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SCREENING FOR TERATOGENIC HAZARDS: NATURE OF THE PROBLEMS

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INTRODUCTION

During the course of in utero life, a large variety of developmental events must occur. Some of these continue postnatally but they are most obvious during embryogenesis when they are proceeding rapidly throughout the body as an orchestrated series of ontogenic sequences. During the passage from zygote to embryo to fetus these events are highly vulnerable and most obviously subject to perturbation. Disruption of any of the myriad events or sequences may precipitate maldevelopment evident as either structural or functional deficits postnatally.

A rapid screening system for adverse effects on development must encompass not just one or two of these events (e.g. cell migration or induction) but as much of the entire spectrum as possible because it is not yet possible to explain the mechanism or triggering event for any teratogenic outcome. In fact, one cannot even be confident that the known and major biochemical action of a substance is the actual precipitator of a particular maldevelopment. Last but not least, a screening system must be able to determine whether developmental events are uniquely vulnerable to a substance (a developmental hazard) or are perturbed only near the dose levels also toxic to the adult (not a marked developmental hazard). Failure to achieve this separation of dose responses inexpensively and as adequately as standard experimental protocols in pregnant rodents is the reason no pragmatic screening system has been developed to date.

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This review explores an avenue that may overcome these obstacles by noting the fundamental developmental problems even though we still do not know the basic mechanisms of abnormal developmental biology.

GOALS

A teratology screening system would detect agents hazardous to the conceptus before they can perturb embryonic development in humans. The presently available alternatives are elaborate studies in rodents or surveillance of human epidemiology. The former is too expensive for evaluation of significant numbers of substances. The two most noted examples of retrospective detection of developmental hazards by surveillance are thalidomide and rubella. Each was identified as a teratogen, at least in part, because (*a*) each gave a high incidence of abnormal offspring from exposed pregnancies and (*b*) the types of malformations seemed to constitute a pattern or syndrome. Such a pair of factors is not to be expected routinely. Unfortunately, surveillance systems are after-the-fact protection, and while they may identify a hazard from which future pregnancies need isolation, they do not help those already afflicted. Experts in the statistics of epidemiology alert us to additional handicaps of epidemiologic surveillance systems. An agent needs to produce a very high incidence of stereotyped or uniform malformations if it is to be recognized against the background incidence of congenitally malformed newborn. It is highly unlikely that retrospective study of human deliveries would detect a 5% increase in nonspecific malformations as attributable to any one factor.

The thalidomide tragedy showed that the developing embryo is uniquely vulnerable to the effects of some substances in the absence of adult toxicity. This occasional differential vulnerability had been known by alert experimental teratologists for many years. Most of their studies were designed to provide an understanding of the basic mechanisms of teratogenesis, which are now considered basic principles of abnormal developmental biology. Such studies generally require a high incidence of abnormal young among the offspring of treated dams and tend not to acknowledge that dams become ill and may even die as a result of the high doses needed to generate the requisitie incidence of abnormal young. Thus, aspirin is classified as a weak teratogen, which really means that aspirin is a coeffective teratogen. It produces abnormal development but only at a dose very near to the dose also toxic or lethal to the adult. Thalidomide was an exception in that it is a non-coaffective teratogen; that is, it disrupts embryogenesis at dose levels far below those toxic, much less lethal, to the adult organism. This type of differential vulnerability to many agents has now been established clearly by studies in laboratory animals by standard protocols which in themselves

constitute a screening system of sorts. In them, a test substance is administered to pregnant animals by specific protocols and one can thereby determine the teratogenic potential of the material by examining the offspring for congenital malformations. Such studies are widely applied in the pharmaceutical industry as a prelude to clearance by federal regulatory agencies and eventual clinical trials and general usage.

The Food and Drug Administration requires that drugs and food additives be preclinically evaluated for safety. Similar requirements exist in other developed nations, varying somewhat in detail from country to country. Such safety evaluations will be even more strikingly evident when the full impact of the Toxic Substances Control Act (TOSCA) becomes operative in the near future.

The guidelines for testing under TOSCA will consist of evaluations in several areas of toxicity. Each of these will consist of a tier system composed of increasingly costly and time-consuming studies as one moves up a particular sequence and gains increasing confidence in the data base supportive of an administrative decision. Tiers are essential elements for implementing the EPA mandate. The lowest level of a tier ideally consists of a single or small group of comparatively inexpensive tests to reveal the most flagrant of the bad actors (substances having adverse effects). This basic screen should give few, if any, false negatives, i.e. indicate that a chemical is not a hazard when it actually is. A chemical being evaluated would not necessarily be examined in higher level tests if no adverse effects are evident in the initial screen for that particular toxic effect. If the initial screen were such that it gives a few false positives, it would still be of value in a tier system of safety evaluations. A substance initially indicated as a bad actor by the initial screen could clear its reputation by a higher level in the tier indicating that the evaluation by the screen was indeed a false positive. The systems of tiers being assembled by the EPA are a realistic and pragmatic approach to the problem of evaluating and selectively regulating huge numbers of environmental chemicals.

From 50,000–70,000 different chemicals are already in the marketplace and some 200–400 new ones are produced each year. The former will be regulated under one section of TOSCA and the latter under another. The tier system approach is an attempt to protect the population from adverse effects by permitting a large number of substances to be tested while keeping costs low enough to not hinder continued research and development by chemical and industrial manufacturers. Proper operation of a tier system requires that the lower levels of screening do not require an excess of highly trained personnel and specialized facilities. This will make possible safety evaluation of the backlog within fewer years and allow for keeping pace with new products as they are generated. The lower levels of well-validated

tier systems have the additional advantage of actually fostering innovation by industry in exploration of new syntheses by allowing rapid identification of substances with low hazard potential.

The only tier system that lacks any kind of basic initial screen is that for teratology. No rapid and inexpensive screening system of teratogenic potential has yet been published indicating documented capability of identifying substances to which developing systems have a heightened vulnerability. This means that a manufacturer can establish that a particular chemical does not adversely affect embryonic development only by intermediate or higher levels of the tier system for teratogenesis and embryo-lethal effects. There are not enough trained people available to serve the forthcoming needs by these rather expensive means, and therefore protection of the developing human will lag far behind safety evaluations of effects produced in adults. That is, substances to which the embryo is uniquely vulnerable will persist in the environment for a long time.

Both industry and the public need a validated screen for detection of developmental hazards. Of what should such a screen consist? That is a difficult question and one on which marked differences of opinion may exist. Most would agree, however, that it must be inexpensive, rapid, and not require a high level of training. Similarly, a screen of teratogenic potential also has to detect embryo-lethal effects as well (1).

THE BASIC PROBLEM

One basic factor that has not been adequately discussed in the published literature is that a developmental test applicable as a screen of environmental agents *must differentially detect agents to which the conceptus is uniquely susceptible.* Dave Karnofsky (2) first stated the concept now established as Karnofsky's law: "Virtually" any substance is capable of adversely affecting the conceptus if given at a high enough dose level. This is an important factor because the situation will be this: If chemical substance X were being evaluated, a dose level will be found at which embryonic development is adversely affected. This level proves to be 250 mg/kg in the rat. Substance Y affects embryonic development at 14 mg/kg in the rat. Do either or both substances require regulation on the basis of being a marked developmental hazard? The only way to answer the question will be to know the answer to another: What dose causes adverse effects in the adult. The low adult lethal dose in the rat is 420 mg/kg in the case of compound X, and the LD_{50} is 650 mg/kg in the case of compound Y. The question can now be answered correctly as "no" for X and "yes" for Y. Note that the logs of the adult effective dose divided by the embryo effective doses are 0.22 and 1.67 for X and Y respectively. X is aspirin and Y is nonacetylated isoniazid.

When one calculates how close the adult effective dose is to the dose adversely affecting the embryo, X proves to be a coeffective teratogen and Y is noncoeffective. If they were environmental agents, X would need regulation only on the basis of adult toxicity (plus a modest safety factor) while Y would have to be regulated to a lower level because the embryo has markedly greater sensitivity to the agent than does either the male or female adult.

Remembering Karnofsky's law that almost anything is a teratogen at some dose level, we can conclude that a chemical substance would need to be regulated as a developmental hazard only if the embryo is uniquely susceptible to the agent. Agents that are coeffective teratogens (adversely affecting the embryo but only at a dose level near that adversely affecting the adult) would not necessarily be regulated as a developmental hazard. The regulatory level would be established on the basis of its toxicity to the adult. Inclusion of a modest safety factor would provide protection to the conceptus from a coeffective teratogen. Only noncoeffective teratogens (those adversely affectin
needed to adversely affect the adult) would require regulation markedly below the adult toxic dose.

The problem is that several hundred rats (the majority of them pregnant), about 3 months of time, and over $50,000 are required to make the determination of each log difference for a previously untested chemical.

DEVELOPMENTAL EVENTS

Let's ask the question again: Of what should a developmental hazards screen consist? This time though we need not be preoccupied with the problem of whether or not an agent can disrupt development. We already have established that almost anything will disrupt development in one way or another; for example, if the maternal trachea is tied off, development of her concepti will surely be disrupted. Tongue-in-cheek, I will let this portray the ultimate in coteratogenesis, for the mother will be dead before the fetuses. A fuller knowledge of teratogenic causation or mechanisms may provide the only shortcut to the task for evaluating the teratogenic potential of environmental chemicals by a screening system. However, there may be an avenue available which we have not yet considered. Instead of waiting for a fuller understanding of teratogenic mechanisms we might be well advised to examine more carefully what is known of normal developmental biology. One could pose the question: What must the embryo accomplish in order to become a fetus? Early developmental events are a self-regulatory system; that is, cells and restricted areas of tissue develop according to their

location and previous experience. Early transplantation of a primordial organ to a different location on the embryo will result in that tissue not developing as it would have if left in its original site. Instead it will differentiate into a normal tissue consistent with the new tissue site into which it has been grafted. Self-regulation can be demonstrated by a different experiment also. Removal of an area of tissue from an early embryo will be overcome and replaced by new cells, and development will quickly return to a perfectly normal pattern and the fetus will show no sign of the damage inflicted on its embryo.

Central to development are phenomena such as induction, organ field formation, and maintenance of developmental potencies. The mammalian embryo, for example, has a large area on the side of its future head representing the lens field. Only the central part will actually become the crystalline lens of the eye. If this central area is removed early enough, the surrounding epithelial cells will increase their mitotic activity and migrate in to repair the wound and proceed to form a normal lens. Place future cutaneous ectoderm in the center of the area and it will not grow hair follicles; it will become the lens. It is as if the surrounding tissue gave directional inclination for differentiation. Formation of such organic fields is one of the things an embryo has to achieve to become a fetus or neonate. Not only must the organ field be developed, it must also be maintained. Maintenance of potency or ability to differentiate into lens must be maintained long enough for the neural optic primordiam to grow into juxtaposition just below the surface. The outgrowing optic bulb is then able to induce lens formation by the surface ectoderm. Once the lens begins invagination, it in turn will assist the neural component in its own normal development. This is but one example and one can as readily examine similar events in other organs such as kidney induction and tubule formation, and branching of the respiratory tree or limb bud outgrowth where pattern formation and polarity phenomena become so obvious.

Organ field formation then is essential to normal embryogenesis. A second essential illustrated in our example is maintenance of potency and the third is induction rapidly followed by a fourth, response (formation of lens), and even an indication of a fifth, reciprocal maintenance.

Other events that must take place include cell migration (e.g. neural crest), cell outgrowth (e.g. neurons maintaining continued limb bud expansion), and cellular morphogenesis (e.g. transition of squamous surface epithelial cells into the tremendously elongated lens fibers of the crystalline lens). Table 1 is a simplified listing of some developmental events characteristic of embryogenesis. Teratogenesis may result from disruption of any one or any combination of two or more. These events must occur throughout the embryo to form the various tissues and organs at the proper moment, continue for the appropriate duration, and be appropriate both in quality

and quantity. This increases the scope of necessary events fourfold and the elaborate interactions expand the list of interrelated specific developmental events beyond comprehension. It should be noted that this list encompasses all the developmental events considered vulnerable to perturbation resulting in frank congenital abnormality (3) and constitutes the table of contents of any good textbook or course in developmental biology.

Which of the myriad events might a potential teratogen alter? Perhaps it is unnecessary to deal with the specifics in order to detect substances uniquely hazardous to developmental events. In fact, it might be wiser if we do not, because deciding ahead of time just what a mechanism of action may be for a compound achieving teratogenesis does not work. Indeed, there are many examples illustrating that the textbook description of mode of action may not be the actually teratogenic trip wire. Cleft palate is an easily recognized endpoint of frank congenital defect and is produced experimentally by diverse agents. In spite of the numerous published reports dealing with this single abnormality, we still do not know its pathogenic etiology. Many developmental events, when altered, have been invoked as causative and these include hypoplastic mandibular rami either primary as a result of disrupted formation and function of Meckel's cartilage or secondarily as a result of hypoplastic osteogenesis from the surrounding mesenchyme as it differentiates. Faulty displacement of tongue due to retarded muscle development or innervation sequence perhaps associated with variations in head width or flexion of the sphenoid bone represent another group of possible causative factors. Some investigators have attempted to explain the defect on the basis of reduced mitotic activity in restricted or localized areas, alteration of turgor or of contractile elements resulting in retarded elevation of the vertically positioned shelves. Factors mediating the necessary programmed death of medial edge epithelial cells may be another causative or associated event used to explain cleft palate formation. There are other possibilities but it is evident that any one could possibly be central in the mode of action of a particular agent in causing this defect.

Table 1 Developmental events required of a zygote in becoming an embryo and then a fetus

Formation of organ fields	Cell migration
Maintenance of organ fields	Formation of cell junctions and selective associations
Development and maintenance of development potency	Intracellular communication
Production of cell-specific organelles and products	Cellular morphogenesis
Induction	Programmed (and induced) cell death
Response to induction	Programmed (and induced) cell division
Regulation of polarity	Formation of intercellular material
Regulation of proportionality	Tissue organization
	Organ formation and function

PREVIOUS ATTEMPTS

The most basic road to follow in attempting to develop a screening system capable of detecting substances having marked developmental hazard potential would be at the level of gene function or cellular expression of differential gene expression. The former does not appear possible at this time and observation of ontogenic sequences of gene product resolution is too difficult an assay; furthermore, one cannot resolve significant perturbances from those which may be teratologically inconsequential (4).

Zone electrophoresis reveals many specific gene products, and their ontogenic sequence can be examined in developing organs and tissues. The molecular sequences are fascinating and when perturbed are at least associated (5) with the pathogenesis of specific structural abnormalities in selected organs. Altered patterns of isoenzyme and multiple molecular forms of enzymes may indicate abnormal developmental events but they are not amenable to screening for developmental hazard potentials. One still needs treated pregnant animals followed by sequential autopsies and expensive analysis of very early embryos and a moderate level of technical expertise.

One can attempt to eliminate the problem of using pregnant rodents by using a single-celled plant such as *Acetabularia,* which can be grafted with a treated portion of a second plant and thereby allow assessment of adverse effects on translational events resulting in resolution of soluble protein patterns. This probably could be developed for some types of agents capable of functioning at this level but unfortunately the pattern of soluble protein is much too complex for facile evaluation. Resolution of just ongoing synthesis of individual proteins by autoradiography is again a step in the direction of complexity and contrary to the desired goal (6). Similar problems have been encountered by investigators using insects as a screening system (7).

Unscheduled DNA synthesis cannot be used, for all teratogens are not mutagens just as surely as all mutagens are not teratogens. We know the former situation is accurate and can surmise that the latter is, because mutation, after all, is central to evolution. It would further appear that factors such as cell function and production of either essential or so-called luxury materials (8) are too easily perturbed and not necessarily directly equated with teratogenesis.

By far the largest number of reported attempts at developing a screen for developmental hazards has centered on in vitro techniques using mammalian embryos. Two general types of systems have been reported. Culture of whole rodent embryos during the initial periods (9) of morphogenesis entails at least two major difficulties. First, the techniques require both pregnant rodents and considerable expertise and, second, even the untreated

embryos develop abnormally and must be considered as hypoxic immediately upon removal from the uterine circulation (10). This latter problem is not avoided by taking advantage of the secondary or yolk sac circulation (11) characteristic of rodent embryos. The second type of system is culture of explanted organs or tissues. This also requires pregnant animals and significant expertise but at least multiple assays can be performed from the contents of one gravid rodent uterus. The real problem encountered is that the range of developmental events achieved is severely restricted. Whereas some manifest several developmental phenomena (12), others may be restricted to one or, at best, very few developmental events (13, 14). The avian embryo has been explored carefully (15) but does not appear to offer a solution because of technical problems associated with it as well as the absence of readily available data concerning significant dose levels.

A TENTATIVE SOLUTION

Two examples (aspirin and nonacetylated isoniazid) were used previously to illustrate that the data of adult acute toxic and teratogenic doses as currently gathered from rodents can be formulated into an index of developmental hazard. Each index number costs about $50,000 to generate; hence, a system to generate the calculation at significantly lower cost is needed.

A recent report (16) used the fresh water coelenterate *Hydra attenuata* which is readily grown in the laboratory (17). The genus is a favorite of developmental biologists; more than 2000 papers have been published since 1744 using various members as subjects. There are many species of *Hydra* but *H. attenuata* appeared to be most amenable to the studies as it is not complicated by associated organisms (i.e. algae). A semiautomated system for their growth was obtained which produced large numbers of adult *attenuata* as a uniform population of large animals with minimal use of time. They were collected and dissociated into their component cells (18) which were then randomly packed by centrifugation and expelled into culture medium as pellets. Within 7 days the randomly associated cells of a pellet normally develop into multiple, attached *attenuata* which separate from one another and become freestanding adult animals.

Studies of regeneration have shown that in order for new adults to be formed, changes in cell size and shape (19, 20), and selective cell death (18) must occur. Cells must become spatially oriented, recognize neighbors, and form specialized junctions (21, 22), form selective adhesive associations and migrate (20), induce differentiation of other cells less differentiated than themselves (23–25), form intercellular matrix (26), respond to inductive stimuli and differentiate (23, 27–31), form cell-specific organelles and products (32), undergo mitotic division, and then differentiate (33,34), form

organ fields (19, 23, 35), regulate organ field size (20, 36), and become associated into tissues (37) capable of functioning as parts of an integrated, coordinated adult. One would not imply that these phenomena are all the same as in higher forms. Perhaps the molecular mechanisms to achieve them, however, are more similar than different and there are a few data supportive of such speculation.

To some extent the system may be considered an artificial embryo. The extent to which this may or may not be true is not immediately relevant to the potential use of the system. For the sake of brevity I refer to this reaggregating group of cells as artificial embryos because the actual pellet stage lasts only a few hours.

The artificial embryos are grown in simple medium containing a test compound at various concentrations. The lowest dose adversely affecting any developmental event and the exposure time needed to produce this effect can be compared to the dose needed to adversely affect adult *attenuata.* The log of the adult toxic/developmental toxic concentration was calculated for several substances (16) and ranged from 0.12 to 2.7. This is considered the index of teratogenic hazard potential; the lower log number represents a coeffective teratogen (aspirin) interfering with developmental events but only in proximity to the adult lethal dose and the larger log (nonacetylated isoniazid) represents a noncoeffective teratogen. Intervening logs represented the expected spectrum between these two extremes. Why can one say "expected"? To illustrate this point one can refer to the open literature or a compendium such as the NIOSH Registry of Toxic Effects of Chemical Substances and obtain both the adult lethal and teratogenic doses for the same substances administered by the same route to the same species. The Registry lists the teratogenic and lowest adult lethal doses for aspirin (mouse) as 250 and 420 mg/kg respectively. The log of this ratio is 0.22. The same two figures for nonacetylated isoniazid (rat) are listed as 14 and 650 (LD_{50}) or a log of 1.67. From experiments in *attenuata* and prepublished data in various rodent species one develops a pair of lists ranked according to increasing developmental hazard (in the absence of adult lethality). Log numbers cost about $50,000 each when developed by experiments in rodents and about $2000 each when developed by experiments in the invertebrate system described. Precise concordance in rank ordering is probably not to be expected from all substances but the ends of the spectrum would be expected to remain predictable from *attenuata* and judgments by regulative agencies will still be needed for the middle ground.

The system under study was chosen because it provided an inexpensive means of examining the ability of substances to disrupt basic developmental events and of identifying the doses required to produce adult toxicity. The differences between toxic dose levels for conceptus and adult would rank

agents according to embryonic susceptibility to adverse effects as adequately as the currently applied safety evaluations in rodents. A simple calculation demonstrates that the system is directly consistent with preexisting data analyzed by the same calculation and thereby can provide inexpensive data separating the two ends of the spectrum of teratogenic potential. It initially appears capable of accurately detecting substances needing regulation only on the basis of adult toxicity plus a safely factor of course, and revealing those to which the conceptus is uniquely vulnerable. These latter would need regulation to levels below that of the embryo's susceptibility. Assuming that such a system is valid, we would still need expert developmental biologists in the regulatory agencies and industry for establishing levels of noncoeffective teratogen exposure acceptable to the conceptus and for the middle ground of the spectrum as well. In other words hazards need evaluation for three groups and not just two. The embryo has vulnerabilities unique unto itself just as do the adult male and female.

This review has dealt closely with its assigned topic and ignored another issue. Detection of exposure of individual women to noxious agents capable of interfering with development may also be possible one day. Klein and co-workers (38) reported that serum from women treated therapeutically with known teratogens would not support development of early rat embryos in vitro.

I hope the reader will realize that the *attenuata* system is still being validated. It should at least be obvious that most investigations of potential screens and even symposia on the topic (39) miss the mark and are precluded from discussion from the onset.

SUMMARY

State of the art teratology is sufficient to develop a screening system of teratogenic hazard potential if one remembers the basic developmental biology underlying teratogenesis. Understanding of teratogenic mechanisms may provide better systems but such knowledge is not yet available. The system described is an interim step and may not prove valid. Hopefully Karnofsky's law will remain in our attention thereby permitting our realization that the question, "Is table salt teratogenic?" is naive and largely irrelevant. The answer is "Yes" from the viewpoint of total well-being of the conceptus. What the question is really asking is: "What is the developmental hazard potential of table salt?" This question can be answered quantitatively and it is, "No, it is not a developmental hazard because it is a coeffective teratogen with a developmental hazard index of < 0.1." If we could all understand these basics, we could realistically proceed to safeguard your grandchildren and mine.

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