TOXICITY STUDIES:

CELL TRANSFORMATION ASSAY (NEOPLASIA) IN EUKARYOTIC CELL CULTURES (BHK-21-C13)

Laboratory: Interlab Group

Test substance: FR-91

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I. INTRODUCTION

When eukaryotic cell cultures are exposed in vitro to chemical substances or tumor viruses, the cultured cells can then be neoplastically transformed (Berwald 1965, Freeman 1971, Styles 1977, Bilthoven Group CEE 1984, etc.).

The term "transformation" indicates that changes have occurred in cell morphology, biosynthetic routes, or changes in cell growth parameters, including the acquisition of tumorogenicity.

In in vitro celtures, there are five general properties which reflect the alterations cited above, which are:

- -Morphological alterations
- -Decrease in density as a consequence of growth inhibition (DNA-RNA-protein alterations)
- -Decrease in the need for fetal calf serum as a requisite for growth in culture
- -Capacity to grow in semi-solid agar suspensions
- -Tumorogenicity

These properties are not entirely independent of one another, and the distinction depends on the experimental method used. When the cells are cultured in vitro, they grow in a monolayer on the surface of the flask or plate which contains them; thus the most convenient method for demonstrating the transforming phenomenon is their growth in semi-solid agar culture (Kakunaga 1973, Di Paolo 1975, Styles 1977), which implies the loss of anchorage with respect to the growth surface and growth in cellular masses (clones) of hundreds of cells. The method of culture in semi-solid agar is in turn complemented by the reduction in the concentration of fetal calf serum required in the culture medium (from 10% to 2%), with which another of the properties described is demonstrated. Tumorogenicity is little used, given that it is an in vivo method, which requires cells susceptible to transformation, antigenically histocompatible with the recipient animal. The method described here is in accordance with the original protocol of Styles (1977) and follows the specifications of the European Community on this

subject (Directive 84/449/CEE, Toxicological Methods for the Characterization of Chemical Substances, Cell Transformation Assay).

II. PRELIMINARY CYTOTOXICITY ASSAY

A toxicological (cytotoxicity) study of the substance FR-91 has been carried out as a preliminary step to determine the concentration of the product to be used in the transformation assay.

In cultures of high-level eukaryotic cells (BHK-21-C13 cells), the cellular toxicity, cytotoxicity, is manifest as cell death. This lethality can be produced by a multitude of distinct effect (membrane damage or rupture, alterations in the synthesis and degradation of macromolecules, interactions in cellular energy metabolism, morphological changes, reproductive incapacity, etc.). Thus, determination of cellular lethality provides adequate toxicological information in reference to the risk generated by chemical substances.

In this type of methodology, the toxicity ranges are in the order of:

SUBSTANCE	LC_{50} (μg/ml)	LD_{50} (oral) (μg/g)
Dinitrophenol	27	30
p-Cl-Hg-benzoic acid	32	25
Cyclohexamide	0.02	2
Vincristin	0.03	1.3

The ranges can be similar to the experimental toxicities obtained <u>in vivo</u>. The assay permits the determination of an assay concentration of the test substance near the 50% lethal concentration (LD_{50}, that concentration which produces the death of 50% of the cultured cells), and from this concentration, four further serial dilutions are obtained such that the lowest concentrations do not provoke cytotoxicity.

The cell line used is widely cited in the appropriate scientific literature.

III. MATERIAL AND METHODS

a) Cultures

Cultures are carried out at 37°C in the dark in a 5% CO_2 atmosphere. The culture medium and reagents, supplied by Flow Laboratories, had the following composition:

-DMEM with 2.5 g/l $NaHCO_3$

-10% fetal calf serum (FCS)

-2% glutamine

b) Positive and negative controls

Positive controls:

-Chloroform, 40 mg/ml and 4 mg/ml

-Trichloroethane, 60 mg/ml and 6 mg/ml

Negative controls:

-Distilled water

c) Test sample

The test sample, given its liquid nature, was used undiluted and diluted at 1/2, 1/10, 1/50, 1/100, 1/500, 1/1000 and 1/10,000. Dilutions were prepared in phosphate-buffered saline, pH 7.2 (PBS).

d) Assay

The samples described were included in the cultures obtained according to the following method:

-Two vials with a 1 ml volume of cells, maintained in liquid nitrogen, are thawed rapidly.

-The contents are added to 20 ml of culture medium at 25°C.

-This is centrifuged for 4 minutes at 800 rpm in a table-top centrifuge.

-The cell precipitate (pellet) is resuspended in 40 ml of DMEM and incubated in four 100 mm diameter plates for three days at 37°C in 5% CO_2.

-The plates are washed with PBS, trypsinized, suspended and counted in a hemocytometer.

-Incubate at 37°C in 5% CO_2 for 24 hours.

-Add 100 μl of sample and its dilutions, as well as control substances, to the corresponding wells.

-All wells were prepared in duplicate with the objective of testing the cytotoxic activity with or without S9 (supernatant at 9000 x g, rat liver metabolic fraction).

-Add 100 μl of the S9 mixture or DMEM to the appropriate wells.

-Incubate at 37°C in 5% CO_2 for 4 hours.

-The cells are trypsinized, centrifuged and dyed with Trypan blue.

-The cells are counted in a hemocytometer, determining viability by exclusion of the colorant (dye exclusion test for cell viability).

The results are presented in tabular form. Quantification is expressed as the number of viable cells = (non-stained cells/total cells) x 10^5.

The cell count is done using a hemocytometer, a recognized and recommended method for this assay.

The values for the negative controls (distiled water) were within the normal ranges found in this laboratory. All of the negative controls were within admissible limits.

The values for the positive controls were, on the other hand, inducers of a cytotoxic response of 50% and 20% lethality. These values are normal for these types of cells in this laboratory.

EXPRESSION OF CELL VIABILITY

Exposure time: 4 hours

		Cell n° (x 10^5/ml)
Distilled water control		10.0
Control S9	Chloroform 40 µg/ml	5.2
	Chloroform 4 µg/ml	8.3
Control	Trichloroethane 60 µg/ml	4.8
	Trichloroethane 6 µg/ml	7.9
FR-91		
	Undiluted	1.2
	1/2 dilution	1.4
	1/10 dilution	2.4
	1/50 dilution	6.1
	1/100 dilution	8.9
	1/500 dilution	Ctrl*
	1/1000 dilution	Ctrl
	1/10000 dilution	Ctrl

*Ctrl = Absence of cytotoxicity (values within the negative control range)

In relation to these results, the following concentration rangewas selected for the cytotoxicity study:

-Dilution 1/40 (approximate LC_{50}) and dilutions 1/100, 1/1000 and 1/10,000.

IV. TRANSFORMATION ASSAY

Assay:

-The cell cultures are prepared as previously described.

-A cell suspension is prepared by trypsinization, centrifugation and resuspension of cells in DMEM with 2% FCS.

-A 1 ml volume of a 1 x 10^6 cell suspension is added to universal tubes.

-To the appropriate tubes, 100 μl of the following substances are added:

-The test substance or its dilutions in the selected range

-Positive controls (chloroform or trichloroethane) or negative controls (distilled water)

-The tubes are prepared in duplicate; to one series 50 μl of S9 mixture is added, and to the other, 50 μl of PBS.

-The tubes are incubated in a 37°C water bath with agitation at 150 rpm for 4 hours.

-To each tube, 9 ml of DMEM with 2% FCS is added and mixed gently.

-From each tube, a sample of 50 μl is withdrawn and mixed with 5 ml DMEM with 10% FCS and 2% glutamine, poured into a 60 mm plate and incubated at 37°C in 5% CO_2 for 5 days (Survival confirmation assay).

-To the remaining contents of the universal tube (approximately 10 ml), 50 μl of 0.5% noble agar is added and maintained liquid at 40°C.

-Agitate rapidly.

-Incubate 21 days at 37°C in 5% CO_2.

-Count clones greater than 500 μm in diameter.

V. RESULTS AND CONCLUSIONS

The results are presented in tablular form, showing the number of transformant colonies. Analysis should be made relative to the number of spontaneous transformants (negative control) and with respect to the positive controls in the presence and absence of metabolic fraction S9.

TRANSFORMANT COLONIES >500 μm IN DIAMETER

Substance: FR-91

	Without S9	With S9
Negative control (distilled water)	19	25
Positive controls:		
Trichloroethane 60 μg/ml (LC_{50})	180	-
Trichloroethane 6 μg/ml	520	-
Chloroform 40 μg/ml (LC_{50})	-	74
Chloroform 4 μg/ml	-	745
Test sample:		
1/40 dilution	6	9
1/100 dilution	15	21
1/300 dilution	21	24
1/1000 dilution	19	25
1/10,000 dilution	20	28

Survival numbers were similar to those obtained in the preliminary cytotoxicity assay, and within the normal values expected.

In conclusion, substance FR-91, submitted by F. Chacón and with reference number 1200-TC in this laboratory, did not show transforming characteristics in this in vitro carcinogenesis asay.

We declare that the results emitted in this report were obtained according to the protocol described.

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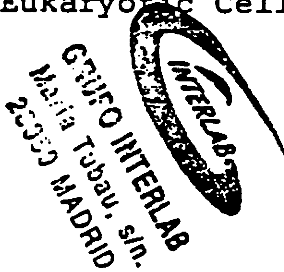

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