EVALUATION OF THE TRANSFORMATION ASSAY USING C3H 10T½ CELLS FOR USE IN SCREENING CHEMICALS FOR CARCINOGENIC POTENTIAL

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In the past few years, considerable effort has been directed to defining batteries or combinations of in vitro test methods which could provide rapid, sensitive, and reliable means for assessing the carcinogenic potential of chemical compounds. These various in vitro test systems can be broadly divided into three major categories, namely (1) those which detect mutagenic or chromosomal changes in micro-organisms or mammalian cells; (2) those in which there is induction of morphological transformation in mammalian cells in culture; and (3) those in which interactions between the chemical and target macromolecules such as DNA can be assessed. Experimental evidence has been accumulated which shows that there is a positive correlation between the in vivo carcinogenicity of many chemicals and their capacity to elicit a response in in vitro systems.

Approximately one hundred different model systems have been identified as having been used, in varying degrees, for assessing the carcinogenic and/or mutagenic potential of chemicals. In some instances, limited numbers of chemicals have been tested and in many cases they have been restricted to well characterized direct-acting agents. Although such a review and such compilations of test data are extremely valuable, certain problems are encountered with data derived solely from the literature. For any single test method, there can be small but critical differences in the methods used which can result in apparent non-reproducibility of results. Another problem relates to the fact that the tests, in general, have been performed with the full knowledge of the in vivo carcinogenic activity of the chemicals. Such a situation can introduce a certain amount of bias.

In the utilization of any in vitro method as a valid indicator of the carcinogenic potential of chemicals, there is a basic requirement to know that (1) the methodology is well-defined and the critical elements of the procedure are recognized; (2) reproducible results can be obtained not only within a specific laboratory but can be obtained equally well among different laboratories; (3) there is experience with a broad spectrum of chemicals of diverse structure and biological activity in order to recognize that certain types of chemicals may give unique negative or positive results in some tests; and (4) there are methods and approaches for analysis and interpretation of the experimental results. The existence of well-defined and evaluated assay methods, which can be exploited for the assay of carcinogenic potential, would then provide the means to examine a large number of chemicals and aid in setting priorities for long-term animal carcinogenicity bioassays.

The purpose of this project is to evaluate and determine the usefulness and reliability of an in vitro transformation assay using C3H 10T ½ cells as a candidate for one of a battery of short-term assays for the initial determination of the carcinogenic potential of chemicals. In addition the reproducibility of the system will be assessed since the studies are being conducted in two laboratories simultaneously.

The workscopes of the two contracts for this effort were sharply defined so as to initially emphasize the methodological aspect. The specific objectives to be approached in parallel were as follows:

1) Propagate and store a large quantity of mycoplasma-free, low passage 10T ½ cells.

2) Characterize the behavior of the cell population with respect to plating efficiency, generation time, saturation density, karyotype stability, cell morphology, cell size distribution, absence of growth in soft agar, and absence of tumor formation in C3H mice.

3) Identify and obtain large lots of fetal bovine serum that yield optimum growth characteristics and transformation response with direct acting carcinogens and polycyclic aromatic hydrocarbons.

4) Establish the 10T ½ cell transformation assay and its concomitant plating efficiency in the absence of an exogenous metabolic activation system, using model chemical compounds.

5) Evaluate the transformation assay using selected known chemical carcinogens and non-carcinogens by scoring for Type III foci and by determining the ability of cells from Type III foci to grow in soft agar and produce tumors when injected into irradiated C3H mice.

6) Develop and characterize a mamalian S-9 activating system for incorporation into the C3H 10T ½ transformation assay.

7) Determine whether the sensitivity and reproducibility of the assay can be further improved by examination of certain baseline factors such as plating density and assay interval, passage number of target cells, and step-down of serum concentration.

8) Test a series of chemicals (supplied under code) consisting of both carcinogens and non-carcinogens for their transforming capacity in 10T ½ cells. The chemicals will be tested with and without an exogenous metabolic activation system.

The progress made in these areas by the two contractors has been satisfactory. Early passage (P-5) cells received from Dr. Heidelberger were subcultured by the prescribed protcol (once every 10 days at 5×10^4 cells/dish) and large aliquots of cells from P6, 7 and 8 frozen in liquid nitrogen to form a uniform stock for future studies. The cells were determined to have the culture characteristics for C3H 10T ½ cells previously reported in the literature. In addition, lots of fetal bovine serum have been identified that provide the expected saturation densitiy and chemically-induced morphological transformation.

These cells have been characterized and it has been found that they have a plating efficiency (100 cells/60 mm dish) of 23%, a saturation density of 5.9×10^5 cells/60 mm dish and a generation time of approximately 16 hours. The cells were also examined by transmission electron microscopy and it was observed that there is overlapping and underlapping of cytoplasm between cells. This suggests that cell contact may not be a controlling variable in the density dependent inhibition of cell division.

Other significant findings have been reported by these two contractors.

Transformation assays with eight known chemical compounds showed that benzo(a)-pyrene (B(a)P), 3-methylcholanthrene (3-MC), dibenz(a,h)anthracene and 7,12-di-methylbenz(a)anthracene gave a positive response by producing Type III foci in the

absence of an exogenous metabolic activation system. Chemicals not active in the assay were the non-carcinogens, anthracene and phenanthrene, and two known carcinogens, 2-acetylaminofluorene (2-AAF) and N-methyl-N-nitro-N-nitroso-guanidine (MNNG). In the assays positive for transformation, the absolute transformation frequency and the dose at which a positive response was induced varied from experiment to experiment. In general, 3-MC induced a more reproducible transformation response and dose-dependent effect than B(a)P, although variation from experiment to experiment was still evident.

An acceptable lot of rat liver S-9 has been prepared from Aroclor-1254 induced Fischer 344 male rats and is being used to determine the critical parameters for chemically transforming 10T ½ cells in the presence of such an exogenously supplied source of mammalian metabolizing activity. Cytotoxicity and transformation assays have been conducted using B(a)P, 2-AAF, diethylnitrosamine (DEN), and 3-MC. When the suspension assay was used, only those cells exposed to B(a)P in the presence of a metabolically active S-9 preparation exhibited morphological transformation. The negative results obtained thus far with the other compounds tested may be attributable to various factors such as (1) the relatively short exposure period (2-4 hours) used; (2) the limitations associated with a suspension assay; (3) failure of the S-9 to activate the chemicals to forms capable of transforming 10T ½ cells; (4) metabolic inactivation and detoxification of the chemicals by the S-9 preparation; or (5) the failure of transformed cells or cells in the process of being transformed to exhibit the transformed phenotype.

An amplificaton assay (Level II transformation assay) is also being assessed to determine whether non-expressed transformed cells are present in the standard

transformation assay. This assay involves replating of the treated cell population when they just reach confluency and scoring the replated cells for Type III foci in 2-4 weeks.

MNNG has been tested using this amplification modification. At a dose of 0.5 ug/MNNG/ml, a single Type II focus was detected in the standard transformation assay. The Level II transformation assay gave rise to numerous Type III foci. The positive control, 3-MC (2.5 ug/ml) induced the formation of 17 Type III foci in the standard transformation assay and no augmentation in transformation was obtained in the Level II assay.

Studies have also been conducted to test various factors which may affect and/or optimize the transformation assay. It was observed with 3-MC treatment that the total number of foci (II and III) increased with increase in exposure time to this chemical, but there was no enhancement of transformation frequency when the treated cultures were maintained in medium containing 2% and 5% serum rather than the standard 10% serum concentration.

The constitutive activity of arylhydrocarbon hydroxylase of the C3H 10T ½ cells was also determined by measuring the conversion of ^{3}H-B(a)P and it was compared to the activity in BALB/C-3T3 clone 1-13 cells. The time course of B(a)P metabolism (0.3 ug/ml) with 2 X 10^{5} cells is linear with incubation time for both 3T3 and 10T ½ cells. However, the 10T ½ cells possess a 15-fold higher activity than the 3T3 cells.

For the next contract period the overall objective will be to further develop and validate a reliable, reproducible assay system for neoplastic transformation using

C3H 10T ½ cells, specifically identifying those factors which influence the assay and then determining those procedures which would lead to their standardization. Emphasis will be placed on developing the exogenous enzyme activation system because of absent or insufficient enzyme levels in the target cells.

Simultaneously, because of the progress already made by two laboratories in standardizing, we are moving ahead on phase II, the initiation of assays of coded samples supplied by the NCI. Cytotoxicity effects have already been run on more than 10 of the coded samples and we expect that this effort will move forward rapidly in the final contract year FY 81.

Dr. Page, EPA: On these assays, does there appear to be any good correlation between the cytotoxicity and transformation capability? Is there any positive correlation you could see?

Dr. Cameron, NCI: Tranformation and the cytotoxicity?

Dr. Page, EPA: Cytotoxicity for the cell cultures?

Dr. Cameron, NCI: I don't have an answer for that. I would mention that the samples came out of the bioassay program.

Dr. Kelsey, NCI: Was I correct in hearing that they did not pick up AAF and MNNG in the standard assay?

Dr. Cameron, NCI: Right.

Dr. Kelsey, NCI: Would that not bother you in terms of giving them coded samples?

Dr. Cameron, NCI: It bothers the contractors.

Dr. Kelsey, NCI: I mean until they can get some knowns to work, or at least some basic knowns, wouldn't it be advisable?

Dr. Cameron, NCI: I think it would bother anybody using the system.

Dr. Kelsey, NCI: Are there plans to use maybe hepatocytes or something like that as an activating system?

Dr. Cameron, NCI: Not in the protocol.

Dr. Hegyeli, NCI: Dr. Alcian made a study to compare in vitro and in vivo toxicity, and he inferred that the distribution of a certain substance between optimal and the aqueous phase has a very important role in determining the ratio that was detected from in vitro to in vivo. My quesiton is whether there was any study done as far as the solubility and what kind of solvent was used for the different kinds of chemicals?

Dr. Cameron, NCI: The solubility factors were determined by a different laboratory, a chemical analysis laboratory, and they were supplied by that laboratory. So that is worked out for the contractor. He receives the chemical and the solubility data simultaneously. I hope that answered the question.

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