

# BELLE NEWSLETTER

*Biological Effects of  
Low Level Exposures*

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## TOXICITY TESTING IN THE 21ST CENTURY- *A View from BELLE*

### *Introduction*

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*The National Research Council of the National Academy of Sciences published a book entitled **Toxicity Testing in the 21st Century: A Vision and a Strategy (2007)**. The vision outlined by the Academy amounts to a radical and systematic overhaul of how hazard and risk assessment has been conducted for the past four decades. It envisions a toxicological world that tests new compounds at a faster rate, with profoundly better toxicological insights and mechanistic understanding and at far less cost. It sees a major shift from the chronic bioassay to high throughput screening studies with primary human cells and human cell lines, greater use of various omic technologies, biologically based modeling and bio-mathematical computational methods as the basis of how decisions will be made on chemicals and their acceptable risks. The Committee sees toxicity testing changing from the goal of generating "apical endpoints" (i.e., the toxic endpoint itself) to that of toxicity pathway identification and possible interactions of multiple contributory pathways. While the NAS Committee was quick to point out that these new approaches would need to be validated, there was little doubt that it sees a seriously limited and/or flawed current governmental risk assessment paradigm, including many hazard assessment procedures that are very prolonged, too costly and still yielding excessive uncertainty. This 40 year testing and risk assessment history*

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has been overly dominated by excessively high doses, too few doses with too narrow a dose range, biostatistical model predictions that can not be practically validated and extrapolation from animals to humans which bring its own version of high uncertainty (Calabrese 2009a,b, 2008, 2005; Calabrese and Baldwin 2003, 2002, 2001). Such concerns have led the Committee to attempt to define a turning point in toxicological and risk assessment history. The Committee argues strongly that society has to do much better in the testing of chemicals and that technological developments and improved scientific understandings can now provide the opportunity to move forward on such improvements.

This issue of the BELLE Newsletter will explore the vision and strategy of the NAS report with a series of independent expert commentaries by leaders in the academic and private sector.

Invited experts were asked to use the following questions as an intellectual starting point in the development of their Commentary but not to be restricted to these questions as they may well have questions of their own.

- Is the current testing scheme and its dependent risk assessment procedures sufficiently flawed as to need a serious and profound overhaul?
- If the current scheme ain't broke, then don't "fix it". Regulatory agencies should simply make a series of minor corrections and refinements as seem necessary.
- If the current scheme is seriously flawed, why did it take 40 years to figure this out?
- If this is the case, then what has Society lost by following such a flawed system.
- If there are important improvements should standards be revised to take this into account?
- Can in vitro systems, even using human primary cell and cell lines, ever satisfactorily be used to offer quantitative predictions of human population responses?
- Is the whole organism simply the sum of a series of human cell lines?
- Can toxicological pathway identification truly replace apical endpoint determination?
- Does the NAS proposal make sense or are we simply throwing the baby out with the bath water?
- Even though the NAS talks about validating their new methods against the "old" methods, the old methods are being criticized because they often could not be validated themselves. Does the NAS suggest to "validate" new approaches against systems that could not be validated themselves? If that is the case then what sense does it make?

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# COMMENTARY ON "TOXICITY TESTING IN THE 21st CENTURY: A VISION AND A STRATEGY"

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## ABSTRACT

The NAS Committee's vision, which focuses on understanding toxicity pathways in *in vitro* systems, represents an important goal for the future of toxicity testing and would contribute to a greater understanding of toxicological effects at low doses. However, implementing this vision for risk assessment purposes requires several intermediate steps, such as establishing that perturbation of toxicity pathways identified in *in vitro* systems are adequately predictive of adverse effects in humans, and that dose and time-course modeling can adequately translate *in vitro* findings to *in vivo* exposures. Despite the multiple challenges that must be overcome before it can be implemented, the vision offers the potential to provide accurate, high-throughput data for toxicological responses that can be used in risk assessment.

## INTRODUCTION

The vision of the NAS Committee<sup>1</sup> represents a transition in toxicology from an emphasis on the study of apical endpoints in whole animals to a focus on the study of pathway perturbations, based primarily on *in vitro* data, from which dose-response models will ultimately be developed. It should be emphasized that this vision does not seek to eliminate whole-animal testing altogether; however, it does aim to eventually use animal testing to a far less extent than in our current system.

Such a change to the current method of toxicity testing represents an important goal for many reasons. For example, toxicity testing needs to incorporate the knowledge that has been developed through the most recent advances in research, particularly at the molecular level. Through the application of mechanistic information resulting from the testing of chemicals by the proposed *in vitro* methods, it may be possible to predict the toxicity of similar chemicals that have not yet

been tested in whole animals. The number of chemicals in commercial use for which we have adequate toxicity information is limited, and higher-throughput assays have the potential to increase this number. While the vision's approach would result in a loss of information from a reduction in testing at high doses, the NAS Committee has proposed that this lack of information would be compensated by a greater understanding of effects at low doses, and that such an understanding can eventually be applied in a practical context. Clearly, however, there are multiple challenges to the actual implementation of the vision.

First, we need to consider the vast biological complexity of whole organisms. Will *in vitro* assays for pathway perturbations, in the absence of some understanding from *in vivo* studies, be able to identify all of the possible toxic endpoints of a particular chemical? The application of *in vitro* methods may be a slow and uncertain process without some initial hypotheses about likely critical apical endpoints for a particular chemical. In contrast, with animal bioassays we can identify toxic endpoints that we may not have originally thought to look for, through the analysis of different target organs.

An understanding of how pathway activation and the corresponding dose-response relationships change with differences in the duration of exposure will be necessary, and yet it may be difficult to distinguish between acute, subchronic, and chronic effects at the pathway level. In addition, *in vitro* assays cannot mirror the metabolism of a whole animal, and the NAS Committee has agreed that much research will be needed to ensure that the new testing methods associated with their vision fully evaluate the effects of both chemicals and their metabolites.

The development of "-omic" technologies (such as genomics, proteomics, and metabolomics) is a key element for advancing the vision of the NAS Committee; however, there are challenges to the use of these approaches for the prediction of *in vivo* toxicity. Rhomberg *et al.*<sup>2</sup> state that:

"Expression systems may show common features of early response between toxicants that are not now believed to operate similarly or to be affecting common cellular targets. On the other hand, when we look at the combinations of genes that are up- or down-regulated by particular substances, we may find it necessary to distinguish among toxicants that we now believe have similar actions."

It will be important to determine which cells in a complex tissue are key to understanding a biologically significant response for the whole organism. Therefore, the isolation of these cells and the determination of which expression changes are causally relevant for a particular adverse effect will be necessary.<sup>2</sup>

The large datasets that -omic experiments can generate make the identification of critical elements of toxicity pathways difficult. The expression levels of hundreds of genes can be significantly altered in response to chemical treatment in a typical microarray study, although the datasets can be somewhat streamlined by categorizing the genes into groups based on their respective biological pathways. It will be important to differentiate adaptive or neutral responses

from toxic responses in these studies. Also, *in vivo* perturbations can be followed by a re-establishment of homeostasis over a particular time course; consideration must be given to ways of mimicking such processes *in vitro*. The application of methods for essential metals, for example, will need to be able to distinguish changes resulting from toxicity due to deficiency or to excess from changes associated with maintenance of homeostasis. Phenotypic anchoring, which will be necessary to relate *in vitro* genomic changes to adverse effects defined by conventional whole-animal testing, was clearly recognized in the NAS report.

An example of the application of phenotypic anchoring is provided in a recent study by Andersen *et al.*<sup>3</sup> in which nasal tissue pathology of formaldehyde-exposed rats was used as a phenotypic anchor for interpreting the results of a genomic analysis of *in vivo* nasal epithelial cell responses to various doses of formaldehyde. In this study, dose- and time-dependent alterations in gene expression were found, and when the genes were grouped together according to their particular biological function, the nature of the alterations was consistent with the observed histopathological effects at the various doses. Interestingly, in this model the genomic changes did not prove to be more sensitive measures of tissue response than the histopathology. This study provides a model for the use of gene expression profiling in dose-response analysis that could potentially be adapted to the genomic studies of *in vitro* responses to toxicants envisioned by the NAS Committee. It should be noted that the toxicity of formaldehyde is relatively well studied, in terms of the dose of formaldehyde to the nasal epithelium at different air concentrations of formaldehyde, the target cell types in the nasal passages, and the plausible mode of action for carcinogenesis.

Extrapolation will continue to be a challenge to toxicologists and risk assessors in implementing the NAS vision for toxicity testing. While *in vitro* assays can employ doses lower than those typically associated with conventional animal tests (and perhaps more similar to typical human doses), it will still be necessary to extrapolate from doses in cells *in vitro* to doses in human tissues *in vivo*. Advances in PBPK modeling in whole organisms which yield estimates of dose to cellular targets may be able to inform selected concentrations for *in vitro* systems as well as appropriate cell types to help facilitate such extrapolations.

Validation of the new assays will be a key component in the implementation of the NAS vision. Careful thought will have to be given to how validation is conducted, including which chemicals to use and which endpoints to study. The NAS Committee suggests that the use of compounds both known to cause and not known to cause a particular adverse effect in humans can be used as reference agents in the validation of the predictive ability of an assay. They also suggest that, in the event that a known positive or negative reference agent is not available for a particular assay, then rodent cell-based assays that are comparable with the human assay would have to be used to establish relevance and to support the use of the human cell-based assay. Several of the challenges that apply to the use of whole animal studies will be issues here, as well. The relevance to humans of the response in rodents, considering inter-species differences,

strain differences, *etc.* will need to be established. Multiple types of assays as well as selection of appropriate positive and negative controls must be considered for the validation process. Potential confounding factors, such as the cell culture conditions or the selective pressure on the cells to evolve, may lead to false positive or false negative results in an assay and must also be identified. A key question in the validation process will be whether the failure to detect an *in vivo* toxicant in an *in vitro* assay is attributable to the toxicity being caused by another pathway/mechanism or because the assay is inadequate. We recommend, as an initial step, that validation efforts use a set of chemicals whose modes of action are relatively well understood and which includes chemicals with modes of action both relevant and not relevant to human responses. For example, the mode of action framework for carcinogenesis, as discussed by Cohen *et al.*<sup>4</sup>, provides an approach for the selection of reference compounds of varying relevance to the human response.

Because the *in vitro* assays proposed in the NAS report may provide a greater understanding of toxic responses at low doses, the NAS Committee state that one goal is to focus resources on the evaluation of the more sensitive adverse effects of exposures of greatest concern rather than on the full characterization of all the adverse effects associated with every chemical. Thus, in terms of relevance to risk assessment, a NOAEL from an *in vitro* assay differs fundamentally from an *in vivo* NOAEL, the highest-tested dose that does not induce an observable, adverse apical endpoint. The *in vitro* NOAEL would indicate the highest-tested dose which does not cause a perturbation of the normal processes in a particular pathway that is ultimately linked to toxicity in the whole organism. As discussed earlier, translation of the *in vitro* NOAEL into a meaningful parameter for risk assessment will involve methods for dose and time-course extrapolation as well as differentiation between adverse and non-adverse responses. And as with all risk assessments, an understanding of the uncertainty and variability in an analysis based on perturbation of toxicity pathways must be conducted.

Thus, before the NAS vision can be used in a risk assessment or regulatory context, it must be established that perturbations of toxicity pathways are adequately predictive of adverse responses in humans, and that dose and time-course modeling can adequately translate the *in vitro* findings to *in vivo* exposures. As with validation efforts for the tests themselves, we recommend that the well-studied chemicals for which quality risk assessments and risk-based criteria exist be evaluated from a “what if” perspective. Specifically, it would be instructive to consider, for these chemicals, what would happen if risk assessments and risk management decisions were made on the basis of *in vitro* data and associated modeling – how would the results compare with existing analyses and decisions?

Another challenge to implementing the NAS vision will be communicating the risk assessment and risk management decisions based on these new approaches to the public. Apical effects in animals are certainly more straightforward to explain than pathway perturbations that are removed from findings of frank toxicity.

In light of recent advances in the understanding of toxicology and biological research in general, the NAS vision is a laudable goal for

toxicity testing. Of course, the long processes of assay development and validation, the evaluation of the toxicity of new classes of agents, the discovery of new endpoints, and the interpretation of *in vitro* findings will still necessitate *in vivo* testing in the intermediate term. Nonetheless, despite its long-range nature and the challenges it may face, the NAS Committee's vision offers the potential to provide high-throughput and accurate data for risk assessment and for a greater understanding of the field of toxicology in general.

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# COMMENTARY ON "TOXICITY TESTING IN THE 21st CENTURY: A VISION AND A STRATEGY"

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## ABSTRACT

*Toxicity Testing in the 21st Century: A Vision and a Strategy* (NRC, 2007) presents a bold plan for chemical toxicity testing that replaces whole-animal tests with cell-culture, genetic, other *in-vitro* techniques, computational methods, and human monitoring. Although the proposed vision is eloquently described, and recent advances in *in-vitro* and *in-silico* methods are impressive, it is difficult to believe that replacing *in-vitro* testing is either practical or wise. It is not clear that the toxicity-related events that occur in whole animals can be adequately replicated using the proposed methods. Protecting public health is a serious endeavor that should not be limited by denying animal testing. Toxicologists and regulators are encouraged to read the report, carefully consider its implications, and share their thoughts. The vision is far too important to ignore.

This reviewer writes from a broad perspective, having served 14 years on the Institutional Review Boards for both human and animal research at a major research university and medical center, and having performed basic and applied inhalation toxicology research involving several species of laboratory animals, cell systems, and computer models for 30 years. This experience has clearly demonstrated the intimate relationship between laboratory animal studies and human studies, as well as the importance of having the very best possible toxicology data available for regulatory and public health purposes. Up front, two aspects of the NRC Committee (The Committee on Toxicity Testing and Assessment of Environmental Agents) report (NRC, 2007) appear to be troublesome: (1) placing a high value on reducing the use of animals, and (2) pressing for cutting the cost and time involved in regulatory

toxicity testing. Neither of these goals seem to be compatible with improving the value of toxicity assessment of chemical agents. In summary, the report proposes a future for regulatory toxicology (the "vision") that involves replacing whole animal studies with a combination of cell culture, genetic and other *in-vitro* methods, computer models, and poorly-specified human monitoring. Certain response pathways in cells, termed "toxicity pathways" are to be the focus of *in-vitro* testing. These pathways will be used to predict diseases with the aid of a generation of emerging computational models. The report appears to have been overly influenced by pressure to discourage animal studies, despite their proven utility. Also, the vision may well increase the cost of regulatory toxicology assessments by possibly requiring vast amounts of new data using unproven methods. On the other hand, the report describes important new emerging technologies which can augment current testing approaches. Incorporating such new technologies in toxicity testing is well defended in the report. Still, the vision is not adequately defended as: (1) being necessary and/or feasible, (2) leading to improvements in the protection of human and nonhuman animal health, and (3) being cost effective. The current rapid evolution of toxicity testing seems to be going well, so it may be premature to consider the proposed new master plan. A proposed long-term goal, to eventually replace *in-vivo* testing, with *in-vitro* testing appears to this reviewer to be unwise, and possibly fatally flawed. The report does acknowledge that novel classes of agents, such as those associated with nanomaterials and biotechnology products, will require maintaining "some whole-animal tests into the foreseeable future" (NRC, 2007, pg. 47).

Toxicologists and other informed readers should be able to follow the NRC Committee's logic, description of the vision, and the key scientific issues and details with ease. Practicing toxicologists should examine this report, as it is likely to have an impact on influential parties that affect future funding opportunities, and establish requirements for regulatory data. A good place to start reading the report is the Appendix, which presents valuable biographic sketches of the report's 22 authors. Knowledge of the training, experience, and current pursuits of the Committee members will help the reader to understand the strengths (and weaknesses) of the report. The report was sponsored by the U.S. Environmental Protection Agency via a contract with the National Academy of Sciences, but the conclusions and recommendations are those of the authors and do not necessarily reflect those of the involved agencies.

In spite of its problems, the report makes interesting reading, and it has several strengths. It eloquently presents a case for augmenting toxicity testing by exploiting many of the new and impressive developments in genetics, cell biology, and physiologic modeling: Developments which, no doubt, are destined to add greatly to understanding the actions of chemical toxicants and significantly contribute to protecting animal, human, and ecosystem health. The vision for the future of toxicology relies heavily on the extensive availability of human, and transgenic laboratory animal cell lines, and to the credit of the report, the inherent artificial nature of cells in culture is acknowledged. Cultured cells do not have the complex

realistic chemical environment that is dynamically provided by the whole animal, and which both modifies and responds to the cell's status. Unfortunately, a solid solution to this severe limitation of cell cultures is not provided by the Committee. The additional problem of extrapolating from subcellular and cellular scales of biological complexity to the whole organism scale (including normal functioning and disease states) is acknowledged, but also not critically evaluated. Also acknowledged is the current inability of *in-vitro* assays, including those other than cell cultures, to mirror the complex metabolic environment in the integrated whole animal. Validation of the vision, and the need for new animal and human studies for such validation of the *in-vitro* assays are discussed. However, the extent and exact nature of such new whole animal and human studies are not well described. The emerging *in-vitro* tools for toxicologists and their promise are more clearly described by experts on the Committee than are the limitations and challenges involved in adapting these tools to chemical toxicity testing for regulatory use. The report discusses implementation of the vision, including many of the research needs, needed perceptual changes (by scientists, regulators, legislators, industry and the public), very substantial institutional changes, and cost requirements for (a) improving and (b) adapting the emerging new tools to regulatory needs.

The weaknesses of the report's vision for the future of toxicity testing are substantial, and this reviewer believes that many of these weaknesses may be insurmountable. A few examples will be described here. First, the apparent assumption that disease processes in complex whole mammals can, even in theory, be understood without extensive on-going whole animal research seems to be seriously flawed. The Committee proposes identifying key toxicity pathways in cells, which can be used to adequately predict whole-animal responses to chemical-agent exposures. Such a bottom-up reductionist approach is not even very successful in the physical sciences, let alone the biological sciences (Patee, 1979). Whole animals are fundamentally different and behave in more complex manners than can reliably be predicted from data in cells, even when kinetic models are used in order to extrapolate the data. Whole animals respond to stress in many ways including hormone secretion, changes in cell replication, changes in metabolism, etc. The current system of integrated *in-vitro*, *in-vivo*, and *in-silico* laboratory approaches complemented by appropriate epidemiologic and clinical data has evolved to be remarkably effective for protecting health. The current system, based on the experience and insight of tens of thousands of scientists, works well, and it does not need to be largely replaced by unproven methods. Countless potentially hazardous chemical agents have been dropped from development programs, and even withdrawn from use, on the basis of either *in-vivo* or *in-vitro* testing: To drop, or even substantially limit, the *in-vivo* tests could be a serious mistake. An intact mammal consists of about 100 trillion cells of about 200 distinct types that are highly coordinated and interdependent. Most, if not all diseases, involve the participation of numerous cell types, significant modifications of chemical environments throughout the body, countless adaptive mechanisms, and the eventual failure of corrective physiological mechanisms. It does not seem to be cost

effective to maintain and use sufficient numbers of cultures, of preferably human cells, for all of the relevant types involved in the important diseases. To attempt to duplicate this complexity and integration using cell cultures and computer models may never be possible. A well-designed whole animal study, by contrast, includes all of the cell types and all of the countless interactions among cell types, tissues, organs and organ systems. Consider a chemical that must be tested in 100 cell types, each with 50 potential toxic pathways, with 50 different modulating hormones and other internal environmental factors, with 5 genetic variations for each cell type, at 3 doses of the tested chemical, and for 3 exposure durations. One must hypothetically set up, use, and evaluate about 100 million separate cell culture tests, which, if even possible, could be enormously time consuming and costly. Superior information might be efficiently obtained from the study of just 300 mice. Interestingly, the report mentions in several places that validation of the cell-level studies will actually require conducting new animal studies. The number of such studies could be enormous, generating a new parallel realm of animal usage.

As previously mentioned, *in-vitro* techniques are inherently artificial, as the dynamic physiological environment of the body cannot be replicated outside of the intact living body. Consider the testing of mixtures in cell cultures, which is at best a formidable task, and at worst not manageable. Mixtures not only interact chemically at many points, but they also often trigger varied physiologic defensive mechanisms which lead to currently unpredictable whole-animal responses. The testing of many types of mixtures very clearly requires the use of laboratory animals. Also, it may not be possible to detect false-positive and false-negative toxicity results for many chemical agents within the limits and constraints of the vision. Therefore, one could expect many promising and/or useful chemicals to be prohibited or withdrawn from use, and unacceptably toxic ones to be put into widespread use by regulators who do not have access to sufficient *in-vivo* data.

To illustrate another problem, consider an aerosol consisting of a broad size distribution of nanosilver particles plus an antibiotic or a pesticide. It is not possible now, or in the foreseeable future, to evaluate the effects of such an aerosol without extensive inhalation studies (Service, 2008). The initial detailed pattern of deposition in the respiratory tract, and the subsequent post deposition phenomena are so complex as to not be currently predictable. Some portion of the deposited material may travel directly to the brain via the olfactory nerve (Dorman, 20002; Gerde, 2008), a poorly-understood pathway that is not yet included in the existing computer models. Whole animal studies are most likely essential for studying many of the future complex, multicomponent and engineered nanomaterials that have unknown distribution in the body, and subsequent potentially widespread effects. This example is but one of many that seem to reach beyond the limits of the Committee's vision for the future of regulatory toxicity testing.

Another problem with the vision can be understood by reference to the Nuremberg Code (<http://ohsr.od.nih.gov/guidelines/nuremberg.html>), which describes the criteria that were developed after

World War II for defining crimes against humanity. The Nuremberg Code was used during the 1940's trials of Nazi scientists who performed human experimentation. Item number 3 of the Code states that "The experiment should be so designed and based on the results of animal experimentation and a knowledge of the natural history of the disease or other problem under study that the anticipated results will justify performance of the experiment". This clearly prohibits (on ethical grounds) intentionally exposing humans to potentially toxic chemicals prior to performing sufficient animal studies. The logic behind this aspect of the code is that all possible adverse events must be explored in whole animal studies prior to permitting human exposures. It should be understood that laboratory animal studies are conducted only when justified as determined by an ethics review committee, and even then only when using means to prevent unnecessary suffering as well as the use of excess numbers of animals (NRC, 1996).

The last chapter of the report (Chapter 6), covering "Prerequisites for Implementing the Vision in Regulatory Contexts", "anticipates continual change over the next 2-3 decades". Such change includes: "far reaching shifts in orientation and perception..."; "congressional funding of agencies to implement the vision,..."; "large expenditures of money..." (possibly more than hundreds of millions of dollars); and the development of test methods that "are in early stages of development..." and "others that will be used eventually (that) are not yet on the drawing board or even imagined." In practical terms, implementation of the proposed vision may not be affordable or feasible, especially for those test methods that are not "even imagined".

To conclude, the report is certainly worthy of being examined and contemplated by all interested parties. Each reader can assess the value and feasibility of the vision on the basis of their own experience and understanding of toxicity testing and emerging regulatory needs. This reviewer is convinced that the report's vision is interesting and of value, but that it is seriously flawed. Perhaps pressure to eventually eliminate all animal research has contributed to the flaws. However, it is clear that many of the new approaches that are described will eventually play significant roles in improving decisions (including regulatory ones) regarding the potential risks of chemical substances. It seems rational to consider the vision as an addition to, rather than a substantial replacement for, the methods by which chemical substances are evaluated for their potential toxic effects.

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# COMMENTARY ON "TOXICITY TESTING IN THE 21st CENTURY: A VISION AND A STRATEGY"

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## ABSTRACT

*Toxicity Testing in the 21st Century: A Vision and a Strategy*, from the National Research Council Committee on Toxicity Testing and Assessment of Environmental Agents, presents a vision wherein toxicology testing moves from feeding test substances to animals for their lifetimes, and assessing clinical laboratory and histopathological changes, to human tissue studies made suitable by recent technological advances in computational biology, toxicogenomics, and the like. This is to be accomplished by elucidating toxicity pathways complemented by targeted testing. The report focuses on the array of available new concepts and attendant technology that the committee considers relevant to its proffer, but, in the final analysis, it describes little in the way of robust strategy for achieving the stated goals. From that perspective, the vision, as described, is no more innovative or far-reaching than goals directed at the utility of cellular metabolism measurements put forth fifty years ago. The report generally lacks the coherence and organization that could have given greater credibility to the committee's deliberative effort.

## OVERVIEW

Toxicity Testing in the 21st Century: A Vision and a Strategy, from the National Research Council Committee on Toxicity Testing and Assessment of Environmental Agents, starts with a Summary that is curiously promotional, and laden with rhetorical excess quite extraordinary for a report of this nature and source. The NRC committee introduces "The Vision" seemingly as a revelation and unique insight, intended as the "transformative paradigm shift" needed to "provide broad coverage of chemicals, chemical mixtures, outcomes, and life stages... reduce the cost and time of testing... use fewer animals and cause minimal suffering in the animals used in... develop a more robust scientific basis for assessing the health effects of environmental agents." Notwithstanding that it is difficult to imag-

ine a non-transformative paradigm shift, and the questionable (and over) use of the terminology, in the first place, it appears that the committee sees its proffer not just as a glimpse of the future, but as a radical change in toxicity testing philosophy -- perhaps so revolutionary as to carry us into the 22nd century. The preamble to the chapter entitled "Vision," quoting the architect, who designed the 1893 Chicago World's Fair, reflects this abundant enthusiasm, to wit (in part):

"... Make big plans; aim high in hope and work, remembering that any noble logical diagram once recorded will never die, but long after we are gone will be a living thing, asserting itself with ever-growing consistency."

Such an exaggerated sense of importance might be excusable if warranted by the product at hand. That is not immediately apparent. The committee's vision is, in its own words

"... built on the identification of biologic perturbations of toxicity pathways that can lead to adverse health outcomes under conditions of human exposure. The use of a comprehensive array of *in vitro* tests to identify relevant biological perturbations with cellular and molecular systems based on human biology could eventually eliminate the need for whole animal testing and provide a stronger mechanistically based approach for environmental decision-making."

Further:

"Although the reliance on *in vitro* results lacks the whole-organism integration provided by current tests, toxicological assessments would be based on biological perturbations of toxicity pathways that can reasonably be expected to lead to adverse health effects. Understanding the role of such perturbations in the induction of toxic responses would be refined through toxicological research."

These are hardly "transformative" concepts; a similarly charged panel could have (and likely did) put them forth two – or even three – decades ago. The committee's toxicity testing concept acknowledges modern components of research, including genetics, genomics, bioinformatics, physiologically-based pharmacokinetic (PBPK) modeling, and other areas of computational biology. Nonetheless, from an historical perspective, the vision is an old conceptual skeleton covered in a new skin. Arguably, the use of *in vitro* and *in silico* technologies can advance the efficacy and efficiency of toxicity testing, presumably a result of better understanding of the biological processes comprising toxicity. The purpose of toxicity testing is, pure and simple, prediction – reliable prediction. Any effort to *understand* the attendant biological processes is an *instrumentalist* one attendant to the purpose of prediction. Almost sixty years ago, we thought that measuring glycolysis and oxidative phosphorylation *in vitro* placed us on the threshold of solving pharmacological and toxicological puzzles, as we also held in high regard the predictive prospects of the *in vitro* genotoxicity battery of tests a decade later. Journals abound with similar illustrative matter. Some of this did lead to better understanding of the underlying toxic mechanisms. But they did not lead to better prediction, or certainly not better than the apical endpoints of experimental animal studies.

Missing from the report is some justification for the committee's optimism – why contemporary theory and technology are likely to produce the envisioned transformative change on scientific grounds, and why such change could pass legislative and regulatory muster. It is not enough to list the rich lode of new technology resources and theory that can or might be exploited in the cause of novel toxicity testing. A perspective how that might be accomplished -- or on the feasibility of doing so in the first place -- demands acuity; this is where the disappointment lies. There is abundance of “maybe this and maybe that” and “some of this” and “some of that” but, once past the Venn chart of the committee's vision, little in the way of real insight is offered. No small part of the problem is that the report is poorly organized, and written in a fashion that frequently belabors the obvious, confuses the old as new, and switches back and forth between being overly specific and confusingly indefinite. But, mostly, the difficulty is incoherence due in good measure to the conflict of purpose. The conundrum that pervades throughout the report is represented in its last paragraph:

“The vision for toxicity testing in the 21st century articulated here represents a paradigm shift from the use of experimental animals and apical end points toward the use of more efficient in vitro tests and computational techniques.”

This might appear to belabor the earlier point, but it reflects the report's repeated emphasis – more efficiency, do away with apical endpoints from experimental animal studies, and in the course, use less experimental animals. Worthwhile? Certainly, but only as they are a covariants of more efficacious predictive power. It is here where coherence is the problem.

Toxicity testing, as conveyed in the report, is seen as two components: toxicity-pathway assays and targeted testing. The first is to look at those cellular and genetic changes leading to dysfunction; the latter is to conduct studies refining the information gained from the first. The required information into an out of the toxicity-testing module includes chemical characterization and dose-response and extrapolation modeling. Related population and exposure data and the contribution of all components to risk assessment (“risk contexts”) complete the vision. The discussion here emphasizes the toxicity testing and dose-response/extrapolation components.

## TOXICITY PATHWAYS AND TARGETED TESTING

The theme of the committee's vision is to focus on toxicity pathways that are defined as “simply normal cellular response pathways that are expected to result in adverse health effects when sufficiently perturbed.” The long-range vision is to identify those pathways and how their perturbations lead to an adverse biological response. Targeted testing is meant to complement toxicity pathway identification by using, for example, in vitro cell models that allow formation of reactive metabolites. This is clearly an important goal, the achievement of which would, indeed, enhance the efficacy of in vitro identification, studies, and exploitation of toxicity pathways. As an objective for regulatory toxicology, it is a new perspective. It has, however,

been one focus of physiologically-based pharmacokinetic and pharmacodynamic modeling for some years now.

Knowledge of toxic pathways, along with dose-response extrapolation modeling, is foreseen as replacing the apical endpoints of experimental animal studies. So far, that is not, as noted, much different than decades-old objectives to harness the mechanistic potentials of mitochondria, microtubules, intermediary metabolism, etc. What is different is the far wider population of candidate pathways for perturbation, which makes the selection process a more daunting one. The committee suggests that high throughput methods could be used as well as integrated cell responses, receptor binding or reactivity of compounds with targets. For the last, cholinesterase inhibition was used as an example. It is a good example – of the question: What is new about this vision? The committee does recognize that toxicity pathways are more likely to be represented by a cellular response network, i.e. the interactions among genes, proteins, and small molecules that are required for normal cellular function. There is, however, another type of “network” that has to be considered in identifying toxicity pathways. This would be the toxicology equivalent of *network pharmacology* or *polypharmacology*. Recent studies have suggested that drug design might be best directed at the phenotypic robustness wherein disease-causing genes form a disease-causing network that in turn contains multiple drug-responsive sites. Drugs acting on these multiple sites are more durable than drugs designed according to the “one gene, one drug, one disease” approach. Hopkins has noted: “[A]s increased understanding of the role of networks in the robustness and redundancy of biological systems challenges the dominant assumption of single target drug discovery, a new approach to drug discovery – that of polypharmacology – is emerging.”<sup>2</sup> This has clear implications for the study of toxic mechanisms, and significantly complicates both the pharmacodynamic and pharmacogenomic bases of the assumptions made by the committee concerning toxic pathway identification.

The advanced phases of the committee's vision focuses on human-cell studies and what has long been a goal of drug and chemical safety evaluation, “... encouraging the integration of toxicological and population data.” But no clear attention is paid to the human genetic variation that can influence biological response to disease or chemicals. One comment is particularly revealing:

“[T]he committee's vision of toxicity testing stands on the presumption that a relatively small number of pathways can provide sufficiently broad converge to allow any moderately sized set of high and medium throughput assays to be developed for the scientific community to use with the confidence and that any important gaps in coverage can be addressed with a relatively small set of targeted essays. That presumption may be found to be incorrect.”

That is quite an extraordinary statement. The consistent theme throughout the report is more human cell assays, less animal usage. Does the committee assume genetic and phenotypic toxic pathway consistency within the human population – particularly in view of the toxicological equivalent of polypharmacology (polytoxicology!)?

Earlier in the report, in discussing its vision, the committee stated:

“Pharmacokinetic and pharmacodynamic models promise to provide more accurate extrapolation of tissue dosimetry linked to cellular and molecular endpoints. The application of toxic of genomic technologies and systems – biology evaluation of signaling networks will permit genomewide scans for genetic and epigenetic perturbations of toxic pathways.”

The phenotypic expressions of single nucleotide polymorphisms (SNPs) within a genome and across a population vary as genetic components of disease risk.<sup>3</sup> As is the case with some diseases, for any specific phenotypic expression of a chemically caused health disturbance, there are potentially numerous SNP variants, wherein alleles rank differently as to the size of the population effect. The importance of such information in identifying at-risk populations is obvious. We know that any single individual will fall somewhere on a population exposure-response curve, but being able to identify *where* on that curve, *a priori* to exposure – that is a worthwhile vision.

## DOSE RESPONSE AND EXTRAPOLATION MODELING

The committee emphasizes empirical dose-response (EDR) modeling and mechanistic, i.e., toxicity-pathway dose response models. Interestingly, the committee does not include quantitative biologically-based dose-response models in its vision because “this type of modeling is in its infancy.” No doubt it is, but just what stage of life does the committee assign to its toxicity pathways proffer. In quite a few places the committee signaled that it assumes that a dose response curve will behave only monotonically. That is discordant with a wide understanding of potential toxic responses to low-dose radiation and chemical exposure, viz. “hockey stick curve” or “J-curve.”

EDR models are seen as ultimately describing *in vitro* data. Apparently, no separate pharmacokinetic studies are prescribed in animals. Instead, tissue concentration data of parent and metabolite compounds would be piggy-backed on targeted testing studies. That appears to eliminate the likelihood of identifying pharmacokinetic parameters of clearance and volume of distribution as well as detecting linear or non-linear pharmacokinetics. Notable is the committee “insisting that the *in vivo* studies have a measure of tissue concentration [so as] to permit comparison with the results from the *in vitro* assays.” However, the EDR models of *in vitro* responses describe the relationship between the concentration in the *test medium*, not the tissue concentration, and the response. The *in vitro* concentration data should include the tissue concentration. Journals and textbooks are rife with erroneous pharmacodynamic modeling output from *in vitro* data because the concentration parameter came from media rather than tissue.

The committee’s emphasis on PBPK modeling is appropriate. It will be a must if extrapolation of *in vitro* toxicity data to the intact human is to achieve the role the committee envisions for it. Such modeling is conducive to meeting physiological, biochemical and genetic variations among humans. However, it is a model, not the real system. For instance, PBPK models generally assume a fixed steady state ratio

between a tissue and its effluent venous blood. This is unlikely to be true in certain tissues such as the liver. It is important to know when such discrepancies matter. The committee is optimistic that quantitative structure activity relationships (QSAR) should allow estimation of blood-tissue partition coefficients and other constants for PBPK modeling “with a minimal research investment in targeted studies in test animals.” As the committee notes, QSAR as an instrument for PBPK modeling is nothing new, but nor is it unequivocally reliable. Further, the soundness of a PBPK – or any model for that matter – is very much influenced by the modeler. It is far from plugging parameter values into a computer, as can frequently be done for analysis of blood data for clearance and volume of distribution values.

## IN SUMMARY

The report, *per se*, is so inconsistent that it is, at times, bewildering. There are instances in the report where gaps in careful thought seemingly jump at the eye. The committee notes, for example, that while it “generally holds true” that test animals biology is similar enough to humans to allow them to be used as models of human exposure, there are cases where this does not hold true. Thus the committee “envision[s] a future in which tests based on human cell systems can serve as better models of human biological responses ...” All well and good. Then the example presented of a human idiosyncratic response, not seen in rats, is thalidomide teratogenicity. Perhaps, sometime in the 21<sup>st</sup> century teratogenicity testing will be possible in human cells other than embryos, but it was a strange example to use in this part of the 21<sup>st</sup> century.

There is so much on which the report could have focused and that would have truly made it a visionary document. A worthwhile comparison is the 2008 Institute of Medicine report, *Emerging Safety Science*. This publication, concerned with preclinical and clinical drug toxicology, includes discussions of investigative toxicology and toxicogenomics, among others, that are clearly relevant to, and missing from, the report under review.

The report does not suffer because of the committee’s inadequacies. That could hardly be the case, considering the committee make-up. The recommendation is that the committee subscribe to its advice contained in the very last lines of the report, speaking of its proposed “paradigm shift”:

“A substantial commitment of resources will be required to generate the scientific data needed to support that paradigm shift, which can be achieved only with the steadfast support of regulators, law-makers, industry, and the general public. Their support will be garnered only if the essence of the committee’s vision can be communicated to all stakeholders in understandable terms.”

## FOOTNOTES

<sup>1</sup> The term paradigm shift is credited to Thomas S. Kuhn (*The Structure of Scientific Revolutions*, Second Edition, University of

Chicago Press, 1962), though the term, per se, is not Kuhn's. He defined paradigm as the structure of ideas that inform scientists and provide the boundaries within which they work. When deviations from the paradigm accumulate, a scientific revolution (i.e., paradigm shift) occurs in which there is a profound revision of what is considered normal science. This is far more reaching than a change in a toxicity-testing algorithm, notwithstanding that some of the testing procedures could reflect revolutionary science.

<sup>2</sup> Hopkins, A.I. Network pharmacology: the next paradigm in drug discovery. *Nature Chemical Biology* 4:682-690, 2008.

<sup>3</sup>Goldstein, D.B. Common genetic variation and human traits. *N Engl J Med* 360:1696-1698, 2009.

# COMMENTARY ON "TOXICITY TESTING IN THE 21<sup>st</sup> CENTURY: A VISION AND A STRATEGY"

## STEM CELLS AND CELL- CELL COMMUNICATION AS FUNDAMENTAL TARGETS IN ASSESSING THE POTENTIAL TOXICITY OF CHEMICALS

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**Keywords:** Adult stem cells; epigenetic toxicants; tumor promotion; gap junctional communication

*"With respect to cancer causation, integration of the analyses suggests that the inhibition of GJIC is involved in non-genotoxic cancer induction or in the non-genotoxic phases of the carcinogenic process (such as inflammation, cell toxicity, cell proliferation, inhibition of cell differentiation, and apoptosis)."*

H.S. Rosenkranz, N. Pollack and A.R. Cunningham (1).

### ABSTRACT

Faced with the reality of our current methods of drug discovery and toxicity assessment of all chemicals is less than perfect, the Report, "Toxicity Testing in the 21<sup>st</sup> Century: A Vision and a Strategy", posed a reality check on all scientific efforts to find new conceptual and technical approaches for being better predictors of potential human health effects. This Commentary is a challenge to both the current paradigms and techniques to test chemicals for their potential toxicities. While, clearly, our scientific understanding of the mechanisms

of chemical-induced toxicity and of the pathogenesis of all human diseases are not complete, the state of scientific understanding seems not only sufficient to know what we are now doing is not sufficient, but that it is adequate enough to make a new paradigm and technological change. Basically, the challenge includes the opinion that human exposure to chemicals, that are associated with one or more health endpoints (birth defects, cardiovascular diseases, cancer, reproductive and neurological dysfunctions), is the result of epigenetic, not mutagenic or genotoxic, mechanisms. In addition, it is postulated that the adult human stem cell should be considered the "target" cell for the important chemical-induced health effects. To test this hypothesis that altering the quantity and quality of adult stem cells by chemical exposures during in utero, neonatal, adolescent, adult and geriatric phases of life can lead to health consequences, it is recommended that 3-D in vitro cultures be used on male and female human adult stem cells from a few major organs (e.g., heart, brain, liver, lung, kidney, breast, prostate). Altered stem cell biology (e.g., increase or decrease in the stem cell numbers in specific organs; altered apoptotic and differentiation frequencies), as well as measured cell-cell communication, should be seriously considered as toxicity endpoints.

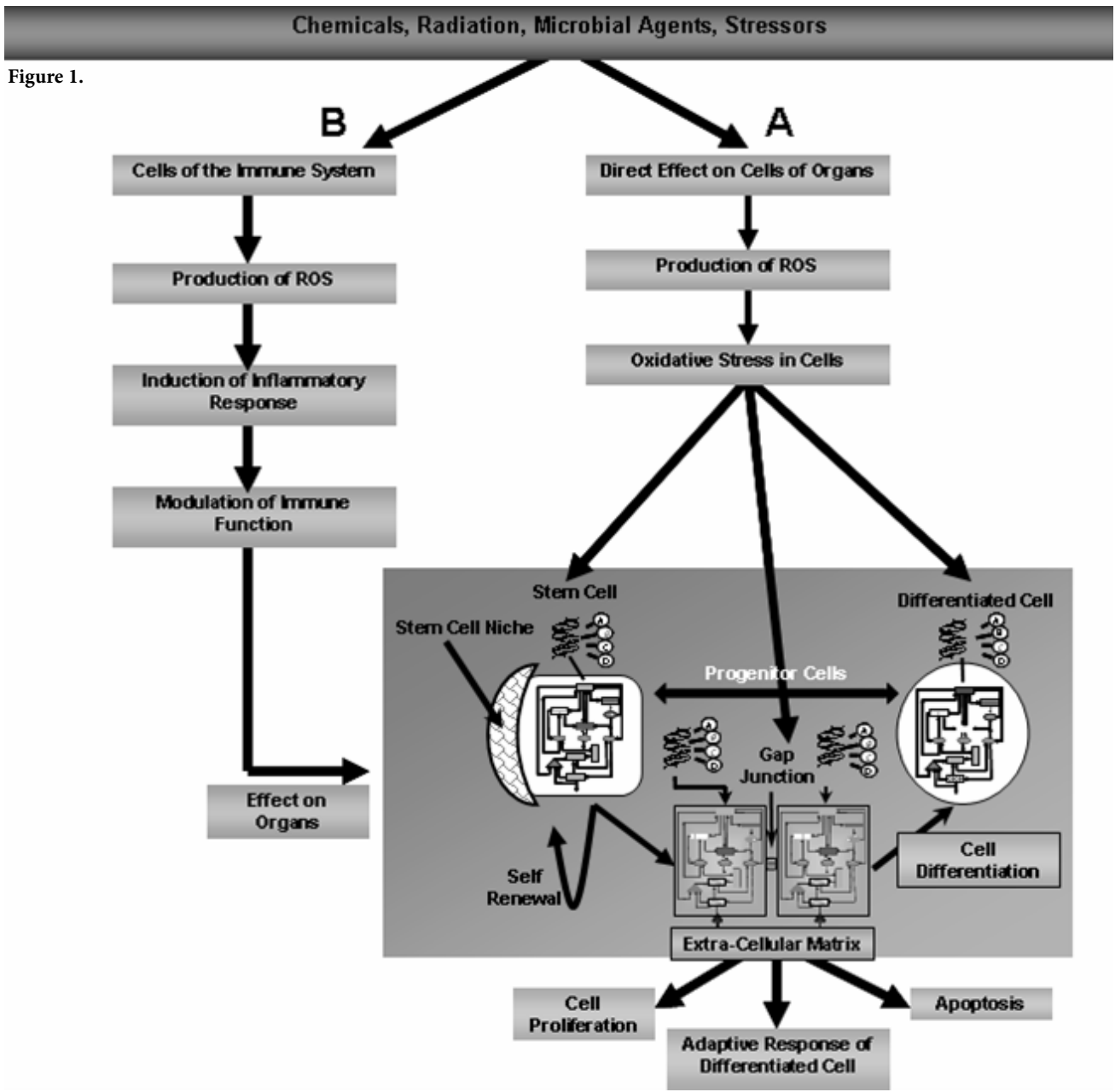
### PREAMBLE:

In order to take up the challenge laid down in the NAS Report, "Toxicity Testing in the 21<sup>st</sup> Century: A Vision and a Strategy" (2), several assumptions are given that underpin the following response to this report.

### ASSUMPTIONS CURRENTLY MADE IN THE PARADIGM THAT CHEMICALS CAUSING "TOXIC" HEALTH ENDPOINTS DO SO VIA NUCLEAR DNA DAMAGE AND MUTAGENESIS

**First**, one must recognize that, starting from the single fertilized egg to the geriatric individual with over 100 trillion cells, organized into organ-specific tissues, consisting of a few adult stem cells, many transit amplifying or progenitor cells and over 200 different specialized or differentiated cell types, a delicate orchestration of cell proliferation, differentiation, apoptosis, adaptive responses of the differentiated cells must exist. This orchestration is mediated by extra-, intra- and gap junctional inter-cellular communication (GJIC) mechanisms (3). This complex communication system is the mechanistic basis for homeostatic control of cell functions directed by the intra-cellular signaling control of specific gene expression controlling cell proliferation, differentiation, apoptosis and adaptive responses of the terminal differentiated cells (4).

**Second**, when a chemical encounters a cell, it (a) could damage the genomic DNA which could lead to a mutation [**mutagenesis**]; (b) cause death of the cell by necrosis, apoptosis, or anoikis; [**cytotoxicity**] and (c) alter gene expression at the transcriptional, translational or posttranslational levels [**epigenetic toxicity**]. It must be noted that



**Figure 1.** The diagram tries to incorporate a “systems” aspect of how a physical, chemical or biological agent could affect a multi-cellular organism. At non-cytotoxic concentrations or doses, an agent could simultaneously trigger oxidative stress in both the cells of the immune tissues and the epithelial/ endothelial/ stromal cells in various organs. Upon induction of reactive oxygen species (ROS) and of oxidative stress induction of intra-cellular signaling in various cell types of the complex immune system, various cytokines would interact on tissues, containing the three fundamental cell types (adult stem cells, progenitor and terminally-differentiated cells). Given that these cells would have been exposed to the toxic agent and that they, also, would have reacted to the agent differentially because of their different physiological/phenotypic state, the interaction of all three types could be very different (e.g., the normal stem cells might be induced to

proliferate asymmetrically; any initiated pre-cancerous stem cell might proliferate symmetrically; the progenitor cells might be induced to proliferate symmetrically and to migrate, as in wound healing; and the terminally differentiated cell might adaptively respond or to apoptose) in response to the inflammatory signal. In summary, each cell type of the immune system and of the various organ tissues, with their different expressed genes and cellular physiology, will respond differently to sub-lethal exposure to agents inducing oxidative stress triggered intracellular signaling and epigenetic alterations. The interaction of inflammatory agents on pre-exposed organ cells could be an additive effect, a synergistic response or possibly, even an antagonistic effect. This could explain the wide range of diseases in which the inflammatory process seems to play a prominent role.

mutations can occur via error-prone repair of damaged DNA. Mutations can also result from error-prone replication of normal DNA. Agents that damage nuclear DNA do so in a random fashion. These mutations that result can be viable or lead to lethality of either the cell or organism.

**Third**, while there is absolutely no question that ionizing and UV radiation can induce oxidative damage, nuclear DNA damage and gene or chromosomal mutations, it has been assumed that chemicals can also induced genomic DNA damage and mutations that can lead to viable cells. However, the major challenge to this fundamental assumption of the mechanism of toxicity of chemicals (particularly, of those chemicals that are associated with tumors, in which are found cells with mutations in the oncogenes or tumor suppressor genes). This author will offer an alternative hypothesis that, while toxic chemicals, in general, and those associated with cancers, can induce reactive oxygen species (ROS), oxidative stress, oxidative damage to macromolecules and cellular structures, at non-cytotoxic levels, they do not damage genomic DNA nor do they induce nuclear mutations in the stem cells or even the progenitor cells. If at high concentrations of the parent compound, in cells that metabolize the compound, massive intra-cellular damage occurs, the cell will die by acute toxicity.

**Fourth**, this leads to this commentator's view of current in vitro assays to measure mutagenesis. From the Ames assay to all the other so-called in vitro "genotoxicity" assays, I feel **none of them** can be predictive of potential mutagenicity of the chemical being tested (5). There are many reasons for this paradigm-challenging statement: **(a)** the target cells for the mutation detection are not reflective of the critical human normal adult stem, progenitor or terminally-differentiated cells of the human body. In addition, those mutation assays, using immortal cell lines, are not reflective of the target cells in vivo; **(b)** most, if not all, of these cells are used in 2-dimensional assays, growth on plastic, at log phase. Cells in vivo are usually in contact inhibited 3-dimensional organization. Published literature has already shown, using any endpoint, that cells in log phase respond differently than the same cells at confluence, and even most differently, when exposed when in 3-dimension (6); **(c)** when testing a chemical's potential mutagenicity, almost universally, recovery of "positive" results occurs when the chemical has induced significant cytotoxicity of the exposed population. If that same cytotoxic level occurs in the human body, it would mean little to the human individual if the chemical actually induced a few mutations in any surviving cell, because the individual would have died of acute toxicity; **(d)** the interpretation of the "genotoxicity" of a chemical in these in vitro assays is not based on a direct measurement of an altered base sequence of the genomic DNA, but a phenotypic surrogate of the presumptive gene target. The best example of this might be the TK- and HPGRT- resistance assays to measure mutations in the thymidine kinase and hypoxanthine guanine ribosyltransferase genes. In both cases, if an agent induces cells that are either the TK- or HGPRT- phenotype, it is interpreted as meaning the cells had those respective genes mutated so that they became dysfunctional. Indeed, if a true point mutation or a deletion mutation did occur in those genes, cells with those phenotypes are true mutations. However, if

the agent induced oxidative stress induced intra-cellular signaling to silence, transcriptionally, those genes, then the surviving cell is not a mutant but has a phenotype shaped by an epigenetic toxicant; **(e)** if we assume that killing of a terminally differentiated cell, such as a neuron in the brain, could be of significance to human health (i.e. Alzheimer's disease), it should not be of any importance in the case of mutagenesis, because these cells would not divide to fix any nuclear damage to form a mutation. Killing a few terminally differentiated cells might not be of any consequence in an adult because they probably can be replaced by the progenitor or stem cells. Using in vitro primary human cells in 2 dimensional and log phase might also not be of significance when the killing effect is minimal. The reason is these cells have a finite life span and there, senescence is evolution's way of riding any mutation from making a significant impact on the organ (7,8).

The real target of concern for a potential mutation is both the **germinal and somatic or adult stem cells**. These cells are fundamentally important for the species and individual, respectively. Only recently have the stem cells been highlighted as target cells for mutation testing. However, a few important features of these stem cells must be brought to light before uncritical application can be made for toxicity testing. The first is coming from the so-called study of "cancer stem cells" (9). In tumors, consisting of cancer stem cells and cancer non-stem cells, one can isolate "side-population" cells when the tumor population is put through a flow cytometer after exposure to fluorescent toxicants (10). The cancer non-stem cell retains the fluorescent toxicant and is separated from the cancer stem cell which pumps out the fluorescent toxicant. These non-fluorescent, side-population cells express drug transporter genes are the cancer stem cells (11). It seems that normal stem cells might also express these drug transported genes to be resistant to toxic chemicals (there might be an evolutionary reason for this, in that if all the stem cells of an organism were equally susceptible to an environmental chemical toxicant as the differentiated cell, the organ would not have the capacity for wound repair and would die). Therefore, the problem of using stem cells to test toxic chemicals might run into problems if the toxic chemical is pumped out of the stem cell.

**Fifth**, after a diseased state is found in a chemically-exposed organism, trying to link an identified lesion in the DNA extracted from that tissue after exposure is complicated by many factors. When one extracts DNA from tissue or from a population of cells, there are two sources of DNA, which might be the target of any reactive chemical electrophile or of ROS's associated with the metabolism of the chemical. Rarely in these DNA lesion studies is there a separation of mitochondrial and genomic DNA. If the lesions are formed in the mitochondrial DNA, they could lead to mitochondrial mutations. However, the likelihood of electrophiles and ROS, formed from a toxic chemicals metabolism, are able to survive defenses within the cytoplasm before reaching the nuclear DNA is small. At cytotoxic levels, nuclear DNA might be damaged, but so would all other vital macromolecules and structures, such as the plasma membrane. These cells would die and dead cells do not give rise to cancers. From the same line of reasoning, in tissues, the cells, expressing the highest expressed metabolizing enzymes, would be the highly differentiated

cells, such as the hepatocytes. In the case of cancers being associated with the exposure to a chemical, one must remember that one cell in that organ led to the cancer. In addition, if one accepts the fact that this one cell was a normal adult stem cell (more on this hypothesis later), one must examine the DNA lesions and mutation frequency of the adult stem cell of that organ, in which the cancer arose, and compare it to the progenitor and terminally differentiated cells' DNA.

**Sixth**, in general, no in vitro assay, designed to measure genotoxicity, can be perfect surrogates of molecular, biochemical or cellular states of a cell in any organ of the intact human body. In addition, because of individual genetic, gender, developmental state differences, and the absence of physiological factors (diurnal factors, immune system, dietary and life style factors), one must remember that the population of primary, immortalized cells or cells from tumors are heterogeneous. Moreover, as pointed out above, these assays use cells grown on plastic, in log phase, in 2-dimension. None of these reflect the fact that chemicals affect cells existing in 3-dimensions, co-existing and interacting with each other via extra-cell factors (i.e., stromal-epithelial factors (12); extra-cellular matrix (13); hormones, growth factors, cytokines, nutrients, oxygen levels (14), etc.) and intercellular factors that can triggered intra-signaling by ions and small molecular weight molecules via gap junctions, cell adhesion molecules, etc. (4). In general, many of these genotoxicity assays have unknown mechanisms responsible for the endpoints being measured, e.g., comet assay; chromosome aberrations, sister chromatid assays, etc. (5). In other words, the sources of potential artifacts leading, especially to false positives, are not known. In a few cases, these endpoints are known to be caused by non-genotoxic mechanisms, such as non-specific nucleases being released by the chemical toxicants in cells killed by the chemical or by membrane leakages (14) or oxidative stress-induced intracellular signaling (4).

**Seventh**, it must be acknowledged that many of the major known environmental, medical, nutrient and dietary chemical "toxicants", that have been associated birth defects, cancer, cardiovascular disease, immuno-toxic, diabetic-inducing, reproductive- and neurotoxic dysfunctions, are not DNA damaging agents or mutagenic examples of this class of toxic chemicals include DDT, TCDD, phthalates, PBB's, PCB's, bisphenol A, phenobarbital, thalidomides, DES, saccharin, TPA, acrylamides, etc.. Yet, scientific manuscripts are still being published, using these misinterpreted "genotoxicity" assays, claiming that these chemicals are genotoxic. The "ghost in the machine" terms that are always used to claim these chemicals cause the diseases, with which they are associated, is that they are "weak mutagens", or, in the case of being associated with cancers, they are "complete carcinogens". In order to develop assays to predict the role of a chemical in the multi-stage, multi-mechanism of carcinogenesis, it is important that the assay measure the mechanism to which it reflects, in the "initiation", "promotion" or "progression" phase of carcinogenesis (15).

## A CHALLENGE TO CHEMICAL "CARCINOGENS AS MUTAGENS"

Given that, operationally, the initiation phase of carcinogenesis is an irreversible event in a single cell (probably, an adult stem cell) that can ultimately lead to the cancer, which, in children, might only take a few months or, in the case of the adult, could take 8 decades. While this irreversible event can be attributed to a DNA damage/mutagenic event, which probably takes place within twenty four hours, **initiation** in children might be due to a relatively stable epigenetic event, as is the case of a teratoma, which has been shown to be potentially reversible (16). Of course, while it is reasonable to assume that one can reduce the risk to "initiation" factors (do not expose oneself to too many UV photons), one can never reduce the risk to zero. In addition, one must remember, spontaneous "initiation" or mutagenesis does occur during an error in replication of a stem cell upon normal DNA replication on non-damaged DNA. The implication of this is usually lost when a chemical is given to an animal and a tumor is formed with a mutation in an oncogene or tumor suppressor gene. It might be that the chemical selects via its mechanism of **tumor promotion**, consisting of mitogenic and anti-apoptotic components, a previously, spontaneously mutated or initiated cell (17). Too often, a well-known chemical, associated with cancer induction in animals or human beings, is acknowledged as having promoting and epigenetic properties. Yet, it is still claimed to be a "weak mutagen" or "complete carcinogen". The implication of that kind of interpretation is that the acute or chronic exposure to this type of chemical can induce all the different mechanisms needed to bring about all the "hallmarks of cancer" (18).

This, then, is getting to the primary effects of chemicals on cells in a human being and generating a "New Vision for Toxicity Testing". In order that a chemical make any kind of impact on a cell and eventually the human being, it must confront the plasma membrane. In doing so, by binding to a receptor, by dissolving into the plasma membrane, by entering in or modifying an ion pore, or by affecting the membrane function in some manner, the chemical will have perturbed the cell's mechanism of being an environmental sensor. The evolution of a cell's ability to be adaptive to inevitable environmental changes required the membrane to be such a sensor. All chemicals will perturb the cells intra-cellular signaling pathways and mechanisms to bring about quick posttranslational modifications of existing gene products, as well as longer-term gene expressional or transcriptional responses. Chemical toxicants do so via (a) the very immediate, chemically-induced- membrane fluidity changes, (b) -changes in pH, - Ca<sup>++</sup> levels, (c) - phosphorylation/de-phosphorylation modification of proteins, (d) -redox states, or (e) -redox-sensitive transcription factors. While each type of cells {stem, progenitor, differentiated} contain the total genome (except normal germ cells, polyploidy cells, enucleated cells), these endogenous and exogenous chemicals must trigger a selective set of genes of the total genome. All chemicals that impact on a cell must, at minimum, trigger intracellular signaling an epigenetic event. If a chemical ultimately destroys a cell's vital function, it could cause necrosis by directly damaging DNA. Any DNA damage in these necrotic cells might demonstrate DNA lesions and DNA damage, but these are



down-stream consequences of prior death inducing effects (membrane destruction). Any agent, such as X rays or UV light can cause DNA damage, but also these can induce redox changes (19). Chemical agents that induce apoptosis can do so without having to damage DNA, i.e., apoptosis requires epigenetic induction of caspase genes. In other words, apoptosis can be induced by agents that do not cause DNA damage or mutations, but that these chemicals can alter gene expression.

Many chemicals, which are not genotoxic, can have a broad range of toxic animal and human health endpoints. Some of these chemicals, such as DDT, TCDD, PCB's, are known to have endocrine-disrupting, teratogenic, tumor promoting, reproductive-, immuno- and neuro-toxic effects. Since all of these adverse health effects represent different tissue/organ site in species, developmental, organ- and gender-specific fashion, the question is: "Do these very different pathological/clinical endpoints share a common underlying mechanism in response to the toxic chemical?". In these cases, since the chemical's mechanism of action is not genotoxicity or, in most cases, not due to cytotoxicity due to necrosis, the only other mechanism of action is an epigenetic mechanism. To modulate a stem cell's ability to proliferate, differentiate or apoptose properly during embryogenesis, fetal or early neonatal development could lead to embryolethality, structural or behavioral teratogenesis or even to long term later effects as predicted with the Barker hypothesis (i.e., DES-induced vaginal cancers) (20-22).

In carcinogenesis, while initiation might involve a mutagenic process, I am assuming that these non-genotoxic "carcinogens" are (a) not damaging DNA or causing error-prone DNA replication by stimulating mitogenesis (although mutagenesis requires DNA replication) and (b) not acting as "weak mutagen-carcinogens" or "complete carcinogens". Operationally, promoters have to cause the clonal expansion of the single initiated cell (23). Mechanistically, this appears to involve stimulating mitogenesis of the initiated cell and by inhibiting the apoptosis of these initiated cells (17,23). To date, most, if not all, of these non-genotypic, tumor promoting chemicals act by reversibly inhibiting cell-cell intercellular communication (either the secreted form or the gap junctional-mediated form) (24). Modulating gap junctional intercellular communication (increasing or decreasing) could affect its function in regulating "contact-inhibition" of GJIC-functioning cells, the differentiation of cells and apoptosis of other cells (25,26). It is now known that GJIC is mediated by 20 different gap junction-coding genes (27,28) and seem to be associated with specific cell types (liver oval cells express Cx43, while hepatocytes in the same organ express Cx26 and Cx32). Gap junction function can be regulated from the transcriptional, translational and posttranslational levels, translocation and assembly, functioning-gating in the membranes (26). Chemicals that affect GJIC do so via many different biochemical mechanisms. Few, if any, of these chemicals act directly on the gap junction protein structure directly.

The fact that chemicals that affect tumor promotion, reproductive toxicity, cardiotoxicity, reproductive and neurotoxicity might seem to be working by different mechanisms, one needs only to examine the fact that gap junctions are required in the gonads for maturation

of sperm and eggs. The heart's action as a finely-tuned pump depends on the synchronized contraction of cardiomyocytes. Arrhythmias could result by disruption of GJIC between the electrotic signal passed through gap junctions (29). The dependence of neuronal functioning, in large part, depends on astrocyte-neural GJIC, as much as chemical neurotransmission (30). The inheritance of various mutant forms of several connexin genes demonstrate their roles in several human diseases (Charcot Marie-Tooth syndrome (31). To make a complex story short, gap junctional communication exists in all human organs and can be modulated by both endogenous physiological chemicals, as well as by a wide range of no-genotoxic or cytotoxic chemicals at non-cytotoxic levels. They can also be modulated by the released endogenous products of cells, such as Kuffer cells, neurophils, macrophages, stellate cells, or cells killed by necrosis.

## LESSONS LEARNED FROM CHEMICAL TUMOR PROMOTERS AND THE TUMOR PROMOTION PROCESS

Basically, these chemicals, which act as tumor promoters can down regulate GJIC at non-cytotoxic concentrations. As promoters, they must act above threshold levels, from hormone- like levels for the chemicals acting with receptors (phorbol ester); at higher concentrations for lipophilic chemicals that do not have receptors (DDT), or at much higher concentrations for those that are aqueous-soluble (saccharin). As promoters, these chemicals must be present in the organism for regular and long term exposures. In addition, if the chemical exposure is stopped or if the exposure is irregular, promotion of the initiated cell does not occur. In effect, tumor promotion is an interruptible and possibly a reversible event during the tumor process. However, if the chemical inhibits GJIC during a critical period of development, when cell communication induces cell differentiation, it could be classified as a teratogen. Lastly, tumor promoters can only inhibit GJIC in the absence of agents that might interfere with its specific mechanism of action. If the chemical promoter induces oxidative stress, which triggers signaling to alter both gene expression and inhibition of GJIC to cause mitogenesis but acts on cells with antioxidants present to negate the redox changes, nothing will happen (32). Chemicals can act via receptor dependent and receptor-independent mechanisms. Estrogens at low concentrations can affect development by acting via specific estrogen receptors. On the other had, at higher concentrations, it can act on cells with receptors, as well as via redox-induced oxidative stress signaling (33). On cells without estrogen receptors, estrogens could simply bring about toxic effects via estrogen receptor independent redox signaling at high concentrations.

If this seems far too complicated for chemical regulation, it gets even more complex when one realizes a chemical can exhibit both the "good news/bad news" label. The fact that thalidomide, a modulator of GJIC (34,35) is an effective sedative, human limb teratogen and anti-angiogenesis factor for cancer therapy seems like a regulator's nightmare (35-37). Retinoids, which can modulate GJIC (38) a known human teratogen (39), can be both a cancer chemotherapeu-

tic agent (40) and a tumor promoter (40-42), depending on the circumstances. The failure of the human intervention trials is to reduce cigarette smoking risk to lung cancer (CARET & ABTC trials) (43,44) might be another example. Even epigenetic compounds, as ethinyl estradiol and TCDD, can be ant-carcinogens under certain circumstances (45,46).

## WHERE DO WE GO FROM HERE?

Today, with the known limitations of current paradigms of chemical toxicity, limitations of animal bioassays, of epidemiological studies, and of in vitro assays, short of testing all new chemicals on human volunteers (both sexes, all developmental stages) and the impossibility of testing them on a representative of all the different human genomic variant, one is faced with a complex dilemma. Clearly, an ex-vivo or 3-dimensional in vitro assay model system has to be developed using normal human cells. However, not just any normal human cell needs to be used, but rather these 3-dimensional organoids must be initiated by adult stem cells. Given that the target cell for many, but not all, chemical toxicants, will be the embryonic and germ line and adult stem cells, creating the niche micro-environment in vitro will have to be done in order to maintain the adult stem cell in its normal quiescent state (low oxygen tension; correct niche substrates, etc.). Each adult stem cell at each state of developmental restriction (embryonic- pluri-potent-multi-potent- bi-polar to uni-polar) from each organ and from both genders, in principle, should be proposed.

## CELL-CELL COMMUNICATION AS AN ENDPOINT IN A “SYSTEMS” APPROACH TO STUDY CHEMICAL TOXICITY

In summary, the evidence is abundantly clear that the current paradigm and assays are inadequate and incomplete, if not just plain incorrect, to predict the wide range of potential toxicities and potential pathological outcomes after animals, humans, non-human species and the biological ecological system. Faced with the impossibility of finding an absolute risk free toxic consequence of any chemical after exposure to all living species and individuals, one must deal with trying to find a mechanistically-based assay, having relevance to one of the three cellular responses of a cell type to a chemical. Using human stem cells from various human organs, brain, liver, kidney, lung, gonads, etc., 3-dimensional organoids (mammospheres, prostatospheres, liver spheres) must be grown in low oxygen atmospheres with cultural micro-environments that come close to the in vivo niches.

Furthermore, given that chemical toxicants, which act as epigenetic agents, trigger intra-cellular signaling within seconds, it must be stressed that the modulation of gap junction function occurs prior to any transcriptional changes in gene expression. Demonstration of altered gene expression is down stream of what the consequences of epigenetic toxicants do to inter-cellular communication. Disruption of homeostatic control of cellular functions can be either adaptive or

mal-adaptive consequences of modulating cell communication, depending on the circumstances of chemical exposure. In other words, linking the chemical triggering of intracellular signaling within a cell and the alteration of gene expression in that cell, cell-cell communication is the critical integrating factor to have an idea what happens on a “systems” view within an organ and organism (47). Obviously, we have a long ways to go.

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# COMMENTARY ON "TOXICITY TESTING IN THE 21st CENTURY: A VISION AND A STRATEGY"

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## ABSTRACT

The report of the National Academy of Sciences entitled "Toxicity Testing in the 21st Century: A Vision and a Strategy", hereinafter referred to as "The Report", is more of a vision than of a strategy. The present article addresses three observations made on The Report; namely, dose response, PBPK modeling, and in vitro testing. An additional observation this author has of the document is that there is missing from the document a role for a scientist who can analyze the big picture. Science today is necessarily composed of specialists in many areas because science today encompasses many diverse, specific fields. Each specialist is in a world of his or her own and unable to integrate all the facts. Must we wait for another Newton or Einstein?

## INTRODUCTION

This is a commentary on the recent report "Toxicity Testing in the 21st Century: A Vision and a Strategy" 1, hereinafter referred to as "The Report", which was published by the National Academies of Science. In the opinion of this author the report is more wishful thinking than it is a strategy. Admittedly, the report was intended mostly to be visionary and thus mostly lacking specifics, but it missed the opportunity of being more specific on certain subjects and therefore more useful. In the opinion of this author, the current scheme of toxicity testing and its dependent risk assessment procedures are sufficiently flawed and need a serious and profound overhaul. This author has been asked to comment on The Report and address certain points. Among those points, this author will address

only those on which he has published or that are of interest to him. Therefore the present article deals only with three topics; namely, dose response, which includes dose selection for experiments; PBPK modeling; and in vitro testing.

## OBSERVATION NUMBER ONE: DOSE RESPONSE.

Dose-response analysis is critical. This is an area of more importance than most toxicologists are aware. For example, currently there is a big debate over whether or not there are thresholds for carcinogenicity of DNA-reactive compounds. Fundamental laws of science have been ignored up to the current time in low dose extrapolation of the linear model for dose response for this particular toxicity<sup>2</sup>. Coupled with this is hormesis; extending the range of doses to include those below the onset of carcinogenesis or toxic effect should always be done; this has rarely been done in the past. This would likely reveal whether or not hormesis is present<sup>3</sup>.

## OBSERVATION NUMBER TWO: PBPK MODELING.

PBPK modeling is currently the subject of many papers. The flaws in this technique have not been widely discussed. Whole-body autoradiography has taught us some potential flaws in PBPK predicted concentrations. Some types of cells in a tissue or subcellular organelles can have much higher affinities for chemicals than other cells in that tissue or other subcellular structures<sup>4</sup>. Calculation of the concentration in a tissue from its partition coefficient, measured in vitro, can be very misleading compared to the in vivo distribution of that chemical or its metabolites. Examples of misleading distributions are lung, adrenal gland, ovary, fat, eye, liver, and placenta. Some cells in these organs take up specific compounds preferentially, depending on metabolic activity in vivo. An attempt is made in The Report to address this point by a discussion of metabolites from chemicals, but mutual interaction from effects on tissues is not addressed. For example, effects on an organ that would change the pH value in other tissues are not addressed. The intracellular distribution of many acids and bases (which includes many compounds considered to be toxic) is influenced by the pH of the extracellular fluid. Furthermore, when a certain cell type takes up a significant amount of the test compound, the concentration in the extracellular fluid (the medium) might decrease causing an error in that measurement.

## OBSERVATION NUMBER THREE: IN VITRO TESTING.

The goal of in vitro testing in order to obviate the need for animal sacrifice is, of course, laudable. It may come eventually, but that day probably is far away. Increments of toxicologic concern will be met stepwise for some toxicologic endpoints, but the whole animal will be needed for the foreseeable future. The whole animal is more

than the sum of human cell lines; the interaction among cells and organs to maintain homeostasis of the entire individual cannot be evaluated currently by studying toxicity to isolated cell lines.

## CONCLUSION

The current scheme for toxicity testing began in the 1950's with the notion that testing for toxicity at high doses in animals and then extrapolating linearly to predict toxicity reliably at the low doses to which humans are commonly exposed. This concept led to many experiments in mice and rats and, unfortunately, still persists today; although some publications currently have begun to question this approach. In the opinion of this author, the concept of testing at high doses in animals and extrapolating linearly to low doses in humans is so severely flawed that a total revision in this concept is needed for risk assessment. Not only does this ignore the fundamental laws of nature, but also it obscures the possibility for the occurrence of hormesis.

This commentary addresses and criticizes three of the points raised in The Report: dose response, PBPK modeling, and in vitro testing to predict whole-body response. In addition, this author finds, like in other sciences, there is a need for someone to see the overall picture. Barring some as yet unrecognized genius, perhaps it will ultimately become evident to a convincing majority of scientists that a better scheme for predicting toxicity to humans than the current scheme is possible.

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# AN INTEGRATED SUMMARY OF COMMENTARY ON THE NATIONAL ACADEMY OF SCIENCES REPORT ON “TOXICITY TESTING IN THE 21<sup>ST</sup> CENTURY: A VISION AND A STRATEGY”

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The purpose of toxicity testing is ultimately to be able to estimate the probability of an adverse event occurring in humans or other defined target organisms after exposure to a potentially toxic material under defined conditions of exposure. Indeed, the report of the National Academy of Sciences entitled “Toxicity Testing in the 21<sup>st</sup> Century: A Vision and a Strategy” (1) (hereafter referred to as “The Vision”) should have made this goal clear. Instead, the report envisions a future scheme that involves the use of systems biology, bioinformatics, and rapid assay technologies assembled in a manner to obviate the current testing paradigm involving numerous animal and *in vitro* tests plus exposure assessments to reach an estimate of the probability of an adverse event.

“The Vision” foresees new tests that define molecular level changes that will better predict how chemical exposures do or do not cause adverse health effects and define sensitive populations. Major goals of these new tests seem to be to reduce animal use and suffering and to reduce the backlog of untested chemicals. Given the need for standardization and validation of the “new” tests, it is likely that the numbers of animals needed for these purposes would greatly exceed the numbers of animals now used in testing.

None of this is to say that “The Vision” would not be extremely valuable in furthering the understanding of how materials perturb biological systems and ultimately lead to adverse events which would clearly provide a strong basis for the interpretation of animal and *in vitro* test results. Most likely, this improved understanding of the mechanisms involved could in itself lead to reduced animal use and better predictions of the likelihood of an adverse event occurring under defined conditions of exposure.

Included in this issue are five independent commentaries on “The Vision” by experienced and well-known toxicologists who offer their views on the merits, and non-merits, of the NRC/NAS report. They are:

1. Toxicity Testing in the 21<sup>st</sup> Century: A Vision and a Strategy. A commentary by Robert F. Phalen, Ph.D., The Department of Medicine, University of California, Irvine.
2. Comments on “Toxicity Testing in the 21<sup>st</sup> Century: A Vision and a Strategy”, William J. Waddell, M.D., Department of Pharmacology and Toxicology, School of Medicine, University of Louisville, Louisville.
3. (A) Challenge to the NAS Report, “Toxicity Testing in the 21<sup>st</sup> Century: A Vision and a Strategy”. “Stem Cells and Cell-Cell Communication as Fundamental Targets in Assessing the Potential Toxicity of Chemicals”, James E. Trosko, Ph.D., Center for Integrative Toxicology, Food Safety Toxicology Center, Department of Pediatrics/ Human Development, College of Human Medicine, Michigan State University, East Lansing.
4. Commentary on “Toxicity Testing in the 21<sup>st</sup> Century”, Sorell L. Schwartz, Department of Pharmacology, Georgetown University Medical Center, Washington, D.C.
5. Commentary on “Toxicity Testing in the 21<sup>st</sup> Century: A Vision and a Strategy” by the National Research Council of the Nation Academy of Sciences, Robyn L. Prueitt and Barbara D. Beck, Gradient Corporation, Cambridge, MA.

What follows is an integration of their collective views on “The Vision”. First of all, there were many positive attributes identified by the commentators, not the least of which was the quality, dedication and commitment of the committee members (1) to find solutions to the cost, time and animal use associated with the current toxicology testing paradigm. In addition, “The Vision” has not gone unnoticed by other commentators who have published their thoughts ranging from the need to consider the definition of an “adverse effect” as distinct from an “adaptive effect”, the importance of broad discussion and debate concerning “The Vision”, that the technologies proposed by “The Vision” not be applied to the process of human risk assessment until the relevance of the data is fully developed, that characterization of exposure is required to translate the toxicology information into the decision making process for risk assessment, and that we avoid the mistakes from *in vivo* approaches such as the use of the maximum tolerated dose (2, 3, 4, 5, 6).

The present commentators were in general agreement on a number of key points raised by “The Vision” document. Among them was that the present integrated *in-vivo*, *in-vitro* and *in-silico* testing complemented with epidemiologic and clinical data may be a currently effective system for protecting health or may be completely inadequate, incomplete, or incorrect to predict a wide range of adverse effects in biological systems. In any case, there will be a requirement for considerable research on unproven methods before replacing the current scheme. The primary basis for this belief is that a whole animal study includes all the cell types and the various interactions that rage throughout the cell types, tissues, organs and organ systems. The biochemical and physiologic processes of the whole body cannot be duplicated by a mixture of cell cultures according to the commentators. For example, there is no genotoxicity assay that perfectly replicates the molecular, biochemical or cellular state of a cell in the intact animal. As one commentator put it, “in order that a chemical make any kind of impact on a cell and eventually the human being, it must confront the plasma membrane. In doing so, by binding to a receptor, by dissolving into the plasma membrane, by entering in or modifying an ion pore, or by affecting the membrane function in some manner, the chemical will have perturbed the cell’s mechanism of being an environmental sensor.” Thus, chemical toxicants may alter intra-cellular signaling pathways and mechanisms and modify posttranslational gene products as well as gene expression or transcriptional events in a manner different from *in-vitro* to *in-vivo*.

“The Vision” foresees two components to the paradigm shift away from use of experimental animals and apical endpoints that consist of “toxicity-pathway assays” and “targeted testing”. The “toxicity-pathway assays” are intended to find cellular and genetic dysfunctions while the “targeted testing” is to design and conduct studies to refine the data from the “toxicity-pathway assays”. To the commentators this raises questions about the determination of the dose-response and the extrapolation modeling. “The Vision” seems to assume that dose-responses will be monotonic which is problematic given the plethora of data demonstrating non-linear dose-response. In general, the commentators acknowledged the importance of PBPK modeling in the extrapolation of *in-vitro* toxicity data to the intact animal. Moreover, the question of human genetic variation seems to be left dangling and it is well known that such variation will alter the response to chemical exposure. Suggestions as to how to address this conundrum would have improved the overall premise of “The Vision”.

A key recommendation from the commentators is that validation of the proposed new “tests” include chemicals that have positive or negative responses that are readily explained by known modes of action that are relevant to whole animal response. A complicating example in this case would be the essentiality of metals in comparison to their toxicity in excess.

In conclusion it seems that the necessary leap from “The Vision” to the regulatory use of the information generated is huge and may well limit the eventual implementation of the foreseen scheme. Moving from known, and understood, apical responses to the complexity of

integrated cell culture and computer model data for the purpose of estimating the probability of an adverse event due to a material under known conditions of use may never be possible.

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