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GUIDE TO THE LITERATURE, RECENT ADVANCES AND LABORATORY PROCEDURES

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IN VITRO CARCINOGENESIS

GUIDE TO THE LITERATURE, RECENT ADVANCES AND LABORATORY PROCEDURES

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Based on presentations made at the Seminar and Workshop on "In Vitro Carcinogenesis", held at the Given Institute of Pathobiology, Aspen, Colorado, July 18-23, 1976, U. Saffiotti and D.W. King, Chairmen

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E R R A T A S H E E T

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Guide to the Literature, Recent Advances and Laboratory Procedures
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Index, page xiv, 5.7 and 5.8
change Andrew R. Peterson to Bruce C. Casto

Page 314 and 315
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PREFACE

In the Fall 1974, plans for a "Workshop on In Vitro Chemical Carcinogenesis" were initiated at the suggestion of Drs. Umberto Saffiotti and I. Bernard Weinstein and developed together with Dr. Donald W. King as part of the conferences held at the Given Institute of Pathobiology, University of Colorado, Aspen. It was felt that this was an important research area on which to hold an initial small workshop that would bring together most of the leading investigators in the field, at a time when several laboratories were attempting to establish criteria for in vitro neoplastic cell transformation following chemical treatment.

The workshop, chaired by Drs. Saffiotti and Weinstein, took place from July 27 to August 1, 1975, and it included 33 participants. It was supported by the National Cancer Institute and consisted of lectures, laboratory demonstrations, and laboratory work actively involving all participants. The workshop was primarily devoted to reviewing laboratory methods, transformation criteria, and markers. The unique environment and intellectual atmosphere of the Given Institute at Aspen were particularly stimulating and the workshop succeeded in fostering friendly, uninhibited, and constructive discussion and cooperation among investigators from different laboratories. The participants found it a unique opportunity to review each other's experimental material and to resolve controversial questions of interpretation directly on site in the laboratory.

The participants in the 1975 workshop expressed their desire to meet again in 1976 for a conference that would allow the findings of the leading research groups to be demonstrated to a larger audience.

On this basis, a "Seminar and Workshop on In Vitro Carcinogenesis" was planned and organized by Drs. Umberto Saffiotti and Donald W. King with advice from several participants, and it was held at the Given Institute of Pathobiology in Aspen from July 18 to 23, 1976. Drs. U. Saffiotti and D. W. King served as chairmen of the conference and Dr. Paul O. P. Ts'o as chairman of the laboratory workshop. This seminar was part of the series held at the Given Institute entitled "Advances in Cancer Biology" and supported by a grant (No. 5R13CA15961) from the National Cancer Institute.

The present publication is based on the presentations made at this conference. The members of the seminar's faculty agreed that it would not be desirable to publish the detailed papers which were presented orally, since many involved discussion of previously published results. Several good reviews were also recently published by some of the participants. A need was recognized, however, to

collect in a single publication the extensive literature that has developed in the area of neoplastic transformation of cells in culture by chemical and physical agents. The editors suggested and the faculty agreed that this publication be devoted to an extensive bibliography on the subject, with brief papers serving as a guide to the literature on different topics. Selected papers give more extensive reports of previously unpublished new advances. In addition, a section is devoted to the publication of detailed laboratory procedures which are not available in the current literature.

The editors have arranged the sequences of papers around main topics and edited their format and bibliography for consistency. They wish to express their appreciation to the authors for their cooperation; to Dr. D. W. King, Director of the Given Institute of Pathobiology, for his sponsorship of the Conference; and to both Dr. D. W. King and Dr. Cecilia Fenoglio, Department of Pathology, Columbia University, for their editorial advice.

The National Cancer Institute Carcinogenesis Technical Report Series, in which this volume appears, was established early in 1976, at the initiative of Drs. U. Saffiotti and N. P. Page, then respectively responsible for the NCI Carcinogenesis Program and for its Carcinogen Bioassays and Program Resources Branch, as a means to publish documentation methods, procedures, and findings in the field of carcinogenesis bioassays and research, particularly when format limitations make it difficult to report detailed data through the conventional scientific journals.

It is hoped that this volume will be a useful contribution to the rapidly developing field of carcinogenesis studies by in vitro culture methods.

Of the participants in the Symposium, four were unable to contribute directly to this volume: J.A. DiPaolo, M.W. Lieberman, F.H. Ruddle, and I.B. Weinstein.

The Editors

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1. INTRODUCTION

1.1. IN VITRO CARCINOGENESIS METHODS IN RELATION TO THE DEVELOPMENT OF CARCINOGENESIS RESEARCH

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A. EFFECTS OF CHEMICAL AND PHYSICAL CARCINOGENS

Evidence of the effects of chemical and physical carcinogens is rapidly expanding from studies on several target levels of biological organization as outlined in the following paragraphs.

1. Human cancers and precancers

The effects of chemical and physical agents are becoming well recognized in the causation of most cancers in the human population. Epidemiologic evidence, even with its limitations, has pointed to clear trends which indicate the major role of environmental agents in the causation of human cancer. Geographic and epidemiologic pathology studies have identified high risk populations and particularly population subgroups, linking them to differences in environmental exposures (6,9,10,15,31,33,34). The vast majority of human cancers throughout the world originates from epithelial tissues, particularly lining epithelia (skin, respiratory tract, digestive tract, urinary tract, pancreas, mammary gland, uterus, prostate).

Research on the histopathogenesis of human cancers has made considerable progress, particularly in the study of the early lesions which can often become useful markers of the initial stages of the response of target tissues to carcinogens, both at the morphologic and at the biochemical levels. A recent symposium organized by Drs. E. Farber and M. B. Sporn for the NCI Carcinogenesis Program reviewed these important developments (63).

Human cancer types have been well defined, characterized and classified and good documentation has been assembled through national and international collaborative efforts, particularly in the Atlas of Tumor Pathology of the Armed Forces Institute of Pathology (3), in the

series of the International Histological Classification of Tumors of the World Health Organization (25) and in several excellent textbooks.

2. Whole animal models

The understanding of the processes by which carcinogens induce neoplasia in their target tissues is largely based on experimentation in animal models. These have allowed us to establish a close similarity between the development of cancers in the laboratory animals and in their human counterpart. The development of animal models for carcinogenesis represents a major methodological step in the investigation of the effects of carcinogens on their target tissues, as well as a powerful tool in defining their metabolic pathways and the permissive host conditions required for a carcinogenic response (51).

Many new animal models have been established in the last decade. Their development was stimulated by the discovery of several new classes of multipotential carcinogens, (e.g. nitrosamines, nitrosamides and substituted hydrazines), by the investigation of new modes of exposure (e.g. respiratory exposure to carcinogens absorbed on carrier dust particles (29,56)) and of new species of laboratory animals (e.g. hamsters (22,61)).

By now, most of the major types of human cancers can be reproduced in animals by chemical induction. Their pathologic characteristics closely resemble those known from human pathology. This close correlation by itself strongly supports the evidence for a chemical origin of a large proportion of human cancers (55).

Examples of recently developed chemically-induced animal models of carcinogenesis include: bronchogenic lung cancer (29,30,41,56,60); carcinoma of the larynx (58); large bowel cancer (11,66,70-72); carcinoma of the pancreas (32,43,46-48); kidney carcinoma (8,19,49,50); urinary bladder carcinoma (20,21,57,67) and mammary carcinoma (16).

Such advances provide us now with a large body of methods for inducing in experimental animals cancers of many specific histologic characteristics, which we can then learn to correlate with functional pathogenetic mechanisms.

The use of animal models for bioassay screening of carcinogens will be discussed below.

The definition, characterization and classification of tumor types by morphological criteria in experimental animals are so far much less extensively documented than their human counterparts. The International Agency for Research on Cancer has undertaken the

publication of a series of volumes entitled "Pathology of Tumours in Laboratory Animals" which are expected to provide extensive pathology reference standards (69). It is hoped that when an even wider range of tumors will be experimentally induced and tumor types in experimental animals will be more precisely defined, the already close correlation between human and experimental tumor pathology will be further strengthened.

3. Cellular level studies

The next step is to correlate the carcinogenic events seen at the tissue level in humans and animals with the events at the cellular and molecular level. A new dimension in carcinogenesis studies has been brought about by the great progress made in the last decade in the development of models for the study of neoplastic transformation induced by chemical and physical agents in cells in culture. The present volume provides an extensive documentation of this progress.

Presently a major task we face is that of evaluating the methods for neoplastic cell transformation in culture to determine their reliability for carcinogenesis research and for the screening of chemicals for carcinogenic activity. The complex issues of developing, defining, and validating short-term screening methods for carcinogenesis were effectively reviewed and debated at a conference organized by the International Agency for Research on Cancer in Brussels in June 1975, the proceedings of which were recently published (36). The report of a previous IARC conference was also published (37).

The research methods needed to provide reliable validation are dependent on several factors: e.g., level of critical quality control of the methods under consideration; extent of their reproducibility; adequacy and accuracy of the in vivo carcinogenesis data against which the in vitro methods are supposed to be validated; number and type of chemicals and chemical groups used in the process of validation.

Much work in this direction has been developed by the NCI Carcinogenesis Program with the collaboration of many investigators and advisors in the United States and abroad. A summary review was given at the Brussels meeting (53). The present volume covers extensive additional bibliography.

4. Mutagenesis and molecular interaction studies

The studies on molecular events in carcinogenesis can be considered under two broad categories:

(a) Events that lead to a critical DNA damage and can be expressed as mutations. They represent the postulated basis of the correlation between mutagenesis and carcinogenesis. The study of the mutagenic effects of carcinogens has rapidly expanded in recent years with studies on different biological targets ranging from bacterial to mammalian systems. We must remain aware of the respective limitations of the mutagenesis and carcinogenesis models used for such correlations, particularly in terms of metabolic activation of carcinogens and susceptibility of the target organisms.

(b) Molecular mechanisms. Particularly important are those affecting the metabolic activation and detoxification of carcinogens, their interaction with target macromolecules and their binding to them, the damage and repair of DNA as well as the effects on cell regulatory control mechanisms.

There is a great need now to correlate what we learn about the effects of carcinogens through all the levels of observation, from the human and the whole animal level to the tissue and cellular level and to the molecular level.

B. DEFINITIONS OF CARCINOGENS AND RELATED AREAS OF UNCERTAINTY

1. Quality of the evidence

We can learn that an agent can be carcinogenic in mammalian species from two main sources, each of which requires critical evaluation of the findings: (a) epidemiologic observations, which require populations of adequate size, exposed under accurately definable conditions and observed over a long period of time; they provide therefore essentially an analysis "after the fact"; and (b) experimental investigations, which require properly conducted long-term animal bioassays with adequate experimental design and well defined experimental exposures (54,55). The experimental method is used for predictive tests capable of detecting the carcinogenic effects of an agent before a large population is exposed.

The concept of pretesting chemicals before a large population is exposed had been recommended by scientists in carcinogenesis over the last two decades, and has been finally established in the United States' law with the passage of the Toxic Substances Control Act of 1976 (68). For carcinogenesis bioassays, the use of long-term animal tests is still the recommended definitive procedure. Growing consideration is being given to a battery of short-term tests as effective prescreening methods.

Much of the bioassay data developed from the 1930s through the 1960s were obtained from small scale bioassays, whose design, conduct, analysis and reporting were often poor. The use of animal models for carcinogenesis bioassays has been recently made much more rigorous by detailed requirements for carefully defined methodologies in the selection of test animals, in their maintenance conditions, in the experimental design, in the clinical observations of the test animals, in the procedures adopted for gross and microscopic pathology and in the collection, analysis and publication of data. These procedures, initially outlined by several expert committees (4,12-14,26,65) were subsequently further developed and specified in much greater detail by the NCI Carcinogenesis Program as guidelines for carcinogen bioassays (62). A model for the fully detailed publication of the reports of such bioassays was also established (38).

I have personally devoted considerable time and effort in the last decade to the development of these standards for conducting and reporting carcinogenesis bioassays, because good bioassays represent our almost exclusive source of direct evidence on the carcinogenicity of chemicals. This kind of evidence is the foundation on which we have to rely for all subsequent studies based on the carcinogenicity or noncarcinogenicity of an agent in terms of mechanisms, predictiveness of structure-activity relationships, development of new biological models, evaluation of susceptibility factors and development of inhibitory and preventive measures.

2. Documentation sources of chemical carcinogenicity data

The following sources provide this information:

(a) "IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man," published by the International Agency for Research on Cancer (24). So far, 14 volumes have been published containing 292 monographs covering a total of about 350 individual chemicals. These monographs are the result of a systematic review by experts of the data on the carcinogenicity of environmental chemicals, and of related data on their characteristics, environmental distribution, human exposure and metabolism. At present, these monographs are the only extensive reference source of carcinogenicity data evaluated by a systematic process of critical review of the experimental conditions, bioassay protocols, standards of pathology and results. The NCI Carcinogenesis Program has extensively collaborated in this initiative, providing extensive data bases for it and participating in the reviews.

(b) "Survey of Compounds Which Have Been Tested for Carcinogenic Activity," published by the National Cancer Institute (39). This is a comprehensive compilation of literature data on

experimental design and results of bioassays on chemicals. It contains no critical evaluation of the information and each entry should, therefore, be evaluated by the reader from the original literature articles. It has become a key documentation source, and has been used to provide a data base for the IARC Monographs.

(c) "Suspected Carcinogens. A Subfile of the NIOSH Toxic Substances List," published by the National Institute for Occupational Safety and Health (40). This is a listing of about 1500 chemicals which have been reported in the literature as having "neoplastic" or "carcinogenic" effects. It gives no critical evaluation of the information, which in many cases derives from totally inadequate reports.

(d) Other lists of carcinogens compiled by various individual authors or groups in several books on carcinogenesis (1,2,5,7,23,64)

I believe that it is very important that these sources be used with great caution, particularly when they have not been assembled through a critical review process examining all the relevant data. In the matter of carcinogenicity data, qualifying the data critically is essential. As I have pointed out before (52,53), many chemicals have been badly or inadequately tested, and yet they have been reported in the literature as being "carcinogens" or "non-carcinogens" (the latter obviously even more difficult to establish). They have been sometimes reported in summary tables and their alleged carcinogenicity has been classified by plus or minus signs: this information has been used in turn as a basis to quote the activity of reference compounds without further questioning the validity of the data.

Particularly in the evaluation of the predictiveness of short-term methods in relation to the animal carcinogenicity data, it is essential that the latter be critically evaluated from the original reports.

The number of "known carcinogen" will vary considerably, depending on the degree of critical stringency adopted for accepting evidence of carcinogencity.

3. Classification of results of carcinogenicity studies

It is important to recognize that the evidence of carcinogenicity for an agent, obtained from epidemiologic observations and/or from experimental bioassays, does not fall necessarily into two sharply distinct categories of positive and negative. In many cases the evidence is insufficient for a definitive assessment. I have

therefore proposed (54,55) the following classification for the evidence of carcinogenicity.

(a) Positive, when exposure to the test agent is conclusively shown to cause the formation of malignant or benign neoplasms, or a combination thereof, in humans or in animals of one or more mammalian species, by adequately designed and conducted studies.

(b) Negative under the conditions of observation, when no indication is obtained that there is any positive influence of the exposure on the induction of tumors; such negative results are only valid within the conditions of the observation. However, the extent and quality of the observations can add predictive value to the findings. If the observations meet a minimum standard of adequacy with negative results, then operationally the test agent can be considered negative. The currently recommended standard is for the agent to be tested in animals of both sexes, in two species, at levels including the maximum tolerated dose (62).

(c) Inconclusive, when neither of the two preceding classifications applies either because adequate observations are lacking, or because they are of poor quality, or of excessively limited extent. This gray area includes results which range from uninformative to highly suggestive. I believe that a similar three-way classification on the results should be used for the evauation of in vitro studies.

4. The so-called "potency" of carcinogens

Estimating the so-called "potency" of an individual carcinogen is in my view, at least at the present time, still an unreliable exercise, unless it is taken in a limited context of well defined experimental conditions. The difficulty stems from the fact that "potency" implies a measurement of a biological interaction and is therefore dependent on different biological variables.

We know several classic examples of chemicals that can be rated either as "negative" or as "very potent" depending on the biological test system used (e.g., aflatoxin in adult mice and rats; 2-fluorenylacetamide in different strains of rats; 2-naphthylamine in rats and humans). The "potency" is only a measure of the intensity of the response, which is in turn conditioned in most cases by host factors such as metabolic activation systems, tissue susceptibility, age and diet.

At present I therefore recommend great caution in any attempt to extrapolate "potency" estimates from one biological system to another. We need much more data and more work to analyze

dose-response patterns and to define control mechanisms, in order to approach the issue of "potency" on a more quantifiable and reliable basis.

Organ and tissue specificity for carcinogens are highly variable in different species and exposure conditions, so that we cannot predict that a tissue or a cell type will respond in a given way to a chemical simply on the basis of the fact that the same tissue responds in another species or set of conditions.

The rapidly expanding knowledge on metabolic activation requirements and pathways is expected to bring this whole area into much sharper focus.

C. CORRLEATIONS AMONG DIFFERENT BIOLOGICAL SYSTEMS

What has been said above is meant to stress how important it is to establish well defined correlations on the effects of carcinogens as observed in several different biological systems.

I would like to repeat some points I made at the IARC Brussels conference (53). A revolutionary advance has occurred in chemical carcinogenesis studies with the development of biological models in which a response to chemical carcinogens can be identified by short-term observations of specific biochemical reactions, mutational events or neoplastic cells transformation.

I hope that the extensive development, thorough evaluation and careful validation of short-term test systems will lead to the establishment of procedures by which positive results, obtained in a battery of short-term tests, will be considered equivalent or better in predictive value to positive results now obtained in a long-term animal bioassay, for the purpose of predicting possible carcinogenicity in humans. The future short-term tests may include bioassays directly on human cells or tissues in culture.

If these short-term systems are to be accepted as valid indicators of a carcinogenic event, they must show a high degree of positive correlation with the results of observations on long-term systems for tumor induction. This requires an extensive process of validation or "testing the test systems," to determine whether they consistently and reliably provide a response analogous to the response obtained in long-term bioassays in animals and, whenever possible, to the observation of carcinogenic effect directly in human populations.

Direct observations of carcinogenic effects in humans remain severely limited by the requirement for long observation periods, well defined exposures, and adequate control populations. Therefore, in

assessing the carcinogenic potential of the thousands of chemicals to which humans are now exposed and of the thousands of new products proposed for use, we must rely on experimental bioassay systems. The consideration, which will determine the acceptability of the short-term systems as indicators of carcinogenicity, will be their correlation with the animal bioassay results. Therefore, the whole process of validating the predictiveness of the short-term systems will depend largely on how valid and reliable are the results obtained in long-term animal bioassays. In other words, if we are willing to accept or reject the validity of the short-term test systems, depending on whether or not they match the results of long-term bioassays, then we must first of all accept reliable standards for the performance and evaluation of long-term bioassay systems.

Until the predictive value of the short-term methods (individually or as a battery) will have been sufficiently validated, the finding of positive results in short-term tests, in the absence of adequate long-term results, may give rise to controversial interpretations. The controversy may become particularly intense with chemicals of major economic value and involving major human exposure.

Positive results obtained with the short-term tests that have been more extensively studied may now be considered as useful indications of priority for further testing and possibly as warning signals suggesting that the test compound may be found carcinogenic when tested by long-term bioassays. Such warnings, in the absence of definitive bioassays, will provide an opportunity for planning long-term bioassays and related studies, such as metabolic studies. An opportunity will also be provided for planning the long-term bioassays of chemicals representing technological alternatives.

On the side of the technological uses of the substances in question, the time between the first positive findings in short-term tests and the completion of subsequent long-term tests (several years) can be effectively used in studying and preparing for technological alternatives and technical approaches to the reduction of exposure (53). Identifying and evaluating technological alternatives should become an integral phase of the process of assessing preventive approaches against the effects of environmental carcinogens (54).

I believe that the process of testing the short-term systems for their predictive value in identifying carcinogens should include the following approaches:

(a) Selection of several short-term test systems, including tests for mutagenesis and for neoplastic transformation of cells in culture.

(b) Definition and standardization of laboratory procedures and measurement of their reproducibility, using a small number of test chemicals for each test method.

(c) Selection of a large list of chemicals as reference compounds, including direct carcinogens, procarcinogens and noncarcinogens. About 100 compounds have been used by the NCI Carcinogenesis Program in the evaluation of mutagenesis test systems (35,44,45); the Ames test has been evaluated using an additional 200 chemicals in various laboratories (35). In the hamster embryo cell transformation system, a list of about 75 chemicals has been tested so far (42). The lists of reference chemicals need to be evaluated for their reliability in terms of in vivo carcinogenesis data. It is important to include a sufficiently large number of noncarcinogenic reference chemicals. These lists should be periodically extended and revised to include a representative range of chemical classes. I believe that, in a second phase, each biological test system should be screened using nearly all the available chemicals for which extensive reliable data exist on carcinogenesis bioassays in animals (perhaps 500 to 600 compounds).

(d) Definition of biological metabolic activation systems and of the metabolic requirements for each of the chemicals of the reference list.

(e) Systematic screening of the selected chemicals in the selected bioassay systems, including tests for reproducibility and by a double blind procedure.

(f) Comparison of the response of different test systems, with particular consideration for the susceptibility of different cell systems with regards to host and tissue of origin.

(g) Concurrently, much new imaginative research is needed in the following areas: development and definition of new cell transformation systems, with emphasis on epithelial cells and on human cells; metabolic activation mechanisms; relation of mutagenic to carcinogenic events in the same target cells; biochemical mechanisms involved in transformation; markers of the transformed state; and definition of culture conditions.

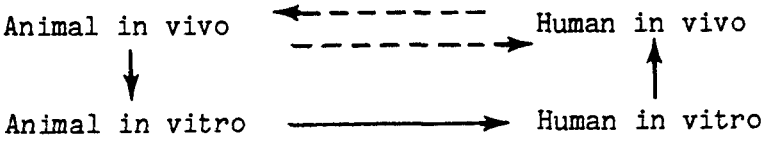
The NCI Carcinogenesis Program has undertaken an extensive effort towards the development and validation of short-term methods for carcinogenesis screening, and towards their definition and interpretation along these lines.

The Aspen Workshop held in July 1975 and the Seminar and Workshop on which the present volume is based have provided an exciting analysis of the rapid and remarkable progress made in this field.

A particularly exciting advance in the field of in vitro chemical carcinogenesis is the development of methods for studying the effects of carcinogens directly on human cells and tissues in culture. A method for the effective induction of neoplastic transformation in human cells in culture by chemical carcinogens was first reported at this conference by Kakunaga (27,28).

Other studies with human cells and tissues, also reported here, show that methods are being developed for defining the effects of carcinogens directly by using human target tissues.

The establishment of organ culture systems for various human epithelial tissues in our laboratory by Harris and co-workers (17,18) is, in my view, a major further step toward providing a basis for a much closer correlation between animals and humans in their response to carcinogens. Thus the study of chemical carcinogenesis can now be visualized at four levels of biological targets:



The problem of correlating the in vivo responses to carcinogens in animals and in humans ("extrapolating from mouse to man," as toxicologists say) can now be approached much more directly by selecting measurable parameters that can be defined in closely comparable conditions in animal and human tissues.

This approach will, I expect, bring us to a much better understanding of the response of human tissues to carcinogens. Therefore it will give us a much more precise basis to attain two main research goals: a) to identify carcinogens representing particularly important hazards for the exposed populations; and b) to identify individuals in the population who are particularly susceptible to given types of carcinogens. Knowledge of both these factors will represent a solid basis for a most effective effort toward cancer prevention.

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2. CARCINOGENESIS STUDIES IN CELL CULTURE SYSTEMS

2.1 REVIEWS ON IN VITRO CARCINOGENESIS AND SELECTED GENERAL REFERENCES

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2.2.2 PROMOTION OF ONCOGENESIS IN CELL CULTURE BY PHORBOLESTERS

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Berenblum (5) and later Mottram (27) demonstrated two stage carcinogenesis of mouse skin by polycyclic hydrocarbons followed by croton oil. They initiated the process of carcinogenesis by application of 7, 12-dimenthylbenz(a)anthracene (DMBA) or benzo(a) pyrene (BP), and promoted the process with repeated application of croton oil. Later Hecker (17) isolated compounds from croton oil, some of which are active promoters, e.g., TPA (12-0-tetradecanoyl phorbol-13-acetate), PDD (Phorbol-12, 13-didecanoated) and some of which are inactive as promoters, e.g., phorbol. These pure compounds facilitated investigations of the two stage carcinogenesis: "initiation" and "promotion." Since the original work, many laboratories have studied the two stage process of carcinogenesis and the subject has been reviewed periodically (5,10,11,35,42).

For the study of carcinogenesis in a well-controlled uniform environment, various quantitative systems for obtaining chemical oncogenesis in cell cultures have been developed, and important mechanistic informations have been obtained, as reviewed by Heidelberger (18,19) and Casto and DiPaolo (12). Sivak and Van Duuren (37,38) demonstrated some effects of phorbolestors on cultured 3T3 cells and Lasne et al. (23) reported on experiments suggesting a promotion effect by TPA on oncogenic transformation of rat embryo cells in culture.

We have demonstrated two stages of chemical oncogenesis in culture C3H/10T1/2 mouse embryo cells (25,26). This cell line has been developed in our laboratory by Rezinkoff et al. (32,33). Treatment of the cells with a subeffective concentration (0.1 ug/ml) of 3-methylcholanthrene (MCA), DMBA, or BP, followed 4 days later by a nontransforming and nontoxic amount (0.1 µg/ml) of TPA or PDD produced transformation. Phorbol was inactive. When TPA was added immediately after hydrocarbon treatment there was a significant inhibition of transformation. When TPA treatment preceded MCA treatment, no enhancement of transformation was observed, corroborating the in vivo experience (7,34). Another experiment showed that the promoting action of TPA could not be attributed to a stimulation of cell division, though cell division was essential but TPA had a property specific for promotion. In mouse skin it has been found that all inducers of hyperplasia are not promoters (6,11,17). Inhibition of transformation, when TPA was added immediately after

hydrocarbon treatment was attributed to the initial inhibition of cell division by TPA preventing the transformed state to get "fixed" which requires at least 2 cell divisions (21).

UV light was shown to be carcinogenic by repeated exposures of rat and mouse skin (15). When UV was given in a single exposure followed by repeated application of croton oil, there was high incidence of tumor development in mouse skin, whereas the single UV exposure was ineffective (31). We showed that UV in single or repeated exposures at different dose levels did not transform C3H/10T1/2 cells (25), but acted solely as initiator, i.e., when UV treated cells were further cultured in the presence of TPA, there was high frequency of transformation.

So, it is clear that the "initiation" and "promotion" stages of carcinogenesis occur in our mouse embryo C3H/10T1/2 cells in culture, just as in mouse skin. When the higher concentration of MCA, BP or DMBA were used, which by themselves produced considerable transformation, no further enhancement of transformation by TPA was observed. This is in agreement with mouse skin experiments (6,8,10). It was also observed that initial treatment with the carcinogenic hydrocarbon, i.e., "initiation" is essentially irreversible (8,10). When the hydrocarbon or its metabolites are not oncogenic to a particular species or cells, there is no "initiation" and so there is no tumor development after repeated application of "promoters." This was evident in the experiments where the cells were treated with the MCA11, 12 oxide (an active metabolite of MCA) and then with TPA, there was no transformation. This epoxide is highly oncogenic in mouse prostate and hamster embryo cells (20,24) but completely ineffective in C3H/10T1/2 cells. Bereblum et al. have recently proposed that such a 2 stage process occurs in the production of mammary tumors and leukemia in female Wistar rats (2) and of liver and lung tumors in AKR mice (1). Peranino et al. have also produced evidence that the chemical induction of rat hepatomas is a 2 stage process (29).

The mechanisms of "promotion" is still unknown. After application of TPA to the adult mouse skin it was found that there was an initial depression of DNA synthesis for first 10-12 hours followed by an increased extent of synthesis (3,9,22). Similar effect was found in the growth rate of different cell lines in culture (14). We have also similar observations (unpublished data). TPA has been found to inhibit semiconservative and repair DNA synthesis (3,9,22). Sivak and Van Duuren showed that it enhanced RNA synthesis in stationary cultures of 3T3 mouse fibroblasts (30) and Baird et al. found the same in mouse skin (4). The protein synthesis was also increased in the cells of mouse skin after TPA application (3). These increases in the maceromolucular synthesis was similar as observed following a single

application of polycyclic hydrocarbons in sufficient quantity to result in tumor development (40). TPA binds weakly with skin proteins and such binding has been considered to be important for its promoting activity (36,41). O'Brien et al. found a good correlation between the stimulatory action on the induction of ornithine decarbonxylase by a series of phorbol esters and their promoting activity after topical application on the mouse skin (28). Wigler and Weinstein (43) reported that TPA is a potent inducer of plasminogen activator in chick embryo fibroblasts and hela cells.

None of the above work explains clearly the biochemical pathway of "initiation" and "promotion." Most of the work was done on mouse skin which is not an ideal tissue for the study of the cellular events that occur at each stage because of the obvious difficulty in obtaining a homogenous population of target cells. Our study of 2 stage oncogenesis in cell culture opens up new possibilities for investigating the cellular and molecular mechanisms of both "initiation" and "promotion" and for developing a relatively rapid prescreening system for environmental promoting agents.

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2.2.3 RADIATION TRANSFORMATION IN VITRO

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In the past there has been conflicting evidence with respect to the induction by X-rays of oncogenic transformation in mammalian cells in vitro. Borek and Sachs (1,2) and later Borek and Hall (3) reported inducing malignant transformation in primary hamster embryo cells with X-ray doses as low as 1 rad. Their work has been questioned by some owing to the lack of firm evidence that morphologically transformed cells formed persistent viable tumors upon reinjection into hamsters. Pollack et al. (16) observed X-ray-induced transformation in 3T3 cultures following exposure to 1500 rads; tumorigenicity of transformed clones was found to be associated with the appearance of C-type viral particles. A number of other investigators, however, were unable to induce oncogenic transformation in vitro by radiation alone (8,11,17), although radiation enhanced the transformation induced by chemical or viral agents. Klein (12,13) presented evidence that the morphologic transformation she observed in vitro was an indirect effect, due to the close contact of the radiation killed and damaged cells with viable cells and presumably mediated by an as yet unidentified cellular transforming factor. Recent studies utilizing the 10T1/2 C1 8 mouse embryo fibroblast cell system (18,19) support those of Borek et al. Direct X-ray induced malignant transformation has been induced in this cell line. Transformation is independent of the presence of dead cells or feeder layers, and upon injection in syngeneic hosts transformed colonies form large viable tumors which will eventually kill the animal.

Studies of the dose-response relationship for in vitro transformation (3,19) indicate that an exponential rise in the transformation frequency per viable cell occurs with increasing radiation dose up to 300-400 rads. At this point a plateau is reached and the transformation frequency per surviving cell remains constant with doses up to 1500 rads (19). The incorporation of the thymidine analogue BUdR into cellular DNA enhanced their sensitivity to X-ray-induced transformation (Terzaghi and Little, unpublished) implicating DNA as the target molecule in the cell. Splitting the radiation dose into two equal fractions separated by several hours enhanced the transformation frequency at low total doses (50-75 rads) (4), but led to a decline in the transformation frequency at higher total doses (300-800 rads) (20). Neutron irradiation appears to be more efficient per rad of dose than x-irradiation in inducing transformation in vitro (5).

The development of malignant transformation in vitro can be thought of as involving at least two steps: 1) The fixation of the transformed state (presumably irreversible DNA damage); and 2) its subsequent expression in terms of morphologically altered cells. The fixation of X-ray damage appears to require that one cell division occurs within 24 to 48 hours after exposure (1,2,18). The expression of the transformed state in 10T1/2 cells, on the other hand, requires approximately 12 subsequent cell divisions (19). Recent evidence implicates DNA repair processes as an important factor in fixation of transformational damage (18). In liquid holding recovery type experiments, confluent 10T1/2 cell cultures were x-irradiated, then held in confluency for varying periods of time (thus preventing cell division) before subculture at low density. Survival was markedly enhanced, reaching a maximum with a repair interval of 3-6 hours. Transformation was initially enhanced reaching maximum at 3 hours, but declined with longer repair times. This result has been interpreted as offering evidence for the action of two repair processes after x-irradiation: The first a rapid, error producing (error-prone) process; and the second a slower error correcting mechanism (18). We are currently investigating the nature of these processes.

Transformation has also been induced in Syrian hamster cells (9) and in 10T1/2 mouse embryo fibroblasts (7) by exposure to 254 nM ultraviolet light. The shape of the dose-transformation frequency curve in 10T1/2 cells was similar to that for X-rays, reaching the plateau at a dose of 75-100 ergs/mm^2 (7). During the exponential rise at lower doses, the transformation frequency per viable cell was similar to that seen following X-ray exposures which yielded similar survival levels, but the frequency on the plateau (100-300 ergs/mm^2 was lower by a factor of 2-3 than that for X-rays. Several reports have indicated that treatment with caffeine following exposure to UV light or UV-like chemical agents suppresses the induction of tumors in animals. Caffeine is thought to block a specific DNA repair process in UV-irradiated cells. We have very preliminary data (Chan and Little, unpublished) which suggest that post-irradiation incubation with 1 mM caffeine significantly suppressed UV-induced transformation in 10T1/2 cells.

There have been a number of investigations of the interactions between x-rays and other agents in the induction of transformation in vitro. These agents include viruses (11,15,17), chemical carcinogens (8,10,21), and noncarcinogenic compounds (6,14,19). DiPaolo et al. (8) demonstrated clear synergistic interaction between X-rays and benzo(a)pyrene (BP) in primary hamster embryo cell cultures. X-irradiation alone did not induce transformation in these cells, and the enhancement of BP-induced transformation was maximal when x-irradiation preceded BP exposure by an interval of

48 hours. This finding has since been confirmed with mouse 10T1/2 cells (21) though maximal enhancement occurred with an interval of 18 hours between exposures. Such enhancement did not occur when exposure to BP was preceded by UV irradiation (9). Treatment of cells with Amphoterecin-B after x-irradiation suppressed transformation (19). Mondal and Heidelberger (14) showed that a high frequency of transformation resulted when cells were exposed to the phorbol ester TPA after irradiation with 254 nM UV light in doses which yielded no transformation by UV alone. TPA is a classical promoting agent which by itself does not induce transformation. Brouty-Boye and Little (6) showed that post irradiation treatment with interferon greatly enhanced X-ray induced transformation. They hypothesize that this effect may be due to a selective action of interferon in suppressing cell proliferation among the normal cells, allowing potential transformants to multiply preferentially and be expressed as transformed foci.

These results stress the importance of the proliferative state of the cell population, and of the interactions between agents in the induction of transformation.

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2.2.4 FACTORS MODIFYING TRANSFORMATION FREQUENCY IN THE C3H/ 10T 1/2 CL8 CELL LINE

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Carcinogens are drugs since they alter the behavior of biological systems. As such their mechanism of action can be investigated by pharmacological methodology similar to that successfully used for other more conventional drugs. Just as many studies of conventional pharmacology required the development of in vitro systems, so too detailed investigations into the pharmacology of carcinogens required the availability of in vitro systems which can be closely controlled and quantitated. Several such systems are now available and have been described at this Conference. This paper is a review of factors modifying transformation frequency in the C3H/10T1/2/CL8 cell line, developed as an assay system for chemically induced neoplastic transformation in culture by Reznikoff et al. (14,15). Transformation frequency (TF) is here defined as the number of transformed foci per dish divided by the number of surviving cells in that dish x 100 (5). This value is thus normalized for variations in cell survival from treatment group to treatment group.

A. PHARMACODYNAMIC MODIFICATIONS OF TRANSFORMATION FREQUENCY

1. Dose. Straight line log dose response relationships have been obtained for malignant transformation induced by polycyclic hydrocarbons (14) X-rays (16) and FUdR (8). TF's of up to 10% have been obtained (14).

2. Time. Within limits, increasing the time of exposure to the carcinogen increases the TF (8,14).

3. Cell Cycle Dependency. The TF decreases progressively if cells are held in the non-cycling state for up to 48 hours post-treatment with X-rays. A transient rise in TF after brief (2-4 hours) holding periods has been attributed to the introduction of errors during repair of potentially lethal damage (17).

4. Cell Cycle Phase Dependency. The methylating carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) induces maximum TF in cells exposed in late G1 of the cycle (5), whereas the chemotherapeutic agents FUdR (8) and ara-C (1,7) exhibit maximum transformation when applied to S phase cells. No direct correlation was observed among the rate of repair of MNNG induced DNA damage and TF or cytotoxicity (6,12,13).

5. Cell Density at the Time of Treatment. Increasing the number of cells from 500 to 10,000 cells/Petri dish reduces the TF resulting from a standard dose of methylcholanthrene. Furthermore, relatively more of the foci appearing in heavily seeded dishes develop at the edges of those dishes (14). Studies with X-rays have indicated a 10-fold decrease in TF on increasing the number of viable cells/100 mm Petri dish from 400 to 1000 cells (16). This finding requires confirmation with other transforming agents.

6. Cell Density Post-treatment. Expression of the malignant phenotype can be reversibly inhibited by an increase in the post-treatment saturation density as a result of exposure to high serum levels. Conversely low saturation densities obtained with low concentrations of serum (2.5 - 5.0%) allow maximum expression of the malignant phenotype (2,3).

B. CARCINOGEN/DRUG INTERACTIONS

1. Actinomycin D. Exposure of 10T1/2 cells to low concentrations of Actinomycin D (1 ng - 10 ng/ml), prior to, simultaneously with, or after, treatment with dimethylbenzanthracene caused an enhancement of TF over the entire concentration range studied (0.1 - 10.0 ug/ml). DMBA induced cytotoxicity was enhanced in cultures treated with the higher concentrations, but not the lowest concentration of Actinomycin D (4, J. S. Bertram, paper in preparation).

2. Tumor Promoters. Marked enhancement of transformation was observed in cultures treated with non-transforming concentrations of methylchlolanthrene or benzpyrene, and treated 4 days later with a concentration of tetradecanoylphorbol acetate (TPA) which by itself produced little transformation. Treatment earlier than 4 days, or prior to, carcinogen treatment reduced this response (9). A similar enhancement of transformation has also been observed for ultraviolet radiation (10).

3. Modifiers of Carcinogen Metabolism. Inhibition of aryl hydrocarbon hydroxylase inhibits methylcholanthrene transformation, while stimulation of this enzyme enhances the TF. Inhibition of epoxide hydrase enhances TF. Simultaneous enhancement of aryl hydrocarbon hydroxylase activity and inhibition of epoxide hydrase activity gave the expected large increase in TF (11).

4. Interaction Between Two Carcinogens. In 10T1/2 cells treated with benzpyrene an increase in TF was observed when cultures were subsequently exposed to X-rays. The enhancement was maximal when about 20 hours separated the two treatments, and was approximately 5 fold higher than that to be expected from the sum of the individual exposures (18).

5. Amphotericin B. The polyene antibiotic has been used as an antimycotic agent in cell culture. It has been found to inhibit TF induced by both methylcholanthrene (14) and X-rays (16). Its mechanism of action in this respect is not understood.

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2.3 MOUSE EPITHELIAL CELLS

2.3.1 MOUSE EPIDERMAL CELL CULTURES AS AN IN VITRO MODEL FOR THE STUDY OF CHEMICAL CARCINOGENESIS

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The use of cell culture systems for the study of chemical carcinogenesis has been an important advance in recent years. Much of the exciting information which has rapidly accumulated using such models has been recently reviewed (7). In general most of the existing models have concentrated on the use of cell strains or cell lines of fibroblastic origin. These have the obvious advantages related to their ability to adapt and grow well in monolayer culture. However the majority of human cancers are of epithelial origin as are most of the malignancies derived from animal models for chemical carcinogenesis. Thus the development of epithelial cell systems for in vitro transformation would have the distinct advantage of a large in vivo data base upon which to validate results obtained under the highly artificial conditions of in vitro growth. Additionally epithelial cell model systems may divulge specialized markers associated with the transformed state which could be useful as experimental tools as well as diagnostic aids for the early detection of disease in human populations. Such specialized culture systems also promise a potential approach to understanding the relationship between normal differentiation and malignant transformation.

Certain problems are posed by the use of epithelial cultures for carcinogenesis studies. The specialized cells may have strict nutritional requirements for successful growth in vitro. The differentiated state may be difficult to maintain. Adequate criteria for recognizing the transformed state in vitro have been difficult to establish for epithelial cells (25,26).

The use of mouse epidermal cell cultures seems especially promising for in vitro studies since many of our current concepts concerning mechanisms of chemical carcinogenesis were derived from studies of mouse skin. Thus there is an enormous in vivo data base (27) to validate findings in vitro. Assuming a parallel exists, one should be able to separate and study at least two distinct stages of carcinogenesis, initiation and promotion. A single dose of carcinogen should be sufficient to induce transformation. Both premalignant and

malignant lesions should be produced and available for study. The information gained might give insight toward developing an analogous human cell model system using a tissue which also appears readily transformable in vivo, the human epidermis.

This report will serve as an overview of the progress made in the development, characterization and application of mouse epidermal cell cultures for the study of chemical carcinogenesis. The culture techniques and biological behavior of the cells in vitro will first be outlined. The response of cells to both initiating and promoting agents will be reviewed and compared to similar studies in vivo. Finally data will be presented which may shed light on the cellular mechanisms involved in response to carcinogenic or promoting chemicals and the relationship between differentiation and carcinogenesis.

CELL ISOLATION AND GROWTH CONDITIONS

We have modified a trypsin-flotation technique first described by Szabo (23) to isolate epidermal cells from newborn Balb/c mice (28). Such cells are isolated as small cell aggregates, probably representing superficial hair follicles, and single cells which migrate into colonies to form large sheets of round to polygonal epidermal cells (28). Cell attachment is completed by 4-6 hours after plating with a plating efficiency of about 40-50%. The standard culture conditions employ Medium 199 plus 11% fetal calf serum and 1% antimycotic antibiotic solution (GIBCO) and growth is maintained in a humidified atmosphere of 5% CO2 at 37oC. In certain experiments it is useful to have dermal fibroblasts or hair follicles as controls. To isolate these fractions the dermis, left behind after separation of epidermis, is dissociated by stirring in 0.35% crude collagenase. The cell suspension containing fibroblasts and intact hair follicles can be separated into its component parts using a linear 4-10% Ficoll gradient (29).

CHARACTERISTICS OF EPIDERMAL CELLS IN VITRO

Within 72 hours of culture initiation, the central areas of epidermal foci begin to accumulate birefringent amorphous material which stains red with Kreyberg stain (12,28) and is presumed to be keratin. If the cells are heavily seeded, this material forms a second layer above the cell monolayer and sheds into the medium by day 6 or 7 leaving behind larger cells with poorly delineated borders. Electron micrographs of such cultures reveal many typical epidermal markers including desmosomes, cytoplasmic filaments, thickened cell membranes and keratohyaline granules (28). When attached cells or sloughed material is extracted with urea buffers (20) and the extracts

electrophoresed on SDS polyacrylamide gels, several bands in the 50,000-60,000 molecular weight range are detected in extracts from attached cells by 1-1/2 days in culture which disappear by 3 days. These bands are identical to those found in urea extracts of stratum corneum (21). The sloughed material, on the other hand, displays this stratum corneum pattern at all times studied indicating that keratinization is proceeding in a relatively normal way in these cultures. Of interest also is the appearance in the attached cells of a band which co-migrates with mouse muscle actin and comprises up to 15-20% of the extracted proteins by the 6th day of culture (22). The functional integrity of these epidermal cell cultures can also be demonstrated by grafting cells grown in vitro for 4 days to syngeneic hosts. Such grafts result in the formation of donor skin at the graft site (30). Similar results have been reported by Worst et al. (24).

The maturation and terminal differentiation occurring in epidermal cultures results in a limited lifespan in vitro under standard conditions. Thus growth curves reveal a rather stable population density for the first 5 days in culture while there is a balance between proliferation and differentiation, followed by a considerable drop in population density associated with the rapid keratinization and sloughing on day 7 or 8 (31). A stable low population density is maintained for 1-2 months but ultimately most cultures die. If 1.25% DMSO is included in the culture medium, a similar growth curve is produced but the stable population achieved after 7 days is 4-5 times higher than in untreated cultures and remains intact for 6 months (28,32). Likewise, 12.5 µg/ml retinyl acetate in the medium prevents keratinization and increases the population density (28,32). Lowering the growth temperature from 37° to 31° without altering medium constituents also seems to delay the maturation process (8). Jepsen reported that conditions of low temperature prolonged the growth of rat oral epithelium in vitro (11).

Under standard culture conditions rather unusual growth kinetics are observed which make these cells valuable for the study of proliferation control as well as carcinogenesis. When DNA synthesis is studied every 24 hours by a one hour pulse with (methyl-^{3}H) thymidine (^{3}H-TdR) and subsequent autoradiography, a pattern of partially synchronous growth is noted with peaks of DNA synthetic activity on the 2nd and 8th day in culture (31). A more intensive study utilizing multiple ^{3}H-TdR pulses (every 2 hours) during the first 96 hours in vitro demonstrated a natural cell cycle synchrony with peaks of DNA synthesis occurring about 20 hours apart, starting 20 hours after culture (5). Mitotic peaks occur approximately 9-12 hours after synthesis peaks. Labeling continuously with ^{3}H-TdR demonstrated that the growth fraction represents initially about 10-15% of the attached cells and is a single cohort of cycling cells which at its maximum after 8 days in culture comprises about 70% of

the attached population (5). Thus it appears that the epidermal cell model system could be useful for studying the effects of chemicals on the cell cycle without further manipulation. This synchrony however varies somewhat in timing and degree from experiment to experiment so that cell cycle studies on any primary isolate would have to include a validation of synchrony in that population. When cells are grown at 31o in 2% serum, the first peak of DNA synthesis is delayed but an increased number of cells (1.5-2 times) are cycling suggesting either that lower temperature enhances the degree of synchrony or allows a group of non-cycling cells to enter the proliferating pool.

ENZYMATIC INTEGRITY OF CULTURED CELLS

Several enzyme systems believed to be involved in cellular growth control and chemical carcinogenesis have been studied in mouse epidermal cell cultures. A remarkable and transient rise in activity of ornithine decarboxylase (ODC), the first enzyme in the polyamine biosynthetic pathway, has been demonstrated in a number of biological systems that are stimulated to proliferate (18). ODC activity in freshly isolated uncultured epidermal cells is almost undetectable low but begins to rise by 4 hours after plating, peaking at 12 hours, with a return to almost undetectable levels by 24 hours. These low values remain unchanged for at least the next 3 days even after a medium change (14). Whether this early activity is related to the synchronous proliferation observed subsequently remains to be established.

Carcinogen methabolism reflects an enzymatic activity which must be present in a model system to be used for chemical carcinogenesis studies. One enzymatic pathway involved in such metabolism is the aryl hydrocarbon hydroxylase (AHH) system (6). During their first several days in culture, epidermal cells have easily measurable levels of AHH; moreover, when the cells are exposed to 3 µg/ml benz-(a)anthracene (BA) for 20 hours before harvest and assay, enzyme activity increases 12-15 fold (31). After the cells have been cultured for 10 days, the activity of both the constitutive and induced enzymes decreases significantly. This decline can be prevented by growing the cells in the presence of 1.25% DMSO (31), a condition which also appears to prolong the lifespan of these cultures. The presence of 12.5 µg/ml retinyl acetate in the medium for 10 days appears to preserve the levels of constitutive AHH seen in younger cultures but prevents the induction of this enyzme by polycyclic hydrocarbons (32).

RESPONSE OF EPIDERMAL CELLS TO CARCINOGENS

In vivo, chemical carcinogens have been shown to effect the state of proliferation of mouse epidermis and to bind to epidermal macromolecules and induce DNA repair. Similar studies can be performed in vitro. When epidermal cultures in vitro for 24 hours are exposed to 0.1 µg/ml or 1 µg/ml 7,12 dimenthylbenz(a)anthracene (DMBA) for 20 hours there is an inhibition of DNA synthesis which increases gradually during the exposure time. DNA synthetic activity returns to control levels by 3 days after treatment and is followed by a stimulation of proliferation for the subsequent 48 hours prior to returning toward normal on day 6 (Yuspa - unpublished results). When epidermal cells in vitro for 24 hours are exposed to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG - 1 µg/ml and 5 µg/ml) for 1 hour, an immediate inhibition of DNA synthesis occurs which returns to control values by day 4 for the lower dose but the higher dose suppression persists for at least 6 days. MNNG induces no stimulation of proliferation (Yuspa - unpublished results). These effects on epidermal proliferation kinetics are identical to those reported in mouse skin in vivo for DMBA (1) or MNNG (4).

Binding of DMBA to epidermal cell DNA can be measured by exposing the cells to 14C-DMBA for 20 hours, isolating DNA and determining specific activity (31). Such studies reveal that carcinogen binding is 2 to 4 times greater in 3 day old cells than in 10 day old cells and is in the range (53 molecules DMBA per 106 nucleotides) seen for binding of this carcinogen in other nontransformed mammalian cells in culture (13). Inclusion of 1.25% DMSO in the culture medium increases the amount of DNA bound carcinogen in 10 day old cultures back to the 3 day level. The presence of 12.5 µg/ml retinyl acetate in the culture medium for 10 days decreases the amount of carcinogen bound to DNA but increases the amount bound to protein (32) relative to control cultures.

Using mouse skin cell cultures we have been able to show that DMBA binding to DNA was not increased when DNA was in the replicative phase during exposure (33). In addition DNA which had the carcinogen bound to it was still capable of functioning as a template for new DNA synthesis and the parent strand to which the carcinogen was attached was carried over into the daughter cell (2). These cells also respond to carcinogen damage to the genome with an induction of DNA repair synthesis. At least two types of repair occur, excision repair after ultraviolet light or high doses of MNNG and B-propiolation (BPL) and guanine specific repair after low doses of MNNG and BPL (9,10).

RESPONSE OF EPIDERMAL CELLS TO PROMOTING AGENTS

The concept of tumor promotion as a distinct entity during carcinogenesis was derived from studies on mouse skin. The epidermal culture system should be a useful model to extend the in vivo studies on mechanisms of tumor promotion. All promoters have in common the ability to stimulate proliferation (3). When epidermal cell cultures are exposed to the phorbol ester and strong tumor promoter 12-O-tetradecanoyl phorbol-13-acetate (TPA), DNA synthesis is stimulated 5-15 fold 72 to 96 hours after initiation of treatment (29). This response is dose dependent between 10^{-5} and 10^{-9} M and occurs even after a TPA exposure of only 1 hour's duration. The stimulation is preceded by an inhibition of synthesis for 24 hours. The maximum response is observed when cells are treated after 24 hours in culture, a time when many cells are in the late G_1 phase of the cell cycle (5). Dermal fibroblasts are not stimulated to proliferate by TPA. There is an excellent correlation between the in vivo tumor promoting potency of a series of phorbol esters and their ability to stimulate epidermal cell proliferation in vitro (34). The addition of the antiinflammatory steroid fluocinolone acetonide to the culture medium prior to or during TPA exposure inhibits the DNA synthetic response (15). This steroid has also been shown to inhibit stimulated proliferation and tumor promotion when applied with TPA on mouse skin in vivo (19).

TPA treatment of epidermal cell cultures at 24 hours after plating results in a transient 5-15 fold rise in ODC activity compared to untreated controls (14). The peak occurs between 6-10 hours after treatment and the magnitude of the response is dose dependent. A 10 minute exposure to TPA is sufficient to stimulate ODC maximally. The degree of ODC responsiveness varies with the time exposure begins after plating. In cells older than 3 days in culture the response is minimal. However, in cultures previously treated with TPA on day 1, the responsiveness is maintained in older cultures suggesting a selection of a sensitive cell type by the initial treatment. There is an excellent correlation between the skin tumor promoter potency in vivo of a series of phorbol esters and their ability to stimulate ODC activity in epidermal cells in vitro (34). Recent data from mouse skin in vivo (16) show that TPA induces ODC activity within 6 hours of painting and that the degree of stimulation correlates well with the promoter potency of phorbol esters. Similarly repeated TPA treatments in vivo increased the magnitude of the ODC response (17).

The mouse epidermal cell culture system appears to be a valid and valuable tool to extend studies on chemical carcinogenesis to the cellular level. These cells appear to differentiate in vitro in a near normal manner. They grow synchronously, at least for several

days, without exogenous treatment. Media additives such as DMSO and retinyl acetate appear to modulate both growth and differentiation. The cells can metobolize polycyclic aromatic hydrocarbon carcinogens and activated products bind to cellular macromolecules. Carcinogen induced DNA damage can be repaired by at least two mechanisms, excision repair and guanine specific repair. These cells also respond to promoting agents in vitro in a fashion highly analogous to mouse skin in vivo. Thus the in vivo-in vitro comparisons necessary to validate this culture system to date are highly positive. The development of a quantitive transformation assay seems to be the next logical refinement necessary for maximum usefulness of the model, and progress is being made in that direction.

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2.3.2 CHEMICAL TRANSFORMATION OF EPIDERMAL CELL CULTURES

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The newborn mouse primary epidermal cell cultures currently being used as model systems for in vitro carcinogenesis studies are characterized by 1) the capacity to undergo spontaneous vertical stratification analogous to keratinization, 2) the capacity to produce stratum corneum as indicated by histochemical staining and electrophoretic detection of keratin proteins, 3) a cell population which decreases with time in culture, 4) the lack of capacity to be subcultured, and 5) responses to carcinogens and tumor promoters which mimic in vivo responses (10,11,31,41,42,43). The conditions used in these studies include growth on plastic dishes in the absence of fibroblast feeder layers or collagen.

Although growth of epidermal cells in contact with dermis or dermal components has been shown to enhance the organization, growth rate, or time in culture of skin epithelial cells (2,3,8,14,15,20,29), attempts to subculture mouse epidermal cells have been largely unsuccessful. Recently Rheinwald and Green (29) have reported that human epidermal cells grown on irradiated fibroblasts form Rhodamin B staining material, show ultrastructural evidence for keratinization and can be subcultured for 50 to 100 generations (100 or more in the presence of epidermal growth factor; H. Green personal communication). Epithelial cells of human (8,29) and guinea pig (16,26,28) skin appear to be unique in their amenability to subultivation.

Although a number of laboratories have reported neoplastic transformation of liver (17,24,37,40) and bladder (12) epithelial cells in culture after treatment with chemical carcinogens, there have been relatively few reports of chemical transformation of skin epithelial cells. Fusenig and coworkers (9) have reported on 2 morphologically transformed cell strains obtained after treatment of primary newborn mouse epidermal cell cultures with 7,12-dimethylbenz-(a)anthracene (DMBA). These cell strains can be subcultured indefinitely, produce carcinomas (one keratinizing and one anaplastic) on injection into syngeneic hosts, and show a substantial decrease in the levels of an epidermal cell surface antigen (38,39). These workers found no spontaneous transformation, but they used conditions in which the transformation of treated cultures was rare (N. Fusenig

and W. Thon, personal communication). These reports contained only brief preliminary descriptions of tumorigenicity data, histopathology, and quantitation of epidermal antigen in tumorigenic cells.

Elias et al. (5) have treated mixed cultures of epidermal and dermal cells from newborn mouse skin with DMBA to obtain morphologically transformed epithelial-like cell strains. These cells produced anaplastic tumors on injection.

Recently Colburn et al. (4) have obtained some 10 morphologically transformed continuously subculturable cell strains 3 months after treatment of mouse epidermal cell cultures with N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG) with and without additional phorbol ester treatment. Two spontaneously transformed strains have also been obtained. These cells along with 3 other strains provided by S. Yuspa et al. (2 produced after DMBA treatment and one spontaneously produced) have been assayed for growth rate, colony formation in 0.33% agar medium (21,22), fibrinolytic activity (19,33) and tumorigenicity. All strains which were tumorigenic also formed colonies in soft agar while all non-tumorigenic strains did not form colonies. Two spontaneously transformed strains were tumorigenic while one was not. The tumorigenic cells grew in soft agar with colony forming efficiencies (CFE) which varied from 7-75%. Doubling times varied from 29 to 78 hrs. Preliminary studies in collaboration with W. Benedict and coworkers indicate that 2 tumorigenic epidermal strains have high fibrinolytic activity while 2 nontumorigenic strains show no or very low activity. Although growth in soft agar is a highly reliable correlate of tumorigenicity for epidermal cells, as it is for liver epithelial cells (25,34,40), it appears that high fibrinolytic activity, a parameter which is not a reliable tumorigeniciity correlate for liver epithelial cells (40), may be a second useful tumorigenicity indicator for transformed epidermal cells.

Growth in semi-sold medium (anchorage independent growth) appears to be the only consistently reliable in vitro correlate of tumorigenicity for a wide variety of cell types (7,13,30,32,35). At this writing we are aware of no reports in the literature in which a false negative has been obtained. There have been reports of apparent false positives (i.e., growth in soft agar of cells that could not be shown to be tumorigenic (19) and DiPaolo and coworkers, personal communication). However, failure to produce a tumor could occur for reasons other than lack of tumorigenicity such as antigenicity (even in nude mice) host response, etc.

Another recent result of our studies (4, and manuscript in preparation) is the production by chemical carcinogen treatment of transformed cell strains which in early passages failed to grow in soft agar or to produce tumors, but which in later passages shifted in

both properties. The transitional passage(s) was characterized by low colony forming efficiency in soft agar ($\leq 1\%$) while subsequent passages showed much higher CFE's. These strains in early passages can then be said to be preneoplastic and may be useful for studying late stages in the conversion to the neoplastic phenotype. The observation that a number of passages may be required for manifestation of tumorigenicity has been reported for chemically transformed guinea pig fibroblasts by Evans and DiPaolo (6) for rat fetal brain cells by Laerum and Rajewsky (18) and more recently for hamster embryo fibroblasts by J.C. Barrett (this volume). These findings imply a multistage process of carcinogenesis involving some minimum number of cell divisions and suggest that it may be difficult to test directly the induction (vs selection) hypothesis in so far as this entails limiting the number of cell divisions.

Most of the questions which have been posed regarding the molecular and cellular mechanism of carcinogenesis have not been answered in experiments using epithelial cell culture systems. With the exception of the report of Yamaguchi and Weinstein (40) on the production of conditional mutants for growth in soft agar, the induction theory, the somatic mutation theory, and the 2-stage hypothesis have not been tested. Other unanswered questions for epithelial cells include the cell cycle specificity of transformation, the role of cell proliferation, the role of DNA repair, and the role of differentiation. The possible role of viruses in liver epithelial transformation has been discussed by Weinstein and coworkers (36) who isolated A-type and C-type viruses from N-acetoxy acetylaminofluorene transformed liver cells and cell lines established from heptomas. Virus particles have also been found by Yuspa and coworkers (44) in cell cultures established from DMBA-induced epidermal carcinomas, and by us (Colburn et al., unpublished) in some MNNG and spontaneously transformed mouse epidermal cell strains. These viruses have not been tested for their ability to produce neoplastic transformation. However, the results of Rapp et al. (27) indicate that at least for 10T1/2 mouse embryo fibroblasts, virus production and chemical transformation are independent processes. Virus production in tumorigenic epithelial cells as well as in most non-epithelial cells may turn out to be symptomatic, i.e., occurring concommitantly but inert in the process of transformation as suggested by Weinstein et al. (36).

Efforts are underway to produce modifications in the epidermal cell culture system which will permit one to ask more questions about the mechanism of chemical carcinogenesis. These include attempts to increase the transformation frequency and decrease the latent period for transformation in carcinogen treated cells while decreasing the spontaneous transformation frequency. Efforts are also directed at increasing the size and stability of the proliferative

(stem cell) population and developing a quantitative transformation assay. Regarding the issue of decreased latent period the possibility exists that a long lag time may be inherent in the process of carcinogenesis in differentiating epithelial systems since carcinoma production in mouse skin in vivo requires some 4 to 10 months (1) and transformation of liver and bladder epithelial cells in culture thus far reported requires 2 to 9 months (12,35).

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2.4 RAT CELLS

2.4.1 A BIBLIOGRAPHY

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2.5 HAMSTER CELLS

2.5.1 THE USE OF EARLY PASSAGE HAMSTER EMBRYO CELLS FOR THE IDENTIFICATION OF CHEMICAL CARCINOGENS

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A number of model systems for studying in vitro carcinogenesis have been described (43). Several of these lend themselves to quantitation and may have potential use for the routine bioassay or identification of chemical carcinogens. In general, two morphological endpoints for activity are measured, i.e., the formation of altered colonies when small numbers of cells are seeded and challenged with carcinogen or the formation of foci of altered cells when mass cultures are employed. Models employing altered colonies have been limited to the use of early passage cells derived from hamster embryos (3,4,10-14,24,32,33). Cells from various strains of mouse (8,15,26,35,36), guinea pig (16) and rat (28,42) have exploited the use of focus formation in carcinogenesis studies.

In vitro systems employing cells derived from rat (17,18,34,39), mouse (37,38) and hamster cells have been used for studying the cocarcinogenic interactions of viruses and chemicals. Parameters other than abnormal morphology, such as the growth of temperature sensitive mutants in semisolid agar (9), may serve as useful tools for studying carcinogenesis in vitro. The system described here, which we are evaluating for use as a routine bioassay, is a modification of the system described by Berwald and Sachs (3), DiPaolo (10,14) and their associates. The system relies upon the use of early passage cultures derived from golden Syrian hamster embryos. Approximately 500 target cells are seeded onto a sparse lawn of x-irradiated feeder cells. The cells are then challenged with carcinogen and monitored for the appearance of morphologically altered colonies indicative of transformation. Cultures derived from morphologically transformed colonies induce sarcomas in hamsters. Variation in response among experiments, which can result in false negative results with standard control carcinogens, can be minimized by using aliquot samples of cells stored frozen in liquid nitrogen as the source of target and feeder cells (32,33).

The reproducibility and reliability of the standard bioassy requires stringent control of the quality of culture medium, serum and other reagents to insure the absence of mycoplasmas or other adventitious agents that may interfere. Samples of these as well as the plastic culture vessels must be pretested for their capacity to support optimum growth.

To reduce the observed subjectivity when altered morphology is used as the endpoint parameter for activity, a number of properties of transformed cells are available for study (1,5,6,19,22,23,26,27, 29-31,40). Results with hamster embryo cells indicate that the ability of morphologically transformed cells to grow in semisolid agar correlates completely with their tumorigenic potential in hamsters. Normal cells failed to grow in agar or induce tumors. Other criteria showing good correlation include the increase in cloning efficiency, extended life span, loss of contact inhibition, and relative growth in factor-free medium or medium supplemented with serum substitute. However, varying degrees of activity with these criteria were seen with normal cell cultures. Similar subjective results were seen with plasminogen activation or cytotoxicity by activated macrophages.

To verify the reliability of the system to identify carcinogens, a large number of standard reference chemicals with known carcinogenic activity were bioassayed using morphologic transformation as the endpoint for activity. These included carcinogenic and noncarcinogenic analogs of direct alkylating agents, polycyclic hydrocarbons, nitrosamines, aromatic amines and aminoazo dyes, metal carcinogens and a number of miscellaneous compounds. A very high correlation (89%) with reported carcinogenic activities was obtained when 77 chemicals were bioassayed. Transformation was not observed when cells were treated with a few carcinogens requiring metabolic activitation. No false positive results were obtained when known noncarcinogens were bioassayed and no spontaneous transformation was observed in untreated control cultures.

Dose responses for transformation have been obtained with many of the chemicals (10), however, this was not always the case.

In the standard bioassay, run in the absence of liver microsome enzymes, N-2-acetylaminofluorene routinely failed to transform hamster cells. However, the inclusion of hamster liver enzymes to the assay resulted in the metabolic conversion of AAF to an active carcinogen which transformed the cells. Preliminary evidence indicates that 2-aminofluorene is the major active metabolite in this case.

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2.5.2 SOMATIC MUTATION AS THE BASIS FOR IN VITRO TRANSFORMATION OF BHK CELLS BY CHEMICAL CARCINOGENS

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The extensive mutagenic activity of carcinogens which has recently been demonstrated in a number of systems (4,16,17,25,26), has been interpreted by many (2,5,25) but not all (31,37) investigators as supportive of the mutational origin of chemically induced cancers. The alternative explanation, that these cancers result from an epigenetic change in gene function rather than from a mutational change in gene structure is also supported by a number of experiments (see 31,37) including the demonstration that markers from embryonic tumor cells can reappear in normal adult tissues (28). To differentiate between these two hypotheses we have studied the transformation process itself and asked if it is or is not a mutational event.

Using a subclone of the quasidiploid line BHK21/cl 13 (38), BHK Supernormal clone 10, which was selected for the exceedingly normal phenotype it displays when cultured in liquid and in semi-solid media, we have investigated in vitro transformation by the chemical carcinogens nitrosomethylurea (NMU) and 4-nitroquinoline-1-oxide (NQO). To insure that in the in vitro study we are truly measuring malignant changes, we have used the soft agar assay for transformation (23), which is the only selective characteristic of transformed cells which shows nearly complete correlation with in vivo tumorigenicity (12,35). To determine if the change induced by the carcinogens in the BHK line from a normal to a transformed phenotype indeed represents a genetic mutation it is not possible to apply the techniques of classical genetics (11). Rather one must rely, as do others (22,36,39), on a set of criteria for mutagenesis which are operationally applicable to somatic cells to determine if a new phenotypic variant is a mutant. These criteria are as follows:

a. The new variant must arise with low frequency spontaneously. Transformed variants arise in the BHK Supernormal line at the 7th passage following cloning with a frequency of 2.2×10^{-7} per viable cell. This frequency is well within the range observed for spontaneous mutations at other loci in mammalian cells (6,15).

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b. The frequency of the new variant must increase in response to mutagenic agents. When the BHK Supernormal line is treated with either NMU or NQO, both potent mutagens in bacterial systems (3,26), the frequency of transformants per survivor increases in a dose-dependent manner to a maximum of 4×10^{-4} and 2×10^{-5} respectively. These frequencies are also within the range commonly encountered for the mutagenesis of other better characterized mammalian cell loci (6,15). In similar experiments Kakanuga has found NQO to be even more effective at the in vitro transformation of his BALB/3T3 mouse cell line (19,20,21). In vitro transformation by NMU has been reported previously by Sanders and Burford (33).

c. The new variant phenotype should be associated with an altered gene product. When the transformants induced by NMU and NQO are isolated, recloned, and grown and tested for the expression of the transformed phenotype at high and low temperatures, it is found that 44% (17/39) of the independently arising clones express the transformed phenotype at both temperatures. The remaining clones are either normal at low temperature and transformed at high temperature or are transformed at low temperature and normal at high temperature. These temperature-restricted phenotypes are indicative of a temperature-restricted gene product which appears to have resulted from the initial treatment with the mutagenic carcinogens.

A similar high frequency of temperature sensitive mutants is found at a few loci in other organisms (1,13,18,34) and also among revertents of both bacterial and mammalian cell mutants (7,9,24), and presumably reflects an unusually high proportion of temperature sensitive sites among the recoverable or permissible lesions. Temperature limited transformed clones similar in some respects to those reported here have occasionally been isolated before, both this laboratory (12), and in others (30,32,40) although in the latter cases data is not yet available to show that it is transformation and not just cloning ability in general which is modulating with temperature.

d. The variant phenotype should be stable. The BHK transformants have been extensively tested and found to stably retain their original temperature-limited transformed phenotype both in vitro and in vivo.

e. Reversion of variants to the normal phenotype should be low but demonstrable. Reversion frequency has been measured for one of the temperature restricted transformants, Me2N4 (12), by using lethal growth in methylcellulose containing flurodeoxyuridine and uridine to enrich for normal reverants after ethylmethylsufonate mutagenesis. The induced reversion rate is 1×10^{-5} revertants per survivor, similar to reversion frequencies observed for other temperature sensitive mutants in the BHK cell line (27).

f. The variant phenotype should be localized on a chromosome. This final criteria has not yet been approached using the BHK transformants.

Although a chromosomal location remains to be demonstrated, the above evidence strongly suggests that chemically induced BHK transformants originate as somatic mutations. Epigenetic alterations can be demonstrated to occur in hybrids of cultured cells (10), but most epigenetic explanations for somatic cell variants have been found unnecessary (8) and remain uproven (11,36). Our observation in this work that temperature sensitive, cold sensitive, and unrestricted transformants can all arise simultaneously in a cloned line in response to the same single short carcinogen treatment is hard to explain by epigenetic mechanisms whereas it is easily understood as mutations induced at different sites in the gene or genes controlling normal growth.

As recent reviews demonstrate (14,29) chemical carcinogenesis is far from being clearly understood, but the evidence now available in the case of BHK cells is consistent with the hypothesis that a somatic mutation is the primary event by which chemicals transform normal cells into ones capable of malignant growth.

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2.6 GUINEA PIG CELLS

2.6.1 NEOPLASTIC TRANSFORMATION BY CHEMICALS OF GUINEA PIG FETAL CELLS IN CULTURE

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Carcinogens from diverse chemical classes induce neoplastic transformation of guinea pig fetal cells in culture. The transition to the neoplastic state often occurs in a step-wise manner (1). Morphological alterations in carcinogen treated cultures appear shortly after treatment, but transformation, a loss of cell orientation does not appear for four or more months, is not seen in cultures treated with a noncarcinogenic chemical and may precede by 2-6 months development of the capacity of transformed cells to grow as tumors in irradiated syngeneic newborn guinea pigs or in nude mice (2). Alterations in plating efficiency, growth rate, serum dependence, cell surface antigens (4), endogenous virus expression (5), and chromosome number and structure (6) are not necessary for the development of neoplastic transformation. The ability of transformed cells to grow as colonies in agar (1), susceptibility of the cells to the cytotoxic effects of lymphokines produced by nonimmune syngeneic leukocytes (3,7), intradermal skin reactivity of transformed cells in nonimmune syngeneic guinea pigs (3), and secretion of large amounts of plasminogen activator (I. B. Weinstein, personal communication) are characteristics that correlate well with neoplastic transformation. These cell properties are useful indicators of the tumorigenic potential of guinea pig cells transformed by chemical carcinogens. Development of this model system offers an additional avenue for studying the process of carcinogenesis using a species with established tumor biology in which spontaneous transformation has not been seen, which possesses well defined immunological parameters, and in which discrete stages in the transition from the growth controlled to the neoplastic state may be studied.

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2.7 HUMAN CELLS

2.7.1 CHEMICAL TRANSFORMATION OF HUMAN AND RODENT CELLS

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The human risk to environmental carcinogens has been assessed mainly by epidemiological methods and by carcinogenesis experiments in animals. Recently other rapid assay systems have also been developed using various species such as prokaryote and methods such as mutagenesis and transformation of cultured rodent cells. However, the evolutionary aspects of biological functions such as repair activity and endogenous virus indicate that there may be some discrepancy between the results obtained with animals or prokaryotes and the actual carcinogenicity of substances in human. In order to solve this problem of the extrapolation of the experimental results into human risk, it would be very useful to develop a system for the transformation of human diploid cells by chemical substances (see: Saffiotti, in this Volume).

The system of cultured human diploid cells has many advantages for genetic and biochemical studies such as the stability of the ploidy and the abundant availability of well analyzed cell mutants, i.e., the cultured cells derived from genetic disease patients. Development of a system for the transformation of human diploid cells by chemicals is very important to establish a better method for understanding the mechanisms of carcinogenesis as well as for the assessment of human risk to enviromental carcinogens.

Although two cases of chemical transformation have been reported by using human cell strains derived from tumor tissues (7,13), there has been no success in the chemical transformation of normal human diploid cells. Many explanations were considered to explain this difficulty before starting my experiments. Most of these explanations were based on the problems dealing with the methods and the conditions of the experiments such as the following: 1) Usual culture conditions used for mammalian cells are not suitable for human cells. This was generally observed by the low plating efficiency of human cells. 2) The transformed phenotype of human cells may be different from those of the experimental animals. 3) Human diploid cells, especially fibroblasts, lack the enzyme activity needed to convert the chemicals into active forms.

First, we examined the culture conditions and established a good system which routinely produced a cloning efficiency of 80 to 95% with most of the human diploid fibroblast strains available. The second explanation seemed unlikely, because it had been reported that all the cells in culture obtained from human tumor tissues show similar biological properties to the transformed rodent cells (4,5). The third problem could be easily solved by using directly acting chemicals or the chemicals which are activated in the cells of all species and all tissues. Thus, these three problems were satisfied, and then we had a chance to try to transform human diploid cells by chemical carcinogens. Here I described the outline of our experimental procedures and results of the chemical transformation.

CHEMICALS

4-nitorquinoline-1-oxide (4NQO) and 4-amino-quinoline-1-oxide (4AQO) were obtained from Daiichi Pure Chemical Co. (Tokyo), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) from City Chemical Co. (New York), and 3-methylcholanthrene (MCA) from Eastman Kodak Co. (Rochester). All chemicals were dissolved in dimethylsulfoxide (DMSO) spectranalyzed (Fisher Scientific Co. (Pittsburgh)) at the appropriate concentrations and then added to culture medium in the dark. The chemical solution was prepared immmediately before usage. Final concentration of DMSO in culture medium was 0.2 - 1.0%.

TYPE OF CELLS

A human diploid cell strain, KD, derived from a skin biopsy taken from a female adult was used in this experiment. The following points were considered: 1. It is desirable that the cells are the most available type of human diploid cells taken from the individual. This would be an advantage for the further development of a comparative study of the sensitivity to chemical carcinogenesis of cells derived from normal patients and various genetic disease patients who are predisposed to the higher incidence of cancer. 2. The normality or abnormality of the individual from whom the cells are taken is demonstrable. 3. The cells of the same genetic background can be obtained repeatedly from the same individual. This strain was initiated by Dr. R. Day (2) from a skin biopsy taken from the lip of an adult female who has not shown any genetic disease. The primary culture was made by plating the cells dissociated by stirring the biopsy in a trypsin-collagenase solution. The grown cells were subcultured at 1:2 dilution and stored in LN_2 freezer as stock cells. For the transformation experiment, the cells were used at the 6th to 34th passage after isolation.

Although the original biopsy contained epidermal tissue, the KD cells used in the experiment showed the typical morphology of fibroblast in mass culture (Figure 1) and did not produce any colony

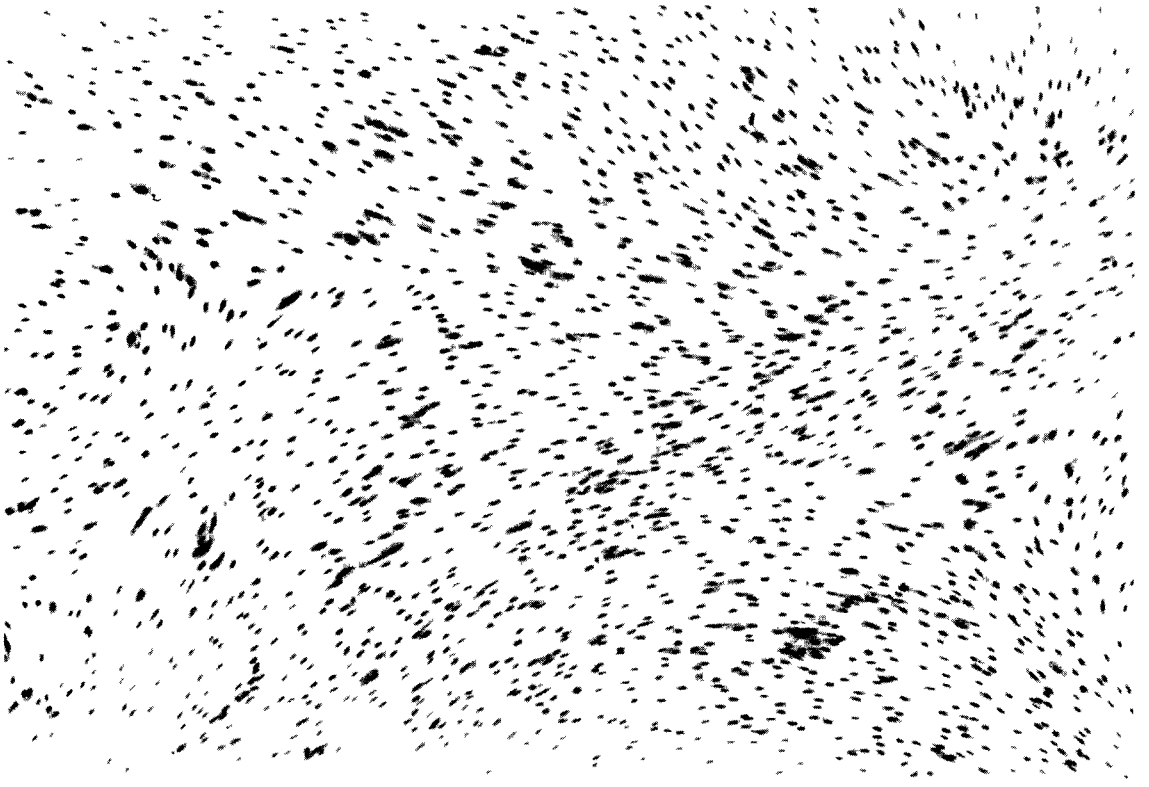

Figure 1. Photomicrograph after fixation and Giemsa-staining of untreated KD cells

of epithelial-like morphology. Throughout the experiment a total of more than 2 x 10^5 colonies of KD cells were observed. The major population of the KD cell stock seemed to be fibroblastic in morphology and preliminary examination of the karyotype indicated that the KD cells are diploid and that their diploidy is stable as long as they grow actively.

CULTURE CONDITIONS

The cells were cultured in 100 plastic dishes (Falcon Plastics (Oxnard)) containing 10 ml culture medium in a humidified CO_2 - incubator at 37°C. The regular culture medium consisted of Eagle's minimum essential medium (MEM) (Grand Island Biological Co. (Grand Island)) supplemented with 10% fetal calf serum (Flow Laboratories (Rockville)). The serum used was selected by pre-screening the serum samples from various sources for the cloning efficiency and the effect on the morphology of cultured KD cells. The culture medium was changed 2 or 3 times a week. The subcultivation was made by adding 0.25% trypsin solution (Grand Island Biological Co. (Grand Island)) to

the culture for 3-5 min, removing the trypsin solution, letting the culture stand at room temperature until the cells showed some change in morphology, a sign of digestion, then adding fresh regular culture medium, aspirating and dispensing into new dishes.

MARKER OF TRANSFORMATION

As described before and will be discussed later, many diagnoses of neoplastic transformation of cultured cells have been reported. Among them, the morphological change in growth pattern and the anchorage independence of cell growth seem to be the most common markers of the neoplastic fibroblast in culture as well as the pathological diagnosis of in vivo tumors. As an initial step in the development of the transformation system, any kind of change in the morphology of the gowth pattern was taken as a primary diagnosis to be observed.

ASSAY PROCEDURE

Using the established assay system of the chemical transformation of rodent cells as reference, several methods were applied to detect the chemical transformation of KD cells.

First, the simplest assay method was tried. That is, the growing cells were treated with 4NQO (0.01 - 1.0 μg/ml) or MNNG (0.1 - 1.0 μg/ml) for 30 or 60 min in suspension, and then plated at an appropriate concentration of cells so that each surviving cell formed a separate colony. Ten to 16 days after plating, the cultures were fixed, stained, and the morphology of the colonies was examined under the stereomicroscope. No change has been observed in more than 200,000 colonies observed so far.

Second, the focus assay method was used. The growing cells were treated with chemicals and the surviving cells were allowed to divide 4 or 5 times before attaining a confluent state. After reaching confluence, the cultures were maintained without subculturing for 3 to 4 months until they were fixed and stained. About 5×10^8 cells were treated with 4NQO or MNNG and 600 dishes containing approximately 10^8 surviving cells were tested. No transformed focus has ever been found in these experiments.

Colony formation of the cells in soft agar was used as an indicator of transformation in a third assay method. The cells were allowed to divide for 4 or 5 cell generations after the chemical treatment and then were plated into a soft agar layer. No colony formation was observed in the soft agar layer into which a total of 3.3×10^7 surviving cells were inoculated.

The fourth was a classical method to obtain transformation in culture. Actively growing cells were treated with various concentrations of 4NQO or MNNG for 30 min and were then subcultured at 1:4 dilution ratio whenever they became confluent. Because of the limitation of capacity of the incubator, the experiment was designed so that only one of four subcultured dishes was used for the subsequent subculturing. All the cultures were examined for morphologic change under the microscope at least once a week.

Seven to 14 weeks and 5 to 10 passages after the treatment with carcinogen, morphologically altered cells were found in small area of a very small number of dishes. The main morphologic alteration was the formation of an area with a high density of cells showing frequent mitosis. When these cultures containing the morphologically altered cells were maintained without subculturing, the dense area became visible by the naked eye as a focus (Figure 2). The shape of the focus was dependent on the stream of the surrounding unaltered cell. The edge of the focus followed the direction of the surrounding cells (Figure 3). Disorganization and a criss-cross pattern of cell arrangement, which is the typical morphology in most of the transformed cells in culture, was not distinctive although some disorder of cell arrangement was easily found by careful observation (Figure 4). It appears that the density-dependent inhibition of "cell growth" was lost whereas the loss of the sensitivity to the density dependent inhibition of "cell movement" is not marked in these morphologically altered cells. When the cells were picked up from the dense area and transferred into new dishes, they retained the property of dense growth and formed heavily multilayered sheets (Figures 5 and 6).

Five out of 6 separate experiments resulted in the development of morphological alteration in subcultures derived from the chemical-treated cultures. The morphologically altered cells appeared only in the cultures treated with about 30% survival dose of 4NQO or MNNG and did not appear in the other cultures including those which were untreated, treated with MCA or 4AOO, and treated with a low concentration of 4NQO, as far as tested.

REQUIREMENT OF CELL DIVISION FOR THE DEVELOPMENT OF TRANSFORMATION

In the transformation of mouse or hamster embryonic cells by 4NQO, we found that approximately 4 or 5 cell generations after chemical treatment are required for the development of the transformation (10,11). As described above, however, no morophologically altered

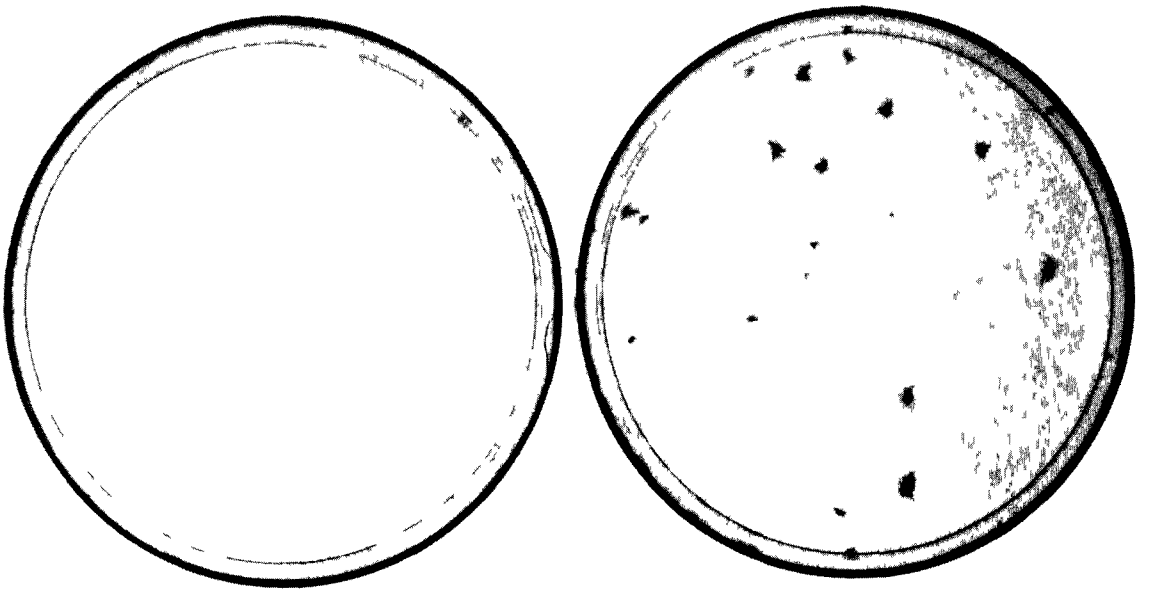

Figure 2. Photograph after fixation and Giemsa-staining of dishes as an example of the focus formation by the morphologically altered cells. They were derived from the cultures which were treated for 30 min with 0.3% DMSO (left) and 0.1 μg/ml of 4NQO (right), then subcultured 11 times at 1:4 dilution ratio whenever they became confluent, and maintained for 24 days without further subculturing.

Figure 3. Focal area of morphologically altered cells

Figure 4. Higher magnification of Figure 3.

Figure 5. A transformed line, 4NQ.T-3.

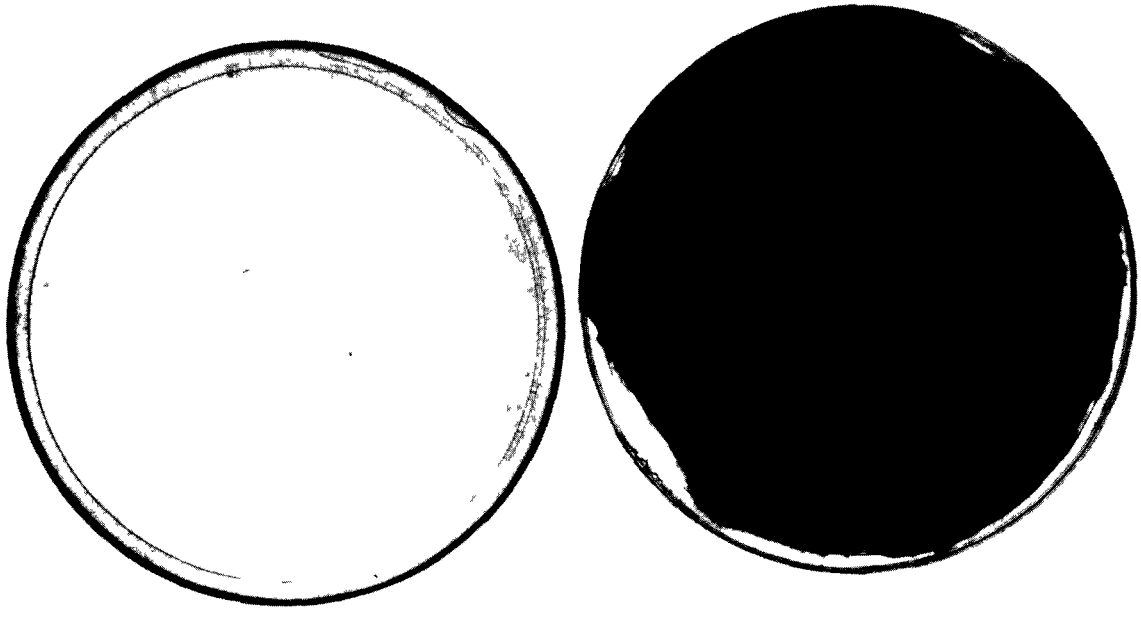

Figure 6. Photography after fixation and Giemsa-staining of dishes as an example of the formation of dense cultures by the morphologically altered cells. The culture of untreated KD cells (left) and the culture in which the cells picked up from foci area were grown (right).

cells were found in tests using the first three assay procedures, i.e., clonal morphology assay, focus assay, and soft agar assay. In these three methods, the surviving cells were allowed to divide 4 to 8 times after chemical treatment. The morphologically altered cells appeared only after extensive subcultivation. When a small number of the morphologically altered cell lines were plated on the monolayer sheet of untreated KD cells, they grew and formed clear foci at the efficiency of approximately 80%. These findings indicate the possibility that an increased number of cell divisions is necessary for the development of the transformation of human diploid cells. In order to test this possibility and to confirm the chemically-induced transformation, the experiments were done by using cloned KD cell. Many clones were isolated from the original KD cell strain and then each clone was increased to have a sufficient number of cells to start the transformation experiment. All the clones isolated showed the fibroblastic morphology. Each clone was treated with 30% survival dose of 4NQO, and subcultured as described above. Regardless of the duration of time the cultures were maintained in the confluent state, the morphologically altered cells were found only after many repeated subcultivations. The average number of cell generations required for the development of morphological alteration was calculated to be at least 13. This is based on the assumption that all the surviving cells divided equally. No morphological alteration was observed in the control cultures in which the same clone was treated with DMSO only and subcultured (until it degenerated) in the same manner as the treated cultures. This supports the idea that morphological alteration was induced by treatment with 4NQO in the human diploid fibroblast.

There was a variation in the time of the first observation of morphological alteration between cultures of separate experiments in which a different clone of KD cells was used as well as between cultures of the same series of experiments. It is uncertain whether there was a significant difference in susceptibility to alteration between the clones of KD cells.

The morphologically altered cells appeared in the sub-cultures derived from only a few of the 10 initially treated cultures. If it is assumed that there was no segregation of the transformable cells, the transformation frequency is calculated to be approximately 3.3×10^{-7} per treated cell. It was impossible to test the possibility that further cell generations after 4NQO- treatment may result in a higher transformation frequency because the morphologically unaltered cells began to degenerate at 8 - 10 passages after treatment in these experiments.

Although it may be dangerous to simply compare the different transformation systems, the transformation frequency obtained in this

experiment is of quite low magnitude compared to those obtained in rodent cells. The maximum frequency of chemical transformation per surviving cell is reported to be 6×10^{-1} - 10^{-3} in Syrian hamster embryonic cells, 10^{-2} - 10^{-3} in the subclone of BALB/3T3, (3,9), and 10^{-2} in mouse prostate cell lines (1), varying between different cell sources, laboratories, criteria of the transformed phenotypes, and procedures, etc. The magnitude of the difference in the chemical transformation frequency between human diploid cells and rodent cells may be related to the difference in the stability of the ploidy in cultures as well as the difference in the cell sources and experimental conditions. It is known that the karyotype of the cultured rodent cells are generally unstable while human diploid cells are stable. It is possible to speculate about variations in the DNA repair activity (12) and variations in the presence and expression of the oncogene between the cells of the differnt species. It is unlikely that this difference in transformation frequency is due to the difference in the permeability across cell membrane of 4NQO or in the total amount of 4NQO bound to DNA. Thirty percent survival dose of 4NQO gave 3×10^{3} fold transformation frequency in A31-714 cells derived from BALB/3T3 (9). We have found that the amount of 4NQO-adduct to guanine or adenine base was similar between human diploid fibroblast and A31-714 cells (8).

BIOLOGICAL PROPERTIES OF THE TRANFORMED CELLS IN CULTURE

Saturation density and the ability to grow in soft agar were examined in the morphologically altered lines derived from each focus and the untransformed cells of chemical-treated cultures. These cells were compared with the untreated and untransformed KD cells (table 2).

Saturation density was determined after inoculating 5×10^{4} cells into 60 mm plastic dishes containing 5 ml regular culture medium. The medium was changed every 3 days, and the number of cells was counted daily. The saturation density was expressed as the cell number per cm^2 when counts on 3 successive days did not change significantly. Saturation density was increased in all the morphologically altered cells but not in the treated, morphologically unaltered cells.

Growth of the suspended cells in soft agar was examined by the procedure as described previously (9) unless otherwise specified. The ability to grow in soft agar was also increased in the morphologically altered cells, although the increase was slight in the lines derived from cloned KD. The increase in the saturation density was not quantitatively parallel to the increase in the ability to grow in soft agar.

All the morphologically altered cells picked up from foci grew exponentially and have not shown any sign of a limit to their life span. Preliminary examination indicated that all the morphologically altered cells showed aneuploidy.

The results indicate that the morphologically altered cells obtained possess the properties which are usually correlated with neoplastic transformation of cells in culture.

TRANSPLANTABILITY INTO NUDE MICE

It has been reported that human tumor transplants or cells freshly cultured from human malignant tumors give rise to tumors in Nude mice (6,14,15). Cellular tumorigencity in nude mice has been reported to be correlated with cell growth in semi-solid medium (4). Nude mice are thought to be one of the best available systems for testing the malignancy of human cells.

To examine the transplantability of the cells, the cells were suspended in 0.2 ml Eagle's MEM serum-free medium and then injected subcutaneously into 5 to 16-week old female nude (athymic) mice, which were maintained under conditions of strict sanitation and segregation from other strains of rodents. Groups of 10 mice with the same distribution of ages in each group were designated. The mice were checked for the development of tumors weekly. Biopsy samples were removed surgically from one-half of the tumor-bearing mice in each experimental group. Each biopsy sample was divided into two portions, one portion being fixed for histological examination and the other portion prepared for subsequent growth in vitro. Autopsies were performed on each mouse that died or was sacrificed in moribund conditions during the course of the study. Six months post-injection, all surviving mice were sacrificed and autopsies were performed.

Two concentrations each of 7 different morphologically altered and unaltered human cells were injected into 14 groups of 10 nude mice each. All the morphologically altered cells grew as tumors at the site of injection (table 3). The latent period for tumor appearance varied from 2 to 21 weeks (average 6 weeks) depending on the cell lines and the number of cells inoculated. The tumors tended to grow progressively. Spontaneous regression of tumor was observed only in 2 cases. No tumor was produced by injection of morphologically unaltered cells. Examination of chromosome preparations of some of the tissue culture transplants showed that the tumor cells were of human origin. Taken together with the results of the biological properties in culture, it is likely that the morphologically altered cells are malignantly transformed cells.

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Table 1. Saturation density and colony forming ability in soft agar

Cells	Days in culture after treatment with chemical	Saturation density ($\times 10^4$ cells/cm^2)	Colony forming efficiency in soft agar
Untreated	--	3.2	$< 5 \times 10^{-7}$
Solvent-treated	4	3.0	$< 1 \times 10^{-6}$
	95	2.6	$< 1 \times 10^{-6}$
4NQ.NT[1]	95	2.7	$< 1 \times 10^{-6}$
4NQ.T-1[2]	95	7.2	9×10^{-4}
4NQ.T-2[2]	95	5.4	1×10^{-3}
NG.T-3[3]	105	9.4	2×10^{-3}
4NQ.TR-1[4]	97	12.7	2×10^{-5}
4NQ.RT-2[4]	97	11.7	1×10^{-5}
4NQ.RT-3[4]	97	10.7	1×10^{-6}

[1]The cells of the current cultures which were treated with 4NQO and did not show any morphological changes.
[2]The cell lines isolated from each focus area in the subcultures at 79 days after the treatment of KD cells with 4NQO.
[3]The cell line isolated from a focus area in the subculture at 90 days after the treatment of KD cells with MNNG.
[4]The cell lines isolated from each focus area in the subcultures at 83 days after the treatment of KD-1 clone with 4NQO.

Table 2. Transplantability of the cultured cells into nude mice

Cells	Days in culture after treatment with chemicals	No. of cells injected	No. of mice with tumors/No. of mice given injection
Untreated	--	2×10^5	0/10
		2×10^6	0/10
4NQ.NT[1]	107	2×10^5	0/10
		2×10^6	0/10
4NQ.T-1[2]	107	2×10^5	3/10
		2×10^6	9/10
4NQ.T-2[2]	107	2×10^5	0/10
		2×10^6	7/10
NG.T-3[3]	125	2×10^5	6/10
		2×10^6	10/10
4NQ.RT-2[4]	120	2×10^5	0/10
		2×10^6	4/10
4NQ.RT-3[4]	120	2×10^5	0/10
		2×10^6	6/10

[1]The cells of the cultures which were treated with 4NQO and did not show any morphological changes.
[2]The cell lines isolated from each focus area in the subcultures at 73 days after the treatment of KD cells with 4NQO.
[3]The cell line isolated from a focus area in the subculture at 90 days after the treatment of KD cells with MNNG.
[4]The cell lines isolated from each focus area in the subcultures at 83 days after the treatment of KD-1 clone with 4NQO.

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2.7.2 HETEROPLOID CONVERSION OF HUMAN SKIN CELLS BY METHYLCHOLANTHRENE: POSSIBLE ROLE OF HYDROCARBON METABOLIZING EPITHELIAL CELLS

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A. QUESTIONS AND ANSWERS THAT SERVED AS A BASIS FOR THE RATIONALE IN DEVELOPING HUMAN CELL MODELS OF TRANSFORMATION[3]

Q - Why is it important to develop in vitro assays for chemical carcinogens?

A - There are two major reasons. First, we have the problem of identifying and eliminating environmental carcinogens that may be the direct cause of much human cancer. This project must include many chemical agents already widely disseminated as well as all of the new agents developed by industry each year. The cost of such a study in laboratory animals is staggering. In vitro assays, hopefully, would be quicker, more inexpensive, better controlled, and more reliable than the equivalent test animals. Secondly, if we can induce cell transformation predictably and under controlled conditions, we may also be able to determine the molecular basis by which a cell becomes malignant and this, in turn, may lead to better methods of cancer cure and prevention.

Q - But why mammalian cell transformation? Aren't bacterial mutagenesis assays quicker, cheaper, and better?

A - Whether or not a potential carcinogen induces a cell to become malignant depends on at least two genetic factors. First, does the cell possess the necessary metabolic pathways for converting distal carcinogens to an ultimate form and secondly, once this ultimate carcinogen is produced, is the cell susceptible, i.e., is it the specific target cell predisposed to becoming malignant?

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Now let us take these two factors, metabolism and susceptibility, and ask whether or not all cells within a single individual are equally prone to develop cancer. How about differences between individuals within a species, between species, between genera, between phyla? Obviously, it is very difficult to be sure that mutagenesis in bacteria has relevance to cancer in man. By extending the data base to include in vitro transformation of several species of mammalian cells, we hope to discover which tests are relevant and which are not. If all tests should be equally effective and relevant, we could utilize the cheapest and quickest tests with confidence.

Q - Would carcinogen testing in animals be eliminated?

A - Certainly not at the present time because comparative in vitro-in vivo tests are needed to confirm the relevance of the in vitro tests. The presence of a functional liver and many hormonal modulations are just two factors that make the living animal an ideal test system for carcinogenesis. Of course, if different chemicals have varying carcinogenic potentials in different animals, they may also have different potentials in man--and man cannot be used as a test animal.

Q - Can a data base designed to correlate in vitro and in vivo tests among the species be extended to include man?

A - The plan would be to correlate in vitro and in vivo tests in nonhuman mammalian cells and then to compare in vitro transformation of nonhuman mammalian cells with transformation of human cells. When the data base is sufficiently complete, we would have to assume that in vitro tests with human cells were predictive of carcinogenic activity in man. Some verification of this could be and indeed has been made with epidemiological studies.

Q - What are the results of in vitro carcinogenesis studies with human cells?

A - It has been frequently observed and is generally known that human cells are resistant to the transforming effects of agents known to be carcinogenic for rodents and for rodent cells in culture, however, few papers have documented this phenomenon (3,5,6,12,14,18). The comprehensive studies by Igel, et al. (14) subjected over 50 diploid human fibroblasts cell strains, derived from normal tissues and from benign and malignant tumors, to a number of carcinogens including methylcholanthrene, N-methyl-N'-nitro-N'nitrosoguanidine, and urethane. No evidence of cell transformation was noted with the following exceptions.

Morphologically altered foci were seen in urethane treated cultures derived from two siblings with von Recklinghausen's disease. Many of these foci were isolated, but only two developed into continuous cell lines. Each culture was heteroploid and contained unique marker chromosomes indicating they were derived from separate transforming events occurring in two original parent cells (15). In addition to indefinite life span in culture and heteroploidy, the cultures grew in soft agar, had increased fibrinolytic activity, and produced nodules, but not progressively growing tumors in newborn mice (14,15). More recently, Rhim, et al., have induced further changes in an aneuploid cell culture derived from a human osteosarcoma (21). In this case, the transformed cells attained an important characteristic of malignant cells, i.e., they produced malignant tumors in nude mice. Both of these studies (14,21) are subject to the same valid criticism. Since the cultures were derived from human tumors, it is possible that treatment with chemicals selected out a pre-existing population of malignant tumor cells. An attractive alternative interpretation is that each cell culture was predisposed to transformation. Von Recklinghausen's disease is transmitted by an autosomal dominate gene and is characterized by formation of neurofibromas which often transform in vivo to neurofibrosarcomas. Thus, diploid cells derived from the neurofibroma could also be genetically predisposed to transformation in vitro. In the case of the osteosarcoma cell, it should be noted that they are derived from tumors and are aneuploid and thus could represent a tumor cell culture with incomplete expression of full malignant potential, thus perhaps, could become fully transformed by a single mutation induced by a carcinogen. In any event, it is evident that chemically induced transformation of human cells is a rare event and has never been reported with diploid human cells derived from normal tissues.

Q - Why are human cells so resistant to chemically-induced transformation?

A - If a cell is to become transformed as a result of chemical treatment, a number of events have to occur. The chemical must reach the cell and enter it. Distal carcinogens have to be metabolically activated to ultimate carcinogenic forms. Once the ultimate carcinogen is formed, correct quantities of it must reach the target sites where a "transformation potential" lesion is produced. The target cell must be capable of transcribing and translating the "transformation potential" lesion into phenotypic expression. Obviously, these events occur readily in rodent cells. We can speculate that certain human cells may be impermeable to certain chemicals. If permeable, human cells may not metabolize certain chemicals and, if so, may produce only

nontoxic noncarcinogens rather than carcinogenic products. If an ultimate carcinogen is formed, it may be inactivated before reaching the target site. The DNA of certain species may contain fewer of the nucleotide sequences susceptible to a specific carcinogen and thus may be more resistant to formation of a lesion. Should a lesion be formed, DNA repair mechanisms may correct it; and/or the cell may contain enzymes that destroy defective messenger RNA. Further, certain virologists would point out that mammalian cell species that are easily transformed by chemicals are also the ones that possess readily demonstrable C-type RNA viral genomes whereas this type of viral information is extremely difficult to demonstrate in human cells. Although this observation proves nothing, it leaves open the suggestion that viral information may play a role in chemical carcinogenesis and that human cells may be resistant to transformation due to powerful cell regulation over viral expression.

Q - Is there evidence to support any of these proposed mechanisms?

A - In 1971, Deitz and Flaxman (5) wrote, "It is tempting . . . to consider the possibility that susceptibility (by cells) to the toxic effects (of chemicals) might indicate the potential for achieving transformation because evidence suggests that both processes may involve metabolism of the hydrocarbons by an inducible aryl hydroxylase enzyme system (4,10,11)." In that same report (5), 1 µg per ml of benzo(a)pyrene or methylcholanthrene induced giant cell formation and a disorderly pattern of growth in human skin epithelial cells, but not fibroblasts, however, no evidence of malignant transformation was noted. Diamond (3) related cytotoxic effects with metabolism of polycyclic hydrocarbons in mammalian cell cultures. Of particular interest was her finding that most of the human cell strains studied had low levels of hydrocarbon metabolism and this was reduced further upon subculture. In a study that effectively combined and confirmed work of Dietz and Flaxman (5) and Diamond (3), Huberman and Sachs (12) studied the hydrocarbon metabolizing activity of epithelial cells derived from human embryos. They found that epithelial cells metabolized 3-25 times more benzo(a)pyrene than did fibroblasts from the same embryo. This observation suggested to them that "the higher activity of epithelial cells might be related to the higher incidence of carcinoma rather than sarcoma in humans." This work has been extended and confirmed in human skin by Fox, et al. (18). Somewhat more recently Kouri, et al. (17) found a wide diversity in hydrocarbon metabolizing activity in human fibroblast strains derived from human embryos or adult human skin. All human strains, however, had low hydrocarbon metabolizing activity relative to Fu5-5 rat hepatoma cells.

All of this work taken together, leads to the following conclusions. Human cell cultures have low levels of hydrocarbon metabolizing activity relative to rodent cell cultures; human fibroblasts have low levels of hydrocarbon metabolizing activity relative to human epithelial cells. This specific increased metabolic activity of epithelial cells may account for the predominance of carcinoma rather than sarcoma in man. Human epithelial cells with high hydrocarbon metabolizing activity may be the targets of choice for cell transformation studies.

Q - It there evidence that certain viruses might play a role in chemical carcinogenesis?

A - There is no evidence for this in human systems. Transformation of rat cells, however, is greatly enhanced by murine C-type RNA leukemia viruses. That is not a topic for discussion here. Those seeking more information are referred to a recent publication (8).

Q - Do you have any new information regarding chemically-induced transformation in human cells?

A - Yes, and it is presented and discussed in this paper.

B. MATERIALS AND METHODS

ESTABLISHMENT OF PRIMARY FORESKIN EXPLANT CULTURES

Portions of circumcised foreskin tissue were trimmed to remove fat and connective tissue and minced with scissors in a small volume of growth medium (Eagle's Minimum Essential Medium containing 1mM sodium pyruvate, 0.1mM nonessential amino acids, 2mM glutamine, 100 U penicillin per ml, 100 µg streptomycin per ml, 50 µg kanamycin per ml, and 10% heat-inactivated fetal calf serum). The skin mince was washed at least three times in growth medium to remove blood. For most experiments, ten to twenty skin mince pieces (1-8mm^3) were placed on the medium-rinsed surface of 90mm plastic petri dishes and incubated at 37°C, 95% humidity, 5% CO_2 in air. After 2 hours, taking care not to dislodge the adhering skin pieces, the cultures were fed 3 ml of growth medium and reincubated until about 1 cm of cell outgrowth was obtained, usually by the tenth to fourteenth day after planting. Most colonies, presumably derived from epidermis, consisted predominantly of epithelial cells but fibroblasts were seen between colonies. Some colonies, presumably derived from bits of dermis, seemed to consist entirely of fibroblasts. For two other experiments, a 0.014mm split thickness skin biopsy and full thickness foreskin tissue were minced as described and planted on the dermal bed of dead sterile pigskin

(7,9,13). A 1-2 cm diameter clearing of the pigskin substrate, corresponding to the diameter of virtually pure epithelial cell outgrowth, occurred around the skin explants within 17 days after planting.

COLONIAL ASSAY OF HYDROCARBON METABOLIZING ACTIVITY

Fifteen skin mince pieces (1-8mm^3) were planted per 75 cm2 Falcon plastic flask by identical methods described above for petri dishes. When individual colonies of fibroblasts or epithelial cells attained a diameter of 16mm, plastic cylinders >(16mm diameter) were cemented over each island with sterile silicone grease. In order to do this, sections of the flask opposite the cells were cut out with a hot scalpel blade. An aluminum foil cover was then used to maintain sterility. Each cylinder was filled with 2 mls of medium freshly supplemented with ^{3}H-Benzo(a)pyrene (Amersham Searle generally labelled, 20 Ci per mmole diluted to 1 g per ml, 2 µCi per ml). After a 3-day incubation period at 37°C, water-soluble metabolites in the medium were extracted as described by Kouri, et al. (17). The cells growing in each cylinder were removed with a mixture of 0.25% trypsin and 0.02% disodium ethylenediaminetetraacetic acid in calcium-magnesium free Hanks' Balanced Salt Solution, diluted in 0.4% trypan blue, and counted in hemocytometer. Background aqueous-soluble ^{3}H-benzo(a)pyrene was determined by extraction of medium inoculated without cells for 3 days. This background, representing 1-3% of total input counts, was subtracted to determine net counts. The hydrocarbon metabolizing activity (HMA) was expressed as pmoles of benzo(a)pyrene converted from an organic-soluble to water-soluble form per 10^6 cells per number of days. Generation of water-soluble benzo(a)pyrene metabolites was linear over a 3-day incubation period.

TREATMENT WITH MCA

Thirteen to 17 days after planting cultures were treated for 7 days (3 medium changes) with growth medium containing the appropriate concentration of MCA, or other chemical, dissolved in acetone. Control cultures were exposed to the equivalent concentration of acetone (0.1%) diluted in growth medium. All changes of media and incubation were carried out in semi-darkness during the week of treatment. After seven days, dishes and pigskins were rinsed three times in growth medium to remove unbound MCA. Then growth medium was replaced, and the cultures were incubated until confluency was reached.

ESTABLISHMENT OF SECONDARY CULTURES

When grown on plastic, confluent primary cultures, consisting of 1-2 cm diameter epithelial islands surrounded by

fibroblasts, were removed with 0.25% trypsin in Hanks' Balanced Salt Solution and subdivided 1:2 and/or 1:4 into Falcon plastic 75 cm^2 flasks. When grown on pigskin, areas containing epithelial cell outgrowth (as evidenced by clearing of the pigskin due to digestion of the connective tissue matrix) were excised, minced with scissors in a small volume of growth medium, and transferred to plastic flasks. Further subdivisions were adjusted to 1:2, 1:4, or 1:8 split ratios so that physical subculture could be carried out weekly.

In our terminology, D refers to the approximate number of population doublings based on the split ratios. Primary cultures are those that have never been physically subdivided. Since skin epithelial cells continuously maturate to form keratin, it is obvious that the true number of population doublings always exceeds D. In spite of this error, we consider the use of D representing the approximate number population doublings to be preferable to the use of P representing the number of physical subpassage.

SELECTION OF MORPHOLOGICALLY ALTERED FOCI

In some experiments, morphologically distinct colonies were noted in MCA treated cultures. These were scraped from the surrounding cell sheet with a sterile wire, removed with a capillary pipette, and planted in a test tube in 1 ml of growth medium. Cell lines derived from these colonies bear the number of the parent cell line, followed by C (for colony) and a numerical designation to identify the individual colony. For example, A566C-1 is colony 1 derived from A566.

OTHER METHODS

Growth in soft agar was done by the method of MacPherson and Montagneir (19). Chromosome preparations at The Children's Hospital of Akron were made by the method of Kajii (16). Chromosome preparations at The Children's Hospital of Los Angeles were made by the method of Paul (20). Banding of human chromosomes was done by the technique of Seabright (23). Plasminogen activation was monitored as described by Jones (15). To determine absolute plating efficiencies the cell cultures were trypsinized and suspended in growth medium containing the lowest concentration of serum included in the test. One hundred cells were added to replicate plates containing medium with fetal calf serum at the concentrations listed in table 8. The cultures were inoculated without change of medium for 7-10 days. Colonies were fixed in 10% buffered formalin, stained with Giemsa, and counted.

C. RESULTS

TABLE 1. Skin epithelial cell and fibroblast colonies derived from 13 different donors were tested for HMA after 21 to 50 days in culture. A continuous spectrum of activity ranging from 1250 to 20,087 units (pico moles BP/10^6 cells per 3 days) was found in epithelial colonies. Epithelial colonies always had more activity than fibroblasts from the same donor. In 9 of 13 donors, the relative levels of HMA in epithelial cells and fibroblast correlated very well but in the other 4 cases, the fibroblasts had low or undetectable activity. Perhaps HMA of epithelial cells and fibroblasts are controlled by different genes, or perhaps the low levels of HMA in certain fibroblast colonies are artifactual, caused by, for example, changes in culture conditions or culture age. Based on the limited number of donors tested, sex, race, or age of donor do not seem to be factors in HMA expression.

TABLE 2. To see if HMA of epithelial cells was affected by culture age, some cultures were maintained (with weekly medium changes) for periods up to 55 days. Periodic monitoring of representative epithelial colonies demonstrated that HMA was independent of culture age. It should be emphasized that epithelial cells constantly maturate through cell division and thus, an "old colony" may be physiologically equal to a "young colony." Note that viable cell count increased over the 55-day period of the experiment, but specific HMA remained fairly constant.

TABLE 3. Although age of the epithelial cultures does not affect HMA, upon subdivision both the epithelial cell population and the HMA decreased precipitously.

TABLE 4. Epithelial colonies of rabbit, hamster, and mouse cells have more HMA than fibroblastic colonies from the same donors. However, due to the limited number of individual animals tested, HMA levels between species cannot be compared because of the possibility that there arevariations between individuals (even in these inbred strains) that are similar to that seen for humans.

NIH Swiss mouse and guinea pig cells did not form epithelial colonies on plastic but did grow on pigskin where their HMA was measured. Usually, however, cultures grown on pigskin had less HMA than epithelial colonies grown on plastic. This may be due to physical separation of carcinogen and cells by the dense layer of keratin that forms when epithelium is grown on pigskin.

TABLE 5. Petri dishes containing individual colonies of rabbit or human epithelial cells and fibroblasts were exposed to 12 chemicals for 4 days. After an additional 10 days, the cultures were screened

microscopically for evidence of cytotoxicity. Certain chemicals were selectively toxic to epithelial cells. These were BAP, DMBA, DBA, and MCA--all polycyclic hydrocarbons. Thus the relative level of HMA in epithelial and fibroblastic cells correspond with relative toxicity. On the other hand MNNG was selectively toxic to fibroblasts. Those chemicals that were toxic at a dose of 6.25 µg or less are carcinogens. DBA, which was toxic at 25 mg per ml is a weak carcinogen. The other chemicals tested are noncarcinogenic and are not toxic.

TABLE 6. This table is a summary of five experiments in which we tried to transform human skin cultures with methylcholanthrene. In the beginning we planned to look for morphological alteration as the primary indication of transformation. We also planned monitoring of chromosomes and fibrinolytic activity, growth in soft agar, and plating efficiency. However, only the morphological examinations and karyotypic analyses were made routinely. In none of the experiments did we notice any striking morphological changes that were definite and consistent from experiment to experiment or culture to culture. None of the small morphological changes that we did observe could be identified when we read the cultures as coded cultures. However, when karyotypic analyses were made we found that heteroploid conversion occurred in each experiment but only in those cultures which had been treated with methylcholanthrene. In the first experiment 2 of 2 cultures treated with 1 µg MCA per ml became heteroploid. Experiment 2--the single culture treated with .5 µg MCA per ml became heteroploid. Experiment 3--heteroploid conversion was noted only in 1 of 4 cultures treated with 1 µg MCA per ml. Experiment 4--we tested MCA at doses of 0 and 1 µg per ml. None of 6 control cultures, but 2 of 6 MCA treated cultures became heteroploid. In the last experiment, which is still in progress, we treated a number of primary cultures of epithelial and fibroblast mixtures with varying doses of MCA and phenanthrene and compared the degree of heterploid conversion in these culture to similarly treated D3 fibroblasts which were free of epithelial cells. At present, we have 2 cultures which have become heteroploid, and both of them had been treated with MCA. Note that the HMA level of the primary cultures was over 9,000 U for the epithelial cells and over 2,000 U for the fibroblastic. D3 fibroblasts, however, had only 574 U.

TABLE 7. This table presents the actual ranges of chromosome counts of a number of selected cultures listed in Table 6. No heteroploid conversion was detected in any culture not included in Table 7.

In experiment 1, heteroploid conversion occurred in both cultures treated with 1 µg MCA per ml but not in the 2 control cultures. Two cell colonies, which were physically removed from the parent culture and grown into mass cultures again, were also heteroploid.

In experiment 2, heteroploid conversion occurred in the culture treated with 0.5 µg MCA per ml. This culture and its control were sent to Dr. William Benedict for detailed karyotypic analysis by chromosomes banding techniques. Marker chromosomes unique to this cell culture were found in a number of metaphases at D40 and again at D47.

In experiment 3, unique marker chromosomes were found at D18 and again at D38. Other marker chromosomes were found in MCA induced heteroploid cultures in experiment 4. Thus, in each of these experiments, unique marker chromosomes were found in MCA induced heteroploid cultures. This means that there were proliferating aneuploid cells and that heteroploidy was not due to cell senescence. More heteroploid cultures were produced by MCA in experiment 5, but Dr. Benedict has not yet completed his karyotypic analyses of these cultures.

TABLE 8. Some cultures have been tested for other characteristics typical of transformed cells. The cell lines presented in this table were derived from experiment 3 (see Table 6). The MCA treated heteroploid culture had a higher plating efficiency than the control culture at all levels of serum tested from 0.5 to 10%. In 0.5% serum, colonies were formed only by the heteroploid culture. We do not yet know the plating efficiency in low serum of cultures derived from these colonies.

TABLE 1

METABOLIC CONVERSION OF BENZO(a)PYRENE
TO A WATER SOLUBLE FORM BY HUMAN SKIN
EPITHELIAL CELLS AND FIBROBLASTS FROM THE SAME DONOR

SOURCE OF SKIN				HYDROCARBON METABOLIZING ACTIVITY (pmoles BP/10^6 cells/3 days Plastic Substrate	
Sex	Race	Age of Donor (year)	Culture Age (days)	Epithelial[a,b] Colonies	Fibroblast[a,b] Colonies
M	NC	15	32	20,087 ± 3,155	7,618 ± 4,503
M	C	NB	23	18,336 ± 2,878	531 ± 350
M	C	.05	32	17,329 ± 9,425	1,665 ± 2,840
M	C	NB	24	17,212 ± 2,560	2,287 ± 684
M	C	19	30	14,581 ± 4,334	0[c]
F	C	11	24	11,751 ± 5,750	2,778 ± 754
M	C	.5	50	10,118 ± 4,346	2,540 ± 1,946
M	NC	9	25	9,127 ± 3,717	2,142 ± 819
M	C	.8	24	7,491 ± 2,800	0[c]
M	NC	15	42	3,874 ± 1,497	0[c]
F	C	.12	29	2,810 ± 678	699 ± 400
M	C	NB	28	2,100 ± 583	436 ± 150
M	C	NB	21	1,250 ± 400	219 ± 89

[a]Colony type is identified morphologically on plastic. Upon transplantation back to pigskin, epithelial colonies form epithelium, fibroblast colonies do not.

[b]Average and standard deviation from 3-5 colonies derived from the same culture flask.

[c]Below background ABBREVIATIONS: NB-newborn; C-Caucasian; NC-Noncaucasian (Negro)

TABLE 2

EFFECT OF CULTURE AGE ON THE
BENZO(a)PYRENE METABOLISM OF HUMAN SKIN EPITHELIAL CELLS[a]

Primary Culture Age (Days)	Description	Average Viable Cell Count/60 mm dish ($X10^6$)	Hydrocarbon Metabolizing Activity pmoles BP/10^6 cells/1 day (N=3 plates)
6		.04	3,970 ± 788
13	Subconfluent (Epithelial)	.07	2,940 ± 980
34		.78	2,389 ± 475
41		2.1	2,244 ± 838
	- - - - - - - -		
47	Confluent (75-90% Epithelial) (10-25% Fibroblasts)	12.8	2,855 ± 296
55		19.3	2,786 ± 894

[a]Cultures derived from foreskin of donor 1.25 years old.

TABLE 3

EFFECT OF SUBPASSAGE ON EPITHELIAL CELL
SURVIVAL AND BENZO(a)PYRENE METABOLISM OF HUMAN SKIN CULTURES

Culture Subdivision Level*	Culture Age at Time of Assay (Days)	Percent Epithelial Cells/Dish	Hydrocarbon Metabolizing Activity pmoles BP/10^6 cells/1 day (N=3 plates)
D_0	40	98	2,877 ± 701
D_1	3	21	485 ± 120
D_2	3	0	555 ± 180
D_3	3	0	448 ± 48
D_4	3	0	586 ± 24
D_5	3	0	736 ± 10

*Cultures derived from foreskin of donor 0.75 years old, grown to confluence (40 days), split 1:2 weekly thereafter with assay of triplicate dishes 3 days after last split.

TABLE 4

METABOLIC CONVERSION OF BENZO(a)PYRENE
BY MAMMALIAN SKIN CELLS OF VARIOUS SPECIES

DONOR	Age of Culture/days		Specific Metabolic Rate pmoles BP per 10^6 cells per 3 days		
	Plastic	Pigskin	Type of Colony on Plastic		Cultures on Pigskin
			Epithelial	Fibroblast	
Rabbit, Strain III	19	24	4,826	1,976	706
Rabbit, Strain WH	19	24	9,045	2,415	287
Rabbit, N.Z. White	35	38	12,872	6,410	4,446
Hamster, Syrian	30	14	1,391	162	1,844
Mouse, C57 B1/6	30	30	52,305	1,420	6,910
Mouse, NIH Swiss	30	30	No colonies	754	6,405
Guinea Pig, Hartly albino	30	30	No colonies	3,901	2,686

TABLE 5

TOXICITY OF CHEMICALS TO RABBIT OR HUMAN SKIN EPITHELIAL CELLS AND FIBROBLASTS GROWN ON PLASTIC

Chemical	Abbrev.	Skin Carcinogen	Skin Species	Input Concentration in 4 ml of medium - µg per ml										
				0.97	.195	.39	.78	1.56	3.125	6.25	12.5	25	50	100
anthracene	A		rabbit							-	-	-	-	-
			human							-	-	-	-	TEF
benzo(e)pyrene	BeP		rabbit						-	-	-	-	-	-
			human										-	TEF
benzo(a)pyrene	BaP	+	[1]rabbit				-	-	TE	TE	TE	TE		
			[1]human					TE	TE	TE	TE	TE		
dibenz(a,h)anthracene	DBA	±	[1]rabbit						-	-	-	-	-	
			[1]human						-	-	-	TE		
7, 12-dimethylbenz(a) anthracene	DMBA	+	[1]rabbit	-	TE	TE	TE	TE						
			[1]human		-	-	TE	TE	TE	TE				
dimethylnitrosamine	DMNA		rabbit							-	-	-	-	-
			[1]human										-	TE
diphenylnitrosamine	DPNA		rabbit						-	-	-	-	TEF	
			human									-	TEF	
methylazoxymethanol acetate	MAMA		rabbit	-	-	-	-	-	-	-	-	-	-	-
			human										-	-
3-methylcholanthrene	MCA	+	[1]rabbit					-		-	TE	TE	TE	
			[1]human					-	TE	TE	TE	TE	TEF	
N-methyl-N-nitro-N-nitrosoguanidine	MNNG	+	[2]rabbit	-	-	-	-	-	-	TF	TF			
			[2]human							TF	TFE	TFE		
pyrene	PY		rabbit							-	-	-	-	-
			[1]human									-	-	TE
phenanthrene	PH		[1]rabbit							-	-	-	-	TEF
			[1]human									-	-	TE

-non toxic*; T toxic, E epithelial; F fibroblasts [1]Selectively toxic to epithelial cells [2]Selectively toxic to fibroblasts
[3]Rabbit, New Zealand white; human, foreskin from 14 year old *Toxicity--gross cell alteration leading to death by 14 days after treatment

TABLE 6

HETEROPLOID CONVERSION OF HUMAN SKIN CELL CULTURES BY METHYLCHOLANTHRENE

Experiment Number	Treatment	Number of Cultures Per Test	Number Converted to Heteroploidy	Comments
1	Acetone	2*	0	From thighs of adult male
	MCA - 1.0 µg per ml	2*	2*	
2	Acetone	1	0	Foreskin
	MCA - 0.5 µg per ml	1	1	
3	Acetone	4	0	Foreskin
	MCA - 0.1 µg per ml	4	0	
	MCA - 0.5 µg per ml	4	0	
	MCA - 1.0 µg per ml	4	1	
4	Acetone	6	0	Foreskin
	MCA - 1.0 µg per ml	6	2	
5	Acetone	2	0	Primary Foreskin
	MCA - 1.0 µg per ml	2	0	HMA (pmoles/10^6 cells/3 days)
	MCA - 3.0 µg per ml	2	1	- epithelials cells 9,127 $\pm$ 3,717
	MCA - 5.0 µg per ml	2	0	
	MCA -10.0 µg per ml	2	1	- fibroblasts 2,142 $\pm$ 819
	Phenanthrene - 1 µg per ml	2	0	
	Phenanthrene - 10 µg per ml	2	0	
	Phenanthrene - 50 µg per ml	2	0	
	Phenanthrene -100 µg per ml	2	0	
	Acetone	2	0	D3 fibroblasts
	MCA - 1.0 µg per ml	2	0	HMA (574 pmoles/10^6 cells/ 3 days)
	MCA - 3.0 µg per ml	2	0	
	MCA - 5.0 µg per ml	2	0	
	MCA -10.0 µg per ml	2	0	

*One culture was also treated with 10 µg BdU per ml

TABLE 7

CHROMOSOMAL CHANGES IN SKIN CELLS TREATED WITH MCA[a]

Experiment # (Conditions)	Culture Code	D#	Treatment	Metaphases with Indicated Chromosome Count									
				<46	46	47-49	50-59	60-69	70-79	80-89	90-91	92	>92
Expt. #1 (Primary on Plastic)	A564	15	Acetone	2	8								
	A565	15	MCA - 1.0 µg	3	3				1	1	1		1
	A566	17		6			1		1	3			
	A566C-1	+11	MCA - 1.0 µg +BdU -10.0 µg	3			1	2	1	3			
	A566C-2	+11		4				2	4				
Expt. #2 (Primary on Pigskin)	A802	40*	Acetone	3	18							2	
		43-50		20	14								
		53*		5	3					1			
		54		5	5								
	A804	40*	MCA - 0.5 µg	2	17	2			11[b]				7[b]
		47-57*		6	6				1[c]	3[c]	1[c]		1[c]
Expt. #3 (Primary on Plastic)	A1040	6	Acetone	4	16								
		18*		3	19								
		21-31		9	30	1							
		35-38*		3	22								
	A1047	6	MCA - 1.0 µg	13	6	1							
		18*		2	5					2[d]	3[d]	14[d]	2[d]
		21-35		3	22		2	2					
		38-39*			4				1[e]	3[e]	5[e]	22	3[e]
Expt. #4 (Primary on Plastic)	A1113-A1118	3	Acetone	11	45	1						3	
		17		3	59						1	7	
		29*		1	28							4	
	A1121-	3	MCA - 1.0 µg	1	9								
		17		1	7	1	2	1[f]	2[f]	1	2	3	
		29*		1	11			1[f]	1[f]	1		1	4[f]
	A1124	3	MCA - 1.0 µg	1	8							1	
		17			3				1	1	1	4	
Expt. #5 (Primary on Plastic)	A1171, A1172	27	Acetone	5	14								
	A1175	27	MCA - 3.0 µg		6	1	1		3	3	3	3	
	A1179	27	MCA -10.0 µg		4			1	1	3		1	
	A1188	26	Phenanthrene - 100.0 µg		9							1	

Footnotes on next page

FOOTNOTES TO TABLE 7

[a]No heteroploid conversion was noted in culture lines listed in table 4 but omitted from this table,

[b]A total of 12 metaphases contained at least one similar dicentric marker chromosome.

[c]Metaphases contain the same dicentric marker chromosome seen at D40.

[d]A total of 6 metaphases contained a common dicentric marker chromosome not seen in the A804 cell culture.

[e]At least a total of 5 metaphases contained the same dicentric marker chromosome seen at D18.

[f]A total of 4 metaphases contain an abnormal marker chromosome not seen in the A804 or A1047 cell culture.

*Coded culture analyzed by W.B. using a blind protocol.

TABLE 8

PLATING EFFICIENCIES OF

CELL CULTURES A1040 and A1047

Line #	Treatment	Passage	Plating Efficiency[a] Serum Concentration 10%	5%	1%	0.5%
A1040	Acetone	28	13.5	16	12.5	N.D.
		32	N.D.	15	7.0	N.D.
		36	N.D.	12	7.0	0
A1047	MCA - 1.0 µg per ml	28	58	56	57.5	N.D.
		32	N.D.	55	43.5	N.D.
		36	N.D.	57	43.0	14

[a]Number of colonies per 60 mm petri dish, 100 cells planted, duplicate dishes at passages 28 and 32, triplicate dishes at passage 36.

N.D. - Not done

D. DISCUSSION

Human fibroblasts may become aneuploid during their senescent phase (22). This, however, is not the basis for heteroploidy in our experiments because we have observed the proliferation of cells with unique marker chromosomes over a number of population doublings. In view of the well documented karyotypic stability of presenescent human cells in general and of our control cultures in particular, we consider it to be highly significant that heteroploid conversion occurred after MCA treatment in 8 separate actively growing cultures derived from 5 different human donors.

Further study is needed to determine whether there is a relationship between in vitro heteroploid conversion of human cells and malignant transformation. There is, however, considerable evidence in other mammalian cell transformation systems that such is the case (1,2,24). In our experiments, there is some evidence that heteroploid conversion of human cells correlated with the acquisition of other characteristics of cell transformation. In experiment 2, the MCA treated heteroploid cell culture produced colonies in soft agar; in experiment 3, the MCA treated heteroploid cell culture had increased plating efficiency. In no case, however, has a continuously growing cell line been established. It should be noted that aneuploid human tumor cells usually have a short in vitro life span. In this sense, the reluctance of human aneuploid cells to proliferate under in vitro conditions is similar whether the cells are derived from tumors or from MCA treated cultures.

We do not know how universal the tendency toward heteroploid conversion is in human diploid skin cells treated with carcinogens of various classes versus noncarcinogenic agents with similar chemical structures. However, in 5 experiments we have shown that MCA induces heteroploidy and in 1 experiment we have shown that a noncarcinogenic polycylic hydrocarbon (phenanthrene) does not have this effect even at a hundred fold increase in dose.

The high frequency with which MCA induced heteroploid conversion in mixed skin fibroblast and epithelial cell cultures is in sharp contrast to the total lack of heteroploid conversion in MCA treated skin fibroblasts. Indeed, we have previously reported that MCA and other carcinogens only rarely induced any signs of cell transformation, including heteroploid conversion, in extensive attempts to transform a number of human fibroblasts derived from normal and malignant tumors (14). It seems likely that the epithelial cells either become heteroploid themselves and dedifferentiate to fibroblastic forms or that the epithelial cells, through their higher level of HMA, convert MCA to an ultimate carcinogenic form that causes

heteroploidy in cocultured fibroblasts. Either interpretation is consistent with the data, but we prefer the latter. If this is correct, MCA will induce heteroploidy in pure cultures of skin fibroblasts cocultivated with irradiated feeder layers of epithelial cells.

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3. CARCINOGENESIS STUDIES IN ORGAN CULTURE SYSTEMS

3.1 ANIMAL TISSUES

3.1.1 IN VITRO CARCINOGENESIS IN TRACHEAL ORGAN CULTURE

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Fragments of tracheal epithelium and associated connective tissues have been maintained in culture for studies of tissue function (2,39,56,69), infectious processes (1,5-9,11-13,16,25-28,31-37,49,52, 53,60-67,72-76), toxicity of various agents (20,22,23,54,51,70) and carcinogenesis (17-20,24,40,42,44,46-48,54,58). For these investigations a number of species have been employed including rat (17,18, 24,40-45,50,51,58,59), rabbit (22,23,56), mouse (49,59), cow (12,13, 61-63), hamster (14,15,19,21,26,38,55,66,68,76), chicken (2,5-8, 11,67), pig (60), ferret (27) and human (4,9,10,16,20,25,27,29-36 46-48,53,57,71-75). Fetal (2,6,8,9,11,12,16,25), young (17,48,60) and adult tissues (16,55,56) have been found to be suitable.

In most instances, lengths of airway have been cut transversely by hand with a scalpel into ring-shaped segments lined by mucosa. For some studies whole or split tracheas (14,17,48), squares of mucosa and cartilage (4,16,25), mucosa alone (45) and strips prepared by longitudinal sectioning of airway (75) have been used. Mechanical devices for rapid production of large numbers of uniform ring cultures have been developed (43).

The tissue fragments have been incubated in many different media, including Eagle's Minimal essential medium (8), Basal medium of Eagle (8,11), Parker's Medium 199 (13,49), Leibovitz's L-15 (4,5,56), CMRL 1066 (4,21), McCoy's 5a (modified) (43) and Waymouth's MB752 (50,54). Comparison of several of these has demonstrated that complex media sustains cellular proliferation more effectively than minimal essential media and its modifications (55). The buffer for these media has usually been sodium bicarbonate or HEPES. HEPES is more convenient because it does not have to be equilibrated with a particular gaseous environment, but it has been shown to alter glycoprotein synthesis by the cultures (21). Most incubating media contained serum or serum albumen although a number of studies have been done with serumless media (55) or without adding serum to media

to which it is normally added (43). When serum has been deleted, various additives including insulin and hydrocortisone have been used (4,14). Maintenance of normal epithelial histology and cellular differentiation is enhanced by addition of retinoids to the culture medium (2,51). Retinoids also reverse squamous metaplasia (14,68).

Conditions of incubation have included free floating (59), steel mesh supported (48,59), clot supported (19) and partially immersed rocker culture (14). The range of temperatures has been 35°-37°C. While high O_2 levels in the environment have commonly been used (20,58) 5% CO_2 in air has been shown to be effective for cultures which can be sustained for periods of months (42,55).

Cultures have been evaluated by autoradiographic technique for cellular proliferation (55,57), protein synthesis (51) and glycoprotein synthesis (21). Measures of mitochondrial function (26,51) have been made. There have been extensive studies of the histochemistry and ultrastructure of cultures (4,71). Secretory activity has been assayed (21,69) and the retention of ciliary function has been used in chemical cytotoxicity studies (51) and infection assays (6,12,36,60).

When injured mechanically (41) or chemically (42), the epithelium or organ cultures responds with mitotic activity. The response is limited however and eventually the cultures degenerate. Continuous exposure to carcinogens therefore results in atrophy or death of cultures if the concentration of carcinogen is sufficient to induce hyperplasia (42). Short term exposure to carcinogen dissolved in the medium produces a sustained hyperplasia (18,58) and this effect occurs more rapidly and at lower concentrations of carcinogen if serum is deleted from the medium (44). Focal hyperplasia can also be induced by local application of carcinogen using fibers coated with the agent (54). Carcinogen has been shown to bind to epithelial cell nuclei exposed in this way (38) and ultrastructural features of epithelium exposed to carcinogen in vitro (7,44) include nuclear and cell periphery alterations seen in vivo.

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3.1.2 IN VIVO – IN VITRO STUDIES WITH RESPIRATORY TRACT EPITHELIUM EXPOSED TO CARCINOGEN

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I. METHODS OF CARCINOGEN EXPOSURE AND ATTEMPTS TO DEFINE NEOPLASTIC PROGRESSION IN VITRO

In Vivo Exposure to Carcinogens

In vivo-in vitro experimental systems are particularly useful for investigating neoplastic progression in a target tissue: (a) because exposing the intact animal to the carcinogen allows for any necessary activation of the carcinogen, (b) the tissue of interest can be sampled at various selected times after injection or exposure to the carcinogen. With respect to (a), the carcinogen has been delivered transplacentally to fetal hamster cells in a host mediated in vivo-in vitro quantitative assay for chemical carcinogens (11). Long term cell cultures were derived from the brain cells of rat fetuses after a transplacental pulse of the nervous-system-specific carcinogen, N-ethyl-N-nitrosourea (19). The progression toward neoplasia in these cell lines was defined in five stages. The first four stages were phenotypic changes and the last stage was identified by growth in soft agar and the ability to form tumors. Organ cultures, made from the kidney of mouse embryos exposed transplacentally to several carcinogens, showed longer survival in culture than unexposed controls, as well as hyperplasia and the formation of cystadenomas in vitro (28). Kidney cells isolated as early as 20 hours after injection of dimethylnitrosamine had a prolonged growth span in vitro, and exhibited a morphological transformation during subculture (4). The resulting cell lines acquired the properties of colony formation in methycellulose gels and Concanavalin A agglutination during subculture.

In Vivo Exposure of the Respiratory Tract to Carcinogens

A major limitation to in vivo chemical carcinogenesis studies is the lack of knowing the actual amount of carcinogen delivered and the areas of the target tissue exposed. This is also true of the respiratory system. After a carcinogen is administered intratracheally or by inhalation exposure, it is distributed unevenly in the tracheo-bronchial tree and alveolar parenchyma (see 10,25 for reviews). Subsequently, a redistribution of the material occurs as it is cleared by various mechanisms. Several investigators have tried to

deal with these problems (16,18). Two new localized tumor-induction systems recently developed in our laboratory have overcome many of these difficulties (14,17,27). In one of these systems (27), the carcinogen is topically delivered to a circumscribed region of the trachea by a means of a special catheter. The second system is a tracheal transplant model (14,17), which is particularly well suited for quantitative studies, and is the system used for the in vivo-in vitro studies to be described in detail here. With the tracheal transplant system, the target tissue is well-defined, and uniform exposure of the entire tracheal epithelium at predetermined dose rates for various periods of time can be carried out (15).

Neoplastic Progression in the Respiratory Tract

The difficulty in distinguishing, morphologically preneoplastic lesions in the respiratory tract from lesions caused by other toxic effects of various substances has been discussed (24). Biological characteristics other than morphology are needed for identifying preneoplastic lesions, as well as detecting progressive changes in the epithelial cells during the evolution to neoplasia. One approach to this problem has been to collect biopsy specimens from human respiratory tract, and to grow the lesions in vitro as organ cultures to detect differences in growth behavior (8,26). The highly replicative state of dysplasias was maintained for 2 weeks, but squamous metaplasias were poorly sustained. The development of improved culture conditions for maintaining respiratory tissue in organ culture for long periods of time as reviewed in earlier presentations and also developed by us (20) should make this approach rewarding. A more experimental approach has been taken by Shabad, et al. (29). In their experiments, benzo(a)pyrene was delivered transplacentally to mice and the lungs of the fetuses maintained in organ culture. Hyperplastic preadenomatous and adenomatous type alterations of the lung epithelium appeared in culture. Administration of the noncarcinogen pyrene did not produce these effects.

We are studying the progression of neoplasia in the respiratory system by attempting to detect preneoplastic changes and indicators of neoplastic transformation by identifying alterations in the in vitro growth behavior of epithelial cells derived from respiratory tissue exposed to carcinogens in vivo (21). We use the tracheal transplant system described earlier (14,17) to uniformly expose the trachea to carcinogen. In the current studies two approaches are followed: (a) After exposure to carcinogen for brief periods of time explants are grown as organ cultures to determine the fate of the lesions in different nutritional environments; (b) In the second approach, the explants are maintained under culture conditions which stimulate outgrowth of epithelial cells. The cultures are monitored for the number of explants that produce outgrowths, the rates of

epithelial cell outgrowths and changes in epithelial cell morphology. This is correlated with the number of outgrowths that can be maintained as primary cell cultures under media conditions suboptimal for that required to maintain unexposed tracheal epithelial cells in primary culture for long periods of time (22). Early epithelial changes are manifested by an increased survival potential. Further indicators of changes in biological behavior due to carcinogen exposures are sought after in the cell lines established from such cultures.

II. METHODS FOR DEFINING ALTERED BIOLOGICAL BEHAVIOR OF EPITHELIAL CELL POPULATIONS

Introduction

The study of oncogenesis in epithelial cell systems introduces some variables different from those encountered in fibroblast models. Amongst these are (a) the possible involvement of differentiation and de-differentiation in epithelial cell transformation (3), (b) differences in the proliferation kinetics of epithelial cell populations (5), and differences in the phenotypic "markers" which can be used as indicators of transformation in epithelial cells (33). Such factors need to be taken into consideration when attempting to describe altered behavior of epithelial cells in vivo or in vitro.

There are several ways of approaching the problem of defining those characteristics of epithelial cell populations which are specifically associated with the oncogenic process. Cell cultures, amenable to detailed characterization in vitro, can be established from in vivo tumor populations. This approach yields useful information about the final stage or stages of oncogenesis, but yields little information about the processes involved. Information about earlier stages must be gained through a more detailed examination of changes occurring prior to the appearance of a frank carcinoma. Towards this end, cell populations can be exposed to a carcinogen in vivo and in vitro and then observed at regular intervals for carcinogen induced alterations in behavior in vitro and in vivo. The above general approaches to defining the altered behavior of epithelial cell populations will be discussed and referenced below.

Characterization of Tumor Derived Cells in Culture

Information obtained through this approach is in general as follows: (a) proliferation kinetics and plating efficiency under varying culture conditions (7,30,31,32), (b) morphology (light microscopy and EM) (2,30,32), (c) TD_{50} (tumor dose-50%) as a measure

of oncogenicity in vivo, (d) attempts to correlate the in vivo behavior of altered epithelial cell populations (1,34) or adjacent fibroblast populations with the behavior of same in culture (6). The above mentioned parameters have been described over variable lengths of time passed in vivo and in vitro. This allows some investigation into the effects of proliferation in vivo or in vitro on the phenotypic make-up of a cell population.

Characterization of Epithelial Cell Populations During the Development of the Transformed Phenotype

This approach to the characterization of transformed epithelial cells allows for the identification of "early" markers. Cell populations can be exposed to a carcinogen in vivo or in vitro and then described at intervals thereafter in vitro (12,13,23,35). Tumorigenicity of the cell population can be used as the final marker of oncogenic transformation (12,13,23,35). Criteria described are (a) increased capacity for continued proliferation in vitro relative to normal non-exposed cell populations (12), (b) changes in morphology (EM and light microscopy) (12,13,23,35,36), (c) growth in agar (36), and (d) tumorigenicity (12,13,23,35).

Other points are of particular note. Colburn (9) observes changes in the differentiated state of epithelial cells during the transformation process. Yamaguchi and Weinstein (36) have described details of the transition from wild-type to transformed-type behavior in a temperature sensitive mutant epithelial cell line.

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3.1.3 INDUCTION AND SUPPRESSION OF DYSPLASTIC GROWTH IN ORGAN-CULTURED URINARY BLADDER EPITHELIUM

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In view of the general lack of improvement in survival rates for various forms of epithelial cancer over the last 25 years, it is apparent that the traditional approaches for treating epithelial cancer - chemotherapy, surgery, or radiation intervention after the onset of invasion - have not been satisfactory. A new approach, in which incipient disease is detected and treated by novel means well before invasion, is clearly needed. (For a detailed discussion of the rationales for this approach see ref. 25.) It will be necessary to rely heavily on experimental animal models for this approach. Before work of this nature can be pursued, however, it is essential that we have a better understanding of the early (preneoplastic) phase of cancer development in experimental animals.

Although there is some disagreement on the interpretation of data (10), a considerable body of evidence (5,7,9,16,18,21-24) has accumulated on the preneoplastic phase of human bladder cancer which suggests to most investigators that the disease is a multifocal process of long duration which begins as hyperplasia and progresses through increasing degrees of dysplastic growth and cytological atypia before invading. (These characteristics are not exclusively those of bladder epithelial cancer but are probably features common to many, if not all, types of epithelial cancer, human and experimental (8)). There is general agreement that many cases of human bladder cancer, like human cervical cancer, are preceded by a carcinoma in situ stage of sometimes considerable duration. (For a discussion of the significance and implications of bladder carcinoma in situ and earlier lesions see ref. 11.) A well-defined carcinoma in situ stage in the bladder epithelium has important diagnostic significance because it signifies a readily identifiable stage in cancer development which, by definition, is non-invasive but which shows a high probability of progressing to invasive cancer if intervening measures are not taken. Histologically, human bladder carcinoma in situ is characterized by dysplastic growth and cytological atypia (9,17). Unfortunately, a well-defined bladder carcinoma in situ stage has not, as yet, been identified in experimental animals. Precancerous changes have, however, been identified. In rats fed the carcinogenic nitrofuran, N-[4(5-Nitro-2-furyl)-2-thiazolyl] formamide (FANFT), hyperplastic foci of endophytic and exophytic growth are seen by 8 weeks of FANFT

feeding (27). Recent evidence suggests that these lesions which exist at 8 weeks may be irreversible precancerous lesions (4,15). Were it not for the absence of severe cytological atypia, these lesions would thus fit the criteria for carcinomas in situ. In mice, to which the carcinogenic N-nitrosamime, butyl-(4-hydroxybutyl)-nitrosamime was administered continuously in the drinking water, endophytic growths which eventually became isolated as nests of Brunn were observed 4-6 weeks prior to the appearance of identifiable cancer and were considered by the authors to represent the last precancerous stage prior to the appearance of overt cancer (2). In humans, endophytic growth with the formation of Brunn's nests is frequently observed in association with bladder carcinoma in situ (7,9), however it is not clear whether these abnormalities constitute precursor lesions or whether they are merely manifestations of an unstable epithelium (17). Some investigators do conclude that endophytic growth "probably indicates aggressiveness even though actual infiltration has not begun" (12).

It is not known at this point if the dysplasias which are characteristic of the precancerous changes observed in the experimental bladder cancer systems described above are in fact precursor lesions. It is clear, however, that dysplastic hyperplasia in the transitional epithelium is cause for concern because it is a salient feature of precancerous bladder epithelium and because there is a direct correlation between dysplastic growth and the degree of malignancy in human (1) and experimentally-induced (13) tumors. We think, therefore, that an analysis of the process by which dysplastic growth arises and how it can be suppressed in the transitional epithelium can provide valuable information for understanding and devising means for controlling both the preneoplastic phase of cancer development and the malignant progression of tumors. A recently-developed organ culture system for bladder is being used to facilitate this analysis.

We have demonstrated (19) that urinary bladders from the rat can be maintained in organ culture for prolonged periods of time in chemically-defined protein-free, culture media. It was further shown that while two culture media, Eagle's MEM (6) and Waymouth's MB752/1 (26) allowed maintenance with only a small degree of non-dysplastic growth, Ham's F12 medium (14) produced extensive dysplastic hyperplasia which included the loss of normal transitional structure of the epithelium and endophytic growth culminating in Brunn's nests. These epithelial abnormalities mimic very closely the precancerous changes which are seen in the experimental bladder cancer systems discussed above (2,27). Recently, it has been found that F12-induced hyperplasia is suppressed by greater than 70% when hydrocortisone is added to the culture medium at a concentration of 2.1μM (20). Dysplasia

was also suppressed under these conditions. Nuclear-cytoplasmic ratios, which are elevated in F12-cultured epithelialcells (as well as in malignant cells), are also maintained at nearly normal levels by 2.1 μM hydrocortisone (26).

Although hyperplasia is often a prerequisite to dysplastic growth, we do not think that excessive hyperplasia is the sole explanation for dysplasia. Increased levels of hyperplasia can be induced in Waymouth's MB752/1 medium by elevating the concentrations of all media components without causing dysplastic growth (Reese, unpublished). Dysplasia may be caused, in large part, by an aberration in the normal maturation process of the transitional epithelium. This is suggested by recent pulse-chase experiments with ^{3}H-thymidine on F12-and MB752/1-cultured epithelia (Reese, unpublished) which indicate that the rate of cell loss from F12-cultured epithelia is only 20% of that observed for MB752/1-cultured epithelia. This suggests an abnormal accumulation of cells in F12-cultured epithelia which could result in the displacement of cells from their normal territories. Cells out of their normal territories may no longer be subject to the controls which determine ordered cell growth. This could thus explain dysplastic growth in F12 media. It may also explain the dysplastic characteristics of malignant growth (3).

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3.2 HUMAN TISSUES

3.2.1 EXPLANT CULTURE AND XENOTRANSPLANTATION OF HUMAN BRONCHI

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Epidemiologic studies of migrating groups, religious sects, occupational populations and tobacco smokers as well as numerous experimental investigations identifying carcinogenic chemicals found in man's environment have strongly suggested human cancer is caused by environmental agents, including chemical carcinogens (17). The epithelia of the respiratory and gastrointestinal tracts are the most important sites of these environmentally-related cancers. One of the major problems with these studies is that they provide important, but retrospective, information, i.e., "after the fact." Therefore, experimental model systems have been developed in attempts to study chemical carcinogenesis in target tissues. For example animal models of human lung (27), colon (6,33) and pancreatic (22) carcinoma have been developed during the last decade. While these animal models successfully mimic many of the features of the human disease, the extrapolation of carcinogenesis data from experimental animals to man is a difficult problem (9,32). The extrapolation of data from both in vitro carcinogenesis and mutagenesis studies is an even more complex problem. Model systems for studying carcinogenesis in human tissues have been developed (10). These systems provide an opportunity to investigate chemical carcinogenesis in the primary target tissues for human cancer. These model systems have three major facets: 1) obtainment of viable human tissues which involves all of the medical, legal and ethical aspects of human experimentation; 2) maintenance of these tissues in vitro; and finally 3) xenotransplantation of human tissues into immunodeficient animals.

This guide to the literature will focus on the methodology required to culture explants of human bronchus, how these explants can be used to investigate the metabolic activation of chemical carcinogens, and lastly, the xenotransplantation of both normal and neoplastic tissues into the athymic nude mice.

A. EXPLANT CULTURE OF HUMAN BRONCHUS

The in vitro maintenance of explants of human bronchi under controlled experimental conditions is an essential aspect of our attempts to develop model systems using human target tissues; the advantages of organ and explant culture are listed in Table 1.

Lasnitzki (19) is credited with being the first to maintain human respiratory epithelium in culture. Since then other investigators (2,4,20,21,28) have cultured lung tissue, including human bronchus, for periods up to 3 weeks. These investigators used culture medium containing serum, and/or the bronchial explants were maintained on plasma clots. Based on studies using organ cultures of hamster tracheas (3), a chemically defined medium for culturing human bronchial explants has been developed (1,15,29; Table II). Human bronchial specimens can be successfully cultured in this medium for periods of 1 month, and for 6 months in medium supplemented with 5% fetal bovine serum. Other conditions important for the culture of human bronchi are also listed in Table II.

B. METABOLIC ACTIVATION OF CHEMICAL CARCINOGENS BY CULTURED HUMAN BRONCHI

Tobacco smoke contains several chemical classes of known carcinogens (10,34). Of these, polynuclear aromatic hydrocarbons (PAH) were chosen for our initial studies. Previous reports by Lasnitzki (19) and Crocker et al., (4) indicated that both tobacco smoke condensates and PAH cause morphologic lesions, e.g., cytotoxitoxicity, hyperplasia and squamous metaplasia, in cultured respiratory epithelium. Using the chemically-defined medium described above to eliminate the complicating factor of serum, we reported that human bronchial explants bind carcinogenic PAH (7, 12-dimethylbenz(a)-anthracene, 3-methylcholanthracene, benzo(a)pyrene and dibenz(a,h)-anthracene) to cellular macromolecules (14). The most potent carcinogen in experimental animals of the four tested, 7,12-dimenthylbenz(a)anthracene, had the highest level of binding to DNA. Recently we have investigated the interaction of benzo(a)pyrene (BP) with bronchial explants (10,12,13,15,31). Binding of BP to cellular macromolecules is dependent on concentration of and duration of exposure to benzo(a)pyrene and to the temperature of incubation. Preliminary studies (8) suggest that the adducts formed between benzo(a)pyrene and DNA in cultured human bronchus have similar chromatographic properties as those adducts formed in both mouse skin and cultured hamster embryo cells. Autoradiographic analysis showed that bronchial epithelial cells bind approximately four times more BP than fibroblasts (15). While the intraindividual variation due to methodology is small (15), the variation of BP binding levels to DNA among individuals is quite marked (12); this 75-fold interindividual

TABLE I

ADVANTAGES OF ORGAN AND EXPLANT CULTURE

1. Maintains intercellular relationships between

   a. epithelial cells
   b. epithelium-mesenchyme

2. Maintains differing state of differentiated state in epithelial cells

3. Maintenance of tissue architecture allows

   a. morphological studies of tumorigenesis including possible identification of preneoplastic lesions
   b. correlation to histological lesions found in vitro to those observed in vivo

Table II

EXPLANT CULTURE OF HUMAN BRONCHUS

A. Culture Medium

   1. CMRL 1066, insulin (1 μg/ml), hydrocortisone (0.1 μg/ml), β-retinyl acetate (0.1 μg/ml), penicillin G (100 units/ml), Streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml)
   2. with or without heat-inactivated fetal bovine serum

B. Atmosphere and Incubation Temperature

   1. 50% O_2, 45% N_2 and 5% CO_2
   2. 36.5°C

C. Other Conditions

   1. submerge 50% of the time
   2. rock at 10 cycles per min

variation in the metabolism of BP is not surprising and may be due to genetic factors. Man is genetically a heterogeneous population, and pharmacogenetic studies in mono- and dizygotic twins have shown that the metabolism of drugs is controlled more by genetic factors than by exogenous agents (30). Since most environmental carcinogens, including PAH, require metabolic activation, one can hypothesize that genetic differences in the metabolic activation of chemical procarcinogens may be very important in determining an individual's risk of developing cancer. While indirect data supporting this hypothesis linking metabolic activation and individual susceptibility has accumulated in studies using cells in culture and experimental animals (16,32) it has not been adequately tested in man.

A number of naturally-occurring and synthetic compounds both alter the metabolism of PAH and to have an anticarcinogenic action in experimental animals by decreasing tumor incidence and/or by increasing the latency period of induced tumors (reviewed in ref. 16). Several of these chemicals have been tested for their ability to alter the level of BP binding to DNA in cultured human bronchial explants (13,15). Binding of BP was significantly inhibited by 7,8-benzoflavone and to a lesser extent by disulfiram, a nonphenolic antioxidant containing sulfur, and vitamin E. Co-incubation with either nicotine or β-retinyl acetate did not alter the level of binding under the experimental conditions tested. These studies indicate the possibility to both quantify and modify the interaction of chemical carcinogens with macromolecules in human target tissues.

In addition to polynuclear aromatic hydrocarbons, the metabolic activation of other chemical classes of carcinogens are being investigated. Ongoing studies (Harris et al., unpublished results) have shown that dimenthylhydrazine are metabolized in cultured human bronchi. While the metabolism of 1,2-dimethylhydrazine had not been previously studied in human tissue, slices of cultured human lung have been shown to metabolize dimethylnitrosamine (5). Matos et al., (24) have described hyperplastic and squamous metaplastic lesions in human fetal bronchial epithelium exposed to diethylnitrosamine. The morphological effects of dimethylnitrosamine and of 1,2-dimethylhydrazine on cultured human bronchus are as yet unkown.

One objective of these studies is to determine if the metabolic pathways of carcinogen activation are the same in human target tissues as it is in animal models; if so, the extrapolation of carcinogenesis data among species is more likely to be valid than if the pathways of metabolic activation are different. While such comparative studies have only recently begun, the metabolic activity pathway of BP in cultured human bronchus appears to be similar to that found in several experimental animals. Other classes of chemical carcinogens are currently being studied.

C. XENOTRANSPLANTATION OF HUMAN BRONCHIAL SPECIMENS IN ATHYMIC NUDE MOUSE

The ability of cells exposed to carcinogens in vitro to produce tumors when transplanted into appropriate hosts is considered to be the ultimate criterion for the assessment of malignant transformation. While human cells transformed to malignant cells in vitro cannot be transplanted into humans to test their tumorigenic properties, they can be xenotransplanted into immunodeficient animals; of the several immunodeficient recipients tested, the athymic nude mouse seems the most useful. A monograph (25) has recently been published which describes the genetics, husbandry and experimental utility of these animals. Adult and fetal normal human tissues have been successfully maintained as xenotransplants (23,26). In addition, human tumors grow and occasionally metastasize in athymic nude mice. Surgical specimens of cancers do not grow as frequently in athymic nude mice as cells from human cancers that have been initially cultured as monolayers prior to xenotransplantation (7). This observation may be due to ischemia in the solid tumor.

We have found that adult human bronchial specimens can be maintained as subcutaneous xenotransplants in athymic nude mice for periods in excess of 5 months (Valerio, M. et al., unpublished results). After an ischemic crisis and revascularization, the bronchial epithelium regenerates and attains a normal morphology by approximately 3 weeks. Using the approach described by Kendrick et al., (18) for exposing heterografts to carcinogens containing beeswax pellets, we have found that pellets containing 7,12 dimethylbenz(a)anthracene, inserted into the lumen of subsegmental human bronchi, cause hyperplastic and squamous metaplastic lesions in the bronchial epithleium (Valerio, M., Harris, C., et al., unpublished results). Therefore, xenotransplantation is a useful technique both for assays of the tumorigenicity of human cells exposured to carcinogens in vitro and for exposing human xenografts to carcinogens in vivo.

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4. MECHANISMS, METABOLISM, CO-FACTORS AND MARKERS

4.1 METABOLIC ACTIVATION OF CHEMICAL CARCINOGENS

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The number of chemicals known to induce cancer in animals is large and it is constantly increasing as more carcinogens are discovered, often as a result of new observations relating certain cancers with occupational or other exposure of human populations to synthetic industrial chemicals. A relatively small proportion of the known carcinogens has been studied in any detail. These compounds include various different chemical classes including certain polycyclic aromatic hydrocarbons, aromatic amines, aromatic azo compounds, biological alkylating agents, N-nitroso compounds, aliphatic azoxy compounds including cycasin, the naturally occurring glucoside of methylazoxymethanol, aflatoxins, pyrrolizidine alkaloids, urethane, 4-nitroquinoline-N-oxide, various chlorinated hydrocarbons and ethionine, the ethyl analogue of methionine. The chemical structures of these different types and classes of carcinogen are very different making it virtually impossible to predict potential carcinogenic activity from chemical structure except where there is close structural resemblence with known carcinogens.

Chemical carcinogens can be divided into two main groups based on their capacity or lack of capacity to induce cancer at the site of injection or when applied to the skin. Those compounds that are active at the site of application are described as direct, or locally acting carcinogens, while those that are active in organs or tissues remote from the injection site are described as indirect or remotely acting carcinogens. These contrasting patterns of activity can be readily illustrated by reference to the biological behaviour of the chemically simplest carcinogenic N-nitroso compounds, dimethylnitrosamine and N-methyl-N-nitrosourea. Dimethylnitrosamine which is chemically stable under physiological conditions, does not induce subcutaneous sarcomas at the injection site or tumors of the skin

after repeated topical applications. In contrast N-methylnitrosourea is unstable under physiological conditions and is a potent locally acting carcinogen.

After administration to rats, dimethylnitrosamine is rapidly and virtually uniformly distributed throughout the body but it induces tumors only in the liver and kidney, with occasional lung tumors. These observations are most readily explained by assuming that the parent nitrosamine is not itself carcinogenic but that it is converted by metabolism in the body to yield a carcinogenically active metabolite or metabolites. It is now generally recognized, largely from the work of James and Elizabeth Miller of the University of Wisconsin, that most chemical carcinogens require similar metabolic activation. The Millers introduced the terms precarcinogen, proximate carcinogen and ultimate carcinogen to describe, respectively, the original, relatively or completely inactive molecule, various metabolites of the original compound with greater carcinogenic activity and the active molecular species that is responsible for conversion of normal cells into tumor cells. All the ultimate carcinogens so far suggested are highly reactive electrophiles that can react with cellular macromolecules under physiological conditions in vitro and are mutagenic in suitable test systems. Examples of extensively studied precarcinogens are the polycyclic aromatic hydrocarbon 7,12-dimethylbenz(a)anthracene, the aromatic amine 2-naphthylamine, the aromatic azo dye p-dimethylaminoazobenzene (DAB) and the nitrosamine N-nitrosodimethylamine or dimethylnitrosoamine. Most precarcinogens are activated to form ultimate carcinogens by the enzyme systems known as microsomal mixed-function oxidases; occasionally activation is affected by other tissue enzymes; for example, 4-nitroquinoline-N-oxide by diaphorase. Another distinct mechanism of activation is mediated by microsomal enzymes of the intestinal flora. The best understood example of this mechanism is activation of the naturally occurring glycoside of methylazoxymethanol or cycasin, found in cycad plants.

Although the liver had long been recognized as the main organ for metabolism of foreign compounds, it was not until 1955 that Brodie and his colleagues at the National Institutes of Health demonstrated that the enzyme system concerned was located in the microsome fraction of liver homogenates. Subsequent work revealed that the enzyme activity was concentrated in the smooth membranes of the endoplasmic reticulum and there was the unusual requirement for reduced pyridine nucleotide (NADPH) and oxygen for activity.

A variety of reactions with foreign compounds are catalysed including aromatic hydroxylation, oxidation of alkyl side chains, O-dealkylation, N-dealkylation, N-oxidation, sulphoxidation and replacement of S by O. The basic mechanism of several of these

reactions is hydroxylation and the enzyme systems are, therefore, widely described as microsomal hydroxylases. Studies with isotopically labelled oxygen as the gas phase in hydroxylation reactions has indicated that one atom of oxygen from the atmosphere is incorporated into the hydroxyl group added to the foreign compound leading to the naming by Mason of the enzymes as mixed-function oxidases. It is well established that this microsomal enzyme system is involved in hydroxylation and other reactions that modify steroids and fatty acids as well as foreign compounds.

The mixed-function oxidases are membrane bound multicomponent enzyme systems containing cytochrome P-450, cytochrome b_5, NADPH- and NADH cytochrome reductases and various lipid and other components. The system is inhibited by carbon monoxide, which binds to cytochrome P-450 and by a number of other compounds of which SKF-525A (2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride) is one of the most extensively used. Substrates of the microsomal mixed function oxidases cause spectral changes in cytochrome P-450 of two types. Type I spectra, exemplified by ethylmorphine, hexobarbitone and most foreign compounds show peaks at 385 mμ and troughs at 420 mu. Type II spectra, produced by the addition of aniline and some other bases, show a reverse type of change, with a trough at 390 mμ and a peak at 430 mμ.

Microsomal mixed-function oxidases are induced by treatment of animals with a variety of chemicals. There appear to be at least three different mechanisms of induction including that produced by phenobarbitone and most other drugs, that produced by polycyclic aromatic hydrocarbons and that by anabolic steroids. Most experimental work on mechanisms of stimulation has been done with phenobarbitone and either 3-methylcholanthrene or 3,4-benzo(a)pyrene. Recently, various polychlorinated biphenyls (PCBS) such as Aroclor 1254 have been found to be potent inducers and the most powerful agent known is a dioxin originally discovered as an impurity of PCBS. Induction of microsomal mixed function oxidases is accompanied by morphological changes in the liver characterized by marked increase in the smooth endoplasmic reticulum. The enzyme systems can be induced in cells in culture. Reactions catalyzed by the mixed-function oxidases that are important for chemical carcinogenesis include epoxidation, N-hydroxylation and N-dealkylation.

Epoxidation is now thought to be an important metabolic pathway in the activation of polycyclic aromatic hydrocarbons, aflatoxins and some chlorinated hydrocarbons. Epoxidation of the polycyclics was first shown to occur at the so-called K-region of the molecule. The K-region is a bond having double bond character, like the 9,10 bond of phenanthrene, rather than aromatic character, the name being derived from Krebs, the German word for cancer.

Chemically, the K-region is a bond which takes part in addition reactions and it has been claimed to be essential for carcinogenic activity on theoretical quantum chemical grounds by A. and B. Pullman.

Extensive studies on metabolism of benz(a)anthracene and other polycyclics by Sims and Grover in the United Kingdom and by Heidelberger and his colleagues in the United States have shown that epoxidation occurs at the K-region and at other bonds in the molecule and that this is followed by various competing reactions including conversion to the dihydrodiol by the microsomal enzyme epoxide hydrase, non-enzymic rearrangement and conjugation with glutathione by cytoplasmic glutathione-S-epoxide transferase, all of which lead to deactivation, and reactions with cellular constituents, thought to be responsible for the induction of cancer and other biological actions of the compound. More recent work suggests that epoxidation at the 8,9 bond with formation of 8,9-dihydro-8-9-dihydroxybenz(a)anthracene may be more closely related to the carcinogenic action of benz(a)anthracene. The activating enzymes, usually called polycyclic aromatic hydrocarbon (PAH) hydroxylases, can be induced in vivo and in vitro by various inducers and can be inhibited by 7,8-benzoflavone. There is evidence for a similar type of activation by epoxidation for aflatoxin B_1 with the formation of the 2,3-epoxide, and for certain chlorinated hydrocarbons, including vinyl chloride, with formation of the corresponding epoxide or oxirane.

Another important metabolic pathway for the activation of chemical carcinogens is N-hydroxylation, again discovered in the laboratory of the Millers in Wisconsin. This pathway is followed by the aromatic amine and azo carcinogens. Metabolic hydroxylation occurs on the carbon atoms of the aromatic rings (C-hydroxylation) and on the nitrogen of the amine groups (N-hydroxylation). C-hydroxylation is thought to represent deactivation and N-hydroxylation activation of the carcinogens. The details of these pathways were largely worked out with the aromatic amine, N-acetylaminofluorene (N-2-fluorenylacetamide; AAF). The hydroxylation product, N-hydroxyacetylaminofluorene (N-hydroxy AAF) is a good example of a proximate carcinogen, being more active than the parent molecule but not mutagenic or capable of reaction with macromolecules. Esterification of the hydroxyl group produces more reactive and mutagenic products. N-acetoxyacetylaminofluorene (N-acetoxy-AAF) is mutagenic and will transform cells in culture without further activation.

Carbon tetrachloride, a relatively weak carcinogen, is activated by the microsomal enzymes, and the trichloromethyl radical has been suggested as a possible active species. Free radical formation may also be involved in the activation of other carcinogens.

N-dealkylation with formation of alkylating intermediates has been suggested as the mechanism of metabolic activation of the carcinogenic nitrosamines, dialkylhydrazines, alkyl azo- and azoxy compounds and the dialkylaryltriazenes. Dimethylnitrosamine is thought to undergo hydroxylation of one methyl group followed by release of formaldehyde, rearrangement to the diazohydroxide followed by decomposition to the methyldiazonium cation and then the methylcarbonium ion with release of molecular nitrogen and methylation of cellular macromolecules and other components, 1,2-dimethylhydrazine and azoxymethane appear to be metabolized in a similar manner. The carcinogenic nitrosamides such as N-methylnitrosourea, mentioned above, and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) do not require enzymic activation for biological activity. MNNG has been widely used for in vitro cell transformation.

Cycasin, the naturally occurring glucoside of methylazoxymethanol (MAM) is activated in quite another manner, i.e., by glucosidases of the microbial flora of the gastrointestinal tract. There is evidence that the further metabolic steps after release of the glucose moiety become identical with those for dimethylnitrosamine and N-methylnitrosourea. It is clearly improbable that cycasin would be active in transformation in vitro but the synthetic compound, methylazoxymethanol acetate (MAM acetate) is activated by tissue esterases and does have effects on cells in culture.

From the above considerations, it is clear that in vitro transformation will not occur if the cells exposed to precarcinogens do not contain appropriate activating enzymes. An obvious way to circumvent this difficulty is to use agents such as MNNG, that do not require metabolic activation, or to prepare proximate carcinogens synthetically, e.g., epoxides of polycyclic hydrocarbons or N-acetoxy AAF. Another method, exploited by Heidelberger and his colleagues, is to use a "feeder" layer of irradiated cells, capable of activating the precarcinogen, upon which the target cells for transformation are grown.

Activation may also be achieved by the so-called in vivo/in vitro method, developed by DiPaolo and his colleagues, in which pregnant animals are treated with the precarcinogen and cultures obtained from the progeny. Tissues for culture may also be taken from the treated animals themselves shortly after exposure to the precarcinogen.

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4.2 CERTAIN ASPECTS OF CHEMICAL CARCINOGENESIS

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I. THE POSSIBLE INVOLVEMENT OF FREE RADICALS IN BENZO(a)PYRENE CARCINOGENESIS

INTRODUCTION

A substantial amount of evidence has been accumulated in support of the notion that metabolic activation is often a prerequisite for the action of chemical carcinogens, particularly the polycyclic hydrocarbons. One hypothesis postulates that metabolic activation of carcinogens produces electrophilic intermediates which react with nucleophilic sites of vital macromolecules in cells, thereby initiating neoplastic transformation. This notion has often been interpreted to mean that the crucial, causative, chemical event is a two-electron reaction resulting in a stable, covalent bond between the carcinogen and the suspected target molecule, DNA.

There has been other evidence, however, which indicates that one-electron, or free radical, mechanisms may also be related to the action of chemical carcinogens (27) as well as perhaps being responsible for radiation damage to tissue. Free radicals as a group are generally considered to be electrophilic reagents. They are capable of altering important macromolecules in cells, not only by covalently binding with the macromolecules, but also by cleavage of chemical bonds leading to their degradation. The propagation reactions between free radicals, produced either by carcinogens or radiation, and cellular macromolecules are complex. One principal mode, which has been documented for radiation-induced DNA damage (7) is hydrogen atom abstraction. This process produces a reactive free radical site in the macromolecule which can undergo many further reactions leading eventually to degradation or strand scission. In this section, the radical species produced by metabolism of benzo(a)pyrene and the reactive reduced oxygen species produced in one-electron autoxidations will be described. These molecules can cause demonstrable breakage in nucleic acids and inflict cytotoxic effects on treated cells.

ENZYMIC FORMATION OF THE 6-OXO B(a)P RADICAL

Benzo(a)pyrene is metabolized by microsomal mixed function oxidases to a number of products. The sixth position of B(a)P is chemically more reactive than the other sites on the hydrocarbon (6),

but 6-OH-B(a)P often is not reported as a metabolite although its formation has been implied by the detection of the 6-OH-B(a)P glucuronide in vivo (9). Negata et al. (21) demonstrated that 6-OH-B(a)P can be detected in the metabolism of B(a)P in rat liver homogenate, as well as in mouse and rat skin homogenates, via its phenoxy radical, 6-oxo-B(a)P. This observation has been confirmed and extended by our laboratory (16).

Upon incubation of B(a)P in uninduced rat liver homogenates fortified with a NADPH-generating system at 37°, a metabolite was formed which gave rise spontaneously to an EPR signal. The metabolite and the radical were extracted quantitatively into benezene in which they were relatively stable. The metabolite also could be quantitatively converted to the radical by shaking with aqueous solutions of 2,6-dichloroindophenol or $K_3Fe(CN)_6$. The EPR signal obtained with B(a)P was identical to that observed after incubating 6-OH-B(a)P in fortified rat liver homogenates and has been identified as the 6-oxo-B(a)P radical by its characteristic hyperfine structure (18,11). No EPR signal was seen when B(a)P or cofactors were eliminated from the incubation mixture or when the homogenate was heated at 65° for 10 minutes. These observations indicate that the oxidation of B(a)P to 6-OH-B(a)P was enzymic in nature.

The decay of added 6-OH-B(a)P in rat liver homogenates showed first order kinetics with a rate constant of 0.29 $minutes^{-1}$. The half-life of added 6-OH-B(a)P in this system, thus, was about 2.4 minutes. The rate of degradation of 6-OH-B(a)P was not decreased by either elimination of the NADPH-generating system or by heating the homogenate at 65° for 10 minutes, indicating that this reaction was nonenzymic in nature.

The products formed by incubating 6-OH-B(a)P in rat liver homogenates were extracted into benzene and isolated by aluminum oxide chromatography (18). The data show that 6-OH-B(a)P was oxidized in this system and the following were the only products detected after 40 minutes of incubation: 6,12-B(a)P dione (15%), 1,6-B(a)P dione (41%) and 3,6-B(a)P dione (44%).

We have estimated the percentage of B(a)P metabolism proceeding through the 6-OH-B(a)P pathway by comparing the initial rate of 6-OH-B(a)P formation (measured via the 6-oxo-B(a)P radical) with the initial rate of total B(a)P metabolism. The comparison indicated the percentage of metabolism proceeding through 6-OH-B(a)P was about 18% and 20% for the liver homogenates of female Sprague-Dawley and female ACI rats respectively. The reaction rates were measured at relatively high substrate concentration to ensure zero-order kinetics. This value of about 20% of B(a)P metabolism proceeding through the

6-OH-B(a)P pathway agrees reasonably well with the proportion of B(a)P diones produced from B(a)P metabolism (14). Apparently, the bulk, if not all, of B(a)P diones formed during B(a)P metabolism have 6-OH-B(a)P as a precursor.

AUTOXIDATION OF 6-OH-B(a)P

We have synthesized 6-OH-B(a)P from B(a)P using modifications of the procedure of Fieser and Hersberg (1939). It is a labile compound. In aqueous buffer-ethanol (1:1) solutions, synthetic 6-OH-B(a)P is autoxidized to a mixture of three stable B(a)P diones, 6,12-; 1,6-; and 3,6-, plus a small amount of an unidentified paramagnetic, violet-colored material (18). In this reaction molecular oxygen is consumed. Some of this oxygen is reduced by electron transfer which is evident by the apearance of H_2O_2 during the autoxidation as determined by enzyme assay. H_2O_2 is the most stable reduced oxygen species (besides H_2O) and the easiest to determine. But the paramagnetic nature of ground state molecular oxygen virtually ensures that autoxidations proceed by one-electron steps, a notion supported in this specific case by the formation of 6-oxo-B(a)P radical in the autoxidation process. Therefore, it is virtually certain that other very reactive reduced oxygen species, such as the superoxide and hydroxyl radicals, are formed transiently in this autoxidation as well. The free radical derived from 6-OH-B(a)P by one-electron oxidation, the 6-oxo-B(a)P radical, is an obligatory intermediate in the autoxidation as indicated by its kinetics for formation and decay (18).

The 6-oxo-B(a)P radical could be produced quantitatively in benzene by oxidation with aqueous $K_3Fe(CN)_6$ and isolated in the absence of oxygen. Like 6-OH-B(a)P, this isolated free radical was autoxidized in aqueous buffer-ethanol solutions and yielded identical products. In addition to this data, recent experiments reveal the incorporation of ^{18}O into B(a)P dione products from 6-OH-B(a)P autoxidation with $^{18}O_2$. All of this suggests a reaction mechanism for autoxidation of 6-OH-B(a)P in which molecular oxygen couples directly with the 6-oxo-B(a)P radical at the positions of high spin density (11), producing ketoperoxyl radicals. Several subsequent pathways from the ketoperoxyl radicals to B(a)P diones are conceivable. The most likely would involve the formation of hydroperoxides or disubstituted peroxides, followed by the decomposition of these peroxides to produce B(a)P diones.

ACTION OF B(a)P DIONES OF DNA AND CELLS IN CULTURE

As noted earlier, the products of 6-OH-B(a)P autoxidation are the 6,12-; 1,6- and 3,6- B(a)P diones. These quinones are routinely identified products of B(a)P metabolism in various tissues and

generally account for about 20% or more of observed products. Although easily isolated, they are not totally unreactive products of B(a)P metabolism. Incubation of B(a)P diones with T7 DNA produces DNA strand breakage as measured by alkaline sedimentation. This ability to damage DNA is inhibited by removing oxygen and by the presence of either superoxide dismutase or EDTA. On the other hand, it is increased by the presence of a cellular reducing agent, NADPH, or a transition metal ion, Cu^{++}. The observations suggest that the DNA strand scission is caused by reactive, reduced oxygen species produced in an one-electron redox reaction facilitated by B(a)P diones.

The B(a)P diones are quite toxic to cells in culture. This is indicated by the inhibition of uptake of $\{^3H\}$ thymidine into the DNA of Syrian hamster embryonic (SHE) fibroblasts in the presence of B(a)P diones. This inhibition is greater by 1,6 and 3,6 B(a)P diones than 6,12 B(a)P dione. Cloning efficiency of SHE cells is also reduced by B(a)P diones; the most cytotoxic is 1,6 B(a)P dione, but all three diones are considerably more cytotoxic than B(a)P.

PROPERTIES OF THE B(a)P DIONE/B(a)P HYDROQUINONE REDOX COUPLES (19)

Quinones in general, including B(a)P diones, can undergo a wide variety of chemical reactions, but are best characterized by their ability to participate in reversible, one-electron, redox reactions involving the hydroquinone and semiquinone radical forms. B(a)P diones can be easily reduced to semi-quinones and hydroquinones as monitored by ESR and absorption spectroscopy. We have observed that even such cellular reducing agents as NADH, NADPH, cysteamine, and glutathione can reduce, wholly or partially, B(a)P quinones to hydroquinones in the absence of oxygen as shown below:

$$\text{B(a)P hydroquinones} + O_2 \xrightarrow{\text{Very fast}} \text{B(a)P quinones} + H_2O_2$$

$$\uparrow \text{reducing agent} \uparrow$$

Reducing agents: $NaBH_4$, $Na_2S_2O_4$ (dithionite), NAD(P)H, cysteamine, gluthathione

This reduction of the B(a)P diones is readily reversible. Thus, B(a)P hydroquinones prepared from diones under anaerobic conditions are autoxidized very rapidly back to B(a)P diones by air in aqueous-alcohol solutions. This cycle of quinone→hydroquinone→quinone can be repeated many times as shown above. As a result of each autoxidation of B(a)P hydroquinone to B(a)P dione, hydrogen peroxide is produced in near stoichiometric amounts. In parallel with other autoxidations, superoxide and hydroxyl radicals are likely to be produced as well. The semiquinone radicals are reaction intermediates in this cycle.

There is considerable evidence in the literature to suggest that quinones of widely varying structure may act as electron acceptor substrates for many respiratory enzymes in cells (20). In the case of B(a)P diones, this enzyme reduction to B(a)P hydroquinones would be immediately followed by autoxidation to produce the dione and reduced, reactive oxygen species (H_2O_2, O_2, ·OH). Using a commercially available diaphorase {NADH dehydrogenase from Cl. Kluyveri (1.6.99-)} we have demonstrated the operation of this cyclic process.

THE METABOLISM OF BENZO(a) PYRENE BY PURIFIED RAT LIVER NUCLEI AND SYRIAN HAMSTER EMBRYO HOMOGENATES

In view of the possible significance of metabolic activation of benzo(a)pyrene within nuclei to the problem of chemical carcinogenesis and the extensive use of Syrian hamster embryos as the experimental system for in vitro carcinogenesis, we have studied the biochemistry of B(a)P metabolism in purified rat liver nuclei and nuclear membranes as well as the homogenates of the Syrian hamster embryos. The main approach adopted in this study is to investigate whether the nuclei preparations and the microsomal preparations have independent and different metabolic pathways for B(a)P. The ideal situation would be to investigate the metabolic profiles of these two preparations and to compare them.

Various reports have appeared in the literature which indicate the activation of B(a)P by nuclei preparations. However, it was difficult to rule out the possibility that nuclear activity resulted from contamination by microsomes (1,13,22,24,30,31). In fact, in a recent article (1) it was claimed that BP activation by these two fractions is indeed very similar. In our approach, in the first step of investigating this problem, i.e., to establish the possibility of differences between nuclear AHH versus microsomal AHH, we emphasize the metabolism at the sixth carbon of B(a)P versus other carbon positions of this hydrocarbon. This approach can be supported by two types of experimental systems:

(1) By measurement of tritium released from generally labelled and selectively labelled 6-^{3}H-B(a)P in the presence or absence of a modulator of AHH activity.

(2) By electron paramagnetic resonance measurements of the radicals generated from phenolic metabolites of B(a)P after chemical oxidations by a series of selection one-electron oxidants. The ratio of the amount of radicals produced by these different oxidants corresponds to the proportions of phenols in the metabolic products.

The nuclear preparation was first made (3,28) and then the nuclear membrane preparation was made from the purified nuclei. The

removal of DNA and other nuclei contents makes the preparation of the nuclear membrane much more manageable and far less contaminated (2). The data from the tritium release studies clearly indicate that the metabolic pathway at the 6 position is twice as extensive in the nuclear membrane preparation as opposed to that of the microsomal preparation. In addition, the inhibitory effect of 7,8-benzoflavone is very large in the microsomal preparation but is minimal in the nuclear membrane preparation. Preliminary results based on esr measurements of the 6-oxo radicals also substantiate this finding that there exists a higher percentage of metabolism at the 6 position of B(a)P, compared to all of the other positions of B(a)P, in nuclear membrane preparations than in microsomes.

Research has indicated the appearance of the 6-oxo B(a)P radical upon the metabolism of B(a)P in Syrian hamster embryo homogenates. In addition, the early results suggest that the metabolic pathway at the 6 position as compared to all other positions of B(a)P is two-three times higher in the embryo preparations than in the liver preparations. This result implies that the metabolism at the 6 position is a predominant pathway in the embryo preparation.

Thus, research results on the metabolism of B(a)P in the nuclear membrane preparation and the Syrian hamster embryo homogenates are in accord with each other: nuclear preparations could have a pathway which is different and independent from that of the microsomes; biological materials which are rich in microsomal activities such as liver would therefore be expected to have a different profile of metabolism of B(a)P from that of biological material enriched in nuclei such as embryonic tissue. The nuclei preparation and the homogenates from biological material enriched with nuclei all show that the metabolic pathway at the 6 position of B(a)P could be a predominant pathway. This finding could have important implications for carcinogenesis research.

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II. THE RELATIONSHIP BETWEEN NEOPLASTIC TRANSFORMATION AND SOMATIC MUTATION

To study the role of somatic mutation in carcinogenesis, a system of normal, diploid Syrian hamster embryo (SHE) cells has been developed in which both processes can be investigated concomitantly. For somatic mutation, 8-azaguanine resistance ($8AG^r$), 6-thioguanine ($6TG^r$) (1,2,8,9,11,19,21,24,29,31-36,39-41) and ouabain resistance (oua^r) (3,10,20,25,26) markers have been characterized quantitatively with SHE cells and a subdiploid transformed cell line (BP6T) derived from SHE cells. The mutation frequencies of $8AG^r$, $6TG^r$ and oua^r of the normal SHE and BP6T cells are within a 4-10 fold range. Isolated mutant clones of both cells are stable and have similar cloning efficiencies in normal and appropriate selective media. Isolated $8AG^r$ and $6TG^r$ clones have very low levels of HGPRT activity (33) and low reversion frequencies as measured by their growth in HAT medium (35,36). The uptake of potassium or rubidium by the Na^+/K^+ ATPase

system of the cells is inhibited by ouabain (20,26). The uptake of ^{86}Rb by oua^r clones is less inhibited by ouabain than wild type cells, a finding consistent with the notion that the ouabain resistance trait is due to a mutation in the Na^+/K^+ ATPase enzyme. Transformation of SHE cells (6,7,12-18,22) has been quantitated by a morphological assay and isolated transformed clones are tumorigenic. The concomitant investigation of morphological transformation and somatic mutation of SHE cells revealed that the morphological transformation frequency is 25-540 fold greater than the mutation frequency.

It has been postulated by Foulds (18) and others that oncogenesis in vivo characteristically occurs as a progressive event, exhibiting numerous qualitatively different stages. It is our aim to determine whether neoplastic transformation of the Syrian hamster embryo system in vitro proceeds through such a series of progressive changes. The experimental design involves the examination of the temporal acquisition of several phenotypes of the transformed state following exposure of the cells to benzo(a)pyrene.

It was found (4,5) that somatic mutation and morphological transformation can be obtained within 3-7 days after carcinogen treatment. However, attainment of other transformation properties, such as fibrinolytic activity (23,27,28,30,37,38), growth in soft agar and tumorigenicity requires additional growth of many weeks in culture.

These results suggest that transformation in vitro of Syrian hamster embryo cells occurs in a progressive manner, therefore correlating the process of in vitro transformation with in vivo transformation. Thus, neoplastic transformation is a continuous, multistep, progressive process, while single-gene somatic mutation is a single-step process. Neoplastic transformation may be initiated by a mutational change, but cannot be adequately described by this mutational event.

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III. CHANGES IN DNA SEQUENCE ORGANIZATION AND GENE EXPRESSION IN NEOPLASTIC TRANSFORMATION

In order to determine the role of nucleic acid changes in neoplastic transformation, we are investigating alterations in DNA sequence organization and the extent of new transcription in B(a)P transformed Syrian hamster embryo (SHE) cells (83,84). Ultimately, we hope to answer two fundamental questions. First, is DNA sequence loss or DNA sequence organizational change, either specific or nonspecific, causally related to neoplastic transformation? In other words, is it necessary to change the DNA, in some gross manner, for transformation to occur? Furthermore, even if DNA organization changes are not important for transformation, it seems obvious that transformation, whether virally or chemically induced, must involve the qualitative or quantitative change in the expression of at least one gene. Our second immediate objective, therefore, was to determine the extent of new transcription in transformed cells. Are ten genes "turned on" - or 10,000?

We have begun our analysis by comparing a newly transformed sub-diploid cell line (18 CL 10), transformed in vitro by B(a)P, and its

highly malignant tumor cell derivative (BP6T) to normal diploid SHE cells. As few as ten BP6T cells produce tumors in 100% of injected newborn hamsters. Karyotypic studies indicate that both 18 CL 10 and BP6T have a modal chromosome distribution of 40 in comparison to the normal 44 chromosomes of cultured diploid SHE cells (37-39,92). This loss in chromosome number appears to be the result of actual chromosome loss and not due to Robertsonian fusions and translocations. Losses of B and D group chromosomes are apparent, and further studies utilizing various chromosome banding techniques (72) are in progress to determine if this line exhibits the "balance" of specific chromosomes found associated with malignancy by various investigators (4,14,61,113).

These karyotypic studies are being extended to the molecular level by analysis of DNA-DNA reassociation kinetics (19-22,40,70,93,104,109,110). If one isolates DNA from normal SHE cells, denatures it, and allows the sheared DNA to reassociate, at least three kinetic components are apparent (34,36,45,56,74,98,115). Using T7 and E. coli as standards, approximately 5% of the genome consists of highly repetitive or fold-back DNA sequences (27,43,90,99,103) with a $Cot\frac{1}{2} < 10^{-3}$; 35% behaves as moderately repetitive sequences (15,23,24,26,28,34,77,107) with an average $Cot\frac{1}{2}$ value of 2.5. These sequences consist of between 250 and 2,000 families repeated 500 to 5,000 times per genome; and 60% behaves like nonrepetitive sequences present only once per genome (34). These various kinetic components can be isolated in pure form. While the reassociation kinetics of tumor cell DNA are not yet complete, preliminary data indicate a 10% decrease in DNA sequence complexity in BP6T. This loss is in both repetitive and single-copy DNA sequences. Flow-microfluorometric analyses of normal and tumor cells indicate a decrease of 5-10% in DNA content in BP6T (32,71). Other probes of specific DNA sequences such as the rRNA cistrons (12,42,47,52, 64,87,112) and poly(A) tracts (9,100) are also being exploited. The relevance of these intriguing DNA changes to neoplastic transformation is currently under investigation.

The extent of new transcription in transformed cells is currently being investigated by examining polysome-associated poly(A) mRNA, a cellular component whose role in protein synthesis is well defined (1,2,18,33,41,58,65,66,75,76,82,86,88,89,95). The sizes of poly(A) mRNAs in SHE cells, 18 Cl 10, and BP6T appear similar, having a heterogeneous distribution with a maximum at approximately 2,000 nucleotides. The gross turnover rate, measured by the approach to steady-state labeling with ^{3}H-Uridine in logarithmically growing cells (16,17,57,67,91,100,102), indicates a similar turnover rate in all three cell types, with a half-life approximately equal to the doubling time of the cells.

There is also no apparent gross qualitative change in the types of mRNAs produced in these three cell types. Trace amounts of steady-state ^{3}H-labeled polysomal poly(A) nRNA were hybridized to a vast excess of hamster organ DNA, and hybridization was assayed with RNase (5,6,8,25,35,51,59,62,69,81,105). Under these conditions, the probability of forming a hybrid is related only to the concentration of the complementary sequence in the DNA. Repetitive sequence transcripts will hybridize at lower Cot values than will single-copy sequence transcripts. Fourteen to fifteen percent of the mRNA in all three cell types is complementary to mid-repetitive DNA sequences, while the remainder of the hybridizable mRNA appears to be complementary to single-copy DNA sequences. No significant differences are apparent between normal and transformed cells.

Early investigations of gene transcription changes during transformation (30,31,73,85,107) were limited to changes in repetitive sequence transcripts (13,50,68,80,93,106,108). Recent advances in hybridization technology, however, have made reactions with single-copy transcripts possible (3,7,10,11,44,46,48,49,53-55,60,63, 96,97,111,114). Under conditions of mRNA excess, the sequences complementary to the mRNA (mDNA) can be isolated from the remaining sequences (nmDNA) by hydroxyapatite chromatography. The hybridization conditions used are sensitive enough to detect down to one mRNA per cell. Normal, transformed, and tumor cell mRNAs saturate 0.64% of the ^{3}H-normal cell single-copy DNA probe, indicating a complexity of 2.5×10^{7} nucleotides or approximately 12,500 genes of average size. Total poly(A) mRNA from 13 day hamster embryos saturates at least twice this amount of DNA. If one mixes normal and transformed cell poly(A) mRNAs, the percent saturation is a measure of the difference in sequence complexity of the two cell types. If all the mRNAs were different, the saturation value would be additive. If all the mRNAs are the same, no increase would be observed. When SHE and BP6T cell mRNAs are mixed, no increase is observed. Taking into consideration the accuracy of the technique, no more than 4% of 12,500 mRNAs are different between normal and transformed cells.

Thus, the maintenance of malignant transformation can be accompanied by relatively small changes in the number of polysome-associated poly(A) mRNA sequences; massive gene activation need not be invoked to account for the transformed state. Recycling experiments, using isolated mDNA and nmDNA, are in progress to detect, isolate, and identify any small amounts of new mRNA existing in transformed cells which have escaped notice by the simple addition experiments.

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4.3 A STRATAGEM FOR EXPERIMENTS ON ONCOGENESIS IN VITRO

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Studies of oncogenesis in vitro were made possible by the development of techniques for culturing oncogenically transformable fibroblasts (4). Using such cells, an ideal approach to experimental oncogenesis would be to take them to pieces, to modify the pieces under carefully controlled conditions, to reassemble the cells in stages, and so to trace, step by step, the process of oncogenesis. Experiments with nuclear transplantation (18) are moves in this direction, but the methods are still at an early stage of development.

An alternative to the above ideal approach is to look, in cultured cells, for correlations between the class of phenomena known as oncogenic transformation and other classes that are better understood at the molecular level. At best, this strategem will produce uniform associations under a limited number of conditions. At least, such associations will provide a basis for deciding whether oncogenic lesions belong in the same set as those that lead, by known processes, to other phenomena.

A precondition for the use of the latter stratagem is a detailed investigation of the techniques employed to establish the associations. Wide usage of a procedure is not a sufficient guarantee that such scrutiny has been applied. For example, the method of measuring mutagenesis in eukaryote cells using 8-azaguanine (AG) resistance as a marker has been widely used, but it is only recently that the importance of replating monolayer cells before selection, of using high concentrations of AG, and of using dialyzed serum has been pointed out by a number of people (cited in 17). Procedures that incorporate these details yield results quite different from those of earlier studies (see below).

Alkaline sucrose sedimentation analysis of DNA molecular weight is another technique that can lead to anomalous results. We previously used this method to study the DNA of transformable mouse fibroblasts (C3H/10T½ cells) treated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). On that occasion we concluded that the single strand breaks that we found were produced not by enzymes, but by the action of alkali on methylation lesions (14). In this we were probably correct, as others have provided evidence of a molecular basis for the phenomenon (6,8,21,23). Nevertheless, our measurements, which suggested that the lesions were apurinic sites, suffered from an error due to an anomaly of sedimentation of large DNA molecules at high speed (11,14,24,25).

Lately, we have applied the techniques of alkaline sucrose sedimentation analysis, and of measurement of mutagenesis using AG resistance as a marker, to look for correlations between DNA damage, mutagenesis, and oncogenic transformation produced by methylating agents in cultured mammalian cells. We have not been able to study mutagenesis in 10T1/2 cells, so we have used Chinese hamster fibroblasts (V79 cells) for those studies. We have been able to measure the production of alkali labile lesions and their repair in both cell lines.

For the mutagenesis studies, monolayers of V79 cells were treated for 2 hours with a range of doses (LD5-LD80) of MNNG or methyl methane sulfonate (MMS). The treated cells were allowed to divide at least four times, and were then trypsinized and replated at low density (10^5 cells per 100 mm dish), and exposed to 40 μg of AG per ml in medium containing 10% dialyzed, heat-inactivated, fetal calf serum. Colonies of AG resistant cells were fixed, stained, and scored after 9 to 10 days.

For the alkaline sucrose sedimentation studies, monolayers of V79 or 10T1/2 cells were labeled for 24 hours with ^{14}C-thymidine, incubated in ^{14}C-thymidine-free medium for 90 minutes treated for 2 hours with a range of doses (LD5-LD80) of MNNG or MMS, labeled for 4 hours with ^{3}H-thymidine at various times after treatment, and lysed for 30 minutes to 1 hour on the surface of alkaline sucrose gradients (5-25% sucrose, 1.7 M sodium ions, 1 mM EDTA, 0.06 M p-amino salicylate, pH 13). The gradients were centrifuged at 13,000 rpm for 7 to 12 hours, fractionated, and the radioactivity in each fraction was determined. The number of alkali labile lesions in the parental DNA was determined from the molecular weight of the ^{14}C-DNA, the effect of these and other lesions on DNA synthesis was determined from the molecular weight of the ^{3}H-DNA.

We and others have shown that the above mutagenesis procedure measures mutations at loci that control the activity of hypoxanthine: guanine phosphoribosyl transferase (HGPRT) in the V79 cells (cited in 17,22). Furthermore, dose response curves for a variety of agents are linear over large ranges of dose (2,5,17,22). We found that MNNG is about 700 times more mutagenic than MMS on a molar basis, and about 35 times more mutagenic than MMS per lethal hit (Table 1). These findings suggest a relationship between the mutagenic potencies of MNNG and MMS that is similar to that observed in bacteria (9), but quite different from that suggested by experiments on V79 cells on a previous occasion (19).

In our system, the DNA sediments as single strands with a weight-average molecular weight of 7×10^8 daltons, and is uncontaminated with proteins or lipids. The DNA does not sediment anomalously at the low

speed (13,000 rpm) that we now employ. The criteria by which we have determined the molecular weight, the extents of contamination and anomalous sedimentation have been discussed by ourselves and others (cited in 13,14). We found in V79 cells that single-strand break production by MNNG is about 700 times greater, on a molar basis, than that with MMS. Furthermore, MNNG produced about 35 times more breaks per lethal hit than did MMS (Table 1). These data are not consistent with a mechanism of single-strand breakage at alkali labile apurinic sites, because the methylating agents produce similar numbers of such sites per lethal hit in V79 cells (18). The results are consistent with a mechanism of breakage at alkali labile phosphotriesters (1,6,10,21,23).

Our latest results are summarized in table 1. It can be seen in V79 cells, that the relationships between the mutagenic potencies of MNNG and MMS on one hand, and single-strand breakage by these agents on the other, are very similar. These findings suggest that the lesions responsible for both phenomena belong in the same subset of methylation lesions in the DNA. That this subset is the set of all O-methylations is suggested by studies of the mechanisms by which these agents methylate DNA (6,7,12). The relationship between single-strand break production by MMS and MNNG in 10T1/2 cells appears to be virtually identical with the associations found in V79 cells. Therefore, careful measurements of the extent of oncogenic transformation produced by MNNG and MMS in 10T1/2 cells will indicate whether lesions in the set of O-methylations of DNA are responsible for that transformation. Experiments aimed at obtaining those measurements are in progress.

The present data suggest several avenues of approach for future experiments. One consists of determining whether the lesions responsible for the single strand breaks are identical with those responsible for mutagenesis. Another approach is to look for parameters of DNA structure and function that change, on treatment with MNNG and MMS, in a manner related to single strand break production, mutagenesis and oncogenic transformation. A third line of attack is to look for the above relationships in cells treated with chemical carcinogens that are not methylating agents.

We have begun to examine two new parameters of DNA structure and function, namely, the number and size of newly synthesized DNA molecules made in cells treated with MNNG and MMS. We find that the size of newly synthesized DNA molecules is reduced to a greater extent in MNNG treated cells than in MMS treated cells (16). This is consistent with results obtained by others (20). By contrast, the number of newly synthesized DNA molecules is reduced by the same extent in cells treated with MMS and in cells treated with MNNG. The interpretation of these data in relation to oncogenic transformation and mutagenesis must await further scrutiny of the procedures that we used.

TABLE 1. Associations among DNA damage, mutagenesis, and cytotoxicity produced by methylating agents in V79 Chinese hamster cells and in 10T1/2 transformable mouse fibroblasts. Data are the values of slopes from straight line curves fitted by linear regression analysis. Standard errors are determined from the standard errors of the estimates as described in reference 25.

a) The units for mutation frequency are AG_1 resistant colonies x 10^{-4} survivors x control mutation frequency^{-1}. See reference 24 for details of the calculation.

b) The units for single strand breaks are breaks x 10^{-8} daltons. See reference 4 for details of the calculation.

c) Mutagenesis data from 10T1/2 cells are not available at this time.

d) At least 2 measurements were taken with at least 3 doses, ranging from LD5 to LD80, of MNNG or MMS.

CELLS	AGENT	CYTOTOXICITY	MUTATION FREQUENCY[a]		SINGLE STRAND BREAKS[b]	
		D_0(μM)	per μM	per D_0	per μM	per D_0
V79	MNNG	7.99 ±1.04	1.10 ±0.20	8.76±1.81	1.03 ±0.29	8.25±2.56
V79	MMS	154 ±26.2	0.0015±0.0006	0.23±0.09	0.0015±0.008	0.23±1.30
10T1/2	MNNG	8.36 ± 1.56	?[c]		1.01 ±0.25	8.48±2.54
10T1/2	MMS	210 ±25.2	?[c]		0.0029±0.0013	0.61±0.29
Number of measurements[d]:						
V79	MNNG	29	28		9	
V79	MMS	10	30		9	
10T1/2	MNNG	8	0		29	
10T1/2	MMS	8	0		14	

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4.4 THE USE OF DIPLOID HUMAN FIBROBLASTS TO MEASURE THE EFFECT OF DNA REPAIR ON THE CYTOTOXIC AND MUTAGENIC ACTION OF CARCINOGENS

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Although it has not yet been possible to prove that chemical and physical carcinogens cause cancer by producing mutational changes in cells, this theory, first proposed by Boveri in 1914 (5), has become widely accepted in the last eight years since the mutagenic action of "active forms" of carcinogens was reported (10,33,35,36). This is because a wide range of data point to mutations as the common denominator in the etiology of cancer caused by chemicals of widely different structure and by physical agents such as ionizing radiation and ultraviolet radiation. In this paper, we will review the use of cytotoxicity and mutagenicity experiments with diploid human cells in culture as a tool to dissect various aspects of this problem. It is obvious that such procedures can be used in conjunction with biochemical, biophysical, or genetic techniques to yield additional information on the mode of action of carcinogens in cells and many of the cited papers, in fact, do this.

Normal human fibroblasts are now routinely grown in a large number of laboratories. Usually they are obtained from skin biopsies or from foreskin tissue removed at circumcision, but may also be obtained from other body tissues or from cell banks such as The American Type Culture Collection (Rockville, Md.). The methods for growing and handling such cells have been described in detail (17-19). With a little care, such fibroblasts routinely give cloning efficiencies of 20 to 60%. Normal human cells are not immortal but die off after 50 to 70 passages in culture as has been established by Hayflick (24). However, this is generally not a limitation since a single culture produces enough cells for a large number of experiments of the type described below. Normal human fibroblasts can be made into "immortal" lines by transformation with SV40 virus (53) if one wishes to preserve cells with particular characteristics, although this may prove objectionable in some cases because the cells are no longer "normal."

One special advantage of normal human fibroblasts is that one can obtain a wide variety of cells defective in various biochemical pathways including DNA repair pathways. These latter are of particular value in examining questions such as the relationship between DNA repair, cytotoxicity, and mutagenesis. In animal cell systems only rarely has a DNA repair-defective cell been reported (20,21).

One reason for using biological parameters such as cytotoxicity or mutagenicity as end points, rather than biochemical or biophysical, is that they are generally much more sensitive indicators of carcinogen interaction with cells. For example, ultraviolet irradiation survival curves for normal cells show that killing begins at about 12 ergs/mm^2 and one reaches 10% survival at about 60 ergs/mm^2 (32). Repair-defective human cells show cytotoxicity at even 1 erg/mm^2 (32). Biochemical experiments designed to measure excision repair following ultraviolet irradiation (including our own) generally use doses of 75 to 150 ergs/mm^2 because of the insensitivity of the assay (6,7). Chemical carcinogen effects on human fibroblasts can also be determined at lower doses by biological parameters than by biochemical or biophysical parameters.

ARYL HYDROCARBON HYDROXYLATING (AHH) ACTIVITY OF HUMAN FIBROBLASTS

Diploid human fibroblasts in culture seem to have little or no AHH activity which is necessary to activate polycyclic hydrocarbon carcinogens (McCormick, unpublished data). Foreskin tissue of newborns, as well as some early cell passages of cells derived from foreskin, contain measurable levels of the enzyme (28). After foreskin derived cells are in culture for 2 or 3 passages, they are, with rare exception, negative for this enzymatic-activity. It is not clear whether any of the enzymatic activity found in foreskin or early passage cells from foreskin cultures is contributed by fibroblasts since homogenates from whole skin are used for the assays of foreskin and early passage of cells contain epithelial cells (which are later lost) as well as fibroblasts and epithelial cells generally possess AHH activity. Practically, this means that human fibroblasts in culture can be utilized to test for active derivatives of compounds such as polycyclic hydrocarbons. They show no cytotoxicity from the parent polycyclic hydrocarbons (59). Therefore, in our studies we have utilized chemically synthesized active forms (32,34). It is also possible to generate active forms of such polycyclic hydrocarbons by adding a rat liver microsomal fraction prepared according to the method of Frantz and Malling (22), to the human cells along with the parent hydrocarbon molecule (Maher, et al., unpublished data). It is probably also possible to use irradiated feeder layers of cells which are able to metabolize carcinogens such as has been done by Huberman, et al., (25), but we know of no examples of such experiments with normal human fibroblasts.

EFFECT OF DNA REPAIR ON THE CYTOTOXICITY OF DIPLOID HUMAN FIBROBLASTS BY CARCINOGENS

Cytotoxicity experiments are usually the first method of measuring a biological response in cells. This can be done by cloning

experiments or by following cell numbers (by hemocytometer or electronic counter) after treatment of cells with an appropriate agent. Such experiments give base-line data on the sensitivity of cells to various agents. They are quick and provide reliable data on the sensitivity of cells to the toxic action of agents and when they are coupled with the use of cells with different characteristics, etc. (see below), they can provide evidence as to the mechanism(s) by which an agent kills cells. Such experiments have been carried out with ionizing radiation (3,54,55) with ultraviolet radiation (31,32,34,37,53), and with a variety of mutagenic, carcinogenic, or otherwise toxic compounds (23,31,32,34,39,46,47,52,61). Cytotoxicity studies can be especially useful when one wishes to examine the interaction of two agents. We and others have examined the synergistic effect of caffeine on the cytotoxic effect of ultraviolet radiation (4,32,39).

If cells are available with a defect in a pathway which affects the uptake or the metabolism of a cytotoxic compound or the repair of lesions caused by an agent, such defects will generally result in a change in survival. When such defective cells are compared with normal cells, for cytotoxicity, one can learn something of the biological effect. One of the clearest examples of this situation is from studies on cells from *xeroderma pigmentosa* (XP) patients (32). Such patients develop multiple carcinomas of the skin when exposed to sunlight (45). Cleaver found that fibroblasts from such patients were unable to excise pyrimidine dimers from DNA (7). More recently, it has been found that such excision-repair defective XP cells (classical) fall into 5 complementation groups based on their ability to restore excision repair capacity when heterokaryons are formed by fussion of XP fibroblasts (26,45). In addition, there exists one small group of XP patients which are clinically indistinguishable from the classical XP patients, but whose cells do not have a defect in the rate of excision repair. These patients have been called "XP variants" by Cleaver (8). Recently, Lehmann, et al., (27) have shown that such variant XP cells have a defect in some post-replication repair process so that such cells after ultraviolet irradiation, take abnormally long to convert low molecular weight DNA into high molecular weight DNA.

Figure 1 shows a series of survival curves demonstrating the loss of cloning ability by normal cells, classical XP cells from various complimentation groups, and variant XP cells following treatment with ultraviolet radiation. It is clear from the data that XP cells are more sensitive than normal cells. Furthermore, the survival of various classical XP cells correlates with their excision repair capacity, i.e., those with the least repair capacity have the greatest toxicity, etc. From this type of experiment along with biochemical measurements of repair capacity, one can determine the effect of DNA

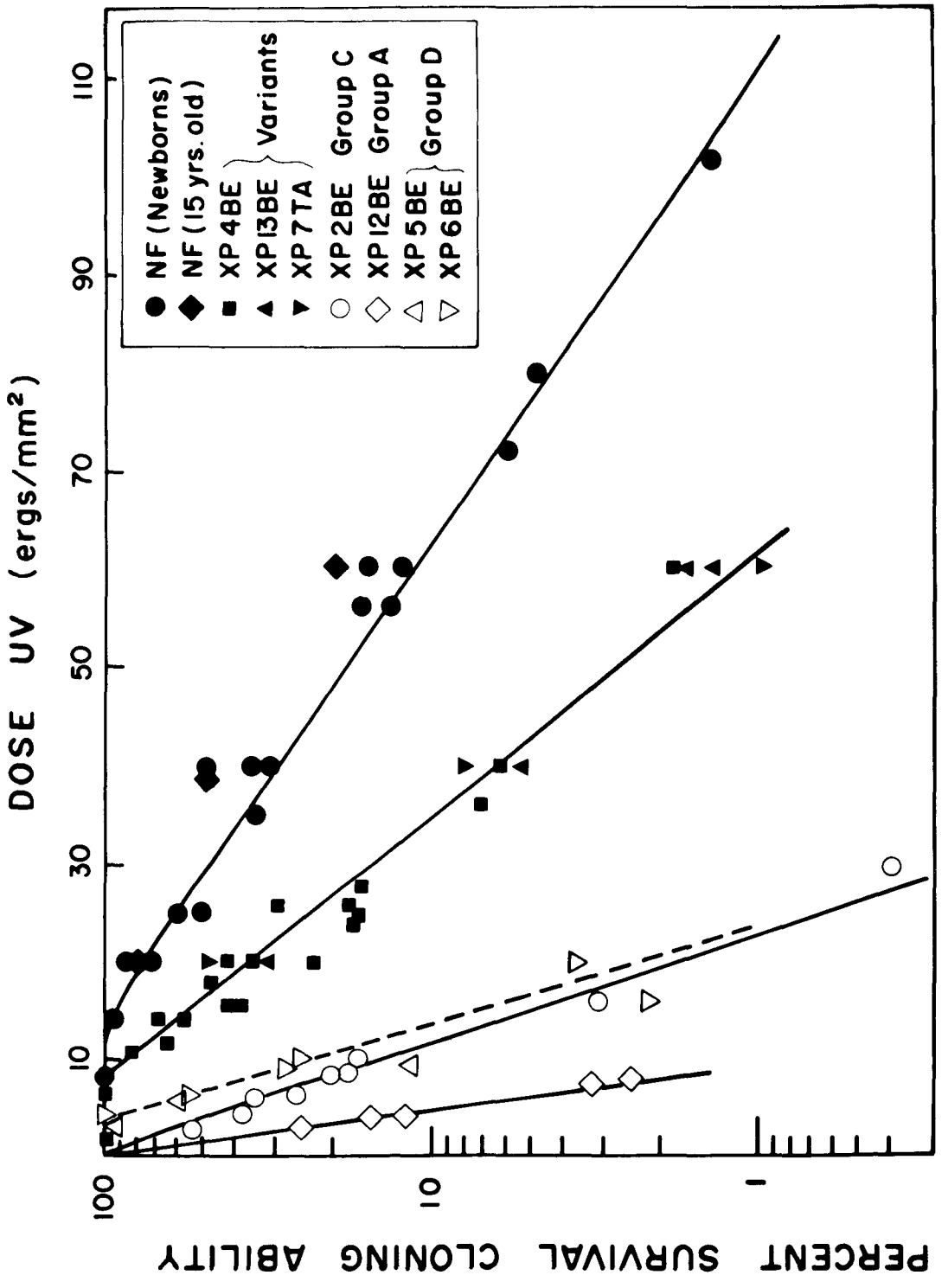

Figure 1. Survival Curve for Cloning Ability Versus Dosage

repair processes on cell survival. In our studies, we have found that similar survival curves are found when cells are treated with active derivatives of some of the carcinogenic polycyclic hydrocarbons and aromatic amides (31,32,34,40), but not with carcinogenic chemical such as N-methyl-N'-nitro-N-nitrosoguandine (MNNG) (Maher, et al., unpublished results). These data indicate that DNA excision and post-replication repair pathways are utilized for DNA damage resulting from these polycyclic hydrocarbons and aromatic amides as well as ultra-violet radiation, but not that from methylating carcinogens such as MNNG. Similar conclusions have been reached by Stich et al., (47) who compared the cloning ability and DNA repair capacity of normal cells with one strain of XP cells following treatment with 4-nitroquinoline 1-oxide (4NQO), some of its analogs, and MNNG. The effect of 4NQO on the survival of one of the XP strains has also been studied by Takebe et al.,(52). Regan and Setlow, who used a biochemical approach, arrived at similar conclusions regarding repair in XP cells (44). Research on XP cells has recently been reviewed by Cleaver and Bootsma (9).

OTHER DNA REPAIR-DEFICIENT CELLS

Patients with the autosomal, recessive defect, ataxia telangiectasia (AT) have an increased incidence of cancer of various types (49). Fibroblasts from such patients are abnormally sensitive to ionizing radiation as seen in cytotoxicity curves. Some of the AT cell lines tested lack the ability to excise lesions in the DNA produced by the ionizing radiation (42). However, cells from other AT patients show a similar increase in cytotoxicity when exposed to ionizing radiation but as yet no defect in DNA repair has been identified.

Cells from patients with Fanconi's anemia (FA), a disease characterized by a predisposition to leukemia, have recently been shown to be about 6 times more sensitive to Mitomycin C treatment than normal cells. These cells seem to have a failure in removing the cross-link in DNA caused by this agent (23).

HOST CELL REACTIVATION OF VIRUSES

Host Cell reactivation of plaque-forming DNA viruses which are treated with carcinogenic agents is a method of determining the ability of cells to repair damage caused by such agents without treating cells directly. It yields data that are similar to survival curves. Various viruses have been used by different workers to study reactivation by normal and XP cells as well as caffeine effects (1,12-15,29,30,43,53). In general, the work of these workers agrees with the survival data for the cells discussed above.

CONCLUSION REGARDING CYTOTOXICITY ASSAYS IN REPAIR-DEFICIENT CELLS

From the data we have reviewed, it is apparent that in human cells there are various DNA repair enzyme pathways that are responsible for the repair of DNA photoproducts as well as DNA carcinogen adducts. On the basis of the present data, one would assume that there are at least three partially independent pathways. It seems likely that other repair pathways remain to be identified. One extremely important area that as yet has received little attention is "post-replication repair." Most generally, this term refers to the ability of cells to replicate DNA containing a lesion by some sort of by-pass mechanism. The XP variant cells described above are the only known human cells with a defect in this mechanism (63). Such mechanisms are especially important since our data on XP cells demonstrate that excision repair in such cells is essentially error-free (32) (see below). Thus, it appears that mutations are caused when cells replicate DNA containing lesions, i.e., carry out post-replication repair.

EFFECT OF DNA REPAIR ON THE FREQUENCY OF MUTATIONS INDUCED IN DIPLOID HUMAN FIBROBLASTS BY CARCINOGENIC AGENTS

It is now possible to carry out mutagenicity studies with diploid human fibroblasts (16,56). Although such studies are much more laborious because of the numbers of dishes involved (see below) than the cytotoxicity studies described above, they are capable of providing a quantitative determination of spontaneous mutation rates (17) and of the ability of chemical (40,41,59-61) or physical carcinogens to induce mutations (3,11,37,38,62). Two types of selection systems have been successfully used for such studies:

(1) resistance to purine analogs such as 8-azaguanine (8AG) (3,37,38,40,59-61) and thioguanine (TG) (11,62), etc. and

(2) resistance to the membrane active drug, ouabain (41).

The advantages and disadvantages of the first system have been outlined in considerable detail in recent reviews (16,56). Briefly, resistance to 8AG or related analogs can be obtained in a "single step" selection (50,51). It is usually, but not always (16), accompanied by a reduction in the level of activity of the enzyme hypoxanthine (guanine) phosphoribosyl transferase (HGPRT, E.C. 2.4.2.8). In addition, a "single step" back selection system is potentially available for assaying the frequency of revertants. This is because when endogenous purine synthesis pathways are blocked by the presence of a folic acid antagonist, such as aminopterin, cells

lacking HPRTase activity should not be able to grow if the only source of purines is exogenous hypoxanthine even when exogenous thymidine is supplied (HAT medium) (16). This situation can be complicated, however, by the acquisition of resistance to the blocking agent itself. (M. Fox personal commun.) Use of 8AG resistance as a genetic marker has the advantage that the HGPRT locus is located on the X-chromosome and, therefore, is essentially present as a single gene (monosomic). This allows the selection of a single mutation to a recessive trait without the need for two independent mutational events or the requirement of starting with cells which are heterozygous for the gene in question. (A related system developed and described by DeMars (16) involves loss of the enzyme, adenine phosphoribosyl transferase (APRT, E.C. 2.4.2.7).) The gene for this latter trait is on somatic chromosome #16 which restricts the use of this marker to cells heterozygous for this trait.

Because of the phenomenon known as "metabolic cooperation" (48,57) in which non-mutant cells transfer material to 8AG-resistant mutant cells and thus make the latter susceptible to killing by 8AG, it is not possible to select for mutants if the cell density in the dishes is too high (3,38,40,57). Reconstruction experiments have demonstrated the need for correction factors to take into account this phenomenon as well as the effect of the different cloning efficiencies of the cells used (57,58).

The second system, i.e., resistance to ouabain, has been described in detail by Thompson and Baker (56). Ouabain is a specific inhibitor of the Na^+-K^+-activated ATPase of the plasma membrane, the enzyme responsible for the active transport of K^+ into the cell and the extrusion of Na^+. The many advantages of this system have been outlined (56). It also gives stable mutants in a "single step" selection. However, there is no back selection system for assaying revertants. The trait is co-dominant and, therefore, it is possible to select ouabain resistant mutants at reasonable frequencies. It has been used by Mankowitz, et al., (41) to quantitate the frequency of mutations induced in diploid human fibroblasts by the carcinogen, ethylmethanesulfonate.

Resistance to purine analogs, such as 8AG or TG, has to date been the genetic marker used most widely for studies on the induction of mutations in diploid human cells in culture (3,37,38,40). With the noted exception (41), we know of no published reports concerning the induction of mutations in such fibroblasts using any other marker. This selection system, developed by Szybalski and his co-workers (50,51) was successfully adapted and perfected to quantitate the induction of mutations to 8AG resistance in normal diploid human fibroblasts by Albertini and DeMars (2,3) using X-rays as the mutagenic agent. We further adapted their techniques in order

to quantitate the induction of mutations by the chemical carcinogen N-acetoxy-2-acetylaminofluorene (40).

More recently, we have revised our own techniques and investigated the effect of DNA excision repair capacity of human fibroblasts on the frequency of mutations induced by UV-radiation by comparing normal cells with several strains of classical XP cells (32,34,37,38,40). Our results indicate that the excision repair process itself is essentially "error free" since the frequency of UV-induced mutations are much higher in XP cells which lack excision repair than in normally repairing cells. What is still more significant, at equicytoxic doses in the normal and classical XP strains, the induced mutation frequencies are equal.

We have investigated this question using "K-region" epoxides of carcinogenic polycyclic hydrocarbons and obtained essentially identical results. The frequency of mutations is higher in the excision repair defective cells than in the normal (59).

It has also been possible to use such mutagenicity studies to investigate the effect of a defective post-replication repair process which has been identified in XP variants (27). We find that these cells, like classical XP's, have higher UV-induced mutation frequencies than normal. Since such XP variant patients are equally as subject to sunlight-induced carcinoma of the skin as classical XP patients, such results add strength to the link between mutagenicity and carcinogenicity.

CONCLUSION

This brief review demonstrates that the use of diploid human fibroblasts has become an established method for examining the cytotoxicity and mutagenicity of carcinogenic agents. The unique availability of a series of DNA repair-defective cells (XP) has contributed greatly to our knowledge of how various carcinogenic agents affect cells. It is to be hoped that the recent discovery that AT and FA cells have DNA repair deficiencies (different from XP) will lead to further insights into the relationship between DNA repair, and the cytotoxic and mutagenic action of carcinogens.

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4.5 ENHANCEMENT OF DNA VIRAL TRANSFORMATION BY CHEMICAL CARCINOGENS AND MUTAGENS[1]

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Enhancement of viral carcinogenesis by chemical carcinogens in vivo was first described by Rous and Kidd (20), who demonstrated an increase in virus-related papillomas following treatment of rabbits with Shope papilloma virus and the horizontal retort tar of the Oster-Gasfabrik of Amsterdam. Subsequently, Rous and Friederwald (19) and Rogers and Rous (18) observed the development of malignant carcinomas in sites jointly treated with Shope papilloma virus and chemical carcinogens that were not observed when virus or chemical were applied independently.

Tumors induced by fibroma virus in animals pretreated with tar or benzo(a)pyrene (B(a)P) were larger and regressed at a slower rate in contrast to tumors induced by virus alone (1,2). In addition, some of the tumors demonstrated neoplastic characteristics, including invasiveness. The enhancement of tumor formation did not occur if chemicals were applied several months before virus infection nor if chemical treatment was begun 10 days after virus innoculation (2). In the preceding studies, the increase in viral-induced tumors was restricted to the site of chemical application. This latter observation, and the temporal relationship between virus and chemical addition suggested that the chemical carcinogen acted directly upon the eventual target cells and thereby increased the susceptibility of a population of individual cells to virus transformation. Subsequently, treatment of cells in vitro with x-irradiation (13,17,21) BCdR, BUdR (3,23) or IUdR (3,13,16), 4NQO (14,22), and with UV-irradiation (3) was shown to increase the frequency of transformation induced by the carcinogenic DNA viruses SV40, polyoma, human adenovirus types 12 and 31, and the simian adenovirus, SA7.

In a series of experiments utilizing hamster embryo cells (HEC) and SA7, it was shown that cells pretreated with chemical carcinogens followed by infection with SA7 demonstrated an increased sensitivity to SA7 transformation. The polycyclic hydrocarbons B(a)P, DB(a,h)A, DB(a,c)A, DMBA and MCA enhanced SA7 transformation but the non-carcinogens phenathrene, pyrene and perylene did not (3,4,5,6,8).

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Similarly, HEC treated with Ac-AAF, AFB_1, ara-C, B-PL, caffeine, MNNG, MMS, and MAM-Ac enhanced SA7 transformation as evidenced by an absolute increase in the number of SA7 foci per plate and a relative increase in the transformation frequency (4,5,6,9,10). Recently, the inorganic metal salts $BeSO_4$, $CdCl_2$, $CaCrO_4$, $K2CrO_4$, $NiSO_4$, $NaAsO_2$, $PbAc_2$, PbO, and $PbCrO_4$ were shown to increase the sensitivity of HEC to SA7 transformation following 18 hours exposure of the cells to the above metals; treatment with TiO_2 was ineffective (11).

Although chemical exposure for 1 to 2 hours prior to virus addition was sufficient to increase SA7 transformation by AcAAF, B-PL, EMS, MNNG, MMS, and PS (9,10) longer exposures were necessary for B(a)P, B(e)P, DB(a,b)A, DB(a,c)A, DMBA, MAM-Ac, and MCA (4,8,9) presumably because the latter compounds require more extensive metabolic activation than the former. Not only was the time of incubation with chemical found to be critical, but also the sequence of addition of chemical and virus. B(a)P, DMBA and AFB_1 were shown to enhance when added prior to SA7 inoculation and cell transfer (5,6,8), but inhibited transformation up to 20-fold when added after cell transfer (5,6,8,10). In contrast, ara-C, 6-Ac-B(a)P and caffeine enhanced SA7 transformation more efficiently when added after virus inoculation and cell transfer (6,10). A third group of chemicals including Ac-AAF, B-PL, EMS, MMS, MNNG, and PS enhanced equally well regardless of time of addition with respect to virus inoculation (5,9,10).

Simultaneous treatment of HEC with B(a)P and benz(a)anthracene (BA) or 7,8-benzoflavone (ANF) 24 hours prior to SA7 inoculation inhibited the lethal effects associated with B(a)P exposure and resulted in an increase in the absolute number of SA7 foci. However, because of the significant increase in cell survival, the frequency of SA7 transformation decreased relative to that observed in cells treated only with B(a)P (6). Similar results were observed when cells were treated with DMBA and BA or ANF, but were not as pronounced when MCA was substituted for B(a)P or DMBA (6). Pretreatment with BA or ANF 24 or more hours prior to B(a)P, DMBA or MCA treatment had little or no effect on enhancement of SA7 transformation by the latter 3 chemicals (6, unpublished data).

The enhancement of viral transformation by chemicals is probably not due to an effect on virus or viral DNA, as the cells are treated 2 to 24 hours prior to virus addition. Further, an effect on viral adsorption or penetration is unlikely since many of the chemicals enhance when added 5 to 8 hours after virus. It is also improbable that the chemicals act by selecting cells sensitive to viral transformation. If the latter were true, one would expect only a relative increase in the proportion of transformed foci among surviving cells. However, with the majority of chemicals tested, there

has been an absolute increase of 2- to 5-fold in the number of SA7 foci per plate where either negligible or extensive cell death occurs following chemical treatment. It has been suggested that irradiation or chemical treatment of cells enhance viral transformation by creating additional attachment sites for virus in cell DNA (3,5,9,13,17). These sites could be expressed during scheduled DNA synthesis opposite regions of unrepaired damage in parental DNA (12) or as a result of the excision-repair system of the cell (6,10). In either case, host cell repair of these damaged areas prior to the availability of viral DNA, should abrogate the expected increase in viral transformation.

Hirai et al. (15) have shown that treatment of Chinese hamster cells with 4NQO increased both the frequency of SV40 transformation and the integration of SV40 DNA into cell DNA. Similarly, Casto and Miyagi (7) found increased amounts of SA7 DNA in Syrian HEC DNA following 2 hour treatment of the cells with MMS. With both 4NQO or MMS treatment, the period of enhancement was directly correlated to the time of repair (B.C. Casto and H.F. Stich, unpublished data; 4). Additionally, Coggin (13) showed that a delay of 10 hours in adding SV40 to x-irradiated hamster cells reduced enhancement by 70%. Alternatively, chemicals that are slowly removed by excision-repair, such as B(a)P or DMBA (10) and chemicals that are not removed such as BCdR (5) enhance transformation up to 72 hours after treatment. In the case of DMBA, enhancement declined progressively over 72 hours (unpublished data) with the highest level of enhancement occurring when virus was added immediately after treatment. With BCdR, enhancement was undiminished when SA7 was added 24, 48, or 72 hours after treatment (5). All chemicals that cause DNA repair increase SA7 transformation of HEC (6,10); however, 57% of all chemicals tested that enhance transformation do not induce demonstrable DNA repair synthesis. It is suspected that these chemicals enhance by being bound to cell DNA and creating gaps for attachment of viral DNA during scheduled DNA synthesis as suggested by Cleaver (12).

The enhancement of viral transformation provides a mammalian-cell assay that not only detects chemical carcinogens and mutagens, but also provides a system for recognition of those compounds that act as co-factors for carcinogenic viruses and chemicals that may not be detected by microbial systems.

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4.6 MORPHOLOGICAL TRANSFORMATION, CHROMOSOMAL CHANGES AND INCREASES IN PLASMINOGEN ACTIVATOR IN IN VITRO CARCINOGENESIS

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A. MORPHOLOGICAL TRANSFORMATION BY CANCER CHEMOTHERAPEUTIC AGENTS IN THE C3H/10T½CL8 CELL LINE

We have chosen to study whether or not various cancer chemotherapeutic agents produce morphological transformation in the C3H/10T½CL8 mouse cell line developed in the laboratory of Dr. Charles Heidelberger (21) for several reasons. First, the molecular mechanism(s) of action of many of these agents have been studied extensively. Secondly, there is considerable data on the in vivo carcinogenicity of most of these chemotherapeutic agents and finally, the majority of these drugs are presently being used for the treatment of malignant and often nonmalignant conditions in man. Therefore, these studies should have relevancy not only to help investigate the actual mechanism(s) of oncogenic transformation in vitro and to compare results obtained in cell culture with in vivo carcinogenicity data, but also would be important in our attempt to determine the significance of short-term assays as potential screens for hazards to man. Since there are many long-term survivors for various types of malignancy following treatment with chemotherapeutic agents, we should be able in the future to determine whether those same agents which cause morphological transformation in cell culture also produce primary or secondary malignancies in man. Thus, cancer chemotherapeutic agents may be a unique group of compounds, particularly since the actual plasma level of the majority of these agents is already known. In general, the concentrations used in cell culture for the transformation studies are actually achieved in the plasma level in man (5).

Various subclasses of chemotherapeutic agents have been studied including alkylating agents, antitumor antibiotics, antimetabolites, and miscellaneous synthetics. Those agents which have been shown to be carcinogenic in vivo also produced morphological transformation, whereas those agents which thus far have not been shown to be carcinogenic in vivo did not cause transformation in vitro (5 and Table 1). Thus, there appears to be a good correlation between known carcinogenesis in vivo and transformation in vitro utilizing these agents. Consequently, it would seem that this system as well as others hold promise in screening for potential carcinogens, especially

TABLE 1

SUMMARY OF CHROMOSOMAL DAMAGE, TRANSFORMATION AND CARCINOGENICITY PRODUCED BY CANCER CHEMOTHERAPEUTIC AGENTS[a]

Chemotherapeutic Agent	Chromosomal Damage In $A(T_1)C1-3$ Cells	Morphological Transformation In C3H/10T 1/2CL8 Cells	Known Carcinogenicity In Animals
Alkylating Agents			
Melphalan	+	+	+
Thiotepa	+	+	+
Antitumor Antibiotics and Natural Products			
Actinomycin-D	+	+	+
Bleomycin	+	+	+
Vincristine	-	-	-
Antimetabolites			
Methotrexate	+	+	+[b]
6-Mercaptopurine	-	-	-[c]
Miscellaneous Synthetics			
Hycanthone	+	+	+
5-aza-C	+	+	+
Tilorone	-	-	-

[a]These findings are presented in detail in Reference 5.

[b]Unknown at high dose exposure.

[c]Produces lymphomas only, possibly secondary to immunosuppression.

when methods for metabolic activation of all types of chemicals can be incorporated into the transformation system such as those now being used for mutagenicity studies (1,13).

B. CHROMOSOMAL CHANGES AND THE EXPRESSION OF MALIGNANCY

Our present feeling about the importance of chromosomal changes in relationship to the expression of malignancy has recently been reviewed (3). We originally showed that aromatic polycyclic hydrocarbons produced aneuploidy in cells within 24 to 72 hours after exposure (4) and suggested at that time that the rapid occurrence of chromosomal changes might be important in cell transformation. Subsequently, various chromosomal banding techniques became available (9,22) which allowed individual chromosomes to be identified with certainty. Thus, it became possible to study the relationship between specific chromosomal changes and malignant expression.

The first evidence for specific chromosomal imbalances being related to the expression of malignancy came from the laboratory of Dr. Leo Sachs (25). It was shown in chemically transformed hamster cells that the number 5_7 and 5_{10} chromosomes appeared to have genes for the expression of transformation and/or malignancy and the number 7_2 and 7_3 chromosomes were thought to have genes for the suppression of these same properties. Likewise, in polyoma virus transformed hamster cells, it was reported that genes for the expression of malignancy were located on the 5_6 chromosome and genes for the suppression of malignancy were located on the 5_3 and 7_2 chromosomes (24).

At this time, we were also studying in the same hamster embryo system the relationship between specific chromosomal changes and the expression of malignancy (7). In cells transformed by ara-C, tumorigenicity was associated with an increased number of 5_7 chromosomes compared to the number of 7_3 chromosomes, i.e., tumorigenicity increased when the ratio of 5_7 to 7_3 chromosomes was greater than one. Additional independent studies also published on a hamster embryo cell line transformed with dimethylnitrosamine again suggested that genes for the expression of malignancy and transformation were located on the 5_7 chromosome and to a lesser extent on the 5_{12} chromosome (8). The 7_3 chromosome was also felt to contain genes for the suppression of malignancy in these cells. Thus, although there appears to be variation at times in the specificity of chromosomes with genes for the expression or suppression of malignancy, the implication from these studies is that chromosomes which contain genetic information involved in oncogenesis are far from random.

We have also found a high correlation between rapid chromosomal damage produced in the $A(T_1)C1\text{-}3$ hamster cell line by various cancer

chemotherapeutic agents and morphological transformation in vitro or carcinogenicity in vivo. This is summarized in Table 1. The chromosomal damage was produced within 24 hours after treatment with various concentrations of the drugs. The A(T_1)C1-3 hamster cell line is being utilized by us as a potential screen for human mutagens and/or carcinogens, since it has a pseudodiploid chromosomal complement (7), rapid growth in cell culture (6), and also has a unique property for fibroblasts in that the cells have highly inducible aryl hydrocarbon hydroxylase activity (unpublished data). This enzyme system is important in the activation of many carcinogens to their ultimate carcinogenic form(s), particularly in the case of aromatic polycyclic hydrocarbons. Thus the cell line may be excellent for testing many types of potential mutagens and/or carcinogens, because the parent compound can be metabolized to its active form(s) when most classes of compounds are exposed to the cells. However, a microsomal activation system or some other activation system may have to be incorporated into this cell line for certain classes of compounds to be evaluated adequately.

Specific chromosomes involved in the suppression of malignancy have also been reported in mouse cells (10). Malignancy appeared to be related to whether or not one or two of the same marker chromosome was present in the cell. When mixed populations containing cells with either one or two of this particular marker chromosome were injected, only cells containing one complement of the specific marker chromosome were found in the tumors. This marker was formed by a translocation of the number 7 chromosome onto the number 19 chromosome, and it was concluded that either the 7 or 19 chromosome contained genetic information for the suppression of malignancy in these cells, since the addition of an extra marker chromosome seemed to inhibit the expression of tumorigenicity.

Finally, chromosome changes have been found in human diploid cells which have been transformed either with chemicals or viruses (11,17). Abnormal chromosomal patterns have also been a consistent finding following treatment of human diploid epithelial cells with methylcholanthrene (12). Therefore, possible specific chromosomal imbalances related to the expression of malignancy can now be studied in human diploid cells after in vitro transformation. We have, in fact, outlined a general hypothesis on how specific chromosomal changes can be related to malignant expression following transformation with all carcinogens including DNA oncogenic viruses, RNA oncogenic viruses, and chemical or physical agents (3).

C. INCREASES IN PLASMINOGEN ACTIVATOR FOLLOWING IN VITRO TRANSFORMATION

Many in vitro characteristics of transformed cells, including a crisscross morphological pattern and growth in semi-solid medium, have been shown to be at least partially related to the presence of a serine protease which is usually produced by malignant cells at a higher level than their normal counterparts (19). This protease, named plasminogen activator (20,23), has also been shown to be increased in many chemically transformed mammalian cells (18). Presently, fibrin overlay methods have been developed to detect single transformed cells or colonies of transformed cells (14). Since the transformed cells can not only be identified using these techniques, but also can be isolated (15), early transformed cells hopefully could be cloned for further investigations regarding the nature of the transformation event(s). An increase in plasminogen activator may, in addition, be useful to identify chemical transformants prior to detection by more standard techniques.

In general, it would appear that an increase in plasminogen activator is an early event following chemical transformation in several systems, including secondary hamster embryo cells (2), and the C3H/10T½CL8 cell line (16) which are now in wide use as model systems for evaluating chemical carcinogens.

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4.7 CELL SURFACE ANTIGENS AS MARKERS FOR THE TRANSFORMED PHENOTYPE [1]

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CELL SURFACE ANTIGENS AS MARKERS FOR THE TRANSFORMED PHENOTYPE

One potential approach for understanding neoplastic transformation is the study of the plasma membrane of cells. Alterations in this cellular structure may result in many of the physiological properties which are associated with neoplastic transformation. Probably the major impetus in stimulating studies on the cell surface was the report that some plant lectins agglutinate transformed cells better than untransformed cells (1,17). Later it was found that many tumor cells contain altered transplantation antigens (2) or embryonic antigens (6). Each of these discoveries suggests that the surface membranes of tumor cells are different from normal cells. However, even after extensive study, the mechanism by which membrane changes are related to other phenotypic characteristics of transformed cells or tumorigenicity is not known.

Limitations in our present technology have made the study of plasma membrane difficult. Methods developed for isolation of plasma membranes are largely unsatisfactory because of contamination by endoplasmic reticulum and other cellular components. However, this may be because these cellular components are intimately associated with the membrane. Another difficulty is that the membrane must be subjected to drastic procedures before the specific activity of certain membrane associated enzymes appears to rise. These procedures often allow the loss of certain membrane components. The most effective reagents for the dissociation of membranes are detergents such as sodium dodecylsulfate (SDS). This frequently used detergent

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has proven useful for analytical procedures where the molecular size is to be analyzed, but may ruin the functional properties of the proteins. The use of nonionic detergents may allow preservation of enzyme or antigenic activity, but frequently the membrane is not sufficiently dissociated to allow biochemical analysis of many of the components.

One technique, which has aided greatly in membrane study, is the surface labeling of viable cells in culture (4,10,21). These reactions are catalyzed by enzymes that are too large to enter the cell. Using these techniques, interesting differences between normal and neoplastic cells have been found (3,4,5,7-15,18,20,22,24).

This paper will describe some of the methods we have used to study the cell surfaces of neoplastic cells and discuss some of the potential uses of similar techniques in studying genesis and evolution of neoplastic cells.

CELL SURFACE LABELING

Lactoperoxidase catalyzed iodination of cells primarily results in labeling of the available tryosine moieties of proteins (21). It has been reported that this reaction may also label cell membrane lipids (polar and neutral) (16). The method can be restricted to labeling the cell surface proteins when the cells are exposed for a short period of time, when there are no extensive intracellular peroxidases, and when the cells are intact.

We use a ten-minute incubation at $22^{o}C$ in this procedure and believe the cell surface proteins are selectively labeled for the following reasons: (1) There is a marked selectivity of labeling. That is, certain proteins show a very high or low specific activity. (2) Pretreatment of the cells with trypsin (1.0 µg/ml for ten minutes) removes most of the proteins which are usually labeled. (3) Postiodination trypsin treatment markedly alters the proteins which are labeled. Glucose oxidase is used to generate H_2O_2 directly because we feel that cells are more viable during this treatment.

In the studies to be described, the cells are iodinated when they are confluent and washed three times with PBS prior to iodination. After the iodination, the cells are scraped, washed, and lysed in the presence of phenylmethylsulfonylfluoride (PMSF), a protease inhibitor. The nuclei and DNA are removed by centrifugation. The samples are boiled in the presence of β-mercaptoethanol and SDS, are count matched, and electrophoresed on a 7.5% polyacrylamide gel (PAG) with a 4.2% stacking gel. E. coli and $T4$ phage proteins are used as molecular weight markers. The I^{125} autoradiograph and Coomassie

blue stained protein profiles are compared to those obtained when the cells are metabolically labeled with C^{14} L-amino acids.

Fig. 1 A and C show the gels of normal tertiary Syrian hamster cells. The most intensely labeled iodine band is approximately 220,000 daltons (Fig. 1C). This protein has been termed LETS (Large External Transformation Sensitive) by Hynes (13). Other names which have been given to this protein are CSP (cell surface protein) (25), SF antigen (fibroblast surface antigen) (23), galactoprotein a (4), Zeta (22), and band 1 (10). Comparing the labeling pattern of transformed hamster cells (Fig. 1D) (a gift from W. Benedict, Los Angeles Children's Hospital) to the untransformed cells demonstrates the lack of this protein on the transformed cell surface. However, when metabolic C^{14} labeling is used, both transformed and untransformed cells are labeled in the 220,000 MW region (Fig. 1 A and B). Analyses of several other chemically transformed Syrian hamster cell lines (obtained from J.C. Barrett, Johns Hopkins School of Medicine) were made and the results are shown in Fig. 2. The A-6, T-6, and U-19 lines show loss of the I^{125} labeling in the LETS region. A-12 shows a reduced amount of label as compared to the normals (U-18, E-11, B-14). Dr. Barrett, earlier in this volume, has described the biological characteristics of these lines. A-6, T-6, and U-19 are tumorigenic, grow in soft agar, and produce extensive zones of fibrinolysis in fibrin overlay experiments. A-12 is less tumorigenic, grows poorly in agar, and has decreased fibrinolytic activities. Table 1 illustrates that for the lines analyzed there appears to be a correlation between the amount of labeling of LETS and the other markers of transformation in this study. In support of this, iodination of SV_{40} transformed human WI-38 cells and untransformed WI-38 cells reveals that the transformed cells lose the 220,000 MW iodinatable protein (unpublished observations). However, studies on the transformation of mouse C3H101/2 cells (15) did not reveal any correlation between the amount of LETS protein and the other characteristics of transformation (Fig. 3).

ANTIBODY PREPARATION AGAINST 220,000 MW CELL SURFACE PROTEIN

Because we felt that assays had to be developed which could detect phenotypic characteristics of single cells, we decided to prepare an antiserum against specific cell proteins and test whether this antiserum could be used to detect altered cells in populations.

Gels containing lysates of 1.5×10^8 cells were run and stained with Coomassie blue. The region which corresponded to the I^{125} 220,000 MW band was cut out, emulsified in PBS, diluted in complete Freund's adjuvant (FA) and injected subcutaneously into rabbits. This was repeated at day 14 with incomplete FA. Lysates of cells containing the 220,000 dalton protein were used to boost the animals

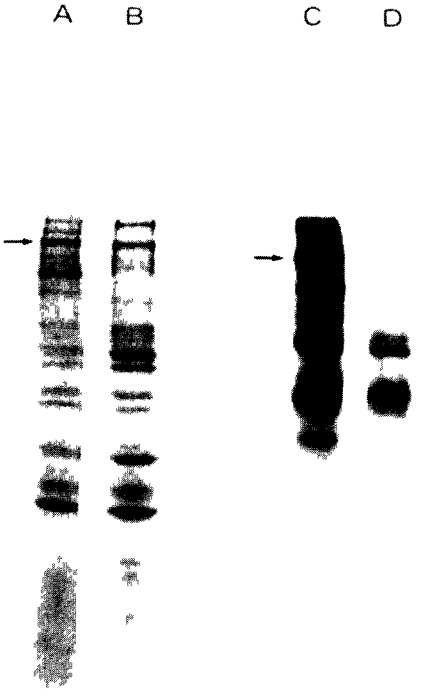

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Fig. 1 SDS polyacrylamide gel autoradiograms of I^{125} and C^{14} labeled normal and transformed Syrian hamster embryo fibroblasts.

A. Normal fibroblasts labeled with C^{14} L-amino acids
B. Transformed fibroblasts [A(T_1)C13]labeled with C^{14}L-amino acids
C. Normal fibroblasts lactoperoxidase I^{125} labeled
D. Transformed fibroblasts lactoperoxidase I^{125} labeled

The arrow denotes the 220,000 MW protein.

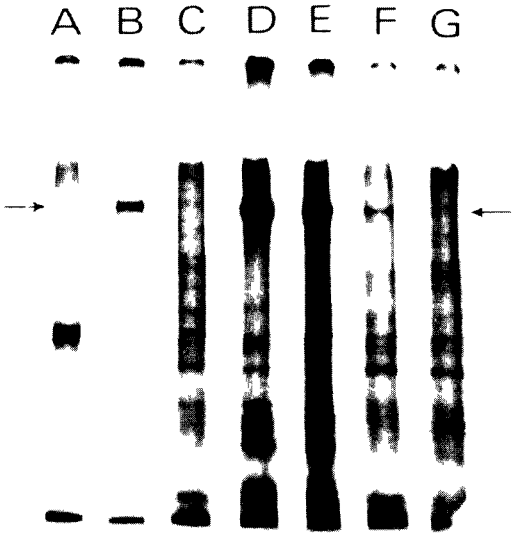

Fig. 2 SDS polyacrylamide gel autoradiogram of lactoperoxidase I^{125} labeled normal and transformed Syrian hamster embryo fibroblasts.

A. U-19 -- spontaneously transformed
B. U-18 -- late passage fibroblasts
C. T-6 -- tumor cell lines
D. E-11 -- early passage fibroblasts
E. B-14 -- late passage fibroblasts
F. A-12 -- transformed after treatment with benz(a)pyrene
G. A-6 -- transformed after treatment with benz(a)pyrene

The arrow denotes the I^{125} 220,000 MW protein.

Table 1

CHARACTERISTICS OF TRANSFORMED AND UNTRANSFORMED SYRIAN HAMSTER EMBRYO (SHE) CELLS

CELLS	ORIGIN	LETS PROTEIN	TUMORIGENICITY	SA[a]	PA[b]
E-11	Early passage, embryo culture from inbred animals	4+	Negative, with 10^7 cells inj.	Neg.	1+
A-12	B(a)P transformed cell line	1+	50% pos. at 10^4 cells inj.	3%	2+
A-6	B(a)P transformed cell line	-	75% pos. at 10^2 cells inj.	51%	4+
T-6	Tumor cell line	-	100% pos. at 10^1 cells inj.	96%	3-4+
B-14	Late passage SHE cells	4+	Neg. at 10^6 cells	0%	2+
U-18	Late passage SHE cells	3+	Neg. at 10^7 cells	0%	2+
U-19	Spontaneously transformed cell line	-	100% pos. at 10^7 cells	N.T.[c]	N.T.

a. Colony Growth in Soft Agar

b. Plasminogen Activator Activity - 0-4+ scale

c. Not Tested

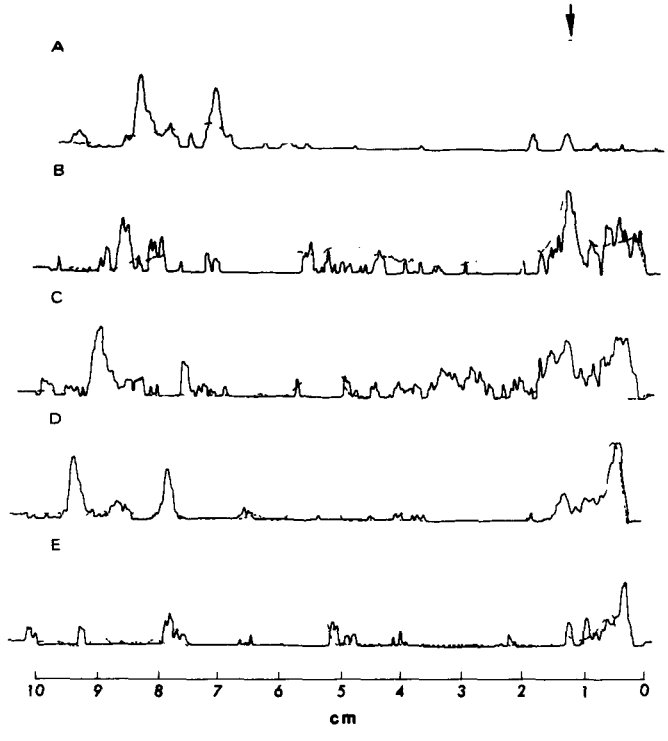

Fig. 3 SDS polyacrylamide gel patterns of lactoperoxidase I^{125} labeled mouse cell lines. Densitometer tracings are given for Coomassie blue staining (——) and I^{125} autoradiographs (---) of the gels.

A. 13H — transformed after treatment with 5-fluorodeoxyuridine
B. J2BB — transformed after treatment with 5-fluorodeoxyuridine
C. B_1b-C11 — transformed after treatment with cigarette smoke condensate
D. MCA-TC115 — transformed after treatment with 3 methylcholanthrene
E. C3H/10T½CL8 — untransformed parental cell line

Arrow indicates the position of the I^{125}-labeled protein (MW 250,000).

on day 21, 22, and 23. The animals were exsanguinated on day 30 which is prior to a time at which they could respond to new antigens in the crude lysate. This antiserum contains an antibody directed against an iodinatable 220,000 MW cell surface protein as determined by immunochemical methods.

IMMUNOFLUORESCENT STAINING OF CELLS WITH ANTI-220,000 DALTON ANTISERUM

In these experiments, tertiary cultures of Syrian hamster fibroblasts and transformed cells were stained with the antiserum and fluorescein-labeled goat anti-rabbit IgG to detect the antigen-antibody complexes. In all studies, non-immunized or pre-immunized rabbit serum was used as a control. Fig. 4 shows the staining pattern of unfixed, untransformed Syrian hamster fibroblasts and their corresponding phase microscopy. The staining seems to be at the surface of these cells. Unfixed transformed Syrian hamster cells ($A(T_1)Cl3$), prepared in an identical manner, have little to no staining. Fig. 5 demonstrates the staining of fixed transformed cells. Most of the cells show a diffuse staining pattern. We believe that acetone or ethand fixation allows penetration of the antibody and the staining suggests that there is an intracellular form of the antigen in transformed cells. This may be true in some transformed cells and not in others. The gels show 220,000 MW C^{14}-amino acid labeled or Coomassie blue stained protein in some transformed lines but not others. From studies on several transformed lines it appears that the decrease or loss of I^{125}-220,000 cell surface protein may occur by more than one process, i.e., decreased synthesis, failure to be retained on the cell, or by allowing a cryptogenic form to occur.

Other experiments on the staining of cells with the antibody suggest that the antigen is not actin, myosin, actin-binding protein or cold insoluble globulin. Adsorption of the antiserum with intact normal cells removes the antibody with resultant loss of its staining abilities. When a transformed cell line was used to adsorb the antiserum, only a slight reduction in the staining intensity was observed.

CYTOTOXICITY AS A METHOD OF SCORING A PARAMETER OF THE TRANSORMATION PHENOTYPE

It has previously been shown that Chinese hamster cells are killed by treatment with rabbit antiserum containing antibodies against cell surface proteins of Chinese hamster cells in the presence of complement (19). Since Syrian hamster cells are either devoid or contain a markedly reduced amount of the I^{125}-220,000 MW cell surface protein, we tested the capacity of our antibody in the presence of complement to kill normal cells. Table 2 demonstrates that a 1:800 dilution of antiserum is cytotoxic to untransformed cells in the presence of 1% complement.

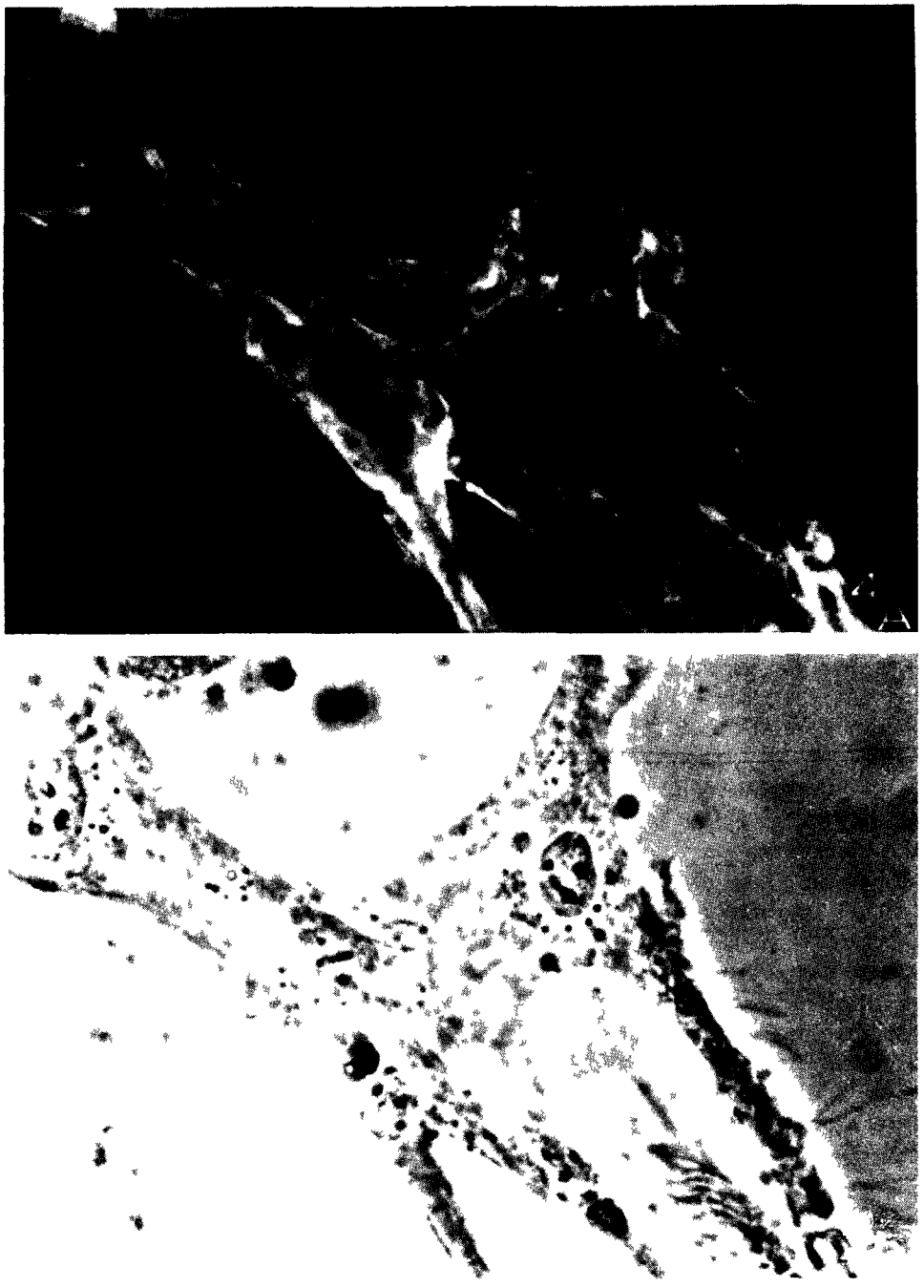

Fig. 4 Indirect immunofluorescent staining of Syrian hamster cells (unfixed) with anti-220,000 MW antiserum and goat anti-rabbit fluorescein conjugate.

A. Epifluorescence (420X)
B. Phase contrast (420X)

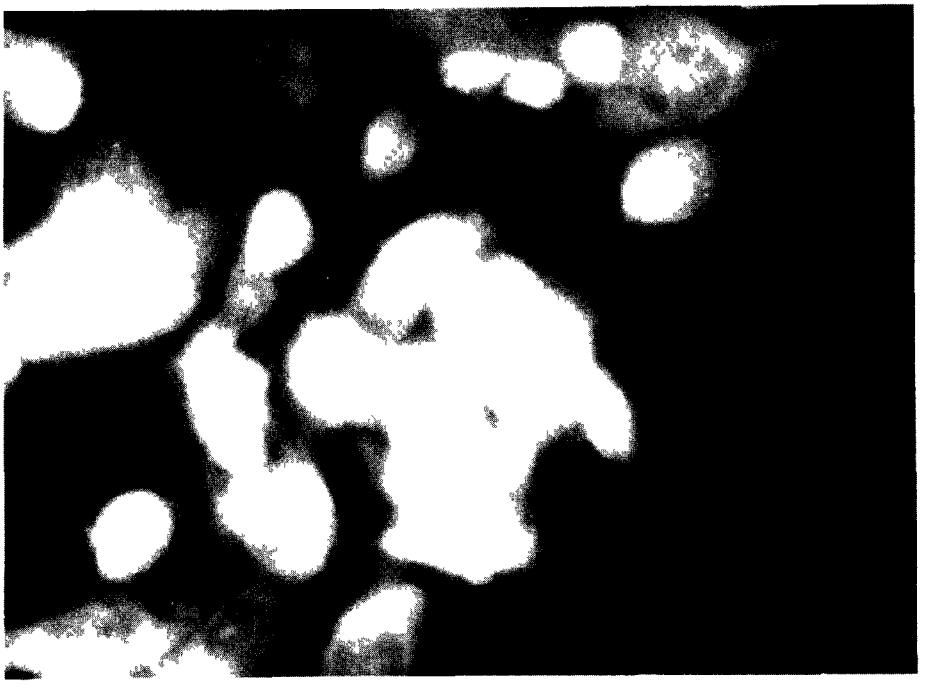

Fig. 5 Indirect immunofluorescent staining of transformed Syrian hamster cells treated with formalin and acetone (420X).

Table 2

Effect of Anti 220,000 Dalton Cell Protein

on Tertiary Syrian Hamster Cells

Treatment of Cells	Number of Surviving Colonies[a]
Media alone	19
Rabbit complement	25
Antiserum	23
Complement and normal rabbit serum	23
Complement and antiserum	1

[a]Colonies macroscopically scored after 7 days incubation. Initial plating was 2,000 cells per 35 mm plate. Average of 12 separate experiments performed in duplicate.

The controls (media alone, rabbit complement, antiserum, and 1:800 dilution of normal rabbit serum plus complement) show no effect on the cells. Table 3 shows that the antiserum has much less toxicity on transformed cells. Table 4 compares the cytotoxic effects of the antiserum on three transformed Syrian hamster cell lines with the untransformed hamster fibroblasts. The differences in the killing observed between untransformed and transformed cells indicate that each type of cell shows a variability either in the synthesis and/or the availability of this protein on its surface.

SUMMARY

Cell surface labeling techniques have allowed the examination of changes in the distribution of a 220,000 MW protein in normal and transformed cell populations. Antiserum produced against this protein has made possible the study of the distribution in individual cells by indirect immunofluorescence and the scoring of the transformed phenotype by selective killing in the presence of complement.

The problems we are now attempting to study are:

1. What are the properties of the clones which allow different percentage killing, e.g., is there heterogeneity within clones?

2. Are the survivors different in other phenotypic properties (other than resistance to the antibody); i.e., what is their (a) growth in agar, (b) lectin agglutinability, and (c) tumorigenicity?

3. If there is a true heterogeneity, is it reestablished after the selective pressure of the antibody? What modulates the amount and regeneration of heterogeneity?

4. Can the selective killing of untransformed cells be used in assays of chemical carcinogenesis to allow quantitation by counting surviving clones? Is this an early or late even in the progression of events which seems to occur after exposure to carcinogens?

Table 3

Effect of Anti 220,000 Dalton Cell Protein

on Transformed Syrian Hamster Cells[a]

Treatment of Cells	Number of Surviving Colonies[b]
Media alone	208
Rabbit complement	228
Antiserum	222
Complement and normal rabbit serum	231
Complement and antiserum	172

[a]Hamster cells were transformed after treatment with 1-B-D-arabino-furanysylcytosine (Ara-C).

[b]Colonies macroscopically scored after 5 days incubation. Initial plating was 1,000 cells per 35 mm plate. Average of 7 separate experiments performed in duplicate.

Table 4

Comparison of the Cytotoxicity of Anti 220,000 Dalton Cell Protein on Untransformed and Transformed Syrian Hamster Cells

Cells treated	Percent of Killing[a]
Untransformed	96
$A(T_1)Cl3$	25
P-MCA-75	17
A-6	59

[a] $\text{Percent of killing} = 1 - \frac{\text{Number of surviving colonies in the presence of antiserum and complement}}{\text{Number of surviving colonies in the presence of normal rabbit serum and complement}} \times 100$

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4.8 BIOPHYSICAL CYTOLOGICAL APPROACHES TO IN VITRO CARCINOGENESIS

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Biophysical cytology deals with the manipulation, measurement, identification, and sorting of cells and subcellular components by physical proceses (77,80). Differential centrifugation, optical scanning, and flow cytometry are the principal approaches of relevance to in vitro carcinogenesis. General sources of information are found in the literature on automated cytological diagnosis, automated cytogenetics, computer image processing, and biophysics (40,64,65,68,69,77,80,83,94,100-102).

The measurement and interpretation of cells and chromosomes by optical scanning is now a highly developed and commercialized capability. Cells can be recognized on the basis of content and distribution of stainable constituents, as well as size and shape of cytoplasm or nucleus. Both fluorescent and absorption stains are used (15,47,69,83,94,103). Recently developed but yet to be published approaches promise major increases in processing speeds, approaching those of flow cytometry.

In flow cytometry, objects in monodisperse suspension flow past optical or electrical detectors. Initially this technique was used to count cells and estimate cell volume by the change in electrical resistance in a minute aperture. The main emphasis today is on fluorescence measurement of cytochemically deposited fluorochromes. Precise measurements of the distribution of properties can be assembled automatically and objects are processed at rates of about 1000 per second. In flow sorting, the objects are separated on the basis of their cytometric values. Several commercial instruments are now available for both processes (12,27,45,46,51,63,68,90,91, 95-97,99). Light scatter signals can be generated simultaneously with fluorescence and are useful as a rough indicator of size (14,46,63,70,96).

The key to both scanning and flow approaches is the availability of a rich assortment of relevant cytochemical probes. In addition to the large classical histochemical literature (which is not referenced) and the specific techniques referred to below, there are several general methods and sources of information (12,15,18,28,29,33,40,48, 53,64,65,67,68,74,77,78,80,98,100,101).

Differential centrifugation and gravity separation are widely practiced at the cellular and subcellular level. Several

techniques are directly relevant to bulk separation of specific types of cells or chromosomes (30,34,62,104).

The application of biophysical cytology to in vitro carcinogenesis can take several forms: 1) the definitive assay of the transformed cell; 2) enrichment of transformed cells; 3) separation of transformed cells for biochemical, biological, or visual evaluation; 4) detection of transformed cells in vivo in blood or other accessible sites. These are potential, not yet achieved capabilities and their fruition will depend on the discovery of suitable markers for the transformation state.

Perhaps the best studied candidate markers are those involving DNA and the genetic apparatus. DNA content is the most quantitative cytochemical property of mammalian cells; it has been extensively measured and is known to be frequently abnormal in cancer cells (1,5,8,12,15,18,25,28,37,39,47,56,58,67,71,85,86,92,99,103,107). Qualitative differences in DNA have also been described in abnormal, malignant and transformed cells (2,8,11,13,19,26,29,35,41,54,61,67,71,85,86). Also, it is generally recognized that malignancy and perhaps transformation are highly correlated with chromosomal changes (4,9,37,56,60,69,75,79,81,82,92,95,104,105). Biophysical cytology is able to address these properties with currently available methods.

The identification and quantitation of enzymes is a richly developed cytochemical capability which is just beginning to be adapted to flow systems. Many enzymes have been linked to cancer and a few to transformation; it should soon be possible to measure one or more rapidly and accurately in single cells (24,31,33,42,44,48,50,51,53,55,59,72,76,78,88,89).

Fluorescence depolarization measures the rotational mobility of molecular probes in solution or in situ in living cells. Recently, the technique has been extended to flow cytometry allowing further testing of the evidence that transformed cells may have altered fluidity of membranes or other cell components (3,16,87).

Immunofluorescence has been successfully applied to flow cytometry. With further development of specific probes, it should be possible to study the membrane, chromosomal, and intracellular antigen-antibody reactions that mark the transformed cell (17,21,23,26,32,35,48,49,57).

In addition to the potential for direct identification of the transformed cell by biophysical cytology, there are several indirect applications worth listing briefly. Flow systems are excellent tools for cytokinetic analysis. Thus presently available methods can be used to study the early and late kinetic changes of the

evolving transformation (6,7,20,22,36,38,40,43,58,61,73,86,93,106); viable cells can be identified and sorted (45,66); and with impending developments such as vital stains for DNA content, there is the potential to synchronize cells by sorting prior to carcinogen treatment (3,7,8,10,34,38,43,54,86). Also, as markers for cell differentiation are developed, there is the exciting possibility of using sorting to enrich for the very cells that are most vulnerable to transformation (3,8,15,21,23,29,30,32-34,42,44,48,50,52,53,55,59,74, 84,89).

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4.9 AUTOMATIC TECHNIQUES IN CELL BIOLOGY: POSSIBLE APPLICATIONS TO IN VITRO CARCINOGENESIS

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I. INTRODUCTION

Rapid progress in many biological and medical research fields depends on observing growth of colonies of microorganisms or animal cells on solid surfaces. Very often the pace and even the ultimate success of the work is limited by the number of colonies that can be observed under well-defined conditions in order to find a rare mutant, measure the rate of mutation induced by some chemical, or determine the probability that a virus causes a neoplastic transformation in a growing animal cell. In addition to the need for examining very large numbers of colonies for these purposes, it is extremely useful to be able to make accurate quantitative measurements of the response of these colonies to a variety of environmental factors. Our methods are designed to apply modern automation methods including materials handling and computer aided pattern recognition to make possible large experiments and quantitative observations on microorganisms and animal cells in tissue culture. A wide variety of studies in fundamental biology and applications to practical biological and medical problems should be aided by these methods. The work in progress ranges from the study of the genetics and physiology of E.coli bacteria and other microorganisms to studies of mammalian cell genetics and the response of such cells to a variety of hormones, mutagens, and potential carcinogens and teratogens. It is expected that these various projects will provide large numbers of mutants of various cell lines very difficult to obtain by conventional hand methods for detailed study in a number of laboratories. They will aid in the mapping of genetic markers in a variety of microorganisms and animal cells which will be important in understanding the biochemical genetics and regulatory systems of these organisms. Feasibility studies for screening programs testing the effectiveness of putative chemotherapeutic agents and examining suspected environmental mutagens, carcinogens and teratogens can be carried out. These methods are applicable to a wide range of difficult medical and biological problems now being attacked in many places but they also make possible an attack on problems usually considered intractable by conventional methods.

II. ADVANTAGES OF A LARGE-SCALE AUTOMATED SYSTEM FOR THESE APPLICATIONS

What does this kind of large-scale automation have to contribute to biomedical science? In general, many experiments profit from the economy of scale and the need for precise control of environmental conditions for the possibility of accurate quantitative measurements. In particular:

(a) Accurate Control of Biological Conditions. With the use of suitable sensors and control circuitry cheaply and reliably monitored with small computers, growth conditions can be controlled very accurately over a large biological sample for extended periods of time and can be changed on a precisely repeatable schedule so that all portions of the biological sample will have experienced exactly the same conditions of growth.

(b) Accurate quantitative measurements of growth and other biological responses to the environment are possible which cannot be done at all by manual methods in many cases.

(c) Accurately timed measurements of biological response can be made on a schedule optimized for the experimental result desired without regard to personal fatigue and convenience of the experimenters.

(d) Advantages of Large Scale

1. Rare events for which no biological selection can be devised can be discovered for study.

2. Extensive statistics can be collected for known or for commonly occurring classes of events.

3. Many similar experiments can be done at the same time in parallel so certain kinds of experimental results can be achieved more rapidly than when serial experimentation is required.

4. The cost of developing sophisticated technology can be spread over many projects which can share the technology even when no single project can justify the necessary investment of time and funds.

5. Mutants and special strains can be provided for many small groups which would otherwise be unable to attack

problems requiring highly specialized starting strains of cells.

III. TECHNIQUES OF OBSERVATIONS

Optical methods of observation were chosen in order to make rapid and accurate measurements on very large numbers of colonies. Time-lapse photographs of growing colonies are taken with 35 mm black and white film using five different colors of illuminating light in sequence. Between successive photographs, the colonies can be exposed to a wide variety of temperature, gaseous environment and radiation regimes in addition to treatment with drugs, nutrients, enzyme substrates that show a color change if the enzyme is present, and various other types of vital stains. Quantitative measurements of these photographs are carried out by a flying-spot scanner (similar in operation to a very high resolution television camera) connected directly with a sizeable computer. These measurements can produce curves describing rate of growth, colorimetric response, changes in appearance and other optically detectable consequences of the known treatment and allow by this means the quantitation of the response and the selection of individual colonies that respond in a particularly specified way.

IV. TECHNIQUES OF COLONY INCUBATION AND MANIPULATION

Colonies are grown in conventional 100 mm petri dishes or on large glass sheets (about 40 x 80 cm) covered with agar or other growth substrate. The dishes or trays are handled manually in a presently operating prototype and will be handled automatically in a large machine now under construction. Temperatures are held constant and uniform to better than $0.1^{\circ}C$ and other biologically important parameters are similarly controlled. Inoculation, colony picking, restreaking, replica plating, administration of drugs, nutrients and other liquids are all carried out by automated equipment under computer control. The capacity of the final system will be about 10^8 colonies per batch.

V. RESULTS

The system has been used successfully for isolating large numbers of E.coli mutants for particular studies. For example, 1.4 million colonies were examined to isolate 2266 heat sensitive mutants which included 110 mutants unable to synthesize DNA at $41^{\circ}C$ but apparently normal at $30^{\circ}C$. These have been mapped and shown to include 11 genes located at different sites on the E.coli map. A number of other nutritional and regulatory mutants of E.coli and Salmonella have been isolated. Present experiments include extension of this work to cold

sensitive mutants of E.coli, to heat and cold sensitive mutants of yeast and to a variety of other bacterial projects.

By analysis of the appearance of colonies growing on agar, it has been possible to identify bacterial pathogens that cause human urinary infections so that an error rate of less than about 1 in 1000 is experienced in identifying such pathogens from a panel of about 12 unknowns.

Chinese hamster ovary cells grown on agar in this system reveal subtle differences in colony morphology not detected when the same cells are grown in the conventional way on plastic under liquid nutrients. These subtle differences breed true in many cases but the colony morphology and growth rate is found to depend very sensitively on small amounts of insulin, testosterone, and cyclic AMP. This combination of stability of clonal appearance with sensitivity to hormonal (and also mutagenic) agents will be the basis for a large scale mutant hunt in which we will try to find large numbers of heat sensitive Chinese hamster ovary cells including those defective in DNA synthesis and its control. Other animal cell experiments designed to furnish the basis for a test method for carcinogens, teratogens and mutagens in mammalian cells are being developed.

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5. WORKSHOP ON LABORATORY PROCEDURES

5.1 THE USE OF EARLY PASSAGE HAMSTER EMBRYO CELLS FOR THE IDENTIFICATION OF CHEMICAL CARCINOGENS

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METHODOLOGY

Culture Medium. Dulbecco's modified Eagle medium (DMEM) containing sodium pyruvate and a low glucose content (Grand Island Biological Company, Grand Island, N.Y.), supplemented with 2mM L-glutamine and 20% sterile fetal bovine serum (FBS) (Rehatuin, F.S., Reheis Chemical Company, Phoenix, Arizona), heat-inactivated at 56°C for 30 minutes was used in all bioassay experiments. DMEM was prepared either from powdered stock with deionized double-distilled water and sterilized by pressure filtration through 0.22μ Millipore filter, or purchased as sterile liquid medium. No antibiotics were used.

Quality Control. The reproducibility of the bioassay employing early passage hamster embryo cells, requires stringent control of the quality of serum, culture medium, and plastic culture vessels. Only those lots of FBS specified by the supplier to be free of adventitious microbial or viral agents were considered for use. To confirm the absence of mycoplasma or bacteriophage, aliquot samples were retested by independent laboratories. Aliquot samples of trypsin solution were similarly monitored for contamination by mycoplasma.

All lots of DMEM, FBS, as well as plastic vessels (Falcon Plastics, Oxnard, Ca.) were pretested for their ability to support optimal growth of standard pools early passage hamster cells for two subcultures when seeded to form individual colonies or monolayers.

Hamsters. Embryos from timed-pregnant Golden Syrian hamsters of the outbred, systematically randomized LVG/LAK strain, purchased from Lakeview Hamster Colony, Newfield, N.J., served as the source of primary embryo cells subsequently used as target or feeder cells in all experiments.

Target Cells. Pregnant hamsters were sacrificed by carbon dioxide asphyxiation, usually on the 13th day of gestation. The uteri were removed aseptically and the embryos were transferred to a sterile petri dish containing warm calcium-, magnesium-free tissue culture diluent (TCD) prepared from 20X stock containing: 160g NaCl, 8g KCl, 0.71g Na_2HPO_4 and 2 ml 1% phenol red in 1 liter of deionized double-distilled water. For use, the concentrate was diluted to 1X with deionized double-distilled water, supplemented with 0.1% glucose and adjusted to pH 7.4 with 7.5% $NaHCO_3$.

The embryos were decapitated, eviscerated, and the remaining tissue was minced finely with scissors or apposing scalpels, and then washed with TCD to remove extraneous red blood cells and debris. After a short preliminary wash with 0.25% trypsin solution to further remove any remaining red blood cells, the tissue fragments were disaggregated by treating the sample with trypsin at least twice for 20 minutes at 37°C and pooling the supernatant cell suspensions. The cells were washed once by centrifugation at 200 x g for 10 minutes, resuspended in a minimum measured amount of TCD and counted. Approximately 1 X 107 cells were planted per 75 cm^2 flask in DMEM and incubated at 37°C in a humidified atmosphere containing 10% CO_2 and air. When approximately 80-90% confluent (usually 2-3 days) the cells were trypsinized, washed and dispensed into glass ampoules at 2.5 or 5 X 10^6 cells/ml in DMEM containing a final concentration of 7.5% dimethylsulfoxide (DMSO) (Crown-Zellerbach Corp., Camas, Wa.) and 20% FBS. If necessary, the cell suspension was adjusted to pH 7.4 by gassing with pure CO_2 immediately prior to flame-sealing the ampoules. The ampoules were placed into a mechanical freezer (Revco) at -85°C for 1 1/2-2 hours and immediately transferred to a liquid nitrogen freezer and stored in the liquid phase at -195°C until needed.

Cells from aliquot samples were tested for their ability to undergo morphological transformation by the reference carcinogen, 3-methylcholanthrene (3MC). Suitable responsive pools of cells were then used in bioassay experiments. Nonresponding cultures were discarded.

For use in the bioassay, an ampoule of frozen target cells was rapidly thawed by immersion in a 37°C water bath and seeded into a 75 cm^2 flask containing 20 ml DMEM and incubated at 37°C. The medium was changed daily until the culture was approximately 80-90% confluent when the cells were trypsinized and seeded at 500 cells per dish in 2 ml of DMEM into 50mm plastic petri dishes previously seeded with x-irradiated feeder cells.

Feeder Layer Cells. Primary cultures of cells subsequently used as feeder cells were prepared in a manner similar to that used for target cells.

Chemicals. Chemicals were stored over silica gel in a dessicator at either -20°C or 4°C, as required to maintain stability. Bioassays were performed in rooms illuminated by yellow lamps (Sylvania F40G0) to minimize photodecomposition of chemicals.

All test chemicals were coded prior to bioassay to eliminate bias when stained dishes were monitored for transformation. Stock solutions were always prepared immediately prior to use. Water-soluble chemicals were dissolved in DMEM, while water-insoluble chemicals were initially dissolved or suspended in DMSO at 1-10 mg/ml. These were further diluted with DMEM to desired concentrations.

Safety Precautions. To minimize exposure of personnel to potentially biohazardous materials, all test chemicals were weighed out in an approved chemical fume hood and stock solutions were prepared in a vented laminar flow safety cabinet. Respirator masks, disposable gloves, and protective outer garments were worn. All cell culture manipulations were performed in laminar flow cabinets (Baker, Sanford, Me., or Contamination Control Inc., Kulpsville, Pa.). Chemically contaminated spent culture media and wash solutions were collected by vacuum aspiration onto absorbant material and subsequently incinerated. To avoid contamination from recycled glassware, only disposable labware was used and this was incinerated after use. Stained dishes were handled with gloves to avoid contamination by residual carcinogens.

Bioassay. The procedure previously described (Berwald and Sachs, Nature, 200, 1182, 1963; DiPaolo, et. al., J.N.C.I. 42, 867, 1969, Nature 235, 278, 1972) was modified to incorporate cryopreserved primary cell cultures as sources of target and feeder cells. Usually four days before use as a feeder culture, an ampoule of cryopreserved primary cells was rapidly thawed by immersion in a 37°C water bath. The cells were seeded into a 75 cm^2 flask containing 20 ml DMEM and were refed daily. The day before x-irradiation, the cells were trypsinized and reseeded into 75 cm^2 flasks at a concentration appropriate to obtain 75-80% confluence on the next day. The cells were then irradiated with 5000R by means of a Picker Portable Industrial x-ray machine fitted with 0.55 mm copper and 0.25 mm aluminum filters and with the cell monolayer 30 cm from the x-ray source. The cells were immediately washed with TCD, trypsinized and seeded at 6 X 10^4 cells per 50 mm dish in 2 ml DMEM in the number of dishes required for the bioassay.

For use as target cells in the standard bioassay an ampoule of cryopreserved cells from a pretested culture was rapidly thawed and seeded into a 75 cm^2 flask containing 20 ml DMEM and incubated at 37°C. The medium was changed daily and when the culture was approximately 80-90% confluent it was trypsinized. Approximately 500

cells contained in 2 ml DMEM were seeded into 50 mm plastic dishes when had been seeded 24 hours previously with x-irradiated feeder cells. On the next day, graded doses of test chemicals, prepared double-strength in DMEM, were added to the cultures in 4 ml amounts to obtain the desired final concentrations of the chemicals in the culture medium. Control cultures were similarly treated with DMEM alone, or DMEM containing a final concentration of 0.2% DMSO.

The cultures were incubated for 8 days without disturbing or refeeding. They were then washed twice with TCD to remove residual unbound chemical remaining on the dishes, fixed with methanol, and stained with Giemsa. After air-drying, the stained dishes were monitored by means of an Olympus Model JM zoom stereomicroscope (7-40X magnification) to identify normal and transformed colonies.

Criteria for In Vitro Transformation. Although a heterogeneous population of colonies is observed in this system, the endpoint used for determining carcinogenic activity is the presence of fibroblast-like colonies morphologically altered beyond that observed in normal cultures. Cells in a transformed colony exhibit three-dimensional growth with extensive random-oriented growth and crossing-over at the periphery of the colony (DiPaolo, et. al., Cancer Res. 31, 1118, 1971, Pienta, et. al., Proc. 3rd. Int. Symp. on Cancer Detect. Prevention, 1976 in press). These cells usually have an increased ratio of nucleus to cytoplasm, are more basophilic, and are variable in size (Sanford, J.N.C.I. 53, 1481, 1974). The center of transformed colonies usually exhibits dense piling up of cells. These criteria are summarized in the chart.

SOME CRITERIA FOR MORPHOLOGIC TRANSFORMATION OF HAMSTER EMBRYO CELLS

Example	Colonial Morphology		Evaluation
	Center	Periphery	
A	P+	X+	Transformed (Contiguous Colony)
B	P+	X-	Normal
C	P-	X-	Normal
D	P±	X+	Transformed (Semidispersed Colony)

P- = No piling up of cells
P+ = Dense piling up of cells
P = Moderate piling up of cells
X- = Little or no crossing over of cells
X+ = Extensive crossing over of cells

STANDARD HAMSTER EMBRYO CELL IN VITRO CARCINOGENESIS BIOASSAY

Day 0

Reconstitute frozen standard feeder cells from liquid nitrogen

Day 1

Refeed feeder cells

Day 3

Seed feeder cells onto 50 mm dishes at 10^6 cells/dish; reconstitute frozen standard target cells

Day 4

X-irradiate feeder cells with 5000_4R, then refeed onto 50 mm dishes at 6 X 10^4 cells/dish; refeed target cells

Day 5

Seed target cells onto feeder layer cells at 500 cells/dish

Day 6

Add chemicals and incubate dishes without disturbing or refeeding

Day 13

Fix with methanol, stain with Giemsa and monitor dishes for transformation

5.2 QUANTITATIVE FOCUS ASSAY FOR TRANSFORMATION OF SYRIAN HAMSTER EMBRYO CELLS BY CHEMICAL CARCINOGENS

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Cell Cultures. Primary hamster cell cultures (HEC) were prepared by trypsinization of eviscerated and decapitated embryos after 13-14 days of gestation. Cells were resuspended in Dulbecco's MEM medium or BioLabs Modified Dulbecco's medium (Casto, 1973) herein referred to as BMD, supplemented with 5% heat-inactivated Reheis fetal bovine serum (FBS), and 0.22g% $NaHCO_3$. Approximately 1.0 X 10^7 cells in 20 ml of medium were plated into 100-mm LUX plastic Petri dishes and incubated in a 5% CO_2 atmosphere at 37°C. Total cell counts after 3 days usually ranged from 1.0 to 1.2 X 10^7 cells per plate. Secondary HEC were prepared by transferring 5 X 10^6 cells into 100-mm dishes in 20 ml of the above medium, but with 10% FBS.

Chemical Transformation. Stock solutions of chemicals were prepared at concentrations of 1 or 10 mg/ml in acetone. Appropriate dilutions were made in complete medium to give the desired final concentrations.

Secondary HEC were plated at 5 X 10^4 cells/60-mm dish in 4 ml of BMD containing 10% FBS and 0.11g% $NaHCO_3$. After 24 hours, 4 ml of chemical dilution (as 2X concentrations) were added to each of 5 to 10 plates per dilution and incubated for 3 days at 37°C in 5% CO_2. The culture fluid was then replaced with fresh medium (0.22g% $NaHCO_3$) containing chemical dilutions at 1X concentrations for an additional three days. After a total of 6 days exposure, the chemical containing medium was removed and the cultures fed with 6 ml of BMD, 10% FBS, and 0.22g% $NaHCO_3$ at 3 to 4 day intervals. After 21 to 25 days from the time of chemical addition, the cells were formalin-fixed, Giemsa stained, and scored for transformed foci. Verification of the transformed morphology was made with a stereomicroscope at 10X to 30X magnification.

Cell lethality due to chemical was determined on plates seeded with 1000 cells and treated as above. The surviving colonies were formalin fixed and stained with 0.02% crystal violet approximately 8 days after seeding. The number of surviving colonies among cells treated with chemical was divided by the number of colonies in solvent treated cells to give the surviving fraction of chemically treated cells.

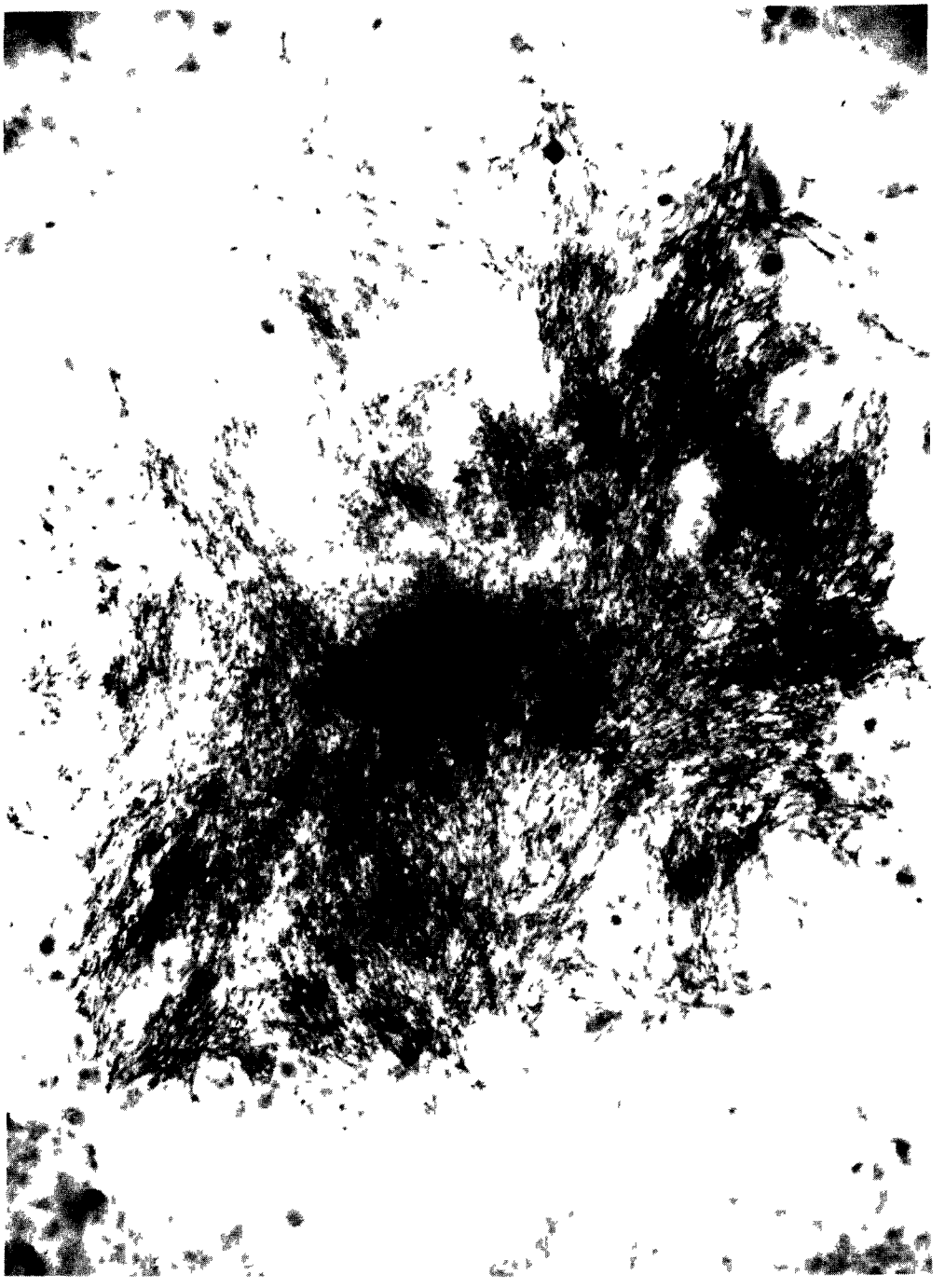

Figure 1. Transformed cell focus in hamster embryo cells following 6 days of exposure to 250 ug/ml of phthalazinone. Cells were fixed and stained 21 days after removal of the chemical. x40

Figure 2. Transformed cell focus in hamster embryo cells following 6 days of exposure to 2 ug/ml of 3-methylcholanthrene. Cells were fixed and stained 21 days after removal of the chemical. x80

To obtain cell lines from the transformed foci, selected Petri dishes, after 17-20 days incubation, were overlayed with medium containing 1% methylcellulose (Methocel). Five to 7 days later, individual foci were transferred with a Pasteur pipette and each placed into a 60-mm dish in 3 ml of medium. The cells were fed every 3 to 4 days until a sufficient number was present for passage into fresh dishes. The cultures were first passaged from one dish into 5 dishes and after establishment in culture were passaged weekly at 1:10 split ratios.

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5.3 IN VITRO CULTURE OF DIPLOID HUMAN SKIN EPITHELIAL CELLS FOR CHEMICAL TRANSFORMATION STUDIES

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Three problems are common to the culture of primary epithelial cells from a variety of epithelial tissues. These are:

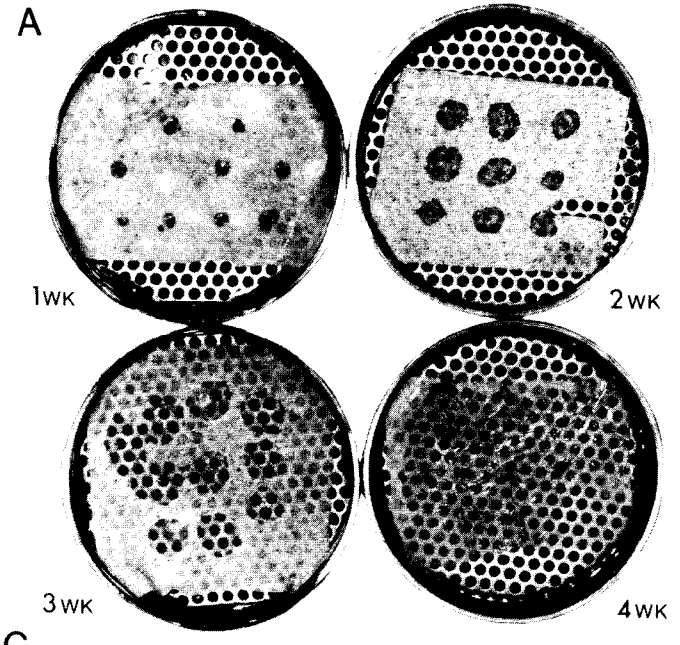
(1) Nutritional and cultural conditions for growth and maintenance of epithelial elements

(2) Physical or selective removal of fibroblasts

(3) Conditions for subculture with long-term retention of epithelial character.

No one culture method yet exists which solves all of these problems. In this demonstration we show three culture methods currently being developed for in vitro carcinogenesis studies with human skin epithelium. Each of these methods for the in vitro culture of adult human skin has distinct advantages or disadvantages as outlined in Table 1.

The first method is the growth of skin explants on a substrate of sterile dead pigskin (1). In this system, skin mince pieces are planted on pigskin which rests dermal side up on an organ culture type grid (Fig. 1A). Medium is added to the level of the grid such that the mince is nourished through the pigskin. After a period of 5-9 days, epithelial cells are seen to migrate from the mince onto the pigskin dermis and have commenced replication (Fig. 1B). A continuous process of replication and differentiation ensues over the following weeks as clearing of the pigskin through digestion of the dermal substrate occurs. These clear areas represent digested pigskin dermis covered with the four layers of skin found in vivo -- basal layer (germinative layer), squamous layer, granular layer, and horny layer (keratin) (Fig. 1C). Expansion of the epithelium continues until the pigskin surface is confluent with new epithelium. Several subcultures to new pigskin substrate can be achieved by either scraping new growth from the digested pigskin, mincing and replanting onto new pigskin, or by cutting out cleared areas and inverting on new pigskin.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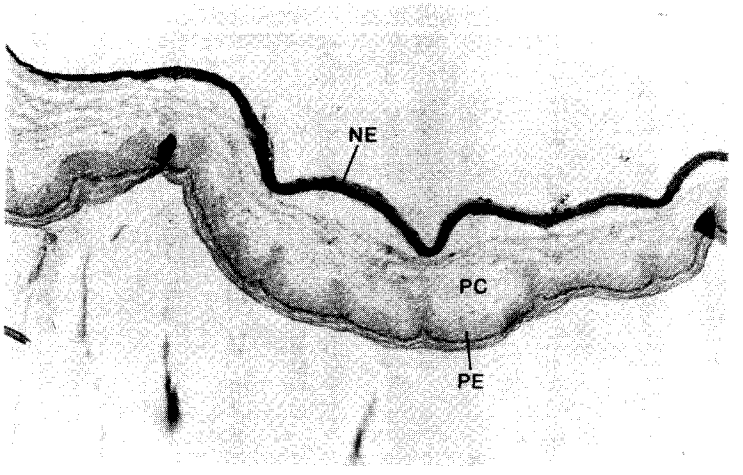
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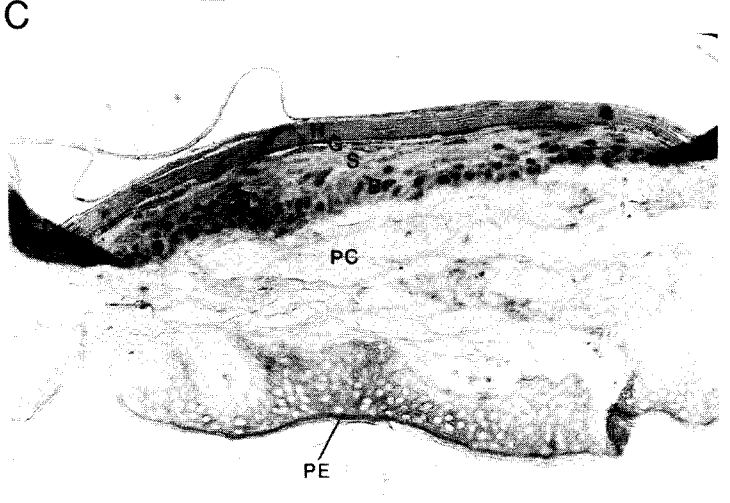
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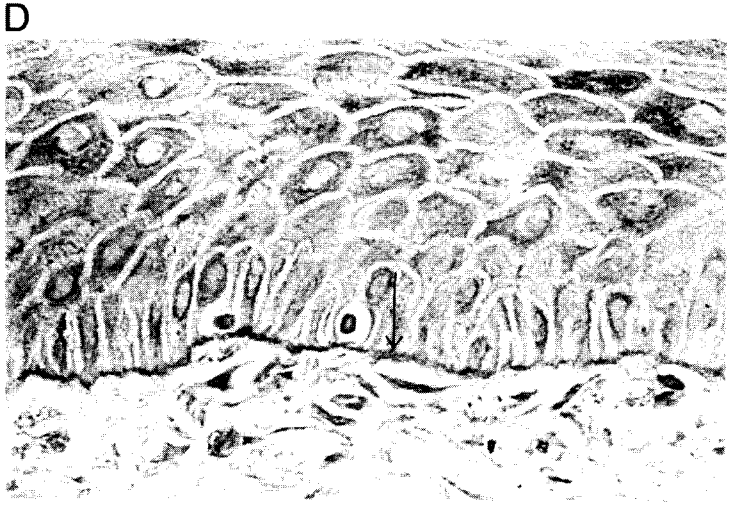
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Figure 1 (A) Progressive clearing of pigskin associated with human skin epithelial cell growth. Each clear zone is derived from one mince piece.*

(B) Histological section of a 14-day outgrowth of human skin epithelial cells (NE) on pigskin. Pigskin collagen fibers (PC) under the epithelium have been partially digested while the pigskin epithelium (PE) remains intact. (120X) Hematoxylin-Eosin stain.*

(C) Histological section of a 21-day outgrowth of human skin epithelium on pigskin. Skin cells organize into the basal (B), squamous (S), granular (G), and horny (H) layers characteristic of in vivo skin. (240X) Hematoxylin-Eosin stain.*

(D) Biopsy of an in vitro grown human epithelial skin graft three months after surgery. Note reformation of true epithelium with intercellular bridges and a basement membrane (arrow) separating the dermal and epidermal layers (640X) Hematoxylin-Eosin stain.

* Figure A, B, C from Freeman et al. (1) with permission from In Vitro.

Grafting of new epithelium to an isogeneic host is achieved by inverting the pigskin onto a prepared graft site so that the in vitro grown human cells are in direct contact with the wound bed and the pigskin serves as a protective dressing. After several days, new characteristic epithelium is found over the wound (Fig. 1D).

For in vitro carcinogenesis studies the pigskin system has the disadvantage that biochemical analysis is difficult because of interference of pigskin protein and dead dermal elements. In addition, periodic microscopic examination of the epithelium in a horizontal plane is impossible; however, vertical sectioning of fixed pigskin yields histological sections with excellent definition.

The second method involves growth and differentiation of skin explants or trypsin-collagenase digests of skin on a layer of 6000R-irradiated mouse 3T3 fibroblast feeders (2). In this system, (Fig. 2) the feeders serve to accelerate the growth of epithelial cells (keratinocytes) while simultaneously retarding the growth of dermal fibroblasts which invariably contaminate primary skin cultures. Subculture is achieved by EDTA removal of feeders and a small number of fibroblasts followed by complete dissociation of epithelium with trypsin-EDTA. Upon replanting on new mouse 3T3 feeders, new epithelial islands arise with approximately 1% plating efficiency. Two to four subcultures are feasible. The disadvantages of this system are the possible contamination of human cells with mouse endogenous viruses or other mouse genetic material and interference of mouse cells with biochemical analysis of the donor epithelial elements. Due to the low plating efficiency it is difficult to increase the total population of epithelial cells on subculture.

The third method is conventional explant growth on a tissue culture grade plastic. Superior growth and longevity of primary cultures is obtained in a closed or low gas exchange vessel (flask T-75 or T-25). Open dishes for unknown reasons yield slower growth and premature keratinization. Foreskin or biopsy skin mince pieces are placed on a dry vessel surface and allowed to adhere for 0.5-3 hours without medium (dried on). Dulbecco's Modified Eagle's Medium (DMEM) containing 5%-10% fetal bovine serum (FBS) is then added. After 20-50 days of incubation at 36.5°C in a humidified incubator at 10% CO_2 certain mince pieces yield exclusively fibroblast colonies and others yield true keratinizing epithelial colonies (Fig. 3). If the original skin sample is cleaned exhaustively by scraping away most of the dermis and underlying connective tissue, 70 to 90% of the mince pieces will yield epithelial colonies free of fibroblasts. Each donor sample will vary with respect to the relative growth of epithelial versus fibroblastic elements; this is dependent on the amount of dermis in each mince piece and the number of mince pieces per unit area of the culture vessel.

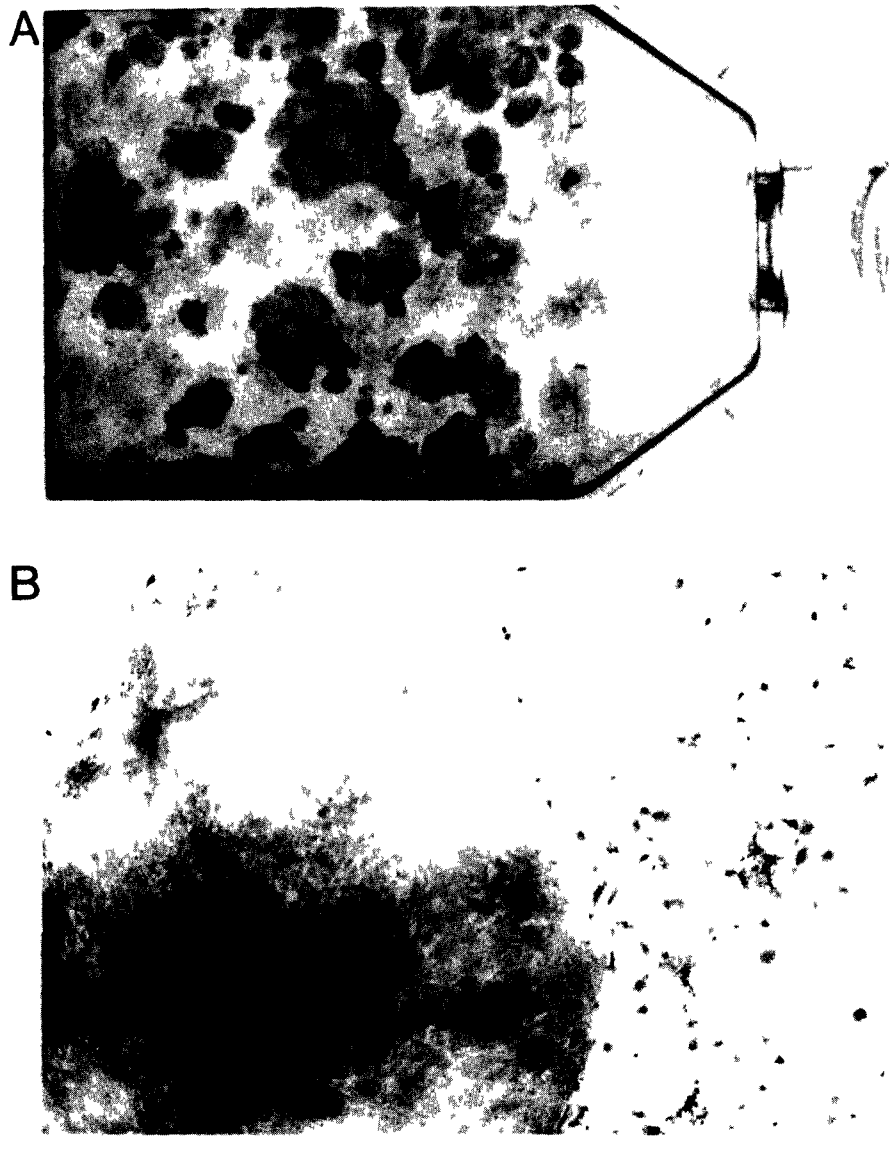

Figure 2 (A) Thirty days of human skin epithelial cell growth on a feeder layer of 5 X 105 irradiated 3T3 mouse fibroblasts. Foreskin was digested overnight in 0.25% trypsin, 0.125% collagenase, and seeded at 1 X 105 viable cells per 25 cm2 Falcon flask. Rhodanile blue stain.

(B) Microscopic view of an epithelial cell island in the above flask. (125X) Most islands emanate from clusters of input cells as seen in the center of this island. Surrounding cells are 3T3 mouse fibroblast feeder cells.

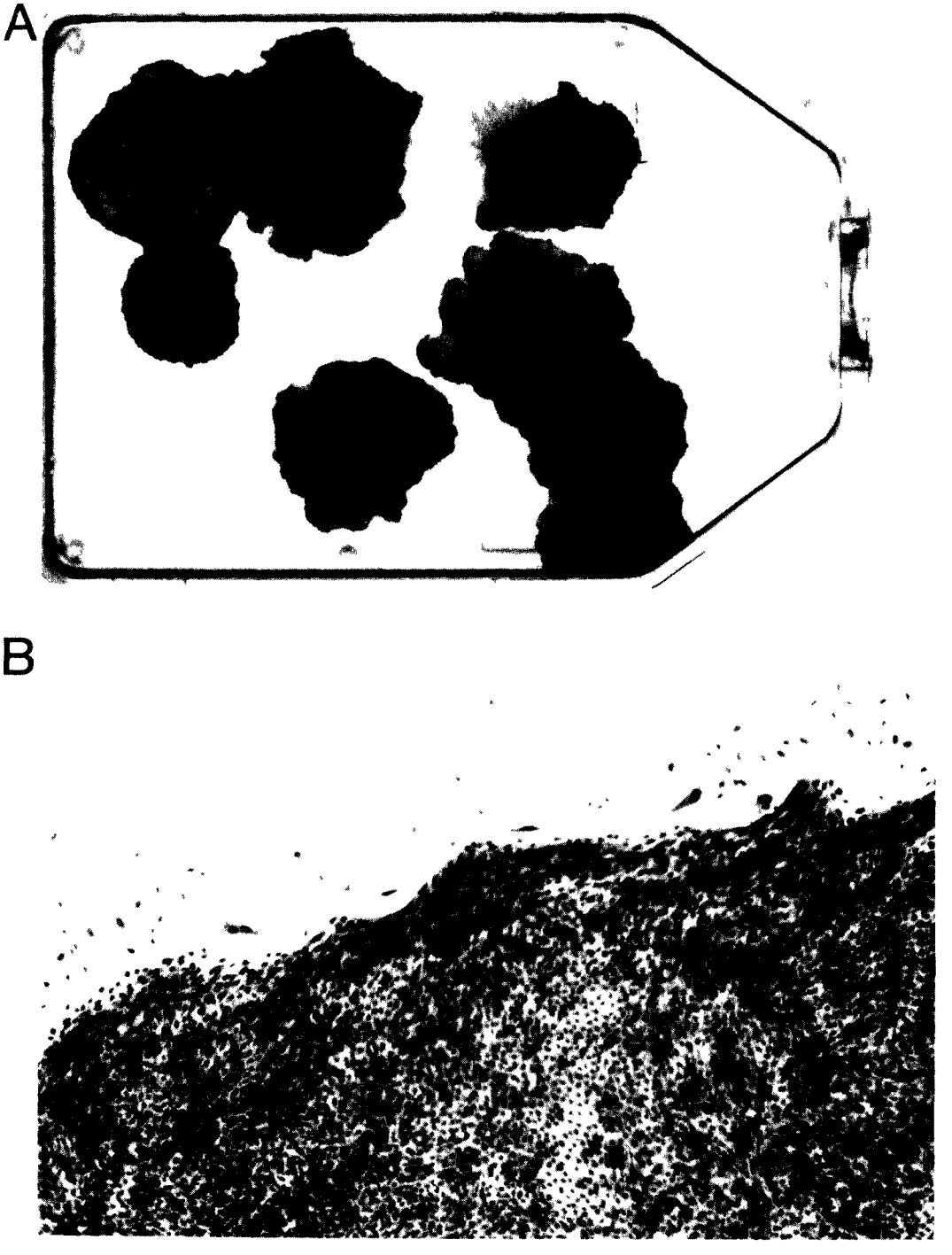

Figure 3. (Sheet 1 of 2)

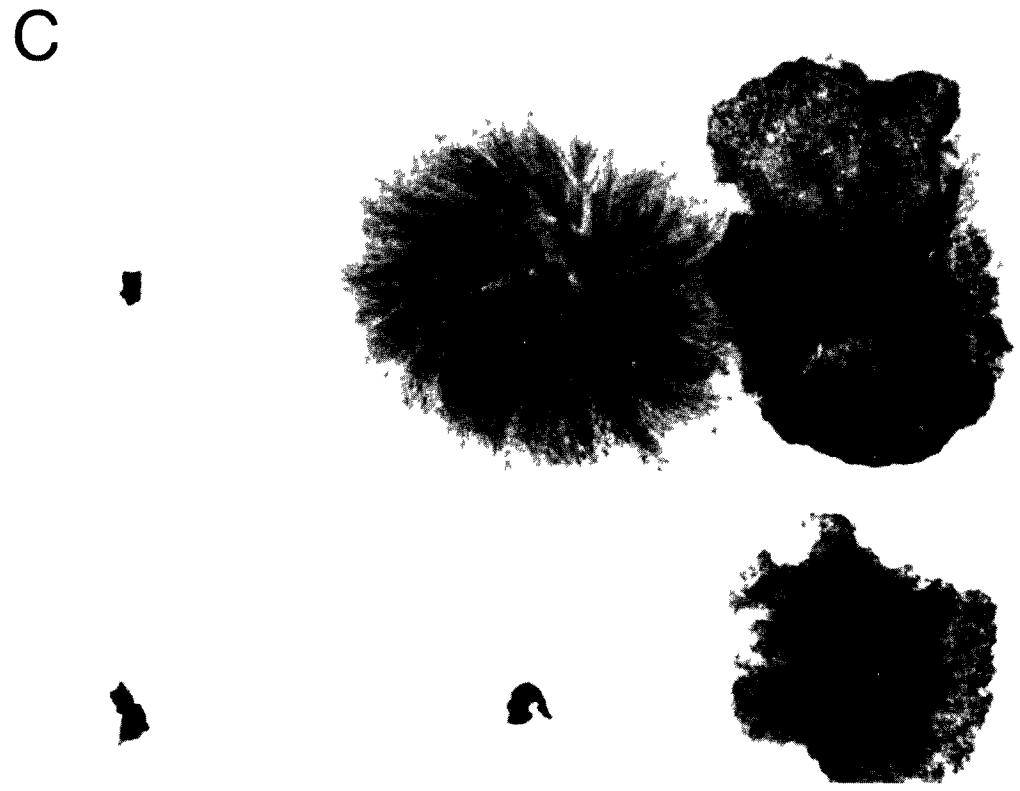

Figure 3 (A) Forty-three days of epithelial cell growth from skin explants grown in plastic flasks. Giemsa stain.

(B) Microscopic view of an epithelial cell island in the above flask. (125X) Note the fibroblasts apparent at the periphery of this island.

(C) Twenty-five days of growth from skin explants on plastic. Two islands are keratinizing epithelium (right); the other is dermal fibroblasts (left). Origin mince pieces to the far left have no cell outgrowth; these are usually pieces of fat. Giemsa stain.

Figure 3. (Sheet 2 of 2)

The obvious advantage of this method is that direct phase microscopic examination is feasible, and individual colonies can be examined for some biochemical property such as macromolecule synthesis or carcinogen metabolism free of interference from feeder cells and pigskin proteins. A major disadvantage of this system of skin growth is that subculture by conventional trypsinization and replanting is uniformly a failure since only fibroblasts remain after 1 or 2 subcultures.

For in vitro chemical carcinogenesis studies, we hope to combine these methods to characterize critical variables and develop a transformation assay in some animal model. With increased survival of epithelial cell types on a feeder system there will be the potential of retaining more target cells with the requisite number of cell divisions during and after treatment with chemicals. Using plastic grown mixed epithelial and fibroblast cultures we have demonstrated that skin epithelial cells have greater carcinogen metabolism than dermal fibroblasts from the same donar (3). With the advantage of being able to graft epithelium from pigskin to an isogeneic host with reformation of true skin epithelium it may be possible to demonstrate carcinogenicity if the target cells are of epithelial origin.

TABLE 1. SKIN EPITHELIAL CELL CULTURE METHODS

METHOD	ADVANTAGES	DISADVANTAGES
Pigskin Substrate	Grafting Slow Fibroblast Take Over Histopathology Subculture	Biochemical Analysis Microscopic Examination
Plastic with Feeders	Growth From Dispersed Cells Survival of More Epithelial Cell Types Subculture Slow Fibroblasts Take Over Microscopic Examination	Agents From Feeder Cell Biochemical Analysis Low Plating Efficiency
Plastic	Explant Colonies of Epithelial or Fibroblast Origin Biochemical Analysis Microscopic Examination	Subculture Rapid Fibroblast Take Over

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5.4 PROCEDURES AND PROTOCOLS FOR USING DIPLOID HUMAN FIBROBLASTS TO MEASURE THE EFFECT OF DNA REPAIR ON THE CYTOTOXIC AND MUTAGENIC ACTION OF CARCINOGENS

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PROCEDURES

Cell Cultures

Stocks of normal fibroblasts derived from foreskins were established, cultured, and stored until use in liquid nitrogen as described previously (2,3). Excision repair defective fibroblasts, derived from skin biopsy specimens of XP patients as well as Lesch-Nyhan patients were obtained from the American Type Culture Collection (Rockville, Md.) and similarly cultured. Cells used in these experiments were from stocks in passages 6-18.

Culture Medium

Stocks were cultured in 250 ml plastic flasks in a humid atmosphere of 5% CO^2 and air at 37°C in Eagle's medium with Earle's salts supplemented with 15% fetal bovine serum (GIBCO, Grand Is., N.Y.) and gentamycin (50 µg/ml) (Scherring Corp., Union, N.J.). Hams F10 lacking hypoxanthine (GIBCO) supplemented with 15% fetal bovine serum and streptomycin (100 µg/ml) and penicillin (100 units/ml) was the medium used for experiments unless otherwise stated.

Selective Medium

The medium for selecting and culturing 8-azaguanine (AG)-resistant cells was Ham's F10 lacking hypoxanthine and supplemented with 15% calf serum (GIBCO, Grand Is, N.Y.) and 8AG at 2×10^{-5}M (Sigma, St. Louis, Mo.). The conditions were standardized by choosing lots of serum which not only supported high cloning efficiencies in XP strains, but also did not interfere with selection. The concentration of 8AG used for the selection (viz., 2×10^{-5}M) was demonstrated to be high enough to insure that surviving colonies would represent 8AG-resistant cells in the population (see Fig. 1). Although 8AG and thioguanine give very similar dose response curves, 8-azaguanine was preferred to thioguanine as a selective agent in the present studies because, under our conditions, it was demonstrated that 8AG prevented a logarithmically growing cell population from undergoing even a partial doubling, whereas

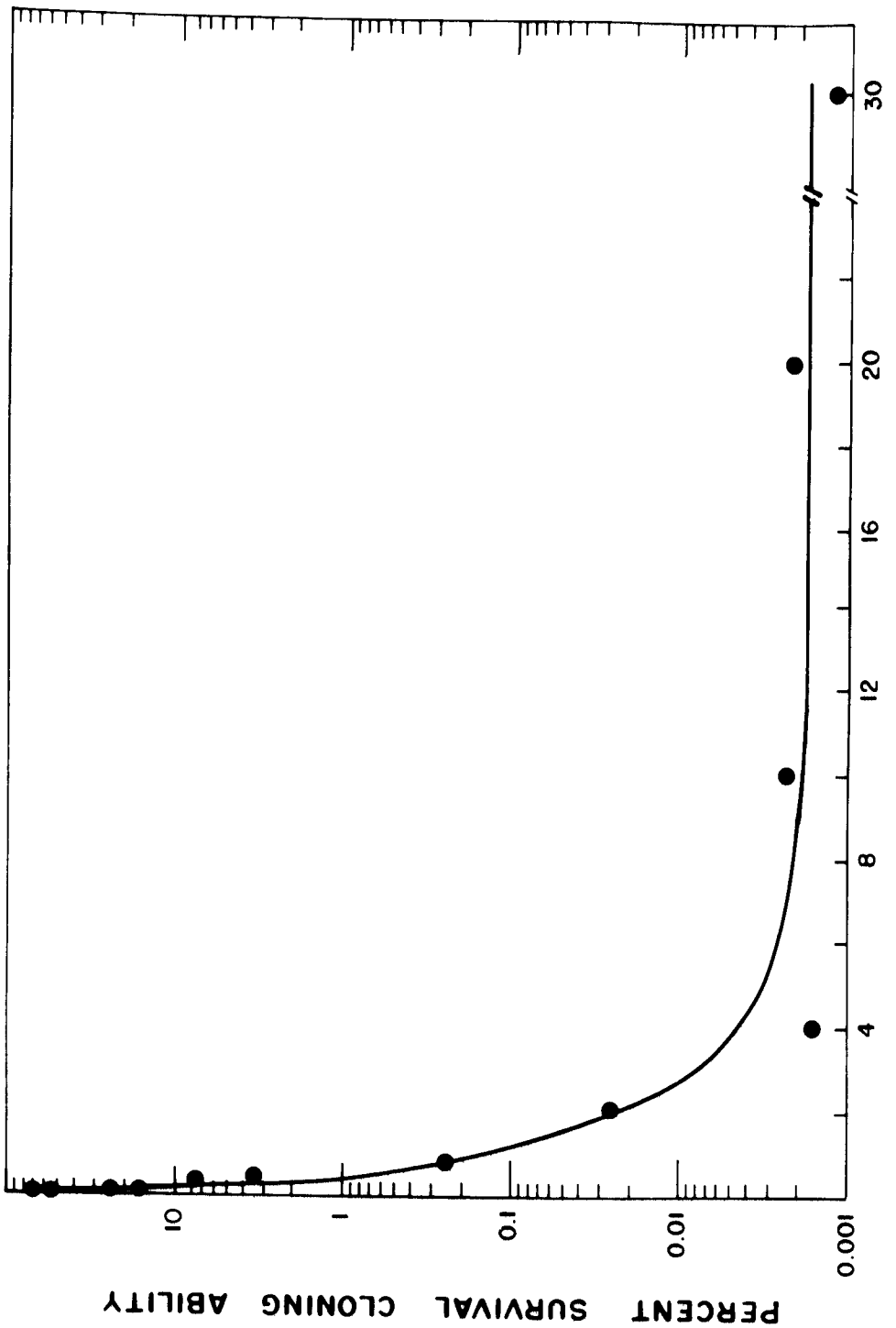


Figure 1. Cytotoxicity of 8-azaguanine in human fibroblasts (from Ref. 7)

thioguanine allowed one or more population doublings before growth cessation (Maher, et al, unpublished data). This phenomenon would lead to complications caused by high cell density (see below, "metabolic cooperation").

Cytotoxicity Assay (Survival of Cloning Capacity) (See Fig. 2A)

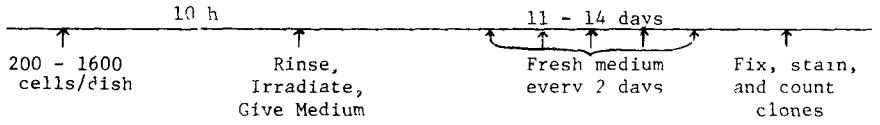
The techniques for determining the % survival of the cloning capacity of the cells following exposure to these compounds have been described (3,4). Briefly, appropriate numbers of cells (250-3000) were plated in 60 mm dishes and allowed time (ca. 10-12 hours) for attachment. The culture medium was replaced with serum-free medium and the compound to be tested was introduced by micropipette into the individual dishes. After 3 hours the medium was removed and the cells were refed with culture medium. Since proteins possess strong nucleophilic centers, the latter medium containing 15% fetal bovine serum should inactivate any remaining traces of electrophilic reactants. All operations with the compounds were carried out in subdued light and cells were protected from light until they had undergone several cell divisions. Cells were refed 3 times weekly until clones developed to macroscopic size (ca. 12-14 days), (see Fig. 2). Cloning efficiencies for the untreated cells ranged from 20-35% for normal fibroblasts, 10-25% for XP2BE and XP4BE, and 10-15% for XP12BE. The cloning efficiency of the treated cells, divided by the cloning efficiency of the control cells which received solvent only determined the cytotoxicity of the compound and is expressed as a percent. The final concentration of acetone in the medium (0.45%) was not cytotoxic to the cells.

For ultraviolet irradiation (254 nm) of cells, the protocol was as described above with the following modifications. When the medium was removed after 10-12 hours, the cells were rinsed in 0.9% saline, exposed to a known dose of ultraviolet irradiation, and medium containing 15% serum was added to each dish. Cells were refed 3 times weekly until clones developed to a macroscopic size. Ultraviolet irradiation was carried out with a UVS-12 or UVS-54 ultraviolet lamp (Ultraviolet Products, San Gabriel, CA). The dose was determined with a J-225 meter from the same manufacturer.

Mutagenicity Assay (See Fig. 2B)

For each dose, a population of 1.0-2.4 X 106 cells was plated in a total of 120 60 mm dishes. After a 12-14 hour cell attachment period, the medium was replaced with serum-free medium and the cells exposed to the carcinogen as described above. A cytotoxicity experiment was carried out in parallel with each mutagenicity experiment to determine the number of survivors. After 3

A. CYTOTOXICITY



B. MUTAGENESIS

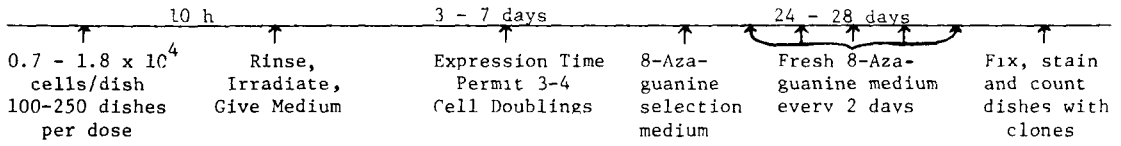


Figure 2. Experimental protocol for UV exposure of human fibroblasts (from Ref. 6)

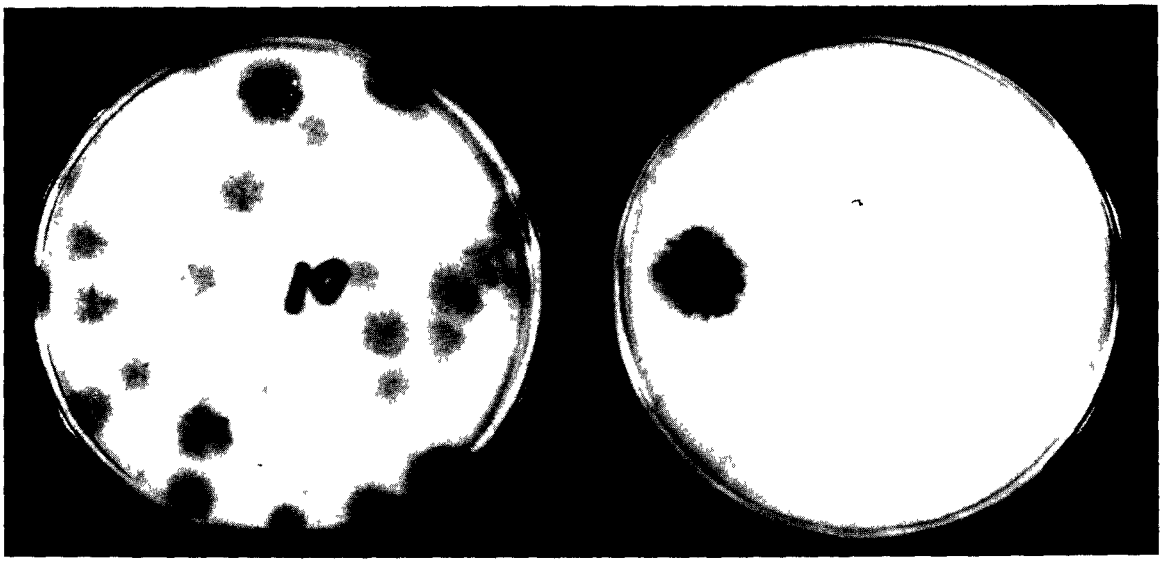


Figure 3. Left: normal skin fibroblast colony Right: azaguanine resistant colony (from Ref. 5)

hours the medium was removed and the cells refed culture medium containing 15% fetal bovine serum. Each population was allowed sufficient time for the surviving cells to undergo 3 doublings which were required to overcome phenotypic lag of cellular expression of azaguanine resistance before 8-azaguanine selection was begun. Because azaguanine has been shown to be unstable in the presence of serum (10), the cells were refed selection medium 3 times weekly for ca. 21 days until macroscopic resistant colonies could be detected by examining the petri dishes in a darkened room with a lamp which gave off a focused beam of light. Dishes in which a macroscopic resistant colony (500 or more cells) was detected were maintained; the rest were rinsed, fixed, stained, and examined for undetected resistant colonies composed of at least 100 cells. The frequent refeeding of the petri dishes might be expected to lead to the formation of satellite colonies which would result in an overestimation of the number of mutants. That this is not the case is demonstrated by the fact that the number of 8AG-resistant colonies formed follows a Poisson distribution (see example below). Furthermore, the P(o) method used to determine the number of mutants (see below) prevents one from making such an overestimation. A control population of 1-1.5 X 106 cells was included with every experiment to determine the background mutation frequency. The frequencies of spontaneous or preexisting mutants in each of the experimental populations used in this study were closely matched so that a valid comparison between different strains could be made.

Reconstruction Experiments to Determine Efficiency of Recovery of Mutants

Since overcrowding of cells in the dishes can result in an apparent reduction in mutation frequency caused by "metabolic cooperation" (9), in every experiment a known number of 8AG-resistant Lesch-Nyhan cells was seeded into ten of the control and ten of each set of experimental dishes as well as into a set of dishes lacking a monolayer of cells to provide an estimate of the efficiency of the recovery of mutants under the conditions of each experiment. The average number of colonies of Lesch-Nyhan cells in each series of dishes containing a non-resistant monolayer divided by the average number of colonies of Lesch-Nyhan cells found in the dishes which lacked such non-resistant cells yielded the efficiency of recovery and was expressed as percent. The relationship between the efficiency of recovery of azaguanine resistant cells and the total number of cells viable or non-viable) attached to the surface of the 60 mm dishes at the time selection was begun has been determined from a large series of such reconstruction experiments with human fibroblasts and is shown in Fig. 4. This empirically-generated data was used to pre-determine the number of cells to be exposed to the various doses of the hydrocarbon to epoxides to insure that the surviving population could

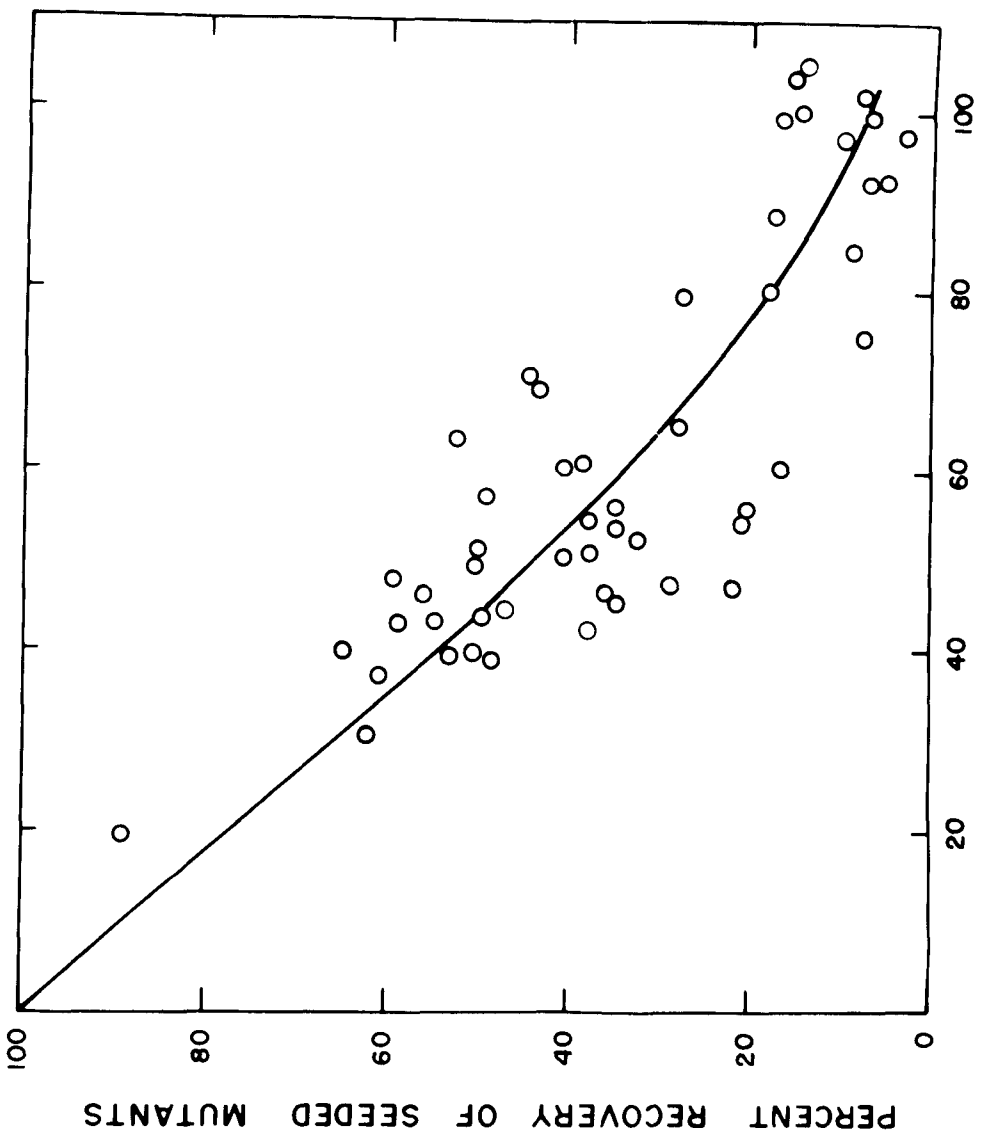


Figure 4. Relationship between efficiency of recovery of 8-AG resistant colonies and the density of non-resistant cells in the dish at the beginning of selection (from Ref. 7)

undergo three doublings without the total number of cells in the dish at the beginning of selection (6 days later) exceeding that which would permit an efficiency of recovery of at least 30-40%. That Lesch-Nyhan cells can validly be used to estimate the efficiency of recovery of AG-resistant human cells has been recently demonstrated by Van Zeeland and Simons (9). They published a similar curve based on the density of untreated cells.

Determining the Frequency of Induced Mutations

The method used to quantitate the frequency of mutations has been described in detail by Albertini and DeMars (1) and by us (3, 7). In summary, this consists of determining the probability of a mutational event occurring per experimental culture dish from the number of culture dishes which contain no clones: $P(0) = \exp(-x)$ where x is the probability of a mutant event per dish. For each experiment this value is corrected first, for the efficiency of recovery of mutants and secondly, for the small contribution of the background mutation frequency. From the probability of mutational event occurring per dish, the number of viable cells per dish, and the cloning efficiency of the cells in each particular experiment, the frequency of induced mutational events per 10^5 survivors is determined. The need for correcting for cloning efficiency has been demonstrated (10). (See example in Table I.)

Analysis of Azaguanine Resistant Colonies

At the end of 21 days of selection, resistant colonies were detected as described above. A representative proportion of such colonies were isolated and cultured in selection medium containing 8AG until they reached sufficient size for the cells to be tested for levels of hypoxanthine guanine phosphoribosyltransferase (HPRT, E.C. 2.4.8.2.) enzyme activity as described by Albertini and DeMars (1). A second group of resistant colonies was isolated, and cultured in non-selective medium for 11-13 generations. At this time they were replated at low number and tested for ability to form colonies in normal culture medium, in azaguanine selection medium, and in medium containing hypoxanthine, aminopterin, and thymidine (1) which selects against cells with little or no HPRT enzyme activity.

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TABLE I. EXAMPLE OF CALCULATING THE FREQUENCY OF INDUCED MUTATIONS FROM THE DISTRIBUTION OF RESISTANT CLONES IN THE POPULATION [P(0) METHOD]

Cloning efficiency of the cells = 0.2	Control	Dose 1	Dose 2	Dose 3
Number of cells plated into dish	10,000	12,000	15,000	18,000
Percent cell survival	100	60	35	18
Viable cells per dish	10,000	7,200	5,250	3,240
Dishes with 0 clones	91	87	80	82
Dishes with 1 clone	3	5	5	4
Dishes with 2 clones	1	1	1	1
Dishes with 3 clones	0	1	1	1
Total mutant clones	5	10	10	9
Total dishes scored	95	94	87	88
$P(0)^a = \frac{\text{(Dishes with 0 clones)}}{\text{(Total dishes scored)}}$	0.958	0.925	0.920	0.932
x^b (chance of mutant event per dish)	0.043	0.077	0.084	0.071
Efficiency of recovery (data from reconstr.)	50%	60%	50%	50%
Corrected x^c	0.086	0.129	0.167	0.141
Chance of a spontaneous or pre-existing mutant per dish[d]	0.086	0.062	0.045	0.028
Chance of an induced mutant per dish[e]	—	0.067	0.122	0.113
Frequency of induced mutants per 10^5 viable cells[f]	—	0.93	2.32	3.49
Frequency of induced mutants per 10^5 viable cells corrected for 20% cloning efficiency	—	4.6	11.6	17.4

[a]Probability of a dish containing no clones.

[b]Calculated from $P(0)=\tilde{e}^x$; Poisson Distribution Function.

[c]Chance of a mutant event per dish corrected for efficiency of recovery (see text).

[d]Obtained from control population dishes and calculated for each population.

[e]Calculated by subtracting the chance of a spontaneous mutant from total chance, x^c.

[f]Calculated from chance of induced mutant event per dish divided by number of viable cells.

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APPENDIX A:

Survival Curves

1. Compute the average number of clones per dish.

2. Chart the number of cells plated originally and the dose of agent.

3. Compute cloning efficiency as follows:

$$\text{Cloning efficiency} = \frac{\text{Average clones per dish}}{\text{Number of cells plated}}$$

4. Compute percent survival as follows:

$$\text{Percent survival} = \frac{\text{Cloning efficiency (experimental)}}{\text{Cloning efficiency (control)}}$$

5. On semilogarithmic paper plot percent survival (log axis) versus dose.

APPENDIX B:

Procedure for Staining Dishes

1. Pour medium off into sterilizing bucket.

2. Put bottom of dish containing cells into perforated bucket

3. Plates in buckets go into:

   a) 0.8% - 0.9% saline for quick rinse (app. 30 seconds)
   b) 95% methanol for fixing app. 5 minutes
   c) 2% methyline blue 10 minutes
   d) running tap water in sink for few minutes

4. Each plate is then individually rinsed in tap water for several minutes.

5. Plates then turned upside down on large trays and placed in an incubator at 37°C to dry, taking care to keep sets separated and identifiable.

APPENDIX C:

Standard Procedure for Starting Primary Cultures (Mincing)

1. Wash tissues 2 times with 8-10 ml F-10 containing 500 units/ml Pen-Strep in 60 mm dish.

   a) discard medium after each wash

2. Hams F-10 liquid medium is added back to the dish, but not so as to float the tissue.

3. Tissue is then minced up using aseptic technique; the tissue to be minced refers to pieces ca. 1 cm square which are cut from the whole foreskin. These pieces are then minced into ca. 1 mm pieces.

4. The minced tissue is then transferred to a dry 60 mm dish where:

   a) it is held firmly in place by pressing down lightly with a small amount of sterile silicone grease on one corner

   b) this dish then receives app. 5.0 ml of medium (Ham's F-10, 15% fetal calf serum, and pen strep)

5. After two days this medium is discarded and new growth medium is added.

   a) the culture should then be refed every day

6. Epithelial cells will be seen in @ 3-5 days.

7. Fibroblasts will be seen in @ 3-5 days.

8. When good colonies of fibroblasts are seen, cells may then be transferred. Epithelial cells are lost within the first or second transfer of these cells.

APPENDIX D:

Procedure for Transfer of Cells

1. Have receiving vessels (dishes, flasks) prelabeled and containing proper amount of growth medium (20 ml/250 ml flask, 5 ml/60 mm dishes).

2. Trypsin and serum free medium for rinsing should be warmed to 37°C for best results. Trypsin, 0.25% (Gibco).

3. Have canulas attached to syringe in hood set-up and ready to use.

4. Drain off growth medium by aspiration.

5. Cover cells with normal amount of warm 37°C serum free medium (20 ml/250ml flask, 5 ml/60 mm dish) and allow to stand for a few minutes, discard.

6. Replace warm 37°C serum free media with trypsin (3 ml P-60, 7-8 ml T-75).

   a) observe culture under scope

   b) when 80-90% of the cells have rounded up, remove trypsin

   c) rinse culture with minimal amount of serum-free media to dilute trypsin

7. Replace trypsin with 6.0 ml regular growth medium (3.0 ml for 60 mm dishes) and force cells off bottom using canula with syringe and gently pipetting them.

   a) bottom of dish may be seen to be free of cells by reflecting in light at proper angle

8. Transfer suspended cells to prelabeled culture flasks.

APPENDIX E:

Procedure for Freezing Cells

1. See cell transfer steps 2-6.

7. Replace rinsing medium with enough growth medium containing DMSO Freezing Medium=DMSO growth medium = F-10 + FCS 15% + DMSO 5% to fill the respective freezing vials - but only in one culture vessel [eg. 4 T-75's to freeze each freezing vial≃1.5 ml 1.5 x 4 ≃ 6.0 ml - this 6.0 ml is put in only 1 of the T-75's and this is used to force off the cells from the bottom of the culture vessel]. This medium containing cells is then transferred to the next flask and so on until all 4 flasks have been completed.

8. This 6.0 ml of cells is then placed in freezing vials.

Note: This technique is for a 1:1 ratio regardless of the no. of cells of T-75 freezing vial, but may be easily adjusted if a different ratio is wanted.

5.5 FIBRINOLYTIC ACTIVITIES AND QUANTITATIVE SOFT AGAR ASSAYS

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PREPARATION OF FIBRINOGEN

References: (1,4)

IMPORTANT: Use no glass!

1. Dissolve 10 grams fibrinogen (60-70% clottable) in 500 ml of 0.1 M phosphate buffer (pH 6.41) in a plastic beaker.

2. Dilute solution with 500 ml double distilled H_2O and allow to stand overnight in the refrigerator.

3. Filter off formed precipitate and obtain clear supernatant (fibrinogen I)

4. To supernatant, add slowly (stirring at room temperature) 330 ml of saturated ammonium sulfate, then stir approximately 30 minutes.

5. Centrifuge down the flocculent white precipitate.

6. Dissolve pellet in $\sim$100 ml 0.6 M NaCl and 0.003 M EDTA. May take several hours. Pellet should be dissolved before moving to cold room.

7. Dialyze overnight against 0.6 M NaCl and 0.003 M EDTA in the cold.

8. Dialyze against PBS (Ca^+, Mg^+ free) in cold.

9. Filter sterilize through 0.22μ Nalgene sterile filter. May need to prefilter 1-3 times through fluted filter paper and may need to dilute and warm (37°C) before filter sterilizing.

10. Determine protein concentration and freeze at 10 mg/ml (16 OD 280/ml).

PREPARATION OF PETRI DISHES COATED WITH ^{125}I-FIBRIN

1. ^{125}I-Fibrinogen prepared by the method of Helmkamp, et al. (3).
2. Pipette radioactive fibrinogen solution into a petri dish ($10\mu g/cm^2$ at 2000-5000 cpm/μg of fibrinogen).
3. Bake at $45^{\circ}C$ overnight.
4. Convert the fibrinogen to fibrin by incubating the plates with 2 ml E MEM with 5% fetal calf serum 2 hours at $37^{\circ}C$.
5. Wash 2 times with PBS.
6. Add 2 ml assay buffer (Tris HCl - 0.1M pH 8.0).

PREPARATION OF HARVEST FLUID

1. Wash the cultures 2 times with PBS.
2. Add fresh medium without serum (E MEM, or E MEM with 5% acid treated fetal calf serum).
3. Incubate for 18 hours at $37^{\circ}C$.
4. Collect the medium and freeze ($-20^{\circ}C$).

PREPARATION OF INTRACELLULAR PLASMINOGEN ACTIVATOR

1. Wash the cultures 2 times with PBS.
2. Scrape the cells into buffer with rubber policeman.
3. Centrifuge and wash 2 times (1000 xg 5 minutes).
4. Add $\sim$10 vol. of 0.5% Triton in Tris-HCl 0.1M pH 8.0.
5. Aspirate with Pasteur pipette.
6. Centrifuge to pellet the nuclei (1000 xg, 10 minutes).
7. Freeze the supernatant ($-20^{\circ}C$).

PREPARATION OF PLASMINOGEN AND PLASMINOGEN FREE SERUM

Reference: (2).

1. Couple cyanogen bromide activated sepharose-4B with L-lysine (1 gm of lysine per 5 ml of activated, moist sepharose).

2. Prepare a column (1 ml of bed volume/5 ml of serum), slowly pass the serum through and then elute with 0.3 M phosphate buffer.

3) Elute the plasminogen with 0.1M EACA in phosphate buffer.

4) Repeat and dialyze the plasminogen extensively against PBS.

PROTOCOL FOR FIBRIN OVERLAY ASSAY

1) Mix 2x EMEM (or 2 MEM) with dog serum (4:1).

2) Tube out 2x EMEM with 20% dog serum in 2 ml aliquots, incubate at 42°C. (One tube/plate).

3) To each tube add 2 ml of 2.5% agarose at 42°C.

4) Suction off medium from plate and wash with medium without serum.

5) To one tube, add 0.5 ml Fibrinogen II (10mg/ml and mix. Replace in water bath.

6) Add 20-50 µl thrombin to tube, mix rapidly, and quickly pour on plate.

7) Let solidify 5-10' at room temperature, then put into 37° CO^2 incubator.

8) Read at 3 hr and 5 hr and 24 hr for lysis.

PROTOCOL FOR GROWTH IN SOFT AGAR

1) Prepare 1.2% agar, 0.4% bacto-peptone. Melt in boiling water bath, equilibrate to 45°C.

2) Prepare 2x medium with 20% serum. Equilibrate to 45°C.

3) Mix the above 1:1 to get 0.6% agar, 0.2% peptone in 1x medium and 10% serum at 45°.

4) Plate 4ml of this mixture onto 60mm culture dishes for base layer. Let firm at room temperature for 15 minutes.

5) For testing 10^5 cells, to the mixture prepared in step (3), add an equal volume of cell suspension at $5x10^4$ cells/ml in 1x medium (90%)/fetal calf serum (10%). This gives a suspension of 2.5 x 10^4 cells/ml at 0.3% agar and 0.1% peptone.

6) Immediately place 4 ml of this suspension over base layers to complete agar plates at 10^5 cells. Let firm in 24° incubator for 1 hour, then switch to 37°C incubator for 2 weeks when they are examined for macroscopic and microscopic colony formation.

7) If additional incubation time to allow further enlargement of clones is desired, incubation at 37° is continued with weekly additions of 1 ml growth medium to preclude dehydration of the agar.

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5.6 ASSAYS FOR SOMATIC MUTATION STUDIES ON SYRIAN HAMSTER EMBRYONIC CELLS

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A. PROTOCOL FOR THE INDIRECT POPULATION ASSAY FOR SOMATIC MUTATION STUDIES OF SYRIAN HAMSTER EMBRYONIC CELLS

1. $1x10^6$ Syrian embryonic fibroblasts of passage 2 to passage 3 (same cells used for in vitro neoplastic transformation) are seeded in a 75 cm^2 flask.

2. After 15 hours, the cells are treated with MNNG at a concentration of $1x10^6$ or 5x10-6 M for 2 hours, or with B(a)P at a concentration of 1 µg/ml or 10 µg/ml for 24 hours.

3. After the exposure time, the flasks treated with MNNG are washed thoroughly with phosphate buffer-saline (PBS) and the flasks treated with B(a)P are washed five times by the complete medium (containing 5% serum), the same washing procedure used in the neoplastic transformation assay.

4. The flasks are then allowed to reach confluency in 4-7 days.

5. The flasks at confluency are subcultured at a split ratio of 1:10 and allowed to grow to confluency. Confluency is normally obtained in 7 days with untreated cells but $5x10^{-6}$ M MNNG or 10 µg/ml B(a)P treated cells require 2-3 weeks to reach confluency.

6. At each passage, 10^5 cells are seeded in a 100 mm petri dish. After 15 hours, 8-azaguanine containing medium is added to the petri dish to a final concentration of 40 µg/ml.

7. The cells in the petri dish are cultured further for 3-4 weeks with intermittent changes of medium containing 40 µg/ml of 8-azaguanine.

8. At each passage, concomitantly, $1x10^4$ cells from the same preparation are seeded in 100 mm petri dishes. These cells are incubated for 7 days, then fixed, stained, and are scored for neoplastic transformation as described in the previous section.

B. PROTOCOL FOR THE DIRECT CLONAL ASSAY FOR SOMATIC MUTATION STUDIES ON SYRIAN HAMSTER EMBRYONIC CELLS

1. 10^5 Syrian hamster embryonic fibroblasts are seeded in a 100 mm petri dish.

2. After 15 hours, these cells in the petri dishes are treated with MNNG and B(a)P as described earlier in the indirect population assay. Parallel experiments with 2 x 10^4 cells/100 mm petri dishes are conducted to determine toxicity as measured by the decrease in cloning efficiency.

3. These cells are then washed, refed with complete medium for a recovery period of 1-3 days.

4. After the recovery period, 40 μg/ml of 8-azaguanine is added to the medium and the cells are grown in this selective medium for 3-4 weeks with refluiding before fixed, stained, and scoring for mutated colonies.

5.7 ANALYSIS OF DNA STRAND BREAKS BY ALKALINE SUCROSE GRADIENTS

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Five to 30% sucrose gradients were prepared by 4 cycles of freeze-thawing (1) of tubes containing 18% sucrose in 0.1M EDTA which was adjusted to pH 12.6 with 10M NaOH. Primary hamster cell cultures, after 3 days' incubation, were passaged to 60-mm plastic dishes using 4 x 10^5 cells per dish. Immediately, or after 24 hours, the cells were pulsed with 0.5 μCi/ml of ^{3}H-labeled thymidine (^{3}H-TdR) for 24 hours. After ^{3}H-TdR labeling, all cultures were changed to GERM with 0.5% FBS and 0.22 gm% NaHCO3, and held for an additional 24 hours. At this time each dish contained approxi- mately 10^6 cells. The test chemicals were usually prepared as 1-10 mg/ml stocks in acetone, acetone-water, or DMSO and added to prewarmed medium to give the desired final concentration. The HEC were treated for 2 hours or for 18 hours, washed 1x, and removed from the dish with EDTA. Following centrifugation, the cells were resuspended in EDTA to give 10^5 cells per 0.2 ml. Two-tenths ml of the cell suspension was then added to the top of a sucrose gradient tube layered with 0.3 ml of lysing solution (1% sarkosyl in 0.0.5% EDTA). The cells were then lysed at room temperature for 1 hr, placed in an SW-50 rotor, and centrifuged for 1 hr at 30,000 rpm in a Model L-2 ultracentrifuge at 20°C. Three drop fractions were collected directly into scintillation vials following bottom puncture, neutralized with 1.0 ml of 0.2N HC1 and prepared for counting by addition 5 ml of Bray's scintillation fluid to each vial. Counts were made in a Packard Tri-Carb scintillation spectrometer, 10 minutes per sample. Data were plotted as percent of the highest count in each gradient. A shift in the peak of radioactivity greater than 3 fractions (more than 6 mm) from the control peak was considered positive evidence for chemical-induced DNA strand breaks.

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5.8 ANALYSIS OF DNA REPAIR SYNTHESIS BY AUTORADIOGRAPHY

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Primary HEC were passaged to 60-mm petri dishes, containing a 22-mm square glass coverslip, at 500,000 cells per dish. After 24 hr incubation, the cultures were changed to medium without arginine, containing 5.0% dialyzed FBS, to inhibit scheduled DNA synthesis (Freed and Schatz, 1969). After incubation in the arginine-free medium (AFM) for 48 hr, the medium was withdrawn an appropriate concentrations of the test chemicals in AFM were added to each of 4 plates of cells. Depending upon the particular experiment and the chemical being tested, the exposure period was either for 2 or 18 hr. After treatment, the cells were rinsed once in AFM and 2.5 ml of AFM with 1.0% FBS was added to each plate. To each of 2 plates per chemical concentration was then added ^{3}H-TDR at a concentration of 10 uCi/ml. The coverslip cultures were pulsed for 6 hr (0-6 hr), rinsed 3x, fixed 15 min in Carnoy's, rinsed 2x with 20% ethanol and 2x with 10% ethanol, (10 min per rinse) and air-dried. The remaining 2 plates were labeled as above with ^{3}H-TdR beginning 6 hr after treatment and continuing through 24 hr (6-24 hr). The coverslips were fixed to slides (cell side up) with mounting medium (Boyd, 1955), dipped in subbing solution (Boyd, 1955) and air-dried. The coverslips were then coated with Kodak Nuclear Track Emulsion (NTB2). Exposure was accomplished by either of two methods: (1) placed in a foil covered slide box and held at 4C for 14 days, or (2) impregnation of the partially dried film with scintillator (Durie and Salmon, 1975) and incubated overnight at -20°C. After developing, the cells were stained for 5 min with 0.1% crystal violet in 0.1M citric acid. The number of grains per nucleus was counted on 100 cells per slide for each of the controls nd chemical concentrations.

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