**Meeting Report**

**Cellular and Molecular Mechanisms of Cell Transformation and Standardization of Transformation Assays of Established Cell Lines for the Prediction of Carcinogenic Chemicals: Overview and Recommended Protocols**

**IARC/NCI/EPA Working Group**

**Introduction**

Although it has long been recognized that the end point of *in vitro* cell transformation has direct relevance to carcinogenesis (4), its use for screening potential carcinogens has not been fully exploited, due mainly to the following factors: (a) our knowledge of molecular and cellular mechanisms is still limited; (b) cell transformation assays are technically more difficult than most other *in vitro* screening tests; (c) scoring of morphologically transformed foci has been somewhat subjective; and (d) the method has not been validated internationally as a screening test for potential carcinogens.

A Working Group was therefore convened to discuss molecular and cellular mechanisms of cell transformation, biological similarities between *in vitro* cell transformation and *in vivo* carcinogenesis, and the feasibility of screening environmental carcinogens using *in vitro* transformation assays in established cell lines. The Working Group identified the methodological and technical problems associated with these assays, prepared recommendations for practical procedures in the performance of cell transformation assays, and developed standard methods for scoring morphologically transformed foci.

The Working Group discussed the problems involved in the use of only 2 established cell lines, BALB/c 3T3 (3T3) and C3H/10T1/2 (10T1/2), since these 2 systems share several common features. In preparing the recommendations for criteria to be used in scoring transformed foci, the Working Group actually examined fixed and stained Petri dishes containing various foci.

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1 This workshop was held at the International Agency for Research on Cancer, Lyon, France, February 15 to 17, 1984. It was organized by the International Agency for Research on Cancer in collaboration with the National Cancer Institute, NIH, Bethesda, MD, and Environmental Protection Agency, Research Triangle Park, NC, and was partly sponsored by the National Cancer Institute. The organizers of the Workshop were H. Yamasaki (International Agency for Research on Cancer) and T. Kakunaga (National Cancer Institute); J. Little (Harvard University School of Public Health, Boston, MA) and Kakunaga acted as Chairspersons; Little, Kakunaga, and L. M. Schechtmann (Microbiological Associates, Bethesda, MD) acted as rapporteurs. The participants were G. A. Bannikov [All-Union Cancer Research Center, Moscow, USSR (unable to attend)], J. M. Bechet (International Agency for Research on Cancer), J. Bertram (Rosewell Park Memorial Institute, Buffalo, NY), I. Chouroulinkov (Institut de Recherches Scientifiques sur le Cancer, Villejuif, France), E. Cortesi (Istituto Patologia Generale, Universita di Roma, Rome, Italy), T. Enomoto (International Agency for Research on Cancer), L. Haroun (International Agency for Research on Cancer), T. Kurori (Institute of Medical Science, University of Tokyo, Tokyo, Japan), J. R. Landolphi (Cancer Research Laboratory, University of S. California, Los Angeles, CA), R. Montesano (International Agency for Research on Cancer), S. Nesnow [Environmental Protection Agency (unable to attend)], R. Newbold (Chester Beatty Institute, London, United Kingdom), A. Sivak (Arthur D. Little, Inc., Cambridge, MA), M. Umeda (Yokohama City University, Yokohama, Japan), and J. C. M. van der Hoeven (Notoxy, Hertogenbosch, The Netherlands).

2 Address reprint requests to H. Yamasaki, International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon, France.

3 The proceedings of the Workshop including the full version of the recommendations is to be published (9).

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suggested the existence of activated oncogenes, and DNAs extracted from different chemically transformed lines were also differentially sensitive to digestion by restriction endonucleases (5). It is evident that further studies in this area are urgently needed to understand molecular mechanisms of chemical induction of transformation in immortalized cell lines such as 3T3 and 10T1/2 cells.

Application to Environmental Carcinogen Screening

The major aims of this Workshop were to identify the problems pertinent to transformation assay procedures and to try to improve the assay protocol so that cell transformation could be used reliably for screening environmental carcinogens. The discussions of the Working Group made it possible to formulate recommendations for the procedures of cell transformation assays. It is hoped that use of these recommended protocols will facilitate international validation of the results of cell transformation assays. However, since much of the recommended protocol is derived empirically from laboratory experience, it should be updated as our experience increases.

As with the majority of indicator mammalian cells used for in vitro studies, the 3T3 and 10T1/2 cell lines possess reduced inherent levels of single-function oxidase activity (7). Although the available data suggest that use of S9 may be better than hepatocytes, the Working Group concluded that the data base is still too limited to recommend incorporation of the system into the standard protocol of cell transformation. It was recommended, however, that if a test compound gave negative data without metabolic activation, an assay using 3T3 cells with rat liver S9 be performed.

It is theoretically desirable to establish a cell transformation system in which a very low spontaneous transformation frequency is observed, but which is highly sensitive to the induction of transformation by a broad spectrum of carcinogenic chemicals. The very low transformation frequency of 10T1/2 cells has been confirmed in a number of laboratories. All subclones of 3T3 cells recommended for screening chemicals show spontaneous transformation; however, this is not a disadvantage in the use of this cell line, since high sensitivity to induced transformation usually accompanies spontaneous transformation.

Transformation Assay and Scoring of Foci. Recommended procedures for maintaining cells, selecting doses of test compounds for inducing cell transformation and cytotoxicity, selection of negative and positive controls, and screening of FBS are described below. Because of the difficulty of obtaining large supplies of different lots of FBS, however, the Working Group considered that development of a synthetic medium suitable for cell transformation assay should be stimulated.

In order to minimize subjectivity in scoring transformed foci, the Working Group looked at a number of transformed foci on a television screen and formulated consensus criteria for what constitutes a transformed cell. Interindividual differences among Working Group members in scoring transformed foci clearly existed. The criteria formulated by the present Working Group are similar to those of the late C. Heidelberger (14), although more details have been added. Differentiation of Type II and Type III foci is the most difficult problem; while in the 10T1/2 cell system this differentiation was considered to be relatively easy, the Working Group recommended that no differentiation be made for 3T3 cells and that foci be scored as either transformed or nontransformed in these cells. Correlation of type of morphologically transformed foci with their ability to produce tumors is an important problem. For the 2 cell lines discussed by the Working Group, there is a good correlation between these 2 parameters (7), but it was noted that only a few studies have been reported on this subject.

Recommended Protocol for BALB/c 3T3 Cell Transformation Assay³

Sources of Cells

Cells used are BALB/c 3T3 clone A31-1 and those of its subclones, i.e., A31-1-1 and A31-1-13, shown to be Mycoplasma-free.

Maintenance of Stock Culture

Cells should be grown in Eagle's minimum essential medium with Earle's salt containing 10% FBS (complete medium). Cultures should be kept in a subconfluent state and passaged at 1:10 to 1:25 split ratio before reaching 80% confluence. Cultures should be treated with trypsin (0.25%) for the minimum time required to dislodge cells. Residual trypsin can be inactivated by addition of excess complete medium that was used to suspend the cells. The range of passage levels of BALB/c 3T3 cells within which they remain useful and responsive and exhibit limited spontaneous transformation must generally be determined empirically.

Selection of Serum

FBS should be preselected by testing its plating efficiency (>30%) and its ability to maintain the contact-inhibited monolayer and to support 3-methylcholanthrene-induced transformation. A large amount of the selected serum should be stored in an ultralow-temperature freezer (<60°C) and can be used for at least 5 years.

Experimental Design

The assay is performed by exposing BALB/c 3T3 cells to a test compound for 72 h in the absence of a S-9 activation system (8) or for at least 2 h in the presence of a S-9 system (15). Positive and negative controls should be included. The transforming potential of a test compound is determined by its ability to induce an increase in the number of transformed foci when compared to the negative (solvent) control. The test material should first be assayed by the standard assay in the absence of a metabolic supplement. Test materials that give negative or inconclusive results for inducing transformation in the standard assay should be retested using the modified assay in the presence of an exogenous S-9 fraction. Recommended protocol with exogenous metabolic activation is described in the "Proceedings" (9).

Dose Levels. The optimal dose levels for the transformation assay should be selected following a preliminary toxicity test based upon colony-forming efficiency. Approximately 100 to 500 cells/60-mm dish should be exposed to solvent alone and to increasing concentrations of test compound for 72 h at 37°C. Cloning efficiency is determined 7 to 10 days later. Only colonies

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³ The abbreviations used are: EBME, Eagle's basal medium with Earle's salts; FBS, fetal bovine serum.
comprising >50 cells are scored. Whenever possible, the high
dose should be selected to give 80 to 90% toxicity. The low
dose should be the maximal nontoxic dose or one of minimal
toxicity. At least 2 intermediate doses should be selected.

Controls. Negative Control. The solvent vehicle for the test
compound is used as the negative control. The solvents com-
patible with this test system are distilled water, acetone, dimethyl
sulfoxide, and ethanol.

Positive Control. 3-Methylcholanthrene or N-methyl-N'-nitro-
N-nitrosoguanidine is used as the positive control for the standard
assay and benzo(a)pyrene for the modified activation assay.

Methods

Preparation of Target Cells. Exponentially growing BALB/c
3T3 A31-1 cells are seeded for each treatment at 100 to 500
cells/60-mm dish in 5 dishes/treatment for determination of
cytotoxicity, and at 1 x 10^6 cells/60-mm dish in at least 12
replicates for determination of morphological transformation. The
plates are incubated at 37°C in a humidified atmosphere of 5%
CO_2 in air for 20 to 24 h.

Treatment of Target Cells. The time of initiation of chemical
treatment is designated as Day 0. Concentrations of the test
compound should be prepared immediately prior to use, and
these should be dissolved in the appropriate solvent and added
to culture medium. Cells are exposed to the test compound, as
or solvent and positive controls, for 72 h at 37°C. The final
concentration of solvent should be equal for all treatments that
are within the nontoxic range. Following the exposure period, all
treatment media are removed and the cells are washed once
with Hanks’ balanced salt solution and refed with 5 ml of complete
growth medium. The medium is changed twice a week for the
duration of the assay.

Estimation of Cytotoxicity. Cells treated at a density of 100
to 500 cells/dish are maintained in culture for 7 to 10 days. After
this incubation period, concurrent cytotoxicity dishes are fixed with
methanol, stained with 10% aqueous Giemsa, and scored for
colonies. Formation only colonies larger than 50 cells/colony
should be counted.

Expression of Transformed Phenotype. Cells treated at a
density of 10^4 cells/dish are maintained in culture for 4 to 6
weeks. At the end of this incubation period, the transformation
plates are fixed with methanol, stained with 10% aqueous
Giemsa, and scored for morphologically transformed foci accord-
ing to the criteria described in the paragraph below.

Scoring of Foci. Foci that show deep basophilic, dense mul-
tilayering of cells, random cell orientation at any part of the focus
edge, invasion into the surrounding contact-inhibited monolayer,
and domination of spindle-shaped cells are scored as positive
transformed foci. Cell aggregates that do not meet these criteria
should not be counted as transformed foci.

Evaluation of Test Results. The cytotoxic effects of each
treatment should be expressed relative to those of the solvent-
treated control (relative cloning efficiency). The transformation
frequency for each treatment is expressed as the total number of
transformed foci per total replicate dishes and the number of
dishes with foci.

Criteria for Determination of a Valid Test

The cloning efficiency of the solvent control must be equal to
or greater than 30%. Assays that yield negative transformation
activity should have included at least one dose that induced
significant reduction in relative cloning efficiency. The maximum
number of transformed foci in the negative control should not
exceed 0.5 focus/dish. The positive control should induce a
significant (P < 0.05) number of transformed foci relative to the
negative control.

Recommended Protocol for the C3H 10T1/2 C1.8 Cell Trans-
formation Assay

Sources of Cells

C3H 10T1/2 C1.8, at a passage as low as possible (preferably
below passage 8) and shown to be Mycoplasma-free, should be
obtained from a laboratory dedicated to the use of this assay.
Because of the necessity of using low passage cells, it is strongly
recommended that cells obtained originally be immediately ex-
panded and cryopreserved in liquid nitrogen.

Maintenance of Stock Culture

Stock cultures of cells should be grown in EBME containing
10% FBS without antibiotics. Cells should be seeded at 5 x 10^4
cells/75 sq cm flask and grown in a humidified incubator contain-
ing 5% CO_2 in air at 37°C. They should be maintained in a
subconfluent (<80% confluent) state by passing the stocks
approximately every 7 days. Cells should be passaged by treating
with 0.1% trypsin in phosphate-buffered saline for approximately 5 min and quenching the trypsin with 2 volumes
or more of EBME containing 10% FBS. This procedure minimizes
spontaneous transformation and maximizes plating efficiency.

An additional flask of cell stock should be maintained in the
confluent state for an additional week, fixed and stained, and
examined by microscope for spontaneous transformation, cell
morphology, and attachment of the monolayers. Cell stocks
containing spontaneously transformed foci or exhibiting poor
attachment properties should be discarded. Cells should be used
up to passage 15 and then discarded.

Cell Growth and Serum Screening

Plating (colony-forming) efficiency should optimally be 30%
and no less than 20%, at a seeding density of 200 cells/60-mm
dish, after approximately 10 days of growth. Growth rate in the
exponential growth phase should be 17 to 20 h/population
doubling. Saturation density should be approximately 8 x 10^6
cells/60-mm dish when cells are grown in EBME medium con-
taining 10% FBS. The monolayer of confluent cells should be
uniform, nonmottled, well-adherent to the Petri dish surface, and
devoid of zones of high cell density.

Only sera that support the cellular behavior described above
should be accepted. Experience suggests strongly that only FBS
is suitable for use in this assay. Additionally, it is crucial to
prescreen serum batches for ability to support transformation
after a 48-h treatment with 2.5 μg of 3-methylcholanthrene/ml
using the standard assay procedures described. Optimally,
approximately two type II or type III foci can be found per 60-mm
dish under these conditions. It has been reported that use of
serum concentrations giving a saturation density of 5 to 6 x 10^6
cells/dish enhances the expression and visualization of foci (2).

Experimental Design

The 10T1/2 transformation assay is performed by exposing
C3H 10T1/2 C1.8 cells to the test compound, as well as positive
and negative controls, for 48 h, after which the cells are cultured
for estimation of the cytotoxic effects of treatment and induction
of phenotypic transformation. The transforming potential of a
test compound is determined by its ability to induce an increase in the number of type II and type III foci when compared to the solvent control.

**Dose Levels.** The optimal dose levels for the transformation assay should be selected following a preliminary toxicity test based upon colony-forming efficiency. Approximately 200 to 500 cells/60-mm dish should be exposed to solvent alone and to increasing concentrations of the test compound for 48 h at 37°C. Cloning efficiency is determined 8 to 12 days later, prior to merging of colonies. At this time, the colony size is 5 mm in diameter, clearly filled out, and easily visible microscopically. Colonies comprising >50 cells are scored. Cell survival among the treated groups is expressed relative to that of the solvent control (relative cloning efficiency).

Whenever possible, the high dose should be selected to give 80 to 90% toxicity. The low dose should be the maximal nontoxic dose or one of minimal toxicity. At least 2 intermediate doses should be selected.

**Controls. Negative Control.** The solvent vehicle for the test compound is used as the negative control. The solvents compatible with this test system are distilled water, acetone, dimethyl sulfoxide, and ethanol.

**Positive Control.** 3-Methylcholanthrene is used as the positive control, at a concentration of 2.5 μg/ml.

**Methods**

**Preparation of Target Cells.** Exponentially growing C3H 10T1/2 clone 8 cells are seeded for each treatment at 200 to 500 cells/60-mm dish in 5 replicates for determination of cytotoxicity, and at 2 × 10^3 cells/60-mm dish in 20 replicates for determination of phenotypic transformation. The plates are incubated at 37°C in a humidified atmosphere of 5% CO₂ in air for 20 to 24 h.

**Treatment of Target Cells.** The time of initiation of chemical treatment is designated as Day 0. Concentrations of the test compound should be prepared immediately prior to use, and these should be dissolved in the appropriate solvent and added to the culture medium. Cells are exposed to the test compound, or as solvent and positive controls, for 48 h at 37°C. Following the exposure period, all treatment media are removed and the cells refed with 5 ml of complete growth medium. The medium is changed at least once a week for the duration of the assay.

**Estimation of Cytotoxicity.** Cells treated at a density of 200 cells/dish are maintained in culture for 8 to 12 days. After this incubation period, concurrent cytotoxicity dishes are fixed with methanol, stained with 10% aqueous Giemsa, and scored for colony formation. Only colonies comprising >50 cells should be counted.

**Expression of Transformed Phenotype.** Cells treated at a density of 2 × 10^3 cells/dish are maintained in culture for 4 to 6 weeks. At the end of this incubation period, the transformation dishes are fixed with methanol, stained with 10% aqueous Giemsa, and scored for morphologically transformed type II and type III foci according to the criteria described in the paragraph below.

**Scoring of Foci.** Transformed foci that display the aberrant phenotypic characteristics designated types II and III should be counted independently. The differences between type II and type III foci are related to the degree of morphological aberrations associated with each, and recommended criteria for scoring these foci are detailed later.

**Evaluation of Test Results**

The number of type II and type III foci per total dishes scored should be presented for each treatment. The transformation frequency for each treatment is expressed as the total number of transformed foci per total replicate dishes and the number of dishes with foci.

**Criteria for Determination of a Valid Test**

The cloning efficiency of the solvent control must be equal to or greater than 20%. The number of type III foci in the negative control should not exceed one focus per total number of replicate dishes. The total spontaneous type II and type III foci should not exceed 0.15 foci/dish. However, historically, negative controls have exhibited spontaneous transformation levels of <0.07 foci/dish (types II and/or III); therefore, for screening purposes, it is preferable that this serve as the maximum allowable value. The positive control should induce a significant (P < 0.05) number of type III foci relative to the negative control.

**Recommended Criteria for Scoring Morphologically Transformed Foci in BALB/c 3T3 and C3H 10T1/2 Cells**

**General Comments.** The criteria set forth by Reznikoff et al. (14) for describing transformed foci in C3H10T1/2 cells are a valuable starting point for characterizing stained foci in transformation assays, and they were also used as the criteria for BALB/c 3T3 cells. Since the morphological characteristics of transformed foci cover a wide range of possibilities, most probably in a continuous manner, the categorization of foci in the BALB/c 3T3 and C3H 10T1/2 systems as type I, II, or III is only an approximate and conventional means of designating the morphological properties of such foci.

With respect to the differences in cell behavior between C3H 10T1/2 and BALB/c 3T3 cells, it has been questioned whether the same system should be used to score foci in both assays. This view is based primarily on the observation that transformed BALB/c 3T3 foci are largely of type III morphology, and segregation of foci into multiple categories for identification of carcinogens does not appear to be a useful procedure. It may, however, be of considerable value to define clone types more closely in studies of mechanisms.

With regard to the C3H 10T1/2 assay, the observation that type I foci grow poorly, if at all, in soft agar and do not give rise to tumors in vivo indicates that only type II and type III foci be counted. The considerable experience already accumulated in scoring transformation of C3H 10T1/2 cells has demonstrated that foci can be segregated into more and less aggressive types in vitro, although these types span a wide and continuous range of morphological variants. Further, it appears that modification of the definitions of type II and type III foci is appropriate on the basis of the decade of experience with this assay and the practices of investigators now using it.

**Criteria for Type II and Type III Foci.** Type III foci have the following properties: dense; multilayered; basophilic; random orientation at focus edge; and invasion into the monolayer; transformed cells are predominantly spindle-shaped.

Type II foci are distinguishable from type III foci primarily by their more ordered and defined edge. Other properties are that they are dense, multilayered, and less basophilic than type III.

Note should be taken of unusual transformed foci (e.g., corded, banded, poorly attached), especially if they represent a
substantial fraction of the foci, and caution should be exercised in including these in the total number of transformed foci. They should also be reported independently of the standard calculations.

Focus size depends on culture conditions and assay duration. Generally, foci of less than 1 to 2 mm should not be scored; however, small foci with striking type III transformed morphology could be counted as transformed at the discretion of the investigator.

Recommendations for Scoring Foci in the BALB/c 3T3 Transformation Assay.
1. For the purposes of identifying carcinogens, foci should be scored as positive or negative.
2. Positive foci should have characteristics of type III morphology in any area, as described above.
3. Cell aggregates that do not meet these criteria should not be counted as transformed foci.

Recommendations for Scoring Foci in the C3H 10T1/2 Transformation Assay.
1. For the purposes of identifying carcinogens, type II and type III foci should be counted as transformed.
2. Type I foci should not be counted as transformed.

Problems in Scoring.
1. Because of the continuum of focus morphology observed, a number of "uniform" foci are usually found that are intermediate (I/II, II/III) in character. These should be counted conservatively and assigned to the category of lower aggressive behavior.
2. "Mixed" foci that have both type II and type III morphologies should be scored as type III. Mixed foci that have both type I and type II properties should be scored as type II.

REFERENCES


Cancer Research

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