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Comparability of in vitro and in vivo Systems for Carcinogenesis Evaluations in Different Species, Tissues and Cells

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ABSTRACT

Mechanisms of carcinogenesis appear to be complex and multifactorial. Wide qualitative and quantitative variations are known for the effects of individual carcinogens *in vivo* in different target cells, organs and species. Therefore, quantitative correlations of the tumour incidence induced by a carcinogen on a specific *in vivo* target with the results of a given *in vitro* test may be of questionable general value. The detectability threshold for a given endpoint, in each bioassay system *in vivo* or *in vitro*, is dependent on experimental conditions and is complicated by concurrent factors. Markers of carcinogen interactions with target cells are needed. Much progress has recently been made in studying the effects of carcinogens directly on human target cells.

Bioassays for neoplastic transformation of mammalian cells in culture have been developed both for fibroblastic and epithelial cells, but further characterization of these systems is needed to interpret their response quantitatively. Recent methods of DNA transfection for the investigation of induced gene changes involved in transformation make use of a highly selected, abnormal, aneuploid cell line (NIH 3T3); therefore extreme caution is needed in their interpretation. Studies are in progress in a sequentially related series of biological models linking organ, tissue and cellular responses.

1 INTRODUCTION

Continuous progress in research on carcinogenesis and in the development of biological models for its study, both *in vivo* and in isolated tissues and cells in culture, suggests the need for frequent reviews of the criteria for estimating human cancer risk on the basis of experimental studies. Extensive reviews of the current scientific bases for such evaluations were conducted in recent years and many critical problems involved in the comparison of biological effects occur-

ring in different biological systems were discussed (IRLG, 1979; OSHA, 1980; Saffiotti, 1980a,b, 1983a,b,c). Human cancer risk assessment from appropriate data in animal bioassay systems was considered to be possible in qualitative terms but usually very difficult or impossible in precise quantitative terms.

Recent reviews by Purchase (1980 and this volume) have emphasized the lack of reliable quantitative correlations found in interspecies comparisons of carcinogenicity and in the comparison of mutagenicity with *in vivo* carcinogenicity.

The present discussion is addressed to the problem of comparing *in vitro* and *in vivo* systems, and particularly the response to carcinogens observed in target tissues of the human population and that observed in the tissues of experimental animals, both *in vivo* and in culture. This problem has motivated a research approach in our laboratory aimed at comparative studies on the interaction of different carcinogens connecting molecular, cellular, tissue, organ and whole organism levels.

2 MULTIFACTORIAL CAUSES AND MECHANISMS IN CARCINOGENESIS

Increasing attention needs to be directed to the interplay of many different factors in the causation of cancers in human populations. Practically all cancers arise in individuals who have been affected by several different causative factors; it is therefore difficult and often impossible to identify, accurately and separately, the contributing role of each factor considered by itself.

A multifactorial model has been recently proposed (Saffiotti, 1983a,c) that suggests consideration of several categories of factors in cancer causation. The contribution of genetic factors, nutritional factors and of unknown factors should be accounted for when evaluating the causation of all clinically manifested cancers. Physical factors, inhaled agents and dietary and other environmental contaminants are part of the exposure history of practically all cancer cases but are likely to have a causative role only in a fraction of cases. Occupational factors, non-genetic hormonal and sexual factors and infectious agents are present only in the history of a portion of all cancer cases and, within that portion, are causally involved only in a smaller fraction. The total sum of these overlapping categories of factors adds up to several times 100 %, and if each category of factors is further subdivided into individual factors or single agents, the total sum of the relevant percentages will increase well above 1000 %. In other words, each cancer will be related to several concurring causative factors.

An open-ended series of quantitative combinations of these different factors can be visualized by considering minimal increments of each factor, combined with the whole range of values for each of the other factors. If all the factors combine together to produce a total effect infinitesimally close to the level needed

for a detectable manifestation of cancer occurrence in a population, then an infinitesimal increment in any single factor can put the total effect above the detection level. Conversely, in a situation where the total causative 'burden' is above the detection level for a cancer but relatively close to it, the decrease of any single factor may lower the total effect below the detection level.

The existence of synergistic combinations of factors suggests that some factors play a proportionally greater role than expected from their relative amounts, so that small increments or decrements may significantly contribute to determining the total effect.

The identification of individual carcinogenic agents and the study of carcinogenic effects pursued in laboratory models *in vivo* and *in vitro* as well as by methods of human pathology and epidemiology were almost always based on studies with single compounds, isolated from their realistic interaction with a wide range of other factors. Not surprisingly, the results of these studies point to a wide variability of quantitative effects of single agents in a variety of test conditions, in different species and in different human subjects.

Mechanisms of carcinogenesis, particularly if induced by exogenous chemical agents, are complex and appear to include many steps, each subject to variable biological regulatory mechanisms. An initial series of steps concerns the events preceding the critical interaction between carcinogens and target cells, and includes: penetration of the carcinogen into the organism; tissue distribution and retention; penetration through cellular and nuclear membranes; metabolic activation and detoxication; interaction with various ligands and with target macromolecules; damage to specific informational molecules and the repair, misrepair or fixation of their specific alterations. A second series of steps is involved in the actual establishment of a neoplastic transformed state in the target cells, and may require fixation of DNA changes at the site of one or possibly several concurring genes, their transcription and translation, and the synthesis of specific gene products, in the presence of permissive epigenetic conditions. A subsequent series of steps is needed for the expression of a transformed state as a diagnosable neoplasm, including replication of the transformed cells, controlled growth of transformed cells during the 'latent period', growth into a tumour mass, invasion and metastasis. Different carcinogens, cofactors and host factors variously affect each of these different steps. Selection of target organs and target cell types for a carcinogenic response also appears to depend on several factors, such as routes of exposure, specific mechanisms of certain carcinogens, combinations with certain cofactors, growth rate and nutritional state of the target tissues, age, hormonal conditions and other factors. It is therefore often observed that the target organs and target cell types responding to carcinogens vary considerably from species to species and under different biological conditions, making it difficult to predict the response in a given human target organ only on the basis of the response observed in experimental animals.

3 PROBLEMS IN EVALUATING THE EFFECTS OF SINGLE CARCINOGENS

Most of the evidence presently available on chemical carcinogens and their biological effects is derived from experimental studies of individual carcinogens in animal model systems. The carcinogenesis literature shows that a large number of carcinogens are reported to induce different effects in diverse biological systems, both in terms of target site and in terms of quantitative level of the effect. In experimental animal studies, wide response differences are known to occur as a result of differences in species, strain, sex, age, diet and other variables.

3.1 Target Sites

The target site may be influenced by many different mechanisms, some directly related to carcinogen transport and localization, and some to tissue differences in metabolic activation. There are many examples of carcinogens that have a broad spectrum of target tissues if tested in a sufficiently wide range of biological model systems or in an appropriately sensitive single system. An example is given by the directly acting carcinogen ethylnitrosourea whose target organ distribution is markedly modified by host factors such as strain, sex and age (Vesselinovitch *et al.*, 1974, 1977). Clearly target tissue selection depends on a complex of susceptibility factors which is not limited to tissue differences in metabolic activation.

Quantitative aspects of carcinogenic responses are, therefore, conditioned by variations in target site. Some organs appear prone to develop a multiplicity of tumours (for example, skin tumours and pulmonary adenomas in mice) while others respond mostly with a single tumour. The biological nature of response, even within the same tissue, can be diverse (for example, degree of malignancy, invasion, cell differentiation) and these differences are usually not accounted for in mathematical models for risk estimation. An attempt was recently made by Squire (1981) to introduce consideration of qualitatively different factors, each evaluated by rough quantitative indicators, in a matrix to be used in ranking carcinogenesis bioassay results. The proposed numerical indices, however, are arbitrary and depend more on the selection of bioassay models than on intrinsic properties of the test compound.

A specific problem concerns the correlation of the levels of activity of carcinogens in different biological systems, where the carcinogenic effect may be expressed by tumours of substantially different biological types. Such different responses range from tumour types that have a high degree of inducibility but also a high incidence in untreated controls (for example, lung adenomas in strain A mice) to tumours that may be induced only under certain test conditions or by selected carcinogens but which are not found to occur significantly in untreated controls (for example, bronchial carcinoma in hamsters, oesophageal carcinoma

in rats). An index has been proposed for quantitative estimation of carcinogenic activity of different chemicals based on the level of chronic carcinogen administration in the diet that would cause a cancer response in 50 % of treated animals (Meselson and Russell, 1977). Such a gross index appears to provide no qualitative distinction between a 50 % incidence of tumours which are very easily inducible such as pulmonary adenomas in mice, and a 50 % incidence of certain unusual tumours such as nasal cavity carcinomas, pancreatic carcinomas or colonic adenocarcinomas, which represent a highly remarkable biological response.

These qualitative differences in the types of tumours induced are not only related to their frequency, but also to their degree of malignancy, a property which is presently hard to evaluate objectively and even harder to correlate to a particular single causative factor. Most benign tumour types can be induced experimentally by the same agents that also induce malignancies, although certain two-stage models of tumour induction using classic promoting agents seem to induce a response mostly limited to benign tumours that often regress.

The fact that there are marked qualitative differences in the response to carcinogens in different species and strains appears particularly noteworthy when contrasted with the quantitative responses obtained in specialized *in vitro* systems (for example, mutagenesis and neoplastic cell transformation of primary embryo cells or of established cell lines) which do not give a qualitatively modulated response. Single quantitative response values obtained in standardized *in vitro* tests for a given compound cannot therefore be expected to correlate with each of the responses obtained in a range of *in vivo* bioassay models.

3.2 Levels of Effect: Thresholds of Detectability

Other problems are encountered when considering the quantitative levels effect. In any observed population, whether a human population in epidemiological studies or an animal population in experimental studies, there is indeed a clear threshold to be considered; not the elusive dose threshold for the effect, which has been so vainly pursued, but the detectability threshold of the bioassay system, which limits our ability to observe an effect which is below a certain level.

Detectability of carcinogenic effects depends on experimental design (such as the size of test and control groups), competing risks, depth and extent of pathological observation (for example, gross diagnosis, microscopic diagnosis on single or serial sections) and on methods for statistical analysis. However, detectability can be markedly altered by concurrent factors, whether fortuitous or controlled by the study design. The role of concurrent factors contributing to the susceptibility of an observed population can include genetic and other heritable factors, as well as a variety of acquired factors. The multitude of acquired exposure factors makes the human populations very difficult to characterize for purposes of risk assessment.

4 PROBLEMS IN EVALUATING CELLULAR STUDIES

It has been often recommended that the level of effect of carcinogens should be estimated at the target cell. While theoretically important, this estimation is still fraught with difficulties, ranging from those encountered in defining the target cell population to those involved in the measurements of dose in the target cell.

The difficulties encountered in attempting to estimate carcinogenic risks across species have emphasized the need for more research on comparative mechanisms of carcinogenesis and on methods for evaluating target tissue effects. A great deal of progress has been made in this direction in the last decade, but much work is still needed to define the conditions under which a reliable estimation of carcinogenic risk can be made from the interaction of carcinogens with target cells or from the study of specific cellular responses to carcinogens, particularly neoplastic transformation of cells in culture.

4.1 Cellular Markers of Carcinogenic Effects

Some of the quantitative interactions of carcinogens with target tissues have been measured by a variety of methods and the resulting measurements have been discussed as indices of possible value in the process of comparative risk assessment. The following determinations are among the most common indices that have been used to characterize the susceptibility of target tissues to carcinogens: metabolic activation of carcinogens by the tissue and rate of formation of specific proximate or ultimate carcinogenic metabolites; binding of carcinogens or their metabolites to target cells, particularly to their DNA, RNA and proteins; formation of specific carcinogen–DNA adducts in target tissues; DNA repair.

These markers provide measurable characteristics that may become useful components of the spectrum of data that are needed to reach a meaningful evaluation of critical species differences. Each of these measurable characteristics, however, represents only a step in the complex multistep process of carcinogenesis. Marked variability is found in the quantitation of these characteristics from tissue to tissue and under various experimental conditions. Therefore their quantitative values, determined in selected conditions, need to be interpreted with great caution as general indices of the activity of a carcinogen. At the present state of our knowledge, none of these measurements is sufficient as a single index of the biological level of activity of a carcinogen.

An important contribution was recently provided by the development of methods for the maintenance of human organ explants and cells in culture (Harris *et al.*, 1980). These methods made it possible to investigate experimentally the direct interaction of carcinogens with human target cells, including epithelia, the major target for human carcinogenesis, such as the epithelia of the bronchus, colon, pancreas, mammary gland, oesophagus, bladder, and uterine

mucosa. A considerable body of data has already accumulated on the measurement of markers of carcinogen interaction in human cells.

At this time, two major generalizations are suggested by comparative studies on carcinogen metabolism and interactions in human and animal tissues:

(1) there is a close qualitative similarity between human and animal response patterns but there are also quantitative species and strain differences; and
(2) human populations present wide inter-individual variations in the quantitative response.

The range of individual variation was often found to be about 100-fold. Since the quantitative variation of response among different animal species and strains often also spans two orders of magnitude, a quantitative correlation between a particular set of experimental conditions and a particular group of human subjects may easily be supposed to be as wide as three or four orders of magnitude. This wide variability range limits the possibility of estimating quantitative differences in the level of activity attributed to different carcinogens.

4.2 Neoplastic Transformation of Cells in Culture

With the establishment of methods for the induction of neoplastic transformation of mammalian cells in culture, a new phase began in the investigation of mechanisms of carcinogenesis at the cellular level. Rapid advances are now taking place in this area of carcinogenesis research, including initial success in the transformation of certain human cells in culture by chemical or physical agents.

Methods that are currently employed for transformation bioassays make use of rodent cells and two main types of assay procedures: primary cell cultures derived from whole embryos, of which the main model is the Syrian hamster embryo cell system (Berwald and Sachs, 1965) and fibroblastic embryo cell lines selected for growth properties and susceptibility to induced transformation. The best established fibroblastic cell line models are those derived from inbred mouse strains, i.e. the C3H/10T1/2 cell line (Reznikoff *et al.*, 1973) and the cell lines derived from the BALB/3T3 clone A31 (Kakunaga, 1973; Kakunaga and Crow, 1980; Sivak *et al.*, 1980; Cortesi *et al.*, 1983). Both of these are highly selected, aneuploid cell lines and they are capable of spontaneously expressing neoplastic transformation, especially if further passaged in culture. Another selected fibroblastic cell line used for transformation studies is the BHK 21 clone 13, a diploid line which can be transformed by carcinogens, involving a recessive mutational mechanism (di Mayorca *et al.*, 1973; Bouck and di Mayorca, 1976, 1980, 1982; Styles, 1981). The C3H/10T1/2, the BALB/3T3 clone A31 and the BHK 21 clone 13 systems have each been tested with a broad range of carcinogens. The responsiveness of these systems varies for different carcinogens. Specific culture and exposure conditions may influence the response, and particularly its quantitative aspects. Other transformation models have been

developed for epithelial cells, including rat liver cells (Williams *et al.*, 1973) and mouse epidermal cells (Colburn *et al.*, 1978; Kulesz-Martin *et al.*, 1980).

The current developments in the methods for epithelial cell transformation, including methods based on both the use of primary epithelial cell cultures and on the development of epithelial cell lines, suggest that a broader battery of transformation models will soon become available. So far the epithelial transformation systems have not been investigated extensively enough to be used as bioassay screening models for a broad spectrum of carcinogens.

It is difficult to interpret the bioassays for neoplastic cell transformation, in any of the systems used so far, in comparison with qualitatively different carcinogenic effects *in vivo*. The mechanisms required to induce the expression of transformation in the target cells in culture are not necessarily the same that are required to convert cells under normal *in vivo* conditions all the way to a neoplastic cell population. The mechanisms of neoplastic transformation of cells in culture suggest a multifactorial origin of the neoplastic state, even at the cellular level, where genetic changes in the target cells are superimposed with those induced by exogenous carcinogens and modulated by nutritional, growth, regulatory and other factors (for example, serum level, cell density, retinoids). The multifactorial mechanism appears further extended by the possibility of synergistic effects of concurrent exposures to different carcinogens, as suggested by preliminary results recently obtained in cell transformation experiments in the course of our studies on combined effects of carcinogens (unpublished observations).

4.3 DNA Transfection and Identification of Altered Genes

A very recent and exciting research approach in chemical carcinogenesis is represented by the application of DNA transfection techniques that allow the transfer of DNA from chemically transformed cells into recipient cells capable of expressing neoplastic transformation (Weinberg, 1982). Altered genes that can induce such transformation have recently been identified in human tumours (Parada *et al.*, 1982; Pulciani *et al.*, 1982).

Work in our laboratory is now addressing questions such as whether different carcinogens affect the same or different genes in the same target cells, and whether different genes may be affected in the same or in different cells by the same carcinogens. If different carcinogens are found to affect different genes, it will be possible to investigate molecular conditions that determine synergistic interactions and explore the molecular basis of multifactorial carcinogenesis.

Extreme caution, however, is needed here in the interpretation of these new findings based on DNA transfection methods. As the recipient cell for detecting the expression of transformation, the NIH 3T3 cell line (Jainchill *et al.*, 1969), derived from NIH Swiss mouse embryo cells, is now generally used. This is an

aneuploid cell line, prone to transformation and capable of expressing the transforming effect of transfected DNAs only under special culture conditions. In other words, the genetic information brought by the transfected DNA sequence into these recipient cells is far from having been shown to be capable of converting a normal cell to a cancer cell. The recipient cells are already well advanced toward a neoplastic phenotype and the transfected DNA appears to provide a 'completing step' in the complex series of changes involved in the process of transformation.

5 A RESEARCH APPROACH: CARCINOGENESIS STUDIES IN A SEQUENCE OF BIOLOGICAL MODELS LINKING MOLECULAR, CELLULAR AND ORGAN LEVELS

There is a fundamental need to relate the process of carcinogenesis to the specific characteristics of the tissues and cells from which the induced tumours originate. Experimental chemical carcinogenesis is the result of chemical and biological interactions resulting in pathological responses that are typical of the different tissues and cells of origin. Human cancer is characterized by a similarly wide variety of pathological response patterns.

In order to correlate mechanisms of carcinogenesis investigated at the cellular and molecular level with the corresponding events in tissues and organs in animal and human organisms, it is important to connect different levels of observation. An approach that is particularly promising in this respect consists of the study of interactions of carcinogens in a series of biological systems of increasing complexity, but fairly closely related to each other in a step-by-step sequence linking together the response mechanisms from the subcellular to the organismic levels. A suitable sequence of biological targets for correlative studies on the response to carcinogens must represent stepwise connections linking macromolecular targets, unicellular targets in controlled microenvironments, organized cell systems and target tissues in culture and *in vivo*, and finally organs and whole organisms, including not only models of animal pathology but also human pathology. Such an approach requires the development of a range of biological models related to each other and ultimately to human cancer pathology (Saffiotti, 1983a,b,c). A great deal of progress has occurred in this direction in the past two decades through major advances in experimental pathology, cell biology and biochemistry. Our laboratory has often contributed to these advances.

By continued systematic comparative studies on the mechanisms of action of carcinogens in these interconnected systems, it is hoped that a much more meaningful comparative evaluation will become possible between the effects of carcinogens on human subjects and those in various test systems *in vivo* and *in vitro*.

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