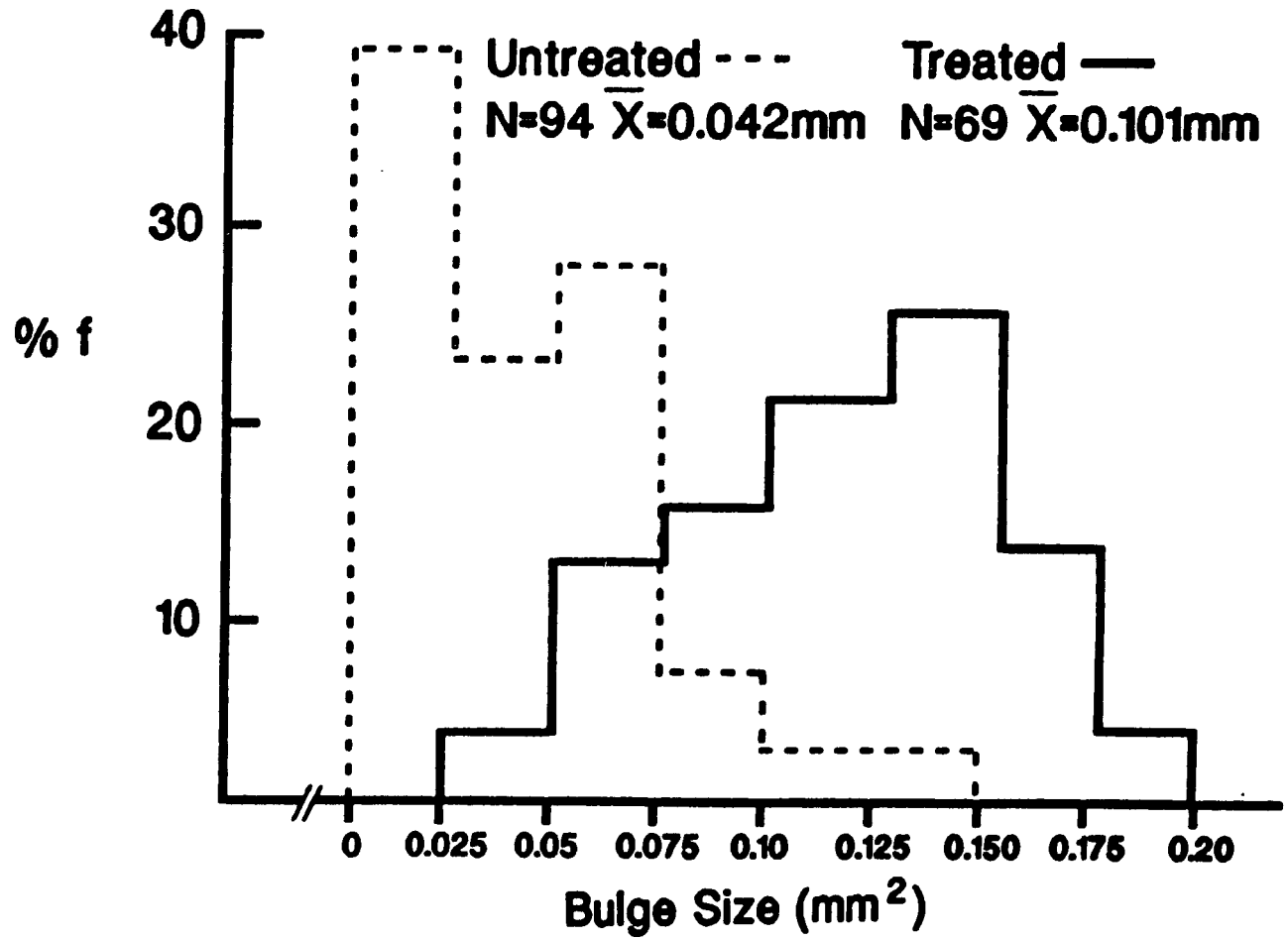

%f
FM1 TRT
50
40
30
20
10
N=33
X̄=32.8
21 25 29 33 37 41 45 49
# of necrotic foci
%f
FM5 TRT
50
40
30
20
10
N=22
X̄=32.6
21 25 29 33 37 41 45 49
# of necrotic foci
%f
FM1 Control
50
40
30
20
10
N=25
X̄=28.3
21 25 29 33 37 41 45 49
# of necrotic foci
%f
FM5 Control
50
40
30
20
10
N=23
X̄=28.7
21 25 29 33 37 41 45 49
# of necrotic foci

The fmV control had mean of 28.69, SD of 3.4, CV of 12.09% and a s.e. of 0.72 while the fmV treated had a mean of 32.6, SD of 5.9, CV of 18.3% and a s.e. of 1.27.

On day 13, the first indications of the INZ were observed. Tissue necrosis was noted in the mesoderm between the third and forth digital rays and the fourth and fifth digital rays. No significant differences were evident on day 13 between the control and treated fetuses. By day 14, there was mesoderm necrosis between all the digits and the INZ was at its maximum in both the control and treated groups. The AER had regressed on day 12 and necrotic cells were scattered throughout the epidermis.

It was noteworthy that a prominent squarish bulge developed on the postaxial side of the forepaw in a majority of the control group and in almost all of the treated group on day 14 (Bynum and Dagg, '87). Measurement of the bulge size on day 14 indicated the treatment resulted in a statistically significant larger tissue mass in the postaxial region (Figure 7). Also, while the control group was generally almost completely necrotic (Bynum and Dagg, '88), a large number of the bulges in the treatment group had less than 60% necrosis (Figure 8). This cell death in the bulge was connected to part of the INZ that normally shaped the postaxial portion of the forepaw (Figure 9). Of the control fetuses that had bulges, they ranged in size from 0.004 to 0.145 millimeters squared, had a mean of 0.042, a SD of 0.026, a CV of 60.4% and a s.e. of 0.003. The distribution for the control

Figure 7: Measurements of postaxial bulge size on day 14. Bar graphs show frequency distributions of the area (millimeters squared) in both control and treatment groups.


40
30
20
10
% f
Untreated - - -
N=94 X=0.042mm
Treated ——
N=69 X=0.101mm
0
0.025
0.05
0.075
0.10
0.125
0.150
0.175
0.20
Bulge Size (mm²)

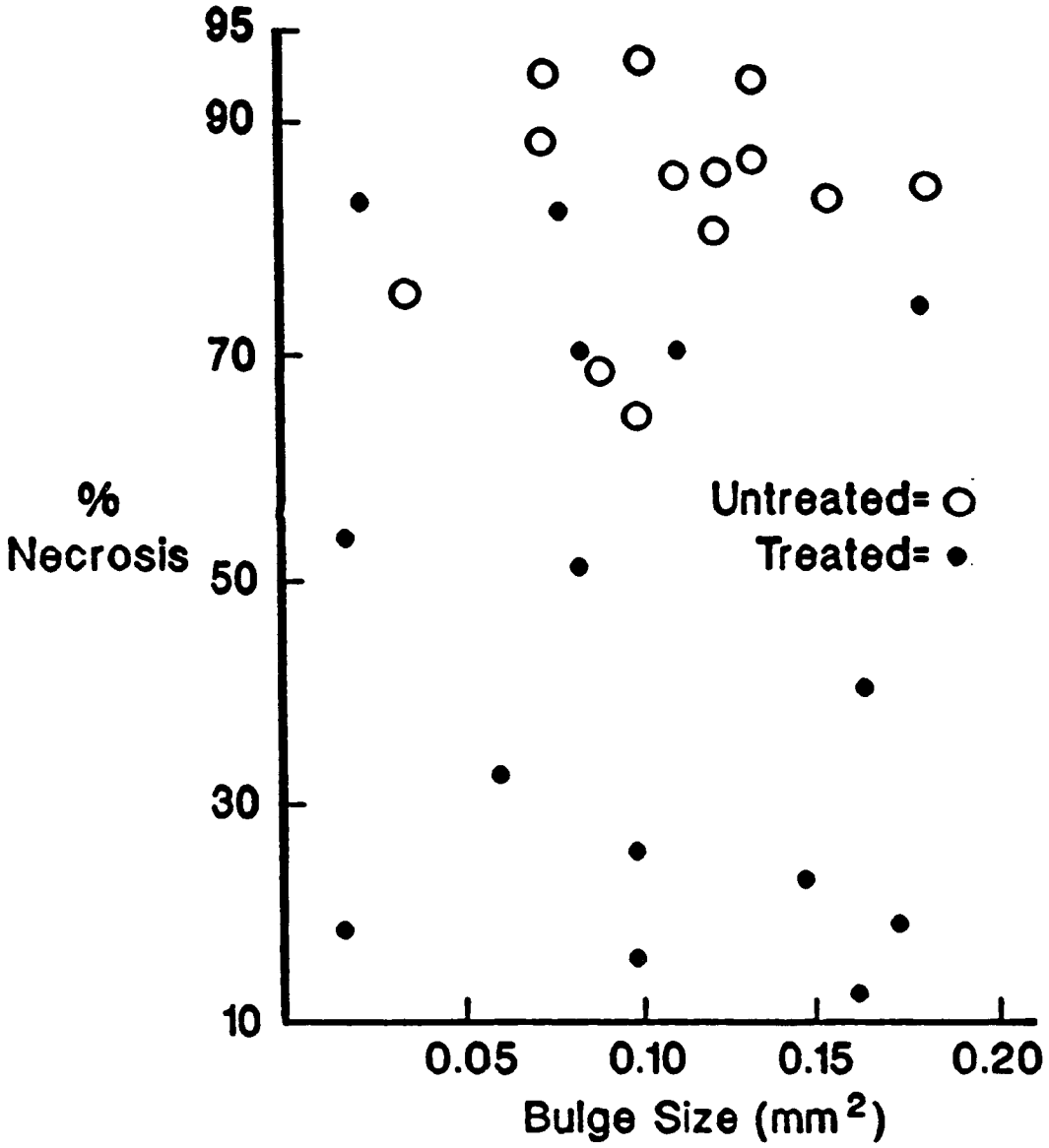
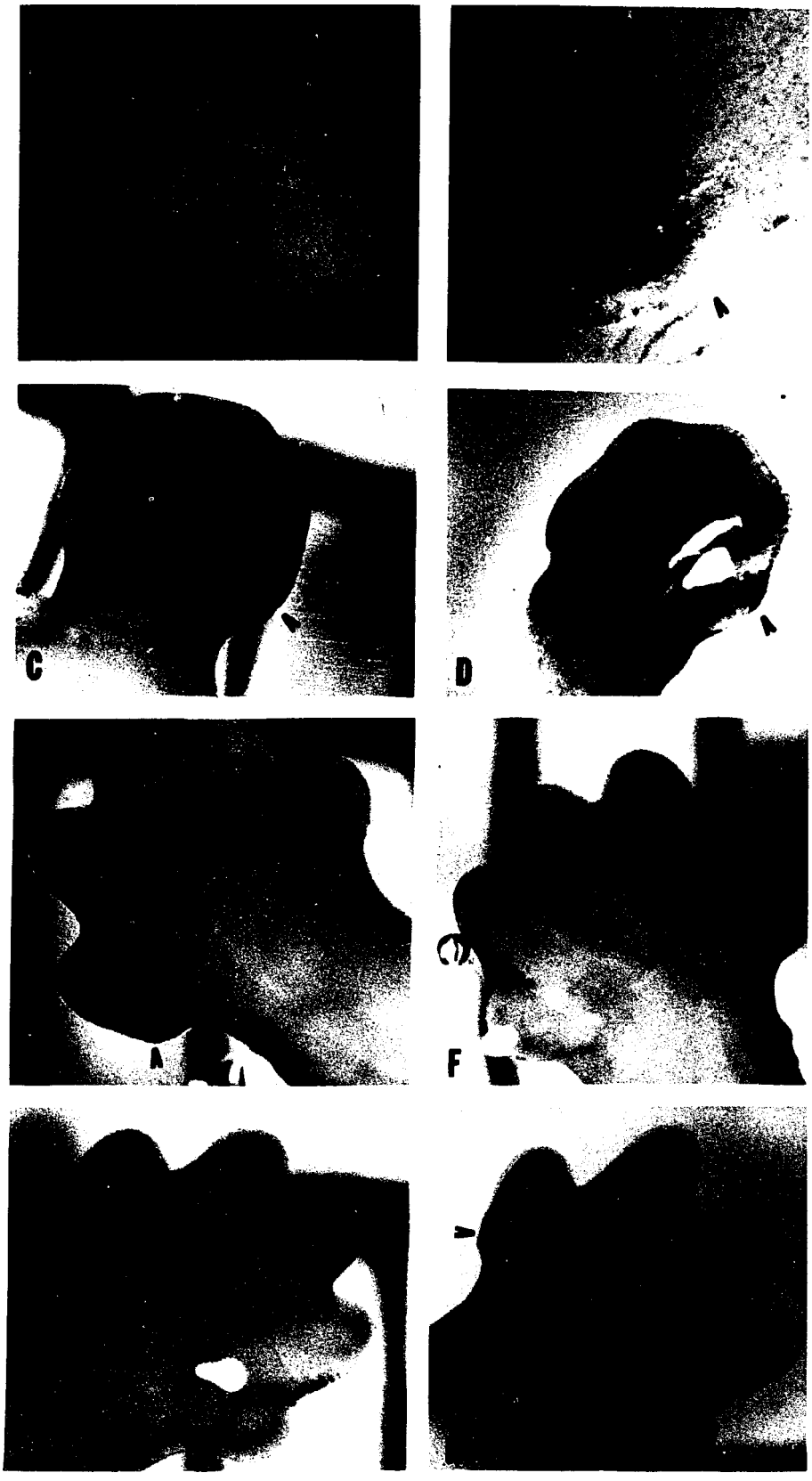


Figure 8. Plot of % necrosis versus bulge size. Data points represent selected control and treated postaxial bulges on day 14 on a scatter plot.

Figure 9: Variously staged forelimbs showing vitally stained areas of cell death. A: Day 11 control with absence of necrosis. X50. B: Day 11.5 treated showing fpp (at uppermost portion of limb). X50. C: Day 12 control with fmI and fmV. X20. D: Day 12 treated with fmI and fmV. X20. E: Day 14 control with small bulge that is mostly necrotic. X30. F: Day 14 treated with bulge that is mostly necrotic. X30. G: Day 14 treated with bulge that has moderate necrosis. X30. H: Day 14 treated with bulge that has relatively less necrosis. (Arrow (>) indicates the postaxial side.)

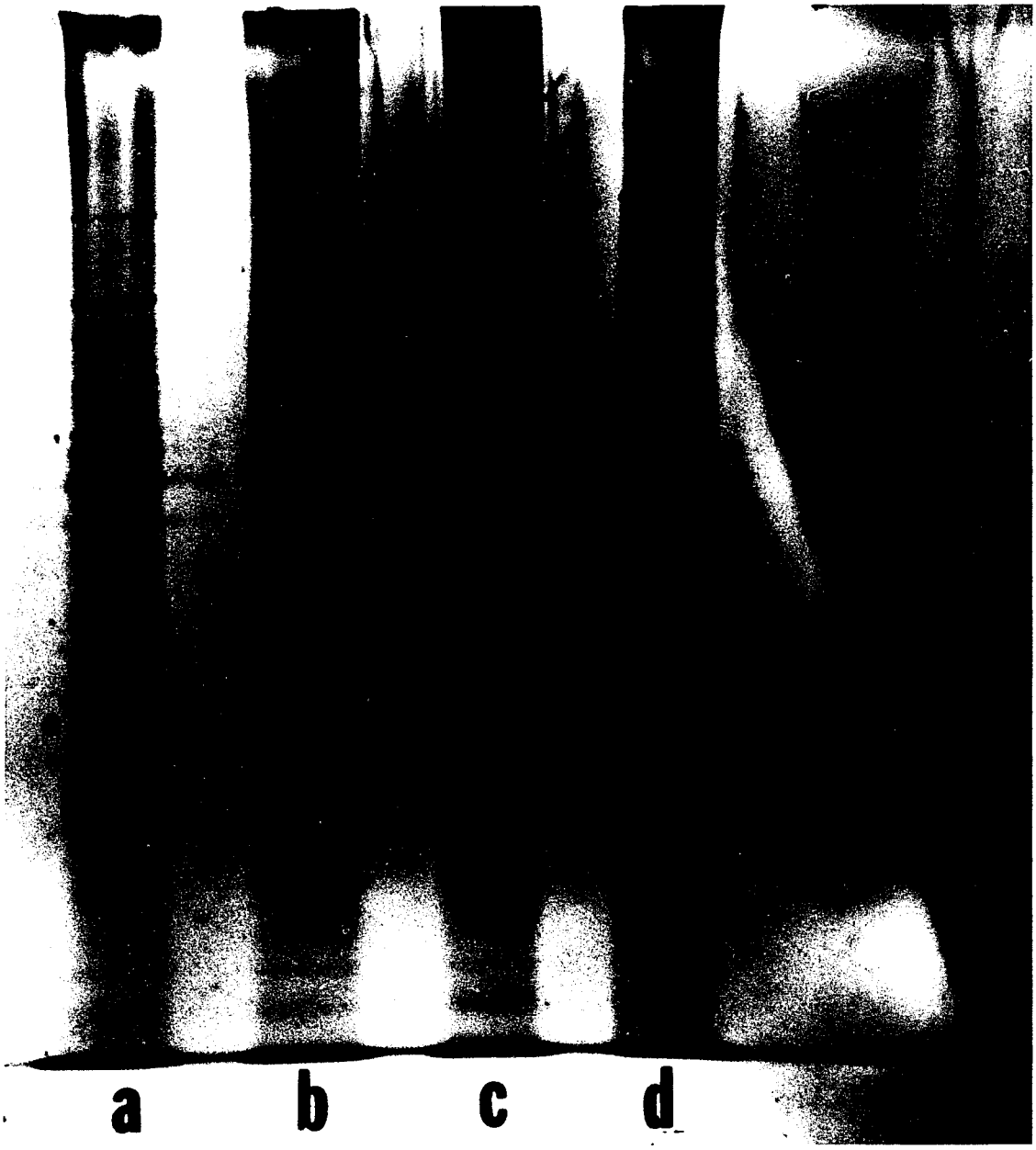

C
D
A
F

group was skewed right and had positive kurtosis-leptokurtic. The treated group had a range of 0.026 to 0.187 millimeters squared, had a mean of 0.1006, a SD of 0.039, a CV of 39.52 and a s.e. of 0.005. The treated group was negative for kurtosis-platykurtosis and was skewed left.

SDS-PAG electrophoresis

Conventional sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed on limb bud tissue form embryos of days 10, 10.5, 11, 11.5, 12, 13 and 14 to evaluate molecular weight pattern of proteins. The protein patterns from day 10 and 10.5 were basically the same, as indicated by the IDI values. But by day 11, there were some differences that were apparent between the control and the treatment group. Some bands that were in the treated group were qualitatively different in that they were more intense than the control group equivalent. Other bands were either absent or present in the treatment groups when compared to the control group conterpart and were interpreted as being quantitatively different. The differences on day 11.5 were discernible, but was not as noticeable as those on days 12-14 (Figure 10). The control group had bands more intensely stained than the treated group at a molecular weight of 47,000 on day 12. The treated group had novel protein bands at a molecular weight of 90,000 and 22,000 on day 12. Generally, the treated group had more bands present than the control group on days 12 and 14. Another relationship

Figure 10. Silver stain of SDS-PAGE on day 12 limb buds. The gel shows duplicates of day 12 control (left), day 12 treated (middle), and molecular weight standards (right).


a
b
c
d

that was obvious was as the limb matured (or as the days of development increased), the number of proteins present in the limb bud increased. The increase of proteins was predominantly in the higher molecular weight range (Figure 11). This progression or elaboration of proteins has been reported in other developmental systems (Meade, '90; Knecht et al., '85).

Isoelectric focusing

Limb buds were also analyzed by IEF to determine if new proteins appeared with development and if any proteins were lost. Initially, a 20 microliter sample was taken from the 100 microliter homogenate of single limb buds. From the isoelectric focusing experiments that were done on the 20 microliter aliquots, several results were interesting and significant. The day 12 control had 12 major and minor bands present in the silver stained protein pattern (Figure 12). The day 12 treated had more bands, usually up to 17. Most of the new or novel bands occurred at the anodic end of the gel at a pH range of 4.2 to 4.8. Besides these extra anodic proteins, two bands were present in the day 12 treated at a pH of 5.4 that were absent in the controls. About 10 protein bands focused in the day 14 control limb buds versus about 20 bands in the day 14 treated. Again, most of these proteins were located in the anodic end of the gel (pH 4-7). Comparisons of IEF patterns of day 12 with day 14 showed the disappearance of 5 major bands (pH 6.0-6.5) during the two days of development.

Figure 11. Silver stain of SDS-PAGE on various days of limb development. The lanes correspond to molecular weight standards in duplicate (on left), day 10, day 11, day 12, blank, blank, day 13, day 14 and standards.

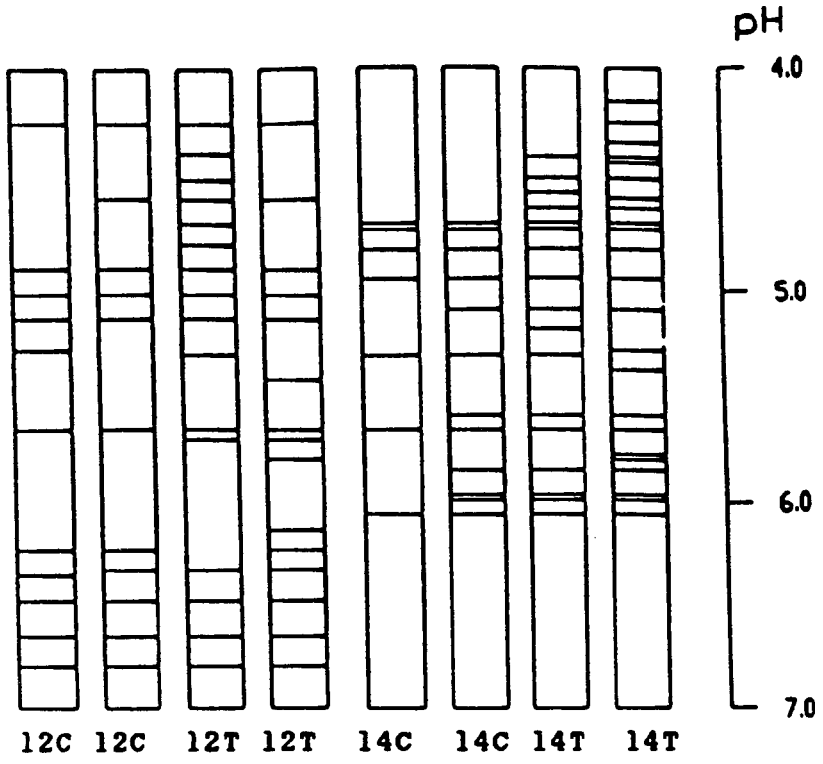


Figure 12. Silver stain of limb bud proteins that have been isoelectrically focused. The lines represent the bands found in a 20 microliter aliquot from a limb bud homogenized in 100 microliters on either day 12 or 14.

When whole single limb buds prepared by the tissue squash method were examined by IEF, little variation between control and treatment groups was detected until day 12. Most of the proteins were of an intermediary pH on days 12-14. There were so many bands present in the range 4.8 to 6.5 that they were visually indistinguishable and difficult to analyze. The day 13 control and treated protein patterns were qualitatively and quantitatively very similar in appearance. The day 13 control had 27 major and minor bands while the day 13 treated had less than 27 bands. On day 14, there was several prominent anodic bands present in the treatment group that were absent in the control group (Figure 13). The day 14 control had about 30 bands while the day 14 treated limb buds had well over 30 bands. Due to the increased concentration (and proliferation of bands) in this limb squash, the 5 bands in the intermediate range that were absent on day 14 in the homogenized limb bud-20 microliter study were not absent with the squash-prepared sample.

Isozyme studies

The data matrix, similarity, and isozymic difference indices for each isozyme are found in Table 5. A typical zymogram was photographed in Fig. 14. Multiple forms of acid phosphatase (Fig. 15), hexokinase (Fig. 16), phosphoglucomutase (Fig. 17), and phosphohexoseisomerase (Fig. 18) were found in every case. The pI values of the various bands are also located in table 5. The IDI

Figure 13. Photograph of limb bud homogenates electrophoresed on a LKB Immobiline gel. There are standards on either end. Lane 2 = day 12 control, lane 3 = day 12 treated, day 13 control, lane 5 = day 13 treated, lane 6 = day 14 control, lane 7 = day 14 treated, lane 8, day 14 treated..

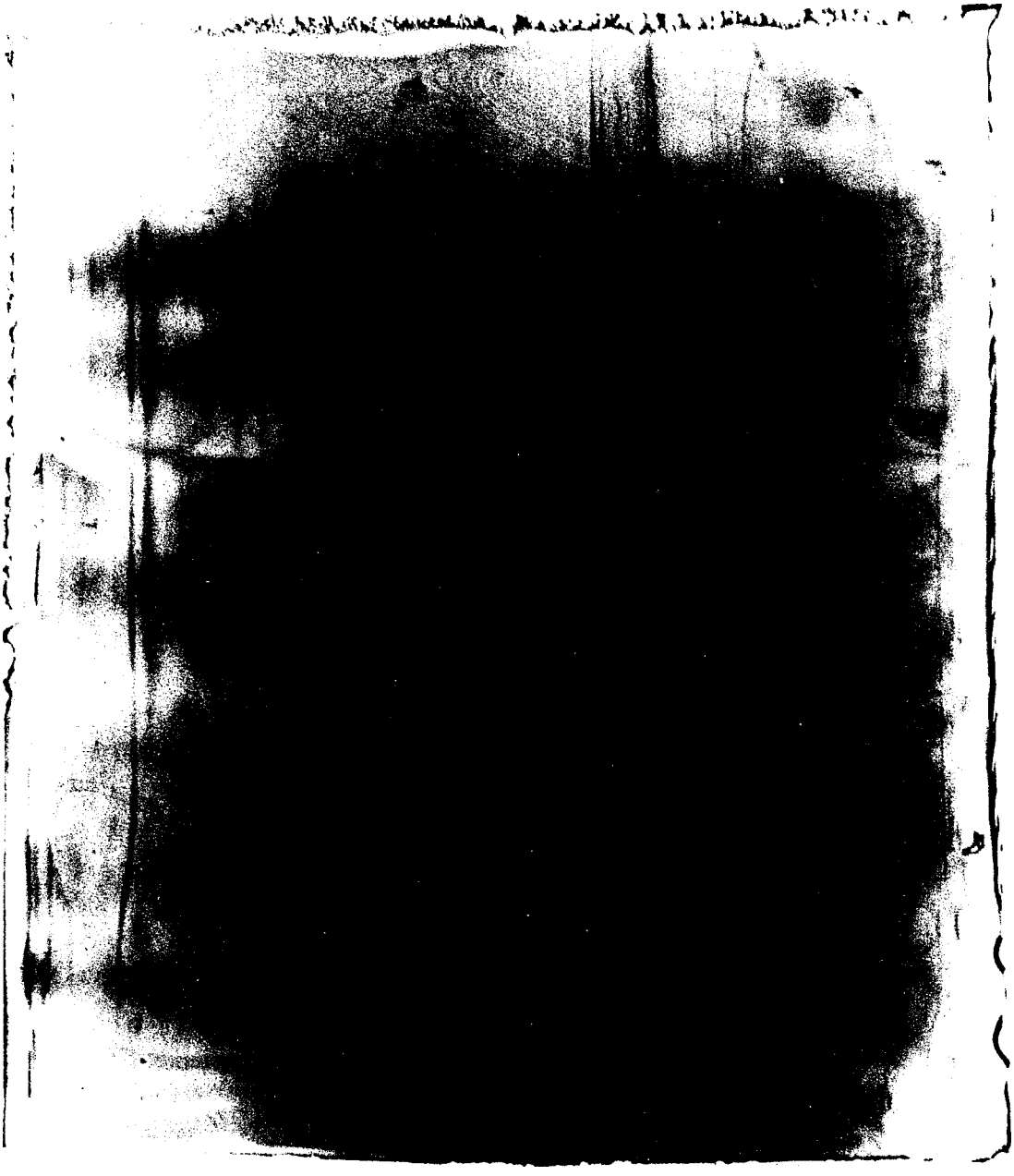
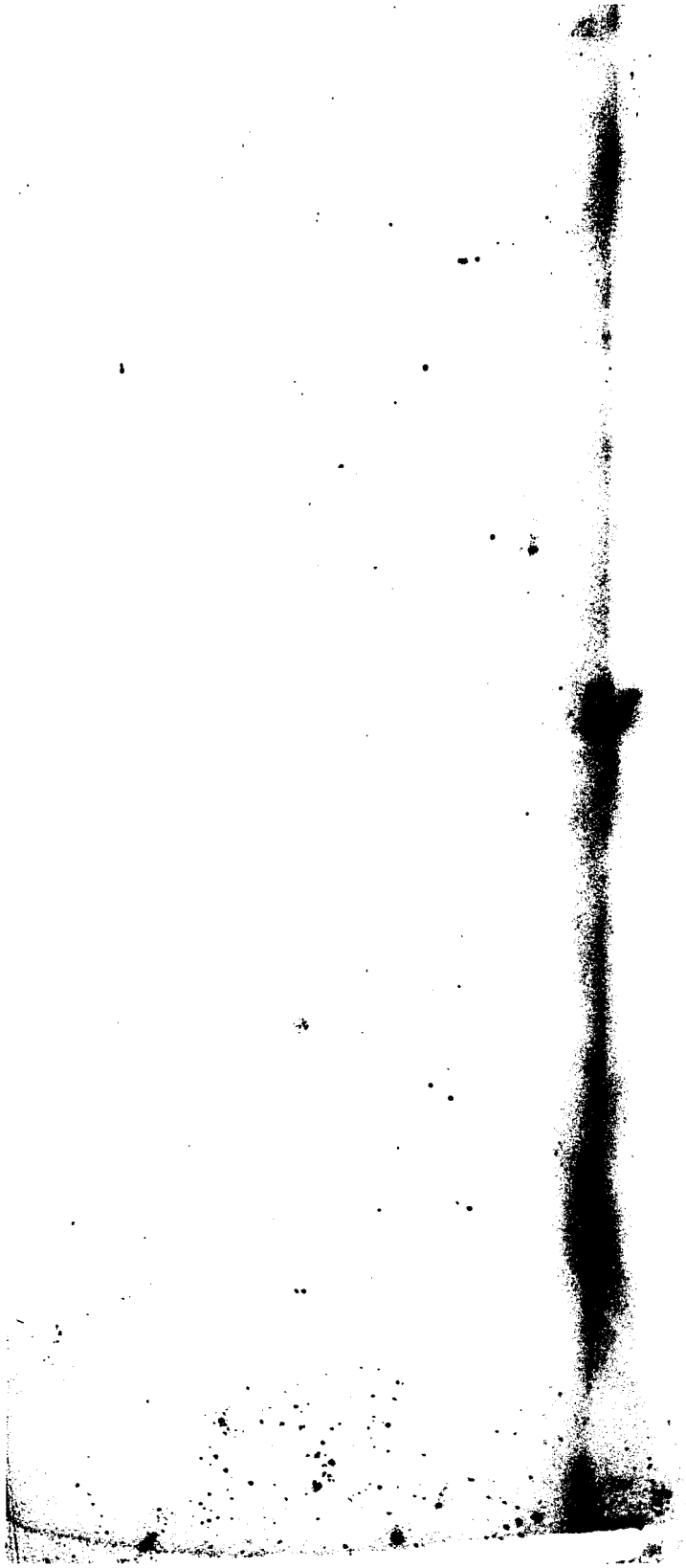


Table 5 - Isozymic difference indices

enzyme	data matrix		Gelfand's similarity		Gelfand's difference	
acid phosphatase						
6.5	1	1	1	1	0	0
6.2	.16	.16	1	1	0	0
5.4	.16	.16	1	1	0	0
phosphogluco-mutase						
4.8	1	1	1	.94	0	.06
5.0	.66	1	.94	1	.06	0
5.5	.86	.86	1	1	0	0
5.6	.71	.71	1	1	0	0
hexokinase						
4.6	.87	.87	1	1	0	0
5.2	.87	.87	1	1	0	0
6.0	1	1				
6.0	.5	.5	1	1	0	0
5.2			1	1	0	0
phosphohexose isomerase						
6.2	.43	.29	1	.97	0	.03
7.8	.43	.29	.97	1	.03	0

Figure 14. Photograph of LKB Immobiline gel that had been eletrofocused for detection of acid phosphatase.



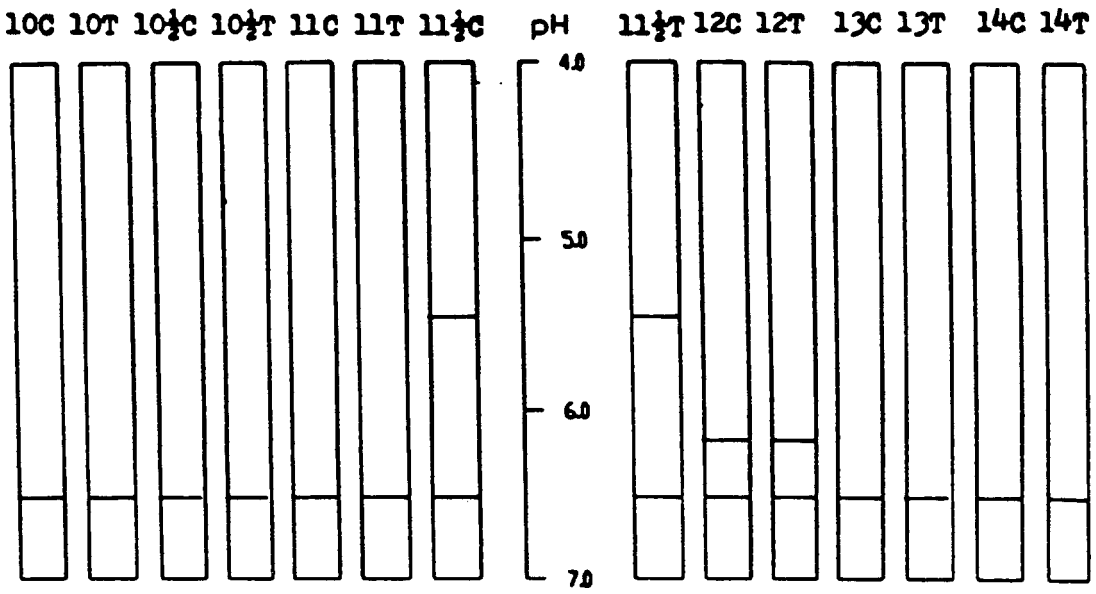


Figure 15. Acid phosphatase isoforms of developing mouse limbs. Horizontal lines represent the location of isoforms.
10C = day 10 control
10T = day 10 treated, etc.

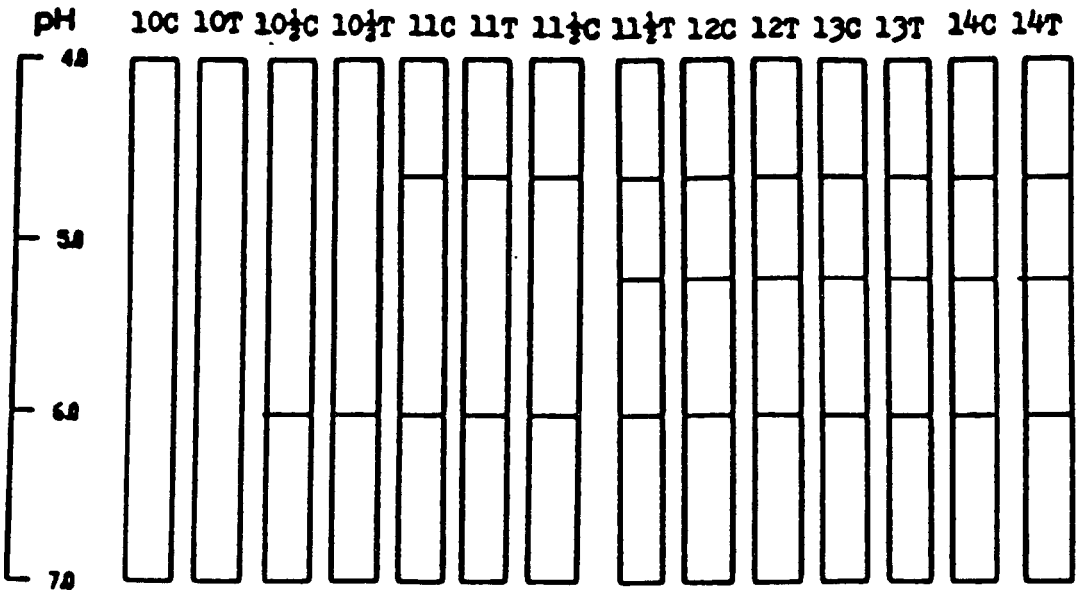


Figure 16. Hexokinase isoforms of developing mouse limbs. Horizontal lines represent the location of isoforms.
10C = day 10 control
10T = day 10 treated, etc.

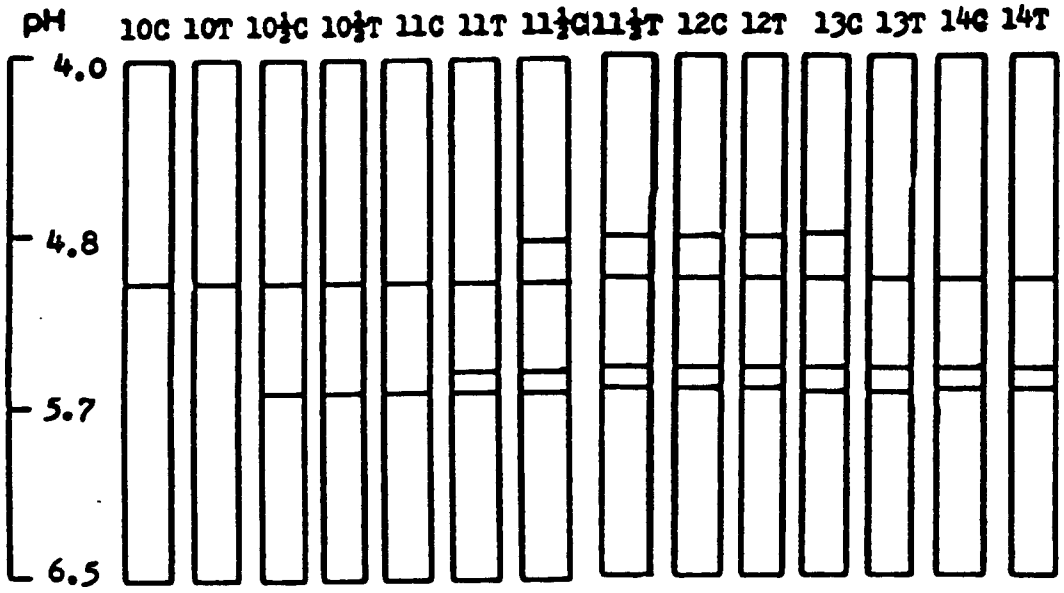


Figure 17. Phosphoglucomutase isoforms of developing mouse limbs. Horizontal lines represent the location of isoforms.
10C = day 10 control
10T = day 10 treated, etc.

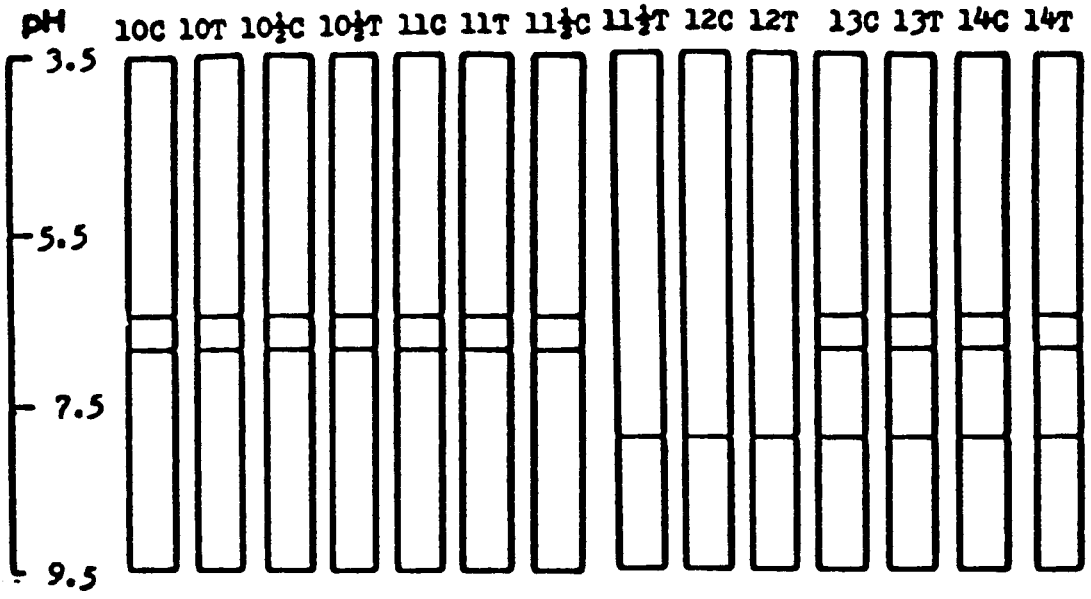


Figure 18. Phosphohexose isomerase isoforms of developing mouse limbs. Horizontal lines represent the location of isoforms.
10C = day 10 control
10T = day 10 treated, etc.

Table 6. Summary of isozyme analysis.

day	10		10.5		11		11.5		12		13		14	
	C	T	C	T	C	T	C	T	C	T	C	T	C	T
acid phosphatase	ND		ND		ND		ND		ND		ND		ND	
hexokinase	ND		ND		ND		−	+	ND		ND		ND	
phosphoglucomutase	ND		ND		−	+	ND		ND		+	−	ND	
phosphohexose isomerase	ND		ND		ND		+	−	ND		ND		ND	

ND = no difference
− = band absent
+ = band present
C = control
T = treated

indicated there were no significant differences between the control and treatment groups for the four enzymes tested.

DISCUSSION

Congenital deformities occur with significant frequency in man and inbred mice. After teratogenic insult, the mechanisms or initial types of changes in developing cells or tissues include gene mutations, chromosomal breaks, nondisjunction, mitotic interference, altered nucleic acid integrity or function, altered energy sources, changed membrane characteristics, osmolar imbalance, enzyme inhibition or lack of normal precursors and substrates (Wilson, '77). Teratogenic agents act in specific ways on developing cells to initiate abnormal embryogenesis by causing such events as excessive cell death, altered cell interactions, reduced biosynthesis, impeded morphogenetic movement and mechanical disruption of tissues. The pathogenesis of a defect may involve one or more types of these abnormal developmental patterns. It appears retinoic acid-induced defects are in general a result of more than one type of pathogenetic event (i.e., excessive cell death and altered cell interactions). The cell death varied from being a very localized area of necrosis in the limb bud to death of the entire embryo. Although proof is lacking, these varied responses are assumed to be due to unrecognized differences in genetic make-up of the embryos.

The 100 mg/kg dose was definitely embryotoxic. About five times more implantation sites were resorbed in the treated group than in the control group (2.5% for the control group versus 13% for the treated group). Only 25% of the control litters exhibited resorption while over 65% of the treated litters exhibited resorption. Therefore the treatment with 100 mg of retinoic acid per kg of body weight on day 10 did increase fetal wastage.

The treatment decreased the crown-rump length on day 12 and day 13. On day 14, there was no significant difference in the crown-rump length. The treated group was larger than the control group on day 18. A range of 0.57 grams to 1.12 grams was reported as being a normal distribution for day 18 fetuses by Rugh ('68). When compared to this range, the control group had 24% of the fetuses above the upper limit of the range while over 63% of the treated fetuses were greater than 1.12 grams. These results might be explained in part by the treated group being skewed as in a platykurtic distribution (Sokal and Rohlf, '81). Another consideration is that the high number of large fetuses could have been related to less competition due to the increase in resorptions.

The safranin/toluidine stain detected signs of cytotoxicity in the central mesodermal cells of the forelimb 12 hours after treatment. This scattered necrosis peaked 24 hours after treatment. After 36 hours post-treatment, most cells appeared healthy except for the cells

located in the fpp. This first episode of necrosis was characterized as being relatively immediate and diffuse in location. Retinoid toxicity has been reported by other investigators. Nakamura et al. ('73) noted retinol caused a remarkable increase in the number of necrotic cells in the central part of the preaxial mesoderm. Also, in experiments using an ICR strain and the same dose of retinoic acid as in this study, retinoic acid was shown to be lethal to prechondrogenic cells located only in the proximal-central core of the limb as early as 4 hours after oral administration (Kochhar & Agnish, '77). Genetic defects (such as oligosyndactyly shown in Fig. 1) are also known to affect necrotic areas as the fpp.

The fpp was present in both the control and treated groups on day 11.5. Analysis with Nile blue of the fpp revealed the treated group possessed significantly more necrosis than the control group. Excessive cell death in areas of physiological cell death has been reported by others to be associated with digital defects induced by retinoic acid in mice (Sulik and Dehart, '88; Alles and Sulik, '89). A similar increase in necrosis was also evident in the subectodermal mesoderm on day 12. The treated group (both the fmI and the fmV) had significantly more dead cells and macrophages than the control group on day 12 (Fig. 6 and Fig. 9). While there was some similarity in the frequency distributions on day 12, the treated fetuses showed a definite tendency to possess more

necroses than the control fetuses. The relative increase of necrotic cells was greater in the treated fmI than the treated fmV. The predominance of reduction defects on the preaxial side and the tissue excess defects on the postaxial side of the forepaw was related to this comparatively asymmetrical necroses. This second episode of necrosis was characterized as being delayed and confined to areas where cell death was already present and physiologically normal. According to Rooze ('83), teratogens may result in either "dead cells appearing diffusely scattered throughout large areas without apparent relationship with the normal necrotic pattern, or they may be concentrated in particular sites where they most frequently contribute to enlarge normal necrotic sites." In this particular situation, both of these conditions were attributable to the retinoic acid treatment.

There has been a recent report contrary to this report of cell death being caused by retinoic acid. Abbott et al. ('90) reported retinoic acid did not cause an increase of cell death. Instead, they found an inhibition of cellular proliferation in the limb bud by measuring the mitotic index. This effect of decreased mitotic index was suggested to contribute to retarded development and malformations of the limb. But, in the same mouse strain as Abbott et al. ('90), using the same isomer (all-trans) and same dose (100 mg/kg), Alles and Sulik ('89) reported a "retinoid-induced cell necrosis in the mesenchymal core of

the limb." Furthermore, Sulik ('89) saw "the patterns of malformations induced by retinoic acid appear to be most consistent with pathogenesis involving excessive cell death in regions of programmed cell death." Also, evidence indicated 13-cis retinoic acid (Sulik and Dehart, '88) results in excessive cell death in regions of programmed cell death and subsequent malformations.

There was not a substantially different amount of cell death detected between the groups with respect to the ectoderm or apical ectodermal ridge (AER). Similar results of no apparent deleterious effects on the AER of retinoic acid treated limbs have been reported by Kochhar ('85). Dead cells were occasionally evident on days 10.5 through 12 but this observation was expected since a few dead cells occurred in the AER long before the AER is at its maximum (Scott, '79). This finding was contrary to the recent report of retinoic acid-induced postaxial polydactyly being linked to cell death in the AER (Sulik and Dehart, '88). This discrepancy could be related to the different time of treatment, strain differences (Biddle, '88; Center, '55), or the use of the isomeric form of retinoic acid. It was noted that the postaxial portion of the ectoderm near the margin of the fifth digit was hyperplastic on day 12 in the treated group. The delayed involution of the AER (Naruse and Kameyama, '82) and the retention of a thickened appearance of the ectoderm (Scott et al., '80) have been observed in cases of preaxial polydactyly.

On day 14, measurement of the bulge size showed the treated bulges had a tendency to be larger. The control group had bulges that were for the most part completely necrotic. The treated group could be classified into those fetuses which were like the controls and those fetuses which had less than 60% necrosis. It was surmised that those fetuses which had a supernumerary digit of significant size on day 14 with less than complete necrosis would possess postaxial polydactyly at term. The size of the bulges on day 14 were variable and continuous over a small range. These parameters were similar to the description of the development of the third molars in mice. Gruneberg ('63) observed the morphogenesis of the third molar tooth bud and suggested a critical size was necessary for that structure to proceed normally in development. If the size was below a certain developmental threshold, cell death ensued and the structure would be absent in the adult.

Yasuda ('75) described a condition of preaxial polydactyly in the hands of human embryos with several remarkable similarities to the postaxial polydactyly encountered in mice. There was an abnormal extension and delayed involution of the ectoderm on the preaxial border of the handplate. He considered the disorder to be a pathogenetic event resulting from an interaction of the limb ectoderm and mesoderm. In another human embryo study by Nishimura et al. ('66), polydactyly was found to be ten

times more prevalent in embryos than the same population at term. Thus, the development and subsequent removal of a polydactylous defect is not limited to murine development.

A persistent problem in teratology is the question of why some embryos respond to the treatment by forming defects and others remain unaffected. An attractive and recurring explanation would lie in the differences due to the sex or gender. One point related to this sexual difference is the fact there exists as much as a 12 hour differential (Theiler, '72) in either direction from the mean gestational age. Also, since males are generally developmentally advanced and retinoic acid acts upon some critical point of differentiating cells (Kochhar and Agnish, '77; Kochhar et al., '84), perhaps the teratogenic insult is related to the difference in developmental maturity. Besides the male gender causing a generally advanced developmental maturity, it is even a more complex situation when considering limb development. For example, while the mean body weight of male embryos was greater than that of female embryos on day 14, "both digit and palatal formation were found to be more advanced in females (Wanatabe and Endo, '89) than in males when compared according to their body weight." This finding is contrary to their finding of digit formation on day 12 where digit development was more advanced in males than in females even when the sexes were compared with a similar body weight. Therefore, female embryos somehow catch up with and pass their male counterparts at later stages of gestation. When

mothers are exposed to teratogens, some sexual difference that is correlated with the incidence of digital defects might be produced, but this theory does not occur with retinoic acid teratogenecity (L. Threlfall, A.M. Cusic - personal communication). Perhaps it is relevant that in experiments using pure and hybrid crosses a significant difference exists in their developmental stage in the different crosses (pure ICR crosses lagged behind the hybrids approximately 8-9 hours). This finding indicates that the differences were due possibly to embryonal genes rather than differences in developmental age (King et al., '86). Significant litter effects also exist (Yasuda and Nakamura, '77). And it has been noted that even noninbred lines of mice may be extremely variable in teratogenic responsiveness (Kalter, '82).

Another salient feature of retinoic acid-induced teratogenecity is the involvement of cellular retinoic acid-binding protein (cRABP). The cRABP is a 14.6 kD protein (Maden et al., '89). Retinoic acid does not react with the genome directly, but rather binds to the cRABP before interacting with DNA. It is interesting to note that the embryonic distribution of cRABP varies with development. For example, in early mouse embryos, the expression of cRABP is mainly in the neural crest and neural crest-derived cells in addition to some neuroepithelial cells (Annerwall et al., '89). They suggested the "specific teratogenic effect caused by exogenous retinoids may thus involve binding to cRABP."

Also relevant to the issue at hand is the temporal and spatial distribution of cRABP in limb development. In chick limbs (Maden et al., '89), levels of cRABP are high while the levels of cellular retinol binding protein are low. Using immunocytochemistry, it was shown cRABP was present at high levels in the progress zone and was distributed across the anteroposterior axis in a gradient with highest amount being at the anterior margin. Though the role of cRABP in the mechanism of retinoic acid teratogenecity remains to be delineated, cRABP has been demonstrated in the mouse forelimb bud at a time of susceptiblity for the production of limb malformations by retinoic acid (Kwarta et al., '85). Tissue levels of cRABP range from 0.2 to 30.4 micrograms per gram wet weith of tissue (Blaner, '90). At these levels, it is not possible to detect changes in cRABP since there were only milligram quantities of tissue available in these experiments.

An alternate proposal that has been put forth is the idea of retinoids being teratogenic when the specific binding proteins (Hixson and Harrison, '81) become saturated and the remainder or excess retinoid causes deleterious effects by cellular membrane perturbations. Schweichel and Merker ('73) described the morphology of three various types cell death in prenatal control tissues. In type I cell death, there was condensation and fragmentation of single cells undergoing phagocytosis, with lysosomal disintegration of the fragments in neighboring cells. In type II cell death, there was a primary

formation of lysosomes in dying cells with activation and subsequent destruction and phagocytosis of the fragments by neighboring cells. This type of cell death was usually found in the destruction of organs and large cell units. In the type III cell death, there was disintegration of cells into fragments without the involvement of lysosomes such as occurred in ossification. After administration of vitamin A, cell death was correlated with labilization of lysosomes and type II necroses. This finding was supported by ultrastrucural studies (Schweichel, '71) showing that vitamin A disturbed the regular cytoplasmic and phagolysosomal liquification of necroses in limb buds (as seen in the interdigitial necrotic zones).

The results of the isozyme data are summarized in Table 6. There were no significant differences found in this study between the control and treatment groups with regards to the isoelectrophoresis of the lysosomal enzyme acid phosphatase. It might be that the method used was not sensitive enough to detect small aberrations of this enzyme. Another possibility was that the amounts of acid phosphatase did not change but membrane labilization caused by the treatment made acid phosphatase (or other enzymes not tested for) more active. It was noteworthy that a similar negative correlation between acid phosphatase and retinoic acid treatment was found in other toxicological studies (Hodges, '77). The analysis of isozymic differences with the computer program showed there were slight differences between the control and treated groups

in relation to the glycolytic enzymes. On day 11.5, with phosphohexose isomerase and hexokinase, the treated group developed a band not present in the control group. On day 11, phosphoglucomutase zymograms indicated the treated group developed a band not present in the control group. There was also the presence of bands in the control but not treated groups in two instances. On day 11.5, phosphohexose isomerase had two isoforms to develop in the control group that were absent in the treated group. On day 13, an isoform of phosphoglucomutase was present in the control group which was absent in the treated group. These differences could be due to the treatment or they might be due to individual variation.

Retinoids are known to exert well documented effects on RNA and protein synthesis (Kistler, '86). The data indicate that retinoic acid influences genomic expression in vivo by activation as well as suppression of the genome. One hour after dosing animals with retinoic acid, a polypetide band of 22,000 Daltons disappears and a band of 55,000 Daltons intensifies (Omori and Chytil, '82). Retinoic acid has been shown to have numerous gene-teratogen interactions in mice. Heterozygotes for the splotch mutation have a white belly spot but are otherwise normal. Maternal treatment with retinoic acid of these heterozygotes causes severe spina bifida like that occurring in splotch homozygotes (Kapron-Bras and Trasler, '88). Retinoic acid was found to alter the phenotype of gene mutation in the mouse including curly tail , splotch

and legless (Wegner et al., '90). Here, the heterozygotes for legless (which usually are normal) turned out to have a severe tail defect and the homozygote legless mutant's malformations had more missing elements when exposed to retinoic acid. Also, retinoic acid has been shown to alter glycoproteins of the cell surface in early development of retinoic acid-induced caudal axial defects (Griffiths and Wiley, '90). Other investigators have reported that treatment with retinoids caused an increase of protein kinase activity without a concomitant increase in adenyl cylase activity or cyclic AMP levels (Kumar et al., '87).

More relevant to limb defects were the finding of "stress proteins" found in mouse embryo limb buds in vivo (Laborde and Young, '90). Target tissues (limbs) showed synthesis of an 88,000 Dalton, a 90,000 Dalton, and two 20-25,000 Dalton proteins in the forelimbs 2.5 hours post-dosing. Non-target tissues (heart and tail) showed no stress protein synthesis. Control embryos lacked stress protein synthesis also. These results showed that teratological anomalies correlate well with the stress proteins in target tissues and suggest that stress proteins may be involved in the teratogenic process. A similar synthesis of the 90,000 Dalton and one 20-25000 Dalton proteins were noted in the treated limbs on day 12 (Fig. 10).

An important advance toward understanding pattern formation in development was made possible by the discovery

of a 180 basepair homeobox that encodes the DNA-binding domain of a multigene family of transcriptional regulators. Studies have demonstrated that most homeobox genes are clustered in the genome, that they are regionally expressed, and that their spatiotemporal expression pattern is developmentally regulated. Similar to the situation of homeobox genes in Drosophila , the expression pattern of homeobox gene products along their anteroposterior axis is reflected by the position of the genes in the cluster. The homeobox XlHbox 1 protein has been noted to occur in a pattern which is the reverse of retinoic acid concentrations (Smith et al., '89). Using an antibody probe, Oliver et al. ('89) recently characterized the expression pattern of a homeobox gene (Hox 5.2) in early mouse, frog and chick limb buds. They found that Hox 5.2 expression is predominantly in the progress zone. It was suggested that its expression could be involved in the regulation of limb outgrowth. Another example of a homeobox gene that is non-uniformly expressed in the limb bud is Hox 7.1. Hox 7.1 is expressed at high levels along the posterior margin, distally in the progress zone and later in the tissue of the interdigital space in those cells destined to die (Hill et al., '89). They noted the initial pattern of certain homeobox gene expressions "may pertain to mesenchymal-ectodermal interactions, but may also be a preparatory stage for later, more focused expression in the interdigitial spaces" (Smith et al., '89).

The conclusions put forth here do not preclude this same malformation being produced in a number of different ways. Retinoic acid has been shown to have many effects on development including disrupting skeletal morphogenesis via hyaluronidase (Kochhar, '85) and being a morphogenetically-active chemical agent in limb bud organogenesis (Eichele and Thaller, '87; Thaller and Eichele, '87). Another point deserving consideration is the time delay between the treatment and the appearance of the defect four days later. Since retinoic acid reaches a maximum concentration in the fetus at 12 hours post-treatment (Kochhar, '76) and is metabolized completely within 48 hours (DeLuca, '79), it might seem tenuous to relate the treatment to a developmental abnormality on day 14. But retinoic acid is known to affect genes both early and later after administration (Chytil and Sherman, '87). "Some of the genes are influenced very soon in a matter of a few hours. Effects on other genes requires considerable time." Perhaps a cascade of events would help explain the development of the phenomenon described here.

This hypothesis concerning primary and seconadary respones to retinoic acid has recently been confirmed. The levels of mRNA's for proteins ERA-1 and Hox 1.3 were shown to increase 30 to 50 fold in a matter of a few hours and as such could be considered a primary response. On the other hand, genes encoding for proteins such as beta-micorglobulin, laminin B1, H-2 and collagen IV were considered to be a secondary response since their mRNA's

levels increased relatively late after treatment, i.e., 24 to 48 hours after the treatment (Gudas, '90).

Several similarities were obvious between the polydactyly caused by a nitrogen mustard mutation (Center, '55; Nishimura and Takagaki, '59) and the polydactyly induced by retinoic acid treatment. Both drugs are cytotoxic and resulted in a postaxial polydactyly. These defects were both first apparent as a squarish bulge on the postaxial boundary of the fifth digital ray on day 14. Interstrain crosses and backcrosses suggested several independently segregating genes were involved in the nitrogen mustard-induced postaxial polydactyly. A proposed mechanism for several drug-induced defects has been an interaction of mutant genes and the teratogen (Dagg, '67).

The idea that "many induced malformations represent an exaggerated incidence of an otherwise infrequently occurring one" has been proposed (Wilson, '64; Landauer, '57). These reports cited examples of strains of animals which without treatment exhibited a low incidence of spontaneous malformations and showed a marked increase in defect frequency when subjected to a teratogenic agent. The ICR mouse strain used in this study fits this description since it had about 5% of day 18 fetuses with spontaneous polydactyly which increased to over 40% with retinoic acid treatment. Though no proof exists, the results support the suggestion that teratogens act on or in conjunction with an existing unstable phenotype.

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Name of Candidate Steve Virgil Bynum

Major Subject Developmental Biology

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Dissertation Committee:

Charles P. Daggs, Chairman

Gary R. Pourier

Jerry N. Thompson

Larry Soots

Lloyd D. Kelly

Director of Graduate Program Daniel W. Jones

Dean, UAB Graduate School Arthur Bonner

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