The Effects of Drugs on Embryonic Development*

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ABSTRACT

A review of the literature demonstrated the difficulties in evaluating the teratogenic effect of drugs in man. Since epidemiologic studies provide suggestive rather than definitive data and results of the current drug testing in laboratory animals may not be applicable to man, the need to develop alternative methods of predicting teratogenicity was apparent. To develop such techniques by studying a possible mechanism of teratogenesis, experiments were performed using a teratogenic folic acid-deficiency and antagonism with 9-methyl pteroylglutamic acid in the pregnant rat. Embryos developing abnormally in response to this regimen consumed oxygen at a significantly decreased rate. Similar significant reductions in oxygen consumption were found both in rat embryos malformed in response to maternal vitamin A acetate administration, and in mouse embryos in response to teratogenic doses of diphenylhydantoin. It was suggested that such measurements of oxidative metabolism or related techniques may have application in predicting drug teratogenicity and could aid present epidemiologic and empiric approaches to identification of human teratogens.

Introduction

Studies show that drugs can be identified as the causative agent in only two to three percent of all congenital malformations in man. However, there is good reason to believe that their actual contribution to altered morphogenesis is considerably more significant. The widespread exposure of pregnant women to potentially teratogenic drugs, on one hand, plus the fact that the etiology of a significant number of malformations has not yet been determined, lends credibility to this hypothesis.

The effect of drugs directly, or in combination with other environmental or genetic factors, may play an important role in the causation of congenital defects. A model relating environmental influences and hereditary susceptibility to disease has been proposed by Carter and Falconer. Their postulate of polygenic or multifactorial inheritance suggests a
threshold of genetic predisposition beyond which there is a risk of malformation upon exposure to yet unidentified environmental agents. Conditions which appear to be inherited in this manner account for at least 50 percent of congenital malformations and are a predominant cause of stillbirth and infant mortality. The importance of the interaction of drugs in causation of these common disorders may be considerable.

Combinations of drugs with other environmental factors, including other drugs, may also be instrumental in causing malformation. Synergistic and potentiative effects of two or more drugs on embryonic maldevelopment are known for experimental animals. Similar multiple drug exposures occur in man and may contribute significantly to causation of the approximately 70 percent of developmental defects which presently have no known etiology.

Shepard has catalogued over 600 drugs and chemicals which can produce congenital anomalies in experimental animals. Although extrapolation of these animal data to man is impossible, the number of drugs involved and the ease with which drug-induced malformations are produced in lower animals should strongly indicate the potential for similar responses in the human.

Numerous epidemiologic studies have indicated that pregnant women are exposed to drugs with sufficient frequency to consider malformations of their offspring as potentially drug-related. Some of these reports even state that commonly used medications such as aspirin, barbiturates, antacids, and nicotinamide cause congenital defects. However, such studies, which only correlate the use of drugs with the occurrence of malformations, do not separate the actual effects of the medications from the effects of the disease for which they were prescribed. Similarly, data which suggest no association between drugs and congenital malformations may also be misleading if the drug exposure occurred only following the period of embryogenesis. Since most teratogenic effects of drugs in man are identified by epidemiologic methods, it is not surprising that only a few drugs have been definitely identified as human teratogens. These drugs are included in table I and will be discussed.

Definite Teratogenic Agents

THALIDOMIDE

Thalidomide serves as an example of a classic human teratogen identified by epidemiologic studies. This tranquilizer, taken during pregnancy, was shown to produce a congenital syndrome of musculoskeletal deformities in several thousand children after three or four years of widespread use. This emphasizes the need for both an active, coordinated surveillance of clinical drug use during pregnancy and better laboratory methods of predicting the human teratogenicity of drugs. Rodents, the laboratory animals used most often in drug testing, are relatively insensitive to this potent human teratogen. Later, Wilson showed that certain simian primates produce malformed offspring following thalidomide exposure.

FOLIC ACID ANTAGONISTS

The use of folic acid antagonists as abortifacients in human pregnancies in

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the early 1950's resulted in a therapeutic abortion rate of only 70 percent. Of the unsuccessful attempts, between 10 and 30 percent of the products of conception which reached term were deformed. The drug most commonly implicated was aminopterin (4-amino pteroylglutamic acid). Methotrexate produces similar results. The folic acid antagonists have been termed universal teratogens in laboratory animals, since almost any organ can be malformed, depending on the time in gestation during which the compound is administered. Similar effects on the human embryo probably account for the wide variety of clinical presentations in reported cases.

**Androgenic steroids and progestins**

Androgens are capable of producing ambiguous or frankly masculinized external genitalia in female infants. Compounds which have been implicated include the 19-nortestosterone derivatives, methyltestosterone and methylandrostenediol. The period of pregnancy in which these compounds are administered influences the degree of masculinization of the genitalia. Labioscrotal fusion results from administration of the hormone to the mother between the 8th and 13th week of gestation. It may be of such a severity that complete fusion with formation of a penile urethra occurs in the female infants. Treatment following the 13th week of pregnancy produces clitoromegaly. Of the infants at risk, probably only 1 or 2 percent will be affected. Although synthetic progestins are not androgens, they possess significant androgenic activity. Their use in the treatment of threatened abortions has resulted in some 600 female babies with virilization similar to that produced by known androgens.

**Suspected Teratogenic Agents**

**ANTICONVULSANT MEDICATIONS**

Groups of drugs which are suspected of being teratogenic agents, but for which conclusive data is lacking, are also listed in table I and include the anticonvulsant medications. Earlier reports in 1968 and 1970 documented the occurrence of 38 cases of cleft lip with or without cleft palate in infants of mothers with convulsive disorders who continued to be treated with anti-seizure drugs during their pregnancies. Later Speidel and Meadow reported a two-fold increase in the expected incidence of major congenital anomalies in women taking anticonvulsant medications. These anomalies included central nervous system malformations, congenital heart disease and cleft lip with or without cleft palate. The most commonly implicated medications were phenobarbital and diphenylhydantoin, taken either singly or in combination, and trimethadione. Further evidence for their teratogenicity derives from the facts that they readily cross the placenta in man and have been shown to be highly teratogenic in mice. Folic acid deficiency may be the mechanism by which these compounds produce congenital anomalies in humans. Women taking anticonvulsant drugs for long durations have subnormal folate levels and pregnancy may further deplete folic acid.

**Sympathomimetics**

The central nervous system sympathomimetics, used in man for appetite suppression, have been demonstrated to have profound teratogenic effects in laboratory animals. Although circumstantial evidence exists that women taking an increased number of these amphetamine-like compounds during pregnancy have produced infants with increased numbers in malformations, this evidence is obviously not conclusive.

**ALKYLATING AGENTS**

The fourth group of suspected teratogenic agents, the alkylating agents, are known to be teratogenic, radiomime-
tic and cytotoxic in experimental animals. Their use in human pregnancies has resulted in intrauterine death and abortion, particularly with higher dosage. However, because of their effect on rapidly dividing and differentiating cells, they must remain suspect as teratogens during pregnancy.

ETHANOL

Ethyl alcohol was generally believed to be of minor significance relative to teratogenic capacity until Jones et al in 1973 and subsequent reports suggested a syndrome of craniofacial, cardiovascular and limb defects associated with chronic maternal alcoholism. The recognition of the association between this common-place, widely-used compound and significant malformations serves to emphasize the hazards of yet unsuspected teratogenic agents.

OTHER DRUGS

Many drugs have been omitted from this discussion since data concerning their teratogenic potential is conflicting and their embryopathic effects remain to be determined. Also, change in the usage of drugs which are not currently associated with human malformations may belie their apparent safety.

Limitations of Surveillance to Identify Human Teratogens

Determination of the teratogenic effect of drugs by surveillance is inefficient and may be inaccurate. The time lag of six to nine months between the use of drugs during pregnancy and the opportunity to observe their effects may make the recognition of teratogenicity difficult. This is indicated by the delay in the recognition of thalidomide as a teratogen, despite conditions which were ideal for the early identification of this compound. These included the facts that the drug was extensively used by pregnant women after it was widely advertised for its safety and effectiveness, and the type of limb abnormality produced by this compound had previously been of such rare occurrence that a particular type of anomaly, tetraphocomelia, had never been described before the introduction of thalidomide.

Despite these favorable epidemiologic circumstances, it took three to four years of use before the cause and effect correlation was noted. It is easy to see how the recognition of teratogenic drugs less frequently used or possessing low teratogenicity, and which might already be causing particular types of defects in a community, would be made more difficult. The recent recognition of the fetal alcohol syndrome emphasizes the point that many teratogenic drugs may have escaped early recognition.

Experimental Method of Identifying Teratogens

Because the information provided by surveys is not immediately available and is suggestive rather than definitive, it is not the best solution to prevention of drug-induced malformations. Studies of the mechanism of teratogenesis, unknown for almost all of the drugs previously discussed, may provide clues to prevention in man. To accomplish this, the laboratory animal must be used, since only under experimental conditions can the variables be adequately controlled and manipulated.

Compounds which are effective as teratogens in man and lower animals are the folic acid antagonists. In the rat, 9-methylpteroylglutamic acid (9-methyl PGA) is a potent teratogen, and has been shown to produce malformations in 95 percent of the littermates. Because most of the litter is involved, the response of the embryonic rat to 9-methyl PGA is a good model to study the mechanism of teratogenesis. This same compound was
described as altering rat embryonic lactate dehydrogenase isozymes, including LDH-5. The latter enzyme is known to catalyze the conversion of aerobic to anaerobic metabolism, and its abnormal persistence made it attractive to postulate that the teratogenic effect of 9-methyl PGA is related to changes in embryonic oxidation metabolism. This prompted a study of cellular respiration as a means of predicting or monitoring teratogenesis. Initial studies in embryos surrounded by their extra-embryonic membranes and in dispersed embryonic cells were equivocal. The purpose of the experimental part of this paper is to present new data which suggest a strong relationship between 9-methyl PGA teratogenesis and alterations in embryonic cellular respiration.

MATERIALS AND METHODS

Virgin Long-Evans female rats were bred with males of the same strain. The presence of sperm in the vaginal smear on the following morning indicated day 0 of pregnancy, and subsequent days of gestation were numbered sequentially. The rats were fed Purina Laboratory Chow supplemented with lettuce twice weekly. Experimental pregnant animals were divided into three groups. The first two groups were exposed to 9-methyl PGA; the third was a comparison group treated with a teratogenic regimen of vitamin A acetate.

Group 1. PGA-deficiency was begun on day 10 by administering 9-methyl PGA by gastric intubation (one mg in one ml of saline) and replacing the above stock diet with a folate-deficient one containing, in addition, 10 mg of 9-methyl PGA per 100 gm of diet. All animals consumed at least 10 gm of this diet per day. This dietary regimen was continued until the pregnant rats were sacrificed by cervical dislocation on days 11, 12 or 13 of gestation. Embryos were immediately obtained after laparotomy and suspended in a phosphate-buffered Ringers solution containing 0.2 percent D-glucose. The embryos were prepared for oxygen consumption determinations following the methods of Spielmann and Lücke.

Day 11—embryos were divested of their yolk sacs and amnions (naked embryos) and used intact at 38° with room air as the atmosphere.

Day 12—embryos were prepared as in the day 11 embryos, but the medium was gassed for five minutes with 100 percent O2 and cellular respiration was measured using oxygen as the atmosphere.

Day 13—embryos were dispersed by a single passage of an embryo through a 1 ml Mohr pipet (Corex No. 7085-A), since naked embryos at this age are too thick for reliable measurement of respiration even in 100 percent O2 at 38°. Substitution of 100 percent O2 instead of room air as the atmosphere failed to increase the respiratory rate of dispersed cells (12.0 ± 0.6 vs 12.6 ± 0.9 µl O2 per hr per mg protein). Thus, dispersion of the embryos facilitated oxygen diffusion maximally and permitted the use of room air at 38° with the Day 13-embryos.

Group 2. Experimental embryos were obtained, as in Group 1, on day 12 of gestation, but the naked embryos were dispersed by passing two of them at a time through a 0.05 ml Mohr pipet. Room air was used and the incubation temperature was 38°.

Group 3. Vitamin A acetate in daily dose of 60,000 international units was instilled by gastric intubation on days 10 through 12 of pregnancy in the experimental rats. On day 13, dispersed cells were obtained as in Group 1 Day 13-embryos.

Control embryos were prepared either intact or dispersed, depending on their experimental counterparts. Oxygen uptake was measured by the Warburg direct technique. The Lowry method for pro-
tein determination was used.\textsuperscript{18} Average respiratory rates were expressed plus or minus their standard errors in $\mu l$ $O_2$ per hr per mg of embryonic protein ($Q_{O_2}$) and compared for significant differences using the Student's t test or its modification if the variances were unequal.\textsuperscript{6}

**DILANTIN EXPERIMENTS**

In a separate series of experiments, primagravid Swiss albino mice were injected intraperitoneally on gestational day 9 with 75 mg of diphenylhydantoin per kg body weight, a teratogenic dose.\textsuperscript{11} Oxygen consumption of embryonic cells (dispersed by a single passage through a 1 ml Mohr pipet) on day 11 was determined by the direct Warburg technique and compared to respiration of normal control embryonic cells.

**RESULTS**

**Experimental Group 1:** PGA-deficiency beginning on day 10 of gestation. In figure 1 are compared the experimental with the control $Q_{O_2}$'s on days 11, 12 and 13 of gestation.

*Day 11—*After only one 24-hour period of PGA-deficiency-antagonism, the mean $Q_{O_2}$'s were not significantly different: experimental versus control $= 10.1 \pm 0.7$, $10.4 \pm 1.1 \mu l$ $O_2$ per hr per mg protein.

*Day 12—*After two days of treatment with the teratogenic regimen, the experimental and control mean $A_{O_2}$'s were $18.5 \pm 1.0$ and $18.8 \pm 1.0 \mu l$ $O_2$ per hr per mg protein, respectively, and have no statistical difference.

*Day 13—*After the full 36-hour teratogenic time course of the experiment, the embryos from maternal rats which were treated with 9-methyl PGA had a statistically significant ($P < 0.01$) decrease in their oxygen consumption when compared to controls. ($Q_{O_2} = 12.4 \pm 0.6$ vs $17.6 \pm 0.5 \mu l$ $O_2$ per hr per mg protein).

**Experimental Group 2:** PGA-deficiency beginning on day 10 of gestation; dispersed day 12-embryos.

Day 12-embryos in experimental Group 2 and their controls were dispersed to determine if this procedure itself might account for the observed changes in cellular respiration of experimental day 13-embryos. The $Q_{O_2}$'s of the experimental and control Group 2 embryonic dispersed cells did not differ significantly ($13.8 \pm 0.6$ vs $15.6 \pm 0.7 \mu l$ $O_2$ per hr per mg protein, $n = 26$ and 25 respectively).

**Experimental Group 3:** An entirely different teratogenic regimen using hyper-vitaminosis A instead of folic acid.

![Figure 1. Mean oxygen consumptions on Days 11, 12 and 13 of gestation for normal control rat embryos and embryos from maternal animals PGA-deficient beginning on Day 10. The number of individual determinations is indicated to the left of the standard error brackets and significant differences between experimental and control $Q_{O_2}$'s are noted by a $P$ value limit.](image-url)
deficiency on days 10, 11, and 12 of pregnancy.

The vitamin A acetate procedure used was identical to that described by Kochhar and Johnson and shown by them to result in high percentages of malformed offspring at term. The present authors studied dispersed embryonic cells on day 13 in this regimen and again found significantly decreased experimental $Q_{O_2}$s (15.8 ± 0.7 and 17.6 ± 0.5 μl O₂ per hr per mg protein), which differ at greater than the 95 percent level (figure 2).

**Dilantin Experiments**

The teratogenic dose of diphenylhydantoin used in these experiments was 75 mg drug per kg body weight. When pregnant Swiss-Webster mice were thus injected intraperitoneally on day 9 of gestation, they developed ataxia for 24 hours, but appeared recovered neurologically by the day of the experiments (day 11). Higher doses (i.e., 150 mg Dilantin per kg) resulted in uniform maternal death by day 11 and prevented use of these embryos. The average $Q_{O_2}$ in experimental embryos was decreased and differed from controls at greater than the 99 percent level of significance (experimental vs control $Q_{O_2} = 5.03 ± 0.45$ vs $10.7 ± 1.0$).

**Discussion**

Since Day 13-embryos were dispersed to facilitate maximum oxygen diffusion and uniform cellular respiration, the dispersion technique itself was investigated. Day 12 Group 2 experimental and control embryos were dispersed similarly to the day 13 animals, rather than being used intact as in Group 1 Day 12. The $Q_{O_2}$s of experimental and control Day 12-embryos remained statistically identical despite the dispersion procedure. Thus, it is unlikely that similar handling of Day 13 embryos contributes to the experimentally decreased embryonic $Q_{O_2}$s noted.

The findings of statistically similar $Q_{O_2}$ values for folate-antagonized and control embryos compared on gestational Days 11 and 12, and the significantly decreased $Q_{O_2}$'s for experimental embryos on day 13, correlate well with the morphologic findings. Far fewer malformations and resorptions occur if less than the full 72-hour period of PGA-deficiency and antagonism is used. The significant decrease in experimental respiration occurred only after the entire 3-day 9-methyl PGA treatment.

A possible and plausible mechanism for 9-methyl PGA teratogenesis is depression of cellular respiration, as shown in the present report, and a resultant decrease in the available energy which is necessary for proper differentiation. Depressed oxygen consumption on Day 13 in the 9-methyl PGA-treated group is consistent with the decreased adenosine di- and triphosphate (ADP and ATP) levels found by Chepenik et al using the same folate-antagonist regimen. These findings suggest that coupling of oxidative phosphorylation was not affected in the 9-methyl PGA-antagonized system. Other studies have demonstrated coupling of oxidation to phosphorylation using succinate as the substrate and by adding dinitrophenol in the same experimental model. Depressed cellular respiration in
an apparently coupled system suggests a slowing of the citric acid cycle. One of the reactions in this cycle important in controlling its rate is the synthesis of citrate from oxaloacetate. The latter is formed from malate via the enzyme malate dehydrogenase (MDH). Johnson and Spinuzzi have reported deletions in MDH isozymes in embryonic tissues from 9-methyl PGA-treated rats. Such deletions may limit the formation of oxaloacetate and, therefore, citrate, thus slowing the citric acid cycle.

The decreased cellular respiration during 9-methyl PGA teratogenesis may be a secondary phenomenon. If the teratogen were to interfere with the reaction of adenosine triphosphate (ATP) and adenosine monophosphate (AMP) by possibly affecting the enzyme adenylate kinase, less product, adenosine diphosphate (ADP), would be formed. Since the most important factor in determining the rate of oxidative phosphorylation is the level of ADP, the rate of oxygen consumption would diminish as ADP content decreased.

The reduced oxidative rate noted during 9-methyl PGA teratogenesis is common to more than just this compound. A similar significant reduction in the experimental QO2 values was noted when a teratogenic regimen of vitamin A acetate was used in rats from days 10 through 12 of pregnancy. Likewise, our preliminary results indicate that when Swiss-Webster mice are treated with a teratogenic dose of diphenylhydantoin, embryonic cellular respiration is decreased. Kaplan and Johnson have demonstrated altered QO2 values in chicken embryos treated with teratogenic doses of trypan blue. It is possible that alterations in oxidative metabolism are more widespread responses to chemical teratogens than those already reported.

These data support either a cause-and-effect relationship or predictive association between altered oxidative metabolism and induction of congenital malformations.

There is at the present time no simple method of predicting which drug, increasing in number yearly, may induce defects either in the human embryo or in the animal model. The experiments concerning alterations in oxidative metabolism and studies of the generation of high energy compounds such as ATP by developing embryos may provide a solution to this problem. Measurements of oxygen consumption or embryonic ATP may well be simple but useful tools in predicting teratogenicity. Variability in teratogenic sensitivity among species might be eliminated and the results made pertinent to man by testing the effects of these compounds directly on cultured human embryonic tissue. If confirmed, the effect of drugs on the oxygen consumption of embryonic cells in tissue culture would prove simpler than the conventional studies in assessing the potential of drug-induced embryopathy. Should altered oxidative metabolism prove to be a common response to most chemical teratogens, such studies would be a powerful aid to the present epidemiologic surveys in identifying related malformations. Until that time, an organized system of responsible reporting of clinical observations must be established to avert another disaster such as thalidomide and to identify less potent human teratogens.

Acknowledgments

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References

Abstracts

Abstract continued from page 364.

Adjuvant Chemotherapy in Breast Cancer. ENRIQUE VÉLEZ-GARCÍA, M.D. ANTONIO GRILLO-LÓPEZ, M.D. JOSÉ J. CERCINO, M.D. AND LUIS J. SUAU, M.D. (Medical Sciences Campus, University of Puerto Rico, San Juan, PR)

Most patients with breast cancer eventually die of disseminated disease. High risk groups have been identified and include patients with regional adenopathy in the ipsilateral axilla at the time of mastectomy. The recurrence rates in patients with >3 positive nodes approximates 80 percent at 10 years and survival in these patients has not been improved with methods of therapy used heretofore. Recent kinetic data suggested that foci of microscopic disease might be eliminated by the early use of chemotherapy after elimination of the bulk of the tumor by surgery. These experiments led to the current surge of interest in adjuvant chemotherapy of many neoplastic diseases. Twenty-six consecutive patients with breast cancer and more than 3 + nodes in the axilla were carefully evaluated to rule out the presence of occult foci of metastatic disease. They were given, as soon as possible after mastectomy, the following cyclic chemotherapy combination every month: cyclophosphamide, 400 mg per M² IV, Fluorouracil, 400 mg per M² IV, methotrexate, 25 mg per M² IV, vincristine, 1.4 mg per M² (maximum = 2 mg) IV on days 1 and 8 of each cycle and prednisone, 40 mg per M² PO from days 1 to 8 of each cycle. Treatments were repeated monthly x 12 months or until disease recurred or patient intolerance made treatment impossible. In addition, 16/26 patients received concommitant radiotherapy to the chest wall and internal mammary area. Significant nausea and Vomiting occurred in 8/26 patients. Schedule adjustments were necessary in 21/26 patients because of granulocytopenia, thrombocytopenia, severe paresthesias and/or gastric intolerance. In general, the regimen was well tolerated, even when accompanied by radiotherapy. Ten of 26 patients have completed a full year of therapy without evidence of recurrent disease. Median follow up time from chemotherapy is 8 + months and so far, only one recurrence has been documented. The clinical implications of this mode of therapy will be analyzed after presentation of the data.