"Establishment Of A Bioassay System For Cancer Risk Assessment In Energy Technology"

A Report on The Conference
Held in May, 1982

Health Effects Research Division

Office of Health and Environmental Research

U.S. Department of Energy

and

Division of Biophysics

School of Hygiene and Public Health

The Johns Hopkins University

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"Establishment of a Bioassay System for Cancer Risk Assessment

in Energy Technology"

May 3 - 5, 1982

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DISCLAIMER

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A Report on the Conference On

"Establishment of a Bioassay System
for Cancer Risk Assessment in Energy Technology"

May 3 to May 5, 1982

The School of Hygiene and Public Health
The Johns Hopkins University
Baltimore, Maryland

Organized By

Health Effects Research Division
Office of Health and Environmental Research
U.S. Department of Energy

With Scientific and Technical Assistance From

Division of Biophysics
School of Hygiene and Public Health
The Johns Hopkins University

I.   Introduction

For several years the Department of Energy (DOE), Office of Health and
Environmental Research (OHER), has supported a research program aimed at
developing new experimental approaches for the improvement of cancer risk
assessments. The central issue is to overcome the organizational, species and
other barriers that make it difficult to extrapolate laboratory-based data to
predict risk to man. One approach for accomplishing that goal has been
proposed by Dr. Paul Ts'o at The Johns Hopkins School of Hygiene and Public
Health. A test of the feasibility of the proposed strategy is the substance
of an OHER research grant to Dr. Ts'o and is the reason why it was decided
that the meeting should be organized by and held at that institution. The
main objectives of the meeting were to review the status of knowledge and
methodologies available from relevant research in this country and abroad.

Emphasis of the Meeting

The emphasis of the meeting was restricted to consideration of cancer risk
from exposure to chemical agents. That subject reflects a major national need
and is relevant to most of the technologies of interest to DOE. National
programs, other than DOE, are concerned with individual chemical compounds and
most of the participants at the meeting are involved in research aimed at
understanding the mechanism(s) of chemical carcinogenesis. Complex mixtures
of chemicals are associated with many energy technologies, and DOE's initial
program emphasis focused on semi-applied research aimed at quantitative
evaluation of carcinogenic activity of complex materials. Since much progress
has been made in DOE integrated technology-specific chemical-biological
characterization studies, the number and kinds of chemicals of concern has

been reduced to a relatively few well-defined classes. Although the classes of compounds seem to be unique to some of the synfuel technologies, they are quite similar to compounds of general interest, for example, poly-nuclear aromatic hydrocarbons. Special emphasis was placed on molecular and cellular dosimetry as one of the key requirements for quantitative comparison of effects at the cell level in vivo and in vitro. Although it is relatively easy to measure cell, tissue, organ and whole organism doses associated with radiation exposures, we are just learning how to do this for chemical agents. Several methods have been developed in the past several years which can be used.

Participation at the Meeting

Thirty-six scientists attended and participated in the various sessions of the meeting. All of the participants were selected for their expertise in one of the following research areas: viral and molecular oncology, cellular oncology, metabolism and dosimetry of carcinogens, animal carcinogenesis and human cancer epidemiology. The investigators were representatives of DOE laboratories, departments of several universities and other national and international groups concerned with cancer research.

Organization of the Meeting

The first day was composed of eight scientific reports covering a number of new findings and methodologies in cancer research. Almost two full days were spent in workshops devoted to the key subjects and issues related to the general subject of the meeting. For example, each of the following subjects constituted a workshop session: target cell concepts, human cell experimentation, DNA modification and metabolism, molecular biology and virology, and assessment methodology. Those subjects were discussed in detail by experts in the relevant scientific disciplines. Each workshop session was followed by a plenary session wherein the several chairpersons summarized the deliberations of the individual workshops prior to general discussion of key scientific issues.

The second major discussion session attempted to cut across three major issues considered central to the conference: host factors that control or regulate carcinogenesis; ways to differentiate between initiation and promotion of tumor developments as well as chemicals that have those properties; the need for quantitation and calibration if one expects to intercompare results obtained at various levels of biological organization. The limitation to three issues allowed a greater spread of expertise with two to twelve experts per group.

The third session was meant to review the progress which has been made in developing basic information, new test systems and new methodology for implementation of a comparative multilevel bioassay network for assessing cancer risk.

An OHER Perspective for the Major Meeting Goals

The major goals of the meeting can be described in a number of ways depending on the perspective of the organizers and participants. The ultimate establishment of a systematic bioassay network system to better assess human cancer risk is the goal of a research program that is only partly implemented in DOE and other agencies. The major purpose of the conference was to evaluate progress being made toward that goal and thus assess the strengths and the weaknesses of the concept. From an OHER point of view, the goals of the meeting conform with the expressed goals of the office's health research program, namely:

1. To advance fundamental scientific knowledge so that predictions of redundant effects of energy-related pollutants will be used on a sound data base.

2. To develop on a continuing basis new short and long term bioassay systems to improve the cost-effectiveness of conducting research aimed at risk assessment.

3. To conduct human epidemiological studies for human populations that relate in a meaningful way to an assessment or risk.

4. To support theoretical and experimental efforts that facilitate information collection, synthesis, analysis and interpretation especially relating to late effects and low level exposure.

II. CONFERENCE DESCRIPTION

This conference was organized to obtain the most useful information and the best advice for the establishment of the desired bioassay system for cancer risk assessment in energy technology. Twenty specific questions pertinent to this goal were sent to the participants prior to their attendance at the meeting. Answers were collected and presentation and discussion were held in a three day conference toward the following objectives:

I. Goals for a New and Quantitative Bioassay System in Cancer/Mutation Risk Assessment for Energy Technology

1. The system will establish a quantitative bioassay for cancer risk for a few technology options (such as nuclear, fossil, shale).

    a. The system can be used to rank these options from the most safe to the most unsafe.

    b. The differences between the options should be evaluated with a degree of certainty for as small a difference as one-fold, in risk (or safety) between each option.

    c. The cancer risk in these options can be compared to some commonly accepted standards which then can be used for policy decisions.

    d. The bioassay system is designed to assess risk in the energy technologies for production and consumption. For instance, it should be designed to measure complex mixtures at low dosage.

2. The bioassay system is not designed to handle a large number (100-1000) of unknown compounds for consumer protection or for detecting a large variety of environmental carcinogens.

3. The bioassay system is not designed to locate the major cause of cancer in the population, so as to reduce the current incidence of cancer.

4. The quantitative results from the bioassay system should be obtainable within a reasonably short time, i.e. 3-6 months.

5. The bioassay system which is adopted can be continuously improved in a stepwise, modular manner without necessitating a complete change.

6. The bioassay system to be developed at present in the laboratory should, upon improvement, be capable of testing human populations in the field.

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II. Rationale for the Development of New Bioassay

1. It is recognized by those in various fields of economic activity, including energy production and consumption, that quantitative cancer risk assessment must be made. To make this assessment, a reliable bioassay system is a necessary and major component. Other components, such as assessment of exposure, pharmacokinetics, genetic variability, and so on, are also important. Nevertheless, a reliable and quantitative bioassay which can give information directly relevant to humans, is an absolute necessity.

2. Because of the problems of low dose and the problem of extrapolation to humans, the use of current animal bioassay systems is woefully inadequate and inappropriate. Equally serious problems are heavy cost ($300,000/compound) and the time requirement (1-2 years).

3. While epidemiology has been very powerful in demonstrating the plausible causes of cancer, it is not possible to make a very quantitative assessment of risk to humans among several options with relatively small differences in risk. It is particularly inapplicable for policy determination on the development and employment of new technology, especially since such a policy has to be formulated within a very short period of time, say from 1-3 years.

4. For these reasons, a new bioassay system must be developed which will use human material directly, and which can be used to obtain information on a quantitative basis within a short duration.

III. Characteristics

1. The major emphasis of the new bioassay system is on cells in culture, but the system will also utilize subcellular measurements as well as intact animals in an integrated fashion. Considerable emphasis will be given to the requirement of duration in completing the experiments, such as within a period of a few months.

2. The bioassay system will have a main emphasis on human material, particularly normal diploid cells. The fibroblast is likely to be the first system to be applied, but hematopoietic tissue and epithelial cells will also be included.

3. The bioassay system will include cells from various stages of differentiation and development. Embryonic cells and newborn tissue are likely to be used first, and these will then be extended to adult tissues.

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4. In this bioassay system, one or two animal systems will also be used in parallel in order to:

    a. Learn about the extrapolation from cells in culture to organisms _in vivo._

    b. Serve as host in human cell tumorigenicity assays as well as to serve as a chimera for the study of human cell transformation in the appropriate animal host.

5. The bioassay system should consist of several informational and correlated matrices. The assay system should be designed in a modular pattern with the results highly correlated and integrated. Many laboratories can participate in and develop this system together. Within the original developmental framework, continuous modifications and improvements can be made. The bioassay system should gain in efficiency with time and each individual unknown agent to be investigated can be studied at various levels of thoroughness and accuracy of assessment.

6. A set of cancer risk reference units needs to be established for this bioassay system. The assessment of an unknown agent for cancer risk can always be compared to a set of external references and possibly a set of internal references for damage. This comparison to known cancer risk units allows quantitative assessment and the basis for policy decisions.

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IV. QUESTIONNAIRE

Introduction:

The objective of this conference (see Section II), is to develop a general strategy in cellular and subcellular studies which can provide biological data relevant to quantitative cancer risk assessment in energy technologies which usually deal with chronic, low dosage exposure of a complex mixture. Special emphasis should be on the biological significance, the interpretation, and the extrapolation of the data obtained from cellular and molecular studies. We hope to articulate this challenge by raising 20 specific questions described below. Attached also is a chapter which may also be very useful as a reference. For instance, you are welcome to comment upon the answers and strategy proposed in this chapter.

Questions:

1. Comparison of the advantages and disadvantages of in vitro bioassay (cellular and molecular system) versus whole animal bioassay and epidemiological approach in order to reach the outlined goals: competition and complementation of those approaches.

2. Comparison of advantages and disadvantages of using human materials (tissue and cells) versus animal materials (mostly rodents).

3. What are the current status and problems in human cell transformation? What are the current problems in the in vitro culture of normal and transformed human cells?

4. Which animal species should be used: Mouse, rat, Syrian hamster, Chinese hamster, guinea pig, rabbit, dog, primate, etc.? How many, which ones, and priority; which strain or breeds within the species, if there is special recommendation?

5. Comparison between human cell neoplastic transformation and rodent cell neoplastic transformation.

6. Which tissue systems/cell types should be used: fibroblast, epithelial cells, hematopoietic tissue, germ cells, etc.? How many, which ones, and priority.

7. Tissue/cells from which developmental stages should be used? Embryonic (early or late), newborn, young adult, adult, aged? How many, which ones priority.

8. How to integrate and connect the bioassay system to the metabolic activation system, the pharmacokinetic/detoxification/repair system, etc. What are the cellular and molecular criteria/parameters for determining neoplastic transformation (or tumorigenicity) of the human cells to be transformed in vitro? Which molecular and cellular endpoints/measurements should be adopted? Can these endpoints/measurements be built into a matrix system of in vitro bioassay? What are the best procedures for such measurements?

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9. Selection of topics for meassurements or as parameters:

    a. DNA/RNA/protein/membrane damage (alkaline elution, sister chromatid exchange, DNA synthesis, etc.). Special emphasis should be given to the possible use of monoclonal antibodies for the identification and quantitation of DNA damage and attachment of carcinogens to DNA.

    b. Chromosomal damage and/or karyotypic changes (banding, automation, flow cytometry, etc.)

    c. Cytotoxicity and early senescence.

    d. Somatic mutation (microbial/insect/plant mutation) (Which trait: dominant, sex-linked recessive, autosomal recessive? Which locus: Na^+/K^+ ATPase, HGPRT, TK, APRT, etc?).

    e. Gene expression changes (2D gels, immunological identification, monoclonal antibody, mRNA [DNA library/mapping, in situ hybridization], expression of "oncogenes", etc.). Special emphasis should be given to the search for neoplasia-related mRNA through the recombinant DNA technique.

    f. Neoplastic transformation: loss of contact inhibition, reduction in nutrient requirements [low serum, low calcium, etc.], anchorage-independent growth [cloning in soft agar], etc..

    g. Organ/tissue/germ cell changes.

    h. Tumorigenicity (newborn animals, immunologically deficient animals [nude mouse], metastasis assay). Special emphasis should be given to the quantification and the time required for the assay.

10. Quantitative correlation and coordination of all the molecular and cellular measurements in a bioassay system. Can a matrix system be established?

11. Procedure and choices for the establishment of the carcinogen/mutagen reference unit. External reference unit? Internal reference unit?

12. What are the relationships between viral carcinogenesis versus chemical/physical carcinogenesis as related to this bioassay system? Are there common mechanisms? Will these two perturbations work synergistically? What is the relationship between the viral transforming genes and the presumptive host (human) "oncogenes"?

13. What are the basic mechanisms of neoplastic transformation/carcinogenesis as related to the establishment of this bioassay system? What are the genetic and developmental determinants in neoplastic transformation: DNA damage? chromosomal changes? developmental (epigenetic) factors? initiation and promotion? role of promoters?

14. How to coordinate the application of this bioassay system with the animal bioassay approach and epidemiological approach? How to correlate the animal data with the human data? How to extrapolate the data on tissue/cells _in vitro_ to whole organisms (animal and human)?

15. What type of basic research is needed for the development of this bioassay system?

16. How to correlate the bioassay system to other components of risk assessment, such as exposure, other types of toxicity (neurological disorders, for instance), etc.?

17. How to standardize procedures, correlate results and efforts of all participating laboratories, etc.

18. How much manpower, material resources are needed to develop this bioassay system and put this system into operation?

19. How to develop measurements which can be used to monitor humans in the field?

20. For the application of the bioassay system in risk assessment for energy technology, there are important questions about the management of the system, such as the selection, the procurement, the storage and the distribution of the test substances, as well as the reference standards; the management of the information about the test substances before and after testing; proper interpretations of the data for policy decisions, etc.

21. Other pertinent questions/comments.

SELECTED EXCERPTS FROM REPLIES TO THE QUESTIONNAIRE

Question #1.

Comparison of the advantages and disadvantages of in vitro bioassay (cellular and molecular system) versus whole animal bioassay and epidemiological approach in order to reach the outlined goals: competition and complementation of these approaches.

Answer: Richard J. Albertini

"I see the following as some of the advantages and disadvantages of (A) in vitro cell systems; (B) whole animal systems, and (C) epidemiology:

A. In vitro cell systems

    Advantages:

    a. Mammalian cells provide some sort of genetic 'target realism'.
    b. The systems are easy to manipulate experimentally.
    c. Large numbers of units (cells) are studied.
    d. Can potentially dissect compound events by cloning.

    Disadvantages:

    a. Lack of metabolic or pharmacokinetic realism.
    b. Lack of whole animal homeostatic or protective mechanisms.
    c. Potential for bias because of in vitro selection.

B. In vivo whole animals systems

    Advantages:

    a. Metabolic and pharmacokinetic realism.
    b. Includes whole animal homeostatic and protective mechanisms.
    c. Provides a better correlate of human diseases than do established cell systems.

    Disadvantages:

    a. Large cost.
    b. Long duration.
    c. Ultimate certainty concerning extrapolation to man.

        i.   Different genetic structure
        ii. Extrapolation must assume homogeneity of susceptibility in humans.

C.   Epidemiological studies (human)

   <u>Advantayes:</u>

   a.   The most realistic endpoint for measuriny human disease.
   b.   The ultimate in taryet, metabolic and pharmacokinetic realism.
   c.   Determines the effects of environmental mixtures.
   d.   Can potentially detect populative heteroyeneity reyarding susceptibility.

   <u>Disadvantayes:</u>

   a.   Classical epidemioloyy is the weakest when detecting small effects
   b.   Can not manipulate experimentally.
   c.   Would hope to use other than the sick individual as the endpoint for monitoring studies.

Clearly, these three systems describe a scale, with 'realism for human disease' best attained by human epidemioloyical studies, and 'understandiny of basic mechanisms' best attained by <u>in vitro</u> cell and subcellular systems. The latter, of course, can be experimentally manipulated with relative ease. Ultimtely, when understandiny is complete, it will allow realistic quantitative human health risk assessments to be made from <u>in vitro</u> assays. However, until then, risk assessments derived from <u>in vitro</u> data are based on models, which may or may not be correct. I feel that, at present, to avoid a serious loss of credibility, pronouncements reyarding human health risks must have some relationship to human epidemioloyical studies. The trick is in linkiny laboratory test results with the results of epidemioloyical and clinical studies."

Answer: Antone L. Brooks

"The advantayes of <u>in vitro</u> assays are obvious since they are cheaper, faster, and it is possible to provide better control of experimental variables. They can identify potential hazards or environmental health problems. However, the information derived has limited application since disease processes involve many systems in the whole animal. The real need is to understand the adverse health effects in man from contact witn elements, compounds and complex materials produced in energy extraction and conversion processes. 'Health effects', as they appear in man, are manifested as functional and morpholoyical alterations occurriny as the result of the integration of many processes: uptake, translocation, metabolism, cell injury and repair. The sequence of events and conditions determininy whether a 'health effect' is manifested, are only partially understood, but it is certain that a multitude of host factors play important roles in the process.

In vitro assays operate on the assumption that sinyle cells or yroups of cells may be affected by the material in question in an observable way which can be correlted with the expression of a health effect in man. Thus, it is assumed that cytotoxicity <u>in vitro</u> relates to cell killiny or injury <u>in vivo</u> and that chromosomal or yenetic alterations <u>in vitro</u> relates to yenetic abnormalities and tumoriyenesis <u>in vivo</u>. In a few yenotoxicity assay systems,

these correlations have been demonstrated, while in others they have not.

At present, it appears that the in vitro systems can play a particularly useful role in the ranking of potentially toxic materials, with respect to specific biological activities, i.e. chromosomal alterations, mutations, cell killing or other forms of cell injury. The in vitro test systems are characteristically more rapid and less expensive than whole animal assays; thus a broader spectrum of materials can be more quickly ranked with respect to toxicity than would be practical with whole animals. Most of the energy processes in question involve large numbers of individual compounds and mixtures of compounds, and it is in the screening and ranking of these materials that the in vitro tests excel.

Given the present state of knowledge, the in vitro tests cannot by themselves yield the answers that are ultimately sought. These essays simply do not include many of the mechanisms and factors which determin the manifestation of health effects in man. Whole animals assays, despite the time and cost required, are the only "test systems" in hand which allow these mechanisms and factors to operate in the way that they do in man. There is still much to be learned about the correlations between the expression of health effects in animals and man; however, our current understanding in this area allows a much more confident extrapolation than from in vitro tests to man.

In objectively reviewing the toxicological assay presently in hand, two facts stand out. First, pitting in vitro and in vivo assays against one another and attempting to choose between the two is absurd. These classes of assays are best used in concert to provide different answers and both are vital to accomplish the primary task, estimating adverse health effects in man. Although there is some overlap, which will hopefully increase as our understanding increases, the in vitro assays are best used as a first line of investigation of the overwhelming number of compounds in question and the whole animal assays are best employed to study a few, well-chosen representatives of classes of compounds or combination of materials. It is presently impractical to expect either type of assay to accomplish the entire task alone.

The second fact is that one of the most important (if not the most important) scientific problem facing the field of toxicology today is the need for a better understanding of the relationships between in vitro test results and the expression of adverse health effects in man. The desirability of accurately predicting a material's potential for inducing health disorders in man, by performing relatively simple, rapid and inexpensive assays which minimize the discomfort and death of animals, is obvious. This should be a primary goal of investigators working with both types of assays. The increased pressures recently being brought to bear to induce legislative action limiting the experimental use of whole animals has merit in its stated intent; however, it is naive to believe we have reached the point where whole animal assays are unnecessary. Indeed, we are only approaching the point where our experimental abilities will let us establish the very correlations which may let us move in the direction that those factions (and hopefully all of us) desire. The time is ripe for inidividuals having their careers couched in in vitro and whole animal assays to redouble their interactive efforts to

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understand the relationships between their results. The extrapolation of in
vitro test results to health effects in man can only be established using the
pathway passing through whole animal studies.

Another very important area begging for improved understanding and
methodology is that of human epidemiology and an understanding of the
relationship between the expression of health effects in whole animals studies
to that in human populations. Most whole-animal studies are, after all,
carefully planned and controlled prospective epidemiological studies.
Although the specific actions may be yet undefined, it seems apparent that
there needs to be closer interaction between individuals studying the animal
and human populations. These interactions should include both investigators
working 'hands on' with clinically-, occupationally- and environmentally-
defined populations and those whose stock and trade is the statistical
manipulation of data."

Answer: Joseph DiPaolo

"One very important consideration in assessing risk assessment is the
element of time. After all, if deleterious pressures are being placed on our
environment more frequently than they were ever in the past, it is important
that some sort of answer be obtained as quickly as possible as to their
potential effect. In vitro bioassays, be they cellular, subcellular, or
molecular, have the advantage over whole animal bioassay models in that a
definitive endpoint is attainable much more quickly than long-term animal
studies. Whereas animal bioassay systems in order to be really valuable must
involve whole life studies and require a large quantity of personnel, space,
and expenses, the in vitro assays should be completed in a relatively short
period of time. In vitro bioassays can serve as a pivotal point for selection
of epidemiological and whole animal studies. In vitro studies can suggest to
the epidemiologist where he should focus his efforts and when the results are
particularly unique they can serve as a basis for considering long-term animal
bioassays. To me, there are at leat two fundamental disadvantages to in vitro
bioassay systems. The first is that although one is considering
target-carcinogen interactions, there is, as yet, no way to determine whether
any detoxifying mechanism would make a particularly potent compound innocuous.
The second problem with in vitro bioassay systems is that for the most part
they cannot be considered as being organ specific. Therefore, their primary
purpose would be to determine whether or not a potential hazard exists."

Answer: Gareth M. Green

"Question 1: There are several factors to consider in the selection of in
vitro bioassay versus whole animal or epidemiologic approaches. (a) Ethical
consideration are less of a concern for in vitro bioassays. In whole animal
approaches there is an increasing concern for pain, suffering, and perceived
cruelty to animals as the animal rights concerns receive increasing attention
and weight in our society. Epidemiologic studies involve questions of primary
and perceived psychological risk. Where biological materials are utilized,
biolgical risk is a consideration. In vitro bioassays involve these
consideration during the sampling procedures only. Society has clearly moved
toward acceptance of manipulation of biological materials in vitro, even to

the level of in vitro fertilization. For example, in vitro fertilization has encountered far less opposition than abortion or even contraception. Ethical considerations, therefore, favor the in vitro approach. (b) Cost: whole animal toxicology, particularly for low frequency events such as carcinogenesis, is extraordinarily expensive. Similarly, human epidemiological studies, even when sharply focused on selected population groups, are extraordinarily expensive. In vitro assays offer low cost bioassessment opportunities. (c) Replication in human epidemiologic studies is difficult, costly, and involves ethical considerations. Replication in whole animal models is possible, but again involves cost and ethical considerations. Replication in vitro is comparatively simple. (d) Confounding variables: the use of whole animals and human populations for study of risk assessment involves multiple (hidden) contributory and uncontrollable factors of both genetic and environmental origins. These are impossible to control even with carefully selected human populations and genetically bred and homogenized animal populations. These hidden variables are undoubtedly responsible for biologic variation in risk considerations but render it difficult to assign risk to single exposures. On the other hand, the removal of biologic material from whole organisms to in vitro conditions isolates the test system from biological control mechanisms and defenses against exposure and biological effect. Neither system, therefore, has a clear-cut advantage. These differences are essentially the cause of extrapolation difficulties. (e) Homogeneity/heterogeneity: whole animal and human popultions are intrinsically heterogeneous from the standpoint of genetic background, lifetime experience, and environmental exposures. In vitro bioassays can be homogenized from the standpoint of biological inheritance, lifetime experience, and environmental exposure. The latter is more convenient for risk assessment in a purely biological sense, but the former is more 'real life'. (f) Extrapolation: in vitro bioassays require extrapolation across barriers of biologic organization from molecule to cell to organism. The higher the level of extrapolation, the more is the problem of species extrapolation. By contrast, whole animal and human epidemiologic studies involve species extrapolation, extrapolation from population to population within species, and extrapolation from population to individual. The latter is a classical limitation for the application of population studies to medicine. (g) Social acceptance: although society is more reluctant to have studies performed on whole animals and human populations than on molecules or cells, there is much greater acceptance of significance of data obtained in human populations or with human materials than with whole animals or purely molecular or in vitro results. This is not an insurmountable barrier to the utility of in vitro bioassay techniques, but public education for policy-making purposes will be required."

Answer: Takeo Kakunaga

"1. Epidemiology provides very reliable and direct information on the cancer risk assessment in energy technologies. At this moment, however, epidemiological studies cannot assess the risk of the new technologies to which humans have not been long exposed. The materials which have been epidemiologically found to be harmful to humans should be used as standard materials for the positive control in a bioassay system. On the other hand, the data from an in vitro bioassay will give useful information that the epidemiologists will be ble to use as the basis of a survey.

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The <u>advantages</u> of an <u>in vitro</u> bioassay versus whole animal bioassay are:

1) time; resources and monetary effectiveness,

2) higher reproducibility (simpler system),

3) availability of using human materials,

4) possibility of surveying individual variation in the sensitivity of reaction to environmental materials,

5) easy comparison with the data from different sources,

6) maneuverability of the experimental conditions and designs, and

7) ease of modification or improvement in the assay system.

The major <u>disadvantages</u> of an <u>in vitro</u> bioassay are:

1) incomplete covering of <u>in vivo</u> carcinogenesis process,

2) difficulty in predicting exactly the tissue specificity and the assay system.

3) not observing development of original tumor formation as an assay end point."

Answer: George Klein

"This is a very large question. Clearly, whole animal bioassay, epidemiological approach and <u>in vitro</u> bioassay must continue in parallel. Neither one can replace the other. There are enormous differences in cost and efficiency, but I see no way out from the conclusion that all three must be pursued. All of them give indirect information only. Animal bioassay is close. Epidemiology is closest, but can only give hints, not answers. <u>In vitro</u> bioassay is precise and relatively inexpensive, but its relevance <u>is</u> <u>much less</u> immediate."

Answer: Manfred F. Rajewsky

"The epidemiological approach is powerful to assess the risk of agents to which human subpopulations had been, or are being exposed, but it is of course not suited to <u>predict</u> whether or not new (or thus far rarely produced) agents are potential carcinogens or mutagens. Furthermore, epidemiological methods cannot be used for short-term (i.e. within months) prediction. Thus, in the context of the present risk assessment approach, epidemiological information can be used only in a restricted number of cases. Contrary to <u>in vivo</u> (whole animal) systems, <u>in vitro</u> bioassay systems (both at a molecular <u>and cellular</u> level) have the advantage of being less costly, and are intended to be much less time-consuming (although at least for <u>in vitro</u> primary cell systems with

malignant transformation as the endpoint, it remains to be established whether they can indeed be carried out in a less time-consuming way than the corresponding in vivo systems; e.g. primary epithelial cells exposed to an agent either in vivo before transfer to culture, or in culture, (see for example p. 320 in the article by Professor Ts'o or Rajewsky et al. in Origins of Human Cancer, pp. 709-726, Cold Spring Harbor Conference on Cell Proliferation, Vol. 4, 1977). The basic unknown in the use of tissues/cells sampled, e.g. from humans for in vitro bioassay, is whether the behavior of cells under in vitro conditions (profoundly altered microenvironmental controls; although methodology in this respect is gradually improving) indeed reflects their real behavior in vivo. In view of the difficulties to simulate in culture the true in vivo 'activation' and exposure conditions for different target cell populations and for different agents, cell (organ) culture assays should be considered where exposure of the particular target cell population in question to the respective agent(s) is affected in the intact animal ('in vivo-in vitro systems'; see, e.g., Laerum and Rajewsky, JNCI 55, 1177-1187, 1975). Molecular bioassay systems (again, if appropriate in vivo or in vivo-like exposure conditions and target cell populations are used) are well-suited to determine known types of molecular effects (e.g., on structural components of DNA, or the expression/suppression of known 'indicator' genes). However, at present such effects cannot serve as proof for carcinogenicity since molecular alterations obligatory for malignant transformation have not yet been definitively established. For the time being, these molecular effects must, therefore, be recorded as what they indicate, namely genotoxicity, mutagenicity, gene/chromosome rearrangements, altered patterns of gene expression, cell surface alterations, etc. The difficult task here will be not to accumulate too many data indiscriminately."

Answer: Benjamin F. Trump

        "In neoplastic transformation normal cells proceed to tumor cells through initiation and subsequent progression. Many factors, e.g., host immunity, nutrient conditions, genetic factors, and biological properties of living cells will affect tumor progression and expression. It is difficult to include these factors in in vitro assays to mimic these conditions in whole animal assays to evaluate tumor progression. However, whole animal assays are time-consuming, and expensive. Metabolic activation and cellular damage, particularly DNA damage or genetic changes by carcinogens are believed to be important steps in the initiation of neoplastic transformation and can be measured in vitro. By equalizing the conditions for tumor progression, cell initiation can be decisive factors in cell transformation. I believe it is a great idea to establish a matrix system by combining both in vitro and in vivo assay for risk assessment of environmental carcinogens. Because of species difference in the response to carcinogens, the interpretation of the results from whole animal assays to humans can be difficult. However, with the matrix system, the possibilities to correlate the animal results to humans exist. Our recent experiments with hepatocyte mediated mutagenesis may be an example. We know aflatoxin B_1 has a wide variation in the induction of liver tumor in different animal species. It is a potent hepatocarcinogen in rat but not in mice. The hepatocytes from rat, mouse, and human can activate aflatoxin B_1 (AFB_1) and cause mutation in Chinese hamster V79 cells. However, the mutation frequency mediated by rat hepatocytes is much higher than that by

human and mouse hepatocytes indicating that rat hepatocytes are much more effective to activate AFB_1 and cause genetic damage. The mutagenesis results are correlated with the whole animal experiments in that AFB_1 is potent hepatocarcinogens in rats but not in mice. If genetic damage by AFB_1 is the decisive factor in liver carcinogenesis and the conditions for progression and expression of tumorigenicity of liver cells (human, mouse, and rat) are similar, then human will probably be as resistant as mouse in liver carcinogenesis by AFB_1."

Answer: H. Yamasaki

"At the present time, epidemiological study is the most relevant method to assess the risk of certain chemicals to human. However, when our goal is to establish a bioassay system to predict the agents which may be hazardous to man, the prediction should be made before the introduction of such agents into to our environment, it is not possible to use any classical epidemiological approaches. Since a significant number of the people already exposed to and/or receiving the effects of such agents is necessary to establish a risk assessment from epidemiological studies, it would be too late to apply the results for policy-making purposes.

Thus, it became necessary to develop whole animal or in vitro bioassay systems. Obviously, the greatest disadvantage in using in vitro bioassay is the fact that there is no guarantee that isolated biological materials (cells, tissues or molecular systems) will behave as when they are in vivo. Moreover, the end-points of most of in vitro bioassay, with a possible exception of cell transformation, are not tumorigenicity. These assay systems had been developed based on the limited current knowledge of mechanisms of carcinogenesis and therefore as we begin to know more about the mechanism, one needs to improve the bioassay method accordingly. It is clear, however, that in vitro bioassay has a great advantage in its simplicity and therefore one can save time and money.

Because of the above reasons, a whole animal short-term bioassay may be an alternative which overcomes several disadvantages of isolated in vitro system. Again, the choice of biological endpoints would be a difficult task."

Answer: Stuart H. Yuspa

Only two advantages to an in vitro approach are clear: time and cost. In vitro approaches fail to detect tissue specific factors, indirect effects (e.g. hormonal imbalance), pharmacological determinants such as uptake, distribution and excretion of agents, sex differences and promoter-like activity where initiation is constitutive or induced. Epidemiological approaches where data is solid have high validity but low sensitivity and are not useful for screening. Molecular systems are generally so specific that extrapolation to a biological process may not be valid. Molecular systems may detect only a portion of the biological activity of an agent while missing an equally important activity."

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Question #2

Comparison of advantages and disadvantages of using human materials (tissue and cells) versus animal materials (mostly rodents).

Answer: Richard J. Albertini

"A.   Rodent Materials

Advantages:

a.   Relatively easy and inexpensive
b.   Mammalian target realism
c.   Can study multiple endpoints
d.   Can construct in vivo-in vitro comparative systems where the whole animal can be manipulated experimentally, e.g. dose response curves.

Disadvantages:

a.   Repair systems different than humans
b.   Uncertainty as to how to extrapolate to man
c.   No way to assay for human population heterogeneity regarding susceptibility

B.   Human Materials:

Advantages:

a.   Relatively easy and inexpensive
b.   The ultimate in target realism
c.   Multiple endpoints
d.   Can study multiple endpoints
e.   Can develop in vivo-in vitro systems
f.   Can potentially define human population heterogeneity regarding susceptibility
g.   Can "validate" in terms of human health risk predictor

Disadvantages:

a.   Cannot manipulate the whole organism for comparative in vivo-in vitro stud   .

Answer: J. Carl Barrett

"Human materials have the obvious advantage of being the most relevant; however, assays for a variety of effects are already well established for animal cells. Many of these assays can be or already have been developed for use with both human and rodent cells (examples: UDS, gene mutations, SCE, alkaline elution, etc.). The level of effort that needs to be applied to human versus rodent cells, therefore, varies according to the endpoint studied. If the endpoint is novel and its significance is unknown, rodent

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cells may be appropriate. If the endpoint is well understood, few additional studies with rodent cells are required and emphasis should be placed on development of assays with human cells and to the comparison of the response of human cells to selected rodent cells. This assumes that the responses among rodent cells is predictable. If this is not true, comparisons between human and rodent cells may not be warranted. Therefore, both human and rodent cells have complementary advantages and studies with both should be continued."

Answer: Antone L. Brooks

"There would be several advantages if all in vitro testing could be done using human cells. The data could better be related to exposed human populations, since the cells have human genes, metabolism, DNA repair systems, cell proliferation repair systems and membrane characteristics. However, in the real world it is difficult to get human materials, especially cells of prime importance. It is almost impossible to get human cells that have been exposed to the pollutants in vivo. After obtaining human cells, they are harder to maintain in culture, only a limited number of relevant cell lines have been established and much of the tissue comes from individuals with diseases. This makes it difficult to compare the same cell type exposed in vitro and in vivo. Using animal cell systems in vitro, observations can be validated in whole animal exposures using controlled environments. This provides a direct in vitro/in vivo comparison. The experimental animal approach also limits the wide range of pollutants which complicates the human data."

Answer: Brian D. Crawford

"Progress in in vitro mutagenesis with diploid human cells (fibroblasts) is sufficient to allow establishment of these cells as in vitro indicators of genotoxicity. In particular, single locus mutation, cytotoxicity, DNA damage and repair, and chromosome damage all can be measured in these cells with reliability. Thus, these cells should be employed in a bioassay designed to measure the mutagenic/genotoxic potential of energy-related pollutants. Such an assay ideally should provide a reliable indication of the somatic genotoxic potential of such agents. However, animal studies (e.g. mouse or Drosophila melanogaster) must be encouraged to ensure that germ-line mutations would not be overlooked. That is, genotoxicity related to meiotic processes is an important aspect if one is concerned about the effect of energy-related by-products on future generations. Such studies would be of considerable significance with regard to occupational (as opposed to "consumer") exposure, and can best be achieved using laboratory animals.

With regard to carcinogenesis assays, at present, rodent cell assays would appear to be, perhaps, even more sensitive indicators than human cells. This is due, in part, to the lack of adequate in vitro assays for human tumor cells, but also reflects an apparent sensitivity of rodent cells to carcinogenic insult, in contrast to human fibroblasts. Clearly, however, human cells of epithelial origin should be developed as an in vitro assay for carcinogenesis, in light of the contribution (>90%) of carcinomas to human cancers. To date, progress in the growth and characterization of human epithelial cells is minimal; such studies should be fostered.

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It is perhaps significant that only in rodent systems can somatic mutation, cytotoxicity, and neoplastic transformation be measured concomitantly with any degree of reliability. With regard to the interrelationship of these test parameters, clearly rodent cells offer the best alternative at present. The question remains: should a bioassay therefore use rodent cells, or should the development of human cells as an in vitro carcinogenesis assay, to compliment existing mutagenesis assays, receive high priority? Perhaps both strategies should be pursued."

Answer (Questions 2 and 3):   Joseph DiPaolo

"Until recently, a very embarrassing situation existed in regards to human material versus animal material. Namely, whereas a large number of chemicals and environmental agents exist that are known to cause cancer in humans as well as animals, none was effective on human material. This discrepancy is slowly being changed; a number of laboratories are now reporting the ability to demonstrate that carcinogenic insults on human cells produce a variety of changes that are considered indices of malignancy and/or transformation. A great deal of information must yet be acquired concerning the culture requirements of human cells and conditions for proving that malignant transformation has occurred. Some years ago, Tomatis (IARC) and his associates demonstrated a positive correlation between mouse skin tumors and rat liver cancers for a variety of chemical carcinogens. I am of the opinion that this correlation can now be extended to rodent cells in culture. When one considers the problems in validating an in vitro animal transformation system, it becomes obvious to attempt such a similar study using human material would require a heroic undertaking."

Answer: Gareth M. Green

"Some of this response has been covered in the response to Question 1. Advantages and disadvantages involve ethical, cost, extrapolation, relevance and public persuasion, as in Question 1. The differences are narrower because of the intact level of biological organization in both instances. However, this gap is narrowing as interest in animal rights increases. There is, however, increasing skepticism of the relevance of animal findings to human population exposures. One may project greater acceptability of extrapolation across levels of biological organization than across species from animals to man. The contrasting of animal versus human sources of biologic information implies that humans can be studied only from the epidemiologic standpoint and animals only from the experimental. Obviously, there are opportunities for cross-over and the use of both approaches in an integrated fashion to increase the power of risk assessment. This is the area the conference should explore carefully."

Answer: Takeo Kakunaga

"The advantages of using human materials versus animal materials:

1.   No species barrier in the extrapolation of the data to human risk assessment

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2. Possible use of the materials from various genetic disease
       patients including those who are genetically predisposed to
       higher incidence of cancer
   3. Possible survey of individual variations in the response to the
       probes
   4. Possible studies in close relation with epidemiological
       invesigations
   5. Higher stability in culture

Disadvantages

   1. Genetic heterogeneity
   2. Limitation in genetic approach
   3. Some difficulty in obtaining the suitable materials
   4. Difficulty in culturing some type of the cells
   5. Unavailability of syngeneic host for transplantation of
       transformed cells
   6. No complete model of experimental carcinogenesis."

Answer: George Klein

   "Human materials ought to be used, whenever practicable. Animal
materials may often give misleading results. The demonstrated relationship
between transformability, spontaneous and induced, and shortness of life span
(as opposed to the low transformability of long lived species like humans)
makes it even more imperative that human cells are used whenever possible."

Answer: J. Justin McCormick

   "The biggest advantage of rodent transformation assays is that syngeneic
animals exist which allow the direct testing of the tumorigenicity of
presumptively neoplastically transformed cells. By comparison, the only
widely-used assay for testing the tumorigenicity of presumptively-transformed
human cells is injection into athymic mice. These animals provide a reliable
measure of tumorigenicity, but are expensive to purchase ($13.95 a mouse) and
are expensive to care for since their water, bedding, food, and cages must be
sterilized and they must be housed in laminar flow hoods. Furthermore, the
persons handling these athymic mice must be specially trained and cannot
simultaneously be caring for other animals, especially rodents. Because of
the sensitivity of athymic mice to bacterial and viral diseases, epidemics may
occasionally sweep through the animal colony and force one to eliminate all
animals and begin again. Regardless of these problems, athymic mice provide a
reliable and proven tumor assay system.

   The availability of human tissues is frequently cited as a problem.
While this may be true in very small communities, our experience suggests that
this is not likely to be a limiting factor in any community with one or more
moderate size hospitals. The main problem in procurement of human tissues is
that one must gain the cooperation of physicians and nurses which we find
requires diplomatic negotiations. Then, one must develop a system for

immediate transfer of the tissue to the laboratory and processing. It is our observation that scientists who complain about difficulty in obtaining human tissues are often those who are unwilling to take the trouble to develop the procedures necessary for obtaining such tissues routinely.

There is no significant difference between human fibroblasts and animal fibroblasts in their ability to grow in mass culture. However, in our hands diploid human fibroblasts routinely clone at 40-90%, whereas most rodent cells in culture that are not cell lines are reported to clone with much lower efficiencies. For example, Barrett et al. report that Syrian hamster embryo cells clone at 2-5% efficiencies (1). It is important to achieve high cloning efficiencies since quantitative studies on transformation almost always require cloning assays. Low cloning efficiency such as 2-5% will require large correction factors to be applied to the data giving much more uncertainty. Another important difference is that human fibroblasts in culture maintain their diploid karyotype until senescence, whereas rodent fibroblasts, particularly mouse, lose their diploid karyotype after a few passages in culture. Since the development of heteroploidy is apparently a random process, the use of heterploid cells leads to uncertainties regarding just what cell populations are at risk, etc. A further problem with mouse cells is that their excision repair capacity rapidly decreases after the cells are placed in culture. I do not know whether this is true with other rodent cells in culture, but the low levels of excision repair reported for various rodent cell lines suggests that this may be true. If so, this means it will be difficult to extrapolate from the data obtained in such cells to the risk in the intact animal or in humans.

Overall, human fibroblasts in culture retain many of the characteristics of the cells in the tissues from which they were derived. Therefore, data obtained from studies on such cells may be easier to extrapolate to man than comparable data obtained with rodent cells.

1. Barrett, J.C., Bias, N.E., and Ts'o, P.O.P. A mammalaian cellular system for the concomitant study of neoplastic transformation and somatic mutation. Cancer Res. 50, 121-136 (1978)."

Answer: Manfred F. Rajewsky

"Fresh human tissues/cells are often difficult to obtain, particularly if standardized conditions (prior to culture) have to be maintained. The advantages of using human cells are obvious when parameters are to be measured which may be different in, e.g., rodent cells (such as metabolic activation, excision repair, etc.). Disadvantage: much greater inhomogeneity in terms of genetics and previous exposure to unknown agents, as compared to inbred laboratory animal strains."
Answer: Helene S. Smith

"Many valuable and relevant assays have been developed to evaluate the carcinogenicity of various compounds, including in vivo studies with mice, and in vitro studies assaying mutagenicity of bacteria or transformation of murine tissue culture cells. However, one of the major sources of concern regarding

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carcinogens is evaluating the applicability of these various tests to humans. Since many differences have been observed between human and murine cells which may be relevant to carcinogenesis assays (i.e. variability in enzyme populations, metabolic pathways, and growth properties, as well as differences in the incidence of cancer among various species), it is extremely important to develop human cell substrates to directly validate results obtained with other transformation systems.

There are three major disadvantages to using human tissues. First, it is difficult to obtain human material immediately from operating rooms or from autopsies. It is expensive in both time and money to develop liasons with physicians, to do the record keeping required for human use certification, for messengers to run to various hospitals when tissue is available, and for clerical help to obtain pathology reports and other patient follow-up information. One way that DOE could expedite obtaining human tissue for various laboratories would be to establish tissue procurement banks. While it is true that one needs tissue soon after removal from the patient, in our experience, it is possible to ship tissue minced and in media with antibiotics. In addition, from personal experience with breast and colon tissue, I would guess that many cell types can be isolated, cryopreserved, and shipped as frozen ampoules.

A second problem is that the technology for culturing and studying the biology of most human organs is in its infancy. Except for fibroblasts, as yet there are no well established quantitative assays for evaluating carcinogenicity using human cells. Clearly, in the initial establishment of a matrix of tests, very little could be included using human epithelial cell. However, the field is moving rapidly, and I predict that such assays will begin to be available within 1 to 2 years.

A third problem is an inherent limitation of working with human cells. The ultimate test of transformation is to reinject the altered cells back into a syngeneic host and obtain a tumor. Obviously, this is impossible for humans. As discussed in Question 9, nude mice have many limitations and interpretations of malignancy data with inoculated human material is difficult. Therefore, it seems to me that for any given organ system, it will be important to study both the rodent and human cell biology. An example of this approach can be seen in the work of Dr. Yuspa with rodent epidermal cells and Dr. Rheinwald with human epidermal cells where they both come to the conclusion that faulty terminal differentiation is a relevant assay for epidermal cell transformation (for details, see answer to Question 5)."

Answer: Benjamin F. Trump

Human materials and animal materials are all required to establish a matrix system (see answer to Question #1). Certainly, human materials have the advantages that genetic damage or tumor initiation can be measured directly in the cells of human. However, the biological significance of such tumor initiation may only be validated through the matrix system with the results from animal tissues, whole animals and epidemiological studies. Disadvantages include availability and interindividual variation. Cells may have already been initiated."

Answer: H. Yamasaki

"Advantages: The parameters in metabolic pathways, distribution of metabolites, DNA repair system, etc., may be more similar to that of in vivo human cells than when one uses rodent cells.

Disadvantages: Availability of human materials, especially from normal people, is limited.   Freshly prepared cells (or tissue) are difficult to obtain.   One cannot expect to have homogeneous material from human population, as we usually do from an inbred strain of animals.   Human individuals are also exposed to different environmental factors which might affect the physiological state of cells and tissues."

Answer: Stuart H. Yuspa

"Based on the comparative data obtained to date, there are no or few advantages to using human material for screening purposes.   Comparative metabolism studies among species have indicated few qualitative differences, while quantitative differences among individual human subjects is great.   Transformation studies have confirmed that chromosomal abnormalities, growth requirements, and markers of neoplasia are similar if not identical among rodent and human species.   Human tissues are hard to obtain, genetically variable and slower to transform (maybe) than rodent tisues, thus reducing sensitivity and increasing cost.   Human tissues are most useful for comparative analyses, characterization of markers of preneoplasia which may be species specific (for diagnostic purposes) and for studies of the molecular basis for genetically determined states of enhanced susceptibility to carcinogenesis."

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QUESTION #3.

What is the current status and what are the problems in human cell transformation? What are the current problems in the in vitro culture of normal and transformed human cells?

Answer: J. Carl Barrett

"The area of human cell transformation is very exciting and making excellent progress. However, it is still in the phenomenological stage (most qualitative) and until a better theoretical understanding of this area exists, caution could be exercised in the use of these assays for risk assessment. Some questions that exist in this area are:

(1) What is the significance of regressing tumor nodules in nude mice?
(2) What is the relationship between anchorage independent growth and tumorigenicity?
(3) What is the relationship between morphological transformation and anchorage independence?
(4) Why are early passage cells required?
(5) What is the role of gene mutations in human cell transformation?
(6) What is the role of chromosome changes and rearrangements in human cell transformation?
(7) What is the role of oncogene activation in human cell transformation?
(8) Are human cell transformation assays good predictors (qualitatively and quantitatively) of risk to humans from carcinogens?

Answer: Brian D. Crawford

"The most persistent problems in human cell transformation are:

(a)   The identification of reliable in vitro markers of neoplastic change, to delineate neoplastic progression in cells of this species.

(b)   The validation of in vitro test systems for the evaluation of tumorigenicity of human cells.

        With regard to problem (a), oncologists perhaps have been somewhat biased in their approach toward the identification of in vitro markers of neoplasia, in that cellular and biochemical alterations associated with neoplastic rodent cells tacitly have been assumed to work as predictors in the human cell system. Clearly, such has not been the case. Given the multi-step and multi-factorial nature of neoplastic development in vivo, this result is not surprising, i.e., many cellular changes studied in rodent cells in vitro are essentially phenotypic end-products of the neoplastic process. A remaining challenge is the identification and characterization of changes related to pre-neoplastic cellular disorders. Such changes would be more reliable indicators of agents capable of initiating the process of carcinogenesis. Such studies would then enable more reliable screens for so-called 'promoters' of carcinogenesis.

With regard to problem (b), it is apparent that tumorigenicity assays are needed to validate in vitro tests. Accordingly, if tumorigenicity assays are themselves deficient, their correlations wit in vitro bioassays will suffer. This was the thrust of the challenge, still not answered, that was offered by Charles Boone in 1975. Specifically, he found that cells deemed "normal" as far as tumorigenic change was concerned, were tumorigenic if assayed in an unconventional manner (i.e., implanted, rather than inoculated in suspension). More generally, the problem with tumorigenicity assays is that a "negative" result does not distinguish adequately cells that are truly "normal" from cells that are pre-neoplastic (i.e., at some early stage in the neoplastic progression) and which may, by virtue of their alteration, be killed by specific host mechanisms (e.g., NK cell activity, activated macrophages). At Los Alamos, Paul Kraemer and his colleagues are using gelatin sponges, implanted in nude mice, as a "retrievable arena" for studying the host-response to cultured cells (personal communication). It is anticipated that this approach will lead to the development of a tumorigenicity assay that will distinguish the alternative fates (i.e. maintenance in vivo as opposed to host-mediated killing) of implanted cells, and that will also provide data within a shorter period of time (days/weeks versus months/years). (Thus, this assay relates to question 9(h) of the questionnaire). Clearly, a significant challenge in the study of human cell transformation is the development of reliable "xenotumori-genicity" assays. Basic research in this area should continue to be supported, to enable more reliable, quantitative biomedical risk assessment in the future."

Answer: Takeo Kakunaga

"1.   Qualitative systems of neoplastic transformation have been developed.

2.   Quantitative systems have been developed using diploid fibroblasts as the target cells and the induction of using anchorage independent cell growth as an assay end point.

3.   No quantitative assay system using permanent cell lines has been developed.

4.   Malignancy of the cells has been measured by their ability to form tumors in nude mice when they were inoculated subcutaneously.

5.   No in vitro marker (transformed phenotype) has been found that closely associates with the transplantability in nude mice. Anchorage independent growth, a phenotype which is usually associated with tumorigenicity in rodent cells, is not necessarily associated with the transplantability in human cells.

6.   It is likely that neoplastic transformation of human diploid cells is a multistep process. However, there has been no concrete evidence that the human cells which show some partially transformed phenotype such as anchorage independent growth have higher probabilities to be converted to neoplastic cells compared to normal diploid cells.

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7. Acquisition of immortality, i.e., unlimited cell growth is rare in human cells.

8. Success rate in establishing cell lines from human tissues is not high. However, it is yet unclear whether the low frequency is due to the difficulty in obtaining suitable and fresh tumor tissues".

Answer: George Klein

"I am not competent to comment on this, except by pointing out that at least one type of human cell transformation is superior to all animal systems in transformation efficiency, namely the EBV-transformation of human B-lymphocytes. Since this is a very special case, however, I shall not elaborate further. I would like to point out, however, that EBV-transformed lymphoblastoid cell lines of normal human origin are and remain diploid through 6-9 months or more, while being at the same time immortal. During the period of diploidy, they provide many advantages for genetic and cytogenetic studies."

Answer: J. Justin McCormick

"Transformation of human fibroblasts can now be carried out in a quantitative manner. Following treatment of cells with adequate doses of radiation or direct acting carcinogens, the cells are allowed to replicate for 9-11 population doublings to allow expression of the transformed state and then assayed for anchorage-independent growth. A linear dose-dependent increase in the frequency of anchorage-independent colonies is seen. When colonies demonstrating anchorage-independent growth are isolated, and the progeny cells grown to large populations and injected into athymic mice, fibrosarcomas composed of fibroblasts with a human karyotype arise at the site of injection. Alternative in vitro markers can also be used to indicate the induction of the transformed phenotype. Kakunaga, Borek and our laboratory have observed foci develop on monolayers of cells which were propagated from the original target population receiving carcinogen treatment and have obtained fibrosarcomas from cells isolated from such foci. However, none of the above workers have found foci formation to be a quantitative assay. This is probably because of the subtle nature of such foci. Morphological transformation following carcinogen treatment, i.e., criss-crossed, piled up growth, has also been reported (A. Kennedy, personal communication), but this criterion is also quite subtle and no one has reported being able to utilize it as a quantitative basis for transformation.

While it might be presumptive to say that human cell transformation (i.e. induction of anchorage-independent growth in a dose-dependent manner) can be routinely carried out by any laboratory, we are aware of three laboratories in the U.S. where the scientists have personally communicated to us their success in carrying out such assays since our publication of the procedure in 1981. In addition, Sutherland has published data which show dose response at lower doses and Little's laboratory has published data showing a dose response using

a different anchorage-independent assay. Thus, there are at least 6 laboratories in the U.S. that have succeeded in inducing anchorage-independent growth in human fibroblasts in a dose-dependent manner following carcinogen treatment.

The tumorigenicity assay with transformed human fibroblasts has, to our knowledge been successfully carried out in five laboratories. The assay (s.c. injection of cells) is quite simple if one has access to well-cared-for athymic mice. Some workers who have successfully carried out the in vitro part of the assay may not wish to carry out tumor studies since animal research, especially with athymic mice, requires special expertise which they may not have. Furthermore, if the athymic mice are not available to them as part of as an institutional commitment, it requires a substantial outlay of space and personnel as well as equipment which must be committed solely to this purpose for a substantial period. It should be remembered that many workers in the area of virus- or carcinogen-induced animal cell transformation rarely, if ever, carry out tumorigenicity studies. In fact, the custom among many workers is to demonstrate early on in their studies that cells with a particular phenotype (e.g., anchorage-independent growth, ability to form foci) are, indeed, tumorigenic and then to utilize only the in vitro end point from that time on. The necessity of carrying out tumorigenicity studies with anchorage independent human transformed cells, therefore, remains unclear (see below).

The major unanswered question in human cell transformation is the relation of the anchorage independent phenotype to the tumorigenic phenotype. The work of Milo (2) and our own work clearly shows that tumors arise in animals only when cells that exhibit the anchorage-independent phenotype are isolated, grown to large populations and injected into athymic mice. Because anchorage-independent colonies of 200 to 10,000 cells must be isolated and grown to populations of 5×10^7 cells for injection into 4 animals, it is not clear whether the anchorage-independent cells are already tumorigenic, or whether during expansion of this cell population a further change may take place which converts anchorage independent cells into tumorigenic cells. This question can be directly addressed by developing a tumor assay requiring fewer cells. Such studies are now underway.

Since metastasis of tumors is such an important aspect of human tumorigenicity, the question has been asked whether transformed human cells produce metastatic lesions in athymic mice. We have not seen metastatic lesions. However, cells derived from human tumors, even from metastatic lesions, are rarely metastatic in athymic mice. Therefore, it is probably not surprising that human fibroblasts neoplastically transformed in vitro do not metastatize in these animals.

Questions have been raised regarding the stability of the anchorage-independent phenotype in human fibroblasts. We have cultures which have continued to maintain this phenotype over a longer period, as well as others which exhibited the phenotypes, but have lost it during passage in culture. The fact that the anchorage-indepedent phenotype is maintained in a stable manner is compatible with it being the result of a genetic trait. The populations which lose this phenotype may represent cells which acquired anchorage-independence by some means other than a genetic trait or they may represent mixed cell populations in which the anchorage dependent cells have a

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selective advantage for growth on plastic. The methods we used to isolate colonies from semi-solid media as a source of our anchorage-independent populations were not designed to rigorously exclude the inclusion of normal cells. Therefore, we consider the latter possibility to be quite reasonable and have undertaken further studies on the question. Biochemical characterization of transformed cells is just beginning in our hands, so no data are available.

In our earlier tumorigenicity studies using normal or X-irradiated athymic mice, we found that the tumors appeared, grew to a size of 0.7-1.0 cm in diameter and then regressed. Those excised before regression were diagnosed as fibrosarcomas and the cells isolated from them gave rise to similar regressing fibrosarcomas upon reinjection. Recent studies have given fibrosarcomas which grow progressively and kill the mouse. We do not as yet fully understand the regression phenomenon, but some studies indicate that it results from using a tumor inoculum which contains non-anchorage-independent cells as well as anchorage-independent cells".
Answer: Benjamin F. Trump

"Most of the problems involve epithelia. Methods of long-term culture and transformation need to develop better markers, especially early markers. Need markers for promoters. Presently, very few early assays of tumorigenicity exist and they are by no means generally acceptable."

Answer: Stuart H. Yuspa

"Major problems include: obtainment of tissues; genetic variability of donors; long latency period for transformation; lack of markers for transformation including difficulty with heterotransplantation. Recent studies indicate that culture of cells from specific target organs wil require customization of media. Markers to assure normal growth and differentiation have been established for only a few tissues. Of course some of these problems are similar for tissue specific epithelial cell culture of rodent cells."

Question #4.

Which animal species should be used? Mouse, rat, Syrian hamster, Chinese hamster, guinea pig, rabbit, dog, primate, etc.? How many, which ones, and priority; which strain or breeds within the species, if there is a special recommendation?

Answer: Richard J. Albertini

"It would seem that the criteria to be used in choosing an animal species for genetic hazard bioassays should include:

A.   A good knowledge of the genetics of the species
B.   An established data bank of mutagenicity/carcinogenicity test results for both in vivo and in vitro studies
C.   A short reproductive time so that studies determining carcinogenicity hazards can be linked to studies determining reproductive (transmissible genetic) risks
D.   The possibility of using parallel systems in man (e.g., specific locus mutagenicity studies, transformation in vitro, etc.).

At present, it seems that rodents--hamster and mouse--are the best animal species to use."

Answer: J. Carl Barrett

"There is no simple answer to which non-human species should be used. Part of this answer will come from an analysis of the status of bioassays with non-human cells. The ones that work should be used! Another question may arise in this context. Can one do risk assessment for rodents based on a "matrix" approach as suggested for human risk assessment? The reliability of this type of approach should be testable by doing rodent to rodent extrapolation. If this fails, the possibility of doing rodent to human extrapolations would be questionable."

Answer: Antone L. Brooks

"Although this is a difficult question to address, certain facts seem apparent. First, advantage needs to be taken of the different characteristics of the various species to answer different types of questions. Second, in order to provide maximal interaction among investigators in establishing links among assays, the numbers of species used should be limited and there should be as much commonality of use as possible. Third, as much as possible, there should also be a commonality of strains used within species. Fourth, there should be as much commonality as practical between species/strains used for whole animal studies as those used for sources of cells/tissues for in vitro study. Fifth, all other factors being equal, the species/strain should be selected for which there already exists the largest data base. Sixth, a certain limited amount of effort needs to be expended toward studying differences among species and their similarity to man with respect to endpoints of interest. Finally, it should be recognized that animals might be

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chosen for their potential as models of common human diseases as well as for their characteristics in the normal state.

Many characteristics of the various species enter into their choice for use in toxicological studies. Let us consider inhalation toxicological studies as an example. The smaller rodents (rats, mice and hamsters) can be used in large numbers at less cost than dogs or primates. The rat and Syrian hamster are large enough for detailed evaluation as clinical subjects, while the smaller mice and Chinese hamsters do not lend themselves well to organ-level function studies (e.g. respiratory function) and assays requiring substantial amount of tissue. Syrian hamsters may be more resistant to pulmonary tumor induction than rats and have a shorter life span than "clean" rats and mice. For these reasons, one might choose the rat as a subject for life span health effect studies of inhaled toxicants in large numbers of subjects. On the other hand, dogs have changes in lung structure and function with age, pulmonary particle deposition and clearance patterns, and pulmonary immunological responses which more closely resemble those of man than do rodents. Primates are likely to share many of these characteristics of dogs; however, they are more difficult and expensive to obtain and maintain than dogs; and many of the characteristics known in dogs and man are yet unknown in primates. For these reasons, the dog might be used for limited comparative studies in addition to the large-scale studies on rats. Other species used for specific characteristics include Chinese hamsters for their low chromosome number and guinea pigs for their sensitivity to airborne irritants. The message is that more than one species will likely have to be used in the comprehensive study of a given class of materials.

Reasons for selection of strains vary as they do for selection of species. Within some species, particularly the mouse, large numbers of strains are available with well-developed sensitivities to different kinds of toxicological insults. In other species, such as the dog, only one strain (the Beagle) has been used widely enough for research that large data bases and established experimental breeding colonies exist. As an example of another basis for selection, the Fischer-344 rat does not undergo the magnitude of body growth after reaching sexual maturity as do several other rat strains. It would seem logical that, other factors being equal, strains should be chosen for their similarities to man.

In selecting species/strains, it would seem reasonable to first estimate the kinds of toxic manifestations likely to be induced by a material, and then to select species and strains best suited to providing answers within the context of the studies to be done. As stated above, there should be an effort toward commonality of species/strains among laboratories and investigators."

Answer: Brian D. Crawford

"Of the animal species suggested, mouse, rat, and Syrian hamster would appear to be best suited for bioassay. In particular, physiologic and pharmokinetic data are extensive for mouse and rat, making these species invaluable in laboratory studies. However, neoplastic transformation can be studied more reliably in the Syrian hamster than in mouse or rat, due to the

problem of spontaneous transformation. The expense of primates makes their use unattractive, particularly if large numbers of animals are used. Dogs are not genetically well-suited for laboratory studies of genotoxicity. Special carcinogen-susceptible strains of rodents (mouse, rat, hamster) would be useful for in vitro/in vivo correlative mutagenesis/carcinogenesis assays. These animals provide a unique approach toward correlating such test systems."

Answer: Joseph DiPaolo

    "The animal species used should include those that are inbred and possibly share some metabolic overlap with humans. In addition, consideration should also be given to a species which is widely used for other purposes. For example, much is known about the immunological responses of mice and guinea pigs. The Syrian hamster is often used, not only for transformation studies, but for studying the aging phenomenon and the dog, particularly the inbred beagle, is used for irradiation studies. Therefore, in terms of animal species, I would recommend the mouse, rat, Syrian hamster, guinea pig, and dog in that order for in vivo studies, but I would recommend the Syrian hamster as the first choice for in vitro transformation studies."

Answer: Manfred F. Rajewsky

    "Depends on the parameters to be investigated. If, e.g., chromosome alterations or changes in the expression of specific genes are to be studied, then the species and strains should be used for which most details have already been worked out in regard of the normal control situation. If an end-point such as malignant transformation is used, then in principle human primary cells are the optimum ones. However, they require (somewhat) artificial conditions for proving tumorigenicity (nude mouse). Which rodent or other animal species are to be considered the most similar to human cells in terms of carcinogenesis, is largely a matter of (personal) philosophy. In principle, the best system is the one that requires the least artificial experimental conditions."

Answer: Stuart H. Yuspa

    "Species should be selected which have been extensively studied in vivo, so that a data base useful for confirming the validity of in vitro data is assured. In most cases this would be rodents, (mice and rats), preferably inbred. Hamsters may also be used. The most sensitive strains should be selected, enhancing the sensitivity of assays for relative evaluation. We must begin to develop tissue specific assays in vitro. Thus cultures of mouse skin, rat liver, rat or mouse mammary gland, hamster trachea or bronchus, rat prostate, rat colon, hamster pancreas, rat or mouse esophagus, and rat bladder would be appropriate systems to study as a battery of tests. A variety of criteria in each cell type will be needed to evaluate any compound (see below)."

QUESTION #5.

Comparison between human cell neoplastic transformation and rodent cell neoplastic transformation

Answer: Brian D. Crawford

"It is enigmatic that human cells appear refractory to in vitro neoplastic transformation, both spontaneous and induced, in comparison to rodent cells. The question remains, is this difference due to inherent genetic/cellular differences, or merely an artifact of the test systems employed? For example, rodent systems use cells derived from embryos at various stages of development. In contrast, human cells used typically are neonatal or adult fibroblasts of a more 'uniform' origin with regard to 'developmental stage'. Perhaps scientists should urge the use of abortuses from early stages of gestation for the study of human cell transformation.

Alternatively, the stable ploidy of human cells grown in laboratory culture may be a significant factor in the inability to transform these cells. Studies of induced karyotypic variability in human cells would be of value in this context. Particularly, if karyotypic instability is a major factor in carcinogenesis, assays to detect agents which affect mitotic processes should be developed and included in any bioassay to assess carcinogenic potential. For example, J.C. Barrett's results with DES, which is carcinogenic, but not overtly mutagenic, would fall in this category.

Finally, as mentioned in answer to question (2), human cell transformation in vitro has been impeded by the lack of reliable in vitro indicators of pre-neoplastic and neoplastic change. Basic research into the detection of pre-neoplastic cellular/biochemical changes should be encouraged."

Answer: Joseph DiPaolo

"Transformation systems used should be well defined. By this, I mean it is important to know whether transformation is an inductive or selection phenomenon involving activation of a virus, and that a dose-response relationship is obtainable. There is evidence in some rodent cell systems and in the few studies representing successful demonstration of transformation of human cells in vitro that the phenomenon is inductive. However, whereas in the Syrian hamster colony assay, transformation is independent of the number of cells used and a dose-response relationship exists for a wide variety of known chemical and physical carcinogens, transformation of human cells be they either epithelial or fibroblast, does not shown this strict quantitative relationship. The best that can be done with human cell transformation is a demonstration of anchorage-independent growth that roughly follows carcinogen concentration. The latter has been obtained by first growing the carcinogen-treated cells in mass cultures for a varying period of time and then suspending them in agar supplemented medium. In contrast to rodent systems, there have been other extenuating circumstances that make the human cell transformation system less than satisfactory. First of all, it has been next to impossible to demonstrate that experimentally transformed human cells have

an indefinite lifespan which is characteristic of many human tumors in culture and is one hallmark of rodent cell transformations. Furthermore, it is also difficult to demonstrate the neoplastic property of experimentally transformed human cells. This is also true at this time for primary human tumors. More study is required to determine how to condition an animal host so that it will accept transformed human cells. Currently, the nude mouse is the animal of choice and the routes that have been used have included intracranial or eye and ear chamber inoculations and subcutaneous injections. The first two routes of administration require a certain amount of patience proficiency."

Answer: Takeo Kakunaga

"1.   Human cell neoplastic transformation was not extensively investigated until a relatively recent time. Thus, many aspects of the transformation such as characterization of the transformed phenotype, optimum experimental conditions for transformation and selection of useful cell lines, need further studies to be defined,

2.   No spontaneous transformation has been observed with normal human diploid cells in culture, thus giving really clean background frequency,

3.   No permanent human cell line has been obtained from normal cells or tissues in culture,

4.   Karyotype is relatively stable.

The diploid mode of untransformed cells is maintained until near senescence. Even the human cells transformed in vitro or in vivo generally show unchanged karyotype throughout many generations.

5.   The induced frequency of anchorage-independent cell growth by chemicals in human foreskin fibroblasts has been reported to be as high as that of morphological transformation of hamster embryonic cells. However, conversion rate of such partially transformed cells into neoplastic cells appears very low in human cells.

6.   Not only the frequencies of each conversion but also the sequential order of multistep progress of neoplastic transformation differ. For example, acquisition of anchorage-independent cell growth occurs at a relatively late stage in the transformation of rodent cells, generally associated with the acquisition of tumorigenicity in the syngeneic host, while human cells acquire anchorage-independent growth in the early stage of transformation. Precise sequential order of acquiring different transformed phenotypes are yet unknown."

Answer: J. Justin McCormick

"While various cell lines have been used for transformation studies, for purposes of the present objectives, it seems necessary to limit studies to assays that utilize normal cell strains. The only two systems that are

developed enough for consideration are the Syrian hamster embryo cells (SHE) and the human fibroblast system. Schematically, the assays are shown below (Fig. 1).

The strong point of agreement between the SHE and human cell system is that anchorage-independent growth is a marker for tumorigenicity, i.e., cell populations with this characteristic are able to form tumors. In human cells, induction of anchorage-independent growth has all the characteristics of an induced mutation. For example, it is induced in a dose-dependent manner with a variety of carcinogens; for maximal induction, an expression period is necessary; the dose response for induction of anchorage-independent growth with various agents is identical to that seen for induction of thioguanine resistance in normal human fibroblasts and in xeroderma pigmentosum (XP) fibroblasts; induction is highest when cells are synchornized and treated with carcinogen at the beginning of S period, just as is true for induction of thioguanine-resistance; repair-proficient cells treated with UV radiation 16 hours before S-phase are not transformed, whereas repair-deficient cells irradiated in early G_1 are transformed as easily as when irradiated at the G_1/S border (3). In contrast, in SHE cells anchorage-independence does not arise as the direct result of induction by mutagenic agents, but rather occurs randomly 32-75 population doublings following treatment (4). Nevertheless, studies by Ts'o and associates suggest that a somatic mutation is responsible for the anchorage-independent phenotype in SHE cells (4). Thus, although both the SHE and human cell transformation assays use anchorage-independent growth as the ultimate in vitro criterion and tumorigenicity as the ultimate in vivo criterion, it is not possible to reach these end points in the two systems using parallel procedures. The hamster cell situation appears to be more complex than the human. Further studies are necessary to understand the processes that occur within hamster cells and whether the temporal acquisition of various phenotypes actually represents a 'progression' toward tumorigenicity."

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FIGURE 1. Comparison of Two Systems

Human Cell Transformation Assay:
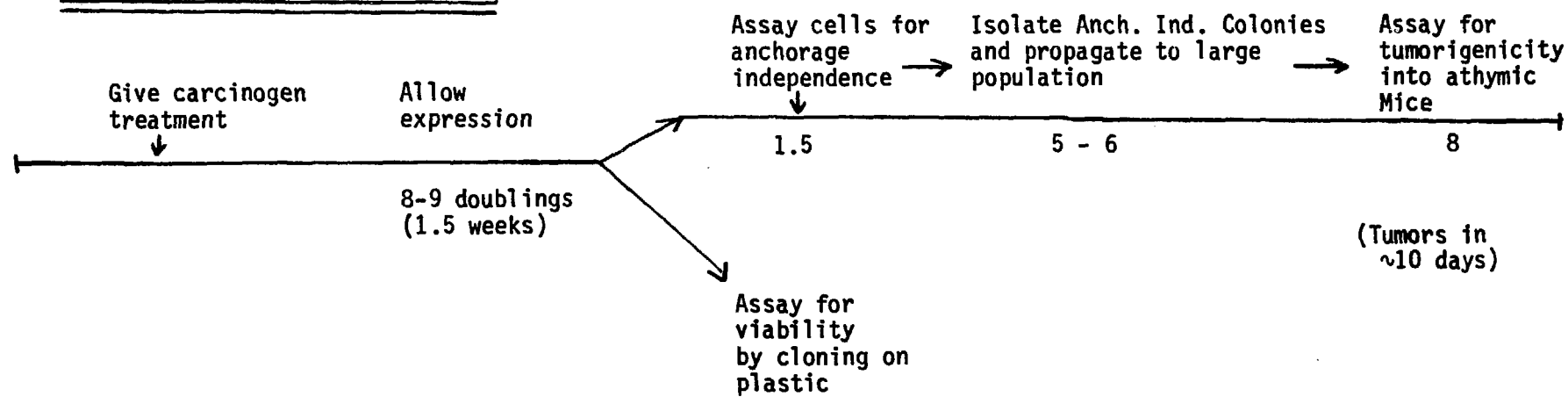
Syrian Hamster Embryo Cell Transformation Assay:
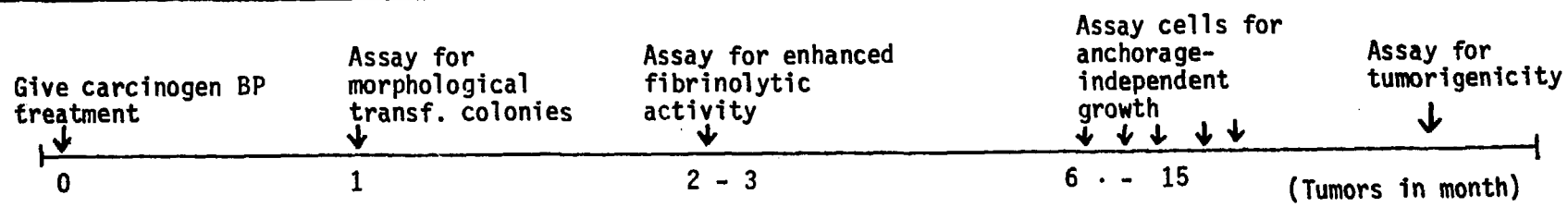
Time in weeks after carcinogen

(from Barrett et al. 1980, REF. 4)

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Answer: Helene S. Smith

"Again, I will confine my answers to epithelial cell transformation and only to those organ systems where there is some progress in the human system as well.   In general, even where there are data on both human and rodent cells of a particular organ system, the approaches from various laboratories are so diverse that it is difficult to make any generalizations.

1.   Epidermal Keratinocytes

Since there is such a large data base on mouse skin carcinogenesis, in vitro transformation studies using mouse epidermal cells are particularly valuable.   The development of a technique for readily obtaining primary cultures of pure epidermal cells has made such studies possible (Yuspa and Harris, 1974; Fusenig et al., 1973).   In standard media, untreated newborn epidermal cells divide and differentiate in primary culture but do not survive subculturing.   After a few weeks, the cells enlarge, show signs of senescence and then die after about three months.   Primary cultures treated with carcinogens go through a similar crisis; however, small populations of the remaining cells again proliferate and subsequently can be passaged.   After a number of passages, these carcinogen treated cultures usually induce undifferentiated malignancies when inoculated into mice (Slaga et al., 1978; Colburn et al., 1978); however, an earlier report of epidermal cell transformation identified the tumors induced by carcinogen-treated epidermal cells as keratinizing squamous cell carcinoma (Fusenig et al., 1973). Occasionally cell lines develop from untreated or promoter treated cultures; however, these lines which have not been treated with carcinogen rarely produce malignancies (Colburn et al., 1978).

Since most epidermal cells cannot be passaged under the standard culture conditions, one can think of the growth in these conditions by carcinogen treated cells as an early change in the progression to malignancy.   Colburn et al. (1978) studied some of the later steps in malignant progression by comparing the in vitro properties of those lines which did and did not induce tumors.   Colony formation in agar medium consistently correlated with malignancy while growth rate did not.

Recently, Yuspa and his colleagues (Hennings et al., 1980) have discovered that the reason normal keratinocytes do not grow under the above culture conditions is because the cells terminally differentiate.   If the calcium concentration is lowered, terminal differentiation is inhibited and the normal cells proliferate rapidly and can be subcultured.   If the proliferating normal cells are switched to high calcium medium, they will terminally differentiate and cease proliferation.   Transformed cells are defective in terminal differentiation and thus continue proliferation in medium containing high calcium concentrations.   Yuspa and his associates have developed a quantitative assay for carcinogen-induced transformation based on this faulty differentiation.   Cells are grown in low calcium to allow expression of transformation and then switched to high calcium medium.   The number of colonies formed under these conditions are proportional to the amount of carcinogen added.   In a most important experiment, Yuspa and Morgan (1981) showed that mouse skin painted with carcinogen in vivo already contained cells incapable of terminal differentiation (as defined by growth in

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high calcium) while untreated skin did not. The fact that malignant human keratinocytes in culture also show defects in terminal differentiation using a different criterion (i.e. survival in methocel) suggests that faulty terminal differentiation may be a relevant assay for keratinocyte transformation.

2. Bronchus and Trachea

A great deal of work has been done on rodent trachea. However, unlike the current studies by Dr. Lechner (Lechner et al., 1981) where primary cultures of human bronchus are treated with carcinogen, studies on rat tracheas have initiated carcinogenesis either in vivo (Marchok et al., 1977, 1978) or in organ cultures (Steel et al., 1977). After carcinogen treatment, the epithelial cells grew more rapidly and survived under nutritional conditions that did not support growth of explants from untreated tracheas. Only the carcinogen preexposed cells could be continuously subcultured; thus these cells seem to have acquired the capacity for "unlimited" growth in vitro. Such changes seem to precede malignancy since tumorigenicity was observed only after extensive passaging. In most cases the lines gained the capacity to grow in soft agar sometime before becoming tumorigenic suggesting that growth in soft agar might be necessary but not sufficient for malignancy. However, there was one case where the line became tumorigenic prior to gaining the ability to grow in agar. In this system, neither doubling time nor saturation density correlated with malignancy.

In vitro exposure of tracheal explants to phorbol ester tumor promoter markedly increased the growth capacity and the development of epithelial cell lines; however, none of these lines either grew in soft agar or became tumorigenic (Steele et al., 1978)."

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Question #6.

Which tissue systems/cell types should be used: fibroblast, epithelial cells, hematopoietic tissue, germ cells, etc.? How many, which ones, and priority.

Answer: Richard J. Albertini

"I believe that, in constructing a matrix of test systems, all three of the somatic cell types listed should be used. Fibroblasts should be used because there is the greatest collective experience to date with these cells. This includes both in vitro mutagenicity and in vitro transformation experience. An effort should be made to develop epithelial cell systems since most human cancers are epithelial in origin.

However, at present, I am making a plea to consider lymphocytes--whether otained from blood or from tissues--for mutagenicity assays in both humans and test animals. With the development of T cell growth factors, cultured T lymphocytes can be developed rapidly from virtually any individual animal or human. These cells constitute a population for in vitro studies that is rapidly proliferating and does not contain EB virus (as do lymphoblastoid cells, which are transformed B cells). Of even more importance, peripheral blood T lymphocytes can be cloned in vitro directly from the blood. It is possible to develop direct mutagenicity assays designed to detect somatic cell mutations occurring in vivo in these cells. Such direct mutagenicity tests can be developed for both animals and humans. Clearly then, parallel in vitro-in vivo systems can be developed with these cells in both test animals and individual humans. A variety of genetic endpoints can be incorporated in the systems developed. A matrix can be developed employing:

A.   In vitro studies of somatic cell mutation
B.   In vivo studies of somatic cell mutation
C.   Whole animal manipulation
D.   Clinical studies of individual humans tested

At present, transformation studies in human cells require fibroblasts. It would be possible to incorporate in vitro mutagenicity and transformation studies using human fibroblasts into this matrix. Ultimately, it may be possible to define markers of transformation in the lymphocytes."

Answer: Antone L. Brooks

"The selection of tissues and cells for study should be driven by the problem or questions addressed. In general it is important to select cell types for in vitro use which are 1) exposed to the pollutant in question, 2) have been shown to be involved in the disease process in vivo and 3) have the ability to metabolize, activate or detoxify the pollutants in much the same way as observed in vivo. For example, the cells of prime importance following inhalation of airborne pollutants would be the lung epithelial cells.

If on the other hand you are concerned about the genetic hazard it is

important to look in vivo at the reproductive tissues. This will make it possible to determine if the chemical can reach the target tissue, if it can interact with the genetic material and finally if it produces changes that are transmitted to the offspring. For detecting genetic damage in vivo measures of reproductive tissue must rank number one. Other systems are appropriate to detect changes which may be related to late occurring disease including cancer induction. I would rank these as 1) epithelial cells, 2) blood cells, 3) hematopoietic cells, 4) secretory epithelial cells and 5) fibroblasts."

Answer: Joseph DiPaolo

"Probably the more important cell types to be used would be fibroblasts and epithelial cells. In terms of relevance to human cancer, there is epidemiological evidence that the fibroblast types of cancer are the predominant ones for the first two plus decades of human life. Subsequently, the fibroblast types of cancer do not really decrease, but the epithelial types of cancer increase in the adult. Therefore, in my opinion, they are both relevant endpoints in terms of a bioassay system. If it were possible to tailor-make specific bioassay systems for a specific organ system, then the target cell would become much more relevant. Perhaps an attempt should be made to use organ cultures that are particularly susceptible to cancer in the human adult. These obviously would include trachea as well as lung."

Answer: Gareth M. Green

"In general, for reasons cited in Questions 1 and 2, tissues should be selected not only on the basis of how well they are currently characterized and can be handled in vitro in the laboratory, but also their relevance as to primary exposures. For example, the bulk of neoplasia involves epithelial tissues of the lung, GI tract, breast, bone marrow and lymphoid tissue. On perceived relevance grounds, these cell and tissue sources would receive priority. On purely biologic grounds, this consideration may not be important. Neoplasia is related to sources of energy and involves primarily the lung, skin, GI tract, breast, hematopoietic tissue and germ cells. There should be a concerted effort to determine whether differentiated or undifferentiated cells are more indicative of biohazard."

Answer: Takeo Kanunaga

"There has been no evidence indicating that there is cell or tissue type specificity in the neoplastic transformation by carcinogens in vitro except cell or tissue specificities in the carcinogen-metabolizing activity. Most of the factors restricting or determining the cell or tissue specificity in carcinogenesis in vivo seem to be absent or equalized in vitro. Thus, target cells should be chosen based on their convenience and easiness in handling and their growing capability with careful consideration of complementing with carcinogen-metabolizing systems."

Answer: J. Justin McCormick

"In my judgement there is sufficient experience only with fibroblast cultures to support any sort of beginning on a bioassay."

Answer: Helene S. Smith

"In the human population, greater than 90% of human cancers are carcinomas, tumors of epithelial origin. For this reason, (and because of my own personal bias since I work with epithelial cells) my initial response to this question is to recommend focusing on epithelial cell transformation. In particular I suggest it will be important to study a number of different epithelial organ systems because differences among systems will exist. A recent example can be seen in studies on BaP metabolism by epithelial cells from various human organ systems. We have found that human mammary epithelial cells metabolize BaP and form DNA adducts to a much greater extent than mammary fibroblasts from the same donors (Stampfer et al., 1980). Similar results were reported by Theall et al., (1981) for epidermal keratinocytes. In contrast, cultured bronchial epithelial cells metabolize BaP to a much lesser degree (Lechner et al., 1981).

Despite my own biases, upon thinking futher, I've concluded that it is essential to include many other cell types. Just because most cancers are of epithelial origin now, doesn't mean that a particular new agent will necessarily affect epithelial cells, rather than fibroblast. For example, vinyl chloride induces mainly angiosarcomas; while radium causes mainly osteosarcomas. In addition, data from survivors of Hiroshima indicate that radiation induces some but not all types of malignancies and the proportion of each type of tumor differed in the general population."

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Answer: Benjamin F. Trump

"I believe that target tissues or cells that are exposed to environmental carcinogens should be used. These tissues or cells in cultures should have enzyme activities or biological functions similar to that in cells of the whole animals. The following results from human cell mediated mutagenesis

experiments will probably show some significance with target cells or tissues in the studies. Human pulmonary alveolar macrophages (PAM) activate benzo(a)pyrene (B[a]P) very effectively, but not dimethylnitrosome (DMN) to cause mutation in indicator V79 cells. However, mutation frequencies mediated by hepatocytes in the presence of DMN, and B(a)p are just the opposite. DMN induces more mutation than B(a)p when human or rodent hepatocytes (the same numbers as PMN) are used to mediate mutation. Liver S9 or hepatocytes are the most common materials to study carcinogen metabolism. If only hepatocytes are used to evaluate the carcinogenic potential, DMN will probably be a more potent carcinogen than B(a)p. Indeed in animal experiments, B(a)p is not an important liver carcinogen. Whereas DMN does induce mainly liver tumors. Since PMN activates B(a)p so effectively, to cause genetic damage the potential of cancer initiation by B(a)p in lung tissues should not be ignored. Much more emphasis is needed on epithelium."

Answer: Stuart H. Yuspa

    "A battery of epithelial cell systems as described in answer to question 4 seems the minimum requirement for a valid bioassay system. Even if all of these models could be easily developed, used with facility and quantitative, indirect action of specific agents would not be detected."

Question #7.

Tissue/cells from which developmental stages should be used? Embryonic (early or late), newborn, young adult, adult, aged? One, or all of them and priority.

Answer: Antone L. Brooks

"The developmental stage used is dependent on the type of experiment you propose and the question to be addressed. Much of our work is related to exposure of worker populations so we select tissue from young adults. If you need to know the influence of age or development on the endpoint of interest of course you could select other tissues of cells. I would rank them in order or priority for study as young adult, embryonic, newborn, adult and aged."

Answer: Joseph DiPaolo

"Evidence concerning the relative susceptibility of cells from various developmental stages to chemical carcinogens is nonexistent. I do not know if it is easier to culture cells from embryonic stage than it is from an adult, but there is no reason to assume that one is more transformable than another. In terms of viral transformation, I recall a study done at Wistar Institute using WI-38 cells of different ages (stages) and although the first conclusion was that stage 3 cells transform more readily with SV-40, later studies demonstrated that it was the younger cells that were undergoing transformation. On the basis that most of the work has been done using embyronic or fetal-derived material from rodents and that these respond readily and that another cycle of a large baby boom is expected, it might be advisable to continue to use this sort of material which we know does respond very well."

Answer: Gareth M. Green

"As best as can be determined, the origins of energy-associated neoplasia occur in the adult organism. Certainly, tumor promotion is largely an adult phenomenon, and perhaps even more associated with the process of aging. From these considerations, prioritization should go to the selection of adult and aged stages of development. Biologically, however, questions should be explored in the newborn, perhaps particularly when looking at the process of initiation."

Answer: Takeo Kakunaga

"Embryonic tissues/cells would be the first choice, because:

1)   usually they have the highest growth ability, high plating efficiency, short generation time and long life span; 2)   they have metabolizing activity for a wide range of carcinogens; 3)   experimentally, in general, they have shown higher transformability."

Answer: J. Justin McCormick

"One can achieve neoplastic transformation of human fibroblasts obtained from embryonic material, newborns or young adults. Material derived from embryos or newborns is preferred as a source for fibroblasts since such cells grow more vigorously and have a longer in vitro lifespan. The easiest material to obtain is skin samples from circumcision of newborns since such material is routinely available and discarded if not used for research."

Answer to Questions #6 and #7:   Manfred F. Rajewsky

"Not only different cell types (preferentially epithelial but also others) should be used, but also different developmental (embryonal/fetal/postnatal) and differentiation stages of a given cell type (cell lineage) must be considered. However, we are still at an early stage of work in trying to prove and to characterize "high transformation risk stages" of development/differentiation for defined cell types. Such "high risk stages" may in fact vary for different cell types and for different types of agents (with differing mechanisms of action). If "high risk" subpopulations can be defined with the use of specific phenotypic markers, such subpopulations may be isolated or at least highly enriched (cell sorting) and used as primary "indicator" cell populations, rather than the complete heterogeneous cell populations."

Answer: Helene S. Smith

"To my knowledge, there are no systematic studies comparing transformation of cells from a single organ system at various stages of development from fetus through newborn to aged adult. Without knowing this information, it is impossible for me to predict whether it will be necessary to include each stage in order to assay risks."

Answer: Stuart H. Yuspa

"This will be determined by the facility of cell culture for a particular model system. A culture model which is biologically analogous to the adult tissue in vivo would be preferred."

Question #8.

How to integrate and connect the bioassay system to the metabolic activation system, the pharmacokinetic/detoxification/repair system, etc.?

Answer: Richard J. Albertini

"The matrix scheme proposed in partial answer to question #6 automatically integrates the metabolic/pharmacokinetic/detoxification systems into bioassays when direct mutagenicity studies detecting somatic cell mutations occurring in vivo are undertaken. When combined in vitro-in vivo studies are undertaken, measuring the same endpoints in the same cells, it may be possible to dissect direct effects of agents from those requiring metabolic activation."

Answer: Antone L. Brooks

"To integrate and connect the bioassay systems it is essential to know the mechanism of action in the bioassay system. It is important to understand the influence of chemical type, concentration and duration of exposure on the response. The exposure concentration, dose to the tissues and if possible the "dose" that reaches the target organs, cells or macromolecules in an active form should be defined if possible.

If a set of compounds or a class of chemical can be defined as representative of those with the biological activity in complex mixture they should be studied to determine the distribution and metabolism both in vitro and in vivo. This should be done in the matrix and exposure model which is realistic for environmental exposure to the compound. If metabolism profiles are similar then the effects measured in vitro should be of use to predict the in vivo response."

Answer: J. Justin McCormick

"Metabolic activation/detoxification of carcinogens is one of the most difficult aspects of in vitro bioassays. There are no universal activation systems which can be applied without regard to the nature of the material to be assayed. For example, benzo(a)pyrene and many other compounds require oxidative metabolism, whereas nitroaromatic compounds require reductive metabolism. Some polycyclic aromatic compounds require only a single step oxidative activation, others such as benzo(a)pyrene require a three-step activation process. As has been shown for nitrotoluene, in vivo activation is sometimes carried on sequentially in two separate tissues. Considering these problems, it is illusory to believe that use of an S9 fraction or any similar preparation, or of a feeder layer of metabolizing cells will successfully mimic in vivo metabolism of mutagens or carcinogens in all cases. Testing materials whose chemical composition is unknown is most reasonably carried out by an investigator who has some idea as to what type of chemical agents might be present and assay conditions are set up to test for such agents. DNA repair processes in cells are not readily manipulated so one must accept the repair capabilities of the cells used."

Answer to Question #8 and #9:   Benjamin F. Trump

"We recommend a matrix approach of <u>in vitro</u>:

      a)   Metabolism
      b)   DNA binding
      c)   Mediate mutagenesis
      d)   DNA repair
      e)   Morphological assessment of pre-neoplastic lesions, including tumor markers
      f)   Tumorigenicity in nude mice
      g) Comprison with animal models; obviously better models are needed.

Question #9.

What are the cellular and molecular criteria/parameters for determining neoplastic transformation (or tumorigenicity) of the human cells to be transformed in vitro? Which molecular and cellular endpoints/measurements should be adopted? Can these endpoints/measurements be built into a matrix system of in vitro bioassay? What are the best procedures for such measurements?

a. DNA/RNA/protein/membrane damage (alkaline elution, sister chromatid exchange, DNA synthesis, etc.). Special emphasis should be given to the possible use of monoclonal antibodies for the identification and quantitation of DNA damage and attachment of carcinogens to DNA.

b. Chromosomal damage and/or karyotypic changes (banding, automation, flow cytometry, etc.)

c. Cytotoxicity and early senescence.

d. Somatic mutation (microbial/insect/plant mutation) (Which trait: dominant, sex-linked recessive, autosomal recessive? Which locus: Na^+/K^+ ATPase, HGPRT, TK, APRT, etc?).

e. Gene expression changes (2D gels, immunological identification, monoclonal antibody, mRNA-cDNA library/mapping, in situ hybridization, expression of "oncogenes", etc.). Special emphasis should be given to the search for neoplasia-related mRNA through the recombinant DNA technique.

f. Neoplastic transformation (loss of contact inhibition, reduction in nutrient requirements [low serum, low calcium, etc.], anchorage-independent growth [cloning in soft agar], etc.).

g. Organ/tissue/germ cell changes.

h. Tumorigenicity (newborn animals, immunologically deficient animals [nude mouse], metastasis assay). Special emphasis should be given to the qualitative aspect and the time required for the assay."

Answer: J. Carl Barrett

"The area of human cell transformation is, in my opinion, too new to make a decision on the best molecular or cellular endpoints to use. All of the suggested changes should be and will be studied further. From a practical standpoint, for a matrix system the endpoints established for non-human cells should be developed for extrapolation to human cells."

Answer: Antone L. Brooks

"(9b) Chromosome changes in terms of aberrations seem to be a useful parameter in predicting the interaction of radiation with cells. It has been demonstrated that the relative efficiency producing aberrations can be related to cancer incidence. This is true when the cells at risk for both endpoints are involved. For example in the liver the relative effectiveness of alpha particles and beta particles in producing chromosome aberrations and cancer are similar. For detecting numerical changes and stable karyotypic changes flow cytometry has great promise. However, since many cell lines have very unstable karyotypes without changing their ability to produce tumors this is not a useful application in vitro.

(9c) Cytotoxicity and early senescence.

Cytotoxicity in vitro is a reflection of potential toxic effects in the whole animal. The level of cell killing can be quantitated and used to rank technologies. The measuring of these rankings in terms of the levels of exposure in the environment is difficult to evaluate since there seems to be a threshold for cell killing with most agents. Cell killing does not reflect genetic damage or cell transformation but may play role in degeneration diseases and tumor promotion. This makes it useful as an endpoint for study.

Early senescence may or may not reflect genetic damage. It seems to be a questionable endpoint since it may reflect culture conditions rather than the genetic status of the cells.

(9d) We recommend the use of Na^+/K^+ ATPase and HGPRT mutants since they have been detected and used successfully in variety of cell lines. TK, APRT and traits which require selection of +/- heterozygous cells and seem to be harder to apply and less useful for routine testing.

(9e) We recommend the use of anchorage-independent growth as the most useful and rapid test to detect cell transformation.

(9f) I will restrict these comments to organ and tissue changes, although similar considerations are relevant to germ cell changes. There are four basic approaches to evaluting changes in organs and tissues. First, indicators of tissue injury can be evaluated without examining the tissue itself or allowing the injury to progress to an outward manifestation of a "health effect". An example would be the study of enzymes and other compounds in serum or airway fluid to determine tissue injury. Second would be the removal by biopsy or necropsy of a portion of the tissue of interest and examining it microscopically. Third would be the performance of organ-level tests of physiological function, such as cardiovascular, respiratory, hepatic, renal or thyroid function tests. Measurements of immunological, particle clearance, and bacterial defense functions also fall into this category. The fourth method is to simply allow the tissue/organ injury to progress until it either becomes clinically evident or results in death. Examples would be gross observation of tumors or respiratory distress.

Each of the above methods have their advantages, disadvantages and proper roles in toxicological studies. The first (biochemical) often lends itself to detection at times before organ-level dysfunction is evident, but

depends on an established relationship and experience on subsequent manifestations of disease for interpretation. Also, the ultimate manifestation of disease (e.g. tumorigenesis, fibrosis) may not be predictable by the early indicators. Direct examination of tissue is an important means of confirming morphological alterations, but requires biopsy or sacrifice, usually includes evaluation of only a small portion of the total tissue, and does not necessarily depict the final manifestation of disease. Physiological function tests are useful in that they are a direct link to symptomatology and disability in man, and are usually nondestructive. These tests, performed in animals by methods similar to those used in man, are unique in that they indicate the impact of a health effect on the individual and results can be directly related to data collected clinically and epidemiologically in man. Function tests characteristically, however, reflect the integrated function of the entire organ or system and do not always detect pathological alterations. For example, a lung tumor of considerable size can be "silent" in respiratory function assays. Finally, observation of animals for outward manifestations of tissue/organ injury is simply done and gives information on ultimate outcome; however, it yields little information on the pathogenesis of the disease.

The position which becomes obvious from reviewing the above information is that all four types of approaches are necessary in a comprehensive study of toxic manifestations in tissues and organs. Each of these approaches can be used singly in addressing specific questions in well-targeted studies; however, it would be inappropriate to completely dispense with any of them."

Answer: Brian D. Crawford

"(9b) The precise role of chromosomal abnormalities in cancer remains to be determined. Detection of cytogenetic abnormalities in mitotic chromosomes from human lymphocytes will require the examination of hundreds of cells to obtain the desired level of sensitivity. Current estimates are that one cytogenetic abnormality such as a ring or dicentric chromosome in 200-300 cells is expected at the desired level of detection. Thus, a minimum of 1000 (46,000 chromosomes) cells will have to be examined to obtain a reasonable degree of confidence in the rate of occurrence of the abnormalities. Since only manual methods are presently available, automated methods of detecting cytogenetic abnormalities are a necessity. Progress in flow cytometric measurements of chromosomes indicates that, with further development, the technique will be able to detect chromosomal abnormalities with high through-put rates. By flow cytometry one can measure the amount of DNA (or other stained moiety) in single cells or chromosomes rapidly (10^4/min) and thereby analyze large numbers of cells or chromosomes from an individual in a short time (30 minutes). With improved resolution, a flow karyotype based on the DNA content of 50,000 chromosomes from an individual could resolve the chromosome types, count the frequency of occurrence of each chromosome with high statistical accuracy and detect abnormal chromosomes. The information conventionally obtained by banding analysis of metaphase spreads (usually less than 50) can potentially be obtained in less time with much greater confidence by flow cytogenetics. Comparisons between exposed population and control population can be reliably made owing to the quantitative and statistical properties of flow cytogenetic data.

The current state of the art in chromosome analysis by flow cytometry and sorting, pioneered at the Los Alamos and Lawrence Livermore Laboratories, has established the potential of the technique. Difficulties that limit widespread application, such as resolution, sensitivity, and speed of sorting remain to be overcome. The resolution of chromosomal DNA content measurements will have to be improved to further resolve the normal human karyotype. In model animal systems, a coefficient of variation (CV) on the order of 1.5% has been fairly routinely achieved. With this resolution, the Chinese hamster chromosome complement can be completely resolved. This achievement has already led to discoveries of chromosomal abnormalities in the study of progression to tumorigenesis before they were detected with classical cytogenetic techniques. Once the abnormalities were suspected in a small fraction of the cells using flow karyotype analysis, they were found by conventional techniques when enough spreads were examined. This example points up the need to examine large numbers of chromosomes. If human chromosome measurements were routinely made with a CV of less than 1%, most of the human karyotype will be resolved. With resolution of this magnitude, it could be possible to detect a large number of cytogenetic abnormalities.

However, to completely resolve the human karyotype, it will be necessary to measure another chromosomal parameter in addition to measuring DNA content to better than 1.0%. One possibility is the centromeric index. In order to measure the centromeric index of a chromosome it will be necessary to locate the centromeric dip in the waveform generated by a chromosome as it passes through the laser beam or to mark the centromere with a specific antibody and measure the time relationship of the centromere marker to the signal from the whole chromosome. Either method requries pulse shape analysis to determine the relevant parameters.

Another cytogenetic signature of exposure to toxic agents is the appearance of double minute chromosomes. Double minutes are characteristic in some cancers such as neuroblastoma. The size of double minutes is estimated to be on the order of 1/10th of the smallest human chromosomes. In order to determine if it is feasible to detect double minutes in a flow cytometer, it is necessary to understand the present sensitivity limitations and which components of the system are limiting.

The result of low level clastogen exposure is likely to be subtle karyotype rearrangements. Thus, one novel and potentially important use of the capability to resolve chromosome types is the ability, based on flow cytometric measurements, to sort large quantities of chromosomes with better than 95% purity for construction of chromosome specific DNA libraries. If chromosomes are resolved by flow measurements, the measurements can be used as a basis for sorting to obtain large amounts (greater than 1 microgram) of specific chromosomes. Once obtained, the chromosomal DNA can be amplified by recombinant DNA techniques to produce a library of chromosome specific DNA. When a complete library for the human chromosome complement is obtained, a host of more subtle questions concerning the translocation of genetic material among chromosomes can be asked. For example, some cancers are associated with specific chromosomal abnormalities. With a DNA library, the specific sequences which are transferred from one chromosome to another can be determined. Chromosome translocations occurring as the result of exposure to

toxic agents can be quantitated and the specific sequences which are moved can be determined. Such studies are already in progress within the Genetics Group at the Los Alamos National Laboratory.

Improvement in the resolution of fluorescence measurements will also facilitate more accurate cellular DNA content measurements which will immediately (1) lower the level of detectability of abnormal cellular DNA content and (2) improve cell cycle quantitation of perturbed cell populations.

Thus, in response to the biological needs stated above, research programs are focused on advancing the state-of-the-art of flow cytometry in the following areas: (1) improving the resolution and sensitivity of fluorescence measurements, (2) analysis of fluorescence pulse shapes, (3) increased chromosome sorting speeds and (4) experiment modeling and data interpretation.

(9d) In the development of any bioassay, considerable emphasis should be given to the quantitation of somatic mutation as an indicator of genotoxicity. Such assays compliment microbial test systems (e.g., "Ames" assay) by providing an appropriate level of genetic complexity. Indicator loci should include both recessive and dominant mutations, sensitive to a variety of mutagenic insults (point, frameshift, deletion, insertion). The hprt locus is ideal for the study of point, frameshift, deletion, and insertion mutation. Moreover, the recent availability of cDNA clones for this genetic locus makes it amenable to analysis at the molecular level. Studies of autosomal loci governing recessive phenotypes appear to offer little additional information. Dominant mutations, such as those at the Na^+/K^+ ATPase locus, are useful primarily for detecting agents capable of "subtle" (e.g., single base-pair transition/transversion) mutational lesions, and must be buttressed by other mutation assays to avoid the problem of "false negatives".

(9e) Any modern genotoxicity assay should include measurements of changes other than single locus mutation. This relates to question 9(e), i.e., "gene expression" changes. Specifically, assays should be developed:

1. To screen for agents which alter DNA methylation, and thereby alter transcriptional regulation. One possibility would be to use a gene under transcriptional regulation by methylation/demethylation. Flow cytometry might be used to quantitate the repression/derepression of this gene, if fluorescent "tags" (e.g., antibodies) can be developed for the gene product of interest. For example, abnormal turn-on of globin synthesis in fibroblast cells could be monitored in this manner. Such an assay would be quantitative and rapid.

2. To utilize nucleic acid probes, or appropriate immunologic probes, for the detection of expressed "oncogenes". This area holds promise as an approach toward both screening and the mechanistic study of gene expression as related to neoplasia. Considerable emphasis should be given to this area of basic research, in consideration of the benefit to be derived for applied studies in the future. These studies also would interface viral and chemical/physical carcinogenesis. Again, flow cytometry affords the possibilities of rapid and accurate quantitation.

3.   To detect karyotic abnormalities such as non-disjunction. Flow cytometric analyses would, as discussed above, be of particular value/necessity in this area. These studies are warranted in light of the non-mutagenic nature of some carcinogens. Large deletions or chromosome rearrangements also could be resolved by flow cytometric analyses.

4.   To measure specific gene rearrangement. For example, in _Drosophila_, movement of known (e.g., _copia_) transposable elements could be monitored, particularly when such movement affects an observable phenotype. In yeast, mitotic recombination and mitotic gene conversion would be reliable indicators of gross genetic alterations which would escape detection in conventional assays for single-locus gene mutation. The value of these test systems is scientifically well-recognized, but has not been emphasized in most recommendations for genotoxicity assays in _applied_ programs. The key here is to provide a direct tie between basic and applied research."

Answer: Joseph DiPaolo

"I am of the opinion that acute chromosomal damage correlates very well with cytotoxicity and at the same time we have evidence that transformation and lethality are independent phenomena. Therefore, I am inclined to minimize the importance of acute chromosomal damage in relation to transformation leading to malignancy since visable chromosomal damage will probably result in lethality. On the other hand, sister chromatid exchange induction is a representative event of carcinogen-DNA interaction and is a very sensitive assay for detection of DNA damage. Practically all carcinogens (except for X-ray) that have been demonstrated to transform _in vitro_ also induce sister chromatid exchanges. In the Syrian hamster system, there is a positive correlation between the induction of transformation and the frequency of sister chromatid exchanges. The induction of sister chromatid exchanges by various carcinogens will provide information concerning the types of DNA lesions responsible for transformation as well as the ability of the target cells to repair the carcinogen-induced lesions. The sister chromatid exchange assay is probably the most rapid one that can be done with mammalian cells. If proper attention is paid to adequate controls, the sister chromatid exchange assay could be the "early alert" that something is amiss in the human population. For this purpose, human lymphocytes could be obtained readily in the field."

Answer: Takeo Kakunaga

"The only convincing criteria for determining neoplastic transformation of the human cells _in vitro_ is the tumorigenicity test in immunologically deficient animals (nude mice). Loss of contact inhibition, reduction in nutrient requirements (low serum, low calcium, etc.) and anchorage-independent growth (cloning efficiency in soft agar, etc.) should be adopted after their correlation with the tumorigenicity is examined. Molecular criteria would be useful and important if they can be adopted as endpoints marker. However, it seems, so far, none of them can serve as general parameters for neoplastic transformation of human cells. For example, we do not know how many different oncogenes exist in human cells."

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Answer: Stephen A. Lesko

"Bioassays for comparing agents from various technologies should be carried out at four levels of sophistication, viz. at the molecular, biochemical, cytological and cellular levels. Molecular damage can be measured as DNA strand scission, DNA-protein crosslinks, DNA interstrand crosslinks, DNA adducts or base alterations and lipid peroxidation.

a.   Molecular Damage

i.   DNA strand scission and DNA-protein crosslink

The alkaline elution technique is a versatile and sensitive procedure which can be used to discriminate DNA single-strand sizes in mammalian cells. Variations in the technique can be used to measure single-strand breaks, alkali-labile sites and DNA-protein crosslinks. The sensitivity of the method is of the order of one DNA lesion per 10^7 nucleotides.

The procedural manipulations allow flexibility in handling cell DNA without mechanical fragmentation. This is accomplished by depositing cells on filters prior to lysis with a detergent solution. Most of the cell protein and RNA can be washed through the filter leaving the DNA intact on the filter and still in double-stranded form. An alkaline solution at about pH12 is then slowly pumped through the filter and the rate of elution of the DNA in single-stranded form is measured. Single-strand breaks are detected on the basis of an increase in the DNA elution rate. DNA-protein crosslinks cause a reduction in elution rates due to an effective increase in strand length as a result of adsorption of proteins to the filter. The frequencies of strand scission and DNA-protein crosslinking can be quantitated in terms of rad-equivalents.

ii.   DNA adducts, base alterations and interstrand crosslinks

Recent advances in the area of monoclonal antibodies/hybridoma research afford a revolutionary new approach for detection and quantitation of lesions in DNA. Monoclonal antibodies have the unique capability of recognizing a specific damage to nucleic acids with a high degree of affinity and specificity. For the monoclonal antibody approach to be successful one must have the capacity to synthesize relatively large quantities of short DNA helices containing specific lesions and be able to fully characterize any lesion introduced into a DNA molecule. The purity of the probe will determine the specificity of the monoclonal antibody preparation. Specific monoclonal antibodies will allow the detection and quantitation of DNA adducts, base alterations or interstrand crosslinks induced in cellular systems as compared to those found in vitro. Furthermore, monoclonal antibodies should allow the detection of low levels of DNA damage in human cells with the use of biopsy material or blood samples. This degree of sensitivity is possible as it has been recently reported that monoclonal antibodies can reliably detect one aflatoxin B_1 residue per 250,000 nucleotides of DNA isolated from the liver of rats treated with aflatoxin. This demonstrates that DNA adducts can be detected at levels of biological significance using monoclonal antibodies and competitive enzyme-linked immunosorbent assays.

iii. Lipid peroxidation

Peroxidation of biological membrane lipids alters both the integrity and biochemical properties of various subcellular organelles. In addition, toxic products (free radicals, hydroperoxides and malonaldehyde) can be released and may damage cellular macromolecules at some distance from the site of peroxidation. There are reports that malonaldehyde can induce DNA protein crosslinks and DNA interstrand crosslinks.

The lethality of certain compounds whose reduced forms are readily autoxidized to generate oxygen radicals is enhanced by the presence of oxygen. A few examples are paraquat, streptonigrin and benzo(a)pyrene quinones. The lethality may result from peroxidation products formed by reaction of oxygen radicals with cellular membranes.

Lipid peroxidation can be detected by measuring the formation of thiobarbituric acid reactive material or conjugated diene formation at 237 nm using UV spectroscopy."

Answer: J. Justin McCormick

"(9)   As explained above, the linear dose-dependent induction of anchorage-independent growth in human fibroblasts is the best in vitro criteria for transformation. Tumorigenicity in athymic mice is the ultimate criterion for transformation. Whether this criterion is equivalent to anchorage-independent growth is not yet clear as discussed above. In our experience, tumors arise 16-22 weeks after s.c. injection of mice. However, in sublethally irradiated animals, tumors arise in 1 to 3 weeks.

(9a)   It is clear that there is a very high correlation between the induction of DNA damage, repair, and transformation. Therefore, assays such as the measurement of DNA damage by alkaline elution, alkaline unwinding or unscheduled DNA synthesis will usually be found to correlate with transformation. Monoclonal antibodies are useful in monitoring the DNA repair of known agents to which one possesses antibody, but are of no use in monitoring DNA damage by unknown compounds. The other methods noted above are non-specific (i.e. satisfactory for known or unknown compounds or agents) and so can be generally applied. Techniques measuring RNA or protein damage or membrane damage are unlikely to be useful.

(9b)   Chromosomal damage, SCE induction and/or karyotypic changes are likely to correlate with transformation. However, since such studies are slow and tedious, they are more likely to be of interest for mechanistic studies or as a secondary assay. To my knowledge, flow cytometry cannot yet be applied to such problems in human cells.

(9c)   We have not found any agent that induces transformation of human fibroblasts without simultaneously inducing cytotoxicity (loss of replicative ability as measured by cloning). However, many agents are cytotoxic and yet do not induce transformation. Since cytotoxicity assays are necessarily carried out as part of the transformation protocol, no further discussion of this subject seems required.

The relationship between neoplastic transformation of human fibroblasts to senescence is unknown.

(9d) In our experience there is a direct relationship between the induction of mutations in the forward mutation marker resistance to thioguanine (resulting from loss of an active HGPRT enzyme) and the induction of anchorage independence (1,2). Although the nature of the gene product or products involved in anchorage independence are not yet identified, the use of forward mutation assays seems wiser than to rely on markers which exclude agents which interrupt or eliminate a protein (e.g. ouabain resistance).

(9e) This is an area of high potential, but essentially no data exist at this time.

(9f) In our hands, human cells induced to exhibit anchorage-independent growth also form foci and vice versa. The relationship of these properties to growth under reduced nutrient conditions is as yet unknown, but is under study."

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Answer: Manfred F. Rajewsky

"Most possibilities for molecular and cellular end-points are listed under (a)-(h). The choice needs thorough discussion at the conference. Regarding point (f): we want to assay for malignant (not only neoplastic) conversion. Regarding point (h): should be called "Tumorigenicity, Invasiveness, Metastasis". Interesting assays for invasiveness (in vitro) are becoming available".

Answer: Helene S. Smith

"For human epithelial cells, too little information exists at this time to make any correlations between various assays as predictive or risk assessment. What little data exist is mostly on biological parameters--cytotoxicity, increased lifespan, loss of anchorage dependence, decreased requirement for growth factors, and defective terminal differentiation. The only quantitative assays that have been reported are for cytotoxicity and for survival in methocel (keratinocytes only). Of particular interest to me are the many reports beginning to appear in the literature on

monoclonal antibodies that recognize tumor, but not normal cells. If any
antibodies become available which can be used immunocytochemically (with
fluorescence of peroxidase assays) such antibodies could be used for
developing a rapid and inexpensive transformation assay.

Of particular concern to me is the problem of evaluating malignancy of
human cells using nude mice. Many people believe that the ultimate test of
malignancy is reinjecting the transformed cells into an isogenic host (or a
nude mouse, for human cells) and thereby causing a malignancy. However, there
is an important reason why this point of view may not be relevant for
evaluating risk. Human malignancy arises as a multistep phenomenon. Even
within a primary invasive carcinoma, not all cells may be capable of all the
steps required to seed and grow at a metastatic site. When one dissociates
cultured cells and then inoculates them subcutaneously into nude mice, one
requires that the cells be capable of growing without anchorage at a distant
tissue site. In many ways the nude mouse assay is more representative of a
metastasis than a primary carcinoma. From what we know about human malignant
progression, it is not necessarily obvious that a particular carcinogen will
do all of the steps necessary to induce metastic growth starting with a normal
cell. Yet a carcinogen that efficiently does only some of the initial steps
of malignant progression may be an enormous biohazard to the human population.
There are other, more often recognized limitations of the nuce mouse assay
mostly relating to the degree of immune competence present in nude mice."

Answer: Stuart H. Yuspa

"9a. An assessment of DNA damage for each system would be useful.
Perhaps alkaline elution and unscheduled DNA synthesis would be broad
screening methods. Antibody technology would not seem indicated at this point
since it would require adducts to prepare antibody. For poorly defined
compounds or mixtures, preparation of adducts would be difficult or impossible
and would take many months under the best of circumstances.

9b. Monitoring of chromosomal damage - useful adjunct screening test.

9c. Cytotoxicity - should be part of any dose-response study.

9d. Somatic mutation - should be part of separate studies on test
compounds but not a necessary component of a bioassay system.

9e. Gene expression changes - should not be included in screening
protocols.

9f. Neoplastic transformation. If epithelial systems are to be used, a
series of endpoints may be more valuable than those listed above. These would
include: (1) Persistant growth in vitro or focal colonial growth emerging out
of a monolayer culture; (2) resistance to a signal for terminal differenti-
ation; (3) stimulation of proliferation which may detect a promoting or
cocarcinogenic agent; (4) transformation demonstrated by tumor development in
animals transplanted with cells which have demonstrated above criteria.

9h. Tumorigenicity - Syngeneic newborns and nude mice are the best recipients; metastasis assay is a separate question and should not be used; the invasion-transplant assay being developed by Norbert Fuseniy at the German Cancer Center should be considered."

QUESTION #10.

Quantitative correlation and coordination of all the molecular and cellular measurements in a bioassay system. Can a matrix system be established?

Answer: Antone L. Brooks

"A number of matrix systems can and should be established. The way these systems can be useful is to relate the unknown to something you know about in humans. For example, we have a great deal of information linking smoking to the induction of cancer. If you want to know the impact of inhaling a pollutant with unknown health effects it would be useful to test extracts of this pollutant in a range of short-term tests at the same time that you test extracts from cigarette smoke. By calculating dose, distribution and retention for unknown pollutant and measuring the relative potency of the extracts it can then be related to the smoking extracts. This technique has been applied and found to be useful in predicting which of the short-term tests predicts what happens in the human populations."

Answer: J. Justin McCormick

"I propose a human bioassay system shown schematically in Fig. 2. Human fibroblasts using suitable activation systems as needed are used to measure cytotoxicity and induction of anchorage-independent growth and induction of thioguanine-resistance. The tumorigenicity of the anchorage-independent cells is confirmed in athymic mice.

Since human T-lymphocytes can now be induced to exhibit thioguanine-resistance these cells are to be utilized in a parallel mutational assay. In addition, one can make use of T-lymphocytes isolated from humans who are suspected of having been exposed to mutagenic and/or carcinogenic agents to measure in vivo induced mutations (1). This matrix would allow one to quantitate thioguanine resistance in vivo and in vitro and to extrapolate from the relationship between induction of thioguanine-resistance and anchorage independence in fibroblasts to the risk for people. As epidemiological studies of tumor frequencies become available to persons exposed to known levels of carcinogenic agents (Question 15), the correlation between induction of thioguanine resistant T-lymphocytes and tumorigenicity could be made at the in vivo level to match the in vitro data."

Reference

1. Albertini, R.J., Silwester, D.L., and Allen, E.F. The 6-thioguanine resistant peripheral blood lymphocyte assay for direct mutagenicity testing in man. In: Mutagenicity-New Horizons in Genetic Toxicology. J.A. Heddle (ed.), Academic Press (in press, 1982).

FIGURE 2. Proposed In Vitro-In Vivo Bioassay System - Human Cells

	Human diploid fibroblasts treated with carcinogens	Human diploid T-lymphocytes taken from non-exposed persons and treated with carcinogens	Human T-lymphocytes from persons exposed to carcinogens in vivo
1. Assay for biological effects			
a. Cytotoxicity	+	+	-
b. Frequency of TG^r mutant cells	+	+	+
c. Frequency of anchorage-independent cells and tumorigenicity	+	+	*
2. Assay for biochemical effects			
a. U.D.S.	+	+	+
b. Alkaline elution or unwinding, etc.	+	+	+
3. Assay for chromosomal effects			
a. Aberrations	+	+	+
b. Loss or gain, etc.	+	+	+

*Long-term epidemiology studies of tumor frequencies.

QUESTION #11.

Procedure and choices for the establishment of the carcinogen/mutagen reference unit. External reference unit? Internal reference unit?

Answer: J. Justin McCormick

"As an external reference unit, UV (290-320 nm, sunlamp) and ionizing radiation seem preferable. Because of the availability of sensitive physical monitoring instruments, cells can be treated with easily-reproducible doses of these agents. Quantitation of doses of chemical agents that require metabolism is necessarily less certain because of the differences in the rates of metabolic activation and deactivation as well as non-specific binding to serum, etc. Although it is possible to avoid some of these problems by using a mutagen that does not require activation, such compounds are usually unstable, sensitive to small differences in protocol and in general give more variable results that the physical agents named above. Use of a 290-320 nm sunlamp instead of a more traditional 254 nm source would allow extrapolation to sunlight effects which might be a help in putting risks into perspective.

The slopes of cytotoxicity, mutagenesis and transformation (agar growth) dose response curves are the most reliable internal reference units. Biochemical reference units (DNA breaks, crosslinking, photoproducts in DNA, adducts in DNA, etc.) are less reliable because of the measurements involved and are not a good starting point for an internal standard. They could be developed into internal reference units by the use of standardized protocols."

Answer: Manfred F. Rajewsky

"In addition to agents requiring enzymatic activation, agents which do not require enzymes to be converted to their ultimate reactive derivatives in vivo (e.g., alkylnitrosoureas) should be included as reference units."

Answer: Stephen A. Lesko

"Ionizing radiation would be the best choice for an external reference unit. It has been thoroughly investigated, is omnipresent and dose-response curves have been established. Dosage and dose rates can be carefully controlled.

Before an internal reference unit can be established it will be necessary to determine if an intrinsic background rate exists for the bioassay system due to a low but constant rate of reaction of oxygen radials with critical macromolecules. The ubiquitous distribution of enzymes such as superoxide dismutase, catalase and glutathione peroxidase attest to the presence of reduced oxygen species in aerobic cells. Cellular senescence and spontaneous cancers may arise from a malfunction or perturbation of the oxygen defense system by environmental agents."

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Answer: H. Yamasaki

1.   One of the criteria suggested by Dr. Ts'o (1) for choosing the reference unit, "omnipresence-universal-environmental" is listed. If an agent is a known human carcinogen and if the mechanism of action of that agent is different from others, this criterion is not necessarily filled. However, such a reference agent should always be easily available for those who would like to test it.

2.   Related to comment 1, diethylstilbesterol can be used as a reference compound. It seems that this compound will serve as a reference compound for the detection of carcinogenic hormones or hormone-like carcinogens.

3.   With respect to the internal reference unit, a cautious approach is desirable for its decision, since at present our mechanistic knowledge does not allow us to evaluate which biochemical or cellular measurement is close to prediction of carcinogenicity. The importance of a mechanistic approach should again be emphasized."

Reference

P.O.P. Ts'o and J.C. Barrett, "Mechanistic Studies of Neoplastic Transformation of Cells in Culture" In: Polycyclic Hydrocarbons and Cancer, Vol. 2, (eds. P. Ts'o and H. Gelboin), Academic Press, Inc., N.Y., 1978, pp. 235-267.

QUESTION #12:

What are the relationships between viral carcinogenesis versus chemical/physical carcinogenesis as related to this bioassay system? Are there common mechanisms? Will these two perturbations work synergistically? What is the relationship between the viral transforming genes and the presumptive host (human) 'oncogenes'.

Answer: Joseph DiPaolo

"Co-carcinogenic experiments involving viral and chemical or chemical and physical agents provide conclusive evidence that enhancement or synergism of transformation frequency is possible. Chemical carcinogens from a variety of chemical classes can be detected in model systems in conjunction with either known DNA or RNA virus types; the results can be differentiated from noncarcinogenic analogs. Furthermore, it is possible to discriminate between damage inflicted upon cellular DNA by mutagen such as iododeoxyuridine and laterations induced by frankly carcinogenic compounds. The quantifiable nature of these assays lends them to investigation of the in vitro and in vivo potency of carcinogenic compounds. Although in all systems that I am knowledgeable about, proof of viral involvement has been obtained from "T" antigens, it might be possible that the cocarcinogenic effect might involve a triggering of changes that are associated only with a chemical transformation. Elevated levels of src-related transcripts in rat cells can be induced by exogenous infection with murine leukemia virus or extended passage in tissue culture and these levels resemble those found in chemically transformed cells. This suggests a correlation between elevated levels of src-related transcripts and enhanced susceptibility of rat cells to transformation by chemical carcinogens. It might be speculated that chemical treatment causes an induction of endogenous src-related transcript which in conjunction with exogenously added src sequences results in an increase in the frequency of viral transformation. On the other hand, it is quite possible, particularly with DNA viruses, such as SA-7, that chemical treatment causes an increase in the rate of DNA synthesis which increases the efficiency of viral integration that results in an elevated frequency of viral transformation. The value of cocarcinogenic bioassays to risk assessment is not dependent upon understanding the mechanism of action."

Answer: Takeo Kakunaga

"It is likely that some cellular "oncogenes" are integrated into the viral genes functioning as viral transforming genes. It is probable that transformations by chemical/physical carcinogens occur via activation of such cellular 'oncogenes', thus chemical transformation mechanisms are overlapping with those of viral transformation. However, we do not know the total number of 'oncogenes' which human cells harbor and how many of them are evolved as viral genes. We also do not know whether the activation of an 'oncogene' is a necessary or sufficient condition for expression of the transformed state.

It is likely that the transforming genes carried by many viruses had been

derived from mRNA of the cellular 'pro-oncogenes' via reverse transcription to cDNA and recombination with retrovirus genomes. Some viral transforming genes directly code for transforming proteins and others turn on the cellular 'pro-oncogenes'. I think the viral oncogenes and 'pro-oncogenes' so far identified represent only a fraction of the genes involved in the induction of oncogenic transformation by chemical/physical carcinogens. On the other hand, it is possible that viral oncogene expression and the effects of chemicals result in the additive or synergistic expression of the transformed phenotypes and could be integrated into an bioassay system."

Answer: George Klein

"It depends on what viral carcinogenesis system is considered. The retrovirally carried v-onc genes are not directly related, but, according to all that we know, are identical with the cellular oncogenes (c-onc). To what extent activation of the same c-onc as carried by the v-onc is involved in chemical carcinogenesis is at least open for study."

Answer: J. Justin McCormick

"In my opinion, viral carcinogenesis and chemical/physical carcinogenesis in human fibroblasts cannot be united at this time. There is no evidence as yet on the interaction of these two types of transformation within cells. While it seems reasonable to believe that human "oncogenes" may be related to viral transforming genes, no data are available on this point."

Answer: Manfred F. Rajewsky

"The common denominator for chemical/physical and viral oncogenesis is, in my opinion, an interference with the genetic program of the particular target cell. I believe that the expression of malignant phenotypes can be brought about not by only one particularly mechanism of interference with the correct expression of a specific gene, but rather in a variety of ways, depending, e.g., on the type and developmental/differentiation stage of the target cells, and on the mode of action of the particular type of agent used. There are a great variety of malignant phenotypes, and perhaps the most outstanding feature of a malignant phenotype is its 'plasticity' (possibly thus far the only generalizable distinction from non-malignant cells) which renders it hyperadaptable to all kinds of microenvironmental conditions and selective pressures. If cells in early developmental/differentiation stages (still to be precisely defined in terms of gene expression) are indeed characterized by an elevated risk of malignant conversion, then we may find that the so-called oncogenes are not only characteristic of specific cell types but also that they are genes normally expressed during specific stages of development/differentiation. In fact, a 'clustering' in terms of the frequency of oncogenes expressed in tumor cells that have arisen from a particular normal cell type may provide clues as to the developmental/differentiation stage with a high transformation risk for the particular cell lineage in question."

QUESTION #13.

What are the basic mechanisms of neoplastic transformation/
carcinogenesis as related to the establishment of this bioassay system? What
are the genetic and developmental determinants in neoplastic transformation?
DNA damage? Chromosomal changes? Development (epigenetic) factors?
Initiation and promotion? Role of promoters?

Answer: J. Carl Barrett

"This question is too basic to be discussed in detail here. Many reviews
on this area have been written by numerous investigators, many of which are
attending this conference. For my latest indepth thoughts in this area, I
refer you to my latest review chapter and I will not repeat any details here.
There are three conclusions, however, that I would like to mention here:

   a.   Neoplastic transformation is a multistage process. I feel that this
        is well established for rodent cells in culture, and while
        definitive experiments are lacking, some experimental evidence
        suggests that human cell transformation in vitro is also a
        multistage process. This conclusion is also supported by
        epidemiological and genetic findings with cancers in humans.
        If this conclusion is accepted, then risk assessment cannot be based
        on a single cellular or molecular change, since carcinogens/pro-
        moters may act at one or more stages by differing mechanisms.

   b.   Our recent results with transformation of Syrian hamster embryo
        cells has led us to conclude that chromosomal changes are very
        important in this process.

   c.   The role of promoters in in vitro transformation and the relevance
        of these in vitro results to in vivo findings should be criticlly
        discussed. It is important to recognize that promotion is a
        phenomenon, not a mechanism. While the effects of tumor promoters
        on cells in culture (under certain conditions) can be demonstrated
        to mimic the phenomenon or tumor promotion in vivo, this does not
        prove that the mechanism by which tumor promoters enhance tumor
        growth in vivo is the same as the mechanism by which they enhance
        transformed foci formation in vitro or any other in vitro effect.
        As Farber has discussed, there are two basic mechanisms by which
        tumor promoters may act: (1) tumor promoters result in the
        differential amplification of initiated cells vis-a-vis normal cells
        (i.e., quantitative change only) or (2) tumor promoters modulate
        initiated cells (i.e., qualitative change) plus cause differential
        amplification of initiated cells. It is not known whether tumor
        promotion in mouse skin is a quantitative or a qualitative change or
        both. The same uncertainty exists for 'tumor promotion in vitro'.
        These basic issues need to be addressed before risk assessment of
        tumor promoters can be attempted with cell culture bioassays."

Answer: Antone L. Brooks

"I feel this is one of the most important remaining questions. To date a great deal of work has been conducted on DNA damage, chromosomal changes, mutation and initiation. These data seem useful in predicting carcinogenesis. The area that has been neglected and requires some real effort is the development of in vitro systems that will detect the presence of promoters in complex mixtures. It seems that there are so many initiations in our environment that promotion of initiated cells may be the limiting factor in development of cancer."

Answer: Stephen A. Lesko

"Oxygen radicals may also play an important role in promotion. TPA is a potent tumor promoter, an inflammatory agent when applied to skin, a stimulator of the respiratory burst in phagocytic cells and an inhibitor of catalase and superoxide dismutase activities. The TPA-stimulated respiratory burst in human leukocytes is associated with extensive DNA strand scission. The following model has been postulated. Repeated applications of TPA to an initiated site on skin leads to inflammation, migration of phagocytic cells to the area, and then triggering of these cells to produce DNA damaging oxygen radicals at a site which has low superoxide dismutase and catalase activities. DNA damage induced at the initiated site may be the process that eventually promotes neoplastic development. Benzoyl peroxide is a potent tumor promoter which can directly cause DNA strand scission. In support of this model, antioxidants such as BHA are potent inhibitors of TPA-induced ODC activity in mouse epidermis."

Answer: J. Justin McCormick

"As discussed above, it seems likely that induction of anchorage-independence in human cells is a mutational event. Since DNA repair modulates the effect, DNA damage is clearly involved. It is not clear to me that chromosomal damage or epigenetic factors have a necessary role to play. The concept of initiation-promotion cannot be applied to human fibroblast transformation. No systematic examination has been made of the action of promoting agents in this system, but it is clear such agents are not necessary."

Answer: H. Yamasaki

"Several lines of evidence already indicate that neoplastic transformation is a multi-stage process and that probably multi-factors are involved. Cell cultures studies to determine the correlation between 'mutation' and 'cell transformation' showed that 'mutation' is not the sole mechanism of 'cell transformation'. In fact, a two-stage model of in vitro cell transformation has recently been established using C3H 10T 1/2 cells, BALB 3T3 and Syrian hamster cells. Thus, one may conclude that 'cell transformation' proceeds by a multistage process.

As far as an early stage of transformation (initiation) is concerned, a large body of evidence has been accumulated to support the idea that DNA may be the primary target. Whatever the precise process of this stage is, it is generally agreed that 'genetic damage' is involved in this process. The mechanism of later stage (promotion) is known to a less extent and a vague term 'epigenetic action' is used to explain this process. In some experiments, tumor promoters, especially phorbol esters, have indeed been demonstrated to exert some reversible epigenetic effects, i.e., modulation of cell differentiation, inhibition of cell-cell communication, modulation of gene expression, etc. However, it is not clear whether these actions are universal to tumor promoters other than phorbol esters.

With respect to the aim of bioassay system, it is, nonetheless, important to realize this complex situation. Different bioassays may be developed so that one system would detect presumptive initiators and the other would predict tumor promoters. On the other hand, a more complex system, such as cell transformation system, may be used to detect both of these factors. Since we do not know about the precise mechanism of carcinogenesis, it is important that we do not rely on a few end points of tests. For example, mutation is a clear-cut end point to measure and therefore much work has already been done; but we feel that the use of cell transformation systems should also be encouraged because the end point is more proximate to carcinogenicity".

Answer: Stuart H. Yuspa

"If I had to guess now I would say (1) an alteration in the commitment to differentiate terminally; (2) responsiveness to a proliferative stimulus (promotion); (3) a change in responsiveness to extrinsic negative regulatory factors (e.g. cell contact, chalones). This question is intimately linked to the validity of the bioassay system and is why the development of short term assays are so difficult."

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QUESTION #14.

How to coordinate the application of this bioassay system with the arrival bioassay approach and the epidemiological approach? How to correlate the animal data with the human data? How to extrapolate the data on tissue/cells in vitro to whole organism (animal and human)?

Answer: Richard J. Albertini

"Again, the matrix system proposed in partial answer to Question #6 allows the correlation of in vitro bioassay data (in this case, using genetic endpoints), in vivo bioassay data (direct mutagenicity testing in animals and humans), whole animal studies, and clinical and epidemiological followup studies of the individual humans studied in these assays. (See also the proposal made in answer to Question #21)."

Answer: Antone Brooks

"This question, of course, is critical and has been partially addressed in answers to questions 1, 4 and 9. Aside from discussions of methodologies, there remains a very important area which has received insufficient attention. In order to establish the relationships between toxicological test systems and human disease, we should start with known toxic manifestations in man and work backward through the test systems. An example might be cigarette smoking. The manifestation in man is as clear as any we know about and includes tumorigenesis, developmental defects, noncarcinogenic cardiopulmonary disease and impairment of defense mechanisms. Although considerable effort has been expended on subhuman toxicology, what do we know about the interrelationships among the toxicological 'signals' we have obtained? Do we have information from endpoints and methodologies that are currently being employed? Do further, well-coordinated studies need to be done in order to reconstruct the best line of 'prediction' from toxicological assays to man? The same questions can be asked in regard to irradiation, lead, asbestos, thalidomide, diethylstilbesterol, coal dust, or any other material for which human manifestations have been observed. Little "toxicology" is being done on many of these because they are already regulated or controlled and it is generally felt that the 'answer', with respect to human toxicity, is already in hand. If so, then these are precisely the materials we should be studying to 'forge the toxicological links'. How can the links be forged using materials with unknown human health effects? Two benefits could result: 1) a better understanding of the use of assays to predict toxicity and 2) a base of data against which 'new' materials can be compared and ranked. In the words of my favorite risk assessor, 'You've got to start with something you know.'"

Answer: W. Gary Flamm

"A recent conference on this subject came to the conclusion that the correlation and utilization of 'in vitro bioassay' data is at present possible only on a case-by-case basis. The reason for this being the importance of the individual properties and characteristics of the chemical under examination as well as the importance of knowing the nature and level of human exposure to the substance at issue. The conference also concluded that for in vitro tests to be really useful they must somehow be capable of providing quantifiably reliable data. Just knowing that the compound in question is positive in the in vitro tests used is often not enough."

Answer: Gareth M. Green

"Definitive risk assessment will require coordinated in vitro, in vivo, and population assessment. Opportunities for combined in vivo/in vitro studies will be required. Carefully defined and focused exposure groups such as in specific occupations will require more sophisticated molecular/cellular in vitro studies in order to confirm the extrapolation of in vitro findings. Experimental whole animal toxicological models exposed to known agents will require more sophisticated molecular/cellular bioassay measurements. Step 1 will be to identify significant molecular/cellular bioassays which respond to the tumor-promoting stimulus. Step 2 will be to conduct defined experimental exposures in whole animals at high- and low-dose levels utilizing these bioassay procedures. Step 3, in parallel, will be to identify known exposure groups to high and low levels and conduct limited and highly focused population studies. Thus, risk assessment will require the measurement of cellular and molecular bioassay batteries in both whole animal toxicologic studies and selected human populuation studies. The strategy combines, in a systematic matrix, measurements at the epidemiologic, toxicologic, cellular and molecular levels."

		Epidemiological	Toxicological
Matrix For Risk Assessment	Cellular		
	Molecular		

Answer: J. Justin McCormick

"Direct correlation with epidemiological studies on persons exposed to known doses of carcinogens (cancer chemotherapy patients) is proposed as a method of standardizing the in vivo assay. Epidemiological studies on persons exposed to energy-related emissions could be correlated via T-lymphocytes mutation assays carried out on these workers.

The proposal given in response to Question 10 does not relate directly to animal bioassay studies since it is validated by use of human exposure to chemotherapeutic agents. A parallel scheme to that given in Question 10 could be carried out in rats since thioguanine-resistant T-lymphocytes can be induced in such animals by mutagens and/or carcinogens. Furthermore, rat fibroblasts appear to transform to neoplastic cells in a manner formally nalogous to that seen with human fibroblasts, although infection of the cells with a virus is claimed to be necessary before chemical carcinogens can induce this effect in rat cells at early passage (1). However, recent studies have been carried out using the rat fibroblast assay which suggests this assay would need reexamination if one wished to use it."

Reference

1.   Albertini, R.J., Silwester, D.L., and Allen, E.F. The 6-thioguanine resistant peripheral blood lymphocyte assay for direct mutagenicity testing in man. In: Mutagenicity-New Horizons in Genetic Toxicology. J.A. Heddle (ed.), Academic Press (in press, 198).

Answer: H. Yamasaki

"Since our goal is to establish a bioassay system for carcinogenic risk assessment to human beings, one should admit that concrete 'positive' epidemiological results should be a reference point for this aim. Any bioassay system or combination of several of them which cannot detect known human carcinogens will not fulfill its purpose and therefore one needs to improve its procedure. (IARC has recently considered various short-term test results and compared them with animal and human carcinogenicity data (Feb. 5-12, 1982). Based on these results, it is envisaged to call a working group (April 1983) to further discuss about possible mechanisms of action of different carcinogenic agents)."

Question #15.

What type of basic research is needed for the development of this bioassay system?

Answer: Antone L. Brooks

"Two of the basic research areas that are needed are to 1) develop additional epithelial cell lines in culture using cells that are the target for transformation in vivo and 2) define the characteristics of transformed epithelial cells in vitro. It would of course be a benefit if these cells could be of human origin."

Answer: J. Justin McCormick

"The basic research needed to activate the schema shown in Figure 2 (Question 10) is:

A. Development of data with a number of different types of agents on the relationship between induction of anchorage-independent growth and the induction of thioguanine-resistance. Such data are available only for UV 254 nm, benzo(b)fluoranthene and propane sultone at present.

B. Standardization of the best conditions for quantitation of thioguanine-resistant human T-cells induced in vitro.

C. Quantitation of the induction of thioguanine-resistant human T-cells induced in vivo in patients undergoing chemotherapy. Determination of the relationship between the frequency of such mutants and the amount of DNA damage by use of biochemical techniques.

D. Generation of dose response curves for in vitro treatment of T-lymphocytes and fibroblasts with the same chemotherapeutic agents and measurement of DNA damage induced by these same agents.

E. Determination in human fibroblasts of the correlation between anchorage-independent growth and tumorigenicity in athymic mice."

Answer: Helene S. Smith

"We need much more information on the biology of human epithelial cells and the ability to culture cells from various organs. The fact that there have been major advances recently in ability to culture some organ systems suggests that the problems can be solved for other organs as well. We need much more information on properties of cells representing various stages of malignant progression and also on the effects of various carcinogenic agents on cells derived from specimens already at various stages of that progression. We will need to compare results from human with rodent models. We need to develop rapid, inexpensive assays that are relevant biologically. We will need to compare epithelial cells, fibroblasts and hematopoetic cells from specimens derived from different developmental stages".

Answer: H. Yamasaki

"As repeatedly emphasized in our answers, much more basic research is needed for the development of a reliable bioassay system. For this purpose, we still need multi-disciplinary approaches and it may not be possible to specify what type of basic research is needed. However, more emphasis may be needed in the field of: (i) mechanism of multi-stage carcinogenesis; (ii) molecular understanding of gene structure and expression, and their changes during carcinogenesis; and (iii) development of a sub-animal; system (i.e., organ culture) and its use in cancer research, in order to fill the gap of our knowledge and apply it to the establishment of bioassay system."

QUESTION #16.

How to coordinate the bioassay system to other components of risk assessment, such as exposure, other types of toxicity (e.g. neurological disorders), etc.?

Answer: Gareth M. Green

"Correlation of tumor risk assessment with other effects is a very important dimension to biologic risk assessment. First, one must identify key organ toxicities at the organ, tissue, cellular, and molecular levels. Second, the commonality or coordinative toxicities should be defined by mechanism. For example, an agent that produces both oxidant injury to lung tissue, cells, and biochemical systems, and is also associated with tumor initiation or promotion. Or in the neurological system, the signficance, for example, of cholinesterase inhibition. Or, in another example, the occurrence and significance of immunosuppression for both infection and tumor promotion. The third step, then, in certain instances may be to look for these common or associated mechanisms for other biological effects."

Answer: Antone L. Brooks

"This question, of course, is critical and has been partially addressed in answers to questions 1, 4 and 9. Aside from discussions of methodologies, there remains a very important area which has received insufficient attention. In order to establish the relationships between toxicological test systems and human disease, we should start with known toxic manifestations in man and work backward through the test systems. An example might be cigarette smoking. The manifestation in man is as clear as any we know about and includes tumorigenesis, developmental defects, noncarcinogenic cardiopulmonary disease and impairment of defense mechanisms. Although considerable effort has been expended on subhuman toxicology, what do we know about the interrelationships among the toxicological 'signals' we have obtained? Do we have information from endpoints and methodologies that are currently being employed? Do further, well-coordinated studies need to be done in order to reconstruct the best line of 'prediction' from toxicological assays to man? The same questions can be asked in regard to irradiation, lead, asbestos, thalidomide, diethylstilbesterol, coal dust, or any other material for which human manifestations have been observed. Little "toxicology" is being done on many of these because they are already regulated or controlled and it is generally felt that the 'answer', with respect to human toxicity, is already in hand. If so, then these are precisely the materials we should be studying to 'forge the toxicological links'. How can the links be forged using materials with unknown human health effects? Two benefits could result: 1) a better understanding of the use of assays to predict toxicity and 2) a base of data against which 'new' materials can be compared and ranked. In the words of my favorite risk assessor, 'You've got to start with something you know.'"

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QUESTION #17.

How to standardize procedures, correlate results and efforts of all participating laboratories, etc.?

Answer: Gareth M. Green

"The first step, of course, would be the development of standardized and published protocols. The second step would be to hold a series of workshops or courses on procedural and laboratory methods. The third step would involve blind testing protocols where samples would be periodically submitted blind to participating laboratories. A computerized surveillance system for quality control nationally might be the role of central coordinating facility."

Answer: J. Justin McCormick

"Standardized protocols would have to be developed by the groups described in the answer to Question 18. They would then be field tested by other workers. I presume correlation could be achieved by having investigators meet frequently in addition to use of the telephone."

Answer: Manfred F. Rajewsky

"I recommend the establishment of (rather small!) working groups for the particular questions; i.e., experts for specific procedures plus experienced, critical scientists who should together engage in inter-laboratory evaluation of procedures and data. The representatives of the various working groups should then try to establish and optimize the bioassay system(s) for human risk assessment."

Answer: H. Yamasaki

"Inter-laboratory validation of procedures is important and to have a small working group for each bioassay end-point may be the best way to achieve it. Such an effort in the field of cell transformation is especially urgent, since the end-point of this assay is influenced by both experimental and subjective factors. We believe there are already several attempts to standardize the procedures for other end-points."

QUESTION #18.

How much manpower, material resources are needed to develop this bioassy system and put this system into operation?

Answer: W. Gary Flamm

"You must have very strong management and the manager of the program needs to be a scientist who is highly respected in this field. The other participants must believe that working together as a team is in their and everyone else's best interest. The success or failure depends upon how well the "management" relates to the researcher. Creating obvious interdependence and making the program work to the clear benefit of the participants is essential."

Answer: Gareth M. Greene

"I do not know how much manpower, material resources, etc. are needed to develop this bioassay system, but clearly the important feature is a coordinated effort among molecular and cellular biologists, animal toxicologists, and human epidemiologists. One will also need environmental chemists and hygienists to work in the animal toxicological and human epidemiologic studies."

Answer: J. Justin McCormick

"Development of the data described in 18A for 6 agents would require 3-man years.

Development of the data described in 18B would require 2-man years.

Development of the biological data described in 18C is difficult and would perhaps require 6-man years.

Development of the biochemical data would require an additional 3-man years.

Development of the data described in 18D would require 6-man years.

Development of the correlation desribed in 18E would require 2-man years.

Each of the above workers requires about $12,000 a year in supplies. If the research were of a high priority, all of the above projects could be developed simultaneously at two locations."

QUESTION #19.

How to develop measurements which can be used to monitor humans in the field?

Answer: Richard J. Albertini

"There are now several measurements (human mutagenicity monitoring tests) which can be applied to humans in field studies. These include:

1.   Standard cytogenetic tests.
2.   Tests of sister chromatid exchanges.
3.   Assays of sperm head morphological changes.
4.   Determinations of double Y bodies in sperm.
5.   Assays of protein alkylation (hemoglobin) to establish molecular doses of environmental alkylating agents.

Answer: Gareth M. Green

"The development of measurements which can be used to monitor humans in the field requires the interaction of the clinical toxicologist, the occupational physician, and the industrial or enviromental hygienist. First will be required the development of biological assays involving obtainable samples such as urine, feces, sputum, tears, sweat, or other secretions, blood and possible cellular or tissue samples such as punch biopsies of skin, mucosal scrapings, fat biopsies, etc. Secondly would be the development of environmental sampling which can provide accurate and relevant assessment of environmental exposure. Thirdly, the molecular and cellular biologists will be involved for the preservation, handling, and treatment of the biological samples. What is most needed is to modernize epidemiologic study by the introduction of sophisticated cellular and molecular bioassays into systematized population surveillance."

6. When fully developed, quantitation of mature red blood cells containing mutant hemoglobins (as a measure of specific locus somatic cell mutations occurring in vivo).

7. The quantitation of mutant peripheral blood T lymphocytes arising in vivo (as a measure of a specific locus somatic cell mutations occurring in vivo).

Although different laboratories now perform these different studies, they have never been performed as a battery on the same human populations. These several assays form the basis of a specific proposal for validation in terms of their relevance as predictors of human health hazards."

Answer: Benjamin F. Trump

"There are several measurements, e.g. sister chromatid exchanges and mutation (variation) in human lymphocytes which have been used to monitor human exposure to carcinogens. Recently, immunological assays have been developed for measuring carcinogen binding to DNA. We have developed an enzyme immunoassay that can detect 1 B(a)p residue in 1-2 x 10^6 nucleotides. It is the most sensitive assay to determine nonradiolabeled B(a)p bound to DNA, and has a sensitivity nearly enough to detect B(a)p adducts in DNA isolated from tissues exposed to environmental, nonradioactive B(a)p. Enzyme immunoassays can be further improved by using better antibodies, e.g., purified antibodies or more specific monoclonal antibodies and can be extended to detect other environmental carcinogens."

Answer: H. Yamasaki

"Use of immunological techniques as detailed in 'Development and Possible Use of Immunological Techniques to Detect Individual Exposure to Carcinogens', Joint IARC/IPCS Working Group Report, IARC, Lyon, France, February 1982, IARC Internal Technical Report No. 82/001."

Answer: Stuart H. Yuspa

"It is this area where antibodies to carcinogen-macromolecular adducts would find their greatest usefulness."

QUESTION #20.

   For the application of the bioassay system in risk assessment for energy technology, there are important questions about the management of the system, such as the selection, the procurement, the storage and the distribution of the test substances, as well as the reference standards; the management of the information about test substances before and after testing; proper interpretations of the data for policy decisions, etc.

Answer: Brian D. Crawford

   "With regard to the storage and distribution of test substances, the national laboratories are well-suited to fill this role. Specifically, these laboratories have a record in the safe handling and distribuiton of toxic materials, and are perhaps best-equipped to provide this service to the various laboratories providing the actual tests. Centralized resource facilities would also permit more reliable comparisons of test data.

   Furthermore, the establishment of a national or even international data-base is essential. Management of this information would require considerable effort, expense, and computing capability. Again, the national laboratories could serve this role, acting as resource centers for the scientific community. Within the context of DOE/OHER funding, such data management should be easily addressed by the national laboratory system. Information storage and retrieval are essential to any meaningful outcome of bioassays. At present, no such centralized data management system exists for the investigators involved in health-effects research. Clearly, this is a deficiency which should be addressed."

Answer: W. Gary Flamm

   "It would seem that the establishment of a prime contractor who could devise, plan and conduct a program which would satisfy all the logistical needs is necessary."

Answer: Richard A. Griesemer

   "Administrative network required for the establishment of a bioassay system in the Department of Energy: See SPECIAL COMMENTS by R.A. Griesemer.

QUESTION #21.

        Other pertinent questions/comments?

Answer: Richard J. Albertini

        "The following is a specific proposal for the validation of human
mutagenicity monitoring tests in terms of their relevance as predictors of
human health hazards.

        This proposal, made elsewhere, is appropriate also for consideration
here. It will be paraphrased from an earlier publication.

        As mentioned, one advantage of human mutagenicity monitoring tests is
that they provide data on individuals that can later be correlated with the
health outcomes of those same individuals. Mutagenicity tests can be
performed repeatedly and clinical observations made over time. This advantage
should be exploited. Long term, cooperative studies designed to validate
mutagenicity monitoring tests in terms of their ability to predict human
disease should be implemented. Once validated, mutagenicity monitoring tests
will be truly useful for making direct, quantitative individualized human
health risk assessments.

        There are now effective chemotherapies and x-irradiation therapies for
many human cancers. Also, the selective use of adjunctive chemotherapy
following apparent surgical removal of cancer has reduced recurrence rates for
some malignancies. However, with the benefits of therapy have come some
apparent late sequelae of the genetoxicity of agents used in treatment--many
of which are known to be mutagens. The incidence of second malignancies has
increased, sometimes dramatically, in some treated patient groups.

        The benefits of treatment of these cancer patients clearly outweigh the
attendant genetic risks. Nonetheless, these treated patients constitute a
group of individuals who are knowingly and ethically exposed to potent
mutagens. Many of these patients are young; many will have children following
their successful therapies. Many of these patients are treated in large,
cooperative chemotherapy or radiotherapy trials, in which there exist
excellent clinical and epidemiological followup as well as infrastructures
capable of sharing information and samples.

        These treated cancer patients are a precious resource for study and
should be studied repeatedly and with as many mutagenicity monitoring tests as
are available. A repository should be created to store at least cryopreserved
blood cells and possibly sperm or other materials. A computerized data bank
should be developed, which can be continually updated, to contain the results
of the mutagenicity tests performed, as well as the clinical and reproductive
outcomes of the individuals concerned. Clearly, a cooperative study of this
magnitude is not designed to confirm again the mutagenicity of agents used to
treat cancer. Rather--and most importantly--it is designed to demonstrate
unequivocally which mutagenicity monitoring tests do and which do not have
validity as predictors of human health.

Epidemiology provides the traditional and presently, most realistic endpoint for documenting human health hazards. Mutagenicity monitoring tests may allow the recognition of genetic damage occurring in vivo in humans. It must be demonstrated that quantitation of the latter allows predictions regarding the former. The ideal and goal of validation of mutagenicity monitoring tests is to replace the occurrence of disease with a test result as a useful indicator of the health or hazard associated with a human environment."

Answer: Gareth M. Green

"A major area not addressed in the preceding questions relates to biologic cofactors that may determine the expression of the mutagenic effect as carcinogenesis. The theme of the conference suggests single-factor etiology in tumorigenesis and risk assessment. Yet in vivo considerations involve preceding or simultaneous cofactors such as simultaneous exposure to mutagenic hydrocarbons and oxidant conditions incuded by associated or simultaneous exposures. Related to that is the whole question of the role of immune surveillance and immunosuppression. The questions listed do not clearly address how in vitro molecular and cellular bioassay systems will test for modulating and control factors that may be metabolically or immunologically determined in the intact host. Although the references indicate the use of the nude mouse model at this stage, the ability to assign risk at the molecular or cellular level on an immunological basis would be desirable."

RESEARCH STATUS REPORT

Mutagenicity and Carcinogenicity Tests of
Products and Wastes from Fossil Fuels

Michael R. Guerin

The Office of Health and Environmental Research has sponsored a major effort to identify and mitigate adverse health and environmental effects associated with products, wastes, and inadvertant emissions resulting from advanced fossil fuels technologies. The liquefaction and gasification of coals, the liquefaction of oil shales, fluidized bed combustion, and diesel fuel utilization have received primary attention. Occupational environments (of immediate concern to technology developers and where exposures are likely to be most severe) have received first attention through studies of products, process streams, and concentrated wastes. The urgency of estimating the biological hazard associated with a multitude of novel technologies required the simultaneous application of existing bioassay methods, validation of those methods, and development of more suitable methods. Samples from conventional fossil fuels technologies (e.g., coal combustion and petroleum processing) were tested along with those from the advanced technologies to provide a comparative measure of biological potency.

Cancer risk has been addressed by studies of the bacterial mutagenicity (presumably related to tumor initiation) and mouse skin carcinogenicity of samples derived from the new fossil fuels technologies. The gross potency of the samples has been determined by biotesting the crude samples. Chemical agents responsible for the biological activity have been identified by reiterative chemical class separation and biological testing. Alkaline and polar-substituted neutral polycyclic aromatic components of the materials are found responsible for the bacterial mutagenicity of petroleum substitutes. Polycyclic aromatic hydrocarbons and their neutral nitrogen analogues appear to be primarily responsible for the mouse skin carcinogenicity of petroleum substitutes. Coal-derived products tend to exhibit greater mutagenicity and carcinogenicity than do shale-derived products. Both petroleum substitutes exhibit greater mutagenicities and carcinogenicities than do their petroleum equivalents. Bioactivity is found to increase with increasing boiling range and to decrease with increasingly severe hydrotreatment.

Definitive conclusions about the cancer hazard associated with new fossil fuels technologies have been hampered by: (a) uncertainties accompanying bioassay results on complex mixtures, (b) the unavailability of accurate measures of the dose reaching target organs, (c) uncertainties associated with extrapolating the results of in vitro tests to in vivo effects and (d) uncertainties associated with extrapolating the effects observed in the laboratory to those observed in man. Fundamental information is required to resolve these issues, especially as regards exposure to complex mixtures of chemical agents as is typically associated with fossil energy technologies.

RESEARCH STATUS REPORT

Metabolic Activation of Carcinogens

Harry V. Gelboin

A primary and essential consideration that is central to any system of risk assessment is the now well-established fact that most carcinogens require metabolic activation to exert their carcinogenic activity. The metabolic activation as well as the detoxification of carcinogens are carried out by several enzyme systems. The primary or initial interaction of carcinogens with mammalian organisms is with the cytochrome P-450 containing mixed function oxidases. Other metabolically subsequent enzyme systems are epoxide hydrolases, and several transferases including glutathione, sulfate and uridinediphosphoglucuronyl transferase. In any system used for risk assessment it is imperative that the apprpriate activating enzymes be present. The cytochromes P-450, for example, are present in different forms and each isozyme of cytochrome P-450 may display a unique but overlapping specificity with other cytochromes P-450. Thus, the profile of the latter enzymes may govern the relative amounts of activation or detoxification in a tissue or organ. A risk assessment system will fail if the proper mix of enzymes is lacking. The recent use of monoclonal antibodies specific for different cytochromes P-450 will permit a detailed analysis and phenotyping of tissues able to catalyze the activation reaction for a given carcinogen. Activation systems need be better defined than simply S-9 supernatant. Perhaps this will be better achievable when the latter enzymes are characteized as to structure and enzyme specificity.

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THE METABOLISM AND ACTIVATION OF BENZO[a]PYRENE

Glucuronides
1-O-G   β-Glucuronidase
3-O-G ─────────────────→ DNA
7-O-G
9-O-G
6-O-G
7,8-Diol-G
9,10-Diol-G
4,5-Diol-G

?
MFO

DNA Binding

Phenols
1-OH
3-OH
7-OH
9-OH
6-OH

MFO

UDPGA-T

DNA ← 6 oxy-BP Radical

MFO
NE

Quinones
3,6
1,6
6,12

ST

Sulfates
1-S
3-S
7-S
9-S
6-S
4,5-S
7,8-S
9,10-S

ST

NE

UDPGA-T

ST

GSH Conjugates
4,5-SG
7,8-SG
9,10-SG

12 1
11   2
10   3
9
8   4
7   6   5

MFO
ER

BP

Epoxides
[2,3-O]
[4,5-O]
[7,8-O]
[9,10-O]

EH
SS

GSH-T

6-OH-Me

Tetrol-1(7,10/8,9)
Tetrol-2 (7/8,9,10)

Triol
(7/8,9)

H2O

NADPH
or
NADH

Diols
(-)t-4,5
(-)t-7,8
(-)t-9,10

MFO

MFO

Diol Epoxide I
r-7, t-8-diol-t-9,10-oxy

DNA
RNA
Protein
Mutagen
Carcinogen

Diol Epoxide II
r-7,t-8-diol-c-9,10-oxy

DD

Quinones?

H2O   NADPH
or
NADH

Tetrol 1 (7,9,10/8)   Triol
Tetrol 2(7,9/8,10)   (7,9/8)

MFO-Mixed Function Oxidases
EH-Epoxide Hydratase
ER-Epoxide Reductase
DD-Dihydrodiol Dehydrogenase
GSH-T-Glutathione S-Epoxide Transferase
UDPGA-T-Glucuronic Acid Transferase
ST-Sulfotransferase
NE-Non-Enzymatic
SS-Stereospecific
t-Trans

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RESEARCH STATUS REPORT

Immunological Detection and Quantification of DNA Components Structurally Modified by Alkylating Carcinogens, Mutagens and Chemotherapeutic Agents

Manfred F. Rajewsky

The detection and quantification of defined reaction products of chemical mutagens and carcinogens (and of many cancer chemotherapeutic agents) with DNA require highly sensitive analytical techniques. Thus far, synthetic, high specific activity radiolabelled agents have been used in the respective studies and the DNA has been analyzed mainly by radiochromatography. The sensitivity of radiochromatographic methods is limited by the specific radioactivity of the agents in question and by the relatively large amount of DNA (cells) required for analysis. Since, with the exception of recently developed "post-labelling techniques", the application of radiochromatography is necessarily restricted to experiments with radiolabelled agents, it does not, therefore, permit analyses in small samples of cells (ideally in individual cells) exposed to low concentrations of non-radioactive (e.g., environmental or chemotherapeutic) agents. To circumvent these shortcomings, immunoanalytical methods are of particular relevance. This is based on the exceptional capability of immunoglobulins to recognize subtle alterations of molecular structure (especially when monoclonal antibodies are used to maximize specificity), to the outstanding sensitivity of immunoanalysis by high-affinity antibodies, and to the fact that radioactively-labelled agents are not required. Besides representing refined tools for the analysis of the molecular mechanism of carcinogenesis, mutagenesis and cytotoxicity, antibodies specifically directed against structurally modified components of DNA are obviously also important in terms of providing "fingerprints" of the exposure of cells, tissues, or individual to agents affecting cellular DNA (dosimetry of exposure to chemicals).

We have recently developed a set of high-affinity monoclonal antibodies (secreted by mouse x mouse as well as by rat x rat hybridomas; antibody affinity constants, 10^9 to $>10^{10}$ lmol) specifically directed against several DNA alkylation products with possible relevance in relation to both mutagenesis and malignant transformation of mammalian cells. These alkylation products include O^6-N-butyldeoxyguanosine, and O^4-ethyldeoxythymidine. Monoclonal antibodies specific for other DNA alkylation products are currently being produced. The best of the available monoclonal antibodies recognize the respective alkyldeoxynucleosides with an extraordinarily high specificity (i.e., exhibit an about 7 orders of magnitude lower cross-reactivity towards normal DNA constituents). When used in a radioimmunassay (RIA), an antibody specific for O^6-ethyldeoxyguanosine, for example, will detect this product at an O^6-ethyldeoxyguanosine/deoxyguanosine molar ratio of $\sim 3 \times 10^{-7}$ in a hydrolysate of 100 ug of DNA. The limit of detection can be lowered further if the respective alkyldeoxynucleosides are separated by HPLC from the DNA hydrolysate prior to the RIA. The anti-alkyldeoxynucleoside monoclonal antibodies can also be used to visualize, by immunostaining and fluorescence microscopy combined with electronic image intensification, specific alkylation products in the nuclear DNA of individual cells, and to localize structurally modified bases in double-stranded DNA molecules by transmission electron microscopy.

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RESEARCH STATUS REPORT

Role of DNA Repair in Carcinogenesis

Richard B. Setlow

DNA damage resulting from exposure to chemicals or mixtures of chemicals in the environment is determined by:

1. the environmental exposure level;

2. the metabolism of the chemical and, as a result,

3. the concentration of the ultimate carcinogen in the neighborhood of DNA;

4. the formation of DNA adducts;

5. the lifetime of the adducts relative to the onset of replication or transcription.

We expect large differences in the acute versus the chronic effects of exposure because of expected differences in steps 2 (3,4), and 5, because the metabolic activation and repair systems can be saturated at high acute doses but not at low chronic ones. Some chemicals result in an adaptive response in which the levels of metabolism or repair enzymes may be enhanced. If one is to make predictions in such a complicated system, one must know the basic biochemistry, biophysics and biology of it.

A complication in extrapolating from animals to humans is that the level of repair activity is different in the conventional animal models and in humans. For example, nucleotide excision repair is much better in humans than in rodents. This repair system works not only on ultraviolet radiation, but on its kinetics, such as the polycyclic aromatic hydrocarbons.

Moreover, recent reports indicate that the dealkylation reaction in which an alkyl group is transferred from O^6-alkylguanine to a protein is much more rapid in human cells than in rodent cells. The latter type of reaction is also very dependent on the tissue.

We know from a comparison of the cancer susceptibilities in humans with the disease xeroderma pigmentosum (XP) and in the average normal population that a defect in DNA repair in XP of approximately 70% to 80% results in a 10^4-fold increase in cancer susceptibility. Hence, it is reasonable to infer that a 10% to 20% change in repair capability could result in at least a 10-fold change in cancer susceptibility. The 10-20% change is the range of normal variability in repair activity. A number of recent measurements on unscheduled DNA synthesis in human lymphocytes and their ability to remove alkyl groups from DNA shows variations appreciably greater than this value in the normal population.

The above considerations indicate that an important part of assessing risks is a knowledge of the variability in the normal population. Such measurements are easily made on lymphocytes, but any variations observed in

such cells should be validated by comparisons with other tissues. There are important key questions to be answered. If an individual is on the high end of the repair distribution, does he always stay there? Is the normal capacity for DNA repair governed only by genetic background or is it under control of lifestyle factors. The confident answers to such questions are not available at present. Obtaining such answers should be a high priority.

RESEARCH STATUS REPORT

Human Cell Transformation Studies

J. Justin McCormick

It is important to realize that human cell transformation (i.e., induction of anchorage-independent growth) has characteristics very similar to the induction of thioguanine-resistance in these same cells. The similarities include the dose response with various agents; the need for expression time; a similar response of XP and normal cells in both assays; a similar response of synchronized cells treated with agents at S and far from S (early G1), and the fact that a background of naturally occurring anchorage-independent and thioguanine-resistant cells exist in both assays (Silinskas et al., Cancer Res., 41:1620; Maher et al., PNAS, May 1982). These similarities of response between induction of thioguanine-resistance, a known mutagenic marker, and induction of anchorage-independence suggest that the latter occurs as a mutagenic event. Since most carcinogens are mutagens, this conclusion is perhaps not surprising. However, previous transformation assays, including human, either did not show this correlation or showed it much less clearly. The lack of correlation relates, in part, to the failure to set up transformation assays as quantitative clonal assays so that results are directly comparable to those obtained in mutagenesis assays.

The relationship of the anchorage-independent phenotype to tumorigenicity is currently under intensive investigation. Either cells with this phenotype are aleady tumorigenic in athymic mice or they require a further change to become tumorigenic. What is clear is that cells with the anchorage-independent phenotype will produce tumors in athymic mice when expanded to the large populations required for injection into animals, whereas normal (non-anchorage-independent) cells do not produce tumors under comparable conditions.

RESEARCH STATUS REPORT

The Transformation of Epithelial Cells

Stuart H. Yuspa

The transformation of epithelial cells in vitro has been slow to develop but is an essential research area in chemical carcinogenesis. The difficulties in the establishment of reproducible in vitro epithelial cell models result from the requirement to develop new culture methods (particularly customized media), new markers of transformation and novel transformation protocols in an attempt to gain quantitation. These efforts are just now yielding promising results, and it can be anticipated that in the next 1-2 years, major advances toward solving a number of persisting problems will occur. We can also feel confident that parallel models with human epithelial cells will develop quickly.

Virtually all scientists working in the area of epithelial transformation have established rigorous goals for optimizing culture systems. These goals have evolved from the firm belief that the complexity of epithelial tissues in vivo is relevant to the mechanism of transformation for a particular tissue and must be reproduced in vitro to obtain valid data on carcinogenesis Thus, most models under development utilize primary cells or early passage cells rather than cell lines. A diploid chromosome number is desirable, but certain tissues (liver, bladder, skin) contain a significant tetraploid population in vivo and such populations should be included in the cell culture model. Maintenance of functional (differentiative or secretory) competence is an important goal as is maintenance of metabolic competence for carcinogen activation. It is desirable to have a short assay time and for the systems to be quantitative. This has resulted in the search for and development of selection protocols to provide recognition for early transformants and to enhance their growth. A significant problem has been the development of specific markers for transformation. Most laboratories have utilized selection markers since specific phenotypic changes often are present only very late in the transformation of epithelial cells. Thus, many of the models used are studying a preneoplastic event.

Currently, the model systems under study consist of mouse skin, rat trachea, mouse and rat mammary gland, rat liver, rat bladder, rat submandibular gland, and rat brain. Only the first three are quantitative to any degree, and these largely depend on dose-response data for quantitation rather than yielding a true transformation frequency on a cellular basis. An early and consistent change in epithelial cells exposed to carcinogens is escape from senescence or an alteration in terminal differentiation. This is also an early and consistent change observed when epithelial tissues are cultured from hosts exposed to carcinogens in vivo. This change has formed the basis for most of the selection protocols used in epithelial transformation models. Few satisfactory markers of transformation in epithelial cells have been defined. Morphology does not change significantly in epithelial transformation. Agar growth is acquired late in the transforma-tion process with some cell types (trachea, liver) but is not correlated at all in other cells (skin).

Progress in the further development of these models is dependent upon the refinement of culture conditions. Currently there is significant promise that, with media or substrate modifications, clonal growth of functioning epithelial cells can be achieved. The development of serum-free growth conditions is occurring rapidly. We can expect the development of highly quantitative clonal assays within the next several years. In general, these will rely on the selection for a preneoplastic trait. Improved selection conditions for both early preneoplastic and later preneoplastic and neoplastic changes are likely to be available within five years and will probably depend on enhanced understanding of the mechanisms of progression toward malignancy. The development of specific markers for intermediate states of carcinogenesis is a high priority and markers will likely be tissue-specific. In this regard, monoclonal antibodies will probably play a major diagnostic role in vitro. The development of such antibodies could be useful for screening the analogous tissues in humans in vivo to assist in defining preneoplastic changes in a particular tissue. As these epithelial models and markers are developed, we can anticipate a logical approach to understanding the tissue-specific role of tumor promoters and cocarcinogens. Such studies cannot be logically performed with the current mesenchymal cell lines available. Finally, the study of genetic changes in specialized function related to neoplasia will be approachable in these models within several years. By the use of gene transfection protocols, the elucidation of tissue-specific alterations should provide insight into the mechanism of transformation in epithelial tissues.

RESEARCH STATUS REPORT

Mechanisms of Tumor Promotion

J. Carl Barrett

Tumor promoting factors have been suggested to play an important role in the development of human cancers (Doll and Peto, 1981). Therefore, quantitative risk analysis of this class of cocarcinogens is very important. The mechanism of two-stage carcinogenesis, described originally nearly forty years ago as the ability of certain treatments to promote tumor formation initiated by a non-carcinogenic dose of a chemical (Boutwell, 1964, 1974), remains undefined today. There are two possible cellular mechanisms for tumor promotion (Farber, 1981):

1) Tumor promoters act only to amplify the number of initiated cells relative to the normal cells resulting in the appearance of a visible lesion (i.e., tumor promotion is a quantitative change only in cellular populations). (Trosko & Chang, 1980; Potter, 1980).

2) Tumor promoters modulate the initiated cell to a state which allows subsequent clonal proliferation of these cells relative to the normal cells in the tissue (i.e., tumor promotion is a qualitative plus a quantitative change in the initiated cells). Surprisingly, little definitive work is available to distinguish whether tumor promotion is a quantitative or qualitative process. Any model of tumor promotion in vivo has to include an amplification of the initiated cell vis-a-vis the non-initiated cell. The importance of this mechanism in tumor promotion of mouse skin is strongly supported by the observations that: (1) all known mouse skin tumor promoters are hyperplastic agents (Boutwell, 1974, Barrett and Sisskin, 1981) and (2) regenerative hyperplasia by wounding or abrasion is sufficient to cause tumor promotion (Argyris, 1980; Argyris and Slaga, 1981). It has been stated that hyperplasia and tumor promotion are unrelated (see Barrett and Sisskin, 1981). However, these results are often difficult to interpret because the hyperplastic response of a single treatment of promoter is related to tumor formation after many months of treatment. These studies assume that the hyperplasia after a single treatment is related to the sustained hyperplasia after multiple treatments. This is clearly not the case (Barrett and Sisskin, 1981; Sisskin et al., 1982). In mouse skin treated with the potent tumor promoter 12-O-tetradecanoylphorbol-13-acetae (TPA), there is a potentiation of the hyperplastic response with additional TPA treatments (Raick, et al., 1972). With other promoters (Frei, 1977) and with other species (Sisskin and Barrett, 1981), this potentiation is not observed and sometimes the epidermis adapts to the promoter and fails to respond hyperplastically (Sisskin and Barrett, 1981). The differences in sustained hyperplasia with multiple TPA treatment can also be related to different sensitivities of different strains of mice to tumor promotion (Sisskin, et al., 1982).

It is important to realize that hyperplasia and amplification of the initiated cell are not necessarily related. In fact, stimulation of growth of the entire epidermis would not allow expansion of the initiated cell unless the initiated cell is altered in its differentiation potential and therefore is amplified relative to the non-initiated cell which terminally

differentiates after its stimulation to proliferate. Recent data
(Kulesz-Martin et al., 1980; Yuspa and Morgan, 1981) support this hypothesis
for mouse skin carcinogenesis. Amplification of the initiated cells in other
organs might occur by a specific stimulation of growth of initiated cells or
by a differential inhibition of growth of the normal cells (Farber, 1981).
Neither of these mechanisms would be associated with general hyperplasia of
the tissue.

        The evidence for a modulation mechanism for tumor promotion in vivo is
limited. We assume that this is what most people are referring to when they
state that tumor promotion in mouse skin must involve something in addition to
hyperplasia. However, the nature of the additional effect is ill-defined at
the cellular or molecular level. The best evidence that tumor promoters cause
a qualitative modulation of initated cells is the identification of different
stages in tumor promotion (Boutwell, 1974; Slaga et al, 1980a). These
experiments demonstrate that croton oil or TPA can effect changes in initiated
cells which allow them to be amplified by compunds ("secondary stage
promoters"), which are inactive or weak promotors by themselves (Slaga et al,
1980a). For example, mezerein, which is as potent at TPA in inducing a
variety of phenotypic effects on cells, is active only as a second stage
promoter. Also, different inhibitors of promotion can be shown to be
effective in inhibiting only the first or the second stages of promotion
(Slaga et al, 1980b). These results suggest that potent promoters can
modulate or convert (Boutwell, 1974) initiated cells qualitatively to allow
them to amplify or proliferate into a clone of tumor cells. An alternative
possibility (Yuspa, personal communication) is that the first stage of
promotion is also a quantitative change. First-stage promoters may allow
initiated cells to escape some but not all growth restraints. Second-stage
promoters then enable small clones of initiated cells to continue to expand.
These agents may be less effective than first-stage promoters on individual
initiated cells.

        A major aspect of tumor promotion is certainly the quantitative effects
of the promoter on the population dynamics in the tissue. Whether or not
tumor promoters also cause a qualitative change in initiated cells and the
nature of these changes are important questions to be answered.

        It is important to recognize that the two stage model of epidermal
carcinogenesis may involve only one step or qualitative change in the cells.
The promotion stage may be a quantitative change only, i.e. the amplification
of the clone of initiated cells. Of course, the identification of multiple
stages in promotion suggests qualitative changes in this process as well.
Regardless of the number of cellular changes during initiation and promotion,
this model only describes the development of benign lesions on mouse skin. A
further progression of the cells to the malignant stage has to occur for this
model to describe the complete, multistep process of carcinogenesis.

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RESEARCH STATUS REPORT

Special Note on Human Tissue Experimentation

Benjamin F. Trump

The utilization of human tissues in toxicologic and carcinogenic research (Harris et al., 1977a,b,c, 1978a,b, 1979; Yang, 1977, 1978, Jones et al., 1977, Autrup et al., 1978a,b,c, 1979, 1980) is attended by a number of ethical, logistical and economic problems. The legal problems vary with the state. In some states, Medical Examiners' material may be used (Trump et al., 1973a), while in others it may not. Tissues obtained at the time of the surgery can ordinarily be handled with informed consent forms and approval of the human volunteers committee although, again, this may vary with the institution and rules of the Federal Government. However, at the present time there appears to be tendency to simplify these rules for tissues removed for treatment and not for research. For example, many tissues can be obtained at the surgery adjacent to the sight of the lesion, i.e., resection for colon cancer with margins of normal colon on each side (Shamsuddin et al., 1981).

In any case, it is essential that any investigator wishing to embark on such studies seek the strong collaboration of the Director of the Department of Pathology and the Director of the Department of Surgery. Failure to achieve such approval could result in serious problems because tissues could be obtained which interfere with appropriate medical care and diagnosis of the patient; this cannot be overemphasized.

Tissues may be obtained at the time of surgery, immediate autopsy, or intermediate autopsy. To do this requires coordinated collection teams who work in close coordination with the Departments of Pathology and Surgery (Cowley et al., 1979). In the case of surgical resections, it means anticipatory checking of operating room schedules, clearance with the surgeon and operating room staff and close liaison with the pathologist who will be working up the specimen. Typically uninvolved tissue may be obtained which does not interfere with the diagnosis, i.e., surgical margins. These tissues then may be placed in media such as L-15 and transported at 0 to 4°C to the laboratory. The time of storage in L-15 at 4°C varies with the tissue, in the case of bronchus, tissue may be stored for at least 24 hours and possibly even several days; in the case of mammary tissue, storage times of up to one week at 0 to 4°C have been reported. The main problem with tissues obtained at surgery when the surgery is done for neoplastic diseases is that preneoplastic lesions and initiated sites may or may not exist in the putatively normal tissue. For that reason, we (Trump and Harris, 1979) have not used such tissues for studies of reactions to carcinogens in vitro.

Tissues may also be obtained at the time of autopsy, although the interval between death of the patient and tissue obtainment is critical depending on the temperature. At 37°C tissues have various survival times. We have determined that bronchus (Barrett et al., 1977) and pancreatic duct (Jones et al., 1981) survive approximately three hours while kidney and liver survive between 30 minutes and one hour (Trump et al., 1975). Different tissues therefore survive for varying times and more work needs to be carried

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out to systematically explore this. In addition, studies should be funded to investigate freezing preservation. We, for example, have observed that pieces of human bronchus can be frozen and then reconstituted and cultured many months later.

Immediate Autopsies. These autopsies are defined as autopsies carried out within 30 minutes after death (Trump et al. 1973a,b; Cowley et al., 1981). The ability to obtain such tissues depends on the logistical support, the

state laws, the availability of traumas and so on. This can be done in connection with organ transplant teams, with permission from the family. These tissues again are placed in media such as L-15 and ice, and transported to the laboratory.

Intermediate Autopsies. We have found that tissues can be obtained from autopsies several hours after death if the bodies are transported to a morgue refrigerator soon after death. Again, tissue such as bronchus, bladder, prostate, pancreatic duct and skin can be cultured for much longer periods of time following death than heart, liver and kidney. It is also very likely that fibroblasts could be cultured for even longer periods following death.

Hazards. In addition to the chemical hazards involving the use of carcinogens, there are substantial biological hazards from infectious disease when dealing with human tissues. These include hepatitis, tuberculosis, but many other possible infectious agents will be present. Therefore, strict control to protect the laboratory workers needs to be ensured.

Logistics and expense. It can be seen from the above sections that to maintain a large facility with teams of individuals on 24 hour call on beepers necessitates a considerable investment. However, once such a center is established, it is possible for that center to work in collaboration with other research teams. It is essential that all individuals involved be full collaborators in the research.

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RESEARCH STATUS REPORT

Human Cell Transformation Studies with Emphasis on Epithelial Cells

Helene S. Smith

Introduction

Most human cancers develop as a progressive series of changes from normal through various degrees of premalignant changes to frankly carcinomatous lesions. Even after development of invasive potential, tumor progression continues as the tumor cells become more dedifferentiated and capable of rapid proliferation at various metastatic sites (Foulds, 1969, 1975). One way to study this progression in culture and to gain insights into the etiologic agents involved in malignant progression is to develop a transformation assay. One can treat cultured normal cells with various carcinogens (i.e. viruses. chemicals, x-rays) and/or potential promoters (retinoids, lipids) to determine which agent or combination of agents alter the behavior of normal cells to that characteristic of malignant cells (i.e. transform cells).

However, to develop relevant transformation assays, we need to know what properties characterize the malignant state in vitro. Numerous studies have been done to characterize the transformed state by comparing the properties in culture of normal vs. tumor-derived cells. Most of these studies have used cultured fibroblasts (Ponten, 1976; Barrett and Ts'o, 1978; Kakunaga, 1977); however, recently, studies on transformation of rodent epithelial cells have appeared (Marchok et al., 1978; Colburn, 1978; Montesano et al., 1977; Borenfreund et al., 1975). From all these studies, it is reasonable to conclude that the properties relevant to defining transformation vary depending on the organ system and perhaps even the species involved. Complicating matters, it is possible that there are many different pathways to malignancy for a given cell type. Figure 1 schematically illustrates this point. In the chart, each arrow represents a change in cellular physiology that could potentially be measured in culture. Depending on either the agent involved or the physiologic state of the cell, a cell might follow alternate pathways to malignancy. There may be properties like "A" which appear on some pathways to malignancy but not on others. If one examined the properties of tumor-derived cultures, some specimens would be positive for the "A" phenotype while others would be negative. There may also be properties like "B" which appear early on some pathways and later on others. Such properties would always be present in tumor derived specimens and would sometimes be seen in cultures of premalignant lesions. Hopefully one could also find properties like "C" which always and only accompany the development malignancy and would thus be present in all tumor derived cultures and absent in all cultures of normal and premalignant specimens. Finally, there may also be properties like "D" which confer increased malignancy and metastasizing ability on already invasive cells. Such properties would characterize cultures derived from metastatic lesions but might only be seen in a small subpopulation of cells from a primary carcinoma.
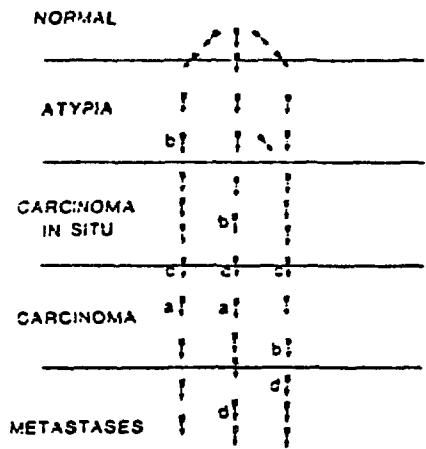
Schematic diagram of pathways of tumor progression.

A fundamental prerequisite for understanding the progression to malignancy of any human tissue is the ability to isolate, culture and identify the particular cell type involved regardless of its stage in malignant progression. Because there may be different pathways by which cells from even a single organ become malignant, it will be necessary to culture every specimen reproducibly. There are currently numerous well-characterized human cells lines derived from primary and metastatic cancer lesions (Engle and Young, 1978; Fogh and Trempe, 1975; Smith and Dollbaum, 1981). One might initially think that the properties of such lines would be representative of the malignant state for that organ. However, cell lines develop from only a small percentage of tumor specimens. As illustrated in Figure 1, the cell lines may represent one pathway to malignancy wherein the cells gain the ability to grow continuously in culture using currently existing techniques and media. In order to understand the other 90-95% of the cells of any given type of cancer, we must develop new techniques that allow us to culture all cancers as well as nonmalignant specimens from that tissue. In addition, it is likely that there will be heterogeneity within a single cancer specimen (Heppner, 1979). Within a single primary carcinoma specimen, there may be some cells that have progressed only to the premalignant state, other cells that are also invasive into the surrounding stroma, and perhaps only small subpopulations capable of the complete process of homing to and growth at another organ site. Therefore, we must also develop techniques that allow us to culture specimens with high efficiency so that we can detect the heterogeneous subpopulations within a given specimen.

State of the Art for Various Systems

Because my area of expertise is in the biology of cultured human epithelium, I will comment mainly on epithelial cell culture and transformation.

1.   Epidermal Keratinocytes.

Probably the most widely studied human epithelial cell culture system is the epidermal keratinocyte culture system developed by Rheinwald and Green (1975). In previous attempts to grow epidermal cells, some multiplication took place but the cells could not be passaged and high initial inoculation densities were required (for a summary of references see Green et al., 1979). Rheinwald and Green (1975) noted that cells could be serially cultivated from small inocula by supporting them with cocultivated lethally irradiated fibroblasts. The cultured epidermal cells showed differentiated behavior which imitated to a remarkable degree that of the epidermis (Sun and Green, 1978a,b; Rice and Green, 1977). Higher plating efficiency and long culture life-times were obtained by the addition of epidermal growth factor (Rheinwald and Green, 1977) and agents known to increase cellular cyclic AMP levels such as cholera toxin (Green, 1978).

More recently, Rheinwald and his colleagues have begun to study the growth properties of cultured malignant epidermal cells (Rheinwald and Beckett, 1980; 1981). They found that approximately 50% of the squamous cell carcinoma specimens that they tested had the capacity to grow as permanently established cell lines. This observation suggests that the property of infinite life in culture is associated with a large proportion of squamous

carcinomas. Some, but not all of the carcinoma lines grew without fibroblast feeders or without anchorage. In contrast, all of the carcinoma lines were somewhat defective in commitment to terminal differentiation (Rheinwald and ᴿeckett, 1980) as measured by ability to survive in the absence of anchorage.

To date, I know of no work on the biology in culture of premalignant keratinocyte lesions or on comparing properties of primary and metastatic lesions. In addition, another aspect of this system to be explored should be studies to transform normal keratinocytes with various agents.

Mammary Epithelial Cells

The human mammary gland is a good system for studying malignant progression since specimens representing at least some stages of progression are readily available as discard material from surgical specimens. Reduction mammoplasties are an excellent source of normal epithelial cells since there is little, if any, pathology of the epithelial cells in such cases. It is difficult to obtain specimens of premalignant lesions because these lesions are usually small and required by a pathologist to rule out possible areas of frank malignancy. However, it is known that breast tissue peripheral to a carcinoma contains numerous atypias and hyperplasias (Wellings, 1980). Hence, such tissue which is readily available from mastectomies, represents a plentiful, albeit heterogeneous source of abnormal cells. Malignant lesions usually provide sufficient discard tissue for culturing carcinoma cells. Unfortunately, sources of metastatic breast cancer cells are limited since the metastatic lesions are rarely treated surgically.

There have been a number of reports on culturing human mammary epithelial cells. Unfortunately, the techniques used still result in low efficiencies of growth. The use of feeder layers to culture human mammary epithelial cells was first reported by Taylor-Papdimitriou et al., (1977). However, even under optimal conditions, they needed to plate approximately 10^6 cells in order to obtain 75 to 100 epithelial patches in primary culture. Similar low plating efficiencies have also been reported for primary cultures of normal cells (Yang et al., 1981), benign tumors (Hallowes et al., 1977) and primary carcinomas (Buehring and Williams, 1976; Hallowes et al., 1977; Kirkland et al., 1979; Klerjer-Anderson and Buehring, 1980). In most cases the carcinoma cultures did not proliferate after subculture.

At Peralta Cancer Research Institute, we have recently developed techniques for readily isolating, culturing and identifying human mammary epithelial cells from both nonmalignant and malignant specimens (Stampfer et al., 1980; Smith et al., 1981). To isolate the epithelial cells free from contaminating fibroblasts, grossly dissected breast tissue is treated with collagenase and hyaluronidase to degrade the stromal matrix and basement membrane. Under these conditions the epithelial cells remain associated in large clumps while the stromal fibroblasts are dissociated to single cells. The clumps which are easily filtered to separate them from remaining single cells can be cryopreserved at this stage. The epithelial clumps can then be plated onto tissue cultures dishes in an enriched medium. Epithelial cells migrate from the attached clump and begin extensive proliferation. At this stage, the cells can be trypsinized and passaged either as mass cultures or as single cells for a highly efficient clonogenic assay. The cells can be

identified as being of epithelial origin by a number of criteria including cuboidal morphology, synthesis of domes and ridges, ultrastructural evidence of junctional complexes and secretory activity, presence of mammary epithelial antigen, presence of keratin, and presence of a distinct pattern of surface fibronectin. All of the tumor derived cultures can be distinguished from the cultures derived from nonmalignant specimens (both reduction mammoplasties and peripheral mastectomy tissue) by the presence of a tumor specific glycoprotein (Stampfer et al., 1982) defined using antisera described by Edgington and his associates (Leung, et al., 1978; 1981). These studies suggest that appearance of the mammary tumor glycoprotein may be a property like "C" illustrated in Figure 1 which is always and only present when cells become malignant. In contrast, loss of anchorage-dependence may be a marker of an earlier stage in malignant progression since cultures from peripheral mastectomy tissue as well as from carcinomas grew in methocel, while most cultures from reduction mammoplasties did not (Hancock et al., 1982; Smith et al., 1982; Stampfer et al., 1982). This is in contrast to work with rodent epidermis, trachea, and fibroblasts where loss of anchorage is a later step in malignant progression.

We have just begun to study the effects of x-rays on the normal mammary cell cultures. We found that the survival curves for epithelial cells from normal breast tissue of various donors were unshouldered and exponential and showed no evidence of sublethal damage repair (Yang, Stampfer and Smith, submitted for publication). These results indicate that there may be no radiation threshold dose for killing normal mammary epithelial cells. Transformation studies using this system are currently in progress.

Human mammary milk cells have also been transformed in culture by SV40 (Chang, S. presented at the Imperial Cancer Research Fund workshop on Immunological Markers for Human Mammary Epithelial Cells, March 1981). The transformed cells were positive for T antigen and showed increased proliferative ability in culture.

Bronchus

Recent work by Lechner et al., (1981) has resulted in major advances in culturing human bronchus. Utilizing a new medium which is reduced in calcium, they can now reproducibly grow bona fide bronchus epithelium in culture. At least some cells in the culture will undergo as many as 35 population doublings. Although these cells grow somewhat more slowly than mammary cells, these authors obtain as many as 5×10^7 cells per specimen every 2-3 weeks. The bronchal cells also grow in a clonal assay when seeded with irradiated feeder cells. In contrast to normal cells, very little work has been done on culturing the three types of malignancies of the bronchus (mucoid epidermal, squamous cell, and adenocarcinoma). Only an occasional specimen develops into a cell line and no one, to my knowledge, has undergone a systematic attempt to culture tumor specimens in the medium developed by Lechner et al. (1981) (personal communication, J. Lechner, NCI). In contrast, a great deal of success has been reported on culturing and studying the biology of oat cell carcinoma of the lung (Carney et al., 1981). However, oat cell carcinomas are not derived from bronchial epithelium but rather from a neural crest derived cell. The normal counterpart of this carcinoma has not yet been cultured.

Dr. John Lechner (personal communication) has begun studies on chemical carcinogenesis of the bronchial epithelium, but no information is yet available.

Other Systems

Much less work has been done on culturing other organ systems. There has been some progress in culturing colon carcinomas in serum-free medium (Murakami and Masui, 1980), but still no one has had success in culturing any normal colon except fetal intestine (Smith et al., 1979). While bladder would be particularly important for industrial carcinogen studies, I know of no studies on culturing normal bladder. Possibly a major difficulty for bladder is the availability of specimen material which could only come from immediate autopsy or organ transplant donors. Normal fetal prostate has been cultured and transformed by the criteria of increased growth in culture after treatment with SV40 (Kaign et al., 1980) but adult prostate did not grow well under these conditions. There is an isolated report of pancreas transformation by dimethylbenz(a)anthracene (DMBA). After 12 weeks in the presence of DMBA, organ cultures that were dissociated into single cells produced tumors in nude mice (Parsa et al., 1981). Kaufman reported an effect of carcinogens on endometrial cells; however, only the stromal cells became transformed (presented at the NCI Workshop on Carcinogenesis Research on Human Cells, Tissues and Subcellular Fractions, Jan. 7-8, 1982).

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RESEARCH STATUS REPORT

Current Progress of the International Agency for Cancer Research

H. Yamasaki

In February 1982, an International Working Group of experts in chemical carcinogenesis met in Lyon to reevaluate the carcinogenic risk to humans for all chemicals, group of chemicals, industrial processes or occupational exposure considered in IARC Monographs, Vol. 1 to 29, and for which data on cancer in humans are available. The conclusions of this Working Group will be published in Supplement 3 to the IARC Monographs and will update the previous Supplement 1: Chemicals and Industrial Processes Associated with Cancer in Humans. Each evaluation was based upon all available published data from human epidemiological studies and animal experiments summarized previously and published in the respective IARC monograph, and all new data published since the appearance of that monograph. In addition, data from short-term tests were summarized, again on the basis of both previously evaluated and more recent data.

In view of the limitations of current knowledge about mechanisms of carcinogenesis, the following cautions were emphasized in relation to the use of short-term test results: (1) at present, these tests should not be used by themselves to conclude whether or not an agent is carcinogenic; (2) even when positive results are obtained in one or more of these tests, it is not clear that they can be used reliably to predict the relative potencies of compounds as carcinogens in intact animals: (3) since the currently available tests do not detect all classes of agents that are active in the carcinogenic process (e.g., hormones, promoters), one must be cautious in utilizing the currently available tests as the sole criterion for setting priorities in carcinogenesis research and in selecting compounds for animal bioassays.

Based on the evaluation of human, experiment animal and short-term test data (Table 1), the chemicals and industrial processes were considered to be carcinogenic (Group 1, Tables 2-4), or highly suspected to be carcinogenic (Group 2A, Table 5) to humans.

TABLE 1

WORKING PROCEDURES

DESCRIPTIVE EVALUATION	DESCRIPTIVE EVALUATIONS	DESCRIPTIVE EVALUATIONS
EPIDEMIOLOGICAL DATA	ANIMAL DATA	SHORT-TERM TEST DATA
DEGREE OF EVIDENCE	DEGREE OF EVIDENCE	DEGREE OF EVIDENCE
OF CARCINOGENICITY	OF CARCINOGENICITY	OF ACTIVITY
IN HUMANS	IN ANIMALS	IN SHORT-TERM TESTS

OVERALL SUMMARY EVALUATION

OF HUMAN CANCER RISK

TABLE 2

GROUP 1. ESTABLISHED HUMAN CARCINOGENIC CHEMICALS AND INDUSTRIAL PROCESSES

PROCESSES

1. Auramine manufacture
2. Boot and shoe manufacture and repair (certain exposure to dusts)
3. Furniture manufacture (certain exposure to dusts)
4. Isopropyl alcohol manufacture (strong-acid process)
5. Nickel refining
6. Rubber manufacturing industry (certain occupations)
7. Underground haematite mining (radon)

TABLE 3

GROUP 1. ESTABLISHED HUMAN CARCINOGENIC CHEMICALS AND INDUSTRIAL PROCESSES

A.	OCCUPATIONAL EXPOSURES	INDUSTRY	SITE OF CANCER
1.	4-aminobiphenyl (Aromatic Amine)	Rubber	Bladder
2.	Asbestos	Insulation	Lung, pleura, peritoneum
3.	Benzene	Solvent	Marrow (leukemia)
4.	Benzidine (Aromatic Amine)	Rubber & Dyes	Bladder
5.	Bis(Chloromethyl) Ether (BCME) and Technical CMME	Chemical Industry	Lung
6.	Chromium and Chromium Compounds	Metal Plating Pigments	Lung
7.	Mustard Gas	War Gas	Larynx, Lung
8.	2-Naphthylamine (Aromatic Amine)	Rubber & Dyes	Bladder
9.	Soots, Tars, and Certain Mineral Oils	Rubber, Coal Liquefaction, Engineering	Skin, Scrotum
10.	Vinyl Chloride	Plastics	Liver

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TABLE 4

GROUP 1. ESTABLISHED HUMAN CARCINOGENIC CHEMICALS

B.	IATROGENIC EXPOSURES	USE OR SOURCE OF EXPOSURE	SITE OF CANCER
1.	Arsenic and Arsenic Compounds	Tonics - Water - Dematology	Lung, Skin
2.	Analgesics Containing Phenacetin	Analgesics	Renal Pelvis
3.	Azathioprine	Immunosuppressive Agent	Skin, Liver, Non-Hodgkins Lymphoma
4.	Chlorambucil	Chemotherapy	Marrow
5.	Chlornaphazine	Chemotherapy	Bladder
6.	Combined Chemotherapy Including MOPP	Chemotherapy	Marrow
7.	Conjugated Estrogens	(Menopausal Syndrome)	Endometrium
8.	Cyclphosphamide	Chemotherapy	Marrow, Bladder
9.	Diethylstilbestrol	Threatened Abortion	Vagina, Uterine Cervix
10.	Melphalan	Chemotherapy	Marrow
11.	Methoxsalen with UVA (PUVA)	Psoriasis	Skin
12.	Myleran	Chemotherapy	Marrow
13.	Treosulphan	Chemotherapy	Marrow

TABLE 5

GROUP 2A. HIGHLY SUSPECT HUMAN CARCINOGENS

	COMPOUND	SOURCE OF EXPOSURE	PROBABLE SITE OF CANCER
1.	Acrylonitrile	Plastic Industry	Lung
2.	Aflatoxins	Food Contaminant	Liver
3.	Benzo(a)Pyrene	Rubber Industry, Coal Liquefaction, Engineering, Air Pollution	Skin, Lung
4.	Beryllium and Beryllium Compounds	Metal Industry	Lung
5.	Diethyl Sulphate	Chemical Intermediate	Larynx
6.	Dimethylsulphate	Chemical Intermediate	Lung
7.	Manufacture of Magenta	Dye Industry	Bladder
8.	Nitrogen Mustard (Chemotherapy)	Iatrogenic	Marrow, Skin
9.	Combined Oral Contraceptives	Iatrogenic	Liver
10.	Oxymethalone (Anabolic Steroid)	Iatrogenic	Liver
11.	Phenacetin (Analgesic)	Iatrogenic	Renal Pelvis
12.	Procarbazine (Chemotherapy)	Iatrogenic	Marrow
13.	Ortho-Toluidine	Dye Industry	Bladder

VI. SPECIAL COMMENTS

SPECIAL COMMENTS

On the Development of Predictive Short-Term Assays

Mortimer Mendelsohn

A. Definition of a Short-Term Assay

1. Given a process:

Exposure → → → → Target Dose → → →   → → → → Effect

*Assay

2. A short-term assay either:

a. Measures some step in the sequence, in some arbitrarily short time interval (minutes, days, even months for some very delayed effects), as a surrogate for measuring the full effect; or

b. Measures an alternate process as a surrogate for the process and effect of interest; the alternate may be in a related or unrelated system and organism.

	Dose	Effect
E		
	Dose	Alternate Effect

B. Definition of a Predictive Short-term Assay

1. Given a process and either a universe of agents or a universe of doses and dose schedules:

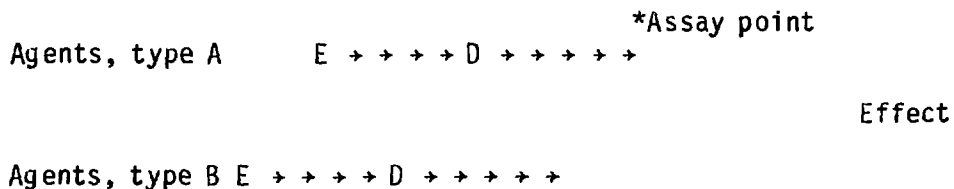| Agent	Dose	
1	1	
2	2	E → → → D → → → → → → → → → Effect
3	3	
.	.	
.	.	
n	n	

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B.2. Predictive assays extrapolate from the particular to the general, i.e. from experience with one or more agents, or from experience with one or more doses, they predict what will happen with other agents or other doses sampled from the two universes.

C. Creation of Assays

Assays arise from clinical or laboratory experience based on:

1. Empiricism - discovery of correlation without insight into mechanism, or

2. Mechanism - knowledge of the process leading to identification of relevant intermediate stages for assay; or knowledge of parallel systems which relate appropriately to the desired process.

D. Predictivity of assays depends on the quantitative and qualitative linkage (or parallelism in the case of A2b) between the assay point in the process and the effect point. When the ultimate effect can be reached through multiple paths (e.g. genotoxic and nongenotoxic carcinogens), then the assay depending on its position in the sequence may predict for only one subset of the universe of chemicals.

                                                  *Assay point
Agents, type A   E → → → → D → → → → →

                                                                    Effect

Agents, type B E → → → → D → → → → →

For quantitative prediction, in addition to being on the correct pathway, the assay point must have a suitable range and functional form of coupling to the effect point. Problems can arise due to differences in saturation background, thresholding, transiency and non-linearity between the assay and the effect, or to changed form such as continuous response of the assay versus all-or-nothing response of the effect.

E. Validation of Assays

1. Nonpredictive validation involves the simple correlation between the assay and the endpoint for the agent being studied.

2. Predictive validation

a. For the universe of doses and dose schedules involves sufficient knowledge of mechanism or of dose-response relationships to allow the assay to be a credible substitute for the full effect. Typically problems arise when the effect is difficult or expensive to assay and hence has a limited data base. Examples: low dose interpolation, chronic exposure extrapolation.

b. For the universe of chemicals involves a data base with sufficient chemical generality containing correlational data between the assay and the effect. This correlation requires an already validated or generally accepted measure for the effect. To achieve chemical generality one must either

   i. Sample randomly from the chemical universe, or

   ii. Sample on the basis of sound knowledge of

        chemical structure relevant to both the assay

        and the effect.

   To estimate fully the predictivity of an all-or-nothing assay and effect one must estimate the true and false positive rate, the true and false negative rate, and the prevalence of positive agents in the universe for which the prediction will apply.

F. Applications to Environmental Carcinogens and Human Risk Analysis

1. The human data base is sparse and, except for radiation carcinogenesis, is non-quantitative.

2. The rodent cancer bioassay is a non-validated surrogate for human carcinogenesis. It has a limited data base, heavily biased toward positive chemicals and certain chemical classes. It is probably not representative of environmental chemicals. It focuses on long-term repeated exposure at high (near lethal) doses. At $0.5 million per test the demand for expediency necessarily leaves unresolved the serious problems of high-dose non-linearities and non-generality of the chemical universe being tested. Outright disagreements with human data and between rat and mouse are well known for the cancer bioassay.

3. The better short-term assays for carcinogensis probably perform against the cancer bioassay in the 50 - 90% range of correct assignments depending on chemical class. This estimate is controversial and by and large involves very small data bases. True predictivity is unknown. Order of magnitude quantitation between short-term assay and cancer bioassay applies for a small subset of standard carcinogens-mutagens, although dramatic exceptions are known. Few chemical carcinogenic dose-responses are well described by cancer bioassay.

4. In spite of its shortcomings, the cancer bioassay has reasonable credibility and legal acceptability. The short term assays, although widely used as operational surrogates for the slow and expensive cancer bioassay, do not share this credibility or acceptability. Any new bioassay will face the same problems and unless dramatically superior to what is available will go through a long period of data accumulation and familiarization.

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SPECIAL COMMENTS

Plasminogen Activator, DNA Repair, and the Rapid
Detection of Human Cellular Responses to DNA Damaging Agents

Mark MacInnes and Brian D. Crawford

Urokinase-plasminogen activator (PA) synthesis is induced profoundly by inflammatory and DNA damaging agents (1). PA is also constitutively expressed at high levels in most virally transformed rodent cells (2) and in many, but not all, human transformed cells (3,4). Hence, measurement of PA induction in vitro has potential utility as part of an integrated, multifaceted, bioa say system designed to detect early events concomitant with human cell transformation and other responses to DNA damage. We will focus this discussion on the role of PA induction in DNA damage responses and its relationship to DNA repair.

There are at least two major biological constraints on PA induction by DNA damage: a) developmental stage and cell type and b) DNA excision-repair capacity. PA is inducible by ultraviolet light (UV 254 nm) in human fetal amniotic and skin fibroblasts, whereas normal adult skin fibroblasts are refractory (1,5). Since both fetal and adult fibroblasts are excision repair-proficient, this differential PA inducibility represents an apparent developmental canalization of PA regulation.

In contrast, adult skin fibroblasts derived from patients with xeroderma pigmentosum syndrome fail to repair U.V. damage of various kinds, are hypersensitive to cytotoxic and mutagenic responses, and are hypersensitive for PA induction (5). The appearance of PA is at present a unique enzymatic marker of cellular responses to DNA damage in repair-deficient, adult human fibroblasts. Hence, we propose to test the involvement of PA induction in a general cellular protective response to DNA damage. We will examine the mechanism of its regulation by DNA repair functions, and its possible direct regulatory impact on DNA repair (as an analog of the bacterial, protease induced, recombination repair system). We will discuss briefly some of the advantages, deficiencies and unknowns of PA induction as part of a potential bioassay system for risk assessment.

The major potential advantages of monitoring PA induction are several fold:

1. Ease of measurement. It is assayed in several ways, adaptable to large scale, both directly and indirectly, e.g., by protease zymograms of PA activation of fibrinolysis (6), or by PA-radioimmune assay.

2. Rapidity of the response to inducing agents. Induction of the enzyme in human and rodent cells is relatively rapid (2-days) compared to other genotoxic endpoints, measurements loss of cloning efficiency (2 weeks), mutagenesis (5 weeks), or transformation (2 weeks).

3. General hypersensitivity (for PA induction) of human repair-deficient cells. This point remains to be established in human cells, but there is some evidence (1) that ionizing radiations, and mionetic drugs

and DNA crosslinking agents may cause PA induction. PA induction may provide a useful test for detecting DNA repair heterozygotes that are estimated to comprise about 1% of the population.

4. PA induction may be a marker for detecting inflammatory, and tumor-promoting agents; and for individuals with a diathesis towards inflammatory disease.

5. Applicability to multiple tissue samples. PA induction is measurable not only in fibroblasts but in erythropoietic, immunogenic, and endothelial tissues. The relationship of PA induction to DNA damage has not been evaluated in these target cells, as yet.

The major deficiencies, unknowns and directions for future research in the PA bioassay system are:

1. Assessment of the potential for non-specific (false positive) PA induction in tester cells. PA is induced by a wide variety of toxic agents, physiologically active hormones, growth factors, etc. (7). Development of a differential sensitivity test using DNA repair-competent vs. deficient human cells in vitro should alleviate this problem somewhat.

2. Assessment of the potential for false negative responses (or critical-dose phenomena) caused by certain metabolic inhibitors. PA induction by DNA damage requires both new mRNA and protein synthesis under conditions of some acute cytotoxicity. Purified agents or components of complex mixtures that arrest concertedly either of these metabolic functions will suppress PA induction. This phenomenon is seen with U.V. (a potent transcription inhibitor) in repair deficient human cells (5). PA induction is maximal for incident doses of 1-2-5 J/M^2, whereas doses >5 J/M^2 produce no induction.

3. The more fundamental questions of the mechanisms of PA induction by DNA damage are unanswered.

Although desirable, it is not necessary to have a detailed molecular knowledge of this response mechanism to begin validation of this bioassay. There is now a plausible relationship between DNA damage, DNA repair and PA induction. More research is required to substantiate these relationships. In this context, we will characterize PA induction in fibroblasts deficient not only in UV repair, but also, repair of X-ray and chemical cross-link damaged. These latter cells are derived from individuals with Louis-Bar and Fanconi's anemia syndromes, respectively.

In conjunction with our program to clone repair genes by DNA transfer, we will study the initiating steps for PA induction by direct injection of damaged DNA into responsive cells. The interactions between repair gene expression and PA induction will be probed directly with cloned DNA repair, and PA gene DNA sequences to detect their mRNA and protein expressions. Finally, DNA damage-induced PA responses may be studied in other cell types, e.g., macrophage, that are important for tissue regeneration in response to inflammatory insults.

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SPECIAL COMMENTS

Viral Oncology as Related to Cancer Risk Assessment

Susan M. Astrin

As a virologist, I choose to comment on the following parameters of the proposed bioassay system:

1. What are the basic mechanisms of oncogenesis? Can mutations at a single genetic locus trigger neoplastic transformation?

2. What are the cellular and molecular criteria for determining neoplastic transformation? Can expression of the cellular homologues of viral oncogenes be used as a parameter?

3. What is the relationship between chemical carcinogenesis and viral carcinogenesis? Is there a common pathway or mechanism?

4. What type of basic research is needed for the development of this bioassay system?

Studies on RNA tumor viruses over the past decade have yielded an insight into possible mechanisms of oncogenesis not only as related to the viruses themselves but also in terms of the general problems of the triggering of neoplasia by other agents. The acute transforming viruses of vertebrates cause tumors in 2-3 weeks after inoculation of a susceptible animal, are often tissue specific for tumor induction, and transform appropriate target cells in culture. These properties are due to the presence in the viral genome of a specific oncogene - a gene which codes for a protein capable of transforming cells. Over 15 distinct oncogenes have already been described - each of which is carried by one or more oncogenic retroviruses. A list of the viruses and their oncogenes is given in Table I. In the best studied cases, it appears that expression of the viral oncogene is both necessary and sufficient for tumor formation.

What is the relationship between the viral oncogenes and the cell? It now appears extremely probable that the viral transforming genes are derived by a recombinational process from the cell genome. That is, each of the 15 or more oncogenes has a homologue present in normal vertebrate cells. Each of these genes is present in all vertebrates tested to date. Thus, the genes are highly conserved in evolution, leading one to believe that they perform an essential function. Preliminary information on the expression of oncogenes in normal tissue indicates that they may be expressed early in development of selected tissues and organs. It is possible that the expression of each of these genes is tissue specific and that expression is linked to proliferation and/or differentiation of a given cell lineage. Given the fact that these genes, when carried by a retrovirus, induce neoplastic transformation, is it likely that activation of the appropriate "oncogene" in a target cell can trigger neoplasia? In other words, do viruses transform cells by "overloading" them with what is otherwise a normal cellular product?

Can this situation also be achieved by enhanced expression of the

cellular gene? Several lines of evidence indicate that the answer to the above questions may be "yes".

First, available evidence indicates that avian leukosis virus (ALV), a retrovirus that causes bursal lymphomas in the chicken, does not carry its own oncogene but rather induces tumors by activation of a cellular gene called myc - the homologue of the oncogene carried by MC29 virus. The level of myc gene expression is enhanced 30-100 fold in the ALV-induced tumors as compared to the level in normal bursal tissue. The enhancement is due, in a majority of cases, to insertion of the provirus adjacent to the myc gene thereby providing a strong upstream promoter (the viral long terminal repeat or LTR) for transcription of myc (1). This mechanism has been termed "promoter insertion". The observations that bursal lymphomas can also be induced by MC29 virus, that the viral myc gene is essential, and probably sufficient for that process, and that the levels of viral myc expression in MC29-induced tumors and cellular myc expression in ALV-induced tumors are comparable, lead to the conclusion that activation of cellualr myc is likely to be sufficient for tumor induction.

Second, in vitro reconstruction studies in which viral oncogenes are linked to strong promoters for RNA polymerase (viral LTRs for example) demonstrate that the combination of an oncogene plus a strong promoter can cause malignant transformation when "transfected" into NIH 3T3 cells (2). These data confirm the idea that enhanced expression of cellular "oncogenes" is oncogenic.

Third, recent experiments using DNA from human tumor cell lines (from bladder, lung and colon carcinomas) to transform NIH 3T3 cells have identified several "transforming genes" to be present in the human tumor cell DNA (3,4,5). Strikingly, these genes have been recently reported to correspond to the human homologues of the viral oncogenes Harvey ras and Kirsten ras (6). These data are the most striking evidence to date of the direct involvement of "oncogene" expression in human neoplasia.

Taking the above into account, it appears that mutation at a single genetic locus, i.e. one affecting "oncogene" expression, may be sufficient to trigger neoplastic transformation. While enhanced expression of one or more oncogenes may not be the primary transforming event in all cases, it appears likely to figure strongly in the oncogenic process. Ultimately it should be possible to use measurement of the level of oncogene products as a molecular criterion for neoplastic transformation. In situ hybridization for detection of RNA or reaction with monoclonal antibodies for detection of proteins could foreseeably be used for these measurements.

What types of genetic changes could affect "oncogene" expression? Can these changes be induced by chemical or physical agents? The following types of events might lead to oncogene activation:

1) "Promoter insertion" by a retrovirus. If oncogenic viruses exist for humans, one possiblity for their mode of action is by promoter insertion. Chemical and physical agents (most notably halogenated pyrimidines and X-ray) are known to affect expression of endogenous viral genomes and could play a role in this type of mechanism.

2) Inactivation of a mechanism for repression of oncogene expression. The endogenous oncogenes appear to be expressed only at low levels, if at all, in most terminally differentiated tissues. Activation could be accomplished by mutation in a gene coding for a "repressor" or by genetic rearrangement resulting in relocation of the oncogene to an active transcriptional unit. Chemical agents may cause point mutations as well as genetic rearrangements. Physical agents could also conceivably induce such changes.

3) Mutation in the regulatory sequences of the oncogene. Such mutation could result in more efficient recognition of the promoter sequence by RNA polymerase or in less efficient recognition by a repressor molecule. Again both chemical and physical agents might induce such changes.

4) Amplification of the oncogene sequence at the DNA level. Gene amplification of an oncogene sequence, if extensive enough could elevate expression from a level of 2-3 RNA transcripts per cell (the level seen in differentiated tissue) to several hundred per cell (the level seen in tumors). The relationship of chemical and physical agents to such a process is not known.

Finally, what type of basic research is needed to further explore the above possibilities? Experiments utilizing both viral and cellular systems should be aimed at identifying oncogenes and their RNA and protein products and determining their mode of expression and regulation. A more difficult task will be to determine their function. Are enzymatic functions associated with "oncogene" protein products? What are the targets in the cell for these activities? How do these products influence cell proliferation and other functions?

A more immediately obtainable goal is to gather as much information as possible about the expression of the cellular "oncogenes" in both normal and neoplastic tissue. Is expression tissue-specific? Is elevated expression of particular oncogenes peculiar to certain types of tumors? These and a host of other questions need to be answered before we can completely assess the importance of the cellular "oncogenes" in human neoplasia.

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TABLE I. Viral and Cellular Oncogenes

Oncogene	Virus carrying the gene	Probable animal origin
abl	Abelson murine leukemia virus	mouse
erb-A	avian erythroblastosis virus	chicken
erb-B	avian erythroblastosis virus	chicken
fes	Snyder-Theilen feline sarcoma virus	cat
	Gardner-Arnstein feline sarcoma virus	cat
fms	McDonough feline sarcoma virus	cat
fps	Fijinami sarcoma virus	chicken
	PRCII sarcoma virus	chicken
	PRCIV sarcoma virus	chicken
mos	Moloney murine sarcoma virus	mouse
	Gazdar murine sarcoma virus	mouse
myb	avian myeloblastosis virus	chicken
myc	avian myelocytomatosis virus MC29	chicken
	avian myelocytomatosis virus CMII	chicken
	avian myelocytomatosis virus MH2	chicken
	avian myelocytomatosis virus OK10	chicken
ras, Harvey	Harvey murine sarcoma virus	rat
ras, Kirsten	Kirsten murine sarcoma virus	rat
rel	avian reticuloendotheliosis virus	turkey
ros	avian sarcoma virus UR-2	chicken
sis	Simian sarcoma virus	woolly monkey
src	Rous sarcoma virus	chicken
	B77 avian sarcoma virus	chicken
	Prague strain Rous sarcoma virus	chicken
yes	Y73 avian sarcoma virus	chicken
	Esh sarcoma virus	chicken

SPECIAL COMMENTS

Speculations on Mechanisms of Multistage Hepatocarcinogenesis - Models for Promotion and Initiation

Carl Peraino

The following remarks will be restricted to what I perceive as a conceptual problem common to most current discussions of multistage carcinogenesis, namely the ambiguity associated with the identification of the mechanistic boundaries between initiation and promotion. The following discussion compares the two alternative initiation-promotion models that I presently visualize as defining this issue.

A.   Model I - Initiation produces phenotypically altered cells that acquire a selective growth advantage through promoter action.

The underlying premise in this case is that the process of initiation encompasses events related both to the creation of the primary tumorigenic molecular lesion and to aspects of its expression. Thus, histochemically detectable foci of phenotypically and behaviorally altered hepatocytes are categorized as clones of initiated cells (1). In this context promoters presumably confer a selective growth advantage on such cells encouraging their accumulation to a critical mass that is then intrinsically resistant to host-imposed growth constraints. Postulated mechanisms for such promoter action include: (a) the direct and selective proliferative stimulation of the cells within these foci (1), (b) interference with the flow of growth regulatory information by disruption of intercellular communication (2), and (c) the creation of conditions in which generalized proliferative stimulation is signalled in a mitoinhibitory environment to which initiated cells are relatively resistant, thereby favoring the differential proliferation of the initiated cells (1).

The two central predictions emerging from the foregoing arguments are: (a) Initiated cells should embody detectable phenotypic alterations that render them uniquely responsive to direct or indirect promoter action, and (b) the chief manifestation of promoter action should be an acceleration to tumorigenesis (rather than a de novo increase in tumor incidence) as exemplified by increases in the growth rates of preneoplastic hepatocyte foci, with consequent reductions in tumor latency and possible increases in tumor growth rates.

B.   Model II - Initiation is restricted to the creation of a tumorigenic molecular lesion; initiated cells are phenotypically normal until stimulated to express their altered phenotypes and neoplastic potential through promoter action.

In this model the sequence of events involved in the acquisition of neoplastic potential (initiation) is mechanistically completely distinct and isolatable from the processes involved in the phenotypic expression of this potential (promotion). Following such expression, promotion per se has ended, and subsequent events involve progression of the neoplastic cells and/or the dynamics of their interaction with the host. Predictions generated by this model include: (a) An initiated cell would be directly detectable only

through the identification of the tumorigenic molecular lesion that it carries (e.g., site-specific carcinogen binding and/or DNA modification) and not by any criteria based on phenotypic change. Indirect identification would be possible on the basis of promotion-mediated expression of the transformed phenotype. (b) Promotion would be manifested only as an increase in the incidence rates for tumors and preneoplastic hepatocytes and would not involve an acceleration of the growth rates of these entities.

C. Evaluation of Models I and II

Although Model I currently dominates considerations of hepatic tumorigenesis mechanisms (1), we believe that the weight of evidence accumulating from recent in vivo studies of skin and liver tumorigenesis, and from in vitro initiation-promotion experiments, would appear to necessitate a re-evaluation of this position. The basis for this viewpoint derives from an evaluation of the degree to which the foregoing predictions generated by each of the models coincides with existing experimental evidence.

A systematic assessment of the validity of the first prediction for each model has not yet been undertaken in the liver system, possibly in part as a result of the prevailing presupposition that initiated hepatocytes are phenotypically altered, and also because detection of phenotypically unaltered initiated hepatocytes might be prohibitively difficult. At present, therefore, the most feasible approach would appear to be an indirect evolutionary one involving the development and application of increasingly sensitive tests for phenotypic change in concert with the use of progressively milder initiating stimuli (to approach as closely as possible "pure" initiating conditions) and an optimum promoting regimen that maximizes the expression of the tumorigenic lesions without introducing such extraneous effects as cytotoxicity or additional initiating activity. Under such sensitive conditions the first prediction of Model I would require that the incidence of the minimal detectable phenotypic change associated with the expression of neoplasia be dependent only on initiator dosage, and should be unaffected by promoter treatment. On the other hand, the first prediction under Model II suggests that the inclusion of promoter treatment would significantly lower the initiator dose required for the production of the first detectable phenotypic changes associated with neoplasia.

Although experiments of this nature remain to be conducted in liver, evidence accumulating from skin tumorigenesis studies indicates that initiator dosages can be reduced to the extent that no phenotypic changes are detected in initiated skin; the presence of initiated cells under such conditions can be revealed only following the application of promoter with the consequent emergence of phenotypic changes characteristic of neoplasia (3). These observations are in accord with the first prediction generated by Model II.

In contrast to the experimental difficulties associated with the first prediction, the second prediction generated by each of the models has been relatively accessible to investigation, and consequently a somewhat more definitive evaluation of the alternative viewpoints is possible. Recent liver tumorigenesis studies have shown, as indicated earlier (Section IV, B), that tumor promotion by phenobarbital involves an increased incidence of tumors without an increase in growth rate or a decrease in latency. This indication

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that tumor promotion does not involve an acceleration of neoplasia, but only an increase in the probability of its expression, is in accord with the second prediction from Model II. Additional support for this position comes from a study showing that phenobarbital added in vitro, to cultured hepatocytes from in vivo AFF-initiated livers, increased the incidence but not the growth rates of the malignantly transformed hepatocyte foci appearing in the cultures (4).

A number of in vitro initiation-promotion studies also bear on the relationship between proliferative stimulation and the promotion of transformation. In experiments establishing the initiation-promotion phenomenon in cultured C3H/10T1/2 mouse embryo cells it was determined that the promoting effect of TPA on cells initiated by prior exposure to polycyclic aromatic hydrocarbons could not be explained in terms of TPA's mitogenic activity (5,6). Additional studies with these cells, utilizing X-radiation as the initiating stimulus, have shown that the promotion of transformation by TPA involves the enhancement of expression of the transformed phenotype in a given initiated cell rather than the creation of a selective growth advantage for the progenitors of transformed clones (7). Evidence that TPA induces the expression of the tumor cell phenotype (manifested as the acquisition of anchorage-independence), as opposed to providing a selective growth stimulation for preexisting transformants, was also obtained with JHB6 mouse epidermal cell cultures (8,9).

On the basis of the foregoing collective evidence from liver, skin, and cell culture studies, we suggest that the initiation-promotion Model II described above is superior to Model I as a working hypothesis defining the mechanistic limits of initiation and promotion, and is applicable to liver carcinogenesis, skin carcinogenesis and in vitro transformation.

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Recommendation

The establishment of initiation-promotion systems in a variety of tissues not only supports the general applicability of the multistage concept of tumorigenesis, but also suggests an approach to the development of a more systematic analysis of possible tumorigenesis mechanisms than has been possible thus far. The approach envisioned would involve comparisons among different systems in which tumor production or cell transformation has been optimized through the use of the most effective system-specific agents, administered according to the most appropriate protocols. When each tumor system was operating optimally, inter-system comparisons could then be undertaken with respect to carefully selected biochemical parameters (e.g. ornithine decarboxylase induction). Such comparisons would permit the identification of those responses that are common to the various systems, and hence potentially relevant to the tumorigenic process; responses that are system-specific could be eliminated from further consideration in this context. Since, as a rule, each of the tumorigenesis systems is under study in a different laboratory, cross-comparisons among different systems would require cooperative interactions among several research groups.

Extrapolation from Animals to Humans using Dose Response Curves

Richard Setlow

A key question is how to extrapolate the results obtained on simple test systems, or even animals, to risks to humans from environmental pollutants. The problem is even more difficult than it may seem if one is dealing with mixtures of reactive components.

A first problem is how one should compare experiments on the transformation of animal cells in culture to the results of skin painting experiments and to experiments in which animals are fed carcinogens. How should one compare the dosimetry in these situations? One way would be to compare the dose response curves obtained with the unknown substance (X) to that of a known compound (C). The known compound would be used as a positive control. The comparison is shown in Figure 1, and the reason for the use of controls is indicated by the different exposure scales in part, A, B, and C. If it were to turn out that the differences between the curves C and X for the three possibilities shown had the same dose ratios, one would have confidence in the extrapolation from work on cells in culture to whole animals. Obviously, for such an extrapolation procedure to work, the control substance must be chosen so that it at least approximates the unknown. It should have chemical characteristics of X. For example, mixtures of polycyclic aromatic hydrocarbons could use benzo(a)pyrene as a control, but it would not be useful to compare the hydrocarbons to a control of dimethylnitrosamine since the mode of activation of the latter is completely different from that of the hydrocarbons and its repair is completely different from that of the hydrocarbons. If the dose ratios in Figures 1A, B and C are not identical, then a more careful examination and more basic understanding is needed. Part of this need arises because some of the experiments may be done using acute exposures and other chronic exposures. Moreover, the effects of tumor promoters could conceivably alter the relative effectiveness of C versus X. Thus, even for this simple case, a great deal of information is needed if reliable assessments are to be made.

A step of equal difficulty is how to extrapolate from animal cells in culture to human cells in culture. One way of doing this is indicated in Figure 2 where conceivable dose response relations are shown for cytotoxicity, mutation and transformation for both C and X. The probability of the relative sensitivities of C and X having the same ratios for these three end points is small because cases are known in which the mutagenic lesion in DNA is different from the cytotoxic one. However, we would expect that the ratio of the cytotoxicity of animal cells to human cells (Figures 2A and D) would be the same as the ratios for mutation in animal cells and human cells and transformation in animal cells versus human cells. If such ratios are constant, then we have confidence that the control substance mimics the unknown mixture and that data on the effects of the unknown mixture on animal cells could be used to predict the effect on human cells provided we have data on the effects of a control substance on human cells.

The system described has a great deal of redundancy to it, but since the molecular mechanisms for the effects observed are, at best, poorly understood, it is necessary to have such redundancy to give credibility to the entire extrapolation procedure. If the ratios of sensitivities are not the same for animal and human cells for particular endpoints, then further research is called for to determine the reasons for the lack of consistency. Some of these reasons may have to do with the effects of different modes of activation or, in chronic experiments, on different effects of promoting agents.

It would be useful to have basic knowledge to permit the construction of theories that would guide one in the extrapolation from cells in culture to whole animals. The construction of such theories is a goal to put the extrapolation procedure on a firm foundation. Until such theories are available, we might make the extrapolation by using the data in Figures 1A, B and C to permit extrapolation from animal cells and culture to animals. If from a comparison of Figures 2A, B, C and D, E, F, we knew the relations between the effects on animal cells in culture and in human cells in culture, then we would be in a position to predict the effect of both the control, C, and the unknown mixture, X, on human beings. To do this, we have to assume that the activation system in humans is similar to that in animals; that is, the important exposure parameter is µg/kg. Thus, given all the data in Figures 1 and 2, the big remaining unknown is the activating system and the levels of endogenous or exogenous promoters and some idea as to why it takes a long time for cancers to develop. An additional factor that must be considered, but may be taken care of automatically in the comparisons between animal and human cells, are the rates of repair of DNA lesions in animal and human cells and the rates of cell proliferation.
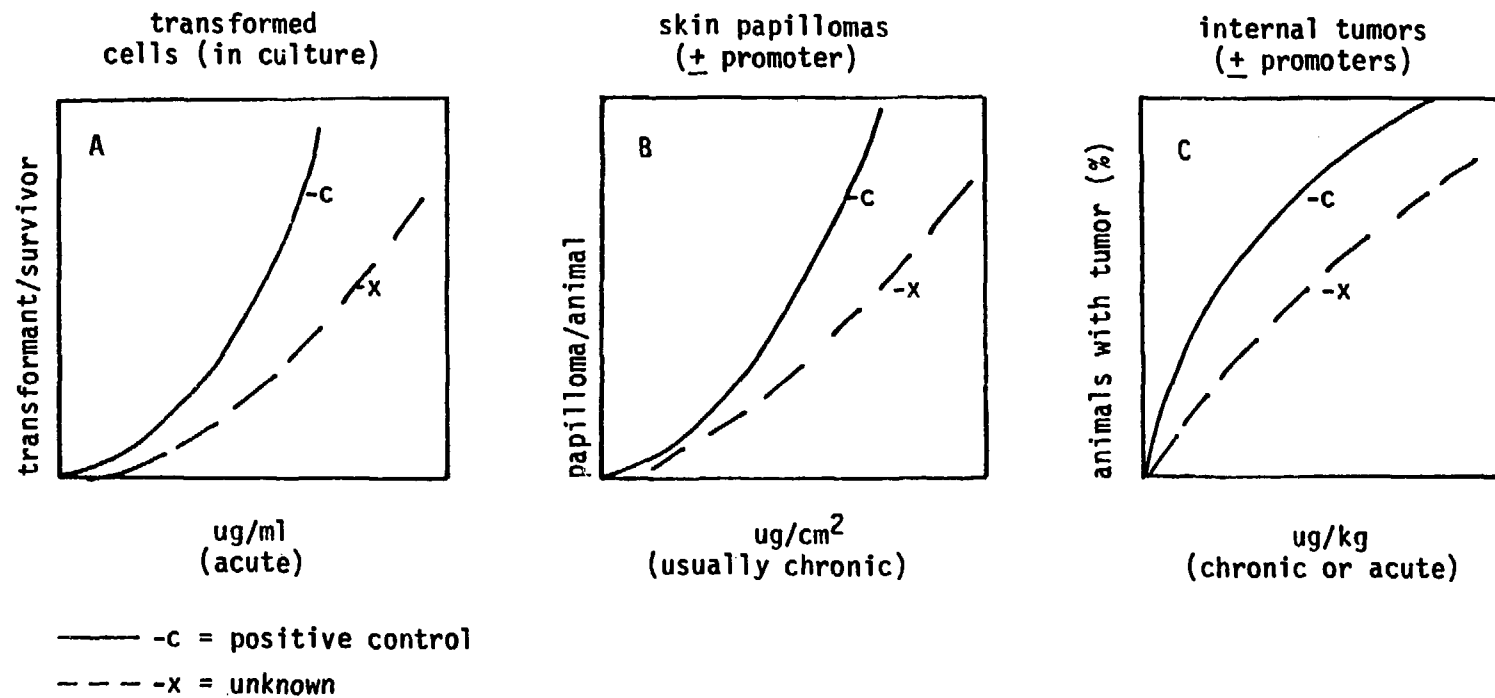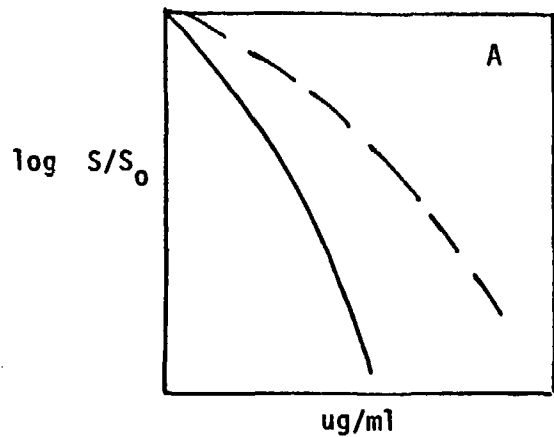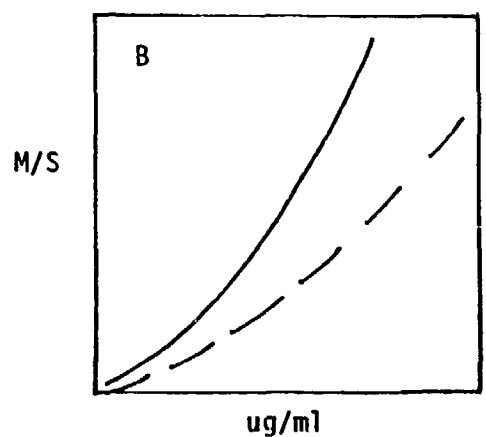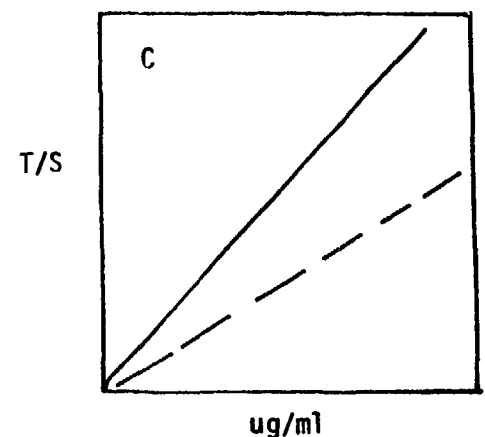
ANIMALS

FIGURE 1

ANIMAL CELLS IN CULTURE

cytotoxicity · mutation · transformation

HUMAN CELLS IN CULTURE

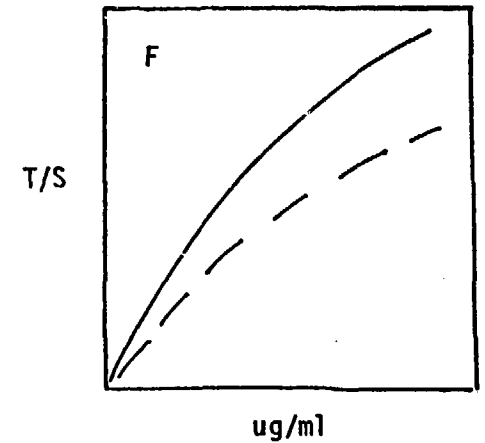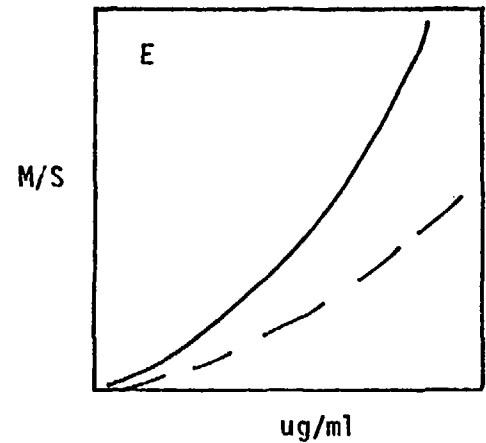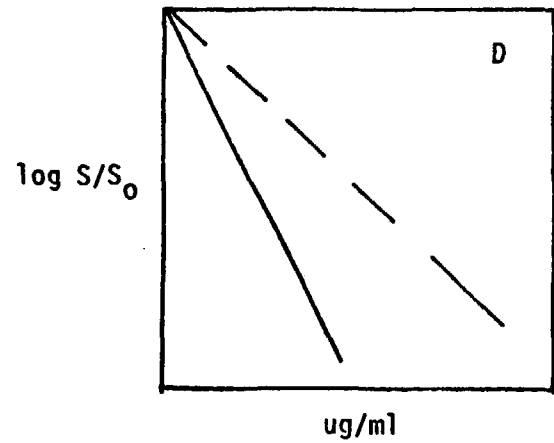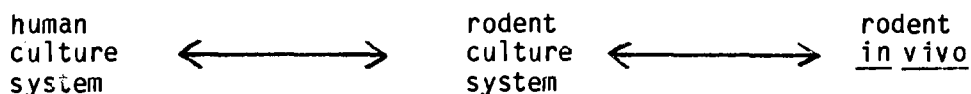FIGURE 2

(± activation, ± promoters)

SPECIAL COMMENTS

Studies of Human Epithelial Cells

Helene S. Smith

One of the major difficulties with evaluating chemical carcinogenesis on human cells is the fact that one cannot readily correlate in vitro data with effects modulated by the whole host. One suggested approach was to utilize chimeras (cultured human cells grafted into rodents). Progress along these lines has been reported for tracheal cells implanted onto prepared rodent tracheal beds. Concern was expressed that the rodent metabolic profile might be very different from the human one; hence the chimera might not accurately reflect the carcinogenicity in humans.

A second promising approach is to develop human cell culture systems that parallel rodent cell culture systems. Since the rodent in vitro assays can be correlated with in vivo carcinogenicity, and with in vitro human assays, a 3-way network of correlations can be established:

```
human                    rodent                    rodent
culture    <------->     culture    <------->      in vivo
system                   system
```

At present, the only quantitative transformation assays using human cells are with fibroblasts. However, since over 90% of human malignancies are of epithelial origin, it is important to develop human epithelial transformation assays. Until recently, the major stumbling block has been the inability to culture human epithelial cells. The development of new culture procedures now makes it possible to readily culture normal keratinocytes, mammary cells and bronchus. Within the next 5 years, these systems will clearly be developing along the line of quantitative transformation assays. It will be necessary to define criteria for the many stages of malignant progression. This must be done by culturing specimens derived from normal premalignant and malignant lesions and then describing the properties of these cultures. Ideally, a number of criteria will be found which distinguish each stage in progression. Some criteria will be useful for selecting transformed clones (i.e., growth without anchorage) so that their ultimate malignancy in nude mice can be assayed. Other criteria should be established which can be used to develop rapid and inexpensive transformation assays. Progress in developing mono-clonal antibodies against tumor specific antigens seems most promising. An assay detecting foci of antigenically-altered cells would be simple, inex-pensive and applicable to screening multiple samples. Different steps in progression could be defined by treating cultures from normal or premalignant lesions and then assaying for an antigen present at the next step in the pro-gression. It is very important to define the early steps in malignant pro-gression since agents which induce early stages of transformation could be very harmful to the human population. Even though the agents might not be shown to induce carcinomas when treated cells are inoculated into nude mice, within the natural human lifetime such "initiated" cells might progress toward cancers in vivo.

SPECIAL COMMENTS

Administrative Network Required for the Establishment of Bioassay System in the Department of Energy

Richard A. Griesemer

1. Selection of test substances. In addition to discussing how to test chemicals for adverse health effect, it is important to address what to test. The Department's health effects research differs from that of most other federal agencies in that the substances of importance tend to be complex mixtures rather than pure substances. For the more costly and long-term evaluations such as animal bioassays for carcinogenicity or for heritable effects, few substances can be tested with the resources available (both private and public). I believe what is needed is a national committee of scientists, with representatives from government and industry, to (a) develop criteria for selecting test substances and (b) establish priorities for testing. The Conference could consider the desirability and feasibility of a coordinated Government-industry approach to testing.

2. Procurement of test substances. Since multiple tests will be performed in many laboratories to take advantage of the special skills and techniques in University and National Laboratories, it is desirable that all the tests be conducted on the same test substance. I propose that one or several contract laboratories be charged to (a) purchase or synthesize test substances, (b) characterize them chemically and physically, (c) identify impurities, (d) store them, (e) provide aliquots as needed to research laboratories, and (f) maintain a chemical repository (library) so that representative materials would be available for follow-up experiments on the same substances.

3. Information about test substances. It is not cost effective for scientists in every research laboratory to review the published and unpublished literature. A contractor could be charged with providing all investigators bibliographic lists, hard copies of articles, and possibly analyses or monographs when warranted. The resulting bibliographic data base would serve as a national resource. The use of proprietary information requires review and safeguards.

4. Management information system and release of data. It is desirable for government and industry to coordinate its testing efforts so that (a) unnecessary duplication is avoided, (b) high priority work gets accomplished, and (b) the results are rapidly shared. A possible conference topic is release of data. How does one go about informing workers or the public of potential dangers in a timely way but without unnecessary alarm? I recommend a contractor be given responsibility for tracking the test substances and the experiments. I recommend further that a joint government-industry committee be established to review test results and to make test data and interpretations available to the public.

5. Reference standards. Reference reagents and standards should be procured and distributed to all investigators in the program in the same way as the test substances. Paticularly important is the creation of a battery of

coded substances for use in validating the tests. The Conference might consider the use of external standards and test batteries to assist in evaluating interlaboratory variability as well as for quality control.

6. Repository for test data and materials. For both scientific and regulatory purposes it is necessary that paper files and test materials (such as histologic sections) be available for extended periods of time (up to 12 years at present) for review by anyone in the public and private sectors. The Department should consider a central repository where materials can be secured and stored as well as made available upon request. The Conference members may want to maintain their own collections but should be reminded of the additional costs and inconveniences that may be imposed on them. For what kinds of research should records be kept? The Conference could address principles and decision rules to meet scientific and regulatory needs with the least cost and disruption to the flow of research.

7. Chemical structure-activity relationships. Because it is not feasible to test all substances to which people may be exposed, it is desirable to systematically approach the world of chemicals through better understanding of the significance of structural differences on biologic systems. While this long-term effort can be conducted retrospectively, I propose prospective analysis and incorporation of type compounds in the test batteries.

SPECIAL COMMENTS

Comments on the Use of Molecular and Cellular Assays
for Cancer Risk Assessment

R. Griesemer

A proposed scheme would be as follows:

Dose → Molecular Studies → Cell Transformation → Animal Bioassays

At present only two approaches for detecting carcinogens are generally accepted, the study of exposed humans or lifetime bioassays in animals. Human studies have several advantages including obtaining the data directly from the species of interest. (Parenthetically there is considerable agricultural economic importance in preventing cancer in domestic animals, too.) But there is an overriding concern since human studies cannot be performed until people have been exposed to potentially hazardous chemicals. Animal bioassays have emerged as a suitable method to detect carcinogens, but here too, are significant limitations. The sensitivity of the assays is known for only a few chemicals. The data base of substances both adequately tested in animals and adequately studied in man is very small. Moreover, animal bioassays are expensive and time-consuming and it is not feasible to test all the substances of importance in animals. Nevertheless the animal bioassay data constitute the most important base against which other tests must be validated.

The proposed scheme indicates that the most likely short-term substitutes for animal bioassays are in vitro cell transformation assays. The enthusiasm of the Symposium participants for cell transformation assays must be tempered, however, by the present limitations of cell transformation assays. With a few exceptions, most cell transformation assays have been performed in only one or a few laboratories. In general, they have not been standardized. Intralaboratory and interlaboratory reproducibility have not been adequately measured. Batteries of unknowns have not been tested for validation. Activation and detoxification systems have scarcely been examined. Most experiments have used only a few, very potent, well-known carcinogens. Age, sex, tissue, organ, and species factors have scarcely begun to be examined. As stated elsewhere in the Symposium, epithelial cells have been difficult to work with, human cells have been somewhat difficult to obtain, and a major confirming endpoint, tumor development in nude mice, is not without its problems. Unambiguous markers of transformation are still urgently needed. Nevertheless, cell transformation assays still hold considerable promise to screen chemicals for selection for animal bioassays and in time may supplant whole animal tests.

The molecular studies referred to in the proposed scheme are all of unknown significance so far as their correlation with carcinogenic activity. The validation process will proceed slowly so long as the molecular studies must be compared with lengthy animal studies. Cellular studies should be given a high research priority with the expectation that validating molecular studies against cell tranformation studies will permit rapid exploitation of molecular approaches to testing and to understanding mechanisms of carcinogenesis.

SPECIAL COMMENTS

Research Directions and Effects in
Inhalation Toxicology Research Institute

Antone L. Brooks

Our prime interest at the Inhalation Toxicology Research Institute is in the health impact of airborne pollutants. Because of this we have concentrated our research efforts on the respiratory tract. The research approach has been to collect relevant samples, to characterize their chemical and biological properties using standard chemical and bioassay techniques and to identify model compounds that play a major role in the biological activity. These compounds are then synthesized and studied in more relevant respiratory tract cell systems and in whole animal experiments. This approach allows us to build a matrix of dose-response information on several biological endpoints for each technology. It also provides a useful set of in vivo and in vitro data to bridge the gap between experimental animals and cells in culture.

ITRI Approach to In Vitro and In Vivo Genotoxicity

The major objective of genotoxicity research at ITRI is to provide data from short-term biological tests and chemical analysis that will be useful in predicting potential health hazards from inhaled energy effluents. Research with well-defined cell systems coupled with chemical analysis can help us to understand the mechanism of action by which energy pollutants produce disease. To understand the genotoxic action of pollutants it is essential to determine the chemicals involved, their metabolism, the interaction of these chemicals with the DNA in simple cell systems, and the relationship between the response in cell systems with that in whole animals. This approach provides needed links between the in vivo health effects and the large in vitro data base.

Our research approach in the study of complex environmental pollutants is to collect well-defined relevant samples, characterize their chemical composition and determine the biological activity of the crude extracts and fractions. During this phase the biological tests are conducted with the well characterized Ames bacterial mutagenesis test and the Chinese hamster ovary mammalian cell test. The endpoints routinely measured in the CHO cells are cytotoxicity and mutations.

Many samples require some chemical preparation before they can be successfully tested in a biological assay. Samples which are highly toxic, contain insoluble materials, or are too inactive to test accurately are common. Extraction and fractionation procedures have been developed to separate components which might mask bioactivity, to remove insoluble materials and to concentrate bioactivity. These preparative techniques enhance the assessment of a complex mixture by making the sample compatible with the specific test system.

This approach requires close interaction between chemical and biological testing. The advantage of stepwise fractionation and testing is that the compounds responsible for the bioactivity of the complex chemical mixture can be characterized. In an approach which relies merely on analysis for suspect carcinogens, unknown compounds can be overlooked. The inorganic chemicals associated with the particles are also evaluated and elements which are present in higher concentrations identified.

After testing crude extracts and fractions the research is to determine the genotoxic effects of model compounds, on lung cells in vitro. In addition to the standard biological tests using bacterial and CHO cells additional cell systems were developed to include epithelial cells from different locations in the respiratory tract. We have established methods for culturing primary lung cells from the Chinese hamster, tracheal explants from the rat and type II epithelial cells from the rat. Methods to evaluate chromosome damage and cell killing have been developed for each of these cell systems. The type II lung cells can be grown on an air collagen gel interface and be exposed to gases and aerosols. This provides an important link between air concentration and biological effects which can be used in extrapolation between in vitro and in vivo exposures.

Additional endpoints are also being developed to address a wider range of problems. The first of these is the interaction of pollutants with carcinogens or mutagens to alter the observed mutagenic response. This synergistic interaction was observed for a range of pollutant sources and could result in an underestimate of the potential health problems.

Another question being addressed with new techniques is the role of pollutants as tumor promoters. At the present time, there are few short-term tests for agents that act as tumor promoters. A method for growing tracheal explants was developed and evaluated as a potential assay for tumor promoters. When fully developed it will provide a rapid estimate of which crude extracts and fractions can act as tumor promoters.

The final question which can be addressed with model systems is to develop a method to detect cell transformation in vitro using epithelial cells derived from the respiratory tract. The current established cell systems used to detect transformation are fibroblasts. Since tumors primarily occur in epithelial cells it is important to develop an in vitro epithelial cell systems to detect cell transformation.

The research at ITRI is now relating the observations made in vitro to changes observed following whole animal exposures. To determine mutagenic changes the same cell type is exposed both in vitro and in vivo to known mutagenic or carcinogenic compounds and genotoxic changes evaluated in cultured explants. These results are related to those obtained when the same cell types are exposed to the same chemical in vitro. The endpoints measured in this combined in vitro/in vivo approach will be unscheduled DNA synthesis and sister chromatid exchanges. In addition, SCE's can be measured in blood lymphocytes of animals exposed in vivo or in cultures after in vitro exposure.

The other approach is to use the same endpoints induced in different cell types. We have measured SCE's in CHO, primary lung cell cultures and lung type II epithelial cells in vitro and compared the response to that observed in vivo in bone marrow and liver. Chromosome aberrations and cell killing can be measured in vitro with macrophages, CHO cells or type II epithelial cells and the response related to aberrations or cell killing in macrophages and aberrations in primary lung cell cultures or to the cytokinetic response of lung epithelial cells.

In all this research we must be reminded that cancer is a disease of the whole animal and cannot be modeled with certainty in vitro. The relationships between chromosome aberrations, unscheduled DNA synthesis, sister chromatid exchanges and cancer induction in the lung following exposure to a known mutagen can be determined. When testing complex mixtures or unknown compounds, all the endpoints can be measured in vitro except cancer formation. Comparative information derived for each endpoint can be used to predict the unknown in vivo response to the complex mixture or suspect carcinogen.

SPECIAL COMMENTS

Use of Cell Transformation Assays for Quantitative Risk Estimation

J. Carl Barrett

The data from cell transformation systems obviously can be used for risk estimation in the same manner as results from other short-term assays. We will restrict our comments to the more unique aspects of cell transformation assays.

1.   Detection of Carcinogens with Cell Transformation Assays.   Several cell transformation assays have been used as qualitative screens for detecting carcinogenic agents (see Tables 1 and 2). In particular, extensive studies with Syrian hamster embryo cells, BHK cells, C3H 10T 1/2 cells, and Balb/c 3T3 have been completed. In contrast, very few compounds have been analyzed with epithelial cell transformation systems. The results with the fibroblasts systems are very promising and indicate that cell transformation assays can detect >90% of known carcinogens with few false negatives. Many carcinogens, not detected in other short-term tests, are positive in cell transformation assays (deSerres and Ashby, 1981; Pienta, 1980). These results indicate the promise of these assays as screening tests for carcinogens, but also provide further evidence for a relationship between cell transformation in vitro and neoplastic development in vivo. Further detailed comparisons of the responsiveness of different cell transformation systems to a variety of chemicals can be found in a number of recent reviews of these studies (Hollstein et al., 1979; IARC Monograph, 1980; Heidelberger et al., 1982; deSerres and Ashby, 1981).

2.   Quantitative Results of Cell Transformation Assays. Berwald and Sachs (1965) were the first to quantitate cell transformation by scoring the number of morphologically transformed Syrian hamster embryo cells following carcinogen treatment. Later studies demonstrated that the number of morphologically transformed colonies observed is dependent on the dose of the carcinogen employed and the number of cells at risk (DiPaolo et al., 1971a). Statistical analysis of the dose response curve of the chemically induced morphological transformation indicates that it fits very well with a "one hit" model for this transformation (Gart et al., 1979 and section 3). Results with this system also demonstrate that this process results from the induction of transformed cells as opposed to selection of pre-existing neoplastic cells (DiPaolo et al., 1971b).

Dose response analysis of induction of enhanced growth variants of rat tracheal epithelial cells also suggests a "one-hit" mechanism for this transformation (Barrett et al., 1982).

It should be kept in mind that these results only relate to the first phenotypic change in a multistep process for the neoplastic development of Syrian hamster embryo cells and rat tracheal epithelial cells.

In contrast to the results with the above two cell types, certain aspects of the morphological transformation of C3H 10T 1/2 cells recently have become

Table 1. Quantitative systems for studying early, carcinogen-induced Changes in cell culture: Fibroblast systems

Species of origin (cell name)	Source of cells	Cell line or strain	Ploidy	Phenotype scored	Time of scoring (posttreatment)	Units of qualification	Development of tumorigenic potential	Comments	Reference
Syrian hamster (SHE cells)	embryos	Early passage strain	diploid	colonies of morphologically altered cells	10 days	foci/total colonies	yes		Berwald & Sachs (1965)
Balb/c mouse (3T3 mouse)	embryos	Line	aneuploid	colonies of morphologically altered cells	10 days	foci/total colonies	yes		DiPaolo et al (1972) Kakunaga (1973)
C3H mouse (10T½ cells)	embryos	Line	subtetraploid	foci of cells at high density on monolayer	6 weeks	% positive dishes	yes		Reznikoff et al (1973)
Syrian hamster (BHK cells)	kidneys	Line	aneuploid	colony formation in semisolid medium	2-3 weeks	colonies/colony formed surviving treatment	yes	Preneoplastic cells	Bouck and diMayorca (1976) Styles (1980
C3H mouse (B1 prostate cells)	ventral prostate	Line	aneuploid	colonies of morphologically altered cells	3-4 weeks	foci/total colonies	yes		Chen and Heidelberger, 1969
human	embryo	strain	diploid	colony formation in semisolid medium	3-4 weeks	colonies/total cells plated	not during experiment	transient module formation in nude mice	Freedman and Shin 1977
human	embryo	strain	n.a.	colony formation in semisolid medium	3 weeks	colonies/dye excluding cell plated	n.a.		Sutherland et al., 1980
human	foreskin	strain	n.a.	colony formation in semisolid medium	6-9 weeks	colonies/total cells plated	yes	cells subcultured 1-6 times before assay	Silinskas et al., 1981

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Table 2. Quantitative systems for studying early, carcinogen-induced changes in cell culture: Epithelial cell system

Species of origin	Source of cells	Cell line or strain	Ploidy	Phenotype scored	Time of scoring (posttreatment)	Units of qualification	Development of tumorigenic potential	Comments	Reference
rat	kidney cortex	primary strain	n.a.	colonies of morphologically altered cells	5-7 weeks	colonies/cells seeded	n.a.	treatment in vivo	Borland and Hard 1974
rabbit	bladder	primary strain	n.a.	colonies of morphologically altered cells	3-6 weeks	% positive dishes	yes		Summerhayes et al., 1981
mouse	submandibular gland	primary strain	n.a.	colonies or morphologically altered cells	10-14 weeks	% positive explants	yes	explant cultures	Wigley, 1979
mouse	epidermis	primary strain	n.a.	focal proliferation in differentiation inducing medium	6-9 weeks	% positive dishes	not after 8 passages	high cell density cultures; selection imposed at ≥ 3 weeks	Kulesz-Martin et al. 1980
mouse	epidermis	early passage strain	n.a.	clonal proliferation in differentiation inducing medium	7-9 weeks	frequency of altered cells	n.a.		Kulesz-Martin et al. 1981
mouse	epidermis	primary strain	n.a.	foci of proliferating cells	3 months (visible at 6-8 weeks)	% positive dishes	yes	high cell density cultures	Ananthaswamy and Kripke 1981
rat	liver	early passage strains	diploid	dense colonies of morphologically altered cells	6-8 weeks	% total colonies altered	yes		Borenfreund et al. 1975
rat	trachea	primary strain	n.a.	foci of proliferating cells with high cell density	3-4 weeks	foci/dish	n.a.	line formation also measured	Pai et al., in preparation
rat	trachea	primary strain	diploid	colonies of proliferating cells under selective conditions	4-6 weeks	colonies/colony forming cell surviving treatment	n.a.	selection (nonpermissive for normal cells) imposed 1-7 days posttreatment	Barrett et al. 1982

evident which complicate the quantitation of the frequency of transformation of these cells and suggests that this conversion is not a one-step process. Haber et al. (1977), Kennedy et al., (1980), and Fernandez et al. (1980) have followed up the original observation by Reznikoff et al. (1973) that the transformation frequency (i.e., number of transformed foci per cell treated after correcting for cell killing) is dependent upon the initial number of cells at risk. Extensive studies by Kennedy et al. (1980) with X-ray induced transformation have demonstrated that the absolute yield of transformed cells is constant over a wide range of initial cell number. For example, if an expression time of 12-14 population doublings after carcinogen treatment is completed and the cells are then resuspended and plated at different densities from 1 to 10,000 cells per plate, the number of transformed foci per dish is approximately 1 for all groups; hence, the apparent transformation frequency varies from 0.01-100% depending on the conditions employed. Also, for a given dose of carcinogen, the number of transformed foci per dish is independent of the number of cells treated. This is in contrast to the results with the Syrian hamster embryo cells (DiPaolo et al., 1971b).

According to Kennedy et al. (1980), these observations suggest that the transformed clones do not occur as the direct consequence of carcinogen treatment. Rather, these authors propose a two-step model to explain the results. The initial change induced by the carcinogen apparently occurs in a large number, perhaps in all of the cells. This change does not directly result in the transformation of the cells, but rather, increases the probability that the transformation of these cells will occur as a rare, secondary event. Since the initial cell number and the number of population doublings do not appear to influence the number of transformed foci, Kennedy et al., (1980) suggested that this process occurs at confluence, because the number of cells at confluence is constant in the density inhibited C3H 10 T1/2 cell line regardless of the initial cell density. The authors further propose that these observations are inconsistent with a mutational mechanism and suggest that an epigenetic process is involved in the carcinogen induced transformation of C3H 10 T1/2 cells.

Heidelberger and co-workers (Fernandez et al., 1980) have made similar observations with the C3H 10T 1/2 cells and have proposed a "probabilistic theory" to explain the formation of transformed foci by these cells following 3'-methylcholanthrene treatment. Their theory is similar to that proposed by Kennedy et al., (1980) in that two steps must occur for cell transformation. The first step is the "activation" of a large percentage of the cells by the carcinogen, which occurs with a probability p_1 and the second step is the transformation of the activated cells, which occurs with a probability p_2 per cell generation. The authors have derived a mathematical equation which predicts the frequency of foci formation based on the probability of these two steps (p_1 and p_2) plus the probability of deactivation per cell generation of the carcinogen activated cells, which is termed p_3. This approach has the advantage of allowing the determination of these probabilities based on experimental results. The equation derived to describe focus formation is $\log(F/N) = \log [2p_1p_2(1-p_3)/2(1-p_3)-1] + n\log(1-p_3)$, where F = mean number of foci per dish, and N = number of cells in a dish at confluence, and p_1, p_2, and p_3 are the probabilities described above. This equation has been verified experimentally with 3'-methylcholanthrene-induced C3H 10T 1/2 transformation and the values of $p_3 = 0.24$ and $p_1p_2 = 3.8 \times 10^{-6}$ were calculated.

Unfortunately, the values of p_1 and p_2 could not be determined uniquely (Fernandez et al., 1980). However, based on the results of these authors as well as Kennedy et al., (1980) the probability of activation, p_1, must be nearly equal to 1, since transformation is commonly observed with 1-5 cells at risk. This means that following carcinogen treatment, most, if not all, of the cells are activated, but only a few of the activated cells subsequently transform ($p_2 \geq 3.8 \times 10^{-6}$ per cell generation).

Unfortunately, the probabilistic theory of Fernandez, Mondal, and Heidelberger (1980) for 3'-methlycholanthrene-induced transformation is insufficient to describe the results of Kennedy et al. (1980) with X-ray induced cell transformation of C3H 10 T1/2 cells. The reasons for this are not clear. However, Barrett and Elmore (1982) have reanalyzed the data of these two groups and have calculated a "transformation rate" for the second step, which is relatively constant. This calculation assumes that the "first step" (activation of the cells) occurs with a high probability (approaching unity), while the second step is a spontaneous transformation of the activated cells which occurs randomly during the growth of the cells. The calculated spontaneous rate of this transformation is $1-6 \times 10^{-7}$ transformants per cell per generation (Barrett and Elmore, 1982) which is found from the results of Fernandez et al., (1980) or Kennedy et al., (1980). In fact, good agreement is found between the results of the different groups. This spontaneous transformation rate is also similar to that reported for a subtetraploid, preneoplastic Syrian hamster embryo cell line (FOL$^+$) (Crawford et al., 1980) which is not enhanced by mutagens, suggesting that they are equivalent to "activated" C3H 10T 1/2 cells (Barrett and Elmore, 1982).

A preliminary report (Mordan et al., 1982) suggests that at least part of the difficulty in quantitating focus formation of C3H 10T 1/2 cells is due to the suppressive effects of normal cells on the expression of focus formation by the transformed cells. These authors suggest that a mimimum colony size of ~128 transformed cells at confluence is required for the formation of a transformed focus. The suppression or reversion of morphological transformation of C3H 10T 1/2 and Balb/c 3T3 cells has been reported previously (Sivak and Van Durren, 1967; Brouty-Boye et al., 1979; and Brouty-Boye and Gresser, 1981). If a single transformation event in a C3H 10T 1/2 has to be amplified 100 times to be detected, this means that quantitation of this process will be very difficult, have a low level of sensitivity, and be subject to many factors inherent in the cell culture methodology. Haber and Thilly (1978) originally suggested that carcinogens affect two parameters in C3H 10T 1/2 transformation. The first was the induction of the potential for transformation which occurred in a large percentage of these cells. This is analogous to the activation step proposed by Fernandez et al., (1980). This induction occurs in nearly all of the cells and according to Haber and Thilly is not dose dependent. The second effect of carcinogen treatment suggested by Haber and Thilly is to influence cell-cell interactions in a dose dependent manner to allow for the expression of the transformed potential of the cells.

At present, it is apparent that the expression of morphological transformation of C3H 10T 1/2 is not a one-step process. The first step appears to be rapid event (Backer et al., 1982) that occurs in a high

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percentage of the cells (Haber and Thilly, 1978; Fernandez et al., 1980; Kennedy and Little, 1980). The second step could either be a second, qualitative change in the cells that occurs at a low frequency during the growth of the cells or at confluence (Fernandez et al., 1980; Kennedy and Little, 1980; Barrett and Elmore, 1982) or the second step could be an amplification of the transformed cells to overcome the suppressive effects of the non-transformed cells (Haber and Thilly, 1978; Mordan et al., 1982). Further experiments are needed to elucidate the mechanism of transformation with C3H 10 T 1/2 and the relevance of carcinogen-induced events on these cells to neoplastic progression in vivo. The use of this system for quantitative risk assessment should be done with caution until these issues are resolved.

It is not known whether the transformation of Balb/c 3T3 cells is also a two-step process, like the other subtetraploid murine cell line, C3H 10T 1/2. However, the results with a preneoplastic hamster cell line, BHK, are more consistent with a one-step process (Bouck and DiMayorca, 1976). It is intriguing to speculate that this difference is related to the near diploid karyotype of this cell line (Barrett and Elmore, 1982).

3. Dose Response Data with Cell Transformation Assays. Few detailed dose response curves have been published with cell transformation systems. Two carcinogens, benzo(a)pyrene and X-ray, have been studied extensively. Transformation of Syrian hamster embryo cells by polycyclic hydrocarbons does not correlate with the cytotoxicity of the carcinogen treatment (DiPaolo et al., 1971; Umeda and Iype, 1973). Huberman and Sachs (1966) and DiPaolo et al., (1971) reported that the logarithm of the frequency of morphological transformation of Syrian hamster embryo cells increased linearly with the logarithm of dose. The slope of this line was approximately unity in both studies suggesting a one-hit model for this change. Gart et al., (1979) have developed statistical methods to analyse these data and have confirmed that the results from the two laboratories are fitted very well by one-hit curves at all but the highest doses. The deviation at high doses is possibly due to cytotoxicity of the chemical. Two-hit and multi-hit models were rejected by their analysis. The one-hit model also holds for the results with X-ray plus benzo(a)pyrene-induced transformation.

Borek and Hall (1973) determined the dose response of morphological transformation of Syrian hamster embryo cells following X-ray treatment. The logarithm of the transformation frequency per surviving cell increased curvilinearly with the logarithim of dose from 1-150 rad. However, a linear response with a slope of 1 also fits within the standard deviation of the data points, which is consistent with the one-hit model of transformation observed with benzo(a)pyrene-induced transformation (Huberman and Sachs, 1966; DiPaolo et al., 1971). Interestingly, the authors were able to detect cell transformation with an X-ray dose of only 1 rad. The X-ray dose response curve reached a plateau from 150-300 rads and declined at 600 rads. The surviving fraction with 150 and 300 rads of X-ray was 0.86 and 0.75 respectively. Therefore, the plateau of transformation was not associated with a high level of cell killing. The surviving fraction with 600 rads of X-ray treatment was only 0.12-0.13, which may account for the decline in the dose response curve (Borek and Hall, 1973).

The use of comparative dose response studies for carcinogen risk analysis can be illustrated by the study of Borek et al. (1978) who have compared the relative biological effectiveness of X-rays and 430-keV monoenergetic neutrons in terms of cell killing and transformation of Syrian hamster embryo cells. Parallel dose response curves for cell transformation by neutrons and X-ray were obtained. When the data was plotted as transformed colonies/surviving cell, neutrons induced cell transformation at lower doses and to a higher maximum than X-rays. However, neutrons were also more effective in inducing cell killing. When the dose curves for cell transformation per initial cell at risk were compared, neutrons and X-rays had similar maximum carcinogenic potential and dose response curves although neutrons were effective at doses approximately 10-fold lower than X-rays. Borek et al. (1978) discussed the significance of their results in terms of the relative risk for secondary tumors of X-ray versus neutron therapy. They conclude that the increased effectiveness of neutrons in terms of cells transformation is offset by increased efficiency for cell killing, so that proportionately smaller doses of neutrons may be used without increased risk relative to X-ray therapy.

The effect of split doses of X-rays on the morphological transformation of Syrian hamster embryo cells has been studied. Borek and Hall (1974) observed that if an X-ray dose was divided into two fractions, more cell transformation was observed than if the same dose was given in a single exposure. Cell killing, on the other hand, generally decreased with split doses versus single exposures. These results are important in risk estimates because humans are generally exposed to low chronic doses rather than large, acute doses of carcinogens.

The results with the Syrian hamster embryo cell transformation systems demonstrates the applicability of cell transformation systems for risk assessment. However, risk assessment requires comparative dose response studies and such results are sometimes difficult to obtain with this system.

Pienta (1980) has screened over 100 chemical carcinogens and mutagens with the Syrian hamster embryo transformation assay and a dose response was not always observed in these experiments. The results mentioned above clearly indicate that dose response curves can be obtained with this system; however, it should be realized that this assay is not a selective assay like a mutational assay. Therefore, large numbers of colonies have to be screened independently to obtain sufficient numbers of transformed colonies to determine dose reponse curves. For example, over 10^5 colonies were scored in the experiments of Borek and Hall (1973) to obtain the dose response curve for X-ray induced transformation. This represents a tremendous amount of effort. The experiments of Pienta were equally heroic to screen the large number of chemicals necessary to validate the qualitative response of this system. His studies were not intended to be quantitative and have limited use for relative risk assessment.

This illustrates the advantage that selective assays for transformation have over non-selective assays. In the Syrian hamster embryo system microscopic examination of every colony (normal and transformed) is required. Other assay systems employ selective conditions, such as anchorage independent growth for BHK cells (Bouck and DiMayorca, 1976; Ishii et al., 1977; Styles,

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1980) and human fibroblasts (Silinskas et al., 1981; Milo et al., 1981) or enhanced growth under conditions that induced the terminal differentiation of normal epithelial cells (Yuspa and Morgan, 1981; Barrett et al., 1982). These assays require one to score only the transformed cells and hence are technically much easier. Dose response data have been obtained for both fibroblasts and epithelial cells using selective assays.

Several detailed studies on the transformation of C3H 10T 1/2 cells have been completed. In particular, the effect of irradiation has been extensively examined with this transformation assay (Terzaghi and Little, 1975; Terzaghi and Little, 1976a and b; Miller and Hall, 1978; Elkind and Han, 1979; Miller et al., 1979; Han and Elkind, 1979). These studies were completed before the problems with the quantitation of transformation in this system were realized (see Section 2). The relevance of these studies is, therefore, difficult to determine. However, it should be noted that the results with this system are very consistent with the known effects of radiation in experimental animals including dose-response relationships and the effects of dose rate, linear energy transfer and modifying agents (Kennedy and Little, 1980; Kennedy, 1982).

4. Mechanisms of Tumor Promotion. See RESEARCH STATUS REPORT by J.C. Barrett.

5. Initiation-Promotion Experiments in Cell Culture. Based on the two-stage model of carcinogenesis (Boutwell, 1974) developed in mouse skin with the potent tumor promoter TPA, several laboratories have attempted and the cell culture experiments succeeded in demonstrating phenomena in cell culture similar to those observed in mouse skin (Kennedy, 1982). That is, following a low dose of carcinogen which is weak or inactive in transforming the cells, TPA causes an increase in the transformation frequency greater than the sum of the two treatments alone. TPA alone is generally weakly active or inactive as a transforming agent. Kennedy (1982) has recently reviewed the current literature in this field and the reader is referred to this paper for details of the experiments in which TPA promotes cell transformation and also of the other agents which have promoting activity in cell cultures.

The cellular mechanism(s) of cell culture models of tumor promotion have not been defined. The two models which are most widely studied are TPA-enhanced transformation of C3H 10T 1/2 cells and Syrian hamster embryo cells (see Kennedy, 1982). In the latter assay, individual cells are exposed to the carcinogen, allowed to form colonies for 6-8 days, and then scored for morphological transformation. If TPA is added to the cultures during colony formation, an increase in the number of transformed colonies is observed if the cells are initiated with a low dose of carcinogen. Since the colony forming efficiency of the cells is unaffected by the tumor promoter, amplification of initiated cells is an unlikely mechanism for this effect. (One can also consider that growth in culture allows the cellular amplification necessary for promotion). It is possible that the tumor promoter affects cell-cell interactions in the emerging colony and allows the expression of transformed cells which are otherwise suppressed. A more likely possibility is that TPA modulates the initiated cells and allows its expression. It will be of interest to clarify if cellular modulation by TPA occurs in this system and the nature of this effect.

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Although "tumor promotion" has been studied more extensively in C3H 10T 1/2 cells (Kennedy, 1982), the qualitative or quantitative nature of this process has not been addressed. Recent studies (Mordan, et al., 1982) have suggested that TPA reduces the minimal colony size of transformed cells required to form a transformed focus. These results imply that tumor promotion in C3H 10T 1/2 cells is a quantitative change which alters the population dynamics and allows the amplification of clones of initiated cells. Further confirmation of these results are necessary.

Are cell culture models of tumor promotion relevant for risk analysis? This is a critical question which is difficult to answer because of our lack of understanding of the mechanism of the cellular changes during tumor promotion in vivo.

Most tumor promoting agents, including substances in the environment which are important in human cancers, are tissue-specific (Doll and Peto, 1981). For example, phorbol, the inactive control compound for tumor promotion on mouse skin, is an effective promoter of lung, liver and mammary carcinogenesis (Armuth and Berenblum, 1972; Armuth and Berenblum, 1974; Armuty and Berenblum, 1979). In contrast, TPA, the model tumor promoter employed in most cell culture studies, is a very active tumor promoter in vivo with a broad range of activity in a variety of tissues. Furthermore, TPA is a very potent biological substance and affects gene expression, morphology, growth, and/or differentiation of a large number of cells in culture. While the potency of TPA makes it an attractive model compound to study, it may also induce cellular phenomenon unrelated to tumor promotion. Thus, the assumption that all effects induced by tumor promoters (particularly TPA) on cells in culture are related to tumor promotion is not justified. This caution also applies to the relevance of "tumor promotion" studies using cell culture systems.

Cell culture promotion experiments were somewhat predetermined, that is the phenomenon of tumor promotion was defined by the in vivo experiments and cell culture experiments were designed to mimic these results. While the anticipated results were observed, it is also common knowledge (i.e. unpublished) among workers in the field that these experiments are difficult to repeat and highly dependent on the lot of serum and sometimes even the lot of TPA used. It should also be noted that TPA-induced promotion has not been observed with mouse epidermal cells in culture (Yuspa, S., personal communication), the target cells for classical two-stage carcinogenesis. In fact, no cell culture model system exists for tumor promotion of epithelial cells, the major target cells in vivo for these substances. Furthermore, the effects of TPA on promotion of cell transformation in fibroblast systems is contradictory to the effects of TPA on fibroblasts in vivo. Bhisey and Sirsat (1976) demonstrated that if one initiates by subcutaneous injection of carcinogen (which induces sarcomas) and then treats with TPA topically, inhibition rather than promotion of sarcomas is observed.

Cell culture experiments are of great utility in understanding the effects of tumor promoters on cellular growth, phenotypes, and differentiation and the mechanisms of these effects. The nature of the modulation of initiated cells by tumor promoters also may be elucidated by cell culture studies. However, if tumor promotion in vivo is primarily an effect on population dynamics within the tissue, i.e. amplification of the initiated

cell, it will be very difficult to design an experimental model with cells in culture that will reflect the cell-cell interactions that control cellular growth in vivo.

Interestingly, it has been shown that a variety of known tumor promoters can affect cell-cell interactions in a cell culture assay (Yotti et al., 1979; Murray and Fitzgerald, 1979). This assay is not a cell transformation assay but rather measures intercellular communication by interchange of small molecular weight metabolites. The potential of this assay for detecting tumor promoters should be explored further. Whether these results can be used for risk analysis remains to be determined. A further understanding of the mechanism of tumor promotion in vivo can possibly lead to future use of cell culture assays for risk analysis for promoters.

6. Analysis of Multistep Models of Carcinogenesis in Cell Culture. Neoplastic transformation of diploid cells in culture is a progressive multistep process in analogy to neoplastic development in vivo. The exact number of steps involved is unknown, although at least two steps have been identified. Various multistage models have been suggested based on epidemiological data from human tumors (Armitage and Doll, 1954; Armitage and Doll, 1957; Fisher, 1958; Cook et al., 1969; Moolgaukar and Knudson, 1981), but this analysis has not been extended to the cell culture models. The reason for this is that the latter differs in that generally a single dose of carcinogen is used rather than continual treatment. Therefore, the effect of the carcinogen is only a single step in the process. The influence of carcinogen dose on the overall process of neoplastic progression in cell culture has not been addressed.

The utility of merging multistage mathematical models and multistage cell transformation studies can be illustrated by considering the model of Moolgaukar and Knudson (1981). In this model, the progression to malignancy is controlled by three factors: (i) μl, the rate of transition of a normal cell to a preneoplastic cell (ii) $\mu 2$, the rate of transition of a preneoplastic cell to a malignant cell; and (iii) the rate of growth of the preneoplastic cells, which is controlled by the rate of division ($\alpha 2$) and the rate of terminal differentiation ($\beta 2$) of these cells.

The third factor in this model probably cannot be accurately determined by cell culture models, because of the inability to define the environmental influences on these processes normally present in vivo. Moolgaukar and Knudson (1981) suggest that this factor is influenced by promoters and this further points out the reasons why cell culture assays for promoters are difficult to develop. However, the transition rates, μl and $\mu 2$ are probably intrinsic properties of the cell (which may be influenced by carcinogens). Therefore, these rates can be determined in cell culture if preneoplastic and neoplastic cells can be identified and quantitated.

7. Relationship between Cell Transformation and Mutation. Since other short-term tests for carcinogens, particularly mutagenesis assays, are used for risk analysis, it is important to determine the relationship between mutagenesis, cell transformation and carcinogenesis. Barrett and Elmore (1982) have recently reviewed the current literature and concepts on the comparison between mutagenesis and carcinogenesis based on studies of cells in culture. The following conclusions were drawn:

Neoplastic transformation in cell culture is a multistage process and the role of mutagenesis may vary with different steps in this process. For example, aneuploid cell lines, which are nontumorigenic, have acquired some properties of neoplastic cells and progress to neoplastic cells more readily than normal, diploid cells and therefore are considered preneoplastic cells. The current evidence suggests that transformation of normal, diploid cells to aneuploid, preneoplastic cells may occur by a mechanism different from the transformation of preneoplastic cells to neoplastic cells.

Cell culture studies offer the advantage of directly comparing the processes of mutagenesis and carcinogenesis in the same target cells. Following carcinogen treatment, transformation of fibroblasts of hamster, mouse or human origin occurs with a frequency of 10-100 times that observed for gene mutation at two loci measured concomitantly. (Recent studies indicate that early alterations of rat tracheal epithelial cells in culture occur at a high frequency similar to that observed with fibroblasts (Barrett, et al., 1982). Induction of cell transformation can occur in the absence of measurable gene mutations (Barrett et al., 1981; Landolph and Jones, 1982; Gyi, 1982). Aneuploid conversion and alterations of DNA methylation have been proposed as mechanisms for transformation of these cells.

These studies point out the need to understand better the role in cell transformation and cancer of changes other than gene mutations, for example, chromosomal rearrangements and alterations in DNA methylation. This understanding will be useful in evaluating the risk to humans of chemicals, particularly those agents which appear to act by a mechanism other than direct DNA damage.

8. Conclusion. Cell Transformation assays can be used to study the cellular changes involved in carcinogenesis. These studies can provide information needed for understanding the nature of these changes, the influence of environmental factors on the changes, as well as the possible identification of inhibitors of these changes. Cell transformation studies have demonstrated the multistep nature of neoplastic development and have indicated that environmental factors can act at one or more stages in this process. Furthermore, different steps in malignant progression of cells may be influenced by different environmental factors. Cell culture assays can be used to identify possible carcinogenic hazards to man, to define the step(s) in neoplastic development which are affected by these substances, and to compare the relative risk of these agents in defined steps of carcinogenesis. The complete assessment of carcinogenic risk, however, requires consideration of the role of host factors as well as the cellular events in cancer.

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VII. HIGHLIGHTS

HIGHLIGHTS ON WORKING GROUP DISCUSSIONS

Target Cells

Richard Griesemer

In discussing target cell concepts, the Working Group found it convenient to divide the subject into (a) what is presently known about target cells, (b) what might reasonably be expected to be the status in the near future, and (c) what unexplored areas deserve investigation.

Present - When dealing with a test substance about which little is known, the initial problem is to identify the potential target sites. The Working Group members surprised themselves by agreeing that for this purpose, one would have to start by exposing intact animals to the test substance. Ideally multiple routes of exposure (inhalation, oral, skin, intraperitoneal, etc.) would be used and compared, depending somewhat on the physico-chemical properties of the test material. Information would be needed on the uptake and distribution of the compound so that toxic or carcinogenic effects could be related to the presence of the chemical. Comparative metabolic data would be obtained to the extent feasible. One reason for using whole animals is so that all the potential target sites can be evaluated. An equally important reason for using whole animals is so that indirect effects, such as those mediated by hepatic metabolism or such as those modified by immunologic or endocrine activity, can be identified. In general, the endpoints to be measured in the animal experiments would be biological rather than chemical. Whenever practical, long-term effects would be evaluated.

Concurrently with whole animal studies, the Working Group proposed to apply existing in vitro cell transformation assays. It was recognized that the available target cell systems for in vitro studies are largely fibroblastic and of rodent origin. Moreover, the assay systems are in various stages of the validation process so that the data obtained may have limited usefulness. The Working Group proposed further that tests for mutagenicity and genotoxicity also be performed although there was less agreement on the usefulness of these tests for carcinogenicity. The majority felt that short-term tests might be useful to flag compounds that warrant additional study.

Near future - It should soon be possible to propagate or maintain cells of all types from all parts of the body for in vitro studies of potential targets. Hopefully, ongoing research will clarify the significance of putative preneoplastic events so that one or more preneoplastic endpoints can be used in vitro. Similarly, identification and characterization of preneoplastic structural and chemical lesions in vivo should make possible a battery of short-term in vivo tests. Those presently under development and promising include assays in rat liver, mouse lung, rat bladder, rat mammary gland, mouse skin, and possibly whole fish. It will then be possible to compare the predictive value of in vitro and in vivo tests. Also promising is the development of methods to detect initiators and promoters in vitro in

cells from various organs. The Working Group recommended that a second set of initiated cells be used in each cell transformation assay.

Long-term - A major problem in developing and validating methods to identify target cells for carcinogens is that there are as yet few well-established non-carcinogens. It appears that there will be a continuing need to conduct animal bioassays to identify non-carcinogens and to provide a larger data base for validation of in vitro cell transformation assays. Another problem, for which conceptual or technical breakthroughs are needed, is the development of methods to transform epithelial cells in vitro. Little explored as yet in experimental systems are the effects of host factors, such as age, sex, or genetic constitution, on target site susceptibility. Also little explored is the use of submammalian cells as possible screens for target cell susceptibility so that fewer mammals need be used in research. (Time did not permit addressing species and organ specificity; sensitivity vs. relevance; developmental stages.

Human Cell Systems

Leila Diamond

A. The group agreed that the major advantages of human cell systems are:

   1. Humans comprise the population of interest and human tissue is the ultimate target tissue of interest.

   2. Human tissue is inexpensive to obtain compared to laboratory animal tissue.

   3. Experimental human cell systems can be related to epidemiological data both retrospectively and prospectively.

   4. There are pools of individuals who are genetically susceptible to cancer and a potential source of sensitive target tissue.

   5. There are pools of individuals who have been excessively initiated th. ough chemotherapeutic, industrial or accidental exposure to initiating agents; these, too, are a potential source of target tissue that may be highly sensitive to initiators and/or promoters.

   6. The tissues from individuals in (4) and (5) above are of particular value, compared to normal individuals, for studying the molecular and biochemical bases of transformation and increased susceptibility to transformation, and for identifying transformation markers.

The group agreed that the major disadvantages of human cell systems are:

   1. Ethical problems which in some cases may be considerable.

   2. Sufficiently large quantities of fresh material are usually difficult to obtain, with a few exceptions such as blood and skin.

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3.   Samples will be genetically heterogeneous and show interindividual variation for unknown reasons. The long- and short-term history with regard to diet, smoking, drugs, etc. will usually be incomplete and inadequate.

4.   The cells may already have been initiated.

5.   The whole animal cannot be manipulated to alter the immune, hormonal, etc. status except by grafting to form chimeras with experimental animals.

6.   Until other valid end-points have been identified, validation for malignant transformation requires transplantation into nude mice or immunosuppressed animals.

In contrast to human cell systems, rodent systems have the advantage that (1) malignant transformation can be directly validated by assaying for tumorigenicity; (2) in vitro and in vivo data can be compared; (3) models of tumor promotion and progression can be and have been developed; (4) prior pharmacokinetic analyses can be done; and (5) exposure to the risk can be adjusted to mimic real life situations. Thus, the group concluded that rodent cell systems are needed in order to develop and validate human cell systems and that, until much more is known about malignant transformation, both will be needed.

B.   After considering how to select the tissues or organs to be used, the group agreed that it would be difficult to choose wisely before more is known about culturing specific cell types, the pharmacokinetics of the compounds to be tested, the sensitivity of specific tissues to the biological effects of the test compounds, etc. One cannot assume at this time that the most sensitive or susceptible tissue in vivo will be the most sensitive or susceptible in vitro or vice versa.

The eventual emphasis should be on transformation assays but toxicity, mutation, and DNA and chromosome effects also should be assessed. Assays should include one-step and two-step protocols that can assess both initiating and promoting activity. Both cell and organ cultures should be used because each has specific advantages. The target cells should include both fibroblasts and epithelial cells. Conversion of human diploid fibroblasts to anchorage-independence seems to be a reproducible assay now and an understanding of the relationship in this system between anchorage-independence and tumorigenicity in irradiated nude mice should be forthcoming soon. New procedures for culturing epithelial and hematopoietic cells are being developed very rapidly and it is anticipated that transformation assays with such target cells will be reported soon. In certain organ culture systems, preneoplastic changes can now be readily induced and quantitated.

It was agreed that no conclusions can yet be made as to whether embryonic or adult tissue would be the most sensitive and, therefore, preferable in assessing risk. While there is evidence that embryonic tissue is more sensitive to mutagens than adult, there is as yet no evidence of differential sensitivity to promoters.

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The anticipated route of exposure should be considered but, in the absence of pharmacokinetic and tissue distribution data, may not be too helpful in selecting the target tissue(s) of choice.

The following recommendations were made for selecting target tissue(s):

(1)   Tissues such as skin and blood that are easily available and manipulated have several advantages, e.g., replicate and sequential samples can be obtained; different cell types from the same individual can be compared.

(2)   Previously initiated populations may provide sensitive target issue and reduce the test time. The tissue may be particularly useful in screening for promoting activity. It can be compared with that from uninitiated donors and, as in (1) above, different tissues from the same individual also can be compared.

(3)   Grafting to experimental animals should be used to mimic in vivo exposure and physiologic situations. These can be developed into initiation/promotion assays with a completely in vivo or an in vivo/in vitro protocol.

C.   It was agreed that the validation of end-points for transformation of human cells depends on knowing more about the behavior of primary and metastic human tumors in nude mice and in vitro.

For now, the final validation is tumorigenicity in nude mice, despite its recognized limitations of the expense, the immunocompetence of the animal, and the antigenic and differentiating capacities of the transformants. In vitro assays for cell invasiveness, e.g., in chick skin and chick heart, are now being developed and validated.

A number of potential markers may soon be forthcoming, e.g., specific cytologic and chromosomal abnormalities; acquired independence from feeder cells, hormones or conditioned medium; a block in differentiation; monoclonal antibodies to tumor-specific antigens; and new or amplified expression of specific proteins, enzymes or oncogenes as detected by fluorescent or molecular probes. Immortality of cell lines was thought not to be a requirement for malignant transformation since many primary human tumors do not become established cell lines when explanted in vitro.

Finally, it was agreed that early markers of transformation such as specific morphologic changes or growth in methocel should be considered a warning that the compound(s) was potentially dangerous.

Assessment Technology

Michael Waters

There are no easy and (at the same time) definitive solutions to the problem of cancer risk assessment. At present, we will have to rely on theoretical concepts, rather than on specific data set from any prescribed battery of short term biological test systems. This is so because each assessment situation will be different and will have to be approached on the basis of the available data. It is clear that we will have to rely for the present primarily on animal carcinogenicity bioassay data. For the future, the group suggests the utility of a human in vivo-based approach, i.e. the application to the extent possible of the range of genetic and related bioassays in man and exposure (dose) assessment methods that could provide information on the carcinogenic process. This could perhaps best be accomplished by initially examining exposed human beings in situations where exposure (and ideally, moelcular dose) can be quantitated (e.g. individuals exposed to cancer chemotherapeutic agents). In parallel with these human studies, definitive dose-response studies on the same agents must be performed in mammals, and in vitro in mammalian and especially human cells in culture (where they are applicable). In addressing a human-based in vivo system we should consider:

a. The range of biological variability within the human population - the variability within the human population is great, perhaps ranging from certain endpoints over two to three orders of magnitude. We need to understand what "normal" is.

b. The need to identify and examine sensitive human population groups and to establish to the extent possible the biological test systems based on the use of cells and tissues derived from these sensitive human populations.

c. The need to take advantage of existing clinical and occupational exposures to quantitate exposure dose and molecular dose including specific molecular adducts (perhaps using post-labeling techniques in the case of complex mixtures), and to relate dose to the range of available biological endpoints. (While we may not understand the significance of specific molecular damage at this point we may be able to unravel the problem in the future).

d. The need to consider the influence of additional endogenous factors such as the viruses and confounding exogenous factors such as are generally described by "lifestyle factors".

e. The need to select reference test agents to which man is exposed and for which we already have at least some epidemiological data. We suggest consideration of the IARC known and suspected human carcinogens for this purpose.

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Regarding complex mixtures:

1. There is a need to develop biological activity profiles (using a
    variety of short term tests) on a range of complex mixtures before
    we can really understand their potential carcinogenic activity.

2. Similarly, we need biological activity profiles on the major classes
    of compounds present in complex mixtures of concern. We should be
    able to use our knowledge of individual pure chemicals to develop
    some of this information.

3. Present data suggests that while relative mutagenic potency for pure
    chemicals in short term tests may span six orders of magnitude and
    relative carcinogenic potential may span four or more orders of
    magnitude, the situation may be different for complex mixtures.
    Data from comparative studies of the in vitro mutagenicity, primary
    DNA damage, and short term in vivo carcinogenicity on cigarette
    smoke condensate, coke oven emissions, roofing tar emissions, Diesel
    particulate extracts, suggests that the relative potency of such
    mixtures (as compared to a standard pure chemical such as
    benzo(a)pyrene) may vary over only two orders of magnitude.

    This emphasized the importance of exposure estimation and of
    human variability in decision making regarding alternatives in
    industrial processing conditions or in control technologies
    involving complex mixtures.

Regarding pure chemical studies and short term tests:

1. We need a much more complete data base for reference purposes. The
    EPA Gene-Tox program should assist in identifying the data gaps.

2. We need more careful mechanistic studies in order to develop and
    interpret the results of any short term test or long term test with
    respect to carcinogenicity in men. In addition to studies on the
    range of human carcinogens (mentioned previously), these mechanistic
    studies should concentrate at present on the "non-genotoxic"
    carcinogens and especially on the promoters.

Host Factors

Richard Griesemer

The Working Group found it easy to list a variety of host factors that may affect the susceptibility of the host to carcinogens. In no particular order, they include:

Immune status
Genetic background
Metabolism
Nutrients in the diet
Additives or contaminants in the diet
Age
Sex
Pregnancy
Hormonal status
Intercurrent disease
Exposure to other chemical or physical agents
Stress
Viral infections
Life style, such as alcohol and tobacco use

In a short discussion of the significance of these various factors, it was apparent that they may affect many different parts of the carcinogenic process, from the likeliness and extent of exposure through initiation, the various steps of promotion, and the rate of tumor growth and spread. The least information is available on the effects of host factors on initiation because the initiation events are fleeting and difficult to detect as compared to tumor progression.

In effect, the host factors listed above represent biological risk factors, potential areas of biology that help to define risk. From knowledge of drug sensitivity in humans, it is obvious that individuals may vary widely in their response to chemicals and that human populations therefore are likely to be heterogeneous in their response to chemical carcinogens. Needed to obtain the most understanding are a combination of epidemiologic studies in humans and controlled studies in animls. The Working Group recommended that to the extent possible hypotheses of human heterogeneity be tested. In selecting human populations for study it was recommended that a suitable group would be patients receiving chemotherapy where measured doses of known chemicals are administered and where metabolic data are commonly collected. The Working Group recommended further that special attention be directed to research on the role of histocompatibility factors in carcinogenesis.

In considering host factors, the question arose whether it is possible to improve animal assays for carcinogenicity. It is possible to study the effects of selected host factors in experimental animals under controlled conditions. For cost effectiveness at the present time, however, it was recommended that host factors be studied only after chemicals have been demonstrated to be capable of producing cancer and then for testing specific hypotheses regarding health risk evaluation. The difficulties in designing animal bioassays for quantitative dose extrapolations, including group sizes

and selection of animal strains, were briefly discussed but no new insights disclosed. It is apparent that to detect carcinogens, the animal experiments need to be carefully controlled but that, in turn, the experimental design limits somewhat the relevance of the experiment to heterogeneous human populations. The only solution at hand is to perform multiple experiments but they are so expensive that few can be done.

Initiation/Promotion

Leila Diamond and J. Carl Barrett

Since the major emphasis of the Conference was on initiation and carcinogens/mutagens, the group chose to spend most of its time on a discussion of promotion and its possible mechanisms. They briefly discussed whether separate models of promotion would be needed to assess risk, whether there is organ specificity for individual promoters, and whether all promoters act by the same or different mechanisms. It was agreed that too little is known about the mechanism of tumor promotion and the models of promotion we do have, to be able to answer such questions at this time.

Dr. Barrett proposed that promotion at the cellular level can occur by one of two mechanisms. The first only requires clonal expansion of initiated cells which could occur either by differential stimulation of proliferation (Peraino liver model), differential differentiation and recovery (mouse skin model) or differential toxicity (Farber liver model). The mechanism by which amplification occurred would depend on specific cell-cell interactions and, therefore, be tissue-specific. The second mechanism of promotion involves a modulating effect of the promoter on the initiated cells followed by amplification which may or may not be promoter-dependent.

Dr. Yuspa described new experiments which suggest that in mouse skin 12-0-tetradecanoylphorbol-13-acetate (TPA) promotes only to benign lesions and has little effect on carcinoma production. When mice bearing papillomas induced by the standard two-step initiation/promotion protocol were subjected to a second carcinogen treatment, 100% developed carcinomas compared to only 20% in appropriate controls, suggesting that progression from papillomas to carcinomas involves a second mutational event rather than simply promotion. Dr. Peraino finds that in the rat liver model the promoter, phenobarbital, also does not affect the size, latency or final destiny of the tumors: once enzyme-altered foci appear in response to phenobarbital treatment, further promoter treatment is not necessary for carcinoma development. However, Dr. Peraino did not agree that progression involves a second mutation in the liver model. Dr. Kakunaga mentioned in private that dihydroteleocidin B (DHTB) promotes to carcinomas in mouse skin, showing once again that it is impossible to generalize at this stage in our knowledge.

There was no consensus as to whether or not promotion involves modulation. Dr. Yuspa argued that it was not necessary, rather the target tissue is a continually changing one as the processes of initiation and promotion proceed. Dr. Huberman argued that modulation is necessary, even if reversible. This viewpoint is more in line with the theories of others.

Dr. Huberman pointed out the very interesting analogies between the in vivo and in vitro models. The hamster embryo system with its long period of progression to malignant transformation may require two mutational events, as does mouse skin (according to Yuspa), whereas cell lines such as C3H/10T 1/2 and BALB/c 3T3 have already undergone the first event(s) and may need only a second mutation. Dr. Kakunaga suggested, instead, that the cell lines may have already undergone the second step(s) and require only the first for transformation. Either interpretation might explain the sensitivity of the NIH 3T3 cell line to DNA-mediated oncogenic transformation -- only one more "hit" is needed. It was concluded that cell culture models of both types are needed to study transformation and promotion, just as multiple in vivo models are needed.

The group discussed briefly the problems of assaying biological activity in complex mixtures that might contain initiators, first-stage or second-stage promoters, inducers or inhibitors of activation or of inactivation, etc. They noted that promoters of many different chemical classes will probably soon be identified, that little will be known about their metabolism, activation or detoxification, that a wide range of potencies can be expected (as exemplified by TPA vs. DHTB), and that little, if any, consideration has been given so far as to how exposure to promoters might be monitored in the human population. The only reasonable conclusion is that the best possible models for testing for specific biological activities should be developed and to learn as much as possible from these about the mechanisms of carcinogenesis. There are as yet no valid "short-term" tests to short-cut this.

With respect to human cell models, the group heard from Dr. Albertini about the valuable resource presented by chemotherapeutically-treated cancer survivors who can be expected to have second neoplasms within a relatively short time. Their cells and tissues are excellent material for studying mutagenesis, transformation and promotion.

VIII. RECOMMENDATIONS

During this conference, the participants and the Steering Committee fully recognized that the main problem in establishing a bioassay system for cancer risk assessment lies in overcoming the barriers interfering in the following four extrapolations:

1. from molecules (DNA damage, etc.) to living cells.

2. from the _in vitro_ cellular system to the _in vivo_ organism level.

3. from experimental animal data to the human situation at both the cellular and organism level.

4. from individual human medical data to the public health situation for human populations.

The major programs which can reduce the barriers interfering with the above extrapolations can be summarized as follows:

1. Establishment of reliable markers for neoplastic transformation.

Although reliable markers exist that can be used to measure certain biological responses such as cytotoxicity or genetic/somatic mutation, the reliable markers (or biological endpoints) for neoplastic transformation are far more difficult to obtain and define. So far, tumor development in animals as a result of injection of presumptive tumorigenic cells is the only reliable and universally accepted biological endpoint. Obviously, it is very difficult to obtain these data in a convenient and rapid manner and it is impossible to obtain such data in the human system (therefore, we only have human tumor cells; we never have human tumorigenic cells, as the latter cannot be demonstrated). Thus, it is generally recognized that the development of reliable markers for neoplastic transformation is the most urgent task.

This task relates to all four extrapolations described above. We may obtain neoplastic markers at the molecular level, e.g DNA damage,

expression of oncogenes etc., and these molecular markers could be extrapolated to the cellular level or correlated with the tumorigenicity of the cells. However, it is unclear whether in vitro markers such as foci formation, growth in low serum medium, and anchorage independent growth etc. can really be extrapolated to in vivo tumorigenicity? Most importantly, can neoplastic transformation markers developed from animal cells be extrapolated to the presumptive neoplastic cells from humans? During this meeting, and certainly up to the time this report was written, it is now fairly certain that the neoplastic transformation system developed from rodent embryonic cells cannot be totally adapted to the transformation of human fetal or adult cells in culture. For instance, rodent cells can easily become an immortal cell line long before becoming tumorigenic, while presumptive human transformed cells can form tumors in nude mice under appropriate conditions but then regress and senesce. Finally, developing a neoplasia marker to screen a population is really the ultimate goal. While this goal is not totally out of reach because of new technological developments such as monoclonal antibodies and nucleic acid in situ hybridization, the basic understanding of the mechanism must first be obtained.

Superimposed on the complexity of obtaining neoplastic transformation markers is the problem of progression and promotion in neoplasia. It was clearly brought up at the meeting that promotion is perhaps now a very key problem in human cancer, particularly because humans have a much longer life span than the rodent, and many types of compounds may serve as promoters. In the past cancer risk assessments, the effects of promoters perhaps may not have received sufficient attention. As with the problem of studying neoplasia in the absence of neoplastic transformation markers, the

study of promotion and progression is even more difficult in the absence of well-defined and stage-specific preneoplastic markers which could result from different types of promotion. It cannot be emphasized enough that future great advances in the field of the study of neoplasia hinge on obtaining both *preneoplastic and neoplastic markers. While it is not absolutely required to* obtain these markers prior to a complete understanding of the mechansim, the study of the mechanism and the obtainment of the markers are closely related. While it is possible to obtain reliable markers through statistical correlation within a single system, without a basic understanding of mechanisms, extrapolation of markers in one system to another system cannot proceed with confidence. For instance, in the Syrian hamster embryo (SHE) system, anchorage independent growth has been shown to be a statistically reliable marker for tumorigenicity of transformed cells. However, anchorage independent growth of human fibroblasts is not necessarily a reliable neoplastic transformation marker for human cells.

2. Establishment of reference compounds and their comparative dosimetry for the study of neoplastic transformation.

It is recognized that positive controls in neoplastic transformation experiments (obviously there are always negative controls in experimental design) always should be included in the testing of the induction of neoplastic transformation of unknown compounds. The positive control therefore automatically serves as a quantitative comparison with the unknown compound. The value of positive control in testing was well-received and emphasized in the meeting. Nevertheless, the type of compounds selected to serve this purpose, i.e. as positive controls and for quantitative comparison, was not fully discussed at the conference. A set of considerations for selection and the names of some compounds as examples have been described by Dr. Ts'o in his

article sent to the conferees (Chapter 23, Proceedings of the 3rd ORNL Life Sciences Symposium on Health Risk Analysis, October 1980). It is apparent that a specific subgroup should be formed to go over the considerations for selection, and this list of compounds as candidates for reference, particularly from the standpoint of the DOE. Once the list of recommended reference compounds has been established, a study of the comparative dosimetry of these compounds in various bioassays should be made.

3. <u>Establishment of convenient and standardized tissue and chemical repositories.</u>

This is perhaps particularly important for the complex mixtures uniquely interesting to DOE. These complex mixtures related to energy could be unique from place to place and from time to time. Since this material is not a pure compound, the composition of the mixture perhaps can never be reproduced. Therefore, a storage system should be established for some of these complex mixtures, so that they could be traced for reference. It is also important to develop more tissue and cell systems for testing, particularly from human sources. This material may again be made available to many laboratories for comparative purposes and research. It appears, however, that such responsibilities are not solely that of DOE and perhaps an interagency coordination within the federal government will be more appropriate to handle this task.

4. <u>Establishment of a broad basis of biological knowledge, particularly about differentiation as the foundation of the bioassay for cancer risk.</u>

Except for certain kinds of <u>in vitro</u> tests, such as the Ames Test, CHO somatic mutation test or even rodent cell transformation tests, there is not enough knowledge to develop a more uniform approach to bioassay for cancer risk. It is too early to say whether research on a specific tissue, whether

skin, liver, mammary, lung, etc., will be more advantageous than research done on other tissues, either for the purpose of bioassay or fundamental research. Similarly, it is not yet known which stage of development/differentiation is particularly important for cancer research, though the data strongly suggest that development/differentiation would have a dominant influence on carcinogenesis and cancer biology. It appears that the embryonic and less differentiated cells are more susceptible to a direct carcinogenic attack than those highly differentiated adult cells.

In view of the complex issues described above, the committee together with the conferees made the following recommendations:

1. It is recommended that standardized reference agents/compounds for cancer risk bioassay and research be developed. The dosimetry of these reference compounds in various bioassays should be established and compared Positive controls should always be included in the experimental design. The control should employ these reference compounds for comparison. In addition, dosage curves of the unknown compound should always be established and compared with the dosage curves of the reference compounds to provide an understanding of extrapolation.

2. Resources and efforts should be given to develop further the neoplastic transformation system both in vitro and in vivo with special emphasis on extrapolation between the two systems. In addition, the study of promotion and neoplastic progression should receive sufficient emphasis. In order to have a proper study on promotion and neoplastic progression, normal diploid cells should be used instead of aneuploid immortal cell lines which may have already partially progressed toward neoplastic transformation and also cannot be related to the in vivo system. The research on neoplastic transformation should place great emphasis on quantitative comparison and understanding of mechanisms.

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3.   Resources and efforts should be directed toward the development of molecular and cellular markers for both preneoplastic progression and neoplasia. It is understood that the markers can be developed by the correlation process but can only be generalized reliably to universal application when the mechanism becomes clear. These markers can then be related to treatment/exposure and can become the foundation for the quantitative evaluation of cancer risk.

4.   A much greater emphasis should now be given to the study of human tissue/material. The human material should be chosen with two considerations in mind:

a.   There is human material obtained from populations which either voluntarily (such as medical treatment) or unknowingly (such as in a factory or environment of risk) have been exposed to strong doses of carcinogens. Hopefully, by studying the highly exposed populations, neoplastic progression can be studied with greater facility.

b.   Normal human tissue should be selected so that the comparison of human tissue to animal tissue (particularly the rodent) in neoplastic transformation experiments can be carried out at comparable stages of development/differentiation. For instance, if the rodent system is always being studied at the embryonic stage, then the study on human tissue, if at all possible, should be carried out at a similar embryonic stage. On the other hand, if human tissue can only be obtained at a certain developmental stage, then the rodent model should be studied also at that development stage in order to get an appropriate comparison. In some special situations, a chimera system in which human cells are grafted into an animal system, may have a special advantage.

5. Exploration should now proceed to the previous unknown area of neoplastic transformation/cancer biology. For instance, epigenetic factors (or the so-called non-genotoxic substances) on neoplastic transformation should now be examined. Similarly, as mentioned above, the importance of development/differentiation on carcinogenesis and cancer biology should now receive fresh emphasis and support.

6. To develop a reliable quantitative and universal bioassay for cancer risk, we clearly need to know more about the basic biological phenomenon of gene structure, gene function and gene control at all levels, from molecules to organisms. The progress on chemical identification of carcinogens and on metabolic pathways of carcinogens has been impressive in the last decade. The main challenge now appears to be focused on the central problem of biology. The control of normal development/growth of an organism versus the abnormal or out-of-control growth can only be understood in a parallel study. Therefore, before any definitive procedures for risk assessment can be established, much more high quality, inquisitive, basic research will be needed. Once the objectives are identified, progress in this area is predicted to be rapid, due to the recent advances and momentum in molecular biology. The current interest and progress on oncogenes is one such example.

7. A desire has been expressed that establishment of tissue and chemical repository systems would be very helpful to research. Except in certain specific areas which are of particular interest to the DOE (such as specific complex mixtures related to energy sources and consumption), perhaps a general coordinated effort within all federal government agencies will be needed to establish this system.

IX. RETROSPECTIVE AND PROSPECTIVE

To fully appreciate the advances achieved in the nearly two years since this conference was initially conceived, it is instructive to compare the state of our knowledge then and now. Two years ago, although the scientific community working in the area of cancer risk assessment was still facing several perplexing problems, collectively it had made significant progress.

First, as a recognized technological accomplishment in cell biology and in experimental carcinogenesis, in vitro neoplastic transformation of normal rodent embryonic fibroblasts, particularly Syrian hamster embryo cells, had been described reliably in quantitative and comprehensive terms (1-9). It was believed with confidence that this technological achievement and the general principles discovered from these studies could be extended to other cell types and other species with appropriate variation. Indeed, substantial progress had been made on in vitro neoplastic transformation of epithelial cells (10-14). In addition, certain cell types, such as those of the hemopoietic system, which had been difficult to maintain in culture, could now be maintained and used to investigate in vitro neoplastic transformation (15). Similarly, active investigations were in progress on in vitro neoplastic transformation of human cells in culture (16-19).

Second, recognition of the importance of tumor promoters began to emerge from scientific experiments and human epidemiology (20-23). The diversity in the biochemistry of tumor promoters raised a real challenge about the basic mechanisms of tumor promotion.

Third, considerable advances had been made in tumor virology which made feasible the extrapolation of viral oncogenesis to human cancer. The working hypothesis was that the cellular oncogenes (proto-oncogenes) may have a function similar to the viral oncogenes, whose in vivo activity in

carcinogenesis has been demonstrated. Since the oncogene coding sequences tend to be conserved in evolution, it was thought that these might serve as biochemical markers for extrapolations of experimental data among different species, and since _in vitro_ transformation could be achieved with one or two oncogenes, then cancer risk assessment and therapy might make use of oncogene markers.

Fourth, the advancements in recombinant DNA research and in monoclonal antibody research began to be utilized in carcinogenesis research. These antibody probes were designed to be used to develop highly sensitive assays for very specific cell types and to allow the precise analysis of _in vitro_ cell populations (24, 25).

Fifth, substantial experimental evidence had been collected to indicate that cellular DNA is indeed a critical target. A direct and specific perturbation of DNA was shown to lead to neoplastic transformation without the necessity of perturbing other cellular macromolecules (26-29). While these findings provided evidence supporting the importance of single gene somatic mutation as _one_ decisive factor in neoplastic transformation, data was also accumulating to indicate that the expression of the final neoplastic phenotype required many cell divisions after the initial insult (30) and could also be influenced greatly by other factors, in particular by the developmental stage of the perturbed cells (31) by substances such as tumor promoters (20-22) and by hormones (32).

Sixth, the successful investigation of the metabolism of carcinogens such as polycyclic hydrocarbons led to the identification of various important ultimate carcinogens such as the diol epoxides (33); however, the relative contribution of these ultimate carcinogens in neoplastic transformation

remained unclear. Reactive oxygen radicals emerged as major factors to be considered in both initiation and promotion (34). It became obvious that reactive oxygen species generated through intrinsic metabolic activity in all living systems or through environmental influences, might have a much larger effect on background somatic mutation and cancer risk than was previously recognized.

Last, the inadequacy of animal _in vitro_ assay systems for quantitative assessment of cancer risk was clear, particularly in evaluation of chronic, low dose exposure to mixtures of compounds. Recognition of this situation raised the question of whether the advances cited above can indeed be utilized to develop a quantitative, rapid and efficient bioassay system for energy technology.

Now, in the Fall of 1983, the results of scientific investigations in these areas can be reexamined and reevaluated.

First, although the results obtained in _in vitro_ neoplastic transformation experiments with embryonic rodent fibroblasts are reproducible and predictable within those systems, the exact results appear not to be transferable directly to the study of human fibroblasts under similar conditions. For instance, anchorage independent growth (cloning in soft agar) has been invariably adopted by investigators of the rodent system as a fairly reliable indicator of neoplastic properties (35, 36). However, anchorage independence does not have the same significance in the human cell transformation system (37). In addition, hamster cell cultures readily become established as permanent cell lines after perturbation, a phenomenon which may be one of the early stages of neoplastic transformation in the rodent system. In contrast, putatively transformed human cells rarely develop into immortal

lines and are much more likely to become senescent. More seriously, the validity of assays for tumorigenicity of human cells using nude mice (xenotumorigenicity) has been challenged (38). Thus, it is very difficult to prove the existence of experimentally transformed tumorigenic human cells, while of course, clinical human tumor cells can be readily obtained. There is a clear distinction in the human cell system between the term "tumor cells" and "tumorigenic cells"; a distinction which does not necessarily exist in the experimental animal system in which a cell derived from a tumor can actually be tested for tumorigenicity. Despite these many problems, putatively transformed human cells have been obtained in vitro (16-19), although the time required for such experiments is extremely long and this long progression time may make it difficult to establish the human cell transformation system as a quantitative bioassay.

Second, the complexity of tumor cells in terms of their heterogeneity and their ever-changing growth characteristics is now recognized (39). The real obstacle for cancer chemotherapy is the adaptation of the tumor cells in response to a challenge. Therefore, the control of cancer cells through some inductive process (40) becomes a far more attractive approach than the destruction of cancer cells along with normal host cells with cytotoxic treatments (i.e., taming versus killing approach). In this regard, the need for a clear understanding of phenotypic change as it applies to tumor cells as a basis for this type of chemotherapy now becomes more urgent.

Third, the experimental observation that differentiation indeed plays a major role in carcinogenesis has now gathered momentum. Important and substantial data indicates that (i) there may exist a more sensitive subpopulation in the host and (ii) this more sensitive subpopulation most likely consists of less differentiated progenitor cells or even stem cells (40-44). Such cells contain great potential for growth and for multiple

developmental pathways, and may therefore be much more easily misguided or misdirected by a carcinogenic perturbation than cells which are already highly differentiated along a given pathway and which possess a much reduced capacity for cell division. These investigations bring the importance of developmental factors in the control of the neoplasia into much clearer focus. Can we reguide the tumor cell back to its original pathway of differentiation? Thus, the basic biology both of normal cellular differentiation and of how the malignant state originated has to be understood.

Fourth, while research on oncogenes continues to hold the fascination of investigators, the complexity of the problem emerges. For example, the transformation of cells other than the NIH 3T3 cells, which may themselves be preneoplastic, may require the transfer of more than one oncogene (45). In a gene transfer experiment, most likely many copies of the putative oncogene are transfered to the cells. Although some oncogenes are amplified in cell lines from human tumors, there is no unequivocal evidence that an oncogene is amplified to a large number in an in vivo tumor. At the present stage of oncogene research, proto-oncogene activation is associated with a change in the DNA arrangement, particularly in lymphomas (46). However, the number of oncogenes as well as the relationship between them, and their normal biological function, has yet to be defined.

Possibly related to the involvement of oncogenes in neoplastic transformation is the generally recognized importance of growth factors in neoplastic transformation. It is logical to presume that the neoplastic cell behaves as if it can provide its own growth factors or bypass this regulatory process (47). The most dramatic demonstration is the recent finding that one of the cell oncogenes has a DNA sequence very similar to the gene sequence of

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human growth factor (48, 49). It still remains to be determined how the the control of growth factor response can be lost in the process of neoplastic transformation.

Discussion

While the promise of modern molecular biology persists, the application of these specialized techniques may not easily yield results, unless there exists a clear concept directing cancer research and strong support from cell biology. Therefore, application of these techniques does not necessarily promise new insights but will bring in new data, the interpretation of which might remain difficult. Nevertheless, the study of cell biology, cell transformation, and differentiation at the level of gene expression has great potential. The first phase in this direction may be the development of appropriate methodology and systems and the next major advance awaits a clear direction developed from conceptual understanding.

For instance, one of the current major challenges in carcinogenesis research is the extrapolation from the animal system to the human system. One proposed approach in the past was to build a chimera i.e. human cells in animals. The xenotumorigenicity assay using nude mice is an extension of this approach. Although the approach has limitations, under certain conditions it may still offer a unique advantage, e.g. the study of human hemopoietic tissue in a mouse chimera system. One new approach proposed by our laboratory to overcome the extrapolation obstacle from animal to man is to study genes and the expression of genes, not necessarily identified as oncogenes, which are conserved in evolution from rodents (particularly the Syrian hamster) to man. The objective is to establish the existence of highly homologous genes, clone

the genes, and then determine whether these genes are expressed in a controlled manner related to the phencmena of differentiation, neoplasia, or aging. If the objectives are realized, then extrapolation from the animal system to the human system via the correlation of the behavior of conserved genes under a variety of conditions becomes highly probable.

A second current, major question in the biology of carcinogenesis is the influence of differentiation. After the initial insult, is expression of the neoplastic phenotype controlled by the developmental stage? To begin to answer this question, a clear understanding of the basic mechanism(s) of control of gene expression in higher organisms is essential. The key question in cancer research today is not so much how genes are being damaged, but how in the normal case, the control of gene expression takes place and then, how in neoplasia this normal control is altered? The purpose of this discussion is not to review possible specific mechanisms that control gene expression but to identify the cellular aspects of this concept as shown in Figure 1 and Figure 3 (modified from reference 50). Figure 1 is a diagrammatic representation of neoplastic progression in which the influence of the developmental stage has been ignored by omitting a definition of the stage of development or differentiation at initiation. The influence of developmental stages is diagrammed in Figure 2 which suggests that different neoplastic endpoints may be reached if the carcinogen-treated cells are derived from different stages of development and aging (i.e. blastomeres, embryonic cells, fetal cells, etc.). However, Figure 2 does not indicate the degree of heterogeneity of the tissue at different developmental stages. Clearly, within the embryo or within any regenerating tissue, there are cells at different stages of differentiation. As mentioned earlier, progenitor cells or stem cells appear to be more susceptible to chemically induced neoplastic

transformation than more differentiated cells (42). Thus, a different kind of emphasis must now be constructed as shown in Figure 3. Figure 3 diagrams the heterogeneity of the system in terms of the frequency of stem cells and/or progenitor cell and indicates that this changing heterogeneity may result in different responses to a given carcinogenic perturbation, administered to a given tissue at a specific stage of development. This difference in response may include variation in the frequency of transformation, the length of progression, and also the type of neoplastic endpoint. Thus, in order to understand the entire system, we have to first recognize the complexity and then define a segment which can be investigated. Clearly, stem cells and progenitor cells deserve a prominent position in these considerations.

Thus, the goals of research on cancer risk assessment must include basic research to continue the development of concepts and systems that can lead to a fundamental understanding of differentiation and carcinogenesis and their interrelationships. Through analogy and empirical observation, limited success can be achieved for certain types of bioassays (i.e. the rodent embryo system). However, in order to bring about a much more realistic bioassay (such as that based on human cells), additional understanding of differentiation at the cellular and molecular levels is indeed required. The knowledge so gained is ultimately useful for the establishment of a truly effective bioassay for cancer risk particularly related to energy utilization.

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FIGURE LEGENDS

Figure 1.   A simplistic diagram illustrating the progressive process of
            neoplastic transformation. In this diagram, the developmental
            stage of the cells initiated by exposure to a carcinogen has not
            been specified. The main point of this diagram is to emphasize
            that neoplastic transformation is a continuing process, possibly
            with the characteristics of a cascade.

Figure 2.   In this Figure, the whole developmental process and aging of the
            organism is considered. The diagram also reveals the relationship
            between the in vivo and in vitro process of development and aging
            for cells in several tissue types. Upon passaging of these cells
            in culture, unperturbed cells through a developmental process
            which is similar but not necessarily identical to the in vivo
            process. The main point of this diagram is to suggest that
            carcinogen-treated cells in culture, derived from different
            developmental stages, may undergo neoplastic progression leading
            to a different endpoint. In this diagram, however, the variations
            in responsive subpopulations within these tissue types and the
            influence of this heterogeneity on transformation and progression
            is not defined.

Figure 3.   In this Figure, the importance of the presence or absence of a
            sensitive subpopulation, postulated to be the stem cells or
            progenitor cells in a tissue, is emphasized. This diagram also
            proposes a working hypothesis which is based on recent
            experimental data (Ref. 42) and which suggests that a high
            frequency of stem cells or progenitor cells leads to a shortened
            progression time and an increased frequency of neoplastic
            transformation. The frequencies of the stem cells and progenitor
            cells have also been postulated to decrease during development and
            aging. Such a decrease may have a large effect on the frequency
            of neoplastic transformation, the length of the neoplastic
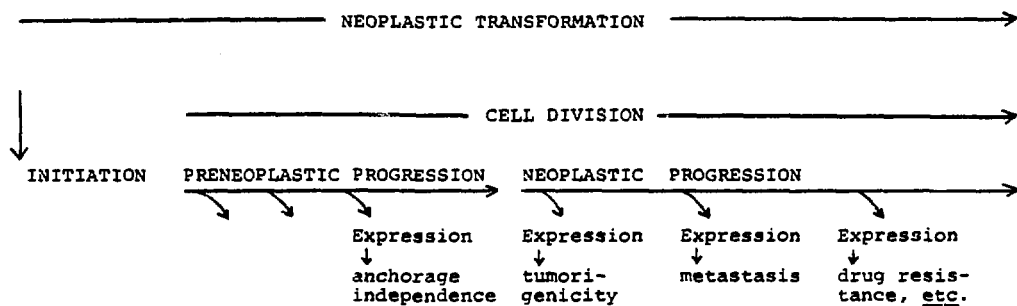   progression, and the neoplastic endpoint achieved.
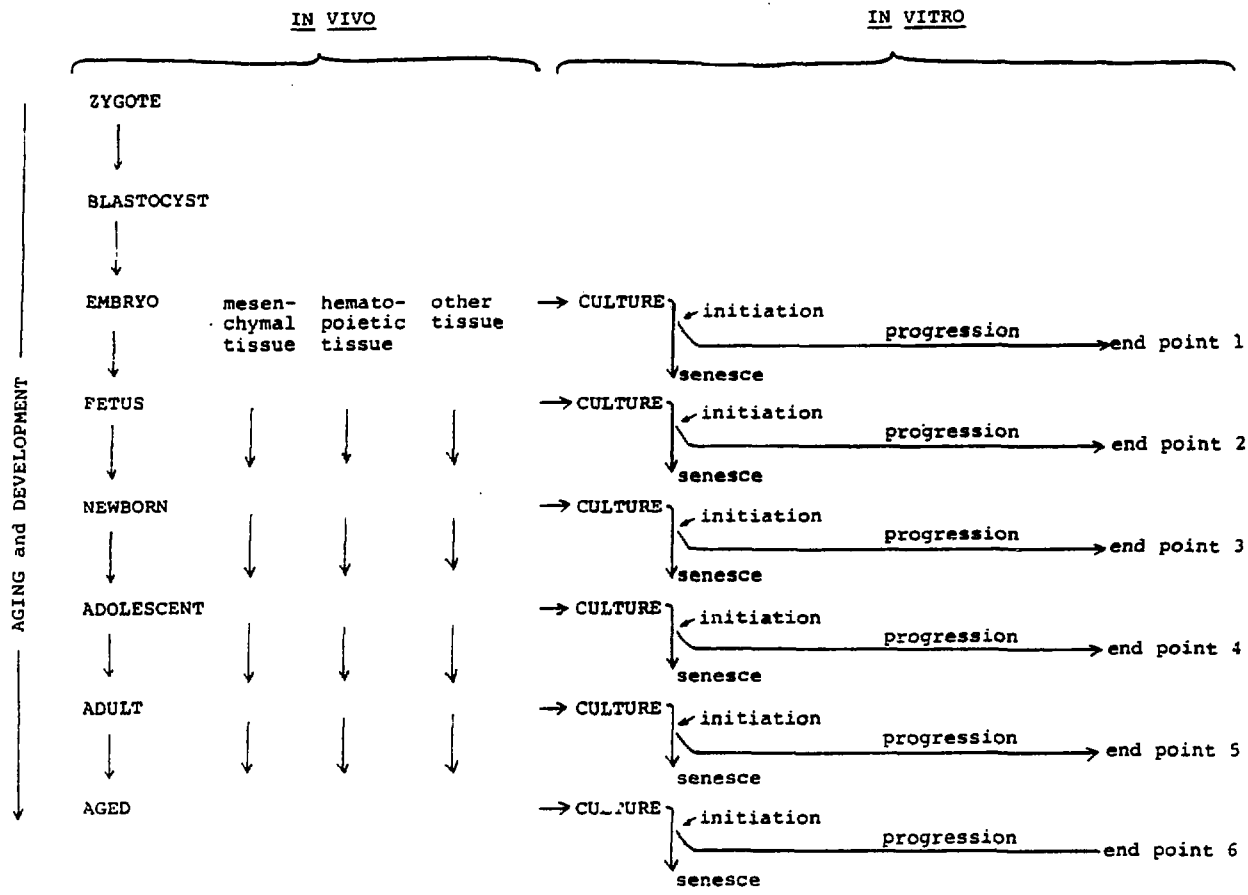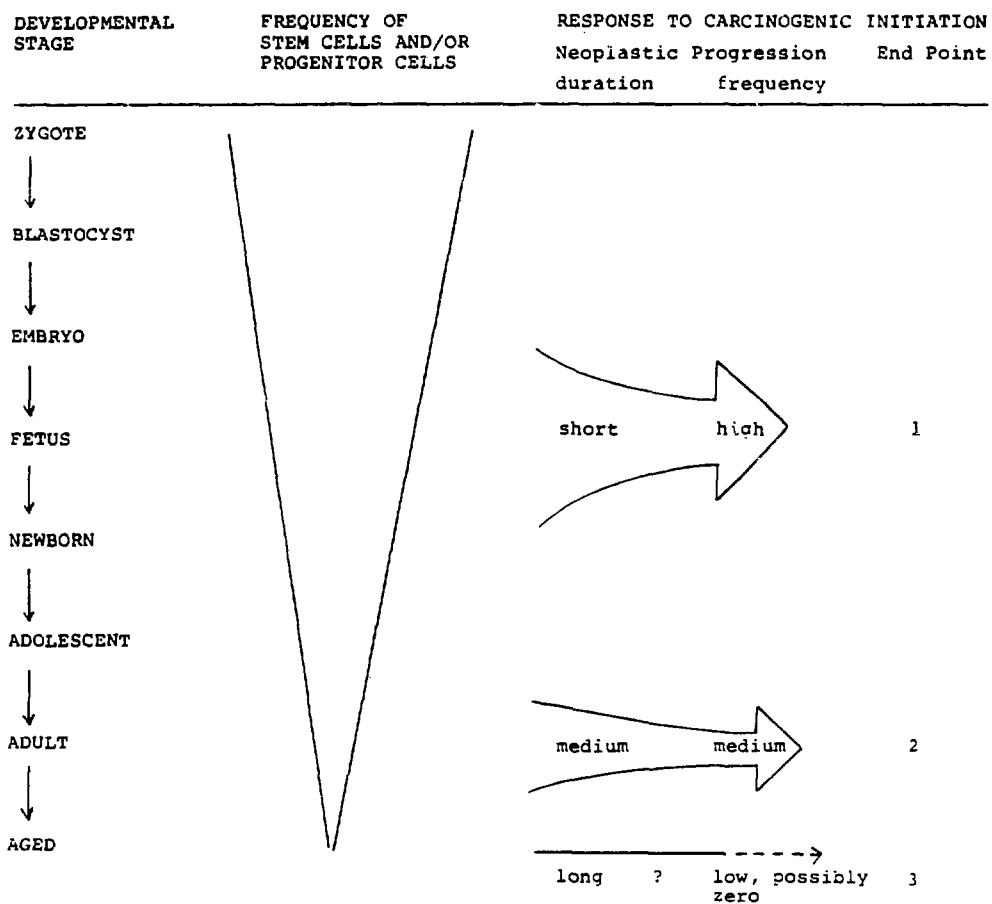
Figure 1

ZYGOTE

BLASTOCYST

AGING and DEVELOPMENT

EMBRYO   mesen-   hemato-   other   → CULTURE   initiation
           chymal   poietic   tissue   progression   → end point 1
           tissue   tissue   senesce

FETUS   → CULTURE   initiation
                                      progression   → end point 2
                                  senesce

NEWBORN   → CULTURE   initiation
                                     progression   → end point 3
                                 senesce

ADOLESCENT   → CULTURE   initiation
                                     progression   → end point 4
                                 senesce

ADULT   → CULTURE   initiation
                                     progression   → end point 5
                                 senesce

AGED   → CULTURE   initiation
                                     progression   → end point 6
                                 senesce

Figure 2

DEVELOPMENTAL STAGE	FREQUENCY OF STEM CELLS AND/OR PROGENITOR CELLS	RESPONSE TO CARCINOGENIC INITIATION		
		Neoplastic Progression		End Point
		duration	frequency	
ZYGOTE				
↓				
BLASTOCYST				
↓				
EMBRYO				
↓				
FETUS		short	high	1
↓				
NEWBORN				
↓				
ADOLESCENT				
↓				
ADULT		medium	medium	2
↓				
AGED		long ?	low, possibly zero	3

Figure 3

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